The effect of probiotic and prebiotic-MOS on the survival, growth and immune competence on spiny lobster *Panulirus homarus* culture

Haryanti¹*, Z Widiastuti¹, Fahrudin¹, S B M Sembiring¹, G N Permana¹ and K Sugama²

¹Institute for Mariculture Research and Fisheries Extension, Ministry of Marine Affairs and Fisheries, Bali, Indonesia
²Research Center of Fisheries, Jakarta, Indonesia

*Corresponding author email : haryanti0423@gmail.com

Abstract. The use of probiotics and prebiotic to improve growth and health status of spiny lobster has been evaluated. The purpose of this study was to obtain the effect of probiotics and prebiotics for the culture of spiny lobster *Panulirus homarus*. The method was initiated with culturing of probiotics (4 strains) and supplemented it in a moist diets. Spiny lobster was collected from Jembrana-Bali waters with an initial body weight of 70.34 ± 4.5 g and cultured in concrete tanks with volume of 4 m³. Initial stock density for each treatment was 15 pcs / m³. The treatments tested were supplemented on moist diets with (A) probiotics, (B) probiotics and prebiotics-MOS (Mannan Oligo Sacharida) and (C) Control e (without probiotics and prebiotic) and each treatment with three replication. The results obtained that the survival rate of spiny lobster was not significantly different (P> 0.05), i.e. (A) 92.70%, (B) 93.33 (%) and (C) 93.33% respectively. However, the results of probiotics supplementation as well as a combination of probiotics and prebiotics showed growth differences compared to control (P<0.05), namely (A) 156.97 ± 6.17 g, (B) 153.75 ± 9.17 g, while (C) control 131.47 ± 7.91 g. The probiotics supplementation on moist diets could increased health status of spiny lobsters, this was expressed by target genes related to immunity (ALFHa -1, ALFHa -4, SAA, ProPO, Tgase and CP). Spiny lobster immunity increased by 2.60 to 42.7 times after challenging with MHD (Milky Haemolymph Disease). The supplementation of probiotics and prebiotics (MOS) could increase immune response by 2.10 to 25.75 times, respectively after challenging with MHD.

1. Introduction

An effort to initiate a *Panulirus homarus* lobster farming business is improved culture techniques that efficiently and environmentally friendly, followed by prevention against disease infections. The first step that have been made to improve health status of lobster are the application of probiotics on lobster culture. Improved performance on lobster through the use of probiotics is likely to be more effective in stimulating better health, survival and growth. Lobster is belonged to crustaceans families, that do not have an adaptive immune response with immunoglobulin [1]. In addition, juvenile and baby lobster only have a natural immune system that is produced from their body themselves (innate immune). Therefore, the immune response system in lobster is very different from fish. The role of action of probiotics was improving indirectly the growth performance, intestinal microbiota, stimulating non-specific immune mechanisms and protecting lobsters from infection by producing compounds that have activities such as bacteriocin (bacteriocin-like activities) [2,3].
Lobster is an export commodity that is exploited from wild capture. Until now the production of lobster cultured is only 2% of the total Indonesian lobster production [4]. Meanwhile, Vietnam is a country with rapid development of lobster culture since 2005, although the seeds source are imported from Indonesia. The condition of seed production from hatcheries has not been successful and is not much interesting by practitioners because of the long production time, low survival rate, which is not economically. Mortality that usually still high in culture is caused by stress, water quality, cannibalism or disease infection. Some diseases in spiny lobster include red body disease, black gill disease, milky hemolymph disease and red tail disease [5,6]. Stress reduction can be done through management of feed and nutrition. Research on feed to increase the health of humpback grouper Cromileptes altivelis, especially those related to stress have been used Fe addition [7] and Se (Selenium) [8]. According to [9] states a theory regarding immune nutrition as an effort to improve immunological function through specific nutrients and feed composition with a level higher than what is needed for growth. Strategies to control diseases infectious can be done by improving environmental conditions, the use of healthy fry and improving health status of lobster by using probiotics. Several studies have been carried out to prevent disease infection and increase growth performance in lobster culture, including the use of probiotics, prebiotics and the application of symbiotic microbes [3,10,11]. According to [12, 13, 14] stated that the use of probiotics could improve the growth performance, enzymatic stimulation, disease resistance, immune gene expression and reduce the prevalence of viral infections in shrimp. The results of the challenge test of Vibrio on Homarus americanus also showed 17-fold greater transcription of antimicrobial protein response and increased expression of immunity genes in larvae [15,16,17].

The proven role of probiotics in aquaculture is to improve water quality, to improve health, and bacterial pathogens and virulence can be controlled, eliminate the use of antibiotics, thereby minimizing the risk of antibiotic resistance, stimulating the immune system, increasing intestinal flora and lowering disease incidence and assimilation increased feed [18,19,20]. Probiotics is not only appear in the mechanism of action in the immune system, but also play a protective role, namely directly blocking pathogenic microbes and increasing mucus production in an integrated manner by stimulating epithelial cells [21,22]. Besides probiotics, the role of prebiotics is also high in the process of digesting diets, the presence of fermentation by bacteria, making it beneficial for the host organism. The combination of MOS (Mannan Oligo Saccharide) and Bacillus spp. on Homarus gammanus larvae feed [2], succeeded for increasing immune response, growth and resistance to disease.

The results obtained from preliminary research at IMRAFE revealed that the use of probiotics, yeast Saccharomyces cerevisiae, Bacillus cereus BC and Alteromonas sp. BY-9 which was supplemented to the semi moist pellets, showed survival, expression of immunity with gene targets of ProPO, ALFHa-1, ALFHa -2 and ALFHa-4, transglutaminase, clotting protein on baby lobster and puerulus of P. homarus were better than control (fresh feed) and without probiotic in moist diets [23]. Research results in 2016 obtained 4 strains of probiotics isolated from intestine of spiny lobster, i.e. Photobacteria damsela N-5, Bacillus subtilis C-1, Bacillus oceanisediminis H-3, and Bacillus amyloliquefaciens I-5. Those probiotics supplemented to the semi moist diets showed the better effect on survival, growth and expression of immunity than control. The aim of this research is to obtain information of the effect of probiotic and prebiotic for spiny lobster P. homarus culture. Therefore, research probiotics supplementation for spiny lobsters culture is expected to improve survival, growth and health status. In future, research results can be used in increasing production lobsters for healthy cultivation and supporting the lobster fisheries program developed in Indonesia.

2. Material and Methods

2.1. Probiotic culture

Four probiotic bacterial strains that were produced in 2016, (Photobacterium damsela N-5, Bacillus subtilis C-1, Bacillus oceanisediminis H-3, Bacillus amyloliquefaciens I-5) have been approved their ability to inhibit the growth of pathogenic bacteria. Those bacteria strains could produce extra cellular enzymes to hydrolyze proteins, fats, carbohydrates. Probiotics bacteria were cultured with media of Marine Broth to reach densities of $10^{10-12}$ CFUmL$^{-1}$. Culture media have to be sterilized at 121 °C for
15 minutes. In probiotics culture, it was equipped with aeration through 0.22 μm filtration to stimulate cell growth. Incubation time was 48 hours. Culture volume of probiotics were 5 L using glass flask.

2.2. Preparation of moist diets with probiotics supplementation

Four strains of probiotic was cultured then added to the feed (moist diets) with a density of 10^5-10^6 CFU/mL (equal with 100 mL/kg-1) and mixture on feed ingredients and air dried. This was to maximize the work of probiotics in stimulating survival, growth and improvement health of lobster. In this research, prebiotics was used as growth media, namely Mannan Oligo Saccharide (MOS). Ingredient of moist diets consisted of a mixture shrimp diets flour (1 kg), small shrimp meal (1 kg), fresh shrimp (1 kg), fresh squid (1 kg), CMC as binder (30 gr) and vitamin mix (7.6 g). All feed were mixed, added with 100 mL of four probiotic strain cultures, ground in a pellet machine and air dried for about 3 hours. Symbiotics moist diets is made by adding a combination of four strain probiotics and prebiotic-MOS (6 g/kg) [24].

2.3. Culture of spiny lobster P. homarus

The study was conducted by applying treatments of feed by supplemented with (A) probiotics on moist diets, (B) probiotics and prebiotics-MOS, and (C) Control (without probiotics or prebiotic) and each treatment with three replication. Moist diets were given 2 times/day. Initial body weight of spiny lobsters between 70.34 ± 4.5 g and reared for 3 months. The concrete tanks with capacity of 4 m^3 were used and filled with filtered seawater, Initial lobster density of each treatment was 60 individuals/tank. During culture, flow through water system was applied and cleaning left over feeds and faeces by siphon, and controlling biosecurity, especially equipments and cleanliness of technicians. Shelter were used with form of a cement block and plastic block with a hole below, and a piece of PVC pipe. At the end of lobster culture, then conducted of challenge test with pathogenic bacteria Milky Haemolymph Disease/MHD) by injection. The injection dose was 100 mL/100 g lobster. Result of challenged challenge were tested by RT-qPCR to determine the relative level of immunity expressed from the immune gene.

2.4. Gene expression related to immunity

2.4.1. Extraction of RNA and cDNA synthesis. Haemolymph that has been collected were then centrifuged at a speed of 12,000 rpm at 4°C for 5 minutes. The haemocytes pellet obtained was then washed once with a cold anticoagulant solution and re-centrifuged at the same speed. RNA extraction was performed using a RNA extraction lysis with IQ-2000 method. Synthesis of cDNA (complementary DNA) was done using the Agilent Affinity Script qPCR cDNA Synthesis kit. The reaction volume was 20 µL, containing of 10.0 µL first strand 2x master mix, 3.0 µL primary oligo (dT), 1.0 µL affinity RT script and 3.0 µg of RNA. The solution in microtube was incubated successively at 25 °C for 5 minutes, 42 °C (15 minutes) and 95 °C for 5 minutes. The cDNA was then placed in ice to stop the synthesis reaction and stored at -20 °C for further analysis.

2.4.2. Analysis of gene expression associated with P. homarus lobster immunity by using RT-qPCR.

Immune status profile analysis by using the expression of gene transcription method associated with immunity quantitatively of RT-qPCR and specific primers [16,25]. Pro PO analysis, Tgase, Protein Clotting [25], while ALFHa-1, ALFHa-4, SAA-Serum Amyloid Protein A [16] as shown in Table 1. Internal control using 18SrRNA. RT-qPCR analysis was performed using the ABI PRISM 7500 sequential detection system with 5x Hot Firepol Evagreen qPCR mix (ROX). The reaction volume for cDNA amplification was 20.0 µL with a final concentration of 1x hot master mix (Rox), primers of 10 pmol F / R (Table 1) each of 250 nM, NFW (Nuclease Free Water) was added up to volume of 20 µl and cDNA (0.01ng / µL). The cycling temperature conditions for RT-qPCR consist of an initial denaturation temperature of 95 °C (15 minutes) followed by 95 °C (15 seconds) and annealing temperature of 60 °C (30 seconds) for 40 cycles and a final extension
temperature of 72 ºC for 1 minute. Calculations of ∆Ct from the PCR cycle threshold (Ct) of the gene under test were normalized relative to Ct of 18sRNA (internal control) in the same sample. The value of ∆∆Ct is calculated from ∆Ct (the sample group tested) - ∆Ct (initial expression). Representations of different relative multiples of the initial expression can be calculated with $2^{-\Delta \Delta Ct}$.

Table 1. Primer sequence used for expression gen related immunity of spiny P. homarus by RT-qPCR

| Immune System                  | Target gen     | Name  | Primer    | Sequence (5”-3”)                            | Gen Bank   |
|-------------------------------|----------------|-------|-----------|---------------------------------------------|------------|
| proPO activating system       | Prophenoloxidase| proPO| proPO-F/R | F : GAGATCGCAAGGGAGAAGCTG                  | EF 565469  |
| Clotting System               | Transglutaminase| Tgase| Tgase-F/R | R : CGTCAGTGAAGTGAGACCA                     | EE 572305  |
| Antimicrobial peptide system  | Clotting protein| CP   | CP-F/R    | F : CTCAGGATCTCCCTCAAC                      | DQ 984182  |
| Anti-Lipopolysaccharide       | ALFHa-1        | ALFHa-1F/R | F : TGGAAAAACCTTCTTACG                      | EE 525516  |
| Antimicrobial peptide system  | ALFHa-4        | ALFHa-4F/R | F : GAGATCGCAAGGGAGAAGCTG                  | DV 772634  |
| Serum Amyloid Protein         | SAA            | SAA  | SAA F/R   | R : CAGTCGTCCTGTTGTTGGAA                    | EH 116055  |
| Internal control              | 18s RNA        | 18s   | 18s-F/R   | F : AGCAGGCTGTTTGTGTCTT                    | AF 186250  |

2.5. Data analysis

Data collected includes growth, survival, lobster health expressed by genes encoding immunity, data presented statistically (Completely Randomized Design).

3. Results and Discussion

3.1. Growth of spiny lobster P. homarus feed the moist diets

The results obtained on growth of spiny lobster P. homarus for 3 months cultured are presented in Figure 1. The treatment of probiotics supplemented in moist diets (A) and (B) probiotics and prebiotics-MOS showed no different growth of body weight 156.97 g and 153.75 g respectively, while on the control (C) was obtained body weight of 141.47 g. This is resulted significant difference among two treatments ($P<0.05$). Apparently, the probiotics supplementation has a positive effect on spiny lobster growth. Probiotics supplemented on moist diets are four strains of bacteria isolated from spiny lobster intestines that exploited from wild. It is possible that the role of probiotics is appropriate for the digestive system of spiny lobsters. Observation of the total length (18.2, 18.56 and 18.49 cm) and carapace length (8.46, 8.63 and 8.48 cm) during three-month culture did not significantly different ($P>0.05$).

![Figure 1](image-url)
When we observed from the percentage of body weight and body length gain, it is revealed difference among treatments supplemented with (A) probiotics and (B) probiotics and prebiotics-MOS have a higher rate, especially in the second and third months of culture respectively, were reached of 65.2 - 73.95% and 101.81 - 102.97% from their initial weight (Table 2).

Table 2. Percentage of growth, total length and carapace length gains of spiny lobster *P. homarus* fed on moist diets with different treatments for 3 month of culture.

| Treatments             | Rearing for (Month) | Body weight gain (%) | Body length gain (%) | Carapace Length gain (%) |
|------------------------|---------------------|----------------------|----------------------|--------------------------|
| Probiotics             | I                   | 43.28                | 10.23                | 22.33                    |
|                        | II                  | 65.2                 | 21.44                | 28.14                    |
|                        | III                 | 101.81<sup>a</sup>   | 32.67<sup>a</sup>    | 35.69<sup>a</sup>        |
|                        | I                   | 39.26                | 18.43                | 21.31                    |
| Probiotics and Prebiotic-MOS | II     | 73.95<sup>a</sup>    | 25.83                | 28.15                    |
|                        | III                 | 102.97<sup>a</sup>   | 33.79<sup>a</sup>    | 31.88<sup>a</sup>        |
|                        | I                   | 33.76                | 14.04                | 17.34                    |
| Control                | II                  | 67.93                | 22.49                | 24.15                    |
|                        | III                 | 87.6<sup>b</sup>     | 30.37<sup>a</sup>    | 30.96<sup>a</sup>        |

Means with different superscripts within a column are significantly different (P<0.05)
3.2. Survival rate of spiny lobster *P. homarus*

The survival rate of lobsters for 3 months cultured with moist diets supplemented with (A) probiotic, (B) probiotic and prebiotic-MOS and (C) Control (without probiotic or prebiotic) was resulted no different values (Figure 4), i.e. at 92.7%; 93.33% and 93.33% respectively. It is able to understand that during culture in the tank was carried out in a controlled manner and the chance of infection with disease is almost non-existent, therefore, mortality is only caused by cannibalism when lobster moultting.

![Figure 4](https://via.placeholder.com/150)

**Figure 4.** Survival rate of spiny lobster fed on moist diets with different treatments for 3 month of culture.

3.3. Gene expression related to immunity

The results of immunity response of spiny lobster with probiotics supplementation after the challenged with MHD expressed from the target gene ALF-1, ALF-4, SAA, ProPO, Tgase, and CP are shown in Figures 5. The results of analysis of gene expression related to immunity in spiny lobster showed that there was an increased ability to fight Milky Hemolymph Disease (MHD).

Result from the challenged test with MHD, it appears that the administration of probiotics and prebiotics-MOS shows a better immune response to 6 target genes on 24-96 hours after exposure to MHD. The immune response vary 2.1-25.75 times. Meanwhile, in spiny lobster with fed of moist diets supplemented of probiotics, the immune response showed better on 24 to 96 hours after exposure to MHD (Figure 5 -10). The ALF-1, ALF-4, SAA, ProPO, Tgase and CP gene targets can express immune multiples of 2.65 - 42.7 times (24 - 48 hours), but after 72 and 96 hours the expression of immunity is seen to decrease.

This proven that the effects of probiotics and prebiotics-MOS have shown to improve the immune system in spiny lobsters, increase intestinal flora and lower disease incidence and increase food assimilation [18,19,20,26].

![Figure 5](https://via.placeholder.com/150)

**Figure 5.** Quantitative relative mRNA anti-lipopolysacharide factor ALF-1 of spiny lobster

![Figure 6](https://via.placeholder.com/150)

**Figure 6.** Quantitative relative mRNA anti-lipopolysacharide factor ALF-4 of spiny lobster
4. Conclusion
The growth response of spiny lobster *P. homarus* were significantly different between probiotic supplementation on moist diets and probiotics and prebiotics-MOS compared control, while the survival rate of spiny lobster in all treatments was not different. Immunity response of spiny lobster also could increases, which was expressed by target genes related to immunity after challenged with MHD.

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