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Optimal dietary protein level improved growth, disease resistance, intestinal immune and physical barrier function of young grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

This study investigated the effects of dietary proteins on the growth, disease resistance, intestinal immune and physical barrier functions of young grass carp (*Ctenopharyngodon idella*). A total of 540 young grass carp (264.11 ± 0.76 g) were fed six diets containing graded levels of protein (143.1, 176.7, 217.2, 257.5, 292.2 and 322.8 g digestible protein kg^{-1} diet) for 8 weeks. After the growth trial, fish were challenged with *Aeromonas hydrophila* and mortalities were recorded for 14 days. The results indicated that optimal dietary protein levels: increased the production of antibacterial components, up-regulated anti-inflammatory cytokines, inhibitor of κBα, target of rapamycin and ribosomal protein S6 kinases 1 mRNA levels, whereas down-regulated pro-inflammatory cytokines, nuclear factor kappa B (NF-κB) P65, NF-κB P52, c-Rel, IκB kinase β, IκB kinase γ and eIF4E-binding proteins 2 mRNA levels in three intestinal segments of young grass carp ($P < 0.05$), suggesting that optimal dietary protein level could enhance fish intestinal immune barrier function; up-regulated the mRNA levels of tight junction complexes, B-cell lymphoma protein-2, inhibitor of apoptosis proteins, myeloid cell leukemia-1 and NF-E2-related factor 2, and increased the activities and mRNA levels of antioxidant enzymes, whereas down-regulated myosin light chain kinase, cysteinyl aspartic acid-protease 2, 3, 7, 8, 9, fatty acid synthetase ligand, apoptotic protease activating factor-1, Bcl-2 associated X protein, p38 mitogen-activated protein kinase, c-Jun N-terminal protein kinase and Kelch-like-ECH-associated protein 1b mRNA levels, and decreased reactive oxygen species, malondialdehyde and protein carbonyl contents in three intestinal segments of young grass carp ($P < 0.05$), indicating that optimal dietary protein level could improve fish intestinal physical barrier function. Finally, the optimal dietary protein levels for the **growth performance (PWG)** and **against enteritis morbidity** of young grass carp were estimated to be 286.82 g kg^{-1} diet (250.66 g digestible protein kg^{-1} diet) and 292.10 g kg^{-1} diet (255.47 g digestible protein kg^{-1} diet), respectively.

1. Introduction

Fish intestines are continuously exposed to foreign substances, such as microbes, pathogens and other toxic substances from food [1]. To prevent foreign substance invasion, fish have developed intestinal immune barriers and physical barriers [2]. Studies from our laboratory have revealed that impaired intestinal immune barriers and physical barriers could decrease the growth performance of grass carp (*Ctenopharyngodon idellus*) [3]. Therefore,

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enhancing intestinal immune and physical barrier functions are crucial to fish. Previous studies in our laboratory have found that nutrients, such as phospholipids [4] and pantothenic acid [5], could improve the intestinal immune barriers and physical barriers of grass carp. In fish, however, protein is the most important nutrient [6], yet. No study has investigated the effects of dietary proteins on fish intestinal immune barriers and physical barriers. Spears et al. [7] demonstrated that dietary protein supplementation increased manganese (Mn) concentrations in the heart of calves. Studies from our laboratory showed that dietary supplementation with Mn could enhance the intestinal immune barriers and physical barriers of grass carp [8]. These data suggest a possible correlation between dietary proteins and fish intestinal immune barriers and physical barriers, which is worthy of investigation.

In fish, the function of the intestinal immune barriers mainly depends on the immune response, which is closely related to antibacterial compounds and cytokines [2,9]. It has been reported that the production of cytokines could be modulated by nuclear factor kappa B (NF-κB) [10] and target of rapamycin (TOR) [11] in humans. However, no reports at present have investigated the role of dietary proteins on the intestinal immune barriers and its possible mechanisms in fish. In humans, dietary protein supplementation could increase serum insulin concentrations [12]. Ehlers et al. [13] found that increasing plasma insulin levels decreased NF-κB activity in human mononuclear cells. Sancak et al. [14] reported that insulin could activate TOR in HEK293E cells. These observations revealed that there may be a relationship between dietary proteins and the intestinal immune barrier function, as well as the related signalling pathway in fish, which needs to be investigated further.

In addition to the immune barriers, fish physical barriers also play a critical role in maintaining their intestinal structural integrity, which is composed of epithelial cells and intercellular tight junction complexes (TJs) [15]. Zhou et al. [16] showed that the disruption of intestinal epithelial cell was closely related to apoptosis and oxidative damage in rats. Studies revealed that the gene expression of TJs, apoptosis and antioxidant capacity could be regulated by myosin light chain kinase (MLCK) [17], c-Jun N-terminal protein kinase (JNK) [18] and NF-E2-related factor 2 (Nrf2) [19] in humans, respectively. However, no study has addressed the effects of dietary proteins on the intestinal physical barriers and its possible mechanism in fish. In humans, supplementation with protein could increase the plasma ghrelin concentration [20]. Cheng et al. [21] demonstrated that ghrelin inhibited MLCK activity in the rat intestine. In addition, Geraedts et al. [22] indicated that protein intake increased glucagon-like peptide 1 (GLP-1) production in humans. GLP-1 could inhibit apoptosis and reduce JNK activity in human umbilical vein endothelial cells [23]. Wu et al. [24] found that dietary protein supplementation could increase nitric oxide synthesis in the placenta of pigs. Nitric oxide could activate Nrf2 in rat pheochromocytoma cells [25]. These data indicate that there may be a correlation between dietary proteins and the function of the fish intestinal physical barriers associated with TJs, apoptosis and antioxidants as well as their related signalling pathways, which require further investigation.

Grass carp are the third biggest contributor to the world's aquaculture production [26]. The culture of grass carp depends on their formulated feed, which relies on the nutrient requirements of this species [27]. However, the nutrient requirements of fish may vary with different growth stages and different indices. Therefore, it is valuable to determine the optimal dietary protein levels for young grass carp based on different indices.

In the present study, we hypothesize that optimal dietary protein levels may increase the intestinal immune and physical barrier functions to improve the global fish intestinal health status. To test this hypothesis, we were, for the first time, able to investigate the effects of dietary proteins on antibacterial components, cytokines, intercellular TJs, apoptosis and antioxidants, as well as the related signalling molecules in the intestines of young grass carp after challenge with *Aeromonas hydrophila*. Meanwhile, the optimal dietary protein levels for young grass carp based on different indices were also evaluated, which may provide a reference for formulating the commercial feed of grass carp.

2. Materials and methods

2.1. Experimental diet and procedures

The formulation and approximate composition analysis of the diets are shown in Table 1. According to Deng et al. [28], fish meal, casein and gelatin were used as dietary protein sources in a particular ratio at 6:16:3. Crystalline amino acid mixtures were supplemented to simulate the amino acid pattern according to the method described by Wang et al. [29] and Gao et al. [30]. Fish oil and soybean oil were used as dietary lipid sources. Six experimental diets with different protein levels (170.0, 210.0, 250.0, 290.0, 330.0 and 370.0 g kg^{-1} diet) were used. The diets were formulated to be iso-energetic according to the method of Garling et al. [31]. According to Kjeldahl method, protein contents in the experimental diets were measured to be 169.2, 204.7, 244.3, 283.2, 323.3 and 366.3 g kg^{-1} diet. After being prepared completely, the diets were stored at −20 °C as described by Takakuwa et al. [32].

2.2. Growth trial and sample collection

The procedures used in this study were approved by the Animal Care Advisory Committee of Sichuan Agricultural University. Grass carp were obtained from fisheries (Sichuan, China). Before starting the experiment, fish were acclimated to the experimental environment for 4 weeks according to Kpogue et al. [33]. Then, 540 fish (mean weight 264.11 ± 0.76 g) were randomly assigned to 18 experimental cages (1.4 L × 1.4 W × 1.4 H m), resulting in 30 fish per cage as described in our laboratory study [5]. Every cage was equipped with a disc of 100 cm diameter in the bottom to collect the uneaten feed according to our laboratory study [34]. For the feeding trial, fish were fed with their respective diets to apparent satiation four times per day for 8 weeks according to Hossain et al. [35]. Thirty minutes after feeding, uneaten feed was collected, dried and weighed to calculate the feed intake according to our laboratory study [36]. During the experiment, water temperature was averaged at 28 ± 2 °C, and pH value was maintained at 7.0 ± 0.2. The dissolved oxygen not less than 6.0 mg L^{-1} according to our laboratory study [3]. The experimental units were under natural light and dark cycle as described by Wen et al. [37].

After the growth trial, fish from each cage were weighed and counted at the initiation and termination of the feeding trial. 18 fish were randomly selected from each treatment, anaesthetized in a benzocaine bath as described by Geraylou et al. [38]. Then sacrificed, the muscle and liver of fish were quickly removed and frozen in liquid nitrogen, stored at −80 °C according to Veiseth-Kent et al. [39], and for later analysis to the determination of glutamate-oxaloacetate transaminase (GOT) and glutamate-puruvate transaminase (GPT) activities.

2.3. Digestibility trial and sample collection

The digestibility trial was performed as described by Takakuwa et al. [32] with slight modification. Briefly, the same experimental diets from each treatment were used as the growth trial, except Cr_2O_3 was added. Each diet was fed to triplicates (30 fish in each