

Research Article



Effects of Zinc oxide Nanoparticles Synthesized by *Bacillus subtilis* on the Performance, Enzymatic Antioxidant Activity, and Neurotoxicity in Tilapia (*Oreochromis mossambicus*)

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Abstract | Antimicrobial resistance (AMR) poses a significant challenge to animal health. One promising strategy to address this issue is the use of zinc oxide nanoparticles (ZnONPs) synthesized from probiotics like *Bacillus subtilis*. This study explored the potential effects of ZnONPs synthesized using *Bacillus subtilis* on the performance, antioxidant activity, and neurotoxicity of tilapia (*Oreochromis mossambicus*). A total of 96 fish were divided into four groups: T₁ (Control), T₂ (3 mg ZnONPs /g diet), T₃ (6 mg ZnONPs /g diet), and T₄ (9 mg ZnONPs /g diet), with four replicates per group arranged in a completely randomized design. The data were analyzed using analysis of variance (ANOVA) to examine the main effects of dietary treatments. The results indicated that the inclusion of ZnONPs in the diet did not produce any significant differences in the average body weight of the fish. Specifically, there was no notable impact on body weight across the different treatment groups ($P>0.05$). However, significant effects were observed in various antioxidant biomarkers ($P<0.05$). Superoxide dismutase (SOD) enzyme activities in the gills showed significant enhancement ($P=0.008$), whereas no significant change was detected in the liver ($P=0.088$). Catalase (CAT) enzyme activities significantly increased in the gills and liver ($P=0.001$). Additionally, glutathione S-transferase (GST) activity was significantly elevated in both the gills and liver ($P=0.006$). Neurotoxicity assessment through acetylcholinesterase (AChE) activity in the brain showed no significant difference ($P=0.952$). The ZnONPs significantly influenced antioxidant biomarkers (SOD, CAT, and GST), while the mortality rate, average body weight, and neurotoxicity of tilapia were not statistically affected. Hence, the addition of ZnONPs in small quantities (3mg/g diet) enhances antioxidant biomarkers and has the potential to serve as a feed additive for improving animal health and nutrition.

Keywords | Probiotic, Health, Antioxidant, Neurotoxicity, Additive, Nutrition

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INTRODUCTION

The aquaculture and livestock industries employ a variety of feed and water supplements or additives to enhance animal farming practices and optimize animal

productivity (Silva et al., 2021). A common strategy involves the incorporation of antimicrobials, particularly antibiotics, to control and prevent disease outbreaks that can negatively impact animal performance. For example, antibiotics are frequently used in aquaculture for both

prophylactic and therapeutic purposes against various bacterial infections (Silva et al., 2021).

In response to the growing concern over antimicrobial resistance (AMR), regulatory bodies like the European Union (EU) have implemented bans on the use of antibiotics as growth promoters in livestock (Przeniosło-Siwczyńska and Kwiatek, 2013). This has spurred the exploration of alternative strategies to combat AMR. One promising avenue lies in the development of nanoparticles with demonstrably effective antimicrobial properties (Khezerlou et al., 2018). However, the effectiveness of these nanoparticles as bactericidal agents is highly dependent on several factors, including their composition, concentration, and morphology (Qi et al., 2017; Liao et al., 2020).

Among various nanoparticles, zinc oxide nanoparticles (ZnONPs) have garnered significant attention due to their promising antimicrobial properties (Mendes et al., 2022). However, the precise mechanism by which ZnONPs exert their bactericidal effect remains a subject of ongoing research. Despite this knowledge gap, several studies have demonstrated the efficacy of ZnONPs as broad-spectrum antibacterial agents, effective against both gram-positive and gram-negative bacteria (Gudkov et al., 2021). ZnONPs have also exhibited antioxidant activities in previous research (Akhaman-Salamat and Ghasemi, 2019; Zhang et al., 2021).

However, the potential benefits of nanoparticle use in various industries are counterbalanced by growing concerns regarding their environmental toxicity (Shanmugam et al., 2023). The presence of hazardous elements or byproducts within certain nanoparticle formulations can significantly limit their safe applications, particularly in sensitive settings like clinical medicine, despite the prevalence of physical and chemical production methods (Shanmugam et al., 2023). To address these concerns, developing well-defined and consistent standards for nanoparticle production, exposure evaluation, and potential toxicity assessment is crucial (Medici et al., 2021).

Synthesis of ZnONPs can be achieved through various methods, categorized as physical, chemical, and biological approaches. However, biological synthesis methods are increasingly favored due to their inherent advantages. These advantages include simplicity, cost-effectiveness, bio-compatibility, and environmental friendliness, as they eliminate the need for harsh chemicals or toxic precursors in the production process (Hamk et al., 2023). Notably, biological methods can utilize various biological entities such as plants, bacteria, fungi, or their enzymes. For instance, *Bacillus subtilis* has been shown to possess both intrinsic antimicrobial activity and the ability to act as a bio-reductant for the synthesis of silver nanoparticles

(Alsamhary, 2020). Additionally, dietary supplementation with *Bacillus subtilis* in poultry has been demonstrated to enhance the activity of antioxidant enzymes, such as glutathione peroxidase (GSH-Px), in laying hens (Liu et al., 2019). Hence, this study investigates the impact of ZnONPs synthesized using *Bacillus subtilis* on performance, antioxidant activity, and neurotoxicity in tilapia (*Oreochromis mossambicus*).

MATERIALS AND METHODS

COLLECTION AND MAINTENANCE OF EXPERIMENTAL ANIMALS

Tilapia (*Oreochromis mossambicus*) were obtained from a reputable fish farm in Karaikudi, Tamil Nadu, India, to ensure the experiment's genetic homogeneity and disease-free status. Upon arrival, the fish underwent a 14-day acclimatization period at the Biomaterials and Biotechnology in Animal Health Laboratory, Department of Animal Health and Management, Alagappa University. During this acclimatization period, the fish were randomly assigned to four treatment groups, each containing four replicates. Throughout the study, a standardized protocol was followed for water replacement, monitoring of key physiochemical water parameters, feeding, and regular observation of fish behavior and mortality (Abinaya et al., 2023).

EXPERIMENTAL DIET FORMULATION

Powdered ZnONPs were used throughout the study for dietary formulation. A commercially available diet was thoroughly mixed with ZnONPs at predetermined concentrations to create four treatment groups: Treatment 1 (Control, no ZnONPs), Treatment 2 (3 mg ZnONPs/g diet), Treatment 3 (6 mg ZnONPs/g diet), and Treatment 4 (9 mg ZnONPs/g diet). The prepared diets were dried and stored at 4 °C following thorough mixing until use.

DATA COLLECTION FOR PRODUCTION PERFORMANCE

Mortality data were collected daily for each treatment group throughout the experiment. On the final day, fish growth performance was assessed. Six fish per replicate ($n = 24$ fish per treatment) were randomly selected for dissection and subsequent analysis of growth parameters in the laboratory.

BIOCHEMICAL BIOMARKERS

The activities of superoxide dismutase (SOD), glutathione-S-transferase (GST), and catalase (CAT) were measured in gill and liver homogenates from fish in all treatment groups. Gills and livers were collected and homogenized in a 0.1 M phosphate buffer (pH 7.2). Following centrifugation at 10,000 revolutions per minute for 30 minutes, the resulting supernatants were utilized for subsequent analyses of oxidative stress markers and enzymatic/non-enzymatic

antioxidant activity.

NEUROTOXICITY

Brain tissue homogenates were prepared from all treatment groups. Brain samples were homogenized in a 0.1 M phosphate buffer (pH 7.5) at a 1:10 (w/v) tissue-to-buffer ratio. The homogenates were then centrifuged at 10,000 rpm for 20 minutes. The resulting supernatants were collected for subsequent analysis of neurotoxic effects induced by ZnONPs. Acetylcholinesterase (AChE) activity, a biomarker of neurotoxicity, was measured in the supernatants following the method described by Ellman et al. (1961). A microplate reader was used to quantify AChE activity at a wavelength of 405 nm.

EXPERIMENTAL DESIGN AND ANALYSIS

The experiment employed a completely randomized design (CRD) with four dietary treatments and four replicates per treatment, with each replicate consisted of six fish. Data were subjected to analysis of variance of the Statistical Analysis System (SAS, 2011, Version 9.3, SAS Institute, Cary, NC, USA) software to assess the main effects of the dietary treatments. Post-hoc comparisons of significant treatment effects were performed using Tukey's Honestly Significant Difference (HSD) test. A p-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

AVERAGE LIVEWEIGHT

As shown in Figure 1, the control group (T₁) exhibited numerically higher average live weight (ALW) at 159.31 g. In contrast, T₄ (9 mg ZnONPs/g diet) displayed the lowest ALW at 107.46 g, indicating stunted growth. The groups T₂ (3 mg ZnONPs/g diet) and T₃ (6 mg ZnONPs/g diet) had ALWs of 134.65 g and 142.70 g, respectively, reflecting lower ZnONPs concentrations. However, statistical analysis revealed no significant differences in ALW among the treatments (P>0.05).

The recent findings on the effects of ZnONPs on the growth performance affirmed those of Abou-Zeid et al. (2023), who reported decreased growth in tilapia. This discrepancy warrants further investigation. However, exposure to the highest ZnONPs concentration (T₄) might have caused stress, potentially affected carbohydrate

or protein metabolism and limiting the energy available for growth (Farag et al., 2021). The intestine plays a vital role in nutrient absorption (Khan et al., 2022) and is sensitive to toxins (Gisbert et al., 2008). Disruption of intestinal function by ZnONPs could lead to oxidative damage and reduced growth, as reported by Bhattacharyya et al. (2014).

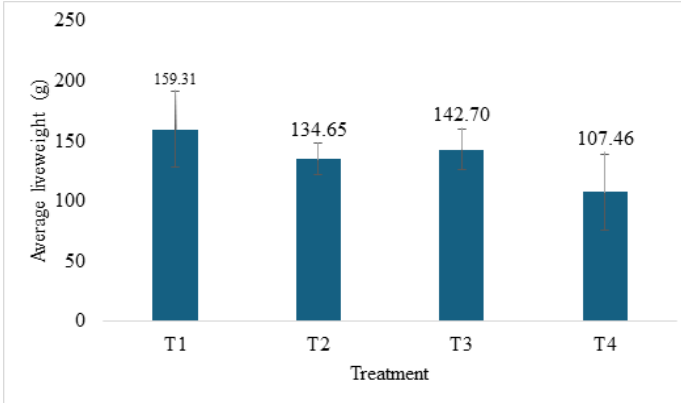


Figure 1: The average live weight of experimental fish with varying concentrations and without supplementation of ZnONPs in the diet.

MORTALITY

No significant differences (P>0.05) in total mortality were observed among the treatment groups throughout the study (n = 96 fish). Overall mortality data are presented in Table 1. While statistically insignificant, the highest average mortality rate (4.25) was observed in T₄ (9 mg ZnONPs/g diet), followed by T₂ (3 mg ZnONPs/g diet) at 4. Moreover, T₃ (6 mg ZnONPs/g diet) exhibited the lowest average mortality (3).

Fish in T₄ showed a gradual increase in mortality, accompanied by behavioral changes such as aggression, abnormal movements, and loss of equilibrium before death. These observations suggest a response to the highest ZnONPs concentration, possibly exceeding tolerance levels and leading to acute toxicity. This aligns with previous findings by Khan et al. (2022), who reported behavioral alterations in tilapia exposed to 20 parts per billion ZnONPs, potentially caused by acute toxicity. These alterations could be linked to immune suppression, performance changes, and oxidative damage, as reported by Chen et al. (2020), Hong et al. (2022), and Abou-Zeid et al. (2023).

Table 1: The average mortality rate of the experimental fish with varying concentrations and without ZnONPs in the diet.

Parameter	Treatments				SEM	P value
	T ₁ , Control	T ₂ , 3 mg ZnONPs/g	T ₃ , 6 mg ZnONPs/g	T ₄ , 9 mg ZnONPs/g		
Mortality	3.75	4.00	3.00	4.25	0.193	0.845

SEM: standard error of the mean.

Interestingly, the control group (T₁) exhibited a higher average mortality (3.75) than T₃. During the study, fish in T₁ displayed clinical signs like red skin patches, suggesting potential deterioration of water quality, which might have contributed to mortality in the absence of ZnONPs exposure (Ali et al., 2020).

SUPEROXIDE DISMUTASE (SOD) IN LIVER AND GILLS

SOD activity was measured in both liver and gill tissues (Figure 2). In the gills, a significant effect of treatment was observed ($P < 0.05$). Fish in T₄ (9 mg ZnONPs/g diet) exhibited the lowest SOD activity (0.17 U/mg protein), while T₂ (3 mg ZnONPs/g diet) and T₃ (6 mg ZnONPs/g diet) displayed the highest activity (0.27 U/mg protein). T₁ (Control) had the intermediate activity of 0.18 U/mg protein, respectively. In contrast, SOD activity in the liver did not differ significantly among treatments ($P > 0.05$). T₄ again showed the highest activity (0.12 U/mg protein), followed by similar results from T₁ (Control) and T₂ (3 mg ZnONPs/g diet) with 0.11 U/mg protein, while T₃ (6 mg ZnONPs/g diet) had the lowest activity of 0.06 U/mg protein.

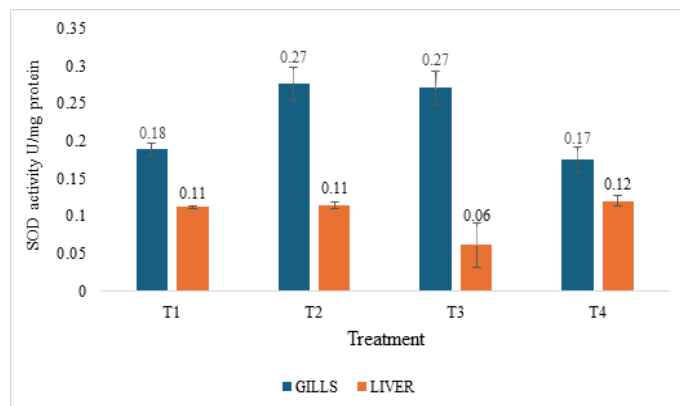


Figure 2: Superoxide dismutase (SOD) enzyme activities in the liver and gills with varying concentrations and without supplementation of ZnONPs in the diet.

Gills displayed a significant reduction in SOD activity ($P < 0.05$) in fish exposed to the highest ZnONPs concentration (T₄), suggesting potential oxidative stress in this tissue. Conversely, liver SOD activity did not differ significantly among treatments, and T₄ even showed the highest activity (not statistically significant). These findings suggest a differential response in antioxidant enzyme activity between these tissues. The results partially align with Meharadiya (2023), who reported that exposure to higher ZnONPs concentrations could influence antioxidant activity.

The decrease in gill SOD activity in T₄ could be attributed to the well-documented ability of ZnONPs to induce reactive oxygen species (ROS) production, as reported by Choudhury et al. (2017). Elevated ROS levels can

trigger an oxidative stress response, potentially leading to the depletion of antioxidant enzymes like SOD (Horie and Tabei, 2020). Excessive ROS production has further downstream consequences, potentially initiating apoptosis and exerting cytotoxic and inflammatory effects, as described by Oyinloye et al. (2015).

CATALASE (CAT) ENZYME ACTIVITIES IN LIVER AND GILLS

CAT activity was significantly affected by dietary ZnONPs concentration in both liver and gill tissues ($P < 0.05$) (Figure 3). In the gills, T₂ (3 mg ZnONPs/g diet) exhibited the highest CAT activity (0.213 U/mg protein), while the control group (T₁) displayed the lowest activity (0.11 U/mg protein). T₃ (6 mg ZnONPs/g diet) and T₄ (9 mg ZnONPs/g diet) showed intermediate activities. These results suggest that ZnONPs exposure at moderate concentrations T₂ (3 mg/g) might stimulate CAT activity in the gills. Conversely, liver CAT activity showed a different pattern. T₁ displayed the highest activity (0.13 U mg/protein), but all ZnONPs treatments (T₂ to T₄) exhibited lower activities. These findings are consistent with Abou-Zeid et al. (2023), who reported reduced hepatic CAT activity in fish exposed to ZnONPs.

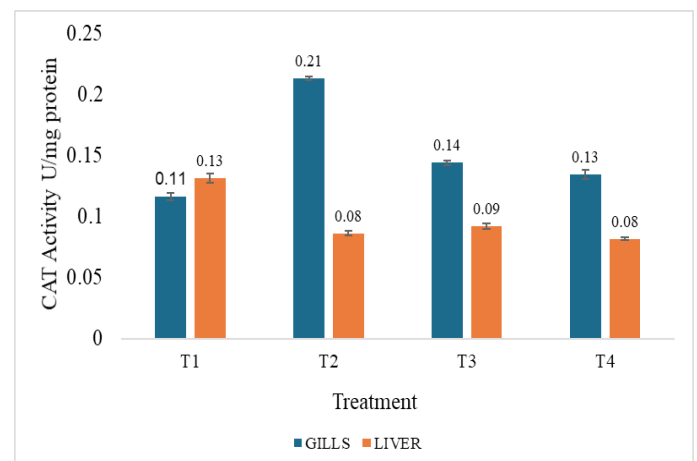


Figure 3: Catalase (CAT) enzymes in the liver and gills with varying concentrations and without supplementation of ZnONPs in the diet.

CAT is a crucial antioxidant enzyme that detoxifies hydrogen peroxide (H₂O₂), a product of superoxide dismutase activity. Reduced CAT activity in the liver suggests its potential depletion due to increased H₂O₂ detoxification demands. Conversely, the observed increase in gill CAT activity might be a compensatory response to mitigate ZnONPs-induced oxidative stress (Goth et al., 2004). CAT regulates various physiological processes like apoptosis, carbohydrate metabolism, and cell proliferation (Goth et al., 2004). Therefore, the observed changes in CAT activity warrant further investigation to understand their potential impact on fish health.

Table 2: The acetylcholinesterase (AChE) activity of the experimental fish in varying concentrations and without supplementation of ZnONPs in the diet.

Parameter	Treatment				SEM	P value
	T ₁ , Control	T ₂ , 3 mg ZnONPs/g	T ₃ , 6 mg ZnONPs/g	T ₄ , 9 mg ZnONPs/g		
AChE	0.47	0.47	0.47	0.49	0.098	0.951

SEM: standard error of the mean.

GLUTATHIONE S – TRANSFERASE (GST) IN LIVER AND GILLS

GST activity was significantly affected by dietary ZnONPs concentration in both liver and gill tissues ($P<0.05$) (Figure 4). In the gills, T₂ (3 mg ZnONPs/g diet) exhibited the highest GST activity (0.26 U/mg protein), while T₃ (6 mg ZnONPs/g diet) displayed the lowest activity (0.15 U/ mg protein). The control group (T₁) and T₄ (9 mg ZnONPs/g diet) had intermediate activities (0.17 U/mg protein and 0.18 U/mg protein, respectively). Conversely, liver GST activity followed a different pattern. The control group (T₁) exhibited the highest activity (0.16 U/mg protein), followed by T₄ (9 mg ZnONPs/g diet, 0.14U/mg protein). T₂ (3 mg ZnONPs/g diet) and T₃ (6 mg ZnONPs/g diet) showed similar activities (0.13U/mg protein each).

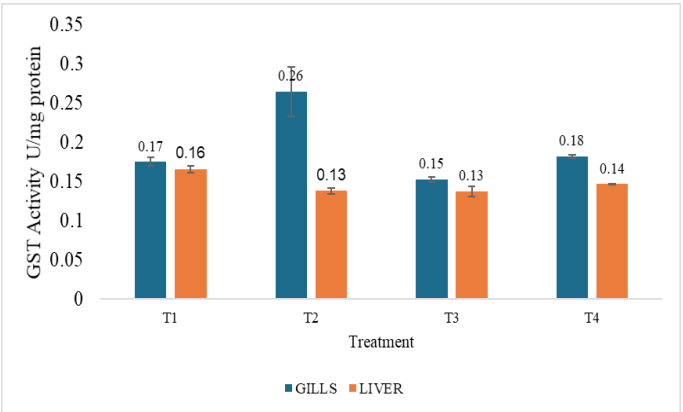


Figure 4: Glutathione S transferase enzyme activities in the liver and gills with varying concentrations and without supplementation of ZnONPs in the diet.

GST enzymes are key components of the phase II detoxification system, responsible for metabolizing and eliminating various toxins, including environmental carcinogens, xenobiotics, reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Dasari et al., 2017).

NEUROTOXICITY

This study investigated the potential neurotoxic effects of dietary ZnONPs exposure in fish by measuring brain Acetylcholinesterase (AChE) activity (Table 2). AChE is a vital enzyme responsible for the nervous system’s acetylcholine (AChE) breakdown. Inhibition of AChE activity is a well-established indicator of neurotoxicity. Although T₄ (9 mg/g ZnONPs diet) exhibited the numerically highest AChE activity (0.4906 U/mg protein)

in Table 2, there were no statistically significant differences among treatments ($P>0.05$). The remaining treatment groups (Control: 0.475 U/mg protein, T₂: 0.4773 U/mg protein, and T₃: 0.4723 U/mg protein) also displayed similar AChE activity levels. Exposure to nanoparticles decreased acetylcholinesterase activity, either in the muscle or brain, depending on the composition of the particles (Miranda et al., 2016).

These findings suggest that ZnONPs exposure at the tested concentrations did not induce overt neurotoxicity in fish, as measured by AChE activity. However, it is important to acknowledge that previous research has linked high-dose ZnONPs administration to neurotoxicity in fish (Affi et al., 2016). This neurotoxicity may manifest as apoptotic and neurodegenerative brain lesions, potentially leading to the observed behavioral alterations in other studies. Therefore, further investigation is necessary to explore the long-term effects of ZnONPs exposure on fish neurochemistry and behavior at environmentally relevant concentrations.

CONCLUSION AND RECOMMENDATIONS

Dietary ZnONPs supplementation at the concentrations evaluated (3-9 mg/g diet) did not significantly improve fish survival or average weight. However, ZnONPs exposure did influence the activity of several antioxidant enzymes. Gill SOD activity was significantly reduced in fish exposed to the highest ZnONPs concentration (T₄), suggesting potential oxidative stress in this tissue. Conversely, liver SOD activity was not significantly affected by ZnONPs treatment. Catalase (CAT) activity displayed a differential response between tissues. Gill CAT activity was highest in fish fed the moderate ZnONPs concentration (3 mg/g diet), while liver CAT activity decreased across all ZnONPs treatments compared to the control.

Similarly, glutathione S-transferase (GST) activity was significantly affected by ZnONPs exposure in both gills and liver. Notably, T₂ (3 mg/g diet) exhibited the highest GST activity in both tissues. Interestingly, AChE activity, a biomarker of neurotoxicity, was not significantly influenced by ZnONPs exposure at any concentration tested. These findings suggest that ZnONPs exposure can modulate antioxidant enzyme activity in fish, with potential tissue-specific effects. The observed decrease in gill SOD activity

and increase in gill CAT activity at moderate ZnONPs concentrations (T_2) might represent compensatory responses to oxidative stress.

Further research is warranted to elucidate the underlying mechanisms of these responses. These studies should validate the current findings under commercially relevant conditions and explore the long-term effects of ZnONPs exposure at environmentally relevant concentrations. Additionally, investigating the mechanisms by which ZnONPs modulates antioxidant enzyme activity in different fish tissues and evaluating the effects on other physiological parameters, such as immune function and reproductive health, would be valuable. Finally, repeating the study using a different fish species would provide insights into the broader applicability of these observations.

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NOVELTY STATEMENT

This study introduces a novel method for investigating the potential antimicrobial effects of zinc oxide nanoparticles synthesized by probiotics in tilapia.

AUTHOR'S CONTRIBUTION

Conceptualization, K.M.B.A., B.V.; methodology, K.M.B.A., R.S., validation, K.M.B.A. and B.V.; statistical analysis, K.M.B.A.; writing—original draft preparation, K.M.B.A.; investigation, K.M.B.A., R.S., B.V.; preparation of manuscript, K.M.B.A.; editing and revision, K.M.B.A.; supervision, B.V. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

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