

The Effectiveness of Extract from Ambon Banana Stem *Musa cavendishii* var. Dwarf paxton for Prevention of *Vibrio parahaemolyticus* Bacterial Infection in *Vannamei* Shrimp *Litopenaeus vannamei*

Geza Intan Septarisa

IPB University

Sri Nuryati

sri_nuryati@apps.ipb.ac.id

IPB University

Sukenda Sukenda

IPB University

Munti Yuhana

IPB University

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Abstract

This study aims to evaluate the effect of administering Ambon banana stem extract through feed in enhancing the immune response, growth, and resistance of vannamei shrimp to diseases caused by *Vibrio parahaemolyticus* bacteria. The research used a completely randomized design consisting of five treatments, namely 2 control treatments (negative control and positive control) and three treatments containing feed with Ambon banana stem extract, namely 0.5 g kg^{-1} , 0.75 g kg^{-1} , and 1 g kg^{-1} of feed. Shrimp were raised for 30 days and fed five times a day. On the 31st day, shrimp were challenged with *V. parahaemolyticus* at a dose of 10^5 CFU mL^{-1} , except for the negative control. The results showed that the dose of 0.75 g kg^{-1} provided significantly higher growth performance ($P < 0.05$) compared to other doses and controls. Then a dose of 0.75 g kg^{-1} was able to increase immune response significantly higher ($P < 0.05$) than other doses and controls. The bacterial abundance in the intestine at a dose of 0.75 g kg^{-1} also significantly suppressed the growth of *V. parahaemolyticus* ($P < 0.05$) compared to other doses and controls. In addition, the survival rate of vannamei shrimp at a dose of 0.75 g kg^{-1} was significantly higher ($P < 0.05$) compared to other doses and controls. The results of this research can be used as a reference for the optimal dosage for preventing *V. parahaemolyticus* bacterial infections using Ambon banana stem extract.

INTRODUCTION

Vannamei shrimp *Litopenaeus vannamei* is one of the aquaculture commodities widely cultivated worldwide and has become one of the leading aquaculture fisheries commodities in Indonesia due to its high economic value. Based on global production value, vannamei shrimp ranks first among crustaceans with a production value reaching 5.81 million tons in 2020. The production value of vannamei shrimp contributes 51.7% to the total production of crustacean groups globally, with Indonesia being the fourth largest producer with a crustacean production reaching 892 thousand tons (FAO 2022). Furthermore, according to data from the KKP (2022), Indonesia's shrimp production value of 860 thousand tons contributed approximately 2 billion USD to global exports in 2019. Despite the average increase in vannamei shrimp production in Indonesia by 12% during the period of 2015–2020 (KKP 2022), vannamei shrimp aquaculture activities are still hindered by disease outbreaks.

Disease is one of the challenges that must be faced as it can lead to production failure and economic losses (FAO 2020). Examples of some disease cases in Indonesia that have caused a decrease in the productivity of vannamei shrimp cultivation are caused by the viruses *Infectious hypodermal and hematopoietic necrosis virus* (IHHNV), *Infectious myonecrosis virus* (IMNV), *Taura syndrome virus* (TSV), *Yellow head virus* (YHV), and *White spot syndrome virus* (WSSV) (Shinn et al. 2018; Arulmoorthy et al. 2020; Suryadi et al. 2021). Besides viral diseases, there are also other diseases caused by bacteria, namely vibriosis. Vibriosis is one of the disease outbreaks that pose challenges in aquaculture activities (Mohamad et al. 2019), one of which is caused by *Vibrio parahaemolyticus* bacterial infection. According to Shinn et al. (2018), this bacterium causes economic losses estimated at 4 billion USD in the Asian region during 2009–2018. *V. parahaemolyticus* bacteria usually infect within 35 days after seed stocking (post-larvae) (Tran 2013; Flegel and Lo 2013). Infected shrimp may experience necrosis, lethargy, loss of appetite and empty guts, hepatopancreas atrophy, and mass mortality exceeding 70% in a short period (Tran et al. 2013; Kongrueng et al. 2014; Navarro et al. 2020).

The handling of vibriosis can be carried out through antibiotic application. However, antibiotics can accumulate in the tissues of organisms or aquatic environments (Zhang et al. 2014; Thiang et al. 2021), thus leading to the emergence of new antibiotic resistant bacterial strains, accumulation of antibiotic residues in the environment, changes in bacterial communities, and food safety issues hazardous to human consumers (Elmahdi et al. 2016; Grenni et al. 2018; Okocha et al. 2018). The use of immunostimulants is currently one of the alternatives in addressing the issue of disease outbreaks caused by several bacteria or viruses. Immunostimulants are substances derived from living organisms or combinations of synthetic materials that can enhance their effects on various components of the immune system (Nurdiansah 2013; Shahbazi and Bolhassani 2017). The use of immunostimulants in the form of herbal ingredients has also been extensively studied for its application in preventing diseases in fish and shrimp aquaculture activities and is known to enhance immune responses, appetite, growth, as well as possessing antistress, antibacterial, antifungal, and antiviral properties (Ramudu and Dash 2013; Pu et al. 2017). Immunostimulants can be applied through injection, immersion, or oral administration of feed, and the administration of immunostimulants through feed is one of the methods that has been widely researched in vannamei shrimp (Wahjuningrum et al. 2013; Simanjuntak 2016).

One herbal ingredient known for its role in enhancing immunity is the banana plant. Bananas are tropical plants that bear fruit only once during their life cycle, so if not utilized, they become waste. Different parts of the banana plant, including the roots, stem,

leaves, and fruit, are known to contain immunostimulant compounds (Haryani et al. 2012; Malangngi et al. 2012). Among these parts, the banana stem is known to have the highest concentration of immunostimulant compounds (Astria et al. 2017). The phytochemical analysis results indicate that the extract of Ambon banana stem contains 4.94% tannins, 8.38% saponins, and 19.52% flavonoids (Simanjuntak 2016; Ramadhan et al. 2017). These active compound contents are beneficial for maintaining or enhancing the health of cultured fish and shrimp (Simanjuntak 2016; Nurjanah et al. 2018).

Several studies on the application of banana stem extract in freshwater commodities through feed or immersion have been conducted, such as in carp (Nuryati 2015; Fitrianingrum 2015), catfish (Lidiawati 2014; Indraswari 2016; Andriani 2017; Astria et al. 2017), tilapia (Sari 2016), and carp (Christine 2019). The use of Ambon banana stem extract as an immunostimulant for controlling white spot disease in vannamei shrimp aquaculture (Simanjuntak 2016; Ramadhan et al. 2017) has also been carried out, but the application of banana stem as an immunity agent for controlling vibriosis caused by *V. parahaemolyticus* bacteria has not been conducted yet. Therefore, this study is conducted to evaluate the effectiveness of different doses of Ambon banana stem extract through feed in enhancing immune response, growth, and resistance of vannamei shrimp to diseases caused by *V. parahaemolyticus* bacteria. This study aims to evaluate the effectiveness of Ambon banana stem extract through feed in enhancing the immune response, growth, and resistance of vannamei shrimp to diseases caused by *V. parahaemolyticus* bacteria.

METHODS AND MATERIALS

Research Design

The research design used was a completely randomized design (CRD) consisting of 5 treatments and 4 replications, including 2 control treatments (negative control and positive control) and 3 treatments of feeding containing Ambon banana stem extract, namely doses of 0.5 g kg⁻¹, 0.75 g kg⁻¹, and 1 g kg⁻¹ of feed.

Preparation of Ambon Banana Stem Extract

The Ambon banana stems were obtained around the IPB Dramaga campus, Bogor, and selected from Ambon banana plants that had already borne fruit. The extraction of Ambon banana stem was conducted using the method described by Ramadhan et al. (2017). The Ambon banana stems were first cut into small pieces. They were then weighed and placed into an Erlenmeyer flask, followed by the addition of 96% ethanol in a ratio of 1:10 (1 g of Ambon banana stem: 10 mL of 96% ethanol). The two materials were mixed and shaken for 24 hours using a water bath shaker at 40°C with a speed of 150 rpm. The extract of Ambon banana stem was stirred and filtered to obtain a filtrate, which was collected in a container. Subsequently, evaporation was carried out using a rotary evaporator at 40°C until a concentrated extract of Ambon banana stem was obtained.

Preparation of Test Bacteria

The bacteria used for challenge testing were *V. parahaemolyticus* bacteria from the Aquatic Organism Health Laboratory collection, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. The *V. parahaemolyticus* Rf^R bacteria, which had been made resistant to *rifampicin* antibiotics, were cultured in *Thiosulfate Citrate Bile Salt Sucrose* (TCBS) agar medium. Subsequently, bacterial colonies were taken with an inoculating loop and inoculated into 15 mL of liquid *Sea Water Complete* (SWC) medium, followed by incubation on a shaker at 160 rpm for 24 hours.

Preparation of Maintenance Container

The containers used were cylindrical tanks with a diameter of 112 cm and a height of 110 cm. The tanks were cleaned using soap, rinsed thoroughly, and disinfected with 30 ppm chlorine, then dried for 24 hours. After drying, the tanks were filled with water to a height of 22 cm and provided with aeration at two points per tank to supply oxygen in the maintenance media and a heater was installed.

Preparation of Animal Test

The test animals used in this study were vannamei shrimp sourced from PT. Suri Tani Pemuka with a body weight of 1.43 ± 0.02 grams shrimp⁻¹. Prior to the maintenance and administration of Ambon banana stem extract for 30 days, the test shrimp were first acclimatized for 14 days in treatment cylindrical tanks with 100 shrimp and fed commercial feed with a protein content of 40% at a

frequency of five times a day at 06:00, 10:00, 14:00, 18:00, and 22:00 with a feeding rate of 10 – 8%. Before treatment, the shrimp were fasted for 24 hours to eliminate any residual feed in their bodies.

Preparation of Test Feed

The preparation of the test feed began with weighing the concentrated extract of Ambon banana stem according to the predetermined treatment doses (0.5 g kg⁻¹, 0.75 g kg⁻¹, 1 g kg⁻¹). The feed used in this study was commercial shrimp feed with a protein content of 40%. Subsequently, the Ambon banana stem extract was mixed with distilled water containing 2% egg white as a binder and then sprayed into the commercial feed while being stirred until evenly distributed. The mixed feed was dried at room temperature. The prepared feed can be used immediately or stored in tightly sealed plastic containers in a refrigerator at 4°C until use, and the feed preparation was done every 3 days.

The Challenge Test

The challenge test was conducted after the administration of treatments to determine the effect of Ambon banana stem extract on vannamei shrimp infected with *V. parahaemolyticus* bacteria in an aquarium measuring 60x30x30 cm³. The challenge test was performed by injecting a density of bacteria into the maintenance tank based on the LD₅₀ determination results. The negative control shrimp (K-) were injected with PBS of the same volume, and each treatment was repeated 4 times (Raharja 2019).

Test Parameters

Growth Performance

The parameters observed in this study include survival rates (SR) $SR(\%) = \left(\frac{N_t}{N_o} \right) \times 100$ (Muharrama *et al.* 2021), specific growth rate (SGR) $SGR (\%/day) = \left[\ln \frac{W_t - W_o}{t} \right] \times 100$ (Zokaeifar *et al.* 2012), feed conversion ratio (FCR) $FCR = F / (B_t + B_m) - B_o$ (Yang *et al.* 2015).

Immune Response

Immune response includes observations of total hemocyte count (THC), phagocytosis activity (AF), respiratory burst (RB), and phenoloxidase (PO). Hemolymph was collected from the base of the swimming legs. Hemolymph was collected using a syringe containing Na-citrate anticoagulant in a 1:2 ratio. Then, the collected hemolymph was placed into an Eppendorf tube for immune response testing in shrimp. Shrimp hemolymph samples collected with the help of a syringe were dropped onto a hemocytometer. The total hemocyte count was calculated by observing under a light microscope at a magnification of 100 times. The total hemocyte count was calculated using the formula (Wang and Chen 2006).

Phagocytosis activity (AF) was measured by mixing 100 µL of shrimp hemolymph sample with 25 µL of *Staphylococcus aureus* suspension (10⁷ CFU mL⁻¹), then incubated for 20 minutes. Subsequently, a smear preparation was made, dried, fixed with methanol for 5 minutes, dried again, then stained with Giemsa stain for 20 minutes and observed using a microscope at a magnification of 400 times (Anderson and Siwicki 1993).

Respiratory burst (RB) was measured by mixing shrimp hemolymph sample with anticoagulant and taking 50 µL of the mixture. The mixture was then incubated for 30 minutes at room temperature. After incubation, the mixture was centrifuged for 20 minutes at 3000 rpm. The pellet obtained was then mixed with 100 µL of 0.3% nitroblue tetrazolium (NBT 0.3%) and incubated at room temperature for 2 hours. Subsequently, the sample was centrifuged for the second time at the same speed for 10 minutes. The pellet obtained from the second centrifugation was mixed with 100 µL of absolute methanol and centrifuged at the same speed for 10 minutes. The pellet was then rinsed twice with 70% methanol. Addition of 120 µL KOH and 140 µL *dimethyl sulfoxide* (DMSO) was performed before the sample was placed in a microplate reader for optical density (OD) measurement at a wavelength of 630 nm. KOH and DMSO were used as blanks. RB describes the reduction activity of NBT per 10 µL of hemolymph (Cheng *et al.* 2004).

Phenoloxidase activity (PO) was measured using the formation of dopachrome produced by L-DOPA. A mixture of 0.2 mL hemolymph and 0.8 mL anticoagulant was centrifuged at a speed of 1500 rpm for 10 minutes. The formed supernatant was discarded, and the pellet was slowly resuspended by adding 1 mL of *cacodylate-citrate buffer solution* (0.01 M *sodium cacodylate*, 0.45 M *sodium chloride*, 0.10 M *trisodium citrate*, pH 7) and centrifuged again at a speed of 1500 rpm for 10 minutes. The formed

supernatant was then discarded, and the pellet was added with 200 μL *cacodylate-citrate buffer* (0.01 M *sodium cacodylate*, 0.45 M *sodium chloride*, 0.10 *trisodium citrate*, pH 7). The resulting suspension of 100 μL was incubated with 50 μL *trypsin* (1 mg mL⁻¹ *cacodylate buffer*) as an activator for 10 minutes at a temperature of 25–26°C, followed by the addition of L-DOPA (3 mg mL⁻¹ *cacodylate buffer*) and incubated for 5 minutes, then 800 μL *cacodylate buffer* was added. The optical density (OD) was measured using a spectrophotometer at a wavelength of 492 nm. The standard solution content was 100 μL of hemocyte suspension, 50 μL *cacodylate buffer* (replacing trypsin), and 50 μL L-DOPA. The optical density of PO activity was expressed as dopachrome formation in 50 μL of hemolymph (Liu and Chen 2004).

Bacterial Abundance In The Intestine

Bacterial abundance was determined using the spread plate method. Shrimp intestines taken from shrimp in each tank were weighed at 0.1 g each, then homogenized in 0.9 mL of sterile *phosphate-buffered saline* (PBS). The bacterial cell suspension was then serially diluted and spread onto each media at 50 μL per plate. TCBS selective media without *rifampicin* was used to observe the total number of vibrios, and TCBS media with 50 mg/L *rifampicin* was used to observe the number of *V. parahaemolyticus*. The plates containing the cultures were then incubated for 24 hours at 37°C, followed by the calculation of total bacterial abundance using the formula (Madigan et al. 2012).

Histopathology

The histopathology was taken from the hepatopancreas tissue of the test shrimp. Test shrimp samples were taken from each treatment to determine the level of tissue damage caused by *V. parahaemolyticus* bacteria and the influence of the treatment given. Organs taken from the test shrimp were first fixed with Davidson's fixative solution for 24 hours. The organs were then cut into 3–5 mm and 1 × 1 cm pieces, followed by dehydration, clearing, impregnation, embedding, and paraffin blocking. The tissue blocks were then sliced using a 5 μm microtome and stained with hematoxylin-eosin (Bell and Lightner 1988).

Gene Expression

Gene expression of the immune system was observed using quantitative real-time reverse transcription (RT) *polymerase chain reaction* (PCR). The samples used were shrimp hemolymph at a ratio of 1:2 with anticoagulant. The hemolymph was centrifuged at 800 rpm for 10 minutes at 4°C. Subsequently, total RNA was extracted and purified using GENEzol™ reagent (Geneaid, Taiwan) according to the manufacturer's instructions. The purity of total RNA was analyzed by spectrophotometry ($\lambda = 260$ nm and 280 nm), and complementary DNA (cDNA) synthesis was performed using the ReverTraAce™ qPCR RT Mastermix with gDNA remover kit (Toyobo Co., Ltd., Japan) according to the manufacturer's instructions. Specific primer pairs for quantifying shrimp immune genes included *peroxinectin* (PE) and *lipopolysaccharide and β -1,3-glucan-binding protein* (LGBP), as well as one control gene, elongation factor – 1 α (EF-1 α), described as follows: PE forward primer 5'-TGG-ACC-TCG-CGG-GAG-AT-3' and reverse primer 5'-GAC-CGA-TAG-CCA-CCA-TGC-TT-3' (Liu et al. 2005); LGBP forward primer 5'-CAT-GTC-CAA-CTT-CGC-TTT-CAG-A-3' and reverse primer 5'-ATC-ACC-GCG-TGG-CAT-CTT-3' (Cheng et al. 2005); and EF-1 α forward primer 5'-CTG-TGG-TCT-GGT-TGG-TGT-TG-3' and reverse primer 5'-TCA-GAT-GGG-TTC-TTG-GGT-TC-3' (Alvarez-Ruiz et al. 2015). qPCR was performed on a Rotorgene 6000 machine (Corbett, USA) using the KAPA SYBR® FAST qPCR kit (KAPA, USA). The amplification was carried out for a reaction volume of 20 μL , containing 10 μL KAPA SYBR® FAST qPCR (KAPA, USA) Green Master Mix, 1.6 μL (0.8 μL each) specific forward and reverse primers, 4 μL template cDNA, and 4.4 μL *nuclease-free water* (NFW). The negative control was NFW. The qPCR program used was pre-denaturation at 95°C for 2 minutes, followed by 40 cycles at 95°C for 15 seconds (denaturation), 60°C for 15 seconds (annealing) and acquiring to Cycling A, 72°C for 20 seconds (extension), and a melting curve in the temperature range of 72–95°C for 15 seconds. RT-PCR data analysis was performed using Rotor-Gene Q software (Rotor-Gene QIAGEN) based on the method by Munaeni et al. (2020). After PCR amplification, melt curve analysis was performed to confirm that only one product was amplified, and standard curves for each gene were prepared. The quantification of gene expression was calculated using the relative quantification method with elongation factor – 1 α (EF-1 α) as the reference gene. The Cycle Threshold PCR (CT) was defined as the cycle number. Significant increases in SYBR® Green fluorescence were observed compared to internal passive dye. The average CT values and standard deviations were used in the $\Delta\Delta\text{CT}$ calculation. The difference in CT values between the immune gene and the endogenous control gene elongation factor – 1 α (EF-1 α), referred to as ΔCT , was calculated to normalize the differences in total RNA added to the cDNA reaction mixture and the RT reaction efficiency. The difference allowing the measurement of changes in immune gene expression in treated samples relative to control samples was expressed as the $\Delta\Delta\text{CT}$ value. The fold change in gene expression was calculated using 2 ^{$-(\Delta\Delta\text{CT})$} (Livak and Schmittgen 2001). The calculation of gene expression is as follows:

- ΔCT (average of normal control) = average target gene/average endogenous control
- ΔCT (average of treatment) = average target gene/average endogenous control
- $\Delta\Delta CT$ = average treatment - average normal control
- Fold change in expression = $2^{(-\Delta\Delta CT)}$

Data Analysis

The analysis of growth performance, immune response and gene expression data was conducted using ANOVA with a 95% confidence interval to determine significant differences among treatments for the test parameters using SPSS 27 (Statistical Program Software System) and Ms. Excel. If significant differences ($P < 0.05$) were found, the Tukey test was conducted. Histopathology parameters were analyzed descriptively.

RESULTS

Growth Performance

The growth performance and survival rate of vannamei shrimp given treatment for 30 days can be seen in Table 1. The initial average weight of the shrimp did not differ significantly ($P > 0.05$) across all treatments. The provision of Ambon banana stem extract (EBPA) showed significantly different results ($P < 0.05$) concerning the final average weight, Specific Growth Rate (SGR), and Feed Conversion Ratio (FCR) compared to the control, while the Survival Rate (SR) of the P2 dose treatment differed significantly ($P < 0.05$) compared to treatments P1, P3, and the control. Overall, the administration of the P2 dose at 0.75 g.kg^{-1} produced the best growth performance, including final average weight, SGR, FCR, and SR.

Table 1

Growth performance and survival of vaname shrimp given different doses of Ambon banana stem extract (EBPA) through feed.

Parameter	Perlakuan				
	K-	K+	P1 (0,50)	P2 (0,75)	P3 (1,00)
W0 (g)	1,43±0,02 ^a	1,43±0,00 ^a	1,43±0,01 ^a	1,42±0,01 ^a	1,42±0,01 ^a
W30 (g)	3,47±0,06 ^a	3,49±0,11 ^a	4,50±0,11 ^c	4,85±0,02 ^d	4,23±0,06 ^b
SGR (%)	2,38±0,07 ^a	2,40±0,18 ^a	3,74±0,12 ^{bc}	4,11±0,02 ^c	3,65±0,07 ^b
FCR	2,24±0,06 ^c	2,22±0,19 ^c	1,65±0,06 ^{ab}	1,55±0,05 ^a	1,78±0,08 ^b
SR 30 (%)	82,00±2,31 ^a	81,00±2,00 ^a	89,00±3,83 ^{ab}	95,00±5,03 ^b	86,00±6,93 ^{ab}
SR CT (%)	92,50±2,89 ^d	47,50±6,45 ^a	71,25±4,79 ^{bc}	80,00±4,08 ^c	65,00±4,08 ^b
Note: different letters at the end (mean value ± standard deviation) in the same row indicate significant differences ($P < 0.05$). W0: average initial weight, W30: average final weight, SGR: specific growth rate, FCR: feed conversion ratio, SR 30: survival rate at the end of rearing and SR CT: survival rate after challenge test.					

Immune Response

The observation results on immune response indicate that the addition of Ambon banana stem extract through feed for 30 days can provide significantly different values of THC, AF, RB, and PO ($P < 0.05$) compared to the control, both before and after the challenge test measured on day 0, 30, H + 1 (1 day after challenge test), H + 7 (7 days after challenge test), H + 14 (14 days after challenge test), H + 21 (21 days after challenge test), H + 28 (28 days after challenge test) (Fig. 1). Generally, the administration of banana stem extract through feed at a dose of P2 0.75 g.kg^{-1} resulted in significantly higher immune response ($P < 0.05$) compared to other treatments.

Bacterial Abundance in the Intestine

The calculation of bacterial populations in the intestines of vannamei shrimp includes total *Vibrio* count and *Vibrio parahaemolyticus* count. The highest populations of total *Vibrio* and *Vibrio parahaemolyticus* after 1 day of challenge test (H + 1)

were significantly different in treatment K + compared to other treatments. Then, at 14 days (H + 14) and 28 days after the challenge test (H + 28), the total *Vibrio* and *Vibrio parahaemolyticus* populations in all treatments showed a decrease. *Vibrio parahaemolyticus* count in each of the treatments P1, P2, and P3 experienced a decrease with lower values ($P < 0.05$) compared to treatment K+, and treatment P2 had lower values ($P < 0.05$) compared to other treatments, including total *Vibrio* count and *Vibrio parahaemolyticus* count (Fig. 2).

Histopathology

The histopathology observed was on the hepatopancreas organ of vannamei shrimp on day 30, H + 1 (1 day after challenge test), and H + 28 (28 days after challenge test). The observation results indicate that treatments P1, P2, and P3 could reduce damage to the hepatopancreas organ compared to the positive control, which exhibited a lot of damage such as vacuolization, tubule necrosis, and B cell necrosis. Additionally, in the P2 dose treatment, results showed a reduction in damage compared to other dose treatments (Fig. 3).

Gene Expression

Observations of *Lipopolysaccharide- and β -1,3-glucan-binding protein* (LGBP) gene expression and *peroxinextine* (PE) gene in vannamei shrimp subjected to treatment with the addition of Ambon banana stem extract (EBPA) and challenged with *Vibrio parahaemolyticus* bacteria (Fig. 4) yielded significantly different gene expression values ($P < 0.05$) compared to the positive control, both before and after the challenge test on day 30, H + 1 (1 day after challenge test), H + 7 (7 days after challenge test). Overall, the administration of banana stem extract through feed at a dose of P2 0.75 g.kg^{-1} resulted in significantly higher gene expression ($P < 0.05$) compared to other treatments.

DISCUSSION

The administration of Ambon banana stem extract (EBPA) through feed in this study provided good growth performance with daily administration frequency for 30 days of maintenance before the challenge test. In Table 1, it can be observed that the highest survival rate was found in treatment P2 (0.75 g.kg^{-1}) at $95 \pm 5.03\%$ before the challenge test and $80 \pm 4.08\%$ after the challenge test. These values indicate that treatment P2 had significantly higher survival rates ($P < 0.05$) compared to all treatments before the challenge test, significantly lower rates ($P < 0.05$) than the negative control but significantly higher rates ($P < 0.05$) than the positive control, P1, and P3 after the challenge test. The specific growth rate and weight values in this study also showed that the administration of EBPA at a dose of 0.75 g.kg^{-1} had significantly higher values ($P < 0.05$), namely $4.11 \pm 0.02\%$ and $4.85 \pm 0.02 \text{ g}$, compared to treatment P1 (0.50 g.kg^{-1}) at $3.74 \pm 0.12\%$ and $4.50 \pm 0.11 \text{ g}$, and treatment P3 (1.0

g.kg^{-1}) at $3.65 \pm 0.07\%$ and $4.23 \pm 0.06 \text{ g}$. Additionally, the feed conversion ratio value in treatment P2 was lower at 1.55 ± 0.05 compared to other treatments.

The high growth and growth rate in treatment P2 (0.75 g.kg^{-1}) compared to other treatments are suspected to be due to the appropriate dosage of EBPA administration, which can stimulate faster and better growth synthesis. Additionally, it is influenced by the active compounds present in EBPA, which can function as immunostimulators. The high survival rate in the negative control treatment is due to the shrimp not being infected with *V. parahaemolyticus* bacteria, the cause of shrimp death. This is also consistent with Awaludin *et al.* (2020), explaining that the lower the feed conversion ratio value, the more effective the feed given will be for shrimp growth, and the feed will be utilized effectively during maintenance.

Qualitatively, EBPA is known to contain several phytochemical compounds such as flavonoids, hydroquinone, saponins, steroids, triterpenoids, alkaloids, and tannins, while quantitatively, it has been shown that EBPA contains 19.52% flavonoids, 8.38% saponins, and 4.94% tannins (Asuquo and Udobi 2016; Simanjuntak *et al.* 2016; Ramadhan *et al.* 2017). Compounds found in EBPA such as flavonoids, saponins, alkaloids, and tannins are known to play a role in maintaining the health of shrimp, enhancing immune function and growth, and inhibiting bacterial growth, which ultimately affects survival, growth, and immune response of shrimp through protection from the immune system acquired after feeding containing EBPA (Malangngi *et al.* 2012; Astria 2017). The growth performance results are also consistent with increased immune response such as total haemocyte count (THC), phagocytosis activity (AF), respiratory burst (RB), and phenoloxidase (PO).

The values of cellular (THC and AF) and humoral (PO and RB) immune responses of shrimp fed with EBPA-containing feed were higher than the control (Fig. 1) and directly contributed to combating *V. parahaemolyticus* bacteria. These results indicate that feeding EBPA with daily frequency for 30 days before challenge testing can enhance shrimp resistance to pathogen attacks, as evidenced by treatments (P1, P2, and P3) fed with EBPA containing feed having higher survival rates compared to the control treatment. This condition is caused by continuous administration of immunostimulants, which can regulate and maintain the immune system in optimal condition until their administration is stopped (Jane et al. 2015). Shrimp immune response consisting of THC, AF, RB, and PO indicates that shrimp fed with EBPA at a dose of 0.75 g.kg^{-1} had the highest values compared to other treatments. The high values of shrimp immune response obtained in treatment P2 occurred not only because of the active compounds present in EBPA but also because the dose of Ambon banana stem extract greatly influences the use of EBPA. Higher doses of EBPA feed cause a decrease in immune response in the test shrimp, and low doses of EBPA feed yield good results, but better results are obtained when used at the optimal dose in this study, namely treatment P2 (0.75 g.kg^{-1}). These results are in line with the statement by Putri *et al.* (2013) that high doses of immunostimulants can suppress defense mechanisms, while low doses affect immune responses less effectively. Additionally, Giri et al. (2023) stated that administration of immunostimulants above the optimal dose can cause immunosuppression in test animals, but can protect fish or shrimp from disease attacks.

Observations of THC in test shrimp after 30 days of EBPA feeding before and after the challenge test indicate that treatment P2 is the best among all treatments. The THC bar graph shows a significant difference between treatment P2 and the control treatment. This significant difference is presumed to be due to the increased production of hemocyte cells in the shrimp's body as a result of EBPA feeding. The increase in hemocyte cells in the shrimp's body plays a crucial role in inhibiting or destroying pathogens that enter the shrimp's body (Ding et al. 2020). THC values were seen to decrease in treatments fed with EBPA containing feed and positive control on day 1 post-challenge test due to the migration of hemocyte cells from the shrimp's body circulation system to tissues where many cells are infected (Simanjuntak *et al.* 2016; Xian et al. 2016). Then, an increase in THC occurred on day 7 and then decreased again on days 14 to 21, followed by an increase on day 28. The increase in THC values on day 7 post-challenge test is due to the infection of *V. parahaemolyticus* bacteria in a critical phase, causing hemocyte cells to react to the body's defense mechanism consisting of pathogen recognition, phagocytosis, melanization, cytotoxicity, and cell communication in fighting pathogens (Azhar and Yudiati 2023; Dhewang et al. 2023; Kemal et al. 2023), and the increase on day 28 is presumably because the shrimp's body has returned to normal, thus it is able to produce hemocyte cells again. There are also parameters of shrimp's immune defense system that can be observed, namely phagocytosis activity (AF), which is the ability of non-specific immune response cells to phagocytose disease agents entering the body. Observations of AF indicate an increase in treatment P2 compared to other treatments. The increase in AF can be interpreted as the administration of EBPA in feed as an immunostimulant has been proven to stimulate or enhance the shrimp's immune system. This is in line with Kumar *et al.* statement (2023) that the mechanism of action of immunostimulants in stimulating the immune system is by increasing the activity of phagocytic cells. The increase in THC and AF values becomes the main defense system in fighting pathogens entering the shrimp's body (Panigrahi et al. 2020).

The activity of hemocytes in shrimp bodies also produces additional antibacterial compounds, such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), which can enhance shrimp's resilience to pathogen infection. Superoxide anion (O_2^-) is the first reaction generated in the process of respiratory burst (RB) activity. RB activity is detected when foreign objects are expelled through phagocytosis; when phagosomes engulf foreign objects, it involves the release of degradative enzymes that produce reactive oxygen intermediates (ROIs) (Risjani et al. 2021). The results of this study indicate that shrimp fed with EBPA for 30 days before the challenge test have higher RB activity compared to the control. Based on the results of the study, RB activity is directly proportional to THC. The decrease in RB activity on day 1 post-challenge test is related to the decrease in the number of hemocytes in the shrimp's body due to the migration of hemocytes to infection sites to perform phagocytosis processes and initiate defense responses (Muharrama *et al.* 2021).

Phenoloxidase (PO) activity is one of the humoral immune responses. In hemolymph, PO is present in an inactive form called prophenoloxidase (proPO). The ability of shrimp to recognize foreign objects entering its body indicates the high value of PO activity and the presence of shrimp's immune defense activity (Mahasri et al. 2017; Barrios et al. 2023). PO will later oxidize phenols to form quinones and produce the end product melanin, a dark brown pigment that inactivates and prevents the spread of pathogens (Amparyup et al. 2013). Measurement results show that the PO value in treatment P2 is higher than in other treatments, both before and after the challenge test. These results indicate that feeding EBPA-containing feed induces the activation of non-specific immune mechanisms in shrimp, thereby triggering the activation of intracellular genes that can increase the production of antimicrobial

molecules (Santhosh et al. 2023). With increased immune response, it can also reduce and suppress the growth of bacteria infecting shrimp as the primary defense against pathogen attacks.

The administration of Ambon banana stem extract (EBPA) through feed in this study also suppressed the growth of bacteria in the intestines of vannamei shrimp, including total *Vibrio* count and *V. parahaemolyticus* count (Fig. 2). Total *Vibrio* count and *V. parahaemolyticus* count in each treatment P1, P2, and P3 significantly suppressed bacterial populations ($P < 0.05$) compared to treatment K+. Treatment P2 was able to significantly suppress bacterial populations ($P < 0.05$) compared to other dosage treatments, including total *Vibrio* count and *V. parahaemolyticus* count up to day 28 after the challenge test. This is because EBPA contains flavonoids as antibacterial compounds, which work by breaking down the bacterial cell membrane, causing a cessation of ion and compound transport into bacterial cells. This transport constraint will result in reduced bacterial nutrition needed for growth (Kusdarwati et al. 2010; Krisnata et al. 2014). Additionally, according to Munaeni et al. (2020), the use of herbal materials can suppress bacterial growth and enhance immune responses in shrimp infected with *V. parahaemolyticus*. This is also emphasized by Rozik et al. (2022), stating that the use of herbal products in fish farming can be the initial step in preventing disease spread and inhibiting the growth of pathogenic bacteria. The reduction in bacterial populations in this study also correlates with the ability to reduce damage to the hepatopancreas organ (Fig. 3). This is evident from the reduction in vacuolization, normal tubules, lysis compared to the positive control. According to Raja et al. (2017), shrimp infected with *V. parahaemolyticus* can cause damage to the hepatopancreas organ. In this study, the use of EBPA was able to enhance immune responses in defense against *V. parahaemolyticus* infection, both through direct and indirect defense mechanisms, thus minimizing damage to the shrimp's hepatopancreas.

Furthermore, this study also observed immunity genes including *Lipopolysaccharide- and β -1,3-glucan-binding protein* (LGBP) and *peroxinectine* (PE) genes. *Lipopolysaccharide- and β -1,3-glucan-binding protein* (LGBP) gene plays a major role in recognizing and responding to entering bacteria (Chen et al. 2016) and plays a crucial role in activating the proPO system (Du et al. 2019). proPO is a key gene in activating the PO enzyme and then induces melanization and phagocytosis (Rowley 2016), while the PE gene plays a vital role in degranulation and phagocytosis, so when pathogens attack, hemocyte cells will undergo degranulation. During the degranulation process, the peroxinectin (PE) enzyme is released and stimulates the phagocytosis reaction. This is also related where peroxinectin (PE) is an essential amino acid in an inactive form and will be activated when the LGBP gene is present, so these two genes are interrelated in influencing the shrimp's immune response (Muharrama *et al.* 2021). In this study, it can be seen that the use of EBPA provided higher results compared to the control for LGBP and PE gene expression parameters (Fig. 4). The increased expression of the LGBP gene in EBPA usage after treatment and challenge tests indicates that the process providing resistance to pathogens by the shrimp's immune system is more effective in treatment compared to the positive control. Treatment P2 (0.75 g.kg^{-1}) in this study provided higher values compared to other dosages that could enhance the expression of immunity related genes. This is also emphasized by Hsieh et al. (2013), indicating that the use of herbal plants can enhance immune responses, immunity gene expression such as LGBP and PE, thereby increasing the survival of vannamei shrimp from bacterial infections.

CONCLUSIONS

The administration of Ambon banana stem extract enhances the immune response and resistance of vannamei shrimp to *V. parahaemolyticus* infection. The dose treatment of 0.75 g kg^{-1} feed is the most effective dose in improving growth performance, immune response, expression of immunity-related genes, reducing the population of *V. parahaemolyticus* in the intestines, and reducing hepatopancreas organ damage in vannamei shrimp before and after the challenge test.

Declarations

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Supplementary Data (Data Availability Statement)

The data underlying this article will be shared upon reasonable request with the corresponding author.

Ethics Approval

This research has been approved by the animal ethics commission of the IPB University veterinary and biomedical school, with registration number: 123/KEH/SKE/X/2023.

Conflict of Interest Statements

The authors declare that they have no further conflicts of interest or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution

All authors contributed to the conception and design of the study. Material preparation, experimental studies, data collection and analysis were performed by Geza Intan Septarisa. The first draft of the manuscript was written by Geza Intan Septarisa. Sri Nuryati, Sukenda and Munti Yuhana provided comments and proof read on the written manuscript. All authors read and approved the final manuscript.

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Figures

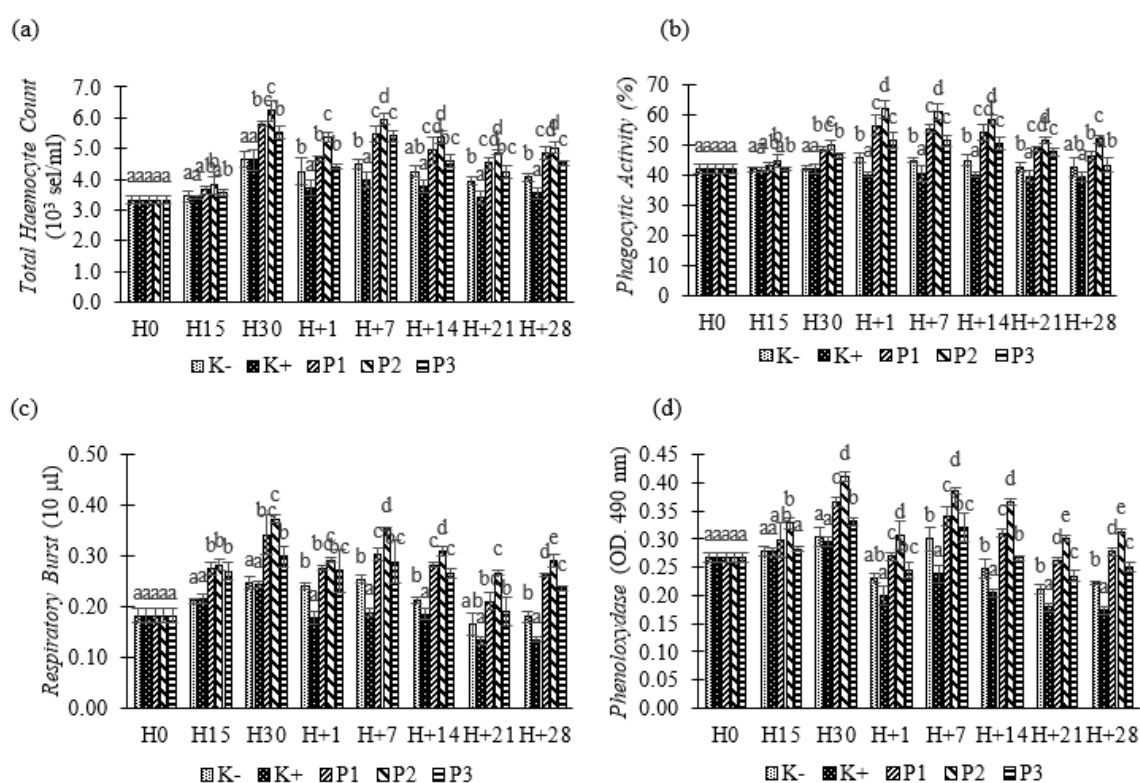


Figure 1

Immune response parameters of shrimp: (a) total haemocyte count (THC), (b) phagocytosis activity (AF), (c) respiratory burst (RB), and (d) phenoloxidase (PO) of vannamei shrimp fed with different doses of EBPA in the feed. H0 (before treatment), H15 (day 15 of treatment maintenance), H30 (day 30 of treatment maintenance or 1 day before challenge), H+1 (1 day after challenge), H+7 (7 days after challenge), H+14 (14 days after challenge), H+21 (21 days after challenge), and H+28 (28 days after challenge). K- (negative control), K+ (positive control), P1 (0.50 g.kg⁻¹), P2 (0.75 g.kg⁻¹), and P3 (1 g.kg⁻¹). Different letters above the bar charts (mean ± standard deviation) in the same row indicate statistically significant differences (P < 0.05).

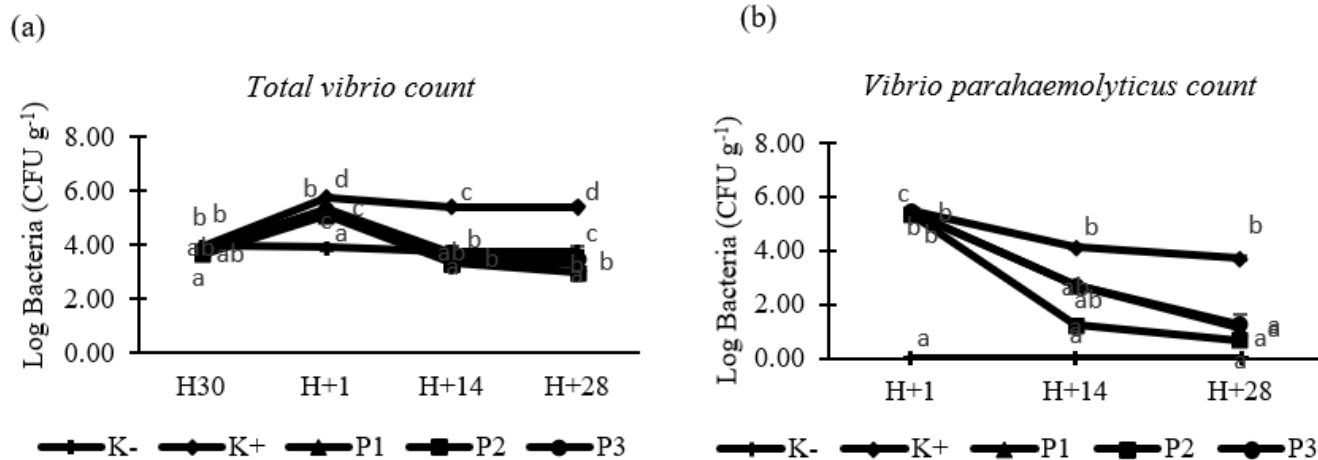


Figure 2

Abundance of bacteria in the intestine consisting of: (a) total *Vibrio* count and (b) *V. parahaemolyticus* count before and after challenge with *V. parahaemolyticus* bacteria. H30 (day 30 of treatment maintenance or 1 day before challenge), H+1 (1 day after challenge), H+14 (14 days after challenge), and H+28 (28 days after challenge). K- (negative control), K+ (positive control), P1 (0.5 g.kg⁻¹), P2 (0.75 g.kg⁻¹), and P3 (1 g.kg⁻¹). Data (mean $\pm$ SD) with different letters on the same day indicate statistically significant differences ($P < 0.05$).

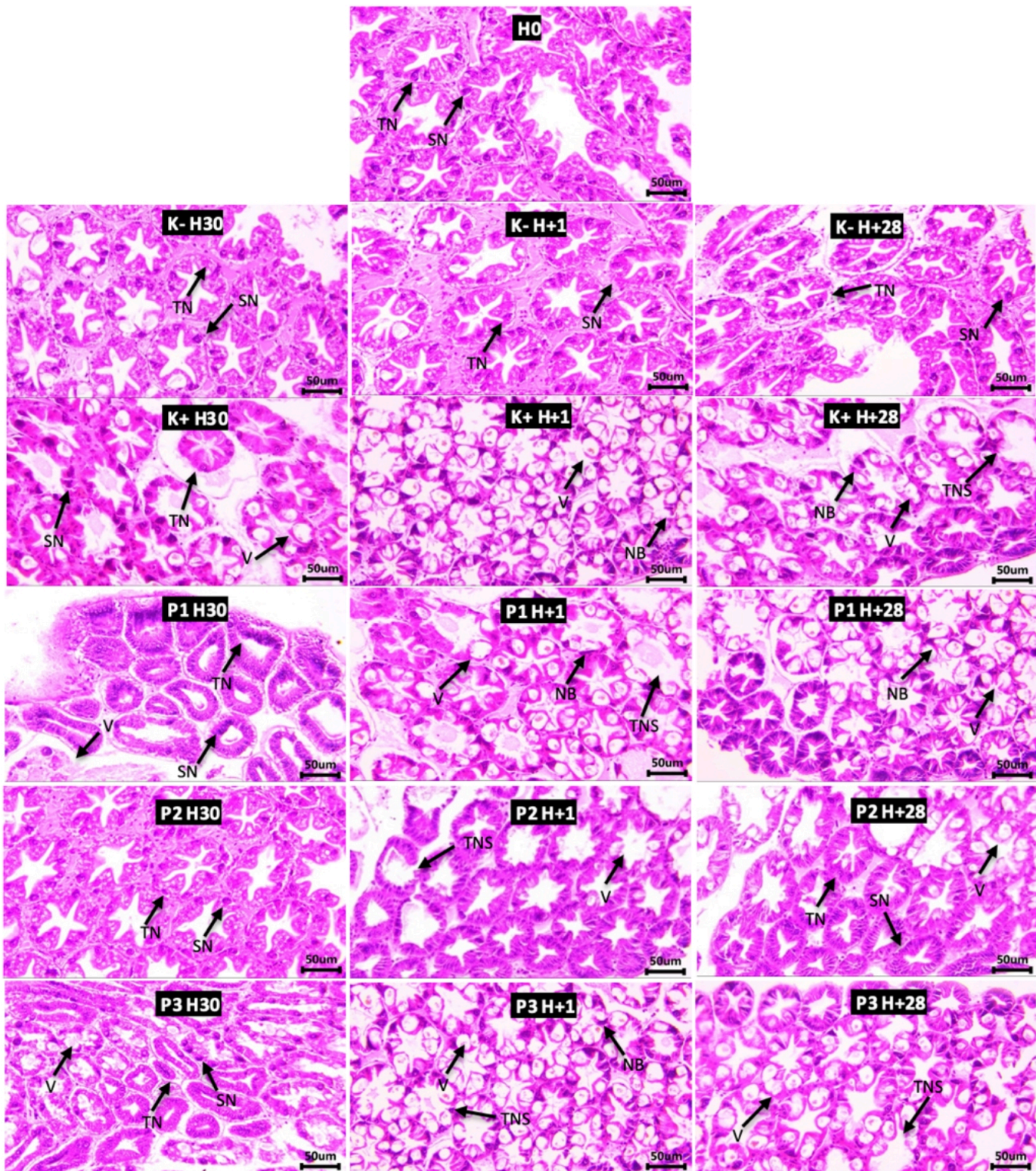


Figure 3

Histopathology before and after challenge with *V. parahaemolyticus* bacteria. H0 (before treatment), H30 (day 30 of treatment maintenance or 1 day before challenge), H+1 (1 day after challenge), and H+28 (28 days after challenge). K- (negative control), K+ (positive control), P1 (0.5 g.kg⁻¹), P2 (0.75 g.kg⁻¹), and P3 (1 g.kg⁻¹). Normal tubules (TN), B cell necrosis (NB), vacuole (V), normal cells (SN), tubule necrosis (TNS).

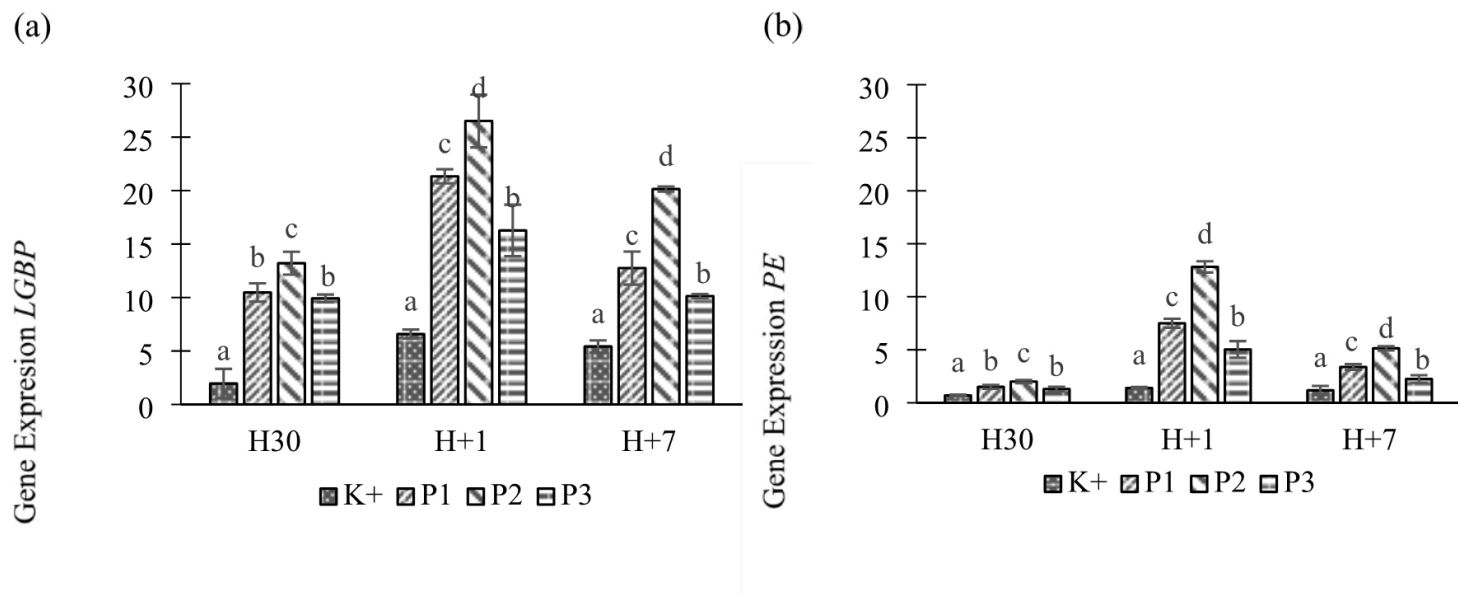


Figure 4

Shrimp gene expression: *Lipopolysaccharide- and β -1,3-glucan-binding protein* (LGBP) and *peroxinextine* (PE). H30 (day 30 of treatment maintenance or 1 day before challenge), H+1 (1 day after challenge), and H+7 (7 days after challenge). K+ (positive control), P1 (0.5 g.kg^{-1}), P2 (0.75 g.kg^{-1}), and P3 (1 g.kg^{-1}). Different letters above the bar charts (mean $\pm$ standard deviation) in the same row indicate statistically significant differences ($P < 0.05$).

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