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Cottonseed protein concentrate (CPC) suppresses immune function in different intestinal segments of hybrid grouper ♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu* via TLR-2/MyD88 signaling pathways

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ABSTRACT

Cottonseed protein concentrate (CPC) has similar amino acid composition compared with fish meal, and has the characteristics of low gossypol and low toxicity. The present study was conducted to investigate the growth performance, antioxidant capacity and different intestinal segments immune responses of hybrid grouper to replacement dietary fish meal ofCPC. Six iso-nitrogenous (50% crude protein) and iso-lipidic (10% crude lipid) diets were formulated: a reference diet (FM) containing 60% fishmeal and five experimental diets (12%, 24%, 36%, 48 and 60%) in which fishmeal protein was substituted at different levels by CPC to feed fish (initial body weight: 11 ± 0.23 g) for 8 weeks. Thena challenge test with injection of *Vibrio parahaemolyticus* was conducted for 7 days until the fish stabilized. The results showed that specific growth rate (SGR) was the highest with 24% replacement level and feed conversion ratio (FCR)was significantly increased when the replacement level reached 48% ($P < 0.05$). The content of malonaldehyde (MDA) in the serum was significantly increased when the replacement level reached 36% ($P < 0.05$). The plica height in the proximal, mid and distal intestine were significantly decreased with the replacement level up to 48% ($P < 0.05$). Hepatic fat deposition wasaggravatedwhen the replacement level reached 36% ($P < 0.05$). The expression of IL-6, TNF-α, and IL-1β mRNAs were significantly up-regulated ($P < 0.05$). The hepcidin mRNA expression was significantly down-regulated ($P < 0.05$). In proximal intestine (PI) and mid intestine (MI), IFN-γ mRNA expression was significantly up-regulated ($P < 0.05$). These results suggested that the CPC decreased hybrid grouper growth performance and inflammation function, and different inflammation function responses in PI,MI, and distal intestine (DI) were mediated partly by the TLR-2/MyD88 signaling pathway. According to the analysis of specific growth rate, the dietary optimum replacement level and maximum replacement level were estimated to be 17% and 34%, respectively.

1. Introduction

The hybrid of brown-marbled grouper and giant grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*) is a new aquaculture fish in Asia following the successful trial production in China. Studies have shown that this hybrid grouper has better growth performance and resistance compared with the parental fish [1]. Therefore, this hybrid grouper has a great potential in the aquaculture industry.

Fishmeal (FM) has been used as a preferred protein source in aquaculture feeds for its high digestibility and palatability [2]. With the rapid growth of aquaculture, demand for aquafeed with less FM has increased because of the cost of this protein source and its limited supply [3]. The typical FM concentration in commercial feeds for grouper is between 480 g/kgand 510 g/kg [4,5]. Consequently, the aquaculture industry has searched for alternative protein sources, such

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as plant proteins, to reduce the dependence on FM and to facilitate the development of a sustainable aquaculture industry [6,7]. However, the use of plant proteinsas a replacement for FM in carnivorous fish still remains questionable due to several factors, such as the presence of anti-nutritional factors [8], low feed availability [9], and imbalanced composition of essential amino acids [10]. Cottonseed protein concentrate (CPC) is one of the potential cottonseed products for partial replacement of FMin fish feeds. CPC (gossypol content is 0.0079 g/kg, tested by SGS, China) is produced by treating the cottonseed flakes to aqueous alcohol extracts in order to reduce the soluble carbohydrate and anti-nutritional factor contents [11]. Thehybrid grouperis a typical carnivorous marine fish, and adding plant proteinto their diet willead to abnormal absorption of nutrients in the intestine [12–14]. The specific components of plant proteinsthat cause intestinal inflammation have not been conclusively identified. Some proposed causes include unidentified antigens that could induce the immune response [15] or cause alterations of intestinal microbial communities [16,17], triggering the inflammation [18].

Previous studies have indicated that theWG, SGR, and SR of grouper (♀*E.fuscoguttatus* × ♂*E.lanceolatu*) decreased as the substitution level increased [1]. Fish growth performance is closely related to intestinal health, which is strongly associated with intestinal immune function [19]. Toll-like receptors (TLRs) expresses several members of novel family of transmembrane receptors which have been demonstrated in various intestinal epithelial cell lines; thus, may play an important role between innate and adaptive mucosal immune response [20]. In mammals, the increase of intestinal opportunistic pathogenic bacteria can affect the activation of inflammatory response by binding to pattern recognition receptors [21]. TLRs are important pattern recognition receptors that activate the intracellular signaling pathway by identifying endogenous ligands, transducing signals into cells and mediating downstream cytokines production [22,23].

In the present study, we proposed a hypothesis that dietary replacement of FMproteins by CPC could impair fish immune function in the liver, serum, and different intestinal segments. To test this hypothesis, we first systemically researched the effects of CPC on fish hepatic fat deposition, serum immunity, inflammatory cytokines, and related signaling molecules in the proximal intestine (PI), middle intestine (MI), and distal intestine (DI). Meanwhile, we determined the dietary optimum replacement level on different indicators, which may provide a practical basis for formulating the most appropriate feed for grouper.

2. Material and methods

2.1. Experimental diets

The cottonseed protein concentrate in this study was supplied by Hunan Xinrui Biological Technology Co. Ltd. (Hunan, China; 67.4% crude protein on dry matter basis). Cottonseed meal was utilized as the substrate for low temperature drying after being milled through 0.45 mm screen, then degreased, dephenolized and desugared under negative pressure and low temperature. The FM was supplied by China National Township Enterprises Corporation (Beijing, China; 73.34% crude protein and 10.90% total lipid on a dry matter basis). Six isocaloric (approximately 50% crude protein) diets with iso-lipidic value (10% total lipid) were formulated to replace 0 (control), 12%, 24%, 36%, 48%, or 60% of FM protein by a corresponding amount of protein with FSM to form the experimental diets (FM, R12, R24,R36, R48, and R60, respectively). Methionine and lysine was added to experimental feed to compensate for imbalance [24]. The experimental feeds were prepared by mixing all the ingredients until homogenous. Then, 300 ml/kg feed of water was added to form moist dough. After pelletized, the feeds were kept in refrigerator (−20 °C) until used. The proximate composition of the experimental feeds contained average of 50.19% and 9.95% of crude protein an d lipid, respectively, without any significant difference. The approximate composition of the test diets and essential amino acid (EAA) content were shown in Table 1 and Table 2, respectively.

Table 1
Ingredient composition and nutrient content of the test diets.

Ingredients (%)	Test diets					
	FM	R12	R24	R36	R48	R60
Fish meal	60.00	52.80	45.60	38.40	31.20	24.00
Cottonseed protein concentrate	0	7.88	15.76	23.64	31.52	39.40
Corn gluten meal	4.89	4.89	4.89	4.89	4.89	4.89
Wheat flour	18	18	18	18	18	18
Fish oil	1.46	2.24	3.03	3.81	4.60	5.38
Phospholipid	2	2	2	2	2	2
Calcium monophosphate	2	2	2	2	2	2
[a]Vitamin mixture	0.5	0.5	0.5	0.5	0.5	0.5
[b]Mineral mixture	0.5	0.5	0.5	0.5	0.5	0.5
Antioxidant	0.05	0.05	0.05	0.05	0.05	0.05
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5
Cellulose microcrystalline	10.10	8.42	6.71	5.04	3.33	1.65
[c]Methionine	0	0.08	0.18	0.23	0.32	0.42
[c]Lysine	0	0.14	0.28	0.44	0.59	0.71
Proximate composition (% air dry matter)						
Moisture	9.89	10.21	10.17	9.95	10.01	10.24
[d]Crudeprotein	50.13	50.21	49.87	50.44	50.39	50.08
[d]CrudeLipid	10.05	9.89	10.12	10.07	9.92	9.64

[a] Vitamin mixture (g/kg mixture): vitamin B1, 17.00 g; vitamin B2, 16.67 g; vitamin B6, 33.33 g; vitamin B12, 0.07 g; vitamin K, 3.33 g; vitaminE, 66.00 g; retinyl acetate, 6.67 g; VD, 33.33 g, nicotinic acid, 67.33 g; D-calcium pantothenate, 40.67 g; biotin, 16.67; folic acid, 4.17 g; inositol, 102.04 g; cellulose, 592.72 g. All ingredients were diluted with corn starch to 1 kg.

[b] Mineral mixture (mg g^{-1} mixture):$CaCO_3$, 350 g; $NaH_2PO_4{\cdot}H_2O$, 200 g; KH_2PO_4, 200 g; NaCl, 12 g; $MgSO_4{\cdot}7H_2O$, 10; $FeSO_4{\cdot}7H_2O$, 2 g; $MnSO_4{\cdot}7H_2O$, 2 g; $AlCl_3{\cdot}6H_2O$, 1 g; $CuCl_2{\cdot}2H_2O$, 1 g; KF, 1 g; $NaMoO_4{\cdot}2H_2O$, 0.5 g; $NaSeO_3$, 0.4 g; $CoCl_2{\cdot}6H_2O$, 0.1 g; KI, 0.1 g; zeolite powder, 219.9 g. All ingredients were diluted with corn starch to 1 kg. (Obtained from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China).

[c] Methionine and lysine were added to balance amino acid with control group.

[d] Crude protein and crude lipid contents were measured value.

Table 2
Essential amino acid profile (%) of the test diets used in the experiment.

Amino acids	Test diets					
	FM	R12	R24	R36	R48	R60
Methionine	1.01	0.98	1.02	1.01	1.03	0.94
Lysine	2.88	2.91	2.90	2.86	2.94	2.92
Threonine	1.89	1.86	1.90	1.92	1.91	1.91
Isoleucine	1.65	1.51	1.49	1.43	1.39	1.35
Histidine	1.63	1.59	1.57	1.65	1.63	1.64
Valine	1.89	1.90	1.75	1.67	1.62	1.55
Leucine	3.19	3.21	3.08	2.95	2.83	2.82
Arginine	2.35	2.87	3.41	3.69	3.88	4.31
Phenylalanine	1.56	1.61	1.63	1.70	1.81	1.84
Tyrosine	1.12	1.09	1.07	0.93	0.88	0.81

2.2. Feeding trial and challenge test

Hybrid grouper juveniles (100% female) with average body weight 11.31 ± 0.12 g were obtained from a commercial hatchery (Zhanjiang, China) and acclimatized to experimental condition for a week while feeding with commercial feed before commencing the feeding trial. The fish were randomly distributed into 500 L fiberglass tank at stocking number of 30 fish/tank. Each experimental feed was fed to triplicate groups of fish twice daily at 08:00 and 17:00 until apparent satiation level and the amount of feed consumed was recorded as described by Wang et al. [5]. The temperature of the water ranged from 28 to 30 °C