Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.243

RESEARCH ARTICLE

The Enhancing Effect of Bacterial Zinc Nanoparticles on Performance, Immune Response, and Microbial Load of Nile Tilapia (*Oreochromis niloticus*) by Reducing the Infection by *Trichodina heterodentata*

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ARTICLE HISTORY (24-396)

Received: July 14, 2024
Revised: August7, 2024
Accepted: August 15, 2024
Published online: September 03, 2024

Key words:
Bacterial Zinc nanoparticles
Bacillus subtilis
Antiparasitic
Antioxidant
Immunity
Trichodina
Nile Tilapia
Performance

A B S T R A C T

Trichodina species can cause extensive fish mortality in an aquaculture system, and extensive use of commercial drugs affects the environment and health. The bacterial zinc nanoparticles (BZN) synthesized using *Bacillus subtilis* MS20 could be a suitable alternative to antiparasitic drugs. This work evaluates the antiparasitic properties of BZN against *Trichodina heterodentata* in Nile tilapia (*Oreochromis niloticus*). Furthermore, it explores BZN's effects on fish health, including growth performance, immunity, and microbial load. The BZN were 45nm in size and negatively charged at -23.6 meV. A total of 180 fish were divided into six groups. G1-G4 were non-*trichodina*-infected groups fed a diet supplemented with 0, 100, 200, and 500 mg/kg of BZN, the G5 was *Trichodina*-infected fish, and G6 was *Trichodina*-infected fish and treated with BZN (500 mg/kg). Fish were carefully observed for clinical alterations and mortalities. Blood and tissue samples were collected from different groups. Survival rates were 100% in the groups (G1-G4), 56.67% (G5), and 88 % (G6). The *Trichodina*-challenged group showed bad performance, especially in the survival rate (56 %) and body weight gain, lower erythrogram (Hb, RBCs, however, higher PCV%, which may indicate the inflammation in fish tissue), significant hypoglycemia, higher ALP activity and ammonia but lower protein content and significant increases were observed in serum amyloid A (SAA) and interleukin-6 (IL-6) values. Applying BZN 500 mg/kg to the challenged group maintains the parameters to control levels and alleviates the effect of *Trichodina* species infection. The histopathology of liver tissues supported the blood biochemistry results. The *Trichodina*-challenged fish showed extensive vacuolar degeneration of hepatocytes and massive necrosis of the pancreas hyperplastic proliferation of the epithelial lining of the secondary lamellae; meanwhile, BZN-treated groups showed normal liver and gill tissue structure. The BZN treatment significantly reduced the total bacterial count by 31-45% and *Aeromonas* count by 30-44%. Finally, it is concluded that using BZN 500 mg/kg significantly mitigates the *Trichodina heterodentata* infection in Nile Tilapia (*Oreochromis niloticus*) and enhances fish health.

To Cite This Article: Alsulami MN, El-Saadony MT, 2024. The Enhancing Effect of Bacterial Zinc Nanoparticles on Performance, Immune Response, and Microbial Load of Nile Tilapia (*Oreochromis niloticus*) by Reducing the Infection by *Trichodina heterodentata.* Pak Vet J, 44(3): 599-610. http://dx.doi.org/10.29261/pakvetj/2024.243

INTRODUCTION

Aquaculture is an important source of animal protein and income in developing societies (Munguti *et al.*, 2022). Tilapia aquaculture has expanded to encompass various regions, including Asia, Africa, and the Americas, commencing in the 1990s (El-Sayed and Fitzsimmons, 2023). Nile tilapia production witnessed a substantial increase in 2019, culminating in global output of 4.6 million tonnes. This development signified a transition from subsistence agriculture to a commercial enterprise, thereby facilitating the international trade of tilapia-related commodities. China, Indonesia, and Egypt emerged as the predominant producers, contributing 1.6, 1.3, and 1.1 million tonnes, respectively (Haenen *et al*., 2023). Notwithstanding the proliferation of tilapia

aquaculture, the emergence of parasitic diseases poses a significant risk to the long-term viability of this food production system (Xiong *et al.,* 2023). The presence of abundant nutrients, high water temperatures, and heavy fish populations in Tilapia farms creates ideal circumstances for the proliferation of parasites, including the emergence of highly pathogenic parasites that have the potential to cause zoonotic diseases (Haenen *et al*., 2023; Shinn *et al*., 2023).

Trichodina heterodentata are ciliated protozoan parasites that frequently infest the skin and gills of fish. Typically, these parasites only provide minor issues for fish when they are only mildly infested. Nevertheless, *Trichodina heterodentata* can potentially induce significant pathological alterations and fatalities in fish extensively infested with parasites. *Trichodina* species may easily infect small fish and fry, making them vulnerable to parasitism (Aly *et al.,* 2020). *Trichodina* species irritate by consuming the epithelial layer of cells that protect fish's skin and gills. Excessive parasitic infestation of *Trichodina* species on fish can promptly result in scratches, lesions, and ulcers, which in turn facilitate the development of secondary bacterial infections in the affected area (Abu-Elala *et al.,* 2021).

The parasitic infections cause considerable mortality between 30-100 % in fish farms. Anshary (2021a) observed that *Trichodina* sp., produced epidermal injuries, leading to bacterial infections. Unfortunately, medications were ineffective in controlling these infections. The use of formalin to control the parasite resulted in a reduction in overall mortality (Anshary, 2021b). Nanotechnology possesses substantial potential to augment aquaculture by leveraging novel nanoparticles, a concept referred to as nano-aquaculture (Kiran *et al*., 2022; Faiz *et al*., 2024).

Using nanoparticles in aquaculture has the potential to promote fish yield, mitigate disease transmission, and optimise water and wastewater treatment. Nanoparticles operate mechanistically to enhance the uptake of nutrients by the intestinal epithelium of fish, consequently diminishing the quantity of unabsorbed feed that would otherwise be eliminated (da Silva *et al*., 2020).

Furthermore, metal nanoparticles found in aquafeeds may be readily absorbed by cells to a greater extent than their bigger counterparts, therefore enhancing growth performance, fish health, and immunological response (Ghazi *et al*., 2021). Previous work has shown that metal nanoparticles exhibit very effective antibacterial properties in laboratory settings (Balderrama-González *et al*., 2021).

Specifically, zinc oxide nanoparticles (ZnO-NPs) have outstanding optical, semiconducting, and piezoelectric properties (Bhadwal *et al*., 2023), making them very useful in several fields, including healthcare. Furthermore, zinc oxide nanoparticles (ZnO-NPs) have several bacterial applications, such as in vitro antibacterial, antifungal, antioxidant, anticancer, and antiparasitic properties (Bondareva *et al*., 2020; Saadatmand *et al*., 2021; Abdelghany *et al*., 2023).

No studies highlighted the *in vivo* efficacy of zinc nanoparticles on the parasites (*Trichodina heterodentata*) of Nile Tilapia; therefore, this work investigates the *in vitro* and *in vivo* antiparasitic activity of bacterial zinc nanoparticles (BZN) and then evaluates its effect on immune response and growth performance of *Trichodina*-infected Nile Tilapia (*Oreochromis niloticus*).

MATERIALS AND METHODS

Isolating and Identifying the Zinc tolerant bacteria: The *Bacillus subtilis* MS20 synthesized bacterial zinc nanoparticles (BZN). This isolate was isolated from the soil rhizosphere. Soil samples were collected and mixed. Serial dilutions (ranging from 10^{-1} to 10^{-7}) using peptone buffer were prepared from the collected soil samples. Subsequently, each dilution was evenly placed on Luria-Bertani (LB) petri plates supplemented with varying concentrations of zinc nitrate [$Zn(NO_3)_2 \cdot 6H_2O$] (1, 2, 3, 4, and 5 ppm). The petri plates were incubated for two days at a temperature of 30°C. The colonies with the highest concentration of zinc nitrate (5 ppm) were selected and purified, then identified using morphological, biochemical, and molecular techniques (Desoky *et al.,* 2020; Saad *et al.,* 2022).

The morphological and biochemical tests were conducted to identify the zinc-producing isolate based on Bergey's Manual (El-Saadony *et al.,* 2021c). Finally, the identification was confirmed by MALDI-TOF spectroscopy.

Synthesis and characterization of BZN: A 100µL of the *B. subtilis* MS20 isolate was homogenized in 100mL of LB broth and incubated in a shaking incubator (250rpm) at 30°C for two days. Next, the flask was centrifuged (8000rpm, 20 min), the pellets were discarded, and the supernatant was obtained. Then, 20mL of supernatant was added to 80 ml of zinc nitrate (5ppm). The mixture was incubated in a shaking incubator (30°C, 250rpm for five days). Following incubation, the flask color transformed from colorless to white. This indicates that the *B. subtilis* MS20 isolate transformed the zinc nitrate into zinc nanoparticles (El-Saadony *et al.,* 2021a, b; Aioub *et al*., 2022).

The BZN produced by *Bacillus subtilis* MS20 was characterized using four advanced instruments. UV determined the optical properties of BZN. UV-VIS-spectroscopy examination utilized a LaxcoTMdual-beam spectrophotometer (Los Angeles, CA, USA) between 200 and 700 nm. A transmission electron microscope (JEOL, 1010, Tokyo, Japan) was used to determine the size and form of BZN. A Nano "Z2Malvern (Malvern Hills, Worcester, UK) was utilized for the DLS analysis. The net surface charge of the BZN was measured by the zeta potential (Malvern Hills, Worcester, UK), ensuring the stability of the generated nanoparticles; meanwhile, the average size was determined by a zeta sizer (Malvern Hills, Worcester, UK) (Alowaiesh *et al.,* 2023).

Biological activity of BZN

The Antioxidant activity: The scavenging ability was determined: 1 mL BZN concentrations (100, 200, and 500 µg/mL) were mixed with 3mL (2,2-Diphenyl-1-picrylhydrazyl) DPPH solution and incubated in the dark for 30min (Saad *et al.,* 2015). The % antioxidant activity was calculated in the equation (Abd Elkader *et al.,* 2022).

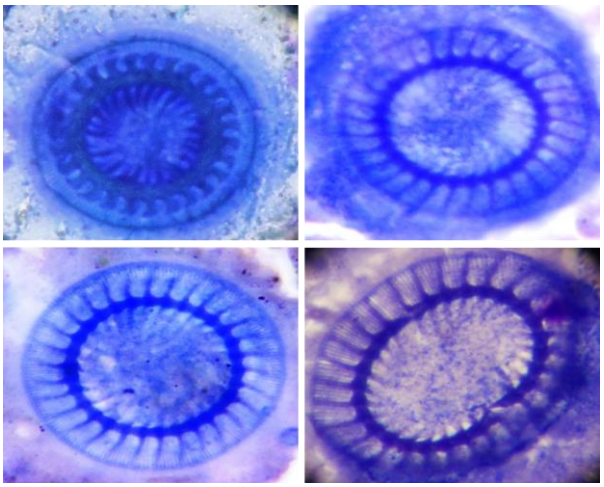
% RSA=(A control-A sample)/(A control) x100

RSA, radical scavenging activity; A, absorbance

The linear plot of %inhibition versus concentration was analyzed. Y= a+bx, where x is the concentration of the measured substance, and y is the % inhibition. Meanwhile, the IC_{50} value was determined as the x value of this equation when y was equal to 50% (Jumina *et al.*, 2019).

Antimicrobial activity: Different concentrations of BZN (100, 200, and 500 µg/mL) and 6 mm paper discs were prepared. The discs were saturated in the BZN concentrations. The saturated discs were placed on the Mueller Hinton agar or potato dextrose agar (PDA) petri plates inoculated with tested pathogenic bacteria (*Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 6538, *Aeromonas hydrophila* ATCC 7966, *Pseudomonas aeruginosa* ATCC 9027) and fungi (*Candida albicans* ATCC 14053, *Candida gelberta* ATCC 2001). The petri plates were incubated on the optimum conditions for tested pathogens, and the antimicrobial activity was expressed as inhibition zones around the discs (mm) (Alsubhi *et al.*, 2022).

Antiparasitic activity of BZN: *Trichodina heterodentata* (1×10^7 parasites/ml) (Figure 1) were cultured in 96-well plates; each well was supplemented with BZN (100, 200, and 500 µg/mL) and then incubated for 24h at 28°C. After that, the wells were supplemented with resazurin for the colorimetric test with parasites (de Freitas Oliveira *et al.*, 2021). The data was graphed as a % of parasite mortality. The selective index was determined by calculating the IC_{50} values of cell lines exposed to BZN against *Trichodina*.



Fig, 1: Microscopic image of *Trichodina heterodentata* in Nile tilapia (*Oreochromis niloticus*).

Experimental design: A group of 180 healthy (the fish was judged healthy from its overall normal morphology, feeding behavior, active breathing, active movement, brightness, and clear eyes) adult Nile tilapia (*Oreochromis niloticus*) fish with an initial weight (88g). Fish were acclimated in glass aquaria containing chlorinated water for two weeks. Water parameters were checked weekly. The quality of water was monitored and stabilized; the sodium, potassium, magnesium, and calcium levels in the water were adjusted to 0.4, 0.05, 0.6, and 0.8 mmol/l, respectively. At a temperature of 25.4±0.3°C, dissolved oxygen level of 7.30±0.13 mg/l, a pH of 7.90±0.06, a total hardness level of 132±4.4 mg $CaCO_3$/L, ammonia (NH_3) from 0.03 to 0.038 mg/l, total salinity of 23.41ppt, total ammonia nitrogen 0.004±0.001g/l, and a photoperiod regime (12:12h light: dark). During acclimatization and experimentation, all groups were fed on a basal diet (20% soybean meal, corn 23.0%, fish meal15.0%, alfalfa hay 14.0%, wheat bran 13.0%, corn gluten meal 11.5%, sunflower oil 1, 1% vitamin mixture, 0.5% mineral mixture, and 1% carboxymethyl cellulose).

The approximate analysis of the diet was 30.32% crude protein, 3.53% crude fibers, 1.79% lysine,0.74% methionine, 0.97% calcium, and 0.86% available phosphorus, with a 9.91% energy expenditure, and a total of 2,905.93 Kcal/Kg of digestible energy.

A total of 180 fish with an average weight of 88±20 g were randomly divided into six groups with three replicates each as follows:

G1: The non-*Trichodina*-infected group was fed a basal diet without BZN supplementation and served as a control group.

G2-G4: The second, third, and fourth groups were non-*Trichodina*-infected and fed basal diet supplemented with 100, 200, and 500 mg BZN powder/kg.

G5: *Trichodina*-challenged fish, and fed basal diet.

G6: *Trichodina*-challenged fish and fed basal diet supplemented with BZN 500 mg/kg.

The experimental duration was ten weeks (2 weeks for acclimatization and eight weeks for the *in vivo* experiment).

To challenge fish with *Trichodina*, fish in G5 and G6 were immersed in buckets containing 5 Liter water with *Trichodina* for 15 min, then the infected fish were placed in aquaria.

Growth performance parameters: To assess the impact of ZnO-NPs on growth performance, the weight of fish was recorded at the beginning and end of the experimental period. The growth parameters, i.e., final body weight (FBW), weight gain (WG), specific growth rate (SGR), and feed intake (FI), were calculated using the method of Kishawy *et al.* (2020). Throughout the experimental period, the fish were meticulously monitored for any deviations from normal behavior and clinical alterations after the *Trichodina* challenge. Moreover, the mortality/survival rate % in each group was recorded.

Blood sampling: To reduce the handling stress, the fish within the containers were lightly anesthetized with sodium bicarbonate-buffered tricaine methane-sulfate (MS-222 at 0.1 gL^{-1}) for 2–3 min until the loss of coordination was visible (Topic Popovic *et al.*, 2012). Afterward, the blood was collected by puncture of the caudal tail vessels, using a 23-gauge needle (Dispovan, A896) attached to a 4mL syringe (HMD, India). A part of the blood sample was transferred into 2 ml heparin-coated vials (Vactube Bio. Ltd. India) for hematological analysis and the assessment of blood cell morphology. The remaining blood was poured into duplicates of 1.5mL serum separating Eppendorf tubes with no anticoagulant for performing the serum biochemistry assays.

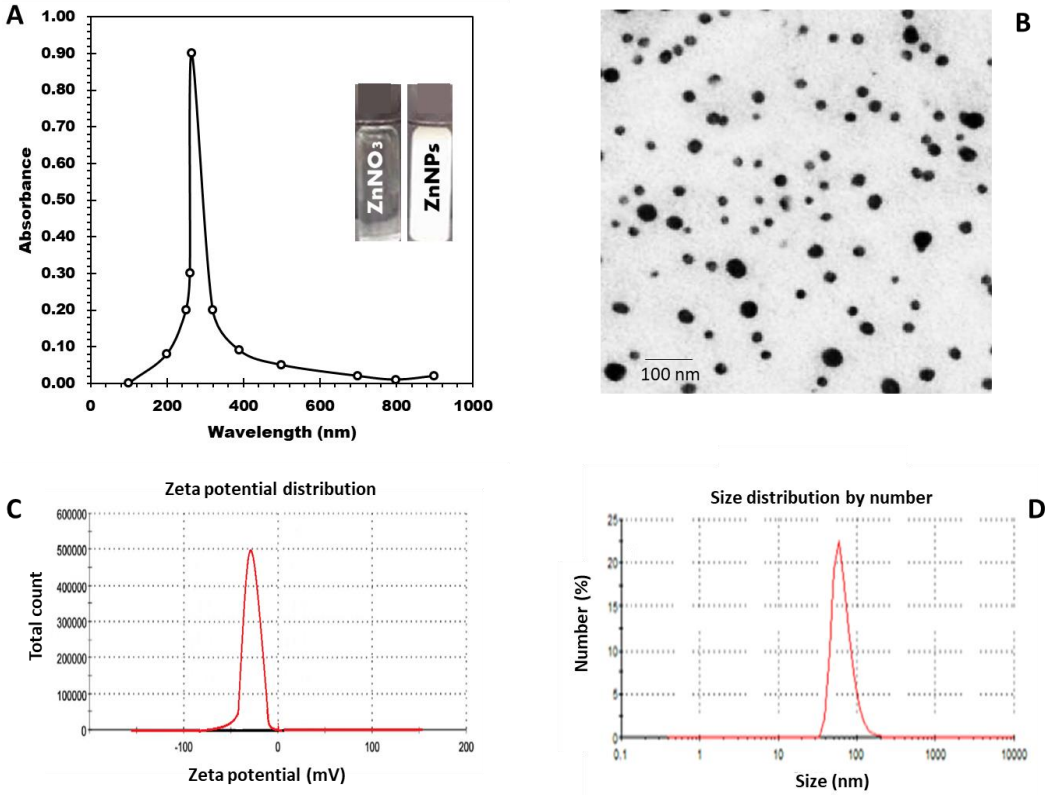


Fig. 2: Characterization of bacterial zinc nanoparticles fabricated by *Bacillus subtilis* MS20 (A), UV absorbance at 285nm, (B) average size of 30-74nm, (C) negative charge of -23.6mV, (D) Size of zinc nanoparticles of 45nm.

Hematological analysis: Blood specimens were obtained from various experimental groups. The blood samples were collected from the caudal vein of fish, utilizing EDTA anticoagulant, with a sample size of five fish per group. These specimens were subsequently employed for the analysis of hematological parameters, encompassing hemoglobin concentration (Hb), packed cell volume (PCV), erythrocyte count (EC), total leucocyte count (TLC), and differential leucocyte count, in accordance with the methodologies outlined by Svobodova and Vykusová (1991) and Feldman *et al*. (2000).

Biochemical analysis: Serum total proteins, albumin, and creatinine, were quantified in accordance with established protocols (Saad *et al*., 2015). The levels of serum total proteins and albumin were assessed utilizing a commercially available kit from Thermofisher Scientific, USA. The globulin concentration was calculated by subtracting the albumin value from the serum total protein value. Creatinine levels were determined following the methodology outlined by Zhou *et al*. (2024). Glucose concentrations were measured in accordance with the protocol described by Bartoňková *et al*. (2017). Furthermore, alkaline phosphatase and CK activities were determined as per the methodology described by Baldissera and Baldisserotto, (2023). The concentration of ammonia was measured following the protocol outlined by Tietze *et al*. (1983) and Shokr (2015).

Immunological parameters: The serum lysozyme activity (SLA, Thermofisher Scientific, USA) was quantified utilizing a methodology described by Hussain *et al*. (2015). Briefly, a 25µl aliquot of serum was combined with 175 µl of *Micrococcus lysodeikticus* in a reaction buffer, at a concentration of 0.75 mg/ml. This mixture was subsequently transferred to a flat-bottomed 96-well plate. The reduction in absorbance at a wavelength of 450nm was monitored over a 15-minute interval at a temperature of 25°C, employing an ELISA reader. A single unit of lysozyme activity was defined as the decrease in absorbance per minute. The levels of serum IL-6 and Serum Amyloid A (SAA, Thermofisher Scientific, USA) were determined by employing the double sandwich ELISA technique. In essence, serum samples were added to a monoclonal antibody-coated ELISA plate, followed by the addition of biotin-labeled IL-6 or SAA antibodies and streptavidin-HRP. The plates were subsequently incubated and washed to remove unbound enzymes. Chromogen solutions A and B were then added, resulting in a color change from blue to yellow upon the addition of acid. The intensity of the color was directly correlated with the concentration of serum IL-6 or SAA in the sample.

Histopathological examination: Fish (n=3) were collected from each group and then euthanized using Na_2CO_3-buffered tricaine methane-sulfate (MS-222 at 0.1 gL-1) for 2–3 minutes until visible loss of coordination occurred (Topic Popovic *et al.*, 2012). The liver and gills tissues were collected at the end of the experimental period for histopathological examination. The tissues exhibiting *Trichodina* cysts were promptly fixed in 10% neutral formalin. The preserved tissues underwent subsequent processing and staining with hematoxylin and eosin, prior to examination under a light microscope (Olympus CX41) Bancroft and Gamble (2008).

Tissue residue determination: The Zn residues were determined by obtaining muscle specimens from fish

(n=3) using the method defined by Christian and Epstein (1980). The muscle samples were digested with 7 ml of Sulfuric acid (H_2SO_4) and 2mL of H_2O_2. The sample was analyzed using a Thermo Scientific iCE 3300 atomic absorption spectrometer from Germany to quantify the zinc concentration.

Bacterial load in Nile tilapia organs: After the experiment, after ten weeks, fish sections, Skins, and gills were obtained from aquaria belonging to each experimental group. Ten grams of gills and skin were dispersed in a sterilized peptone buffer (90mL) to obtain a 10^{-1} dilution. Decimal dilutions were conducted up to 10^{-7}. The total bacterial count (TBC) was enumerated on a plate count agar (PCA) inoculated with different dilutions of skin and gills samples and subsequently incubated at a temperature of 37°C for one day. Similarly, the *Aeromonas* count was determined on an *Aeromonas* agar medium after a 12h incubation period at 37°C. The colonies of *Aeromonas* spp. were characterized by their dark green coloration interspersed with black centers (Sheir *et al*., 2020; Saad *et al*., 2021).

Statistical analysis: The data were assessed for normality and homogeneity of variance using a one-way ANOVA, followed by the least significant difference test, which was used for comparison at P<0.05 (Cardinali and Nason, 2013).

RESULTS

Isolation and identification of zinc-tolerant bacteria: A total of 33 bacterial isolates were obtained from soil samples and labeled as MS1, MS6, MS10, and MS20-MS60. The isolates were assessed for their tolerance to different concentrations of $Zn(NO_3)_2{\cdot}6H_2O$ (1, 2, 3, 4, and 5ppm). All isolates survived at a $ZnNO_3$ concentration of 1ppm. Twenty isolates grow at 2 ppm concentration, where 15 isolates tolerate a $Zn(NO_3)_2{\cdot}6H_2O$ (3ppm), nine isolates grow at 4ppm, and the isolates MS9, MS10, MS20, MS44, and MS55 survived at 4ppm. Only one isolate (MS20) that grows at the $Zn(NO_3)_2{\cdot}6H_2O$ (5ppm) was selected and identified.

Based on the microscopic analysis, the selected isolate was gram-positive, motile, long rod, and capable of generating spores. These observations indicate that the bacterium is a member of the *Bacillus* species. The biochemical tests conducted according to the Beregy manual identified this isolate as *Bacillus subtilis*; thus, the examined bacterial isolate coded as (*Bacillus subtilis* MS20) matches *Bacillus subtilis* DSM 5611 DSM based on the MALDI-TOF analysis.

Characterization of bacterial zinc nanoparticles: The supernatant of *B. subtilis* MS20 efficiently converted zinc nitrate into zinc nanoparticles, exhibiting a color change from colorless to white. The BZN exhibited absorption of UV light at a wavelength of 285 nm (Fig. 2A). They possessed a spherical form (Figure 2B), with a median diameter of 45nm as determined by zeta sizer analysis. Additionally, they had a negatively charged of 23.6mV as determined by zeta potential analysis (Fig. 2C, D).

Biochemical and antiparasitic activities

Antioxidant activity: Fig. 3 shows that zinc nanoparticles have considerable (P<0.05) scavenging activity against DPPH free radicals. The findings demonstrated a positive correlation between the concentration of BZN and its antioxidant activity. Specifically, the maximum concentration of BZN (500 µg/mL) eliminated 88% of DPPH radicals. The IC_{50} of BZN against ROS was 80 µg/mL.

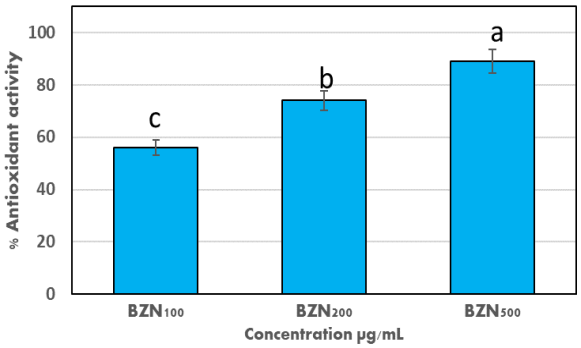


Fig. 3: Antioxidant activity of bacterial zinc nanoparticles fabricated by *Bacillus subtilis* MS20 against DPPH free radicals. The significance of antioxidant activity is indicated by letters above each column at p<0.05.

Antimicrobial activity: Fig. 4 shows that zinc nanoparticles' concentrations (100, 200, and 500 µg/mL) have significant antimicrobial activity (P<0.05) against fish pathogenic bacteria (*B. cereus*, *S. aureus*, *Aeromonas hydrophila*, *P. aeruginosa*) and fungi (*C. albicans*, *C. gelberta*). The inhibition zones' diameters increased in a concentration-dependent manner. *S. aureus* showed the most sensitivity to BZN doses of 500 µg/mL, with an inhibition zone diameter (IZD) of 28mm. *B. cereus* had a slightly lower sensitivity with an IZD of 23mm. On the other hand, *A. hydrophila* exhibited the highest resistance to BZN (17mm), followed by *P. aeruginosa* (19mm). In addition, BZN demonstrated substantial antifungal efficacy against *C. gelberta*, the most resistant fungus to BZN concentrations at 20mm.

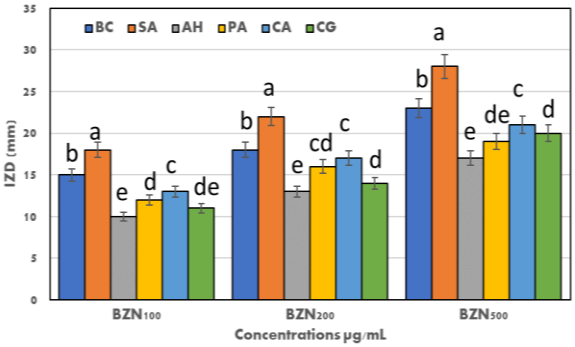


Fig. 4: Antimicrobial activity of bacterial zinc nanoparticles fabricated by *Bacillus subtilis* MS20 against fish pathogenic microbes. *Bacillus cereus* (BC), *Staphylococcus aureus* (SA), *Aeromonas hydrophila* (AH), *Pseudomonas aerogenosia* (PA), *Candida albicans* (CA), *Candida gelberta* (CG). The significance of antioxidant activity indicated by letters above each column at p<0.05.

Antiparasitic activity: Fig. 5 shows that bacterial zinc nanoparticles have *in vitro* antiparasitic activity against *Trichodina heterodentata*, and the percentage of parasitic death positively correlated with the BZN concentrations. The highest concentrations of BZN (500 µg/mL) reduced 88 % of the *Trichodina heterodentata* count.

***In vivo* experiment of BZN positive effect:**

Growth performance: The effect of different BZN concentrations on Nile tilapia's growth performance is illustrated in Table 1. Compared to the control group, there was a significant improvement in the growth performance parameters of fish groups fed on a diet supplemented with BZN, where weight gain increased 5-fold compared to the infected group; also, the survival rate was enhanced by 60% after BZN treatment. The *Trichodina*-challenged group showed bad performance, especially in the survival rate (56%) and body weight gain. Applying BZN 500 to the challenged group maintains the parameters to control levels.

Haematology: The erythrogram findings revealed a significant increase in PCV%, Hb concentration, and RBCs count in fish supplemented with the BZN 100mg /Kg diet compared with the control group. Meanwhile, compared to the control group, non-significant changes were recorded in BZN-supplemented fish (200 mg/Kg).

Nevertheless, the group of fish supplemented with a BZN 500 mg/Kg diet showed significant microcytosis (decreased MCV) with a considerable increase in RBCs count compared with the negative control group (Table 2). The BZN treatments lowered the leukogram results compared to the control. The *Trichodina*-challenged group showed lower erythrogram (Hb, RBCs) but higher PCV%, which may indicate inflammation in fish tissue. On the other hand, there were significant increases in leukogram results ($P<0.05$). Applying BZN 500 to the challenged group maintains the parameters to control levels.

Blood Biochemistry: The biochemical parameters (total proteins, albumin, globulins, creatinine, glucose, ALP, and ammonia) revealed significant enhancements in total protein levels in fish fed on a diet supplemented with different doses of BZN compared to the control group, while no sense in other parameters. Significant hypoglycemia occurred in challenged fish with higher ALP and ammonia values but lower protein content (Table 3).

Zinc content in fish tissue: The Zn content increased in muscle of fish groups fed BZN-supplemented diets compared to the untreated control (Table 4). However, the highest concentration of Zn (19.7 ppm) was shown in the BZN 500 group; this content was lowered by 40% in challenged fish then treated with BZN 500, indicating the antiparasitic activity of BZN.

Table 1: Effect of dietary doses of BZN on growth performance parameters of *Trichodina*-challenged Nile tilapia.

Treatments	IW (g)	FW (g)	WG (g)	SGR	FI	SR
T1	88	92d	7d	0.65c	11.5d	100a
T2	89	102b	13c	0.78b	12.9c	100a
T3	87	105b	18b	0.81ab	13.6b	100a
T4	88	110a	22a	0.85a	14.2a	100a
T5	89	78c	5d	0.66c	11.0d	56.6c
T6	87	100bc	13c	0.79b	12.9c	88b
P value	0.9	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Note: Initial weight (IW), final weight (FW), weight gain (WG), specific growth rate (SGR), feed intake (FI), and survival rate (SR). Lower cases indicate significant differences in the same column at $P<0.05$. T1 basal diet, T2 basal diet+BZN 100 mg/kg diet, T3 basal diet+BZN 200 mg/kg diet, T4 basal diet+BZN 500 mg/kg diet, T5 *Trichodina* challenged group, T6 *Trichodina* challenged group+BZN 500 mg/kg diet.

Table 2: Effect of dietary doses of BZN on haematology parameters of *Trichodina*-challenged Nile tilapia.

Treatments	T1	T2	T3	T4	T5	T6	P value
RBCs $x10^6$/µl	1.45c	1.96b	1.99b	2.2a	1.3d	1.89bc	0.0012
PCV %	26.3c	26.9c	27.1b	27.3b	32.3a	26.8c	0.003
MCV (fl)	184ab	160c	165c	175b	188a	166c	0.0047
MCHC (g/dl)	24.2c	30.5bc	31.6b	33.2a	23.6c	31.9b	0.0085
Hb (g/dl)	6.06c	9.2ab	9.5a	9.7a	6.0c	8.6b	0.0056
TLC$x10^3$/µl	31.3bc	31.9bc	26.5c	24.6c	39.3a	32.0b	0.0041
Heterophils $x10^3$/µl	8.2b	7.8c	7.5bc	6.2c	10.2a	7.7bc	0.0023
Lymphocytes $x10^3$/µl	22.5b	21.5bc	17.6c	14.8d	25.8a	21.3bc	<0.0001
Eosinophils $x10^3$/µl	0.0	0.0	0.0	0.0	0.6a	0.36b	<0.0001
Monocytes $x10^3$/µl	0.85b	0.81b	0.77c	0.69c	1.1a	0.75c	0.0011

Note: Red blood cells (RBCs), packed cell volume (PCV), mean corpuscular volume (MCV), mean cell hemoglobin concentration (MCHC), hemoglobin (Hb), Total Leukocyte Count (TLC), T1 basal diet, T2 basal diet+BZN 100 mg/kg diet, T3 basal diet+BZN 200 mg/kg diet, T4 basal diet+BZN 500 mg/kg diet, T5 *Trichodina* challenged group, T6 *Trichodina* challenged group+BZN 500 mg/kg diet. Different lower cases indicate significant differences in the same raw at $P<0.05$.

Table 3: Effect of dietary doses of BZN on blood biochemistry parameters of *Trichodina*-challenged Nile tilapia.

Treatments	T1	T2	T3	T4	T5	T6	P value
ALP (U/l)	45.6c	43.3c	41.3c	39.8d	56.9a	48.3b	<0.0001
Creatinine (mg/dl)	0.37	0.33	0.32	0.33	0.35	0.33	0.98
Glucose (mg/dl)	86.3b	80.5c	77.3d	71.6d	95.6a	81.3c	<0.0001
Total protein (g/dl)	3.2b	3.5b	3.9ab	4.2a	2.8c	3.1bc	0.002
Albumin (g/dl)	1.37b	1.39b	1.41a	1.45a	1.30c	1.34b	0.031
Globulin (g/dl)	1.8b	1.88b	1.9ab	2.2a	1.5c	1.78bc	0.025
A/G	0.76b	0.74b	0.74b	0.66c	0.87a	0.75b	0.011
Ammonia (mmole/l)	0.45b	0.41b	0.35c	0.33c	0.65a	0.42b	0.023

Note: T1 basal diet, T2 basal diet+BZN 100 mg/kg diet, T3 basal diet+BZN 200 mg/kg diet, T4 basal diet+BZN 500 mg/kg diet, T5 *Trichodina* challenged group, T6 *Trichodina* challenged group+BZN 500 mg/kg diet. Different lower cases indicate significant differences in the same raw at P<0.05.

Table 4: Effect of dietary doses of BZN on the zinc concentration in the tissue of *Trichodina*-challenged Nile tilapia.

Treatments	Zinc concentration (ppm)
T1	8.2e
T2	13.66c
T3	17.68b
T4	19.37a
T5	0.66f
T6	11.69d
P value	<0.0001

Note: T1 basal diet, T2 basal diet+BZN 100 mg/kg diet, T3 basal diet+BZN 200 mg/kg diet, T4 basal diet+BZN 500 mg/kg diet, T5 *Trichodina* challenged group, T6 *Trichodina* challenged group+BZN 500 mg/kg diet. Different lower cases indicate significant differences in the same raw at P<0.05.

Table 5: Effect of dietary doses of BZN on immunity parameters of *Trichodina*-challenged Nile tilapia.

Treatments	IL-6 (ng/ml)	SLA (mg/ml)	SAA (ng/ml)
T1	18.55bc	0.31b	3.1b
T2	15.66c	0.28c	2.9c
T3	12.58d	0.25d	2.5cd
T4	10.66e	0.19e	2.1d
T5	29.5a	0.68a	4.9a
T6	19.65b	0.33b	2.8c
P value	<0.0001	<0.0001	<0.0001

Note: Serum Lysozyme Activity (SLA), Interleukin – 6 (IL – 6) and Serum Amyloid A (SAA). T1 basal diet, T2 basal diet+BZN 100 mg/kg diet, T3 basal diet+BZN 200 mg/kg diet, T4 basal diet+BZN 500 mg/kg diet, T5 *Trichodina* challenged group, T6 *Trichodina* challenged group+BZN 500 mg/kg diet. Different lowercases indicate significant differences in the same raw at P<0.05.

Immunity parameters: The serum immunological parameters revealed a significant increase in SLA concentration in the *Trichodina*-challenged groups (G5) compared to the negative control group. The fish fed on a BZN-supplemented diet significantly decreased SLA comparing the *Trichodina* group. However, fish fed on a BZN-supplemented diet showed a marked tendency to restore SLA. Also, significant increases were observed in IL-6 and SAA values in challenged groups (Table 5). The BZN post-treatment in the challenged group downregulated the IL-6, SAA, and SLA by 30-50%.

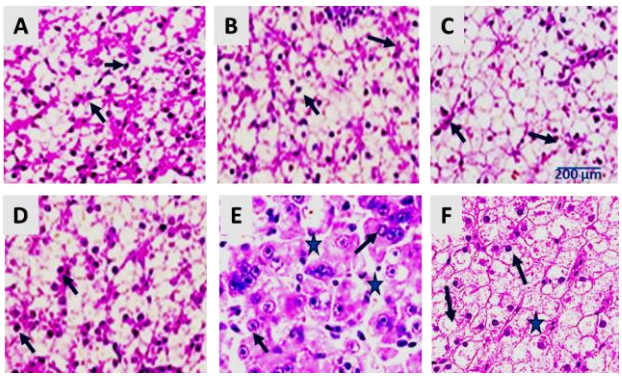


Fig. 5: The liver of *O. niloticus*, of the (a) control negative group showed normal hepatocytes, with normally rounded nuclei and normal hepatopancreas, (b) 100mg BZN group, showing normally vacuolated hepatocytes and focal infiltration of the hepatopancreas with mononuclear cells, (c) 200mg BZN group showing normal hepatocytes with few Melan macrophages infiltrating the hepatopancreas, and (d) 500mg BZN group showing the normal hepatic structure, (e) showing extensive vacuolar degeneration of hepatocytes (blue star), which appeared markedly swollen with the presence of *Trichodina* and massive necrosis of the pancreas (arrow), which is heavily colonized with the *Trichodina* and expansive necrosis with loss of pancreatic acinar cells as well as the aggregation of *Trichodina*, (f) showing mild vacuolar degeneration of hepatocytes (black star) and focal necrosis of the pancreas (Stain: H&E; scale bar=200µm).

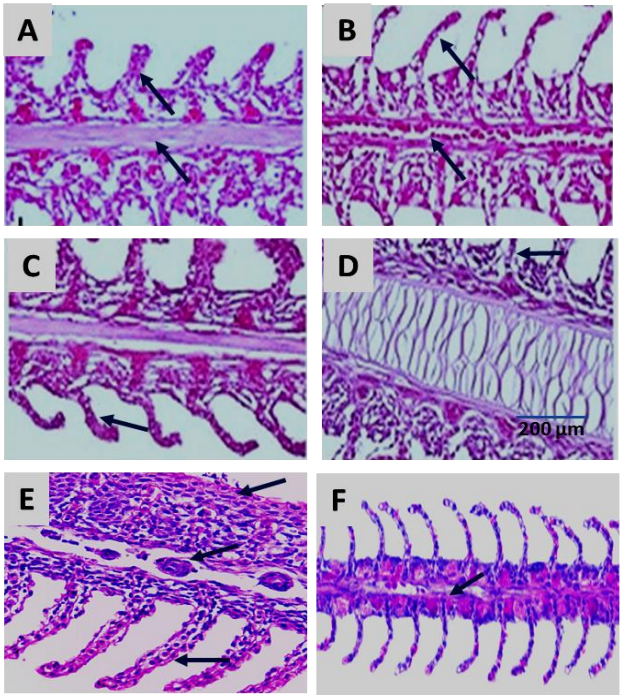


Fig. 6: The gills of *O. niloticus*, of the (a) control negative group showed normal gill lamellae, with mild dilation of the blood capillaries, (b) 100 mg BZN group showing normal lamellar epithelium, with mild congestion of the central venous sinuses, (c) 200mg BZN group showing normal gill lamellae, (d) 500mg BZN group showing normal gill lamellae and focal telangiectasis, e) showing hyperplastic proliferation of the epithelial lining the secondary lamellae (blue arrow), which is associated with the presence of the *Trichodina*, f) normal gill lamellae (blue arrow) (Stain: H&E; scale bar=200 m). T1 basal diet, T2 basal diet+BZN 100 mg/kg diet, T3 basal diet+BZN 200 mg/kg diet, T4 basal diet+BZN 500 mg/kg diet, T5 *Trichodina* challenged group, T6 *Trichodina* challenged group+BZN 500 mg/kg diet.

Histopathological examinations: Fig. 5 shows normal hepatocytes, with normally rounded nuclei and normal hepatopancreas in the liver of control *O. niloticus* (Fig. 5A); the group received 100mg BZN group, showing normally vacuolated hepatocytes and focal infiltration of the hepatopancreas with mononuclear cells (Fig. 5B), while 200mg BZN group showing normal hepatocytes with mild melan macrophages infiltrating the hepatopancreas (Fig. 5C), the 500mg BZN group showing the normal hepatic structure (Fig. 5D). On the other hand, the *Trichodina*-challenged fish showed extensive vacuolar degeneration of hepatocytes, which appeared markedly swollen with the presence of *Trichodina* and massive necrosis of the pancreas, which is heavily colonized with the *Trichodina* and expansive necrosis with loss of pancreatic acinar cells as well as the aggregation of *Trichodina* (Fig. 5E). After the treatment with BZN 500, mild vacuolar degeneration of hepatocytes and focal necrosis of the pancreas was observed (Fig. 5F).

The gills of *O. niloticus*, of the control negative group showed normal gill lamellae, with mild dilation of the blood capillaries (Fig. 6A), normal lamellar epithelium, with mild congestion of the central venous sinuses in 100 mg BZN group (Fig. 6B), meanwhile 200mg BZN group

showing normal gill lamellae (Figure 6C). The 500mg BZN group showed normal gill lamellae and focal telangiectasis (Fig. 6D). The challenged group showed hyperplastic proliferation of the epithelial lining of the secondary lamellae, which is associated with the presence of *Trichodina* (Fig. 6E). After BZN treatment, it showed normal gill lamellae (Fig. 6F).

Bacterial load in Nile tilapia organs: The number of bacteria in the medium indicated the bacterial populations in the fish's organs that were in direct contact with water, such as the skin and the gills (Fig. 7). In the T1 and T5 groups, the fish gills exhibited elevated total bacterial counts, ranging from 8.3 to 8.8 Log CFU ml^{-1}. The BZN treatments (T2, T3, T4, and T6) reduced 31-40% of total bacterial counts in the gills and 37-45% in the skin. The highest *Aeromonas* count recorded in the gills of T1 and T5 was 4.5-5.1 Log CFU ml^{-1}. The BZN treatments reduced 30-35% of total *Aeromonas* counts in gills and 44 % in skin.

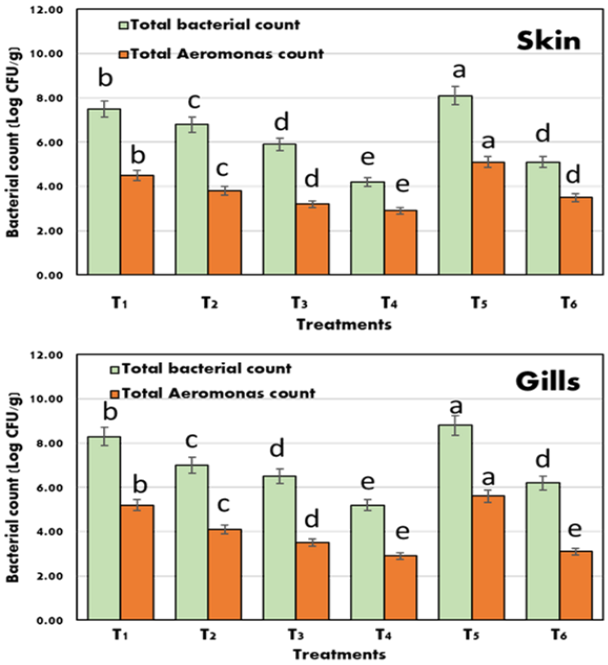


Fig 7: Total bacterial and *Aeromonas* count in surface fish organs (Skin and Gills) after ten weeks of different treatments. a-e letters above columns indicate significant differences P<0.05 between bacterial counts in gills and skin. T1 fed a basal diet, T2 fed a basal diet+100 mg/kg BZN diet, T3 fed basal diet+BZN 200 mg/kg diet, T4 basal diet+BZN 500 mg/kg diet, T5 *Trichodina* challenged group, T6 *Trichodina* challenged group+BZN 500 mg/kg diet.

DISCUSSION

The predominant parasites detected in farmed tilapia and wild water environments were Trichodinids and *Cichlydogyrus* spp. Trichodinids proliferate by binary fission, and their ability to directly transmit makes them capable of rapidly infecting the whole host population, particularly in fish housed in unfavorable environmental circumstances, polluted by chemicals or insecticides, or unsuitable culture settings (Faruk, 2018; Li *et al.*, 2022; Mahmood *et al.*, 2022). The water contaminated with herbicides and insecticides cause toxic effects via disruption of physiological and hematobiochemical reactions of fish (Afzal *et al.*, 2022; Naseem *et al.*, 2022; Wang *et al.*, 2022). For example, BPA and heavy metals induce adverse effects on physical, blood-biochemical parameters and histopathological changes in multiple visceral tissues of exposed fish (Namratha *et al.*, 2020; Akram *et al.*, 2021; Jabeen *et al.*, 2021).

Freshwater fish are more susceptible to Trichodinids and monogenea infections; water quality affects the physiological and biochemical markers (Raza *et al.*, 2022; Ahmad *et al.*, 2023). The prevalence of parasite infections is often greater in fish raised in aquaculture settings than those living in natural water bodies, primarily due to increasing fish density in the aquatic environment. Trichodinids can be found in natural habitats; however, they are not often seen with significant infection levels in healthy animals (Blazhekovikj-Dimovska and Stojanovski, 2020). Various researches have documented increased parasite infection rates in cultivated environments due to greater population density (DeVore *et al.*, 2020; Wilson *et al.*, 2021).

The parasites have a monoxenic life cycle, reproducing by binary division and, in some circumstances, conjugation (Lehmann *et al.*, 2020; Dominguez *et al.*, 2023). The high prevalence of these parasites in farmed fish can lead to severe clinical consequences in affected individuals (Fahmy *et al.*, 2022). The harmful effect of trichodinids is closely linked to their method of infecting their hosts. The parasite firmly adheres to its host by generating a suction motion on the epithelial cell surface, resembling a saucer (Blazhekovikj-Dimovska and Stojanovski, 2020). Hence, a substantial accumulation of these trichodinids on the host, along with their persistent circular movements, might cause severe damage to the epithelium of the infected hosts, resulting in physiological disturbance and elevating the susceptibility to subsequent infection by other pathogenic microorganisms (Lehmann *et al.*, 2020). The presence of *Trichodina centrostrigeata* in Towuti Lake mostly inhabits the gills of cichlid fish around the globe. This parasite is often characterized by 11 to 14 distinct central ridges inside its sticky disc (Jahangiri *et al.*, 2021).

Zinc (Zn) is a necessary micronutrient for fish, as it is crucial for several biological functions (El-Saadony *et al.*, 2021d). Shnawa *et al.* (2024) stated that green zinc nanoparticles synthesized by Ziziphus spina-christi possessed considerable ROS-scavening activity and antimicrobial against *Asperigulls niger*, *Staphylococcus aureus*, and *Escherichia coli*. Also, biosynthesized zinc oxide nanoparticles using *Typha domingensis* flower extract showed anthelmintic activity against *Echinococcus granulosus* protoscoleces (Shnawa *et al.*, 2023a). While composing the zinc with silver in zinc-sliver nanocomposites showed acaricidal efficacy on *Hyalomma marginatum* ticks (Shnawa *et al.*, 2023b).

Because of the abovementioned activities, zinc plays a crucial role in several enzymatic processes involved in fish metabolism. These activities include growth, immunological response, and enzyme activity. It inhibits the ROS production. Zinc cannot be stored in the body; hence, it is necessary to regularly consume it through food (Sherif *et al.*, 2023). Previous studies have shown that catfish, common carp, and rainbow trout can withstand

dietary ZnO-NP levels up to 1.2, 1.7, and 1.9 grams per kilogram of diet, respectively (Onuegbu *et al*., 2018), without experiencing any negative impact on their growth performance and survival. The objective of this study was to assess the possible effects of high dietary dosages of BZN on Nile tilapia fish challenged with Trichodina. The Nile tilapia-fed diets enriched with ZnO-NPs showed better growth and hematological parameters compared to the control group.

These findings are consistent with Yaqub *et al.* (2023), who recorded a significant increase in PCV%, Hb concentration, and RBCs count in Nile tilapia fed on ZnO-NPs supplemented diet at a dose of 40 mg/kg diet for eight weeks. The authors stated that a lack of zinc in the control might potentially increase the oxidation of lipids in the membranes of mitochondria and microsomes, which could cause the erythrocyte membranes to become more fragile due to osmotic stress (Pei *et al.*, 2022). Ghazi *et al.* (2021) recorded a significant increase in Hb concentration and RBCs count of Nile tilapia fed on a ZnO-NPs supplemented 10 mg/kg diet for 60 days.

Safety experiment findings are consistent with reports of Yaqub *et al.* (2023) demonstrating the nontoxic impacts of ZnO-NPs (40 mg ZnO-NPs/kg diet for eight weeks) on hematology and biochemistry of Nile Tilapia. The muscular tissues of *O. niloticus* that were given ZnO-NPs in their meal contained more Zn than the control fish. The Zn content in the muscle was consistently higher in all treatments than in the controls. Nevertheless, it remained below the acceptable threshold (40 ppm) established by FAO. These findings agree with a previous study by Kaya *et al.* (2015), who reported that Zn accumulation occurred in the intestine, followed by the liver, kidney, gills, brain, and muscle tissue.

Regarding the challenge results, the arthrogram revealed normocytic normochromic anemia in the experimentally challenged group compared to the negative control group. This finding may be attributed to the acute blood loss (severe hemorrhages in the skin, base of fins) induced by *Trichodina* infection. At the end of the experimental period, all the infected groups showed significant microcytosis compared to the negative control group. Comparing all infected groups to the negative control group, leukogram analysis showed remarkable leukocytosis with heterophilia and monocytosis. This finding might be explained by the bacteremia that has shown up obviously on the blood film. Significant leukopenia with neutropenia and lymphopenia were recorded in the infected groups, which might be attributed to the migration of white blood cells to the different tissues as a predominant cell in the defense reactions of the organism. The presence of lymphocytes and eosinophilic granular cells in the mesencephalon of the infected groups was verified by extensive infiltration. The presence of blood in a fish's body is linked to the levels of serum proteins and can indicate a strengthened immune system in the fish. Blood is crucial in identifying natural antigens in the fish's skin, organs, and plasma (Assefa and Abunna, 2018).

Concurrently, the rise in leukocytes proves that fish react to viruses invading their bodies, employing several mechanisms to defend themselves against external assaults (bacteria or parasites) (Howell and de Leeuw, 2018). Significant improvement in TLC and absolute lymphocyte count in the treated groups could be attributed to the ability of ZnO-NPs to reduce infection in different examined tissues. The liver showed considerable improvement, and the hepatocytes revealed mild vacuolar degeneration. Meanwhile, the liver of the positive control group showed congestion of the central vein and hepatic sinusoids with severe diffuse vacuolar degeneration of hepatocytes and aggregation of Trichodina. The results are consistent with those of Sahreen *et al.* (2021), who reported a significant decrease in total proteins, albumin, and globulins concentrations in the Nile tilapia-challenged group.

All infected groups showed a significant increase in ammonia levels throughout the study. This was likely caused by the thickened epithelial lining of the secondary lamellae of the gills and the presence of Trichodina cysts. Fish release ammonia into the water through their gills by simple diffusion. This is made possible by their large gill surface area, good blood flow, fast breathing, short diffusion distances, and contact with different mucosal media (Ip and Chew, 2010). Damage to the gills can lead to higher ammonia levels in fish, which can be toxic. Ammonia is known to promote the growth of astrocytes, but the specific ways it does this still need to be fully understood. It is thought that when there is too much ammonia in the blood, astrocytes can make glutamine from ammonia and glutamate. This can cause problems with water balance in the brain and lead to swelling (Ip and Chew, 2010).

Serum lysozymes are a crucial element of the innate immune response to viral infections or injuries. The principal function of lysozyme is to enhance the body's immune response to pathogens by stimulating the degradation of infections.

Several studies have found that the actions of lysozymes augment after infection or the introduction of foreign substances or pathogens. Hence, the increased SLA in the infected groups is strong evidence of *Trichodina*-infection. This finding agrees with Mishra *et al.* (2020), who reported that lysozyme activities following the injection of *Trichodina* in olive flounder were higher than those of the control group 1 and 3 days after the injection.

Interleukin 6 cytokine is crucial in the immune response since it stimulates the differentiation of B cells into plasma cells and enhances antibody synthesis by antibody-secreting cells. A non-significant increase in the values of IL-6 was noticed in the infected groups on the 2nd day post-challenge. Wei *et al.* (2018) reported that IL-6 might get involved in host defense against bacterial infection in Nile tilapia, and the up-regulated expression of IL-6 confirms this; fish organs that were infected with a single administration of *Trichodina* species increased the inflammation in gills, kidneys, and liver tissues. From a histopathological perspective, the damage on the gills and fish surface indicated harm caused by releasing bacterial toxins and septicemia. Furthermore, the presence of significant numbers of melanoma macrophages may suggest that hematopoietic tissues are mounting a defensive reaction against the harmful effects of free

radicals induced by Aeromonas pathogenicity, as noted by Kathirkaman *et al*. (2018).

Water quality facilitates the *Trichodina* infection that promotes the bacterial infection in the parasite fish; this explains the highest total bacterial and *Aeromonas* count in T5 (Ashwath *et al*., 2024).

No direct studies clarify the in vivo effect of zinc nanoparticles on *Trichodina* in fish. However, in this regard, some similar studies showed zinc nanoparticles' in vitro and in vivo antiparasitic efficiency against the aquaculture parasites. Saleh *et al.* (2017) examined the impact of Ag, Au, and ZnNPs on the ability of *Ichthyophthirius multifiliis* free-living stages to reproduce and cause infection. They found that 50% of theronts diminished within 30 min of Ag and Zn nanoparticles (10 and 5ppb); total mortality happened after 2h of treatments. Au nanoparticles at a concentration of 20 ng/ml resulted in the death of 80% and 78% of tomonts and theronts, respectively, two hours after exposure. Studies conducted on live rainbow trout (*Oncorhynchus mykiss*) found that the theronts exposed to zinc oxide nanoparticles exhibited lower infectivity than those theronts in the control group. No deaths were seen in the fish groups that lived with theronts exposed to nanoparticles, in contrast to a 100% mortality rate in the control group.

Further, Dorostkar *et al.* (2017) recorded the anthelmintic effects of FeNPs against *Toxocara vitulorum* through the induction of oxidative/nitrosative stress. However, Gonzalez-Moragas *et al.* (2017) suggested that IONPs might be endocytosed by a clathrin-mediated process, a putative mechanism of nanotoxicity in the nematode *C. elegans*. Similarly, the anthelmintic effects of ZnONPs were evaluated against *Haemonchus contortus*, which induced oxidative/nitrosative damage to the biomolecules of the worm (Esmaeilnejad *et al.*, 2018).

Conclusions: The bacterial zinc nanoparticles synthesized using *Bacillus subtilis* MS20 demonstrate a range of biological uses, including *in vitro* antibacterial, antifungal, antioxidant, and antiparasitic characteristics. This study showed that the prevalence and intensity of parasite infections in *Trichodina*-infested Nile Tilapia were reduced by administering BZN at 500 mg/kg. The administration of BZN effectively decreased bacterial invasion in fish infected with *Trichodina*, reducing bacterial counts in fish tissues and enhancing fish survival rates. This study indicates that preventing and treating fish parasite infections are essential for managing fish health. It will reduce damage caused by parasitic infestation and decrease fish mortality caused by subsequent bacterial infection.

Acknowledgments: This work was funded by the University of Jeddah, Jeddah, Saudi Arabia, under grant No. (UJ-23-DR-233). Therefore, the authors thank the University of Jeddah for its technical and financial support.

Funding: This work was funded by the University of Jeddah, Jeddah, Saudi Arabia, under grant No. (UJ-23-DR-233). Therefore, the authors thank the University of Jeddah for its technical and financial support.

Authors contributions: MNA, and MTES, supervision, conceptualization, methodology, validation, and writing review and editing. MNA, and MTES, conceptualization, methodology, software, investigation, resources, data curation, writing original draft, visualization, and experiment administration. MNA, and MTES, supervision, conceptualization, methodology, validation, and writing review and editing. MNA, and MTES, supervision, conceptualization, methodology, validation, and writing review editing, and discussed the results and approved the final manuscript.

Competing interests: The authors declare that they have no competing interests.

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