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Effects of dietary cholesterol on antioxidant capacity, non-specific immune response, and resistance to *Aeromonas hydrophila* in rainbow trout (*Oncorhynchus mykiss*) fed soybean meal-based diets

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ABSTRACT

This study evaluated the effects of dietary cholesterol on antioxidant capacity, non-specific immune response and resistance to *Aeromonas hydrophila* in rainbow trout (*Oncorhynchus mykiss*) fed soybean meal-based diets. Fish were fed diets supplemented with graded cholesterol levels (0 [control], 0.3, 0.6, 0.9, 1.2, and 1.5%) for nine weeks. The fish were then challenged by *A. hydrophila* and their survival rate recorded for the next week. Dietary cholesterol supplementation generally increased the serum and hepatic superoxide dismutase (SOD), glutathione-peroxidase (GSH-Px), catalase (CAT), and total antioxidant capacity (TAC) activities, but decreased the serum and hepatic malondialdehyde (MDA) contents. Further, the hepatic CAT and serum SOD, CAT, and TAC activities were significantly higher in fish fed diets supplemented with 0.9 or 1.2% cholesterol compared to those fed the control diet, whereas the serum and hepatic MDA contents were significantly lower. The respiratory burst activity, alternative complement activity, and hepatic lysozyme activity increased steadily when the supplemental cholesterol was increased by up to 1.2% and then declined with further addition. The serum lysozyme activity and phagocytic activity increased steadily with increasing dietary supplemental cholesterol level up to 0.9% and then declined with further addition. Dietary cholesterol supplementation generally enhanced the protection against *A. hydrophila* infection, and fish fed diets supplemented with 0.9 or 1.2% cholesterol exhibited the highest post-challenge survival rate. The results indicated that cholesterol may be under-supplied in rainbow trout fed soybean meal-based diets, and dietary cholesterol supplementation (0.9–1.2%) contributed to improved immune response and disease resistance of rainbow trout against *A. hydrophila*.

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1. Introduction

Due to increasing demand, limited supply, and the high price of fish meal (FM), efforts to replace FM by plant-derived protein sources have been increasing in aquafeeds [1]. Among the alternative protein sources for fish feeds, soybean meal (SBM) has been the most intensively studied plant feed ingredient because of its high protein content, relatively well-balanced amino acid profiles, availability and reasonable cost–effectiveness ratio [2]. However, feeding high levels of SBM was reported to have negative effects on growth, feed utilisation and disease resistance of many fish species [2–4]. The main limitations in the use of SBM are generally attributed to its poor palatability, low nutrient digestibility, presence of certain anti-nutritional factors, and lack of some essential amino acids [5]. Thus, some essential amino acids, attractants and minerals are often supplemented when FM is replaced by SBM, but there are numerous essential nutrients that are often overlooked. For example, the increasing proportion of SBM in feed formulations will reduce the level of dietary cholesterol, which is rich in FM but deficient in SBM [6,7]. Feeds traditionally formulated with FM and fish oil will provide at least 1 g cholesterol per kg feed [7]. Thus, the substitution of FM and/or fish oil will greatly reduce the dietary cholesterol level. On the other hand, soy protein and non-protein compounds present in SBM (*e.g.* soy saponins and phytosterol) reportedly lowered the plasma total cholesterol level in fish [8,9].

Abbreviations: ACP, alternative complement pathway; AKP, alkaline phosphatase; DMSO, dimethyl sulfoxide; FM, fish meal; GSH-Px, glutathione-peroxidase; MDA, malondialdehyde; PP, phagocytosis percentage; SBM, soybean meal; SOD, superoxide dismutase; TAC, total antioxidant capacity.

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Thus, the effects on cholesterol metabolism suggest that this may be an area requiring consideration in the future as the proportion of SBM in dietary formulations increases [7].

Cholesterol is a necessary constituent for eukaryotic cell growth and development. It serves as a precursor to many physiologically active compounds, such as sex hormones, adrenal corticoids, bile acids and vitamin D [10]. Vertebrates including fish can synthesise sterol from acetate, thus limited research has addressed the potential need for a dietary supply of cholesterol in fish [11–16]. However, recent studies showed that fish fed diets containing high levels of plant-derived protein sources had lower levels of blood cholesterol, and were more susceptible to infectious disease [17–19] and occurrences of green liver [20,21]. Previous studies also demonstrated that the plasma total cholesterol level was significantly related to fish mortality following bacterial and viral challenge [21–23], and unhealthy fish [24,25], or fish under starvation conditions [26–28], temperature stress [29,30] and low dissolved oxygen [31] have below normal plasma cholesterol level. These investigations indicate that the blood cholesterol level is a good indicator of fish health and innate immunity [20,21]. Therefore, the authors deem a decrease in the plasma cholesterol level of cultured fish caused by replacing FM with SBM to possibly be an indicator of a reduction in fish health. Although there have been a few studies investigating the dietary supplementation of cholesterol [11–16], there is no report on the effects of dietary cholesterol supplementation on antioxidant capacity and disease resistance of fish. The objective of the present study was to determine the effect of graded levels of cholesterol supplementation on antioxidant capacity, non-specific immune response and resistance to *Aeromonas hydrophila* in rainbow trout (*Oncorhynchus mykiss*) fed SBM-based diets.

2. Materials and methods

2.1. Fish and experimental conditions

Rainbow trout obtained from a local commercial farm (Fenghong Fisheries Co., Ltd., Kunming, China) were acclimatised to the experimental conditions for two weeks. Fish were fed twice (08:00 h and 16:00 h) daily with a commercial diet (TR-2242, Salmofood S.A., Castro, X Región, Chile) to satiation during this period. At the end of the acclimation period, fish with an average mass of 57.8 g were randomly distributed into 18 tanks with 30 juveniles per tank (triplicate groups per dietary treatment). Water was recirculated through a 4000 L biological and mechanical filtration system containing a vertical quartz sand filter and an activated carbon purifier to remove solid and nitrogenous wastes. A flow rate in each rectangular tank (1.0 m × 0.7 m × 0.8 m) of *c*. 3 L per min was maintained: the water temperature was maintained at between 14 and 18 °C. All rearing tanks were provided with continuous aeration and maintained under natural photoperiod.

2.2. Experimental diets and feeding

Six isonitrogenous (crude protein 43%) and isoenergetic (gross energy 21 kJ g^{-1}) practical diets were formulated to contain graded levels of cholesterol. A basal diet (C0) was formulated using a combination of FM (accounting for approximately 30% of dietary protein) and SBM (about 70%) as the primary protein sources. The other five diets (C3, C6, C9, C12, and C15) were supplemented with 0.3, 0.6, 0.9, 1.2, and 1.5% cholesterol at the expense of wheat flour in the basal diet, respectively. The ingredients and chemical composition of diets are presented in Table 1. The actual cholesterol contents were 0.38, 0.68, 1.03, 1.37, 1.68, and 1.83% in diets C0, C3, C6, C9, C12, and C15, respectively. The experimental ingredients were ground into fine powder and passed through a 320 μm square aperture mesh sieve. All the ingredients were thoroughly mixed with soybean oil and fish oil; water was added to produce a stiff dough which was then extruded by a pellet feed maker (KS-180, Jiangsu Jingu Rice Mill Co., Ltd., Jiangsu, China) through a 3 mm diameter die. The moist pellets were dried in a forced air oven at room temperature for about 12 h, and then stored at −20 °C until used.

Table 1
Ingredients and proximate composition (% dry matter) of the experimental diets.

	Dietary cholesterol supplementation level (%)					
	0	0.3	0.6	0.9	1.2	1.5
Ingredients						
Fish meal[a]	20	20	20	20	20	20
Soybean meal[a]	57	57	57	57	57	57
Wheat flour[b]	1.5	1.2	0.9	0.6	0.3	0
Soybean lecithin (40%)[c]	5.0	5.0	5.0	5.0	5.0	5.0
Fish oil[a]	5.8	5.8	5.8	5.8	5.8	5.8
Soy oil[a]	5.4	5.4	5.4	5.4	5.4	5.4
Choline chloride (50%)[a]	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin C[d]	0.2	0.2	0.2	0.2	0.2	0.2
$Ca(H_2PO_4)_2$[a]	0.8	0.8	0.8	0.8	0.8	0.8
Ethoxyquin (30%)[a]	0.05	0.05	0.05	0.05	0.05	0.05
L-lysine HCl[e]	0.35	0.35	0.35	0.35	0.35	0.35
DL-methionine[e]	0.4	0.4	0.4	0.4	0.4	0.4
Mineral pre-mix[f]	1.5	1.5	1.5	1.5	1.5	1.5
Vitamin pre-mix[h]	1.5	1.5	1.5	1.5	1.5	1.5
Cholesterol[i]	0.0	0.3	0.6	0.9	1.2	1.5
Approximate composition						
DM (%)	92.74	91.86	93.95	91.35	91.82	93.39
Crude protein (% DM)	43.45	43.72	43.37	43.47	43.49	43.56
Crude fat (% DM)	16.98	17.55	17.29	17.36	18.16	18.64
Ash (% DM)	10.49	10.41	10.05	10.13	10.06	10.09
Fibre (% DM)	5.65	5.54	5.39	5.59	5.60	5.51
NFE[j]	23.43	22.78	23.90	23.45	22.69	22.20
Total energy (MJ kg^{-1})[k]	21.0	21.2	21.2	21.2	21.4	21.5
Cholesterol	0.38	0.68	1.03	1.37	1.68	1.83

[a] Supplied by Kunming Tianyuan Feed Co., Ltd. (Yunnan, China); fish meal, 67.0% crude protein, 11.5% crude lipid; soybean meal, 49.3% crude protein, 2.8% crude lipid.
[b] Supplied by Zhaoqing Four Gardener Flour Co., Ltd. (Guangdong, China), 12.9% crude protein, 2.8% crude lipid.
[c] Supplied by Shanghai Hanhong Chemical Co., Ltd. (Shanghai, China).
[d] L-ascorbate-2-polyphosphate (35%), supplied by Galaxy Chemicals Co., Ltd. (Hubei, China).
[e] Supplied by Shanghai Hanhong Chemical Co., Ltd. (Shanghai, China).
[f] Mineral pre-mix (g kg^{-1} mixture): $MgSO_4 \cdot 7H_2O$, 180.0; KI, 1.0; $FeSO_4 \cdot H_2O$, 260; $ZnSO_4 \cdot H_2O$, 180; $CuSO_4 \cdot 5H_2O$, 25; $Na_2Se_2O_3$, 0.01; $MnSO_4 \cdot H_2O$, 180; $CoCl_2 \cdot 6H_2O$, 0.75.
[h] Vitamin pre-mix (g kg^{-1} mixture): retinyl acetate (2 800 000 IU g^{-1}), 2; cholecalciferol, 0.03; DL-α-tocopheryl acetate, 30; menadione, 3; thiamine hydrochloride, 8; riboflavin, 11; pyridoxine hydrochloride, 8; vitamin B_{12}, 0.02; ascorbic acid, 50; folic acid, 1; biotin 0.1; niacin, 30; calcium D-pantothenate, 32; inositol, 25.
[i] Supplied by Shanghai Shunbo Bioengineering Co., Ltd. (Shanghai, China).
[j] NFE (*nitrogen*-free extract) = 100 − (crude protein + crude fat + fibre + ash).
[k] Gross energy was calculated by using the factors 39.5 kJ g^{-1} for fat, 23.7 kJ g^{-1} for protein, and 17.2 kJ g^{-1} for NFE.

Fish were fed by hand to apparent satiation two times per day (08:00 and 16:00) with one of the six experimental diets over 9 weeks.

2.3. Sample collection and analysis

2.3.1. Sample collection and tissue preparation

At the end of the feeding trial, the fish were fasted for 24 h before harvest. All experimental fish were anaesthetised with eugenol (1:12 000) (Shanghai Reagent Corporation, Shanghai, China) before sampling. Blood samples were collected from the caudal vein of five fish per tank with a sterile 5 ml syringe and withdrawn into Eppendorf tubes without anticoagulant. Blood samples in Eppendorf tubes were allowed to clot for 4 h at 4 °C. Following centrifugation (3000 × *g* for 10 min at 4 °C), the serum