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Vannamei-Shield: Functional Feed Application for Wssv Prevention in Vannamei Shrimp (*Litopenaeus Vannamei)* Through *B-Glucan* Feed and *Bacillus Subtilis* Probiotic

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ABSTRACT

White spot syndrome virus (WSSV) remains a major constraint in *Litopenaeus vannamei* culture because it can cause rapid mass mortality and substantial production losses. This study evaluated the effects of a functional diet containing *β-glucan* and the probiotic *Bacillus subtilis* on growth performance, survival, and non-specific immune responses of vannamei shrimp under natural disease-pressure conditions. A completely randomized design was applied with four dietary treatments and three replicates: control diet without additives (P0), diet supplemented with 0.1% β-glucan (P1), diet supplemented with B. subtilis at 10^8 CFU/g feed (P2), and a combination of 0.1% β-glucan and B. subtilis at 10^8 CFU/g feed (P3). After 28 days of culture, P3 produced the highest absolute weight gain (4.24 ± 0.18 g/shrimp), specific growth rate (5.86 ± 0.12%/day), and survival rate (89.67 ± 2.52%). The combined treatment also resulted in the highest total hemocyte count (8.92 ± 0.43 × 10^6 cells/mL) and phagocytic activity (59.33 ± 2.52%). One-way ANOVA showed that dietary treatments significantly affected all measured biological and immune parameters ($p<0.05$). Water quality remained within acceptable ranges, while the proximate composition of the diets was relatively similar among treatments. These findings indicate that the combination of *β-glucan* and *Bacilus. subtilis* can improve growth, survival, and non-specific immunity of vannamei shrimp. Further validation using WSSV detection by PCR or controlled challenge tests is required to confirm its specific protective effect against WSSV.

INTRODUCTION

Litopenaeus vannamei is one of the leading aquaculture commodity with a strategic role in supporting national fisheries production and exports. The advantages of vannnamei shrimp include relatively fast growth, broad environmental adaptability, and high economic value. Nevertheless, the sustainability of vannamei shrimp aquaculture still faces serious challenges, especially disease outbreaks, particularly White Spot Syndrome Virus (WSSV) (Chen *et al.,* 2025).

WSSV is known as one of the most dangerous viral pathogens in shrimp culture because it can cause mass mortality, with mortality rates reaching 80-100% within a short time after the onset of clinical symptoms (Das *et al.,* 2022). WSSV outbreaks have caused significant economic losses and reduced pond productivity in various regions. To date there is, no effective treatment method is available for WSSV infection; therefore, the most rational and recommended control strategy is prevention through enhancement of shrimp body resistance. One developing and safe preventive effort is the use of immunostimulant-based functional feed.

β-glucan, which is generally derived from the cell wall of yeast (*Saccharomyces cerevisiae*), has been reported to improve the non-specific immune response in shrimp, including increased hemocyte count, phagocytic activity, and survival under disease pressure (Ng *et al.,* 2023). The administration of *β-glucan* through feed is considered practical and applicable because it can be easily implemented in small-scale and semi-intensive shrimp culture systems.

Although the benefits of *β-glucan* and the probiotic *Bacillus subtilis* have been widely reported separately, studies that integrate these two approaches simultaneously as a WSSV prevention strategy in vannamei shrimp remain limited, particularly under culture conditions with natural disease pressure. In fact, an integrative approach between functional feed and probiotics has the potential to provide synergistic effects in increasing shrimp resistance to disease pressure, while also being more realistic and safer to apply in the field without biosecurity risks (Laice *et al.,* 2021). Based on these conditions, several main problems need to be examined: whether the administration of β-glucan-enriched feed can improve vannamei shrimp survival and immune response against WSSV disease pressure; whether the application of the probiotic Bacillus subtilis affects the improvement of shrimp resistance and the stability of the culture environment; and whether the combination of the two treatments provides more optimal results than single treatments in preventing vannamei shrimp mortality caused by WSSV (Khofifah *et al.,* 2023).

This research is highly urgent because WSSV remains a major threat in vannamei shrimp aquaculture and still has no effective curative solution. A preventive approach based on functional feed and probiotics is considered capable of reducing dependence on chemicals and antibiotics while supporting a more environmentally friendly and sustainable shrimp culture system.

The problem-solving approach in this study is arranged in several stages. The initial stage begins with problem identification through a literature review and field observations related to the high mortality rate of vannamei shrimp due to White Spot Syndrome Virus (WSSV) disease pressure. The next stage is the formulation of problem-solving strategies through a scientific literature review on the use of *β-glucan* based functional feed as an immunostimulant and *Bacillus subtilis* probiotic as a support for shrimp health and culture environment quality. The results of this review are used as the basis for developing a safe and applicable disease-prevention strategy.

Litopenaeus vannamei is a leading aquaculture commodity characterized by rapid growth, relatively broad environmental tolerance, and high economic value . However, vannamei shrimp aquaculture is highly vulnerable to disease outbreaks, particularly White Spot Syndrome Virus (WSSV), which is a major cause of production failure, with mortality rates reaching 80-100% within a short period. To date, there is no effective treatment for viral diseases in shrimp, so prevention through enhancement of the shrimp immune system is the most rational and sustainable strategy (Lestari *et al.,* 2022).

One widely studied disease-prevention strategy is the use of natural immunostimulants, including *β-glucan*. *β-glucan* is a structural polysaccharide derived from the cell walls of yeast, fungi, and several plants, and is known to enhance non-specific immune responses in crustaceans. The administration of *β-glucan* in shrimp feed has been reported to increase total hemocyte count, phagocytic activity, and the expression of immune genes involved in defense against pathogens, including viruses (Yu *et al.,* 2022). Several studies have shown that *β-glucan* supplementation in vannamei shrimp feed can increase shrimp survival under disease pressure and improve growth performance (Pratiwi *et al.,* 2024).

Another strategy widely developed in shrimp aquaculture is the use of probiotics, particularly from the genus Bacillus. The probiotic *Bacillus subtilis* is known to produce digestive enzymes and antibacterial compounds and to improve the quality of the aquatic culture environment. The administration of *Bacillus subtilis* in shrimp culture systems has been reported to suppress populations of opportunistic pathogenic microorganisms, improve feed efficiency, and indirectly strengthen shrimp immune responses (Pramudia *et al.,* 2023). Previous studies indicate that a combination of β-glucan and the probiotic Bacillus subtilis has

the potential to be developed as a synbiotic functional feed for aquaculture organisms. Wongsasak *et al.* (2015) reported that supplementing Litopenaeus vannamei feed with β-glucan and B. subtilis enhanced phenoloxidase activity and induced the expression of serine protease and prophenoloxidase genes, which play roles in the innate immune response. Furthermore, Boonanuntanasarn *et al.* (2016) demonstrated that this combination could modulate the intestinal microbial community, characterized by an increase in lactic acid bacteria and a decrease in Vibrio spp. These findings suggest a synergistic potential between β-glucan (acting as an immunostimulant/prebiotic) and B. subtilis (acting as a probiotic) in improving intestinal health and the immune response of aquatic organisms. Most previous studies have focused on challenge tests using artificial viral infection, which have limitations in terms of biosecurity and do not fully reflect field culture conditions (Sui *et al.,* 2024). Therefore, research is needed to examine a preventive approach based on simultaneous enhancement of immunity and environmental health.

METHOD

The research design used is a Completely Randomized Design (CRD). Feed treatments consist of four levels: feed without b-glucan and probiotic/control (P0), feed supplemented with 0.1% *β-glucan* per kg feed (P1), feed supplemented with the probiotic *Bacillus subtilis* at a concentration of 10^8 CFU/g feed (P2), and feed containing a combination of 0.1% *β-glucan* and *Bacillus subtilis* probiotic at 10^8 CFU/g feed (P3). The purpose of these treatments is to determine the most effective functional feed treatment for increasing the resistance and immune response of vannamei shrimp against White Spot Syndrome Virus (WSSV) disease pressure. Each treatment is replicated three times and placed randomly. The culture containers used are aquaria measuring approximately 60×40×40 cm^3 and equipped with an aeration system. The test animals are vannamei shrimp (*Litopenaeus vannamei*) at the post-larval/early juvenile stage, with stocking density adjusted to the capacity of the culture container, at a density of 20 individuals per aquarium of that size. The shrimp were reared under controlled environmental conditions throughout the study period and fed according to the dosages and treatments established for each experimental group. Feeding was conducted regularly at the same frequency across all treatments, while the feed quantity was adjusted based on the shrimp's biomass and requirements during the rearing period. The equipment and materials used during the study include aquaria, blower, aeration stones, dissolved oxygen meter, pH meter, thermometer, digital scale, dip net, buckets, sampling tools, and supporting laboratory equipment. The materials include vannamei shrimp post-larvae, commercial feed as the basal diet, *β-glucan* as an immunostimulant, *Bacillus subtilis* probiotic, and brackish water with salinity adjusted for vannamei shrimp culture, Salinity was maintained within the 15–20 ppt range throughout the study period to provide relatively stable rearing conditions and support osmoregulation, growth, and shrimp survival. The parameters observed in this study include survival rate, shrimp growth, and non-specific immune responses such as total hemocyte count and phagocytic activity (Yang *et al.,* 2023). In addition, water-quality parameters such as temperature, pH, and dissolved oxygen are also observed periodically to support interpretation of the research results. This study is conducted using a prevention approach based on natural disease pressure, without artificial WSSV infection.

Shrimp feed preparation stage

The feed used in this study is commercial shrimp feed as the basal diet, which is modified into functional feed by adding *β-glucan* and *Bacillus subtilis* probiotic according to the treatments. Feed preparation is carried out using a coating method to maintain the stability of the active ingredients and avoid damage caused by heating. The initial step in feed preparation begins with weighing the commercial feed according to the needs of each treatment. Next, *β-glucan* is weighed according to the treatment dosage, namely 0.1% of total feed (1 g/kg feed), and dissolved in warm distilled water until homogeneous. For the probiotic treatment, *Bacillus subtilis* probiotic is weighed according to the dosage at a concentration of 10^8 CFU/g feed and

then dissolved in sterile distilled water. The *β-glucan* and/or probiotic solution is then sprayed evenly onto the surface of the commercial feed while being stirred slowly until the entire feed surface is homogeneously coated. To improve adhesion of the active ingredients, a binder in the form of fish oil or vegetable oil is added at approximately 2-3% of total feed. After the mixing process is completed, the feed is air-dried at room temperature for approximately 12-24 hours until the feed moisture content returns to normal. The dried feed is then stored in a tightly closed container and placed in a cool, dry location to maintain feed quality and probiotic viability. The treatment feed is given to vannamei shrimp according to the feeding schedule and dosage during the research period (Surya *et al.,* 2023).

Shrimp rearing stage

The vannamei shrimp (*Litopenaeus vannamei*) rearing stage begins with the preparation of culture containers in the form of aquaria, which are cleaned and sterilized using a disinfectant solution and then rinsed thoroughly. The aquaria are filled with filtered seawater, and the salinity is adjusted according to the requirements for vannamei shrimp culture. The water is then aerated for 24 hours before shrimp stocking. The vannamei shrimp used in this study are post-larvae/juveniles of uniform size and healthy condition. Before stocking, the shrimp are acclimatized by gradually adjusting temperature and salinity.

The stocking density is adjusted to the capacity of the culture container to avoid stress, and the shrimp are then reared throughout the research period. Feeding is carried out using the treatment feeds according to the research design, with a frequency of 3-4 times per day in the morning, afternoon, evening, and night. The amount of feed provided is adjusted to shrimp biomass and periodically adjusted based on shrimp growth. During rearing, leftover feed and waste are siphoned regularly to maintain water quality.

Parameter observation and sample collection stage

Parameter observations are carried out periodically during the vannamei shrimp (*Litopenaeus vannamei*) rearing period. The main parameters observed include survival rate, shrimp growth, and non-specific immune response. Survival is observed daily by recording the number of live and dead shrimp in each treatment. Shrimp growth is observed by measuring body weight and length through sampling at the beginning and end of the study. Samples for immune response analysis are collected by randomly selecting a number of shrimps from each treatment at the end of the rearing period. Hemolymph samples are carefully collected using a sterile syringe containing an anticoagulant (EDTA) to prevent clotting. The observed non-specific immune parameters include total hemocyte count and phagocytic activity as indicators of shrimp body resistance to disease pressure. The observed water-quality parameters include temperature, pH, dissolved oxygen (DO), ammonia, nitrate, and nitrite, which are measured periodically during the study to support interpretation of the research results (Safina *et al.,* 2024). Water-quality data are used as supporting parameters to ensure that differences in the obtained results arise from feed treatments rather than fluctuations in the culture environment.

Test Parameters

Absolute Weight Growth (W)

Absolute weight growth is calculated using the formula by Sarmin *et al.* (2025) as follows:

$$W = Wt - Wo$$

Note:

W: Absolute growth of test shrimp (g)

Wo: Weight of test shrimp at the start of the rearing period (g)

Wt: Weight of test shrimp at the end of the rearing period (g)

Specific Growth Rate (SGR)

According to Sarmin *et al.* (2025) the specific growth rate (SGR) of Vannamei shrimp is calculated using the following formula:

$$\text{SGR} = \frac{In\ Wt - \ln Wo}{t}\ x\ 100\%$$

Note:
SGR: Specific growth rate (%g/day)
Wt: Total weight of Vannamei shrimp at the end of the rearing period (g)
Wo: Total weight of Vannamei shrimp at the start of the rearing period (g)
t: Rearing duration (days)
Survival Rate (SR)
The survival rate (SR) is calculated to determine the mortality level of the test shrimp during the study; it can be calculated using the formula by (Sarmin *et al.,* 2025) as follows:

$$\text{SR} = \frac{Nt}{No}\ x\ 100\%$$

Note:
SR : Shrimp survival rate (%)
Nt : Number of test shrimp at the end of the study (individuals)
NO : Number of test shrimp at the beginning of the study (individuals)

Data analysis and statistical testing

Data obtained from observations are statistically analyzed using analysis of variance (ANOVA) at a 95% confidence level. If the analysis shows significant differences among treatments, Tukey post-hoc tests are conducted to determine specific differences among treatments. All data are presented as mean values and standard deviations. Statistical analysis is performed to determine the effect of *β-glucan* and *Bacillus subtilis* probiotic-based functional feed on survival, growth, and immune response of vannamei shrimp. The results of data analysis are used as the basis for drawing conclusions in determining the most effective feed treatment for the prevention of White Spot Syndrome Virus (WSSV) disease.

RESULT AND DISCUSSION

Weight Gain

Observations of shrimp weight gain during the study showed a weekly increase. The magnitude of weight gain varied among the tested treatments (Figure 1).

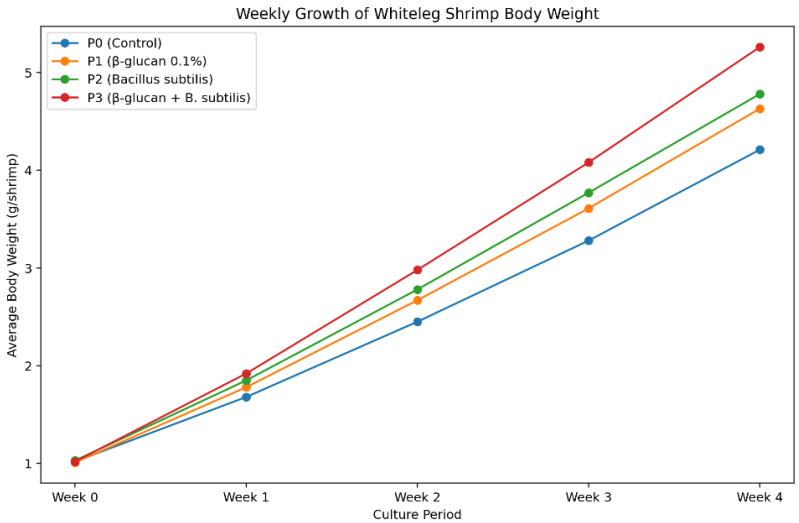


Figure 1. Weekly Growth of Vannamei Shrimp
Source: research result, 2026

Observations of weight gain were conducted weekly throughout the four weeks rearing period.

The results showed a gradual increase in weight across all treatment groups. Differences in growth became apparent as early as the first week and became increasingly pronounced by the fourth week. Treatment P0 resulted in the lowest weight gain during the rearing period. Treatments P1 and P2 showed higher growth compared to the control, whereas treatment P3 yielded the highest average weight at each week of observation. By the fourth week, the average weight of shrimp in the P3 group reached 5.26 g/individual, surpassing that of P2 (4.78 g/individual), P1 (4.63 g/individual), and P0 (4.21 g/individual).

The weight gain pattern observed in P3 is thought to stem from the combined benefits of an enhanced immune response and improved feed utilization efficiency. β-glucan helps prime the non-specific immune system, while *Bacillus subtilis* supports digestion through the production of extracellular enzymes and the stabilization of gastrointestinal microflora. This combination enables feed nutrients to be utilized more effectively for growth (Farhan & Budiyanto, 2025).

Absolute Weight Growth

Absolute weight gain is defined as the difference between the measured final weight and initial weight. A higher absolute weight gain in the cultured organisms indicates superior feed quality, alongside other factors correlated with growth. Research findings show that supplementing the feed with honey resulted in increased absolute weight gain in *Litopenaeus vannamei* as illustrated in Figure 2.

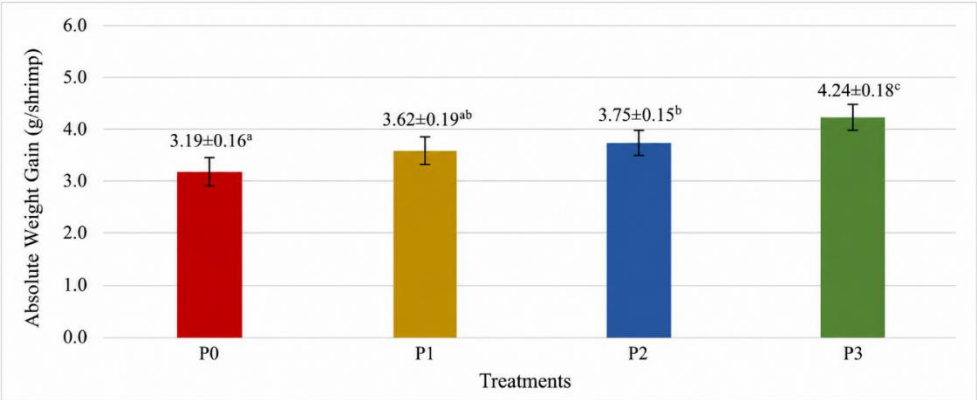


Figure 2. Absolute Weight Gain (g/shrimp)
Source: research result, 2026

The results indicate that the absolute weight growth of vannamei shrimp differed among treatments. Treatment P3 a combination of 0.1% *β-glucan* and the probiotic *Bacillus subtilis* at 10^8 CFU/g of feed—yielded the highest absolute weight growth at 4.24 ± 0.18 g/individual. The lowest value was observed in the control treatment (P0) at 3.19 ± 0.16 g/individual. The results of the one-way analysis of variance indicate that the administration of functional feed had a highly significant effect on the absolute weight growth of vannamei shrimp, with $F(3,8) = 19.26$ and $p = 0.00051$. Since the p-value is less than 0.05, it can be concluded that the feed treatments significantly influenced the absolute weight growth of the shrimp.

Absolute weight gain in treatment P3 increased by 1.05 g/head compared to P0. In relative terms, the absolute weight gain in P3 was approximately 32.92% higher than that of the control treatment. These results indicate that the combination of *β-glucan* and *Bacillus subtilis* elicited a better growth response than the administration of either substance alone.

The high absolute weight gain observed in treatment P3 is thought to result from the combined effects of *β-glucan* and probiotics. *β-glucan* enhances the readiness of the non-specific immune system, thereby reducing the energy shrimp must expend to cope with stress and disease pressure (Sun *et al.,* 2024). Meanwhile, *Bacillus subtilis* aids digestion by producing

extracellular enzymes and improving the microbial balance within the digestive tract. Improved digestive efficiency allows for the optimal utilization of dietary protein, fat, and carbohydrates for body tissue formation. Consequently, shrimp subjected to the combination treatment achieved greater absolute weight gain over the four weeks rearing period (Tegar & Budiyanto, 2025).

Overall, the ranking of absolute weight gain from highest to lowest was P3, P2, P1, and P0. Thus, the combination of 0.1% β-glucan and *Bacillus subtilis* probiotic (10^8 CFU/g of feed) represents the best treatment for enhancing the absolute weight gain of vannamei shrimp.

Specific Growth Rate (SGR)

The study was conducted over a period of four weeks, or 28 days. The results indicate that the specific growth rate of vannamei shrimp ranged from 5.06% to 5.86% per day. The lowest SGR value was observed in treatment P0, while the highest was observed in treatment P3 (Figure 3)

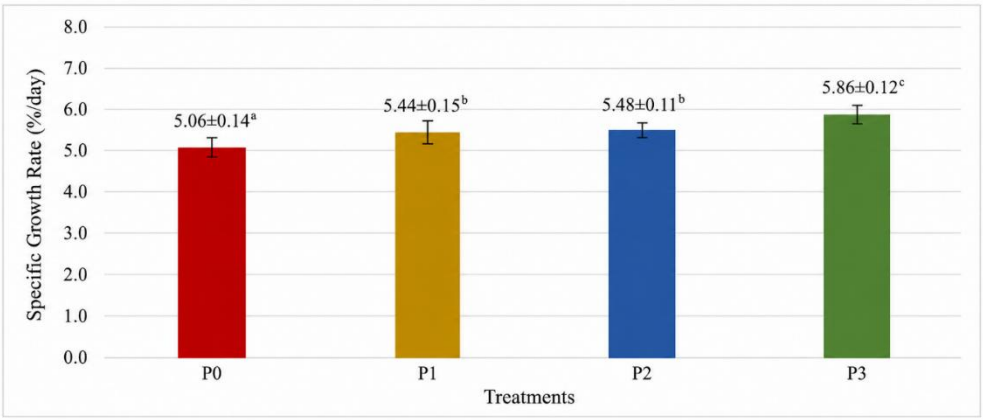


Figure 3. Spesific Growth Rate (%/day)
Source: research result, 2026

Analysis of variance results indicate that the administration of functional feed had a highly significant effect on the specific growth rate of vannamei shrimp, with $F(3,8) = 18.56$ and $p = 0.00058$. A p-value of less than 0.05 indicates significant differences in specific growth rates among the treatments. Tukey's post-hoc test results show that treatment P0 differed significantly from P1, P2, and P3. Treatment P1 did not differ significantly from P2, yet both differed significantly from P3. Thus, the combination of *β-glucan* and *Bacillus subtilis* probiotics resulted in a higher specific growth rate compared to single-additive treatments or the control.

Treatment P3 yielded an SGR of 5.86 ± 0.12% per day, which was higher than that of P0 (5.06 ± 0.14% per day). In relative terms, the SGR value for P3 increased by approximately 15.81% compared to the control. The high specific growth rate observed in P3 is thought to stem from a synergistic effect between *β-glucan* and *Bacillus subtilis*. *β-glucan* helps improve the shrimp's physiological condition and immune resilience, while the probiotic supports digestive processes through the production of extracellular enzymes and the restoration of microbial balance within the digestive tract (Aswinto & Dhengi, 2023). Enhanced digestive efficiency allows feed nutrients to be absorbed and utilized more optimally for growth. Furthermore, the shrimp's improved health status enables a greater allocation of dietary energy toward body tissue formation rather than expending it to cope with stress or disease-related pressures (Kaltsum *et al.,* 2023).

The SGR values for P1 and P2 did not differ significantly. This indicates that the administration of either β-glucan or probiotics alone resulted in relatively comparable growth effects. However, the combination of both substances in P3 yielded a superior response, demonstrating an additive benefit when the immunostimulant and probiotic were administered together. Based on these results, the order of specific growth rates from highest to lowest was P3, P2, P1, and P0. The combination of 0.1% *β-glucan* and *Bacillus subtilis* (10^8

CFU/g feed) was the most effective treatment for enhancing the specific growth rate of Vannamei shrimp over the 28-day rearing period.

Survival Rate

The results of the observations indicate that the survival rate of vannamei shrimp ranged from 68.33% to 89.67%. The lowest survival rate was observed in treatment P0, whereas the highest value was obtained in treatment P3, which received a combination of 0.1% β-glucan and the probiotic *Bacillus subtilis* at 10^8 CFU/g of feed (Figure 4).

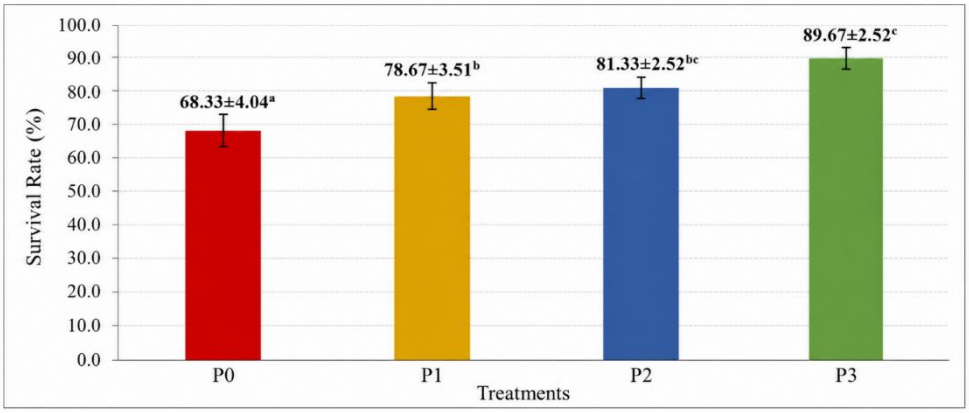


Figure 4. Survival Rate (%)
Source: research result, 2026

The results of the one-way analysis of variance indicate that the administration of functional feed had a highly significant effect on the survival of vannamei shrimp, with F(3,8) = 22.46 and p = 0.00030. A p-value of less than 0.05 indicates that there were differences in survival rates among the treatments. Tukey's post-hoc test results show that treatment P0 differed significantly from P1, P2, and P3. Treatment P1 did not differ significantly from P2 but differed significantly from P3. Meanwhile, P2 and P3 did not show a significant difference at the 5% level, although numerically, the survival rate for P3 was higher than that for P2.

Treatment P3 resulted in a survival rate of 89.67 ± 2.52%, which was 21.34 percentage points higher than the control treatment (68.33 ± 4.04%). In relative terms, the administration of the combination of β-glucan and *Bacillus subtilis* increased survival by approximately 31.23% compared to the control. The high survival rate observed in P3 is thought to be linked to the synergistic effect between *β-glucan* and the probiotic. *β-glucan* acts as an immunostimulant capable of enhancing the activity of the shrimp's non-specific immune system, including hemocyte production and phagocytic activity. This strengthening of the immune defense system helps the shrimp withstand environmental stressors and pathogen attacks.

The probiotic *Bacillus subtilis* is also believed to play a role in maintaining the balance of microorganisms in the digestive tract and the rearing medium. This bacterium is capable of producing digestive enzymes and compounds that inhibit opportunistic microorganisms, thereby helping to maintain the shrimp's health during the rearing period (Kadek *et al.,* 2018). The higher survival rates observed in P1 and P2 compared to P0 indicate that administering either *β-glucan* or the probiotic alone benefited shrimp survival. However, the combination of both substances in P3 yielded the highest survival rate (Basir and Ika Apriliani, 2022).

However, the difference between P2 and P3 was not statistically significant. This indicates that, with three replicates, statistical evidence regarding the superiority of P3 over P2 in terms of survival remains limited. Increasing the number of replicates in future studies could enhance the statistical power of the analysis. Overall, the ranking of vannamei shrimp survival rates from highest to lowest was P3, P2, P1, and P0. The combination of 0.1% β-glucan and *Bacillus subtilis* probiotic (10^8 CFU/g feed) yielded the best survival performance over the 28-day rearing period.

Total hemocyte count and phagocytic activity

Total hemocyte count and phagocytic activity are two important parameters for evaluating the non-specific immune response of Vannamei shrimp. The total hemocyte count reflects the number of defense cells circulating in the hemolymph, whereas phagocytic activity indicates the ability of hemocytes to recognize, engulf, and destroy foreign particles or pathogens. Consequently, these two parameters are interrelated, reflecting the readiness of the shrimp's immune system to withstand environmental stressors and disease challenges. The study results indicate that the administration of functional feed based on β-glucan and the probiotic *Bacillus subtilis* positively influenced the total hemocyte count and phagocytic activity of vannamei shrimp. The lowest values for both parameters were observed in the control treatment (P0), while the highest values were recorded in the treatment combining 0.1% β-glucan and *Bacillus subtilis* at 10^8 CFU/g of feed (P3) (Figure 5).

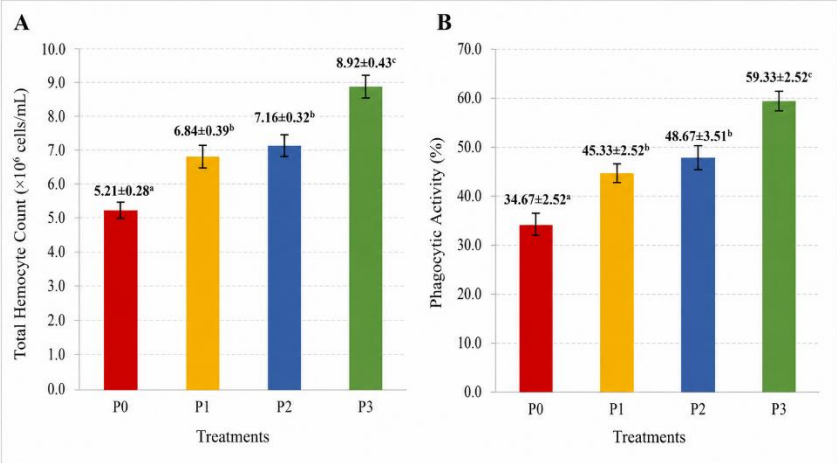


Figure 5. A. Total Hemocyte Count (x 10^6 cells/mL); B. Phagocytic Activity (%)
Source: research result, 2026

Analysis of variance results indicate that the feed treatments had a highly significant effect on both total hemocyte count and phagocytic activity. For total hemocyte count, the ANOVA yielded an F-value of F (3,8) = 53.81 (p = 0.000012), while for phagocytic activity, the value was F (3,8) = 39.55 (p = 0.000038). P-values below 0.05 indicate that the functional feed treatments significantly influenced both immune parameters. Post-hoc Tukey test results show that regarding total hemocyte count, P0 differed significantly from P1, P2, and P3; P1 did not differ significantly from P2; whereas P3 differed significantly from all other treatments. A similar pattern was observed for phagocytic activity, where P0 differed significantly from P1, P2, and P3; P1 did not differ significantly from P2; and P3 exhibited the highest value, differing significantly from the other treatments. These findings indicate that administering either *β-glucan* or *Bacillus subtilis* alone was capable of enhancing the cellular immune response, but the combination of the two yielded the most optimal results.

The total hemocyte count in treatment P3 reached 8.92 ± 0.43 × 10^6 cells/mL, an increase of approximately 71.2% compared to P0, which was only 5.21 ± 0.28 × 10^6 cells/mL. This increase indicates that the combination of β-glucan and *Bacillus subtilis* is capable of stimulating the formation and mobilization of hemocytes within the shrimp hemolymph. Hemocytes play a central role in the crustacean immune system, participating in processes such as phagocytosis, encapsulation, nodule formation, coagulation, and activation of the prophenoloxidase system. Thus, the rise in total hemocytes reflects an enhanced state of immune readiness in the shrimp to withstand pathogen challenges. Phagocytic activity also exhibited an increasing trend that paralleled the total hemocyte count. The highest phagocytic activity was observed in P3 (59.33 ± 2.52%), while the lowest was recorded in P0 (34.67 ± 2.52%). The increased phagocytic activity in P3 indicates not only an increase in hemocyte numbers but also an enhancement in the functional capacity of these immune cells. This is

significant because the effectiveness of the immune system is determined not merely by the quantity of defense cells, but also by the cells' ability to perform their biological functions (Dewi *et al.,* 2022).

The elevation of these two immune parameters is thought to be linked to the role of *β-glucan* as an immunostimulant. *β-glucan* is a polysaccharide recognized by pattern recognition receptors within the crustacean innate immune system. This interaction can trigger hemocyte activation, enhanced phagocytosis, activation of the prophenoloxidase system, and the production of other defense molecules. Consequently, the inclusion of β-glucan in the feed can directly boost the readiness of the shrimp's immune response (Abudi *et al.,* 2023). Meanwhile, *Bacillus subtilis* acts as a probiotic that indirectly supports shrimp health by improving the balance of the digestive tract microbiota, enhancing digestive enzyme activity, and inhibiting opportunistic microorganisms. A healthier digestive tract facilitates more efficient nutrient absorption, allowing available energy and protein to be allocated not only for growth but also for the development and maintenance of the immune system. The presence of probiotics can also reduce physiological stress, thereby enabling a more optimal immune response in the shrimp (Nugraha *et al.,* 2022).

The positive correlation between total hemocyte count and phagocytic activity indicates that an increase in hemocyte numbers is accompanied by an increase in the cells' phagocytic capacity. This implies that treatments capable of boosting total hemocyte counts also tend to enhance the shrimp's cellular defense capabilities. These findings reinforce the hypothesis that the VANNAMEI-SHIELD feed not only increases the quantity of immune components but also improves the quality of the Pacific white shrimp's immune defense functions. Overall, the study results demonstrate that a combination of 0.1% *β-glucan* and the probiotic *Bacillus subtilis* (10^8 CFU/g feed) is the most effective treatment for enhancing the non-specific immune response of Pacific white shrimp. The observed increases in total hemocyte count and phagocytic activity suggest that the functional feed VANNAMEI-SHIELD has the potential to serve as a preventive strategy for strengthening the shrimp's physiological and immunological resilience against disease stress.

Water Quality

Water quality observations during the rearing period indicated that all parameters remained within ranges suitable for *Litopenaeus vannamei* culture (Table 1). Temperature, pH, dissolved oxygen, ammonia, nitrite, and nitrate levels remained relatively stable throughout the study. These stable water quality conditions suggest that differences in survival, growth, and immune response were primarily driven by the dietary treatments rather than by variations in the rearing environment.

Table 1. Water Quality Measurement Data

Parameter	P0	P1	P2	P3	Optimum
Temperature (°C)	28,1–29,4	28,0–29,3	28,2–29,2	28,1–29,3	26–32
pH	7,4–7,8	7,5–7,9	7,5–8,0	7,6–8,0	7,0–8,5
Dissolved Oxygen (mg/L)	4,8–5,6	5,0–5,8	5,2–6,0	5,3–6,1	>4
Amonia (mg/L)	0,08–0,15	0,06–0,12	0,04–0,10	0,03–0,09	<0,1
Nitrit (mg/L)	0,05–0,09	0,04–0,08	0,03–0,06	0,03–0,05	<0,1
Nitrat (mg/L)	1,2–2,4	1,1–2,2	0,9–2,0	0,8–1,8	<10

Water quality is a crucial factor in the successful rearing of *Litopenaeus vannamei*, as fluctuations in temperature, pH, dissolved oxygen, and nitrogen compounds can influence metabolic processes, growth, immune responses, and survival rates. Measurements indicated that water quality conditions across all treatment groups remained relatively stable throughout the study period and stayed within the suitable range for *Litopenaeus vannamei* culture. Water

temperatures during the study ranged from 28.0°C to 29.4°C. This temperature range was relatively uniform across treatment groups and fell within the established optimum range of 26–32°C. Stable temperature conditions indicate that differences in shrimp growth, survival, and immune responses were not attributable to extreme temperature variations. Temperature influences metabolic activity, feed consumption, oxygen demand, and nitrogen excretion processes in shrimp; therefore, temperature stability throughout the study supported normal physiological functioning. Research on *Litopenaeus vannamei* culture further confirms that temperature, pH, dissolved oxygen, and nitrogen compounds are water quality parameters directly linked to shrimp growth, survival, and immune function (Rahmawati & Isabela, 2023).

The pH values across all treatments ranged from 7.4 to 8.0, remaining within the optimal range of 7.0 to 8.5. Treatment P3 exhibited a pH range of 7.6–8.0, showing slightly greater stability than P0, which ranged from 7.4 to 7.8. pH levels ranging from relatively neutral to slightly alkaline support enzymatic activity, osmoregulation, and the stability of microbial communities within the culture medium. pH stability is also crucial because ammonia toxicity is influenced by the interplay of pH, temperature, and salinity; an increase in pH can raise the fraction of un-ionized ammonia, which is more toxic to aquatic organisms. Consequently, maintaining pH within an appropriate range is a factor that helps minimize physiological stress in the shrimp (Pakaya *et al.,* 2022).

Dissolved oxygen concentrations during the study ranged from 4.8 to 6.1 mg/L. The lowest value was observed in treatment P0 (4.8 mg/L), while the highest was in P3 (6.1 mg/L). All treatments met the recommended minimum level of >4 mg/L. Adequate dissolved oxygen supports respiration, nutrient metabolism, aerobic microbial activity, and feed utilization. Favorable oxygen conditions also help prevent hypoxia-induced stress and reduce the risk of reduced feed intake and impaired growth. Research on shrimp culture systems indicates that managing dissolved oxygen a longside temperature, pH, ammonia, nitrite, and nitrate—is crucial for maintaining the production performance of *Litopenaeus vannamei (Ahmad et al., 2022)i.*

Ammonia levels in P0 ranged from 0.08–0.15 mg/L, which was higher than in P1 (0.06–0.12 mg/L), P2 (0.04–0.10 mg/L), and P3 (0.03–0.09 mg/L). This pattern indicates that treatments containing the probiotic *Bacillus subtilis* particularly P3 tended to maintain lower ammonia concentrations. Ammonia in the culture medium originates primarily from shrimp excretion, the decomposition of uneaten feed, feces, and organic matter. Ammonia accumulation can impair gill function, energy balance, antioxidant responses, and the physiological health of the shrimp. Experimental studies demonstrate that ammonia stress can disrupt the physiological homeostasis of *Litopenaeus vannamei* gills; therefore, maintaining low ammonia concentrations is crucial. The observed trend of decreasing ammonia levels in P2 and P3 may be linked to the probiotic activity of Bacillus strains. These bacteria are capable of producing extracellular enzymes that facilitate the decomposition of organic matter and support the microbial communities involved in nitrogen compound transformation. Hu *et al.* reported that the administration of Bacillus based probiotics during the culture of *Litopenaeus vannamei* improved water quality parameters and microbial community structure (Rajalakshmi *et al.,* 2022). Other studies have also found that Bacillus species can increase dissolved oxygen levels while simultaneously reducing total ammonia, un-ionized ammonia, and nitrite concentrations in the culture medium.

Nitrite concentrations followed a pattern similar to that of ammonia. The highest nitrite levels were observed in P0 (0.05–0.09 mg/L), while P3 exhibited the lowest levels (0.03–0.05 mg/L). All values remained below the 0.1 mg/L threshold. Nevertheless, nitrite concentrations require management, as the compound can impair oxygen transport and induce physiological stress if it accumulates. The toxicity of nitrite and ammonia is determined not only by their concentrations but is also influenced by temperature, salinity, pH, and the organism's developmental stage. Low nitrite levels in P2 and P3 indicate a more effective nitrogen transformation process compared to the control. Probiotics can support the proliferation of bacteria involved in nitrification the conversion of ammonia into nitrite and subsequently into

nitrate. Research on *Litopenaeus vannamei* culture ponds demonstrates that the application of Bacillus spore-based probiotics can facilitate ammonia degradation and maintain water quality in intensive culture systems (Mizuta *et al.,* 2023).

Nitrate concentrations ranged from 0.8 to 2.4 mg/L, well below the maximum limit of 10 mg/L. Treatment P0 exhibited the highest nitrate range (1.2–2.4 mg/L), whereas P3 yielded the lowest (0.8–1.8 mg/L). Nitrate is the primary end-product of the nitrification process and generally exhibits lower toxicity than ammonia and nitrite. The low nitrate concentrations across all treatments indicate that the accumulation of nitrogen compounds during the rearing period remained under control (Zhang & Gui, 2023). However, nitrate levels still require monitoring, as long-term accumulation can indicate an increased load of nutrients and organic matter.

CONCLUSION

Research indicates that the administration of functional feed based on β-glucan and the probiotic *Bacillus subtilis* positively affects the performance of *Litopenaeus vannamei*, as evidenced by improvements in absolute weight gain, specific growth rate, survival, total hemocyte count, and phagocytic activity compared to the control treatment. The combination treatment of 0.1% β-glucan and *Bacillus subtilis* at 10^8 CFU/g of feed yielded the best results with an absolute weight gain of 4.24 ± 0.18 g/individual, a specific growth rate of 5.86 ± 0.12%/day, and a survival rate of 89.67 ± 2.52% supported by water quality conditions remaining within optimal ranges and relatively uniform feed proximate composition across treatments. These findings suggest that VANNAMEI-SHIELD feed has the potential to be developed as a functional feed to enhance the growth, physiological resilience, and non-specific immune response of *Litopenaeus vannamei*, although its specific efficacy against WSSV requires further validation through PCR based testing or controlled challenge tests.

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