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Dietary alpha-linolenic acid/linoleic acid ratios modulate intestinal immunity, tight junctions, anti-oxidant status and mRNA levels of NF-κB p65, MLCK and Nrf2 in juvenile grass carp (*Ctenopharyngodon idella*)

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This study was conducted to investigate the effects of dietary alpha-linolenic acid/linoleic acid (ALA/LNA) ratios on the immune response, tight junctions, antioxidant status and immune-related signaling molecules mRNA levels in the intestine of juvenile grass carp (*Ctenopharyngodon idella*). A total of 1260 juvenile grass carp with an average initial weight of 8.78 ± 0.03 g were fed diets with different ALA/LNA ratios (0.01, 0.34, 0.68, 1.03, 1.41, 1.76 and 2.15) for 60 days. Results indicated that ALA/LNA ratio of 1.03 significantly increased acid phosphatase, lysozyme activities and complement C3 contents, promoted interleukin 10, transforming growth factor β1 and κB inhibitor α mRNA abundance, whereas suppressed pro-inflammatory cytokines (interleukin 1β, interleukin 8, tumor necrosis factor α and interferon γ2) and signal molecules (IκB kinase β, IκB kinase γ and nuclear factor κB p65) mRNA levels in the intestine ($P < 0.05$), suggesting that optimal dietary ALA/LNA ratio improved intestinal immune response of juvenile fish. Additionally, ALA/LNA ratio of 1.03 significantly promoted Claudin-3, Claudin-b, Claudin-c, Occludin and ZO-1 gene transcription, whereas reduced Claudin-15a and myosin light-chain kinase mRNA levels in the intestine, suggesting that appropriate dietary ALA/LNA ratio strengthened tight junctions in the intestine of juvenile fish. Meanwhile, ALA/LNA ratio of 1.03 noticeably elevated glutathione contents, copper/zinc superoxide dismutase, glutathione peroxidase, glutathione S-transferase and glutathione reductase activities and mRNA levels, as well as signaling molecule nuclear factor erythoid 2-related factor 2 gene transcriptional abundance in the intestine, suggesting that proper ratio of dietary ALA/LNA ameliorate the intestinal antioxidant status of juvenile fish. Based on the quadratic regression analysis of the complement C3 content in the distal intestine and malondialdehyde content in the whole intestine, optimal ALA/LNA ratio for maximum growth of juvenile grass carp (8.78–72.00 g) were estimated to be 1.13 and 1.12, respectively.

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1. Introduction

Alpha-linolenic acid (ALA, 18:3n-3) and linoleic acid (LNA, 18:2n-6) are considered as the essential fatty acids (EFA) in fish [1]. Our previous study indicated that optimal level of ALA and LNA could improve the growth performance, digestion and absorption capacities of juvenile grass carp (*Ctenopharyngodon idella*) [2].

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Study in Atlantic salmon implied that the digestion and absorption capacities partly rely on the intestinal health [3]. The intestinal health was constituted by immunity, integrity and functionality [4], which was associated with intestinal immune response [5] and physical barrier [6] in fish. However, no systematic investigation reported regarding the effect of dietary ALA and LNA on the intestinal immunity in fish. Study in Arctic charr *Salvelinus alpinus* L. indicated that the limitation of ALA or LNA disturbed the balance of intestinal microflora [7]. Meanwhile, intestinal microflora was closely related to the intestinal mucosal immune in the cecum of broiler chickens [8]. These data implies that dietary ALA and LNA may affect intestinal mucosal immunity of juvenile fish, which is worthy of further investigation.

In common carp, the intestinal mucosal immune barrier partly depends on immune response [9], which is related to the antibacterial compounds [10] and cytokines [11,12]. Cytokines have been shown to be transcriptionally regulated by nuclear factor κB (NF-κB) in the human intestinal epithelial cells [13]. Nevertheless, little information is available regarding on the effects of dietary ALA and LNA on cytokine production via NF-κB signaling pathway in the intestine of fish. In large yellow croaker, optimal dietary ALA/LNA ratio could stimulate peroxisome proliferative activated receptor α (PPARα) expression in the liver and kidney [14]. Mishra [15] found that PPARα could inhibit NF-κB activation in human microvessel endothelial cells. These data indicates that dietary ALA and LNA may affect cytokine production via NF-κB signaling pathway in the intestine of fish, which needs additional investigation.

Fish intestinal health also relies on intestinal physical barrier, which is associated with the tight junction (TJ) complex, such as Occludin, Claudins and zonula occludens 1 (ZO-1) [16]. Study in weaning piglets demonstrated that the enhancement of Occludin and ZO-1 mRNA abundance could improve intestinal barrier function [17]. Gu et al. [18] suggested that TJ protein expression could be modulated by myosin light-chain kinase (MLCK) in the intestinal epithelial of mice. Nevertheless, no information is reported regarding the relationship between MLCK and dietary ALA and LNA in the intestine of fish. It was reported that dietary ALA and LNA could elevate the melatonin activity in the hepatoma of rat [19]. Melatonin reduced the expression of myosin light chain kinase (MLCK) in the intestinal endothelial cell of rabbit [20]. These data implies that dietary ALA and LNA may affect TJ mRNA levels via a MLCK-dependent way in the intestine of fish, which warrants further investigation.

Apart from the TJ proteins, epithelial cells also play important roles in the intestinal physical barrier of fish [21]. Morais et al. [22] reported that fish intestine was prone to be oxidative damaged by ROS, which could disrupt the epithelial structural integrity. To counteract the potential damage of ROS, fish intestine have developed antioxidant system including non-enzymatic compounds and antioxidant enzymes [23]. Study in juvenile Jian carp (*Cyprinus carpio* var. Jian) implied that the antioxidant enzyme activities were partly associated with antioxidant enzyme gene transcriptions, which were regulated by the nuclear factor erythoid 2-related factor 2 (Nrf2) in the intestine [24]. However, little information is available regarding the effects of dietary ALA and LNA on the intestinal antioxidant enzyme activities through modulating their gene transcriptions link to Nrf2 in animal. Yu et al. [25] reported that dietary ALA could promote superoxide dismutase (SOD) and glutathione peroxidase (GPx) gene expression through enhancing Nrf2 mRNA levels in the cytoplasm of rat. Bergamo et al. [26] demonstrated that LNA isomeride, conjugated linoleic acid (CLA), could facilitate Nrf2 gene expression, thereby elevate GST and γ-GCL mRNA levels in the liver of mouse. Above data indicates that dietary ALA and LNA may regulate antioxidant enzyme activities partly through modulating their mRNA levels, which may be related to Nrf2 signaling pathway in the intestine of fish. This possibility is worthy of investigation.

This study was a part of a larger study that involved in the determination of the effect of dietary ALA/LNA ratios on the digestive and absorptive ability in fish using the same growth trial as the previous study [2]. The objective of this study was to further investigate the impacts of dietary ALA and LNA on the intestinal immune response, tight junctions and antioxidant status of juvenile grass carp. Additionally, we further investigated the mRNA levels of cytokines, TJ proteins, antioxidant enzymes and signal molecules, including NF-κB, MLCK and Nrf2 in the intestine of fish. The resultant findings could provide partial theoretical evidence for the effect of dietary ALA and LNA on the intestinal health status of fish.

2. Materials and methods

2.1. Experimental diets and design

Experimental diets and the procedures for diet preparation and storage (−20 °C) were the same as our previous study [2]. The formulation of the experimental diets is shown in Table 1. Seven iso-nitrogenous and iso-lipidic experimental diets varying only in the dietary lipid source were formulated. Meanwhile, the experiment diets contained 5% of lipid and 35% of protein, respectively according to Ji et al. [27] and NRC [28]. Fish meal, casein and gelatin were used as the protein sources. According to Li et al. [29], three different lipid sources (linseed oil, safflower oil and coconut oil) were utilized to formulate the experiment diet containing varying ratios of ALA/LNA (0.00, 0.35, 0.70, 1.05, 1.40, 1.75 and 2.10), with a constant total C_{18} PUFA (ALA + LNA) content. Ethoxyquin was added as the antioxidant. According to the method described by Otsuka et al. [30], final ratios of dietary ALA/LNA of the seven experimental diets were measured to be 0.01, 0.34, 0.68, 1.03, 1.41, 1.76 and 2.15.

Table 1
Diet formulation and composition.[a]

Ingredients	g kg^{-1}
Fish meal	30.0
Casein	280.0
Gelatin	75.0
DL-Methionine (99%)	1.4
Vegetable oil premix[b]	50.0
Alpha-starch	240.0
Corn starch	215.5
Vitamin premix[c]	10.0
Mineral premix[d]	20.0
$Ca(H_2PO_4)_2$ (220 g kg^{-1})	22.6
Choline chloride (600 g kg^{-1})	5.0
Cellulose	50.0
Ethoxyquin (300 g kg^{-1})	0.5

[a] Crude protein and total lipids were measured to be 347.4 g kg^{-1} and 46.9 g kg^{-1}.
[b] Linseed oil and safflower oil were added to achieve different ALA/LNA ratios (0.00, 0.35, 0.70, 1.05, 1.40, 1.75 and 2.10). Each mixture was made isolipidic with the addition of coconut oil.
[c] Per kilogram of vitamin premix (g kg^{-1}): retinyl acetate (500 000 IU g^{-1}), 2.40 g; cholecalciferol (500 000 IU g^{-1}), 0.40 g; D, L-α-tocopherol acetate (500 g kg^{-1}), 12.55 g; menadione (230 g kg^{-1}), 0.80 g; cyanocobalamin (10 g kg^{-1}), 0.83 g; D-biotin (20 g kg^{-1}), 4.91 g; folic acid (960 g kg^{-1}), 0.40 g; thiamin nitrate (980 g kg^{-1}), 0.05 g; ascorhyl acetate (930 g kg^{-1}), 7.16 g; niacin (990 g kg^{-1}), 2.24 g; meso-inositol (990 g kg^{-1}), 19.39 g; calcium-D-pantothenate (980 g kg^{-1}) 2.89 g; riboflavine (800 g kg^{-1}), 0.55 g; pyridoxine hydrochloride (980 g kg^{-1}), 0.59 g. All ingredients were diluted with corn starch to 1 kg.
[d] Per kilogram of mineral premix (g kg^{-1}): $FeSO_4 \cdot H_2O$, 23.110 g; $CuSO_4 \cdot 5H_2O$, 0.010 g; $ZnSO_4 \cdot H_2O$, 0.620 g; $MnSO_4 \cdot H_2O$, 1.640 g; KI, 0.070 g; $NaSeO_3$, 0.005 g; $MgSO_4 \cdot H_2O$, 60.530 g. All ingredients were diluted with corn starch to 1 kg.

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