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

Junming Deng, Bin Kang, Linli Tao, Hua Rong, Xi Zhang

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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.

* Corresponding author. Tel.: +86 871 5227796; fax: +86 871 5227284.
E-mail address: xzhangynau@163.com (X. Zhang).

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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 to be an area requiring consideration in the future as the proportion of SBM in dietary formulations increases [7].

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

<table>
<thead>
<tr>
<th>Dietary cholesterol supplementation level (%)</th>
<th>0</th>
<th>0.3</th>
<th>0.6</th>
<th>0.9</th>
<th>1.2</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients</td>
<td>---</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish meala</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Soybean mealb</td>
<td>57</td>
<td>57</td>
<td>57</td>
<td>57</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td>Wheat flourb</td>
<td>1.5</td>
<td>1.2</td>
<td>0.9</td>
<td>0.6</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Soybean lecithin (40%)c</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Fish oil</td>
<td>5.8</td>
<td>5.8</td>
<td>5.8</td>
<td>5.8</td>
<td>5.8</td>
<td>5.8</td>
</tr>
<tr>
<td>Soy oil</td>
<td>5.4</td>
<td>5.4</td>
<td>5.4</td>
<td>5.4</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Choline chloride (50%)d</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin C4</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Ca(H2PO4)2</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Ethanoxin (30%)e</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>i-lysine HCl</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>α-methioninef</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Mineral pre-mixture</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Vitamin pre-mixture</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Cholesterol*</td>
<td>0.0</td>
<td>0.6</td>
<td>0.9</td>
<td>1.2</td>
<td>1.5</td>
<td>1.83</td>
</tr>
<tr>
<td>Approximate composition</td>
<td>---</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DM (%)</td>
<td>92.74</td>
<td>91.86</td>
<td>93.05</td>
<td>91.35</td>
<td>91.82</td>
<td>93.39</td>
</tr>
<tr>
<td>Crude protein (%) DM</td>
<td>43.45</td>
<td>43.72</td>
<td>43.37</td>
<td>43.47</td>
<td>43.49</td>
<td>43.56</td>
</tr>
<tr>
<td>Crude fat (%) DM</td>
<td>16.98</td>
<td>17.53</td>
<td>17.29</td>
<td>17.36</td>
<td>18.16</td>
<td>18.64</td>
</tr>
<tr>
<td>Ash (%) DM</td>
<td>10.49</td>
<td>10.41</td>
<td>10.05</td>
<td>10.13</td>
<td>10.06</td>
<td>10.09</td>
</tr>
<tr>
<td>Fibre (%) DM</td>
<td>5.65</td>
<td>5.54</td>
<td>5.39</td>
<td>5.59</td>
<td>5.60</td>
<td>5.51</td>
</tr>
<tr>
<td>NFE</td>
<td>23.43</td>
<td>22.78</td>
<td>23.90</td>
<td>23.45</td>
<td>22.69</td>
<td>22.20</td>
</tr>
<tr>
<td>Total energy (MJ kg−1)f</td>
<td>21.0</td>
<td>21.2</td>
<td>21.2</td>
<td>21.2</td>
<td>21.4</td>
<td>21.5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.38</td>
<td>0.68</td>
<td>1.03</td>
<td>1.37</td>
<td>1.68</td>
<td>1.83</td>
</tr>
</tbody>
</table>

* Supplied by Kunming Tianyuan Feed Co., Ltd. (Yunnan, China); fish meal, 67.0% crude protein, 11.5% crude lipids; soybean meal, 49.3% crude protein, 2.8% crude lipid.

b Supplied by Zhaqiao Four Gardener Flour Co., Ltd. (Guangdong, China), 12.9% crude protein, 2.8% crude lipids.

c Supplied by Shanghai Hanhong Chemical Co., Ltd. (Shanghai, China).

d L-ascorbate–2-phosphophate (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): MgSO4, 7H2O, 180.0; KI, 1.0; FeSO4, 1.0; ZnSO4, 7H2O, 180; MnSO4, 8H2O, 260; CuSO4, 5H2O, 25; Na2Se2O3, 0.01; MnSO4, H2O, 180; CoCl2, 6H2O, 0.25.

Table 1 Ingredients and proximate composition (% dry matter) of the experimental 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 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 isoinenergetic (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. All the 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 apertures 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.

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 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...
was removed and frozen at −80 °C for antioxidant-related parameter and lysozyme analyses. Livers were dissected from five fish per tank and pooled for analysis. Liver samples were stored frozen (−80 °C) for subsequent determination of antioxidant-related parameters and lysozyme analysis. To obtain adequate crude enzyme extract solution, the amount of physiological saline solution (0.9% NaCl) needed for addition to the wet liver was determined by a preliminary study. Wet livers plus the four fold volume (v/w) of ice-cold physiological saline solution were added to a 10 ml test tube and homogenised using an IKA homogeniser (IKA Works Asia, Bhd, Malaysia). The homogenate was centrifuged (9000 × g for 30 min at 4 °C) using a high-speed refrigerated centrifuge (MX-160, Tomy, Tokyo, Japan). The supernatant was diluted to an adequate volume with a physiological saline solution (if necessary) and used as a crude enzyme solution.

Head kidney macrophages from five fish in each tank were isolated as described by Ai et al. [32]. Briefly, the head kidney was excised, cut into small fragments and transferred to an RPMI-1640 (Gibco, USA) medium supplemented with 10 IU ml⁻¹ heparin (Sigma, USA), 100 IU ml⁻¹ penicillin (Amresco, USA), 100 μg ml⁻¹ streptomycin (Amresco, USA) and 2% foetal calf serum (Gibco, USA). Cell suspensions were prepared by forcing the head kidney through a 100 μm aperture stainless-steel mesh. The resultant cell suspensions were enriched by centrifugation (1000 × g for 25 min at 4 °C) on 34%/51% (v/v) Percoll (Pharmacia, USA) density gradient. The cells were collected from the interface and washed twice with RPMI-1640. Cell viability was determined by the trypan blue exclusion method and the cell density was determined in a haemocytometer. Then additional RPMI-1640 medium was added to adjust the cell concentration (1 × 10⁷ ml⁻¹) for analysis.

2.3.2. Antioxidant-related parameters assay

Superoxide dismutase (SOD), glutathione-peroxidase (GSH-Px), catalase (CAT), alkaline phosphatase (AKP), and total antioxidant capacity (TAC) activities in serum and liver were determined with a spectrophotometer (UV-2100, Shanghai Jinhua Technology Instrument Co., Ltd., Shanghai, China) at 550, 412, 405, 520, and 520 nm, respectively. Antioxidant-related parameter detection kits (SOD, GSH-Px, CAT, AKP, and TAC) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). One unit of SOD activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the nitroblue tetrazolium reduction rate measured at 550 nm. One unit of GSH-Px activity was defined as the amount of enzyme that reduced the GSH concentration in the reaction system at 1 μmol L⁻¹ per min. One unit of CAT activity was defined as the amount of enzyme that catalysed the decomposition of 1 μmol of H₂O₂ per min. One unit of AKP activity was defined as the amount of enzyme that reacted with the matrix and produced 1 mg phenol in 15 min at 37 °C. One unit of TAC was defined as a 0.01 increment of absorbance of the reaction system caused by serum per millilitre reacting at 37 °C for 1 min.

The malondialdehyde (MDA) content in serum and liver was determined by the thiobarbituric acid method [33] using commercially available kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The protein content in the liver samples was quantified by the Bradford method [34] using a total protein quantification kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with bovine serum albumin as the standard.

2.3.3. Lysozyme assay

The lysozyme activity in serum and liver was determined according to the method of Ellis [35] based on the lysis of the lysozyme sensitive Gram positive bacterium Micrococcus lysodeikticus (Sigma, USA). Briefly, 2 ml of M. lysodeikticus at a concentration of 0.2 mg ml⁻¹ (w/v) in 0.05 M phosphate buffer solution (pH 6.2) was added to 100 μl of serum or crude enzyme solution. The reduction in absorbance at 530 nm was measured after 0.5 min and 4.5 min at room temperature. Results were expressed in units of lysozyme ml⁻¹ serum or mg⁻¹ protein. One unit is defined as the amount of sample causing a decrease in absorbance of 0.001 units per min.

2.3.4. Alternative complement pathway (ACP) activity

ACP activity in serum was assayed according to Yano’s method [36]. Briefly, a series of volumes of the diluted serum ranging from 0.1 to 0.25 ml were dispensed into test tubes and the total volume made up to 0.25 ml with barbitone buffer in the presence of ethyleneglycol-bis (2-aminoethoxy)-tetraacetic acid (EGTA) and Mg²⁺, then 0.1 ml of rabbit red blood cells (1 × 10⁸ cells ml⁻¹) was added to each tube. After incubation for 2 h at 22 °C, 3.15 ml of 0.9% NaCl was added. Then the sample was centrifuged at 1000 × g for 5 min at 4 °C to eliminate unlysed rabbit red blood cells. The optical density of the supernatant was measured at 414 nm. The volume of serum producing 50% haemolysis (ACH50) was determined and the number of ACH50 units ml⁻¹ obtained for each group.

2.3.5. Phagocytic activity

Phagocytic activity was determined by modification of the method of Pulford et al. [37]. Briefly, 100 μl head kidney leucocytes suspension (1 × 10⁷ cells ml⁻¹) was placed onto a sterile slide and allowed to attach for 30 min at 25 °C. Following attachment, 100 μl yeast suspension (Baker’s yeast, Type II, Sigma, USA, 1 × 10⁸ cells ml⁻¹) was added to the cell monolayer. The slide was incubated for 45 min at 25 °C, and then washed three times with phosphate buffered saline to remove any unattached cells. After air-drying, the slides were fixed in ethanol, re-dried and stained with Giemsa. The percentage of phagocytes was calculated by enumerating 200 phagocytes under a microscope. The phagocytosis percentage (PP) was then given by Eq. (1).

\[
\text{PP} = \frac{\text{(number of cells ingesting yeast)} \times \text{(number of yeast ingested cells)}}{\text{number of adherent cells observed}}
\] (1)

The respiratory burst activity of the phagocytes was quantified by reduction of nitroblue tetrazolium (Yanyu Chemical Reagent Co., Ltd., Shanghai, China) following the method of Secombes [38] albeit with minor modification. Briefly, 100 μl cell suspension was deposited in microtitre plate wells, stained with 100 μl of 0.3% nitroblue tetrazolium solution and 100 μl Phorbol 12-myristate 13-acetate (Alexis, USA; 1 μg ml⁻¹) for 30 min at room temperature. Absolute methanol was added to terminate the staining. Each tube was washed three times with 70% methanol and air-dried. Then 120 μl 2 M KOH and 140 μl dimethyl sulfoxide (DMSO) (Yanyu Chemical Reagent Co., Ltd., Shanghai, China) were added

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and the colour subsequently measured at 630 nm with a spectrophotometer using KOH/DMSO as a blank.

2.3.7. Challenge test

*A. hydrophila*, which was identified using the Kaper’s multi test medium [39] and provided by the Marine Culture Collection of China (MCCC, 1A00007), was originally isolated from diseased rainbow trout. Bacteria were grown in tryptic soy broth (Sigma, USA) medium and incubated at 22 °C for 24 h. The seven day LD50 was determined by intraperitoneal injection using graded doses of *A. hydrophila* for 7 days. The cause of death was confirmed by isolating the organism from the liver of dead fish using conventional methods.

2.4. Statistical analysis

Percentage data were subjected to arcsine transformation before statistical analysis. All data were subjected to one-way analysis of variance (ANOVA) followed by the use of Tukey’s method to determine significant differences among treatment groups. The level of significance was set to *P* < 0.05. Statistical analysis was performed using the SPSS 16.0 suite for Windows (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Antioxidant-related parameters

Dietary cholesterol supplementation generally increased the serum SOD and GSH-Px activities, which were significantly higher in fish fed the C12 and C15 diets compared to fish fed the C0 diet (Table 2). The serum CAT activity increased steadily when the supplemental cholesterol was increased by up to 1.2% and then declined with further addition. In contrast, the serum MDA content decreased steadily when the supplemental cholesterol was increased by up to 1.2% and then increased with further addition. The hepatic CAT activity was significantly higher in fish fed the C3, C6, C9, and C12 diets compared to fish fed the C15 diet.

3.2. Immune parameters

3.2.1. Serum and hepatic lysozyme activities

The serum lysozyme activity increased steadily when the supplemental cholesterol was increased by up to 0.9% and then declined with further addition (Fig. 1). Similarly, the hepatic lysozyme activity increased steadily when the supplemental cholesterol was increased by up to 1.2% and then declined with further addition (Fig. 2). However, no significant difference was observed in hepatic lysozyme activity between fish fed the C9 and C12 diets.

3.2.2. Serum ACP activity

The serum ACP activity increased steadily when the supplemental cholesterol was increased by up to 1.2% and then declined with further addition (Fig. 3), but no significant difference was observed among fish fed the C6, C9, and C12 diets.

3.2.3. Phagocytic activity

The PP increased steadily when the supplemental cholesterol was increased by up to 0.9% and then declined with further addition (Fig. 4), but no significant difference was observed among fish fed the C6, C9, and C12 diets.

3.2.4. Respiratory burst activity

Dietary cholesterol supplementation generally increased the respiratory burst activity of head kidney macrophages (Fig. 5).

### Table 2

Effect of dietary cholesterol supplementation on antioxidant capacity parameters in serum of rainbow trout.

<table>
<thead>
<tr>
<th>Dose</th>
<th>0%</th>
<th>0.3%</th>
<th>0.6%</th>
<th>0.9%</th>
<th>1.2%</th>
<th>1.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U ml&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>4.99 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.93 ± 1.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.45 ± 0.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.07 ± 2.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.42 ± 1.93&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55.50 ± 6.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSH-Px (U µl&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.09 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.12 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.11 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.11 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.13 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.14 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT (U l&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>3.79 ± 0.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.55 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.06 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.46 ± 1.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.63 ± 2.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.26 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AKP (U d&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>24.98 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.45 ± 0.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.44 ± 1.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.93 ± 3.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.25 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.21 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAC (U ml&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>3.78 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.22 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.16 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.82 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.62 ± 0.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.03 ± 0.67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDA (nmol ml&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>16.89 ± 1.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.73 ± 2.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.08 ± 0.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.29 ± 0.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.81 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.19 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE (*n* = 3). Values in the same row with different superscript alphabets are significantly different from each other (*P* < 0.05). SOD: superoxide dismutase; GSH-Px: glutathione-peroxidase; CAT: catalase; AKP: alkaline phosphatase; TAC: total antioxidant capacity; MDA: malondialdehyde.

### Table 3

Effect of dietary cholesterol supplementation on antioxidant capacity parameters in liver of rainbow trout.

<table>
<thead>
<tr>
<th>Dose</th>
<th>0%</th>
<th>0.3%</th>
<th>0.6%</th>
<th>0.9%</th>
<th>1.2%</th>
<th>1.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U mg&lt;sup&gt;-1&lt;/sup&gt; protein)</td>
<td>2.69 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.59 ± 0.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.84 ± 0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.17 ± 0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.53 ± 0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.87 ± 0.64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSH-Px (U mg&lt;sup&gt;-1&lt;/sup&gt; protein)</td>
<td>7.98 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.36 ± 0.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.03 ± 0.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.31 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.27 ± 1.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.85 ± 1.45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT (U mg&lt;sup&gt;-1&lt;/sup&gt; protein)</td>
<td>9.49 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.53 ± 0.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.40 ± 1.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.29 ± 1.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.65 ± 0.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.92 ± 0.42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AKP (U g&lt;sup&gt;-1&lt;/sup&gt; protein)</td>
<td>33.89 ± 1.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.07 ± 1.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.82 ± 0.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.71 ± 1.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.10 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.01 ± 0.68&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAC (U mg&lt;sup&gt;-1&lt;/sup&gt; protein)</td>
<td>1.06 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.58 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.82 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.78 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.28 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.96 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDA (nmol mg&lt;sup&gt;-1&lt;/sup&gt; protein)</td>
<td>0.19 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.59 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.58 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.74 ± 0.07&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE (*n* = 3). Values in the same row with different superscript alphabets are significantly different from each other (*P* < 0.05). SOD: superoxide dismutase; GSH-Px: glutathione-peroxidase; CAT: catalase; AKP: alkaline phosphatase; TAC: total antioxidant capacity; MDA: malondialdehyde.
which was significantly higher in fish fed the C6, C9, and C12 diets compared to fish fed the C0 diet.

3.3. Challenge test

The challenge test showed that long-term oral administration of cholesterol-supplemented diets generally enhanced protection against *A. hydrophila* infection for fish fed SBM-based diets (Fig. 6). However, significantly higher post-challenge survival rates were only observed in fish fed the C6, C9, and C12 diets compared to fish fed the C0 diet. Fish fed the C9 (73.3%) and C12 (76.7%) diets exhibited the highest post-challenge survival rate.

4. Discussion

Cholesterol is an integral component in cell membrane maintenance and is involved in the preservation of fluidity within the membrane [40]. Further, cholesterol is also a precursor to several important hormones that protect the body against stress, and vitamin D which is a necessary nutrient for immune system function [41]. Thus, many researchers have suggested that blood cholesterol plays an important role in the immune defence system [42,43]. In fish, the non-specific immune system is more important for disease resistance than its specific counterpart as the latter needs a longer time for antibody production and specific cellular activations [44]. In the present study, the non-specific immune parameters (e.g. lysozyme activity, respiratory burst activity, phagocytic activity, and alternative complement activity) increased steadily when the supplemental cholesterol was increased by up to 0.9 or 1.2% and then declined with further addition. The results indicated that 0.9–1.2% cholesterol supplementation will contribute to improve the immune response of rainbow trout fed SBM-based diets, also implying that inadequate cholesterol might limit the normal immune response. At present, limited information is available concerning the effect of dietary cholesterol on non-specific immune responses in fish [45]. In terrestrial animals, it has been reported that cholesterol may play an important role in modulating the immune response [46–49]. An elevated level of blood cholesterol was associated with increased immune system activities [46]: feeding with a cholesterol-rich diet improved the activation of immune cells [47], spleen and liver functions [49], and serum antibody levels [50]. The authors’ data were consistent with these conclusions. However, the present study also showed that non-specific immune parameters of rainbow trout fed a diet supplemented with 1.5% cholesterol were lower than those of fish fed diets supplemented with 0.9 or 1.2% cholesterol, indicating that excessive dietary cholesterol may depress the normal immune response of rainbow trout. Bowden et al. [45] reported that rainbow trout fed soybean meal-based diets, also implying that inadequate cholesterol might contribute to improve the immune response of rainbow trout fed SBM-based diets, also implying that inadequate cholesterol might limit the normal immune response.
trout fed a diet containing 0.043% cholesterol demonstrated reduced fluidity in their macrophages and depressed macrophage ability compared with those fed a cholesterol-free diet. In fish, macrophages are part of the innate and acquired immune systems [45]. Thus, Bowden et al. [45] suggest that dietary cholesterol reduces both the immune functions and disease resistance of rainbow trout. Similarly, feeding a cholesterol-rich diet suppressed the non-specific phagocytosis of carbon particles in vivo in terrestrial animals [48,51], and in vitro ingestion of latex particles and oil droplets by macrophages [48,52]. These findings suggested that animals fed cholesterol were more susceptible to infections than control animals. The apparent discrepancy among these studies may be attributed to dietary cholesterol levels, dietary constituents, and animal species.

Survival after challenge with certain pathogens is usually assessed as a measure of disease resistance [53]. It has been reported that fish having low plasma cholesterol levels had lower disease resistance compared with fish having high plasma cholesterol levels [21]. Therefore, previous studies suggest that plasma cholesterol can be an indicator of fish health and innate immunity [20,21]. In the present study, dietary cholesterol supplementation (0.9–1.2%) significantly increased the survival rate of rainbow trout to A. hydrophila infection, suggesting that dietary cholesterol may enhance fish resistance to bacterial pathogens. Similarly, Ravnkov [43] also suggested that high cholesterol may protect against infections.

In addition, multiple types of antioxidants (e.g. glutathione, catalase, SOD, and various peroxidases) are needed to maintain the complex immune system of fish. The antioxidant capacity includes enzymatic and non-enzymatic antioxidant activities. Antioxidant enzymes include SOD, catalase, and GSH-Px that constitute the first line of the enzymatic defence mechanism against free radicals in organisms. SOD catalyses dismutation of superoxide radicals to hydrogen peroxide and oxygen; catalase catalyses breakdown of hydrogen peroxide to water and molecular oxygen; GSH-Px decomposes peroxides using the peptide glutathione as their cosubstrate [54]. TAC, as a reliable biomarker of disease, could be useful in the evaluation of the antioxidant capacity of all antioxidants [55]. Lipid peroxidation is one of the major problems associated with failure of the antioxidant system. Malondialdehyde is the end-product of lipid peroxidation, which causes toxic stress in cells and is used as a biomarker to measure the level of oxidative stress [56]. As a monounsaturated lipid (double bond at its 5,6-position), cholesterol is susceptible to free radical- and 1O2-mediated oxidation, which contributes to overall membrane and lipoprotein oxidative damage. Some studies indicated that cholesterol may act as an antioxidant, protecting the body from free radicals and therefore strengthening the immune system [57,58]. In the present study, dietary cholesterol supplementation generally increased the serum and hepatic antioxidant enzymes’ activities and decreased the MDA content: the highest antioxidant capacity was exhibited at the 0.9–1.2% supplemental levels. Previous studies also showed that feeding cholesterol-supplemented diets (0.5–1.0% cholesterol) increased the plasma vitamin E and GSH-Px levels in rabbits [47] and the hepatic GSH-Px and CAT activities in rats [59], while they decreased the hepatic MDA [60] or thiobarbituric acid reactive substances (TBARS) [49,61] contents. These results indicated that high cholesterol diets reduced susceptibility to lipid peroxidation. In contrast, other studies showed that feeding a high cholesterol diet (0.5–1.0% cholesterol) decreased the plasma or hepatic antioxidant enzymes activities or mRNA [59,62,63] and increased the plasma MDA or TBARS content [62–64], the tissue oxidative stress in rats [65] and oxidative DNA damage in rats [66], indicating that high cholesterol diets would decrease the protection of the tissue against lipid peroxidation.

5. Conclusion

In summary, dietary cholesterol supplementation generally improved the antioxidant capacity, non-specific immune response, and disease resistance of rainbow trout fed SBM-based diets: these were relatively higher in fish fed diets supplemented with 0.9–1.2% cholesterol. These results implied that cholesterol may be under-supplied in rainbow trout fed SBM-based diets, thereby affecting their immune responses and disease resistance. Dietary cholesterol supplementation (0.9–1.2%) can alleviate these negative effects in rainbow trout, but excess cholesterol (1.5%) cannot elicit either further immune response or disease resistance.

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References


