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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 15µg$kg^{-1}$ 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 2.2 x $10^4$, 2.2 x $10^5$, 2.2 x $10^6$, 2.2 x $10^7$, and 2.2 x $10^8$ 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.

## 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.

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

| Gene primer | Accession No | Sequence |
|---|---|---|
| IL-10 | MH341526.1 | F-CTGTGAAGGCATGGGTGTG<br>R- ATCACTTTCTTCACCCAGGG |
| TNF-α | No. MH521259.1), | F- CAAGCAATTGGCGAGTGTGT<br>R-CAGTTCCACTTTCCTGATTACTCTGA' |
| β-actin | DQ160229.1 | F- TCACCCACACTGTGCCCATCTACGA<br>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$^{b}$ |

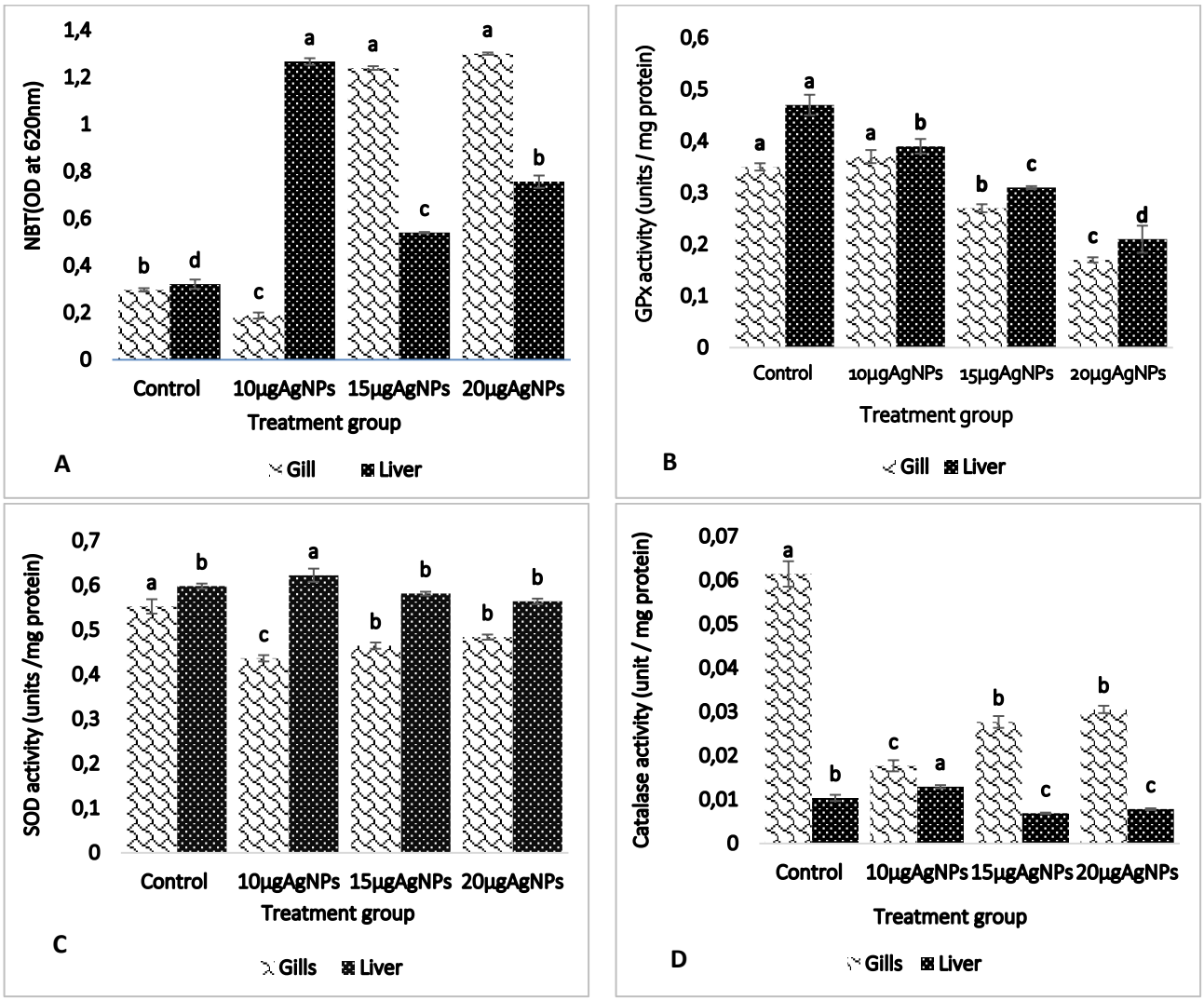


**Figure 1**. Biochemical analysis of *Labeo rohita* infected with *A. caviae* and fed various amounts of dietary silver nanoparticles (AgNPs). (The mean values with contrasting superscripts differ significantly (the significance level is chosen at $p<0.05$). (A) Nitro blue tetrazolium test (NBT), (B)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

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