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By S Supono; M Rahayu; M W Yusuf

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The Effect of *Bacillus Coagulans* as Feed Probiotics on Non-specific Immunity of Whiteleg Shrimp *Litopenaeus Vannamei*

S Supono[1,2] M Rahayu[1] M W Yusuf[1]

[1]*Department of Aquaculture, Universitas Lampung, Bandar Lampung, Indonesia*
[2]*Post Graduate Program, Universitas Lampung, Bandar Lampung, Indonesia*
[*]*Corresponding author.Email :supono_unila@yahoo.com*

ABSTRACT

The study aimed to evaluate the effect of giving a different dosage of *Bacillus coagulans* to feed (feed probiotics) on nonspecific immunity of *Litopenaeus vannamei* (white shrimp). The parameters tested included water quality and shrimp immunity parameters, namely total hemocyte count (THC), differential hemocyte count (DHC) and phagocytosis activity (PA) and carried out for 20 days of culture. The method of administering *Bacillus coagulans* was by spraying it into the commercial feed. White shrimp used were the size of 13.1 ± 0.06 grams in the amount of 10 shrimps per container. The size of the container was 50cm × 40cm × 40cm totaling 12 pieces (4 treatments and 3 replications). The treatments consisted of K (control), a bacterial dose of 10^4 CFU ml^{-1}, a bacterial dose of 10^6 CFU ml^{-1}, and a bacterial dose of 10^8 CFU ml^{-1}. The results showed that *Bacillus coagulans* as a probiotic feed was able to increase the nonspecific immunity of white shrimp. The treatment of *Bacillus coagulans* of 10^8 CFU ml^{-1} produced the best nonspecific immunity in white shrimp.

Keywords: *immunity, hemocyte, whiteleg shrimp, bacillus coagulans*

1. INTRODUCTION

Whiteleg shrimp (*Litopenaeus vannamei*) is an introduction shrimp that is currently widely cultivated in Indonesia. The superiority of the white shrimp has led to higher production and demand for these shrimps [1]. The value of white shrimp exports in 2014 reached 197 thousand tons, an increase of 17.34 percent compared to the previous year [2]. Based on these data the value of white shrimp production in Indonesian fisheries has increased every year.

In line with the increased production of white shrimp, intensive cultivation systems characterized by stocking densities and high feeding have been carried out [3]. However, this intensive cultivation system can cause disease and the risk of decreased white shrimp production [4]. The disease is a major obstacle that needs attention besides feed. This causes a general attack of disease suddenly and can lead to shrimp death [5].

One effort in the prevention of disease is through enhancing the body's defense system against shrimp attack pathogens [6] by using immunostimulants [7] [8]. Shrimp body defense against disease is not only done through feed with a balanced composition but can be accompanied by giving immunostimulants in the feed. Immunostimulants are directly related to cells that can activate the immune system in shrimp [9].

Immunostimulants are now widely used for disease control in aquaculture activities as an alternative to the use of drugs, chemicals, and antibiotics [10] [11]. Immunostimulant can be applied by injection, immersion, or oral methods [12]. Giving immunostimulants through the feed is one method of immunostimulant that is widely studied in shrimp culture.

Much evidence has shown that immunostimulants added to feed can increase fish and shrimp resistance to disease infections through increased non-specific immune responses [13] [14]. In shrimp, hemocyte is a very important factor in a non-specific cellular defense system. The ability of hemocytes in the phagocytic activity that can increase in the incidence of infection shows the body's cellular defense. Increased shrimp body endurance can be known from the increased phagocytic activity of hemocyte cells [15].

The bacterium that has the potential to be immunostimulants is *Bacillus coagulans* [16] *Bacillus coagulans* can produce lactic acid, is resistant to high temperatures, tolerates acidic environment, is antagonistic to pathogenic bacteria, and produces anti-disease compounds [17]. Under these conditions, it is necessary to research to evaluate the use of *B. coagulans* to enhance nonspecific immunity in whiteleg shrimp.

2. MATERIALS AND METHODS

2.1. Materials

The research was conducted at the Fisheries Laboratory, Department of Fisheries and Marine, Faculty of Agriculture, University of Lampung, Indonesia. Whiteleg shrimp used were sized 13.1 ± 0.06 gr as many as 10 shrimps/container. Previously shrimp acclimatized for 5 days. Shrimp culture was carried out for 20 days by feeding 4 times a day at (08.00 am, 12:00 am, 4.00 pm, 8.00 pm).

2.2. Container Preparation

The container for culture was the aquarium, as many as 12 units measuring 50 cm × 40 cm × 40 cm and covered with black plastic to prevent stress on the shrimp. The aquariums were filled with seawater until it reaches the desired salinity (25 ppt) of 30 liters. Strong aeration is installed using a blower that is drained using a 0.5-inch pipe that has been perforated and placed on the bottom of the aquarium.

Bacterium *Bacillus coagulans* was isolated from shrimp ponds in Lampung Province. Bacteria were prepared by the re-culture of *Bacillus coagulans* on 70% seawater tilted TSA (Tryptone Soy Agar) to get a younger bacterial culture. Furthermore, the bacteria were cultured on 70% seawater TSB (Tryptone Soy Broth) so that it could be stored until it was used. Bacteria were cultured on the media until they reached the desired density (10^4 CFU ml^{-1}, 10^6 CFU ml^{-1}, and 10^8 CFU ml^{-1}).

2.3. Feed Preparation

The feed used in this study is commercial feed. The process of preparation of the test feed involved mixing the diluted bacterial isolates with a dilution dose of 10^4 CFU ml^{-1}, 10^6 CFU ml^{-1}, 10^8 CFU ml^{-1} and then mixed into the feed by spray technique. After being mixed evenly, the feed is dried for 5 minutes after which the feed is put into a container and ready to be given to the test shrimp by feeding it by 2% of shrimp biomass every day.

2.4. Observed parameters

2.4.1. Differential hemocyte count (DHC)

Hemocytes that have been taken from test shrimp are dripped on glass and made a review. The samples are dried in the air and fixed with 100% methanol for 5 minutes. After that, it was dried in the air again and colored by soaking it in 10% giemsa solution for 18 minutes dried in the air, washed in running water for 30 seconds and allowed to dry. The preparations were observed using a light microscope with a magnification of 40 times and distinguished according to its type namely hyaline cells and granular cells [18].

2.4.2. Total hemocyte count (THC)

Fresh hemocyte (20 μL) was diluted with PBS (40 μL), then samples were diluted using a micropipette and placed on the surface of the hemocytometer, then observed under a microscope. Hemocyte seen on a microscope was then calculated the number of hemocytes (total hemocyte count / THC) [18].

2.4.3. Phagocytic activity (PA)

As much as 0.1 ml of hemocyte taken from the test shrimp was put into a microplate and then added 25 µl of *Staphylococcus aureus* (10^6 CFU ml^{-1}) mixed evenly and incubated for 20 minutes. Hemocyte as much as 5 µl was dropped on a glass object and made to be prepared and then dried. The preparations were fixed into 100% methanol for 5 minutes and stained with giemsa solution for 15 minutes. Phagocytic activity was measured based on the percentage of phagocytic cells that carry out phagocytosis [18].

2.4.4. Water quality

Water quality parameters observed during the study included temperature, pH, dissolved oxygen, and ammonia. Types of equipment used to control water quality were a thermometer, pH paper, DO meter and spectrophotometer. Media water quality was analyzed descriptively.

2.5. Data Analysis

Nonspecific immunity performance data were analyzed using Anova with a 95% confidence interval using the SPSS software. If there were significant differences in results followed by the LSD test. While the water quality data were analyzed descriptively.

3. RESULTS

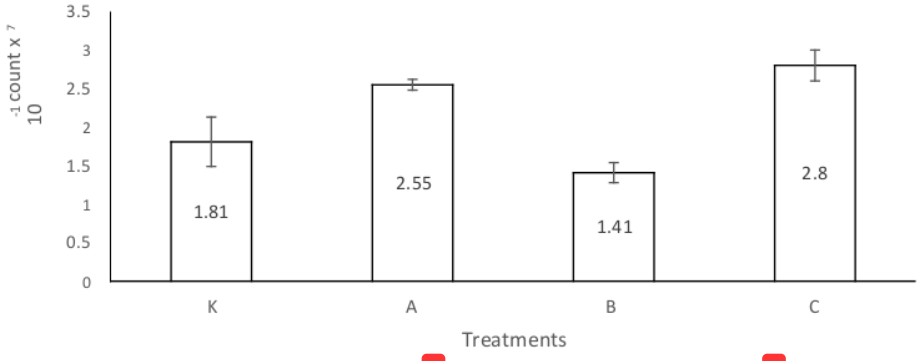
The results of measurements of water quality parameters such as temperature, dissolved oxygen (DO), pH and ammonia were still in the range of whiteleg shrimp culture to grow normally (Table 1)

Table 1. Water quality during the culture of whiteleg shrimp treatments

	K	A	B	C
Temperature (°c)	28	28	28	28
DO (ml^{-1})	5.63	5.29	5.50	5.27
pH	7	7	7	7
Ammonia (ml^{-1})	0.003	0.004	0.003	0.003

3.1. Total Hemocyte Count (THC)

Data on total hemocyte count of white leg shrimp for 20 days of culture is displayed in Figure 1.



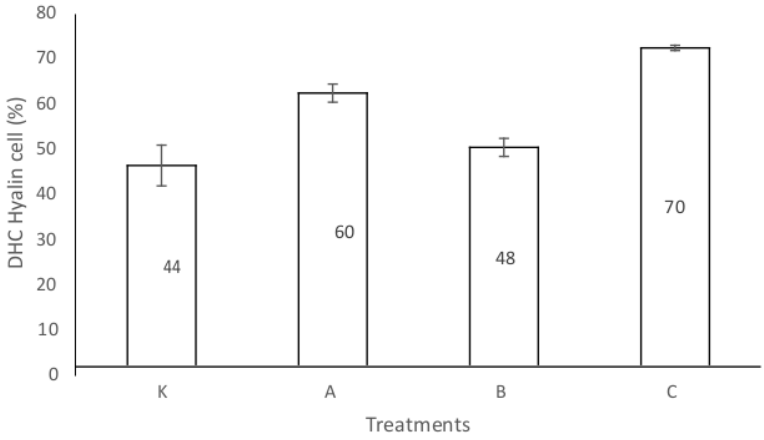
(K) Control; (A) Treatment of B. coagulans dose of10^4 CFU ml^{-1}; (B) Treatment of B. coagulans dose of 10^6 CFU ml^{-1}; (C) Treatment of B. coagulans dose of 10^8 CFU ml^{-1}

Figure 1 Total hemocyte count of whiteleg shrimp

Based on data analysis (Anova), the addition of *B. coagulans* to feed had a significant effect on the total hemocyte count of whiteleg shrimp at a 95% confidence level. Treatment of *B. coagulans* dose of 10^6 CFU ml^{-1} was different in the treatment of *B. coagulans* dose of 10^4 CFU ml^{-1}, treatment of *B. coagulans* dose of 10^8 CFU ml^{-1} and control. While the treatments of *B. coagulans* dose of 10^8 CFU ml^{-1} and treatment of *B. coagulans* dose of 10^4 CFU ml^{-1} were significantly different from the controls.

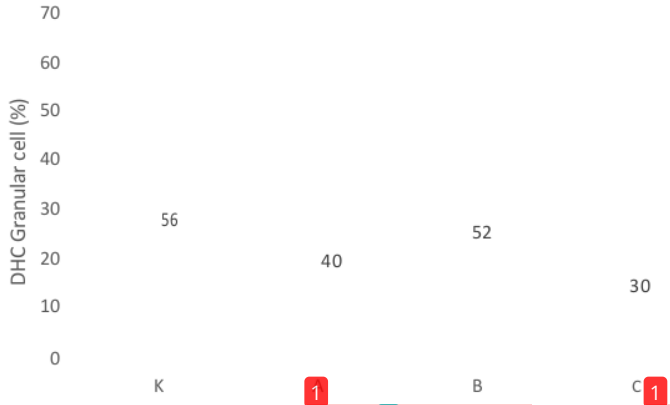
3.2. Differential Hemocyte Count (DHC) Hyalin Cells

Data on differential hemocyte count (DHC) of hyaline cells of whiteleg shrimp for 20 days of culture is displayed in Figure 2. Based on data analysis (Anova), the addition of*Bacillus coagulans* to the feed had a significant effect on the differential hemocyte count of hyaline cells of whiteleg shrimp at a 95% confidence level. Treatment of*B. coagulans* dose of 10^8 CFU ml^{-1} was significantly different ($p < 0.05$) for all treatments. Treatment of*B. coagulans* dose of 10^4 CFU ml-1 was significantly different ($p < 0.05$) on the treatment of*B. coagulans* dose of 10^6 CFU ml-1 and control.



(K) Control; (A) Treatment of B. coagulans dose of10⁴ CFU ml⁻¹; (B) Treatment of B. coagulans dose of 10⁶ CFU ml⁻¹; (C) Treatment of B. coagulans dose of 10⁸ CFU ml⁻¹

Figure 2 Differential hemocyte count of hyaline cells of whiteleg shrimp



(K) Control; (A) Treatment of B. coagulans dose of10⁴ CFU ml⁻¹; (B) Treatment of B. coagulans dose of 10⁶ CFU ml⁻¹; (C) Treatment of B. coagulans dose of 10⁸ CFU ml⁻¹

Figure 3 Differential hemocyte count (DHC) of granular cells of whiteleg shrimp

While treatment of*B. coagulans* dose of 10^6 CFU ml^{-1} was not significantly different from control.

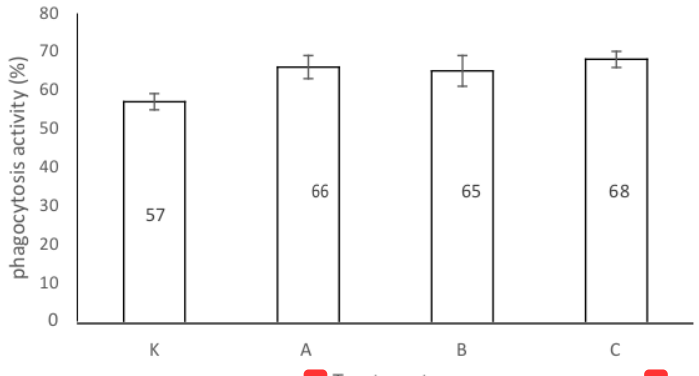
3.3. Differential Hemocyte Count (DHC) Granular Cells

Data on differential hemocyte count (DHC) of granular cells of whiteleg shrimp for 20 days of culture is shown in Figure 3.

Based on data analysis (Anova), the addition of Bacillus coagulans to the feed had a significant effect on DHC granular cells. Treatment of *B. coagulans* dose of 108 CFU ml-1 treatment wassignificantly different for all treatments. Treatment of *B. coagulans* dose of 10^4 CFU ml-1 was significantly different from the treatment of *B. coagulans* dose of 106 CFU ml-1 and control.

3.4. Phagocytosis Activity

Data on the phagocytosis activity of whiteleg shrimp for 20 days of culture is shown in Figure 4. The results of data analysis (Anova) showed that the addition of *B. coagulans* to the feed affects the phagocytic activity of whiteleg shrimp. Treatment of *B. coagulans* dose of 10^8 CFU ml^{-1} was significantly different in all treatments.



(K) Control; (A) Treatment of B. coagulans dose of 10^4 CFU ml^{-1}; (B) Treatment of B. coagulans dose of 10^6 CFU ml^{-1}; (C) Treatment of B. coagulans dose of 10^8 CFU ml^{-1}

Figure 4 Whiteleg shrimp phagocytosis activity

However, treatment of *B. coagulans* dose of 10^4 CFU ml^{-1} and treatment of *B. coagulans* dose of 10^6 CFU ml^{-1} did not show significantly different results on controls.

4. DISCUSSION

Total hemocyte count (THC), differential hemocyte count (DHC) and phagocytosis activity (AP) are immune parameters related to hemocyte used to evaluate the immunostimulatory effect of probiotics on shrimp (19). Shrimp defense mechanism depends on the hemocyte process, the total value of hemocyte obtained in this study ranges from 1.41 to 2.8 × 10^7 CFU mL^{-1}. Total hemocyte in treatment of *B. coagulans* dose of 10^8 CFU ml^{-1} was significantly different ($p < 0.05$) with other treatments. Total hemocyte count obtained reached 10^7 CFU ml^{-1} so that the test shrimp was still in normal condition and even tended to increase its immunity after being given the bacterium *B. coagulans*. The total hemocyte count of normal shrimp is 10^4 CFU ml^{-1} [20].

The increase in total hemocyte in this study means increasing the chances of the formation of hemocyte cells, namely hyaline cells and granular cells. Both of these cells have their respective functions. When the function of each cell increases, the shrimp can defend themselves from incoming pathogen attacks [21].

The results of DHC parameters in hyalin cells ranged from 44% - 70% and granular cells in the range of 30% - 56%. Hyalin and granular DHC cells in the treatment of *B. coagulans* dose of 10^8 CFU ml-1 were significantly different in all treatments. One of the parameters to impr the health status of shrimp with an increase in hyaline cells and granular cells, but in granular cells, the number is l s than in hyaline cells [22]. Hyalin cells have an important role in the activity of phagocytosis in crustacean immunity [23].

The activity of whiteleg shrimp phagocytosis can be seen based on the increase in h mocyte cells that carry out phagocytosis [24] [25]. The mechanism of action of immunostimulants in stimulating the body's immune system is by increasing the activity of phagocyte cells [26].

As the main response mechanism in the shrimp immune system, phagocytic activity parameters indicate how much the shrimp immunity reacts to pathogens that enter the body. An increase in phagocytic activity shows that probiotic bacteria can increase phagocyte cell activity so that when an attack occurs, hemocyte cells are ready to carry out the phagocytosis process [27]. Phagocytosis itself can occur when phagocytic cells are close to the antigen, or the antigen must be attached to the surface of phagocytic cells [28]. The results of the study of phagocytosis activity showed an increase in each treatment ranging from 57% to 68%. The highest value was significantly in the treatment of *Bacillus coagulans* dose of 10^8 CFU ml^{-1}.

5. CONCLUSION

The administration of *B. coagulans* with different density dosages in feed influences the nonspecific immunity of whiteleg shrimp. Bacterium *B. coagulans* as feed probiotic can increase total hemocyte count (THC), differential hemocyte count granular cells and phagocytocis activity. The dosage of *B. coagulans* 10^8 CFU ml^{-1} on whiteleg shrimp feed showed the best results.

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