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Chitosan-Vitamin C Nanoparticles Ameliorate Heat Stress in the Nile Tilapia

Amr Fadel [1], Ahmed H. Sherif [2*], Lamiaa A. Okasha[3], Mohamed Abdelmegeid[4] Awad A. Abdelbaky[5]

[1]National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt
[2]Fish Diseases Department, Animal Health Research Institute AHRI, Agriculture Research Center ARC, Kafrelsheikh 12619, Egypt
[3]Bacteriology Unit, Animal Health Research Institute AHRI, Agriculture Research Center ARC, Kafrelsheikh 12619, Egypt
[4]Internal Animal Medicine Department, Faculty of Veterinary Medicine, Kafrelsheikh, Egypt
[5]Aquatic Animal Medicine and management Department, Faculty of Veterinary Medicine, Cairo University, 12211, Egypt
[*]**Corresponding Author: ahsherif77@yahoo.com**

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ABSTRACT

Climate change has become a global concern, with rising temperature extremities affecting the survival of aquatic animals. In this study, the Nile tilapia (*Oreochromis niloticus*) were divided into seven groups and fed diets for eight weeks: a control group with no feed additives, a group supplemented with bulk vitamin C (420 mg/kg dry diet), and five groups receiving vitamin C nanoparticles (VCNPs) at 20, 40, 60, 80, and 100% of the recommended dose—equivalent to 84, 168, 252, 336, and 420mg/ kg dry diet, respectively. The immune-oxidative status and resistance to *Aeromonas hydrophila* were evaluated following exposure to a low water temperature of 18 ± 0.5°C. All fish fed VCNP-supplemented diets showed significantly improved growth performance and reduced feed conversion ratios (FCR, ranging from 2.25 to 1.89), compared to those fed bulk vitamin C (FCR = 2.43) and the control group (FCR = 2.6). Serum cortisol levels increased significantly after 6 hours of heat stress, and serum glucose rose after 24 hours in all groups. However, VCNP-fed groups showed a significantly faster return to basal levels (0 h). The expression of the SOD gene was the highest in the VCNPs100 and VCNPs80 groups, with fold-changes of 6.8 and 6.5, respectively. No significant changes were observed in CAT gene expression 24 hours post-stress. Tilapia fed VCNP diets exhibited lower mortality rates following *A. hydrophila* infection, with relative protection levels of 33.3% (VCNPs100), 16.67% (VCNPs80), and 16.67% (VCNPs60). Regardless of bacterial infection, expression levels of TNF-β and IL-1β in the head kidney were lower in VCNP groups compared to the bulk VC and control groups. These pro-inflammatory markers significantly increased in response to *A. hydrophila* challenge. Interestingly, the bacterial infection did not influence HSP70 gene expression. Under heat stress, control fish exhibited signs of stress, including greenish-discolored livers, normal-sized spleens, empty intestines, and pale gills. In contrast, VCNP-fed groups maintained normal body coloration and organ appearance. In conclusion, dietary supplementation with VCNPs enhances the Nile tilapia's ability to tolerate low water temperatures and improves resistance to *Aeromonas hydrophila* infection.

INTRODUCTION

In recent decades, anthropogenic activities—particularly industrial operations—have significantly contributed to climate change **(Reinmann *et al.*, 2016)**. Rising ambient temperatures not only induce heat stress in aquatic animals, but also increase the risk of bacterial and viral infectious diseases **(Liang & Gong, 2017; Coppola *et al.*, 2018; Attia *et al.*, 2022; Sherif *et al.*, 2022a; Tawfeek *et al.*, 2024; Fadel *et al.*, 2025)**.

Optimal growth performance and feed utilization in tilapia species typically occur between 25 and 28°C, while temperatures below 16°C result in reduced growth, and mortality may occur when temperatures drop below 10– 12°C. Similar negative effects also arise under elevated temperatures **(Pandit & Nakamura, 2010)**. Changes in water temperature can mediate oxidative stress, reduce survival rates, and induce immunosuppression in fish **(Mariana & Badr, 2019)**. Oxidative stress is a common consequence for aquatic species exposed to cold conditions **(Fan *et al.*, 2013)**. The Nile tilapia (*Oreochromis niloticus*), representing approximately 71% of global tilapia production, is largely farmed in inland aquaculture systems **(L'Engle, 2016)**. This species is known for its resilience to varying environmental conditions, fast growth, high reproductive capacity, and ability to utilize diverse feed types **(Peterson, 2005; Sherif *et al.*, 2021; Okasha *et al.*, 2025a)**.

Maintaining optimal water temperature is critical for normal physiological functions, including feeding behavior, metabolism, consistent growth, and effective immune responses in aquatic animals **(Liu *et al.*, 2019; Wu *et al.*, 2019)**. When water temperature becomes suboptimal, fish immune systems become suppressed, reducing their resistance to infections **(Idris *et al.*, 2014; Hamdan *et al.*, 2015)**.

Vitamin C (VC) is a water-soluble vitamin and an essential antioxidant micronutrient in fish. It plays a vital role in regulating growth, reproduction, and immune-antioxidative responses by preventing lipid peroxidation through the elimination of reactive oxygen species (ROS) **(Padayatty & Levine, 2001; Ming *et al.*, 2012; Wan *et al.*, 2014; Elnagar *et al.*, 2024; Sherif *et al.*, 2024; Sherif *et al.*, 2025)**. In teleosts, VC improves immune responses against various stressors while minimizing oxidative tissue damage **(Shahkara *et al.*, 2015)**.

Additionally, dietary nano-composites containing chitosan nanoparticles have been shown to improve the performance of various aquatic species, including the sea bass (*Dicentrarchus labrax*), rainbow trout (*Oncorhynchus mykiss*), grey mullet (*Mugil cephalus*), gibel carp (*Carassius auratus gibelio*), loach (*Misgurnus anguillicaudatus*), and the Nile tilapia **(Sheikhzadeh *et al.*, 2017)**. Chitosan and nano-chitosan are also known to enhance digestive enzyme activity and to improve intestinal structure in fish **(Sheikhzadeh *et al.*, 2017; Hamidian *et al.*, 2018)**.

Therefore, this study was conducted to evaluate the growth performance and immune status of the Nile tilapia fed diets supplemented with vitamin C nanoparticles (VCNPs) protected by nano-sized chitosan. Additionally, the experimental fish were

exposed to low water temperatures to assess the efficacy of VCNPs under cold stress conditions.

MATERIALS AND METHODS

1. Experimental design

1.1 Accommodation and feeding regime

A total of 210 apparently healthy Nile tilapia (*Oreochromis niloticus*), weighing 71 ± 1.2 g body weight (b.w.), were collected from a local freshwater fish farm and immediately transported to the wet laboratory of the Animal Health Research Institute (AHRI) in Kafrelsheikh City, following the methods described by **Sherif and Zommara (2023)** and **Okasha *et al.* (2025b)**. For a 14-day acclimatization period, the fish were stocked in fiberglass tanks ($3 \times 1.5 \times 1$ m). The water parameters during this period were maintained at a temperature of 27.5 ± 0.5 °C, pH 7.9 ± 0.1, and salinity 0.48 ± 0.1 g/L. Tank water was exchanged daily at a rate of 30% with clean, dechlorinated water to ensure optimal environmental conditions for the experiment. Fish were fed at a rate of 5% b.w. per day, divided into two feedings at 10:00 a.m. and 4:00 p.m. The chemical composition of the diets was as follows: 41.22% crude protein, 3000 kcal/kg digestible energy, 5.74% ether extract, 2.6% crude fiber, 35.3% nitrogen-free extract, and 7.4% ash.

Preparation of chitosan nanoparticles: The preparation method relied on the ionic interaction between the amino groups of chitosan and the phosphate groups of tripolyphosphate. A solution was prepared by dissolving 1.5g of chitosan (Sigma-Aldrich, USA; molecular weight 50–90 kDa; ≥75% degree of deacetylation) in 300ml of acidified distilled water containing 3ml of glacial acetic acid. The mixture was vigorously stirred until a transparent solution with a pH of 4.5 was obtained. This solution was then filtered, and 420mg of bulk vitamin C (VC) (SLA1306, SLA4315, Sciencelab.com *et al.*, Texas, USA) was added and stirred. The mixture was subjected to 10 minutes of sonication and then centrifuged at 14,000rpm at 4°C for 30 minutes. The supernatant was discarded, and the pellet (sediment) was lyophilized **(Raj *et al.*, 2018)**. The resulting nanocomposites were analyzed for particle size and physical characteristics using high-resolution transmission electron microscopy (JEM-1400F HRTEM, 300 keV beam energy) at the Faculty of Agriculture, Cairo University.

Diet preparation: Initially, commercial fish pellets were soaked and thoroughly mixed with clean, dechlorinated water to form a dough-like consistency. The prepared vitamin C nanocomposites were then incorporated into the mixture and blended thoroughly with 5% (w/w) gelatin (Nutri-B-Gel; Canal Aqua Cure, Port Said, Egypt) to ensure even distribution.

Different diets of bulk VC (420 mg/kg dry; **NRC, 2011**) and VCNPs (20%, 40%, 60%, 80%, 100%) equal 84, 168, 252, 336, 420mg/ kg dry diet were prepared and offered to the experimental Nile tilapia in triplicates.

Growth performance and feed utilization were calculated as follows **(Sherif *et al.*, 2021):**

Total weight gain (TWG) = (FW) Final body weight (at the end of trial period) – (IW) Initial body weight (at starting time)
Daily weight gain (DWG) = TWG / Experimental period (day)
Weight gain WG % = TWG/IW X100
Feed conversion ratio (FCR) = Feed intake (FI) / TWG
Relative growth rate (RGR) % = 100 (FW – IW) (g) / IW (g)
Survival rate (SR) = (survivied fish in specific period / total fish number in the same period) X 100

1.2. Heat stress

In the experimental aquaria, the water temperature was adjusted at 27.5 ±1.5°C for eight weeks. The acute heat stress was attained by gradually lowering the water temperature from 27.5 ±1.5 °C to 18±0.5 °C within 3h. The fish were then exposed to 18±0.5°C for 4h.

2. Gene expression of immunity and antioxidative genes

Gene expression in the head kidney of the experimental Nile tilapia was performed for immunological cytokines (interleukin *(IL)-1β, IL-10*, and tumor necrosis factor *(TNF)-α* as well as antioxidant enzymes superoxide dismutase *(SOD),* glutathione peroxidase *(GPx),* and catalase *(CAT).* In Table (1), all primers sequences are present based on National Center for Biotechnology Information (NCBI) database. All primers and kits were supplied from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Quantitative poly merase chain reaction qPCR were carried out in a thermal cycler (AbiPrism 7300) (Applied Biosystems, USA). The quantitative fold alterations in the examined genes was calculated in relation to *β*-actin mRNA (house hold gene) by the 2^{-DD} CT method.

Table 1. List of all primers used in this study

Target gene	**Primer sequence**	**Amplified segment** **Length**	**Annealing temperature**	**Accession number**
β-actin	F: GCATCACACCTTCTACAACGA R: TGGCGGGGGTGTTGAAGGTCT	139 bp	57 °C 30 s	AA566386

TNF−α	F: AGCATGGAAGACCGTCAACGAT R: ACCCTCTAAATGGATGGCTGCTT	131 bp	56 °C 30 s	AJ277604
IL−1β	F: TGGTGACTCTCCTGGTCTGA R: GCACAACTTTATCGGCTTCCA	86 bp	56 °C 30 s	XM_005457887.1
IL−10	F: ACCCCGTTCGCTTGCCA R: CATCTGGTGACATCACTC	70 bp	56 °C 30 s	AM268529
IL−8	F: GCACTGCCGCTGCATTAAG R: GCAGTGGGAGTTGGGAAGAA	273 bp	56 °C 30 s	XM_003455949.2
HSP−70	F: ACCCAGACCTTCACCACCTA R: GTCCTTGGTCATGGCTCTCT	84 bp	59 °C 30 s	FJ213839.1
SOD	F: GGTGCCCTGGAGCCCTA R: ATGCGAAGTCTTCCACTGTC	377	56 °C 30 s	JF801727.1
CAT	F: TCCTGAATGAGGAGGAGCGA R: ATCTTAGATGAGGCGGTGATG	232	56 °C 30 s	JF801726.1
GPx	F: CCAAGAGAACTGCAAGAGA R: CAGGACACGTCATTCCTACAC	180	56 °C 30 s	FF280316.1

Note: *TNF-β*: tumour necrosis factor beta; *IL-1β:* Interleukin-1 beta; *HSP70*: heat shock protein 70; *SOD:* superoxide dismutase; *CAT:* catalase; *GPx:* glutathione peroxidase.

3. Bacterial infection with different modes with re-isolation

To assess the immunostimulatory effects of VC, ten fish from each experimental group were intraperitoneally injected with pathogenic *Aeromonas hydrophila* (AHRAS2, accession number MW092007) at an LD_{50} dose of 2.4×10^5 cfu, as previously isolated and identified by **Sherif and Abuleila (2022)**. A negative control group was included by injecting fish with sterile saline solution (0.65%) **(Boijink *et al.*, 2001)**. The challenged Nile tilapia were monitored for fourteen days to record mortalities and to calculate the mortality rate (MR%) and relative protection level (RLP%) according to the equations provided by **Ruangpan *et al.* (1986)**:

$$\text{MR\%} = \frac{\text{number of deaths in a specific period}}{\text{total population during that period}} \times 100$$

$$\text{RLP\%} = \left(1 - \frac{\%\text{deaths in the treated group}}{\%\text{deaths in the control group}}\right) \times 100$$

4. Statistical analyses

The impact of VCNP nanocomposites on the Nile tilapia performance under heat stress was statistically analyzed using SPSS software (version 2022). Data were assessed through one-way ANOVA, and results are presented as means ± standard errors (SE). Differences between treatment groups were determined using Duncan's Multiple Range Test, with statistical significance considered at $P< 0.05$.

RESULTS

1. Impacts of vitamin C and nano-vitamin C on fish performance

In Table (2), the Nile tilapia fed the dietary nanocomposite (Fig. 1) VCNPs100 showed significantly higher final weight (FW), total weight gain (TWG), daily weight gain (DWG), weight gain (WG), and relative growth rate (RGR), followed by the VCNPs80 group. The VCNPs100 group reached values of 150.15 g (FW), 79.15 g (TWG), 1.41 g/day (DWG), 111.6% (WG), and 211.6% (RGR), while the VCNPs80 group recorded 138.7 g, 67.6 g, 1.21 g/day, 94.97%, and 195%, respectively. No consistent or dose-dependent pattern of growth performance was observed in response to increasing levels of VCNP supplementation. All VCNP-treated groups exhibited significantly lower feed conversion ratios (FCR), ranging from 2.25 to 1.89, compared to the bulk VC group (2.43) and the control group (2.6).

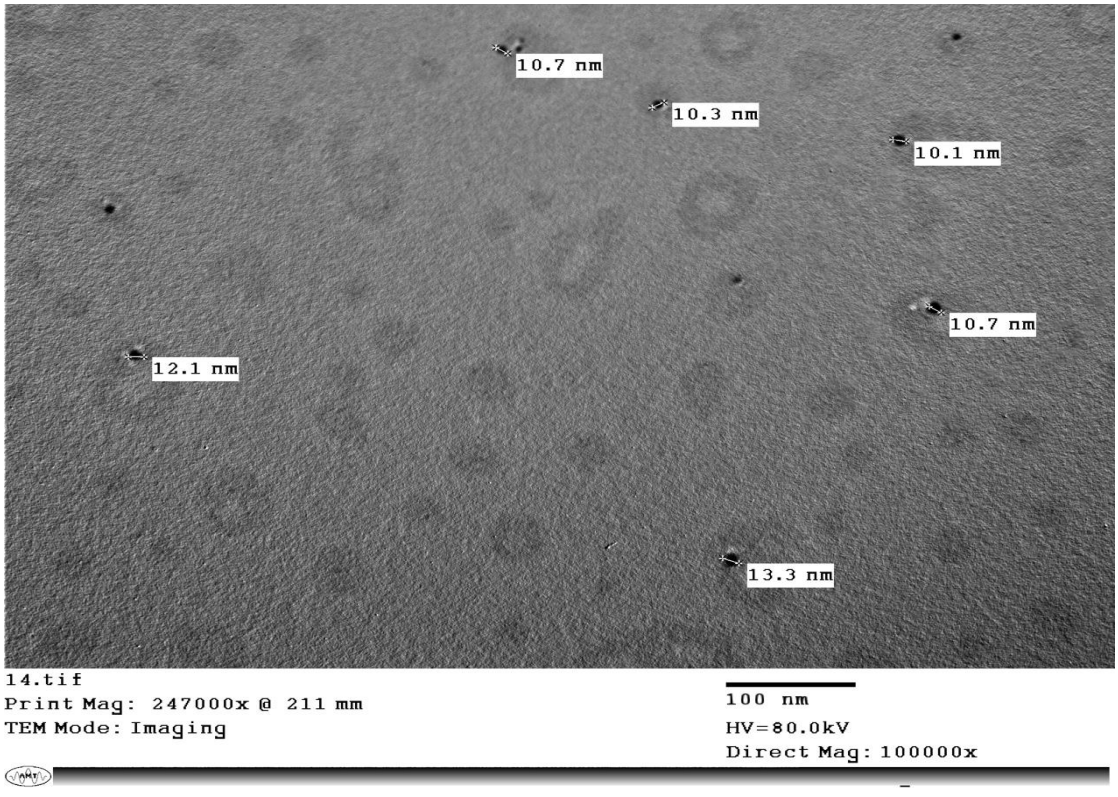

Fig. 1. Nanocomposite of chitosan and VC by transmission electron microscope

Table 2. The growth and feed utilization of the Nile tilapia 8-weeks feeding trial

Items	Control	VC	VCNPs 20%	VCNPs 40%	VCNPs 60%	VCNPs 80%	VCNPs 100%
IW (g/fish)	71.33 ±0.6	70.6 ±0.47	71.5 ±0.45	70.7 ±0.25	71 ±0.6	71.13 ±0.18	71 ±0.6
FW (g/fish)	115.95**E** ±0.5	116.9**DE** ±1.1	123.2**C** ±0.5	122.6**CD** ±0.9	121.4**CDE** ±0.07	138.7**B** ±3.7	150.15**A** ±3
TWG (g/fish)	44.6**E** ±0.8	46.3**DE** ±1.22	51.7**C** ±0.8	52**C** ±0.8	50.4**DE** ±0.65	67.6**B** ±3.6	79.15**A** ±3.6
DWG (g/fish)	0.8**D** ±0.01	0.83**CD** ±0.02	0.92**C** ±0.01	0.93**C** ±0.01	0.9**CD** ±0.01	1.21**B** ±0.06	1.41**A** ±0.06
WG %	62.57**D** ±1.6	65.62**CD** ±2	72.34**CD** ±1.6	73.4**C** ±1.12	71**CD** ±1.5	94.97**B** ±4.9	111.6**A** ±5.9
RGR %	162.6**D** ±1.6	165.6**CD** ±2	172.34**CD** ±1.6	173.4**C** ±1.12	171**CD** ±1.5	195**B** ±4.87	211.6**A** ±5.9
FCR	2.6**A** ±0.02	2.43**AB** ±0.15	2.25**BC** ±0.03	2.05**DE** ±0.03	2.25**BC** ±0.05	2.06**DE** ±0.07	1.89**E** ±0.006
FI (g)	116.2**B** ±3	113**B** ±9.74	116.2**B** ±3.34	106.4**B** ±3.16	113.4**B** ±1.5	139.35**A** ±8.1	149.6**A** ±6.5
SR %	100	90	100	90	90	90	100

Note: Data represented as means ± standard error. Mean values with different letters at the row differ significantly at $P \leq 0.05$. VC; vitamin C, VCNPs, vitamin C and chitosan nanocomposite. IW; initial weight, FW; final weight, TWG; total weight gain, DWG; daily weight gain, WG; weight gain, RGR; relative growth rate, FCR; feed conversion ratio, FI; feed intake, SR; survival rate.

2. Impacts of acute heat stress

2.1. Stress hormone and serum glucose

In Table (3), serum cortisol levels significantly increased in all groups 6 hours after exposure to heat stress compared to baseline (0h). Among the treatments, the VCNPs100,

VCNPs80, and VCNPs60 groups exhibited significantly higher cortisol levels (2.64, 2.57, and 2.57µg/ dL, respectively) compared to the bulk VC (2.3µg/ dL) and control (2.37µg/ dL) groups. However, after 24 hours, cortisol levels in the VCNPs100, VCNPs80, VCNPs60, and VCNPs40 groups declined to basal values similar to pre-stress levels (0h). In contrast, the control, VC, and VCNPs20 groups maintained elevated cortisol levels at 1.23, 1.11, and 0.96µg/ dL, respectively.

Table 3. Serum cortisol level after heat stress

Time	**Control**	**VC**	**VCNPs 20%**	**VCNPs 40%**	**VCNPs 60%**	**VCNPs 80%**	**VCNPs 100%**
0h	0.84 ±0.02	0.86 ±0.04	0.84 ±0.02	0.88 ±0.02	0.85 ±0.06	0.83 ±0.02	0.83 ±0.03
6h	2.37**B** ±0.05	2.3**B** ±0.02	2.45**AB** ±0.08	2.33**B** ±0.07	2.57**A** ±0.04	2.57**A** ±0.03	2.64A ±0.1
12h	1.6**A** ±0.02	1.5**ABC** ±0.03	1.42**CD** ±0.02	1.26**E** ±0.03	1.38**DE** ±0.04	1.54**AB** ±0.02	1.55**AB** ±0.1
24h	1.23**A** ±0.16	1.11**AB** ±0.04	0.96**BC** ±0.07	0.84**C** ±0.02	0.85**C** ±0.01	0.85**C** ±0.01	0.81**C** ± 0.01

Note: Different capital letters within the same column indicate that values are significant differences at $P \leq 0.05$. VC; vitamin C, VCNPs, vitamin C and chitosan nanocomposite.

In Table (4), serum glucose levels significantly increased after 24 hours of heat stress, with the highest elevation observed at this time point. However, by 96 hours, glucose levels in the VCNPs100 and VCNPs80 groups were significantly restored to near-normal values comparable to the pre-stress baseline (0h), followed by the VCNPs60 group. The glucose concentrations recorded at 96 hours were 55.3mg/ dL (VCNPs100), 59.5mg/ dL (VCNPs80), and 64.4mg/ dL (VCNPs60), indicating a faster recovery compared to the other groups ($P \leq 0.05$).

Table 4. Serum glucose level after heat stress

Item	Control	VC	VCNPs 20%	VCNPs 40%	VCNPs 60%	VCNPs 80%	VCNPs 100%
0h	42 ±1.15	44 ±2.1	44.67 ±2.03	45.67 ±1.86	42.7 ±0.7	43.3 ±1.7	43.7 ±2.2
24h	77.3**BC** ±1.8	75.7**BC** ±1.2	76.7**BC** ±0.88	75.7**BC** ±2.85	75**C** ±1.15	81**AB** ±1.5	84**A** ±2.1
48h	91.3**A** ±1.86	78**E** ±1	79.13**DE** ±0.72	82.23**CD** ±0..5	83.8**C** ±0.93	87.7**B** ±1.05	91.7**A** ±0.88
72h	88.3**A** ±2	90.7**A** ±1.2	92.2**A** ±0.6	91.13**A** ±0.94	88.27**A** ±1.6	74.8**B** ±0.99	71.4**B** ±0.54
96h	72.3**B** ±1.2	78**A** ±2.5	72.5**B** ±1.26	70.9**B** ±0.26	64.4**C** ±2.73	59.5**DE** ±0.76	55.3**E** ±1.38
7days	50.3**A** ±1.2	42.2**BC** ±1.3	43.6**BC** ±0.78	44.87**B** ±0.07	41.9**BC** ±0.5	42.3**BC** ±1.18	41.6**C** ±0.9

Note: Different capital letters within the same column indicate that values are significant differences at $P \leq 0.05$. VC; vitamin C, VCNPs, vitamin C and chitosan nanocomposite.

2.3. Gene expression of antioxidants

In Fig. (2), the gene expression of SOD increased at 72 hours post-stress and then declined to pre-stress levels by day 7. Fish that received dietary VCNPs showed a higher and more gradual elevation in SOD expression, ranging from 1.62 to 1.77 fold-change, compared to the control group, which exhibited only a 1.25 fold-change. These results indicate that VCNP supplementation enhanced the antioxidant response more effectively than the bulk VC or control diets.

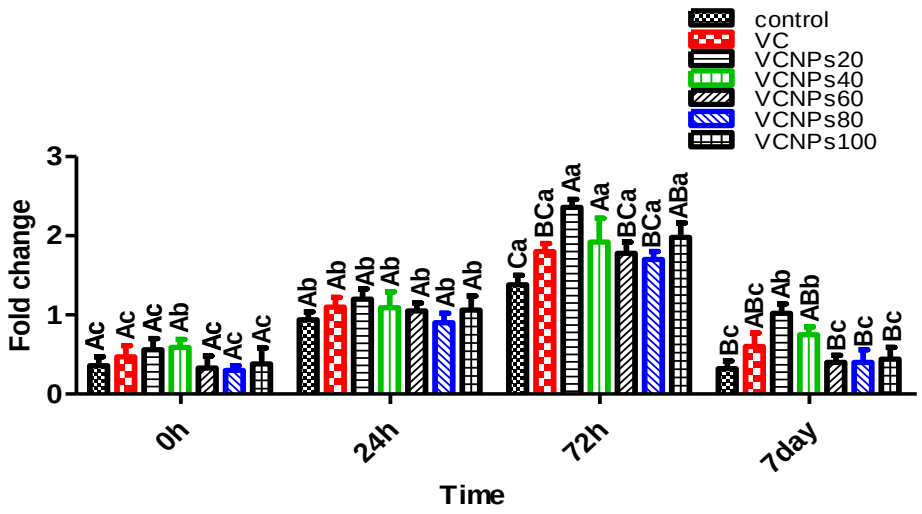

Fig. 2. The expression of superoxide dismutase *SOD* gene after heat stress. VC; vitamin C, VCNPs, vitamin C and chitosan nanocomposite. Different capital letters indicate that values are significant differences at $P \leq 0.05$

After 24 hours post-stress, the gene expression of GPx increased across all groups, with the highest expression observed in the VCNPs100 and VCNPs80 groups, showing 6.8 and 6.5 fold-change, respectively. By 72 hours post-stress, gene expression was elevated in all experimental fish regardless of dietary supplementation. However, the Nile tilapia fed VCNPs100 and VCNPs80 continued to exhibit the highest GPx expression levels, reaching 8.4 and 7.5 fold-change, respectively, compared to other groups (Fig. 3).

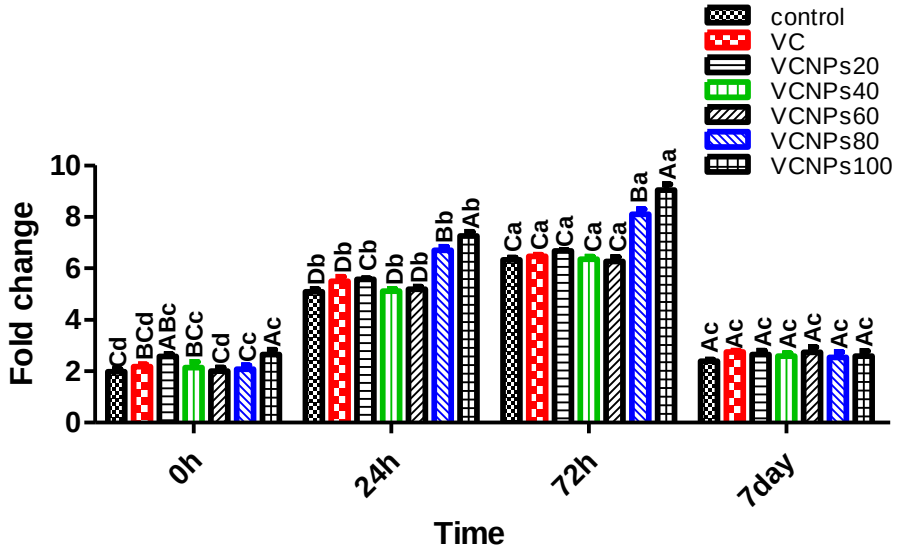

Fig. 3. The expression of glutathione peroxidase *GPx* gene after heat stress. VC; vitamin C, VCNPs, vitamin C and chitosan nanocomposite. Different capital letters indicate that values are significant differences at $P \leq 0.05$.

In Fig. (4), no significant alterations in CAT gene expression were observed at 24 hours post-stress, except in the VCNPs100 group, which showed an increase to 3.8 fold-change compared to the pre-stress level of 2.8 fold-change. At 72 hours post-stress, CAT gene expression was markedly elevated in fish fed VCNPs100 and VCNPs80, reaching 9.85 and 9.2 fold-change, respectively. Fish in the VCNPs60, VCNPs40, and VCNPs20 groups exhibited expression levels of 8.8 fold-change. By day 7 post-stress, all groups returned to baseline (pre-stress) gene expression levels, except for VCNPs100 and VCNPs80, which still showed elevated levels of 3.58 and 3.5 fold-change, respectively.

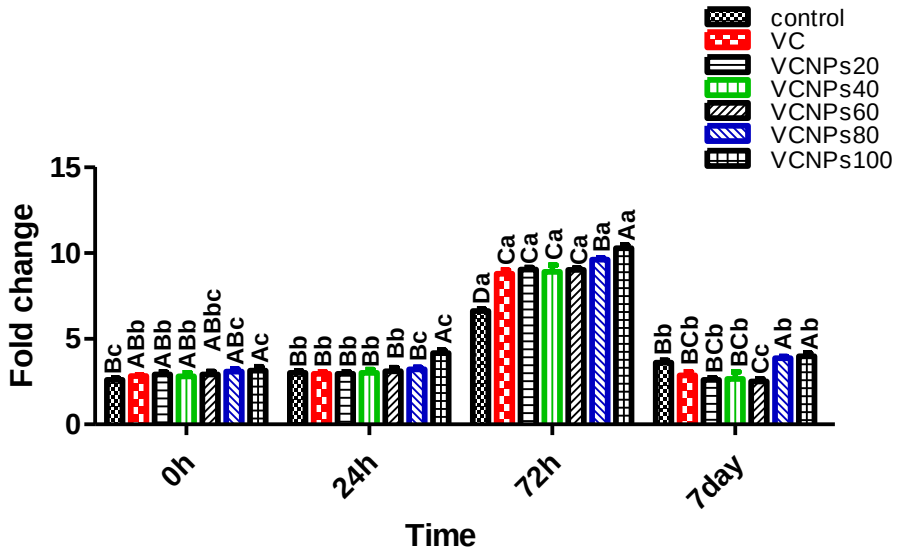

Fig. 4. The expression of catalase *CAT* gene after heat stress

3. Impact of heat stress on cytokines gene expression in the Nile tilapia infected with *Aeromonas hydrophila*

The experimental Nile tilapia were infected with pathogenic *A. hydrophila* via intraperitoneal (I/P) injection at the LD_{50} dose, both before and after exposure to heat stress (Table 5). Following infection, the gene expressions of TNF-β, IL-1β, and HSP70 were measured in the head kidney.

During the pre-stress period, a lower mortality rate (MR) was observed in the VCNPs100 group, followed by VCNPs80 and VCNPs60, with recorded MRs of 40%, 50%, and 50%, respectively. These groups demonstrated relative protection levels (RLP) of 33.3, 16.67, and 16.67%, respectively. In contrast, the VCNPs40 and VCNPs20 groups exhibited a 60% mortality rate, the same as the control group, and provided no protective effect.

During the post-stress period, only the VCNPs100 and VCNPs80 groups were able to reduce mortality rates to 60 and 80%, respectively, corresponding to RLP values of 40 and 20% (Table 5).

Table 5. Bacterial infection of the experimental Nile tilapia (n=10)

Items		**Cont -ve**	**Cont**	**VC**	**VCNPs 20%**	**VCNPs 40%**	**VCNPs 60%**	**VCNPs 80%**	**VCNPs 100%**
Pre-stress &challenge	**MR %**	10	60	60	60	60	50	50	40
	RPL %	-	-	0	0	0	16.67	16.67	33.3
Post-stress &challenge	**MR %**	10	100	100	100	100	100	80	60
	RPL %	-	-	0	0	0	0	20	40

Note: Cont; control, VC; vitamin C, VCNPs, vitamin C and chitosan nanocomposite.

In Figs. (5, 6), the gene expressions of TNF-β and IL-1β were upregulated in response to *A. hydrophila* infection; however, these immune responses were reduced following exposure to heat stress. Additionally, a positive and gradual increase in gene expression was observed with increasing doses of VCNPs in the diet. Notably, in the absence of bacterial infection, the expression levels of TNF-β and IL-1β were not significantly affected by dietary VCNPs and remained lower than those observed in the bulk VC and control groups.

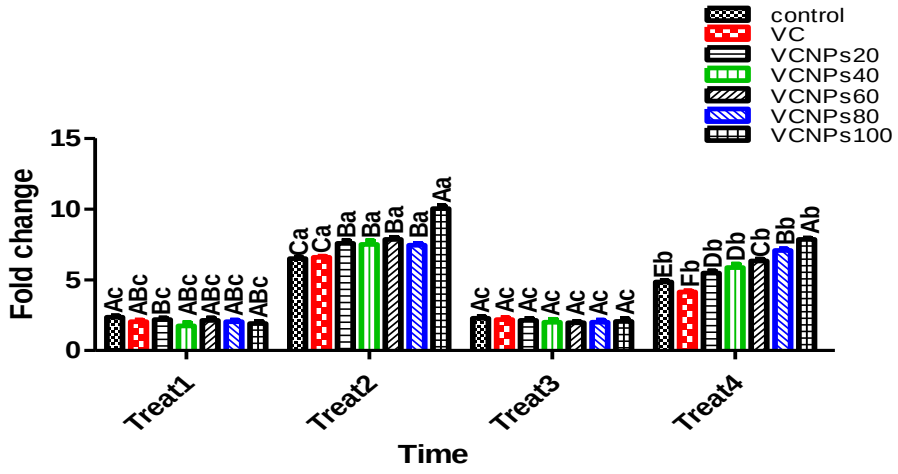

Fig. 5. The expression of *TNF-β* gene after heat stress. Note: Treat1; Pre-stress, Treat2; Pre-stress & challenged, Treat3; Post-stress, Treat4; and Post-stress & challenged.

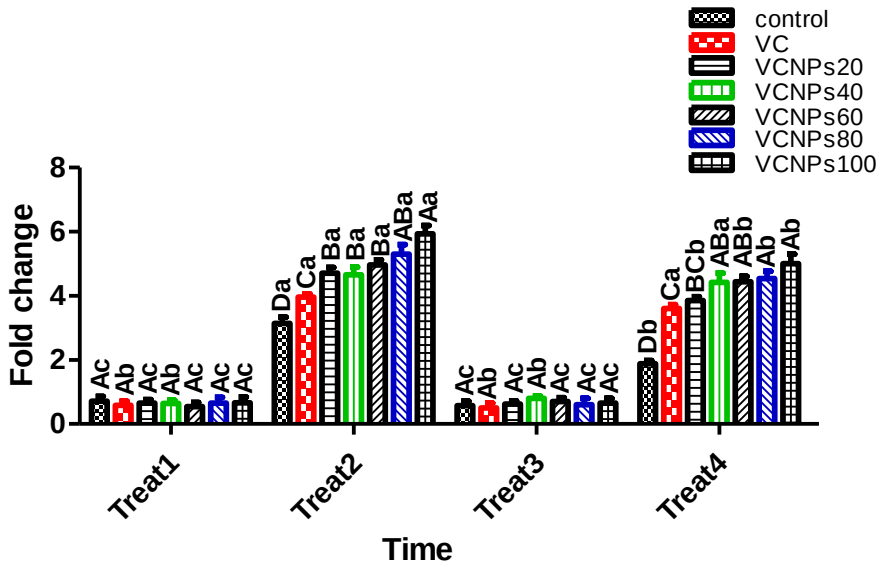

Fig. 6. The expression of *IL-1β* gene after heat stress. Note: Treat1; Pre-stress, Treat2; Pre-stress & challenged, Treat3; Post-stress, Treat4; and Post-stress & challenged. VC; vitamin C, VCNPs, vitamin C and chitosan nanocomposite. Different capital letters indicate that values are significant differences at $P \leq 0.05$.

In Fig. (7), the gene expression of HSP70 was influenced by heat stress, with expression levels ranging between 4.63 and 4.82 fold-change in the head kidneys of fish fed VCNP-supplemented diets. In comparison, expression levels were 4.62 and 4.58 fold-change in the bulk VC and control groups, respectively. Conversely, *A. hydrophila* infection did not induce HSP70 expression during the pre-stress period. Only minimal alterations in gene expression were observed across all groups following *A. hydrophila* infection during the post-stress period.

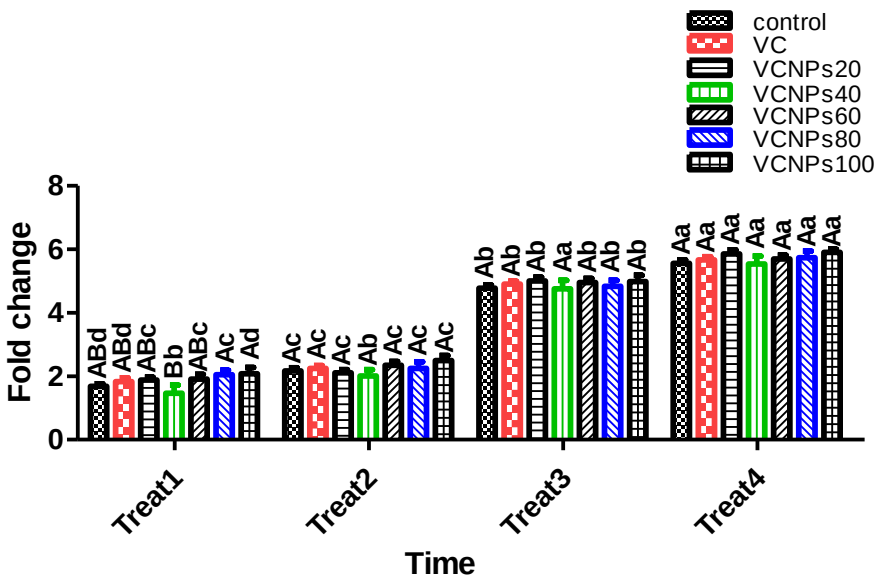

Fig. 7. The expression of *HSP70* gene after heat stress. Note: Treat1; Pre-stress, Treat2; Pre-stress & challenged, Treat3; Post-stress, Treat4; and Post-stress & challenged. VC; vitamin C, VCNPs, vitamin C and chitosan nanocomposite. Different capital letters indicate that values are significant differences at $P \leq 0.05$.

In Fig. (8), the Nile tilapia in the control group lost their body and gills color whereas post-mortem investigation revealed affected liver with distended gall-bladder. Those fed on VC-supplemented diet had lesser signs compared to the control ones (Fig. 9). Meanwhile, fish received VCNPs reserved their body coloration with full intestine.

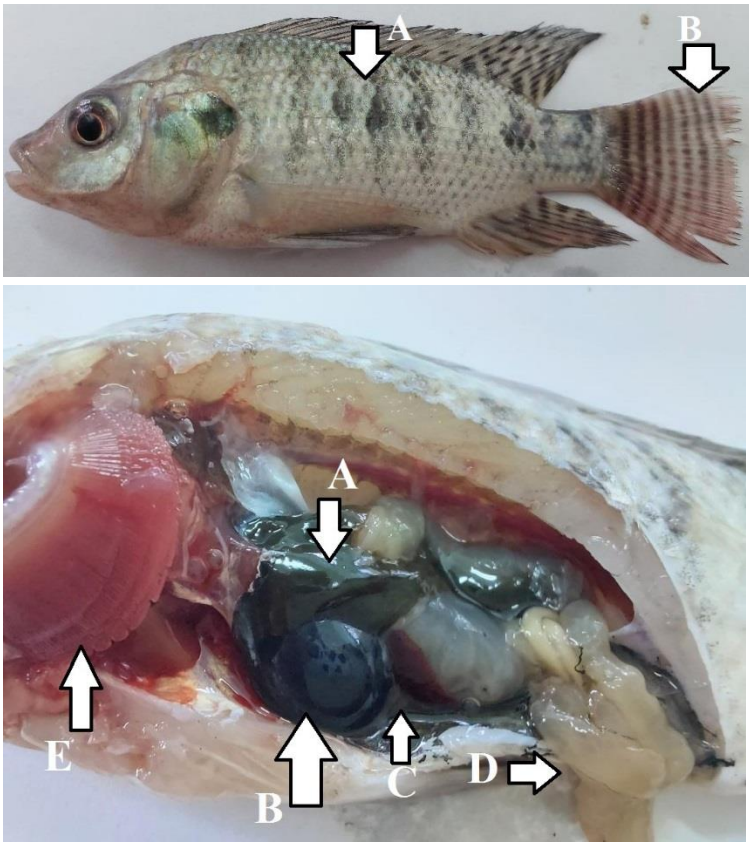

Fig. 8. Heat stressed (control) faint body coloration, postmortem (A) greenish-discolored liver, (B) distended gall-bladder, (C) normal size spleen, (D) empty intestine, and (E) pale gills.

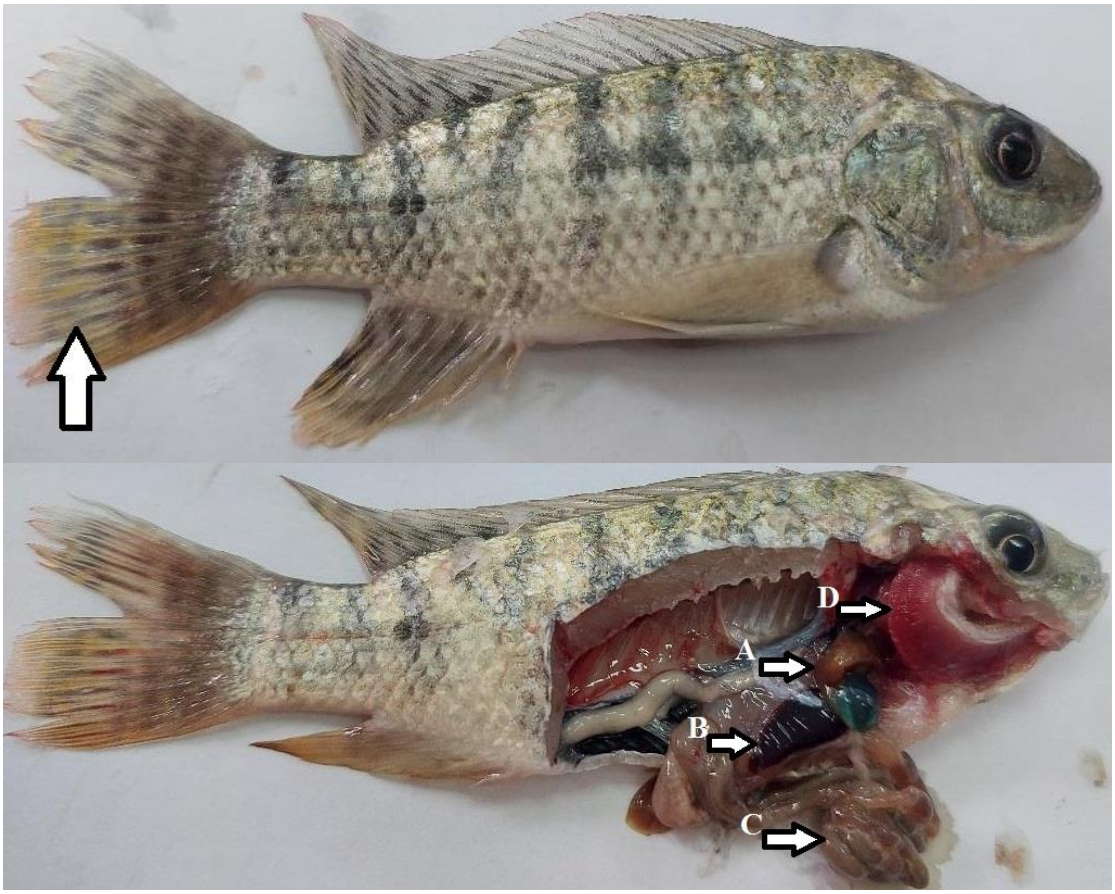

Fig. 9. The Nile tilapia received (VC) heat stressed body yellowish discoloration, postmortem (A) light brownish live, (B)splenomegaly, (C) partial empty intestine, and (D) pale gills

DISCUSSION

Nano-sized vitamin C (VC) can be effectively protected using chitosan nanoparticles. **Minhajul *et al.* (2020)** found that low molecular weight chitosan nanoparticles enhance the availability of free amino groups for protonation, increasing VC adsorption. Conversely, high molecular weight chitosan has also proven effective for encapsulation, as its long-chain amino groups can capture VC nanoparticles **(Caritsa *et al.*, 2020)**. However, nano-sized materials can act as either enhancers or toxic agents for fish health **(Sherif *et al.*, 2022b; Sherif *et al.*, 2023; Sherif & Zommara, 2023; Farag *et al.*, 2024)**.

In this study, the experimental Nile tilapia were supplemented with the recommended dose of VC (420 mg/kg dry diet) or various doses of VC nanoparticles (VCNPs) corresponding to 20, 40, 60, 80, and 100% of the recommended dose (i.e., 84, 168, 252, 336, and 420mg/ kg, respectively). The VCNPs100 group exhibited significantly enhanced growth performance along with the lowest feed conversion ratio

(FCR). Overall, all VCNP-treated groups had significantly lower FCRs compared to the bulk VC and control groups. These findings support the established role of VC as a key micronutrient required for the growth of the Nile tilapia. Previous research demonstrated that dietary VC supplementation at 300mg/ kg improved growth and weight gain **(NRC, 1993; Wang & Li, 2011; Khan *et al.*, 2017; Rathore *et al.*, 2019)**, possibly due to stimulation of digestive enzymes **(El-Basuini *et al.*, 2021)**. However, some studies reported that nanoparticles could negatively affect metabolic processes in fish **(Wang *et al.*, 2015; Naderi *et al.*, 2017)**.

In this experiment, the positive effects of VCNPs may be attributed to nano-chitosan, which activates digestive enzymes and inhibits pathogenic bacteria in marine species **(Qin *et al.*, 2014; Younus *et al.*, 2020)**. Additionally, nano-chitosan offers improved absorption and bioavailability in the fish gut **(Rajesh Kumar *et al.*, 2008)**, and its prolonged retention in the gastrointestinal tract allows for more efficient absorption through the epithelium **(Wang & Li, 2011; Imefon, 2018)**.

In aquaculture, fish frequently encounter environmental stressors such as fluctuations in water quality, handling, high stocking density, and transportation, all of which can trigger stress hormone secretion **(Mommsen *et al.*, 1999; Rubio *et al.*, 2005)**. In the present study, following heat stress, serum cortisol and glucose levels in VCNP-fed groups were rapidly restored to basal levels. Cortisol and glucose are widely recognized as stress biomarkers, reflecting the fish's energy regulation in response to stress **(Bertotto *et al.*, 2010; Martin *et al.*, 2011; Sopinka *et al.*, 2016)**. Similar results were reported for SeNPs- and VC-fed Nile tilapia, where stress biomarkers declined significantly **(El-Basuini *et al.*, 2021; Rathore *et al.*, 2023)**. In teleosts, acute and chronic heat stress activates the hypothalamus-pituitary-interrenal (HPI) axis, leading to cortisol release into the bloodstream **(Mommsen *et al.*, 1999; Yang *et al.*, 2021)**.

Elevated activities of antioxidant enzymes such as SOD, GPx, and CAT indicate stimulation of antioxidant defense and are commonly used as biomarkers of oxidative stress in aquatic organisms **(Sherif *et al.*, 2019; Yousefi *et al.*, 2019)**. In this study, fish fed VCNPs exhibited a gradual increase in the gene expressions of SOD, GPx, and CAT following heat stress, with expression levels returning rapidly to basal values. Similar findings have shown that reactive oxygen species (ROS) production increases in fish exposed to low water temperatures (13–17°C), as cold conditions can impair ROS elimination systems **(Lushchak, 2011; Luo *et al.*, 2014; Lu *et al.*, 2016; Cheng *et al.*, 2018)**. Heat stress has also been shown to induce SOD gene expression based on RNA-seq data **(Chen *et al.*, 2023)**. VC, as a potent antioxidant, plays a critical role in detoxifying ROS and serves as a cofactor in numerous enzymatic reactions **(Arrigoni & De Tullio, 2002; Yousef *et al.*, 2007; Njus *et al.*, 2020)**. VC has also been found to protect the Nile tilapia from acetamiprid-induced oxidative stress and DNA damage by downregulating SOD1 and SOD2 gene expression **(Takisawa *et al.*, 2019; Hathout *et al.*, 2021)**.

Temperature fluctuations significantly threaten fish health, causing physiological disruptions and reducing bacteriolytic activity **(Bly & Clem, 1992; Quiniou *et al.*, 1998; Bowden, 2008)**. In this study, dietary VCNPs provided protection against *A. hydrophila*, even under post-stress conditions. This aligns with previous studies showing that VC combined with chitosan nanoparticles (VC-CSNPs) enhanced phagocytic activity against multiple pathogens, including *Vibrio harveyi*, *A. veronii*, *A. hydrophila*, *V. anguillarum*, and *V. alginolyticus* **(Mohan *et al.*, 2019; Chen *et al.*, 2020)**. The antibacterial properties of chitosan are attributed to its ability to interact with bacterial DNA and chelate metal ions. Additionally, dietary VC-chitosan improved lysozyme activity in the rainbow trout **(Alishahi *et al.*, 2011)** and silver carp **(Younus *et al.*, 2020)**, enhancing bacterial resistance.

In the current study, the expression of TNF-β and IL-1β genes increased in a dose-dependent manner with dietary VCNP supplementation, correlating with reduced mortality rates following *A. hydrophila* infection. Similar upregulation of IL-1β was observed in the head kidney of zebrafish fed silver-chitosan nanocomposites **(Udayangani *et al.*, 2017; Nikapitiya *et al.*, 2018)**. Furthermore, nano-Se, vitamin E, and VC have all been reported to stimulate and regulate pro-inflammatory cytokine expression **(Abarike *et al.*, 2019)**.

In this study, HSP70 gene expression was only slightly increased in VCNP-fed fish compared to the VC and control groups. *A. hydrophila* infection resulted in only minimal changes in HSP70 expression during the post-stress period. Other research has also shown that HSP70 is highly sensitive to heat stress, and changes in its expression can be used as a biomarker for stress severity in fish **(Tomanek & Somero, 1999; Chen *et al.*, 2023)**.

CONCLUSION

Based on the obtained results, dietary VCNPs effectively enhanced the growth performance of the Nile tilapia following an 8-week feeding trial. Compared to bulk VC, VCNP supplementation led to improved antioxidant status, as indicated by lower basal expression of SOD and GPx genes, which increased significantly after *A. hydrophila* infection. Additionally, pro-inflammatory cytokine gene expressions were upregulated in the VCNP-fed groups in response to the bacterial challenge. Notably, fish receiving VCNPs exhibited a faster recovery and restoration of normal physiological status following exposure to heat stress and/or bacterial infection.

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