Modulatory effect of dietary copper nanoparticles and vitamin C supplementations on growth performance, hematological and immune parameters, oxidative status, histology, and disease resistance against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*)

Nik Mojtaba Delavari · Ahmad Gharaei · Harijani Javad Mirdar · Aida Davari · Abolhasan Rastiannasab

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Abstract Copper and vitamin C are micronutrients needed for the living organism’s functions. Vitamin C has a great effect on the immune system of fish. The present study aimed to evaluate the effects of dietary copper nanoparticles (Cu-NPs) and vitamin C (VC) supplementations on rainbow trout (*Oncorhynchus mykiss*) juveniles. So, 216 rainbow trout juveniles were randomly assigned to six groups with trial diets supplemented with Cu-NPs and VC including 0/0 (T1, control diet), 0/250 (T2), 0/500 (T3), 2/250 (T4), 2/500 (T5), and 2/0 (T6) mg Cu-NPs/VC per kg diet. After the feeding trial for 60 days, the fish were challenged with *Yersinia ruckeri*, and the survival rate was calculated for 15 days. Based on the data analysis, weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER), lysozyme, alternative complement activity (ACH50), hematocrit (Hct), hemoglobin (Hb), and mean corpuscular volume (MCV) were significantly ($p<0.05$) increased in the fish fed on T4 and T5 diets compared with the control group. Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) were significantly ($p<0.05$) decreased in the fish fed with diets contain Cu-NPs and VC (T4 and T5). The expressions of TNF-α, IL-1β, IL-10, SOD, CAT, and GPX genes were significantly ($p<0.05$) decreased in the fish fed on T3, T4, and T5 diets versus the control. In addition, the dietary Cu-NPs and VC supplementations significantly enhanced resistance against pathogens and led to the control of infection in rainbow trout. In conclusion, Cu-NPs and VC administered as feed additives at 2/250–500 mg/kg elevated the growth performance, antioxidant capacity, and health of rainbow trout.

Keywords Antioxidant capacity · Resistance to pathogen · Copper nanoparticle · Vitamin C · Growth performance

Introduction

Production costs and quality of fish farming water can be determined by fish nutrition. Nutrition plays a key role in the sustainable development of aquaculture.
A key factor of aquaculture viability in all rearing systems is optimal nutrition. Mineral supplements, such as copper, are used to improve growth and food metabolism, strengthen the immune system and antioxidant capacity, and regulate ion exchange and osmotic balance (El Basuini et al. 2016; Mohseni et al. 2014; Lin et al. 2008).

Copper is an essential trace element that is involved in various physiological and biological processes in fish. It plays an essential role in the structure of liver enzymes, melanin and skin pigments, bone and connective tissue formation, myelin maintenance in the nervous system, and hemoglobin synthesis (Halver JE and Hardy RW 2002; El Basuini et al. 2016). Many studies have investigated the role of dietary copper concentrations and types on various aquatic species including Penaeus vannamei (Davis et al. 1993), Penaeus monodon (Lee and Shiau 2002), Oreochromis niloticus (Shiau and Ning 2003), Haliotis discus hannai (Wang et al. 2009), Ctenopharyngodon idella (Tang et al. 2013), Huso huso (Mohseni et al. 2014), and Pagrus major (El Basuini et al. 2016).

Nanoparticle forms of essential elements have been shown to be more effective and beneficial than their traditional forms in biological systems through promoting bioavailability, thereby facilitating uptake and utilization (El Basuini et al. 2016; Gharaei et al. 2020a). Nanometer dimensions, large active surface, multiple active centers, and greater catalytic efficiency improve bioavailability and some functional aspects of these metals (Izquierdo et al. 2016; Rather et al. 2011). Copper nanoparticles (Cu-NPs) are a new form of copper that has widely been used in dietary supplementation in aquatic nutrition (El Basuini et al. 2016; Wang et al. 2015).

Vitamin C (VC) or ascorbic acid is a vital water-soluble micronutrient that is essential for physiological functions in animals, including aquatic animals (Fracalossi et al. 2001; Grosso et al. 2013). Adel and Khara (2016) reported that adding vitamin C and iron to the basic diet of rainbow trout could improve the growth rate and health status. VC cannot be synthesized from D-glucose due to the lack of L-gluconolactone oxidase and must be obtained from exogenous sources (Wahli et al. 2003; Chahardeh Baladehi et al. 2017). Vitamin C supplementation directly modulated many immune functions and increased the survival of rainbow trout infected with Ichthyophthirius multifiliis (Leal et al. 2017). Harsij et al. (2020) found that diet supplemented with NanoSe, vitamin C, and vitamin E could increase growth performance, antioxidant capacity, and immune responses in juvenile rainbow trout.

VC, as a cofactor of many enzymes, plays a role in synthesizing collagen, tyrosine, cartilage, and endothelium of vessels, iron metabolism, and hematology, improving growth and reproduction, strengthening the antioxidant and immune system, and improving survival rates in aquatic animal (Combs 2008; Dawood and Koshio 2016; Leal et al., 2017). Some previous research has shown that the consumption of VC interferes with the intestinal absorption of Cu and its distribution to tissue enzymes; however, ascorbate makes the absorption of iron through the intestine (Pekiner and Nebioglu 1994). Additionally, it is established that dietary VC can decrease the risk of Cu toxicity, and Cu supplementation reduces the risk of hypervitaminosis (Watts 1989). In a review paper, Dawood and Koshio (2016) state that VC requirements of aquatic animal species depend on species, size, and feeding behavior.

Although extensive research has addressed the effect of VC supplementation, there is limited knowledge on the interaction of VC and Cu-NPs in fish. Therefore, the present study aimed to assess the effect of dietary VC and/or Cu-NP supplements on growth performance, hematological indices, antioxidant status, histological parameters, immune response, expression of some important genes like TNF-α, IL-1b, and IL-10 in kidney, and disease resistance against Y. ruckeri in juvenile rainbow trout as one of the most important aquaculture species in the Pacific Ocean in Asia and North America.

Materials and methods

Experimental diets

The basal diet formulation is presented in Table 1. The results of the chemical analysis of the trial diets are shown in Table 2. Cu-NPs (Sigma-Aldrich, 99% purity, NPs size < 75 μm) were used as the Cu source and Stay-C (L-ascorbyl-2-mono phosphate-Ca/Na, Cayman Co., 95% purity) as the VC source. According to available relevant references, six trial diets were prepared including two levels of Cu-NPs (0 and 2 mg kg⁻¹ dry feed) (El
Basuini et al. 2016) and three levels of VC (0, 250, and 500 mg kg\(^{-1}\) dry feed) (Dawood et al. 2016) (the control diet without Cu-NPs and/or VC (T1), T2, T3, T4, T5, and T6). They were supplemented to the basal diet according to a 23 factorial design. In preparing the trial diets, ingredients were mixed in a blender for 15 min. The Cu source was mixed with the lipid sources for 15 min and then added to the other ingredients. In the next step, the premixed ingredients were mixed with water and then passed through a meat grinder to prepare pellets with 2-mm diameter, which were dried on nylon screens at 45 °C and kept in two-layer plastics at −20 °C until they were consumed. The actual concentration of proximate composition of Cu and VC was measured in each diet (Table 2).

### Treatments and sample collection

Juvenile rainbow trout were taken from a reproduction center (Shahid Motahari, Yasouj, Iran), transported alive to the laboratory, and acclimated to the experimental conditions for 2 weeks prior to the beginning of the feeding trial. The fish (initial average weight 15.1 ± 1.05 g) were randomly distributed into six groups with triplicate tanks within each group (18 tanks (200 L of each) and 12 fish per tank). During 60 days, the fish were fed with various test diets three times a day at 08:00, 13:00, and 18:00 based on 3% of the body weight. The characteristics of water during the experimental period including temperature, pH, and dissolved oxygen were recorded to be 16 ± 2 °C, 7.1 ± 0.3, and 6.4 ± 0.2 mg l\(^{-1}\), respectively. At the end of the trial, the fish fasted, and their growth performance, weight gain (WG%), specific growth rate (SGR%), feed conversion ratio (FCR), survival rate (%), and protein efficiency ratio (PER) were measured by the following equations (Jafarinejad et al. 2020):

\[
\text{WG \%} = \frac{\text{final body weight (g)} - \text{initial body weight (g)}}{\text{initial body weight (g)}} \times 100
\]

\[
\text{SGR \%} = \frac{(\ln \text{final body weight (g)} - \ln \text{initial body weight})}{\text{experimental days}} \times 100
\]

\[
\text{FCR} = \frac{\text{dry feed fed}}{\text{wet weight gain}}
\]

### Table 1 The formulation of the basal diet

| Ingredients                      | g kg\(^{-1}\) dry diet |
|----------------------------------|-------------------------|
| Fish meal                        | 620                     |
| Meat powder                      | 60                      |
| Soybean meal                     | 20                      |
| Wheat flour                      | 80                      |
| Canola oil                       | 40                      |
| Fish oil                         | 40                      |
| Lecithin                         | 30                      |
| Vitamin premix \(^1\) (VC free)  | 30                      |
| Mineral premix \(^2\) (Cu free)  | 29.96                   |

\(^1\)Composition of vitamin premix (IU, g or mg/kg): A (3,600,000 IU), D3 (8,000,000 IU), E (14.4 g), K3 (800 mg), B1 (7 g), B2 (2.64 g), niacin (11.8 g), calcium pantothenate (3.92 g), B6 (1.17 g), B9 (0.4 g), biotin (40 mg), choline chloride (100,000 mg). Aras Bazar Pharmaceutical Co., Mazandaran, Iran

\(^2\)Composition of mineral premix (g/kg): Mn (39.68 g), Fe (20 g), Zn (33.88 g), Co (4 g), I (0.39 g), Se (0.08 g). Aras Bazar Pharmaceutical Co., Mazandaran, Iran

### Table 2 The chemical analysis of the trial diets

| Trial diets (Cu-NPs/VC mg kg\(^{-1}\)) | Analyzed copper (mg kg\(^{-1}\)) | Analyzed VC (mg kg\(^{-1}\)) | Crude lipid (g kg\(^{-1}\)) | Ash (g kg\(^{-1}\)) | Crude protein (g kg\(^{-1}\)) | Gross energy (kj g\(^{-1}\)) |
|--------------------------------------|----------------------------------|------------------------------|-----------------------------|----------------------|-------------------------------|-------------------------------|
| T1 (0/0)                             | 1.59                             | ND                           | 223                         | 101.2                | 417.5                         | 22.21                         |
| T2 (0/250)                           | 1.60                             | 244                          | 226                         | 101.4                | 413.2                         | 22.33                         |
| T3 (0/500)                           | 1.57                             | 491                          | 226.2                       | 102.5                | 413.3                         | 22.19                         |
| T4 (2/250)                           | 3.51                             | 241                          | 230.1                       | 102.3                | 418.1                         | 22.40                         |
| T5 (2/500)                           | 3.48                             | 489                          | 227.8                       | 100.5                | 415.5                         | 22.31                         |
| T6 (2/0)                             | 3.52                             | ND                           | 222                         | 103.1                | 410.6                         | 22.37                         |

ND not detected, Cu-NPs Cu nanoparticles, VC vitamin C
Hematological and biochemical measurement

Blood was collected from the caudal vein of three fishes from each tank by a non-heparinized syringe (3 ml) for hematological analysis. Afterward, partial whole blood was introduced into heparinized microtubes and used to measure red blood cell (RBC), white blood cell (WBC), hematocrit (Hct), hemoglobin (Hb), mean corpuscular hemoglobin (MCh), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV) by the procedures described in Gharaei et al. (2020b) and Adel and Khara (2016). For biochemical analysis, blood sera were separated by centrifuging at 3000 rpm for 10 min (Gharaei et al. 2010). Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and malondialdehyde (MDA) \((n=6 \text{ from each group})\) in serum samples were measured with a commercial chemical calorimetric enzyme assay kit (ZellBio GmbH, Germany) by the procedures described in Gharaei et al. (2020b). The lysozyme activity in serum samples was assayed using the method of Ellis (1990) and Gharaei et al. (2020b). Alternative complement activity (ACH50) in serum samples was assayed using the protocol presented by Yano (1992).

Measurement of gene expression

To compare mRNA expression levels, the posterior parts of the intestinal tissues from each treatment group \((N=5)\) were randomly collected at the end of the trial, frozen, and kept at \(-80^\circ C\) until use. Total RNA was extracted in the intestinal samples by using the TakapouZist Kit (Tehran, Iran) following the manufacturer’s instructions. RNA integrity was verified by ethidium bromide staining of the 28S and 18S ribosomal RNA bands (as a marker) on 1.2% agarose gel. To remove DNA contaminants, the extracted RNA was treated with RNA-free DNase (Takara, Japan), and the reverse was transcribed to cDNA by a superscript cDNA synthesis kit (AccuPawer® CycleScript RT PreMix, Germany) following the manufacturer’s instructions. The mRNA expression levels of CAT, SOD, GPX, TNF-α, IL-1β, IL-10, and β-actin (housekeeping gene) were designed according to the cDNA sequences of rainbow trout in GenBank (Nootash et al. 2013; Hoseini et al. 2020) and thermocycling conditions as indicated in Table 3. All primers were synthesized by TakapouZist Co., Ltd. and amplified fragments length of 70–295 bp. Real-time quantitative PCR was conducted in a quantitative thermal cycler (Mastercycler® eprealplex, Eppendorf, Germany). Three replicates were performed for each sample. The threshold cycle (CT) was determined manually for each run. The PCR efficiency of each set of primers was determined using serial tenfold dilutions of cDNA and the resulting plots of CT vs. the logarithmic cDNA dilution using the efficiency equation \((E)\):

\[
E = 10^{(-1/\text{slope})}.
\]

Gene expression data were analyzed using the \(2^{-\Delta\Delta CT}\) method after it was verified that the primers were amplified with an efficiency of 97–99% (Gharaei et al. 2011). The data for all treatment groups were compared to the control group.

Bacterial challenge test

At the end of the experiment period, the survivals of fish in each treated group (9 fish per group) were challenged by the \textit{Yersinia ruckeri} (BCCM/LMG3279) strain. This test was carried out as described by Gharaei et al.’s (2020b) procedure. The mortality percentage of fish in each group was measured after a 15-day resistance test period, and the relative percentage of survival (RPS) was determined by the following formula:

\[
\text{RPS} = 100 - \left[ \left( \frac{\text{test mortality}}{\text{control mortality}} \right) \times 100 \right].
\]

Histopathological analysis

On the last day of the trial, six randomly selected fish of each treated group were sampled. After anesthetized with 200 mg l\(^{-1}\) MS222 (Faggio et al. 2014), gill, liver, kidney, and intestine tissue samples with the size of \(1 \times 1 \times 0.5 \text{ cm}\) were preserved in 10% neutral buffered formalin for fixation. Afterward, the tissue preparation with the routine protocol (dehydration in ethanol, clearing in xylene, impregnation, and embedding in melted paraffin, sectioning at 5 μm with a rotary microtome, and...

\[
\text{PER} \% = \left( \frac{\text{final body weight (g) - initial body weight (g)}}{\text{dry protein intake (g) x 100}} \right)
\]

\[
\text{Survival rate} \% = \left( \frac{\text{final number of fish}}{\text{initial number of fish}} \right) \times 100
\]
staining with hematoxylin–eosin standard staining method) was performed for the histopathological investigation by light microscopy (Mohammadi et al. 2020).

Statistical analysis

Normality and homogeneity of the data were tested before performed parametric tests. The data (means ± SE) from each group were subjected to one-way analysis of variance (ANOVA), and Tukey’s post hoc test was used to rank the groups in SPSS (version 18) if significant differences (p < 0.05) were detected.

Results

Fluctuations of growth performance including WG, FCR, SGR, PER, and survival rate of juvenile rainbow trout treated with different levels of dietary Cu-NPs or/and VC supplementation for 60 days are shown in Table 4. According to the experiments, WG, FCR, SGR, and PER were significantly affected only by Cu-NPs/VC supplementation. In addition, the WG, SGR, and PER values were significantly higher (p < 0.05) in all treatment groups than in the control group (T1). The mortality rate in all treatment groups did not show any significant differences (p > 0.05). In addition, the FCR value was significantly (p < 0.05) decreased in all treatment groups compared with the control group (T1). The survival rate, which varied from 97.22% to 100%, was not significantly different among the groups (p > 0.05).

As shown in Table 5, the lysozyme activity and ACH50 values were significantly increased by the Cu-NPs/VC supplementation in the fish fed on the T4 and T5 diets when compared with the control group (T1). However, the ACH50 value in all treatment groups was significantly (p < 0.05) more pronounced than that in the control group (T1). The highest level of ACH50 was recorded in the fish fed on the T5 diet (Table 5).

The variations in the activity of antioxidative enzymes (SOD, CAT, and GPX) are presented in Table 6. The one-way ANOVA test revealed that Cu-NPs/VC supplementation was significantly decreased (p < 0.05) for the SOD, CAT, and GPX levels in the fish fed on the T4 and T5 diets when compared with the control group (T1). Furthermore, the MDA value did not vary significantly (p > 0.05) with dietary treatments at the end of the trial.

Table 3 The real-time PCR primer sequences and thermocycling condition

| Genes   | Primer | Primer sequence (5′–3′) | Thermocycling condition | Accession no | Primer efficiency (%) |
|---------|--------|-------------------------|--------------------------|--------------|------------------------|
| SOD     | F      | GTAGTCGTTGGCTCAATGGTAAG | 95 °C 30 s, 35 cycles of 95 °C 5 s, 60.4 °C 30 s and 72 °C 30 s | NM_001160614 | 0.981 |
|         | R      | GCTTTATATTGCGGCCCCTTTATT | | | |
| CAT     | F      | TTAGGTGACATGACATCCTCT   | 95 °C 30 s, 35 cycles of 95 °C 5 s, 54.5 °C 30 s and 72 °C 30 s | 110,490,868 | 0.99 |
|         | R      | ACCTGGTGAAGCCAAAGAACC   | | | |
| GPX     | F      | AATTGCCATTTCCCCCTCCGA   | 95 °C 30 s, 35 cycles of 95 °C 5 s, 60 °C 30 s and 72 °C 30 s | AF281338 | 0.99 |
|         | R      | TCCATCAGGACTGACCCAGAGG | | | |
| IL-1β   | F      | ACATTGCCAACCTCATCATCG   | 95 °C 30 s, 35 cycles of 95 °C 5 s, 62 °C 30 s and 72 °C 30 s | AJ223954 | 0.98 |
|         | R      | TTGACGGTCTCTGGTTCTTC    | | | |
| IL-10   | F      | GCATTGGAACCTTCCATCGAC   | 95 °C 30 s, 35 cycles of 95 °C 5 s, 60 °C 30 s and 72 °C 30 s | AB118099 | 0.98 |
|         | R      | GCATTGGAGATCTTTCTTC     | | | |
| TNF-α   | F      | TGGAGGGTATGCCATCACCTCTG | 95 °C 30 s, 35 cycles of 95 °C 5 s, 60 °C 30 s and 72 °C 30 s | AJ249755.1 | 0.987 |
|         | R      | TGAGGCTTTTCTCTAGCAAGACG | | | |
| β-actin | F      | TCACCCACACTGTGCCCATCTACGA | 95 °C 30 s, 35 cycles of 95 °C 5 s, 60 °C 30 s and 72 °C 30 s | AC006483.3 | 0.99 |
|         | R      | CAGCGGAACCGCTCATGCAATGG | | | |

SOD superoxide dismutase, CAT catalase, GPX glutathione peroxidase, IL-1β interleukin 1β, IL-10 interleukin 10, TNF-α tumor necrosis factor
After 60 days of treatment, there were significant differences in the hematological parameters as shown in Table 7. The Hb, Hct, and MCV values were significantly increased ($p < 0.05$) by Cu-NPs/VC supplementation in fish fed on diets containing 250/500 mg VC and 2 mg Cu-NP supplementation when compared with the control group (T1). Meanwhile, the RBC, WBC, and MCHC values did not significantly ($p > 0.05$) vary with dietary treatments at the end of the trial.

The relative mRNA expressions of TNF-α, IL-1β, IL-10, SOD, CAT, and GPX genes in the intestinal tissue after 60 days are shown in Fig. 1. The analysis of variance demonstrated that the interactive effects of dietary Cu-NPs and VC were significant on all target genes in the intestinal tissue. In the intestinal tissue of the fish fed on the T3, T4, and T5 diets, the expression of TNF-α, IL-1β, IL-10, SOD, CAT, and GPX was significantly decreased versus the control group (Fig. 1).

### Table 4
The growth performance and nutrient digestibility of different experimental groups in the rainbow trout for 60 days (means ± SE, $n = 3$). Means marked by different letters are significantly different ($p < 0.05$)

| Parameters | Trial diets (Cu-NPs/VC mg kg$^{-1}$) | WG% | FCR | SGR% | PER% | SR% |
|------------|----------------------------------|-----|-----|-----|------|-----|
| T1 (0/0)   | 159.24 ± 7.83$^a$                | 1.88 ± 0.06$^a$ | 1.58 ± 0.07$^a$ | 120.52 ± 13.43$^a$ | 100 ± 0.00 |
| T2 (0/250) | 182.04 ± 10.09$^b$              | 1.66 ± 0.1$^b$  | 1.72 ± 0.05$^b$  | 144.61 ± 10.40$^b$ | 100 ± 0.00 |
| T3 (0/500) | 201.50 ± 9.71$^{bc}$            | 1.41 ± 0.03$^{bc}$ | 1.83 ± 0.03$^{bc}$ | 167.46 ± 6.39$^{bc}$ | 100 ± 0.00 |
| T4 (2/250) | 247.96 ± 8.53$^d$              | 1.24 ± 0.03$^d$  | 2.07 ± 0.08$^d$  | 191.54 ± 10.86$^d$ | 100 ± 0.00 |
| T5 (2/500) | 236.56 ± 10.32$^{cd}$          | 1.26 ± 0.07$^{cd}$ | 2.01 ± 0.1$^{cd}$ | 188.69 ± 15.76$^{cd}$ | 100 ± 0.00 |
| T6 (2/0)   | 214.63 ± 11.21$^{bcd}$         | 1.35 ± 0.02$^{bcd}$ | 1.90 ± 0.03$^{bcd}$ | 175.95 ± 11.61$^{bcd}$ | 97.22 ± 0.33 |

Cu-NPs Cu nanoparticles, VC vitamin C

### Table 5
The changes in immunological parameters in the juvenile rainbow trout at different experimental groups for 60 days (means ± SE, $n = 3$). Means marked by different letters are significantly different ($p < 0.05$)

| Parameters | Treatment groups (Cu-NPs/VC mg kg$^{-1}$) |
|------------|------------------------------------------|
| T1 (0/0)   | T2 (0/250) | T3 (0/500) | T4 (2/250) | T5 (2/500) | T6 (2/0) |
| Lysozyme (U ml$^{-1}$) | 50.2 ± 2.1$^a$ | 56.7 ± 2.5$^a$ | 57.1 ± 3.7$^a$ | 85.0 ± 4.5$^b$ | 100.6 ± 5.9$^c$ | 58.4 ± 2.6$^a$ |
| ACH50 (U ml$^{-1}$) | 125.2 ± 1.3$^a$ | 137.9 ± 3.2$^b$ | 140.3 ± 2.3$^b$ | 153.4 ± 4.1$^c$ | 159.1 ± 3.9$^c$ | 134.9 ± 3.8$^b$ |

Cu-NPs Cu nanoparticles, VC vitamin C, ACH50 alternative complement activity

### Table 6
The changes in antioxidant capacity parameters in the juvenile rainbow trout at different experimental groups for 60 days (means ± SE, $n = 3$). Means marked by different letters are significantly different ($p < 0.05$)

| Parameters | Treatment groups (Cu-NPs/VC mg kg$^{-1}$) |
|------------|------------------------------------------|
| T1 (0/0)   | T2 (0/250) | T3 (0/500) | T4 (2/250) | T5 (2/500) | T6 (2/0) |
| SOD (U ml$^{-1}$) | 65.33 ± 2.28$^a$ | 62.21 ± 3.20$^{ab}$ | 53.63 ± 2.91$^b$ | 45.81 ± 3.62$^c$ | 42.13 ± 1.97$^c$ | 59.11 ± 2.81$^{ab}$ |
| CAT (U ml$^{-1}$) | 145.67 ± 12.8$^a$ | 141.0 ± 9.53$^a$ | 120.66 ± 8.96$^a$ | 112.0 ± 11.26$^b$ | 113.5 ± 7.47$^b$ | 146.3 ± 6.50$^a$ |
| GPX (U ml$^{-1}$) | 680.52 ± 17.3$^a$ | 692.66 ± 18.1$^a$ | 601.5 ± 14.9$^b$ | 573.30 ± 21.2$^b$ | 559.4 ± 23.4$^b$ | 673.71 ± 25.7$^a$ |
| MDA (μmol ml$^{-1}$) | 57 ± 6.1 | 56.4 ± 4.9 | 58.3 ± 5.03 | 56.6 ± 4.7 | 55.6 ± 5.15 | 58.3 ± 4.2 |

Cu-NPs Cu nanoparticles, VC vitamin C, SOD superoxide dismutase, CAT catalase, GPX glutathione peroxidase, MDA malondialdehyde
Histopathological evaluation of the gills, kidneys, liver, and spleen revealed no damage in different tissues of fish treated with Cu-NPs and VCs (Figs. 2, 3, 4, and 5).

The resistance of juvenile rainbow trout to the pathogen (*Y. ruckeri*) after treatment with different diets is depicted in Fig. 6. The trend of survival percentage indicated that Cu-NPs/CV supplemen-
tations significantly enhanced (*p* < 0.05) the fish survival.

### Table 7 The hematological parameters of the rainbow trout juveniles in different experimental groups for 60 days (means ± SE, *n* = 3). Means marked by different letters are significantly different (*p* < 0.05)

| Trial diets (Cu-NPs/VC mg kg⁻¹) | Parameters | RBC (×10⁶ mm⁻³) | WBC (×10³ mm⁻³) | Hb (g dl⁻¹) | Hct (%) | MCV (fl) | MCH (pg) | MCHC (g dl⁻¹) |
|----------------------------------|------------|-----------------|-----------------|-------------|---------|---------|---------|-------------|
| T1 (0/0)                         |            | 1.03 ± 0.31     | 52.3 ± 5.7      | 5.55 ± 0.2a | 38.6 ± 2.1ab | 357.4 ± 5.8b | 53.8 ± 8.1 | 14.4 ± 0.4    |
| T2 (0/250)                       |            | 1.1 ± 0.12      | 51.1 ± 6.8      | 5.6 ± 0.51a | 40.6 ± 6.6ab | 369.1 ± 13.7b | 50.9 ± 7.3 | 13.8 ± 1.5    |
| T3 (0/500)                       |            | 0.98 ± 0.15     | 52.4 ± 5.9      | 5.6 ± 0.36a | 35.6 ± 4.3b  | 363.2 ± 14.2b | 57.1 ± 9.2 | 15.7 ± 1.2    |
| T4 (2/250)                       |            | 1.1 ± 0.28      | 49.7 ± 4.7      | 6.2 ± 0.15b | 42.0 ± 1.2a  | 381.8 ± 21.1a | 56.4 ± 4.5 | 14.8 ± 0.8    |
| T5 (2/500)                       |            | 1.12 ± 0.16     | 50.3 ± 4.9      | 6.4 ± 0.4b  | 43.6 ± 3.0a  | 389.3 ± 25.5a | 57.1 ± 6.8 | 14.7 ± 1.5    |
| T6 (2/0)                         |            | 1.11 ± 0.10     | 49.6 ± 5.3      | 5.75 ± 0.3a | 41.0 ± 4.5ab | 369.4 ± 12.6a | 51.8 ± 6.3 | 14.0 ± 0.7    |

*Cu-NPs* Cu nanoparticles, VC vitamin C, *RBC* red blood cell, *WBC* white blood cell, *Hb* hemoglobin, *Hct* hematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration.

Fig. 1 The relative mRNA levels of the rainbow trout intestine at fed on different doses of Cu-NPs and VC including 0/0 (T1, control diet), 0/250 (T2), 0/500 (T3), 2/250 (T4), 2/500 (T5), and 2/0 (T6) mg Cu-NPs/VC per kg diet after 60 days (means ± SE, *n* = 5). Means marked by different letters are significantly different (*p* < 0.05). Cu-NPs Cu nanoparticles, VC vitamin C, TNF-α tumor necrosis factor, IL-1β interleukin 1β, IL-10 interleukin 10, SOD superoxide dismutase, CAT catalase, GPX glutathione peroxidase.

Fig. 6 The relative mRNA levels of the rainbow trout intestine at fed on different doses of Cu-NPs and VC including 0/0 (T1, control diet), 0/250 (T2), 0/500 (T3), 2/250 (T4), 2/500 (T5), and 2/0 (T6) mg Cu-NPs/VC per kg diet after 60 days (means ± SE, *n* = 5). Means marked by different letters are significantly different (*p* < 0.05). Cu-NPs Cu nanoparticles, VC vitamin C, TNF-α tumor necrosis factor, IL-1β interleukin 1β, IL-10 interleukin 10, SOD superoxide dismutase, CAT catalase, GPX glutathione peroxidase.
resistance against *Y. ruckeri* when compared with the control. In addition, 15 days post-challenge with this bacterium, the highest relative percentage of survival was recorded in fish fed on the T5 diet (75%) and followed by those fed on the T4 diet (70%).

**Discussion**

Some studies have demonstrated that minerals and vitamins such as Cu and VC are required for normal cell functioning, improvement of growth performance, and physiological and immunological process of aquatic species (El Basuini et al. 2016; Dawood and Koshio 2016; Chen et al. 2015; Ai et al. 2006; Watanabe et al. 1997). Also, it has been reported that nanominerals such as Cu nanoparticles have novel features such as high surface activity, larger specific surface area, high catalytic efficiency, high surface active centers, and stronger adsorption capacity, making them more capable of crossing biological barriers so that they are rapidly absorbed by cells and exhibit higher bioavailability than mineral salts (Gharaei et al. 2020a; Izquierdo et al. 2016; Rather et al. 2011).

The present study showed that growth indices including WG, FCR, SGR, and PER were significantly affected by Cu-NPs and VC supplementations, which is in parallel with previous studies according to which dietary Cu and CV supplementations improved growth indices, immune response, and antioxidant status in several species of fish (El Basuini et al. 2016; Mohseni et al. 2014; Tang et al. 2013; Faramarzi 2012; Sabatini et al. 2009; Wang et al. 

![Fig. 2](#) Gill histological structure of rainbow trout fed on different doses of Cu-NPs and VC including: 0/0 (T1, control diet), 0/250 (T2), 0/500 (T3), 2/250 (T4), 2/500 (T5), and 2/0 (T6) mg Cu-NPs/VC per kg diet after 60 days. Histopathological alterations were not observed in all treatment groups. H & E; scale bar = 185 µm
The growth-promoting effects of Cu-NPs may be explained by the fact that optimal dietary copper induces growth by improving metabolism and activities of the brush border and preventing lipid peroxidation and protein oxidation in the hepatopancreas and intestine (Tang et al. 2013). It has been explained that Cu-NPs have higher efficiency than the larger size at lower doses due to easy absorption and bioavailability to aquatic animals as well as fish (El Basuini et al., 2016). Also, nanoparticles have better interaction with other materials because of the magnitude of the active surface (Zaboli et al., 2013). One of the reasons for the increased growth rate may be the improvement in the intake of digestible energy. Unlike this study, Gatlin and Wilson (1986) and Lorentzen et al. (1998) reported no significant effect of Cu supplementation on WG and feed efficiency of channel catfish (Ictalurus punctatus) and Atlantic salmon (Salmo salar), respectively. Therefore, the severity of the response to copper supplementation and its different impact on growth performance may be attributed to species, age, Cu dosage, and Cu chemical forms.

Crustaceans and fish species are limitedly capable of VC synthesis, so adding it to the diet of farmed species is crucial for improving growth performance and maintaining normal physiological functions (Dawood and Koshio 2016; Chen et al. 2015). Improved growth performance through the nutrition of VC often appears as a result of the increased feed efficiency of the diet as proven by the previous studies on red seabream (P. major) (Dawood et al. 2016), Caspian roach (Rutilus rutilus caspicus) (Roosta et al. 2014), common carp (Cyprinus carpio) (Liu et al. 2011), cobia (Rachycentron canadum) (Zhou et al. 2012), Oreochromis spilurus (Al-Armoudi et al. 1992), and Korean rockfish (Sebastes schegelii) (Kim
and Kang 2015). In the present study, the growth performance reached a significantly higher level in the fish fed on the T5 diet (2 mg kg\(^{-1}\) Cu-NPs mixed with 500 mg kg\(^{-1}\) VC) compared with the control group. Adel and Khara (2016) presented that the highest WG and SGR and lowest FCR in rainbow trout fingerlings were observed in those fed on 250 mg kg\(^{-1}\) VC. Yousefi et al. (2013) revealed that the growth performance of *Barbus sharpeyi* was improved by VC supplementation. Similarly, Faramarzi (2012) indicated that the growth performance of common carp was increased in the fish fed on dietary VC supplementation at a rate of 800–2000 mg kg\(^{-1}\) diet. Unlike this study, it was reported that dietary VC supplementation did not influence the growth performance in large yellow croaker (*Pseudosciaena crocea*) juveniles (Ai et al. 2006). However, our results in this study revealed that Cu-NPs combined with VC had a synergic effect on the growth indices and the physiological status of rainbow trout.

Lysozyme and ACH50 widely participate as humoral components in the innate defense system, so they are important for fish protection against diseases (Kaya et al. 2016). The antibacterial activity of the complement system, reported in various fish, has been suggested as one of the most important mechanisms of bacterial killing and clearing in fish, which can be activated by various immune stimuli (Srivastava & Pandey 2015). One of the triggers of complement activation is cytokines, some of which are effective in regulating proteins involved in iron metabolism, such as ceruloplasmin (Di Bella et al. 2017). In our study, lysozyme activity enzyme and ACH50 value were enhanced significantly (\(p < 0.05\)) in the fish fed...
Kidney histological structure of rainbow trout fed on different doses of Cu-NPs and VC including: 0/0 (T1, control diet), 0/250 (T2), 0/500 (T3), 2/250 (T4), 2/500 (T5), and 2/0 (T6) mg Cu-NPs/VC per kg diet after 60 days. Histopathological alterations were not observed in all treatment groups. H & E; scale bar = 125 µm.

The survival rate of rainbow trout fed on different doses of dietary Cu-NPs and/or VC including: 0/0 (T1, control diet), 0/250 (T2), 0/500 (T3), 2/250 (T4), 2/500 (T5), and 2/0 (T6) mg Cu-NPs/VC per kg diet supplementation during 15-day post-challenge with Yersinia ruckeri.
on the Cu-NPs and/or CV supplemented diet. This incremental fluctuation may be due to the immune suppressive effects of Cu-NPs (Kaya et al. 2016). This result coincides with the investigation of El Basuini et al. (2016) and Mohseni et al. (2014) who reported increasing lysozyme level in P. major and H. huso fed on dietary Cu-NPs and inorganic copper supplementations, respectively. In addition, Ghareei et al. (2020a) indicated that the lysozyme and ACH50 values were increased in H. huso fed on the dietary Chitosan-Zn NPs supplemented diet. Unfortunately, there is a lack of knowledge on the effect of nanoparticles on the ACH50 level in fish. However, it has been demonstrated that VC is a strong inducer of the immune system, especially non-specific immunity as it results in enhanced lysozyme activity level reported in various fish species including P. major (El Basuini et al. 2016), Oncorhynchus mykiss (Adel and Khara 2016), Pseudosciaena crocea (Ai et al. 2006), Takifugu rubripes (Eo and Lee 2008), Scophthalmus maximus (Lin and Shiau 2005), and Pangasianodon gigas (Pimpimol et al. 2012). Qhuntai et al. (2004) observed increased fish lysozyme and ACH50 values when the dietary VC supplementation was enhanced up to 489.0 mg kg\(^{-1}\). Similarly, Chen et al. (2003) demonstrated that the ACH50 level in golden shiner (Notemigonus crysoleucas) was increased under the effects of dietary VC supplementation.

The antioxidant defense system is highly correlated with the health and safety of fish, and its major enzymes (SOD, CAT, GPX, and MDA) decompose reactive oxygen species (ROS) into a less reactive form (Sheikh Asadi et al. 2018; Dekani et al. 2018). The SOD functions as an antioxidant by catalyzing the alteration of superoxide anions to hydrogen peroxides, which are then used as a substrate by the CAT and GPX enzymes (Saffari et al. 2016). \( \text{H}_2\text{O}_2 \) that is produced by the performance of SODs or the action of oxidases is reduced to water by CAT and GSH-Px (Birben et al. 2012). There are two forms of SOD that contain copper including copper/zinc SOD (existing within most body cells) and extracellular SOD (found in blood plasma) (Gonzales-Egua et al. 2009). CAT is an omnipresent tetrameric heme-containing antioxidant enzyme that speeds up the transformation of two molecules of \( \text{H}_2\text{O}_2 \) into \( \text{H}_2\text{O} \) and \( \text{O}_2 \). GPX catalyzes the conversion of \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) or organic peroxides into their analogous stable alcohols (Ghareei et al. 2020a, b). Therefore, a simultaneous activity induction of SOD and CAT is usually an expected response. However, this relation is not always observed, and it is confirmed to be species dependent. In the present study, high activity values of both SOD and CAT suggest a “cooperative” mechanism of the two enzymatic systems. As shown in Table 5, maximum activities of the SOD, CAT, and GPX enzymes are observed in the fish fed on the T5 (2 mg kg\(^{-1}\) Cu-NPs mixed with 500 mg kg\(^{-1}\) VC) diet compared with the control group. Previous studies have shown that Cu-containing diet, Cu/Zn-SOD enzyme activities in hepatocyte of rainbow trout (Oncorhynchus mykiss) (Osredkar and Sustar 2011; Trenzado et al. 2009) and grass carp (Ctenopharyngodon idella) (Tang et al. 2013), and GPX increases in the plasma of goldfish (Carassius auratus gibelio) (Shao et al. 2010). In fact, copper is positively associated with the antioxidant defense system (Fang et al. 2013), and the effect of dietary copper on stopping oxidative damage may be related to the reaction with ROS such as anion superoxides and hydroxyl radicals (Tang et al. 2013). On the other hand, ceruloplasmin is a Cu-containing protein whose activity increases with appropriate levels of dietary Cu (Shiau and Ning 2003). This Cu-containing protein is capable of stopping superoxide radical production (Valko et al. 2007) and hydroxyl radical formation (Zhang et al. 2013). In addition, it has been reported that the concurrent increase in the activity of SOD and GPX enzymes enhances the activity of NADPH oxidase, which is responsible for scavenging superoxide anion (Sheikh Asadi et al. 2018).

One of the major antioxidant additives in the fish diet and food industry is VC, which alleviates oxidative stress (Dawood et al. 2016; Geo et al. 2013). VC plays an important role in scavenging free radicals (ROS and reactive nitrogen species) by acting as an early electron donor and reducing the agent (Dawood et al. 2016). Many previous studies have stated that dietary VC supplementation increases SOD, CAT, and GPX activities in yellow catfish (Pelteobarus fulvidaco) (Liang et al. 2017), Siberian sturgeon (Acipenser baerii) (Xie et al. 2006), and black carp (Mylopharyngodon piceus) (Hu et al. 2013). Therefore, the results suggested that dietary Cu-NPs + VC supplementation can probably increase the antioxidant level, and they have a synergistic interactive effect on inducing the antioxidant system. Our results showed no significant
variations in the MDA value in all treatments. On the contrary, Jankowski et al. (2020) reported a reduction of MDA concentration under the effects of various forms of Cu (mineral and nanoparticle) in turkeys.

Hematological assessments can provide an indication of the physiological status of fish (Behera et al. 2013). In the present study, the Hb, Hct, and MCV values were increased more significantly in the fish fed on the T5 diet (2 mg kg$^{-1}$ Cu-NPs mixed with 500 mg kg$^{-1}$ VC) than the control fish, suggesting the positive effect of Cu-NPs + VC on physiological responses. The values of these hematological parameters in the control group indicate that adding Cu and VC to the diet improves blood counts. The measured levels of blood variables in the normal range are for trout health, which confirms the effects of non-toxic Cu-NPs used under the present experimental conditions. It was confirmed that increased Hb indicates a stress response or increased hematopoiesis (Clauss et al. 2008). While the fish fed on Cu-NPs/VC were healthier than the control group, which was determined by the level of antioxidant, safety, and survival rates in the bacterial stress test. Thus, an increase in RBC, Hct, and Hb associated with hematopoiesis increased or decreased hemolysis (Hoseini et al. 2018). This may be due to the role of Cu as a combination of many enzymes and glycoproteins that aid in the synthesis of hemoglobin (Nordberg et al. 2015; Dawood et al. 2020). Ceruloplasmin (a liver-derived protein) is required to release iron and transfer it from cells and tissues to plasma. There are several copper molecules in the structure of this protein, and its synthesis in the liver requires the presence of copper. In fact, copper deficiency impairs the ability of iron absorption or release from tissues for hemoglobin synthesis (Haver and Hardy 2008). The same results were recorded by Adel and Khara (2016) and Zhou et al. (2012) for pirarucu (Arapaima gas) and cobia (R. canadum), respectively.

To investigate the effects of Cu-NPs and VC on inflammatory and antioxidant responses, we measured the expression of several gene biomarkers including three pro-inflammatory cytokines (TNF-α, IL-10, and IL-1β) and three antioxidant systems (CAT, SOD, and GPX). The results of the present study showed that the expression levels of TNF-α, IL-10, and IL-1β genes were decreased in the intestine of the fish in the T3, T4, and T5 treatment groups. TNF-α (tumor necrosis factor) is known as a multifunctional cytokine that plays a key role in cell-mediated inflammatory immunity responses (Lykouras et al. 2008; Mocellin et al. 2015). IL-1β acts as a mediator of the inflammatory response and helps reduce inflammatory pain sensitivity in various cellular activities, including cell proliferation and apoptosis by inducing cyclooxygenase-2 (PTGS2 /COX2) in the central nervous system. TNF-α and IL-1β are considered important indicators of phagocytic activity, and they are the first cytokines produced in the early stages of inflammation in fish (Skadberg 2015). IL-10 is known as a cytokine synthesis inhibitory factor that minimizes damage to target cells by suppressing the transcription of pro-inflammatory cytokine (Shafiee-Jahani et al. 2020). The significant reduction in the expression of pro-inflammatory cytokine genes in fish fed on the diets containing Cu-NPs/VC supplements can be interpreted as their significantly down-regulated synergistic effect on the immune response, which was also dose-dependent. IL-10 has also been reported to be capable of degrading pro-inflammatory cytokine mRNA, reducing TNF-α receptor expression, and regulating macrophage-derived TNF-α and IL-1 secretion (Opal et al. 1998; Opal and DePalo 2000). Suska et al. (2003) reported that cellular Cu sites induce the secretion of TNF-α and IL-1β by inflammatory cells ex vivo and in vivo. On the other hand, TNF-α has been shown to increase phagocytosis of neutrophils under apoptosis. Thus, Cu-NPs may reduce the production of pro-inflammatory cytokine because the Cu ion is closely related to RNA and DNA. Antioxidant vitamins, including VC, can increase immune function by increasing the proliferation of lymphocytes and macrophages (Jang et al. 2014). Changes in gene expression reported in this study suggest a low inflammatory potential of the Cu-NPs/VCs tested. In this regard and consistent with our results, Yun et al. (2012) and Jang et al. (2014) reported that dietary supplemental VC significantly reduced TNF-α, IL-1β, and IL-6 mRNA levels in mice and broiler chick, respectively.

Despite the various benefits of copper nanoparticles in aquatic organisms, their toxic effects have also been reported in some cases. In Epinephelus coioides, there have been adverse effects on gut, gill, and liver (Wang et al. 2015), in Cyprinus carpio a sharp decrease in alkaline phosphatase and increased T4 and free T4 in blood plasma (Hoseini et al. 2016).
and in *Oncorhynchus mykiss* reduced hematocrit percentage and the amount of potassium and sodium in the blood plasma (Shaw et al. 2012). Nowadays, the interaction between transition metals, e.g., Cu, with VC is well known (Akbıyık et al. 2012) as the rate of VC oxidation stability increases with the fixed concentration of Cu and prevents catalytic oxidation of VC in the presence of a stable Cu complex. Various studies have shown that dietary VC can produce antioxidants (Biller and Takahashi 2018). VC bonds to ROS in the body and retrieves free radicals through H⁺ donation. VC has also been shown to act as a reducing agent, primarily by reducing the transport of metals such as Cu and Fe ions, which react with H₂O₂ to form hydroxyl radicals (Babior 1997).

Despite reports on inducing increased expression of the SOD, CAT, and GPX genes due to the toxicity of Cu-NPs in aquatic organisms (Ramya et al. 2019; Muralisankar et al. 2014; Dawood et al. 2020), the results of this experiment suggested that the Cu-NP level of 2 mg kg⁻¹ had no adverse effect on fish and, when applied with VC, had the greatest effect on ROS production. SOD and CAT are related to stress management (Li et al. 2010) and are used as important indicators in the early detection of environmental pollution due to immunological function and the protection of cells against free radical damage (Afshari et al. 2021). Decreased expression of the SOD, CAT, and GPX genes in the fish exposed to Cu-NPs/VC could indicate oxidant eradication. The results regarding Nile tilapia (*Oreochromis niloticus*) exposed to ZnO-NPs and vitamins C and E (Abdelazim et al. 2018), *O. niloticus* exposed to Ag-NPs (Afifi et al. 2013), and *Carassius auratus* exposed to a mixture of Cu-NPs and ZnO-NPs and cerium oxide-NPs and pure NP (Xia et al. 2013) are in agreement with our findings. It is confirmed that VC can destroy the superoxide anion by forming radical semidehydra ascorbate (Abdelazim et al. 2018). The results of our experiment showed significant neutralization in the antioxidant system so that the fish fed on a mixture of Cu-NPs and VC exhibited the lowest expression level of the SOD, CAT, and GPX genes. This ability of VCs to combat possible oxidative damage was caused by exposure to Cu-NPs. Othman et al. (2017) explained that they used VC to prepare ligands for cerium oxide nanoparticles as a tool to facilitate the detection of NP in tissues because they suggested that VC could bind tightly to NPs and showed its performance. It has also been reported that the presence of NPs themselves can increase the VC activity (Astete et al. 2011). The maximum resistance to *Y. ruckeri* and survival rate in this study were recorded in the fish fed on the T5 diet. Many previous studies have shown that various dietary additives have increased the survival rate and resistance to the pathogen in rainbow trout (Gharaei et al. 2020b; Yilmaz et al. 2018; Aghamirkarimi et al. 2017). The significant enhancement in survival rate, as well as the reduced expression of pro-inflammatory cytokines and antioxidant genes in this study, may be related to the induction of non-specific immune defenses and antioxidant system by synergistic interaction of Cu-NPs and VC.

In the present study, histopathological alterations were not observed in gill, intestine, liver, and kidney tissues affected by Cu-NPs and Cu-NPs+VC. According to the previous studies, the gill, liver, and kidney are the most sensitive organs to Cu-NP exposure and respond by showing various degrees of necrosis and tissue damage (Ostaszewska et al. 2018). Gills are an important organ with several functions like respiratory osmoregulation and respiratory gas exchange, acid–base balance, and excretion of metabolites. Thus, they are the primary target for a high concentration of Cu-NPs (Ostaszewska et al. 2018).

The intestine is the site of absorption of a huge portion of the nutrients and non-nutrients digested. It has been shown that Cu-NPs are well oxidized to ionic forms in acidic environments and have high adsorption capacity (Pirarat et al. 2011). On the other hand, the height of intestinal villi is an important indicator of the efficiency of digestion and absorption in the gut (Ringoe et al. 2003). Rathore et al. (2019) have reported that dietary VC increases the height of villi in the gut of tilapia (*Oreochromis niloticus*). The concurrent addition of Cu-NPs and vitamin C improved nutrient absorption in the gut by providing a larger surface area because of small size of nanoparticles as well as an acidic environment of the gut more effectively than their individual supplementation. Physiologically, improving the absorption of micronutrients increases the strength of the immune system against the pathogens.

**Conclusion**

In conclusion, our study identified some changes in growth, blood, biochemical, immune, and histopathology parameters of rainbow trout juveniles under the
influence of dietary Cu-NPs and/or CV supplements. Based on the data obtained, a diet containing 2 mg Cu-NPs combined with 500 mg VC per kg food can improve the growth indices including WG, FCR, SGR, PER, and blood indices including Hb, Hct, MCV, and biochemical and immunological indices including SOD, CAT, GPX, ACH50, and lysozyme. It appears that the synergistic effect of Cu-NPs and VC improves feed utilization, metabolism efficiency, and intestine tissue structure and enhances the antioxidant capacity and immune system in rainbow trout. However, additional studies are required to evaluate the other effects of these supplements on the immune responses and blood parameter variations and why some of the results differ from those of the previous studies. This research indicated that a combination of Cu-NPs and CV could be used in the rainbow diet as a growth promoter as it can improve the physiological conditions and resistance against pathogens.

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Author contribution DNM: MSc student; these data are coming from his thesis; GA: Associate professor in the Department of Fisheries and the main supervisor for the student. He provided all the scientific suggestions and grants; MHJ: He is the second supervisor and provided scientific suggestions; DA: She was the advisor for the work and provided scientific comments; RA: He was the advisor for the scientific group.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval All procedures were carried out in accordance with the Animal Care and Use Committee guidelines at the Faculty of Sciences of the University of Zabol.

Consent to participate The authors agree to collaborate and publish this article.

Consent for publication We will transfer the copyright of the article to editorial office for publishing.

Conflict of interest The authors declare no competing interests.

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