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Synbiotic microcapsule dietary supplementation for prevention against co-infection diseases in Pacific white shrimp: a limited field experiment

Suplementasi mikrokapsul dalam pakan untuk pencegahan penyakit koinfeksi pada udang vaname: uji lapang terbatas

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ABSTRACT

This study aimed to evaluate the effectivity of microencapsulated synbiotic (MS), *Bacillus* sp. NP5 and mannan oligosaccharide (MOS) dietary to enhance the immunity of Pacific white shrimp for the prevention against co-infection with WSSV (White Spot Syndrome Virus) and *Vibrio harveyi.* The MS was administered as a feed supplementation in different feeding frequencies. The synbiotic was microencapsulated by the spray dryer method. Shrimps were reared in the floating net cages in the pond. Treatments included the administration of MS at different frequencies i.e, daily (A), twice a week (B), once a week (C), and without MS supplementation (consisting of negative and positive controls) with a feeding rate of 6% of shrimp biomass (shrimps were fed 5 times a day). During the challenge trial, shrimps were removed and further reared in plastic tanks, for 7 days. The shrimps (except for negative control treatment) were intramuscularly injected by WSSV filtrate at the infective dosage of 10-4 copies mL-1. Twenty four hours after WSSV injection the shrimps were immersed in the water-containing cells suspension of *V. harveyi* at the cell's population dosage of 106 CFU mL-1. Immune responses were observed for 7 days after experimental infection. The shrimps that have been treated with daily MS supplementation (A) showed the best immune responses i.e., total haemocyte counts, phenoloxidase, respiratory burst, and the lowest pathogenic cells abundance in the intestine compared to other treatment groups.

Keywords: microcapsule, synbiotic, *Litopenaeus vanamei*, WSSV, *Vibrio harveyi*, co-infection

ABSTRAK

Tujuan dari penelitian ini adalah untuk mengevaluasi efektivitas mikrokapsul sinbiotik (MS), *Bacillus* sp. NP5 dan mannanoligosakarida (MOS) melalui suplementasi pakan dalam frekuensi yang berbeda di percobaan lapangan dalam budidaya udang vaname. MS diberikan sebagai suplementasi pakan dalam rangka meningkatkan kekebalan untuk pencegahan koinfeksi dengan WSSV (White Spot Syndrome Virus) dan *Vibrio harveyi*. Sinbiotik dilakukan mikroenkapsulasi dengan metode spray dryer. Udang dipelihara dalam keramba jaring apung di tambak. Perlakuan meliputi pemberian MS dengan frekuensi yang berbeda yaitu setiap hari (A), dua kali seminggu (B), seminggu sekali (C), dan tanpa suplementasi MS (terdiri dari kontrol negatif dan positif) dengan feeding rate 6% dari biomassa udang (pakan diberikan 5 kali sehari). Selama percobaan uji tantang, udang diangkat dan dipelihara lebih lanjut dalam tangki plastik, selama 7 hari. Udang (kecuali perlakuan kontrol negatif) diinjeksi secara intramuskular dengan filtrat WSSV dengan dosis infektif 10-4 kopi mL-1. Dua puluh empat jam setelah injeksi WSSV, udang direndam di dalam air media yang mengandung suspensi sel bakteri *V. harveyi* dengan dosis populasi sel 106 CFU mL-1. Respons imun diamati selama 7 hari setelah infeksi eksperimental. Udang yang diberi suplementasi MS setiap hari (A) menunjukkan respons imun yang paling baik yaitu jumlah total hemosit, phenol oxidase, respiratory burst dan komposisi bakteri usus yang terbaik dibandingkan dengan kelompok perlakuan lainnya. Selain itu, pemberian suplemen mikrokapsul sinbiotik setiap hari dapat menekan jumlah populasi patogen paling rendah dibandingkan perlakuan yang lain.

Kata kunci: mikrokapsul, sinbiotik, *Litopenaeus vanamei,* WSSV, *Vibrio harveyi*, koinfeksi

INTRODUCTION

Pacific white shrimp *Litopenaeus vannamei* is one of the major commodities of the brackish water culture in Indonesia. The country export value of the white shrimp is promising, globally in position as the 3rd largest exporter after India and Ecuador (FAO, 2020). The intensive Pacific white shrimp farming is objected to increase the mass scale production that can meet the global market demand. However, rapid growth of the intensive shrimp culture is often threatened by various disease outbreaks that can occur simultaneously. Co-infectious diseases in shrimp can be caused by common saltwater bacteria, such as *Vibrio harveyi* infection (Vibriosis) and at the same period often coinfected by viruses, where the white spot syndrome virus/WSSV belongs to the major viral disease (Kawato *et al*., 2019). Mortality rate of the shrimp infected by Vibriosis occurs in all stadia, whereas the WSSV infection resulted 70–90% mortality within three to seven days observation (Verbruggen *et al*., 2016).

Infectious diseases have led to the unfriendly situation where various chemicals or many antibiotics are used. Therefore ecofriendly treatment such as using of synbiotic for improving the immunity is more preferred way to increase the shrimp growth and survival rate. Synbiotic is a mixture of beneficial microbe of probiotics and prebiotics that improve the nonspecific immunity response of the host to infection. Previous studies have showed that probiotic, prebiotic, synbiotic applications have succeeded in improving the shrimp's survival rate, growth, immune response, and resistance against infectious diseases (Nimrat *et al*., 2012; Zokaeifar *et al.,* 2012; Hamsah *et al*., 2017b; Hamsah *et al.*, 2019).

In this study, we applied the probiotic *Bacillus* sp. NP5 which originally was isolated from the Tilapia intestine (Putra & Widanarni, 2015), which showed a broad range tolerance to salinity and was proven to be able to inhibit the growth of pathogenic bacteria such as *Vibrio harveyi* (Munaeni *et al.*, 2012; Zubaidah *et al* 2014). A number of studies have demonstrated that prebiotics can improve growth, survival rate, feed digestibility, feed efficiency, the composition of microflora in the intestines, inhibit the growth of pathogens and improve the shrimp's immune system (Zhang *et al.,* 2012; Aktas *et al.,* 2014). The prebiotic used in this study was mannan-oligosaccharides (MOS) which has been demonstrated able to improve the growth and the survival rate of Pacific white shrimp (Hamsah *et al.*, 2017a.). The study demonstrated that the synbiotic mixture resulted more benefits in shrimp performance compared to those of single probiotic and prebiotic applications (Hamsah *et al*., 2017b, Hamsah *et al.*, 2019).

Microencapsulation is important to protect the probiotic cells from extreme environmental conditions with the coating materials (Morales & Ruiz, 2016). This technology has brought a various range of applications (Morales & Ruiz, 2016). Synbiotic microencapsulation using prebiotic mannan oligosaccharides (MOS) most of the MOS products, especially those that have been scientifically developed, are derived from the cell wall of the yeast, *Saccharomyces cerevisiae*. Microencapsulated of probiotics have been applied in shrimp larviculture (Nimrat *et al.,* 2012). Their studies showed balancing the composition in microbial intestinal tracts, improvement in water quality as well as enhancement of shrimp survival and growth. However, this microencapsulated synbiotic formula needs to be studied for its ability against pathogenic bacterial-viral-coinfection in penaeid shrimp. Therefore, the objectives of this study were to evaluate the effects of mixed microencapsulated *Bacillus* sp., NP5 probiotics and MOS on *L. vannamei* health status and disease resistance against WSSV and *Vibrio harveyi* in a field experiment.

MATERIALS AND METHODS

Bacillus sp. NP5. probiotic preparation

The probiotic bacteria used were *Bacillus* sp. NP5 (Putra & Widanarni, 2015) and was prepared resistant to the antibiotic rifampin. NP5 Rf^R bacterial cells were propagated using SWC agar media (sea water complete: bacto peptone 0.5%, yeast extract 0.1%, glycerol 0.3%, bacto agar 2%, seawater 75% and aquadest 25%) and incubated for 24 hours. The method used in producing this probiotic was the up-scaling method. A single, separate colony was transferred to 25 ml of SWC broth 25 mL cultured and incubated at 25°C-27°C for 20 hours and transferred to 250 mL of SWC broth and incubated with a water bath shaker at a temperature of 25-27°C for 20 h at a speed of 140 rpm. After 20 h of incubation, the culture was centrifuged at 6.000 rpm for 15 minutes to separate the cells pellet and supernatant. The *Bacillus* NP5 cells pellet were then rinsed with 250 mL phosphate buffered saline (PBS: 0.8%

NaCl, 0.15% Na_2HPO_4, 0.02% K_2HPO_4, 0.02% KCl, and 1 L of distilled water) then homogenized and recentrifuged at 6.000 rpm for 15 minutes. The resulting *Bacillus* sp. NP5 Rf^R cells pellet was then added to PBS solution at a ratio of 1:1 to SWC medium. This bacterial cells suspension was then used as a probiotic in the synbiotic microencapsulation process.

Microencapsulation process

Synbiotic composition used in this study is a combination of probiotic *Bacillus* sp. NP5 Rf^R (10^8 CFU mL^{-1}) and 0.4% (w/w) MOS prebiotic (Zhang *et al.,* 2012 with a slight modification). The coating materials used to coat the synbiotic suspension are maltodextrin and whey protein from cow's milk. Whey protein, maltodextrin and synbiotic suspension were mixed in a ratio of 1:0.1:1 (v/w/v) (Munaeni *et al.,* 2014; Zubaidah *et al.,* 2015; Febrianti *et al.,* 2016). Synbiotic were homogenized with coating materials using a mini BUCHI spray dryer with an inlet temperature of 100-110°C and an outlet temperature of 50-60°C at the Pilot Plant Laboratory SEAFAST (Southeast Asian Food and Agriculture Science and Technology) Center, IPB University.

Preparation of shrimps

Specific-Pathogen Free (SPF) vanamei shrimp against white spot syndrome virus (WSSV) were used based on polymerase chain reaction (PCR). Shrimp were obtained from Brackish Water Cultivation Fisheries Center, Situbondo with an average initial weight of 6.32 g/shrimp and a stocking density of 15 shrimps 25 L.

Rearing and feeding of the shrimps

As many of fifteen floating net cage measuring 2.5 m × 1 m × 1m with a mesh size of 0.5 mm were used. During the rearing process, the shrimp were fed commercial feed at-satiation with the frequency of feeding five times a day (at 07:00, 10:00, 13:00, 17:00, and 21:00.) The shrimps were fasted for 24 hours before being used to emptied leftover food from the intestine.

Research design

Synbiotic microcapsule supplementation was mixed manually with commercial shrimp feed (32% protein) with different dose (w/w) with 2% egg white (v/w) as a binder. The shrimps were divided into five treatment groups: negative control (k-), positive control (k+) without synbiotic supplementation and synbiotic supplementation with a frequency of daily (A), once a week (B) and two times a week (C). The preparation of the MS feed was done day before the time of feeding. During the test, the shrimp were fed the test feed every day with a feeding rate of 6% of the weight of the biomass.

Coinfection experiment: challenge test with WSSV and *Vibrio harveyi* (Febrianti *et al.,* 2016)

On day 32 shrimp were infected with 100 uL (10^{-4}) (SID50) WSSV by injection on the abdominal shrimps between the third and fourth segments. The negative control shrimps (k-) were injected with PBS with the same volume. Twenty four hours after the virus injection, the shrimps were infected by immersion with 25 mL of *Vibrio harveyi* MR5339 Rf^R (10^6) (SID50).

Sampling time and parameters observed (Febrianti *et al.,* 2016)

Research parameters such as Total Haemocyte Count (THC), Respiratory Burst (RB), and Phenoloxidase (PO) activity and microbiological analyses of gut bacterial were carried out before MS supplementation (day 0), after synbiotic supplementation (day-30) as well on days 34 and 37. The parameters for bacterial composition analyses included the abundance of bacterial cells in the intestine including Total Bacterial Count (TBC), *Bacillus* sp. NP5 Rf^R count, Presumptive Vibrio Count (PVC), *Vibrio harveyi* MR5339 Rf^R.

Total haemocyte count (THC) (Febrianti *et al.,* 2016)

Total haemocyte count (THC) of white shrimp was carried out by taking 0.2 mL of shrimp blood or hemolymph from the base of the first swimming leg using a 1 mL syringe that already contained 0.2 mL of anticoagulant. The hemolymph-anticoagulant composition is then put into a hemocytometer to calculate the total number of haemocytes. Observations were made at 100 times magnification using a light microscope. Calculation of the total number of haemocytes using the following formula:

Phenoloxydase (PO) activity (Febrianti *et al.,* 2016)

Hemolymph and 1 mL anticoagulant were centrifuged for 10 minutes at 3000 rpm and 4°C. The centrifuged pellet was collected and mix with 1 mL of cacodylate-citrate buffer solution (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7) then centrifuged

again at the same speed, temperature, and time. The pellet was separated and dissolved with 200 µL of cacodylate buffer. A total of 100 µL of cell suspension was added to 50 µL of trypsin (1 mg mL^{-1} in cacodylate buffer) and incubated at 25°C for 10 minutes. Next, 50 µL of L-DOPA was added (3 mg mL^{-1} in cacodylate buffer) and allowed to stand for 5 minutes. A total of 800 µL cacodylate buffer was added to the cell suspension and then inserted into microplate wells. Optical density (OD) reading was measured using a spectrophotometer with a wavelength of 492 nm.

Respiratory burst (RB) activity (Febrianti *et al.,* 2016)

A total of 50 L of the hemolymph-anticoagulant mixture was incubated for 30 minutes at room temperature. Then it was centrifuged at 700x g for 20 minutes and the supernatant was discarded, then 100 µL of NBT was added to HBSS (Hank's buffered salt solution with a concentration of 0.3% and allowed to stand for 2 hours at room temperature. Then th solution was centrifuged at 700x g for 10 minutes, the supernatant was removed and added 100 µL absolute methanol for further centrifugation 700x g for 10 minutes (supernatant removed). The resulting pellet was rinsed 2 times with 70% methanol followed by the addition with 120 µL KOH (2M) and 1409 µL DMSO (dimethyl sulfoxide) were added. This homogeneous mixture was inserted into a microplate and the optical density (OD) was measured using a microplate reader with a wavelength of 630 nm.

Bacterial abundance in the gastrointestinal tract (Febrianti *et al.,* 2016)

As many of 0.1 g shrimp intestine from each treatment was taken and homogenized in 0.9 mL sterile PBS. The bacterial cell suspension was then diluted by serial dilution (1:10) and 50 µL spread to each medium accordingly. Total bacterial count using SWC media without rifampin, *Bacillus* sp. NP5 Rf^R using SWC medium with rifampin. *Vibrio harveyi* MR5339 Rf^R using selective media of TCBS with rifampin TCBS without rifampin for presumptive vibrio count assay. The media were incubated for 24 hours at 37°C.

Data analysis

Data were analyzed using Microsoft Excel 2013 and analyzed by variance (ANOVA) using SPSS version 17 software. If the results obtained were significantly different ($p<0.05$), further tests were carried out using Duncan's test with a 95% confidence interval.

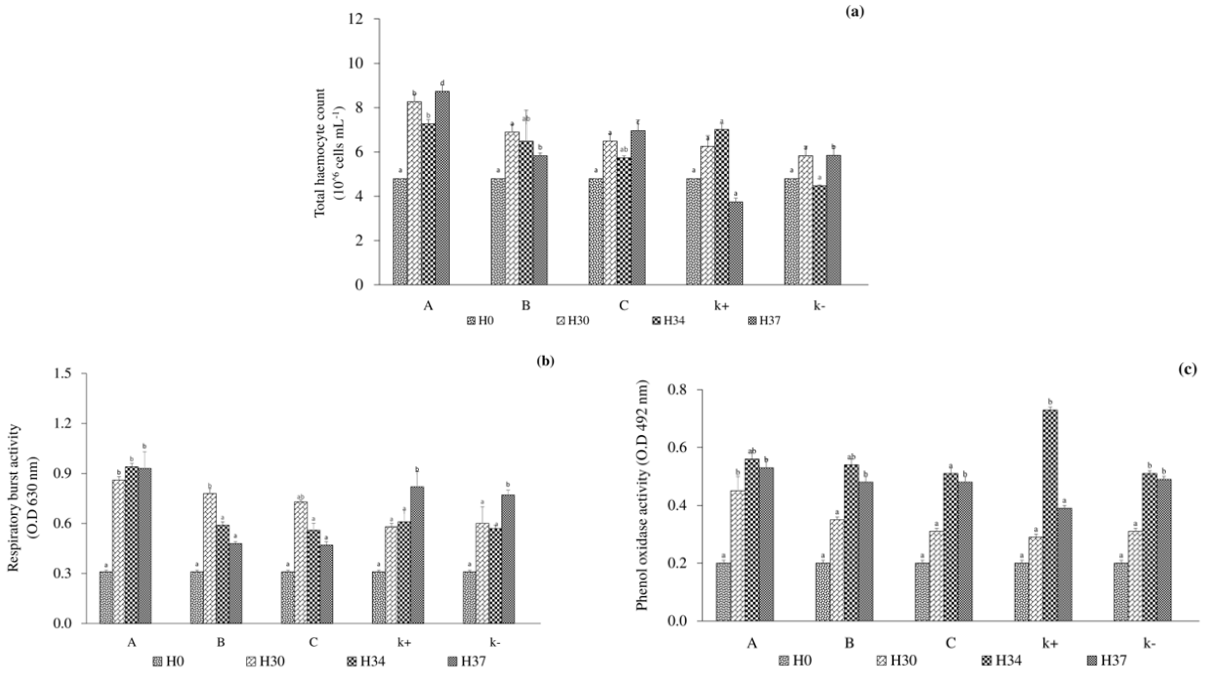

Figure 1. Immune parameters of the shrimp treated with microcapsule synbiotic dietary supplementation at different frequencies and controls in the field experiment. Total Haemocyte Count Activity (THC) (a), Respiratory Burst Activity (b), Phenoloxidase Activity (c).

Notes: A (MS supplementation every day), B (MS supplementation twice a week), C (MS supplementation once a week), k+ (positive control), k- (negative control). Different superscript letters showed significant significant difference ($p<0.05$). The values listed are the mean and standard deviation values.

RESULTS AND DISCUSSION

Results

Observation of immune response parameters after 30 days of application of synbiotic is depicted in Figure 1. Immune parameters showed a significant increase, in THC, RB, and PO in treatment A (daily MS supplementation) 8.27 ± 0.33 x 10^5cells mL^{-1}, 0.86 ± 0.02, and 0.45 ± 0.05 ($p<0.05$) respectively. Observation on day 34, RB and THC still increasing 0.94 ± 0.02 and 7.27 ± 0.20 and PO value of treatment k+ is 0.73 ± 0.11 ($p<0.05$). On day 37 the variation of RB and PO values between treatment A and k+ increased. THC value of all synbiotic treatments were not significantly different, only in the control treatment increased, and the RB value decreased compared to treatment k-. The PO value at the end of the observation decreased in all treatment group. RB value of treatment A until the end of the observation was not significantly different ($p<0.05$) with control.

PO activity in white shrimp is part of the immune response that can be observed (Oktaviana *et al.,* 2014; Arisa *et al.,* 2015; Huynh *et al.,* 2018; Nurhayati *et al.,* 2015; Zubaidah *et al.,* 2015; Roomiani *et al.,* 2018; Hamsah *et al.,* 2019). The highest increase in the PO value is in treatment A was 0.45 ± 0.05 ($p<0.05$) compared to all microcapsules and control treatments. The treatment of H37 synbiotic supplementation was not significantly different from that of k-. After the H37 challenge test, the PO value in the k+ treatment was 0.39 ± 0.01 and the lowest significant was significantly different ($p<0.05$) with all synbiotic and k- treatments.

The microbiological analysis of bacterial abundance in the gastrointestinal tract of the shrimp during the field experiment is shown in Figure 2. Supplementation of MS in feed has increased the toal bacterial count (Figure 2a) and probiotic population in the intestines of all MS treated groups (Figure 2b). The best immune response in treatment A was the effect

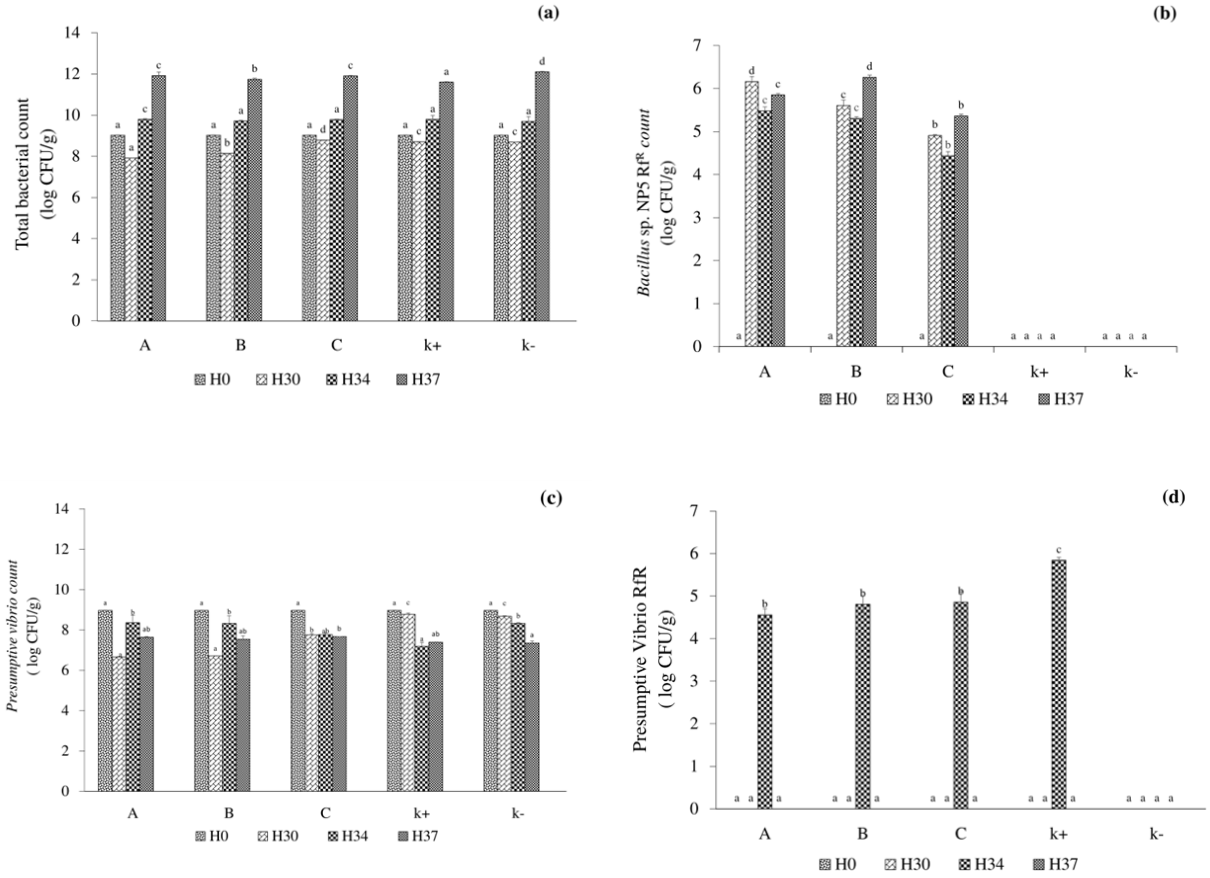

Figure 2. Intestinal bacterial analysis of the shrimp treated with synbiotic microcapsule dietary supplementation at different frequencies and controls in the field experiment. Total Bacterial Count (a) (TBC), *Bacillus* sp. NP5RfR (b), Presumptive *Vibrio* Count (c), Presumptive *Vibrio* Rf^R (d).

Notes: A (MS supplementation every day), B (MS supplementation twice a week), C (MS supplementation once a week), k+ (positive control), k- (negative control). Different superscript letters showed significant significant difference ($p<0.05$). The values listed are the mean and standard deviation values.

of increasing probiotic *Bacillus sp*. NP5 Rf^R. The application of treatment A obviously suppressed the abundance the *Vibrio* sp. i.e the presumptive Vibrio Count (PVC) (Figure 2c) and Presumptive Vibrio Count Rifampin PVC Rf^R (Figure 2d) in the intestine on day H30 compared to control, the increase in bacterial cell abundance in the intestine increased until the end of the observation on all synbiotic treatments.

Discussion

Microcapsule synbiotic supplementation influenced blood profiles of shrimps such as THC (Figure 1a), RB (Figure 1b), and PO (Figure 1c). Haemocyte cells react to immune responses, phagocytosis, encapsulation, nodulation, cytotoxin intermediary, Antimicrobial Peptides (AMPs) synthesis and proteolytic activation in the process of melanization, coagulation, resulting in protein stress responses, and opsonization (Liu *et al.,* 2020). The increase in THC in H30 treatment A, B, C, and control, the highest THC value in treatment A ($p<0.05$) compared to control, after the challenge test (H34) the THC value in treatment A, B, C decreased, compared to k+ treatment. Variations in THC values are a response to the presence of infection (Liu *et al.,* 2020).

Haemocytes in shrimp have an important role as the center of the immune response, in which there are mechanisms of recognition, phagocytosis, melanization, cytotoxicity and cell communication (Liu *et al.,* 2020). RB activity is used as a parameter and evaluation of the immune response direct reaction against pathogens (viruses and bacteria) that enter the host's body (Ji *et al.,* 2011; and Zubaidah *et al.,* 2015, Liu *et al.,* 2020). Observations on (H30) RB activity of treatment A, B, C was higher than both control treatments (k+ and k-). The RB mechanism is a reaction formed from the reactive oxygen intermediates (ROIs) process which functions as a strong microbicidal (Herb & Schramm, 2021). Oxygen is reduced and catalyzed by the enzyme bonding layer, NAD(P)H oxidase and turns into superoxide (O_2^-), then the number changes in the reaction producing hydrogen peroxide (H_2O_2), single oxygen ($1O_2$), hydroxyl radicals (OH), and other products and other reactive (Muñoz *et al.,* 2000). In addition to RB, PO activity was also observed, PO is an important part of the host's defense system against pathogen invasion in the process of melanization and cytotoxin production, the prophenol activation process involves many enzymes and proteinase complex molecules that can be triggered by LPS material, Beta-1,3-glucans, peptidoglycan of microorganisms (Liu *et al.,* 2020).

Bacillus sp. is widely known belonging to the genus of various antibactericidal producer. The existence of intestinal *Bacillus* sp. a natural antimicrobial producer lead the other bioprocesses and microenvironmental functions such as the immune enhancing agent, increase the pathogenic resistance in shrimp (Laksmi *et al.,* 2013). The presence of *Bacillus* sp. in combination with prebiotics produces synergies collaboration against pathogenic infection which increases the resistance (Mohan *et al.,* 2019; Pan *et al.,* 2018). Febrianti *et al.* (2016) reported that the appropriate use of probiotic bacteria can enhance the immune response in the gut, as a stimulus for innate, humoral, and cellular immune responses.

The increase in probiotics given to the treatment can increase number of bacteria and suppress the abundance of vibrio compared to k+, similar results to the abundance of *Vibrio harveyi* Rf^R bacteria. According to Sha *et al.,* (2016) that probiotics given to shrimp will change the intestinal bacterial community which is an important factor in improving health, immune response, and growth performance which is in accordance with research reported by (Hasyimi *et al.,* 2020) in a study of sequencing the content of gut microbiota by application synbiotic in vaname resulted in dominance in the phylum proteobacteria. Proteobacteria are the most dominant microbiota in the vannamei shrimp gut (Zhang *et al.,* 2012).

The decrease in probiotic number after (day 30) was due to discontinuation of synbiotic supplementation in the feed and the presence of infection with WSSV and *V. harveyi* MR5339 Rf^R, according to Wang *et al.,* (2018) WSSV infection alters the homeostasis and abundance of gut microbiota, predominance of Proteobacteria and Fusobacteria. The application of synbiotic microcapsules with a daily frequency of A, was the best result which resulted in a high SR compared to the control, and an efficient FCR and high LPS, resistance to co-infection of WSSV and *Vibrio harveyi* Rf^R for seven days, proving that the immune response of THC, PO, RB in treatment A were significantly different from the control, as well as increased TB, and decreased PVC and PVC in the gastrointestinal tract compared to control.

CONCLUSION

Synbiotic microcapsule supplementation (a combination of *Bacillus* NP5 Rf^R and mannan oligosaccharides) of 1% in feed for 30 days was able to increase the immune response parameters (total haemocyte count, respiratory burst activity, and phenol oxidase activity), vaname shrimp after challenge test with WSSV and *Vibrio harveyi* coinfection The enhancement of the immunity status in shrimps was obviously related to the microbial composition of the bacterial composition in the intestine.

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