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Antibiofilm of metabolites of the *Proteus myxofaciens* JB 20B for the prevention and treatment of vaname shrimp infected with *Vibrio harveyi* bacteria

Antibiofilm metabolit *Proteus myxofaciens* JB 20B untuk pencegahan dan pengobatan udang vaname yang di infeksi bakteri *Vibrio harveyi*

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ABSTRACT

Vibrio harveyi is one of the common bacteria that cause disease in vaname shrimp. The aim of this study is to determine the efficacy of bacterial metabolites *Proteus myxofaciens* JB 20B as an antibiofilm agent in the prevention and treatment of *V. harveyi*-infected vaname shrimp (*Litopenaeus vannamei*). The experimental design included eight treatments and three replicates: K- (negative control), K+ (positive control), PCA and PGA (antibiotic control 50 g/100ml for preventive and treatment), PCE (preventive extract 0.1 ml/kg feed), PCS (preventive supernatant 20 ml/kg feed), PGE (treatment extract 0.1 ml/kg feed), and PGS (treatment supernatant 20 ml/kg feed). On day 15th, vaname shrimp were intramuscularly infected with 10^6 CFU/mL of *V. harveyi*. Examining clinical symptoms, viewing histology, assessing total hemocyte count, phagocytosis activity, phenoloxidase activity, respiratory burst, and growth performance. According to the findings of this study, the treatment of bacterial metabolites *P. myxofaciens* JB 20B (PCE, PCS, PGE, and PGS) is superior to the positive control (K+) treatment in terms of reducing shrimp hepatopancreas necrosis, increasing the value of total hemocyte count, phagocytosis activity, phenoloxidase activity, respiratory burst, and growth performance of vaname shrimp through feeding in both prevention and treatment. The use of *P. myxofaciens* JB 20B bacterial metabolites to control *V. harveyi* infection in vaname shrimp yielded the best results in the supernatant treatment.

Keywords: antibiofilm, biofilm, *Proteus myxofaciens* JB 20B, vannamei shrimp, *Vibrio harveyi*

ABSTRAK

Vibrio harveyi merupakan salah satu bakteri penyebab penyakit pada udang vaname. Tujuan dari penelitian ini adalah untuk mengetahui efektivitas metabolit bakteri *Proteus myxofaciens* JB 20B sebagai agen antibiofilm dalam pencegahan dan pengobatan udang vaname (*Litopenaeus vannamei*) yang terinfeksi *V. harveyi*. Rancangan penelitian ini terdiri dari delapan perlakuan dan tiga kali ulangan: K- (kontrol negatif), K+ (kontrol positif), PCA dan PGA (kontrol antibiotik 50 g/100ml untuk pencegahan dan pengobatan), PCE (ekstrak pencegahan 0,1 ml/kg pakan), PCS (supernatan pencegahan 20 ml/kg pakan), PGE (ekstrak pengobatan 0,1 ml/kg pakan), dan PGS (supernatan pengobatan 20 ml/kg pakan). Pada hari ke-15, udang vaname diinfeksi secara intramuskular dengan 10^6 CFU/mL *V. harveyi*. Memeriksa gejala klinis, melihat histologi, menilai jumlah hemosit total, aktivitas fagositosis, aktivitas phenoloxidase, respiratory burst, dan performa pertumbuhan. Berdasarkan hasil penelitian ini, perlakuan metabolit bakteri *P. myxofaciens* JB 20B (PCE, PCS, PGE, dan PGS) lebih unggul dibandingkan dengan perlakuan kontrol positif (K+) dalam hal mengurangi nekrosis hepatopankreas udang, meningkatkan nilai jumlah hemosit total, aktivitas fagositosis, aktivitas phenoloxidase, respiratory burst, dan performa pertumbuhan udang vaname melalui pemberian pakan pasca infeksi baik pada pencegahan maupun pada pengobatan. Penggunaan metabolit bakteri *P. myxofaciens* JB 20B untuk mengendalikan infeksi *V. harveyi* pada udang vaname memberikan hasil terbaik pada perlakuan supernatan.

Kata kunci: antibiofilm, biofilm, *Proteus myxofaciens* JB 20B, udang vaname, *Vibrio harveyi*

INTRODUCTION

Vannamei shrimp (*Litopenaeus vannamei*) is one of the most popular commodities with high export potential. However, intensive farming systems for vannamei shrimp can have adverse effects, as high stocking densities and poor water quality can increase the risk of diseases caused by bacterial pathogens (Watts *et al*., 2017). Vibriosis, a disease in shrimp, is caused by bacteria from the genus *Vibrio*, including *V. harveyi*, where mortality rates due to *V. harveyi* infection range between 92–100% in a short period (Apriliani *et al*., 2016). Shrimp mortality caused by *V. harveyi* poses significant economic losses for shrimp farmers in both large- and small-scale aquaculture operations in Indonesia (Widanarni *et al*., 2015). Clinical symptoms of shrimp infected with *V. harveyi* include pale hepatopancreas with atrophy, soft carapace, empty intestines, and the presence of reddish-black spots on the body and hepatopancreas (Adam *et al*., 2022).

Vibrio harveyi bacteria are capable of forming biofilms that enhance their virulence and resistance while protecting them from external environmental factors and supplying nutrients to cells, making the bacteria resistant to various environmental conditions (Rasmussen-Ivey *et al*., 2016). The biofilm structure complicates prevention and treatment using antibiotics, as biofilms are difficult to penetrate, necessitating higher antibiotic dosages (Thamburaj *et al*., 2020). Excessive use of antibiotics can negatively impact the environment and increase the risk of antibiotic resistance. This study utilized an antibiofilm agent derived from the metabolites of *Pseudomonas myxofaciens* JB 20B, a bacterium isolated from the surface of guava (*Psidium guajava*) leaves.

The metabolites of *P. myxofaciens* JB 20B have been tested *in vitro* according to Nathalia and Waturangi (2021). The JB 20B isolate effectively inhibited *V. harveyi* biofilm formation by 55% and degraded *V. harveyi* biofilms by 84% at a dose of 20 mg/mL. This finding aligns with other studies, such as Raissa *et al.* (2020), which reported that a 20 mg/mL dose of *Actinomycetes* bacteria exhibited inhibitory activity against *V. harveyi* and *Aeromonas hydrophila*. Further studies by Mauliana (2021) conducted *in vivo* on Nile tilapia with *A. hydrophila* demonstrated that supernatant doses of 2 mL/100 g feed and extract doses of 0.01 mL/100 g feed were effective. However, the application of *P. myxofaciens* JB 20B metabolites *in vivo* on vannamei shrimp has not been conducted. Therefore, such research is crucial to develop preventative and therapeutic measures for vannamei shrimp infected with *V. harveyi*.

MATERIALS AND METHODS

Time and location

This study was conducted from April to July 2023 at the Aquatic Organism Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University.

Tested materials

The test materials used in this study were vannamei shrimp (*Litopenaeus vannamei*), sourced from shrimp ponds in Cidatu Beach, Anyer, with an average weight of 3 ± 0.5 g per shrimp. The *Vibrio harveyi* bacterial isolate was obtained from the collection of the Aquatic Organism Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. The metabolite used was derived from the bacterium *Pseudomonas myxofaciens* JB 20B, provided by the Food Microbiology Laboratory, Faculty of Biotechnological Sciences, Atma Jaya Catholic University of Indonesia, Jakarta.

Experimental design

The study used a completely randomized design with seven treatments and three replications, as described below:

- **K-** : Feed without *P. myxofaciens* JB 20B coating + PBS injection.
- **K+** : Feed without *P. myxofaciens* JB 20B coating + bacterial challenge with *V. harveyi* at 10^6 CFU/mL on day 15.
- **PCA** : Feed coated with oxytetracycline antibiotic at 50 g/100 mL (before bacterial challenge) + bacterial challenge with *V. harveyi* at 10^6 CFU/mL on day 15.
- **PGA** : Feed coated with oxytetracycline antibiotic at 50 g/100 mL (after bacterial challenge) + bacterial challenge with *V. harveyi* at 10^6 CFU/mL on day 15.
- **PCE** : Feed coated with *P. myxofaciens* JB 20B extract at a dose of 0.1 mL/kg feed (before bacterial challenge) + bacterial challenge with *V. harveyi* at 10^6 CFU/mL on day 15.
- **PCS** : Feed coated with *P. myxofaciens* JB 20B extract at a dose of 20 mL/kg feed (before bacterial challenge) + bacterial challenge with *V.*

harveyi at 10^6 CFU/mL on day 15.

• **PGE** : Feed coated with *P. myxofaciens* JB 20B supernatant at a dose of 0.1 mL/kg feed (after bacterial challenge) + bacterial challenge with *V. harveyi* at 10^6 CFU/mL on day 15.

• **PGS** : Feed coated with *P. myxofaciens* JB 20B supernatant at a dose of 20 mL/kg feed (after bacterial challenge) + bacterial challenge with *V. harveyi* at 10^6 CFU/mL on day 15.

Preparation of feed

Feed treatments were prepared using a coating method adapted from Huang and Nitin (2019). The test feed was prepared by homogenizing *P. myxofaciens* JB 20B metabolites (PCE: 0.1 mL/kg feed, PCS: 20 mL/kg feed, PGE: 0.1 mL/kg feed, PGS: 20 mL/kg feed) with sterile distilled water (10% of feed volume) and egg white (2% of feed weight) as a binder. The homogenized mixture was evenly distributed over the feed and oven-dried for 30 minutes at 40–50°C.

Preparation and maintenance of tested shrimp

The vannamei shrimp used had an average size of 3 ± 0.53 g per individual, stocked at a density of 15 shrimp per aquarium (water volume: 32 L). Maintenance containers comprised 24 aquariums, each measuring 60×30×40 cm^3. Shrimp were acclimatized for seven days with a feeding rate (FR) of 10% body weight and a feeding frequency of five times daily (07:00, 10:00, 13:00, 16:00, and 19:00 WIB). During maintenance, water quality was maintained by replacing 50% of the aquarium water every three days.

Preventive feeding was conducted from days 0–14, followed by bacterial injection on day 15, and non-treated feed was provided from days 16–28. For therapeutic feeding, non-treated feed was given from days 0–14, followed by bacterial challenge via injection on day 15, and treated feed was provided from days 16–28. During the maintenance period, water quality was maintained by replacing 50% of the aquarium water every three days. Observations included shrimp feeding response, behavior, mortality, and clinical symptoms.

Observation parameters

Observation parameters included growth performance metrics such as survival rate (SR), specific growth rate (SGR), and feed conversion ratio (FCR). Immune response parameters consisted of clinical symptoms, total hemocyte count (THC), phagocytic activity (PA), phenoloxidase (PO) activity, respiratory burst (RB) activity, and histopathology.

Data analysis

Data were analyzed using MS Office Excel 2019, and analysis of variance (ANOVA) was performed using SPSS 26.0 with a 95% confidence interval. Significant differences among treatments were further analyzed using Duncan's test. Clinical symptom parameters were analyzed descriptively, while histopathology, THC, DHC, PA, PO, RB, AL, and TPC data were analyzed quantitatively.

RESULTS AND DISCUSSION

Result

Clinical symptoms in vannamei shrimp (L. vannamei)

Observations of clinical symptoms in vannamei shrimp post-injection with *Vibrio harveyi* on day 15 of infection are presented in Figure 1. The clinical symptoms observed in shrimp after infection included pale hepatopancreas, empty intestines, pale carapace, reduced activity during feeding, and sluggish swimming movements.

Immune response in shrimp

The total values of total hemocyte count (THC), phagocytic activity, phenoloxidase (PO) activity, and respiratory burst (RB) activity on days 0, 14, 16, 19, and 28 are presented in Figure 2. The total hemocyte count (THC) results from ANOVA showed that on day 0, there were no significant differences among treatments ($P>0.05$). On day 14, the THC value increased, with the PCS treatment showing a significant difference ($P<0.05$) compared to the K+ treatment. On day 16, the PCS and PGS treatments showed significantly different results ($P<0.05$) from the K+ treatment. On day 19, the PGE and PGS treatments showed significant differences ($P<0.05$) compared to the K+ treatment. On day 28, the THC value in the PGS treatment was significantly different ($P<0.05$) from the K+ treatment.

The phagocytic activity (PA) on day 0 did not show significant differences among treatments ($P>0.05$). However, on day 14, the AF value in the PCE treatment was significantly different ($P<0.05$) from the K+ treatment but not from the K- treatment. On day 16, the PCS treatment showed significant differences ($P<0.05$) compared to the K+ treatment but not the K- treatment. On day 19, the PCS and PGS treatments showed

significant differences (P<0.05) compared to the K+ treatment but not the K- treatment. On day 28, the PCS and PGS treatments showed significant differences (P<0.05) compared to the K+ treatment but not the K- treatment.

The phenoloxidase (PO) activity results on day 0 (before bacterial challenge) showed no significant differences among treatments (P>0.05). However, the PCE, PCS, PGE, and PGS treatments showed significant differences (P<0.05) on day 14. On day 16, the PCE, PCS, PGE, and PGS treatments showed no significant differences (P>0.05). On day 19, the PGS treatment showed a significant difference (P<0.05). On day 28, the PO value in the PGS treatment was significantly different (P<0.05) from the K+ treatment, but not from the K- treatment.

The respiratory burst (RB) on day 0 showed no significant differences among treatments (P>0.05). On day 14, the PCE, PCS, PGE, and PGS treatments showed no significant differences (P>0.05). On day 16, the PCS treatment showed a significant difference (P<0.05). On day 19, the PGE and PGS treatments showed significant differences (P<0.05) compared to the K+ treatment but not the K- treatment. On day 28, the respiratory burst value in the PGS treatment showed a significant difference (P<0.05) from the K+ treatment, but not from the K- treatment.

Histopathology

The histopathological observations of the hepatopancreas in shrimp are presented in Figure 3. The hepatopancreas histology in the K- treatment showed no damage, however, the hepatopancreas in other treatments exhibited hypertrophy. Hepatopancreatic cells in the lumen of the hepatopancreatic tubules (black

Figure 1. Clinical symptoms post-challenge in vannamei shrimp; (a) healthy shrimp and (b) diseased shrimp.

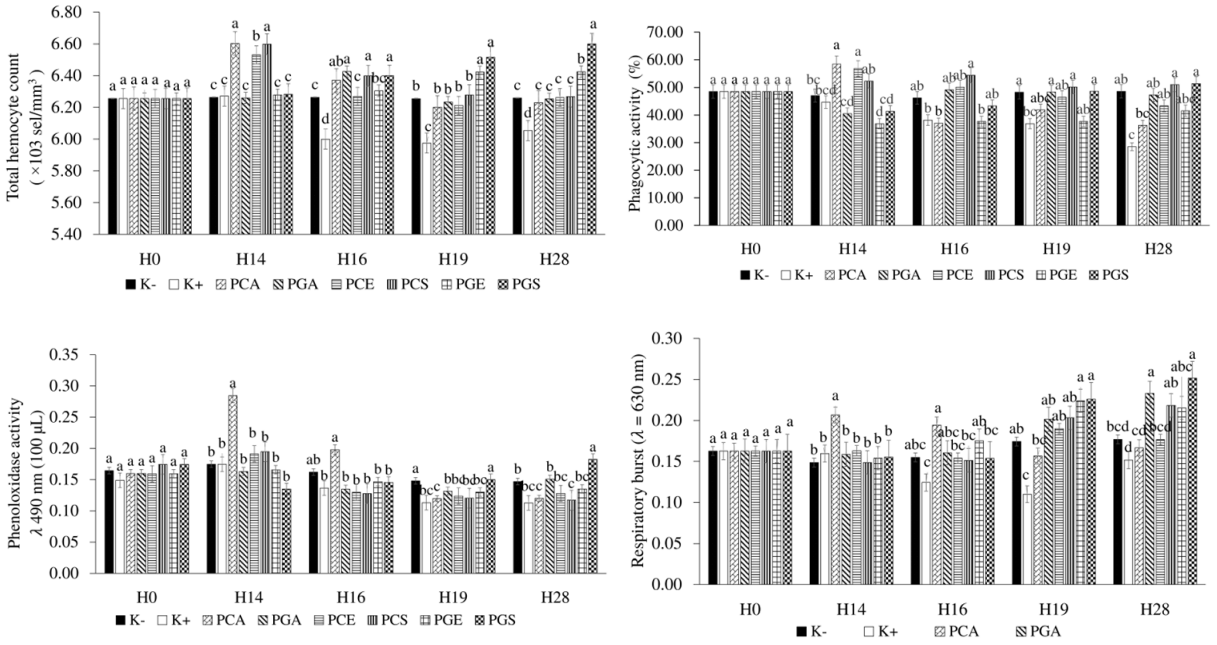

Figure 2. Total hemocyte count (THC), phagocytic activity, phenoloxidase (PO) activity, and respiratory burst (RB) activity in vannamei shrimp on days 0, 14, 16, 19, and 28 post-challenge with *Vibrio harveyi*. Superscripts on the graphs indicate statistically significant differences as determined by Duncan's test at a 5% significance level.

arrow), showed hypertrophy (yellow arrow), fat degeneration (green arrow), cell necrosis (red arrow), and there was a gap between the tubules, with hepatopancreas size enlarged (brown line). The hepatopancreas scoring results are presented in Table 1.

Table 1. Hepatopancreas scoring in vannamei shrimp post-challenge with *Vibrio harveyi*.

Treatments	The percentage of necrosis (%)				
	H0	H14	H16	H19	H28
K-	12.23 ± 1.44^{a}	11.21 ± 0.68^{ab}	11.98 ± 0.00^{c}	13.49 ± 0.86^{c}	13.28 ± 1.56^{c}
K+	12.23 ± 1.44^{a}	11.67 ± 0.88^{ab}	42.14 ± 0.04^{a}	50.52 ± 2.54^{a}	53.77 ± 5.40^{a}
PCA	12.22 ± 1.44^{a}	10.61 ± 0.45^{ab}	17.09 ± 0.01^{c}	37.29 ± 1.01^{b}	48.22 ± 4.19^{a}
PGA	12.22 ± 1.44^{a}	10.43 ± 1.25^{b}	26.67 ± 0.01^{b}	35.29 ± 4.17^{b}	37.42 ± 6.05^{b}
PCE	12.23 ± 1.44^{a}	11.92 ± 0.48^{a}	34.61 ± 0.02^{b}	38.23 ± 1.45^{b}	40.16 ± 2.80^{b}
PCS	12.25 ± 1.40^{a}	11.57 ± 0.54^{ab}	32.89 ± 0.04^{b}	35.14 ± 3.34^{b}	37.81 ± 4.12^{b}
PGE	12.25 ± 1.40^{a}	11.57 ± 0.36^{ab}	32.07 ± 0.04^{b}	37.62 ± 1.86^{b}	40.66 ± 3.57^{b}
PGS	12.25 ± 1.40^{a}	12.19 ± 0.37^{a}	30.57 ± 0.04^{b}	36.42 ± 1.70^{b}	39.07 ± 3.68^{b}

Note: Data (mean ± SD) with different superscripts indicate statistically significant differences at the 5% significance level (Duncan's test). The treatments are as follows: negative control (K-), positive control (K+), PCA (antibiotic prevention), PGA (antibiotic treatment), PCE (extract prevention), PCS (supernatant prevention), PGE (extract treatment), and PGS (supernatant treatment).

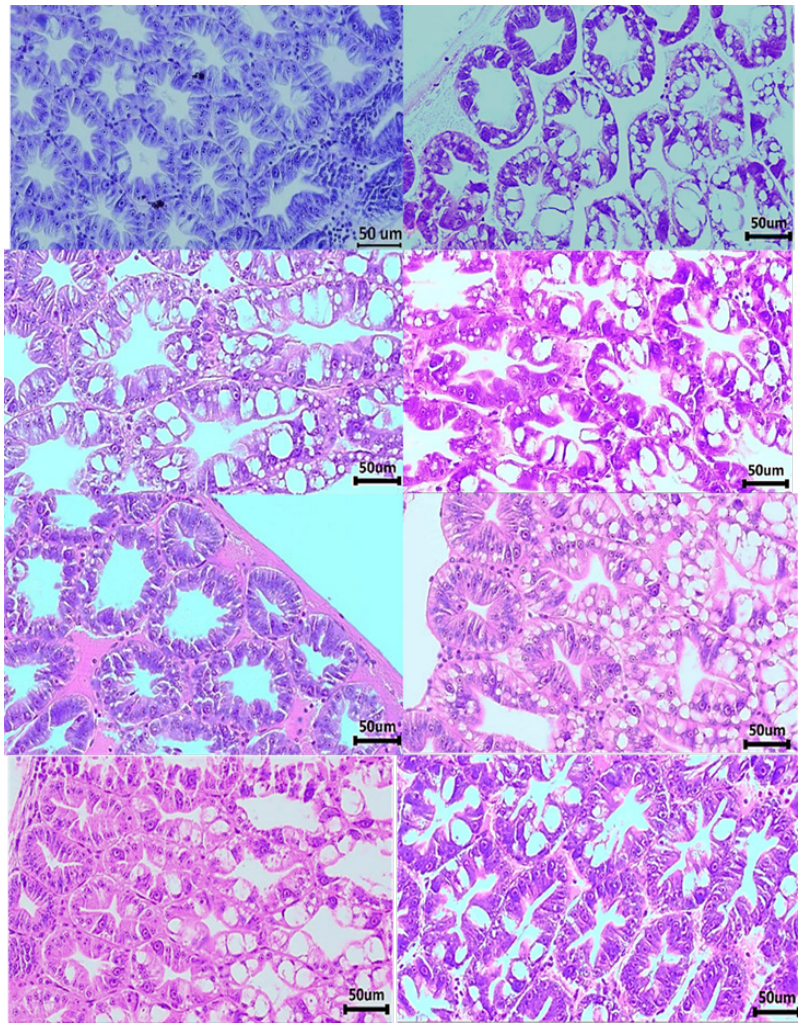

Figure 3. Histopathology of the hepatopancreas in vannamei shrimp post-infection with *Vibrio harveyi*. K- (negative control), K+ (positive control), PCA (antibiotic prevention), PGA (antibiotic treatment), PCE (extract prevention), PCS (supernatant prevention), PGE (extract treatment), and PGS (supernatant treatment).

Bacterial abundance

The results of the *Vibrio harveyi* bacterial density in the hepatopancreas organ of vannamei shrimp during the 30-day maintenance period are presented in Figure 4.

Growth performance of vannamei shrimp (L. vannamei)

The measurement of growth performance in vannamei shrimp treated with bacterial metabolite of *P. myxofaciens* JB 20B for prevention and treatment of vannamei shrimp injected with *V. harveyi* is presented in Table 2.

Discussion

Vibrio harveyi bacteria are capable of forming biofilms, which can enhance virulence traits, resistance, and protect the bacteria from external environments while providing cellular nutrients, making them resistant to various environmental conditions (Rasmussen-Ivey *et al*., 2016). The presence of this biofilm structure complicates prevention and treatment using antibiotics, as biofilms are difficult for antibiotics to penetrate, requiring higher doses (Aurestila *et al*., 2018). However, the use of antibiotics can have negative consequences, as it may lead to resistance in shrimp, the environment, and humans (Lulijwa *et al*., 2020). The bacterial metabolite from *P. myxofaciens* JB 20B is one potential solution for prevention and treatment in this study. The bacterial metabolite of *P. myxofaciens* JB 20B was administered to shrimp feed during the 15-day maintenance period.

Infection of vannamei shrimp in this study was carried out through an intramuscular injection into the third segment of the abdomen with a bacterial dose of 10^6 CFU/mL. Post-injection, the

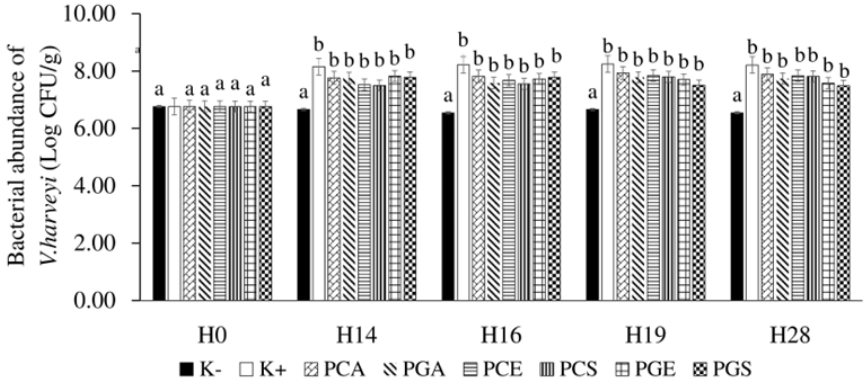

Figure 4. Bacterial density of *Vibrio* in the hepatopancreas of vannamei shrimp infected with *V. harveyi*. The superscripts indicated on the graph showed no significant differences at the 5% significance level (Duncan's test).

Table 2. Growth performance of vannamei shrimp treated with *P. myxofaciens* JB 20B bacterial metabolites.

Treatments	Parameters				
	Initial weight (g)	Final weight (g)	SR (%)	FC (g)	FCR
K-	2.99 ± 0.03^{a}	8.26 ± 0.33^{a}	100.00 ± 0.00^{a}	211.33 ± 9.07^{a}	1.75 ± 0.02^{a}
K+	2.99 ± 0.03^{a}	6.54 ± 0.19^{c}	57.78 ± 3.85^{d}	125.00 ± 5.00^{d}	1.63 ± 0.04^{b}
PCA	2.98 ± 0.02^{a}	7.48 ± 0.72^{abc}	60.00 ± 3.85^{cd}	71.67 ± 7.64^{cd}	1.24 ± 0.14^{ab}
PGA	2.97 ± 0.02^{a}	7.61 ± 0.20^{abc}	64.44 ± 7.70^{cd}	138.33 ± 7.64^{cd}	1.72 ± 0.06^{ab}
PCE	2.90 ± 0.17^{a}	7.05 ± 1.12^{abc}	64.44 ± 10.18^{cd}	70.00 ± 9.01^{cd}	1.18 ± 0.16^{ab}
PCS	2.98 ± 0.02^{a}	6.76 ± 0.65^{bc}	71.11 ± 3.85^{cd}	72.50 ± 6.61^{cd}	1.21 ± 0.10^{ab}
PGE	2.97 ± 0.05^{a}	7.96 ± 0.03^{ab}	66.67 ± 6.67^{c}	155.00 ± 15.00^{bc}	1.67 ± 0.09^{ab}
PGS	2.98 ± 0.02^{a}	8.09 ± 0.13^{a}	82.22 ± 3.85^{b}	173.33 ±12.58^{b}	1.72 ± 0.05^{ab}

Note: Data (mean ± SD) with different superscripts indicate statistically significant differences at the 5% significance level (Duncan's test). SR (survival rate), FC (feed consumption), FCR (feed conversion ratio). K- (negative control), K+ (positive control), PCA (antibiotic prevention), PGA (antibiotic treatment), PCE (extract prevention), PCS (supernatant prevention), PGE (extract treatment), and PGS (supernatant treatment).

shrimp were observed for growth performance, including survival rate (SR), specific growth rate (SGR), and feed conversion ratio (FCR). Supplementation with the bacterial metabolite of *P. myxofaciens* JB 20 in the shrimp feed resulted in significant differences ($P<0.05$) post-challenge for the treatments PCE, PCS, PGE, and PGS compared to K+, PCA, and PGA, but did not show significant differences compared to K- treatments. The growth performance observed in all treatments was suboptimal, suggesting that some of the energy was not fully utilized for growth but rather directed towards recovery from the *V. harveyi* bacterial infection. However, other factors may also influence vannamei shrimp growth, such as environmental stress (DO, temperature, pH, and TAN) and disease occurrence (Mengistu *et al*., 2020), which may have led to variations in survival rates.

The vannamei shrimp post-infection with *V. harveyi* caused significant mortality by day 19, marked by clinical symptoms including reduced appetite, abnormal swimming, soft carapace, absence of intestines, and pale hepatopancreas. This aligns with previous studies, which state that shrimp infected with *V. harveyi* exhibit symptoms such as pale hepatopancreas, soft carapace, absence of intestinal contents, and the presence of reddish-black spots on the body or hepatopancreas (Romano *et al*., 2015). Mortality in shrimp post-infection indicates the spread of bacteria throughout the shrimp's body, including the hepatopancreas. According to Dhar *et al.* (2019), the major damage in infected shrimp occurs in the hepatopancreas. To measure the extent of damage caused by *V. harveyi* in the hepatopancreas, total plate count (TPC) (Sukmawati & Hardianti, 2018) and histopathological necrosis (Tatukede *et al*., 2014) can be used.

The TPC results from this study indicate a bacterial load of 10^8 CFU/g in the hepatopancreas of vannamei shrimp infected with *V. harveyi*. According to Utami *et al.* (2016), infected shrimp with *V. harveyi* in the hepatopancreas exhibit bacterial counts exceeding 10^6 CFU/mL. This is also in agreement with the findings of Sirikharin *et al.* (2015), where the bacterial density of *Vibrio* sp. in the hepatopancreas of shrimp exceeded 10^4 CFU/mL. The major damage caused by *V. harveyi* post-challenge in the K+ treatment showed changes in the lumen of the hepatopancreas tubules, with hypertrophic cells, hepatocyte damage, lipid degeneration in the cytoplasm, necrosis, and a noticeable gap between the tubules, with the hepatopancreas enlarging (Kumar *et al*., 2021). The severity of damage to the hepatopancreas in the K+ treatment resulted in higher shrimp mortality compared to other treatments.

This finding is supported by Pardede *et al.* (2023), where severe hepatopancreatic damage is characterized by enlarged, distanced, and irregular tubules with necrotic cells. In contrast, normal hepatopancreas tissue is marked by clearly structured tubules without necrosis. According to Nadella *et al.* (2018), a normal hepatopancreas in shrimp does not exhibit necrosis, and the tubules appear normal and well-defined. Damage to the hepatopancreas disrupts shrimp physiology, leading to decreased survival rates (Utami *et al*., 2016). In addition to histological examination of the hepatopancreas, necrosis percentage was also observed.

The necrosis percentage in the hepatopancreas of shrimp exhibited the most severe damage in the K+ treatment, while the least damage occurred in the PGS treatment. The lower necrosis percentage in the PGS treatment was due to the inhibitory effect of *P. myxofaciens* JB 20B metabolites on *V. harveyi* growth during feeding and maintenance. According to Nathalia and Waturangi (2021), *P. myxofaciens* JB 20B can inhibit the biofilm formed by *V. harveyi*, showing 55% biofilm inhibition and 84% biofilm destruction in vitro. Hemocytes are a crucial component of the immune defense system in shrimp. They function to combat foreign entities that enter the body, thereby preventing disease outbreaks (Kulkarni *et al*., 2021). During the maintenance period, the utilization of *P. myxofaciens* JB 20B metabolites led to an increase in total hemocyte count (THC) on day 14 (PCE and PCS), 16 (PGE and PGS), 19 (PGE and PGS), and 28 (PGS).

The observed increase in THC values is suspected to be due to the ability of *P. myxofaciens* JB 20B to inhibit the formation and destruction processes of *V. harveyi* bacteria, as part of the shrimp's immune response when the pathogen enters (Hidayatullah, 2019). According to Nathalia and Waturangi (2021), the antibiofilm activity of *P. myxofaciens* JB 20B involves the production of enzymes capable of degrading extracellular polymeric substances (EPS), which in turn inhibits the growth and microbial activity of biofilms. This is further supported by Luo and Song (2021), who state that antibiofilm activity functions in inhibiting biofilm quorum sensing, which plays a role in breaking down bacterial molecules from pathogenic bacterial attacks.

However, the decrease in THC values in the treatments is due to thinning of the hemocyte layer caused by bacterial attack, leading to an inability to phagocytize the invading bacteria (Havanapan *et al*., 2016). According to Mauliana (2021), antibiofilm isolates from Actinomycetes can suppress the formation and destruction of biofilms by *A. hydrophila* bacteria, thereby reducing the virulence of *A. hydrophila*.

Hemocyte cells in shrimp are located in the hemolymph and play a key role in the defense system against pathogen infections (Kulkarni *et al*., 2021). According to Oktaviana and Febriani (2019), hemocytes in shrimp function as part of the immune defense, responsible for phagocytosis, encapsulation, and nodulation, which enhances the shrimp's immune system. Phagocytic activity is a part of the innate immune system in shrimp (Hao *et al*., 2019). The phagocytic activity before the challenge test showed an increase on days 14 (PCE), 16 (PGS), 19 (PCS and PGS), and 28 (PCS and PGS), with statistically significant differences ($P<0.05$). The increased phagocytic activity indicates a beneficial effect of feeding *P. myxofaciens* JB 20B metabolites to the shrimp in eliminating *V. harveyi* bacteria post-challenge.

High phagocytic activity reflects an effective immune system capable of producing phagocytes to combat bacterial attacks by performing phagocytosis (Lin *et al*., 2017). Phagocytosis is the process by which phagocytes recognize foreign substances, then migrate and adhere (chemotaxis) to macrophages, neutrophils, and monocytes, which engulf and kill pathogens attacking the shrimp's body, as well as remove bacterial remnants in the shrimp's hemolymph (Platt & Fineran, 2015). The phagocytic cells then form digestive vacuoles, known as phagosomes. A phagosome is a vesicle formed around material that enters the cell via phagocytosis. After a series of complex events, the phagosome merges with a lysosome (phagolysosome) containing degradative hydrolytic enzymes (Allen, 2016).

The foreign material is then destroyed and expelled through feces. The destruction process during phagocytosis is associated with the breakdown of enzymes and the production of reactive oxygen intermediates and the phagosome. This sequence of events is known as the respiratory burst (Gustilatov *et al*., 2022). The respiratory burst activity plays a significant role in protecting shrimp from foreign entities that invade their bodies. The respiratory burst values showed an increase and significant differences ($P<0.05$) post-infection on days 16 (PCS), 19 (PGE and PGS), and 28 (PGS). The high respiratory burst values are attributed to the prolonged administration of *P. myxofaciens* JB 20B metabolites during the maintenance period.

According to Rudi *et al.* (2019), the duration of treatment affects the respiratory burst activity in shrimp, leading to an increase. An increase in respiratory burst activity during maintenance indicates the activation of immune processes and highlights the important role of phagocytosis in eliminating foreign substances such as bacteria from the shrimp's body (Maftuch *et al*., 2012). However, a drastic increase in respiratory burst during maintenance may trigger oxidative stress induction within the cells, which could be caused by the bacteria or other pathogens (Gorski *et al*., 2012). Phenoloxidase is an inactive enzyme component derived from proPO (prophenoloxidase), acting as an immunostimulant (Ramadhan *et al*., 2017). Phenoloxidase is part of the system that defends against pathogen attacks and plays a role in melanization.

The melanization process involves both enzymatic and non-enzymatic reactions (Hidayatullah, 2019). Phenoloxidase activity increased on days 14 (PCE, PCS, PGE, and PGS), 19 (PGS), and 28 (PGS), with significant differences ($P<0.05$). This indicates an improvement in the shrimp's defense system due to the utilization of *P. myxofaciens* JB 20B metabolites following *V. harveyi* bacterial infection. High phenoloxidase activity suggests that the treatment with *P. myxofaciens* JB 20B metabolites helps the shrimp defend against *V. harveyi* bacterial attacks. The high phenoloxidase activity is believed to result from the shrimp's ability to recognize foreign invaders through phagocytosis (Ramadhan *et al*., 2017). According to Amparyup *et al.* (2013), the melanin produced by phenoloxidase can prevent the spread of foreign entities such as bacteria.

CONCLUSION

The treatment with *P. myxofaciens* JB 20B bacterial metabolites, both in the form of extract and supernatant, outperforms the positive control treatment in terms of reducing hepatopancreas necrosis in shrimp, increasing the total heemocyte count, phagocytosis activity, phenoloxidase activity, respiratory burst, survival rate, feed conversion ratio, and specific growth rate of *L. vannamei* shrimp through dietary supplementation, both in prevention

and treatment. The use of *P. myxofaciens* JB 20B bacterial metabolites to control *V. harveyi* infection in *L. vannamei* shrimp yielded the best results with the supernatant treatment.

REFERENCES

Adam MA, Ernawati IMW, Yayan AY, Insivitawati I, Yuliana, Pakaya RF, Soegianto A, Khumaidi A. 2022. Analysis of white feces disease (WFD) caused by *Vibrio* sp. and Dinoflagellata in Vannamei Shrimp *(Litopenaeus vannamei)* in brackishwater culture pond. Jurnal Ilmiah Perikanan dan Kelautan 14: 160–166.

Allen LAH. 2016. Microbicidal Mechanisms. Encyclopedia of Cell Biology 2: 766 –775.

Apriliani M, Sarjito, Haditomo AHC. 2016. The variety of vibriosis causative agent on Vaname shrimp *(Litopenaeus vannamei)* and bacteria susceptibility to antibiotics. Journal of Aquaculture Management and Technology 1: 98–107.

Amparyup P, Charoensapsri W, Tassanakajon A. 2013. Prophenoloxidase system and its role in shrimp immune responses against major pathogens. Fish Shellfish Immunology 34: 990–1001.

Aurestila BJ, Villaver EAM, Tan EY. 2018. Anti-biofilm activity of chitosan from crab and shrimp species indigenous to the philippines on established biofilms of Pseudomonas aeruginosa and *Staphylococcus aureus.* Journal Pharmacognosy and Natural Products 4: 1–5.

Dhar AK, Piamsomboon P, Caro LFA, Kanrar S, Adami R, Juan YS. 2019. First report of acute hepatopancreatic necrosis disease (AHPND) occurring in the USA. Diseases of Aquatic Organisms 132: 241–247.

Gorski A, Miedzybrodzki R, Borysowski J, Dabrowska K, Wierzbicki P, Ohams M, Korczak-Kowalska G, Olszowska-Zaremba N, Łusiak-Szelachowska M, Kłak M. 2012. Phage as a modulator of immune responses. Practical Implications for Phage Therapy 83: 41–71.

Gustilatov M, Widanarni, Ekasari J, Pande GSJ. 2022. Protective effects of the biofloc system in pacific white shrimp *(Penaeus vannamei)* culture against pathogenic Vibrio parahaemolyticus infection. Fish Shellfish Immunology 124: 66–73.

Hao J, Zhang Y, Fu S, Lu Y, Hua X, Liu Y. 2019. Pathogenicity and protein analysis of photorhabdus insect-related (Pir) toxin PirAB revealed PirABvp is a host-specific toxin. Aquaculture 500: 290–299.

Havanapan PO, Taengchaiyaphum S, Ketterman AJ, Krittanai C. 2016. Yellow head virus infection in black tiger shrimp reveals specific interaction with granule-containing hemocytes and crustinPm1 as a responsive protein. Developmental Comparative & Immunology 54: 126–136.

Hidayatullah M. 2019. Immune Response and Activity of Digestive Enzymes of Vaname Shrimp *(Litopenaeus vannamei)* in Pond During White Feces Disease [Thesis]. Bogor: Institut Pertanian Bogor.

Huang K, Nitin N. 2019. Edible bacteriophage based antimicrobial coating on fish feed for enhanced trearment of bacterial infections in aquaculture industry. Aquaculture 502: 18–25.

Kulkarni A, Krishnan S, Anand D, Kokkattunivarthil Uthaman S, Otta SK, Karunasagar I, Kooloth Valappil R. 2021. Immune responsses and immunoprotection in crustaceans with special reference to shrimp. Reviews in Aquaculture 13: 431–459.

Kumar V, Roy S, Behera BK, Bossier P, Das BK. 2021. Acute hepatopancreatic necrosis disease (Ahpnd): virulence, pathogenesis and mitigation strategies in shrimp aquaculture. Toxins (Basel) 13: 1–28.

Lin SJ, Hsu KC, Wang HC. 2017. Structural insights into the cytotoxic mechanism of Vibrio parahaemolyticus PirAvp and PirBvp toxins. Marine Drugs 15: 9–12.

Luo Y, Shong Y. 2021. Mechanism of antimicrobial peptides: Antimicrobial, anti-inflammatory and antibiofilm activities. International Journal of Molecular Sciences 22: 11401.

Lulijwa R, Rupia EJ, Alfaro AC. 2020. Antibiotic use in aquaculture, policies and regulation, health and environmental risks: a review of the top 15 major producers. Review Aquaculture 12: 640–663.

Mauliana MA. 2021. Actinomycetes Isolate Extract and Supernatant as Antibiofilm for Prevention of Aeromonas hydrophila Bacterial Infection in Tilapia *(Oreochromis niloticus)* [Thesis]. Bogor: Institut Pertanian Bogor.

Maftuch, Toban MH, Risjani Y. 2012. Administration of marine algae *(Gracilaria verrucosa)* immunostimulant enhances some innate immune parameters in black tiger shrimp *(Penaeus monodon Fabricus)* against

Vibrio harveyi infection. Journal of Applied Sciences Research 8: 1052–1058.

Mengistu SB, Mulder HA, Benzie JAH, Komen H. 2020. A systematic literature review of the major factors causing yield gap by affecting growth, feed conversion ratio and survival in nile tilapia *(Oreochromis niloticus).* Review Aquaculture 12: 524–541.

Nadella RK, Prakash RR, Dash G, Ramanathan SK, Kuttanappilly LV, Mothadaka MP. 2018. Histopathological changes in giant freshwater prawn *Macrobrachium rosenbergii* (de Man 1879) fed with probiotic Bacillus licheniformis upon challenge with *Vibrio alginolyticus.* Aquaculture Research 49: 81–92.

Nathalia O, Waturangi DE. 2021. Extract from phyllosphere bacteria with antibiofilm and quorum quenching activity to control several fish pathogenic bacteria. BMC Research Notes 14: 202.

Oktaviana A, Febriani D. 2020. Jumlah hemosit total pada udang vaname *(Litopenaeus vannamei)* yang diberikan tambahan tepung batang pisang pada pakan. Jurnal Perikanan Unram 9: 188–193. (In Indonesia).

Pardede MA, Widanarni W, Sukenda S, Yuhana M. 2023. Evaluation of dietary microencapsulated synbiotics *Pseudoalteromonas piscicida* 1UB, bacillus np5, and mannan oligosaccharides to prevent Vibrio parahaemolyticus infection in pacific white shrimp *Penaeus vannamei*. Aquaculture International 32: 2077–2091.

Platt N, Fineran P. 2015. Measuring the phagocytic activity of cells. Methods in Cell Biology 126: 287–304.

Raissa G, Waturangi DE, Wahjuningrum D. 2020. Screening of antibiofilm and anti-quorum sensing activty of actinomycetes isolates extracts against aquaculture pathogenic bacteria. BMC Microbiology 20: 343.

Ramadhan A, Nuryati S, Priyoutomo NB, Alimuddin A. 2017. Dietary ambon lumut banana stem extract Musa cavendishii var. dwarf paxton as an immunostimulant for white spot disease prevention in Pacific white shrimp *Litopenaeus vannamei.* Jurnal Akuakultur Indonesia 16: 164–173.

Rasmussen-Ivey CR, Figueras MJ, McGarey D, Liles MR. 2016. Virulence factors of Aeromonas hydrophila: in the wake of reclassification. Frontiers Microbiology 7: 1–15.

Rudi M, Sukenda, Wahjuningrum D, Pasaribu W, Hidayatullah D. 2019. Seaweed extract of *Gracilaria verrucosa* as an antibacterial and treatment against *Vibrio harveyi* infection of *Litopenaeus vannamei.* Jurnal Akuakultur Indonesia 18: 120–129.

Romano N, Koh CB, Ng WK. 2015. Dietary microencapsulated organic acids blend enhances growth, phosphorus utilization, immune response, hepatopancreatic integrity and resistance against Vibrio harveyi in white shrimp, *Litopenaeus vannamei*. Aquaculture 435: 228–236.

Sirikharin R, Taengchaiyaphum S, Sanguanrut P, Chi TD, Mavichak R, Proespraiwong P, Nuangsaeng B, Thitamadee S, Flegel TW, Sritunyalucksana K. 2015. Characterization and PCR detection of binary, pir-like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. Journal Plos One 10: 1–16.

Sukmawati, Hardianti F. 2018. Analisis *total plate count* mikroba pada ikan asin kakap dikota sorong Papua Barat. Jurnal Biodjati 3: 72–78. (In Indonesia).

Tatukede P, Loho L, Lintong P. 2014. Gambaran histopatologi hati mencit swiss yang diberi air rebusan sarang semut *(Mymercodia pendans)* paska induksi dengan *carbon tetrachlorida* (CCl4). Jurnal e-Biomedik 2: 459–466. (In Indonesia).

Thamburaj S, Ramaraj E, Sethupathy S, Kamalanathan C, Raji A, Rajasekharan SK. 2020. Antibacterial and antibiofilm activities of diphyllin against fish pathogens. Microbiology Pathogenesis 145: 104232.

Utami W, Sarjito, Desrina. 2016. Effect of salinity on *Vibrio harveyi* infection in white leg shrimp (Litopenaeus vanname). Journal Aquaculture Management Technology 4: 95–100.

Watts JEM, Schreier HJ, Lanska L, Hale MS. 2017. The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. Marine Drugs 15: 1–16.

Widanarni W, Yuhana M, Muhammad A. 2015. Bacillus NP5 improves growth performance and resistance against infectious Myonecrosis Virus in white shrimp *(Litopenaeus vannamei)*. Ilmu Kelautan Indonesia 19: 211–218.