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RESEARCH ARTICLE

Antimicrobial Peptide Nisin targets *Pseudomonas aeruginosa* and mitigates the Inflammation in Zebrafish Larvae

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Abstract

Pseudomonas aeruginosa is an opportunistic gram-negative bacterium known for its high virulence and antibiotic resistance, making alternative therapeutic approaches essential. Antimicrobial peptides like Nisin, a lantibiotic with bactericidal activity, have shown promising effects against various bacterial infections. This study evaluates the antimicrobial and anti-inflammatory effects of Nisin (200 µg/mL) against *P. aeruginosa* infection in zebrafish larvae. Larvae were exposed to *P. aeruginosa* (1×10^6 CFU/mL) for 24 hours, followed by Nisin treatment for an additional 24 hours. Bacterial load, survival rates, and histopathological changes were assessed to determine the peptide's therapeutic potential. Additionally, oxidative stress markers—including reactive oxygen species, nitric oxide levels, and lipid peroxidation were analyzed to assess Nisin's anti-inflammatory effects. Apoptotic and oxidative stress responses were further investigated using acridine orange, diphenyl-1-pyrenylphosphin, and dichlorofluorescein diacetate fluorescence assays. Results demonstrated that Nisin significantly reduced bacterial load and improved survival rates, with notable suppression of ROS, NO production, and lipid peroxidation. Histopathological analysis confirmed that Nisin mitigated tissue damage and inflammatory responses, suggesting its potential as an effective antimicrobial agent against *P. aeruginosa* infections. This study underscores the dual role of Nisin in both bacterial clearance and inflammation reduction, supporting its use as an alternative or adjunct therapy for antibiotic-resistant bacterial infections. Further molecular studies are required to elucidate its mechanisms of action in host-pathogen interactions.

1. Introduction

Pseudomonas aeruginosa is an opportunistic, gram-negative bacterium responsible for a wide range of infections in both humans and aquatic organisms (Algammal et al., 2020). It is particularly known for its ability to form biofilms, exhibit multidrug resistance, and secrete virulence factors that contribute to disease progression. *P. aeruginosa* infections often result in severe inflammatory responses, tissue damage, and oxidative stress (Ruffin and Brochiero, 2019), making them difficult to manage with conventional antibiotics. The rapid emergence of antibiotic-resistant strains has led to an urgent need for alternative therapeutic strategies to combat P. aeruginosa-related infections.

Antimicrobial peptides (AMPs) have gained significant attention as potential alternatives to conventional antibiotics due to their broad-spectrum antibacterial properties and unique mechanisms of action (Wang et al., 2019). Nisin, a lantibiotic produced by Lactococcus lactis, has been widely studied for its potent antimicrobial effects against gram-positive bacteria (Thanjavur et al., 2022). However, recent research suggests that Nisin also exhibits bactericidal activity against certain gram-negative pathogens, including *P. aeruginosa* (Vukomanović et al., 2017), by disrupting bacterial membrane integrity and inhibiting biofilm formation. Additionally, Nisin has been reported to modulate inflammatory responses by reducing cytokine overexpression and oxidative stress (Derakhshan-sefidi et al., 2024), further supporting its potential as a therapeutic agent.

Oxidative stress plays a crucial role in bacterial infections by promoting tissue damage and inflammation (Shastri et al., 2018). Pathogenic bacteria like *P. aeruginosa* induce excessive reactive oxygen species production, leading to lipid peroxidation, protein oxidation, and DNA damage (Vetrivel et al., 2023). These oxidative imbalances trigger inflammatory pathways that exacerbate infection-related complications. Nitric oxide, another key inflammatory marker, is often elevated during bacterial infections, contributing to immune dysregulation and cellular toxicity (Mishra et al., 2017). Therefore, therapeutic agents that can effectively combat bacterial proliferation while simultaneously reducing oxidative stress and inflammation hold significant promise.

Zebrafish larvae provide an excellent in vivo model for studying bacterial infections and evaluating the efficacy of antimicrobial agents (Boucontet et al., 2018). Their transparent embryos enable real-time monitoring of bacterial colonization, inflammation, and oxidative stress responses.

Additionally, zebrafish share key immune system components with mammals, making them a valuable model for investigating host-pathogen interactions and therapeutic interventions (Li et al., 2017). Previous studies have demonstrated the feasibility of using zebrafish to evaluate the antimicrobial activity of various peptides and plant-derived compounds against bacterial infections (Aleksic et al., 2018). By investigating the dual role of Nisin in bacterial inhibition and inflammation reduction, this study aims to provide new insights into the therapeutic potential of antimicrobial peptides against antibiotic-resistant bacterial infections. Understanding the molecular interactions between Nisin, *P. aeruginosa*, and host immune responses could contribute to the development of alternative treatments for bacterial infections in both medical and aquaculture applications.

2. Materials and Methods

2.1. Zebrafish Maintenance and Experimental Setup

Zebrafish embryos were collected from healthy adult fish maintained at 28 ± 1°C under a 14:10-hour light-dark cycle. The embryos were reared in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) in a controlled laboratory environment. At 5 days post-fertilization (dpf), larvae were divided into five groups: (1) Control (untreated larvae), (2) *P. aeruginosa*-infected group (1×10^6 CFU/mL), (3) Nisin 50 µg/mL-treated group post-infection, (4) Nisin 100 µg/mL-treated group post-infection. Bacterial infection was induced by immersing zebrafish larvae in a *P. aeruginosa* suspension for 24 hours, followed by treatment with Nisin for an additional 24 hours. Larvae were monitored for survival, morphological changes, and behavioral alterations (Barreiros et al., 2021).

2.2. Protective Effect of Nisin Against P. aeruginosa in Zebrafish

To assess the protective effect of Nisin, zebrafish larvae were observed for signs of infection such as body deformities, impaired motility, and reduced survival rates. The protective effects were quantified by measuring survival percentages and scoring morphological integrity using stereomicroscopic analysis (Nayak et al., 2023).

2.3. Analysis of Antioxidant Enzyme Activity

Whole-body homogenates were prepared in ice-cold PBS (pH 7.4) using a probe sonicator. The homogenates were centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatants were used for enzymatic assays: Superoxide Dismutase (SOD) activity was measured using a colorimetric assay, where inhibition of nitroblue tetrazolium (NBT) reduction by superoxide radicals was detected at 560 nm. Results were expressed as U/mg protein. Catalase (CAT) activity was analyzed by monitoring hydrogen peroxide breakdown at 240 nm. Enzyme activity was expressed in U/mg protein. Glutathione S-Transferase (GST) activity was assessed using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, and absorbance was recorded at 340 nm. Enzyme activity was expressed as U/mg protein. Glutathione (GSH) levels were measured using Ellman's reagent (DTNB) at 412 nm, and results were expressed as nmol/mg protein (Agraharam et al., 2021; Kuder and Philip, 2017; Wang et al., 2020).

2.4. Lipid Peroxidation and Nitric Oxide (NO) Quantification

Lipid Peroxidation (MDA Levels): Malondialdehyde (MDA) levels were determined using the thiobarbituric acid reactive substances (TBARS) assay. Homogenates were incubated with thiobarbituric acid at 95°C for 30 minutes, and absorbance was measured at 532 nm. Results were expressed as nmol MDA/mg protein (Wei et al., 2018).

Nitric Oxide (NO) Levels: NO production was quantified using the Griess reagent. Supernatants were mixed with Griess reagent, and absorbance was measured at 540 nm to assess nitrite accumulation (Muthulakshmi et al., 2018).

2.5. Fluorescence-Based Assays for Oxidative Stress and Apoptosis

Acridine Orange (AO) assay was used for Apoptosis Detection. Zebrafish larvae were stained with acridine orange (5 µg/mL) for 30 minutes in the dark, washed with PBS, and imaged under a fluorescence microscope. Fluorescence intensity was analyzed using ImageJ software (Murugan et al., 2022b). DPPP Assay was used for Lipid Peroxidation. Larvae were incubated with diphenyl-1-pyrenylphosphine (DPPP, 5 µM) for 30 minutes, washed with PBS, and analyzed using fluorescence microscopy (Guru and Arockiaraj, 2023). DCFDA Assay was used for ROS Quantification. Zebrafish larvae were exposed to 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, 20 µM) for 30 minutes in darkness, washed with PBS, and analyzed under a fluorescence microscope. ROS levels were quantified using ImageJ (Murugan et al., 2022a).

2.6. Statistical Analysis

All experimental data were analyzed using GraphPad Prism 9 software. Results were expressed as mean ± standard deviation (SD). One-way ANOVA followed by Tukey's post hoc test was used to determine statistical significance, with p-values < 0.05 considered significant.

3. Result

3.1. Protective Effect of Nisin Against P. aeruginosa in Zebrafish

Zebrafish larvae infected with *P. aeruginosa* exhibited severe morphological abnormalities, including body curvature, pericardial edema, and erratic swimming patterns. Additionally, a significant decline in survival rates was observed in infected larvae (27%) (Figure 1). However, treatment with Nisin at 200 µg/mL (93%) markedly improved survival rates and reduced infection-related deformities. Nisin-treated larvae exhibited enhanced mobility and improved structural integrity compared to the infected untreated group, demonstrating the protective effect of the antimicrobial peptide against P. aeruginosa-induced damage.

3.2. Effect of Nisin on Antioxidant Enzyme Activity

Infection with *P. aeruginosa* significantly lowered SOD activity, leading to a compromised antioxidant defense system. Treatment with Nisin restored SOD activity, with the highest recovery observed at 200 µg/mL (28 U/mg of protein), suggesting enhanced superoxide radical neutralization. The bacterial infection resulted in a marked suppression of CAT activity (3 µmol/mg of protein), increasing oxidative stress. Nisin treatment significantly improved CAT activity (18 µmol/mg of protein), demonstrating its potential role in enhancing cellular detoxification and protection against oxidative damage (Figure 2). Infected larvae showed a decline in GST activity (14 nmol/mL), indicating impaired detoxification

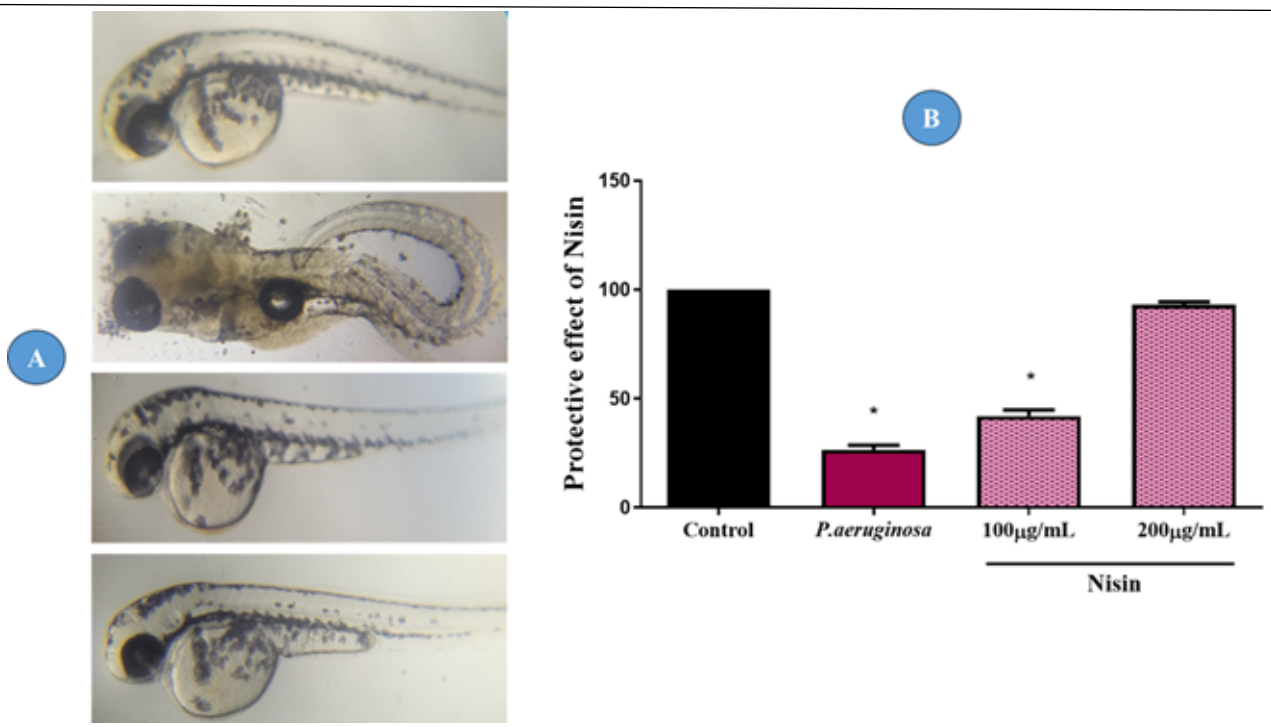

Figure 1: Microscopic images depicting structural integrity of zebrafish larvae post-infection, whereas larvae treated with Nisin at 100 µg/mL and 200 µg/mL (n = 50 larvae/group). * represent significant difference at $p < 0.05$.

capacity. Nisin-treated groups exhibited a significant enhancement in GST activity, with 200 µg/mL (0.33 nmol/mL) showing the greatest improvement, reinforcing its role in cellular defense. Infection depleted intracellular GSH stores (3 nmol/mg of protein), further exacerbating oxidative imbalance. Nisin supplementation restored GSH levels (9 nmol/mg of protein), suggesting that it plays a crucial role in maintaining redox homeostasis.

3.3. Impact of Nisin on NO Levels and Lipid Peroxidation

P. aeruginosa infection caused a significant increase in NO levels (65 µM), indicating an inflammatory response. Treatment with Nisin effectively reduced NO production, with the highest reduction at 200 µg/mL (27 µM), demonstrating its anti-inflammatory potential (Figure 3). Elevated MDA levels in infected zebrafish larvae suggested extensive lipid peroxidation and oxidative damage (61%). Nisin treatment significantly lowered MDA levels, with 200 µg/mL (18%) exhibiting the most pronounced decrease, highlighting its protective role against oxidative membrane degradation (Figure 4).

3.4. Reduction of ROS, Apoptosis, and Lipid Peroxidation

A significant increase in ROS accumulation was observed in infected larvae, as indicated by the strong fluorescence intensity in the DCFDA assay. Nisin-treated groups showed a remarkable reduction in ROS levels, with 200 µg/mL providing the strongest antioxidant effect (Figure 5). Infection with *P. aeruginosa* led to a substantial increase in apoptotic cell numbers, as observed through AO staining. Nisin treatment significantly reduced apoptosis, with 200 µg/mL displaying the highest reduction, supporting

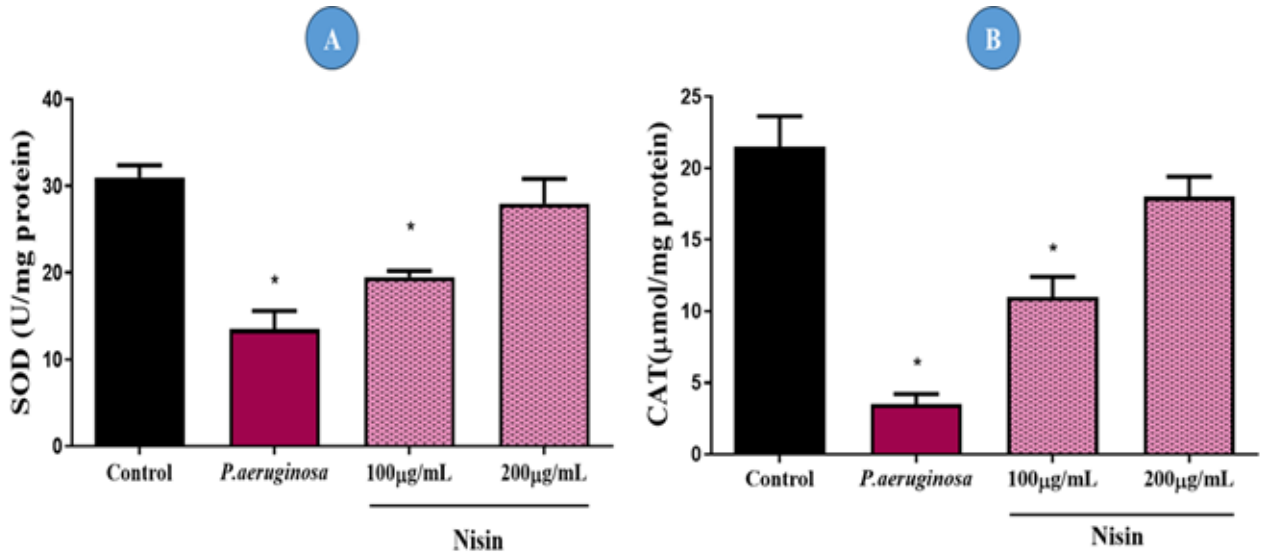

Figure 2: Quantitative analysis showing the impact of nisin treatment on SOD and CAT levels in zebrafish larvae (n = 50 larvae/group). * represent significant difference at $p < 0.05$.

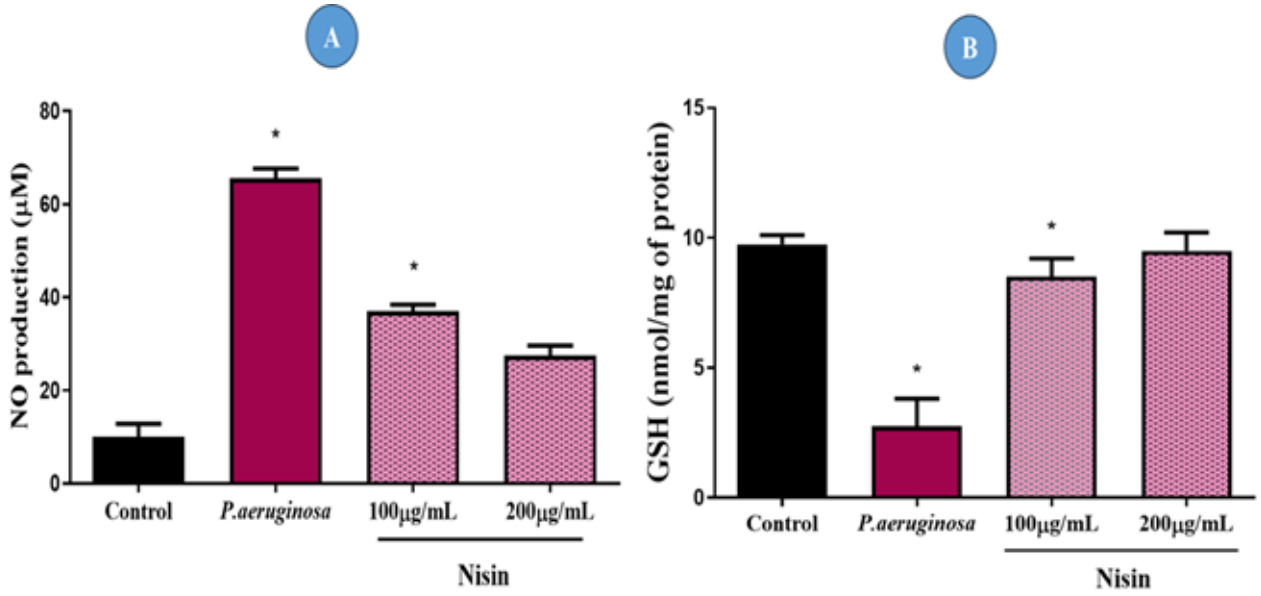

Figure 3: Quantitative analysis showing the impact of nisin treatment on NO and GSH levels in zebrafish larvae (n = 50 larvae/ group). * represent significant difference at $p < 0.05$.

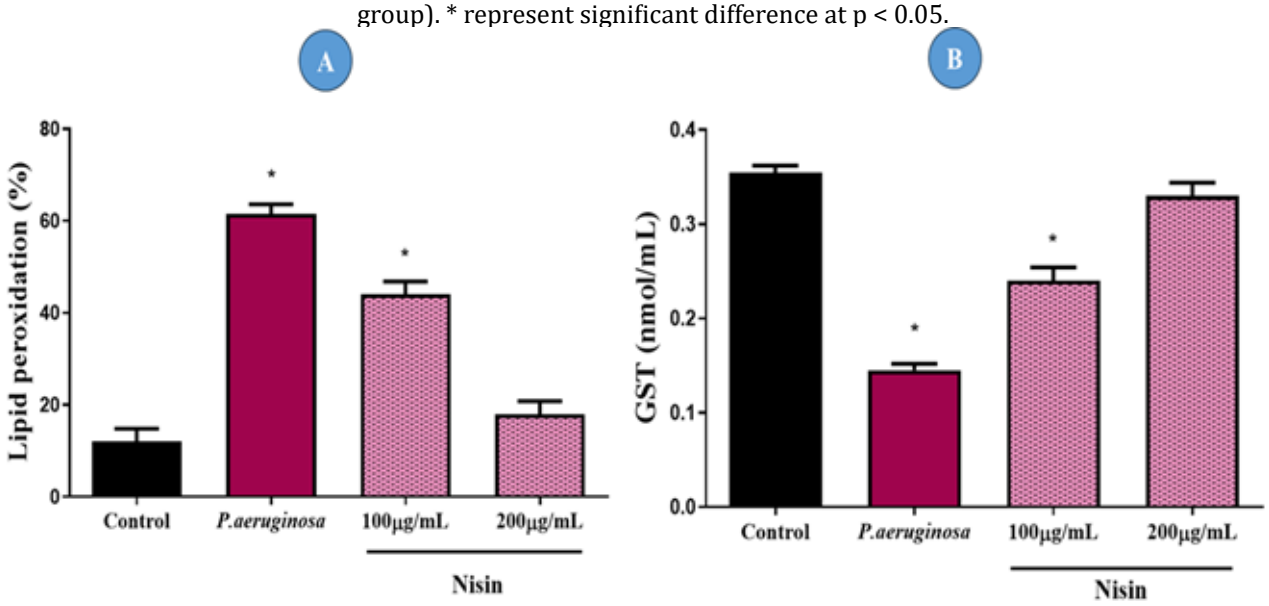

Figure 4: Quantitative analysis showing the impact of nisin treatment on Lipid peroxidation and GST levels in zebrafish larvae (n = 50 larvae/group). * represent significant difference at $p < 0.05$.

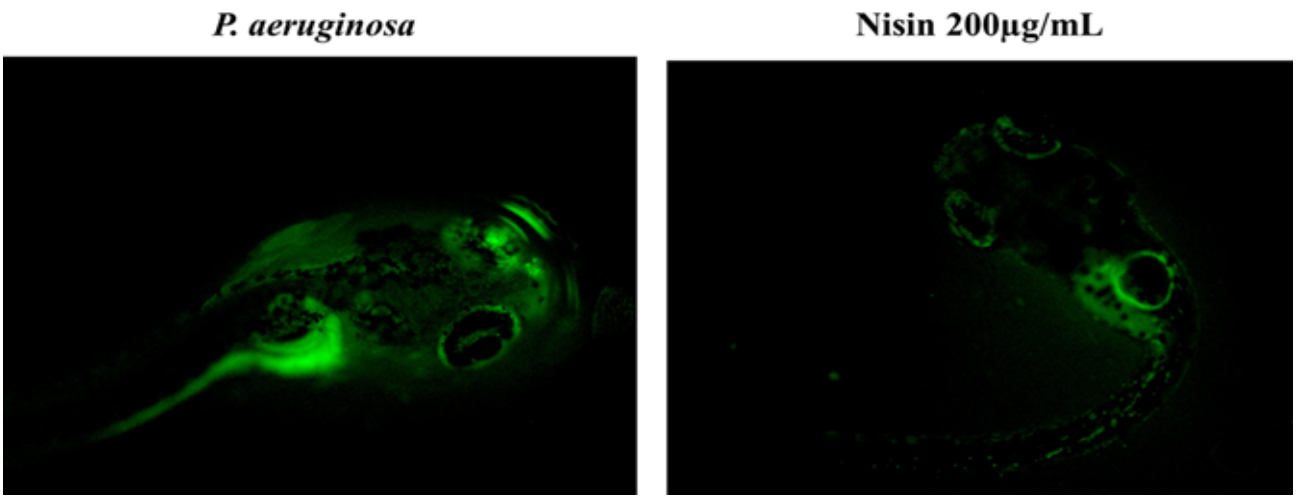

Figure 5: Representative fluorescence images of zebrafish larvae stained with DCFDA to assess ROS accumulation. (A) *Pseudomonas aeruginosa*-infected larvae and (B) Nisin-treated group (200 µg/mL)

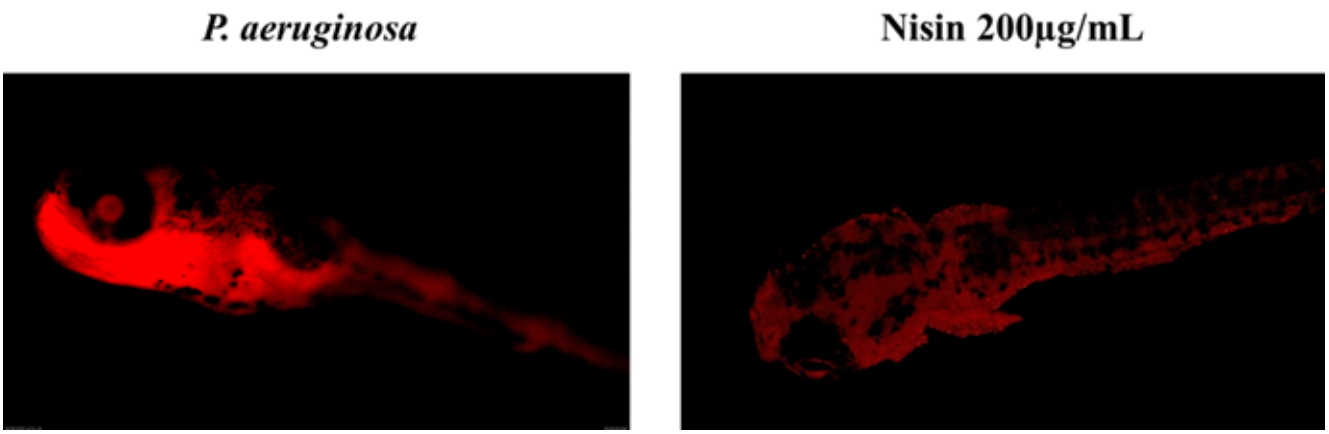

Figure.6 :Fluorescence microscopy images showing apoptotic cells in zebrafish larvae stained with AO. (A) *Pseudomonas aeruginosa*-infected larvae and (B) Nisin-treated group (200 µg/mL)

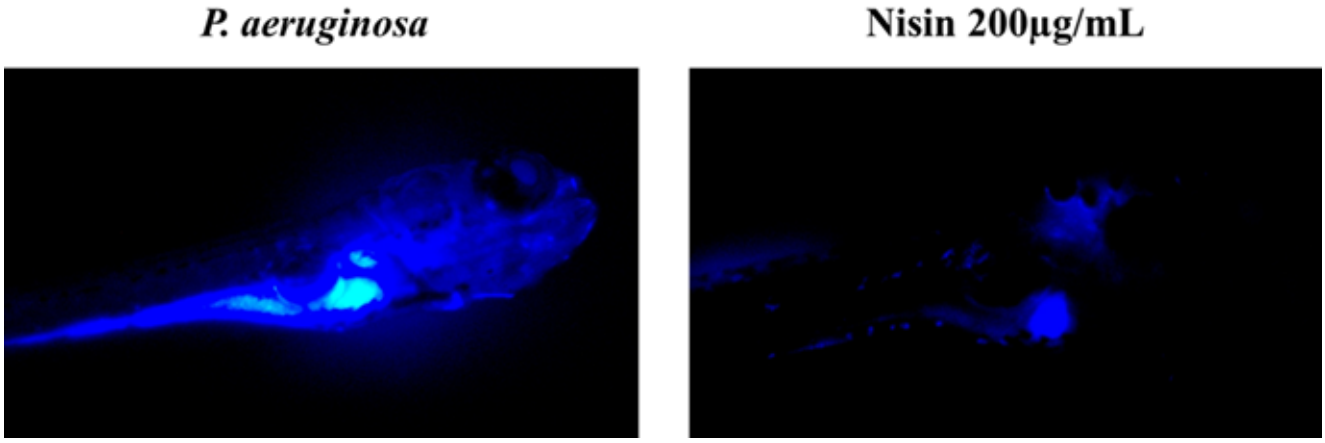

Figure 7: Fluorescence microscopy images of zebrafish larvae stained with DPPP to detect lipid peroxidation. (A) *Pseudomonas aeruginosa*-infected larvae and (B) Nisin-treated group (200 µg/mL)

its role in cellular protection (Figure 6) Infected zebrafish larvae exhibited elevated lipid peroxidation, as evidenced by increased fluorescence intensity in the DPPP assay. Nisin treatment reduced lipid peroxidation, with 200 µg/mL showing the greatest suppression, suggesting its potential in preserving membrane integrity (Figure 7).

4. Result

The findings of this study underscore the potential of Nisin as an effective antimicrobial agent against *P. aeruginosa* infections. The significant improvements in survival rates and reductions in morphological deformities observed in zebrafish larvae following Nisin treatment indicate its protective role against bacterial-induced pathogenesis. These results align with prior research highlighting the efficacy of antimicrobial peptides in reducing bacterial virulence and enhancing host resilience. One of the primary challenges in *P. aeruginosa* infections is the excessive generation of reactive oxygen species, which disrupt cellular homeostasis and lead to oxidative damage (Lam et al., 2020). In this study, Nisin-treated groups exhibited a substantial decrease in ROS levels, confirming its role in mitigating oxidative stress. The elevation of antioxidant enzymes, particularly SOD and CAT, further supports the ability of Nisin to enhance endogenous defense mechanisms. These findings are consistent with previous studies that have demonstrated the antioxidative effects of antimicrobial peptides in bacterial infections (Li et al., 2023).

Inflammation is a critical factor in bacterial infections, often exacerbated by increased nitric oxide production (Uehara et al., 2015). The significant reduction in NO levels in Nisin-treated zebrafish larvae suggests that the peptide plays a role in modulating inflammatory responses. This is in agreement with studies on other antimicrobial peptides that have shown their ability to downregulate inflammatory mediators and reduce infection-induced tissue damage. Lipid peroxidation, measured through MDA levels (Mas-Bargues et al., 2021), was another key parameter evaluated in this study. The elevated MDA levels in *P. aeruginosa*-infected larvae indicate extensive membrane damage due to oxidative stress. The marked reduction in MDA levels following Nisin treatment suggests that it effectively preserves cellular integrity by preventing lipid peroxidation. This aligns with previous studies demonstrating that antimicrobial peptides not only target bacterial cells but also provide protection against oxidative membrane damage in host cells (Choi et al., 2017).

The apoptosis analysis using acridine orange staining revealed a significant reduction in apoptotic cell numbers in Nisin-treated groups. This suggests that Nisin helps to maintain cellular viability by reducing programmed cell death triggered by *P. aeruginosa* infection. These findings support earlier reports that antimicrobial peptides can regulate apoptotic pathways and enhance host survival under bacterial stress conditions (Oyinloye et al., 2015). The results from fluorescence-based assays further confirmed the protective role of Nisin in mitigating oxidative stress,

lipid peroxidation, and apoptosis. The DCFDA assay showed that ROS accumulation was significantly lower in treated groups, while the DPPP assay demonstrated reduced lipid peroxidation, reinforcing Nisin's ability to combat bacterial-induced oxidative damage.

Overall, this study highlights the dual functionality of Nisin as an antimicrobial and anti-inflammatory agent. By inhibiting bacterial proliferation, reducing oxidative stress, and modulating inflammatory responses, Nisin presents itself as a strong candidate for therapeutic applications against antibiotic-resistant bacterial infections. Future research should focus on elucidating the specific molecular pathways through which Nisin exerts its effects and exploring its potential in combination therapies with existing antibiotics to enhance treatment efficacy.

5. Conclusion

The results of this study confirm that Nisin effectively combats *P. aeruginosa* infections in zebrafish larvae by enhancing bacterial clearance, reducing oxidative stress, and mitigating inflammation. These findings support the potential application of Nisin as an alternative antimicrobial therapy for bacterial infections. Further research is warranted to explore its mechanisms of action and clinical applications.

Declarations

Ethics approval statement

No ethical approval was required for the current study as it did not deal with any human or animal samples.

Consent to participate

Not applicable

Consent to publish

Not applicable

Data Availability Statement

The data are available from the corresponding author upon reasonable request

Competing Interests

The authors declare that they have no conflict of interest

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Not Applicable

Author contribution

Conceptualization, Data curation, Investigation: A.O. Formal analysis. Writing—review and editing. All authors have read and agreed to the published version of the manuscript

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Not Applicable

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