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Influence of vitamin C feed supplementation on the growth, antioxidant activity, immune status, tissue histomorphology, and disease resistance in Nile tilapia, *Oreochromis niloticus*

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ABSTRACT

The present study aimed to evaluate the influence of dietary incorporation of vitamin C (L-ascorbic acid phosphate) on the growth rate, antioxidant activity, certain immunological indices, and tissue histomorphology in Nile tilapia (*Oreochromis niloticus* L.) fingerlings. One hundred and eighty fingerlings (14.74 ± 0.016 g each) were randomly distributed into four groups based on basal diets supplemented with 0, 200, 300, and 400 mg kg^{-1} diet of vitamin C, respectively, for ten weeks. The dietary incorporation of vitamin C significantly increased the final body weight, total weight gain (TWG), specific growth rate, and daily weight gain (DWG) in all groups. The serum levels of vitamin C and growth hormone increased in the highest supplementation level group (400 mg kg^{-1}). In a concentration-dependent manner, vitamin C markedly enhanced the serum and hepatic antioxidant activities, the levels of lysozyme, nitric oxide, and interleukin-10, the phagocytic percentage, and the relative percentage survival (RPS%) of the fish against *Aeromonas sobria*. The liver histoarchitecture was also improved in a level-dependent manner with vitamin C supplementation. In addition, intestinal histomorphology improved, as evidenced by the increase in villus height and width. Therefore, vitamin C could serve as a suitable dietary supplement for enhancing the growth, hepatic and intestinal structures, immune status, and resistance against *A. sobria* in Nile tilapia.

1. Introduction

In recent years, the aquaculture industry has expanded dramatically compared to the other food production sectors, mainly to fulfill the increasing demand for fish as food, particularly in a few developing countries where fish is considered the main source of nutrition (FAO, 2018).

Nile tilapia (*Oreochromis niloticus*) is one of the most valuable and widely cultivated fish species worldwide, owing to its beneficial characteristics, including rapid growth rate, tolerance to adverse environmental conditions, and acceptable growth on numerous dietary protein sources (Ng and Romano, 2013; Welker and Lim, 2011).

Intensive aquaculture practices render fish further vulnerable to infectious diseases, resulting in economic losses due to chemotherapy expenses and fish mortality (Hoseinifar et al., 2017). Bacterial diseases may cause great losses in tilapia production, particularly during the wintering period. Aeromonas is one of the main bacteria infecting tilapia (Hernández et al., 2009; Mian et al., 2009; Son et al., 1997). *Aeromonas*

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sobria, a species of motile aeromonads, is highly pathogenic to juvenile tilapia and causes tail rot outbreaks (Li and Cai, 2011). Moreover, excessive application of antibiotics leads to antibiotic resistance in fish, which is considered a global threat to public health as the antibiotic-resistant genes could transfer to humans, animals, and other aquatic organisms (Kraemer et al., 2019). The risk of disease in farmed fish may be reduced by increasing their resistance level against infections with the use of better dietary formulations, vaccines, and immunostimulants (Abdel Rahman et al., 2018a,b; Al-Khalaifah et al., 2020; Amer, 2016; Amer et al., 2018, 2020; Amer et al., 2019; El-Araby et al., 2020; Abdel Rahman et al., 2019).

Vitamins are organic compounds that are essential for life as they are required in trace amounts for normal growth, reproduction, and health (Gasco et al., 2018; Gouda et al., 2020). Vitamin C induces immune responses, including macrophage infiltration, cell proliferation, natural killer cell activity, complement activity, lysozymes levels, phagocytic activity of leucocytes, development of cytokines, and antibody concentrations (Li and Lovell, 1985; Navarre and Halver, 1989). Fish lack L-gluconolactone oxidase enzyme, which is responsible for the de novo synthesis of vitamin C, and therefore, fulfill their vitamin C requirements from an exogenous source (Fracalossi et al., 2001).

L-ascorbic acid phosphate (ROVIMIX®STAY-C®35) is a stable form of vitamin C used in feed. Several studies have reported the use of this form of vitamin C in juvenile Mexican Silverside (*Menidia estor*) (Martinez-Palacios et al., 2007), juvenile yellow catfish (*Pelteobagrus fulvidraco Richardson*) (Liang et al., 2017a), *Sparus aurata* (Amerio et al., 2000), and Nile tilapia (*Oreochromis niloticus*) (Barros et al., 2014).

Dietary vitamin C supplementation is reported to enhance growth and immune response in fish (Abdel Rahman et al., 2018b; Barros et al., 2014; Liua et al., 2016). In this context, the present research was aimed at using different concentrations of vitamin C as a supplement in fish diet and evaluating their efficacy in enhancing the growth, antioxidant activity, and immune status in Nile tilapia. In addition, the intestinal and hepatic structures and the resistance of fish against *A. sobria* were analyzed.

2. Materials and methods

2.1. Fish rearing conditions

A total of 180 Nile tilapia fingerlings (14.74 ± 0.016 g each) were procured from Abbassa Fish Hatchery, Sharkia Province. The fish were placed in well-aerated glass aquaria (size: 80 × 40 × 30 cm) containing 60 L dechlorinated tap water and having approximately 25 % of its water exchanged daily. The fish were maintained in the aquaria until two weeks prior to the beginning of the experiment and were fed with a basal diet (Table 1) during this period. The water quality parameters were measured according to APHA, 1998. In the whole experiment, the same conditions of dissolved oxygen (7.17 ± 0.4 mg L^{-1}), temperature (22.4 ± 1.02 °C), pH (6.7 ± 0.1), ammonia-N (0.035 ± 0.01 mg L^{-1}), and nitrite content (0.12 ± 0.02 mg L^{-1}), and a controlled photoperiod (12 h light, 12 h dark) were maintained in the laboratory. The water parameters were monitored twice each day throughout the experimental period to maintain the parameter values within the recommended range.

2.2. Experimental design and diet preparation

The experiment protocol was reviewed and approved by the 'Ethics of Animal Use in Research' Committee (EAURC), Zagazig University, Egypt (ZU-IACUC/2/F/156/2019). The fish were divided randomly into four groups, each with three replicates (n = 45 fish/group, 15 fish/replicate). Four isoenergetic diets containing equal protein content (Table 1) were formulated (NRC, 2011), and vitamin C (L-ascorbic acid phosphate; ROVIMIX®STAY-C®35, DSM company, Egypt) supplementations (0, 200, 300, and 400 mg kg^{-1}, respectively, for the four groups) were added to the basal diets. The experiment began with the proximate chemical analysis of the fish and the diets, performed in accordance with AOAC (2000). The feed ingredients were mixed mechanically and then pelletized using a meat mincer equipped with a 1.5-mm diameter. The obtained feed pellets were air-dried, with regular turning to ensure uniform drying. The dried pellets were stored at 4 °C in the refrigerator until use. The feed pellets were fed to the fish fed by hand, until complete satiation, three times a day (9:00 am, 12:00 pm, and 4:00 pm) for ten weeks.

Table 1
Proximate chemical composition of experimental diets (g kg^{-1} on dry weight basis).

Ingredients	Vit. C 0	Vit. C 200	Vit. C 300	Vit. C 400
Fish meal (70.7 %)[1]	180	180	180	180
Soy bean meal (49 %)[2]	240	239.8	239.7	239.6
Ground yellow corn	271	271	271	271
Corn gluten	60	60	60	60
Wheat flour	100	100	100	100
Wheat bran	50	50	50	50
Fish oil	65	65	65	65
Vitamin C (l-ascorbic acid phosphate)	0	0.2	0.3	0.4
Vitamin and mineral mixture (premix)[3]	30	30	30	30
Methionine	4	4	4	4
chemical analysis (g/kg)				
Crude Protein	334.69	334.59	334.55	334.50
Crude fiber	34.34	34.33	34.32	34.31
NFE[4]	465.5	465.7	465.7	465.8
Fat	103.73	103.72	103.72	103.72
Ash	61.7	61.68	61.67	61.67
Lysine	19.08	19.08	19.07	19.07
Methionine	10.88	10.88	10.88	10.88
GE MJ/kg[5]	20.64	20.64	20.64	20.64
Vitamin C mg kg^{-1}	333.42	403.45	438.40	473.50

[1] Argentinean fish meal by Coomarpes Ltda, Argentina.
[2] U. S. soybean meal.
[3] Premix: [1]Each 1 kg premix contains: vit A, 550000 IU; vit D, 110000 IU; vit E, 11000 mg; vit K, 484 mg; vit C, 30000 mg; vit B1, 440 mg; vit B2, 660 mg; vit B3, 13200 mg; vit B5, 1100 mg; vit B6, 1045 mg; vit B9, 55 mg; Choline, 110000 mg; Biotin, 6.6 mg; iron, 6.6 gm; copper, 330 mg; Mn, 1320 mg; Zn, 6.6 gm; Se, 44 mg; and iodine, 110 mg.
[4] Nitrogen free extract, determined by the following difference: 100 − (protein% + fat% + crude fiber% + ash%).
[5] Gross energy (GE) (kcal/kg) calculated according to NRC (2011) using the following expression: (5.7 × g protein) + (9.4 × g fat) + 4.1 × (g NFE + g fiber).

2.3. Growth performance and survival rate

The weight and feed intake of the fish were recorded at the beginning, after every two weeks, and at the end of the experiment. The daily weight gain (DWG), total weight gain (TWG), specific growth rate (SGR), and feed conversion ratio (FCR) were determined as described by Castell and Tiews (1980). The protein efficiency ratio (PER) was calculated as described by Stuart and Hung (1989).

Total weight gain (g/fish) = (WT–WI) [where WT = final weight of the fish in grams and WI = initial weight of the fish in grams]

DWG (g/fish/day) = total weight gain/experimental days

SGR (%/day) = 100 × (ln WT–ln WI)/time in days [where ln denotes natural logarithm]

FCR = total feed intake (g)/total weight gain (g)

PER = total gain (g)/protein intake (g)

Percentage (%) survival = (total number of fish in each group after the feeding period of ten weeks/initial number of fish) × 100

2.4. Disease challenge experiment

The lethal dose (LD_{50}) of *A. sobria* was determined, and the fish were injected intraperitoneally (IP) with different doses of 24-h live bacteria. Three days post-injection, the mortality of infected fish was noted. The estimated LD_{50} was 3×10^7 CFU/mL, and a sub-lethal dose of 1.5×10^7 CFU/mL was used for the bacterial challenge. Eight fish/replicate (24 fish/group) were selected randomly for receiving the intraperitoneal injection of 0.1 mL cell suspension of pathogenic *A. sobria* (cell density: 1.5×10^7 cells/mL, calculated using McFarland standard tubes), as described by Khalil et al. (2017). The used strain of pathogenic *A. sobria* was originally isolated from moribund fish at the Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University, under project no. 5589. The challenged fish were monitored twice a day for over two weeks to document the clinical signs and mortalities. Re-isolation of the bacteria from the fish kidney, liver, and intestine was performed to ensure that the fish mortalities were caused specifically by *A. sobria*. The relative percentage survival (RPS) was calculated as described by Amend (1981), RPS = 100 − [(treatment mortality ÷ control mortality) × 100].

2.5. Sampling

At the end of the experimental period of 10 weeks and 14 days after the bacterial challenge, nine fish/group (three fish/replicate) were selected randomly and subjected to fasting for 12 h, following which the fish were weighed and then blood samples were obtained from them using a 1-mL heparinized syringe. Another set of blood samples from these fish were collected without anticoagulant. The sera were obtained through centrifugation at 3000 rpm for 15 min and were then used for the estimation of non-specific immunological and biochemical parameters. Liver and intestinal samples were obtained for histological studies, and the liver samples collected for antioxidant analysis were frozen at −20 °C.

2.6. Serum biochemical assays

Growth hormone (GH) levels were determined using a fish growth hormone ELISA kit (sensitivity 1.0 ng/mL, reaction control 1.56–50 ng/mL; MyBioSource Co., Cat. No. MBS044656), following manufacturer's instructions. The serum levels of vitamin C were estimated as described in a previous study (Papp et al., 1998). The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine were evaluated as described by Reitman and Frankel (1957), Burits and Ashwood (1994), and Fossati et al. (1983), respectively.

2.7. Serum and hepatic antioxidant activities

The serum and hepatic malondialdehyde (MDA) levels and activities of antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), and reduced glutathione (GSH)] were determined. The MDA levels were evaluated using the method described by Uchiyama and Mihara (1978), while the activities of CAT, SOD, and GSH were estimated using a colorimeter and the method of Aebi (1984), Nishikimi et al. (1972), and Beutler (1963), respectively.

2.8. Analysis of immune indices

The estimation of serum lysozyme activity using spectrophotometry was based on the lysis of freeze-dried particles of *Micrococcus lysodeikticus*, originally described by Ellis (1990). The serum levels of nitric oxide were determined using a colorimetric assay, according to Montgomery and Dymock (1961), while the percentage phagocytic activity was measured as described by Kawahara et al. (1991). Interleukin 10 (IL-10) was quantified using fish-specific ELISA kits (MyBioSource, Cat. no. MBS044038), following manufacturer's instructions.

2.9. Histology and morphometric methods

Tissue specimens of the intestine (anterior segment) and liver (three samples/tissue) from each group were collected at the end of the feeding period. Subsequently, the specimens were fixed in 10 % neutral buffered formalin, followed by embedding in paraffin, sectioning at 5 μm thickness using a microtome (Leica®, Wetzlar, Germany), and staining with hematoxylin and eosin (H&E). Slide examinations and photographing, as well as morphometric measurements, were performed under a microscope (Ceti England) equipped with a digital camera (AmScope) (Bancroft and Layton, 2013). Twenty images per fish, covering 10 fields for each slide, were captured at 4x and 40x magnifications for morphometric measurements. All measurements for villus height (from top to base of the villi), villus width (medial region, side to side), and goblet cell count (per field at high magnification, i.e., 400x) were performed using AmScopeToupView software version 3.7 (AmScope, United States).

2.10. Statistical analysis

All data were expressed as a means ± standard error (SE). Shapiro–Wilk's test was used to verify the normality of the data, while Levene's test was used to verify the homogeneity of variance components between the experimental treatments and the assumption ($P > 0.05$). ANOVA was applied on the basis of polynomial orthogonal contrasts. SPSS Version 17 for Windows (SPSS Inc., Chicago, Illinois, USA) was employed to calculate linear and quadratic regression equations to determine the effects of different concentrations of vitamin C in Nile tilapia. Duncan's multiple range test was used to identify the differences among the means at a significance level of 0.05.

3. Results

3.1. Growth performance

As clear from Table 2, the FBW, TWG, DWG, and SGR increased linearly ($P < 0.05$) in the three vitamin C supplementation groups compared to the zero vitamin C level group, while the changes in the

Table 2
Effect of vitamin C (Vit. C) dietary supplementation (mg kg^{-1}) on growth performance in Nile tilapia fingerlings after ten weeks of feeding.

Parameters	Vit. C 0	Vit. C 200	Vit. C 300	Vit. C 400	Regression analysis *	
					Linear	Quadratic
IBW/g	14.79 ± 0.01	14. 61 ± 0.03	14.81 ± 0.02	14.72 ± 0.02	0.09	0.07
FBW(g/fish)	38.71 ± 0.47[b]	40.83 ± 0.26[a]	41.51 ± 0.42[a]	42.42 ± 0.22[a]	0.01	0.50
TWG (g/fish)	23.91 ± 0.47[b]	26.22 ± 0.29[a]	26.70 ± 0.44[a]	27.70 ± 0.26[a]	0.02	0.34
FI (g/fish)	39.81 ± 2.48	39.21 ± 2.55	39.03 ± 2.19	39.94 ± 2.09	0.20	0.35
FCR	1.66 ± 0.10	1.49 ± 0.11	1.46 ± 0.08	1.44 ± 0.04	0.13	0.35
PER	1.79 ± 0.10	1.99 ± 0.15	2.04 ± 0.11	2.07 ± 0.06	0.14	0.31
SGR (%/day)	1.37 ± 0.01[b]	1.46 ± 0.02[a]	1.47 ± 0.01[a]	1.51 ± 0.01[a]	0.04	0.27
DWG (g/fish)	0.34 ± 0.001[b]	0. 37 ± 0.02[a]	0.38 ± 0.01[a]	0. 39 ± 0.01[a]	0.01	0.35

IBW, initial body weight; FBW, final body weight; TWG, total weight gain; FI, feed intake; FCR, feed conversion ratio; PER, protein efficiency ratio; SGR, specific growth rate; DWG, daily weight gain.
* The regressions were considered significant at $P < 0.05$.

feed intake, FCR, and PER among the experimental groups were insignificant.

3.2. Serum biochemical indices

In the group that received 400 mg kg^{-1} dietary supplementation of vitamin C, the serum vitamin C levels (Fig. 1A) increased linearly (P = 0.01), while the serum growth hormone levels (Fig. 1B) increased linearly (P = 0.00) and quadratically (P = 0.01), compared to the non-supplemented group. No significant effect of vitamin C supplementation was observed on the serum levels of ALT, AST, and creatinine (Table 3) at the end of the experiment, while these levels decreased linearly and quadratically ($P < 0.05$) in a concentration-dependent manner in the vitamin C-supplemented groups compared to the non-supplemented group.

3.3. Serum and hepatic antioxidant activities

As presented in Table 4, the serum MDA levels decreased linearly (P = 0.01) and quadratically (P = 0.00) with the increase in vitamin C supplementation levels, and the 400 mg kg^{-1} vitamin C supplementation group presented the lowest value for serum MDA. No significant effect of vitamin C supplementation was observed on the serum CAT activity, while the serum SOD and GSH levels in the vitamin C-supplemented groups presented a linear and quadratic increase ($P > 0.05$) in a concentration-dependent manner. Furthermore, no significant alteration in the hepatic MDA content was detected in the vitamin C-supplemented group, while the activities of hepatic SOD and CAT increased linearly and quadratically. The highest hepatic SOD and CAT activities were obtained in the group that received 400 mg kg^{-1} diet supplementation of vitamin C, followed by the groups with vitamin C supplementation of 300 mg kg^{-1} diet and 200 mg kg^{-1} diet, respectively, compared to the non-supplemented group. The GSH content presented a concentration-dependent linear increase (P = 0.00) in the groups supplemented with vitamin C compared to the non-supplemented group.

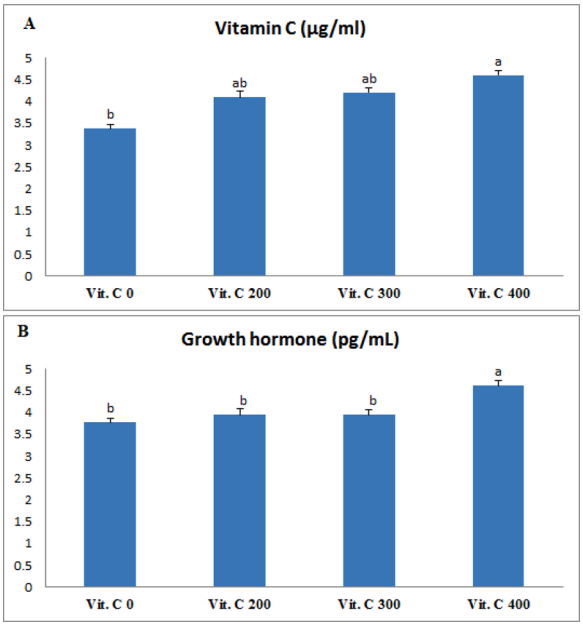

Fig. 1. The effects of vitamin C dietary supplementation on the serum levels of vitamin C (µg/mL) (A) and growth hormone (pg/mL) (B) in *Oreochromis niloticus* fingerlings. Data are expressed as Mean ±SE. n = 3. The groups with different superscripts differ significantly (P < 0.05).

Table 3
Effect of vitamin C (Vit. C) dietary supplementation (mg kg^{-1}) on hepato-renal functions in Nile tilapia fingerlings at the end of the experimental period (10 weeks) and after *Aeromonas sobria* challenge (2 weeks).

Parameters	Vit. C 0	Vit. C 200	Vit. C 300	Vit. C 400	Regression analysis *	
					Linear	Quadratic
At the end of the experiment						
ALT (UL-1)	17.60 ± 0.34	16.98 ± 0.28	16.78 ± 2.6	17.40 ± 0.92	0.95	0.26
AST (UL-1)	14.35 ± 0.20	13.85 ± 1.7	14.65 ± 0.20	14.18 ± 3.4	0.21	0.32
Creatinine (mgdl-1)	0.44 ± 0.02	0.53 ± 0.00	0.47 ± 0.01	0.49 ± 0.10	0.71	0.51
After bacterial challenge						
ALT (UL)	39.0 ± 2.88^{a}	32.50 ± 2.0^{b}	21.50 ± 1.44^{c}	14.50 ± 0.28^{d}	0.00	0.01
AST (UL)	63.50 ± 3.17^{a}	49.50 ± 0.28^{b}	40.50 ± 1.44^{c}	20.10 ± 0.60^{d}	0.01	0.02
Creatinine (mgdl-1)	1.60 ± 0.00^{a}	1.14 ± 0.05^{b}	1.03 ± 0.00^{b}	0.72 ± 0.05^{c}	0.00	0.02

ALT, Alanine aminotransferase; AST, Aspartate aminotransferase.
* The regressions were considered significant at P < 0.05.

Table 4
Effect of vitamin C (Vit. C) dietary supplementation (mg kg^{-1}) on serum and hepatic antioxidant activities in Nile tilapia fingerlings at the end of the experimental period (10 weeks).

Parameters	Vit. C 0	Vit. C 200	Vit. C 300	Vit. C 400	Regression analysis *	
					Linear	Quadratic
Serum antioxidant activity						
MDA (nmolml-1)	17.65 ± 0.25^{a}	14.22 ± 0.14^{b}	12.68 ± 0.10^{c}	11.69 ± 0.05^{d}	0.01	0.00
SOD (Uml-1)	2.81 ± 0.20^{d}	3.39 ± 0.28^{c}	3.46 ± 0.19^{b}	4.07 ± 0.04^{a}	0.00	0.02
CAT (Uml)	1.47 ± 0.23	1.35 ± 0.12	1.53 ± 0.34	1.51 ± 0.19	0.45	0.58
GSH (mmolL-1)	0.74 ± 0.01^{c}	1.02 ± 0.30^{b}	1.25 ± 0.01^{a}	1.48 ± 0.02^{a}	0.03	0.00
Liver antioxidant activity						
MDA (nmol $mL^{-1)}$	11.94 ± 0.67	11. 62 ± 0.41	11.38 ± 0.23	11.77 ± 0.71	0.76	0.52
SOD (Uml-1)	3.44 ± 0.15^{d}	3.85 ± 0.15^{c}	3.99 ± 0.11^{b}	4.09 ± 0.11^{a}	0.00	0.02
CAT (Uml-1)	1.59 ± 0.10^{c}	1.87 ± 0.05^{b}	1.91 ± 0.02^{b}	2.31 ± 0.18^{a}	0.03	0.01
GSH (mmolL-1)	2.14 ± 0.03^{d}	2.45 ± 0.02^{c}	2.68 ± 0.01^{b}	2.86 ± 0.03^{a}	0.00	0.06

MDA, Malondialdehyde; SOD, Superoxide dismutase; CAT, Catalase; GSH, reduced glutathione.
* The regressions were considered significant at P < 0.05.

3.4. Immunological indices

As presented in Table 5, no significant effect of vitamin C supplementation was observed on the serum levels of IL–10 at the end of the experiment. The lysozyme activity increased linearly (P = 0.00) and quadratically (0.01) in the 400 mg kg^{-1} vitamin C supplementation group compared to the non-supplemented group. The phagocytic percentage and nitric oxide levels increased linearly and quadratically ($P < 0.05$) in a concentration-dependent manner in all the vitamin C supplementation groups compared to the non-supplemented group, with

Table 5
Effect of vitamin C (Vit. C) dietary supplementation (mg kg^{-1}) on the immune parameters in Nile tilapia fingerlings at the end of the experimental period (10 weeks) and after *Aeromonas sobria* challenge (2 weeks).

Parameters	Vit. C 0	Vit. C 200	Vit. C 300	Vit. C 400	Regression analysis *	
					Linear	Quadratic
At the end of the experiment						
Lysozyme (UL-1)	18.07 ± 0.27^{b}	25.34 ± 1.99^{b}	25.33 ± 3.7^{b}	54.32 ± 1.93^{a}	0.00	0.01
Nitric oxide (μmoll)	7.14 ± 0.32^{d}	9.04 ± 0.14^{c}	14.1 ± 0.11^{b}	17.55 ± 0.14^{a}	0.02	0.01
Phagocytic%	70.62 ± 1. 2^{d}	72.66 ± 1.5^{c}	75.33 ± 0.95^{b}	82.23 ± 0.85^{a}	0.01	0.04
IL-10 (pgml)	1.63 ± 0.16	1.67 ± 0.06	1.71 ± 0.17	1.64 ± 0.15	0.90	0.85
After bacterial challenge						
Lysozyme (UL)	6.21 ± 0.13^{d}	7.33 ± 0.25^{c}	9.27 ± 0.41^{b}	16.30 ± 0.41^{a}	0.02	0.01
Nitric oxide (μmoll)	29.75 ± 1.76^{d}	29.53 ± 1.70^{c}	42.45 ± 0.72^{b}	48.15 ± 1.29^{a}	0.01	0.03
Phagocytic%	59.66 ± 0.22^{c}	64.25 ± 1.20^{b}	68.33 ± 1.2^{a}	68.60 ± 1.00^{a}	0.44	0.03
IL-10 (pgml)	1.94 ± 0.08^{d}	3.23 ± 1.39^{c}	4.40 ± 0.28^{b}	5.90 ± 0.10^{a}	0.00	0.00

IL-10, Interleukin-10.
* The regressions were considered significant at $P < 0.05$.

the 400 mg kg^{-1} vitamin C group presenting the best values. After the bacterial challenge, the serum lysozyme activity and the levels of nitric oxide and IL-10 increased linearly and quadratically ($P < 0.05$) in a concentration-dependent manner in all the vitamin C supplemented groups compared to the non-supplemented group, with the highest values ($P < 0.05$) recorded for the 400 mg kg^{-1} vitamin C group. The phagocytic percentage exhibited a quadratic increase ($P = 0.03$) in the groups supplemented with 300 mg kg^{-1} diet and 400 mg kg^{-1} diet of vitamin C.

3.5. Challenge with A. sobria

While the non-supplemented group presented the highest mortality % (83.33 %), the mortality rate in the vitamin C supplemented groups decreased in a concentration-dependent manner (54.16 %, 37.5 %, and 20.83 % mortality rate for 200, 300, and 400 mg kg^{-1} vitamin C supplementation groups, respectively). Among all groups of *A. sobria*-challenged fish, the highest value for RPS (75.00 %) was presented by the group supplemented with 400 mg/kg^{-1} vitamin C, followed by the groups with 300 mg kg^{-1} (55.00 %) and 200 mg kg^{-1} (35.00 %) vitamin C supplementation, respectively.

3.6. Histological analysis

The examined sections of the fish intestine (Fig. 2) from the group fed with diet containing 0 mg kg^{-1} vitamin C presented nearly normal intestinal mucosal and sub-mucosal coats, with widened lacteals and mild submucosal inflammatory lymphocytes. Remarkably tall and thick villi with focal inflammatory lymphocytic infiltrations were observed in the fish intestine sections from the group supplemented with 200 mg kg^{-1} vitamin C. The group supplemented with 300 mg kg^{-1} vitamin C presented a few remnant villi that were tall, thick, and short, while healthy-appearing villi with distinct brad cup-shaped tips were observed in the group supplemented with 400 mg kg^{-1} vitamin C.

The examined fish liver sections (Fig. 3) from the group fed with a diet containing 0 mg kg^{-1} vitamin C presented nearly normal hepatic architectures, with mild focal edema and a few inflammatory cells. Distinct normal healthy hepatic histo-structures with congestion sinusoids were observed in the liver sections from the group supplemented with 200 mg kg^{-1} vitamin C, while normal hepatic tissues with a few Kupfer cell hyperplasia appeared in the group supplemented with 300 mg kg^{-1} vitamin. The 400 mg kg^{-1} vitamin C-supplemented group presented normal-appearing hepatic tissues with a few fatty vacuoles.

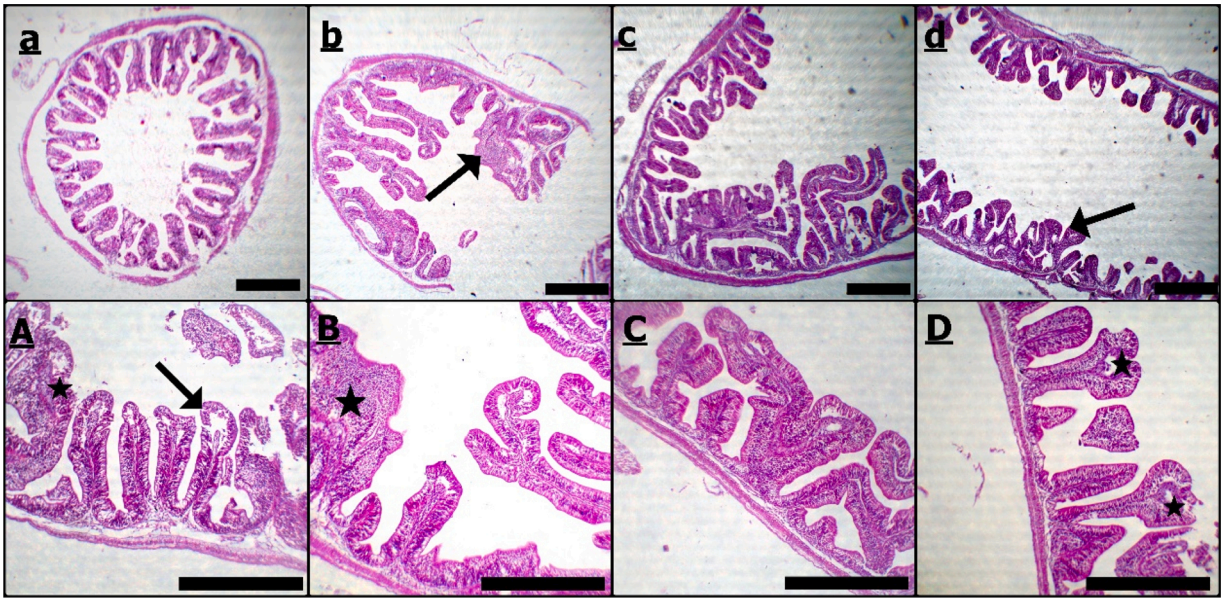

Fig. 2. Low and high magnification (Scale bare 100 μm) photomicrographs of H&E-stained sections from the fish intestine (anterior part) exhibiting **a and A**: nearly normal intestinal mucosal and sub-mucosal coats with widened lacteals (arrow) and mild sub-mucosal inflammatory lymphocytes (star) in the group fed with a diet containing **0** mg kg^{-1} vitamin C; **b and B**: distinct tall and thick villi with focal inflammatory lymphocytic infiltrations (star) in the group supplemented with **200** mg kg^{-1} vitamin C; **c and C**: a few remnant villi that are tall, thick, and short, in the group supplemented with **300** mg kg^{-1} vitamin C; and **d and D**: healthy-appearing villi (arrow) with distinct brad cup-shaped tips (stars) in the group supplemented with **400** mg kg^{-1} vitamin C.

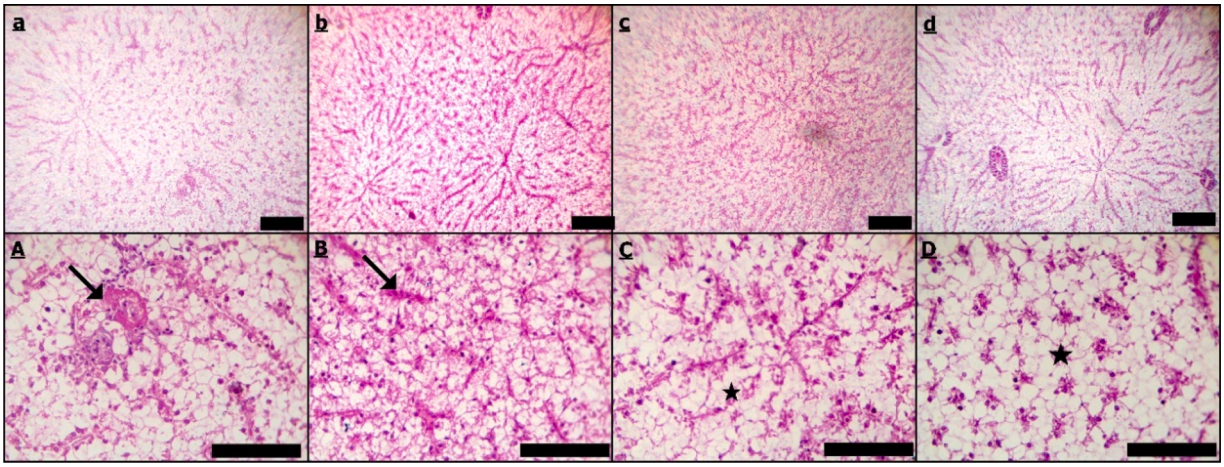

Fig. 3. Low and high magnification (Scale bare 100 μm) photomicrographs of H&E-stained sections from fish liver exhibiting **a and A:** nearly normal hepatic architectures with mild focal edema and a few inflammatory cells (arrow), in the group fed with a diet containing 0 mg kg^{-1} vitamin C; **b and B:** distinct healthy hepatic histo-structures with congestion sinusoids (arrow) in the group supplemented with 200 mg kg^{-1} vitamin C; **c and C:** normal hepatic tissues with a few Kupfer cell hyperplasia (star) in the group supplemented with 300 mg kg^{-1} vitamin C; and **d and D:** apparently normal hepatic tissues with a few fatty vacuoles (star) in the group supplemented with 400 mg kg^{-1} vitamin C.

3.7. Morphometric measurements of the intestine

The results for the morphometric measurements of the intestine are presented in Table 6. No significant difference was observed in the villus width (VW) between the vitamin C supplemented groups and the non-supplemented group, while the villus height (VH) increased linearly ($P = 0.02$) and quadratically ($P = 0.01$) in all the vitamin C supplemented groups compared to the non-supplemented group. Goblet cell count (GCC) and intra-epithelium lymphocyte count (IEL) exhibited concentration-dependent linear and quadratic increases ($P < 0.05$) in the groups supplemented with vitamin C compared to the non-supplemented group.

4. Discussion

The use of vitamins as feed additives is recommended in the diets of farmed fish. Vitamin C has an important role as an immuno-stimulant and an antioxidant scavenger. In the present research, dietary supplementation of different concentrations of vitamin C in Nile tilapia fingerlings significantly improved the growth performance of the fish in comparison to zero-level of vitamin C supplementation. This could be attributed to the role of vitamin C in increasing the serum levels of growth hormone, enhancing the intestinal morphology, and improving the absorptive surface of the intestine in fish (Abdel Rahman et al., 2018b). Enhancement of growth rate and weight gain observed in Nile tilapia upon vitamin C supplementation could be ascribed to the vitamin C-induced stimulation of protein synthesis (Chagas and Val, 2003; Faramarzi, 2012).

Excessive reactive oxygen species (ROS) that cause damaging effects are eliminated by the action of antioxidant enzymes, such as CAT, SOD, and GSH (Liang et al., 2017b). In the present work, the fish fed on a diet supplemented with vitamin C exhibited higher levels of serum SOD and GSH, lower levels of serum MDA, and higher levels of liver CAT, SOD, and GSH. All these results were attributed to the powerful antioxidant activity of vitamin C, as previously reported by Yadav et al. (2015). These findings were in agreement with the results reported by Liang et al. (2017a) for juvenile yellow catfish (*Pelteobagrus fulvidraco*) that received a diet supplemented with 156.5 mg kg^{-1} vitamin C and those by Hu et al. (2013) for juvenile black carp (*Mylopharyngodon piceus*) that received a diet supplemented with 63.0 mg kg^{-1} vitamin C.

Higher phagocytic percentage, as well as higher levels of lysozyme and NO, were observed, in a concentration-dependent manner, in the fish that received vitamin C supplemented diet. Vitamin C is reported to enhance various immune parameters, such as macrophage infiltration, complement activity, lysozyme levels, phagocytic activity of leucocytes, cytokine production, and antibody concentrations (Abdel Rahman et al., 2018b; Tewary and Patra, 2008). Moreover, vitamin C is one of the major antioxidants present in plasma and cell membranes, which may serve as a potent scavenger of free radicals and increase the levels of available nitric oxide via oxidation defense and endothelial nitric oxide synthase activity (Huang et al., 2000; Padayatty et al., 2003). The immune-stimulatory effect of vitamin C against *A. sobria* challenge was evidenced by the increase in RPS, particularly in the 400 mg kg^{-1}supplementation group. The use of vitamin C alone or in combination with *Echinacea purpurea* (EP) successfully enhanced the immune parameters in Nile tilapia (*Oreochromis niloticus*) (Abdel Rahman et al., 2018b). Dietary inclusion of vitamin C for enhancing fish resistance against bacterial infection has also been reported previously for Nile tilapia and juvenile cobia (*Rachycentron canadum*) (Khalil and ElHady, 2015; Zhou et al., 2012).

The intestine plays an important role in the digestion and absorption of nutrients. Moreover, digestive function improves with the improvement in intestinal structure, as evidenced by the increased nutrient absorption surface of the intestines with the increase in the height of the intestinal villus (Zhu et al., 2012). Intestinal mucosa forms a physiological and immune barrier for several pathogens and foreign substances

Table 6
Effect of vitamin C (Vit. C) dietary supplementation (mg kg^{-1}) on the intestinal morphology in Nile tilapia fingerlings at the end of the experimental period (10 weeks).

parameters	Vit. C 0	Vit. C 200	Vit. C 300	Vit. C 400	Regression analysis *	
					Linear	Quadratic
VH (μm)	21.10 ± 3.8^{b}	29.4 ± 5.0^{a}	37.6 ± 5.9^{a}	41.10 ± 9.2^{a}	0.02	0.01
VW (μm)	72.3 ± 6.9	76.66 ± 6.6	79.9 ± 9.3	33.18 ± 6.6	0.09	0.22
GCC	0.66 ± 0.33^{d}	1 ± 0.57^{c}	5 ± 1.1^{b}	1.6 ± 0.33^{a}	0.04	0.01
IEL	7.33 ± 2.0^{c}	10 ± 0.57^{b}	13.0 ± 1.5^{b}	19.66 ± 2.8^{a}	0.03	0.01

VH, villus height; VW, villus width; GCC, Goblet cell count; IELC, intra-epithelium lymphocytes.

* The regressions were considered significant at $P < 0.05$.

through the secretion of polymeric mucin glycoprotein from goblet cell (Leduc et al., 2018), and with assistance from the mucus-gel layer and from IELs, which play an essential role in the mucosal defense mechanisms against pathogens (Kiristioglu et al., 2002).

In the present study, dietary incorporation of vitamin C increased the villus height and the number of goblet cells and IELs, indicating improved intestinal absorption and immune defense, and consequently, the growth efficiency. A previous study by Abdel Rahman et al. (2018b) also reported improved intestinal structure in Nile tilapia fed with vitamin C-supplemented diet for 28 d. In addition, the liver and kidney functions were normal in all the treatment groups in the present study, at the end of the experiment as well as prior to bacterial challenge, as evidenced by non-significant changes in the levels of AST, ALT, and creatinine. On the other hand, elevated levels of AST, ALT, and creatinine after bacterial challenge were observed in the non-supplemented group, and this effect could be modulated with vitamin C supplementation in a concentration-dependent manner. Modulation in the liver function was observed in the 400 mg kg^{-1} diet vitamin C supplemented group, as evidenced by the histopathological analysis, which revealed a concentration-dependent improvement in the tissue architecture in vitamin C-supplemented groups compared to the non-supplemented groups. The liver sections from the fish fed with a diet containing 0 mg kg^{-1} vitamin C presented nearly normal hepatic architectures, with mild focal edema and a few inflammatory cells, while the liver sections from the fish supplemented with 300 mg/kg^{-1} diet of vitamin C presented normal hepatic tissues with just a few Kupfer cell hyperplasia. Kupfer cells are crucial for eliminating macromolecules, immune complexes, toxins, and degenerated cells, all of which are considered hepato-protective at low quantities and under steady-state conditions, from circulation (Deshane et al., 2005; Vickers, 2017). Moreover, the fish supplemented with 400 mg kg^{-1} diet of vitamin C exhibited normal liver architecture with a few fatty vacuoles, which could be attributed to the hepatoprotective effect of the antioxidant activity of vitamin C (Ozmen et al., 2004).

5. Conclusion

The results of the present research suggested that the dietary incorporation of vitamin C exerts a positive effect on the growth performance, immunological indices, oxidative capacity, and tissue histomorphology in fish, as well as on the disease resistance of fish against *A. sobria* bacteria. In Nile tilapia fingerlings, the best dietary level of vitamin C supplementation was determined to be 400 mg kg^{-1}.

CRediT authorship contribution statement

Rowida E. Ibrahim: Conceptualization, Methodology, Resources, Software, Formal analysis, Investigation, Data curation, Visualization, Writing - original draft. **Shaimaa A.A. Ahmed:** Conceptualization, Methodology, Visualization, Writing - review & editing. **Shimaa A. Amer:** Conceptualization, Methodology, Resources, Software, Formal analysis, Investigation, Data curation, Visualization, Writing - original draft. **Naif A. Al-Gabri:** Conceptualization, Methodology, Data curation, Visualization, Writing - review & editing. **Amany I. Ahmed:** Conceptualization, Methodology, Visualization, Writing - review & editing. **Abdel-Wahab A. Abdel-Warith:** Conceptualization, Methodology, Visualization, Project administration. **El-Sayed M.I. Younis:** Conceptualization, Methodology, Visualization, Project administration. **Abdallah E. Metwally:** Conceptualization, Methodology, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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