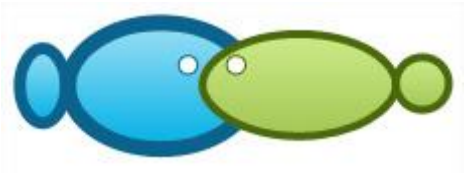

The effect of postbiotic *Bacillus amyloliquefaciens* on the growth performance and immune response of *Litopenaeus vannamei* against *Vibrio harveyi*

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Abstract. Postbiotics are preparations of dead microorganisms and their components that benefit the host. Vannamei shrimp (*Litopenaues vannamei*) is one of the commodities with high consumption value, so its production needs to be developed to maintain its quality and standard. This study was designed to investigate the effects of postbiotics from *Bacillus amyloliquefaciens* on the growth and immune response of the vannamei shrimp in combating *Vibrio harveyi*. This study consisted of three treatments: *B. amyloliquefaciens* postbiotics (fed with *B. amyloliquefaciens* postbiotics and challenged with *V. harveyi*), C(+) (fed with phosphate-buffered saline (PBS) and challenged with *V. harveyi*), and C(-) (fed with commercial feed without challenge). The dose of postbiotics used in this study was 10^6 CFU g^{-1} feed. The concentration of *V. harveyi* used for the challenge test was 10^7 CFU mL^{-1}. The study used post larvae of 12 days specimens (PL-12) and was conducted for 35 days. The challenge test was performed during the last 7 days. In vitro, results showed antimicrobial activity as a clear zone of *B. amyloliquefaciens* against *V. harveyi*. *B. amyloliquefaciens* postbiotics treatment did not show a significant effect on growth parameters (survival rate, weight growth, specific growth rate, and feed conversion ratio. *B. amyloliquefaciens* postbiotics showed a significant effect (Sig<0.05) on immune response parameters (total hemocyte count) after the challenge test, with a value of 4.56×10^6 cells mL^{-1}. This study concludes that *B. amyloliquefaciens* postbiotics have an insignificant impact on improving growth performance, but have proven to act as an immunostimulant in vannamei shrimp against the pathogen *V. harveyi*.
Key Words: aquaculture, disease, growth, postbiotic, resistance.

Introduction. Vannamei shrimp (*Litopenaeus vannamei*) is one of the cultivated shrimp species alongside tiger shrimp (*Penaeus monodon*) and white shrimp (*Penaeus merguensis*). The contribution of frozen vannamei shrimp exports to the total value of fishery exports in 2016 reached more than 27% (Yunarty et al 2022). In many cases, diseases commonly affecting vannamei shrimp are caused by bacteria from the *Vibrio* group. *Vibrio harveyi* is one species within this group that can cause diseases in shrimp (Rafiqie 2021). Disease control methods for *V. harveyi* typically rely on antibiotics. However, the continuous use of antibiotics can lead to antibiotic resistance in pathogenic bacteria (Merrifield et al 2010). Probiotics have emerged as an alternative solution to antibiotics, utilizing live microbial cells that are safe for aquatic animals. Despite the benefits of probiotics, the use of live microbes still poses potential negative impacts, such as the horizontal transfer of virulence genes from pathogenic microbes to probiotics (Choudhury & Kamilya 2019). A new alternative to probiotics is using dead and inactive microbial cells, known as "postbiotics," which can be administered to animals to support growth and immunity (Yassine et al 2021).

Postbiotics are preparations of dead microorganisms or their components that can provide benefits to the host when administered in appropriate amounts. Postbiotics are believed to enhance growth and strengthen the immune system of animals, making them more prepared to combat diseases (De Almada et al 2016). Previous studies have shown that administering heat-killed *Bacillus* sp. and *Lactobacillus plantarum* to *Paralichthys olivaceus* fish improved immune responses such as respiratory burst and lysozyme activity, making them suitable for growth and innate immunity enhancement (Back et al 2020). Additionally, research on heat-killed *Bacillus subtilis* in *Penaeus vannamei* shrimp has proven effective in boosting immune responses and resistance to *V. harveyi* (Purbiantoro et al 2023). However, to the best of the authors' knowledge, no studies have yet investigated the potential of postbiotic *Bacillus amyloliquefaciens* in enhancing growth and immune responses in *L. vannamei* against *V. harveyi*.

This research on the administration of *B. amyloliquefaciens* postbiotics in shrimp feed was conducted to evaluate its effects on the growth and immune responses of *L. vannamei* against *V. harveyi*. This research aimed to determine which postbiotic is more effective in enhancing the growth and immune defense of *L. vannamei* against *V. harveyi*. The study is expected to provide practical applications for addressing health issues in *L. vannamei* within the aquaculture sector.

Material and Method

Description of the study sites. The research was conducted from January to June 2024 at the Integrated Laboratory, Biotechnology Laboratory of the Faculty of Science and Mathematics, and the Wet Laboratory of the Faculty of Fisheries and Marine Science, Diponegoro University.

Tools and materials. The tools included a round inoculating loop, balance scale, microwave, Erlenmeyer flask, measuring cylinder, spatula, autoclave, incubator and shaker, laminar air flow cabinet, Bunsen burner, colony counter, Petri dishes, centrifuge, Falcon tubes, beaker glass, vortex mixer, microtubes, micropipettes, pipette tips, L-shaped spreader, spray bottle, freezer, aerator machine, aeration hose, air stones, counter, sieve, spoon, bucket, sponge, 1 mL syringe, hemocytometer, microscope, pestle, spectrophotometer, bottles, water quality meter, cuvettes, and test tubes. The materials used include *L. vannamei*, shrimp feed, *B. amyloliquefaciens*, trypticase soy broth (TSB) and tryptic soy agar (TSA), nutrient broth (NB) and nutrient agar (NA), Luria Bertani broth (LB) and Luria Bertani agar (LBA), marine broth (MB) and marine agar (MA), distilled water, 70% alcohol, aluminum foil, cotton, gauze, parafilm, phosphate buffer saline (PBS), NaCl, seawater, labels, *V. harveyi*, and anticoagulants.

Purification of isolates. *B. amyloliquefaciens* was purified on marine agar medium and incubated at 37°C for 24 hours. *V. harveyi* was purified on a nutrient agar medium and incubated at 37°C for 24 hours.

Antibacterial test. The antibacterial test was conducted using the paper disc diffusion method (Kirby-Bauer Assay) in duplicate. The pathogen culture density of *V. harveyi* was compared to the turbidity of the McFarland 0.5 standard. A sterile cotton swab was dipped into a test tube containing *V. harveyi* and then evenly spread over the surface of the MA medium in a petri dish. *B. amyloliquefaciens* isolate was applied by placing 20 µL onto a sterile 6 mm paper disc, which was then placed on the petri dish along with the positive control (tetracycline 1,000 ppm) and the negative control (marine broth medium) using sterile forceps. The diameter of the clear zone formed was measured using a caliper.

Inactivation of B. amyloliquefaciens. *B. amyloliquefaciens* was cultivated in Luria Bertani Broth (LB) medium in a shaker incubator for 36 hours at 27°C and 150 rpm (Cho & Kasem 2018). Subsequently, the *B. amyloliquefaciens* culture was plated on TPC to determine its concentration, resulting in 1.2×10^8 CFU mL^{-1}. The bacterial culture in LB medium was then centrifuged at 3,000 rpm for 30 minutes to obtain the pellet, which was

resuspended in 30 mL of sterile PBS and washed twice. A total of 0.2 g of pellet was obtained from 150 mL of liquid culture. Inactivation was performed using an autoclave at 121°C for 15 minutes. Afterward, validation was carried out by growing the inactive bacterial suspension on LBA medium, which was then incubated at 37°C for 72 hours to ensure that the bacteria had been completely killed (Shahbazi et al 2023).

Application of postbiotics. The postbiotic *B. amyloliquefaciens* pellets were applied to the feed at a final dose of 1.0×10^6 CFU g^{-1} of feed (Ballantyne et al 2023). The postbiotic pellets were dissolved in sterile PBS to facilitate the mixing process with commercial shrimp feed. The postbiotic solution was sprayed evenly onto the feed and then left to air-dry. The positive control feed was only sprayed with PBS, while the negative control feed was regular commercial feed. The dried feed could be used immediately or stored in a freezer at -4°C to maintain the quality of the test feed (Shahbazi et al 2023).

Preparation of vannamei shrimp. Aquariums measuring 40x25x30 cm^3 were cleaned and chlorinated. The chlorination process was carried out by soaking the containers overnight in 100 ppm chlorine for disinfection of pathogenic microorganisms (Widanarni et al 2012). The aquariums were then filled with 10 L of seawater and equipped with one aerator to supply oxygen to the shrimp. Post-larvae shrimp at the PL-12 stage (stocking phase) were acclimatized to adjust to the aquarium seawater temperature, after which they were released and acclimatized for 3 days. A total of 20 shrimps were placed in each aquarium, with a stocking density of 200 shrimp m^{-2} (Budiardi et al 2005).

Research design. Postbiotic-treated feed was given to *L. vannamei* for 35 days (day of culture (DOC) 1-35) with a feeding rate of 25-8%. The experiment consisted of three treatments with three replications: postbiotic *B. amyloliquefaciens* treatment (postbiotic *B. amyloliquefaciens* and *V. harveyi* infection), positive control treatment (+) (PBS feed and *V. harveyi* infection), and negative control treatment (-) (commercial feed without infection). The shrimp were fed four times a day.

Challenge test. The infection challenge was conducted during the last 7 days by administering the pathogenic *V. harveyi* bacteria in the form of a liquid culture with a final concentration of 10^7 CFU mL^{-1}. *V. harveyi* was cultured in 200 mL of tryptic soy broth (TSB) + 2% NaCl and incubated in a shaker incubator at 37°C for 24 hours with 150 rpm (Zhu et al 2023). A 1 mL aliquot of the *V. harveyi* culture was then subcultured in the same medium (TSB + 2% NaCl) and incubated under the same conditions. The *V. harveyi* culture was subsequently plated on TPC, yielding a concentration of 2.3×10^8 CFU mL^{-1}. The infection was applied through the immersion method for 30 minutes by adding the bacterial suspension, ensuring a final concentration of 10^7 CFU mL^{-1}, with one aerator. After immersion, the shrimp were returned to their respective treatment aquariums, which had been siphoned and replaced with fresh seawater (Widanarni et al 2012).

Immunological analysis. The immunological analysis performed was the total hemocyte count (THC), conducted on DOC 0, DOC 28, DOC 30, and DOC 35. Hemolymph was aseptically collected using a 1 mL syringe from the ventral sinus and then mixed with a sterile anticoagulant at a 1:2 ratio. The counting was performed using the formula (Joseph & Philip 2007):

$$THC = \frac{The\ number\ of\ cells\ counted}{The\ number\ of\ fields\ of\ view} \times 10^4 \times DF$$

Where:
THC - total hemocyte count (cells mL^{-1});
DF - dilution factor.

Growth performance analysis. Growth performance is a crucial parameter in evaluating the effectiveness of cultivation conditions and feed utilization in aquaculture. This analysis

provides insights into how well the organisms grow in response to the given environmental and nutritional factors. Several key metrics are used to assess growth performance, including weight gain, survival rate, feed efficiency, and growth rate. These parameters help determine the overall health and productivity of the cultured species, ensuring optimal management strategies for sustainable aquaculture. The counting was performed using the formulas (Lugert et al 2016):

Calculation of the absolute weight growth (W):

$$W = W_t - W_O$$

Calculation of the survival rate (SR):

$$SR\ (\%) = \frac{Nt}{No} \times 100$$

Calculation of the feed conversion ratio (FCR):

$$FCR = \frac{Total\ Feed\ (g)}{Total\ Biomass\ Increase\ (g)}$$

Calculation of the specific growth rate (SGR):

$$SGR = \frac{(LnWt - LnWo)}{t} \times 100\%$$

Where:
W_t - final shrimp weight (g);
W_0 - initial shrimp weight (g);
Nt - total shrimp at the end;
N_0 - total shrimp at the start;
t - long maintenance (days).

Data analysis. The data were analyzed using a quantitative method with a One-Way Analysis of Variance (ANOVA) at a 95% confidence level. This was followed by Duncan's test at a 5% significance level, where a p-value<0.05 indicated significant differences. Water quality parameters (temperature, pH, salinity, DO, and ammonia) were analyzed descriptively.

Results

Macroscopic and microscopic characterization of B. amyloliquefaciens and V. harveyi. Microscopic observations were conducted at 1000x magnification using Gram staining. Characterization was performed to ensure that the isolates used matched the characteristics described in the reference (Figure 1 and Table 1).

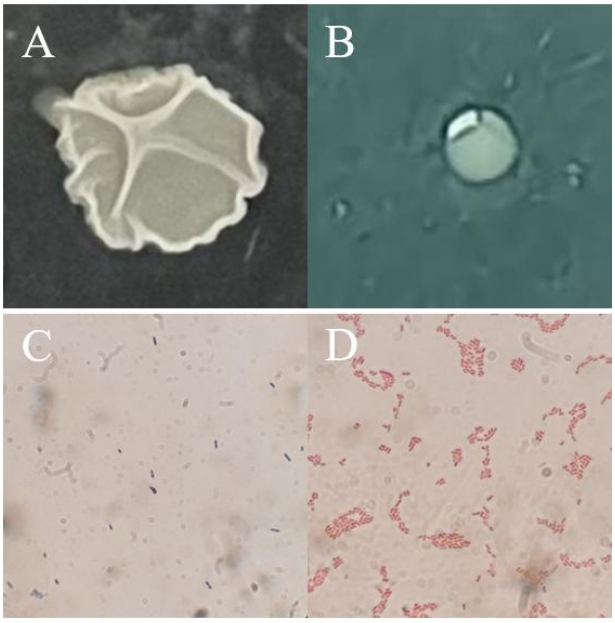

Figure 1. A. Colony of *Bacillus amyloliquefaciens*; B. Colony of *Vibrio harveyi*; C. *Bacillus amyloliquefaciens* as gram-positive bacteria (1000x); D. *Vibrio harveyi* as gram-negative bacteria (1000x).

Table 1

Morphological Characterization of *Bacillus amyloliquefaciens* and *Vibrio harveyi*

Parameter	*Bacillus amyloliquefaciens*	*Vibrio harveyi*
Colony shape	Irregular	Circular
Margin	Undulate	Entire
Texture	Smooth	Mucoid
Elevation	Raised and folds	Convex
Pigmen	Cream	White slightly yellow
Cell shape	Basil	Vibrio
Gram staining	Positive	Negative

The *B. amyloliquefaciens* isolate has the following colony characteristics: irregular shape, undulated margin, smooth texture, raised and folded elevation, cream, and basil cells, and it is classified as gram-positive bacteria. The *V. harveyi* pathogenic isolate has the following colony characteristics: circular shape, entire margin, mucoid texture, convex elevation, whitish-yellow color, and comma-shaped (vibrio) cells, and it is classified as gram-negative bacteria.

Antibacterial test of B. amyloliquefaciens against V. harveyi. The antibacterial test of the *B. amyloliquefaciens* isolate against *V. harveyi* was conducted using the paper disc diffusion method (Kirby-Bauer Assay) (Figure 2 and Table 2). The clear zone produced by *B. amyloliquefaciens* against *V. harveyi* measured 6.6 mm (Figure 2 and Table 2).

Table 2

Measurement of inhibition zone diameter of *Bacillus amyloliquefaciens* against *Vibrio harveyi*

Isolate	*Repetition of antibacterial test (mm)*		*Average (mm)*
	1	*2*	
B. amyloliquefaciens	8.3	8.9	8.6±0.42

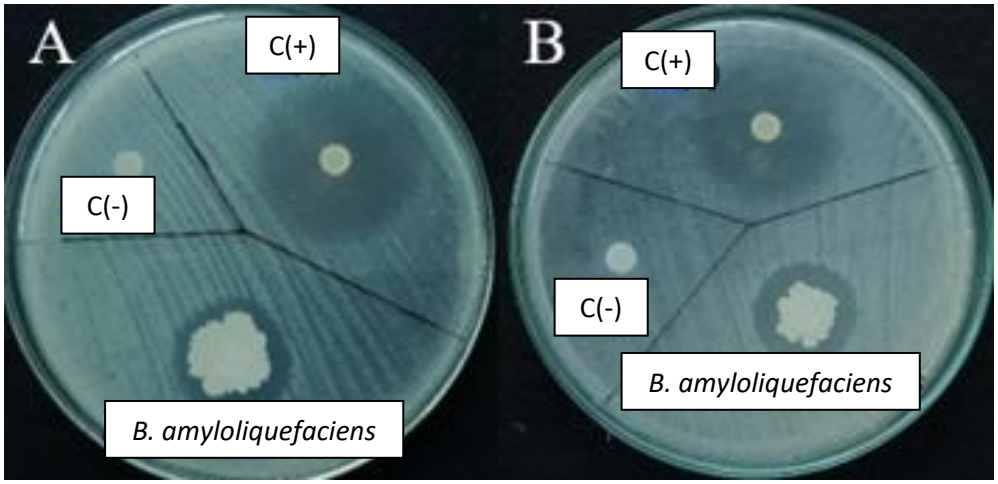

Figure 2. Results of antibacterial test of *Bacillus amyloliquefaciens* to *Vibrio harveyi*; A. First repetition; B. Second repetition.

The Kirby-Bauer method is based on the diffusion of antibacterial compounds into the solid media inoculated with microbes, forming a clear area around the disc, known as the inhibition zone, due to the absence of bacterial growth (Khasanah & Nugraheni 2021). The clear zone produced by *B. amyloliquefaciens* against *V. harveyi* measured 6.6 mm.

Validation of B. amyloliquefaciens postbiotics. Validation is a method used to ensure that the postbiotic product is successful, indicated by the absence of *B. amyloliquefaciens* growth after being incubated on LBA media (Figure 3).

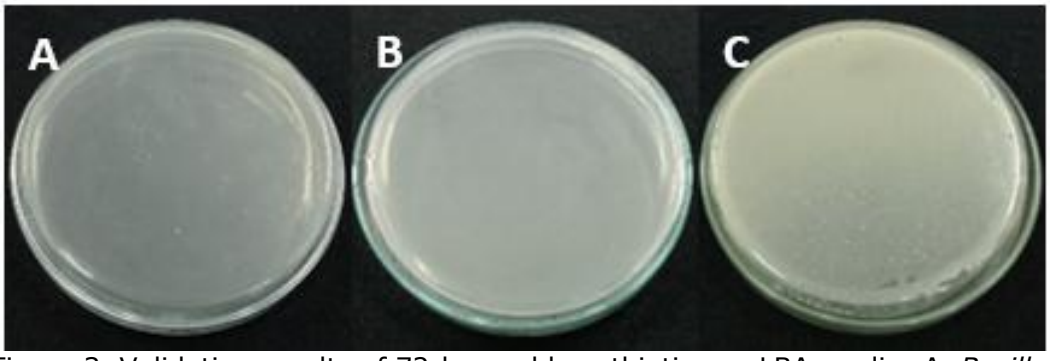

Figure 3. Validation results of 72-hour-old postbiotics on LBA media; A. *Bacillus amyloliquefaciens* postbiotics successfully inactivated at 121°C; B. Control - (PBS); C. Control + (*Bacillus amyloliquefaciens* before inactivation).

The postbiotic process of *B. amyloliquefaciens* inactivated at 121°C was successful, as indicated by the absence of any isolate growth or contamination by other microbes over 72 hours.

In vivo vannamei shrimp. The feed conversion ratio (FCR), specific growth rate (SGR), weight growth (W), survival rate (SR), and total hemocyte count (THC) were obtained and statistically analyzed (Figure 4). Water quality parameters measured during the study included pH, DO, temperature, salinity, and ammonia (Table 3).

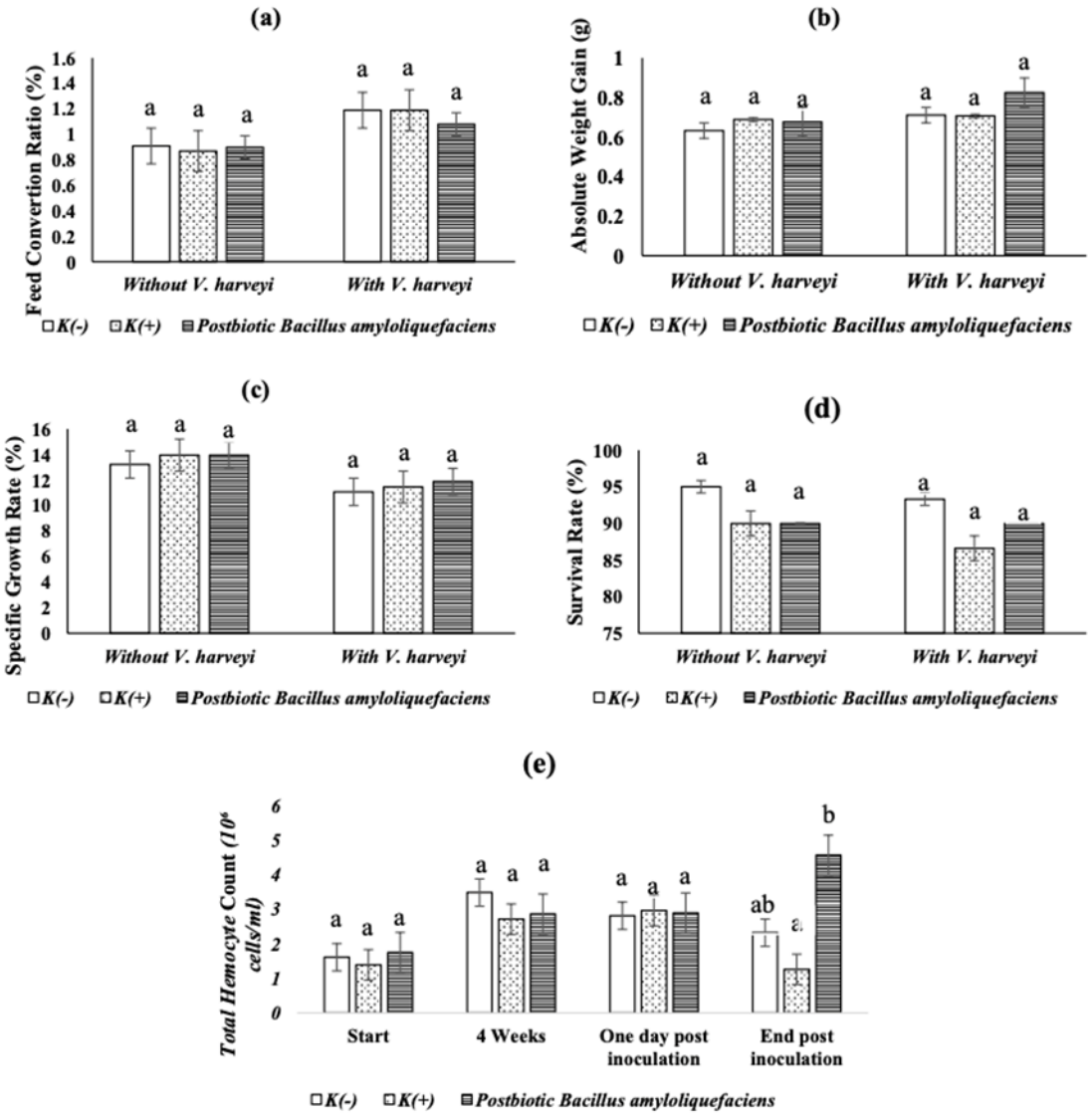

Figure 4. The effect of *Bacillus amyloliquefaciens* postbiotics on growth and immune response *Litopenaeus vannamei* (a) feed conversion ratio (FCR), (b) weight growth (W), (c) specific growth rate (SGR), (d) survival rate (SR), (e) total hemocyte count (THC). Values with different letters indicate significant differences (P<0.05) among treatments.

Table 3

Water quality

Parameter	*Treatment*			
	C(-)	*C(+)*	*PBA*	*Reference values*
pH	7.5-8.5	7.5-8.5	7.5-8.4	7-8.5 (Carbajal et al 2011)
DO (ppm)	5.3-61	5.5-6.1	5.6-6.1	4.5-7 (Komarawidjaja 2008)
Temperature (°C)	27.6-28.2	27.5-28.3	27.5-28.3	26-33 (Supono 2019)
Salinity (ppt)	20.2-21.6	20-21.6	20.1-22.1	10-30 (Supono 2019)
Ammonia (ppm)	0.03	0.04	0.06	<0.1 (Suharyadi 2011)

C(-) (negative control), C(+) (positive control), PBA (postbiotics *B. amyloliquefaciens*)

The feed conversion ratio, weight growth, survival rate, and specific growth rate of *L. vannamei* fed with *B. amyloliquefaciens* postbiotics before and after *V. harveyi* infection showed no significant differences. A significant difference was observed in the total

hemocyte count, with a value of 4.56×10^6 cells mL^{-1}, compared to the positive control treatment. Water quality during the study was consistent with the referenced standards.

Discussion. The *B. amyloliquefaciens* isolate has the characteristics reported by Kim et al (2016): cream-colored, with undulate edges, raised or flat elevation, rod-shaped cells, and it is classified as Gram-positive bacterium. Duan et al (2021) also reported that the morphology of *B. amyloliquefaciens* colonies shows the formation of folds. Paray et al (2023) explained that Gram-positive bacteria have a thick peptidoglycan cell wall, which retains the crystal violet stain and binds more strongly after alcohol treatment, resulting in a purple color observed microscopically. The pathogenic *V. harveyi* isolate has the characteristics described by Hidayat (2014): round, with smooth edges, convex elevation, smooth texture, whitish-yellow color, comma-shaped cells, and it is classified as Gram-negative bacteria. The *B. amyloliquefaciens* isolate has been proven to produce antibacterial compounds against the growth of the pathogenic *V. harveyi* by generating a clear zone. Xiaolong et al (2019) stated that *B. amyloliquefaciens* is capable of producing bioactive compounds that act as antibacterial, antioxidant, and immunocompetence agents. The postbiotic process of *B. amyloliquefaciens* that was killed at 121°C showed success, as indicated by the absence of any growth or contamination by other microbes. Thorakkattu et al (2022) stated that heat is capable of causing bacteria to undergo denaturation, after which they die or can no longer reproduce, causing the microorganism's inactivation.

Growth parameters such as absolute weight growth, specific growth rate, and feed conversion ratio did not show a decrease in shrimp that were given *B. amyloliquefaciens* postbiotics before the infection with *V. harveyi*. The survival rate (SR) in shrimp treated with *B. amyloliquefaciens* postbiotics was found to be 90%, which is considered good according to Arshad et al (2017), with values >70%. However, the growth parameter's results were not significantly different among the treatments. The lack of decrease in growth parameters indicates that the *B. amyloliquefaciens* postbiotic supplement did not have a toxic effect on shrimp growth and survival, being in line with a study by Priya et al (2024), which used a *B. coagulan*t postbiotic supplement formulation and found no toxicity towards post-larvae of *L. vannamei*.

The *B. amyloliquefaciens* postbiotic did not result in an increase in growth parameters in *L. vannamei* in this study, which may be due to factors such as an ineffective dose, differences in species used in the postbiotic, lack of microbial colonization, differences in digestive systems between fish and shrimp, and side effects from inactivation with autoclaving. Saikia (2015) stated that fish have a digestive system consisting of the mouth, throat, esophagus, stomach, intestines, rectum, and anus. Supono (2017) stated that shrimp have a simpler digestive system, which consists of the mouth, esophagus, intestines, and anus. Siciliano et al (2021) stated that thermal inactivation can damage cell membranes, leading to the leakage of nutrients and ions that can cause protein denaturation, ribosome aggregation, and DNA damage. High pressure can also cause membrane rupture, resulting in changes to ribosomes, denaturation, protein coagulation, and the loss of many dissolved substances.

The *V. harveyi* used in this study showed its infectious effects through reductions in parameters such as W, SGR, FCR, SR, and immune parameters (THC) in the positive control group compared to pre-infection levels. The infection by *V. harveyi* increased the FCR value in the positive control group, as consumed feed was utilized to combat the infection rather than being converted into biomass. Ridlo & Subagiyo (2013) stated that a high FCR value indicates a greater amount of feed that is not converted into shrimp biomass. Shrimp in the positive control group exhibited lower specific growth rates and weight growth, with an increase of only 0.0181 g (0.6887–0.7068 g). Aini et al (2018) noted that animals affected by the disease may experience growth reductions because the energy derived from the feed is prioritized for fighting disease, repairing damaged tissues, and the healing process.

The postbiotic *B. amyloliquefaciens* demonstrated a greater ability to combat *V. harveyi* infection, resulting in more optimal parameters for growth (W, SGR, FCR, and SR) as well as immune parameters (THC) in shrimp treated with *B. amyloliquefaciens* postbiotic. Ballantyne et al (2023) stated that strong immune health in shrimp tends to

make them more resistant to diseases, leading to better specific growth rates. The *V. harveyi* strain used in this study showed its infective effects by reducing parameters such as W, SGR, FCR, SR, and immune parameters in the positive control treatment compared to the values of these parameters before the *V. harveyi* infection. Aini et al (2018) stated that animals infected with the disease may experience growth reduction because the energy from food is redirected to combat the disease, repair damaged tissues, and support recovery processes. The *L. vannamei* infected with *V. harveyi* for 7 days and fed with *B. amyloliquefaciens* postbiotics showed a significant increase in total hemocytes, reaching 4.56×10^6 cells mL^{-1} compared to the positive control group. Cooper & Eleftherianos (2017) stated that an increase in hemocyte numbers indicates a rise in immune cells that work to eliminate pathogen cells. Wu et al (2017) also mentioned that heat-killed *B. amyloliquefaciens* can trigger an immune response, particularly antimicrobial activity, thus protecting against pathogens. A research study of Hasan et al (2019) on the administration of heat-killed *Bacillus* sp. to *Paralichthys olivaceus* indicated that the latter exhibit activities similar to the immunostimulants. Immunostimulant activity can reduce pathogenicity and stimulate phagocytic cells to destroy pathogenic antigens. As a gram-positive bacterium, *B. amyloliquefaciens* contains peptidoglycan in its cell wall, which has potential immunostimulatory properties to enhance innate immunity in shrimp (Ang et al 2020; Rattanachai et al 2005). The innate immune system in shrimp can recognize pathogenic microorganisms through pattern recognition receptors. These receptors detect pathogens by binding to common molecular patterns present in all pathogens rather than specific to certain species. In gram-positive bacteria, the recognized patterns include peptidoglycan and lipoteichoic acid (Wang & Wang 2013). The water quality during the study period did not influence the growth and immune response, which were not optimal, because the parameters were within the acceptable standards for *L. vannamei* farming. The pH was within the ideal range for shrimp growth according to Carbajal et al (2011), between 7.0–8.5. The dissolved oxygen level was within the ideal range for shrimp growth, according to Patkaew et al (2024), namely between 5–7 mg L^{-1}. The temperature was within the acceptable range for shrimp farming, according to Supono (2019), namely between 26–33°C. The salinity was within the standard range for *L. vannamei* growth, according to Supono (2019), namely between 10–30 ppt. The ammonia level was still within the normal range for *L. vannamei* farming, according to Suharyadi (2011), namely below 0.1 ppm.

Conclusions. The feed supplemented with postbiotics of *B. amyloliquefaciens* in *L. vannamei* did not show a significant effect on growth parameters both before and after the infection with *V. harveyi*. However, the feed supplemented with postbiotics of *B. amyloliquefaciens* had a significant effect on the immunity of the shrimp, as evidenced by the total hemocyte count, indicating its role as an immunostimulant.

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

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