Inhibitory potential of two brackish water fish species in Bataan, Philippines, against presumptive *Vibrio parahaemolyticus* causing AHPND

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Abstract. The emergence of shrimp diseases has caused a decline in shrimp production and financial losses for top Asian shrimp producers, particularly the Philippines. Among these diseases is the acute hepatopancreatic necrosis disease (AHPND), caused by *Vibrio parahaemolyticus* with virulence $pirAB^{VP}$ genes (VP_{AHPND}). In the past, green water technology (GWT), which utilizes water containing beneficial microorganisms and skin mucus from finfish aquaculture, has demonstrated effectiveness in reducing shrimp diseases. However, in areas like Bataan, where some commonly used GWT species are less viable, locally thriving brackish water fish species may offer a tailored solution. Thus, this study explored the potential of *Leiopotherapon plumbeus* and *Sarotherodon melanotheron* against presumptive VP_{AHPND}. The disk diffusion method confirmed the presence of inhibitory metabolites in the mucus of *L. plumbeus*, while the mucus of *S. melanotheron* showed no inhibitory indication. Thus, *L. plumbeus* specimen were further investigated for their antimicrobial activity through a challenge test in a completely randomized design setup. Sterile water in aquaria was inoculated with presumptive VP_{AHPND} and stocked with varying biomasses (g L^{-1}) of *L. plumbeus* (0, 0.7, and 1.4). After 72 hours, *L. plumbeus* biomass of 0.7 to 1.4 g L^{-1} significantly ($p<0.01$) reduced presumptive VP_{AHPND} concentrations in the water. These findings provide valuable insights into the use of *L. plumbeus* in developing prophylaxes against AHPND, supporting the shrimp industry and its farmers.

Key Words: green water technology, skin mucus, antimicrobial activity, biological control.

Introduction. The acute hepatopancreatic necrosis disease (AHPND) is an emerging disease caused by the bacteria *Vibrio parahaemolyticus* with associated binary toxins $pirA^{VP}/pirB^{VP}$ referred to as "VP_{AHPND}" (Li et al 2017; Tare et al 2023). These toxins lead to the growth of the $pirAB^{VP}$ toxin in the shrimp's stomach (Lai et al 2015), ultimately resulting in lowered shrimp production. In some cases, 100% mortality in shrimps can occur within 30 days of exposure to VP_{AHPND} (Thadtapong et al 2020). To help prevent the spread of shrimp diseases including vibriosis, the National Institute of Molecular Biology and Biotechnology (NIMBB) and the Institute of Aquaculture of the College of Fisheries and Ocean Science (AI-CFOS) of the University of the Philippines Visayas have developed Green Water Technology. With the emergence of AHPND, further investigation on the efficacy of GWT on AHPND must be in the limelight of research efforts.

According to Corre et al (2005), green water technology is a technique of culturing shrimps in water with abundant phytoplankton. The use of phytoplankton-rich culture water discharged by culture tilapia species fishponds or of the traditional green water technology (GWT) were observed to help enhance the water quality and survival rate of shrimps (Chithambaran et al 2017). One of the enticing aspects of green water culture is that tilapia and other fish species secrete mucus that contains chemicals that inhibit several bacteria, including *Vibrios* (Zorriehzahra & Banaederakhshan 2015). The algal and fish components of this technology effectively inhibit the growth of certain pathogens, making it a highly cost-effective means of restoring pond water quality for shrimp (Tendencia & Pena 2003; Bosma & Tendencia 2014). Nevertheless, there is a paucity of research related to the use of brackish water fishes to address the possible osmotic stress for shrimps utilizing freshwater GWT.

Based on the study of riverine fish assemblage in Bataan (Corpuz et al 2019), two promising fish components for GWT with aquaculture potential are the saline-tolerant *Leiopotherapon plumbeus* and *Sarotherodon melanotheron*. The *L. plumbeus* (Kner, 1864), or silver therapon, is an endemic and economically important freshwater food fish in the Philippines. In the early 1960s, *L. plumbeus* was regarded as the most abundant freshwater fishery fish resource in Laguna de Bay, the largest lake in the Philippines (Aya 2021) and are initially considered as by-catch in aquaculture ponds. They are quite hardy and can tolerate a wide range of water quality parameters (Rowland & Bryant 1995). The natural populations of this species have been declining during the past years, mainly due to intense fishing pressure, habitat degradation, and introduction of invasive alien species (Aya et al 2017), thus an ideal species for conservation efforts (Consigna et al 2019). On the other hand, *S. melanotheron* is an exotic species in the Philippines, where it is thought to have been introduced via the aquarium trade. It has since spread within the provinces of Bataan and Bulacan, with the potential to further expand its range. The *S. melanotheron*'s rapid growth rate and ability to reproduce quickly have made it a rapidly proliferating species in Filipino fisheries, providing a valuable, yet untapped resource (Lederoun et al 2020).

The conventional setup of most shrimp ponds in the region and the lack of awareness and means for sufficient biosecurity measures (Flores et al 2015; Rabadon & Corpuz 2021) prompted this research concept. With shrimp being one of the top five commodities in the Philippines and a critical species in the economy, this study is vital in further addressing the problems to improve its productivity. This will be helpful for future studies aimed to properly manage and develop strategies and low-cost technologies or immunotherapies that will be applied for the benefit of the shrimp farmers in the region (Rabadon et al 2022). Thus, the study was conducted to evaluate the inhibitory potential of two locally thriving brackish water fish species against presumptive *Vibrio parahaemolyticus* causing AHPND, and to assess the effect of *L. plumbeus* biomass on reducing $\mathrm{VP_{AHPND}}$ concentrations in the water under controlled experimental conditions following confirmation of inhibitory activity.

Material and Method. Diseased shrimp samples were collected for isolation of presumptive $\mathrm{VP_{AHPND}}$. Immediate molecular testing was performed using the Juan Amplification (JAmp) AHPND diagnostic kit, a loop-mediated amplification-based rapid detection platform for AHPND in shrimps, developed by the University of Santo Tomas, Philippines. Subsequently, bacterial isolation was conducted, then a metabolite assay and a challenge test.

DNA extraction and toxin detection. Shrimp (*Penaeus vannamei*) samples with suspected AHPND were collected from selected ponds in Bataan during the grow-out period. The samples were transported to the Pathology Laboratory at the Center for Research on Aquaculture and Aquatic Resources in Brackishwater Systems (CRAABS) in sterile, chilled containers. Pleopods and hepatopancreas from diseased shrimp were aseptically amalgamated. DNA was extracted using the JAmp-AHPND kit from 1-2 g of shrimp pleopods and hepatopancreatic tissue, placed in reaction tubes. The samples were homogenized, filtered, and processed for DNA extraction. The extracted DNA was then amplified using a Loop-Mediated Isothermal Amplification (LAMP) assay under controlled temperature conditions. Toxic gene detection was performed using a DNA-binding dye. After the LAMP assay, 4 μL of visual dye was added, and the samples were examined under UV light. Fluorescence indicated the presence of $\mathrm{pirAB^{VP}}$ genes in positive samples. Positive and negative controls were included for comparison.

$\mathrm{VP_{AHPND}}$ culture. The *V. parahaemolyticus* causing AHPND was initially enriched in alkaline peptone water (APW) for 16-18 hours at 37°C based on the methods of Karunasagar et al (2018). The $\mathrm{VP_{AHPND}}$ were inoculated on *Vibrio*-selective thiosulfate–

citrate–bile salts–sucrose (TCBS) plates prepared based on the manufacturer's specifications (HiMedia®). The colonies of presumed VP_{AHPND} isolates were confirmed with their inability to ferment sucrose. Sucrose fermenting *Vibrios* turns the TCBS media yellow. Among the *Vibrio* species, only *V. parahaemolyticus* is non-sucrose fermenting, hence will appear as blue or green. Thus, the green colonies were isolated and purified using the streak method. The isolates are replenished every 3-5 days at room temperature. Pure colony isolates were re-inoculated on nutrient agar slants and APW for further storage under 4–10°C.

Skin mucus anti-VP_{AHPND} metabolite assay. Mucus samples were collected from the skin of *L. plumbeus* and *S. melanotheron*, centrifuged to decant the water from mucosal proteins, and tested in triplicates for *in vitro* antibacterial activity against VP_{AHPND}. Anti-VP_{AHPND} metabolites were confirmed based on growth inhibitions through the Kirby-Bauer disc-diffusion method (Lio-po et al 2005) with some modifications (Table 1).

Table 1

Different test disks used for the disk-diffusion method

Disk	*Content*	*Volume and concentration of substance impregnated on disk*
A	H_2O (negative control)	25 μL; 99.9% pure water
B	Commercial antibiotic: AMX (positive control)	25 μL; 50 mg mL^{-1} H_20
C	*L. plumbeus*	25 μL; whole extracted mucus
D	*S. melanotheron*	25 μL; whole extracted mucus

Challenge test of L. plumbeus against presumptive VP_{AHPND}. Sterilized glass aquaria were filled with artificial seawater and aerated. The aquaria were sheltered under metal roofing and covered with black plastic sheets. The study employed a completely randomized design (CRD) following the methods of Tendencia & Pena (2003) with modifications. Three treatments with three replicates were set up, with 0, 0.7, and 1.4 g L^{-1} of *L. plumbeus* biomass stocked in the aquaria, respectively. After fish acclimation, water was inoculated with VP_{AHPND} to a density of 500 CFU mL^{-1}. Throughout the investigation, feeding was restricted and there was no water exchange. To attain an artificial brackish water, solar salt was used to simulate the average salinity of Bataan shrimp ponds amounting to 6.9 ppt (Rabadon et al 2022), ensuring that the necessary minerals from brackish water were present. Water samples were taken to ascertain uniform VP_{AHPND} counts prior to and following the addition of fish. Bacterial counts were quantified at 72 h before the termination of the challenge test.

Statistical analysis. Normality of data (Shapiro-Wilk test) and homogeneity of variance (Levene's test) were verified. Variables did meet the assumptions and mean bacterial counts were subjected to analysis of variance (ANOVA), followed by Tukey's post-hoc test if found significantly different ($p<0.05$).

Results and Discussion. Figure 1 demonstrated the resistant, bacteriostatic, and bactericidal properties of the three different disks. They were observed to maximally inhibit bacterial growth at 12 hours, with the antibiotic being the most potent (1.9±0.36 cm), followed by *L. plumbeus* mucus (0.74±0.22 cm), and sterile water (0.33±0.15 cm). The findings demonstrated that within the first 12 hours, bacterial growth had not seized the entire disc; instead, the mucus had countered the bacterial invasion in the medium. As anticipated, the antibiotic AMX had confirmed its bactericidal action, acting against both Gram-positive and Gram-negative microorganisms by inhibiting the biosynthesis and repair of the bacterial mucopeptide wall (Karaman et al 2015). Based on the study of Ha et al (2023), farms administer six different types of antibiotics for disease treatment and prevention in the first stages of rearing. Among them, amoxicillin was the most frequently used antibiotic (73.33%), followed by ceftazidime (66.67%) and colistin (33.33%), whereas ciprofloxacin, oxytetracycline, and tetracycline were less frequently used.

At 24 hours, the disc containing sterile water had been fully colonized, confirming that the water had not demonstrated any bactericidal or bacteriostatic property. Whereas, the *L. plumbeus* mucus still exhibited some suppression against bacterial invasion (0.69±0.39 cm), though this inhibition was not statistically significant in comparison to the efficacy of antibiotics in suppressing bacterial multiplication in the inhibition zone (1.5±0.5 cm). Notwithstanding, the *L. plumbeus* mucus exhibited bacteriostatic potential against the bacterial lawn.

The *S. melanotheron* replicates were immediately covered with bacterial growth. However, it was unconfirmed if the bacterial growth surrounding the disk were of VP_{AHPND} lawn or an inoculant from the microbiota of *S. melanotheron* mucus. According to the study of Apines-Amar et al (2022), several bacterial isolates from saline-tolerant tilapia exhibited anti-*Vibrio* activity. An isolate demonstrated inhibitory activity with the biggest zone of inhibition against the shrimp pathogens, *Vibrio harveyi* and *V. parahaemolyticus*. Notwithstanding, a high concentration of *V. parahaemolyticus* was detected in the shrimp ponds (Rabadon et al 2022), even with the presence of *S. melanotheron* indicating that this bacterium can tolerate the presence of *S. melanotheron* mucus in low quantities.

In 36 hours, several replicates of *L. plumbeus* mucus discs were colonized by bacteria, albeit zones of inhibitions were still present in three mucus replicates (0.15±0.18 cm). The reduction of size of the inhibition zone of the antibiotic disk was also observable in this culture period (0.6±0.17 cm). At 48 h, all treatment replicates were completely invaded by the bacterial growth (Figure 1).

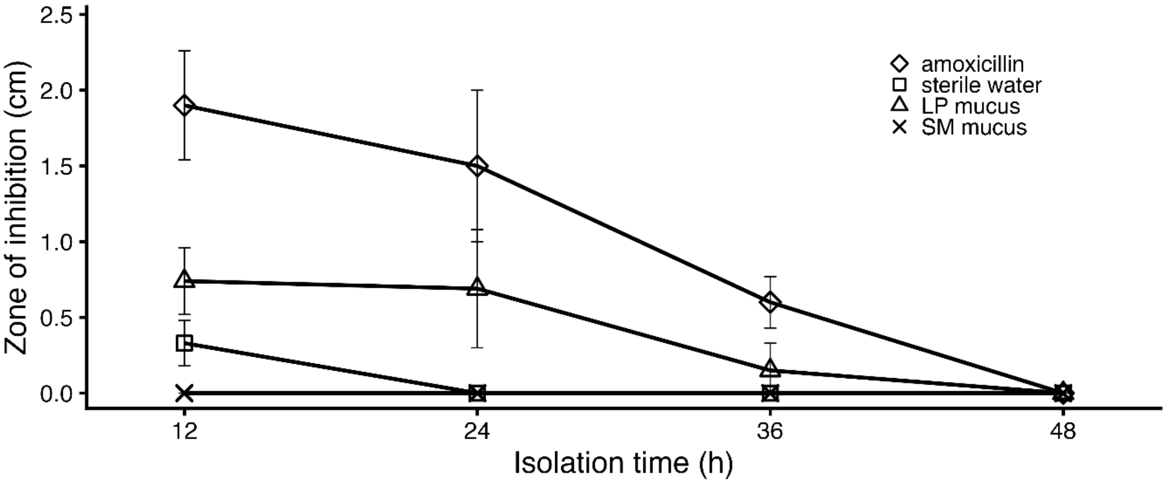

Figure 1. Temporal changes in the zone of inhibition (cm) of the test disks.

This study provides evidence on the potential of *L. plumbeus* mucus in inhibiting the VP_{AHPND} growth *in vitro*, albeit with lesser efficacy relative to the bactericidal actions of commercially available antibiotic AMX. The disk diffusion assay used to determine the antibacterial activity of mucus from *L. plumbeus* showed that the skin mucus has active compounds that are effective for the inhibition of VP_{AHPND}.

The antibacterial activity of L. plumbeus against VP_{AHPND}. Based on the *in vitro* results, the *L. plumbeus* was further tested in an *in vivo* experiment. The experiment revealed a highly significant difference between T1, T2, and T3 after 72 h. T1 (negative control) displayed the highest bacterial concentrations (560±22.71 CFU mL^{-1}). The presence of *L. plumbeus* in T2 reduced the initial concentration into 468±24.43 CFU mL^{-1} VP_{AHPND}. The highest decrease was observed in T3, which was stocked with the highest biomass of *L. plumbeus* and resulted in 280±26 CFU mL^{-1} VP_{AHPND} final density, highlighting the potential of this species in combating VP_{AHPND} in the water. These results suggest that the addition of *L. plumbeus* contributes towards a significant decrease in the VP_{AHPND}

concentration, indicating its potential for GWT or as biological control. This highlights the importance of the study, as it demonstrates the potential of this fish with aquaculture potential to act as a natural VP$_{AHPND}$ reducer, offering a promising alternative to traditional use of antibiotics.

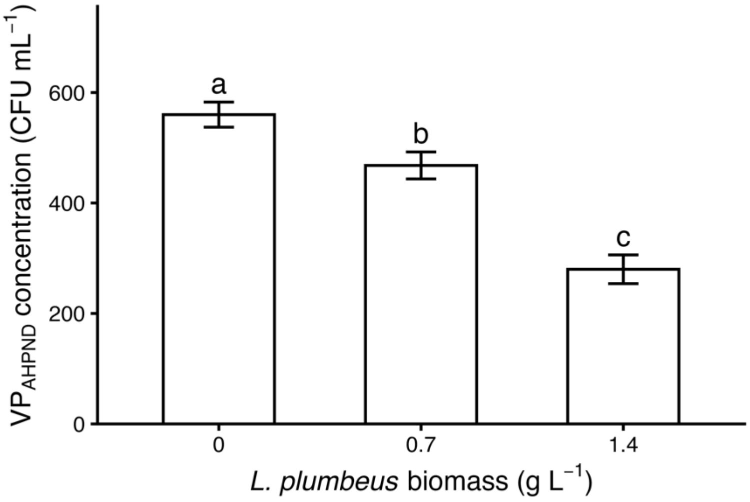

Figure 2. Mean values (± SD) of VP$_{AHPND}$ concentrations within treatment ($p<0.01$).

The findings of this study suggest that *L. plumbeus* is a promising candidate for AHPND prevention due to its efficacy in inhibiting VP$_{AHPND}$. This implies that the presence of *L. plumbeus* in aquaculture ponds can be beneficial for controlling the population of VP$_{AHPND}$*.*

Conclusions. The study suggests that a biomass of 0.7 to 1.4 g L^{-1} *L. plumbeus* can be used to inhibit VP$_{AHPND}$ growth. The range can be utilized depending on the severity of AHPND cases and may be limited on available resources. Further, understanding on the utilization of *L. plumbeus* in shrimp aquaculture could lead to more effective strategies for the management, control, and prophylaxis of this emerging disease. Research into this potential research gap could provide invaluable insights into how to more effectively respond to AHNPD-related challenges and enable us to craft more sustainable and resilient aquaculture systems. Based on the conclusion of this study, some recommendations can be proposed. First, it is endorsed to utilize the results of this research project to ascertain the desirable concentrations of *L. plumbeus* in an actual shrimp pond setting as prophylaxis and possible treatment mechanism against AHPND. Second, it is recommended that a pilot study of *L. plumbeus* and shrimp in a co-culture system (culture of both in a single rearing enclosure) or sub-culture system (culture of both in two separates rearing enclosures) must be conducted. By embracing these recommendations, researchers can further advance the development of effective strategies to mitigate AHPND as well as improve the overall health and sustainability of aquaculture operations. The dissemination of these findings and the collaborative efforts of researchers will facilitate the translation of scientific knowledge into practical solutions, enabling the successful management of AHPND and promoting the progress of the aquaculture industry. This work on the AHPND in Bataan, pioneers further shrimp health related studies that will not only benefit the advancement of science and technology, but also alleviate the problems faced by shrimp farmers.

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

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