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Effects of probiotics *Lacticaseibacillus paracasei* and *Bacillus amyloliquefaciens* on water quality and inhibition of *Vibrio vulnificus* and *Vibrio alginolyticus* in white shrimp (*Litopenaeus vannamei*) culture

Sukkasem, K.[1], Thiankham, S.[1], Srisomwong, K.[2], Kantha, P.[3] and Kitikiew, S.[1*]

[1]Department of Animal Production Technology and Fisheries, School of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand; [2]Chachoengsao Shrimp Farmers Club, Thailand; [3]Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, 10900, Thailand.

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Abstract The infected white shrimp (*Litopenaeus vannamei*) caused by *Vibrio alginolyticus* and *Vibrio vulnificus* pose significant threats to white shrimp farming. Using probiotics for disease prevention and water quality improvement offers a promising eco-friendly alternative as it avoids chemical residues. This study evaluated the effectiveness of a probiotic formulation containing *Lacticaseibacillus paracasei* and *Bacillus amyloliquefaciens* at a concentration of 10^6 CFU/g. The probiotics were applied at concentrations of 0.125 and 0.1875 mL/L. White shrimp (average weight of 6 grams) were stocked at a density of one shrimp per liter for 15 days. Results demonstrated that ammonia levels were significantly lower in the treatments with probiotics compared to the control treatment. However, nitrite levels were elevated in the treatment. The probiotics did not affect temperature, pH, dissolved oxygen and total alkalinity. Notably, the probiotic treatment effectively reduced *Vibrio* spp. in the system with bacterial counts markedly lower in treatment compared to the control. The survival rates of white shrimp following a challenge with *Vibrio* spp. were 90% and 96.67% for the treatments with 0.125 mL/L and 0.1875 mL/L of probiotics while the control treatment exhibited 0% survival by day 13. The probiotic treatments also enhanced the immune response of white shrimp by increased total hemocyte count and clearance efficiency. Thus, probiotics can effectively reduce *Vibrio* spp. and ammonia levels while improving white shrimp survival even under infectious conditions.

Keywords: *Lacticaseibacillus paracasei*, *Bacillus amyloliquefaciens*, *Vibrio vulnificus, Vibrio alginolyticus*, Probiotics, White shrimp

[*]**Corresponding Author:** Kitikiew, S.; **Email:** suwaree.ki@kmitl.ac.th

Introduction

In Thailand's economy, the white shrimp (*Litopenaeus vannamei*) aquaculture industry is extensive and considered a crucial economic aquatic animal for the country. Cultivation occurs in freshwater, brackish water, and saltwater environments. Furthermore, there are both domestic and international exports. White shrimp is a significantly exported aquatic animal and is widely cultivated by farmers. Consequently, farmers need to cultivate in large quantities and high-density farming. As a result, white shrimp farms are impacted by issues such as water pollution, which weakens the shrimp and makes them susceptible to infections. The most frequently infected pathogens are *Vibrio* species. The bacterial infection organs found in white shrimp include the gills, stomach, and hepatopancreas. White shrimp infected with *Vibrio* bacteria can be initially observed with the naked eye as follows slow growth hence progressing to rapid death (Sivakumar *et al.,* 2012), floating on the water surface and decreasing the consumption rate. The main causes of infection are stress from high-density farming systems and changes in external and internal pond environments (Burrell *et al.,* 1991).

The *Vibrio* bacterial species that cause infections in affected white shrimp are *V. vulnificus* and *V. alginolyticus* which are the most frequently found bacteria in white shrimp farming systems (Luis-Villasenor *et al.,* 2015). This results in negative impacts and affects to the production (Yudiati *et al.,* 2021). Consequently, much current research involves using microorganisms in shrimp aquaculture systems to increase efficiency in shrimp cultivation, enhance shrimp immunity, growth, and control the outbreak of these two bacterial species. For these reasons, experiments with *Lacticaseibacillus paracasei* 10^6 CFU/g and *Bacillus amyloliquefaciens* 10^6 CFU/g were conducted to improve water quality increase survival rates, growth rates of shrimp, and enhance immunity response which avoid the problem of accumulation of harmful chemical residues to consumers (Bernal *et al.,* 2016) reduce bacterial content in shrimp aquaculture systems (Kumar *et al.,* 2016) and respond to immune systems and disease resistance (Luis-Villasenor *et al.,* 2015).

However, chemicals and antibiotics are still used instead of probiotics in some cases. Using chemicals and antibiotics has negative effects such as residues in shrimp and consumers and antibiotic resistance (Tank *et al.,* 2018). The probiotic efficacy trial experiments have been conducted to determine the effects and ensure the efficacy of probiotics. It has been confirmed that probiotics are a better treatment method than antibiotics and chemicals in inhibiting bacteria in aquaculture systems (Moriarty, 1999). Therefore, the objective was to test the efficiency of *L. paracasei* 10^6 CFU/g and *B. amyloliquefaciens* 10^6 CFU/g in

improving water quality reducing *V. vulnificus* and *V. alginolyticus* in water enhancing the immune response and increasing the survival rate of white shrimp when infected with *V. vulnificus* and *V. alginolyticus.*

Materials and methods

Experimental design

The experiment was designed using a completely randomized design (CRD) with six experimental treatments and three replications. Experimental treatment 1 (NC) served as the negative control, receiving no probiotic supplementation and no exposure to *Vibrio vulnificus* and *Vibrio alginolyticus*. Experimental treatment 2 (PC) was the positive control treatment which did not receive probiotic supplementation but was exposed to both *V. vulnificus* and *V. alginolyticus*. Experimental treatment 3 (P1-NonV) involved the addition of probiotics at a concentration of 0.125 mL/L without exposure to the pathogens. Experimental treatment 4 (P2-NonV) received a higher concentration of probiotics at 0.1875 mL/L also without pathogen exposure. Experimental treatment 5 (P1-V) included probiotic supplementation at 0.125 mL/L along with exposure to *V. vulnificus* and *V. alginolyticus* and Experimental treatment 6 (P2-V) combined probiotic supplementation at 0.1875 mL/L with exposure to the same pathogens.

Animal

White shrimp (*Litopenaeus vannamei*) with an average weight of 6 g were sourced from a private farm in Chachoengsao Province. Before the experiment, the shrimp were acclimated for two weeks in large tanks containing water with a salinity of 10 ppt maintained with aeration systems. Following acclimatization, 30 shrimp were randomly assigned to each of six 60-liter plastic tanks at a stocking density of one shrimp per liter and cultured in 30 L of 10 ppt seawater. White shrimps were fed to satiation twice daily with a commercial diet (GF555, Charoen Pokphand Group).

Preparation of probiotics for incubation in experimental tanks

A preparation containing *Lactobacillus paracasei* and *Bacillus amyloliquefaciens* at a concentration of 10^6 CFU/g was formulated before the experiment. The mixture consisted of 200 g of *L. paracasei* and *B. amyloliquefaciens*, 400 ml of pasteurized milk, 200 L of distilled water and 500

g of dextrose monohydrate which was fermented in a sterilized 1-liter glass jar. Fermentation was allowed to proceed until the pH of the mixture reached approximately 4.6–5.0 at which point the solution was considered ready for use. The pH of the fermented solution was monitored throughout the experiment and the product was administered daily to the experimental tanks.

Preparation of V. vulnificus and V. alginolyticus

The bacterial strains *V. vulnificus* and *V. alginolyticus* were prepared following a modified protocol based on Sritunyalucksana *et al.* (2005). Each strain was cultured separately in 100 ml of nutrient broth containing 10 ppt of NaCl for 16 hours. Following incubation, the bacterial cultures were prepared as suspensions for use in the subsequent experiments.

Water quality parameter

Water samples were collected before the addition of the product and at intervals of the day 1, 3, 5, 7, 9, 11, 13, and 15 post-applications. Water quality parameters, including dissolved oxygen (DO) and temperature were measured using a YSI Model 57 meter while pH levels were determined with a H198128 pH meter. Ammonia, nitrite- nitrogen, and total alkalinity were analyzed according to the standard methods outlined by the American Public Health Association (APHA, 2005). All water quality measurements were conducted in the morning before feeding.

Survival rate

V. vulnificus and *V. alginolyticus* were introduced to the system at a combined concentration of 10^4 CFU/mL following the challenge method by bath exposure as described by Sudheesh and Xu (2001) and Tran *et al.* (2013). After the challenge, white shrimp were raised for 15 days and survival rates were recorded daily throughout the experiment. The survival rate was calculated using the formula from Hsu *et al.* (2021).

$$\text{Survival rate (\%)} = \frac{\text{The final number of white shrimps}}{\text{The starting number of white shrimps}} \text{x100}$$

Efficacy test of probiotics in reducing the contaminate of V. vulnificus and V. alginolyticus in water

The number of *V. vulnificus* and *V. alginolyticus* in the water was counted using Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar with the drop plate method as described by Herigstad *et al.* (2001). Samples were taken on day 1, 3, 5, 7, 9, 11, 13, and 15 after application of probiotics.

Immune response testing

The immune response assay which included Total Hemocyte Count (THC) and clearance efficiency was conducted using four experimental treatments: Experimental treatment 1 (NC), Experimental treatment 2 (PC), Experimental treatment 3 (P1-NonV), and Experimental treatment 4 (P2-NonV). Each experimental treatment was replicated three times.

Total Hemocyte Count (THC)

Hemolymph was collected from three shrimp in each experimental treatment (NC, PC, P1-NonV, and P2-NonV) on day 0, 1, 3, 5, and 7 of the experiment. Hemolymph was drawn using a 0.5 x 25 mm syringe connected to a 1 mL plastic syringe and coated with an anti-coagulant (ACG). The hemolymph was drawn from the ventral sinus located near the last pair of walking legs on the right side of the white shrimp. A volume of 100 µL of hemolymph was transferred into a 1 mL eppendorf tube containing 900 µL of ACG. During the procedure, the sample tubes and reagents were kept on ice to maintain temperature. Subsequently, 10 µL of hemolymph was counted on a hemocytometer slide in all four corners of the slide to obtain the Total Hemocyte Count (THC) calculated using the following formula (Lee *et al.,* 2024).

$$\text{THC} = \left(\frac{\text{Number of cells counted in each chamber x 10,000 x 10}}{\text{1,000,000 x } 10^6 \text{cells/mL}}\right)$$

Clearance efficiency

Three shrimp from each experimental treatment (NC, PC, P1-NonV, and P2-NonV) were injected with a live bacterial suspension at a concentration of 10^4 CFU/mL on day 0, 1, 3, 5, and 7. One hour after injection, the white shrimp were transferred to aerated tanks for an additional hour before hemolymph samples were collected. A 100 µL aliquot of white shrimp hemolymph was

diluted with 900 µL of saline solution, and 10 µL of this mixture was applied onto Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates. The plates were incubated at 28°C for 24 hours then bacterial colony counts were recorded. Clearance efficiency was calculated according to the formula provided by Lee *et al.* (2024).

$$\text{Clearance Efficiency} = 100 - \left(\frac{\text{CFU of experimental treatment}}{\text{CFU of control treatment}}\right)\text{x}100\ \%$$

This procedure allowed for the assessment of bacterial clearance efficiency in the white shrimp.

Statistical analysis

Data were analysed using Analysis of Variance (ANOVA). Differences between mean values were compared using Duncan's new multiple range test at a 95% confidence level. Statistical analysis was performed using SPSS 28.0 for Windows.

Results

Water quality

During the 15-day experimental period, the salinity of the water in all tanks was maintained at 10 ppt. The temperature ranged between 29.00-30.00 °C, with no significant differences in temperature among the different experimental treatments. The dissolved oxygen (DO) levels in the water ranged from 5.8 to 6.2 ppm across the PC, P1-NonV, P2-NonV, P1-V, and P2-V treatments, showing no significant differences throughout the experiment. In contrast, the PC treatment had the lowest DO level on day 1 after the infection (Figure 1). The pH levels in all experimental treatments were maintained between 8.2 and 8.5 during the experimental period.

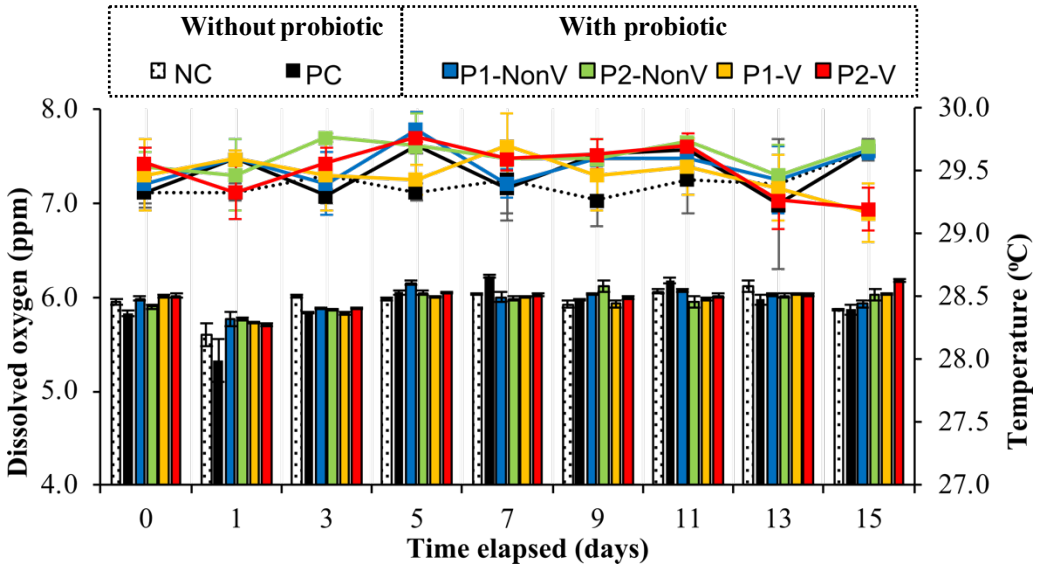

Figure 1. Water quality, dissolved oxygen (DO) is displayed in a bar graph and water temperature is displayed in a line graph

The total alkalinity of all experimental treatments at the beginning of the experiment was between 159.00 and 163.67 which was not significantly differed among the treatments. From the start to the end of the experiment (day 1 to 15), the total alkalinity of the NC treatment ranged from 161.00 to 213.67 ppm while the PC treatment ranged from 185.67 to 340.33 ppm, peaking on day 1 before decreasing to the lowest point on day 7. After day 7, a gradual increase was observed. The total alkalinity of the P1-NonV treatment varied between 182.33 and 251.67 ppm, with the highest levels recorded on day 1 followed by a decrease on day 7 and then an increase on day 9. For the P2-NonV treatment, total alkalinity ranged from 192.33 to 280.00 ppm, also peaking on day 1 then dropping to the lowest levels on day 9 followed by an increase. The P1-V treatment exhibited total alkalinity levels between 193.33 and 308.00 ppm, peaking on day 3 before reaching the lowest point on day 7 which slightly increased on day 9, 13, and 15 with the highest recorded value being 209.33 ppm. The P2-V treatment had the highest total alkalinity at 282.00 ppm on day 1, the lowest values recorded at 237.33 ppm on day 7, 11, and 13. However, the changes in total alkalinity for the P2-V treatment were the least pronounced throughout the experiment (Figure 2).

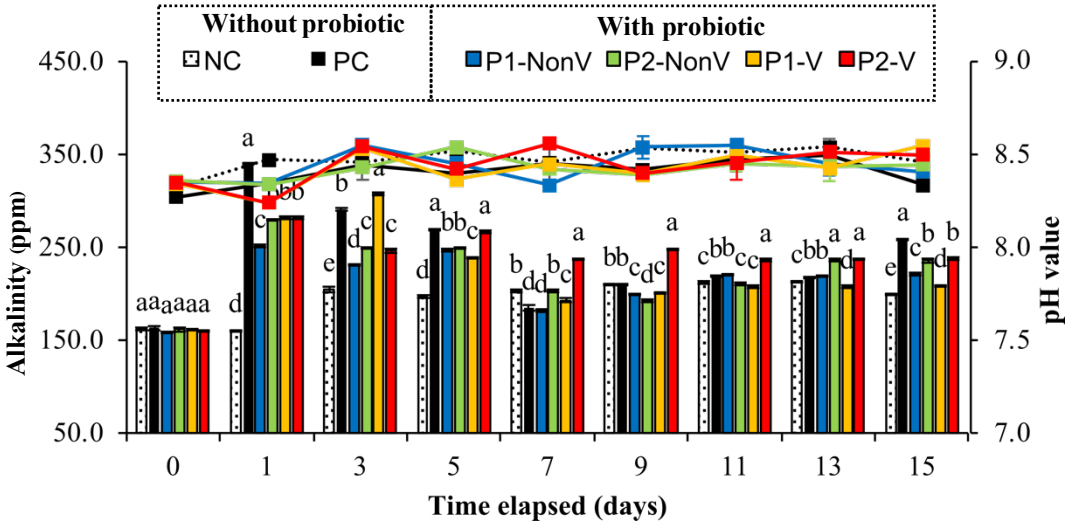

Figure 2. Water quality, Alkalinity is displayed in a bar graph and pH is displayed in a line graph

The initial ammonia levels at the start of the experiment were approximately 0.06 ppm in all treatments. Upon initiation, the ammonia levels in the PC treatment reached the highest at 5.53 ± 0.42 ppm due to stress from the infection and subsequent mortality, leading to increased waste in the farming system. This level subsequently decreased to the lowest point on day 15, where no white shrimp remained. Conversely, the NC treatment recorded increasing ammonia levels starting from day 1, peaking at 3.43 ± 0.23 ppm on day 13. On day 1 of the experiment, the ammonia levels for P1-V and P2-V treatments were significantly differed but were higher than those in the NC, P1-NonV, and P2-NonV treatments, with P2-NonV exhibiting the lowest levels at 0.85 ± 0.03 ppm. From day 5 onward, the ammonia levels in the experimental treatments P1-NonV, P2-NonV, P1-V, and P2-V were lower than NC treatment, continuing until the end of the experiment. Starting from day 7, treatments without infection, P1-NonV and P2-NonV, consistently had the lowest ammonia levels among all treatments were not significantly differed until the end of the experiment (Figure 3A).

The nitrite levels before the start of the experiment and on day 1 were similar (ranging from 0.011 to 0.015 ppm). After that, the nitrite levels increased across all experimental treatments. The NC treatment recorded values between 0.015 and 0.144 ppm, with the highest value observed on day 7 of the experiment (0.144 ± 0.00 ppm). The PC treatment had nitrite levels peaking on day 15

(ranging from 0.015 to 0.156 ppm). However, these values were lower between day 5 and 13 than those in treatments P1-NonV and P2-NonV. Treatments P1-NonV and P2-NonV exhibited higher trends compared to other experimental treatments, with the highest values recorded at 0.156 ± 0.00 ppm and 0.155 ± 0.00 ppm on day 13, respectively. In contrast, treatments P1-V and P2-V had lower nitrite levels than P1-NonV and P2-NonV between day 5 and 9 but their levels increased and became comparable to P1-NonV and P2-NonV between day 11 and 15. On day 13, treatment P1-V had the highest nitrite level at 0.155 ± 0.00 ppm while treatment P2-V highest value was 0.149 ± 0.00 ppm on day 11, 13, and 15. Nevertheless, the nitrite levels in treatments P1-NonV, P2-NonV, P1-V, and P2-V tended to decrease on day 15 while the NC and PC treatments which continued to increase (Figure 3B).

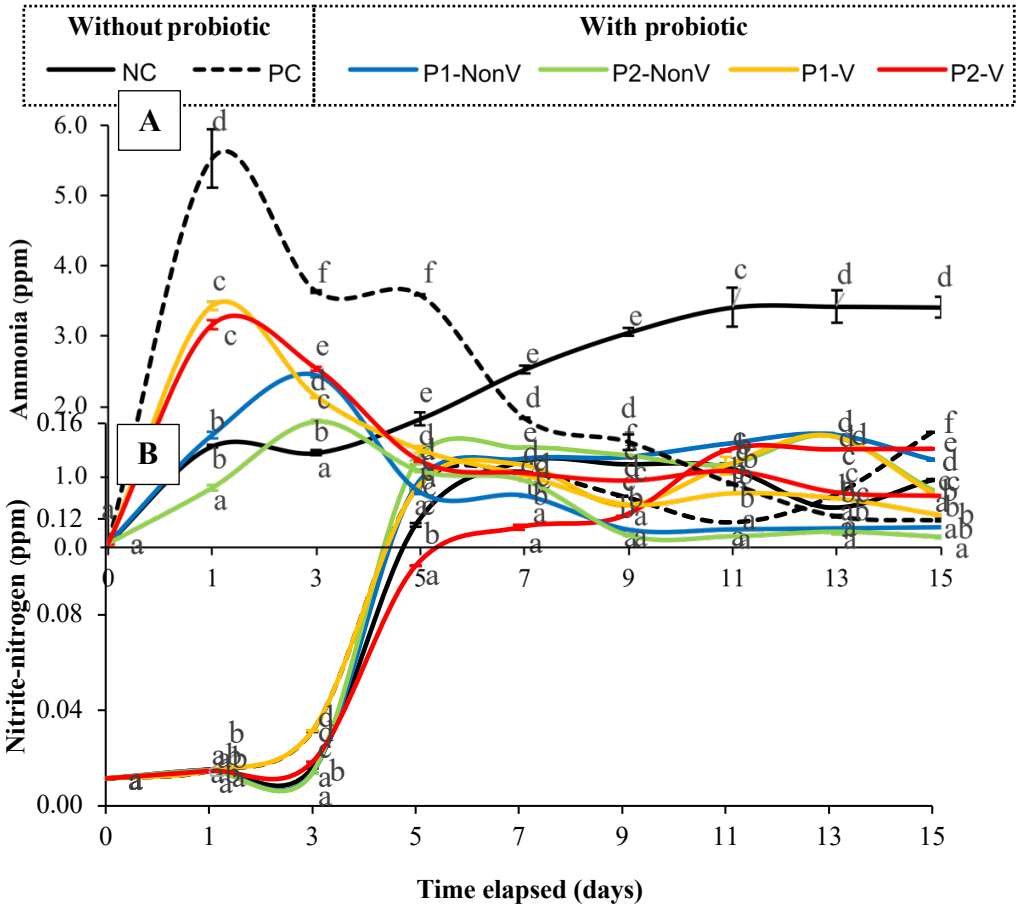

Figure 3. Water quality, the value of ammonia (A) and the value of Nitrite-nitrogen (B)

Survival rate

The survival rates of white shrimp in the NC, P1-NonV and P2-NonV treatments were all 100% which was not significantly differed among the treatments as none of these three treatments experienced an infection with *Vibrio* spp. In contrast, treatments P1-V and P2-V, which received probiotics at concentrations of 0.125 mL/L and 0.1875 mL/L, respectively, and were infected with *Vibrio* spp. showed that treatment P2-V had a survival rate of 96.67% by the end of the experiment which was higher than the survival rate of P1-V for 90.00%. Meanwhile, the PC treatment which had infected but did not receive probiotics and survival rate was 0% starting from day 13 of the experiment with the survival rate of white shrimp decreasing from day 1 after the infection (Figure 4).

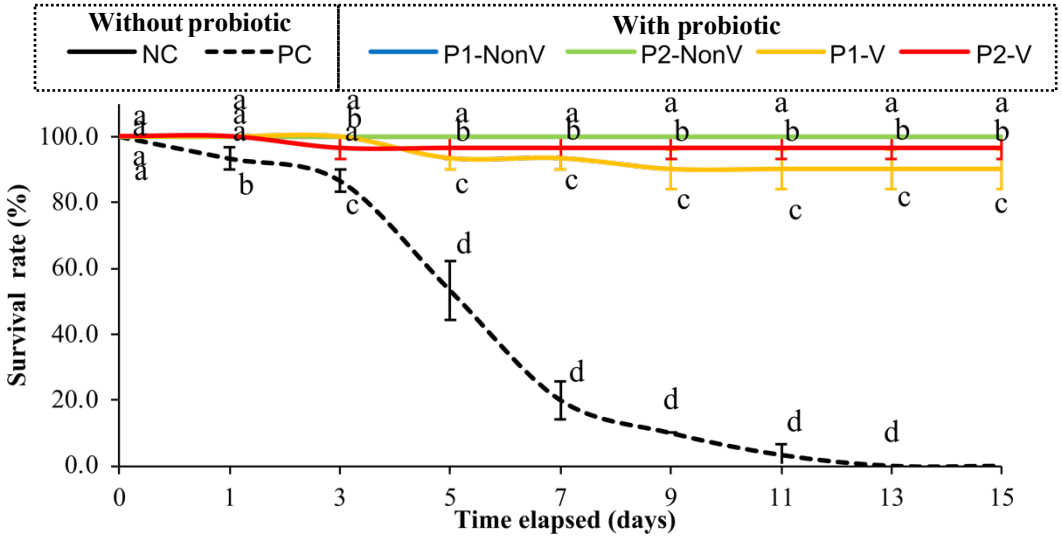

Figure 4. Survival rate of white shrimp

Efficacy test of probiotics in reducing the contaminate of V. vulnificus and V. alginolyticus in water

No colonies of *Vibrio* spp. were detected in the NC, P1-NonV and P2-NonV treatments throughout the experiment because no bacterial challenge was introduced into the water. In contrast, PC, P1-V, and P2-V treatments had *Vibrio* spp. count of 1.00 x 10^4 CFU/mL on day 0 before the experiment. Afterward, the PC treatment increased in bacterial count on day 1 (1.33 to 1.67 x 10^6 CFU/mL)

throughout the experiment. In treatments P1-V and P2-V, *Vibrio* spp. count increased on day 1 (1.01 and 0.97 x 10^6 CFU/mL, respectively). However, the *Vibrio* spp. count in treatment P2-V was significantly lower than in treatment P1-V. Afterward, both experimental treatments decreased in bacterial count. Treatment P1-V exhibited a decline reaching its lowest bacteria count on day 7 (0.14 x 10^6 CFU/mL) before slightly increasing on day 15 (0.23 x 10^6 CFU/mL). Treatment P2-V decreased in bacterial count between day 3 and 5 (ranging from 0.23 to 0.60 x 10^6 CFU/mL). At the end of the experiment, the *Vibrio* spp. count was 0.23 x 10^6 CFU/mL (Figure 5).

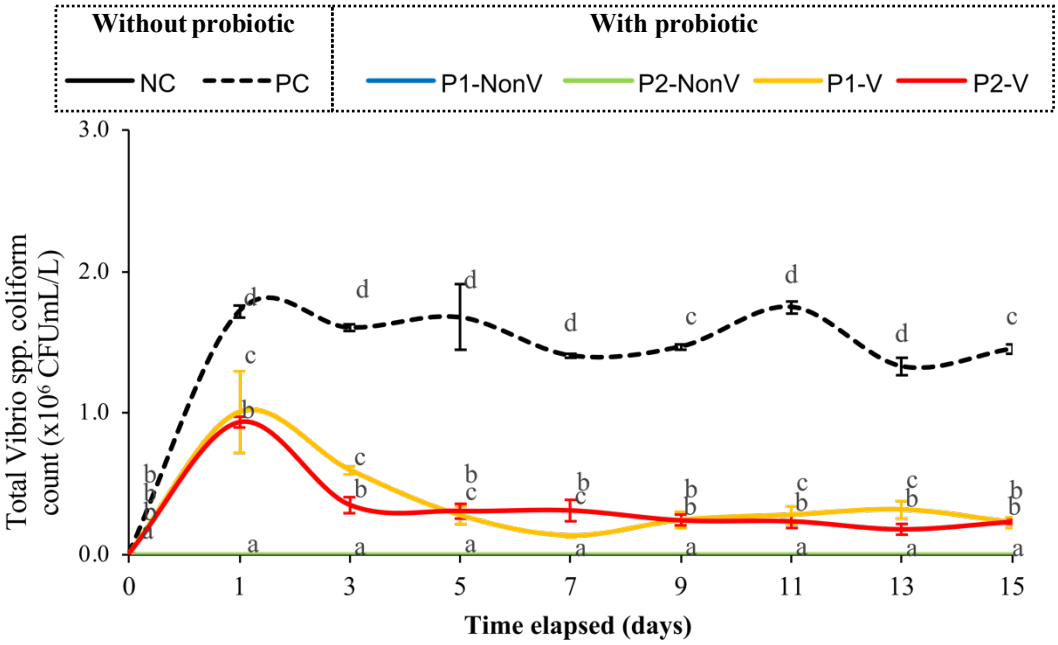

Figure 5. Total *Vibrio* spp. coliform count

Total Haemocyte Count

The Total Hemocyte Count (THC) of the white shrimp in treatments P1-NonV and P2-NonV which were treated with probiotics was higher than in the untreated treatment. The THC values for treatments P1-NonV and P2-NonV was not significantly differed on day 1, 3, 5, and 7 with the highest value recorded at 30.50 ± 0.40 x10^6 cells/mL in treatment P1-NonV on day 7 (Figure 6).

Clearance efficiency

The clearance efficiency of white shrimp in treatments which treated with probiotics (P1-NonV and P2-NonV) was the highest among all experimental treatments which was not significantly differed on day 5 and 7 of receiving probiotics, with the highest value recorded at 91.67 ± 0.86% in treatment P2-NonV on day 7 (Figure 7).

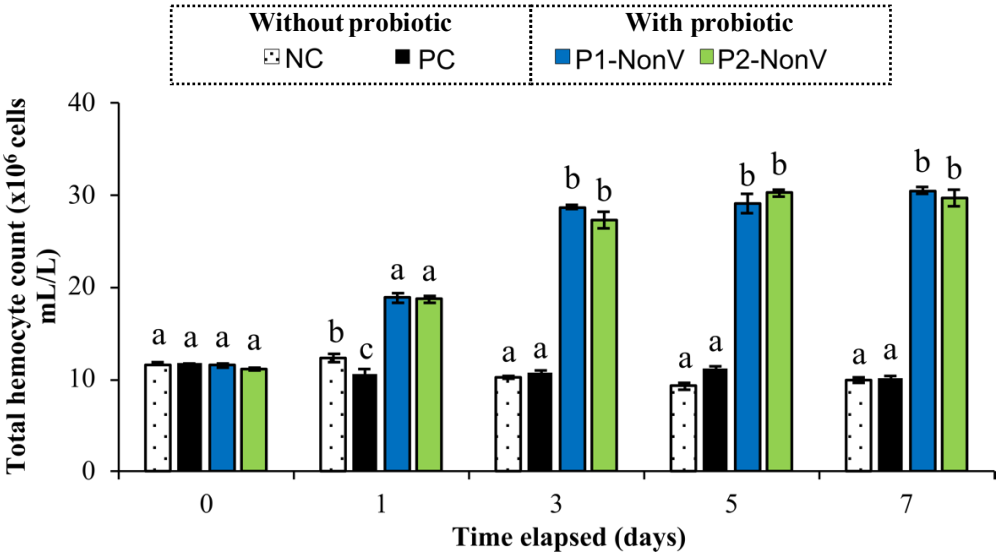

Figure 6. Total Hemocyte Count (THC)

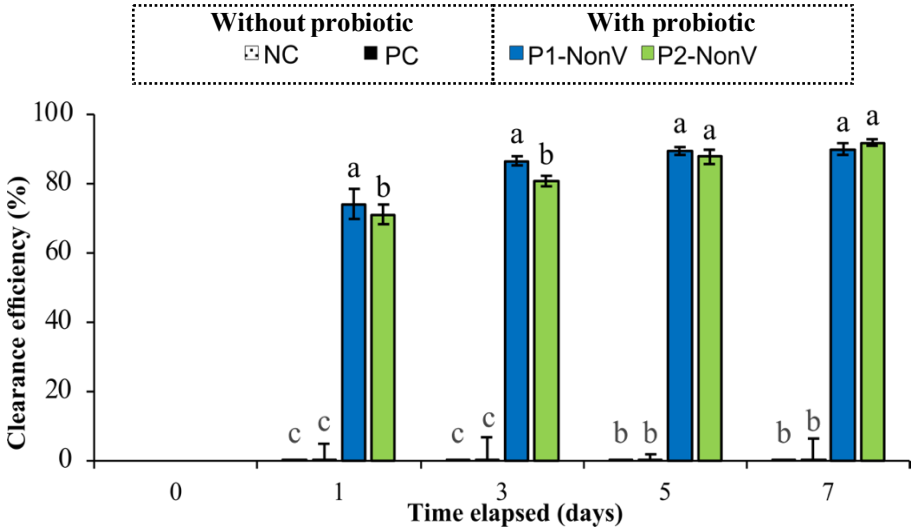

Figure 7. Clearance efficiency

Discussion

In this study, *Lacticaseibacillus paracasei* and *Bacillus amyloliquefaciens* significantly reduced the inhibitory efficiency against *V. vulnificus* and *V. alginolyticus*. The survival rate of *L. vannamei* increased markedly and water quality improvements were within suitable parameters for shrimp aquaculture systems. According to Lee *et al.* (2024), where the addition of *Bacillus* spp. to white shrimp feed resulted in lower ammonia levels compared to those receiving normal feed. Additionally, Llario *et al.* (2020) reported that the addition of *B. amyloliquefaciens* at concentrations of 9.48 x 10^4, 1.90 x 10^5, and 3.79 x 10^5 CFU/mL to post-larval shrimp in a density of 300 individuals per square meter in 30 ppt salinity did not effectively reduce ammonia levels, possibly due to inappropriate concentrations concerning the waste levels in the tank or environmental factors such as high salinity, which may have hindered probiotic function. However, over 45 days resulted to reduce nitrite levels in the treatment with probiotics by day 20 of the experiment. Moreover, the results are consistent with the findings of Amjad *et al.* (2022) added *B. amyloliquefaciens* to white shrimp tanks at a rate of 0.06 grams with a density of 150 shrimp per 200 liters at the salinity of 15 ppt for 40 days. They found that nitrite levels in the system with *B. amyloliquefaciens* were higher than in the control treatment and decreased after day 25 of the experiment. Therefore, in the previous experiment conducted over 15 days, nitrite levels had not decreased. However, it was observed that the addition of *B. amyloliquefaciens* to the water stimulated the immune response in shrimp.

Furthermore, Bachruddin *et al.* (2018) noted that incorporating *Lactiplantibacillus plantarum*, *Lactobacillus fermentum*, *B. subtilis*, *B. licheniformis*, *B. megaterium*, *Nitrobacter* spp. and *Nitrosomonas* spp. The amounts of 1, 2, 3, and 4 mL/L added to the white shrimp farming tanks at a density of 5 shrimp per liter showed that all water quality parameters were within good ranges. According to James *et al.* (2021), the use of *Bacillus* spp. in conjunction with *Lactobacillus* spp. in aquaculture systems can reduce ammonia levels due to their ability to decrease waste levels in the system. These bacteria produce enzymes such as protease, amylase, lipase, and cellulase to break down large waste molecules which can then be utilized as an energy source or carbon source for the bacterial cells. Additionally, these bacteria can convert ammonia into nitrite and nitrate, respectively. Thus, reducing the concentrations of ammonia and nitrite in the farming system.

Freire-Peñaherrera *et al.* (2020) and Xu *et al.* (2014) demonstrated that using *B. amyloliquefaciens* strains A5 and M1 could inhibit *V. vulnificus*. It was found that *B. amyloliquefaciens* can inhibit the colony formation of *V. vulnificus*.

Additionally, *Lactobacillus* spp. shown to inhibit the growth of *Vibrio* spp. by producing organic acids (Koga *et al.,* 1998). This ability to inhibit the growth of *Vibrio* spp. led to a reduction in the concentration of pathogenic bacteria in the water. Wongpracha *et al.* (2024) report that white shrimp treated with *B. subtilis* and *B. cereus* had a higher THC and got a survival rate of 86.7% which was higher than that of the infected treatment without probiotics.

In conclusion, the use of probiotics *L. paracasei* and *B. amyloliquefaciens* at concentrations of 0.125 mL/L and 0.1875 mL/L effectively reduced ammonia levels and *Vibrio* spp. in the water within one day of application without affecting temperature, pH, dissolved oxygen, or total alkalinity. However, nitrite levels were not decreased within 15 days and the treatments treated with probiotics had higher nitrite levels than the untreated treatment. Using probiotics did not have any negative effects on the white shrimp and improved the immune response at both concentrations. The application of *L. paracasei* and *B. amyloliquefaciens* 0.1875 mL/L resulted in the highest white shrimp survival rate in the presence of *Vibrio* spp. infection.

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