Effects of Dietary Cholesterol Content on Growth Performance, Anti-Vibrio Capacity, and Resistance to Nitrite Nitrogen Stress in Freshwater-Cultured Litopenaeus vannamei (Postprint)

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Abstract

To investigate the effects of dietary cholesterol content on growth performance, anti-Vibrio capacity, and resistance to nitrite nitrogen stress of Pacific white shrimp (Litopenaeus vannamei) under freshwater culture conditions, isonitrogenous and isoenergetic diets with cholesterol supplementation levels of 0 (C0 group), 1 (C1 group), 2 (C2 group), 3 (C3 group), and 4 g/kg (C4 group) were formulated based on 10% fish meal (the measured dietary cholesterol contents were 0.78, 1.57, 2.45, 3.43, and 4.18 g/kg, respectively), and fed to shrimp with initial body weight of (0.160$±$0.002) g for 50 days. Each diet was randomly assigned to four replicate cages, with 50 shrimp per cage. At the end of the feeding trial, growth performance, proximate muscle composition, and mortality under acute Vibrio harveyi infection and nitrite nitrogen stress were examined. The results showed that the specific growth rate and survival rate of shrimp were not significantly affected by dietary cholesterol content ($P>0.05$), but the feed conversion ratio of shrimp in the C0 group was significantly higher than those in the C2, C3, and C4 groups ($P<0.05$). With increasing dietary cholesterol content, the crude protein content in shrimp muscle gradually increased, with the C3 and C4 groups being significantly higher than the C0 group ($P<0.05$); the crude lipid content in muscle first increased and then decreased, with the C2 group being significantly higher than the other groups ($P<0.05$). The cholesterol content in hepatopancreas and muscle increased significantly with increasing dietary cholesterol content ($P<0.05$), whereas the serum cholesterol content first increased and then decreased, with the C2 group being significantly higher than the C0 group ($P<0.05$). After acute V. harveyi infection, the cumulative mortality of shrimp in each group first decreased and then increased with increasing dietary cholesterol content, with the C2 group exhibiting the lowest cumula-

tive mortality at 24, 36, 48, 72, and 96 h post-infection. Under acute nitrite nitrogen stress at 8.5–9.0 mg/L, the cumulative mortality of shrimp at 96 h was negatively correlated with dietary cholesterol content, and the C0 and C1 groups were significantly higher than the C2, C3, and C4 groups ($P<0.05$). In summary, under freshwater culture conditions, a dietary cholesterol content of 1.57 g/kg could meet the growth requirements of shrimp, but a dietary cholesterol content of 2.45 g/kg could enable shrimp to achieve optimal anti-Vibrio capacity and desirable resistance to nitrite nitrogen stress.

Full Text

Effects of Dietary Cholesterol Content on Growth Performance, Vibrio and Nitrite Nitrogen Stress Resistant Abilities of Litopenaeus vannamei Cultured in Freshwater

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Abstract: This study investigated the effects of dietary cholesterol content on the growth performance, Vibrio resistance, and nitrite nitrogen stress resistance of Litopenaeus vannamei cultured in freshwater. Five isonitrogenous and isoenergetic experimental diets were formulated based on a 10% fish meal basal diet, with cholesterol supplementation levels of 0 (C0 group), 1 (C1 group), 2 (C2 group), 3 (C3 group), and 4 g/kg (C4 group). The measured dietary cholesterol concentrations were 0.78, 1.57, 2.45, 3.43, and 4.18 g/kg, respectively. These diets were fed to L. vannamei with an initial body weight of (0.160$±$0.002) g for 50 days. Each experimental diet was fed to four net cages, with 50 shrimps per cage. After the feeding trial, growth performance, muscle proximate composition, and mortality following acute Vibrio harveyi infection and nitrite nitrogen stress were evaluated. The results showed that dietary cholesterol content had no significant effect on survival rate or specific growth rate ($P>0.05$). However, the feed conversion ratio (FCR) of the C0 group was significantly higher than that of the C2, C3, and C4 groups ($P<0.05$). Muscle crude protein content increased gradually with dietary cholesterol level, with the C3 and C4 groups showing significantly higher values than the C0 group ($P<0.05$). Muscle crude lipid content initially increased then decreased, with the C2 group exhibiting significantly higher values than all other groups ($P<0.05$). Hepatopancreas and muscle cholesterol contents increased significantly with dietary cholesterol content ($P<0.05$), while serum cholesterol content initially increased then decreased, with the C2 group showing significantly higher values than the C0

group (P<0.05).

Following acute V. harveyi infection, cumulative mortality initially decreased then increased with dietary cholesterol content, with the C2 group displaying the lowest cumulative mortality at 24, 36, 48, 72, and 96 hours post-infection. Under acute nitrite nitrogen stress at 8.5–9.0 mg/L, cumulative mortality at 96 hours was negatively correlated with dietary cholesterol content, with the C0 and C1 groups showing significantly higher mortality than the C2, C3, and C4 groups (P<0.05). In conclusion, under freshwater culture conditions, a dietary cholesterol content of 1.57 g/kg meets the growth requirements of L. vannamei, while 2.45 g/kg provides optimal Vibrio resistance and desirable nitrite nitrogen stress resistance.

Keywords: freshwater; Litopenaeus vannamei; cholesterol; growth performance; Vibrio resistance ability; nitrite nitrogen stress resistance ability

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Litopenaeus vannamei is a globally important cultured shrimp species with a wide salinity tolerance range and can be cultured in freshwater environments after gradual acclimation. In 2013, China' s L. vannamei production reached 1.4299 million tons, with 43% from freshwater culture [1]. However, salinity affects the metabolism and osmoregulation of L. vannamei [2-3], which may influence nutrient consumption and dietary requirements. Cholesterol, a vital sterol in animals, is a cyclopentanoperhydrophenanthrene derivative and a major structural component of cell membranes that reduces membrane fluidity and permeability to protons and sodium ions [4]. Membrane cholesterol also plays important roles in intracellular transport and intercellular signal transduction [5]. Additionally, cholesterol is a precursor for steroid hormones (e.g., estrogen, androgen, ecdysone, adrenocortical hormones) and vitamin D, making it physiologically essential [6]. Arthropods such as shrimp cannot synthesize cholesterol from acetate [7] and require dietary supplementation. Dietary cholesterol requirements for L. vannamei in marine culture have been reported [8-12], with both deficiency and excess reducing growth performance [8-10]. However, Gong et al. [13] suggested that excessive dietary cholesterol and phospholipids improve osmoregulatory capacity, thereby enhancing survival and growth in low-salinity water. Whether dietary cholesterol supplementation improves growth performance in freshwater culture remains unclear. Disease outbreaks have frequently occurred in cultured L. vannamei in recent years, and nutritional modulation is considered an important measure to enhance immune function and disease resistance [14]. Previous studies on dietary cholesterol in L. vannamei have focused on growth, survival, and FCR, with no reports on effects against infection or environmental stress. Moreover, due to fish meal shortages, low-fish-meal diets are a research priority for carnivorous aquaculture species. Studies show that fish meal in L. vannamei diets can be reduced to 6% without affecting growth [15], though commercial diets typically contain 10–25% fish meal depending on life stage and culture mode. Given current disease constraints in L. vannamei culture, investigating the effects of cholesterol supplementation in a 10% fish

meal diet on growth, Vibrio resistance, and stress tolerance under freshwater greenwater (with algae) conditions has practical significance.

1.1 Feed Preparation

Five isonitrogenous and isoenergetic experimental diets with different cholesterol contents were prepared according to the formula in Table 1 . Ingredients were ground to pass through an 80-mesh sieve and mixed using the progressive enlargement method. Oil was added, and water was incorporated to form a dough, which was extruded through a meat grinder into 1.5 mm diameter strands. The strands were cooked at 90°C for 20 minutes, air-dried in a cool, dark place, broken into appropriate sizes for the experimental shrimps, vacuum-packaged, and stored at -20°C until use.

1.2 Culture Management

L. vannamei postlarvae were purchased from Shanghai Xianzhang Fishery Professional Cooperative as desalinated stock (culture water salinity of 5). The postlarvae were stocked in cement tanks (salinity 5) in a greenhouse. Freshwater was added each morning (20 cm) until depth reached 110 cm, then 30 cm water exchange was performed daily until apparent salinity reached 0. Commercial shrimp flakes were fed during desalination, and all freshwater was filtered through 200-mesh silk gauze. After 30 days of acclimation, when juveniles reached $(0.160\$\pm0.002)g, 1,000healthy, uniformlysizedshrimpswereselectedandrandomlydistributedinto20netcages($ m), with 50 juveniles per cage. Five experimental diets were fed, with four cages per diet, at four daily feedings (05:30, 10:30, 16:30, and 22:30) at 5–8% of body weight, adjusted according to weather and feeding behavior. The culture trial lasted 50 days.

To maintain stable algal communities and water quality, heat-tolerant *Scenedesmus quadricauda* and *Golenkinia radiata* were inoculated at the start. Water was exchanged every 5 days using 200-mesh filtered influent, maintaining transparency at 20–40 cm. Dissolved oxygen was maintained >6 mg/L through continuous aeration, ammonia nitrogen <0.2 mg/L, pH 7.8–8.3, water temperature (30$\pm$2)°C, and apparent salinity 0.

1.3.1 Growth Performance Measurement

At the end of the trial, shrimps were fasted for 24 h, then weighed and counted per cage. Eight intermolt shrimps were randomly selected from each cage; hemolymph (1 mL) was withdrawn from the pericardial cavity into 1.5 mL centrifuge tubes, and hepatopancreas and muscle tissues were dissected on ice and transported to the laboratory in an icebox. Hemolymph was centrifuged at 4°C, 10,000 r/min for 20 min to obtain serum, which was stored at -80°C with hepatopancreas samples for subsequent analysis.

Survival rate (SR), feed conversion ratio (FCR), and specific growth rate (SGR) were calculated as follows:

Survival rate = 100 × Nf/Ni
FCR = Wd/(Wf -Wi)
SGR = 100 × (lnWf/Nf -lnWi/Ni)/t

Where: Nf = final shrimp number per cage; Ni = initial shrimp number per cage; Wd = total dry weight of feed fed per cage (g); Wf = final total weight of shrimps per cage (g); Wi = initial total weight of shrimps per cage (g); t = culture duration (days).

1.3.2 Proximate Composition Analysis

Moisture content in feed and muscle was determined by drying to constant weight at 105°C. Crude lipid was measured by chloroform-methanol extraction. Crude ash was determined by incineration at 550°C in a muffle furnace. Crude protein was analyzed by the Kjeldahl method using a KjeltecTM 2300 nitrogen analyzer (Sweden).

1.3.3 Cholesterol Content Determination in Feed, Muscle, Hepatopancreas, and Serum

Cholesterol content was determined according to reference [16]. Briefly, 0.2000-0.5000 g of freeze-dried sample (0.1 mL for serum) was weighed into a 25 mL colorimetric tube, mixed with 0.5 mL of 50% KOH solution, then vortexed immediately. Two mL of absolute ethanol was added, sealed, vortexed, and sonicated in a 65°C water bath for 30 min. After cooling to room temperature, 0.5 mL of 14% boron trifluoride methanol solution and 2 mL absolute ethanol were added sequentially and mixed. The mixture was sonicated again at 65°C for 30 min, then cooled. Three mL of 5% NaCl solution and 10 mL petroleum ether were added, vortexed for 2 min, and allowed to separate. Four mL of the upper petroleum ether layer was transferred to a 10 mL glass tube, dried under vacuum at 50°C, redissolved in 4 mL methanol by sonication, and filtered through a 0.22 m syringe filter. Cholesterol content was analyzed using a Waters e2695 HPLC system with a Waters 2998 photodiode array detector. The column was a Waters XbridgeTM C18 (5 m × 4.6 mm × 250 mm). Following Li et al. [17], the mobile phase was 100% methanol, detection wavelength 205 nm, flow rate 1.00 mL/min, column temperature 38°C, injection volume 50 L, and run time 15 min. Cholesterol standards (Aladdin, $ $99%) were prepared in HPLC-grade methanol at concentrations of 0.001, 0.005, 0.010, 0.020, 0.050, 0.100, 0.200, and 0.500 mg/mL to generate a standard curve and regression equation. Sample cholesterol content was calculated using external standard method.

1.4 Vibrio harveyi Artificial Acute Infection Test

Vibrio harveyi from the pathogen bank of Shanghai Ocean University was used. After the 50-day feeding trial, 30 intermolt shrimps per group were randomly selected and divided into three replicates of 10 shrimps each. Each replicate was held in a 50 cm$\times$30$cm\times$80$cmnetcagesuspendedinthesamecementtank(apparentsalinity0,temperature$27–28$
V.harveyi * suspension(2.5\times10^{7}$ CFU/mL). Mortality was monitored continuously, and cumulative mortality at 24, 36, 48, 72, and 96 h post-infection was recorded.

1.5 Nitrite Nitrogen Artificial Acute Stress Test

After the 50-day feeding trial, another 30 intermolt shrimps per group were randomly selected and divided into three replicates of 10 shrimps each, held in 50 cm$\times$30$cm\times$80$cmnetcages.Allcageswereresuspendedinacementtank(2.8m\times4.5m\times$1.5 m) with nitrite nitrogen concentration maintained at 8.5–9.0 mg/L, measured every 6 h. During stress, water apparent salinity was 0, temperature 27–28°C, with continuous aeration. Mortality was monitored continuously, and cumulative mortality at 24, 36, 48, 72, and 96 h post-stress was recorded.

1.6 Data Processing and Statistical Analysis

Results are expressed as mean ± standard deviation (SD). SPSS 18.0 software was used for one-way ANOVA to analyze relationships between results and dietary cholesterol content. If significant differences were detected, Duncan's multiple comparison test was applied. $P<0.05$ was considered statistically significant.

2.1 Effects of Dietary Cholesterol Content on Growth Performance of Litopenaeus vannamei

As shown in Table 2 , after the 50-day feeding trial, no significant differences in survival rate were observed among groups ($P>0.05$). Final body weight tended to increase with dietary cholesterol content, with the C4 group significantly higher than the C0 group ($P<0.05$). Feed conversion ratio decreased with increasing dietary cholesterol, with C2, C3, and C4 groups significantly lower than the C0 group ($P<0.05$). Specific growth rate tended to increase with dietary cholesterol content, but no significant differences were detected among groups ($P>0.05$).

2.2 Effects of Dietary Cholesterol Content on Muscle Proximate Composition and Tissue Cholesterol Content of Litopenaeus vannamei

As shown in Table 3 , no significant differences in muscle moisture content were observed among groups ($P>0.05$). Muscle crude protein content generally increased with dietary cholesterol level, with C2 and C4 groups significantly higher than C0 and C1 groups ($P<0.05$). Muscle crude lipid content initially increased

then decreased with dietary cholesterol level, with the C2 group significantly higher than all other groups ($P<0.05$). Crude ash content in the C2 group was significantly lower than in the C3 and C4 groups ($P<0.05$).

As shown in Table 4 , cholesterol content in hepatopancreas and muscle increased significantly with dietary cholesterol content ($P<0.05$). Serum cholesterol content initially increased then decreased, with the C2 group significantly higher than the C0 group ($P<0.05$).

2.3 Effects of Dietary Cholesterol Content on Vibrio Resistance of Litopenaeus vannamei

Figure 1 [Figure 1: see original paper] shows cumulative mortality curves of L. vannamei fed different cholesterol diets during 96 h after *V. harveyi* challenge. Cumulative mortality decreased then increased with dietary cholesterol content. The C2 group showed significantly lower cumulative mortality than C0, C3, and C4 groups at 24 and 36 h post-infection ($P<0.05$). At 48, 72, and 96 h post-infection, the C2 group had significantly lower mortality than all other groups ($P<0.05$). No significant differences were observed among C0, C3, and C4 groups at 24, 48, 72, and 96 h ($P>0.05$).

2.4 Effects of Dietary Cholesterol Content on Stress Resistance of Litopenaeus vannamei

Figure 2 [Figure 2: see original paper] shows cumulative mortality curves of L. vannamei fed different cholesterol diets during 96 h of nitrite nitrogen stress. Under nitrite nitrogen concentration of 8.5–9.0 mg/L, no significant differences in cumulative mortality were observed among groups before 48 h ($P>0.05$). At 72 h, C3 and C4 groups showed significantly lower mortality than the C1 group ($P<0.05$). At 96 h, C0 and C1 groups had higher cumulative mortality, significantly exceeding that of C2, C3, and C4 groups ($P<0.05$), while the C2 group was significantly higher than the C4 group ($P<0.05$).

3.1 Effects of Dietary Cholesterol Content on Growth Performance of Litopenaeus vannamei

The effects of dietary cholesterol on growth and survival of L. vannamei have been previously reported. Duerr et al. [8] demonstrated that in seawater culture (salinity 35), optimal growth required 2.3–4.2 g/kg dietary cholesterol (with 10 g/kg phospholipid), with growth inhibition below or above this range. In a seawater recirculating system (salinity 25) using semi-purified diets, optimal cholesterol requirements were negatively correlated with phospholipid supplementation: 0.5, 1.3, 1.4, and 3.5 g/kg cholesterol were required for optimal growth when dietary phospholipid levels were 50, 30, 15, and 0 g/kg, respectively [9]. Under marine culture conditions (salinity 30), optimal dietary cholesterol was 1.10–1.55 g/kg (with 5 g/kg phospholipid) [11]. Excessive dietary cholesterol (>9.2 g/kg) inhibited growth in marine culture [10]. Although reported

requirements vary, optimal dietary cholesterol for marine-cultured L. vannamei generally ranges 0.5–4.2 g/kg, comparable to requirements for other marine shrimp such as *Marsupenaeus japonicus* (5 g/kg) [18], *Penaeus penicillatus* (5–10 g/kg) [19], and *Penaeus monodon* (2–8 g/kg) [20]. Gong et al. [13] reported that excessive cholesterol and phospholipids improve osmoregulatory capacity, enhancing survival and growth in low-salinity water. However, studies in salinity 4 water (indoor and outdoor) showed no significant growth improvement with 2 or 4 g/kg cholesterol supplementation (with 5 or 10 g/kg phospholipid) compared to unsupplemented controls [21]. In the present study, dietary cholesterol ranging 0.78–4.18 g/kg did not significantly affect growth rate, and all groups reached market size after 50 days, indicating that cholesterol supplementation does not significantly improve growth performance in freshwater culture.

In the freshwater greenwater (with algae) conditions of this study, all groups showed high survival rates with no significant differences, consistent with previous reports [8-11,21]. These results suggest that L. vannamei survival is not affected by dietary cholesterol content across marine, brackish, or freshwater conditions. Two possible reasons are hypothesized: first, L. vannamei may be less sensitive to cholesterol deficiency or excess, possessing strong cholesterol regulatory mechanisms that minimize survival impacts; second, algae in the culture system and plant sterols in plant protein ingredients may balance dietary cholesterol effects. Plant proteins and microalgae contain abundant phytosterols such as ergosterol, stigmasterol, and β-sitosterol [22]. Although structurally similar to cholesterol, phytosterols can be converted to cholesterol via dealkylation in some shrimp species [23-24] and may inhibit cholesterol absorption, promote catabolism, and suppress biosynthesis [25]. Research on American lobster (*Homarus americanus*) showed lower total sterol and cholesterol content in whole shrimp fed 1 g/kg cholesterol compared to those fed 1 g/kg cholesterol plus 1 g/kg phytosterols [26].

In this study, FCR in C2, C3, and C4 groups was significantly lower than in C0, indicating that dietary cholesterol affects feed efficiency. Similar results have been reported for marine-cultured L. vannamei and *M. japonicus*. FCR decreased with increasing dietary cholesterol (0.061–0.155 g/kg) in L. vannamei [11], and feed conversion efficiency increased with dietary cholesterol (0.4–4.3 g/kg) in *M. japonicus* [27]. Supplementing low levels of cholesterol to β-sitosterol-containing diets improved feed conversion in *M. japonicus* [28], and decreasing the cholesterol:β-sitosterol ratio reduced feed conversion when total sterol was 10 g/kg [29].

3.2 Effects of Dietary Cholesterol Content on Tissue Cholesterol Content and Muscle Proximate Composition

This study showed that muscle cholesterol content was higher than hepatopancreas cholesterol content. When *Penaeus esculentus* was fed ^{14}C-labeled cholesterol, 47% was recovered in muscle and 40% in hepatopancreas after 72 h [30], indicating that most dietary cholesterol is deposited in muscle considering tissue

mass proportions. Hepatopancreas and muscle cholesterol contents in L. vannamei increased significantly with dietary cholesterol, while serum cholesterol increased then decreased. Whole-body cholesterol content in *M. japonicus* was positively correlated with dietary cholesterol (0.4–4.3 g/kg) [27], and *P. monodon* whole-body cholesterol increased with dietary cholesterol [20]. However, studies on phospholipid-cholesterol interactions in L. vannamei found no significant effect of dietary cholesterol on hepatopancreas or muscle cholesterol, though phospholipid and its interaction with cholesterol significantly affected muscle cholesterol [9], possibly related to phospholipid' s role in cholesterol transport [31]. From a food nutrition perspective, excessive cholesterol intake causes cardiovascular disease in humans, and crustacean muscle contains higher cholesterol than fish [32]. This study suggests that reducing dietary cholesterol appropriately can produce lower-cholesterol shrimp products without compromising growth.

Muscle crude lipid in *P. penicillatus* increased with dietary cholesterol [19], and whole-body crude lipid in L. vannamei increased with dietary cholesterol [10]. However, other studies showed no positive correlation between dietary cholesterol and muscle crude lipid in L. vannamei [9]. In this study, muscle crude lipid increased then decreased with dietary cholesterol, while muscle crude protein generally increased, showing a positive correlation with muscle cholesterol content. This may be related to osmoregulatory mechanisms in freshwater L. vannamei. In freshwater, internal osmotic pressure exceeds external pressure, requiring cells to prevent water influx or actively pump out water. As a membrane component, cholesterol reduces water and sodium permeability by inhibiting fatty acid chain mobility and phospholipid headgroup rotation, conserving ATP and energy for protein synthesis [33-34]. Additionally, muscle is the largest amino acid pool in crustaceans [35], and free amino acids are important for osmoregulation [3]; cholesterol' s osmoregulatory role may reduce the need for free amino acids to balance osmotic pressure, potentially explaining the positive correlation between muscle crude protein and cholesterol in freshwater L. vannamei.

3.3 Effects of Dietary Cholesterol Content on Vibrio and Stress Resistance of Litopenaeus vannamei

No published reports exist on dietary cholesterol effects on antimicrobial ability in aquatic animals. In this study, muscle and hepatopancreas cholesterol increased with dietary cholesterol, yet cumulative mortality after Vibrio challenge decreased then increased, with 2.45 g/kg dietary cholesterol showing strongest Vibrio resistance, while both lower and higher levels reduced resistance. The physiological response to nutrients follows a parabolic relationship, peaking then reversing with increasing nutrient levels [36]. Antimicrobial ability involves multiple effectors including lysozyme, nitric oxide (NO), and superoxide anion. NO effectively kills *V. harveyi* in L. vannamei [37]. In mice, increased dietary cholesterol enhanced erythrocyte xanthine oxidase activity, producing

more superoxide anion for bactericidal effects, while excessive cholesterol inhibited erythrocyte NO synthase activity, which catalyzes L-arginine to L-citrulline and generates NO for antibacterial action [38]. The optimal Vibrio resistance at 2.45 g/kg dietary cholesterol likely represents the combined efficacy of various immune factors.

Nitrite nitrogen is a major toxic pollutant stressing L. vannamei in culture systems. In *M. japonicus*, body moisture increased while hemolymph protein and free amino acids decreased with increasing nitrite concentration and exposure duration, reducing hemolymph osmotic pressure and causing metabolic disorder and death [39]. In this study, cumulative mortality under nitrite stress generally decreased with increasing dietary cholesterol over 96 h, indicating that higher dietary cholesterol enhances nitrite nitrogen stress resistance in freshwater L. vannamei. The mechanism may involve cholesterol' s role in osmoregulation under hypotonic conditions, though detailed mechanisms require further investigation.

In conclusion, under freshwater culture conditions, 1.57 g/kg dietary cholesterol meets the growth requirements of L. vannamei, while 2.45 g/kg provides optimal Vibrio resistance and desirable nitrite nitrogen stress resistance.

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Note: Figure translations are in progress. See original paper for figures.