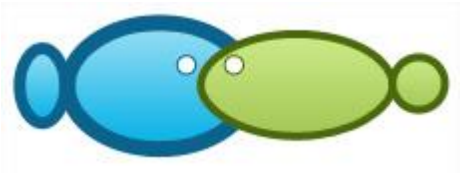

Effects of recombinant LvCTL3 protein supplementation on the MPO activity and hepatopancreas histology in *Penaeus vannamei* challenged with AHPND-causing *Vibrio parahaemolyticus*

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Abstract. This study evaluated the efficacy of dietary supplementation of recombinant LvCTL3 protein in cultured whiteleg shrimp (*Penaeus vannamei*) challenged with *Vibrio parahaemolyticus,* the causative agent of acute hepatopancreatic necrosis disease (AHPND). A controlled experimental design was employed, with monitoring of water quality parameters, myeloperoxidase (MPO) enzyme activity, and histopathological changes in the hepatopancreas. The results demonstrated that dietary administration of recombinant LvCTL3 significantly enhanced MPO activity and improved hepatopancreatic tissue integrity, with the most pronounced effects observed at a supplementation dose of 10 mg kg^{-1} feed, suggesting enhanced immunity and resistance of shrimp against this dangerous pathogen. This study provides strong evidence supporting the potential application of recombinant protein as a functional health-promoting feed additive in shrimp aquaculture.
Keywords: dietary supplementation, disease resistance, innate immunity, shrimp disease.

Introduction. Crustaceans such as shrimp lack an adaptive immune system and therefore depend solely on innate immune mechanisms for protection against pathogens, without the ability to establish pathogen-specific immunological memory (WOAH 2020). Acute hepatopancreatic necrosis disease (AHPND), initially termed early mortality syndrome (EMS), is a severebacterial infection affecting penaeid shrimp, typically manifesting within the first 40 days of culture. Caused primarily by pathogenic *Vibrio* species, AHPND has emerged as a major global threat to shrimp aquaculture, including in Vietnam. First reported in China in 2009, the disease rapidly spread to Vietnam (2010), Malaysia (2011), Thailand (2012), Mexico (2013), and the Philippines (2015), followed by outbreaks in South America and Australia in 2016, and subsequently in the United States and Bangladesh by 2019 (Tran et al 2013; Nunan et al 2014; Kumar et al 2021), AHPND has severely impacted the global shrimp farming industry, with cumulative mortality rates reaching up to 100% in farmed shrimp within 35 days of stocking.

Between 2011 and 2013, AHPND outbreaks led to a global shrimp production decline of over 15%, resulting in economic losses of approximately $3 billion. In Mexico and across the Northern Hemisphere, the disease resulted in estimated economic losses of approximately USD 118 million (De Schryver et al 2014). The causative agent of AHPND in whiteleg shrimp has been identified as *Vibrio parahaemolyticus*, a Gram-negative bacterium belonging to the family Vibrionaceae, order Vibrionales, class Gammaproteobacteria, and phylum Proteobacteria. The bacterium is characterized by a straight or slightly curved rod-shaped morphology, with cell dimensions ranging from approximately 0.3–0.5 μm in width and 1.4–2.6 μm in length (Tran et al 2013). AHPND-causing strains of *V. parahaemolyticus* possess plasmids encoding pirA and pirB proteins, which destroy shrimp hepatopancreatic tissue and impair its function (Nunan et al 2014).

In uninfected whiteleg shrimp, the intestines were filled with food, the hepatopancreas exhibited a dark brown coloration, and the stomach was clearlydiscernible. In contrast, AHPND-infected shrimp displayed characteristic clinical signsincluding a thin shell, pale body coloration, empty intestines, and a hepatopancreas that was pale, atrophied, or liquefied. Histopathological analysis revealed that the hepatopancreas of AHPND-infected shrimp showed sloughed epithelial cells, necrosis, and blood cell infiltration, while the hepatopancreas of uninfected shrimp displayed intact, non-necrotic hepatopancreatic tubules (Kumar et al 2020). Recent studies have demonstrated that recombinant shrimp-derived proteins exhibit significant antibacterial activity ().

Upon bacterial invasion in shrimp, C-type lectin plays a critical role in immune defense by promoting agglutination, destruction, or clearance of bacteria through interaction with shrimp hemocytes (Sun et al 2017; Runsaeng et al 2018; Qin et al 2019). Although our previous studies (Phuong et al 2023, 2024), confirmed the immunomodulatory effects of recombinant LvCTL3 and its role in enhancing resistance to *Vibrio parahaemolyticus* in *Penaeus vannamei* shrimp, information regarding its influence on MPO activity and hepatopancreatic histopathology remains limited. Therefore, the present study was designed to further investigate these unresolved aspects using an experimental challenge model.

Material and Methods

Preparing of protein recombinant. The recombinant LvCTL3 protein used in this study was produced by our research group according to the cloning, expression, and purification procedures previously described by Phuong et al (2024) and was subsequently used for the biological assays conducted in the present study. It was formulated at three concentrations 100 µg mL^{-1}, 200 µg mL^{-1}, and 500 µg mL^{-1} diluted from a 500 µg mL^{-1} standard sample using phosphate-buffered saline (PBS). These concentrations aligned with three treatment levels at 2, 4 and 10 mg kg^{-1} of feed. For each treatment, 20 mL of the recombinant LvCTL3 protein solution was evenly sprayed onto 1 kg of basal diet, following the methodology of Hong et al (2022) and Phuong et al (2024). The control sample was treated with 20 mL of PBS buffer in place of the recombinant protein, followed by air-drying at room temperature for 20 min and storage at 4°C.

Experimental shrimp. Shrimp (*Penaeus vannamei*) were provided from Quoc Thang Aquatic Trading Co., Ltd., Hue City. The shrimp had an initial weight from 0.4-0.6 g/shrimp and were acclimated in 1 m^3 composite tanks for 14 days before the experiment. Environmental factors during acclimation and the experiment were maintained within the following ranges: temperature 22–23°C, pH 7.5–8.0, salinity 22–25‰, and dissolved oxygen 5–6 mg L^1. Shrimp were fed three times daily at 8:00 AM, 1:00 PM, and 5:00 PM with a ration equivalent to 3% of their body weight per day (Phuong et al 2024).

Ethical conduct approval. All shrimp sampling and experimental procedures complied with the ethical standards approved by the Animal Ethics Committee of Hue University (Approval No. HUVN0025; May 4, 2023).

Experimental design. The experiment was set up with four treatments; each replicated three times. The treatments included a control group (Control) fed a basal diet without recombinant LvCTL3 supplementation, and three treatments supplemented with recombinant LvCTL3 at the following levels: P1 (2 mg kg^{-1} feed), P2 (4 mg kg^{-1} feed), and P3 (10 mg kg^{-1} feed). Shrimp were randomly assigned to 12 tanks (120 L, 30 shrimp per tank), with continuous aeration, and fed diets with or without recombinant LvCTL3 for 30 days. At the end of the experiment, 30 shrimp per treatment (10 shrimp/tank) were randomly selected for experimental infection with *Vibrio parahaemolyticus* (Phuong et al 2024).

Water quality parameters monitoring. Water quality parameters such as temperature (°C), pH, dissolved oxygen (DO), alkalinity and total ammoniac nitrogen (TAN) were monitored at regular intervals, and the results are summarized in Table 1.

Table 1

Measurement of water quality parameters

Parameter	*Time*	*Frequency*	*Equipment*
Temperature (°C)	07:00 & 14:00	Twice daily	Mercury thermometer (Vietnam)
pH	07:00 & 14:00	Twice daily	Testkit (Sera, Germany)
DO (mg L^{-1})	07:00 & 14:00	Twice daily	Testkit (Sera, Germany)
Alkalinity (mg L^{-1})	07:00	Every 3 days	Testkit (Sera, Germany)
TAN (mg L^{-1})	07:00	Every 3 days	Testkit (Sera, Germany)

Myeloperoxidase activity. Myeloperoxidase (MPO) activity was assessed using the procedure described by Quade and Roth (1997). Briefly, assays were conducted in a 96-well microplate using Hanks' balanced salt solution without Ca^{2+}/Mg^{2+} (Thermo Scientific, USA). A volume of 35 µL of 20 mM 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich) and 5 mM hydrogen peroxide (H_2O_2) was added to each well. After incubation, the reaction was stopped by adding 35 µL of 4 M sulfuric acid, and absorbance was measured at 450 nm. MPO activity was expressed as units per milligram of total protein (U mg^{-1} protein).

Effect of recombinant LvCTL3 protein on histopathological changes. The *Vibrio parahaemolyticus* strain TTHVP202101001, previously isolated from AHPND-affected white-leg shrimp (Phuong et al 2023), was stored in glycerol at -80°C. It was revived on TSA medium (Himedia, India) supplemented with 2% NaCl. The bacterial density was determined using an optical density meter (Zuzi, Spain) at a wavelength of 620 nm, diluted with physiological saline to achieve an OD_{620} value of 1, equal to 10^9 CFU mL^{-1}. For infection, shrimp were exposed to *V. parahaemolyticus* TTHVP202101001 at a concentration of 1×10^5 CFU mL^{-1} (Phuong et al 2023).

Shrimp preparation. Whiteleg shrimp with individual body weights ranging from 4.12 to 5.77 g were stocked at a density of 15 shrimp per tank, with three replicate tanks per treatment, following the experimental design of Phuong et al (2024). Shrimp were experimentally challenged by immersion in 20‰ brackish water containing *Vibrio* at a final concentration of 1×10^5 CFU mL^{-1}, maintained at 28 ± 1°C for 1 h. Post-infection, shrimp were housed in separate 120 L tanks with continuous 24/24 aeration, maintaining water temperature at 27–29°C. The shrimp were fed a basal diet supplemented with recombinant LvCTL3 three times daily at a dose of 2.5% of their body weight for 15 consecutive days.

Sample collection and bacterial re-isolation. Bacterial re-isolation was performed from the hepatopancreas of 50% of the surviving shrimp showing pathological signs in each treatment using the API 20E identification system (bioMérieux, France).

Histopathological analysis. Three diseased shrimp and two healthy shrimp were dissected to obtain hepatopancreas samples for histopathological examination, which were fixed in Davidson's solution for 48 hours and then transferred to 70% ethanol, following the method described by Lightner (1996). Tissue samples were embedded in paraffin, sectioned at 5 µm, mounted on slides, and stained with hematoxylin and eosin (H&E). Hepatopancreas sections were observed under a light microscope (Leica, Germany) at 10× or 40× magnification.

Statistical analysis. Data are expressed as mean ± SD. Treatment effects were evaluated using a one-way analysis of variance (ANOVA). When overall significance was observed, pairwise comparisons were conducted using Tukey's honestly significant

difference test. All analyses were carried out in SPSS (version 16.0), and statistical significance was accepted at $p < 0.05$.

Results

Water quality parameters. During the experimental period, the water quality parameters in the culture tanks remained within acceptable ranges, with pH, temperature, dissolved oxygen (DO), alkalinity values, and total ammonia ranging between 7.46 – 8.94, 26.67 – 28.5 °C, 4.05 – 5.32 mg L^{-1}, 100 – 130 mg_{CaCO3} L^{-1}, and 0.05 – 1.02 mg L^{-1}, respectively (Table 2). Overall, these conditions were well maintained and did not adversely affect the shrimp's growth or development. Water quality parameters did not differ significantly among the three experimental treatments (Table 2).

Table 2

The fluctuation of water quality parameters

Parameters	*Min – Max*	*Mean ± SD*
pH	7.46 – 8.94	8.40 ± 0.34
Temperature (°C)	26.67 – 28.5	27.53 ± 1.05
DO (mg L^{-1})	4.05– 5.32	4.45 ± 0.53
Alkalinity (mg_{CaCO3} L^{-1})	110 – 130	116.78 ± 9.53
TAN (mg L^{-1})	0.05 – 1.02	0.07 ± 0.03

Myeloperoxidase activity. Myeloperoxidase (MPO) activity represents a key functional element of the innate immune system in crustaceans in general, and specifically in whiteleg shrimp. Following *Vibrio parahaemolyticus* challenge, shrimp fed diets supplemented with recombinant LvCTL3 exhibited higher MPO activity compared with the control group. This trend indicates a strengthened innate immune response in LvCTL3-treated shrimp under experimental infection conditions.

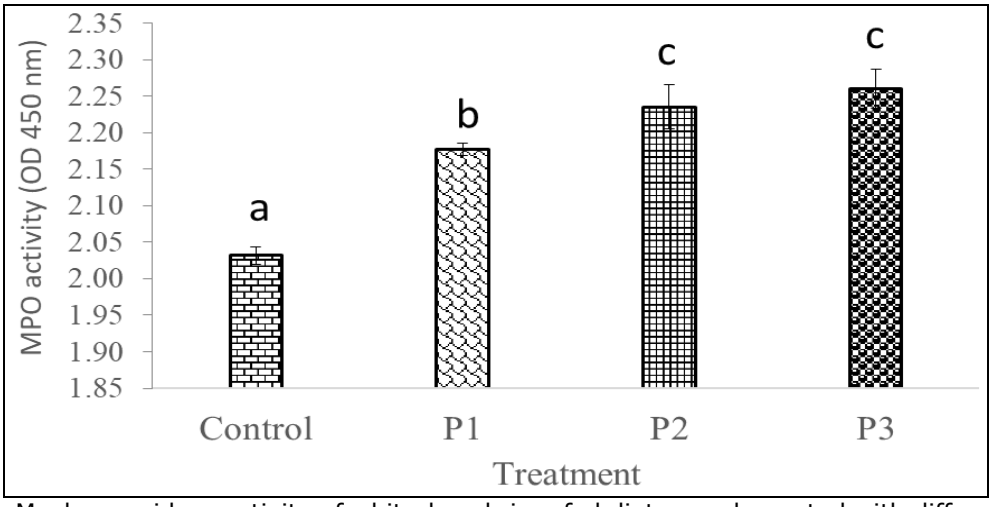

Figure 1. Myeloperoxidase activity of white-leg shrimp fed diets supplemented with different levels of recombinant LvCTL3 protein for 30 days.

The findings indicated that the control group (without recombinant LvCTL3 supplementation) exhibited the lowest MPO activity (2.03 U mg $protein^{-1}$) compared to groups supplemented with recombinant LvCTL3 ($p < 0.05$). Supplementation with recombinant LvCTL3 at varying concentrations influenced MPO activity in shrimp. The MPO level in shrimp supplemented with 2 mg kg^{-1} (P1 treatment) was 2.18, significantly lower than in those supplemented with 4 mg kg^{-1} (P2 treatment) or 10 mg kg^{-1} (P3 treatment) ($p < 0.05$). However, no significant difference in MPO activity was observed between the P2 and P3 treatments ($p > 0.05$) (Figure 1). These results align with the findings of (Song & Hsieht 1994), has shown that when MPO activity increases, it will

help increase the production of atomic oxygen and H_2O_2 shrimp haemocyte, thereby increasing the phagocytosis process. Supplementing recombinant LvCTL3 increases MPO activity more than other immunostimulants such as supplementing β-glucan (2 mg mL^{-1}), shrimp MPO only reaches 0.972, or supplementing Zymosan at the same concentration, MPO reaches 1.141 (Song & Hsieht 1994). Similarly, Bao et al (2022) reported MPO values ranging from 2.05 to 2.18 (OD_{450} nm) in shrimp hemolymph following dietary supplementation with *Pediococcus pentosaceus*, which were lower than those observed in the present study.

Histopathological characteristics. Histological observations showed that LvCTL3-supplemented shrimp displayed less severe hepatopancreatic alterations after bacterial challenge than the control group. The hepatopancreatic architecture in treated shrimp remained comparatively intact, whereas more pronounced pathological changes were evident in untreated shrimp. Analysis of the histopathological characteristics of shrimp showed that after 15 days of infection with *Vibrio parahaemolyticus* bacteria, the control treatment (not supplemented with recombinant LvCTL3) had necrotic, peeling, torn, and atrophied hepatopancreas tissue, showing signs of bacterial invasion in hepatopancreatic cells and the phenomenon of epithelial cells peeling into the hepatopancreatic tubules (Figure 2A&C & 3A) and the epidermis had abnormally large nuclei (Figure 3A, red arrows). These hepatopancreatic tissue changes are characteristic signs of AHPND as described by Kumar et al (2020) and Phuoc et al (2020).

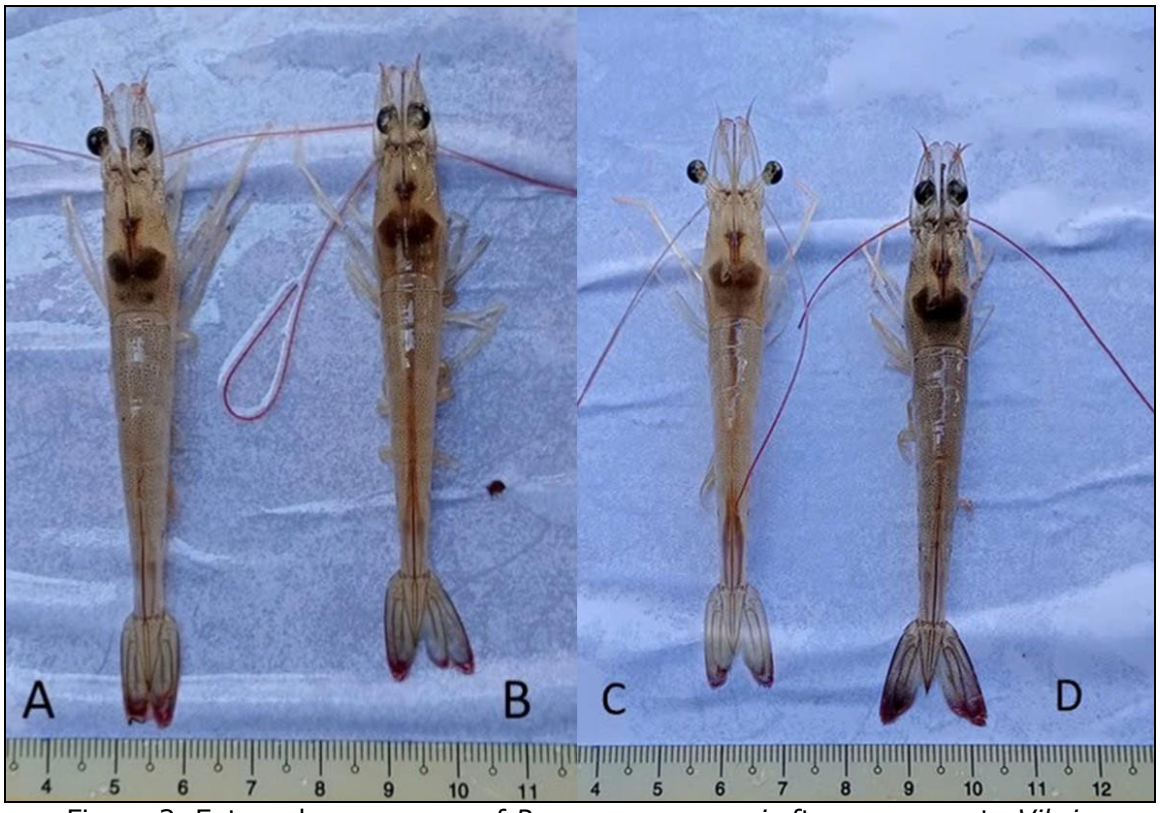

Figure 2. External appearance of *Penaeus vannamei* after exposure to *Vibrio parahaemolyticus*. A & C: Infected shrimp (not fed with feed supplemented with recombinant protein); B & D: *Penaeus vannamei* shrimp showed a dark, well-defined hepatopancreas and intestines filled with feed, shrimp fed with recombinant protein supplementation in feed at the dose of 2 and 4 mg kg^{-1} feed, respectively.

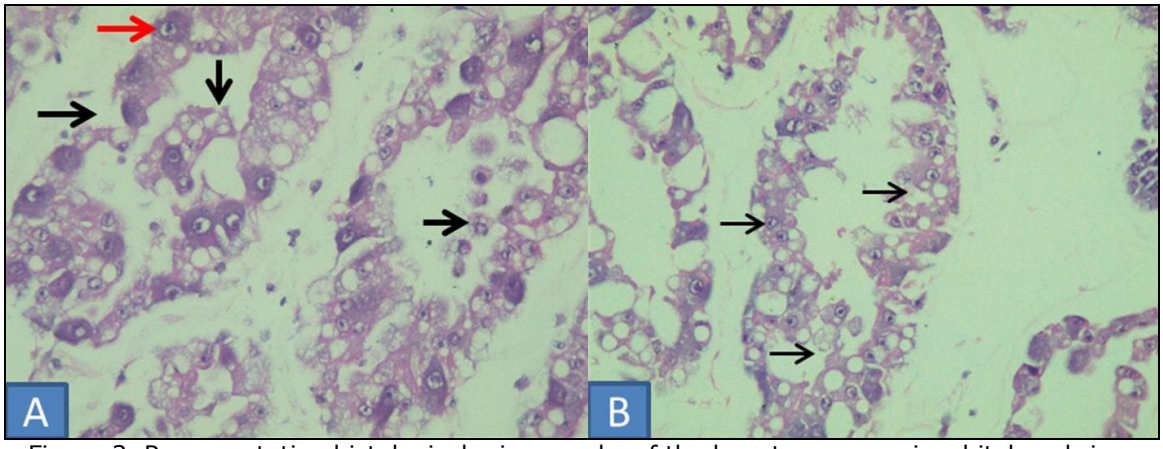

Figure 3. Representative histological micrographs of the hepatopancreas in whiteleg shrimp following experimental infection with *Vibrio parahaemolyticus* (H&E staining). (A) Control group: severe epithelial sloughing, necrosis, bacterial invasion, and reduced numbers of B, F, and R cells (40× mag). (B) Shrimp fed diets supplemented with recombinant LvCTL3 at 2 and 4 mg kg^{-1} feed showing mild histopathological alterations in hepatopancreatic tubules (40× mag).

In shrimp infected with non-AHPND causing *Vibrio parahaemolyticus*, the hepatopancreatic tubules remain normal. In contrast, AHPND infection leads to sloughing and necrosis of epithelial cells in the hepatopancreas, along with haemocyte infiltration. In cases of bacterial infection, the hepatopancreas tissue structure is altered, with a notable reduction in B cells (storage cells), F cells (fibrous cells), and R cells (cytoplasmic cells with numerous small vacuoles). In healthy shrimp, the hepatopancreas maintains an intact structure, with abundant B, F, and R cells, and reduced activity of E cells (embryonic cells) (Lightner et al 2012; Wu et al 2008). In this study, for treatments supplemented with recombinant LvCTL3 at levels of 2 or 4 mg kg $feed^{-1}$, slight changes in hepatopancreas tissue structure were observed, with mild signs of atrophy and slight sloughing of hepatopancreatic tubules (Figure 3B). In the treatment where shrimp were fed a diet supplemented with 10 mg kg^{-1} of recombinant LvCTL3, healthy shrimp (Figure 4B), and the hepatopancreas tissue structure resembled that of healthy shrimp, with clear observation of B, F, and R cells (Figure 5). No bacterial infiltration was detected in any of the treatments supplemented with recombinant LvCTL3 after 15 days of bacterial infection. After 15 days of infection, the re-isolation of bacteria from the hepatopancreas sample of infected shrimp was identified by API 20E test kit as *Vibrio parahaemolyticus.*

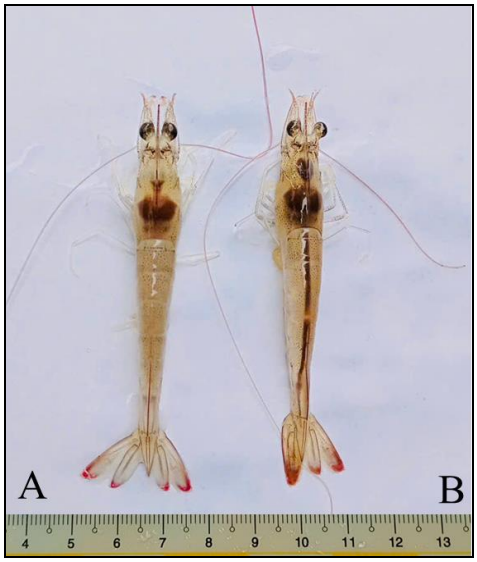

Figure 4. Gross morphology of whiteleg shrimp after challenge test. A: Infected shrimp (not fed with feed supplemented with recombinant protein). B: Healthy whiteleg shrimp showed a dark, well-defined hepatopancreas and intestines filled with feed, shrimp fed with recombinant protein supplementation in feed at the dose of 10 mg kg^{-1} feed.

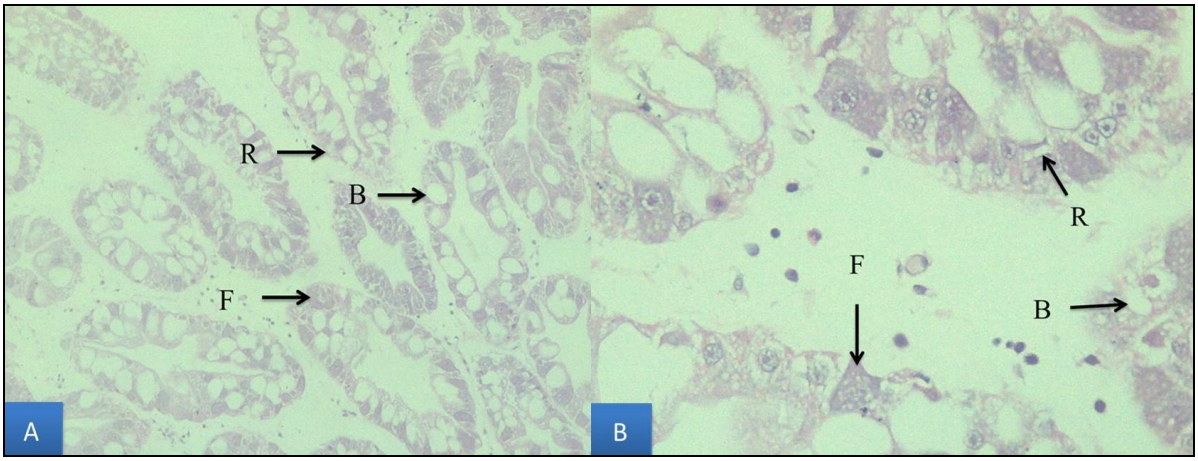

Figure 5. Histological structure of the hepatopancreas of white-leg shrimp fed a diet supplemented with recombinant LvCTL3 at 10 mg kg^{-1} feed after challenge with *Vibrio parahaemolyticus* (H&E staining). A: Normal hepatopancreatic architecture showing clearly defined B, F, and R cells (magnification 10×). B: Higher magnification view of hepatopancreatic tubules showing intact epithelial cells (magnification 40×).

Discussion. This study is consistent with the publication of Nguyen et al (2023), where the hepatopancreatic tissue structure of whiteleg shrimp (*Penaeus vannamei*) infected with *Vibrio parahaemolyticus* bacteria when fed with food supplemented with 1% leaf extract of herbs including *Hibiscus sabdariffa*, *Eucalyptus globulus* and *Mimosa pigra* did not show a decrease in B, F and R cells nor did it show necrosis or sloughing of the hepatopancreatic tubules after 14 days. However, according to the report of Tien and Oanh (2020), the hepatopancreatic tissue of white-leg shrimp infected with *V. parahaemolyticus*, whether fed a diet with or without β-glucan supplementation, exhibited histopathological changes characterized by atrophied hepatopancreatic tubules, a decreased number of B, R, and F cells, enlarged epithelial cell nuclei, and blood cell accumulation around bacterial clusters in necrotic areas. However, supplementing the shrimp diet with β-glucan at a dose of 2 g kg $feed^{-1}$ enhanced non-specific immune parameters and reduced mortality rates in shrimp infected with *V. parahaemolyticus* causing AHPND. Another research indicates that, *Penaeus vannamei* shrimp fed a basal diet supplemented with herbal *Wedelia chinensis* extract at concentrations of 31.25, 312.5, and 625 mg L^{-1} had no adverse effects on the structure or function of the hepatopancreas, while it helped reduce *Vibrio parahaemolyticus* infection (Ngoc et al 2024).

Consistent with the findings of Huyen et al (2020), PCR-based re-identification of bacteria from infected shrimp confirmed that whiteleg shrimp that died within 14 days post-infection were positive for *V. parahaemolyticus* bacteria as in our study results. This shows that mortality accompanied AHPND associated pathological signs were attributable to *V. parahaemolyticus* infection. Moreover, dietary supplementation with recombinant LvCTL3 enhanced immune responses and increased resistance of whiteleg shrimp to AHPND-causing *V. parahaemolyticus*. Notably, no bacterial isolates were recovered from healthy shrimp or from shrimp that survived until the end of the experimental period.

The present findings demonstrate that dietary supplementation with recombinant LvCTL3 protein not only enhanced MPO activity but also preserved hepatopancreatic integrity in white-leg shrimp challenged with AHPND-causing *V. parahaemolyticus*. The pronounced protective effects observed at the 10 mg kg^{-1} feed level suggest a strong link between LvCTL3-mediated immune activation and reduced tissue damage. These results highlight the potential of recombinant LvCTL3 as a functional immunostimulant for mitigating AHPND-associated pathology in shrimp aquaculture.

Conclusions. Dietary inclusion of recombinant LvCTL3 protein improved innate immune responses and increased resistance of white-leg shrimp (*Penaeus vannamei*) to *Vibrio parahaemolyticus* infection causing AHPND. The shrimp fed 10 mg kg^{-1} feed dosage significantly improved MPO activity and hepatopancreas health. These results indicate

that recombinant LvCTL3 is a promising immunostimulant for improving shrimp health in aquaculture. However, further field trials are needed to validate efficacy under intensive farming conditions.

Authors Contributions. Conceptualization: TVP, . Methodology: TVP, YTHP . Validation: TVP, YTHP, PNN. Formal Analysis: TVP, YTHP, PNN. Investigation: TVP, YTHP. Resources: TVP, YTHP. Data curation: TVP, YTHP, PNN. Writing: TVP. Review and Editing: PNN. Visualization: TVP.

Conflict of Interest. The authors declare that there is no conflict of interest.

Data Availability. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Funding. This work was supported by The Vietnamese Ministry of Science and Technology's (Grant number DTDL.CN-56/22).

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Received: 15 January 2026. Accepted: 22 April 2026. Published online: 11 June 2026.
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How to cite this article:
Tran P. V., Pham Y. T. H., Nguyen P. N., 2026 Effects of recombinant LvCTL3 protein supplementation on the MPO activity and hepatopancreas histology in *Penaeus vannamei* challenged with AHPND-causing *Vibrio parahaemolyticus*. AACL Bioflux 19(3):1242-1250.