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Influence of Silver Nanoparticle Supplementation on Growth Performance, Immune Response, Tissue Biopsy, and Gene Transcription in the *Aeromonas carviae* Challenged *Labeo rohita*

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Abstract

The study examined the impact of a diet enriched with nanosilver (AgNPs) on the specific immunity, tissue biopsy, and growth effectiveness of *Labeo rohita* infected with *A. carviae*. The fish were divided into four groups with replications, and three diets were prepared with AgNPs (0, 10, 15, and 20 µg AgNPs kg^{-1} diet) added. The fish in the 15µg kg^{-1} group showed improved growth rates and a 70% survival rate after being challenged with *A. carviae.* Antioxidant indices, as well as the non-specific immune response, provided notable variations in the inclusion values of AgNPs in the liver and gill. The supplementation of 15µgkg^{-1} AgNPs most effectively improved tissue damage caused by *A. caviae* exposure in immunoprotective organs. T3 had higher liver TNF-α transcription than other treatments, while T4 had the highest values for IL-10. The amount of IL-10 in kidneys, gills, and muscles was also higher in T3, T2 and T3 respectively. The research suggests that AgNPs can positively influence fish growth and manage *A. caviae* infections by boosting antioxidant status and immunity while causing minimal harm to the immunoprotective organs.

Introduction

Aquaculture is essential to human existence because it provides sufficient and affordable animal protein. According to FAO estimates, aquaculture accounts for 250 billion of the 401 billion dollars in annual global fish production (State of World Fisheries and Aquaculture 2020).

Because fish farming contributes to the entire food supply for the population, it also has a significant impact on food security. The contribution of aquaculture to the poor, who are most at risk for malnutrition, in terms of food security, must be considered as another impact (Pradeepkiran, 2019).

Due to the rise in food fish production, and the introduction of various commercial fish species either extensively grown in small or confined areas like ponds or tanks under large populations has resulted in adversely influencing the well-being of aquaculture candidates with a latent stressor and transmissible diseases in recent decades (Van Doan et al., 2018; Popoola et al., 2023). Expansion of aquaculture is being hampered by disease outbreaks, the incidents have expanded due to the unrestrained migration of aquatic animals, which has caused the spread of infectious organisms between the regions. The condition known as motile septicemia or hemorrhagic septicemia, which has resulted in significant financial losses in the fish industry, is brought on by a single bacterial infection called *Aeromonas carviae* (Van Hai, 2015).

For over 20 years, antibiotics and chemotherapeutics have been adopted as a means through which bacterial infections are cured or prevented in aquaculture (Sakai, 1999). Unfortunately,

the development of antibacterial drugs, negative effects on the native intestinal flora of cultured fish, and the buildup of antimicrobial deposits in fish tissue and also the habitat, all of which pose risks to human and animal health, render antibiotic application for treatment ineffective and unsustainable. In fish culture, vaccination is an efficient preventative measure against infectious diseases, although it can be costly and traumatic for fish. Due to the complicated antigenic composition, a single vaccination can only be efficacious against a limited number of infections of a particular type (Ardó et al., 2008).

Therefore, it has been considered necessary to explore alternate methods of disease prevention that are environmentally friendly. One of the most promising approaches is the application of immunostimulants in boosting fish immune systems. Immunostimulants' most well-documented effects include improving phagocytic cell performance and raising their fungicidal and bactericidal properties (Sakai, 1999). An immunostimulant is a chemical that either directly boosts immunity or increases the non-specific defense mechanism (Anderson, 1992) and they are employed in aquaculture to prevent the immunomodulatory effects of stresses (Thompson et al., 1993; Barman et al. 2013). They can also be utilised to avoid periodical occurrences of endemic diseases or as a repressive measure against innate or sub-lethal pathogens. Immunostimulants have been reported to aid animals to recover from stress-related immunosuppression (Sakai, 1999). Fish and shrimp farming uses a variety of immunostimulants, including artificial chemicals, biological compounds, dietary components, hormones, and nanoparticles.

To improve immunity, diagnostic capabilities, and antibacterial properties in aquaculture candidates, incorporating additives into fish diets has been reported to yield outstanding success (Percival et al., 2007). Silver nanoparticles (AgNPs) is one of the nanoparticles that are frequently employed in physics, chemistry, medicine, and other fields (Yang et al., 2012; Li et al., 2020). As a result, AgNPs are strong antibacterial substances (Elechiguerra et al., 2005; Shahverdi et al. 2007), that also function as growth and immune system enhancers at low dosages, useful in healing burns and wounds (Samuel & Guggenbichler, 2004). To improve immunity and reduce stress in animals, scientists are searching for a perfect and environmentally friendly technique through which food additives could be added to feed (Aklakur et al., 2016). In this circumstance, nanoparticles are a perfectly sustainable feed inclusion material since they are safe for the environment and satisfy metabolic needs while staying within the boundaries of tissue retention (Chakraborty et al., 2013). According to reports, the micronutrient silver encourages an increase in the concentration of zinc and copper in epithelial tissue, indirectly encouraging favourable effects on metabolism (Lansdown, 2006). Silver nanoparticles have been used in fish farming because of their bactericidal properties, but there are relatively few reports on their inclusion in fish diets. Therefore, it is essential to assess the effectiveness of dietary new nanoparticles administered to culturable fish at a nontoxic level against pathogenic microbes such as *Aeromonas carviae.*

Materials and Methods

The research was carried out following the Guide for the Use of Experimental Animals of the ICAR-Central Inland Fisheries Research Institute (CIFRI), Barrackpore, India.

Silver Nanoparticle Dosage Preparation

AgNPs doses were prepared by dissolving 1 mL of Argovit® (No. 1324458) stock solution in 99 mL of PBS and swirling the mixture slowly for approximately 35 seconds. This stock solution was serially diluted until metallic silver concentrations of 278.9nM, or 30.15ng/mL, were reached. The physicochemical properties of the Argovit® utilised in this investigation were summarised and taken from Bello-Bello et al. (2017).

Experimental Design and Diet Preparation

About 180 *Labeo rohita* (Cypriniformes: Cyprinidae), were purchased from a reputable fish farm in Kolkata, India provided which weighed 40.15±1.4 g. They were acclimated for one week before the experiment and given commercial feed twice daily. The *L. rohita* experimental fish were triplication-stocked in four 25-liter tanks with fifteen (15) fish each at random including the control. Different concentrations of AgNPs (Table 1) were added to the commercial diet, earlier pulverized and later pelleted into a 2mm feed size. The prepared diets were administered separately at 3% of body weight throughout the 72-day feeding period. Consistent with Bowman et al (2012).'s findings, the non-toxic dosages of AgNPs (0, 10, 15, and 20 µg AgNPs kg^{-1} diet) were selected. Every day, the water's pH (7.8±5.2), dissolved oxygen concentrations (5.3 mgL^{-1}), ammonia, (0.09 mgL^{-1}), and temperature (27.10 ±1.1 °C) were measured.

Feed Utilization and Growth Characteristics

After the 72-day feeding study, all fish in the various treatments were weighed to estimate growth as;

$$Weight\ gain = \frac{Final\ weight - Initial\ weight}{Initial\ weight}\ x100$$

$$FCR = Feed\ given\ to\ the\ fish \frac{Dry\ weight}{Total\ wet\ weight\ gain}$$

Table 1. Experimental diet composition

Composition	% inclusion	Proximate composition	
Soybean oil cake	36.0	Protein	31±0.10
Mustard oil cake	45.50	Lipid	10.2±1.31
De-oiled rice bran	2.0	Moisture Ash	9.2±0.41
Fish meal	5.0	Ash	10.1±0.85
Oil mix	1.5		
Vitamin premix	1.0		
Mineral mixture	1.0		
Herbal attractant	2.0		
Probiotics	2.0		
Cysteine	0.25		
Methionine	0.25		
Tryptophan	1.5		
Antox	1.0		
Aquace	1.0		

AgNPs nanoparticles were incorporated into the diet at doses of 0, 10, 15, and 20 µg AgNPs kg^{-1} diet.

The survival rate was calculated after the feeding experiment as follows:

$$Mortality = \frac{Number\ of\ the\ fish\ started\ with}{Number\ of\ fish\ stocked}\ x\ 100\ .$$

***Aeromonas caviae* Source and Preparation**

A. caviae (MK829052), an isolate from an infected fish, was acquired from CIFRI, Barrackpore, India. The bacterium was cultivated to a log phase in a 150ml flask under constant shaking at 37°C using Tryptic Soy Broth (TSB; Merck). The bacterial culture was harvested after centrifuging it at 3500 x g at 4°C for 20 min. The bacterial pellets were then cleaned with germ-free 0.15 M phosphate-buffered saline (PBS) (pH 7.2). The pellets were redissolved in PBS, divided into portions, and stored in TSB that had been added with 15 percent (v/v) glycerol until they were used.

Determination of Lethal Dose 50 (LD_{50}) of *Aeromonas caviae* before the Challenge Test

Ten healthy, acclimated fish were chosen, separated into six groups with one control in each, and kept in a 250 L tank before the challenge test. *A. caviae* MK829052 bacterial cultures that had been grown overnight were spun at 10,000 rpm for five minutes. The recovered pellet was diluted up to 10^5 times in 0.85 percent normal saline solution and rinsed twice with normal saline solution (NSS). Following a 24-hour incubation period at 37°C, the spread plate method was used to calculate the number of cells per mL of suspension. About 0.1 ml of bacterial suspension with a final concentration of $2.2x10^4$, $2.2x10^5$, $2.2x10^6$, $2.2x10^7$, and $2.2x10^8$ CFU/mL was intraperitoneally injected in challenging the fish with the control group injected with 0.1 ml of NSS. For 96 hours, fish mortality was measured every 24 hours. Reisolated bacterial pathogens came from clinically ill fish in the group whose mortality began to meet the Koch postulate. The Reed & Muench (1938) method was used to obtain the LD_{50} values based on mortality data

Challenged Test.

From each experimental group, ten individual *L. rohita* fish were chosen and injected with 0.1 ml of bacterial culture through intraperitoneal mean. The fish were injected at a concentration below the LD_{50} and housed in well-aerated tanks for 15 days.

Antioxidants and Immune-related Parameters

The test and placebo fish's liver and gill samples were minced in a solution containing sucrose (0.25 M) with the aid of TissueLyser II (Qiagen, Hilden, Germany). Subsequently, the clear upper liquid was transferred into sterilised 2-milliliter test tubes and stored at -40 degrees Celsius for the enzyme test. For the activity of SOD (pH 10.2) with sample homogenate, a reaction mix comprising a buffer of carbonate bicarbonate (0.1M, pH 10.2) was utilised. 100 µl of epinephrine was later added to the mixture. For three minutes, the Optical Density was read at 480 nm at 30-second intervals. Caliborne's (1985) method was used to measure catalase activity involving phosphate buffer (50 mM, pH 7.2) and 50 mM H_2O_2. At 240 nm, the reaction rate was measured. The activity of glutathione peroxidase (GPx) was measured using previously acquired tissue homogenates and the usual technique (Noguchi et al., 1973). The respiratory burst activity was determined by treating 100 µl of homogenised tissues (liver and gill) from the fish in each treatment with 0.1ml of Nitroblue tetrazolium (NBT) (0.2%) (Sigma, USA) and left in the incubatory stage for 30 minutes at 25°C. Following incubation, 1000 µl of N, N diethyl methyl formamide (Qualigens, India) was mixed with approximately 50 µl of the aforementioned mixture, and the resulting solution was spun at 6,000 xg for five minutes, with an OD measurement made at 540 nm.

Histopathology

After the 14-day challenge period, the selected organs (liver, kidney, and gill) tissues were excised from

the fish. The tissue samples were kept in 10% neutral buffered formalin (NBF) for histological analysis. The tissues were chopped into small blocks, dehydrated with various alcohol concentrations, and cleaned in xylene (Popoola et al. 2023). Following paraffin embedding, sections (5 µm thickness) were cut with a rotary microtome (RM2125 RTS, Leica, Germany), and stained with haematoxylin and eosin (Luna 1968). Under the microscope (AXIO scope, A1, Carl Zeiss, US), structural abnormalities in stained sections were seen, and microphotographs were obtained.

Total RNA Isolation and Gene Expression Analysis

The TRIzol® method (Invitrogen, India) was used to isolate total RNA from fish kidneys, liver, and muscle, including the gills. Nanodrop (Thermo Scientific, USA) was used to quantify the isolation. RNA integrity was evaluated by electrophoresis in a 1.0% agarose gel, and any leftover DNA was removed using RNAse-free DNAse I (Fermentas, USA). Using a cDNA synthesis kit (Fermentas, USA), reverse transcription was performed to produce first-strand cDNA from total RNA in a 20 µL reaction volume. Subsequently, every sample's cDNA was diluted to achieve a final concentration of 750 ng/mL. The synthesised cDNA was used as the RT-PCR construct for quantitative PCR amplification, and an ABI 7500 device (Applied Biosystems, USA) was utilised along with ChamQTM SYBR® qPCR Master Mix. β-actin was utilised as the reference gene in this study, and the reference primers for fluorescence quantitative PCR were TNF-α and IL-10 (Table 2). The target gene's expression levels were evaluated by applying the $2^{-\Delta\Delta CT}$ approach proposed by Livak & Schmittgen (2001).

Statistical Analysis

One-factor analysis (ANOVA) was used to analyze the obtained data. Where there is a significant difference at 5% ($P<0.05$), Duncan's new multiple range tests were used to separate the mean differences, performed using SPSS 21.

Results

Growth Performance

The growth performance metrics of *L. rohita* administered different dietary amounts of AgNPs (Table 3) demonstrate that the final body weight in AgNPs treated fish was considerably greater ($P<0.05$) than in control fish. However, FCR was observed to be significantly different from the inclusion doses and with control, with better performance in 15µgKg^{-1} AgNPs (Table 3). Moreover, there was a significant difference in mortality across the treatments (inclusion levels).

Immune System Response and Antioxidant Characteristics

The dietary effects of AgNPs significantly ($P<0.05$) affected the activities of catalase (CAT) and superoxide dismutase (SOD) in *Labeo rohita*'s gills. Also, NBT and glutathione peroxidase (GPx) activities in the gills of *Labeo rohita* (Figure 1b & 1c) were seen to vary with inclusion levels of AgNPs. Treatment 2 (10 µKg^{-1}) had the greatest SOD levels in the gills and liver. The value, however, was substantially higher in the gills of the control fish (Figure 1a).

CAT activity (in the liver and gills) of fish administered AgNPs differed significantly ($P<0.05$), with the control treatments having the highest level in the gills (Figure 1d). Treatment 2 has high contents in the sample fish's liver. The NBT values varied significantly ($P<0.05$) across inclusion levels of AgNPs (treatment groups), with treatment 2 having the greatest value in the liver and control having the lowest. The NBT value in the tested gills was higher in treatments 3 and 4, with treatment 2 having the lowest value.

Table 2. Primers list and their sequences used for the gene transcription.

Gene primer	Accession No	Sequence
IL-10	MH341526.1	F-CTGTGAAGGCATGGGTGTG
		R- ATCACTTTCTTCACCCAGGG
TNF-α	No. MH521259.1),	F- CAAGCAATTGGCGAGTGTGT
		R-CAGTTCCACTTTCCTGATTACTCTGA'
β-actin	DQ160229.1	F- TCACCCACACTGTGCCCATCTACGA
		R- CAGCGGAACCTCATTGCCAATGG.

Table 3. Growth and survival of *Labeo rohita* given various levels of AgNPs

Diets	Initial weight (g)	Final weight (g)	Weight gain (WG%)	FCR	Survival (%)
The control diet (0)	29.28±0.01^{a}	44.37±0.31^{d}	55.63±3.14^{d}	2.09±0.10^{a}	80^{c}
10 µgKg^{-1} AgNPs	30.22±0.02^{a}	57.12±0.29^{b}	89.01±3.20^{b}	1.62±0.10^{c}	90^{b}
15 µgKg^{-1} AgNPs	30.78±0.03^{a}	59.49±0.34^{a}	93.27±4.67^{a}	1.48±0.11^{d}	100^{a}
20 µgKg^{-1} AgNPs	29.98±0.05^{a}	51.89±0.29^{c}	73.08±2.47^{c}	1.79±0.12^{b}	90^{b}

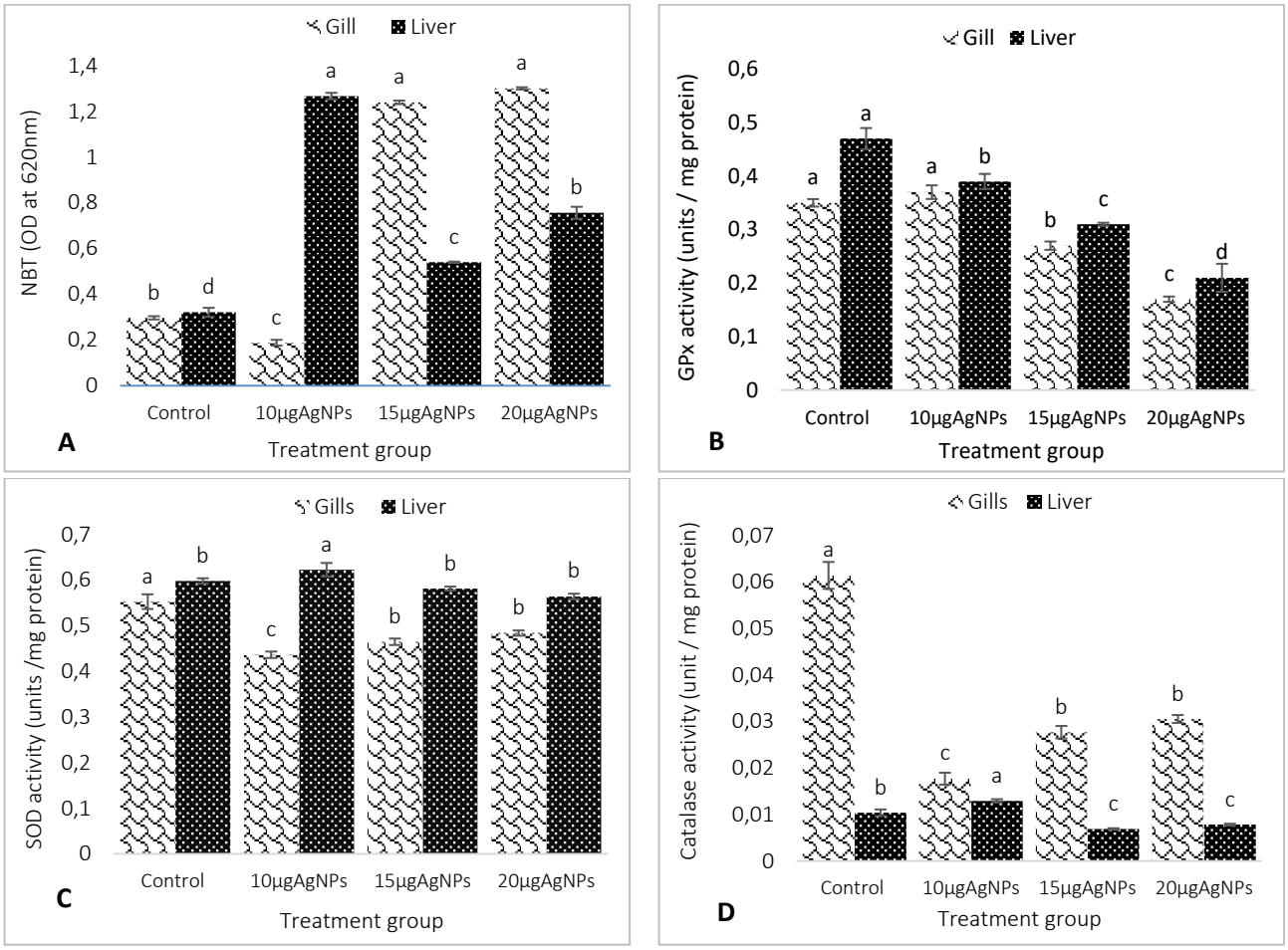

Figure 1. Biochemical analysis of *Labeo rohita* infected with *A. caviae* and fed various amounts of dietary silver nanoparticles (AgNPs). (The mean values with contrasting superscripts differ significantly (the significance level is chosen at P<0.05). (A) Nitro blue tetrazolium test (NBT), (B) Glutathione peroxidase (GPx) activities, (C) Superoxide dismutase activity (SOD), and (D) Activities of catalase (CAT).

Bacterial Challenge

Dose-dependent mortality was monitored for 96 hours, and from the cumulative mortality record, the LD_{50} deduced was 2.20×10^6 CFU/fish for *A. caviae* (Figure 2).

The survival of *Labeo rohita* after 15 days of the challenged test was recorded (Figure 3), with treatment 3 having the highest survival and none recorded for the control.

Histopathological Investigation

Histopathological investigation in *L. rohita* fed AgNPs incorporated diet did not show severe damage to the *A. caviae* infection.

In the *L. rohita* gills fed 10 μkg^{-1} AgNPs, moderate hyperplasia was visible (Figure 4), though, gill samples from fish fed 15 μgkg^{-1} of AgNPs displayed no abnormalities. At the highest and lowest inclusion levels, the liver also showed some minor tissue damage (Figure 5). In the kidney samples of fish-fed AgNPs, there was a slight Bowman's capsule dilatation (Figure 6).

Gene Expression

The physiological investigation was supported by the detectable effects of several genes linked to immunity. The TNF-α was up-regulated in treatment 3 while the IL-10 detectable effect was statistically (P<0.05) higher within the liver of rohu fed an AgNPs-included diet (Figure 7a). Similar findings were made regarding the expression of IL-10 in tissue, which showed no discernible difference between treatments 2 and 3 compared to the control and was down-regulated in treatment 4 (20 μgkg^{-1}) for TNF-α (Figure 7b). Treatment 3 (15 μgkg^{-1}) significantly (P<0.05) increased the reaction of TNF-α in *L. rohita* kidney, in contrast to IL-10, which had a high expression level and a rising response in fish-fed diets containing AgNPs in treatment 3 (Figure 7c). In comparison with the control treatment, IL-10 was down-regulated in the gill of T4 but up-regulated (P<0.05) in the gill of treatment 2 and treatment 3 AgNPs fed rohu (Figure 7d). The TNF-α expression does not significantly change across T1, T2, and T3, with T4 showing a down-regulation.

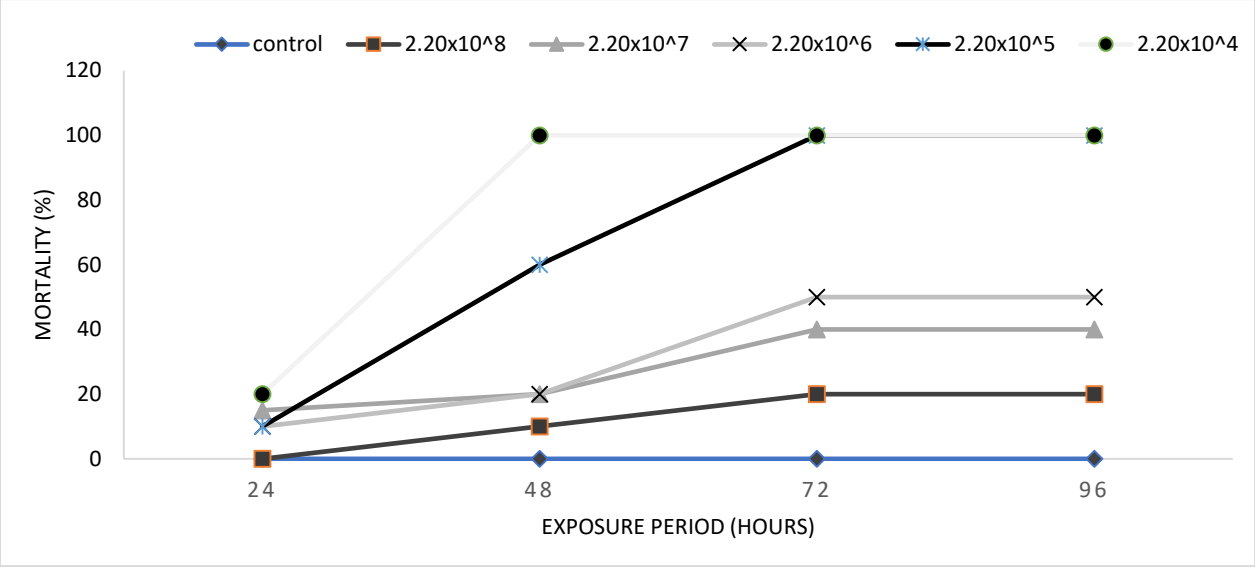

Figure 2. The determination of the LD_{50} value and the mortality curve of *Labeo rohita* challenged with *A. caviae* (MK829052).

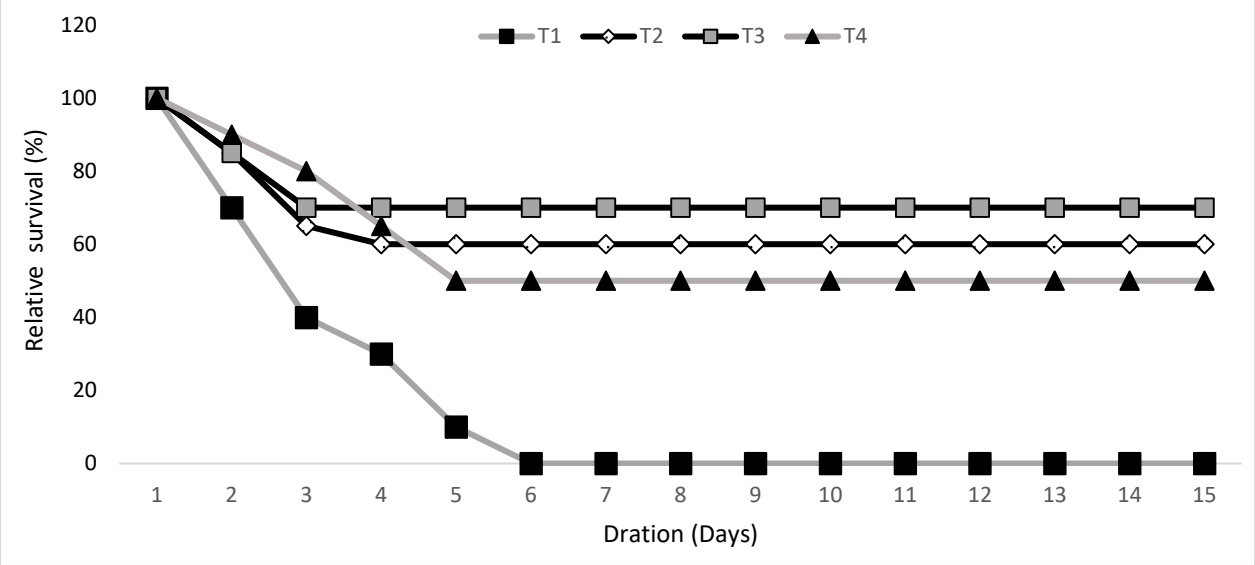

Figure 3. Relative survival (%) of *L. rohita*, challenged with *A. caviae* after feeding with AgNPs containing diets for 72 days.

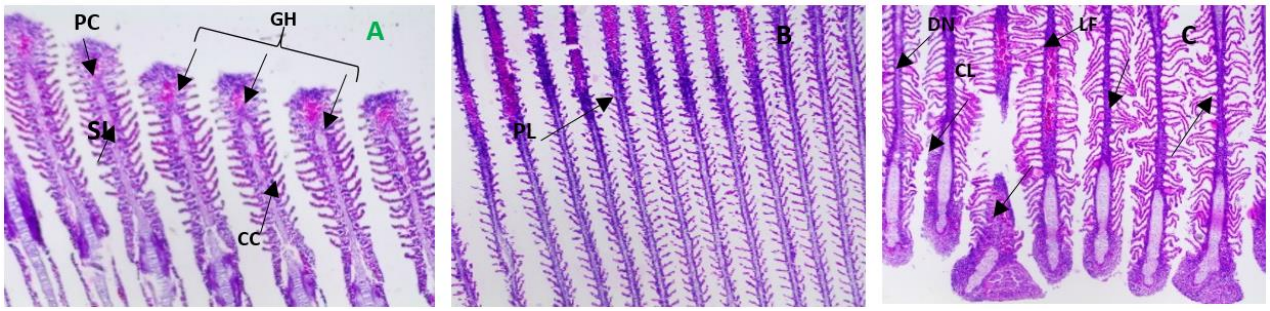

Figure 4. Histoarchitectural changes in gill tissue of *A. caviae* -infested *Labeo rohita* fed with varying levels of dietary Silver Nanoparticles (AgNPs). A:10µgkg^{-1}, B:15 µlkg$^{-1,}$ and C: 20 µlkg^{-1} Chloride cell (CC), Secondary lamellae (SL), Pillar cell (PC) Degeneration and necrosis of pillar cells and dilution of capillary walls (DN), Fusion of Lamellar Layers (LF), Secondary Lamellae Curling (CL), Hyperplasia of the gill (GH). (H&E, ×10).

Discussion

In this study, different levels of inclusion of AgNPs, which were used as immunostimulants, had a discernible impact on the growth of *L. rohita*, with better growth and survival in the diet containing AgNPs compared to the control. However, there was a decline in the growth level at the highest concentration of AgNPs as observed by Mabrouk et al. (2021). The improved growth observed in AgNPs fed Rohu compared with control is comparable to the findings of Vineela et al. (2017), who found that a diet enhanced with AgNPs resulted in better-quality growth in *Catla catla*. Comparable to the increase in feed consumption and weight gain observed in the current study as a result of an AgNPs-included diet, Mahanty et al. (2013) attributed this improvement to the antibacterial action of AgNPs and a change in the intestinal bacteria. AgNPs have been related to growth in nutritional useful bacteria like lactic acid intestinal flora (Vadalasetty et al., 2018). This could lead to better growth by increasing the availability of nutrients and nutrient absorption. Furthermore, dietary AgNPs increased the health, body weight gain, and overall survival of fish subjected to a variety of stressors (Kumar et al., 2019). However, the stunting observed in conjunction with the elevated level of AgNPs in the current study is comparable to that observed in *Oryzias melastigma* larvae that exhibit growth retardation when fed with brine shrimp contaminated with AgNPs (Wang &Wang, 2014). Forouhar et al. (2019) also noted the decreasing length and weight of goldfish, *Carassius auratus gibelio,* which they attributed to rising AgNP levels.

Diseases are significant stressors on organisms and reducing the stress impact, particularly in cultured organisms, results in improved performance in terms of growth and other life parameters. Antioxidant enzymes guard against oxidative stress as part of the animal defense mechanism (Zahran & Risha 2014). When an organism experiences a stressful environment such as a disease, or a change in the chemistry of the water, etc. excess superoxide is produced in the organism, and ROS work as scavengers of this superoxide. The concentrations of AgNPs induced oxidative indices (SOD, CAT, GPx, and respiratory burst) activities in fish fed with various amounts of the substance, according to the ion homeostasis and innate immunity of the animals (Kumar et al., 2018). Antioxidant enzyme activities also showed a decreasing trend as AgNP levels rose, unlike NBT values that were higher in high inclusion levels of AgNPs. The stimulation of antioxidant enzymes at low concentrations of AgNPs suggests that the immune and antioxidant systems may have mitigated the effects of *A. carviae* infection (Gültepe et al., 2014). Rajkumar et al. (2016) found that the liver, gills, and muscles of *Labeo rohita*, given a contaminated diet, increased in antioxidant enzyme activities owing to the induction of immunity against an upsurge in the production of

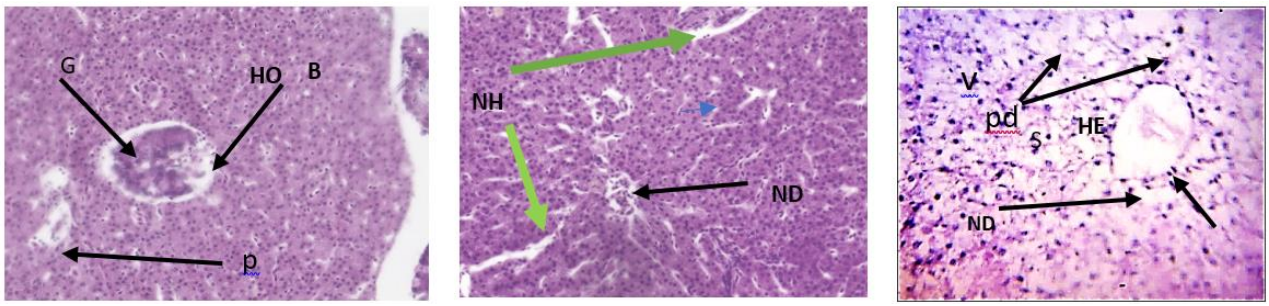

Figure 5. Photomicrograph of liver tissue of *A. caviae* -infested *Labeo rohita* fed with varying levels of dietary Silver Nanoparticles (AgNPs). A:10µlkg-1, B:15 µlkg-1 and C: 20 µlkg-1. (PD) patchy degeneration, Degeneration of nuclear materials (ND), hepatocyte necrosis (NH), and overfilling of hemocytes within the blood vessels (HO). (H&E, ×40).

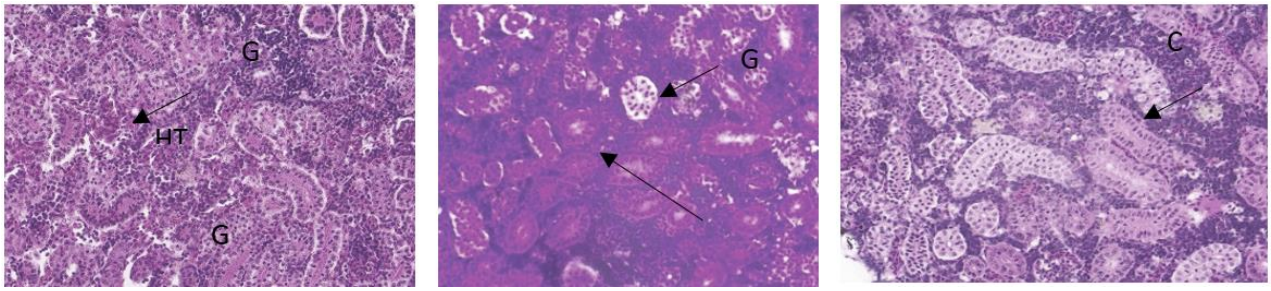

Figure 6. Photomicrograph of Kidney of *A. caviae* -infested *Labeo rohita* fed with varying levels of dietary Silver Nanoparticles (AgNPs). A:10µgkg-1, B:15 µgkg-1 and C: 20 µgkg-1 Glomerulus (G), Complex proximal portion (B), and Complex distal portion (C), hematopoietic tissue (HT) and renal corpuscle with its glomeruli (G). (H&E, ×40).

reactive oxygen species. Aside this, AgNPs-exposed-fish showed immunosuppressive effects at higher AgNP doses and immunostimulatory effects at lower doses (Dobrovolskaia & McNeil, 2007; Kumar et al., 2018).

The antioxidant enzymes were noticed to increase with inclusion in the gills unlike the liver (catalase and SOD). Halliwell & Gutteridge (2015) reported that the enzyme's activity varied in various tissues or organs with high oxidative potential, necessitating the expression of the tissue-specific protein during oxidative stress (Halliwell & Gutteridge, 2015). The functions of these organs can account for the variations in catalase activity. Aside from respiration, fish gills serve a variety of critical tasks such as electrolyte balance, metabolic waste excretion, hormone production and pH control (Herrero et al., 2018). These functions require energy and require a large amount of energy supply from oxidative metabolism (Oliveira, 2008). Following an *A. caviae* challenge, the addition of AgNPs dramatically boosted the activation of three antioxidative enzymes essential in ROS scavenging (SOD and CAT). Fish immunity is triggered by an increase in phagocytes or phagocytes that are active (Gültepe et al., 2014). According to estimates made by the NBT test, phagocytosis, which uses oxidative radical synthesis and respiratory burst activity, is an aquatic animal secondary defence mechanism. (Fischer et al., 2006). Increased respiratory burst activity with inclusion level of AgNPs have been observed in the gill and liver as AgNPs (except T2 gill 10ng/kg AgNPs) levels rise. Fish under stress have been reported to exhibit a decrease in NBT values (Abdel-Tawwab et al., 2014; Haridas et al., 2017). The upsurge values found in this finding may indicate that including AgNPs in the diet assisted in controlling *L. rohita*'s *A. carviae* -induced stress.

The histopathology of the examined organs revealed the effect of *A. carviae* and AgNPs on the *L. rohita* fish. At lower concentrations of AgNPs, the tissues (gill, liver, and kidney) exhibit some negative effects which might be from the bacterial challenges. In *L. rohita* gills fed 10 µkg-1 AgNPs, moderate hyperplasia was visible (Figure 3). Gill samples from fish that had been fed 15 µgkg-1 of AgNPs showed no abnormalities, though. At the highest and lowest inclusion levels, the liver also showed some minor tissue damage (Figure 2). In the kidney samples of fish-fed AgNPs, there was a slight Bowman's capsule dilatation degeneration. AgNPs have been found to cause, at higher doses, histological alterations in the gills of Indian major carp ranging from mild to severe (Scown et al. 2010; Rajkumar et al. 2016). Consistent with Subashkumar & Selvanayagam (2014), similar nanomaterials like zinc nanoparticles caused hyperplasia and epithelial lifting in the gills of *Cyprinus carpio* resulting in respiratory disruption and, eventually, fatality with such inclusion level. Nuclear degeneration observed in the liver of *L. rohita*

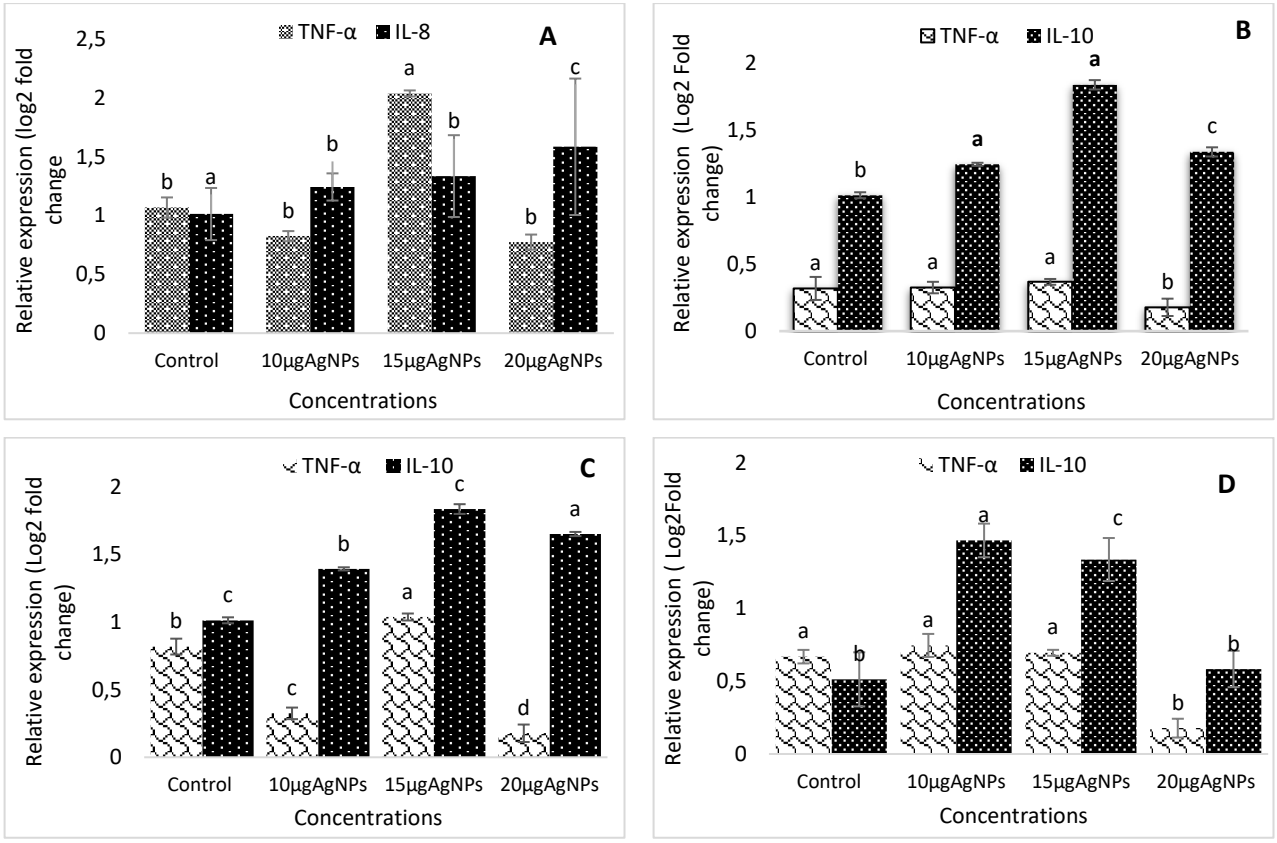

Figure 7. Relative transcription of IL-10 and TNF-α genes in *Labeo rohita* fed various amounts of dietary silver nanoparticles and infected with *A. caviae* (AgNPs). The transcript levels were normalized against β-actin. (The mean values with contrasting superscripts differ significantly (the significance level is chosen at P<0.05). (A-liver B-tissue, C-kidney, and D-gills)

challenged with *A. caviae* might be from bacterial infection through challenge and or the toxicity of AgNPs at higher levels which resulted in tissue damage. These histological alterations were consistent with those observed in silver nanoparticle-exposed Indian main carp and nickel nanoparticle-exposed Mozambique tilapia (Jayaseelan et al., 2014 Rajkumar et al. 2016). The buildup of Ag+ ions in animal tissues may be responsible for the histological alterations observed in the various tissues investigated (Martin et al., 2018).

In reaction to stress, animal cells produce tiny proteins called cytokines, the same thing applicable to inflammation, infection, and immunological response. Based on their capacity to promote or decrease inflammation, cytokines can be grouped as pro- and anti-inflammatory categories (Dinarello, 2000). It is generally agreed that insufficient inflammation might impede pathogen clearance and health restoration, whereas host cells suffer damage from high inflammation. In this environment, a normal immune response is dependent on a balance of pro-inflammatory and anti-inflammatory cytokines; thus, the production of these cytokines is tightly regulated (Opal and DePalo 2000). The liver, kidney, muscle/tissue, and gill of AgNPs supplemented diets given rohu were shown to have higher levels of IL-10 than the control group after being challenged with *A. carviae* in the present study. In comparison to the fish in the other treatments, the kidneys of rohu fed an AgNPs-supplemented diet had higher TNF-α expressions. This study's observations of the up-regulation of IL-10, kidney, liver, tissue, and gills were comparable to those made by Sukumaran et al. (2016). In previous work, Mohanty and Sahoo (2010) found that the expression patterns of IL-10 and TNF-α differed significantly between rohu that had been exposed to *E. tarda* infection and those that had not. A large increase in the transcription of IL-10 and previous research using *A. hydrophila* to challenge *L. rohita* shown a substantial reduction in TNF-α (Swain et al., 2011). This demonstrated how these genes react when a pathogen is present. In the current work, bacterial challenge enhanced the production of IL-10 mRNA in Rohu-given diets containing AgNPs; the expression was particularly strong in T3. According to similar investigations, bacterial infection has been shown to boost the expression of IL-10 (Rojas et al., 2017). AgNPs may have achieved the best results in establishing immunity against *A. carviae* in T3, as demonstrated by the zero-mortality value following infection, which may account for the greater expression level.

Conclusion

According to the findings of the current investigation, adding AgNPs to the diet of *L. rohita* at a level of inclusion of (15 μgkg^{-1}) enhanced growth performance, feed conversion ratio, antioxidant enzyme activities, and specific immunological response. It can also lessen the histological consequences of an *Aeromonas carviae* challenge in the kidney, liver, and gills. However, physiological indicators and growth performance were negatively impacted by increasing the amount of AgNPs in the diets, which is understandable given the potential toxicity AgNPs may have at higher inclusion levels. Therefore, it is imperative to be conscious in administering AgNPs in the fish diet for immunostimulatory purposes to prevent overuse which might have negative effects on the reared fish.

Ethical Statement

The research was carried out following the Guide for the Use of Experimental Animals of the ICAR-Central Inland Fisheries Research Institute, Barrackpore, India.

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Author Contribution

Conceptualization, Funding acquisition, Methodology, Data curation, Formal analysis, Writing – original draft: OMP. Supervision, review, and editing: BKB. There was a mutual agreement between the authors after thorough reading for it to be published.

Conflict of Interest

The authors claim that there are no competing financial interests or personal relationships known that might have an impact on the work reported in the document.

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