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Dietary leucine regulates the intestinal immune status, immune-related signalling molecules and tight junction transcript abundance in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

The role of leucine (Leu) in the regulation of the intestinal immune status, immune-related signalling molecules and tight junction (TJ) protein transcript abundance in the intestine of grass carp (*Ctenopharyngodon idella*) was investigated. Six iso-nitrogenous diets that contained graded levels of Leu (7.1–17.1 g Leu kg^{-1} diets) were fed to the fish for 8 weeks. Compared with the control group, appropriate Leu supplementation increased ($P < 0.05$) the following: (1) the lysozyme activity, acid phosphatase activity and complement component 3 (C3) content in all intestinal segments; (2) the mRNA levels of interleukin 10, inhibitor factor κBα (IκBα) and target of rapamycin (TOR) in the mid and distal intestine as well as transforming growth factor β1 in all intestinal segments; and (3) the transcript levels for claudin b, claudin 3, claudin 15, occludin and zonula occludens-1 (ZO-1) in the intestine of young grass carp. At the same time, appropriate Leu supplementation down-regulated the mRNA levels of tumour necrosis factor α, interleukin 8 and nuclear factor κB p65 (NF-κB p65) in the mid and distal intestine of young grass carp ($P < 0.05$). Interestingly, the transcript levels for claudin c and claudin 12 showed no significant differences among the groups in the intestine of young grass carp. In conclusion, the positive effect of Leu on intestinal health is associated with the improvement of the intestinal immune status and the regulation of immune-related signalling molecules and tight junction transcripts of fish.

1. Introduction

Leucine (Leu) is an essential dietary nutrient for the normal growth of fish (National Research Council, 2011). Our previous study indicated that dietary Leu can improve the growth performance of grass carp (*Ctenopharyngodon idella*) (Deng et al., 2014). Fish growth is associated with intestinal health (Zhang et al., 2013a,b). However, the intestine is known to be an important site that is often targeted by invading antigens that are derived from ingested food, water, commensal flora and pathogens (Ringø et al., 2007). Therefore, it is of utmost importance to maintain the intestinal health of fish.

The intestinal immune response plays a pivotal role in maintaining intestinal health in fish (Wen et al., 2014). Inflammation is considered to be an important part of the immune response and is primarily mediated by cytokines (Secombes et al., 2001). In fish, tumour necrosis factor-α (TNF-α) and interleukin 8 (IL-8) are crucial pro-inflammatory cytokines (Secombes et al., 2001; Stone et al., 2008), while transforming growth factor β (TGF-β) and interleukin 10 (IL-10) are indicated to be anti-inflammatory cytokines (Rymuszka and Adaszek, 2012). Pro-inflammatory cytokines have been shown to be transcriptionally regulated by nuclear factor κB (NF-κB) and its inhibitor IκB (Tak and Firestein, 2001). However, there is no information regarding the relationship between Leu and NF-κB in fish. In rats, Leu supplementation has been shown to increase the concentration of adiponectin in serum (Torres-Leal et al., 2011). Ouchi et al. (2000) found that adiponectin could inhibit endothelial NF-κB signalling in human aortic endothelial cells (HAECs). Accordingly, there could be a close relationship between Leu and NF-κB, and NF-κB could play a crucial role in modulating the pro-inflammatory cytokines that are regulated by Leu in fish. In addition,

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NF-κB is regulated by the mammalian target of rapamycin (mTOR) in human peripheral blood mononuclear cells (Weichhart et al., 2008). Accumulated evidence has pointed to Leu being an activator of mTOR in rainbow trout hepatocytes (Lansard et al., 2011), shrimp *Fenneropenaeus chinensis* (Sun et al., 2015) and skeletal muscles of postabsorptive rats (Anthony et al., 2000). Nevertheless, studies that refer to the effects of Leu on intestinal TOR are scarce in fish.

The health of the intestine is also associated with its structural integrity in fish (Wen et al., 2014). The intestinal antioxidant capacity and tight junction complexes are critical in maintaining the structural integrity of the intestine (Ineu et al., 2013; Liu et al., 2009). Our previous study with grass carp showed that dietary Leu decreased oxidative damage and improved the antioxidant capacity in the intestine of young grass carp (Deng et al., 2014). However, no information has been reported about the effect of Leu on tight junction (TJ) complex proteins in fish. The TJ complex consists of several transmembrane proteins, such as occludin and claudins, and intracellular molecules, such as zonula occludens-1 (ZO-1) (Piche et al., 2009). In Caco-2 cells of humans, Leu reversed the decrease in transepithelial electrical resistance (TEER), which can assess the barrier function (Beutheu et al., 2013). Additionally, in the human forearm, Leu infusion increased glutamine output (Abumrad et al., 1982). Beutheu et al. (2013) indicated that glutamine limited the decrease of ZO-1 and occludin expression in Caco-2 cells. Recently, the cDNAs of ZO-1 (GenBank accession number: KJ000055) and claudin-12 (GenBank accession number: KF998571) of grass carp have been cloned for the first time in our laboratory. These data suggest that Leu could regulate the intestinal TJ proteins in fish.

This study used the same growth trial as the previous study (Deng et al., 2014). Here, we aim to explore the effect of dietary Leu on the intestinal structure, immunological functions and related signalling factors, which could provide a systemic understanding of Leu with respect to intestinal health in fish.

2. Materials and methods

2.1. Experimental diet and procedures

Experimental diets and the procedures for diet preparation and storage (−20 °C) were the same as previously reported by Deng et al. (2014). As presented in Table 1, casein, fish meal and gelatin were used as the main protein sources and fish oil and soybean oil were used as the main lipid sources. The Leu concentration in the basal diet was 7.4 g Leu kg^{-1} diet, which was calculated according to the analysed Leu concentration in the ingredients. Six experimental diets were obtained by supplementing the basal diet with a 0 (un-supplemented control, deficient group), 2, 4, 6, 8 and 10 g Leu kg^{-1} diet, while adjusting glycine to maintain the diets as isonitrogenous according to the method described by Cheng et al. (2011). The final Leu content in the six experimental diets was analysed to be 7.1, 8.9, 11.0, 13.3, 15.2 and 17.1 g Leu kg^{-1}, as described by Zhou et al. (2007), using high-pressure liquid chromatography. The approximate compositions of the feed were analysed according to the standard methods of the Association of Official Analytical Chemists (AOAC (1995)) and Le and Fotedar (2014): briefly, the moisture was determined by drying the samples to a constant weight at 105 °C and the crude protein content (N × 6.25) was determined by the Kjeldahl method after an acid digestion was performed; the crude lipid was obtained by the ether-extraction method using the Soxhlet method; and the ash content was determined by a muffle furnace at 550 °C for 6 h.

Feeding management followed the Animal Care and Use Committee of Sichuan Agricultural University. Grass carp were obtained from a commercial farm (Sichuan, China). Before starting the experiment, the fish were acclimated to the experimental conditions for 2 weeks. At the end of the acclimation period, 540 uniform-sized fish (initial average weight of 295.85 ± 2.07 g) were randomly distributed into 18 experimental cages (L1.4 m × W1.4 m × H1.4 m with volume of 2.74 m^3), each containing 30 fish. The fish were fed with their respective diets to excess satiation four times daily at 07:00, 11:00, 15:00, and 19:00 h for 8 weeks. Uneaten feed was collected after feeding for 30 min. The experiment was completed under natural light (a diurnal:dark cycle of approximately 14 h:10 h). The water temperature and pH were 26 ± 2 °C and 7.0 ± 0.5, respectively. The dissolved oxygen was at least 6 mg L^{-1}.

Table 1
Composition and nutrient content of the basal diet.

Ingredients	g kg^{-1} diet	Nutrient content[a]	g kg^{-1} diet
Fish meal	78.0	Crude protein	306.9
Casein	30.0	Crude lipid	47.7
Gelatin	39.9	n − 3	10.0
Amino acid mix[b]	203.3	n − 6	10.0
Leucine premix[c]	50.0	Available phosphorus	6.0
α-starch	280.0		
Corn starch	118.7		
Fish oil	22.0		
Soybean oil	18.9		
Vitamin premix[d]	10.0		
Mineral premix[e]	20.0		
$Ca(H_2PO_4)_2$	22.7		
Choline chloride (50%)	6.0		
Cellulose	100.0		
Ethoxyquin (30%)	0.5		

[a] Crude protein, crude fat and crude ash were measured values. Available phosphorus, n − 3 and n − 6 contents were calculated values.

[b] Amino acid mix (g kg^{-1}): lysine, 17.132; methionine, 7.778; tryptophan, 3.571; threonine, 11.878; arginine, 12.893; histidine, 7.959; isoleucine, 12.690; phenylalanine, 13.604; valine, 15.330; cysteine, 0.909; tyrosine, 10.863; glutamic acid, 32.323; glycine, 56.364.

[c] Leucine premix (g kg^{-1}) was added to obtain a graded level of crystalline leucine, and the amount of glycine and corn starch was reduced to compensate. Per kilogramme of leucine premix composition from diets 1 to 6 was as follows (g kg^{-1}): L-leucine 0.0, 40.6, 81.2, 121.8, 162.4, and 203.0 g; glycine 115.2, 90.9, 68.7, 44.4, 22.2, and 0.0 g and corn starch 884.8, 868.5, 850.1, 833.7, 815.3, and 797.0 g, respectively.

[d] Per kilogramme of vitamin premix (g kg^{-1}): retinyl acetate (500,000 IU g^{-1}), 0.800 g; cholecalciferol (500,000 IU g^{-1}), 0.480 g; DL-α-tocopherol acetate (500 g kg^{-1}), 20.000 g; menadione (230 g kg^{-1}), 0.220 g; cyanocobalamin (10 g kg^{-1}), 0.100 g; D-biotin (20 g kg^{-1}), 5.000 g; folic acid (960 g kg^{-1}), 0.520 g; thiamine hydrochloride (980 g kg^{-1}), 0.120 g; ascorhyl acetate (930 g kg^{-1}), 7.160 g; niacin (990 g kg^{-1}), 2.580 g; meso-inositol (990 g kg^{-1}), 52.330 g; calcium-D-pantothenate (900 g kg^{-1}), 2.780 g; riboflavine (800 g kg^{-1}), 0.990 g; pyridoxine hydrochloride (980 g kg^{-1}), 0.620 g. All ingredients were diluted with corn starch to 1 kg.

[e] Per kilogramme of mineral mixture (g kg^{-1}): $FeSO_4 \cdot H_2O$ (300 g kg^{-1} Fe), 25.000 g; $CuSO_4 \cdot 5H_2O$ (250 g kg^{-1} Cu), 0.600 g; $ZnSO_4 \cdot H_2O$ (345 g kg^{-1} Zn), 4.350 g; $MnSO_4 \cdot H_2O$ (318 g kg^{-1} Mn), 2.040 g; KI (38 g kg^{-1} I), 1.100 g; $NaSeO_3$ (10 g kg^{-1} Se), 2.500 g; $MgSO_4 \cdot H_2O$ (150 g kg^{-1} Mg), 230.670 g. All ingredients were diluted with corn starch to 1 kg.

2.2. Sampling and tissue preparation

At the end of the feeding trial, the fish were collected from each cage and were anaesthetized with a benzocaine bath and killed by a blow to the head. The proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of 15 fish from each treatment were quickly removed, frozen in liquid nitrogen, and stored at −80 °C until the parameters involved were determined according to the method described by Zuo et al. (2012).

2.3. Intestinal immune parameters assay

The intestine samples were homogenized on ice in 10 volumes (w v^{-1}) of ice-cold physiological saline and centrifuged at 6000 *g* for 20 min at 4 °C, and then, the supernatants were collected to obtain the acid phosphatase activity (ACP), lysozyme activity (LA) and complement component 3 (C3) content assay. The ACP, LA and C3 contents were assayed as described by Zhao et al. (2012b); Ellis (1990) and Wang et al. (1998), respectively.