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Effects of dietary vitamin C on the growth performance, muscle composition, non-specific immunity, and resistance of juvenile ivory shell (*Babylonia areolata*) to ammonia

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ABSTRACT

Vitamin C (VC) plays an essential role in the physiological function and normal growth of aquatic animals. However, the effects and requirements of VC on juvenile ivory shell (*Babylonia areolata*) are still unknown. This study aimed to investigate the effects of VC on growth, muscle composition, non-specific immunity, and resistance to ammonia stress of *B. areolata* and determine its requirement for VC. Six experimental diets (Diet 1, Diet 2, Diet 3 Diet 4 Diet 5, and Diet 6) with different levels of VC (0, 100, 200, 400, 800, and 1200 mg/kg diet, respectively) were fed to juvenile *B. areolate* for 3 months. The weight gain rate (WGR) and specific growth rate (SGR) of *B. areolata* increased in parallel with the VC content, and those of the 800 mg/kg group were significantly higher than those of the control group. There was no significant difference in the survival rate (SR), flesh shell ratio (FSR), viscerosomatic index (VSI), soft tissue index (STI), and muscle composition among the experimental groups. VC (800 mg/kg) significantly increased the total antioxidant capacity (T-AOC) of the hepatopancreas. In addition, VC (400–800 mg/kg) significantly increased the activities of superoxide dismutase (SOD) and alkaline phosphatase (AKP) of the hepatopancreas and significantly decreased the content of malondialdehyde (MDA). VC significantly induced the expression levels of functional genes, such as *SOD*, *GST*, *CYP450*, *ferritin*, *mucin-5AC*, and *CYC* in the hepatopancreas and increased the survival rate of *B. areolata* under ammonia stress. These results indicate that supplementation with dietary VC could increase the growth, antioxidant capacity, immunity, and resistance against the stress caused by ammonia. The optimal dietary VC requirement for juvenile *B. areolata* was 400–800 mg/kg of VC.

1. Introduction

Ivory shell (*Babylonia areolata*) is a carnivorous gastropod species of the phylum Mollusca, class Gastropoda, order Stenoglossa, and family Buccinidae. It is a marine shellfish found in warm water that is widely distributed in the tropical and subtropical waters of the Indo-Pacific Ocean (Zhou et al., 2023). The aquaculture industry of *B. areolata* has been developing rapidly in the coastal areas of Guangdong, Fujian, and Hainan in China, and some Southeast Asian countries, including Thailand and Vietnam (Li et al., 2024). It has become the main economic shellfish along the southeastern coast of China, and the scale of *B. areolata* aquaculture has been expanding yearly (Lü et al., 2020). *B. areolata* is favored by consumers and aquaculturalists because of its quick rate of growth, high economic value, delicate taste, and high content of unsaturated fatty acid (UFA) (Ding et al., 2023). It is considered to be a high-quality marine cultured shellfish that is commercially viable (Liu et al., 2023). The rapid development in aquacultural technology has led to the increasing density of cultivation of *B. areolata* in recent years (Mai et al., 2022). However, intensive farming uses chilled fish as feed, which has resulted in inappropriate

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nutritional intake, frequent aquatic diseases and significant losses (Chen et al., 2010). To ensure the long-term survival of cultured aquatic animals, chemicals and antibiotics have been widely used (Zhang et al., 2009). This has resulted in many hazards, including environmental pollution, resistance to antibiotics, and antibiotic residues in food (Hedberg et al., 2018). Improving the immune ability of cultured animals is important for the development of aquaculture (Lu et al., 2023).

Vitamin C (VC), known as L-ascorbic acid (anti-scurvy) because of its preventive effect against this condition, has strong reducing properties and is a natural antioxidant and an important nutrient for aquatic animals to maintain their normal physiological functions (Yusuf et al., 2021). It is an immunomodulator used as a feed additive to improve the health of farm animals and reduce the use of antibiotics (Laltlanmawia et al., 2019). Most aquatic organisms, such as fish, shrimp, and crabs, are deficient in gluconolactone oxidase and cannot synthesize enough VC by themselves; it can only be provided by food (Asaikkutti et al., 2018; Zou et al., 2020). VC promotes the metabolism, growth, and disease resistance of aquatic animals and regulates the reproductive process by affecting the biosynthesis of sex hormones (Shao et al., 2018). Dietary supplementation with VC promotes the specific growth rate (SGR) in Caspian roach (*Rutilus rutilus caspicus*) (Nikjoo et al., 2023) and significantly improves the antioxidant activity and immunity, such as the activities of alkaline phosphatase (AKP), superoxide dismutase (SOD), and immunoglobulin (lg), in juvenile cobia (*Rachycentron canadum*) (Zhou et al., 2012). Studies on the appropriate requirement for VC have reported that 400 mg/kg VC could serve as a suitable dietary supplement for enhancing the growth, hepatic and intestinal structures, immune status, and resistance against *Aeromonas sobria* in Nile tilapia (*Oreochromis niloticus*) (Ibrahim et al., 2020).

There is limited information concerning the roles of VC in growth, antioxidant status, immunity, and stress resistance of *B. areolata*. Therefore, this study aimed to determine the dietary VC requirement of this mollusk and evaluate its effects on the growth performance, muscle composition, immunity, and ammonia resistance of *B. areolate* following treatment with different concentrations of VC for 3 months of cultivation experiments. In addition, it sought to lay a foundation for the application of VC as a stress protectant and immunostimulant in aquatic feed.

2. Materials and methods

2.1. Experimental diets

The composition and nutrient content of the basal diets are shown in Table 1. The form of VC used in this study was L-ascorbyl-2-polyphosphate (35 % ascorbic acid equivalent) (Shanghai Yuanye Biological Co., Ltd., Shanghai, China). The VC contents of the six experimental diets were 0 (Diet 1, control group), 100 (Diet 2), 200 (Diet 3), 400 (Diet 4), 800 (Diet 5), and 1200 (Diet 6) mg/kg diet. We detected the actual concentration of VC by ELISA using reagent kits (MM-926863O1) according to the manufacturer's instructions (Jiangsu Meimian industrial Co., Ltd., Jiangsu, China). The VC concentrations in these experimental diets were 2.46, 37.68, 71.50, 144.09, 276.33, and 425.71 mg/kg, respectively. The raw materials were crushed and passed through an 80-mesh sieve before formulation. All the ingredients were weighed according to the feed formulation and mixed with a V-mixer. Fish oil and 30 % water were added to the basic feed, and the mixture was kneaded into a dough-like soft pellet feed. These feeds were then divided into small dough-like pieces following the estimated daily feeding amount and stored at −20°C.

Table 1

Composition and level of nutrients in the base diet.

Ingredients	Content (%)	Nutrient levels	Content (%)
Fish Meal	46.00	Crude protein	42.76
Soybean meal	24.00	Crude lipid	13.10
α-Starch	24.10	Ash	9.36
Vitamin premix[a]	0.40	Moisture	8.08
Mineral premix[b]	1.00		
Fish oil	3.00		
Monocalcium phosphate	1.50		

[a] Vitamin premix: 100 g premix contains vitamin A 200000 IU, vitamin D 20000 IU, vitamin B_1 15 mg, vitamin B_2 300 mg, vitamin B_6 200 mg, vitamin B_{12} 0.3 mg, vitamin K 15 mg, folic acid 15 mg, biotin 0.75 mg, vitamin E 2000 IU, inositol 5000 mg, pantothenic acid 500 mg, niacin 1500 mg.

[b] Mineral premix: 100 g premix contains $ZnSO_4{\bullet}7\ H_2O$ 1377.5 mg, Na_2SeO_3 13.5 mg, $CuSO_4{\bullet}5\ H_2O$ 416.5 mg, $FeSO_4{\bullet}7\ H_2O$ 4909.5 mg, $MnSO_4{\bullet}H_2O$ 728.5 mg, $CoSO_4{\bullet}7\ H_2O$ 5.9 mg, $Ca(IO_3)_2{\bullet}7\ H_2O$ 5.9 mg.

2.2. Experimental animal and experimental procedures

The experiment was conducted at the temporary breeding facility at the Chinese Academy of Tropical Agricultural Sciences (CATAS) Research and Experimental Base (Wenchang, Hainan, China). A total of 720 healthy *B. areolata* of similar sizes (average body weight: 0.11 ±0.01 g) were randomly allocated in 18 glass breeding tanks (60 cm×40 cm×50 cm) in a volume of 65 L water. Experimental *B. areolata* was obtained from the Longyuan Aquaculture Farm (Wenchang, Hainan, China). The *B. areolata* were acclimated in the culture system for 2 weeks, and they were fed basal diets (with no VC supplementation) during this period. *B. areolata* was randomly divided into six groups, with three replicates in each group, and 40 individuals for each tank. The bottom was covered with a 3–4 cm thick layer of sand. During the trial, the water temperature (27°C-30°C), pH (7.2–7.8), and salinity (30 ‰-31 ‰) were maintained in the recirculating water culture. *B. areolata* were fed twice a day for 3 months.

2.3. Sample collection

The *B. areolata* from each tank were counted and collectively weighed at the end of the feeding trial after 24 h of starvation. The samples were then quick-frozen in liquid nitrogen and stored at −80°C. For the experimental measurements, *B. areolata* were removed; the snail meat and viscera were removed and weighed separately, and the growth performance and body indices were calculated. The muscle was stored at −20°C to analyze its composition. The hepatopancreas was isolated and washed with saline to determine the immunoenzymatic activity and related analyses of gene expression.

2.4. Data collection

2.4.1. Growth performance

The parameters of growth performance and body indices were calculated using the following formulae:

Weight gain rate (WGR, %) = 100 × (final average weight - initial average weight) / initial average weight

Specific growth rate (SGR, %/d) = 100 × [Ln(final average weight) - Ln(initial average weight)] / duration of experiment

Survival rate (SR, %) = 100 × final snail number / initial snail number

Flesh shell ratio (FSR, %) = 100 × (soft tissue weight / shell weight)

Viscerosomatic index (VSI, %) = 100 × (visceral weight / whole body weight)

Soft tissue index (STI, %) = 100 × (soft tissue weight / whole body weight)

2.4.2. Proximate composition analysis

The muscle composition was determined according to the established methods of the AOAC (AOAC, 2005). The moisture was assessed by drying at 105°C. The crude protein was determined using a Kjeldahl apparatus (nitrogen × 6.25) after the sample had been digested with acid. The crude lipid was extracted with petroleum ether (FOSS-Soxtec

2050, Hoganas, Sweden) and measured using a Soxhlet apparatus (SZF-06A, Shanghai, China). The ash was determined by incineration at 550°C.

2.4.3. *Enzyme activity analysis*

The frozen tissues of *B. areolata* were weighed, thawed at 4°C, and homogenized with pre-cooled and sterilized saline solution (0.9 % NaCl) at a ratio of 1 : 9 (weight : volume). These samples were centrifuged at 2500 rpm for 10 min, and the supernatant was collected in 1.5 mL tubes to assay the enzymes. The activities of hepatopancreas superoxide dismutase (SOD, A001–3–2), catalase (CAT, A007–1–1), acid phosphatase (ACP, A060–2–2), and alkaline phosphatase (AKP, A059–2–2), and the contents of total antioxidant capacity (T-AOC, A015–2–1) and malondialdehyde (MDA, A003–2–2) were determined using reagent kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

2.4.4. *Gene expression level analysis*

Real-time quantitative PCR (RT-qPCR) was utilized to determine the expression levels of *SOD*, *GST*, *Cu/Zn-SOD*, *CYP450*, *ferritin*, *ACP*, *mucin-5AC*, and *CYC* from the hepatopancreas mRNA. The primer sequences of RT-qPCR are shown in Table 2. The TRIzol reagent (Invitrogen, Waltham, MA, USA) was used to extract the total hepatopancreas RNA, and the integrity of the total RNA was demonstrated using 1 % agarose gel electrophoresis. The RNA extracts were treated with a PrimeScript RT reagent kit with a gDNA eraser (Takara, Dalian, China) to remove potential DNA contamination. A NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the quality of RNA extracts. The ratio of absorbance of the RNA extract was 1.80–2.10 at 260/280 nm and 260/230 nm, which indicated its high standard quality. Reverse transcription of the complementary DNA (cDNA) was performed using a Prime Script™ RT Reagent Kit (Takara Bio, Shiga, Japan). The RT-qPCR mixture (20 μL) contained 10 μL of 2 × SYBR® Green qPCR Mix, 1 μL of cDNA, 0.4 μL of each primer, and 8.2 μL of PCR-grade water. The thermal cycling parameters were as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 15 s, 58°C for 15 s and 72°C for 20 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression levels of the genes (Livak and Schmittgen, 2001).

2.5. *Challenge test*

After the end of sampling, 10 *B. areolata* individuals were randomly sampled from each tank for the ammonia stress test. The lethal concentration 50 (LC_{50}) of ammonia was determined to be 400 mg/L for 48 h from the pretest. The acute stress test was conducted with ammonium chloride (NH_4Cl) at 400 mg/L for 48 h, during which the crustaceans were not fed, and the water was not changed. The number of dead individuals was recorded at 12, 24, and 48 h to calculate the final mortality rate.

Table 2
Primers used in the quantitative real-time PCR.

Primers	Sequence (5'-3')
β-actin-F	CGTCCTTTGGTGACTCTGG
β-actin-R	TGGATGTGGTAGCCGTTTC
SOD-F	AGCACGGAAGTCAAAGGAGA
SOD-R	CAAACTGATGGATGTGGAAGC
GST-F	TCTTCTGGGGTTCTGGTAGC
GST-R	CCTGATTCATTGACAATGGTG
Cu/Zn-SOD-F	GACACTTCAACCCCTTCGG
Cu/Zn-SOD-R	TCACTACAGCCTTGCCACTG
CYP450-F	AACCCTCGCCATTTATCG
CYP450-R	GTTGTCAGCAGCATCGGA
mucin-5AC-F	CAACAGGTTCCTCATCTTCG
mucin-5AC-R	AAGGAGGATGCGGGAGA
CYC-F	GCAGGAAATGCCGAGAAG
CYC-R	AGTCTTGCGTCCAATCAGG
ferritin-F	CAACGGTCACAACGATGCT
ferritin-R	TGGTCGCCGATTTCCTT
ACP-F	CAACTTCACCAAGAACACGG
ACP-R	TGAGTGCTGTTGTGGATGGT

SOD: superoxide dismutase; GST: glutathione S-transferase A-like; Cu/Zn-SOD: Cu/Zn-superoxide dismutase; CYP450: cytochrome P450 3A21-like; CYC: cytochrome c; ACP: iron/zinc purple acid phosphatase-like protein.

2.6. *Statistical analysis*

The values were expressed as the mean ± standard deviation (SD). All the data were analyzed using a one-way analysis of variance (ANOVA) followed by the Tukey multiple comparisons test. Levene's and Shapiro-Wilk tests were used to assess the homogeneity of variance and normality of the data, respectively. SPSS 15.0 (SPSS, Inc., Chicago, IL, USA) was used for all the statistical analyses. Differences were considered statistically significant at $P < 0.05$. A broken-line regression was utilized to evaluate the optimal dosage of dietary VC for *B. areolata*.

3. Results

3.1. *Growth performance*

The effects of different concentrations of VC on the growth performance and survival rate (SR) of *B. areolata* are shown in Table 3. The addition of VC to the diet had no significant effect on the SR, soft tissue index (STI), flesh shell ratio (FSR), and viscerosomatic index (VSI) of *B. areolata* when compared with the control group ($P > 0.05$). Compared to the control group, the Diet 5 group increased its WGR by 78.51 % ($P < 0.05$). The rest of the experimental groups did not differ significantly ($P > 0.05$). The SGR in the Diet 5 group was 1.87 ± 0.03, which was substantially higher than that in the control group (1.62 ± 0.08) ($P < 0.05$). A linear regression analysis of the WGR and SGR indicated that the requirements of VC for *B. areolata* were 273.78 and 284.98 mg/kg, respectively (Fig. 1A and B).

3.2. *Proximate composition*

The effects of dietary levels of VC on the muscle proximate composition of *B. areolata* are shown in Table 4. The moisture ranged from 71.20 % to 73.36 %, and the content of crude protein varied from 45.51 % to 54.52 %. The content of crude lipid varied from 7.54 % to 9.24 %, while the content of ash ranged between 10.98 % and 14.41 %. There were no significant differences in the muscle ash, crude protein, crude lipid, and moisture content of *B. areolata* following the addition of VC when compared to the control group ($P > 0.05$).

3.3. *Immune function*

The immune ability of the *B. areolata* hepatopancreas with different concentrations of VC is shown in Fig. 2. The activity of AKP in the Diet 4 and 5 groups with 400 mg/kg and 800 mg/kg were significantly higher when compared to the control group ($P < 0.05$). There was no significant difference in the activity of ACP from *B. areolata* following the addition of VC compared to the control group ($P > 0.05$).

3.4. *Antioxidant function*

The antioxidant enzyme activity of the *B. areolata* hepatopancreas with different concentrations of VC is shown in Fig. 3. The activities of SOD in the Diet 4, 5, and 6 groups were significantly higher than those of the control group ($P < 0.05$). The T-AOC in the Diet 5 group with 800 mg/kg was significantly higher when compared to that of the control group ($P < 0.05$). The contents of MDA from the Diet 4 and 5 groups were significantly lower than that of the control group ($P < 0.05$).

Table 3
Effects of vitamin C on the growth performance of *Babylonia areolata*.

Diet mg/kg	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	*P* value[a]	*P* value[b]	Regression analysis		
	0	100	200	400	800	1200			Linear	Quadratic	Cubic
Initial body weight, IBW (g)	0.11 ± 0.01^{a}	0.11 ± 0.01^{a}	0.11 ± 0.01^{a}	0.11 ± 0.01^{a}	0.11 ± 0.00^{a}	0.11 ± 0.01^{a}	0.831	0.052	0.446	0.755	0.888
Final body weight, FBW (g)	0.40 ± 0.01^{d}	0.42 ± 0.01^{cd}	0.44 ± 0.02^{bc}	0.47 ± 0.03^{abc}	0.50 ± 0.00^{a}	0.47 ± 0.01^{ab}	0.103	0.147	< 0.01	< 0.01	< 0.01
WGR, %	249.36 ± 19.43^{b}	291.11 ± 44.13^{ab}	312.95 ± 49.58^{ab}	308.15 ± 19.23^{ab}	345.72 ± 11.14^{a}	320.37 ± 34.03^{ab}	0.191	0.251	< 0.01	< 0.01	0.021
SGR, %/d	1.56 ± 0.07^{b}	1.70 ± 0.14^{ab}	1.77 ± 0.16^{ab}	1.76 ± 0.06^{ab}	1.87 ± 0.03^{a}	1.79 ± 0.10^{ab}	0.231	0.223	< 0.01	< 0.01	0.017
SR, %	86.67 ± 1.44^{a}	88.33 ± 3.82^{a}	90.00 ± 2.50^{a}	91.67 ± 6.29^{a}	93.33 ± 1.44^{a}	90.83 ± 3.82^{a}	0.231	0.143	0.036	0.061	0.110
STI, %	0.35 ± 0.02^{a}	0.38 ± 0.04^{a}	0.36 ± 0.04^{a}	0.35 ± 0.08^{a}	0.36 ± 0.05^{a}	0.37 ± 0.03^{a}	0.100	0.729	0.840	0.681	0.582
FSR, %	0.37 ± 0.02^{a}	0.40 ± 0.05^{a}	0.38 ± 0.04^{a}	0.37 ± 0.09^{a}	0.40 ± 0.06^{a}	0.39 ± 0.04^{a}	0.106	0.714	0.813	0.782	0.726
VSI, %	5.09 ± 0.60^{a}	6.20 ± 2.72^{a}	5.22 ± 2.53^{a}	5.10 ± 1.53^{a}	7.59 ± 2.25^{a}	4.92 ± 1.70^{a}	0.054	0.071	0.774	0.953	0.952

Means in the same row with different superscripts are significantly different ($P < 0.05$). Orthogonal polynomial contrasts were used to assess the significance of linear, quadratic, or cubic models to describe the response in the dependent variable to the level of VC. FSR, flesh shell ratio; SGR, specific growth rate; SR, survival rate; STI, soft tissue index; VSI, viscerosomatic index; WGR, weight gain rate.

[a] *p* value: p value of the Levene's test.

[b] *p* value: p value of the Shapiro-Wilk test.

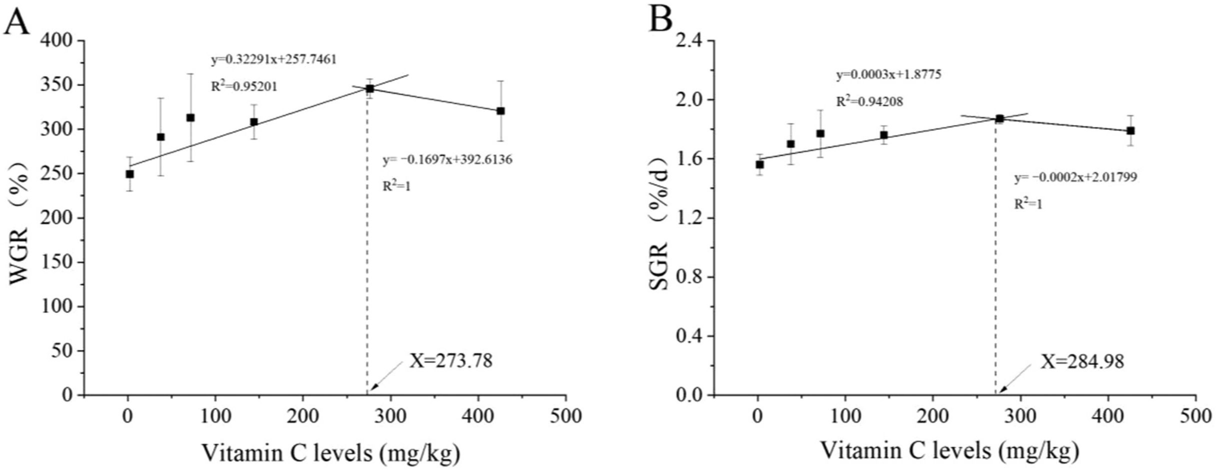

Fig. 1. Scatter regression analyses of WGR and SGR with dietary levels of vitamin C in *Babylonia areolata*. A: weight gain rate; B: specific growth rate.

Table 4
Effects of vitamin C on the proximate composition of *Babylonia areolata* (Dry weight).

Diets mg/kg	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	P value[a]	P value[b]	Regression analysis		
	0	100	200	400	800	1200			Linear	Quadratic	Cubic
Moisture %	72.64 ± 0.40^{a}	71.20 ± 1.07^{a}	71.31 ± 0.46^{a}	72.49 ± 1.38^{a}	73.36 ± 1.43^{a}	72.35 ± 0.94^{a}	0.270	0.709	0.211	0.360	0.120
Crude protein %	53.50 ± 3.02^{a}	46.91 ± 1.17^{a}	48.65 ± 8.07^{a}	49.69 ± 2.22^{a}	54.52 ± 1.43^{a}	45.51 ± 2.94^{a}	0.074	0.202	0.182	0.338	0.089
Crude lipid %	9.11 ± 0.35^{a}	9.22 ± 0.25^{a}	8.36 ± 0.41^{a}	8.73 ± 0.35^{a}	9.24 ± 1.39^{a}	7.54 ± 0.44^{a}	0.080	0.933	0.050	0.070	0.018
Ash %	10.98 ± 0.73^{a}	13.76 ± 1.72^{a}	14.41 ± 1.89^{a}	13.29 ± 0.52^{a}	12.52 ± 1.85^{a}	13.26 ± 0.33^{a}	0.075	0.734	0.722	0.700	0.112

Means in the same row with different superscripts are significantly different ($P < 0.05$). Orthogonal polynomial contrasts were used to assess the significance of linear, quadratic, or cubic models to describe the response in the dependent variable to the level of VC.

[a] *p* value: the p value of the Levene's test.

[b] *p* value: the p value of the Shapiro-Wilk test.

3.5. Gene expression

The relative levels of expression of *SOD*, *Cu/Zn-SOD*, *GST*, *CYP450*, and *ferritin* in the hepatopancreas of *B. areolata* are shown in Fig. 4. The relative levels of expression of *SOD* in the hepatopancreases of *B. areolata* fed Diet 4, 5 and 6 groups increased significantly compared with the control group ($P < 0.05$). The relative level of expression of *CYP450* was significantly higher in the Diet 5 group than in the control group ($P < 0.05$). The relative level of expression of *ferritin* was significantly higher in the Diet 2, 4, and 5 groups than that in the control group ($P < 0.05$). However, there were no significant differences in the expression levels of *Cu/Zn-SOD* among the groups ($P > 0.05$).

The relative levels of expression of *ACP*, *mucin-5AC*, and *CYC* in the hepatopancreas of *B. areolata* are shown in Fig. 5. The relative levels of expression of *mucin-5AC* and *CYC* were significantly higher in the Diet 4 and 5 groups when compared with the control group ($P < 0.05$). The

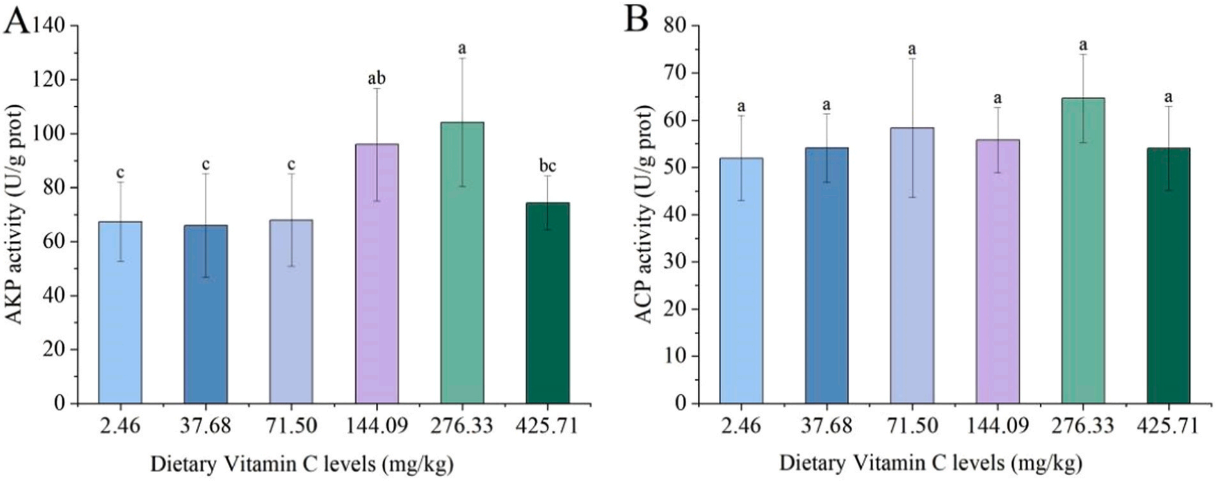

Fig. 2. Immune ability of the *Babylonia areolata* hepatopancreas fed diets that contained different concentrations of vitamin C. Different letters indicate significant differences ($P < 0.05$). A: AKP activity, B: ACP activity.

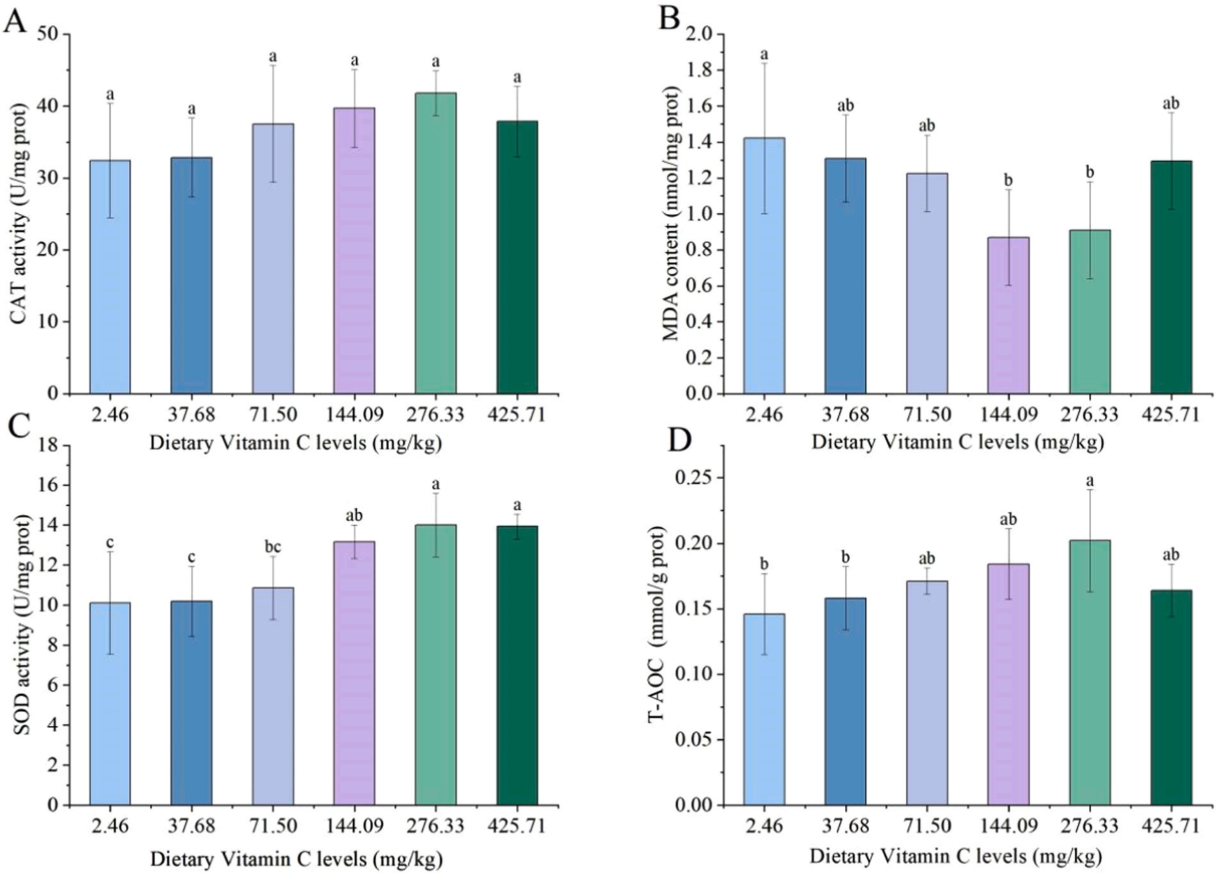

Fig. 3. Antioxidant capacity of the *Babylonia areolata* hepatopancreas fed diets that contained different concentrations of vitamin C. Different letters indicate significant differences ($P < 0.05$). A: CAT activity, B: MDA content, C: SOD activity, D: T-AOC.

relative levels of expression of *ACP* were not significantly different when compared with the control group ($P > 0.05$).

3.6. Challenge test

The results of ammonia stress are shown in Fig. 6. *B. areolata* fed with different concentrations of VC were stressed with 400 mg/L ammonia. Compared with the control group, the addition of 400–800 mg/kg of VC to the feed significantly improved the ability of *B. areolata* to resist ammonia stress ($P < 0.05$).

4. Discussion

Most aquatic animals cannot synthesize VC, and as a type of water-soluble vitamin, it does not accumulate in the body for long periods (Yu et al., 2020). VC, as an essential nutrient for the body, is commonly added to aquatic animal feed (Ghafarifarsani et al., 2022). VC participates in the redox process of the body and plays a positive role in

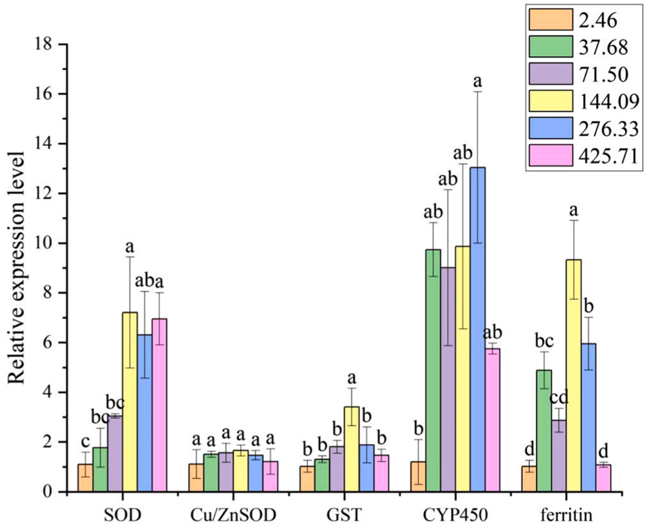

Fig. 4. Effects of vitamin C on the expression levels of antioxidant related genes in the hepatopancreas of *Babylonia areolata*. Different letters indicate significant differences ($P < 0.05$).

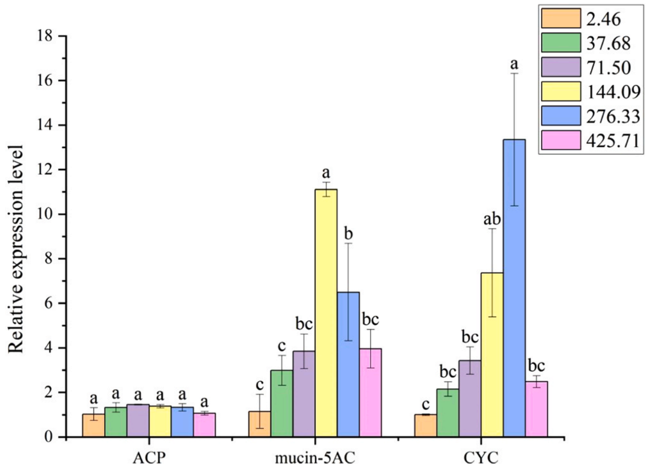

Fig. 5. Effects of vitamin C on the expression levels of the genes related to immunity in the hepatopancreas of *Babylonia areolata*. Different letters indicate significant differences ($P < 0.05$).

immunity, resistance to stress, improvement in meat quality, and detoxification among others and promotes the growth of aquatic animals (Asaikkutti et al., 2016). However, few studies have been conducted on the nutrition of shellfish regarding their needs for vitamins. In this study, the WGR and SGR of *B. areolata* increased significantly as the levels of dietary VC increased from 0 to 800 mg/kg. Based on growth (WGR and SGR), broken-line analysis projected the dietary vitamin C requirement of *B. areolata* to 273.78–284.98 mg/kg, which was similar with those of red swamp crayfish (*Procambarus clarkia*) (265.67 mg/kg) (Kong et al., 2021) and tilapia *Oreochromis spilurus* (200 mg/kg) (Al-Amoudi et al., 1992), but it was higher than those of many other fish species. VC (102.6–147.8 mg/kg) significantly increased the WGR and SGR of largemouth bass (*Micropterus salmoides*) (Chen et al., 2015). Hu et al., (2020) found that the addition of VC (80.66 mg/kg) to the feed increased the WGR of ricefield eel (*Monopterus albus*). The differences are probably due to the differences in aquatic animal species and size, feed formulations, feeding behaviors, nutritional status and rearing conditions of aquatic animal. The improved growth performance of *B. areolata* may be attributed to the increased utilization of protein and the activities of amino acyl transferase and digestive enzymes owing to the supplementation with VC (Cai et al., 2022). The addition of different concentrations of VC did not significantly affect the SR, STI, FSR, and VSI of *B. areolata* compared to the control group. VC significantly increased the hepatosomatic index of Kuruma shrimp (*Marsupenaeus japonicus*) (Nguyen et al., 2012). However, studies regarding the effects of VC on the body indices (STI, FSR, and VSI) in shellfish are still limited. More information on the effects of shellfish nutrition on the body index merits further study.

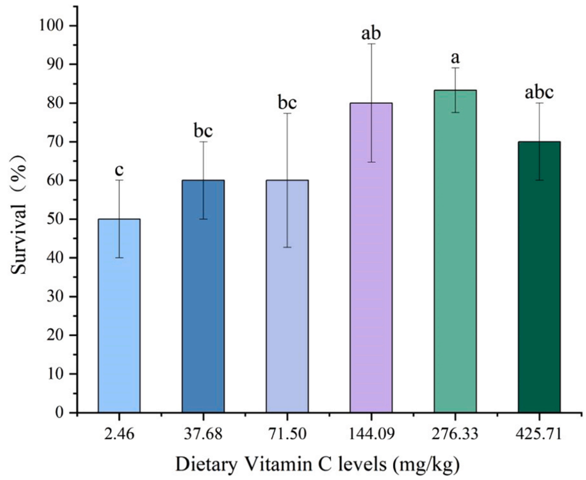

Fig. 6. Survival rate of *Babylonia areolata* fed diets supplemented with vitamin C under the stress of 400 mg/L ammonia. Different letters indicate significant differences ($P < 0.05$).

The muscle composition of an organism reflects its value as a nutrient and its ability to utilize nutrients (Banaee et al., 2015). In this study, the level of supplementation of dietary VC did not have a significant impact on the muscle moisture, crude protein, crude lipid, and ash content of the snail, which is similar to the results obtained in Coho salmon (*Oncorhynchus kisutch*) (Xu et al., 2022), large yellow croaker (*Larimichthys crocea*) (Wei et al., 2020), broodstock Japanese eel (*Anguilla japonica*) (Shahkar et al., 2015), and Chinese soft-shell turtle (*Pelodiscus sinensis*) (Wang and Huang, 2015). Dietary supplementation with VC increased the crude lipid content of rainbow trout (*Oncorhynchus mykiss*) (Eharifzadeh et al., 2017) and Caspian brown trout (*Salmo trutta caspius*) (Arab and Rajabi Islami, 2015) and reduced the moisture content of freshwater prawn (*Macrobrachium rosenbergii*) (Asaikkutti et al., 2018) and Caspian trout (*Salmo caspius*) (Saheli et al., 2021). Several factors may contribute to this discrepancy, including the following: (1) Supplementation of the feed with VC can have differential effects on the metabolism of proteins, lipids, and sugars in aquatic animals; (2) The activities of digestive enzymes in aquatic animals may be affected by dietary VC; and (3) The levels of supplementation with dietary VC may affect the metabolism of nutrients.

Aquatic animals produce reactive oxygen species (ROS) during the oxidation process (Donaghy et al., 2015). However, the increased production of ROS in cells leads to oxidative stress and subsequent cell and tissue damage (Zhu et al., 2022). The body removes ROS through non-enzymatic systems, such as antioxidant enzymatic systems and vitamins with antioxidant effects, which protects the tissues and cells from oxidative damage (Ahmadinejad et al., 2017). The antioxidative ability is normally used to indicate the status of the health of aquatic animals (Yang et al., 2021). The T-AOC is an important comprehensive indicator that reflects the antioxidant capacity of the animal (Xu et al., 2018). This study revealed that dietary supplementation with 800 mg/kg significantly increased the T-AOC compared to the control diet, which is consistent with the results from abalone (*Haliotis discus hannai*) (Luo et al., 2021) and Coho salmon (*Oncorhynchus kisutch*) (Xu et al., 2022). SOD and CAT can eliminate excess ROS and maintain the balance of free radicals, which reduces the damage caused by lipid peroxidation (Liu et al., 2016). MDA is the final product of lipid peroxidation and is toxic owing to its ability to damage the structure and function of cells. Its content can be used to evaluate the degree of lipid peroxidation (Tsikas, 2017). In this study, dietary VC increased the activities of SOD and CAT and decreased the content of MDA. Similar findings were observed in studies of the Caspian roach (*Rutilus rutilus caspicus*) (Nikjoo et al., 2023), juvenile grass carp (*Ctenopharyngodon idella*) (Nasar et al., 2021), and juvenile cobia (*Rachycentron canadum*) (Zhou et al., 2012). All these are considered to be attributed to the powerful antioxidative activity of VC. Acting as an electron donor, VC is an antioxidant that scavenges free radicals, thus inhibiting radical injuries to cellular components (Song et al., 2023).

Glutathione S-transferase (GST) can participate in the regulation of oxidative stress signaling pathways, which can help to limit the amount of cell death caused by hydrogen peroxide (H_2O_2) and other toxic molecules and bind a variety of endogenous and exogenous ligands to participate in the repair of oxidatively damaged biological macromolecules and the regeneration of proteins that contain oxidized sulfhydryl groups (Feng et al., 2023). CYP450 is a mixed-function oxidase that can catalyze a variety of redox reactions and plays an important role in the metabolism of endogenous and exogenous substances (Mohamed et al., 2020). Ferritin is an important defense protein in the antioxidant system and a key protein for iron storage that maintains homeostasis. It can transform the conversion of toxic ferrous iron (Fe^{2+}) into non-toxic ferric oxide (Fe^{3+}) that is stored in the inner cavity of ferritin nanocage, which can reduce the occurrence of the Fenton reaction and inhibit the formation of harmful ROS (Wake et al., 2020). In this study, the levels of translation of the mRNA for *SOD*, *GST*, *CYP450,* and *ferritin* were up-regulated following supplementation with 400–800 mg/kg of dietary VC. Changes in the expression levels of these antioxidant-related genes were similar to the trends in the activities of the antioxidant enzymes. Similar results were found on juvenile largemouth bass (*Micropterus salmoides*) (Yusuf et al., 2020) and rainbow trout (*Oncorhynchus mykiss*) (Delavari et al., 2022). The antioxidant activity was highly enhanced after the mollusks had been fed a diet supplemented with VC compared to one that lacked VC. VC improves the antioxidant capacity in aquatic animals by up-regulating the expression levels of the antioxidant-related genes.

Non-specific immunity is the main form of immunity in crustaceans and is an important line of defense for the body against external pathogens (Huang et al., 2022). AKP is a key indicator of non-specific immune responses in crustaceans (Sunish et al., 2020). This enzyme can alter the surface structure of pathogens by hydrolysis, improve recognition and phagocytosis, and enhance disease resistance (Shao et al., 2018). In this study, the addition of 400–800 mg/kg of VC to the feed significantly increased the activity of AKP in the hepatopancreas of *B. areolate*. The results of this study were consistent with those conducted on fingerling (*Channa punctatus*) (Zehra and Khan, 2021) and Wuchang bream (*Megalobrama amblycephala* Yih) (Wan et al., 2014). *Mucin-5AC*, a secreted mucin, plays an important role in mucosal immunity (Anderson et al., 2022). *CYC* is one of the key enzymes involved in the biosynthesis of ATP in the mitochondria, which is released from the mitochondria by the altered permeability of the cell after stimulation by signaling. Its binding to factors, such as nitric oxide (NO), forms the apoptotic complex, which activates the caspase pathway and mediates apoptotic cell death (Yan et al., 2020). In this study, the relative levels of expression of the *CYC* and *mucin-5AC* genes were significantly up-regulated with dietary VC supplementation. The addition of appropriate VC to the diet was found to enhance the activities of immune enzymes and the expression levels of immune-related genes in large yellow croaker (*Pseudosciaena crocea*) (Ai et al., 2006), northern whiting (*Sillago sihama*) (Huang et al., 2020), and coral trout (*Plectropomus leopardusa*) (Zhu et al., 2022), which is similar to the findings of this study. VC enhances immunity with its functions of reducing the biosynthesis of histamine, scavenging free radicals in the cytosol, hindering the peroxidation of fatty acids, and maintaining the integrity of the immune system (Daniel et al., 2018). In non-specific immune responses, VC accumulates in the neutrophils, enhances phagocytosis, chemotaxis, and the production of ROS, and even kills pathogens (De la Fuente et al., 2020). VC can modulate the immune response by stimulating the expression of transcription factors (nuclear factor kB) through the increased production of ROS (Ellulu, 2017). After the onset of oxidative damage, VC acts on apoptosis to remove ineffective neutrophils and reduce cell necrosis and the oxidative damage to tissue (Washko et al., 1993). These may explain the enhancement of antioxidant and immune responses by VC in *B. areolata* in this study.

One of the most important factors in aquatic environments is ammonia, which originates from the decomposition of nitrogenous organic matter (Zhou et al., 2023). The growth and physiological characteristics of aquatic animals are closely related to the content of ammonia (Zhao et al., 2021). The concentration of ammonia in the water may increase when there is a deterioration in water quality, an increase in the density of aquaculture, and the accumulation of animal excreta and feed residues (Randall and Tsui, 2002; Xing et al., 2016). Owing to its high lipid solubility and lack of charge, ammonia can penetrate the cell membranes to enter tissues and form ionic ammonia (NH_4^+) (Ou et al., 2022). Aquatic organisms are affected by ammonia with effects, such as impaired growth and survival, tissue damage, oxidative stress, and immune suppression (Wu et al., 2023). Moderate amounts of VC can significantly enhance the resistance of Nile tilapia (*Oreochromis niloticus*) to stress from the bacterial pathogen *Aeromonas hydrophila* (Rathore et al., 2023), and red sea bream (*Pagrus major*) that were fed VC survived in greater numbers after a low-salinity stress challenge (Dawood et al., 2016). In this study, the survival rates of the Diet 4 and Diet 5 groups were significantly higher than those of the control group, which suggested that the addition of VC at 400 and 800 mg/kg to the diet enhanced the resistance to ammonia stress of *B. areolata*. Similar results were found in other studies. For example, (Harsij et al., 2020) found that the addition of 200 mg/kg of VC to the feed enhanced the resistance of rainbow trout (*Oncorhynchus mykiss*) to ammonia stress. (Wang et al., 2005) found that supplementation with dietary vitamin C enhanced the capacity of East Asian river prawn (*Macrobrachium nipponense*) to respond to elevated levels of ammonia. Therefore, dietary VC showed positive effects on the protection of *B. areolata* against ammonia stress.

5. Conclusion

This study showed that dietary VC significantly improved the growth performance and enhanced the antioxidant capacity, non-specific immunity, and resistance of *B. areolata* to ammonia stress. Broken-line regression analysis on WGR and SGR indicated that the optimal dietary VC requirement for juvenile *B. areolata* were 273.78 and 284.98 mg/kg, respectively. Therefore, VC is an essential immunostimulant for the enhancement of *B. areolata* growth, antioxidant ability, immunity, and resistance to ammonia stress. This study lays a foundation and provides a theoretical basis for the development of high-efficiency compound feed for *B. areolata*.

CRediT authorship contribution statement

Xiu-Xia Zhang: Validation, Software. **Jun-Tao Li:** Validation, Software. **Pei-Hua Zheng:** Methodology, Investigation, Formal analysis, Data curation. **Ze-Long Zhang:** Methodology, Investigation, Formal analysis, Data curation. **Teng Li:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Yao-Peng Lu:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Hui Guo:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Jian-An Xian:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Yi-Ning Lu:** Investigation. **Jia-Jun Li:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

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