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Analysis of Probiotic Beverage Fermentation Waste Dosage on Phagocytic Activity of Whiteleg Shrimp (*Litopenaeus vannamei*) Following *Vibrio parahaemolyticus* Challenge

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ABSTRACT

Intensive shrimp farming systems are often associated with increased disease risks due to environmental stress and pathogen exposure. This study aimed to evaluate the effect of probiotic beverage fermentation waste supplementation on the phagocytic activity of whiteleg shrimp (*Litopenaeus vannamei*) following challenge with *Vibrio parahaemolyticus*. The research was conducted using a completely randomized design with five treatments and four replications, consisting of negative control (P−), positive control (P+), and probiotic fermentation waste supplementation at doses of 1, 3, and 5 g/kg feed (P1, P2, and P3). Shrimp were fed experimental diets for 14 days followed by V. parahaemolyticus challenge, and phagocytic activity was evaluated at different observation times. The results showed that probiotic fermentation waste supplementation significantly affected phagocytic activity based on repeated measures ANOVA ($p<0.05$). The highest phagocytic activity was observed in the P3 treatment with a supplementation dose of 5 g/kg feed, which maintained the highest phagocytic activity after bacterial challenge compared with other treatments. Survival rate and water quality parameters remained within suitable ranges during the experimental period. These findings indicate that probiotic beverage fermentation waste has potential as a functional feed additive to enhance non-specific immune responses and support disease resistance in whiteleg shrimp aquaculture.

INTRODUCTION

Whiteleg shrimp (*Litopenaeus vannamei*) is one of the most important aquaculture commodities due to its rapid growth, high survival rate, adaptability to various culture conditions, and high economic value. The increasing demand for shrimp production has encouraged the implementation of intensive farming systems to improve productivity. In Indonesia, shrimp production reached 958,464 tons in 2024, with whiteleg shrimp contributing the largest proportion at 786,382 tons (Kementerian Kelatutan dan Perikanan, 2025). However, intensive culture practices with high stocking densities have increased challenges related to environmental degradation and disease outbreaks.

High stocking density and intensive feeding practices can promote the accumulation of organic matter from uneaten feed and feces, which may deteriorate water quality and induce physiological stress in shrimp. These conditions can weaken shrimp defense mechanisms and increase susceptibility to pathogen infections (Samadan et al., 2018; Nurhayati and Ayu, 2023).

One of the major bacterial pathogens affecting whiteleg shrimp farming is *Vibrio parahaemolyticus*, particularly strains associated with Acute Hepatopancreatic Necrosis Disease (AHPND). This disease causes severe hepatopancreatic damage and high mortality through the activity of PirA and PirB toxins encoded by the pVA1 plasmid (Kumar et al., 2021). Therefore, improving the natural immune capacity of shrimp is an important approach for sustainable disease management.

Several strategies have been developed to improve shrimp resistance against pathogens, including the application of probiotics and immunostimulants. The use of antibiotics as disease control agents has become a concern due to the emergence of antimicrobial resistance and environmental impacts associated with antibiotic residues (Milijasevic et al., 2024). Consequently, natural immunostimulants have received increasing attention because they can enhance innate immune responses without contributing to antimicrobial resistance. Previous studies have demonstrated that microbial-derived compounds, such as peptidoglycan and polysaccharides, can stimulate immune responses in aquatic organisms by activating immune recognition pathways (Vijayaram et al., 2022).

In shrimp, hemocytes play an important role in innate immunity by recognizing and eliminating invading pathogens. Among cellular defense mechanisms, phagocytosis is considered one of the most important processes because it directly reflects the ability of hemocytes to capture and destroy foreign particles or microorganisms. Previous studies have reported that immunostimulant supplementation can enhance phagocytic activity, indicating improved cellular defense capacity against pathogens (Teame et al., 2020). However, most studies have focused on purified immunostimulants or live probiotic supplementation, while the utilization of fermentation residues as a potential immunostimulant source remains limited. Fermentation waste represents an underutilized resource that may contain microbial biomass, cell wall components, and bioactive metabolites. In particular, residues from probiotic fermentation may retain microbial-derived compounds such as peptidoglycan and polysaccharides, which can act as microbe-associated molecular patterns (MAMPs). These compounds can be recognized by pattern recognition receptors (PRRs), leading to hemocyte activation and enhanced phagocytic responses (Suissa et al., 2023; Miao et al., 2023). Therefore, probiotic fermentation waste has potential to be developed as a functional feed additive for improving shrimp immune performance.

Although the immunostimulatory effects of probiotics and microbial-derived compounds have been widely investigated, the potential utilization of probiotic beverage fermentation waste to enhance phagocytic activity in whiteleg shrimp remains poorly explored. Previous studies have mainly focused on live probiotic supplementation or purified immunostimulatory compounds, whereas fermentation residues containing microbial biomass and bioactive components remain underutilized. The conversion of fermentation waste into functional feed additives may provide a novel approach by transforming residual materials into valuable resources while supporting sustainable aquaculture practices.

Therefore, the utilization of probiotic beverage fermentation waste as a feed additive represents a potential strategy to enhance the cellular immune response of whiteleg shrimp, particularly through phagocytic activity following exposure to *Vibrio parahaemolyticus*.

METHOD

Time, Place, and Materials

The research was conducted from June to August 2026. Shrimp rearing and feed treatment were carried out at the Laboratory of Anatomy and Aquaculture, Faculty of Fisheries and Marine Sciences, Universitas Airlangga. The preparation of probiotic beverage fermentation waste and *Vibrio parahaemolyticus* challenge test were conducted at the Laboratory of Microbiology and Disease, Faculty of Fisheries and Marine Sciences, Universitas Airlangga. Phagocytic activity analysis was performed at the Central Research and Healthy Animal Diagnostic Laboratory, Malang.

The test animals used in this study were whiteleg shrimp (*Litopenaeus vannamei*) with an initial body weight of approximately 3 g/individual. Shrimp were obtained from a hatchery center in Probolinggo, East Java. The experimental animals were acclimatized before being distributed into experimental aquaria. The feed used was commercial shrimp feed supplemented with probiotic beverage fermentation waste as an immunostimulant candidate. The fermentation waste consisted of sediment from a multistrain probiotic beverage containing lactic acid bacteria, including *Lactobacillus bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, and *Rhodopseudomonas palustris*.

Research Design

The study used a completely randomized design (CRD) consisting of five treatments with four replications. The treatments consisted of a negative control (P−), positive control (P+), and three doses of probiotic beverage fermentation waste supplementation (P1, P2, and P3). The negative control (P−) received commercial feed without fermentation waste supplementation and was not challenged with *Vibrio parahaemolyticus*. The positive control (P+) received commercial feed without fermentation waste supplementation but was challenged with *Vibrio parahaemolyticus*. The treatment groups received commercial feed supplemented with fermentation waste at different doses, namely P1 (1 g/kg feed), P2 (3 g/kg feed), and P3 (5 g/kg feed), followed by challenge exposure. Shrimp were stocked at a density of 13 individuals per aquarium. Each aquarium had a volume of approximately 20 L, resulting in 20 experimental units. Shrimp were maintained for 14 days before challenge exposure, followed by a seven-day observation period after bacterial challenge.

Preparation of Fermentation Waste and Test Feed

The fermentation waste used in this study was obtained from the sediment fraction of probiotic beverage fermentation. The sediment was separated from the liquid fraction and used as a feed additive. The fermentation waste was mixed with commercial feed according to the treatment doses of 1, 3, and 5 g/kg feed. The treated feed was mixed until the fermentation waste was evenly distributed on the feed surface and then air-dried at room temperature before administration. Feed was provided four times daily according to shrimp biomass requirements during the rearing period.

Challenge Test with *Vibrio parahaemolyticus*

After the feeding treatment period, shrimp were challenged with *Vibrio parahaemolyticus*. The bacterial suspension was prepared at a concentration of 10^6 CFU/mL and injected intramuscularly into the second and third abdominal segments at a volume of 100 µL/shrimp. The negative control group was injected with sterile phosphate-buffered saline (PBS) at the same volume. Following the challenge test, shrimp were maintained for seven days to evaluate the immune response after bacterial exposure. Hemolymph sampling was conducted at three observation periods: before treatment (T0), after seven days of feeding treatment (T1), and seven days after challenge exposure (T2).

Research Parameters

The main parameter observed in this study was phagocytic activity. Hemolymph samples were collected aseptically from the ventral sinus using a sterile syringe containing anticoagulant solution. Hemolymph was mixed with bacterial suspension and incubated to allow phagocytosis to occur. Smears were prepared on glass slides, fixed with methanol, and stained with Giemsa solution. Phagocytic activity was determined by counting the percentage of hemocytes showing phagocytic ability from the total observed hemocytes. Supporting parameters included survival rate and water quality. Survival rate was calculated based on the percentage of surviving shrimp at the end of the experiment. Water quality parameters, including temperature, pH, salinity, and dissolved oxygen (DO), were measured periodically during shrimp rearing to ensure suitable culture conditions.

Data Analysis

Data on phagocytic activity and survival rate were analyzed using repeated measures analysis of variance (ANOVA) at a 95% confidence level. When significant differences were observed among treatments, Duncan's Multiple Range Test (DMRT) was performed to determine differences between treatment groups. Water quality parameters were analyzed descriptively to evaluate the suitability of environmental conditions during the experiment.

RESULT AND DISCUSSION

The microscopic observation of hemocyte phagocytosis activity in *Litopenaeus vannamei* under different treatments is presented in Figure 1.

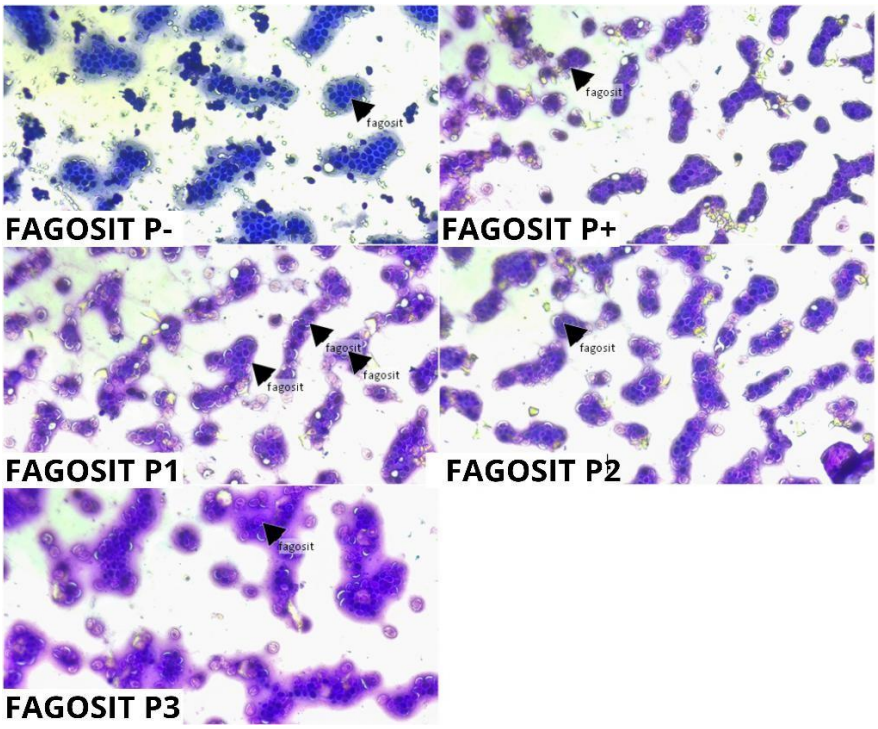


Figure 1. Hemocyte phagocytosis activity of *Litopenaeus vannamei* under different probiotic fermentation waste supplementation treatments. Arrows indicate phagocytic hemocytes. P−: negative control; P+: positive control challenged with *Vibrio parahaemolyticus*; P1, P2, and P3: shrimp fed with fermentation waste supplementation at different doses.

Figure 1 shows the microscopic observation of hemocyte phagocytosis activity in *Litopenaeus vannamei* under different treatments. Phagocytic hemocytes were observed in all treatments, as indicated by the presence of engulfed particles within the hemocyte cells (black arrows). The observation confirmed that hemocytes actively performed phagocytosis as a cellular defense mechanism against foreign particles and bacterial exposure. The presence of phagocytic hemocytes in all treatments indicates that shrimp hemocytes remained functionally active during the experimental period. Hemocytes are important immune cells in crustaceans that function in pathogen recognition, encapsulation, and phagocytosis as part of the innate immune system (Muahiddah et al., 2022).

Figure 2 shows the changes in phagocytic activity of *Litopenaeus vannamei* during the experimental period under different probiotic fermentation waste supplementation treatments. The phagocytic activity showed different responses among treatments from T0 to T2. At the beginning of the experiment (T0), all treatments exhibited relatively similar phagocytic activity values, indicating that the initial cellular immune condition of shrimp was comparable among treatments.

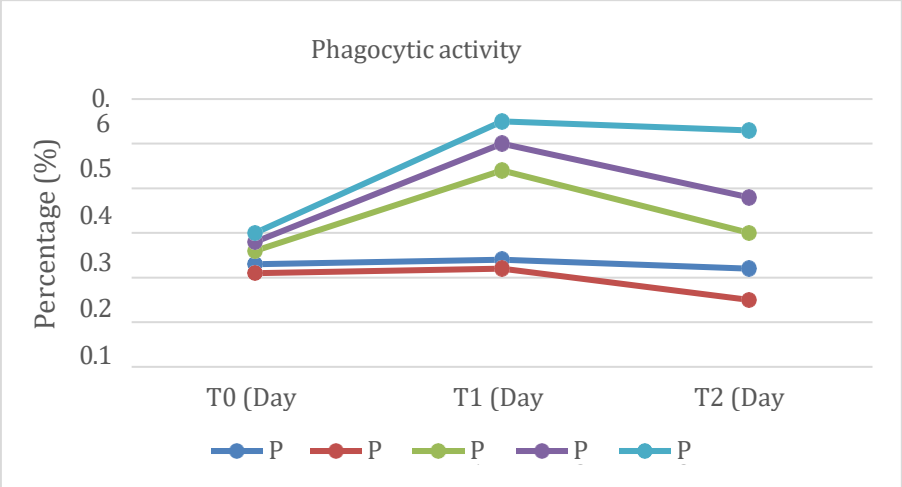


Figure 2. Changes in phagocytic activity of *Litopenaeus vannamei* during the experimental period under different treatments.

After seven days of feeding treatment (T1), phagocytic activity increased in shrimp receiving probiotic fermentation waste supplementation (P1, P2, and P3). The highest phagocytic activity was observed in the P3 treatment (5 g/kg feed) with a value of 0.55, followed by P2 (3 g/kg feed) and P1 (1 g/kg feed) with values of 0.50 and 0.44, respectively. Meanwhile, the control treatments (P– and P+) showed lower values of 0.24 and 0.22. Repeated Measures ANOVA revealed that treatment, observation time, and the interaction between treatment and observation time significantly affected phagocytic activity ($p<0.05$).

Following *Vibrio parahaemolyticus* challenge (T2), phagocytic activity decreased in P1 and P2 treatments, whereas P3 maintained a relatively high response with a value of 0.53. In contrast, P+ showed the lowest phagocytic activity (0.15) at the end of the observation period. Duncan's Multiple Range Test indicated that P3 showed a significant difference compared with other treatments at T2, while no significant differences were observed among treatments at T0. The increased phagocytic activity following probiotic fermentation waste supplementation indicates that fermentation-derived components may stimulate the cellular immune response of whiteleg shrimp. Hemocytes are the primary immune cells in crustaceans that function in pathogen recognition, phagocytosis, and elimination of foreign materials. Therefore, enhanced phagocytic activity reflects improved non-specific immune defense capacity against bacterial infection (Muahiddah et al., 2022).

The immunostimulatory effect of probiotic fermentation waste may be related to microbial-derived components and fermentation metabolites that have the potential to activate innate immune responses. Previous studies have reported that microbial-derived compounds can stimulate immune pathways and enhance disease resistance in aquatic organisms (Nisa et al., 2024).

The survival rate of *Litopenaeus vannamei* after *Vibrio parahaemolyticus* challenge showed different responses among treatments. Shrimp supplemented with probiotic beverage fermentation waste showed survival rates ranging from 75.00–78.85%, indicating that shrimp were still able to maintain survival performance under bacterial challenge conditions. According to Akbarurrasyid et al. (2025), survival rates above 70% in whiteleg shrimp culture are categorized as good, suggesting that the survival values obtained in this study represented a relatively stable condition after pathogen exposure.

The higher survival performance observed in supplemented treatments may be associated with improved immune responses, particularly increased phagocytic activity observed in this study. Immunostimulants derived from probiotic-based materials have been reported to enhance shrimp health by stimulating innate immune mechanisms and improving resistance against pathogenic microorganisms (Nisa et al., 2024). The utilization of probiotic-

derived compounds may support shrimp physiological conditions by enhancing defense mechanisms during pathogen exposure.

Water quality parameters remained within suitable ranges for *L. vannamei* culture throughout the experimental period. Dissolved oxygen values ranged from 6.65–6.72 mg/L, temperature ranged from 26.85–27.45°C, pH ranged from 8.72–8.75, and salinity ranged from 22.02–22.16 ppt. These values were within the recommended range for whiteleg shrimp cultivation according to SNI 01-7246-2006 (Badan Standardisasi Nasional, 2006).

The stable water quality conditions among treatments indicate that environmental parameters did not become limiting factors affecting shrimp immune responses and survival. Stable environmental conditions are important for maintaining shrimp physiological balance, allowing the observed differences in phagocytic activity and survival rate to be associated mainly with probiotic fermentation waste supplementation and *V. parahaemolyticus* challenge.

CONCLUSION

This study demonstrated that probiotic beverage fermentation waste supplementation improved the cellular immune response of whiteleg shrimp (*Litopenaeus vannamei*) following *Vibrio parahaemolyticus* challenge. The supplementation enhanced hemocyte phagocytic activity, with the highest and more stable response observed in shrimp receiving 5 g/kg feed supplementation. The improvement in phagocytic activity indicates that fermentation waste has potential as a functional feed additive to stimulate non-specific immune defenses in shrimp. In addition, the relatively stable survival rate and suitable water quality conditions suggest that the supplementation supported shrimp performance under pathogen exposure. These findings highlight the potential utilization of probiotic fermentation waste as an alternative immunostimulant source in sustainable shrimp aquaculture. However, further studies are required to evaluate the specific bioactive compounds contained in fermentation waste and their mechanisms in regulating shrimp immune responses.

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