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Impairing of gill health through decreasing immune function and structural integrity of grass carp (*Ctenopharyngodon idella*) fed graded levels dietary lipids after challenged with *Flavobacterium columnare*

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ABSTRACT

The current study conducted to investigate the hypothesis that low or excess levels of lipids increased the gill rot morbidity through impairing the immune function and structural integrity in the gill of grass carp (*Ctenopharyngodon idella*). A total of 540 young grass carp with an average initial weight of 261.41 ± 0.53 g were fed diets containing six graded levels of lipids at 0.59%, 2.14%, 3.60%, 5.02%, 6.66% and 8.01% diets for 8 weeks. After the growth trial, fish were challenged with *Flavobacterium columnare* for 3 days. The results indicated that compared with optimal lipids supplementation (2.14%–8.01% lipids diets), low or excess levels of lipids impaired fish immune function through declining the activities of humoral compounds, down-regulated the mRNA levels of anti-inflammatory cytokines, inhibitor of κBα (IκBα) and ribosomal p70S6 kinase (S6K1), and up-regulated pro-inflammatory cytokines, nuclear factor κB p65 (NF-κB p65) (not p52), IκB kinase α (IKKα) (not IKKβ), IKKγ and eIF4E-binding protein (4EBP) in the gill of young grass carp. In addition, low or excess levels of lipids decreased young grass carp physical barrier function through down-regulating the mRNA levels of ZO-1 (rather than ZO-2b), Claudin b, c, 3, 12, 15a, 15b, 7b, 7a and Occludin through MAPKK 6/p38 MAPK/MLCK signaling molecules, decreasing antioxidant ability via Kelch-like ECH-associating protein 1a (Keap1a)/NF-E2-related factor 2 (Nrf2) signaling molecules, and down-regulating the mRNA levels of B-cell lymphoma-2 (Bcl-2) and inhibitor of apoptosis protein (IAP) and up-regulating the mRNA levels of apoptotic protease activating factor-1 (Apaf-1), Caspase-3, -8 and -9 and Fas ligand (FasL) in the gill of grass carp. Based on the quadratic regression analysis for the gill rot morbidity, C3 and MDA contents, the dietary lipids requirements for young grass carp have been estimated to be 5.60%, 6.01% and 4.58% diets.

1. Introduction

Fish gill is a multi-function organ, which is involved in the immune defense against invasion pathogens [1]. Former study in our lab indicated that impairing gill health resulted in reduction of fish growth performance [2]. To our knowledge, fish gill health mainly depend on its immune function and structural integrity [3]. Study report that folic acid can enhance the immune function and maintain structural integrity of grass carp gill [4]. However, lipids, as the second vital nutrition for fish, little massage is available on fish gill immune and structural integrity. It is report that lipids can activate immune cells in fish kidney, thereby promoting immune process [5]. Study indicate that fish gill harbors various immune cells [6]. These data suggest that dietary lipids may be associated with fish gill immune function and physical barrier function, and therefore, these relations warrant investigation.

One important function of fish immune organ is producing antibacterial compounds such as lysozyme (LA), acid phosphatase (ACP), complements, inflammatory cytokines and antimicrobial peptides [7–9]. In terrestrial animals, transcription factors nuclear factor-κB (NF-κB) and mammalian target of rapamycin (mTOR) exhibit to regulate inflammatory response through modulating inflammatory cytokines

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[10,11]. Nevertheless, to date, the effect of total dietary lipids levels on fish gill immune function and inflammatory response is still unclear. In mice, dietary lipids reduce prostaglandin D_2 (PGD_2) levels in the kidney [12]. It is reported that PGD_2 up-regulate the mRNA levels of β-defensin-3, thereby inducing the production of interleukin-8 (IL-8) in human skin keratinocytes [13,14]. Furthermore, Bell et al. [15] reports that fish oil can increase the amount of phospholipids in Atlantic salmon (*Salmo salar*) kidneys. Previous study in our lab indicates that increased phospholipids contents can up-regulate the mRNA levels of NF-κB and TOR in grass carp (*Ctenopharyngodon idella*) gill [16]. According to that, lipids may affect the production of humoral compounds and inflammatory response in fish gill, and the possibility is worthy of investigation.

In addition, the structure integrity is necessary for fish immune function, which is consists of the intercellular tight junction complexes (TJs) and epithelial cells [17]. Study report that disruption of TJ has been considered as an important pathogenesis of human inflammatory diseases [18]. Meanwhile, oxidant damage can induce cell apoptosis, thereby impairing fish gill structural integrity [19]. However, no reports have focused on the effects of dietary lipids on the TJ, antioxidant ability and apoptosis in fish gill. Cameron et al. [20] report that lipids deficiency can decrease the plasma growth hormone (GH) concentration in Arctic charr (*Salvelinus alpines* L.). In Atlantic salmon, GH inhibit Claudin15 gene expression in the gill [21]. Carratelli et al. [22] report that lipids supplementation can up-regulate the protein expression of 70-kDa heat shock protein (hsp70) in mice splenic lymphocytes. Study has indicated that inducing hsp70 could protect human liver cells from apoptosis [23]. These data suggest that there may be a possible relationship between dietary lipids and fish gill structure integrity, which is worthy of further investigation.

In this study, the growth trial was identical to that used in our previous study [24], which is part of a larger study that involve in the determinations of the effects of dietary lipids on the fish growth, health and their potential mechanism. The objectives of the present study are intended to further investigate the effects of lipids on the gill immune function and structure integrity in fish and the finding may reveal the potential regulatory approach for fish gill health influenced by dietary lipids. Additionally, the dietary optimal lipids levels based on different indices for young grass carp are estimated.

2. Materials and methods

2.1. Experimental diet and procedures

As we mentioned above, the formulation and approximate composition analysis of the diets are the same as our previous study [24].” The dietary protein level was fixed at 30% diet, which was reported to be optimum for the growth of grass carp, as described by Khanet al. [25]. Fish oil and soybean oil were added to the basal diet to provide graded concentrations of 0 (un-supplemented), 1.5%, 3%, 4.5%, 6% and 7.5% lipids diets, and the amount of cellulose was reduced to compensate, according to the method of Du et al. [26]. Final lipids levels of the six experimental diets were measured to be 0.59%, 2.14%, 3.60%, 5.02%, 6.66% and 8.01% lipids diets, respectively. After being prepared completely, the diets were stored at −20 °C as described by Zheng et al. [27]. The procedures used in this study were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. The experimental conditions were all the same as our previous study [24].

2.2. Challenge trial and sample collection

After the growth trial, 90 fish were obtained from six groups (15 fish/group with similar body weight) as described by Li et al. [28]. The fish were acclimated in the cages for 5 d. At the end of acclimatize period, fish were challenged by immersion exposure to 1.0×10^8 colony-forming units (cfu) ml^{-1} *Flavobacterium columnare* (kindly provided by the College of Veterinary Medicine, Sichuan Agricultural University, China) for 3 h, and finally returning each experimental cages feed for 3 days. The infection dose was sufficient to activate the immune system, and consequently, an investigation was conducted of effluent on reactivity against a threatening disease, according to our preliminary study data (unpublished data). During the 3 days challenge trial, fish were fed the same diets as the feeding trial four times each day. Experiment conditions were the same as the growth trial. After the challenge trial, all fishes from each treatment were anaesthetized in a benzocaine bath, as described by Geraylou et al. [29]. To investigate the effects of diets containing graded levels of lipids on the gill rot morbidity, a scoring system was designed to evaluate the severity of gill rot morbidity in fish, which was similar to Taylor et al. [30]. At last, the gills of fish were quickly removed and frozen in liquid nitrogen, and stored at −80 °C for later analysis.

2.3. Biochemical analysis

The gill lysozyme (LA) activity was measured based on the turbidimetric assay with a slight modifications, which was according to Ndong et al. [31]. The acid phosphatase (ACP) activity and complement 3 (C3) and complement 4 (C4) contents were assayed according to Zhao et al. [32]. The glutathione (GSH) content was assayed according to Feng et al. [33]. The reactive oxygen species (ROS) content was measured using 2′7′-dichlorodihydrofluorescein diacetate which was oxidized to fluorescent dichlorofluorescein (DCF) by ROS, according to Ko et al. [34]. The lipids peroxidation was analyzed in terms of malondialdehyde (MDA) equivalents using the thiobarbituric acid (TBA) reaction as described by Petrović et al. [35]. The protein carbonyl (PC) residue content was determined according to Armenteros et al. [36] using the 2, 4-dinitrophenylhydrazine (DNPH) reagent. The protein carbonyl content was calculated from the peak absorbance at 340 nm using an absorption coefficient of 22,000/M/cm. The total superoxide dismutase (T-SOD) activity was measured following the method of Lu et al. [37]. To distinguish SOD isoforms, we used the Total SOD (T-SOD) detection kit (Nanjing Jian Cheng Bio Inst., China). The kit can make Cu/ZnSOD activity unchanged and MnSOD activity lost, and then the T-SOD activity minus the Cu/ZnSOD activity is the MnSOD activity. The catalase (CAT) activity was determined by measuring the decomposition of hydrogen peroxide, which as described by Rueda-Jasso et al. [37]. Glutathione peroxidases (GPx) activity was assayed in a coupled enzyme system where NADPH was consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH) Nugroho et al. [38]. Glutathione-*S*-transferases (GST) activity was measured using chloro-2, 4 dinitrobenzene as substrate, according to Bianchi et al. [39]. Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of oxidized glutathione, GSSG [40].

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

The procedures of RNA isolation, reverse transcription and quantitative real-time PCR were similar to those previously described in another study conducted in our laboratory [2]. The total RNA was extracted from the gill using RNAiso Plus kit (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's instructions followed by DNAse I treatment. RNA quality and quantity were assessed using agarose gel (1%) electrophoresis and spectrophotometric (A260: 280 nm ratio) analysis, respectively. Subsequently, RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (TaKaRa), according to the manufacturer's instructions. For quantitative real-time PCR, specific primers were designed according to the sequences cloned in our laboratory and the published sequences of grass carp (Table 1). β-actin was used as a reference gene to normalize cDNA loading, according to the results of our preliminary experiment concerning the evaluation of internal control gene (data not shown). The target and housekeeping gene amplification efficiency were calculated according to the specific

Table 1
Real-time PCR primer sequences.

Target gene	Primer sequence Forward (5′→3′)	Primer sequence Reverse (5′→3′)	Annealing temperature (°C)	Accession number
Hepcidin	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	59.3	JQ246442.1
LEAP-2a	TGCCTACTGCCAGAACCA	AATCGGTTGGCTGTAGGA	59.3	FJ390414
LEAP-2b	TGTGCCATTAGCGACTTCTGAG	ATGATTCGCCACAAAGGGG	59.3	KT625603
β-defensin-1	TTGCTTGTCCTTGCCGTCT	AATCCTTTGCCACAGCCTAA	58.4	KT445868
IFN-γ2	TGTTTGATGACTTTGGGATG	TCAGGACCCGCAGGAAGAC	60.4	JX657682
TNF-α	CGCTGCTGTCTGCTTCAC	CCTGGTCCTGGTTCACTC	58.4	HQ696609
IL-1β	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	57.1	JQ692172
IL-6	CAGCAGAATGGGGGAGTTATC	CTCGCAGAGTCTTGACATCCTT	62.3	KC535507.1
IL-8	ATGAGTCTTAGAGGTCTGGGT	ACAGTGAGGGCTAGGAGGG	60.3	JN663841
IL-4(13)A	CTACTGCTCGCTTTCGCTGT	CCCAGTTTTCAGTTCTCTCAGG	55.9	KT445871
IL-4(13)B	TGTGAACCAGACCCTACATAACC	TTCAGGACCTTTGCTGCTTG	55.9	KT625600
IL-10	AATCCCTTTGATTTTGCC	GTGCCTTATCCTACAGTATGTG	61.4	HQ388294
IL-12 p35	TGGAAAAGGAGGGGAAGATG	AGACGGACGCTGTGTGAGTGTA	55.4	KF944667.1
IL-12 p40	ACAAAGATGAAAAACTGGAGGC	GTGTGTGGTTTAGGTAGGAGCC	59.0	KF944668.1
TGF-β1	TTGGGACTTGTGCTCTAT	AGTTCTGCTGGGATGTTT	55.9	EU099588
NF-κB p52	TCAGTGTAACGACAACGGGAT	ATACTTCAGCCACACCTCTCTT-AG	58.4	KM279720
NF-κB p65	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG	62.3	KJ526214
IκBα	TCTTGCCATTATTCACGAGG	TGTTACCACAGTCATCCACCA	62.3	KJ125069
IKKα	GGCTACGCCAAAGACCTG	CGGACCTCGCCATTCATA	60.3	KM279718
IKKβ	GTGGCGGTGGATTATTGG	GCACGGGTTGCCAGTTTG	60.3	KP125491
IKKγ	AGAGGCTCGTCATAGTGG	CTGTGATTGGCTTGCTTT	58.4	KM079079
TOR	TCCCACTTTCCACCAACT	ACACCTCCACCTTCTCCA	61.4	JX854449
S6K1	TGGAGGAGGTAATGGACG	ACATAAAGCAGCCTGACG	54.0	EF373673
4EBP 1	GCTGGCTGAGTTTGTGGTTG	CGAGTCGTGCTAAAAAGGGTC	60.3	KT757305
4EBP 2	CACTTTATTCTCCACCACCCC	TTCATTGAGGATGTTCTTGCC	60.3	KT757306
Occludin	TATCTGTATCACTACTGCGTCG	CATTCACCCAATCCTCCA	59.4	KF193855
ZO-1	CGGTGTCTTCGTAGTCGG	CAGTTGGTTTGGGTTTCAG	59.4	KJ000055
ZO-2b	TACAGCGGGACTCTAAAATGG	TCACACGGTCGTTCTCAAAG	60.3	KM112095
Claudinb	GAGGGAATCTGGATGAGC	ATGGCAATGATGGTGAGA	57.0	KF193860
Claudin3	ATCACTCGGGACTTCTA	CAGCAAACCCAATGTAG	57.0	KF193858
Claudin7a	ACTTACCAGGGACTGTGGATGT	CACTATCATCAAAGCACGGGT	59.3	KT625604
Claudin7b	CTAACTGTGGTGGTGATGAC	AACAATGCTACAAAGGGCTG	59.3	KT445866
Claudin12	CCCTGAAGTGCCCACAA	GCGTATGTCACGGGAGAA	55.4	KF998571
Claudin15a	TGCTTTATTTCTTGGCTTTC	CTCGTACAGGGTTGAGGTG	59.0	KF193857
Claudin15b	AGTGTTCTAAGATAGGAGGGGA-G	AGCCCTTCTCCGATTTCAT	62.3	KT757304
MLCK	GAAGGTCAGGGCATCTCA	GGGTCGGGCTTATCTACT	53.0	KM279719
FasL	AGGAAATGCCCGCACAAATG	AACCGCTTTCATTGACCTGGAG	61.4	KT445873
MAPKK 6	GAGCATCTCCACAGCAACCT	CTTCGCCACTGAATCCACAA	57.1	KT445869
p38 MAPK	TGGGAGCAGACCTCAACAAT	TACCATCGGGTGGCAACATA	60.4	KM112098
Bcl-2	AGGAAAATGGAGGTTGGGAT	CTGAGCAAAAAAGGCGATG	60.3	JQ713862.1
Bax	CATCTATGAGCGGGTTCGTC	TTTATGGCTGGGGTCACACA	60.3	JQ793788.1
Apaf-1	AAGTTCTGGAGCCTGGACAC	AACTCAAGACCCCACAGCAC	61.4	KM279717
IAP	CACAATCCTGGTATGCGTCG	GGGTAATGCCTCTGGTGCTC	58.4	FJ593503.1
Caspase-3	GCTGTGCTTCATTTGTTTG	TCTGAGATGTTATGGCTGTC	55.9	JQ793789
Caspase-8	ATCTGGTTGAAATCCGTGAA	TCCATCTGATGCCCATACAC	59.0	KM016991
Caspase-9	CTGTGGCGGAGGTGAGAA	GTGCTGGAGGACATGGGAAT	59.0	JQ793787
CuZnSOD	CGCACTTCAACCCTTACA	ACTTTCCTCATTGCCTCC	61.5	GU901214
MnSOD	ACGACCCAAGTCTCCCTA	ACCCTGTGGTTCTCCTCC	60.4	GU218534
CAT	GAAGTTCTACACCGATGAGG	CCAGAAATCCCAAACCAT	58.7	FJ560431
GPx1a	GGGCTGGTTATTCTGGGC	AGGCGATGTCATTCCTGTTC	61.5	EU828796
GPx1b	TTTTGTCCTTGAAGTATGTCCGT-C	GGGTCGTTCATAAAGGGCATT	60.3	KT757315
GPx4a	TACGCTGAGAGAGGTTTACACAT	CTTTTCCATTGGGTTGTTCC	60.4	KU255598
GPx4b	CTGGAGAAATACAGGGGTTACG	CTCCTGCTTTCCGAACTGGT	60.3	KU255599
GSTR	TCTCAAGGAACCCGTCTG	CCAAGTATCCGTCCCACA	58.4	EU107283
GSTO-1	GGTGCTCAATGCCAAGGGAA	CTCAAACGGGTCGGATGGAA	58.4	KT757314
GSTO-2	CTGCTCCCATCAGACCCATTT	TCTCCCCTTTTCTTGCCCATA	61.4	KU245630
GR	GTGTCCAACTTCTCCTGTG	ACTCTGGGGTCCAAAACG	59.4	JX854448
Nrf2	CTGGACGAGGAGACTGGA	ATCTGTGGTAGGTGGAAC	62.5	KF733814
Keap1a	TTCCACGCCCTCCTCAA	TGTACCCTCCCGCTATG	63.0	KF811013
Keap1b	TCTGCTGTATGCGGTGGGC	CTCCTCCATTCATCTTTCTCG	57.9	KJ729125
β-actin	GGCTGTGCTGTCCCTGTA	GGGCATAACCCTCGTAGAT	61.4	M25013

gene standard curves generated from 10-fold serial dilutions. The relative amount of each transcript was normalized to the amount of β-actin transcript in the same cDNA. After verification that the primers amplified with an efficiency of approximately 100%, the expression results were analyzed using the $2^{-\Delta\Delta CT}$ method, according to Livak and Schmittgen [41].

2.5. Statistical analysis

The results were presented as the mean ± standard deviation (SD). All data were subjected to a one-way analysis of variance (ANOVA) to determine whether significant differences occurred among treatments. If a significant difference was identified, differences among means were

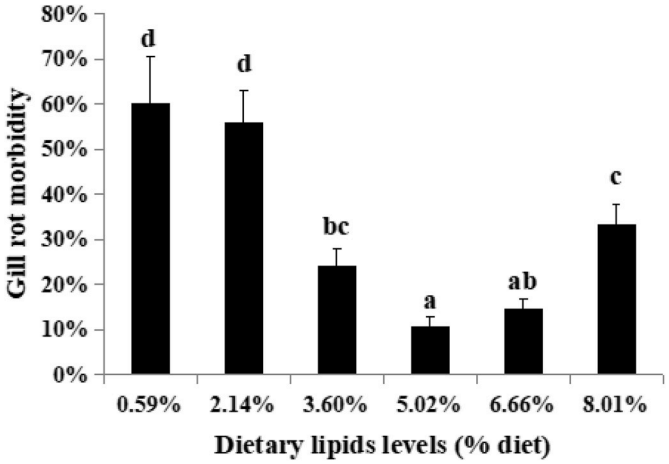

Fig. 1. Low or excess levels of lipids aggravate gill rot under the infection of *Flavobacterium columnare* in young grass carp (*Ctenopharyngodon idella*)[1].
[1]Values having different letters are significantly different ($P < 0.05$).

compared by Duncan's multiple range tests at $P < 0.05$ with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The results are presented as the means ± SD. Quadratic regression was used to estimate the optimal dietary level of lipids for young grass carp according to Jin et al. [42].

3. Results

3.1. Gill rot morbidity of young grass carp after the infection with F. columnare

Effects of graded concentrations of dietary lipids on young grass carp gill rot morbidity are presented in Figs. 1 and 2. Fish fed low or excess levels of lipids (0.59%, 2.14%. 6.66% and 8.01% lipids diets) had significantly ($P < 0.05$) higher gill rot morbidity (Fig. 1) after the infection with *F. columnare* than fish supplementation with optimal lipids level (5.02% lipids diet). Compared with optimal lipids supplementation (5.02% lipids diet), low or excess levels of lipids led to an obvious gill rot symptom (Fig. 2) in young grass carp.

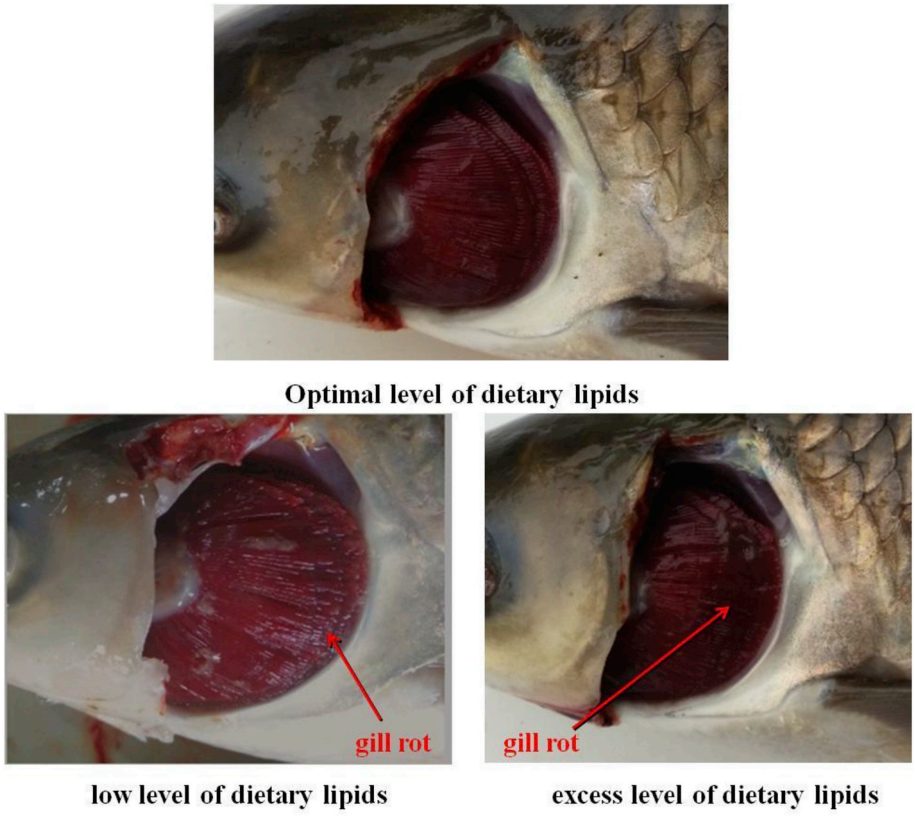

Fig. 2. Compared to optimal lipids supplementation, low or excess levels of lipids led to obviously gill rot after infection with *F. columnare* in young grass carp.

Table 2
Immune parameters in the gill of young grass carp fed diets containing different lipids levels after challenge with *Flavobacterium columnare*.[1].

	Dietary lipids levels (g/kg diet)					
	0.59%	2.14%	3.60%	5.02%	6.66%	8.01%
LA	145.37 ± 13.86^{a}	148.15 ± 7.76^{a}	181.50 ± 9.12^{b}	187.61 ± 12.30^{b}	179.07 ± 6.76^{b}	179.88 ± 9.60^{b}
ACP	241.61 ± 19.98^{a}	369.8 ± 26.22^{b}	365.35 ± 5.19^{b}	354.03 ± 30.86^{b}	370.43 ± 29.27^{b}	362.85 ± 27.82^{b}
C3	10.62 ± 0.64^{a}	12.44 ± 0.95^{b}	15.48 ± 1.24^{c}	20.70 ± 1.25^{e}	17.21 ± 0.97^{d}	17.03 ± 0.72^{d}
C4	1.21 ± 0.11^{a}	1.47 ± 0.09^{b}	1.52 ± 0.10^{b}	1.46 ± 0.12^{b}	1.51 ± 0.07^{b}	1.22 ± 0.08^{a}

[1]Values are means ± SD (n = 6), and different superscripts in the same row are significantly different ($P < 0.05$). LA (U mg^{-1} protein), ACP (U mg^{-1} protein) activities, C3 (mg g^{-1} protein) and C4 (mg g^{-1} protein).

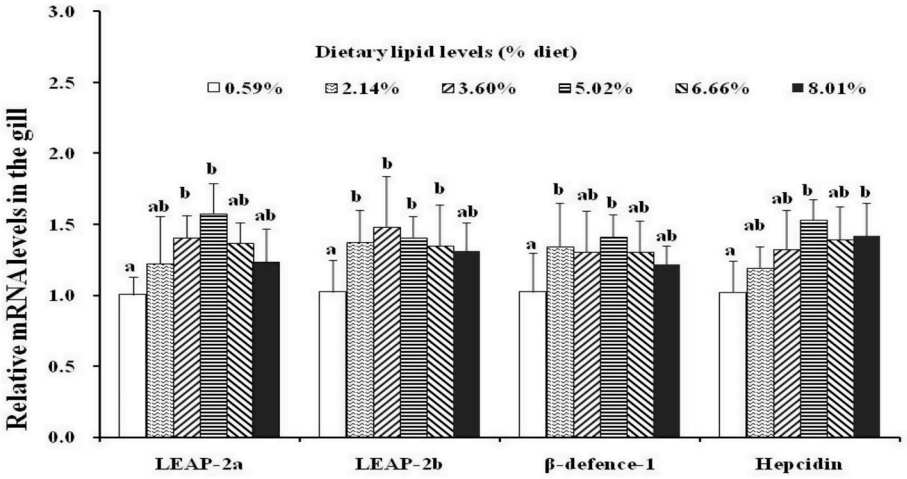

Fig. 3. Relative expression of LEAP-2a, LEAP-2b, Hepcidin and β-defensin-1 in the gill of young grass carp fed diets containing graded levels of lipids[1].
[1]Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). LEAP-2: liver expressed antimicrobial peptide 2.

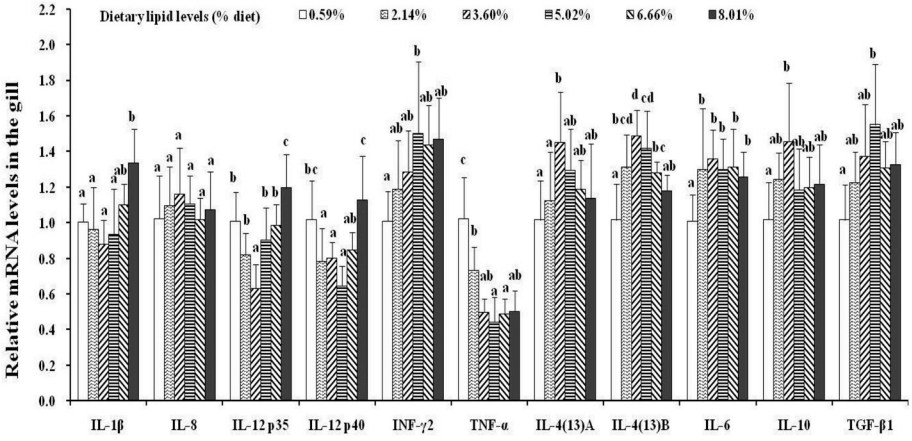

Fig. 4. Relative expression of IFN-γ2, TNF-α, IL-1β, IL-6, IL-8, IL-12 p35, IL-12 p40, IL-4(13)A, IL-4(13)B, IL-10 and TGF-β1 in the gill of young grass carp fed diets containing graded levels of lipids[1].
[1]Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). IL: interleukin; TGF-β: transforming growth factor β; IFN-γ2: interferon γ2; TNF-α: tumor necrosis factor α.

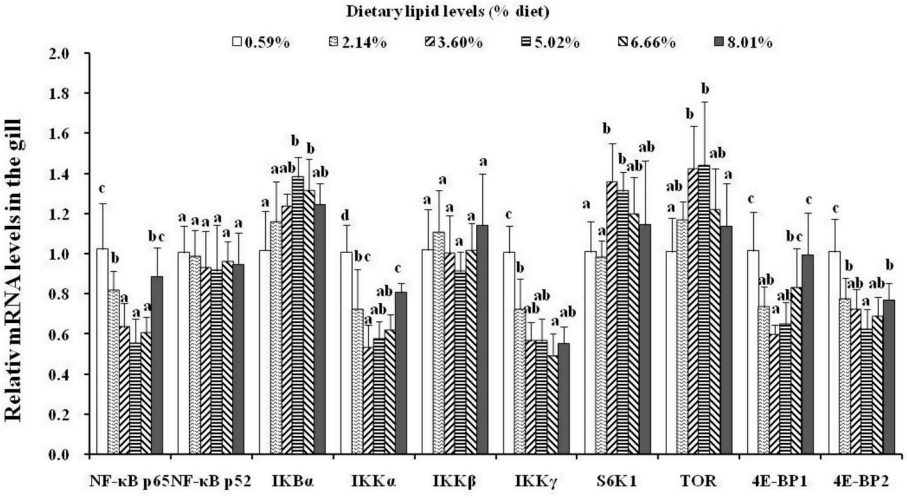

Fig. 5. Relative expression of NF-κB p65, NF-κB p52, IκBα, IKKα, IKKβ, IKKγ, TOR, S6K1, and 4EBP in the gill of young grass carp fed diets containing graded levels of lipids[1].
[1]Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). NF-κB: nuclear factor kappa B; IκBα: inhibitor of κBα; IKK: IκB kinase; TOR: target of rapamycin; S6K1: ribosomal protein S6 kinase 1; 4EBP: eIF4E-binding protein.

3.2. The immune response related parameters in the gill

The effects of dietary lipids levels on the activities of LA and ACP as well as the contents of C3 and C4 in the gill of grass carp are presented in Table 2. The activities of LA and ACP as well as the contents of C3 and C4 were gradually increased with dietary lipids levels from basal diet up to 5.02%, 6.66% 5.02% and 3.60% lipids diets, respectively, and then gradually decreased.

The effects of dietary lipids on liver expressed antimicrobial peptide-2a and -2b (LEAP-2a and -2b), Hepcidin and β-defence-1, inflammatory cytokines, NF-κB p65, NF-κB p52, IκBα, IKKα, IKKβ, IKKγ, TOR, S6K1 and 4EBP in the gill are displayed in Fig. 3, Fig. 4 and Fig. 5. The mRNA levels of LEAP-2A, LEAP-2B, Hepcidin and β-defensin-1 were gradually up-regulated with dietary lipids levels from basal diet up to 5.02%, 3.60%, 5.02% and 5.02% diets, respectively, and then down-regulated gradually.

The mRNA levels of NF-κB p65, TNF-α, 4EBP 2 and IL-12 p40 were gradually down-regulated with dietary lipids levels from basal diet up to 5.02% diet, and then up-regulated gradually. The mRNA levels of IL-1β, IL-12 p35 and 4EBP 1 were gradually down-regulated with dietary

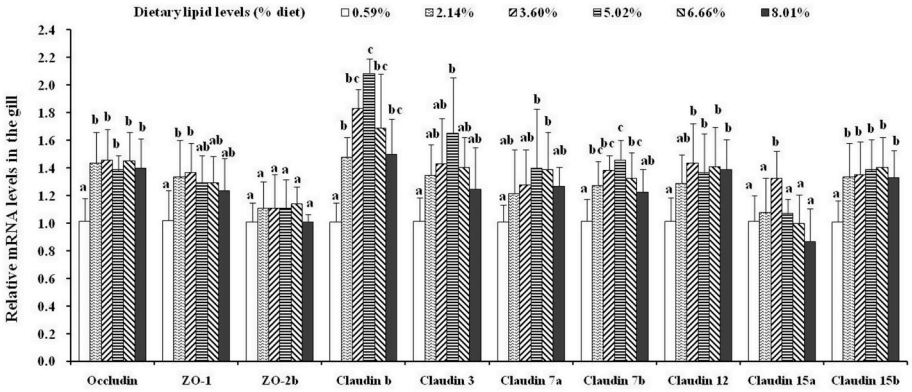

Fig. 6. Relative expression of Occludin, ZO-1, ZO-2b, Claudinb, 3, 7a, 7b, 12, 15a and 15b in the gill of young grass carp fed diets containing graded levels of lipids[1].

[1]Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). ZO: zonula occludens; MLCK: myosin light chain kinase.

lipids levels from basal diet up to 3.60% diet, and then up-regulated gradually. The mRNA levels of IL-4(13)B, IL-4(13)A, IL-6, IL-10 were gradually up-regulated with dietary lipids levels from basal diet up to 3.60% diet, and then down-regulated gradually. The mRNA levels of IFN-γ2, TGF-β1, IκBα and TOR were gradually up-regulated with dietary lipids levels from basal diet up to 5.02% diet, and then down-regulated gradually. The mRNA level of IKKα was significantly ($P < 0.05$) down-regulated with dietary lipids levels from basal diet up to 3.60% diet, and then up-regulated gradually. The mRNA level of IKKγ was gradually down-regulated with dietary lipids levels from basal diet up to 6.66% diet, and then up-regulated gradually. Dietary lipids had no effects on the mRNA levels of IL-8, IKKβ, and NF-κB p52 in the gill ($P > 0.05$).

3.3. TJ complexes, MAPKK6, p-38 MAPK and MLCK mRNA levels in the gill

The effects of dietary lipids on mRNA levels of TJ complexes, MAPKK6, p-38 MAPK and MLCK in the gill are showed in Fig. 6 and Fig. 7. The mRNA levels of Occludin, Claudin 12, ZO-1 and Claudin 15a were all gradually up-regulated with dietary lipids levels from basal diet up to 3.60% diet, and then down-regulated gradually. The mRNA levels of Claudin 3, Claudin b, Claudin 7b and Claudin 7a were all gradually up-regulated with dietary lipids levels from basal diet up to 5.02% diet, and then down-regulated gradually. The mRNA levels of MLCK and MAPKK6 were gradually down-regulated with dietary lipids levels from basal diet up to 3.60% diet, and then up-regulated gradually. The mRNA level of p-38 MAPK was gradually down-regulated with dietary lipids levels from basal diet up to 5.02% diet, and then up-regulated gradually.

3.4. Antioxidant-related parameters in the gill

3.4.1. ROS content, lipids peroxidation, protein oxidant and antioxidant-related parameters in the gill

The gill oxidative status and antioxidant capacity are showed in Table 3. The MDA and PC contents were all gradually decreased with dietary lipids levels from basal diet up to 5.02% diet, and then increased gradually. The ROS content was gradually decreased with dietary lipids levels from basal diet up to 8.01% diet. The activities of MnSOD and GPx were gradually increased with dietary lipids levels from basal diet up to 5.02% diet and then gradually decreased. The activity of CAT was gradually increased with dietary lipids levels from basal diet up to 2.14% diet and then gradually decreased. The activity of GR was significantly increased ($P < 0.05$) with dietary lipids levels from basal diet up to 3.60% diet and then significantly ($P < 0.05$) decreased. The content of GSH was gradually increased with dietary lipids levels from basal diet up to 3.60% diet and then significantly ($P < 0.05$) decreased. The activity of GST was significantly increased ($P < 0.05$) with dietary lipids levels from basal diet up to 3.60% diet, and then gradually decreased.

3.4.2. Antioxidant enzymes, Nrf2, Keap1a and Keap1b gene expression in the gill

The mRNA levels of antioxidant enzymes, Nrf2, Keap1a and Keap1b in the gill are showed in Fig. 8 and Fig. 9. The mRNA levels of CuZnSOD, GPx4a, Nrf2, MnSOD, GSTO-1, CAT, GSTO-2 were all gradually up-regulated with dietary lipids levels from basal diet up to 5.02% diet, and then down-regulated gradually. The mRNA levels of GR, GPx1, and GPx4b were all gradually up-regulated with dietary lipids levels up to 3.60% diet, and then down-regulated gradually. The mRNA level of GPx1b was gradually up-regulated with dietary lipids

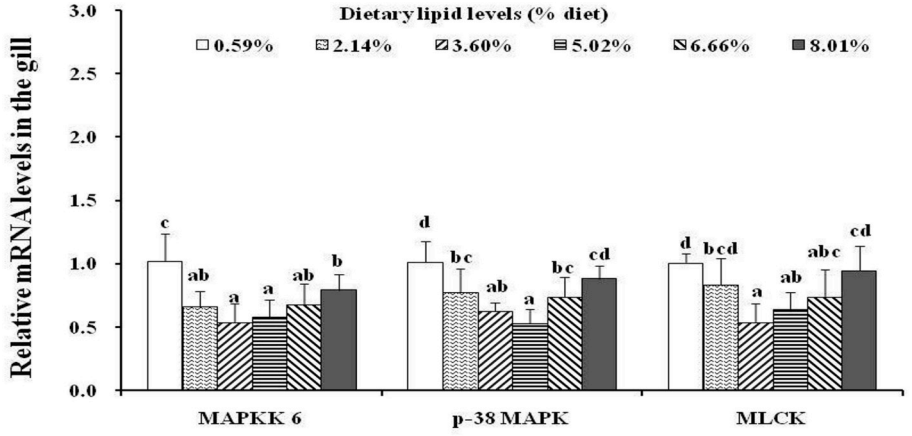

Fig. 7. Relative expression of MLCK, p38 MAPK and MAPKK 6 in the gill of young grass carp fed diets containing graded levels of lipids[1].

[1]Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). p38 MAPK: p38 mitogen-activated protein kinases; MAPKK 6: mitogen-activated protein kinase kinase 6; JNK: c-Jun N-terminal kinases.

Table 3
Antioxidant related parameters in the gill of young grass carp fed diets containing different levels of lipids after challenge with *F. columnare*[1].

	Dietary lipid levels (g/kg diet)					
	0.59%	2.14%	3.60%	5.02%	6.66%	8.01%
ROS	100.00 ± 5.76^{c}	75.71 ± 5.62^{b}	70.81 ± 3.82^{b}	58.79 ± 4.38^{a}	57.69 ± 2.08^{a}	55.98 ± 4.18^{a}
MDA	3.66 ± 0.29^{c}	2.70 ± 0.21^{a}	2.67 ± 0.15^{a}	2.64 ± 0.22^{a}	2.79 ± 0.16^{a}	3.28 ± 0.20^{b}
PC	3.02 ± 0.24^{b}	3.04 ± 0.13^{b}	2.86 ± 0.13^{b}	2.36 ± 0.21^{a}	2.98 ± 0.20^{b}	3.59 ± 0.21^{c}
CuZnSOD	3.34 ± 0.12^{a}	3.40 ± 0.27^{a}	3.43 ± 0.10^{a}	3.40 ± 0.27^{a}	3.44 ± 0.12^{a}	3.36 ± 0.27^{a}
MnSOD	2.47 ± 0.21^{a}	2.45 ± 0.19^{a}	3.36 ± 0.17^{c}	3.48 ± 0.26^{c}	2.81 ± 0.25^{b}	2.89 ± 0.26^{b}
CAT	1.33 ± 0.10^{b}	1.35 ± 0.05^{b}	1.32 ± 0.08^{b}	1.15 ± 0.09^{a}	1.09 ± 0.07^{a}	1.08 ± 0.09^{a}
GPx	67.19 ± 4.41^{a}	74.86 ± 4.94^{a}	94.19 ± 6.07^{b}	112.04 ± 8.46^{c}	108.81 ± 4.58^{c}	110.42 ± 9.13^{c}
GST	44.10 ± 2.90^{a}	50.91 ± 4.16^{b}	58.83 ± 2.76^{c}	49.83 ± 4.68^{b}	48.14 ± 2.38^{ab}	47.69 ± 4.05^{ab}
GR	12.21 ± 1.00^{b}	15.30 ± 0.92^{d}	17.25 ± 0.73^{e}	14.01 ± 1.26^{c}	12.41 ± 0.94^{b}	9.83 ± 0.77^{a}
GSH	1.55 ± 0.11^{bc}	1.69 ± 0.10^{cd}	2.10 ± 0.16^{e}	1.78 ± 0.16^{d}	1.51 ± 0.09^{b}	1.35 ± 0.10^{a}

[1]Values are means ± SD (n = 6), and different superscripts in the same row are significantly different ($P < 0.05$). MDA, (nmol mg^{-1} protein); PC (nmol mg^{-1} protein); ROS (% DCF fluorescence); CuZnSOD (U mg^{-1} protein); MnSOD (U mg^{-1} protein); CAT (U mg^{-1} protein); GPx (U mg^{-1} protein); GST (U mg^{-1} protein); GR (U mg^{-1} protein) and GSH (mg g^{-1} protein).

levels from basal diet up to 2.14% diet, and then plateaued thereafter ($P > 0.05$). The mRNA level of Keap1a was gradually down-regulated with dietary lipids levels from basal diet up to 5.02% diet, and then plateaued thereafter ($P > 0.05$). However, there was no effect on the mRNA levels of Keap1b by dietary lipids ($P > 0.05$).

3.5. Apoptosis related indexes gene expression in the gill

Relative expressions of Apaf-1, IAP, Bcl-2, Bax, FasL, Caspase 3, Caspase 8 and Caspase 9 in the gill are represented in Fig. 10. Fish fed low or excess levels of lipids had higher mRNA levels of Apaf-1, Caspase 3, Caspase 8, FasL, Bax and Caspase 9 in the gill than fish supplementation with optimal dietary lipids level (3.60%, 6.66%, 8.01%, 5.02%, 5.02% and 3.60%diets, respectively). Fish fed low or excess levels of lipids had lower mRNA levels of IAP and Bcl-2 in the gill than fish supplemented with optimal dietary lipids level (5.02% diet).

4. Discussion

This study used the identical growth trial as that in our previous study [24] and was a part of a larger research effort to determine the effects of dietary lipids levels on the growth, immune function and physical barrier function in fish. Former study indicate that dietary low or excess levels of lipids decreased the growth performance of young grass carp [24]. Based on our understanding, fish growth often relies on the healthy gill [43]. Thus, our current study investigated the effects of dietary lipids on the gill immune function and structure integrity in fish for the first time.

4.1. Low or excess levels of lipids promoted gill rot of young grass carp

F. columnare is wide-spread in aquatic environments, which can cause fish gill rot and result in high mortality and losses of fish [44]. Thus, we found a scoring system to evaluate the gill rot morbidity of fish, which is similar to Taylor et al. [30]. The result show (Figs. 1 and 2) that low or excess levels of lipids induced the gill rot morbidity, whereas optimal supplementation (5.02% lipids diet) with lipids can enhance the resistance ability of gill rot in fish. Based on our quadratic regression analysis ($Y_{gill\ rot\ morbidity} = 203.6x^2 - 22.80x + 0.805$, $R^2 = 0.829$), the dietary lipids requirement for protecting young grass carp against enteritis morbidity has been estimated to be 5.60% diet." which is higher than that based on growth performance (43.7 g/kg diet) in our previous study [24].

4.2. Low or excess levels of dietary lipids decrease immune function in the gill of young grass carp

4.2.1. Low or excess levels of dietary lipids reduce the humoral compounds in young grass carp gill

It is currently believed that teleost gill is also a lymphoid organ, which can secret antibacterial compounds (include LA, acid phosphatase, complements and antimicrobial peptides) to protect fish from invasion pathogens [4,43]. In the current study, compared to the optimal dietary lipids supplement (3.60%–6.66% lipids diets), low or excess levels of lipids decrease LA and ACP activities, C3 and C4 contents and down-regulate LEAP-2a, LEAP-2b, Hepcidin and β-defensin-1 mRNA levels in the gill of young grass carp, indicating that low or excess levels of lipids reduce the gill immune function of fish. However, proper lipids supplementation can recover it.

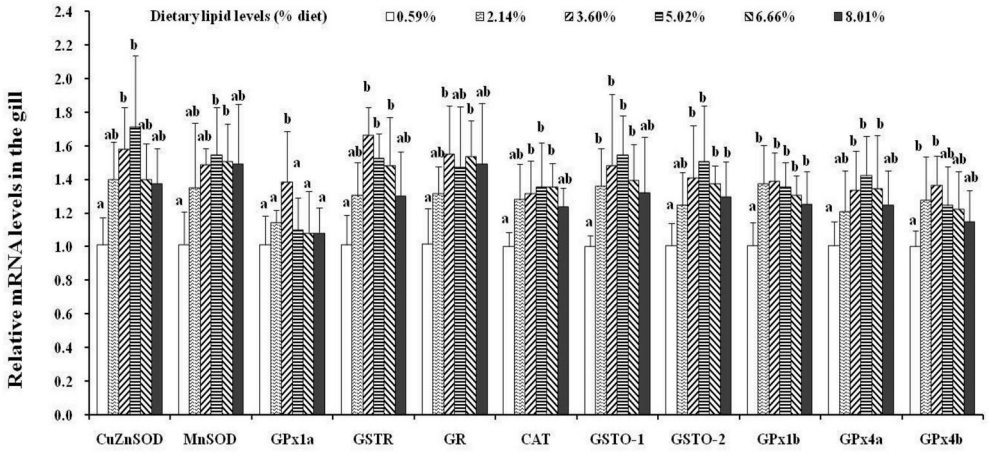

Fig. 8. Relative expression of CuZnSOD, MnSOD, CAT, GPx1a, GPx1b, GPx4a, GPx4b, GSTR, GSTO-1, GSTO-2 and GR in the gill of young grass carp fed diets containing graded levels of lipids[1].
[1]Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). CuZnSOD: copper, zinc superoxide dismutase; MnSOD: manganese superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GST: glutathione S-transferase; GR: glutathione reductase.

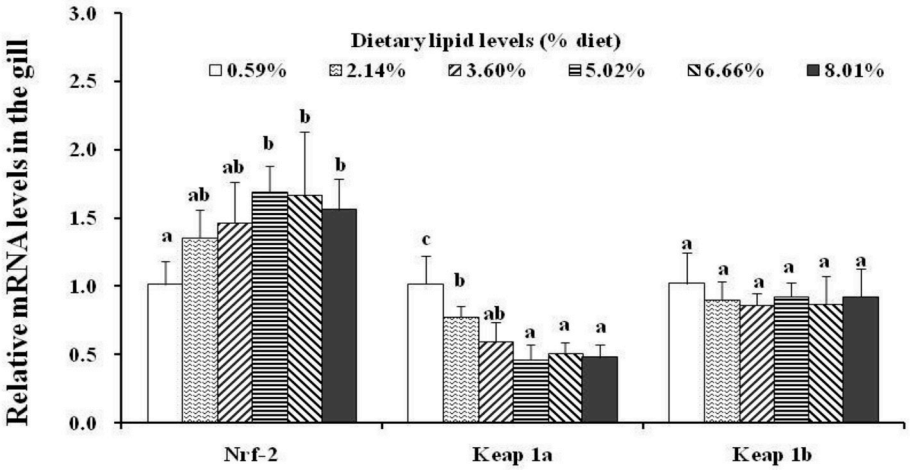

Fig. 9. Relative expression of Nrf2, Keap1a and Keap1b in the gill of young grass carp fed diets containing graded levels of lipids[1].
[1]Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). Nrf2: NF-E2-related factor 2; Keap1: Kelch-like-ECH-associated protein 1.

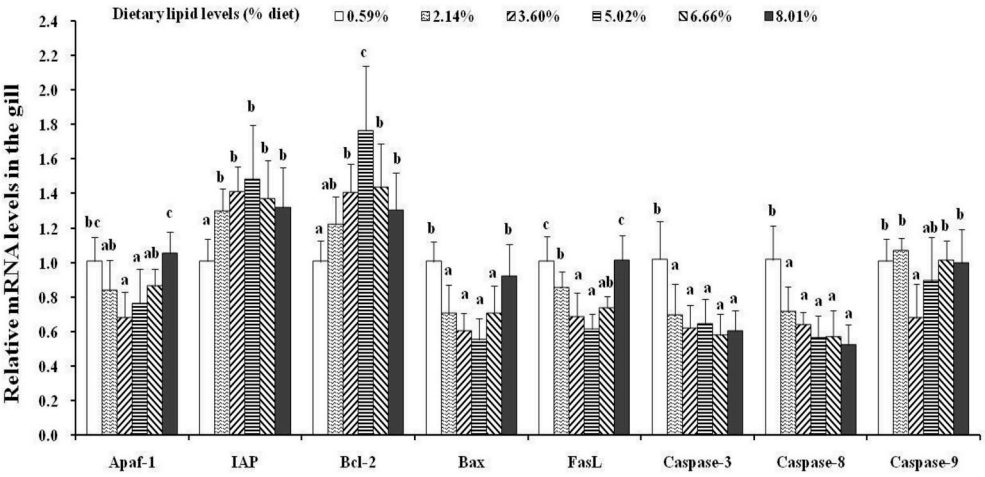

Fig. 10. Relative expression of Apaf-1, IAP, FasL, Caspase 3, Caspase 8, Caspase 9, FasL, Bcl-2, Bax in the gill of young grass carp fed diets containing graded levels of lipids[1].
[1]Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different ($P < 0.05$). FasL: Fas ligand; Bcl-2: B-cell lymphoma-2; Bax: B-cell lymphoma protein 2 associated X protein; Apaf-1: apoptotic protease activating factor-1; IAP: inhibitor of apoptosis protein.

4.2.2. Low or excess levels of dietary lipids induce inflammation in young grass carp gill

Inflammatory cytokines modulate immune function in fish under pathogenic bacterial infection [45], which consist of both pro-inflammatory cytokines (such as IL-1β, IL-12 and TNF-α) and anti-inflammatory cytokines (such as TGF-β1 and IL-10) [46,47]. In our study, low or excess levels of lipids up-regulate pro-inflammatory cytokines IL-1β, IL-12 p35, IL-12 p40 and TNF-α mRNA levels as well as down-regulate anti-inflammatory cytokines IL-4(13)A, IL-4(13)B, IL-6, IL-10 and TGF-β1 mRNA levels in the gill of young grass carp. These results show that low or excess levels of lipids may advance gill rot by up-regulating pro-inflammatory cytokines and down-regulating anti-inflammatory cytokines, whereas optimal lipids levels (3.60%–5.02% lipids diets) can inhibit inflammation in the gill of fish.

However, dietary lipids do not change the mRNA levels of IL-8 in fish gill. The reason may be partly due to the suppression of Na^+/H^+ exchangers(NHEs)to IL-8. In our research, low or excess levels of lipids up-regulate the mRNA levels of TNF-α. In mice, the up-regulation of TNF-α mRNA levels activated NHEs protein [48]. It was indicated that NHEs may suppress the gene expression IL-8 in human colon [49]. Data above indicate that low or excess levels of lipids may through up-regulating mRNA levels of TNF-α to suppress IL-8 and may explain the unchanged IL-8 mRNA level in fish gill. However, this hypothesis needs further investigation.

NF-κB and TOR played the central role in the modulation of inflammation by regulating cytokine [50,51]. Furthermore, it was reported that the NF-κB activation depended on IκB kinase (including IKKα, IKKβ and IKKγ three subunits) activation and led to degradation of the inhibitory protein κBα (IκBα), which functions by inhibiting nuclear translocation of NF-κB in human cells [52]. In this study, low or excess levels of lipids up-regulate NF-κB p65, IKKα and IKKγ mRNA levels and down-regulate IκBα mRNA levels, whereas supplementation with optimal lipids (3.60%–6.66% lipids diets) reverse this result in the gill of young grass carp. Correlation analyses (Table 4) indicate that gene expression of pro-inflammatory cytokines TNF-α, IL-12 p40 are positively related to NF-κB p65 mRNA level, IκBα mRNA level is negatively correlated with IKKα and IKKγ mRNA levels and the mRNA level of NF-κB p65 is negatively correlated with the mRNA level of IκBα in the gill of young grass carp. Data suggest that low or excess levels of lipids may up-regulate the expression of IKK (IKKα and IKKγ), and then inhibiting of IκBα to activating NF-κB p65 to up-regulate the gene expression of pro-inflammatory cytokines in the gill of fish. However, dietary lipids have no effect on the gene expression of the NF-κB p52. Neurath and Pettersson [53] reported that there were canonical and non-canonical activation of NF-κB related to p65 and p52 in mammal, respectively. This result indicate that lipids may regulate the inflammatory response only through a canonical (NF-κB p65), rather than non-canonical (NF-κB p52) pathway in the fish gill. Additionally, Weichhart et al. [54] reported that inhibiting TOR suppressed the anti-inflammatory cytokine production in human cells. Moreover, TOR could activated S6K1 and inhibit 4EBP to initiate the mRNA translation of cytokines [55,56]. In our study, compared with optimal lipids supplementation (3.60%, 5.02%, 3.60% and 5.02% lipids diets), low or excess levels of lipids down-regulate TOR and S6K1 mRNA levels and up-regulate 4EBP 1 and 2 mRNA levels in the gill of young grass carp. Correlation analyses (Table 4) show that IL-4(13)A, IL-4(13)B, IL-10,

Table 4
Parameters correlation coefficient in the gill.

Independent parameters	Dependent parameters	Gill	
		Correlation coefficients	*P*
NF-κB P65	IL-1β	–	–
	IL-8	–	–
	TNF-α	+0.816	$P < 0.05$
	IL-12p40	+0.780	$P = 0.067$
IκBα	NF-κB p65	−0.848	$P < 0.05$
IKKα	IκBα	−0.952	$P < 0.05$
IKKγ	IκBα	−0.788	$P = 0.063$
TOR	IL-6	+0.757	$P = 0.082$
	IL-4/13A	+0.929	$P < 0.01$
	IL-4/13B	+0.946	$P < 0.01$
	TGF-β1	+0.88,290	$P < 0.05$
MLCK	occludin	–	–
	Claudin 3	−0.826	$P < 0.05$
	ZO-2b	−0.799	$P = 0.057$
	ZO-1	−0.768	$P = 0.04$
	Claudin b	−0.863	$P < 0.05$
	claudin 12	−0.923	$P < 0.01$
	claudin 15a	−0.769	$P = 0.074$
p38 MAPK	MLCK	+0.924	$P < 0.01$
MAPKK 6	p38 MAPK	+0.929	$P < 0.01$
Nrf2	CuZnSOD	+0.820	$P < 0.05$
	MnSOD	+0.969	$P < 0.01$
	CAT	+0.911	$P < 0.05$
	GPx1a	+0.713	$P = 0.094$
	GPx1b	–	–
	GSTR	+0.776	$P = 0.070$
	GSTO-1	+0.869	$P < 0.05$
	GST O-2	+0.923	$P < 0.01$
	GR	+0.916	$P < 0.05$
Keap1a	Nrf2	−0.974	$P < 0.01$

Table 5
Regression of immune and antioxidant related parameters in the gill of young grass carp after challenge with *F. columnare*.

Regression	R^2	*P*
$Y_{LA\ in\ Gill} = 1189x + 133.2, Y_{Max} = 176.0040$	0.7930	= 0.30
$Y_{C3in\ Gill} = -2914x^2 + 350.3x + 7.749$	0.8097	< 0.01
$Y_{C4\ in\ Gill} = -129.8x^2 + 9.294x + 1.360$	0.7380	< 0.05
$Y_{MDA\ in\ Gill} = 647.4x^2 - 59.24x + 3.890$	0.9163	< 0.01
$Y_{ROS\ in\ Gill} = -875.1x + 101.1; Y_{Min} = 55.5948$	0.9273	< 0.05
$Y_{pc\ in\ Gill} = 493.9x^2 - 38.50x + 3.396$	0.6596	< 0.01
$Y_{GPx\ in\ Gill} = 1.039x + 57.57; Y_{Max} = 109.7278$	0.9650	< 0.05
$Y_{MnSOD\ in\ Gill} = -475.6x^2 + 47.36x + 2.059$	0.5940	< 0.01
$Y_{CAT\ in\ Gill} = -3.802x + 1.396; Y_{Min} = 1.1983$	0.6360	= 0.202
$Y_{GR\ in\ Gill} = -3477x^2 + 255.3x + 11.21$	0.8820	< 0.01
$Y_{GSH\ in\ Gill} = -345.1x^2 + 26.26x + 1.396$	0.7820	< 0.01

TGF-β1 and S6K1 mRNA levels are positively related to TOR mRNA level, whereas 4EBP 1 and 4EBP 2 mRNA levels are negatively related to TOR mRNA level in the gill of young grass carp, suggesting that low or excess levels of lipids down-regulated anti-inflammatory cytokines may partially ascribe to the down-regulation of TOR and S6K1 mRNA levels and the up-regulation mRNA levels of 4EBP 1 and 4EBP 2 in fish gill.

In addition, dietary lipids also do not alter the mRNA levels of IKKβ in fish gill, with this phenomenon possibly related to keap1. In our research, low or excess levels of lipids up-regulate the mRNA levels of keap1 in fish gill. In rat liver, the important function of keap1 is confirmed as IKK upstream signaling molecules and can inhibit its transcription [57]. Thus, we speculate that the unaltered mRNA levels of IKKβ in fish gill may partly due to keap1, which needs to be further investigated.

4.3. Low or excess levels of lipids impair the TJ complexes in the young grass carp gill

Disruption of tight junction is an important cause of gill disease in fish [58]. In the current study, compared to optimal lipids levels (3.60%–6.66% lipids diets), low or excess levels of lipids down-regulate the mRNA levels of ZO-1, Claudin b, Claudin 3, Claudin 12, Claudin 15a, Claudin 15b, Claudin 7b and Occludin in the gill of young grass carp. These results indicate that low or excess levels of lipids may impair the fish gill structure integrity through down-regulating the mRNA levels of TJ proteins, whereas optimal lipids (3.60%–6.66% lipids diets) can prevent this damage in fish. However, dietary lipids do not alter the mRNA levels of ZO-2b in the gill. Our finding may be partly explained by the different function of ZO-1 and ZO-2b in the gill of fish. In mouse, compared to ZO-1, ZO-2 played a secondary role in tight junction formation. Study indicated that inflammatory disease could enhance mRNA levels of ZO-1 rather than ZO-2 [59]. Thus, data above indicate that in inflammatory process, lipids may regulate ZO-1 rather than ZO-2 to maintain fish gill structure integrity, which requires further study.

Myosin light chain kinase (MLCK) was one important regulatory element of TJ complexes, up-regulation of MLCK expression redistributed the TJ proteins and induced inflammatory disease in human [60]. In this study, compared with optimal lipids supplementation (3.60% lipids diet), low and excess levels of lipids up-regulate the MLCK mRNA level in the gill of young grass carp. Correlation analysis (Table 4) show that the mRNA levels of Claudn 3, Claudin 12, ZO-1, ZO-2b, Claudn b, and Claudin15a in the gill of young grass carp are negatively correlated with MLCK mRNA levels. This result implies that low or excess levels of lipids down-regulating these TJ complexes mRNA levels may be partly related to the increased MLCK mRNA level in the gill of fish. In addition, MLCK expression was regulated by p38 mitogen-activated protein kinase (p38 MAPK) which was also modulated by its upstream molecules mitogen-activated protein kinase kinase (MAPKK) in terrestrial animal [61]. In our research, compared with optimal lipids supplementation (5.02% and 3.60% lipids diets), low or excess levels of lipids up-regulate p38 MAPK and MAPKK 6 mRNA levels. Further correlation analysis (Table 4) indicate that the mRNA level of MLCK is positively related to p38 MAPK mRNA levels which is also positively related to MAPKK 6 in the gill of young grass carp, suggesting that low or excess levels of lipids-induced the up-regulation of MLCK mRNA expression is due in part to the enhancement of p38 MAPK and MAPKK 6 gene expression in fish gill.

4.4. Low or excess levels of lipids decrease the gill antioxidant ability of young grass carp

4.4.1. Low or excess levels of lipids increase ROS, PC and MDA contents, and decease antioxidant ability in the gill of young grass carp

Fish gill enriched polyunsaturated fatty acids (PUFA) which were highly sensitive to ROS and more tended to oxidant damage than mammal [62,63]. Therefore, antioxidant system is crucial for fish gill to combat oxidative damage. In our experiment, compared with optimal lipids supplementation (2.14%–8.01% lipids diets), low or excess levels of lipids increase ROS, PC and MDA contents in the gill, implying that low or excess levels of lipids aggravate oxidative damage, whereas optimal lipids prevent this damage in the gill of young grass carp. The present study also show that low or excess levels of lipids decrease the MnSOD, CAT, GPx, GST and GR activities as well as GSH content in the gill of young grass carp, indicating that low or excess levels of lipids decrease antioxidant ability in fish gill. (Table 5).

4.4.2. Low or excess levels of dietary lipids down-regulate mRNA levels of antioxidant enzymes and Nrf2 and up-regulated Keap1 mRNA levels in the gill of young grass carp

In the present study, compared to optimal lipids (3.60%–5.02% lipids diets), low or excess levels of lipids down-regulate CuZnSOD, MnSOD, CAT, GSTR, GSTP-1, GSTP-2, GPx1a, GPx1b, GPx4a, GPx4b and GR mRNA levels in fish gill, indicating that low or excess levels of lipids may decrease antioxidant enzyme activity partly by down-regulating their mRNA expression in the gill of fish.

In addition, down-regulation of Keap1 gene expression could activate Nrf2, thereby up-regulating antioxidant enzyme gene expression in rat [64]. Our current study show that compared with optimal lipids supplementation (5.02% lipids diet), low or excess levels of lipids down-regulate Nrf2 mRNA levels and up-regulate the mRNA levels of Keap1 in the gill of fish. Correlation analyses (Table 4) show that the mRNA levels of CuZnSOD, MnSOD, GPx1a, CAT, GSTR, GSTO-1, GSTO-2 and GR in the gill are positively related to the Nrf2 mRNA level, whereas Nrf2 mRNA level is negatively correlated with Keap1a (not Keap1b) mRNA level in the gill of young grass carp. This result demonstrate that low or excess levels of lipids down-regulate antioxidant enzymes mRNA levels partly through inhibiting Keap1/Nrf2 signaling molecules in fish gill.

In addition, previous studies in our lab indicated that oxidative damage could induce apoptosis in the gill of young grass carp [17]. Hence, we further examine the effects of lipids on apoptosis in the gill of young grass carp.

4.5. Low or excess levels of lipids induced apoptosis in the gill of young grass carp

Gill cells apoptosis could impair its structure integrity in grass carp [17]. Research has indicated that Caspase-3 as the major effectors to execute apoptosis process, which was mediated by mitochondrial pathway (Bax/Apaf-1/Caspase-9) and death receptor pathway (FasL/Caspase-8) [65–67]. Meanwhile, the B-cell lymphoma-2 (Bcl-2) and the inhibitor of apoptosis proteins (IAP) were important class of regulatory proteins, which inhibited apoptosis at the mitochondrial level [68,69]. In this study, compared with optimal dietary lipids levels (3.60%–5.02% lipids diets), low or excess levels of lipids up-regulate the Bax, Apaf-1, FasL, Caspase 3, Caspase 8 and Caspase 9 mRNA levels as well as down-regulate Bcl-2 and IAP mRNA levels in the gill of young grass carp, suggesting that low or excess levels of lipids can increase apoptosis, whereas supplementation with optimal lipids can reverse it in the gill of young grass carp.

Data above show that fish gill apoptosis maybe partly ascribe to the oxidative damage and inflammatory process which is induced by low or excess levels of lipids diets. Former studies in our lab indicate that oxidative damage and inflammation which is regulated by NF-κB and TOR signaling molecules caused apoptosis in fish gill [17,33]. Meanwhile, TNF-α was shown to lead the apoptosis by binding its receptors to activate caspase 8 in human cells [70,71]. In our current study, low or excess levels of lipids diets enhance ROS, MDA and PC contents, inhibit antioxidant enzymes activities through Keap1/Nrf2 signaling molecules and aggravate inflammation by down-regulate the mRNA levels of TOR and up-regulate the mRNA levels of NF-κB and TNF-α, thereby promoting apoptosis in fish gill. However, diet supplementation with optimal level of lipids could prevent this damage (5.02% lipids diet).

5. Conclusion

In summary, the current study indicated that low or excess levels of lipids increased the gill rot morbidity through impairing the immune function and structural integrity, and in turn, revealing the underlying mechanisms of young grass carp. We found that compared with optimal lipids levels (2.14%–8.01% lipids diets), low or excess levels of lipids impaired fish gill immune function through declining the activities of humoral compounds, down-regulating the mRNA levels of IL-4(13)A, IL-4(13)B, IL-6, IL-10, TGF-β1, IκBα and S6K1, and up-regulating the mRNA levels of TNF-α, IFN-γ2, IL-1β, IL-12 p40, IL-12 p35, NF-κB p65 (not p52), IKKα (not IKKβ), IKKγ and 4EBP. In addition, low or excess levels of lipids decreased young grass carp physical barrier function through down-regulating the mRNA levels of ZO-1 (ranther than ZO-2b), Claudin b, c, 3, 12, 15a, 15b, 7b, 7a and Occludin through MAPKK 6/p38 MAPK/MLCK signaling molecules, decreasing antioxidant ability via Keap1/Nrf2 signaling molecules in the gill, and down-regulating the mRNA levels of Bcl-2 and IAP and up-regulating the mRNA levels of Apaf-1, Caspase-3, -8 and -9 and FasL in the gill of young grass carp. Based on the quadratic regression analysis for the gill rot morbidity, C3 and MDA contents, the dietary lipids requirements for young grass carp have been estimated to be 5.60%, 6.01% and 4.58% diets.

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