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Research article

Dietary Vitamin C Fortification Enhances the Growth Performance, Hematological Status, Defensive Antioxidant Enzymes, and Resistance Against Aeromonas Hydrophila Infection for Bullseye Snakehead (*Channa marulius*)

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Abstract

To evaluate the impact of increased dietary Vitamin C (VC) levels on the survival, growth performance, hematological serum biochemistry, immune system response, antioxidant enzyme activity, stress parameters, and susceptibility of bullseye snakehead (Channa marulius) to Aeromonas hydrophila infection, a 90-day feeding trial was conducted. Four (40% crude protein) isonitrogenous diets with 0 (basal diet), 75, 150, and 300 mg/kg of targeted diet VC were prepared, having analysed VC contents of 4.6, 67.3, 142.5, and 275.8 mg/kg, respectively. At the end of the trial, results showed significant improvements in weight gain % (104.33±6.0 g fish^{-1}), lowest feed conversion ratio (1.67±0.04), and specific growth rate %/day (1.93±0.06) with added VC. Hematological parameters improved dose-dependently, with significant increases in total cholesterol, total protein, and triglycerides, and alanine phosphatase activity at 275.8 mg/kg of VC. Alanine aminotransferase and aspartate aminotransferase activities decreased, suggesting improved liver integrity. Serum electrolyte levels and antioxidant enzymes rose with higher VC incorporation, while serum cortisol and glucose levels dropped, suggesting reduced stress. Liver VC content also increased dose-dependently. Fish on VC fortified diets showed higher survival rates against A. hydrophila challenge. The ideal VC requirement for liver content and weight gain (%) is estimated between 230 and 273 mg/kg based on third-order polynomial (cubic) regression analysis.

Keywords: Channa marulius, vitamin C, aeromonas hydrophila challenge, serum cortisol, antioxidant enzymes, hematology, serum biochemistry.

1. Introduction

Fish require vitamins to survive because they act as enzyme cofactors [1]. L-ascorbic acid, or VC, is a water-soluble vitamin that is crucial for maintaining healthy metabolic processes and immune responses in fish [2,3]. VC is essential for fish growth and reproduction because it is involved in several physiological processes, including the synthesis of collagen, carnitine, and norepinephrine, as well as the absorption of nutrients [4-9]. By improving wound healing, immune system, respiratory responses, inflammatory response, and stressor response, it increases the fish's survival rate [10-15].

Moreover, studies have shown that VC reduces oxidative stress in fish, hence enhancing fish welfare generally [16,17]. Undoubtedly important, some fish species are incapable of producing the enzyme L-gulonolactone oxidase, which is required for the *de novo* synthesis of VC, and as a result, are unable to synthesise VC [18,19]. As a result, VC is added to aquafeeds to promote optimal growth performance and regular physiological processes [20]. Aquafeeds are therefore regularly supplemented with VC to ensure the health and proper growth of farmed fish.

Fish diets low in VC cause several disorders, including low immunity, hemorrhagic exophthalmia, poor development and reproduction, high mortality, anorexia, and anaemia [7,21]. Variations in serum cholesterol and triglyceride levels are examples of nonspecific symptoms that could result from a dietary VC deficiency [1,22]. Growth retardation and ultimately death could result from these issues [13]. Significant dietary differences between species have been found in studies on the VC requirements of farmed fish [1]. However, different fish species have different needs for VC [1]. Variations in fish species, size, growth stages, VC form, feeding habits, feed formulations, and conditions for farming could be the cause of these variations, according to [20]. It has been reported that the growth and development of several cultured fish species depend on VC levels in their diet. According to research findings, the following fish species require various concentrations of VC: 500 mg/kg [23], 142.2 mg/kg [24], and 700 mg/kg [25] for Wuchang bream, yellow drum, and Asian catfish. Therefore, to ascertain the ideal VC requirements research on fish of all kinds is necessary.

[26] stated that fish infections can be avoided by including VC supplements in the diet. In more demanding situations, a higher level of VC is needed to improve stress resistance [27]. In reaction to pesticide stress, VC functions as an antitoxic mitigating agent [28]. It has been noted that when *Heteropneustes fossilis* is stressed by cypermethrin, it benefits from increased tissue reserves of VC through dietary supplementation [29]. The stress brought on by the pesticide fenvalerate was found to be decreased by an increased ascorbic acid (AA) level (100 mg/kg bw) in the diet; however, a low level (50 mg/kg bw) showed no efficacy [30]. According to [31], *Labeo rohita* immune response and growth were stimulated by 100 mg/kg body weight of VC.

In aquaculture environments, fish experience various stressors, such as handling, transport, crowding, and high densities. These stressors disrupt the fish's internal balance (homeostasis) and trigger a cascade of physiological reactions. Similar to other stressors, bacterial stress can result in the production of reactive oxygen compounds and free radicals in fish cells. These can cause oxidative stress and potentially damage cellular structures [32]. Fish, like other vertebrates, have antioxidant defense mechanisms to counteract oxidative damage [33].

The snakehead fish, *C. marulius*, is a predatory freshwater fish with a higher potential for growth, good nutritional content, low quantity of intramuscular spines, and significant market value and customer favorability [34]. Some reports have been reported on the use and effects of various functional dietary additives for snakehead fish, including *Channa punctatus, Channa striatus* and *Channa maculata*♀× *Channa argus*♂ hybrids exposed to *A. hydrophila* bacterial pathogens. Among these additives, seaweeds, VC, vitamin A (VA), probiotics and prebiotics, yeast with β-glucan, kelp powder, aerial roots (*F. benghalensis*), and pod seeds (*L. leucocephala*) are the most salient [35,36], concerning to the resistance of *C. marulius* against *A. hydrophila*. However, information on how VC protects or resists *A. hydrophila* infection in *C. marulius* is not available. The optimal dietary requirement for this species has not been properly verified and remains to be determined accurately for all stages of development. In general, VC requirements for carnivorous warmwater fish such as African and Asian catfish (*Clarias* spp) have been reported as ranging 40-60 mg/kg [1]. *C. marulius* under intensive rearing conditions and subjected to various stressors and potential disease warrants further attention to better qualitatively and quantitatively determine optimum dietary VC inclusion level for production in both laboratory and farm scenarios. In many locally available feeds for aquaculture in developing countries, vitamin and mineral premixes may fall well short of meeting

requirements of fish to the NRC [1] standards due to economic costs and inadequate formulations. Storage issues, high temperatures and humidity can also greatly reduce VC stability. This would be relevant for snakehead fed potentially inferior diets from local feed mills in tropical regions.

To better define the VC requirements for this species, the present investigation was conducted to examine the growth efficiency, hematology and serum marker enzymes, electrolytes, metabolic and physiological stress response, antioxidant enzyme activities, survival, and resistance against *A. hydrophila* infection or stress in *C. marulius*.

2. Materials and methods

2.1. Ethics statement

This study was performed at the University of Veterinary and Animal Sciences, Lahore, Pakistan, with approval granted by the University Ethical Review Committee under reference DR/163, dated 26-04-2021.

2.2. Experimental fish for the experiment

The experimental trial was conducted from April to July 2020. One hundred forty-four bullseye snakeheads (*C. marulius,* 100 g fish^{-1} initial body weight) were procured from a native public hatchery (Fish Biodiversity Hatchery, Chashma, District, Mianwali) and the experiment was carried out in the Fish Seed Rearing Unit, University of Veterinary and Animal Sciences, Lahore, Pakistan.

2.3. Experimental design and diet preparation

In this experiment, a Complete Randomized Design (CRD) was used, incorporating four distinct diets detailed in (Table 1). The diets maintained uniform protein (40% CP, crude protein) and energy content (Gross Energy, kcal/kg ~4,395). Ascorbate (VC) from the phosphate form of L-ascorbic acid (fivevet, Vietnam) was added at levels of 0 (basal diet), 75, 150, and 300 mg/kg, resulting in analyzed VC contents of 4.6, 67.3, 142.5, and 275.8 mg/kg, respectively, named VC $^{4.6}$, VC $^{67.3}$, VC $^{142.5}$, and VC $^{275.8}$.

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Table 1: Feed formulation and chemical composition, and VC contents (% on dry matter basis) of experimental diets.

Feed ingredients	Diets with graded VC levels			
	VC 4.6	VC 67.3	VC 142.5	VC 275.8
Fish meal	35	35	35	35
Soybean meal	25	25	25	25
Corn gluten 60%	17	17	17	17
Wheat flour	14	14	14	14
Vegetable oil (Sunflower oil)	7	7	7	7
[1]Vitamin premix (Free of VC)	1	1	1	1
[2]Mineral mixture	1	1	1	1
[3]Vitamin C (mg/kg)	0	75	150	300
Chemical composition of experimental diets (%)				
Dry matter	90.56± 0.07	90.87± 0.11	90.61± 0.15	90.88± 0.02
Crude Protein	39.81± 0.05	39.83± 0.02	39.81± 0.02	39.86± 0.02
Crude Fat	8.12± 0.01	8.15± 0.00	8.13± 0.01	8.16± 0.01
Ash	7.25± 0.01	7.22± 0.01	7.39± 0.01	7.16± 0.01
Gross energy (GE), kcal/kg	4395± 1.00	4395± 1.00	4395± 1.00	4395± 2.00
VC content (mg/ kg)	4.6	67.3	142.5	275.8

Note: [1](fivevet, Central Veterinary Medicine JSC No. 5, Ha Noi, Vietnam). Vitamin premix (VC free) contains per kg of diet: Vitamin A 3,500,000 IU kg/g, sorbitol 20 g/kg, Zn gluconate 40 g/kg, vitamin E 3.500 mg/kg, vitamin D3 1,750,000 IU/kg, vitamin PP (nicotinamide) 30 g/kg, vitamin B1 3,500 mg/kg

[2]Mineral premix contained the following per kg of diet: Sodium chloride 60 g/kg, ferrous sulphate 25 g/kg, magnesium sulphate 137 g/kg, calcium phosphate 397 g/kg, calcium lactate 327 g/kg, sodium selenite 20 mg/kg, potassium iodide 150 mg/kg, manganese oxide 800 mg/ kg, zinc oxide 1.5 g/kg, copper sulphate 780 mg/kg, manganese oxide 800 mg/kg, cobalt carbonate 100 mg/kg, potassium chloride 50 g/kg

[3]Vitamin C (VC) 300 g/kg (fivevet, Central Veterinary Medicine JSC No. 5, Ha Noi, Vietnam); L-ascorbic acid phosphate (not a stable form).

For VC analysis, reverse phase High-Performance Liquid Chromatography (HPLC) was employed. Sample preparation involved adding 5 ml of 0.1% formic acid to 1 g of the sample, followed by homogenization, centrifugation, and filtration.

The mobile phase was a 0.1% solution of the formic acid, which was used after the removal of impurities and gas release. The HPLC parameters comprised of an injection amount of 5 µL of the solution and a detector with a wavelength of 254 nm, at a 30°C column temperature. Following the protocol described by [37], validation control (VC) measurements were performed using an external standard, a tris (cyclohexyl ammonium)-ascorbic acid 2-phosphate solution. Ingredients for the diets were sourced locally in Lahore, Pakistan, ground finely, and screened (1.0 mm). A mix of mineral supplements, vegetable oil (sunflower oil), and a VC-free vitamin premix were added to the dough with 15% additional distilled water. The resulting dough was processed into pellets (3.0 mm) using a modified meat mincer (ANEX, AG3060). Diets were placed into clearly labelled polythene bags, sealed, and allowed to air dry with 10% moisture before being kept in refrigerators at -20°C until needed. Fish were fed the specially formulated diet twice a day (at 8:00 and 16:00 hours) for the course of the 12-week trial, with an average feeding ratio of 3% of their wet body weight.

2.4. Experimental trial

The fish were acclimated for two weeks with a basal diet (4.6 mg/kg) to indoor conditions in a 2000 L cement tank using a flow-through system and a 0.4 L/mint water exchange rate. After acclimation, bullseye snakehead fish with a 100 L (each tank) water capacity were randomly assigned to tanks with 12 fish each. The fish were fed twice a day and their daily survival rate was recorded in triplicate for each tank. Throughout the feeding experiment, the water quality parameters remained consistent, with temperatures ranging from 28.87 to 30.33°C, dissolved oxygen levels maintained between 5.65 and 6.82 mg/L, and pH values stabilized within the range of 8.01 to 8.17 (measured using YSI Inc, Model 55, Yellow Springs Ohio, USA). The feeding trial lasted for 90 days.

2.5. Growth indices

Prior to stocking, the initial body weight was recorded, and the mean value was documented. Following that, growth performance metrics and parameters including feed conversion ratio (FCR), specific growth rate (SGR), and weight gain percentage (WG) were evaluated. At the end of the trial, these parameters were calculated using the formulas listed below.

WG % = 100 × FW (g) - IW (g)/IBW (g)

FCR = Feed intake (g)/Wet weight gained (g)

SGR (% day^{-1}) =100 × [ln Final mean body weight (g) – ln Initial mean body weight (g)]/ Number of days of feeding

2.6. Blood and tissues collection for further analysis

After the trial termination, each tank's six selected *C. marulius* fish were given 150 mg/L of MS222 to sedate them after they had fasted for 24 hours. Blood was then drawn from the caudal vein using 3-ml syringes and stored in EDTA vacutainers for analysis of hematological parameter analysis. Blood samples were also taken in Eppendorf tubes without anticoagulant with the help of hematological testing, and within 30 minutes of collection, centrifuged at 3000 rpm for 5 minutes at 4°C. The resulting samples were then stored at − 80 °C for analysis [38,39]. Serum was used for serum biochemistry, enzymes, electrolytes, serum cortisol, serum glucose parameters analysis. After the fish's blood was extracted, the liver and muscle tissues were examined to determine selected antioxidant enzymes. Using a solution containing 0.1% Triton X-100, 20 mM phosphate buffer (pH 7.4), and 1 mM EDTA, each 2-g liver and muscle samples were homogenized (1:4 w/v). After centrifugation for 10 minutes at 860 x g and 4°C, the supernatant was kept at -80°C using an Eppendorf centrifuge (5810R) [40].

2.7. Hematology parameters

An automated hematological analyzer (Sysmex KX-21 N) was used to analyse the hematological parameters such as hemoglobin (Hb), hematocrit (HCT), platelets (PLT), red blood cell count (RBC), white blood cell count (WBC), and mean corpuscular volume (MCV).

2.8. Serum biochemistry, enzymes, electrolytes, and liver

VC content

The key parameters, such as triglycerides (TG), total protein (TP), and total cholesterol (T-CHO), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), glucose, and electrolytes like ionised calcium (Ca), sodium (Na), potassium (K), and chloride (Cl), were assessed through serum analysis. For the analysis, a Micro Lab-300 computerized biochemical analyzer was used. A spectrophotometer and a commercial kit (Ascorbic Acid Assay Kit, ab65346-Abcam) were used to determine the VC content of the liver tissues.

2.9. Serum cortisol

Cortisol levels in the serum were assessed using radioimmunoassay with a Coat-to-Count Kit (Diagnostic Products Corporation, Los Angeles, CA, USA), following the method outlined by [41]. Cortisol concentration was measured in nanograms per milliliter (ng/mL).

2.10. Antioxidant enzymes

According to [42] protocol the catalase (CAT) activity was measured. Using a spectrophotometer (Analytik Jena 200 plus Specord, Germany), the reduction of H_2O_2 concentration was determined at a wavelength of 240 nm. Superoxide dismutase (SOD) activity was determined based on the method described by [43]. Blank readings were taken, and the spectrophotometer was zeroed at 560 nm. Cuvettes containing buffer, enzyme extract, riboflavin, nitroblue tetrazole (NBT), and EDTA/NaCN solution were allowed to incubate in a light for 12 minutes. Following the reaction, absorbance was measured, and the percentage inhibition of NBT was used to determine SOD activity. Glutathione peroxidase (GSH-Px) activity was determined following the [44] method. GSH-Px catalyzed the oxidation of glutathione by adding cumene hydroperoxide for 5 minutes at 37°C (pH 7.6). Glutathione levels in the supernatant were measured at 412 nm using 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB).

2.11. Experimental fish were challenge with Aeromonas hydrophila

Whereas the remaining five fish from each treatment were further subjected to a challenge with a virulent strain of *A. hydrophila*, isolated from diseased *O. niloticus*. After culturing on blood agar plates and incubating at 37°C for 24 h [45], confirmation of presumptive Aeromonas colonies was achieved using a Microbact MB 24E identification kit [46]. Identification of *A. hydrophila* followed the criteria set by [47], involving various biochemical tests. *A. hydrophila* colonies were classified based on an acid reaction at the base and beveled edge of the agar, with or without gas formation. The cultures of bacteria kept in phosphate-buffered saline (PBS). Following a 24 h fast, five randomly chosen fish from each experimental group were injected intraperitoneally using 0.2 ml of phosphate-buffered saline (PBS) that contained 1×10^7 CFU/ml live *A. hydrophila*, and then placed in separate tanks [48]. After infection, fish resumed feeding with experimental diets, and continuous monitoring of water quality parameters was maintained. The well-being and survival of the fish were observed for 48 h at a temperature of 30°C. Cumulative survival patterns (%) were determined using a specified formula, and mortality was subsequently assessed.

Survival rate (%) = 100 × (final number of fish/initial number of fish)

2.12. Statistical analyses

The data are displayed using the mean ± standard deviation. The post hoc Duncan's Multiple Range Test was used to identify differences between the treatment means in the tested parameters following one-way analysis of variance (ANOVA). The results were considered statistically significant at $P<0.05$. Graphfbv bvPad Prism version 9.0.0 (121) was utilised to perform third order polynomial regression to ascertain the ideal VC inclusion level for weight gain (%), FCR, and VC liver contents.

3. Results

3.1. Growth performance and survival rate

Dietary VC supplementation had a significant effect on *C. marulius* growth, as indicated by FMW, WG, SGR, FCR, and SR in (Table 2). The FMW, SGR, and SR were elevated linearly ($P<0.05$) in the three VC supplementation groups compared to the basal diet. The weight gain was highest in 275.8 mg/kg (104.33±6.0 %) of VC supplementation compared

to 142.5 mg/kg (94.00±4.00), 67.3 mg/kg (80.00±1.48), and 4.6 mg/kg (66.28±3.53) of VC respectively. Through third order polynomial (cubic) regression analysis (y = -2E-06x3 + 0.0004x2 + 0.1605x + 66.28; R2 = 1), based on WG%, the ideal VC requirement was calculated to be 273 mg/kg (Figure 1). The FCR (1.67±0.04) for the 275.8 mg/kg of the VC diet group was lower than that of the 142.5, 67.3, and 4.6 mg/kg treatments. For FCR, 300 mg/kg of dietary VC inclusion is the ideal level. Third-order polynomial (cubic) regression analysis (y = -7E-09x3 + 7E-06x2 -0.0031x + 2.18; R2 = 1) (Figure 2) provides the best indication of the relationship between dietary VC inclusion levels and FCR. Comparing fish fed the basal diet (4.6 mg/kg) of VC to the other groups (67.3, 142.5, and 275.8 mg/kg of VC, P<0.003), the SGR was lower. Regarding survival rate, there was no significant difference (P>0.05). Also, no external or internal pathology indications of clinical deficiency were observed for bullseye snakehead (*C. marulius*) fed the basal diet (4.6 mg/kg of VC).

3.2. Hematology, and serum biochemistry, enzymes, electrolytes profile

The impact of VC supplementation on serum biochemistry and hematological indices is presented in Table 3. Overall, blood physiological and biochemical parameters varied significantly with dietary treatments. Hematological parameters such as HGB, RBC, WBC, HCT, MCV, PLT, and showed significant (P<0.05) differences. Notably, key blood parameters such as HGB, RBC, WBC, MCV, and PLT demonstrated an overall rise in reaction to VC supplementation. Elevated values of these parameters were linked to VC levels; the highest VC value was 275.8 mg/kg, compared to the basal diet's 4.6 mg/kg of VC. T-CHO, TP, and TG concentrations in serum were significantly (P<0.05) affected by dietary VC levels.

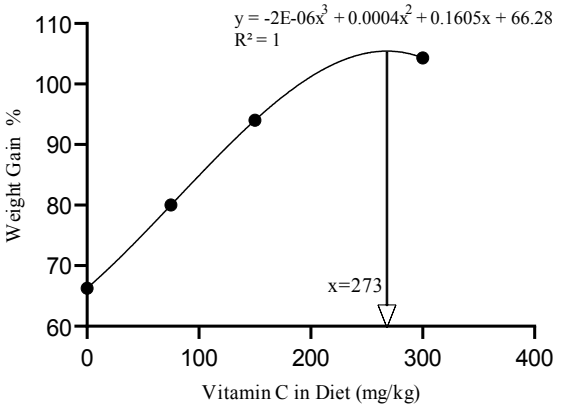

Figure 1. Third order polynomial (cubic) regression illustrates the ideal dietary VC requirement of bullseye snakehead fingerlings with respect to the weight gain (%).

On the other hand, T-CHO in 275.8 mg/kg was significantly (P<0.05) lower than 4.6 mg/kg of VC. Serum biochemical parameters, including enzymatic activities, also exhibited significant variations with incremental VC levels, as presented in Table 3.

Table 2. Growth performance of bullseye snakehead fed practical diets supplemented with various levels of VC for 12 weeks

Parameters	Diets with VC levels				*p* value
	VC 4.6	VC 67.3	VC 142.5	VC 275.8	
IMW (g fish^{-1})	104.0±2.00	107.6±2.51	101.3±2.51	108.6±3.21	>0.05
FMW (g fish^{-1})	170.28±4.43^{a}	187.67±3.29^{b}	195.33±1.52^{b}	213.00±7.0^{c}	<0.001
WG (g fish^{-1})	66.28±3.53^{a}	80.00±1.48^{b}	94.00±4.00^{c}	104.33±6.0^{d}	<0.001
FCR	2.18±0.05^{d}	1.98±0.08^{c}	1.84±0.02^{b}	1.67±0.04^{a}	<0.001
SGR (% day^{-1})	1.54 ±0.04^{a}	1.78±0.10^{b}	1.81±0.11^{b}	1.93±0.06^{b}	<0.003
SR (%)	96.55±4.11	96.32±3.04	96.66±2.31	96.40±2.01	>0.922

Note: Mean comparisons ± SD, n = 3. A row or column's matching letters indicate no statistical significance (P>0.05). Initial mean weight = IMW; final mean weight = FMW; weight gain = WG; feed conversion ratio = FCR; specific growth rate = SGR; survival rate = SR are acronyms

Table 3. Hematological parameters and serum biochemistry profiles of bullseye snakehead fingerlings receiving varying dietary VC levels.

Parameters	Diets with VC levels				*p* value
	VC 4.6	VC 67.3	VC 142.5	VC 275.8	
Hematological parameters					
HGB (g/dL)	9.22+0.29^{a}	10.29+0.18^{b}	10.11+0.12^{b}	11.22+0.21^{c}	<0.000
HCT (%)	48.88+0.11^{a}	52.55+0.27^{c}	50.32+0.27^{b}	54.44+0.32^{d}	<0.000
RBC (10^{6}/μL)	3.91+0.08^{a}	4.77+0.19^{c}	4.28+0.20^{b}	5.16+0.04^{d}	<0.000
MCV (fL)	72.22+0.11^{a}	74.34+0.11^{b}	75.14+0.06^{c}	74.41+0.09^{b}	<0.000
WBC (10^{3}/μL)	60.26+0.14^{a}	60.73+0.19^{b}	61.48+0.17^{c}	61.91+0.06^{d}	<0.000
PLT (10^{6}/ μL)	71.08+0.05^{a}	71.23+0.13^{b}	72.69+0.01^{c}	73.23+0.05^{d}	<0.000
Serum biochemistry					
T-CHO	241.36+0.25^{d}	230.75+0.18^{c}	209.37+0.21^{b}	197.18+0.16^{a}	<0.000
TP	3.38+0.11^{a}	3.85+0.15^{b}	3.54+0.14^{a}	4.01+0.02^{b}	<0.001
TG	26.71+0.17^{a}	28.26+0.15^{b}	41.14+0.07^{c}	47.17+0.04^{d}	<0.000
Serum enzymes					
ALP (U/ml)	18.03+0.04^{a}	19.03+0.04^{b}	20.13+0.05^{c}	22.39+0.17^{d}	<0.000
AST (IU/ml)	229.51+0.41^{c}	227.37+0.05^{b}	224.15+0.04^{a}	223.77+0.15^{a}	<0.000
ALT (IU/ml)	41.36+0.04^{d}	39.88+0.09^{c}	38.36+0.06^{b}	36.56+0.23^{a}	<0.000
Serum electrolytes					
Ca	9.53+0.13^{a}	9.85+0.10^{b}	10.75+0.13^{c}	11.66+0.05^{d}	<0.000
Na	120.30+0.08^{a}	122.14+0.04^{b}	124.36+0.06^{c}	126.17+0.04^{d}	<0.000
Cl	130.22+0.03^{a}	131.26+0.13^{b}	132.22+0.10^{c}	133.40+0.09^{d}	<0.000
K	12.13+0.05^{a}	12.63+0.05^{b}	12.94+0.03^{c}	13.11+0.09^{d}	<0.000

Note: Mean comparisons ± SD, $n = 3$. Statistical significance ($P<0.05$) is indicated by distinct letters within a column or a row. Hemoglobin = HGB, hematocrit = HCT, red blood cells = RBC, mean corpuscular volume = MCV, white blood cells = WBC, platelets = PLT, total cholesterol = T-CHO, total protein = TP, triglycerides = TG, alanine transaminase = ALT, aspartate transaminase = AST, alkaline phosphatase = ALP, sodium = Na, calcium = Ca, potassium = K, chloride = Cl are acronyms.

Table 4. Antioxidants enzyme activities, and liver VC content in the liver and muscle of bullseye snakehead fingerling at various level of dietary VC.

Parameters	Diets with VC levels				*p* value
	VC 4.6	VC 67.3	VC 142.5	VC 275.8	
Liver (U/mg protein)					
GPH-x	23.03±3.59^{a}	26.70±4.67^{a}	37.62±5.56^{b}	44.54±6.40^{b}	<0.003
CAT	31.73±4.16^{a}	46.48±4.03^{b}	58.63±2.45^{c}	67.44±6.73^{d}	<0.001
SOD	36.84±4.02^{a}	54.63±4.94^{b}	82.27±4.19^{c}	93.74±5.54^{d}	<0.001
Muscle (U/mg protein)					
GPH-x	18.92±3.06^{a}	21.22±2.22^{a}	28.47±3.67^{b}	32.29±3.40^{b}	<0.003
CAT	22.92±3.84^{a}	29.15±4.13^{ab}	35.27±6.01^{bc}	40.68±2.62^{c}	<0.005
SOD	29.61±5.12^{a}	46.73±3.89^{b}	51.26±5.69^{b}	65.26±5.35^{c}	<0.001
VC content in liver (μg/g tissue)	18±1.00^{a}	26±1.00^{b}	36.37±0.54^{c}	37±1.00^{c}	<0.000

Note: Mean comparisons ± SD, $n = 3$. A row or column containing different letters indicates statistically significant ($P<0.05$). Glutathione peroxidase = GPH-x, catalase = CAT, superoxide dismutase = SOD are acronyms

Table 5. Effect of dietary VC on bullseye snakehead fingerlings' stress biomarkers.

Parameters	Diets with VC levels				p value
	VC 4.6	VC 67.3	VC 142.5	VC 275.8	
Serum cortisol (ng/mL)	59.15±3.58^{c}	51.59±2.43^{b}	43.31±4.38^{a}	36.85±4.20^{a}	<0.001
Serum glucose (g/dL)	62.19+0.09^{a}	54.28+0.19^{b}	45.37+0.06^{c}	43.86+0.05^{d}	<0.000

Note: Mean comparisons ± SD, n = 3. A row or column containing different letters indicates statistically (P<0.05).

Table 6. The (%) of survival among bullseye snakehead fingerlings following exposure to Aeromonas hydrophila, varied based on the levels of VC administered during feeding.

Parameters	Diets with VC levels				p value
	VC 4.6	VC 67.3	VC 142.5	VC 275.8	
Survival rate after challenge	68.23±2.75[a]	73.38±1.81[a]	88.46±2.48[b]	87.28±9.02[b]	<0.002

Note: Statistical comparison of means ± SD, n = 3. indicated significant differences, as evidenced by distinct letters within a row or column, signifying a confidence level of (P<0.05).

There was a significant (P<0.05) rise in ALP activity in fish that received 275.8 mg/kg of VC. On the other hand, fish that were fed increasing amounts of VC showed notable decreases in their ALT and AST activities in contrast to the basal diet. For Na, K, Ca, and Cl, the serum electrolyte levels were highest (P<0.05) in the VC-supplemented group and lowest (P<0.05) in the basal diet group that received 4.6 mg/kg of VC diet.

3.3. Activities of antioxidant enzymes and liver VC content

Table 4 shows how increasing VC supplementation affects antioxidant enzyme activities in the liver and muscle of *C. marulius*, revealing insights into the modulation of antioxidant defenses in response to varying VC levels. The fish's liver and muscle samples showed a significant increase in antioxidant enzyme activities when compared to the fish fed the basal diet. SOD activity in both liver and muscle also demonstrated a significant increase at 275.8 mg/kg (93.74±5.54 U/mg and 65.26±5.35 U/mg, respectively) of VC when compared to the basal diet. CAT activity was significantly elevated at 275.8 mg/kg (67.44±6.73 U/mg and 40.68±2.62 U/mg, respectively) of VC in both fish liver and muscle samples. These results show how the antioxidant enzyme activities in the liver and muscle tissues of the fish samples under investigation were affected by VC supplementation. Furthermore, the fish liver was found to have a significantly higher GPH-x (44.54±6.40 U/mg) compared to 4.6 mg/kg of VC (basal diet). Bullseye snakehead liver's VC content peaked at 300 mg/kg of VC, increasing proportionately to the amount of VC supplemented. This suggests that the amount of VC in the liver had a dose-dependent interaction. The ideal dietary requirement for the bullseye snakehead was estimated to be 230 mg/kg, based on the third-order polynomial (cubic) of liver VC content (Figure 3).

3.4. Stress parameters

It was found that as VC supplementation increased, serum cortisol and serum glucose levels decreased (P<0.05) (Table 5). The cortisol level of fish fed a basal diet containing 4.6 mg/kg of VC was found to be the highest (59.15±3.58), while fish fed diets supplemented with VC had the lowest level (36.85±4.20). In the same way, the fish fed a basal diet containing 4.6 mg/kg of VC had the highest glucose level (62.19+0.09) in the case of glucose whereas the fish fed diets supplemented with VC had the lowest level (43.86+0.05).

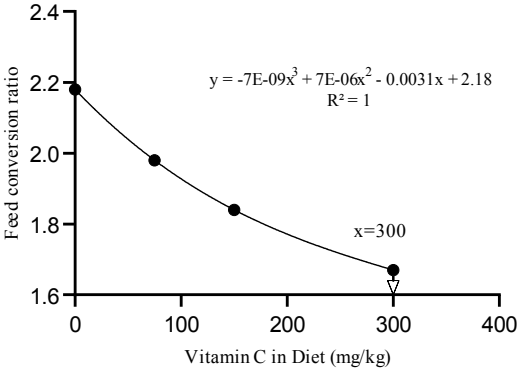

Figure 2. Feed conversion ratio using third-order polynomial (cubic) regression in bullseye snakehead fed VC diets at different inclusion levels

3.5. Challenge trial

Compared to the basal diet, fish exposed to increasing levels of VC supplementation showed significant improvement in survival during the *A. hydrophila* challenge. The fish's rate of survival positively correlated with higher dietary VC levels, peaking at 275.8 mg/kg of VC (Table 6).This heightened resilience highlights the potential of VC supplementation in enhancing the fish's immune response and stress resistance,——

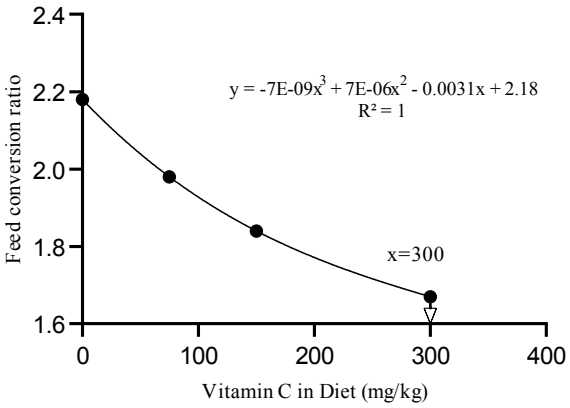

Figure 2. Feed conversion ratio using third-order polynomial (cubic) regression in bullseye snakehead fed VC diets at different inclusion levels

contributing to overall robust health. The findings emphasize the crucial role of optimal dietary VC levels in bolstering the fish's ability to combat pathogens and improve overall survival.

4. Discussion

Dietary VC supplementation affects growth, feed utilization, hematology, serum biochemistry, and oxidative stress parameters in bullseye snakeheads fed a typical diet formulation. In the current study, the addition of various concentrations of VC to the diet of *C. marulius* fingerlings resulted in enhanced growth performance compared with basal diet 4.6 mg/kg. However, this baseline level in the un-supplemented reference diet did seem to meet basic requirements for growth but was evidently insufficient to drive higher growth rate and feed performance for *C. marulius* juveniles under the experimental conditions. There was also no indication of deficiency for snakehead with respect to superficial health and external appearance. A longer-term assessment over an extended growth period would be interesting to observe any clinical effects of low VC status.

Our data depicted higher WG (%) and SGR for fish fed 275.8 mg/kg of VC diet than those fed a basal diet. Based on WG%, the ideal VC requirement for bullseye snakehead was calculated to be 273 mg/kg (R2 = 1). This ideal VC value was determined statistically and is similar to the value stated by [49] in Coho Salmon (224.68 mg/kg of VC). Consistent with findings from other relevant investigations [4] determined optimal VC requirements (109.5 and 102.6 mg/kg) for WG% and SGR in largemouth bass (*Micropterus salmoides*). Similar investigations by [50] in Korean rockfish (*Sebastes schlegeli*), [51] in large yellow croaker (*Pseudosciaena crocea*), [52] in Nile tilapia (*Oreochromis niloticus*), and [53] in golden trevally (*Gnathanodon speciosus*) yielded comparable results regarding the optimum VC requirements. On the contrary, according to [54], juvenile (*Coregonus clupeaformis*) whitefish showed no noticeable growth performance in response to dietary levels of VC.

In our investigation, snakehead fed a diet containing 275.8 mg/kg of VC were found to have the best FCR. These findings indicate that supplementing with VC at a supranutritional level of 275.8 mg/kg enhances the fish's capability to assimilate nutrients in accordance with the specified feed requirements. Our findings align with the research of [52], where the optimal FCR was reported at a supplementation level of 400 mg/kg VC in Nile tilapia (*Oreochromis niloticus*). The different fish species, ages, sizes, energy levels, metabolism, natural food availability, water quality, feeding, and culture management in the studies listed above may be the cause of the variances in the VC requirements and effects. Furthermore, different fish species' VC requirements may also have an impact on how the feed is formulated [4].

Fish health and the harmful effects of different substances on fish can be obtained through hematology studies [55]. Increases in WBC, RBC, HCT, HGB, PLT, and MCV values were indicated in this study in diets supplemented with VC, suggesting a beneficial effect on *C. marulius*. Because VC helps fish absorb iron, including it in the diet is crucial for enhancing blood chemistry [56]. This improvement in blood chemistry demonstrates how VC benefits fish physiological processes in general and nutrient assimilation. These results align with the research published by [5], [21], and [57].

The serum biochemistry of key enzymes, AST, ALP, and ALT are vital biomarkers for assessing kidney and liver health status. Liver enzymes (AST and ALT) released into the bloodstream in stressed fish are usually indicative of either cellular damage

or stress [58,59]. The activities of both ALT and AST were significantly (P<0.05) reduced in our study when VC was added to the diet. Comparable outcomes were also reported by [49] in Coho Salmon (*Oncorhynchus kisutch*) fed VC supplemented diet and by [60] in *Oreochromis niloticus* fed guava Psidium guajava (L.) aqueous extract diet. The decrease in ALT and AST activities may be due to an increase in antioxidant activities. AST activity is necessary to lower lipotoxicity and lipid peroxidation [61].

In our study, VC supplementation dramatically increased the ALP activity in comparison to the basal diet. Similar results were also observed in the cases of juvenile cobia (*Rachycentron canadum*) [26], juvenile grass carp (*Ctenopharyngodon idella*) [62], and juvenile *Megalobrama amblycephala Yih* by [25]. Bullseye snakehead supplementation increased the ALP values in our study, which may be related to better development of liver cells, regular cellular function, and overall growth performance. It's crucial to keep in mind, though, that previous studies, such as [24], demonstrated that there was a contrary ALP response to VC supplementation following a *Vibrio alginolyticus* challenge in yellow drum (*Nibea albiflora*). These contradicting results highlight the complex and situation-specific effects of VC on fish ALP activity.

TG, TP, and T-CHO, which are widely acknowledged as markers of animal health [63], significantly varied in response to different dietary VC concentrations in this investigation. Fish fed the basal diet exhibited significantly lower levels of TG, TP, and T-CHO than fish fed diets supplemented with VC. By improving the circulating nutrient status, these results show how dietary VC levels impact the health of *C. marulius* and support previous research on juvenile cobia [26]. Furthermore, a significant increase in serum TP content was linked to the VC-supplemented diet, which is in line with [64] findings in young Matrina. According to [65], fish fed 200 mg/kg of VC feed exhibited improved immunity; this finding is consistent with the diet group that included VC having higher serum TP levels.

Fish adjust the concentrations of blood ions in their bodies in response to a variety of stress parameters. In our study, we determined the electrolyte content (Na, K, Cl, and Ca) of fish serum. K levels in outside the cell should normally be lower than thosein intracellular fluid. Na and K are the two main electrolytes found in fish, regulating the acid–base balance and maintaining ionic sufficiency for tissue functions [66,67]. Our study emphasized notable changes in fish serum electrolytes when subjected to stress induced by *A. hydrophila*. This aligns with observations by [68],who documented significant changes in serum electrolytes in *Panagasianodon gigas* fish subjected to cold and heat shocks.

Several blood markers, such as hormones and metabolic indicators, are associated with stress, such as cortisol and glucose. Cortisol is widely acknowledged as a chronic stress hormone, and its concentration serves as a key indicator for assessing stress responses in fish [69]. Due to prolonged periods of stress are frequently linked to elevated cortisol levels, measuring cortisol has become a standard method for assessing the physiological stress status of fish populations. Furthermore, dietary VC may alter physiological reactions linked to stress [70]. In the current study, it was found that the glucose and cortisol levels of the diets supplemented with VC were significantly lower than those of the basal diet, which contained 4.6 mg/kg of VC. The present results supported the observations of other researchers, who found that high-VC diets can assist in relieving and reducing fish stress responses, including stress caused by chasing and crowding Nile tilapia and seabream [71]. Serum glucose levels may have decreased to reflect stress in the fish receiving 4.6 mg/kg of VC as part of their basal diet, or they may have shown improvements in health because of the VC supplementation. [72] also noted similar results for the ricefield eel (*Monopterus albus*). [56] reported that Mekong Giant Catfish (*Pangasianodon gigas*) supplemented with VC showed elevated plasma glucose levels. These results are in line with the notable increase in blood glucose noted in *Labeo rohita* fed a diet supplemented with VC [73] and catfish *Clarias gariepinus* [30]. Variations in the blood, plasma, and serum samples used for the glucose analysis could be the cause of the difference.

The teleost innate or nonspecific immune system is their first line of defense against pathogens, according to [74], which emphasises the significance of bolstering fish immune systems to effectively prevent disease. Moreover, it is recognised that the sensitive oxidative stress biomarkers GPH-x, SOD, and CAT act as the body's first line of defense [8]. The antioxidant enzyme activities (GST, SOD, and GPH-x) in the liver and muscle of *C. marulius* exhibited a noteworthy and statistically significant increase on VC supplementation (P<0.05) when compared to the basal diet. The findings of [75], who noticed a similar trend in *O. niloticus* when fed prebiotic mannan oligosaccharides, and who observed elevated levels of SOD, GPH-x, CAT, and in muscle and liver tissues contrasted to the basal diet, are consistent with the observed elevation in enzyme activity. In the current investigation, a diet containing 275.8 mg/kg of VC increased muscle and liver activities such as SOD, GPH-x, and CAT compared with basal diet. Previous studies have shown that *Pelteobagrus fulvidraco* (yellow catfish) and *Ctenopharyngodon idella* (young grass carp) exhibited stronger antioxidant enzyme activities when fed diets supplemented with VC than when fed VC-deficient diets [62,9,76]. However, higher dietary VC levels have not been found to enhance antioxidant capacity in juvenile cobia in specific investigations [26]. The effects of VC supplementation on the antioxidant capacity of integument located enzymes would be of further interest as they would relate to the systemic role of the vitamin and effects on structural integrity of skin, wound healing, and mucous barrier physiology [77,78]. For the reason of determining stress resistance, the fish survival rate following an *A. hydrophila* challenge is crucial. In the snakehead experiment, pre-challenge, VC did not affect *C. marulius* survival rates. However, post-challenge, *C. marulius* fed VC-supplemented diets showed significantly improved resilience, consistent with studies by [79,80, and [24]. Additionally, previous research [81,82, 26] noticed that adding VC to commercially important species such as *Vibrio harveyi*, *Mesanophyrs sp.*, and *Escherichia coli* improved the immune response against various bacterial species.

5. Conclusion

The addition of VC to the diet considerably improved the growth performance of fingerling bullseye snakeheads. According to survival rates, WG%, and liver VC contents, the most effective VC *ranges* were found at 142.5 and 275.8 mg/kg, respectively. This amount of VC is necessary for enhancing growth performance, nutrient absorption, hematology, serum biochemistry, oxidative stress, and survival against infection.

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Data Availability statement

The data used to support the outcomes of this study is available from the corresponding author on request.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Authors Contribution

Sadia Nazir: data curation, investigation, software, writing—original draft. Noor Khan: conceptualization, supervision, writing—review and editing. Dilawar Hussain: writing—review and editing. Mahroze Fatima: Conceptualization, methodology. Hamda Azmat: writing—review and editing. Moazama Batool: Review and editing. Sheeza Bano: Formal analysis, writing—review and editing. Muhammad Asghar: Formal analysis, writing—review and editing. Zahra Hussain: Review and editing. Muhammad Adnan Ali: Review and editing. Summia Perveen: review and editing. Simon J. Davies: writing—review and editing, co-supervision.

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