Pakistan J. Zool., pp 1-11, 2026. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20230603070641

Effect of Ascorbyl-2-Monophosphate (Thermostable Vitamin C) on Growth Performance, Antioxidative Behavior and Immune Response of Grass Carp (*Ctenopharyngodon idella*)

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ABSTRACT

A 60-days feeding trial was carried out to evaluate the effect of vitamin C (VC) on growth performance, serum biochemistry, antioxidant activity and survival rate of grass carp (*Ctenopharyngodon idella*). For this purpose, 5 experimental diets were formulated by supplementing graded ascorbyl-2-monophosphate levels at 0, 75, 150, 300 and 600 mg/kg with analyzed ascorbic acid values of 7.26, 32.85, 59.1, 109.04 and 213.06 mg/kg. Twenty-five fish with average initial weight (4.00±2 g) were stocked in each aquarium (100 liters capacity) after acclimatization and fed with experimental diets @3% body weight ratio for 60 days. After completion of experiment, results showed that VC supplementation enhanced ($P < 0.05$) growth performance up to 59.1 mg/kg. A better feed conversion ratio was noted at 59.1 mg/kg of VC and was constant afterward. However, VC levels did not affect ($P > 0.05$) feed intake and survival rate of grass carp. The whole-body crude protein significantly increased by increasing the level of VC from 7.26 to 59.1 mg/kg, while further addition in the level of VC gave a constant result. VC supplementation significantly improved the crude fat compared to fish fed basal diet. VC supplementation had a significant impact on hematological parameters of grass carp. The fish fed with higher levels of VC had higher muscle superoxide dismutase, catalase, glutathione peroxidase activities and serum lysozyme. Values for serum lysozyme and alkaline phosphatase significantly increased reaching a constant value after 59.1 mg/kg of VC level for ALP and 32.85 mg/kg of VC for serum lysozyme. Moreover, VC supplementation significantly decreased the activity of alanine aminotransferase, aspartate aminotransferase, thiobarbituric acid reactive substances and glucose. Dietary VC concentration in the liver enhanced in dose-dependent manner till 59.1 mg/kg of VC. Furthermore, survival rate of fish fed dietary VC was significantly increased in the challenge trial with *Aeromonas hydrophila*. The best VC level of grass carp, on the basis of weight gain% and liver VC content were estimated as 63.06 and 80.12 mg/kg, respectively. In conclusion, the supplementation of VC improved the growth, body composition, serum biochemistry, antioxidant enzyme activities and survival rate of juvenile grass carp.

Article Information
Received 03 June 2023
Revised 15 April 2026
Accepted 21 April 2026
Available online 11 November 2026 (early access)

Authors' Contribution
MF: Supervision, methodology. AM: Formal analysis, investigation. SZHS: Methodology, software. NK: Conceptualization, supervision. HN: Data curation, writing original draft. WA: Formal analysis, writing review and editing. AF and AT: Data curation, visualization.

Key words
Ascorbic acid, Optimum requirement, Weight gain, Antioxidant status, Serum biochemistry, *Aeromonas hydrophila*

INTRODUCTION

Vitamin C (VC) being an essential micronutrient, is required for proper development and growth of fish (Dawood and Koshio, 2018; NRC, 2011; Varghese *et al*., 2022). It enhances fish survival rate by improving healing of wounds and decreasing the chances of pathogen infection. It enhances the response of fish to stressors, respiratory and immune factors (Naiel *et al*., 2020; Carvalho *et al*., 2021; Harsij *et al*., 2020). It is the necessary element for many body functions including mineralization, nutrient

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0030-9923/2026/0001-0001 $ 9.00/0

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absorption, norepinephrine and carnitine production (Carvalho *et al*., 2021; Zou *et al*., 2020; Corti *et al*., 2010). Furthermore, it is required for collagen biosynthesis (Ogawa *et al*., 2004; Xie and Niu, 2006). Its role as an antioxidant protects the body's metabolism from free radicals and other health deteriorating components (Huang *et al*., 2017; Asaikkutti *et al*., 2016). Its utilization in different physiological processes necessary for health makes the presence of VC vital for fish survival. However, some fish species cannot synthesize VC due to the lack of enzyme involved in VC production (*L-gulonolactone oxidase*) (Fracalossi *et al*., 2001; Xie *et al*., 2006; Moreau *et al*., 1999). VC deficiency has harmful impacts on the fish body including scoliosis, lordosis, fin erosion, spinal deformity, erratic swimming behavior, internal hemorrhaging, anorexia, anemia, suppressed immunity, impaired collagen development and erratic swimming behavior (Ai *et al*., 2004, 2006; Liang *et al*., 2017; Zehra and Khan, 2012). Therefore, VC supplementation is vital for normal fish growth and development (Shah *et al*., 2015).

Supplementation of VC has been studied extensively and its supplementation varies with the change in fish species, culturing environment, feeding nature, developmental stages, size, VC form and feed formulation (Xu *et al*., 2016; Chen *et al*., 2015; Zhou *et al*., 2012). Best level of VC supplementation has been determined by Zhou *et al*. (2012) in cobia (13.6 mg/kg), Wang *et al*. (2017) in yellow drum (142.2 mg/kg), Lin and Shiau (2004) in juvenile grouper (8.3 mg/kg), Nasar *et al*. (2021) in grass carp (67.1667 mg/kg), Huang *et al*. (2016) in tilapia (45 mg/kg), Zehra and Khan (2012) in mori (35.65 mg/kg), Huang *et al*. (2017) in Chinese sucker (84.6 mg/kg), Zou *et al*. (2020) in Chu's croaker (102.28 mg/kg), Ming *et al*. (2012) in Wuchang bream (700 mg/kg), Shiau and Hsu (1999) in tilapia (63.37 mg/kg), Xu *et al*. (2016) in grass carp (92.8-129.8 mg/kg), Ai *et al*. (2006) in large yellow croaker (28.2 mg/kg), Chen *et al*. (2015) in largemouth bass (109.5 mg/kg) and Gouillou-Coustans *et al*. (1998) in carp (270 mg/kg).

Ctenopharyngodon idella, commonly known as grass carp, has an annual production of over 5 million tonnes (FAO, 2012). By weight, its production rate is much higher than other fish species (Nasar *et al*., 2021). It is native to China and southeastern Russia and was introduced in Pakistan in 1994. It can be cultured in both mono and polyculture practices. Its culturing can be enhanced by improving the quality of feed. As the supplementation of optimal VC level has proven to be a beneficial inclusion in the fish diet so its addition is usually made to boost growth performance and immunity. Previously, effects of VC on grass carp have been determined using L-ascorbic acid which is an unstable form of VC and degrades quickly (Xu *et al*., 2016; Nasar *et al*., 2021). However, no study has been conducted on the effects of VC in grass carp using ascorbyl-2-monophosphate which is a stable form of VC. So, this experiment was conducted to assess the efficacy of VC supplementation levels on growth performance, serum biochemistry, antioxidant activity and survival rate in grass carp.

MATERIALS AND METHODS

Experimental diet

This experiment was carried to evaluate dietary effects of VC dietary in grass carp. For this purpose, five experimental diets were formulated by increasing the supplementation levels of VC (Ascorbyl-2-monophosphate) at 0, 75, 150, 300 and 600 mg/kg levels. The ascorbic acid level in diets was analyzed as 7.26, 32.85, 59.1, 109.04 and 213.06 mg/kg through high performance liquid chromatography (Yusuf *et al*., 2021) and the diets were named $C_{7.3}$, $C_{33.9,}$ $C_{59.1}$, $C_{109.0}$ and $C_{213.1}$, respectively. Table 1 provides the basal diet composition determined following the protocol of AOAC (2016). Diet components were

Table I. Experimental diet and proximate composition.

Ingredients (g/kg)	$C_{7.3}$	$C_{32.9}$	$C_{59.1}$	$C_{109.0}$	$C_{213.1}$
Fish meal	250.0	250.0	250.0	250.0	250.0
Soybean meal	250.0	250.0	250.0	250.0	250.0
Wheat flour	195.0	194.92	194.85	194.70	194.40
Rice polish	200.0	200.0	200.0	200.0	200.0
Fish oil	60.0	60.0	60.0	60.0	60.0
Vitamin premix (vitamin C free)+	20.0	20.0	20.0	20.0	20.0
Mineral mixture§	20.0	20.0	20.0	20.0	20.0
Monocalcium phosphate	5.0	5.0	5.0	5.0	5.0
L-Ascorbyl- 2-monophos-phate (mg/kg)	0	75.0	150.0	300.0	600.0
Proximate composition (g/kg)					
Moisture	97.9	99.9	100.4	98.6	98.9
Crude protein	298.7	299.1	298.6	301.2	299.7
Crude fat	111.5	113.4	113.8	113.2	111.5
Vitamin C content (mg/kg)	7.26	32.85	59.1	109.04	213.06

+ Each gram of vitamin premix (vitamin C free) contains: Vitamin A 15 M.I.U; Vitamin B_1 5000 mg; Vitamin B_2 6000 mg; Vitamin B_6 4000 mg; Vitamin B_{12} 9000 mg; Vitamin D_3 3 M.I.U; Vitamin E 6000 IU; Vitamin K_3 4000 mg; Folic acid 750 mg; Nicotinic acid 25000 mg; Calcium pantothenate 10000 mg. § Each gram of mineral mixture contains: KH_2PO_4 479 mg/g; $MgSO_4.7H_2O$ 153 mg/g; NaCl 51 mg/g; $COCl.6H_2O$ 0.0816 mg/g; $AlCl_3.6H_2O$ 0.255 mg/g; $CuSO_4.5H_2O$ 210.67 mg/g; $FeSO_4.H_2O$ 100.6 mg/g; $MnSO_4.5H_2O$ 116.67 mg/g; $ZnSO_4.7H_2O$ 121.33 mg/g; $CaCO_3$ 316 mg/g

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purchased from market and were finely ground in a grinder (Kenwood, AT283). They were screened (0.05 mm) and analyzed according to AOAC (2016) for proximate composition before diet formulation. Vitamin premix (VC free), fish oil and mineral mixture were added in horizontal double ribbon mixer for uniform mixing. Water was added, and the dough was passed through a modified meat mincer fitted with cutter for pellet formation. The resulting pellets were shade-dried till 10% moisture content. They were stored in labeled self-sealing bags and kept at -20°C.

Experimental fish and feeding trial

Grass carp (*C. idella*) juveniles were procured from Fish Farms, Ravi Campus C block, Pattoki, Pakistan. To reduce the risk of pathogen infection, the fish were given salt bath (5 g/L $KMnO_4$). Before trial, the juveniles (4.00±2 g) were acclimatized in 2000 L cemented tanks with 0.4 L/min water exchange rate and were fed with basal diet. After this duration, 25 juveniles were distributed randomly in experimental aquaria (100 L) and initial weight was recorded. The juveniles were hand-fed twice a day @3% body weight for a duration of 60 days. Survival rate of each aquarium was checked daily. All treatments were assessed in triplicates. The uneaten diet was siphoned off the aquaria bottom after 3 h for determining the feed intake (FI) and feed conversion ratio (FCR). Water quality parameters like dissolved oxygen, temperature and pH were monitored daily and were in optimum culture ranges throughout the experimental trial.

After feeding trial, 10 fish were kept for challenge trial with *Aeromonas hydrophila*, while remaining 15 juveniles from each aquarium were starved for 24 h and harvested. The juveniles were euthanized by clove oil overdose (3000 mg/L) for 60 sec (Khajepour *et al*., 2012). For chemical analysis, 3 juveniles were used for proximate whole-body analysis and 4 for hematology and serum analysis. Muscle samples of 3 fish were separated for antioxidant enzymes analyses. Body indices (viscera and liver) of 3 fish were analyzed to efficacy of VC supplementation. Liver of 2 fish were collected to determine VC concentrations.

Data collection

The data regarding growth performance was assessed by calculating weight gain (WG), % weight gain (%WG) and specific growth rate (SGR). FI and FCR were calculated to determine feed utilization. Moreover, the viscera and liver weight were used for viscerosomatic index (VSI) and hepatosomatic index (HSI) analysis (Zou *et al*., 2020).

$$WG = \text{final weight (FW)(g)} - \text{initial weight (IW)(g)}$$

$$WG(\%) = 100 \times \frac{FW\ (g) - IW\ (g)}{IW\ (g)}$$

$$SGR = 100 \times \frac{\ln(FW, g) - \ln(IW, g)}{\text{Time (days)}}$$

$$FCR = \frac{FI\ (g)}{WG\ (g)}$$

$$VSI(\%) = 100 \times \frac{\text{Viscera weight (g)}}{\text{Whole body weight (g)}}$$

$$HSI(\%) = 100 \times \frac{\text{Wet weight of liver (g)}}{\text{Wet weight of fish (g)}}$$

Survival rate (SR) was estimated as,

$$SR(\%) = 100 \times \frac{\text{Final fish number}}{\text{Initial fish number}}$$

Proximate composition analysis

Samples were analyzed following AOAC (2016). Dry matter (DM) was examined by oven drying (105 °C for 24 h). Crude protein (CP) of the samples was calculated by estimation of nitrogen after acid digestion (N×6.025) using Kjeldhal's apparatus. Crude fat (CF) was measured by using the soxhlet apparatus (Sr. no. 70861). Furthermore, the crude ash (CA) was assessed by flaming the sample in an electric furnace at 660 °C.

Hematology and antioxidants enzymes activity

At the trial termination, blood was collected from caudal vein of non-anesthetized fish with the help of EDTA rinsed sterile syringe. Hematological parameters HGB (hemoglobin), HCT (hematocrit), WBC (white blood cell), RBC (red blood cell) and MCHC (count mean corpuscular hemoglobin) were determined by an automated hematology analyzer (MEK-655) (Reddy, 2018).

The activities of antioxidant enzymes were determined from fish muscle samples. Superoxide dismutase (SOD) was estimated through calculating its capacity to prevent photo reduction of Nitroblue tetrazole (Giannapolitis and Ries, 1997). Catalase (CAT) was measured at 240 nm, where it reduces the level of H_2O_2 in tissues (Chance and Maehly, 1995). Glutathione peroxidase (GPx) was measured by calculating its ability to decline H_2O_2 concentration at the wavelength of 470 nm by Civello method (Civello *et al*., 1995). Lastly, thiobarbituric acid reactive substances (TBARS) were estimated at 530 nm through Gatta *et al*. (2000) method.

Serum biochemistry

Collected blood samples were used for analyzing alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), glucose and serum lysozyme. Samples were assessed through kits (CHEMELEX, S.A, Pol. Ind. Can Castells, Spain). Serum lysozyme activity was assessed by using the procedure of Shugar (1952) and following formula:

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$$\text{Units/mL enzyme} = \frac{(\Delta\ A_{450nm}/\text{min Test} - \Delta\ A_{450nm}/\text{min Blank})\ (df)}{(0.001)\ (0.1)}$$

0.001 is the absorbance change at $A_{450\ nm}$; 0.1 is the enzyme solution.

Moreover, VC content in liver tissues was determined by following Rutkowski and Grzegorczyk (1998).

Challenge trial

The juveniles (10 per aquarium) were challenged with *A. hydrophila* (5×10^5 cfu/ml) via intra-peritoneal injection (Garcia *et al*., 2007). The juveniles were fed with their respective diets during challenge trial and the water quality parameters were checked as in the feeding trial. The challenge trial was carried out for 10 days, during which the mortality was determined.

Statistical analysis

Data was assessed through one-way analysis of variance (ANOVA) under CRD (completely randomized design) by using Costat Computer Software (version 6.303). The means having significant differences were compared by Tukey's Honestly Significant Difference test. At $p < 0.05$ the means were taken as significant. Broken-line regression analysis was used to estimate the best dietary level of VC.

RESULTS

Growth performance, feed utilization and body indices

Impacts of VC levels on grass carp growth performance, feed utilization and organ indices are presented in Table II. VC levels significantly affected the growth parameters (FW, WG, WG% and SGR%). Compared to basal diet, an increase ($P < 0.05$) in growth performance was noted at higher VC levels till 59.1 mg/kg, becoming constant afterward. Based on WG%, best level of VC was estimated as 63.06 mg/kg in grass carp (Fig. 1A).

VC supplementation had a significant effect on FCR. Increase in the VC supplementation level yielded a better FCR at 59.1, 109.04 and 213.06 mg/kg (these levels were non-significant compared to each other). VC supplementation did not affect ($P > 0.05$) FI.

Among body indices, only VSI was significantly affected by the VC supplementation, while HSI remained non-significant even at higher VC levels. An increase in VC levels resulted in higher VSI values till 32.85 mg/kg and remained constant afterward. However, the survival rate remained independent of the VC supplementation levels.

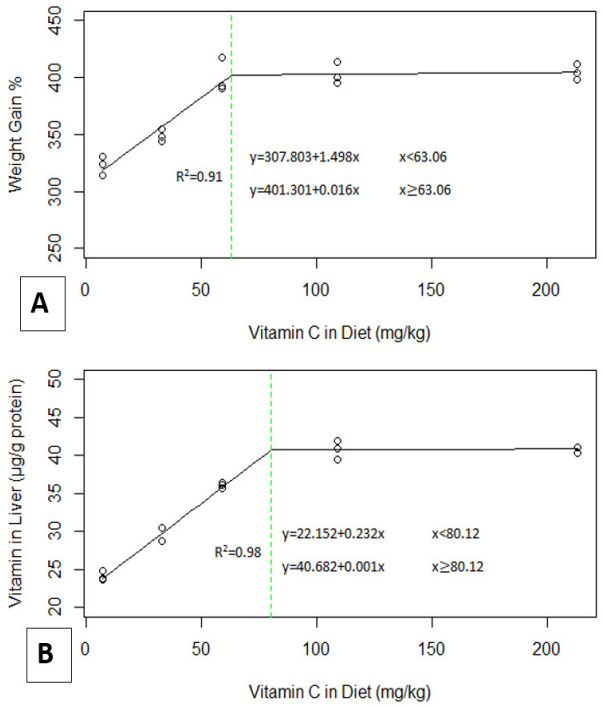


Fig. 1. Optimum VC level for grass carp on the basis of % WG (A), and lever content (B) calculated through broken line regression.

Table II. Growth performance and body indices of *C. idella* fed with different levels of vitamin C.

Diets	FW (g)	AWG	WG (%)	SGR (%)	F1	FCR	SR (%)	HSI (%)	VSI (%)
$C_{7.3}$	17.73±0.33^c	13.53±0.33^c	322.81±8.7^c	2.40±0.03^c	20.38±0.2	1.50±0.04^a	100±0	2.35±0.03	4.07±0.05^b
$C_{32.9}$	18.83±0.18^b	14.64±0.19^b	348.85±5.5^b	2.50±0.01^b	20.30±0.0	1.38±0.02^b	100±0	2.37±0.01	4.89±0.02^a
$C_{59.1}$	21±0.64^a	16.80±0.64^a	400.38±14^a	2.68±0.04^a	20.4±0.04	1.21±0.04^c	100±0	2.35±0.05	4.92±0.02^a
$C_{109.0}$	21.16±0.36^a	16.95±0.37^a	403.01±9.5^a	2.69±0.03^a	20.35±0.0	1.20±0.02^c	100±0	2.36±0.00	4.89±0.03^a
$C_{213.1}$	21.16±0.3^a	16.96±0.3^a	404±7.08^a	2.69±0.02^a	20.48±0.2	1.20±0.03^c	100±0	3.22±0.81	4.92±0.02^a

Superscript. Different superscript in one row indicates significant difference and same superscript show non-significance. IW, initial weight; FW, final weight; AWG, absolute weight gain; SGR, specific growth rate; FI, feed intake; FCR, feed conversion ratio; SR, survival rate; HIS, hepatosomatic index; VSI, viscerosomatic index.

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Whole-body composition

The supplementation of VC had a non-significant effect on whole-body moisture. The whole-body CP significantly increased as VC level increased from 7.26 to 59.1 mg/kg and any further increase gave a result similar ($P > 0.05$) to fish fed VC level of 59.1 mg/kg. Dietary VC significantly enhanced CF content when compared to basal diet. Moreover, the highest whole-body CA was observed in fish fed 109.04 mg/kg of VC (Table III).

Table III. Whole body proximate (g/kg) of *C. idella* fed with different levels of vitamin C.

Diets	Moisture	CP	CF	Ash
$C_{7.3}$	708.4±0.35	169.0±0.34^{c}	56.5±0.10^{b}	41.2±0.02^{d}
$C_{32.9}$	709.3±0.05	174.2±0.04^{b}	60.2±0.00^{a}	41.9±0.01^{bc}
$C_{59.1}$	711.1±0.40	177.1±0.31^{a}	60.3±0.05^{a}	41.6±0.00^{c}
$C_{109.0}$	710.9±0.04	177.0±0.01^{a}	60.9±0.04^{a}	42.3±0.00^{a}
$C_{213.1}$	710.3±0.14	176.5±0.01^{a}	61.0±0.05^{a}	42.0±0.00^{ab}

Superscript. Different superscript in one row indicates significant difference and same superscript show non-significance. CP, crude protein; CF, crude fat.

Hematological parameters

Effects of VC levels on the hematology parameters of *C. idella* are given in Table IV. VC supplementation decreased ($P < 0.05$) the HGB content in fish fed 59.1, 109.04 and 213.06 mg/kg of VC compared to 7.26 and 32.85 mg/kg of VC. The highest RBCs were observed in fish-fed 59.1 mg/kg of VC, however, VC supplementation had an overall non-significant effect on RBC levels. Increase in VC levels significantly affected WBC and HCT levels. In comparison to the above-mentioned hematology parameters, VC supplementation had a non-significant effect on MCHC values at all levels.

Table IV. Hematology of *C. idella* fed with VC levels.

Diets	HGB (g/dl)	RBC (10^6/dl)	WBC (10^3/dl)	HCT (%)	MCHC (g/dl)
$C_{7.3}$	9.6± 0.17^{a}	1.67± 0.13^{b}	439.66± 1.52^{c}	25.6± 1.34^{d}	26.03± 1.15
$C_{32.9}$	10.10± 0.45^{a}	1.80± 0.03^{ab}	450± 2^{bc}	36.26± 1.22^{b}	24.56± 1.15
$C_{59.1}$	7.86± 0.30^{b}	1.97± 0.06^{a}	458± 2.64^{b}	32.96± 0.11^{c}	26.13± 1.16
$C_{109.0}$	8.13± 0.37^{b}	1.67± 0.03^{b}	522.33± 8.50^{a}	37.26± 0.90^{ab}	25.9± 1.2
$C_{213.1}$	7.8± 0.34^{b}	1.84± 0.06^{ab}	532.66± 6.65^{a}	39.64± 0.66^{a}	26.2± 1.99

Superscript. Different superscript in one row indicates significant difference and same superscript show non-significance. HGB, hemoglobin; RBC, red blood cells; WBC, white blood cells; HCT, hematocrit; MCHC, mean corpuscular hemoglobin concentration.

Antioxidants enzyme activities

Significantly higher muscle SOD, CAT, and GPx activities were observed in fish fed higher VC diets (Table V). Lowest ($P < 0.05$) SOD and GPx activities were noted in fish fed basal diet and these values significantly increased as the VC level increased to 32.85 mg/kg, however, higher VC levels did not further increase the values. Increase in the dietary VC enhanced CAT activity till 109.04 mg/kg of VC, however, further increase resulted in a constant value. Dietary VC significantly reduced the TBARS values when compared to fish fed basal diet.

Table V. Antioxidants enzyme activities of *C. idella* fed with different levels of vitamin C.

Diets	SOD (u/mg protein)	CAT (u/mg protein)	GPX (mu/mg protein)	TBARS (mg/g)
$C_{7.3}$	6.28±0.02c	76.88±0.0^{d}	101.47±0.7^{b}	3.49±0.02^{a}
$C_{32.9}$	6.48±0.03^{a}	79.87±0.00^{c}	113.44±1.16^{a}	3.21±0.01^{b}
$C_{59.1}$	6.50±0.00^{a}	79.97±0.00^{b}	113.53±0.68^{a}	3.19±0.00^{b}
$C_{109.0}$	6.49±0.01^{a}	80.23±0.03^{a}	115.00±0.30^{a}	3.22±0.03^{b}
$C_{213.1}$	6.37±0.00^{b}	80.21±0.02^{a}	114.69±1.09^{a}	3.16±0.04^{b}

Superscript. Different superscript in one row indicates significant difference and same superscript show non-significance. SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; TBARS, thio barbituric acid reactive substance.

Serum biochemistry

The means of serum lysozyme and ALP significantly increased, reaching a constant value after 59.1 mg/kg of VC level for ALP and 109.04 mg/kg of VC for serum lysozyme. However, VC supplementation significantly decreased the values for ALT, AST and glucose (Table VI).

Table VI. Serum biochemistry f *C. idella* fed with VC levels.

Diets	ALP (U/ml)	ALT (IU/ml)	AST (U/ml)	Glucose (mg/dl)	Serum lysozyme (U/ml)
$C_{7.3}$	14.93± 0.17^{c}	71.34± 0.83^{a}	126.62± 0.79^{a}	6.58± 0.27^{a}	213.25± 10.33^{c}
$C_{32.9}$	17.85± 0.48^{b}	29.83± 1.02^{b}	81.59± 1.52c	5.03± 0.29^{b}	276.12± 8.90^{b}
$C_{59.1}$	22.15± 0.50^{a}	29.61± 0.55^{b}	104.06± 3.09^{b}	3.47± 0.32^{c}	279.47± 8.97^{b}
$C_{109.0}$	23.16± 0.36^{a}	30.72± 0.94^{b}	82.28± 2.26^{c}	3.60± 0.08^{c}	320.09± 7.23^{a}
$C_{213.1}$	23.46± 1.00^{a}	30.29± 0.78^{b}	103± 0.4.54^{b}	3.58± 0.11^{c}	327.65± 5.33^{a}

Superscript. Different superscript in one row indicates significant difference and same superscript show non-significance. ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase.

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Vitamin C concentration in liver

Dietary VC concentration in the liver enhanced in a dose-dependent manner till 109.04 mg/kg of VC (Table VII). The mean values for fish fed VC levels of 109.04 and 213.06 mg/kg showed a non-significant result. According to the regression analysis, the optimal VC content in the liver was 80.12 mg/kg (Fig. 1B).

Table VII. Vitamin C concentration in liver of *C. idella.*

Diets	Vitamin C concentration in liver (µg/g protein)
$C_{7.3}$	24.07±0.61^{d}
$C_{32.9}$	29.31±0.99^{c}
$C_{59.1}$	36.11±0.35^{b}
$C_{109.0}$	40.79±1.25^{a}
$C_{213.1}$	40.88±0.43^{a}

Survival rate after challenge trial

After *A. hydrophila* infection, VC supplementation significantly increased the survival rate of *C. idella* (Table VIII) compared to juveniles fed basal diet. The survival rate improved till 109.04 mg/kg and was constant afterward.

Table VIII. Vitamin C concentration survival rate after challenged with *A. hydrophila* in *C. idella.*

Diets	Survival rate (%)
$C_{7.3}$	37.77±3.84^{c}
$C_{32.9}$	55.55±3.84^{b}
$C_{59.1}$	66.66±6.66^{b}
$C_{109.0}$	91.11±3.84^{a}
$C_{213.1}$	88.88±3.84^{a}

Superscript. Different superscript in one row indicates significant difference and same superscript show non-significance. SR, survival rate.

DISCUSSION

In this experiment, dietary VC increased growth performance of juvenile grass carp when compared to basal diet. Past experimental studies conducted by Nasar *et al*. (2021), Chen *et al*. (2015), Ai *et al*. (2006), Liang *et al*. (2017), Zhou *et al*. (2012), Zou *et al*. (2020), Khan *et al*. (2017) and Shiau and Hsu (1999) concluded a significant impact of dietary VC on growth performance. According to the regression analysis, best level of VC based on WG% was estimated 63.06 mg/kg (R^2= 0.91) which is comparable to statistical value determined by Nasar *et al*. (2021) as 67.1667 mg/kg in grass carp. However, higher values of VC supplementation were reported in Chinese sucker (84.6 mg/kg) (Huang *et al*., 2017), in Chu's croaker (102.28 mg/kg) (Zou *et al*., 2020) and largemouth bass (109.5 mg/kg) (Chen *et al*., 2015). This variation might be because of fish size, metabolism, source of VC, feed formulation and nature of the feed ingredients used (Chen *et al*., 2015; Dabrowski, 1991, 1994; Lin and Shiau, 2005).

In this study, the improvement in growth performance was because of enhanced feed utilization and whole-body nutrient composition. Improved FCR value was recorded at 59.1 mg/kg of VC, indicating that at this level of VC supplementation the fish showed higher ability in utilizing feed ingredients and any further increase in VC levels did not further enhance FCR. Similar to our result, Nasar *et al*. (2021) observed an increase in FCR value in juveniles fed 100 mg/kg of VC diet. Moreover, VC supplementation also improved feed utilization in largemouth bass and mori (Zehra and Khan, 2012; Chen *et al*., 2015). As the VC level improves the feed utilization, so more nutrients are available for absorption and deposition, consequently improving the fish's whole-body nutrient composition. Moreover, VC also acts as a cofactor for promoting protein synthesis (Chen *et al*., 2015; Chagas and Val, 2003; Biswas *et al*., 2013), which further increases whole-body CP content, which can be observed in the present study. Furthermore, VC also improved the whole-body CF in this study. These findings were in line with the results of Zou *et al*. (2020), Huang *et al*. (2017), Chen *et al*. (2015), Nasar *et al*. (2021) and Tewary and Patra, (2008). This improvement might be because of antioxidant characteristic of VC which lowers unsaturated fatty acid peroxidation. Lower levels of CF were observed in juveniles fed VC unsupplemented diet (basal diet) which may be because of impaired carnitine and insulin-like growth factor secretion (Hu *et al*., 2009; Chen and Jenn, 1991), both of which are related to the body lipid synthesis.

Organ indices HSI and VSI are often used to evaluate nutritive and physiological status of fish (Dawood *et al*., 2017; Lee *et al*., 2002; Goede, 1990). In this study, VC levels significantly improved VSI when compared to fish-fed basal diet. Similarly, Chen *et al*. (2003) and Daniel *et al*. (2018) also reported similar results. Higher levels of body indices indicate that VC supplementation supports normal physiological processes of the fish body (Chen *et al*., 2015; Zhou *et al*., 2012).

Blood parameters (HGB, RBC, WBC and HCT) are indicators of fish health and immune response. In response to VC supplementation, HGB, WBC and HCT significantly increased in this study. Previous studies by Andersen *et al*. (1994) and Hamre *et al*. (1997) also noted a significant improvement in the hematological parameters. Dietary VC plays important role in iron absorption (Pimpimol *et al*., 2012) consequently improving the blood chemistry in fish.

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Antioxidant enzymes (CAT, GPx, SOD and TBARS) are crucial in the degeneration of ROS (reactive oxygen species), lowering oxidation stress (Tabrez and Ahmad, 2009) and mechanism leading to protection during tissue injury (Wu *et al*., 2017; Fontagne-Dicharry *et al*., 2014). In the current experiment, fish fed dietary VC had higher values of CAT, GPx and SOD when compared to juveniles fed basal diet. Similar to our study, Xie and Niu (2006) and Hu *et al*. (2013) also reported a significant impact of dietary VC on antioxidant enzyme activities. In contrast, TBARS values were higher in juveniles fed with basal diet than in VC supplemented diets. Higher levels of TBARS are an indicator of biotoxicity and lipid peroxidation (Dawood *et al*., 2017). VC supplementation protects fish body from lipid peroxidation by reducing levels of TBARS. Past studies have indicated that VC supplementation significantly lowers the content of TBARS in fish body (Nasar *et al*., 2021; Shahkar *et al*., 2015; Lee and Dabrowski, 2003). This decrease might be because of the nature of VC in increasing activities of antioxidant enzymes. Moreover, formation of vitamin E (lipid-soluble antioxidant) from alpha-tocopherol radicles in presence of VC (Jimenez-Fernandez *et al*., 2015) further helps in the prevention of lipid oxidation and enhancing growth performance of fish (Fatima *et al*., 2019).

The ALP, AST and ALT are important indicators of liver physiology. They are important in oxidative stress responses and health status of fish. In this research, dietary VC increased ($P < 0.05$) serum ALP activity. This was similar to past studies that observed a positive increase in activity of ALP in response to dietary VC (Huang *et al*., 2016, 2017; Zhou *et al*., 2012; Ming *et al*., 2012; Wan *et al*., 2014). According to our findings, the ALT, AST and glucose content significantly decreased when the level of VC was increased. The increase in these enzymes (ALT and AST) and the serum glucose indicate stress conditions in fish. In his study, the decrease in ALT, AST and glucose indicates that the role of VC in increasing the activities of antioxidants might be responsible for decreasing stress, consequently improving fish health.

The liver is considered a major organ for catabolism and storage of VC (Dabrowski, 1994; Xiao *et al*., 2010). In liver, its content indicates the status of VC in fish (Zhou *et al*., 2012; Ai *et al*., 2006). In the present study, best VC concentration was estimated as 80.12 mg/kg in grass carp liver, which was much higher than the value estimated for WG%. Similar results regarding VC supplementation were also reported by Ai *et al*. (2006), Xiao *et al*. (2010) and Wu *et al*. (2015), indicating that optimal VC level needed to sustain physiological functions in fish is much higher than VC level required for growth.

A. hydrophila challenge method is often used in experimental designs to check the effectiveness of a dietary inclusion regarding disease resistance. VC supplementation significantly increased fish survival rate during challenge trial when compared to juveniles fed with basal diet. Past studies by Hu *et al*. (2020), Han *et al*. (2020), Liang *et al*. (2017) and Tewary and Patra (2008) have observed that VC supplementation significantly enhances survival rate in fish. Additionally, VC supplementation improves the resistance of fish against pathogenic species like *Escherichia coli*, *Vibrio harveyi*, *Vibrio alginolyticus* and *Edwardsiella tarda* (Ai *et al*., 2006; Zhou *et al*., 2012; Wang *et al*., 2017; Misra *et al*., 2007; Leal *et al*., 2017).

CONCLUSION

In conclusion, VC significantly increased growth performance of juvenile grass carp by improving feed utilization, whole-body composition, hematological parameters, antioxidant enzyme activities and serum biochemistry. The best dietary VC level for grass carp based on WG% and liver VC content was estimated as 63.06 mg/kg and 80.12 mg/kg, respectively.

DECLARATIONS

Funding

This research did not receive any grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical statement

This experiment was carried out in accordance with the standard guidelines for care and use of laboratory animals in research and procedures were approved by the Ethical Review Committee of University of Veterinary and Animal Sciences, Lahore Pakistan (No. Dr/161). During experiments, the fish were inspected several times a day and precautions were taken to minimize stress by keeping the fish in opaque tanks, minimizing handling and monitoring water chemistry. Humane endpoints were used and any fish that showed signs of disease or abnormal behavior (lethargy and disoriented swimming) was quickly euthanized. At all sampling times, fish were almost completely exsanguinated by blood sampling and euthanized using an overdose of clove oil before collection of organ samples.

Supporting information

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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