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*Research Article*

# Antibacterial properties of rainbow trout (*Oncorhynchus mykiss*) pro-hepcidin as a new approach against bacterial disease in fish

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**Abstract**

This study aimed to produce and evaluate the antibacterial activity of recombinant rainbow trout pro-hepcidin (rpro-RtHep). The gene sequence encoding rainbow trout pro-hepcidin was synthesized and cloned into the pET28a+ expression vector. The recombinant vector pET28a+/pro-RtHep was transformed into *Escherichia coli* BL21 (DE3) Rosetta for protein expression. The recombinant bacteria were cultured at 37°C and induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The presence of a 10 kDa band on SDS-PAGE confirmed successful peptide expression; subsequently, purification was carried out using affinity chromatography, and the single 10 kDa band was verified by SDS-PAGE. The biological activity of purified rpro-RtHep was evaluated by examining its inhibitory effects on the growth of selected *Aeromonas hydrophila, Streptococcus iniae* and *Yersinia ruckeri*. The results showed that rpro-RtHep effectively inhibited the growth of these bacterial species. Additionally, the survival rate of zebrafish (*Danio rerio*) and with the rpro-RtHep (25 µg $g^{-1}$) was assessed and the results revealed that survival rate was significantly higher compared to that of the control group exposed to *A. hydrophila*. Immune factors, including lysozyme levels, ACH50, total protein, and total immunoglobulin demonstrated significant increases in fish injected with rpro-RtHep compared to the control group ($p$<0.05). The study demonstrated that rpro-RtHep protects zebrafish against *A. hydrophila* infection via regulating of immune factors.



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## Introduction

The rapid expansion of intensive aquaculture has raised growing concerns over disease outbreaks and associated public health risks (Bhat *et al*., 2024). Among the pathogens affecting cultured fish, bacteria represent a major group responsible for severe disease manifestations (Korzekwa *et al*., 2022; Naiel *et al*., 2023) Conventional treatment involves the use of antibiotics, but their efficacy is variable, and concerns regarding antibiotic residues in fish tissue pose potential health risks to consumers. Moreover, the widespread and often indiscriminate application of antibiotics has driven the emergence of antibiotic-resistant bacterial strains, complicating disease management and posing a significant threat to both aquaculture and public health. Developing alternative strategies to control resistant bacteria has become a critical priority in veterinary and human medicine. One promising approach involves replacing traditional antibiotics with antimicrobial peptides (AMPs) (Bhat *et al*., 2024).

AMPs are integral components of the innate immune defenses in a broad range of organisms, including mollusks, crustaceans, and vertebrates. These small, typically cationic peptides exert antimicrobial activity primarily through interactions with and disruption of microbial membranes (Valero *et al*., 2020). Their structural diversity has evolved in tandem with pathogen evolution, enabling them to counteract a wide array of microbial threats and reduce the risk of resistance development (Katzenback, 2015). Additionally, AMPs can modulate host immune responses by recruiting immune cells to infection sites and stimulating immune activity (Valero *et al*., 2020).

Hepcidin is a cysteine-rich, positively charged antimicrobial peptide primarily produced in the hepatopancreas. Hepcidin plays a key role in iron regulation: it decreases iron levels by binding to ferroportin, the only known protein responsible for iron export in vertebrate cells, leading to its internalization and degradation (Chen *et al*., 2021). Additionally, hepcidin exhibits antiviral and antibacterial activities within the immune systems of various animals (Shirdel *et al*., 2019).

The most cost-effective method for producing AMPs involves recombinant expression in bacterial hosts, as natural extraction is often economically impractical and raises ethical concerns, particularly at larger scales. Recombinant production has been successfully utilized to generate various AMPs from diverse plant and animal sources (Kalbassi *et al*., 2024). So that this study contributes to the expanding body of research on recombinant protein production and provide valuable insights into developing efficient methods for producing functional recombinant pro-Rainbow trout hepcidin (rpro-RtHep) as a new strategy against *A*. *hydrophila*.

## Materials and methods

All experiments were conducted in accordance with the protocol approved by the ethics committee at the Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran.

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*Production of recombinant rainbow trout pro-hepcidin (Rtpro-Hep)*

A full length of pro-hepcidin of rainbow trout (*Oncorhynchus mykiss*) was acquired from the National Center for Biotechnology Information (XP_021450828.1) database and synthesized by Shainegene Company (Shanghai, China) and cloned into the pET28a+ expression vector. The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) cells. Post-transformation, expression conditions were optimized, and the recombinant pro-hepcidin was purified via affinity chromatography.

*In-vitro antibacterial activity*

Gram-negative bacteria including *Aeromonas hydrophila* (IBRC-M10814) and *Yersinia ruckeri* (IBRC-M11507) and a Gram-positive bacterium, *Streptococcus iniae* (GQ850377.1) were chosen for the experimental analysis. The bacteria strains were prepared from the Iranian Biological Resource Center (Tehran, Iran) and subsequently transferred to a microbiology laboratory (Amol, Iran). Following an incubation period in brain heart infusion broth (BHI), the bacterial cells were collected and adjusted to a concentration of $1\times10^8$ CFU/mL using physiological serum, then further diluted to $1\times10^6$ CFU/mL with BHI medium. The purified rpro-RtHep was utilized to prepare the desired concentrations (400, 200, 100, 50, 25, 12.5, and 6 µg/mL) in 96-well plates filled with BHI medium. Subsequently, 50 µl of prepared bacterial suspension was introduced into the wells and the microplate incubated at 37°C for 20 h (Shirdel *et al*., 2019). The minimum inhibitory concentration (MIC) of the peptide was determined as the lowest concentration at which bacterial growth was completely suppressed, resulting in a clear culture medium within the well. The minimal bactericidal concentration (MBC) was investigated by using l0 µL of samples from wells with no visible growth on LB agar plates. The plates were incubated at 37°C for overnight and bacterial growth was evaluated after 24 h.

The disc diffusion methodology for assessing antimicrobial susceptibility was conducted in accordance with the established protocol to determine of antibacterial efficacy of rpro-RtHep. A bacterial culture (adjusted to 0.5 McFarland standard) was used to Muller-Hinton agar plates uniformly using a sterile swab. The plates were dried for 15 minutes and then used for susceptibility testing. The discs coated with rpro-RtHep (100 and 200 µg/mL) were placed on the surface of Mueller-Hinton agar. Each test plate consisted of three discs including one positive control (standard commercial antibiotic disc, ciprofloxacin (CP5) for *A. hydrophila*, and Gentamicin (GM10) *Y. ruckeri* and two treated discs (100 and 200 µg/ml rpro-RtHep). The bacteria were incubated at 37°C for 24 h. After incubation, the plates were examined for the zone of inhibition. The zone of inhibition was then measured and recorded using a caliper. To ensure reliability, the experiment was repeated three times. Based on Clinical & Laboratory Standards Institute (CLSI, M100, VET01, and M45), *Aeromonas.* spp*, Y. ruckeri (Enterobacteriaceae)*, and *Streptococcus.* spp with the zones of inhibition ≥21, ≥16, and ≥24 mm was reported to be sensitive to CP and GM, respectively.

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*FE-SEM*

Prior to field emission scanning electron microscopy (FE-SEM) imaging, the slides were coated with a poly-L-lysine solution and allowed to dry at room temperature. After sample collection, bacterial cells were initially fixed with 2.5% glutaraldehyde at 4°C, followed by post-fixation with osmium tetroxide ($OsO_4$) to further stabilize cellular structures. Once fixation and en bloc treatment were completed, the fixatives were discarded and the samples were thoroughly rinsed with 0.1 M phosphate-buffered saline (PBS). A graded ethanol dehydration series (30%, 50%, 70%, 90%, and 100%) was then carried out, with each step lasting 5 minutes and repeated three times. Approximately 100 µL of the dehydrated sample was placed onto the prepared coverslips. The samples were stored in absolute ethanol until critical point drying, which was performed using $CO_2$ to remove extracellular fluid while preserving intracellular components. During drying, the chamber was kept at 10 °C, and six exchange cycles were performed to eliminate ethanol, after which the temperature was raised to 42°C and the pressure maintained above 85 bar until drying was complete. The dried specimens were mounted on aluminum stubs and coated with a thin platinum layer using a sputter coater to enhance conductivity. Finally, imaging was carried out using a FESEM instrument (FE-SEM, TESCAN, Brno, Czech Republic)

*In vivo protective efficacy of recombinant rpro-RtHep against* A. *hydrophila in zebrafish*

A total number of 60 healthy zebrafish (*Danio rerio*) with a mean weight of 0.4±0.01 g were prepared from a fish farm (Mazandaran, Iran) and moved to the research unit of the Faculty of Veterinary Medicine (Amol, Iran). The fish were kept in two tanks (20 L) and adapted to the experimental condition for two weeks. After two weeks, the fish were divided into two groups with 30 fish for each group (10 fish per tank) and were injected with PBS (control group) and 25 µg $g^{-1}$ rpro-RtHep. To challenge with prepared *A. hydrophila*, the amount of water was reduced to half, and the fish were immersed in a concentration of $1.7\times10^6$ CFU/mL of the bacterium (Esfahani *et al*., 2025). The fishes were then challenged with prepared *A. hydrophila* at 12 h post-injection (Mohapatra *et al*., 2019). The mortality rate was reported after 14 days of challenge.

*Sampling and measurements*

After 14 days, two fish per tank were randomly selected and were euthanized with 50 µl $l^{-1}$ clove oil. The heads and fins of the fish were detached and then placed in a 25 mM Tris-HCl solution at a pH of 7.2 for the assessment of immunological factors. Lysozyme activity in body homogenates was evaluated according to the methodology outlined by Joibari *et al*. (2025). The quantification of total protein was conducted utilizing an assay kit procured from Pars Azmun Company in accordance with established protocols. The measurement of total immunoglobulin was

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performed following the methodology delineated by Siwicki *et al*. (1994). The assessment of alternative complement activity (ACH50) in fish homogenates was conducted following the procedure proposed by Joibari *et al*. (2025).

*Statistical analysis*

One-way analysis of variance (ANOVA) and a Tukey post hoc test was employed to evaluate the statistically significant differences among means in the *in vitro* antibacterial assay (inhibition zone), while a T-test was utilized for the analysis of the *in vivo* antibacterial test conducted in zebrafish using SPSS software version 2023. All data were presented as mean±standard deviation (SD).

## Results

*Production of rpro-RtHep*

The rpro-RtHep was cloned into the pET28a+ expression vector and the recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) cells (Fig. 1). It was expressed as in the *E. coli* BL21 and the band of peptide matched the predicted molecular mass of 10 kDa (Fig. 2). SDS-PAGE analysis of purified peptide showed a clear band of rpro-RtHep at about 10 kDa (Fig. 3). The concentration of rpro-RtHep was 2 mg/mL.

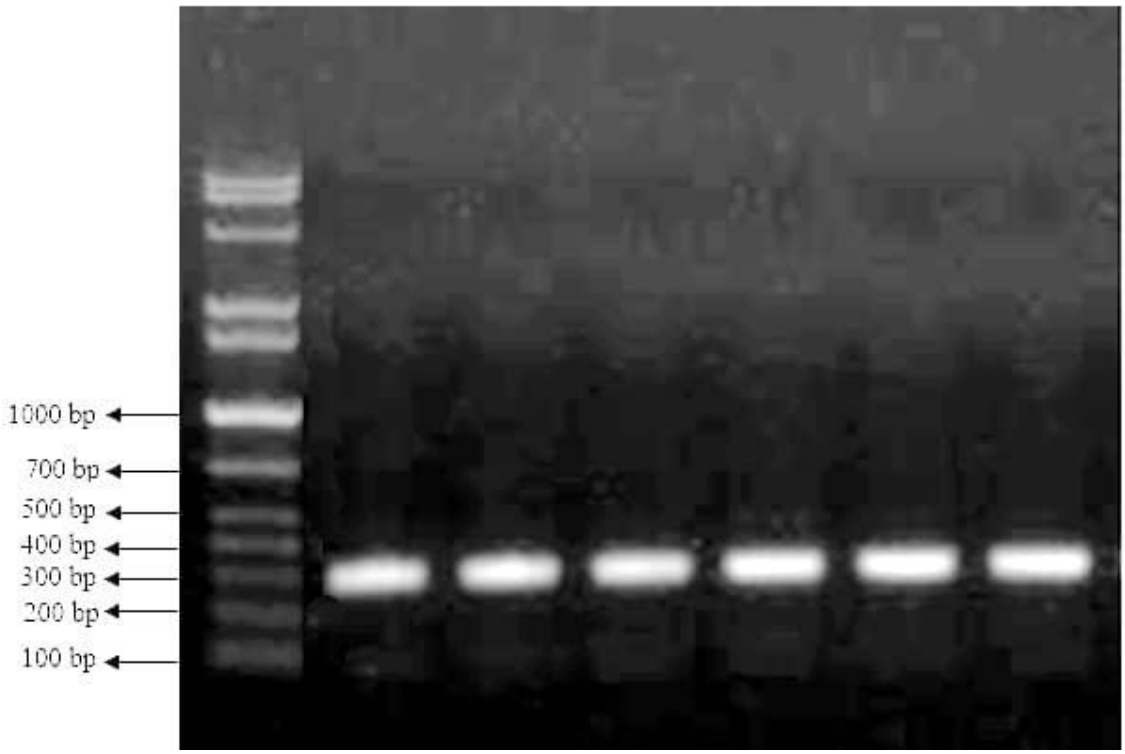


**Figure 1: Agarose gel electrophoresis of PCR colony. Line 1, DNA molecular weight marker; Line 2-6, the band of 294 bp of pro-hepcidin gene.**

*In vitro antibacterial activity*

The MIC values of rpro-RtHep were reported as 50 µg/mL for *S. iniae*, 100 µg/mL for *A. hydrophila*, and 200 µg/mL for *Y. ruckeri* . The MBC values of rpro-RtHep were 50 µg/mL for *S. iniae*, 100 µg/mL for *A. hydrophila*, and 400 µg/mL for *Y. ruckeri* (Table 1).

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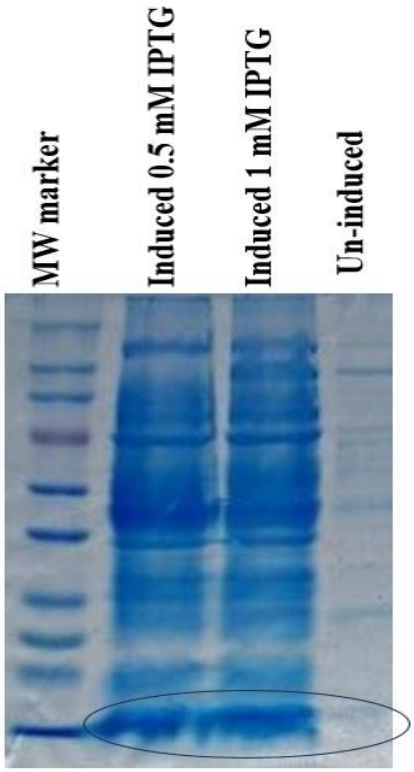


**Figure 2: SDS-PAGE gel electrophoresis of the rpro-RtHep.**

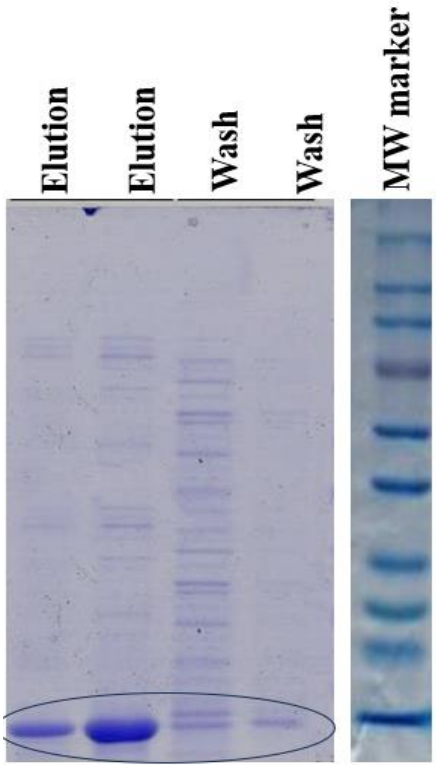


**Figure 3: SDS-PAGE gel electrophoresis of the purified rpro-RtHep.**

**Table 1: The MICs and MBCs of recombinant pro-RtHep.**

| Bacterial strain/Diluting concentration (µg/mL) | 400 | 200 | 100 | 50 | 25 | 12.5 | 6 | MIC[1] (µg/ml) | MBC[2] (µg/mL) |
|---|---|---|---|---|---|---|---|---|---|
| *A. hydrophila* | - | - | - | + | + | + | + | 100 | 100 |
| *Y. ruckeri* | - | - | + | + | + | + | + | 200 | 400 |
| *S. iniae* | - | - | - | - | + | + | + | 50 | 50 |
| Negative control | + | + | + | + | + | + | + | / | / |

MIC[1]: Minimal inhibitory concentration of the antimicrobial peptide; MBC[2]: Minimal concentration of antimicrobial peptides required to kill 99.99% of bacteria.
+: bacterial growth observed
–: no bacterial growth observed

The inhibition zone of *S. iniae* against rpro-RtHep at the concentration 100 and 200 µg/mL and P10 were 14, 18, and 12.1 mm and notable bactericidal effects were observed for different concentrations of rpro-RtHep and antibiotics ( $p<0.05$). The inhibition zone of *A. hydrophila* against rpro-RtHep at the concentration 100 and 200 µg/mL and CP5 were 24.3, 28, and 27.6 mm respectively and no significant difference was found between the different concentrations of rpro-RtHep and the CP5 ($p>0.05$). The zone of inhibition of growth in *Y. ruckeri* against different doses of rpro-RtHep and antibiotic GM10 was 19, 24.8, and 14.1 mm, respectively, and there was a significant difference between antibiotic GM10 and different peptide concentrations (Fig. 4, $p<0.05$).

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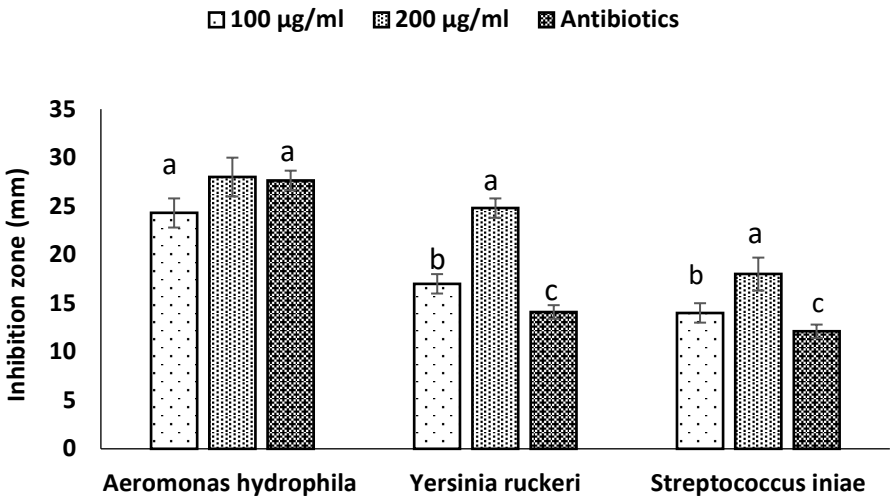


**Figure 4: Comparative analysis of inhibition zone in response to different rpro-RtHep concentrations (100 and 200 µg/ml) and antibiotics. Different letters indicate significant differences by One-way Anova and Tukey's post-hoc tests.**

*FE-SEM*

In the control group of *S. iniae* treated with PBS (Fig. 5A), the bacterial cells have a regular spherical shape and a smooth and healthy surface. The cell wall is clearly intact and the cells tend to aggregate or form small colonies in some areas, which indicates their normal growth state. No signs of damage, wrinkling or leakage of cellular contents are observed. In the group treated with recombinant pro-Hep at a dose of 50 µg/mL (Fig. 5B), the cells show a clear deformation. Their surface is uneven and wrinkled and some cells have partial or complete destruction of the cell wall. In several places, signs of leakage of cellular contents and collapse of the surface structure are observed. These changes are indicative of severe membrane damage and the cytolytic effect of hepcidin peptide on bacterial cells.

*In vivo antibacterial activity in zebrafish*

The findings from the challenge experiment are presented in Figured 6 and 7. It was observed that fish injected with rpro-RtHep demonstrated a lower mortality rate to infection than the control group ($p$<0.05). Immune factors including lysozyme activity, ACH50, total immunoglobulin, and total protein were significantly higher in zebrafish injected rpro-RtHep (T) compared to control (T0) (Fig. 8, $p$<0.05).

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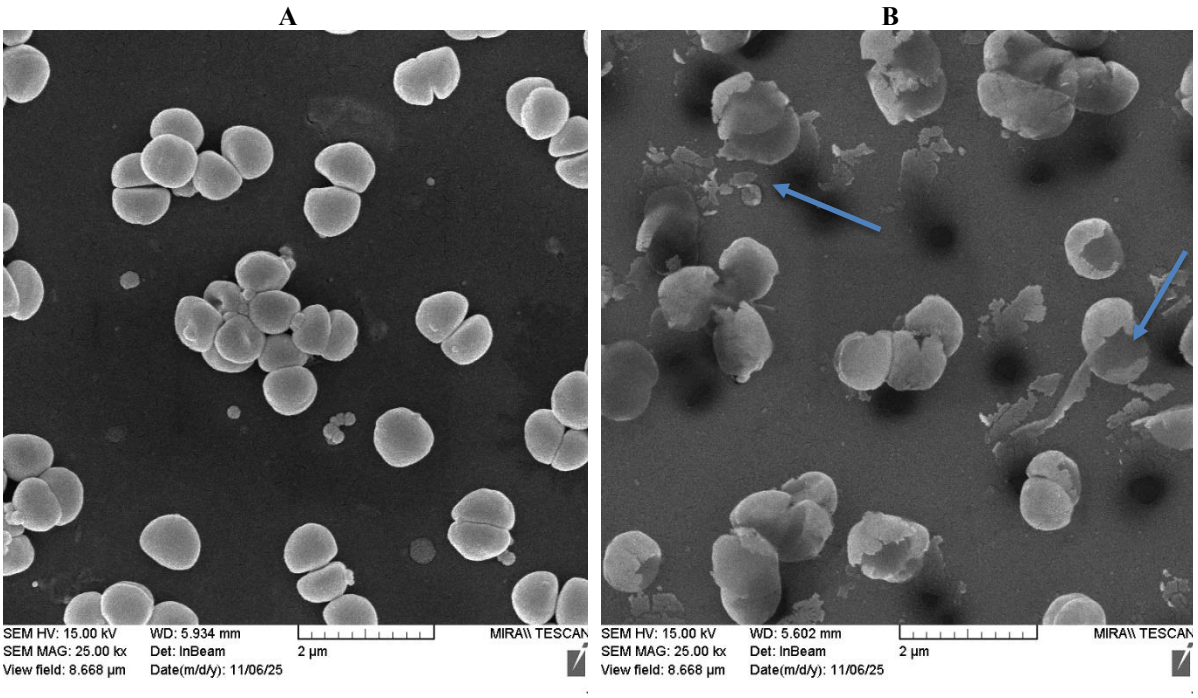


**Figure 5: Field emission scanning electron microscopy (FE-SEM) images of *Streptococcus inaei* after treatment with PBS (control, A) and recombinant pro-hepcidin (B).**

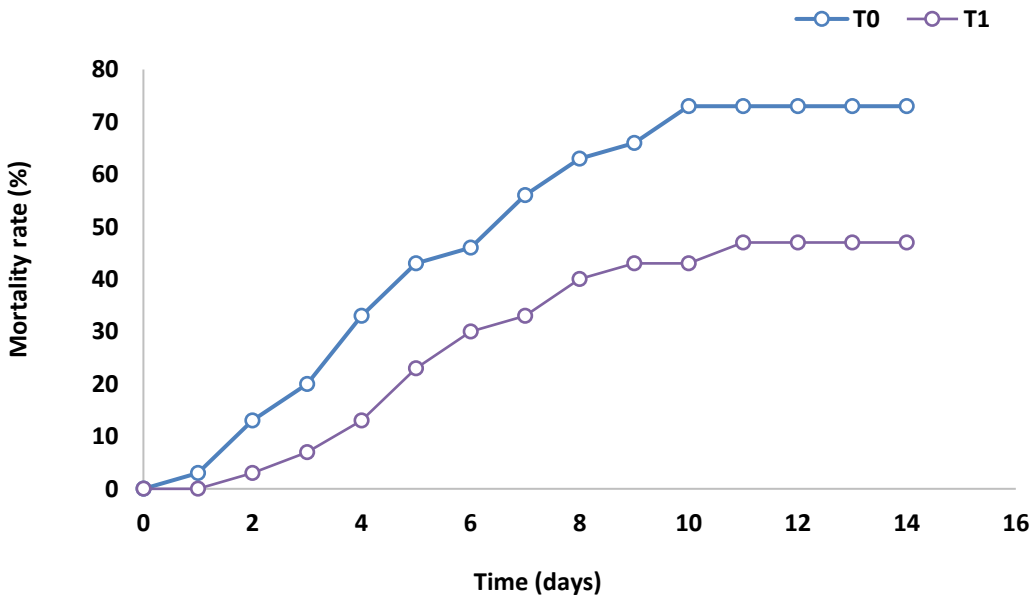


**Figure 6: Mortality rate of zebrafish injected by PBS (T0) and rpro-RtHep (T1) and subsequently challenged with *A. hydrophila* for 14 days.**

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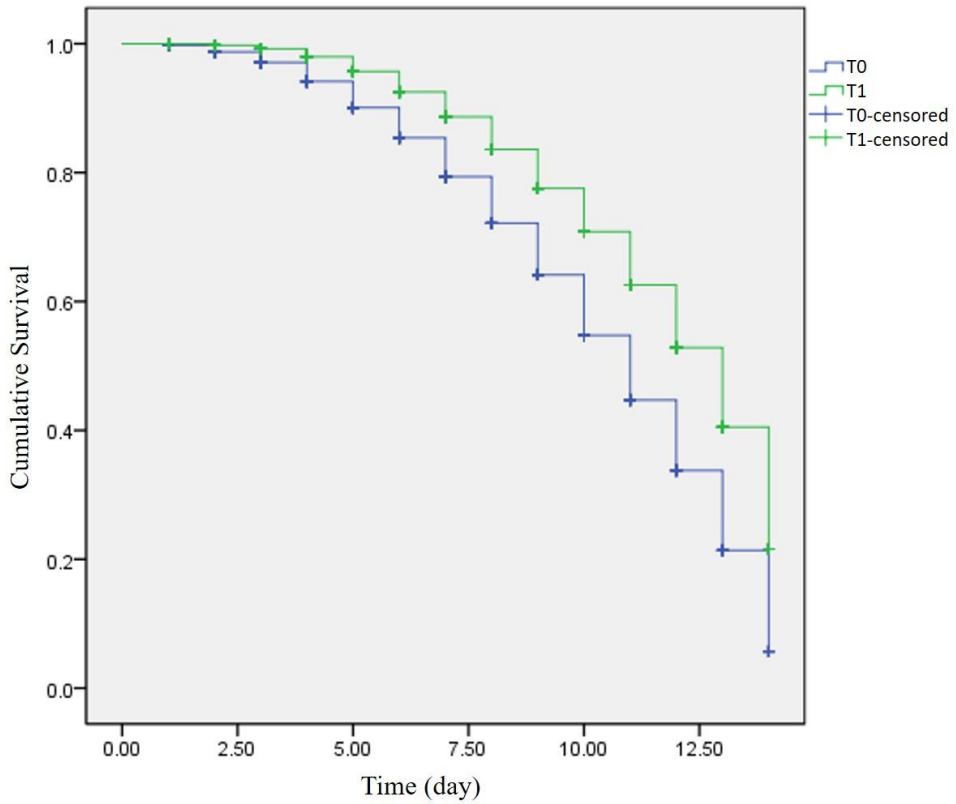


**Figure 7: Kaplan –Meier survival cure of zebrafish injected by PBS (T0) and rpro-RtHep (T1) and subsequently challenged with *A. hydrophila* for 14 days.**

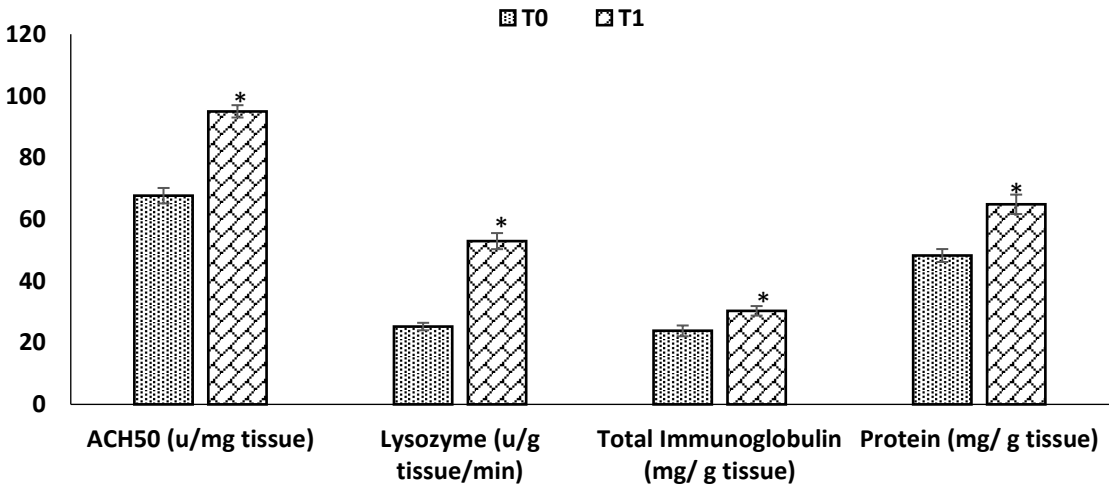


**Figure 8: Immune factors of zebrafish injected by PBS (T0) and rpro-RtHep (T1) and subsequently challenged with *A. hydrophila* for 14 days. * Indicate significant differences by T- tests.**

## Discussion

Direct antibacterial activity from prokaryotic-expressed hepcidin has been reported in several research (Luo *et al*., 2020; Liu *et al*., 2022). In this study, rpro-RtHep demonstrated direct antibacterial activity. Synthetic and recombinant mature and full length hepcidin showed broad antibacterial activity against Gram positive and Gram-negative bacteria (Shirdel *et al*.,

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2019; Liu *et al*., 2022; Zhang *et al*., 2024). AMPs change as pathogens evolves to be able to defend the body against a variety of microbes. Thus, they prevent the creation of mutant pathogen strains resistant to antimicrobial compounds (Katzenback, 2015). So far, there has been no report of pathogens becoming resistant to AMPs, and therefore, they can be used as a suitable alternative to antibiotics in the field of medicine and veterinary medicine (Katzenback, 2015; Garvey, 2023).

Antimicrobial resistance in various Aeromonas species has been commonly documented in aquaculture, and a recent increase in this resistance trend has been noted. In this study, a higher survival rate of zebrafish injected with rpro-RtHep against *A. hydrophila* was observed than that of the control group. Similar to the present study, recombinant hepcidin injection into Indian major carp resulted in 73% survival against *A. hydrophila* (Mohapatra *et al*., 2019). Elsewhere, the presence of recombinant hepcidin in the diet of common carp (*C. carpio haematopterus*) improved survival against *A. hydrophila* (Qiao *et al*., 2023). This may have occurred due to the entry of recombinant hepcidin through the bacterial membrane, resulting in the lysis of bacterial cells, thus highlighting the crucial role of this recombinant molecule in host immunity (Mohapatra *et al.*, 2019). Recombinant hepcidin is suggested to increase survival rates probably by inhibiting exaggerated inflammation and enhancing antibacterial immune responses (Qiao *et al*., 2023).

AMPs play a key role in the innate immune system and modulate the defense response. In the present study, immune factors (lysozyme, ACH50, total protein, and total immunoglobulin) were higher in the rpro-RtHep-injected treatment than in the control group. Likewise, synthetic hepcidin injection improved the immune response in European sea bass (*Dicentrarchus labrax*) (Álvarez *et al*., 2022). The immunomodulatory properties of some cationic peptides have been shown to be essential for the coordination of defense mechanisms in different organisms (Álvarez *et al*., 2022). These compounds can regulate the immune response by increasing or decreasing cytokine expression while inhibiting the growth of microorganisms (Haney and Hancock, 2013; Hilchie *et al*., 2013; Álvarez *et al.*, 2022). AMP has demonstrated immunomodulatory features in zebrafish and protects them against infection with *Vibrio vulnificus* (Pan *et al.*, 2011).

## Conclusion

In summary, SDS-PAGE analysis confirmed the successful purification of recombinant pro-hepcidin via affinity chromatography. The investigated antimicrobial properties of pro-hepcidin revealed that the produced peptide could inhibit pathogenic Gram-positive and Gram-negative bacteria in fish. Furthermore, injection of recombinant pro-hepcidin into zebrafish improved their immune factors and survival against *A. hydrophila*. The method used in the present study to express the peptide as a soluble protein in a prokaryotic host can be used in future studies on recombinant hepcidin production.

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**Conflicts of interest**

Authors declare no conflict of interest**.**

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