Aquatic Science and Fish Resources
http://asfr.journals.ekb.eg
Print ISSN: 2682-4086 Online ISSN: 2682-4108

Effect of Silver Nanoparticles Biosynthesized by *Azolla Pinnata* in Preventing *Clarias Gariepinus* Infection with *Aeromonas Hydrophila*

Mohamed Sayed [1,*] and Rehab M. Reda[2]

[1]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt.

[2]Department of Physiology, Faculty of Veterinary Medicine, Beni-Suef University, Beni- Suef 62511, Egypt.

*Correspondence: Mohamed Sayed, mohamed_omar@vet.bsu.edu.eg

ARTICLE INFO

Received: june. 01, 2025
Revised: July. 16, 2025
Accepted: Sept.04, 2025
Available online: Sept.11, 2025
Doi: 10.21608/asfr.2025.391089.1083

Keywords:
Aeromanas,
Antioxidant,
Azolla,
silver nanoparticles.

Abstract

To counteract the infection caused by the *Aeromonas hydrophila* (*A. hydrophila*) pathogen, current study suggests adding *Azolla pinnata*-biosynthesized silver nanoparticles (AP-AgNPs) to fish diet. *Clarias gariepinus* were kept in four experimental groups: groups 1 and 2 were raised on supplemental diets at 0.6 and 0.3 mg AP-AgNPs/kg feed, whereas groups 3 and 4 were the positive and negative controls, respectively, and were fed non-supplemented feed. Following a 21-day feeding trial, lethal dose of *A. hydrophila* isolated from local outbreak with LD_{50} of 1.5×10^6 was administered to all groups (except the control negative). Following bacterial challenge, fish sera were biochemically investigated. Additionally, transcriptional investigations of particular immune and antioxidant-associated genes in the anterior kidneys were performed at all levels before and right after *A. hydrophila* challenge. Results demonstrated that fish groups treated with 0.6 and 0.3 mg AP-AgNPs/kg feed displayed survival rates of 72.22% and 44.44% against *A. hydrophila* with normal serum levels of both hepatic and renal indictors. However, groups 1 and 2 showed a modest increase in serum levels of triglycerides and cholesterol when compared to control negative. Further, especially following the challenge, higher serum levels of the measured lysozyme (LZM) and complement 3 (C3) were noted. Additionally, gene expression investigations revealed that the anterior kidneys of the experimental fish had up-regulated transcriptional levels of the IL-1β, MHC-IA, GPX, and SOD1 genes. This signifies that African catfish fed supplemental diet containing 0.6 mg of AP-AgNPs/kg of feed for three weeks had higher intrinsic immune responses.

1. Introduction

Disease outbreaks in aquaculture and/or wild fish stocks are caused by a variety of bacterial pathogens. A common gram-negative motile bacillus in aquaculture, *Aeromonas hydrophila* (*A. hydrophila*) causes septicaemia in warm-water fish by causing diffuse haemorrhages, ascites, and degenerative changes in the parenchymatous organs, which results in high losses and mass mortality **(Janda and Abbott, 2010)**. Mass fish deaths from microbial illnesses pose a significant challenge to aquaculture **(Sudheesh *et al.*, 2012)**. Natural resources are under a lot of strain right now. Because more human activity is causing the water to become contaminated, which stresses fish **(Amoatey and Baawain, 2019)**. As their

health regularly deteriorates, fish that live in water with low quality are more susceptible to infection. Several medications, including growth stimulants and antimicrobials, are used to improve their health and manage microbial illnesses in order to prevent financial losses. However, there are a number of reasons why drug use has been restricted. The primary justification for restricting the use of antibiotics in the aquaculture industry is the increased danger of antibiotic resistance in bacteria and the build-up of antibiotics in the muscles of commercialized fish **(Islam *et al.*, 2023)**. The development of infectious diseases in fish is mostly caused by environmental stress, weakened immune systems, and microbes. It is challenging to eradicate all germs from the aquaculture system. Improving water quality and preventing excessive stocking can reduce environmental pressures. But, the most effective way to lower the incidence of disease is to use immune stimulants to strengthen their immune system, including both innate and adaptive immunity **(Barman *et al.*, 2013; Wang *et al.*, 2017)**. This includes substances that influence the immune system and increase the host's resistance to infectious diseases, such as chemicals, probiotics, polysaccharides, plant or animal extracts, cytokines, and dietary components. According to **Luis *et al.* (2019)**, the application of nanotechnology has demonstrated efficacy in preventing infectious diseases. However, the widespread use of nanoparticles in aquaculture systems may be limited by their potential to have negative effects **(Mahboub *et al.*, 2021)**. As a result, new developments in environmentally friendly nanomaterial synthesis have emerged recently. The production of abundant metallic nanoparticles depends on the usage of plant resources as bioactive mediators, which lessen ecological contaminants linked to chemical ones **(Kumari *et al.*, 2021)**. According to **Khan *et al.* (2024)**, plant-based synthetic nanoparticles provide aquaculture with a safe, sustainable, and more economical tool as they can be used for disease control, growth enhancement, and water quality improvement. They serve as dietary supplements, antibacterial agents, and pollution removers, which promote healthier aquatic life and a more ecologically responsible aquaculture sector **(Khan *et al.*, 2023; Khursheed *et al.*, 2023; Bhushan *et al.*, 2024)**. Mostly found in Africa, Southeast Asia, and Australia, *Azolla pinnata* (*A. pinnata*) is a free-buoyant aquatic water velvet **(Lumpkin and Plucknett, 1980)**. In addition to fixing atmospheric nitrogen through a symbiotic association with *Anabaena azollae*, it thrives quickly, multiplying its biomass in a matter of days. It also has a high capacity for eliminating heavy metal ions from aqueous mixtures and is one of the best entrants for the making of nanoparticles, especially silver nanoparticles (AgNPs) **(Korbekandi *et al.*, 2014)**. Researchers are interested in metal nanoparticles (NPs), particularly silver ones, because to their potential uses in fish medicine and pharmaceutical sciences **(Iravani *et al.*, 2014; Popoola *et al.*, 2022)**. *Azolla pinnata*'s high biomass yield, quick growth, and simplicity of cultivation make it a superior choice for nanoparticle synthesis when compared to other plant-based techniques **(Korsa *et al.*, 2024)**. These qualities result in a production method that is more scalable and economical. Additionally, during the manufacture of nanoparticles, *Azolla pinnata* contains

a variety of bioactive chemicals such as flavonoids that function as stabilizing and reducing agents, producing more stable and distinct nanoparticles **(Zhu *et al.*, 2025)**. The present study examined the immunomodulatory activity and potential significance of *Azolla pinnata*-biosynthesized silver nanoparticles (AP-AgNPs) in preventing infection caused by *A. hydrophila* bacterium in the native African carnivorous fish, *C. gariepinus*, which has been presented to numerous countries.

2. Materials and Methods

1. Biosynthesis of silver nanoparticles

The preparation of silver nanoparticles followed **Anjana *et al.* (2020)**. In short, five grams of fresh, green *A. pinnata* leaves were gathered and properly cleaned with flowing tap water and then double-distilled water to get rid of any dirt particles that might have stuck to them. They were placed in a round-bottom flask with a water condenser and heated with 100 mL of double volume-deionized water for 20 minutes. After cooling, it was filtered using Grade 1 Whatman filter paper (Sigma-Aldrich, Missouri, US). According to **Joseph and Mathew (2015)**, the resulting extract was kept at 4°C for storage. Furthermore, 75 ml of 1 mM $AgNO_3$ solution was occupied in an Erlenmeyer flask. To this 0.5 ml *A. pinnata* extract diluted to 10 ml was added and combined thoroughly. This was sited in an ordinary microwave oven (IFB Solo 24PM2S, India) running at 750 W power and 2130 MHz frequency and was imperiled to microwave irradiation for 390 seconds. The produced nanoparticles (AP-AgNPs) can then be disseminated in distilled water and subjected to centrifugation. The isolated particles were desiccated and were used for further examination. A transmission electron microscope (TEM) and a zetasizer (Malvern Panalytical, Ltd. UK) were used to assess the size and shape of the generated silver nanoparticles.

2. Ethical statement and fish rearing settings

The Institutional Animal Care and Use Committee (IACUC) of Beni-Suef University in Egypt provided approval number (025-016) for the fish study protocol, which complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) standards **(Percie du Sert *et al.*, 2020)**. By mean weight of 130 ± 20 g, *C. gariepinus* were supplied by a saleable farm in Egypt's Beni-Suef Governorate. Large plastic tanks, 200 L capacity per each, in which the fish were housed, have continual oxygen supply and water without chlorine. The fish were acclimated for 14 days and then regularly checked for health before the tests began. The fish were fed at a proportion of three percent of their mean weight on a simple diet. The studies were conducted by redistributing the fish onto glass aquaria of 85 × 35 × 55 cm after they had acclimated. Water settings were retained at 32 ± 1 °C, 7.5 ± 0.5 pH, 3.5 ± 0.61 mg/L dissolved O2, 0.016 ± 0.004 mg/L NO2, 0.03 ± 0.005 mg/L NH3, and comparable dark to light hours, 12:12 hours, throughout the entirety of the experimental process.

3. Molecular identification of isolated *A. hydrophila* strain

An outbreak at a catfish farm in the municipality of Beni-Suef, Egypt in the summer of 2024 led to the isolation of a virulent strain of *A. hydrophila*. It underwent first biochemical identification. Following that, it was molecularly identified by detecting the hemolysin gene (hly) in accordance with **Rozi *et al.* (2017)**, using sequence sets (5′–3′), F:GGCCGGTGGCCCGAAGATACGGG R:GGCGGCGCCGGACGAGACGGGG with the following modifications to the cycling

conditions: primary denaturation at 94 °C for 5 minutes, secondary denaturation at 94 °C for 45 seconds, annealing at 56 °C for 30 seconds, extension at 74 °C for 40 seconds, and final extension at 72 °C for 7 minutes, and a thermos-cycler programmed for 30 cycles. 50% glycerol solution was used to preserve the isolate at -80°C.

4. LD_{50} of the isolated *A. hydrophila* strain

An inoculum of 1.5×10^8 CFU/mL was obtained by serially diluting a microbial suspended solution in 0.65% sodium chloride solution to go with McFarland tube 0.5 (bioM´erieux, France). The dilutions ranged from $1.5x10^8$ to 1.5x104 CFU/mL. The pathogenicity and LD_{50} determinations were made promptly using the inoculums **(Reed and Muench, 1938)**. 108 *C. gariepinus* were chosen at random and split up into 6 groups, 3 repeats, which were then sited in 18 aquariums with a combined water volume of 70 L. Six groups were allocated: five were marked as investigational and one as negative control. According to **Roubach *et al.* (2005)**, fish were put to doze by dissolving 5% eugenol (Sigma, USA) in 45 ppm of dimethyle sulfoxide (DMSO, Sigma-Aldrich, USA). The fish were subsequently intraperitoneally (IP) infected with 200 µL of serial microbial suspended solution (0.65% sodium chloride), from 1.5x108 to 1.5x104 CFU/mL of *A. hydrophila*. The three replicates, related to the control negative, were given just 200 µL of saline. Throughout the two-week trial period, fish from every group were watched for signs of recovery and tracked for cumulative mortality and clinical anomalies. Tissue samples, head kidneys, were taken from fish that were moribund and streaked over BHI. The plates were then kept at 29 °C for 2days in order to isolation of the inoculated bacterium. In parallel, PCR assays were used to recognize the re-isolated bacterial strain.

5-Fish Feed preparation

With a mortar, the commercial pelleted fish diet (Table 1) was milled into a fine powder. To generate four fish diets, the fine powder that had been previously milled was combined immediately with the Ag-*Azolla pinnata* nanoparticles. Diets 3 and 4 included no additives, representing control groups; diet 2 contained 0.3 mg/kg feed; and diet 1 contained 0.6 mg/kg feed. The components of the fish diet were completely combined and then immersed in purified water. After sixty-five minutes, the feeds were ground into pastes. The pastes were individually ground in a mechanical grinder and then pelleted using a paste extruder with a diameter of 2 mm. The meals were broken down into pellets that were between three and five millimetres long and kept in plastic bags at 4 °C after being air-dried for a day at room temperature **(Alishahi *et al.*, 2011)**.

Table 1. Fish diet composition.

25% protein, Diet ingredients	Percent
SBM	35
YC	33
FM	7
RB	21
MP	1
VP	1.5
Cysteine	0.5
Methionine	0.5
Tryptophan	0.5

SBM, Soya bean meal, YC, Yellow corn, FM, Fish meal, RB, Rice bran, MP, Mineral premix and VP, Vitamin premix.

6. Feeding, microbial challenge and gathering samples

At random, 96 fish were split up into four even groups, each containing 24 fish (eight in each replicate, and three in duplicate). For 21 days, Ag-*Azolla pinnata* nanoparticles were added to G1 and G2 at levels of 0.6 and 0.3 mg/kg feed, respectively, whereas G3 and G4 received a standard feed supplement during the same time frame. Every day, at three regular interval time periods, each experimental group received 3 % of their body weight. At day 21 and according to **Roubach *et al.* (2005)**, six fish from each group were anesthetized using eugenol solution and had their caudal arteries blood drawn. To separate the serum, blood samples were drawn without the use of an anticoagulant (centrifugation at 4 °C, 15,000 rpm for 20 minutes). The serum samples were deposited at -20°C for consequent biochemical and antibodies examination. The anterior kidneys were cut, thereafter dipped in RNA, and stored at -80 °C for gene transcription studies. The rest of the fish in each group were subjected to bacterial challenge. A lethal dose of *A. hydrophila* (1.5×10^8 CFU/mL) was inoculated intraperitoneally to groups 1, 2, and 3 at the ending of the feeding trial, while PBS was injected to group 4. Along with survival rates, as shown by **Ibrahim *et al.* (2022),** clinical symptoms, mortality %, and post-mortem anomalies were documented every day for 14 days of monitoring. After the end of the observation period, the survival fish were subjected to anaesthesia to collect blood and their anterior kidneys for further immune and gene expression analyses.

7-Biochemical assessments

The serum levels of alanine aminotransferase (ALT, catalog number (Cata. N.) EK12287), (Biotrend Corporation, Maryland, US), aspartate aminotransferase (AST, Cata. N. EK12276), and lactate dehydrogenase (LDH, Cata. N. MBS013278) (BioSource Corporation, California, US) were detected. Additionally, total levels of serum bilirubin, total protein and albumin were estimated using (Bilirubin Kit, Cata. N.: MBS169250), (BCA Protein Kit II, Cata. N.: MBS481734) from BioSource Corporation, California, US and (Albumin Assay Kit cata. N. MAES0066) from Assay Genie, Dublin, Ireland. Total globulins calculated as following: globulins concentration = total protein - albumin. In addition, the levels of urea and creatinine were measured using kits from Abbexa LLC in Houston, Texas, US and Abcam Inc, Cambridge, U K, respectively (Urea Nitrogen Kit, Cata. N. abx090684 and Creatinine Kit, Cata. N. ab65340). Furthermore, serum levels of cholesterol (Cata. N. STA-390), triglycerides (Cata. N. STA-396) and glucose (Cata. N. STA-680) were determined using assay kits provided by Cell Biolabs Co, San Diego, USA. Furthermore, lysozyme (LYZ) and complement 3 (C-3) activities were measured using commercial kits (Cusabio biotech, Cata. N. CSB-E17296Fh and CSB-E09727s, China) pursuing the manufacturer instructions.

8. Titration of specific antibodies

The specific IgM against *A. hydrophila* was measured quantitatively in the serum of experimental fish groups using the agglutination assay. Using a microtiter plate, the assay was carried out in accordance with the **Biller-Takashi *et al.* (2014)** protocol. In a nutshell, the combined serum from the experimental and control groups were diluted ten times using phosphate buffered saline (PBS). Labels attached to microtiter plates that corresponded to the sera of the experimental and control groups were placed. The first well of each microtiter plate was filled

with 50 µL (1:10) of each collective serum group that had been diluted two times with phosphate buffer saline. Each microplate's remaining wells were thereafter serially diluted twice using PBS. The corresponding microtiter plate was then labeled, and each well was filled with 50 µL of freshly prepared *A. hydrophila* 9×10^8 CFU/mL suspended solution, McFarland tube #3. Each plate was rotated and waggled on a level surface, then enclosed with para-films and kept for 20 hours at 26 ◦C. microtiter plates with both positive and negative control groups were determined. PBS was supplemented in place of the suspended bacterial solution to establish a negative control, and a dosage of 9×10^8 CFU/mL of *E. coli* bacterial suspension was used as a positive control. The endpoint for agglutination is considered to be the final serum dilution at which agglutination seen. To compute the agglutination antibody titer, which was represented as Log_2 of reciprocal, the maximum serum dilution at which agglutination was detectable was employed.

9. Transcription assessments of some immune and antioxidant associated genes

With 1 mL of Trizol (Invitrogen), total RNA was extracted from 75 mg of head kidney tissues from three fish per replication (N = 12 per group), in accordance with the manufacturer's instructions. RNA concentration at 260 and 280 nm was calculated by a UV-Vis Spectrophotometer, NanoDrop (Thermo Sci., US) to assess the amount of total RNA that was collected. As directed by the manufacturer, the cDNA was generated from the isolated RNA using cDNA reverse transcription kit (Maxima, Thermo Sci., US). In 25-µl reaction tubes, 7 µl of RNA sample, 2 ng of total RNA, 4 µl of maximum enzyme combination, 5 µl of 6X buffer mixture, and 9 µl of purified (MilliQ) water were combined to create cDNA, which was then stored at -80 °C for later use. The IL-1β, MHCIA, GPX and SOD1 genes in the anterior kidneys were computed using real-time quantitative PCR (RT-qPCR) with particular primers (ShineGene Molecular Biotech) for the genes listed above (Table 2). The Rox Universal SYBR Green Master Kit was used for the RT-qPCR processes. Each 25 µl qPCR experiment contained 10 µl of ROX Syber Master Mix, 2 µl of cDNA, 1 µl of forward and reverse primers, and 11 µl of highly filtered water. RT-PCR from Applied Biosystems, US, was used for the qPCR tests. The thermal cycler was configured to execute 35 cycles at 94 °C for 7 minutes, 94 °C for 15 seconds, 56 °C for 30 seconds, and 74 °C for 15 seconds, respectively, for initial denaturation, denaturation, annealing, and extension. The transcriptions of the genes of interest were computed as a relative value of fold change regarding to β-actin, the reference gene, as described by **Kordon *et al*. (2019)**.

10. Statistical assessment

The ANOVA, one-way analysis of variance (SPSS 18, Illinois, US) was used to examine data from biochemical assays and fold change values of immune and antioxidant-associated genes. The mean differences were determined at a significance level of $P \leq 0.05$.

Table 2. Primer sets of selected immune and antioxidant-associated genes for RT-qPCR.

Gene	Sequence	GenBank Accs.Numb.	Reference
IL-1β	F: TGCAGTGAATCCAAGAGCTACAGC R: CCACCTTTCAGAGTGAATGCCAGC	MH341527.1	**(Nasrullah *et al.*, 2021)**
MHC-IA	F: AACAAGTGGGATCCTGATAGTG R: AACAAGTGGGATCCTGATAGTG	MG545605.1	**(Nasrullah *et al.*, 2021)**
GPX	F:ACAACCAGGGACTACACTCAAGTG R: CACACCCAAAATAACGAGACCTT	GQ376155.1	**(Swaleh *et al.*, 2020)**
SOD1	F: TGCTCCCGTAGTGGTTAAAGGG R: TTCATCAAGTGGCCCACCATG	MK112879.1	**(Nasrullah *et al.*, 2021)**
β-actin	F:ACCCCCGCCATGTACGTT R:CCGGAGTCCATGACGATACC	XR_002012167.1	**(Swaleh *et al.*, 2020)**

Interleukin 1β, MHC-IA, major histocompatibility complex, GPX, glutathione, SOD 1, superoxide dismutase and β-actin, beta actin.

3. Results

1. Characterization of AP-AgNPs

The average size of the synthesized AP-AgNPs was 243.6 ± 3.68 nm, according to the particle size distribution curve (Fig. 1). The ovoid and spherical shape of the AP-AgNPs was shown by the TEM micrograph (Fig. 2).

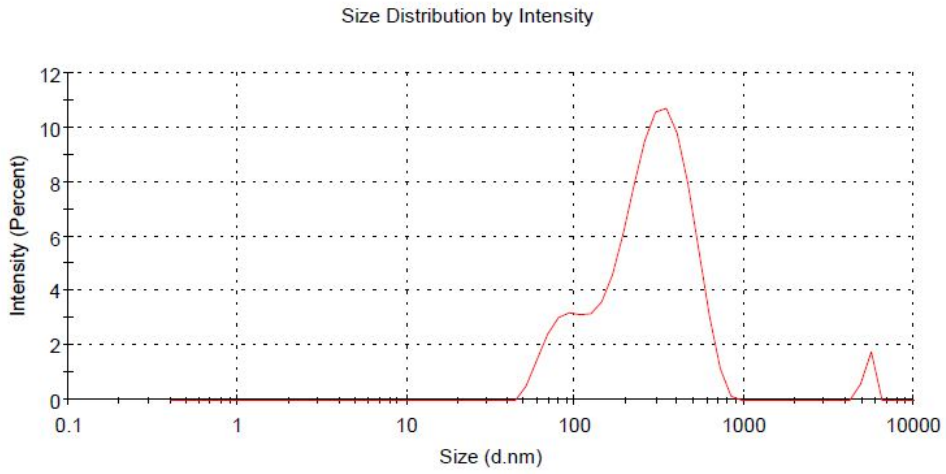

Figure 1. Particle size distribution of A-AgNPs. The average particles size was 243.6 ± 3.68 nm.

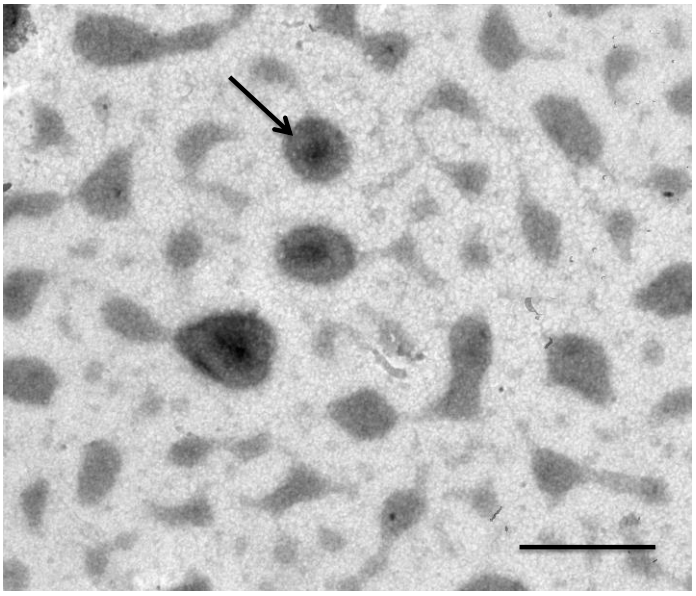

Figure 2. TEM image of *Azolla pinnata*-AgNPs, scale bar = 500 nm.

2. Molecular identification of isolated *A. hydrophila* isolate

Virulent gene detection in the samples of *C. gariepinus* showed that the isolated strain is positive for the hly gene, which was amplified at 592 bP (Fig. 3).

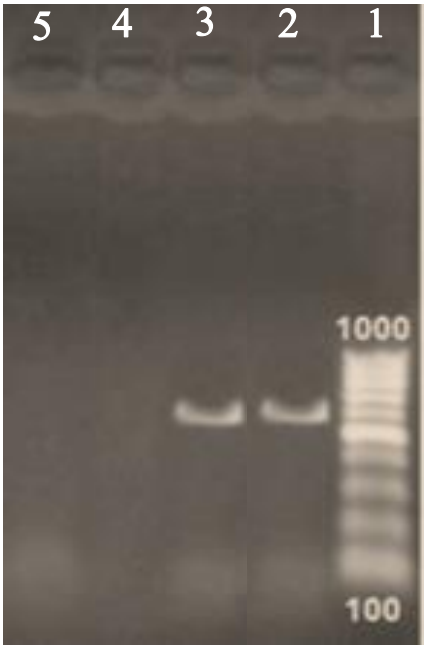

Figure 3. PCR product of *A. hydrophila* hly gene (592 bp). Lane (1): 100 bp DNA ladder, lanes (2 & 3), isolated strain and lanes 4 & 5 negative control.

3.Virulence and LD_{50} determination of *A. hydrophila* strain

The experimentally infected *C. gariepinus* mortality rate was noticed for 14 days subsequent to intraperitoneal administration of variable dosages of the pathogenic *A. hydrophila* isolate. According to (Fig. 4), the LD_{50} was 1.5×10^6 CFU/ml, and the mortalities happened in the first 10 days after microbial exposure.

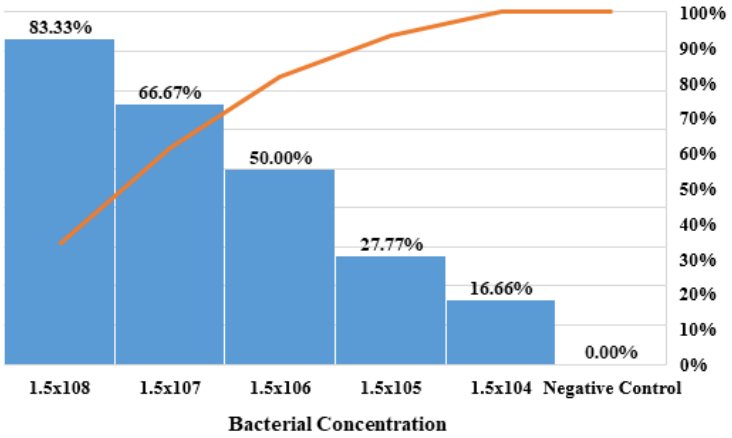

Figure 4. Mortality percentage and LD_{50} of virulent *A. hydrophila* isolated from *C. gariepinus* outbreak.

4. Prevention of *A. hydrophila* infection in *C. gariepinus* using AP-AgNPs

Group 1 supplemented with AP-AgNPs at 0.6 mg/kg diet had the highest relative percentage of survival (72.22%) following intraperitoneal injection with the pathogenic *A. hydrophila* strain. Group 2 had a lower concentration of AP-AgNPs at 0.3 mg/kg diet, with 44.44 percent RPS (Fig. 5). Group 3, that was fed a basic meal beforehand delivery an inoculation of the virulent *A. hydrophila* isolate, had a mortality of 83.33%. In contrast, the control negative group (G 4), which was fed a basic meal, displayed zero percent mortality following a physiological saline injection.

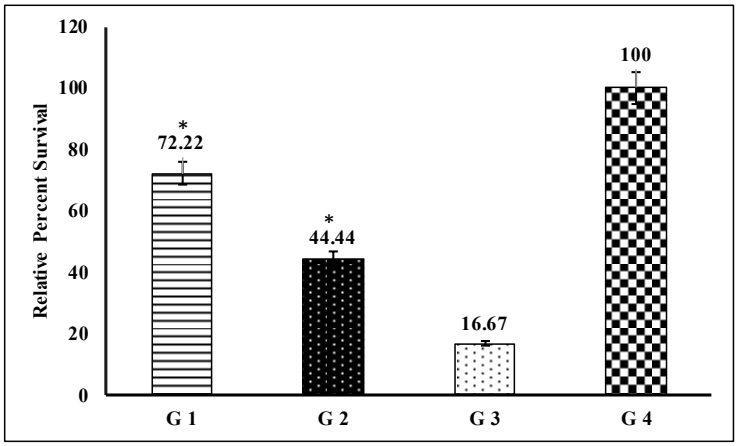

Figure 5. Survival rates post nurturing trial. G 1 and G 2 denote fish groups supplemented with AP-AgNPs at 0.6 and 0.3 mg/kg diet. G 3 and G 4 were supplemented with basic meal. All groups were inoculated with 1.5×10^8 CFU/mL of *A. hydrophila* except control negative, group 4 was inoculated with saline. * Indicates to noteworthy RPS $P \leq 0.05$.

5. Effects of AP-AgNPs on hepato-renal, protein profiles, stress and some immune indicators of *C. gariepinus* following *A. hydrophila* challenge

G1, G2, and the control negative group, represented by G 4, did not substantially differ in terms of alanine amino transferase, aspartate transferase, lactate dehydrogenase, or total bilirubin, as shown in Table 3. The greatest detected levels of these markers were found in G 3. Group 3 showed the largest increase, but G 1, G 2, and G 4 did not differ significantly in urea and creatinine, which are indicators of renal function. As seen in Table 4, there was no discernible difference between G 1 and G 2 in total proteins, albumin, or globulin. In the meantime, these factors were significantly reduced ($P < 0.05$) in G 3. At the level of cholesterol and triglycerides

G 3 had the highest values, followed by G 1 and G 2 (Table 4). None of the experimental groups showed a significant change in glucose levels. Regarding to lysozyme and complement activities, before challenge, the highest activities were recorded in G 1 that fed on AP-AgNPs 0.6 mg/kg feed while there was no significant difference among groups 2 that fed on AP-AgNPs 0.3 mg/kg, G 3 and G 4 that denote control groups, positive and negative regarding to lysozyme activity as shown in (Fig. 6. A & B). After challenge, the uppermost activities were recorded in G 1 pursued by G 2 at both lysozyme and complement levels (Fig. 6. C & D).

Table 3. Impact of AP-AgNPs on hepato-renal functions of *C. gariepinus* intraperitoneal infected with *A. hydrophila*.

Indicators	**Fish groups**			
	Gr 1	**Gr 2**	**Gr 3**	**Gr 4**
ALT (U/L)	30.38 [a] ±0.19	30.85 [a] ±0.85	45.58 [b] ±0.25	30.57 [a] ±0.23
AST (U/L)	43.64 [a] ±0.57	44.01 [a] ±0.65	70.46 [b] ±0.81	43.26 [a] ±1.53
LDH (U/L)	89.81 [a] ±0.66	90.62 [a] ±0.5	190.45 [b] ±0.69	90.35 [a] ±0.30
Total bilirubin (mg/dL)	0.52 [a] ±0.02	0.50 [a] ±0.01	1.02 [b] ±0.06	0.51 [a] ±0.02
Urea (mg/dL)	8.69 [a] ±0.38	8.38 [a] ±0.35	10.31 [b] ±0.33	8.49 [a] ±0.22
Creatinine (mg/dL)	0.40 [a] ±0.01	0.41 [a] ±0.03	1.93 [b] ±0.04	0.42 [a] ±0.02

Group 1 and group 2 fish were infected with *A. hydrophila* and were supplemented with AP-AgNPs 0.6 mg/kg and 0.3 mg / kg feed, respectively. Group 3 represents fish infected with *A. hydrophila* and was supplemented with plain diet. Group 4 symbolizes non infected fish and supplemented with plain diet. Values are expressed as mean ± standard error (±SE). At $p \leq 0.05$, values in the same line with different superscript letters differ significantly.

6. Specific antibody response

With the exception of group 4 (control negative) that recorded no specific antibody titres against *A. hydrophila* (< 2), the antibody titre of fish in other groups increased significantly ($P \leq 0.05$) after intraperitoneal challenge. G 1, supplemented by AP-AgNPs 0.6 mg/kg feed recorded the highest specific antibody titre (64). While G 2, supplemented with AP-AgNPs 0.3 mg/kg feed and G 3 (control positive) showed the same specific antibody titre of 32.

Table 4. Impact of AP-AgNPs on protein profile and stress indicators of challenged *C. gariepinus* with *A. hydrophila*.

Parameters	G 1	G 2	G 3	G 4
Total proteins (g/dL)	5.82[a] ± 0.11	5.79[a] ± 0.11	4.51[b] ± 0.03	6.82[c] ± 0.06
Albumin (g/dL)	3.88[a] ± 0.08	3.85[a] ± 0.07	3.01[b] ± 0.02	3.44[c] ± 0.03
Globulin (g/dL)	1.94[a] ± 0.03	1.93[a] ± 0.03	1.50[c] ± 0.01	3.38[b] ± 0.04
Cholesterol (mg/dL)	158.43[a] ± 1.02	159.28[a] ± 0.80	187.02[b] ± 2.13	150.93[c] ± 1.04
Triglyceride (mg/dL)	112.21[a] ± 0.81	111.96[a] ± 1.03	131.62[a] ± 1.52	97.02[b] ± 0.55
Glucose (mg/ dL)	49.24[a] ± 1.07	50.31[a] ± 0.71	52.45[a] ± 2.61	51.05[a] ± 0.91

Group 1 and group 2 fish were infected with *A. hydrophila* and were supplemented with AP-AgNPs 0.6 mg/L and 0.3 mg / kg feed, respectively. Group 3 represents fish infected with *A. hydrophila* and was supplemented with plain diet. Group 4 symbolizes non infected fish and supplemented with plain diet. Values are expressed as mean ± standard error (±SE). At $p \leq 0.05$, values in the same line with different superscript letters differ significantly.

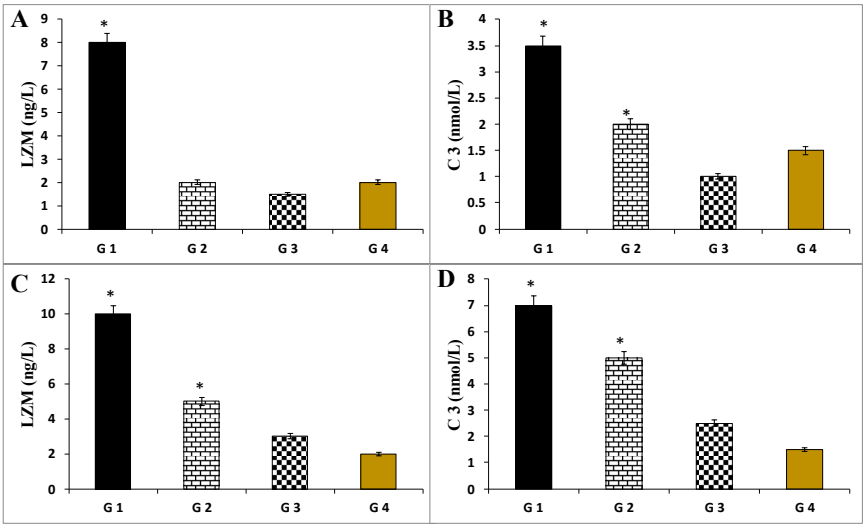

Figure 6. (A & B) represent lysozyme and serum complement C 3 activities before *A. hydrophila* challenge. (C & D) denote lysozyme and serum complement C 3 activities after *A. hydrophila* challenge. * Indicates to noteworthy RPS $P \leq 0.05$.

7. Transcriptional levels of some immune and anti-oxidant genes in response to AP-AgNPs in feed

Both the IL-1β and MHC-IA genes displayed increased expression levels at the immune gene level, especially after the *A. hydrophila* challenge. The IL-1β gene in G 1 that was fed 0.6 mg AP-AgNPs/kg of food had the greatest transcription level (Fold change 6) (Fig. 7). However, as shown in Figure 7, the fish group that was fed a basic diet and challenged with *A. hydrophila* showed the lowest up-regulated expression level for MHC-IA (Fold change 1.5). The same array was observed at the level of antioxidant-associated genes, GPX and SOD1, with more pronounced up-regulated transcription, especially for SOD1 (fold change 10), in the fish group that was fed 0.6 mg AP-AgNPs/kg diet, as shown in (Fig. 7).

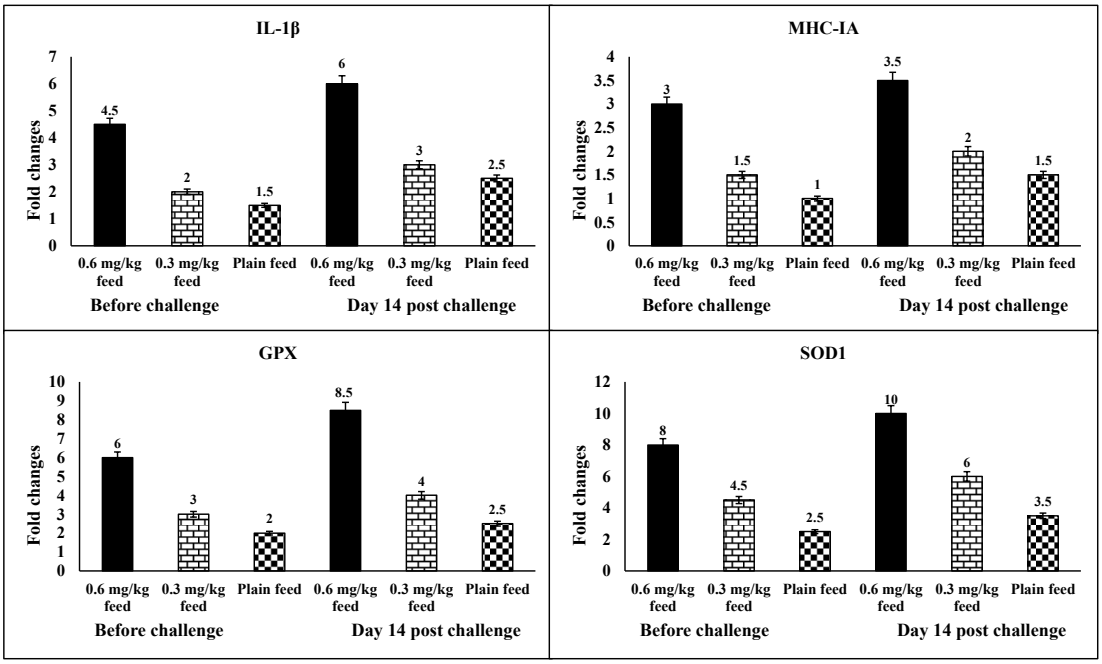

Figure 7. Noticeable surges in transcription up-regulated levels of IL-1β, MHC-IA, GPX and SOD1 genes in fish groups that supplemented with AP-AgNPs at 0.6 and 0.3 mg/kg diet particularly after *A. hydrophila* challenge.

4. Discussion

It seems that using nanomaterials as food additives to boost immune responses is the most effective way to prevent fish infections. Their unique properties come from their small size and highly specialized surface area. It has been observed that the incorporation of nanoparticles into fish diets increases the proportion of nutrients in fish food that pass through the fish's gut and into its system rather than just passing through its digestive system unchanged **(Eleraky *et al.*, 2016; Chris *et al.*, 2018)**. The assimilation of dietary minerals into fish tissue may be accelerated by their ability to penetrate cells more easily than minerals of a bigger size **(Sallam *et al.*, 2020)**. The general health and growth of fish may also be significantly impacted by the frequent addition of mineral elements at the nanoscale to their diets. Due to the use of toxic and dangerous chemical compounds, conventional synthetic methods that involved reducing silver ions with reducing agents and then surface-modifying the resulting particles using appropriate capping ligands and organic solvents raised concerns about the environment **(Korbekandi *et al.*, 2014)**. These restrictions encourage the development of novel green chemistry-based, ecologically acceptable methods for creating silver nanoparticles with the

appropriate shapes and sizes. For pharmaceutical, medicinal, and biomedical applications, it is crucial to produce metal and metal oxide nanoparticles using these environmentally friendly processes and nontoxic ingredients. The green biogenesis of silver nanoparticles is currently a major concern, and the roots, stems, fruits, and leaves of many plants, herbs, and spices have been employed for this purpose **(Velmurugan *et al.*, 2014)**. The leaves of plant *A. pinnata* were utilized in the current work to produce ovoid AP-AgNPs with an average particle size of 243.6 ± 3.68 nm through green biosynthesis (Fig. 1 & 2). Similarly, **Korbekandi *et al.* (2014)** produced relatively small spherical silver nanoparticles with a mean size of 6.5 nm using the entire *A. pinnata* plant.

At the level of pathogenic bacteria, hemolysins are among the several virulence factors that *A. hydrophila* produces and/or secretes, contributing to its multifactorial pathogenicity **(Beaz-Hidalgo *et al.*, 2015)**. In order to verify the pathogenicity of the isolated strain, the hly gene, which codes for the conserved portion of hemolysin, was employed in the current investigation (Fig. 3). The virulence of the isolated strain was also confirmed by the pathogenicity test that showed LD_{50} 1.5×10^{6} (Fig. 4). The increased survivability in the current study following the IP challenge using the isolated *A. hydrophila* strain could be explained by the silver nanoparticles' antibacterial properties **(Shaalan *et al.*, 2018)**. G1 and G2 showed higher survival rates, indicating that both concentrations (0.6 and 0.3 mg AP-AgNPs/kg feed) could be used to increase immunity in *C. gariepineus*. However, 0.6 mg/kg feed performed better (RPS 72.22%). Similarly, according to **Popoola *et al.* (2022)**, *Labeo rohita* showed 90% survival rates after receiving 15 μg of silver nanoparticles per kilogram of food for 56 days. **Jha *et al.* (2022)** reported that oral administration of 2 mg/kg *Avicennia marina* mangrove based-selenium nanoparticles followed by 0.2 mg/kg of *Avicennia marina* mangrove based-silver nanoparticles generated relative percent survival over 70 % in *Cyprinus carpio* against *A. hydrophila* infection. Furthermore, **Ibrahim *et al.* (2022)** recorded the mollifying effect with high survivability in *O. niloticus* exposed to silver nanoparticles synthetized by moringa for 7 days against *Saprolegnia* spp. challenge with survivability over 80%. These findings may be attributed to the microbicidal effect of bio-synthetized nanoparticles according to **Wrońska *et al.* (2023)** who reported the bactericidal impact of bio-synthetized silver nanoparticles against pathogenic E. coli at a concentration of 7 μg/mL.

Concerning to biochemical analyses, in contrast to negative control and AP-AgNPs supplemented groups, the infected and non-supplemented group (G 3) exhibited elevated liver function biomarkers linked to *A. hydrophila* infection, as evidenced by lower levels of total proteins, albumin, and globulin, as well as higher levels of renal indicators (urea and creatinine) and hepatic indicators (ALT, AST, LDH, and total bilirubin) (Table 3 & 4). The effects of *A. hydrophila* toxins may be responsible for elevated indicators of liver and kidney function **(Sellegounder *et al.*, 2018)**. AP-AgNPs supplementation of the *A. hydrophila* challenged fish might enhance the hepatic function indicators that may result in avoiding the disease development. AP-AgNPs might raise protein content, allowing organs to acquire endurance to microbial load and promote enzyme synthesis for detoxification **(Vineela and Reddy, 2015)**. Fish blood contains lipids that are changeable, such as lipoproteins like cholesterol and triglycerides, which are the body's main source of energy. Their quantities are used as moderately sensitive stress biomarkers **(Abarra *et al.*, 2017)**. Furthermore, blood glucose levels are a crucial physiological indicator of fish health **(Polakof *et al.*, 2012)**. *A. hydrophila* infection causes pathological stress in the present investigation, as evidenced by elevated triglyceride and cholesterol levels in the supplemented and infected group (G 3), whereas blood glucose levels do not significantly change (Table 4). The utilization of lipid from the liver and other organs for succeeding exploitation to deal with the microbial hassle and the host tissue injury caused by

the tissue inflammatory response may be the reason for the increase in triglyceride and cholesterol levels in G 3. The administration of 0.6 and 0.3 mg A-AgNPs /kg diet to G 1 and G 2 did not result in any modulation of these stress parameters (Table 4), suggesting that the obtained silver nanoparticles may alleviate the stress condition caused by the microbial infection. According to **Magnadottir (2006)**, fish's instinctive immunity is regarded as their primary defense mechanism and is a major component of their adaptive immune responses. Leucocytes secrete lysozyme, which is considered an essential mucolytic enzyme. It is found in various bodily fluids of fresh and marine fishes, including plasma, lymphoid tissue, and mucus, and its activity is an essential indicator of innate fish immunity **(Saurabh and Sahoo, 2008)**. The complement system plays a crucial role in warning the host about potential infections and eliminating them. Additionally, the development of an acquired immune response depends heavily on complement activation **(Boshra *et al.*, 2006)**. Likewise, immunoglobulin M (IgM) is a vital immunological component that is essential for protecting fish from many infections **(Yang *et al.*, 2020)**. In comparison to G 1 and G 2, which supplemented with 0.6 and 0.3 mg A-AgNPs/kg feed, the *A. hydrophila*-infected group G 3 supplemented with plain diet showed lower levels of immunological parameters, such as lysozyme, C3 (Fig. 6), and IgM. According to **Reyes-Becerril *et al.* (2011)**, fish leukocytes may have been able to identify the polysaccharides in the cell wall of *A. hydrophila*, which caused a strong response to tissue inflammation in its host and significantly suppressed the gene transcription levels associated with acquired immune response in fish by down-regulating the release of immunoglobulins and T-helper cell chemokine's. This may account for the immunosuppressive effect in G 3. **El-Houseiny *et al.* (2021)** found that *O. niloticus's* immunological function was changed by *A. hydrophila* infection, particularly in relation to the complement system and lysozyme level. The immune function may be modulated by adding silver nanoparticles to the *A. hydrophila*-infected groups. This might lead to AgNPs halting the evolution of the disease. likewise, *O. niloticus* and *Penaeus vannamei* have both been shown to exhibit the immune-modulatory impact of the silver nanoparticles **(Ochoa-Meza *et al.*, 2019; El-Houseiny *et al.*, 2021)**. Recognizing the generation of immune molecules and how they react to infections is essential to comprehending the basic mechanisms of immune responses and developing reliable tactics for improved administration in the aqua farming area. Related disease issues, such as infections caused by *A. hydrophila*, have caused a major setback for the fish farming industry in recent years **(Elgendy *et al.*, 2024)**. The current study demonstrates that diet augmentation with AP-AgNPs can effectively up-regulate the transcription of particular genes in *C. gariepineus*, in addition to enhancing immune response. After *C. gareipineus* was challenged with *A. hydrophila* in the current study, the anterior kidneys of the fish fed AP-AgNP-enriched diets showed higher levels of IL-1β, MHC-IA, GPX, and SODI than the negative control group that fed on plain diet. Associated with inflammation, a key protein that is necessary for drawing immune cells like neutrophils to the spot of infection is IL-1β, which could originate from endothelial, epithelial, smooth muscle cells and macrophages **(Swain *et al.*, 2012)**. Numerous biological processes, including angiogenesis, tissue remodeling, and cell proliferation, have been shown to be influenced by this chemokine **(Gauglitz *et al.*, 2008)**. In our investigation, *C. gariepineus* that was given AP-AgNP and affected by *A. hydrophila* exhibited elevated relative IL-1β expression in the head kidney (Fig. 7). Similarly, after receiving silver nanoparticles, *Labeo rohita* expresses IL-8, suggesting that it plays a major role in the early innate immune response **(Popoola *et al.*, 2022)**. In a similar pattern MHC-IA gene showed up-regulated expressed levels, particularly after bacterial challenge (Fig. 7), and this could be explained by the fact that the major histocompatibility complex molecules play a crucial role in triggering immune responses to invasive infections **(Grimholt, 2016)**. A direct contributor to the pathogenesis of infectious

illnesses, fish bacterial agents produce oxidative damage by the overproduction of reactive oxygen species (ROS), resulting in inhibition of enzymes associated with antioxidant activities and decline of non-enzymatic molecules with no relation to antioxidants **(Ibrahim *et al.*, 2022)**. SOD1 and GPX are among the group of genes that express the SOD and GPX that represent a class of metallo-enzymes. According to **Kohen and Nyska (2002)**, they serve as the initial line of defense against reactive oxygen species' harmful effects (ROS) and have a significant anti-oxidant role in aerobic pathogens. In the current study, mRNA transcriptional levels of renal antioxidant genes, SOD1 and GPX, showed higher up-regulation levels in AP-AgNP-supplemented groups compared to the infected, non-treated group in the head kidney of the challenged fish groups (Fig. 7). Similarly, oral administration of moringa-synthesised colloidal silver nanoparticles to Nile tilapia prevented Saprolegnia infection **(Ibrahim *et al.*, 2022)**. It is possible that the increased ant oxidative role in the group supplemented with AP-AgNPs is due to the antioxidant action of silver nanoparticles, which has been shown in earlier studies to mitigate all negative effects related to ROS generated by the infection **(El-Houseiny *et al.*, 2021; Ibrahim *et al.*, 2022)**. The increased capping agents that are adsorbed onto silver nanoparticles may be the cause of their antioxidant action **(Gomaa, 2017)**.

5. Conclusions

In summary, the results of this study showed that, without endangering the fish, a basic meal containing 0.6 mg/kg of *Azolla pinnata*-AgNPs enhance the intrinsic immune system and antioxidant capacity of *C. garipeineus*. According to the findings, silver nanoparticles could be a useful substitute for traditional pharmaceuticals and vaccines in the fight against harmful *A. hydrophila* infection in fish farming sector.

Author Contributions: Mohamed Sayed designed the experiments; maintained and performed the fish challenge experiments; Mohamed Sayed and Rehab M. Reda carried out the biochemical and gene expression analyses, wrote and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Institutional Review Board Statement: The Institutional Animal Care and Use Committee (IACUC) of Beni-Suef University in Egypt provided approval number (025-016) for the fish study protocol, which complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) standards.

Informed Consent Statement: Not applicable.
Conflicts of Interest: There are no conflicts among authors.

References

Abarra, S. T., Velasquez, S. F., Guzman, K. D. D., Felipe, J. L. F., Tayamen, M. M., & Ragaza, J. A. (2017). Replacement of fishmeal with processed meal from knife fish Chitala ornata in diets of juvenile Nile tilapia Oreochromis niloticus. *Aquaculture reports*, *5*, 76-83. https://doi.org/10.1016/j.aqrep.2017.01.001

Alishahi, A., Mirvaghefi, A., Tehrani, M. R., Farahmand, H., Koshio, S., Dorkoosh, F. A., & Elsabee, M. Z. (2011). Chitosan nanoparticle to carry vitamin C through the gastrointestinal tract and induce the non-specific immunity system of rainbow trout (Oncorhynchus mykiss). *Carbohydrate polymers*, *86*(1), 142-146. doi:10.1016/j.carbpol.2011.04.028.

Amoatey, P., & Baawain, M. S. (2019). Effects of pollution on freshwater aquatic organisms. *Water Environment Research*, *91*(10), 1272-1287. https://doi.org/10.1002/wer.1221

Anjana, V. N., Koshy, E. P., & Mathew, B. (2020). Facile synthesis of silver nanoparticles using Azolla caroliniana, their cytotoxicity, catalytic, optical and antibacterial activity. *Materials Today: Proceedings*, *25*, 163-168. https://doi.org/10.1016/j.matpr.2019.12.250

Barman, D., Nen, P., Mandal, S. C., & Kumar, V. (2013). Immunostimulants for aquaculture health management. *J. Mar. Sci. Res. Dev*, *3*(3), 134. https://doi.org/10.4172/2155-9910.1000134

Beaz-Hidalgo, R., Hossain, M. J., Liles, M. R., & Figueras, M. J. (2015). Strategies to avoid wrongly labelled genomes using as example the detected wrong taxonomic affiliation for Aeromonas genomes in the GenBank database. *PloS one*, *10*(1), e0115813. https://doi.org/10.1371/journal.pone.0115813

Bhushan, D., Shoran, S., Kumar, R., & Gupta, R. (2024). Plant biomass-based nanoparticles for remediation of contaminants from water ecosystems: Recent trends, challenges, and future perspectives. *Chemosphere*, *365*, 143340. https://doi.org/10.1016/j.chemosphere.2024.143340

Biller-Takahashi, J. D., Montassier, H. J., Takahashi, L. S., & Urbinati, E. C. (2014). Proposed method for agglutinating antibody titer analysis and its use as indicator of acquired immunity in pacu, Piaractus mesopotamicus. *Brazilian Journal of Biology*, *74*(1), 238-242. https://doi.org/10.1590/1519-6984.21312

Boshra, H., Li, J., & Sunyer, J. O. (2006). Recent advances on the complement system of teleost fish. *Fish & shellfish immunology*, *20*(2), 239-262. https://doi.org/10.1016/j.fsi.2005.04.004

Chris, U. O., Singh, N. B., & Agarwal, A. (2018). Nanoparticles as feed supplement on Growth behaviour of Cultured Catfish (Clarias gariepinus) fingerlings. *Materials Today: Proceedings*, *5*(3), 9076-9081. https://doi.org/10.1016/j.matpr.2017.10.023

Eleraky, W., Ibrahim, D., & Mahmoud, R. (2016). Effects of rare earth elements and exogenous multienzyme supplementation to plant protein enriched diet on growth. *Japanese Journal of Veterinary Research*, *64*(2), S73-S78. Supplement, http://hdl.handle.net/2115/62023

Elgendy, M. Y., Ali, S. E., Dayem, A. A., Khalil, R. H., Moustafa, M. M., & Abdelsalam, M. (2024). Alternative therapies recently applied in controlling farmed fish diseases: mechanisms, challenges, and prospects. *Aquaculture International*, *32*(7), 9017-9078. https://doi.org/10.1007/s10499-024-01603-3

El-Houseiny, W., Mansour, M. F., Mohamed, W. A., Al-Gabri, N. A., El-Sayed, A. A., Altohamy, D. E., & Ibrahim, R. E. (2021). Silver nanoparticles mitigate Aeromonas hydrophila-induced immune suppression, oxidative stress, and apoptotic and genotoxic effects in Oreochromis niloticus. *Aquaculture*, *535*, 736430. https://doi.org/10.1016/j.aquaculture.2021.736430

Gauglitz, G. G., Finnerty, C. C., Herndon, D. N., Mlcak, R. P., & Jeschke, M. G. (2008). Are serum cytokines early predictors for the outcome of burn patients with inhalation injuries who do not survive?. *Critical Care*, *12*(3), R81. https://doi.org/10.1186/ cc6932

Gomaa, E. Z. (2017). Antimicrobial, antioxidant and antitumor activities of silver nanoparticles synthesized by Allium cepa extract: A green approach. *Journal of Genetic Engineering and Biotechnology*, *15*(1), 49-57. https://doi.org/10.1016/j.jgeb.2016.12.002

Grimholt, U. (2016). MHC and evolution in teleosts. *Biology*, *5*(1), 6. https://doi.org/10.3390/biology5010006

Ibrahim, R. E., Elshopakey, G. E., El-Rahman, G. I. A., Ahmed, A. I., Altohamy, D. E., Zaglool, A. W., Younis, E. M., Abdelwarith, A. A., Davies, S. J., Al-Harthi, H. F., & Rahman, A. N. A. (2022). Palliative role of colloidal silver nanoparticles synthetized by moringa against Saprolegnia spp. infection in Nile Tilapia: Biochemical, immuno-antioxidant response, gene expression, and histopathological investigation. *Aquaculture Reports*, *26*, 101318. https://doi.org/10.1016/j.aqrep.2022.101318

Iravani, S., Korbekandi, H., Mirmohammadi, S. V., & Zolfaghari, B. (2014). Synthesis of silver nanoparticles: chemical, physical and biological methods. *Research in pharmaceutical sciences*, *9*(6), 385-406.

Islam, S., Riman, M. M., Mannan, S., Lawrence, M. L., & Abdelhamed, H. (2023). Characterisation and mobilisation of IncA/C plasmid-mediated antibiotic resistance in Edwardsiella ictaluri. *Journal of Global Antimicrobial Resistance*, *33*, 177-185. https://doi.org/10.1016/j.jgar.2023.03.005

Janda, J. M., & Abbott, S. L. (2010). The genus Aeromonas: taxonomy, pathogenicity, and infection. *Clinical microbiology reviews*, *23*(1), 35-73. https://doi.org/10.1128/CMR.00039-09

Jha, N., Annamalai, A., Essakiraj, P., Balamurugan, R., Lakra, A. K., Tilwani, Y. M., & Arul, V. (2022). Effects of polysaccharide-based silver and selenium nanoparticles on growth performance, biochemical parameters, and immune response of Cyprinus carpio. *Fish and Shellfish Immunology Reports*, *3*, 100062. https://doi.org/10.1016/j.fsirep.2022.100062

Joseph, S., & Mathew, B. (2015). Facile synthesis of silver nanoparticles and their application in dye degradation. *Materials Science and Engineering: B*, *195*, 90-97.

Khan, S. K., Dutta, J., Ahmad, I., & Rather, M. A. (2024). Nanotechnology in aquaculture: Transforming the future of food security. *Food Chemistry: X*, *24*, 101974. https://doi.org/10.1016/j.fochx.2024.101974

Khan, S., Rukayadi, Y., Jaafar, A. H., & Ahmad, N. H. (2023). Antibacterial potential of silver nanoparticles (SP-AgNPs) synthesized from Syzygium polyanthum (Wight) Walp. against selected foodborne pathogens. *Heliyon*, *9*(12), e22771. https://doi.org/10.1016/j.heliyon.2023.e22771

Khursheed, S., Dutta, J., Ahmad, I., Rather, M. A., Badroo, I. A., Bhat, T. A., Ahmad, I., Amin, A., Shah, A., Qadri, T., & Habib, H. (2023). Biogenic silver nanoparticles: Synthesis, applications and challenges in food sector with special emphasis on aquaculture. *Food Chemistry: X*, *20*, 101051. https://doi.org/10.1016/j.fochx.2023.101051

Kohen, R., & Nyska, A. (2002). Invited review: oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicologic pathology*, *30*(6), 620-650. https://doi.org/10.1080/01926230290166724

Korbekandi, H., Chitsazi, M. R., Asghari, G., Najafi, R. B., Badii, A., & Iravani, S. (2014). Green biosynthesis of silver nanoparticles using Azolla pinnata whole plant hydroalcoholic extract. *Green Processing and Synthesis*, *3*(5), 365-373. https://doi.org/10.1515/gps-2014-0042

Kordon, A. O., Abdelhamed, H., Ahmed, H., Baumgartner, W., Karsi, A., & Pinchuk, L. M. (2019). Assessment of the live attenuated and wild-type Edwardsiella ictaluri-induced immune gene expression and langerhans-like cell profiles in the immune-related organs of catfish. *Frontiers in immunology*, *10*, 392. https://doi.org/10.3389/fimmu.2019.00392

Korsa, G., Alemu, D., & Ayele, A. (2024). Azolla plant production and their potential applications. *International Journal of Agronomy*, *2024*(1), 1716440. https://doi.org/10.1155/2024/1716440

Kumari, S. C., Dhand, V., & Padma, P. N. (2021). Green synthesis of metallic nanoparticles: a review. In: R.P. Kumar & B. Bharathiraja (Eds.), *Nanomaterials*, Academic Press, pp. 259–281.

Luis, A. I. S., Campos, E. V. R., de Oliveira, J. L., & Fraceto, L. F. (2019). Trends in aquaculture sciences: from now to use of nanotechnology for disease control. *Reviews in Aquaculture*, *11*(1), 119-132. https://doi://dx.doi. org/10.1111/raq.12229

Lumpkin, T. A., & Plucknett, D. L. (1980). Azolla: botany, physiology, and use as a green manure. *Economic Botany*, *34*(2), 111-153. https://doi.org/10.1007/bf02858627

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish & shellfish immunology*, *20*(2), 137-151. https://doi.org/10.1016/j.fsi.2004.09.006

Mahboub, H. H., Khedr, M. H., Elshopakey, G. E., Shakweer, M. S., Mohamed, D. I., Ismail, T. A., Ismail, S. H., & Rahman, A. N. A. (2021). Impact of silver nanoparticles exposure on neuro-behavior, hematology, and oxidative stress biomarkers of African catfish (Clarias gariepinus). *Aquaculture*, *544*, 737082. https://doi.org/10.1016/j.aquaculture.2021.737082

Nasrullah, H., Yanti, D. H., Faridah, N., Hardiantho, D., Nababan, Y. I., Sukenda, S., & Alimuddin, A. (2021). Early immune gene development and expression in African catfish Clarias gariepinus after challenged with Aeromonas hydrophila. *Aquaculture International*, *29*(2), 595-607. https://doi.org/10.1007/s10499-021-00645-1

Ochoa-Meza, A. R., Álvarez-Sánchez, A. R., Romo-Quiñonez, C. R., Barraza, A., Magallón-Barajas, F. J., Chávez-Sánchez, A., García-Ramos, J. C., Toledano-Magaña, Y., Bogdanchikova, N., Pestryakov, A., & Mejía-Ruiz, C. H. (2019). Silver nanoparticles enhance survival of white spot syndrome virus infected Penaeus vannamei shrimps by activation of its immunological system. *Fish & Shellfish Immunology*, *84*, 1083-1089. https://doi.org/10.1016/j.fsi.2018.10.007

Du Sert, N. P., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., . . . Würbel, H. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLoS biology*, *18*(7), e3000410. https://doi.org/10.1371/journal.pbio.3000410

Polakof, S., Panserat, S., Soengas, J. L., & Moon, T. W. (2012). Glucose metabolism in fish: a review. *Journal of Comparative Physiology B*, *182*(8), 1015-1045. https://doi.org/10.1007/s00360-012- 0658-7

Popoola, O. M., Behera, B. K., & Kumar, V. (2023). Dietary silver nanoparticles as immunostimulant on rohu (Labeo rohita): Effects on the growth, cellular ultrastructure, immune-gene expression, and survival against Aeromonas hydrophila. *Fish and shellfish immunology Reports*, *4*, 100080. https://doi.org/10.1016/j.fsirep.2022.100080

Reed, L., & Muench, H. (1938). A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS12. *American Journal of Epidemiology*, *27*(3), 493–497. https://doi.org/10.1093/oxfordjournals.aje.a118408

Reyes-Becerril, M., López-Medina, T., Ascencio-Valle, F., & Esteban, M. Á. (2011). Immune response of gilthead seabream (Sparus aurata) following experimental infection with Aeromonas hydrophila. *Fish & Shellfish Immunology*, *31*(4), 564-570. https://doi.org/10.1016/j.fsi.2011.07.006

Roubach, R., Gomes, L. C., Leão Fonseca, F. A., & Val, A. L. (2005). Eugenol as an efficacious anaesthetic for tambaqui, Colossoma macropomum (Cuvier). *Aquaculture Research*, *36*(11), 1056-1061. https://doi:10.1111/j.1365-2109.2005.01319.x

Rozi, N., Rahayu, K., & Daruti, D. N. (2018). Detection and analysis of hemolysin genes inAeromonas hydrophilaisolated from Gouramy (Osphronemus gouramy) by polymerase chain reaction (PCR). *IOP Conference Series Earth and Environmental Science*, *137*, 012001. https://doi.org/10.1088/1755-1315/137/1/012001

Sallam, A. E., Mansour, A. T., Alsaqufi, A. S., Salem, M. E. S., & El-Feky, M. M. (2020). Growth performance, anti-oxidative status, innate immunity, and ammonia stress resistance of Siganus rivulatus fed diet supplemented with zinc and zinc nanoparticles. *Aquaculture Reports*, *18*, 100410. https://doi.org/10.1016/j. aqrep.2020.100410

Saurabh, S., & Sahoo, P. K. (2008). Lysozyme: an important defence molecule of fish innate immune system. *Aquaculture research*, *39*(3), 223-239. https://doi.org/10.1111/j.1365-2109.2007.01883.x

Sellegounder, D., Gupta, Y. R., Murugananthkumar, R., & Senthilkumaran, B. (2018). Enterotoxic effects of Aeromonas hydrophila infection in the catfish, Clarias gariepinus: Biochemical, histological and proteome analyses. *Veterinary immunology and immunopathology*, *204*, 1-10. https://doi.org/10.1016/j.vetimm.2018.08.008

Shaalan, M., El-Mahdy, M., Theiner, S., Dinhopl, N., El-Matbouli, M., & Saleh, M. (2018). Silver nanoparticles: Their role as antibacterial agent against Aeromonas salmonicida subsp. salmonicida in rainbow trout (Oncorhynchus mykiss). *Research in veterinary science*, *119*, 196-204. https://doi.org/10.1016/j.rvsc.2018.06.019

Sudheesh, P. S., Al-Ghabshi, A., Al-Mazrooei, N., & Al-Habsi, S. (2012). Comparative pathogenomics of bacteria causing infectious diseases in fish. *International Journal of Evolutionary Biology*, *2012*, 1–16. https://doi.org/10.1155/2012/457264

Swain, B., Basu, M., & Samanta, M. (2012). Molecular cloning and characterization of nucleotide binding and oligomerization domain-1 (NOD1) receptor in the Indian Major Carp, rohu (Labeo rohita), and analysis of its inductive expression and down-stream signalling molecules following ligands exposure and Gram-negative bacterial infections. *Fish & Shellfish Immunology*, *32*(5), 899-908. https://doi. org/10.1016/j.fsi.2012.02.018

Swaleh, S. B., Banday, U. Z., Asadi, M., & Usmani, N. (2020). Biochemical profile and gene expression of Clarias gariepinus as a signature of heavy metal stress. *Environmental Pollution*, *264*, 114693. https://doi.org/10.1016/j.envpol.2020.114693

Velmurugan, P., Anbalagan, K., Manosathyadevan, M., Lee, K., Cho, M., Lee, S., Park, J., Oh, S., Bang, K., & Oh, B. (2014). Green synthesis of silver and gold nanoparticles using Zingiber officinale root extract and antibacterial activity of silver nanoparticles against food pathogens. *Bioprocess and biosystems engineering*, *37*(10), 1935-1943. https://doi.org/10.1007/s00449-014-1169-6

Vineela, D., & Reddy, S. J. (2015). Effect of Silver Nanoparticles on Haematological and Protein Metabolic Indices of Carp Fish, Catla catla, treated with Lihocin and A. veronii. *International Journal of Pharmacy & Life Sciences*, *6*(4), 23–35.

Wang, W., Sun, J., Liu, C., & Xue, Z. (2017). Application of immunostimulants in aquaculture: current knowledge and future perspectives. *Aquaculture Research*, *48*(1), 1-23.

Wrońska, N., Płaczkowska, S., Niedziałkowska, K., & Lisowska, K. (2023). The Synergistic Effect of Biosynthesized Silver Nano-particles and Phytocompound as a Novel Approach to the Elimination of Pathogens. *Molecules (Basel, Switzerland)*, *28*(23), 7921. https://doi.org/10.3390/molecules28237921

Yang, S., Chen, W., He, F., Fu, S., Jin, Z., Zheng, C., Zhang, X., Ye, Z., Jin, H., Qian, S., Sun, Y., Huang, M., Lv, Z., & Fei, H. (2020). Comparison of the roles of IgM in systemic and mucosal immunity via tissue distribution analysis in largemouth bass (Micropterus salmoides). *Aquaculture*, *527*, 735488. https://doi.org/10.1016/j.aquaculture.2020.735488

Zhu, Y., Li, W., Ahmed, W., Mahmood, M., Ali, H. M., Rizwan, M., Bundschuh, J., Akmal, M., & Mehmood, S. (2025). Study on application of green-synthesized ZnO and Si nanoparticles in enhancing aquaculture sediment quality. *Chemical and Biological Technologies in Agriculture*, *12*(1), 6. https://doi.org/10.1186/s40538-024-00716-4