Jurnal Akuakultur Indonesia 18 (2), 130–140 (2019)

Original article

DOI: 10.19027/jai.18.2.130-140

Microencapsulation of probiotics and its applications with prebiotic in Pacific white shrimp larvae through *Artemia* sp.

Mikroenkapsulasi probiotik dan aplikasinya dengan prebiotik pada larva udang vaname melalui *Artemia* sp.

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(Received January 31, 2019; Accepted July 4, 2019)

ABSTRACT

This study aimed to produce microencapsulated probiotic *Pseudoalteromonas piscicida* (1Ub) and evaluate it with prebiotic mannan-oligosaccharide (MOS) through the enrichment of Artemia sp., on bacterial population, growth performances, immune responses, and disease resistance of Pacific white shrimp larvae. Microencapsulation of probiotic was done by the freeze-drying method. The shrimp larvae were reared for 13 days and fed by the *Artemia* sp. enriched with microcapsule of probiotic 1Ub (10 g/L), prebiotic MOS (12 mg/L), synbiotic, and control without administration of microencapsulated probiotic and prebiotic, including negative (C-) and positive (C+) control. On the day 14, all of the experimental shrimp larvae except C- were challenged through immersion method with *Vibrio harveyi* MR5339 (107 CFU/mL). This study showed that the administration of microcapsule of probiotic 1Ub, prebiotic MOS, and synbiotic through the enrichment of *Artemia* sp. could increase the bacteria population, growth performances, immune responses, and disease resistance of Pacific white shrimp larvae. Moreover, synbiotic treatment demonstrated the best result compared to other treatments.

Keywords: probiotic, prebiotic, synbiotic, Pacific white shrimp, microencapsulation

ABSTRAK

Penelitian ini bertujuan untuk membuat mikrokapsul probiotik *Pseudoalteromonas piscicida* (1Ub) dan mengevaluasinya dengan prebiotik *mannan-oligosaccharides* (MOS) melalui pengayaan *Artemia* sp. terhadap populasi bakteri, performa pertumbuhan, respons imun dan resistensi penyakit pada larva udang vaname. Mikroenkapsulasi probiotik dilakukan dengan metode *freeze-drying*. Larva udang dipelihara selama 13 hari dan diberi pakan *Artemia* sp. yang telah diperkaya dengan mikrokapsul probiotik 1Ub (10 g/L), prebiotik MOS (12 mg/L), sinbiotik, dan kontrol tanpa penambahan mikrokapsul probiotik dan prebiotik, termasuk kontrol negatif (C-) dan positif (C+). Pada hari ke-14, seluruh larva udang percobaan kecuali C- diuji tantang melalui metode perendaman dengan *Vibrio harveyi* MR5339 (107 CFU/mL). Hasil penelitian menunjukkan bahwa pemberian mikrokapsul probiotik 1Ub, prebiotik MOS, dan sinbiotik melalui pengayaan *Artemia* sp. dapat meningkatkan populasi bakteri, performa pertumbuhan, respons imun, dan resistensi penyakit pada larva udang vaname. Selain itu, perlakuan sinbiotik menunjukkan hasil terbaik dibandingkan perlakukan lainnya.

Kata kunci : probiotik, prebiotik, sinbiotik, udang vaname, mikroenkapsulasi

INTRODUCTION

Pacific white shrimp *Litopenaeus vannamei* is one of the important aquaculture commodities that are widely cultured both in Indonesia and around the world. Indonesia is the fourth largest shrimp producer after China, India, and Vietnam, which accounts for about 4.6% of the world shrimp production (FAO, 2014). Production of Pacific white shrimp needs a high-quality larva in adequate quantity and time. However, bacterial disease induced by *Vibrio harveyi* has remained a great challenge in the production of Pacific white shrimp larvae in Indonesia and generally called as vibriosis. Recent study (Huang *et al.,* 2016) reported that *Vibrio* bacteria were predominant in the digestive tract of Pacific white shrimp during the postlarvae (80%) and juvenile stages (89.1-94.2%). Vibriosis can cause serious mortality in Pacific white shrimp (Raja *et al.,* 2017) and the mortality rate can reach 100% (Karunasagar *et al.,* 1994).

The utilization of antibiotics has been proposed to control the disease outbreak. However, the technique was reported to promote serious problems associated with antibiotic-resistant bacteria, residual existence, and food safety issues (Zhang *et al.,* 2014). Stalin *et al.,* (2016) reported that *Vibrio harveyi* has resistant to various antibiotics such as ampicillin, cefaclor, ciprofloxacin, penicilin, rifampicin, chloramphnicol, and vancomycin. The administration of probiotic, prebiotic, and synbiotic could be a preventive alternative approach that was more eco-friendly and beneficial in shrimp culture.

Probiotic is a living microorganism that has beneficial effects on the host and enhances microbial balance in the digestive tract, feed efficiency, and environmental condition (Nayak, 2010). Several studies reported that probiotics can improve the survival rate and immune responses in Pacific white shrimp (Zokaeifar *et al.,* 2014; Liu *et al.,* 2010), digestive enzyme activity, nutrient digestibility and growth performances in Tilapia (Putra *et al.,*2015; Utami *et al.,* 2015). Meanwhile, prebiotic is a non-digestible food component and promotes advantageous effects to the host through inducing intestinal bacterial growth and activity, which may improve the host health (Cerezuela *et al*., 2011). Prebiotics known for aquaculture includes arabinoxylan-oligosaccharide (AXOS), fructo-oligosaccharide (FOS), galactooligosaccharides (GOS), mannan-oligosaccharide (MOS), xylooligosaccharides (XOS), inulin, and β-glucan (Akhter *et al.,* 2015; Hoseinifar *et al.,* 2019). Some researchers reported that prebiotic MOS can improve health and fish production (Torrecillas *et al.,* 2014), growth, survival rate, intestinal flora and gut surface area of lobster *Panulirus homarus* (Huu *et al.,* 2014), as well as protection in the Pacific white shrimp upon pathogen exposure (Rungrassamee *et al.,* 2014). Other studies reported that a combination of probiotic and prebiotic, recognized as synbiotic, could exhibit synergistic action (Huynh *et al.,* 2017). Merrifield *et al.,* (2010) suggested that synbiotic may produce great beneficial effects rather than the application of individual prebiotic or probiotic. Several studies showed that synbiotics can improve health and growth of *Sebastes schlegelii* (Rahimnejad *et al.,* 2017), digestibility, feed absorption, specific growth rate, and digestive enzyme activities in common carp (Dehaghani *et al.,* 2015), growth performances and immune responses in Pacific white shrimp (Oktaviana *et al.,* 2014; Zubaidah *et al.,* 2015).

The present study reported that probiotic *Pseudoalteromonas piscicida* 1Ub (fresh culture) improved the growth performances of Pacific shrimp larvae (Hamsah *et al.,* 2017a). However, its fresh culture still showed some disadvantages such as limited storability and difficult application. Microencapsulation is one of the alternative techniques to protect probiotic against extreme conditions. In this approach, bacterial cells were surrounded by an encapsulated membrane which reduced degradation and loss of the cells, thus the bacteria would survive and could be released at appropriate sites in the digestive tract of the host (Martin *et al.,* 2015). The present study aimed to investigate the effects of microencapsulated probiotic *P. piscicida* 1Ub and prebiotic mannan-oligosaccharide (MOS) through *Artemia* sp. enrichment on bacterial growth, growth performance, immune responses, and resistance of Pacific white shrimp larvae.

MATERIALS AND METHODS

Preparation of probiotic, prebiotic, synbiotic, and *V. harveyi*

Probiotic *P. piscicida* 1Ub was isolated from Pacific white shrimp nauplii (Widanarni *et al*., 2009) and was marked with antibiotic rifampicin at a dose of 50 µg/mL (1Ub Rf^R). Probiotic *P. piscicida* 1Ub was cultured in 50 mL of seawater complete broth (SWC, 0.5 g bactopeptone, 0.1 g

yeast extract, 0.3 mL glycerol, 75 mL seawater, 25 mL distilled water), incubated in the thermoshaker at 140 rpm for 18 h at 25°C, and followed by upscaling (1:10). Bio-MOS (Alltech Inc., KY USA) was used as prebiotic which contained mannan-oligosaccharide (MOS) derived from the cell walls of *Saccharomyces cerevisiae* with a composition of 30% crude protein, 1.4% crude fat and 13% crude fiber). Probiotic and prebiotic were combined to produce synbiotic. Antibiotic-resistant *V. harveyi* MR5339 (*V. harveyi* MR5339 Rf^R) was used in the challenge test. *V. harveyi* was cultured in TCBS (thiosulphate citrate bile-salt sucrose) media (HiMedia Laboratories) for 24 h, and then cultured in SWC broth and incubated in the thermoshaker at 140 rpm for 10 h at 25°C.

Probiotic microencapsulation

The microencapsulation process included the preparation of probiotic bacteria and coating materials. The coating materials used were 10% sterilized maltodextrin (100 g maltodextrin and 1 L distilled water) and whey protein. The proportion of probiotics, whey protein, and maltodextrin was made at 1:1:0.1 (v/v/w), respectively (Munaeni *et al*., 2014). Furthermore, the probiotic was dried by using a freeze dryer (LABCONCO) at -50°C for 24 h. The microcapsule of probiotic was then transferred in a container and stored in the refrigerator at -20°C.

Feeding treatments

The feed was *Artemia* sp. with appropriate size to shrimp larvae, high nutrition, and high digestibility. The treatments were positive and negative control (C+, C-: without enriched *Artemia* sp.), probiotic *P. piscicida* 1Ub (10 g/L; 10^6 CFU/g), prebiotic MOS 12 mg/L, and synbiotic.

Hatching and enrichment of *Artemia* sp.

Artemia sp. cysts were hatched in 2 g/L seawater (salinity 30 g/L), and the *Artemia* sp. were harvested after 24 h. *Artemia* sp. were enriched at the instar 2 stage (approximately 4 h after harvesting) in a plastic tank containing 1 L seawater (salinity 30 g/L) at densities of 100 individual/mL (Hamsah *et al.,* 2017a). Microencapsulated probiotic, prebiotic MOS and synbiotic were added and aerated for 4 h. The enrichment dose was determined as previously described by Hamsah *et al.,* (2017a). *Artemia* sp. were harvested using a plankton net and washed with disinfected seawater, while remaining *Artemia* sp. were stored in the refrigerator at 4°C for further use on the same day.

Larvae rearing

Pacific white shrimp larvae (mean initial length of 4.72 ± 0.25 mm) were obtained from PT. Suri Tani Pemuka (STP), Carita, Banten. The larvae were reared in 15 aquariums (60×30×35 cm^3; volume 10 L) at densities of 200 individuals per aquarium. Completely randomized design (CRD) was arranged with triplicates. The larvae were reared from mysis 3 (M3) to postlarvae (PL) 12 and fed by enriched *Artemia* sp. 3−4 individuals for M3 larvae and 8−10 individuals for PL1−PL12 at five times daily (06.00 am, 10.00 am, 02.00 pm, 06.00 pm, and 10.00 pm). The dose of *Artemia* sp. was determined as previously described by Nimrat *et al.,* (2011). During rearing, water quality was controlled at 29−30°C, pH 8.33−8.53, salinity 30−33 g/L, and total ammonia nitrogen (TAN) 0.58−0.69 ppm. To maintain the water quality, water was replaced at 5−10% by disinfected seawater and siphoned every three days. At the end of the experimental period, a challenge test using *V. harveyi* MR5339 Rf^R (10^7 CFU/mL) was infected to PL 13 of all treatments using the immersion method, which was conducted in a container containing 1 L seawater at densities of 20 individual/L. Meanwhile, a negative control was immersed with an equal volume of the SWC broth medium. During the challenge test (5 days), shrimp larvae were fed by non-enriched *Artemia* sp., and the number of their deaths was monitored.

Determination of product percentage and probiotic viability

Determination of product percentage and probiotic viability included product percentage after drying, bacterial viability after drying, the percentage of bacterial viability after microencapsulation, and probiotic viability after storage. All these parameters were determined according to the method constructed by Utami *et al.,* (2015).

Determination of bacterial population

The spread plate method was used to determine bacterial count, total probiotic *P. piscicida* 1Ub Rf^R, persumtive *Vibrio* and *V. harveyi* (Ludemann *et al*., 2015). Five shrimp larvae 0.1 g were crushed and homogenized in 0.9 mL PBS (phosphate buffer saline; 0.8 g NaCl, 0.02 g KH_2PO_4, 0.15 g Na_2HPO_4, 0.02 g KCl, 100 mL distilled water).

The serial dilution was then made (1:10). The suspension (50 µL) was spread onto SWC-agar to count total bacteria and onto SWC-agar+Rif onto count total probiotic *P. piscicida* 1Ub Rf^R, TCBS medium to count total *Vibrio* and TCBS+Rif medium to count *V. harveyi* MR5339 Rf^R.

Determination of growth performance

Growth performance was determined at the end of the rearing period, including survival rate (Nimrat *et al.,* 2011), specific growth rate (Nimrat *et al.,* 2011), and absolute length growth (Dehaghani *et al.,* 2015).

Immune responses

The immune responses were determined at the end of the experimental period (PL12) and the fifth days after the challenge test. The observed parameter included total haemocyte count (THC), phenoloxidase activity (PO), and respiratory burst activity (RB). The procedure for the determination of THC followed the method previously described by Tampangalloo *et al.* (2013), while the procedure for PO and RB assay follows the method from Hyunh *et al.* (2011), respectively.

Larval resistance against *V. harveyi*

The larval resistance was determined by enumeration of shrimp larvae survival, presumptive *Vibrio* and *V. harveyi* MR5339 Rf^R during five days of challenge test.

Statistical analysis

Data on growth performances, immune responses, and survival rates were statistically evaluated by one way-ANOVA in SPSS (version 16). Significant differences between means were compared using Duncan multiple range tests (DMRT) at a confidence interval of 95%. Descriptive analysis was used to evaluate the product percentage and bacterial viability, bacterial population in the Pacific white shrimp larvae, and daily mortality.

RESULTS

The analysis on product percentage and bacterial viability resulted in product percentage after drying 10% (100 g dried probiotic obtained from 1 L probiotic suspension), bacterial viability after drying 7.38 × 10^5 CFU/g, probiotic viability after microencapsulation 63%, and probiotic viability during four months storage 91.04%.

The results on the bacterial population demonstrated that synbiotic treatment showed the highest total bacteria (4.26 × 10^8 CFU/larvae) and probiotic *P. piscicida* 1Ub Rf^R (1.51 × 10^5 CFU/larvae). Meanwhile, the highest persumtive

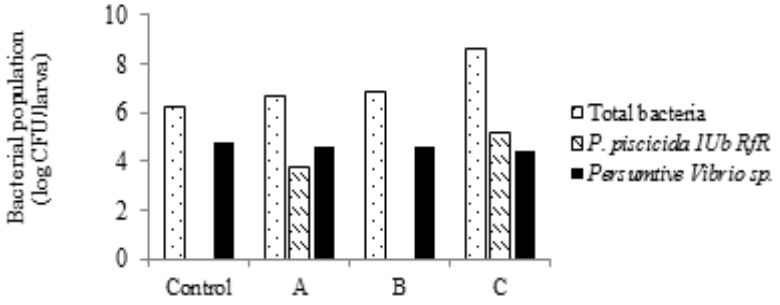

Figure 1. The bacterial population in Pacific white shrimp larvae after treatment; administration of microcapsule probiotic *P. piscicida* 1Ub 10 g/L (A), prebiotic MOS 12 mg/mL (B), and synbiotic (C) through the enrichment of *Artemia* sp.

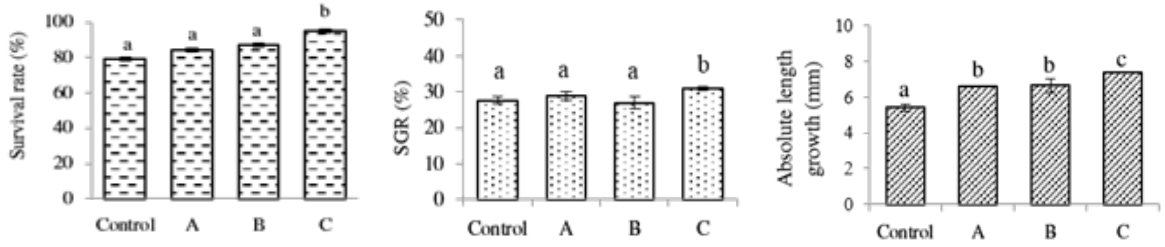

Figure 2. Growth performance; survival rate (a), SGR (b), absolute length growth (c) of Pacific white shrimp larvae after treatments. Different letters on the same bar (mean±SD) indicated significant differences (DMRT; P<0.05). Administration of microencapsulated probiotic *P. piscicida* 1Ub 10 g/L (A); prebiotic MOS 12 mg/L (B), synbiotic (probiotic *P. piscicida* 1Ub 10 g/L + prebiotic MOS 12 mg/L) (C) through enrichment *Artemia* sp.

Vibrio was attributed to control treatment (C+ and C-) 6 × 10^4 CFU/larvae, and synbiotic treatment resulted in the lowest *Vibrio* population 2.8 × 10^4 CFU/larvae (Figure 1).

The results on growth performances showed that synbiotic treatment had highest survival rate after treatment (95.00 ± 1.72%) and significantly different (P<0.05) with control (79.00 ± 0.01%), probiotic (84.00 ± 0.05%), and prebiotic (87.00 ± 0.04%). Whereas between control, probiotic, and prebiotic there was not significantly different (P>0.05). The highest specific growth rate (SGR) was shown by synbiotic treatment (31.00 ± 0.50%) and significantly different (P<0.05) with control (27.60 ± 0.94%), probiotic (28.80 ± 1.19%), and prebiotic (26.90 ± 1.72%). Whereas the other treatments there was not significantly different (P>0.05). Synbiotic treatment also showed the highest absolute length growth (7.35 ± 0.01 mm) and significantly different (P<0.05) with control (5.41 ± 0.18 mm), probiotic (6.58 ± 0.04 mm), and prebiotic (6.65 ± 0.39 mm). Whereas probiotic and prebiotic treatment was not significantly different (P>0.05), but it was significantly different (P<0.05) with control (Figure 2).

The highest total hemocyte count (THC) after treatment was shown by synbiotic (4.80 ± 1.04 × 10^5 cell/mL) and significantly different with control (3.47 ± 0.12×10^5 cell/mL), probiotic (2.30 ± 0.92 × 10^5 cell/mL), and prebiotic (3.00 ± 0.00×10^5 cell/mL). Whereas the other treatments there was not significantly different (P>0.05). THC has increased after being challenged with *V. harveyi* MR5339 Rf^R. The highest THC after challenged was synbiotic (10.83 ± 1.27×10^5 cell/mL) and significantly different (P<0.05) with negative (4.30 ± 1.25× 10^5 cell/mL) and positive

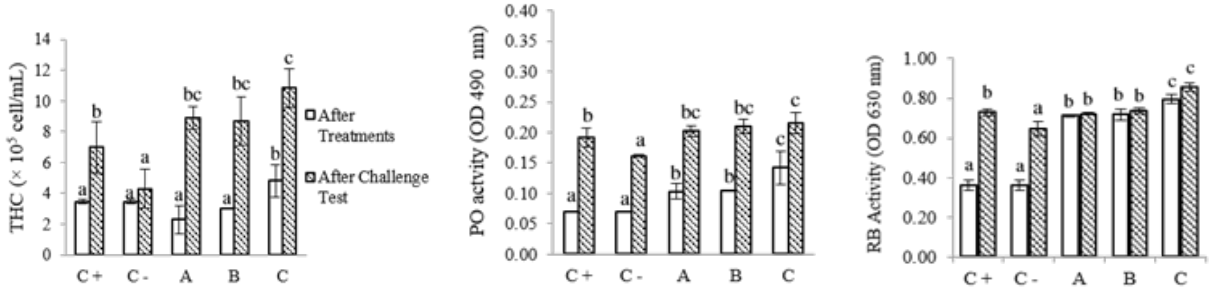

Figure 3. Immune responses; THC (a), PO activity (b), RB activity (c) of Pacific white shrimp larvae after treatments and after challenged by *V. harveyi*. Different letters on the same bar (mean±SD) indicated significant differences (DMRT; P<0.05). Administration of microencapsulated probiotic *P. piscicida* 1Ub 10 g/L (A); prebiotic MOS 12 mg/L (B) , synbiotic (probiotic *P. piscicida* 1Ub 10 g/L + prebiotic MOS 12 mg/L) (C) through enrichment *Artemia* sp.

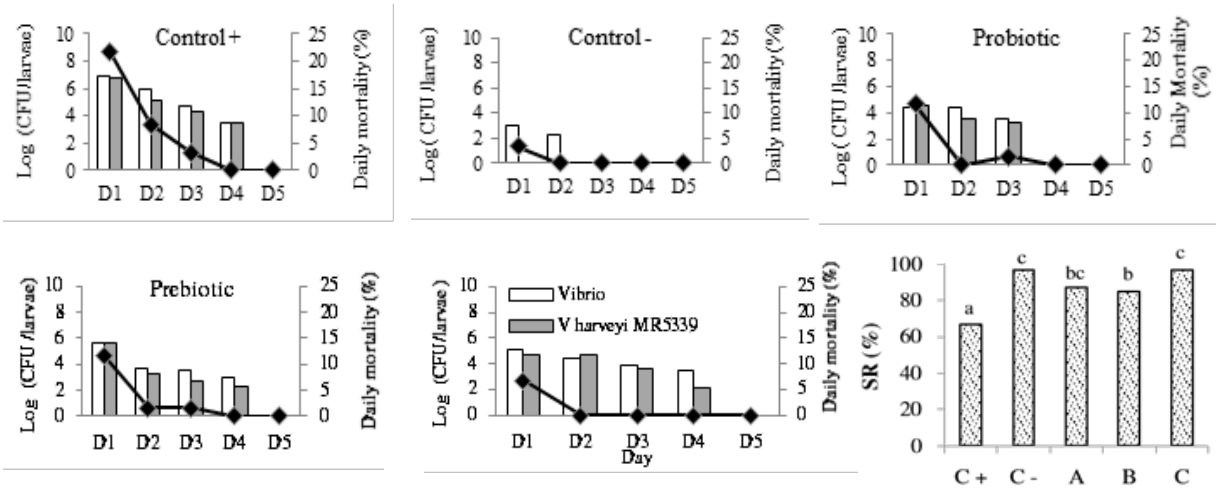

Figure 4. After challenge test using pathogenic bacteria *V. harveyi* MR5339 RfR; daily mortality pattern, presumptive *Vibrio* and *V. harveyi* in Pacific white shrimp larvae, as well as the survival rate of Pacific white shrimp larvae for 5 days. Different letters on the same bar (mean ± SD) indicated significant differences (DMRT; P<0.05). Administration of microencapsulated probiotic *P. piscicida* 1Ub 10 g/L (A); prebiotic MOS 12 mg/L (B), synbiotic (probiotic *P. piscicida* 1Ub 10 g/L + prebiotic MOS 12 mg/L) (C) through enrichment *Artemia* sp.

(6.97 ± 1.67× 10^5 cell/mL) control. Whereas probiotic, prebiotic, and synbiotic there was not significantly different (P>0.05) (Figure 3).

Synbiotic treatment also showed the highest phenoloxidase (PO) activity (0.28 ± 0.03 OD 490 nm) after treatment and significantly different (P<0.05) with positive and negative control (0.14 ± 0.00 OD 490 nm), probiotic (0.21 ± 0.01 OD 490 nm), and prebiotic (0.21±0.00 OD 490 nm). Whereas probiotic and prebiotic treatments there were not significantly different (P>0.05). However, both are significantly different (P<0.05) with the positive and negative control. PO activity has increased after challenged by *V. harveyi* MR5339 Rf^R. The higher PO activity after challenge test were shown by synbiotic (0.45 ± 0.02 OD 490 nm), prebiotic (0.43 ± 0.01 OD 490 nm), and probiotic (0.38± 0.01 OD 490 nm) treatments as well as significantly different (P<0.05) with negative (0.36 ± 0.00 OD 490 nm) and positive (0.37 ± 0.02 OD 490 nm) control (Figure 3).

The highest respiratory burst (RB) activity after treatment was shown by synbiotic treatment (0.79 ± 0.02 OD 630 nm) and significantly different (P<0.05) with probiotic (0.71 ± 0.00 OD 630 nm), prebiotic (0.72 ± 0.01 OD 630 nm), positive and negative control (0.36 ± 0.01 OD 630 nm). Whereas probiotic and prebiotic treatments there were not significantly different (P>0.05). However, both are significantly different (P<0.05) from the positive and negative control. RB activity also increased after challenged by *V. harveyi* MR5339 Rf^R. The highest RB activity after challenge test was shown by synbiotic treatment (0.86 ± 0.12 OD 630 nm) and significantly different (P<0.05) with probiotic (0.72 ± 0.01 OD 630 nm), prebiotic (0.74 ± 0.01 OD 630 nm), and positive (0.73 ± 0.04 OD 630 nm) as well as negative (0.64 ± 0.01 OD 630 nm) control (Figure 3).

After the challenge test, the highest number of the shrimp larvae death, total *Vibrio,* and *Vibrio harveyi* were attributed to control positive, while the lowest one was attributed to the synbiotic treatment. After challenge test, the highest survival rate (P<0.05) was obtained in synbiotic treatment (97.00 ± 0.06 %) and significantly different (P<0.05) with positive control (67.00 ± 0.03 %) and prebiotic treatment (85.00 ± 0.05 %). Whereas probiotic treatment (87.00 ± 0.06 %) was not significantly different (P>0.05) with prebiotic and synbiotic treatment (Figure 4).

DISCUSSIONS

The results of probiotic microencapsulation demonstrated high product percentage and probiotic viability after microencapsulation and four months of storage. The high yield of encapsulated probiotic indicates a protective effect of coating materials namely maltodextrin and whey protein. The coating material might contribute to stabilizing cell viability during the storage process. Maltodextrin is one of the coating materials that had water-soluble properties and the ability to absorb water, as well as maltodextrin contains polyanion that only able to protect bio-active compounds on the first layer (Kurniasih *et al*., 2018). Recent research reported that whey protein can interact with a wide range of active molecules and can protect probiotic bacteria before their targeted release in the digestive tract of the host (Martin *et al.,* 2015). Moreover, whey protein is a polycation that able to protect as the second layer after maltodextrin (Mishra, 2016). The coating materials from this polyanion and polycation will form spiral tissue to protect bio-active content (Saloko *et al.,* 2014) so that encapsulated probiotic had a long-time release.

The present study revealed that the administration of synbiotic showed a lower population of *Vibrio* compared to control (C+ and C-), indicating that synbiotic treatment could inhibit the development of *Vibrio*. The high total bacteria and probiotic *P. piscicida* 1Ub in synbiotic treatment indicate that the probiotic bacteria could utilize MOS for their growth and successfully adapted through attaching and colonizing in the digestive tract of shrimp larvae. Wongsasak *et al.* (2014) reported that probiotic encapsulation could enhance probiotic colonization in the digestive tract of Pacific white shrimp larvae. Goh *et al.* (2015) reported that during synbiotic administration, prebiotics that has been incorporated into synbiotics will be hydrolyzed in the digestive tract of the host and will be used as a carbon source to increase the probiotic bacteria biomass. Therefore, the high proliferation of probiotic bacteria supported by prebiotic MOS causes the growth of *Vibrio* to decrease. This result is related to the ability of probiotic bacteria *Bacillus* spp. can inhibit the proliferation of *Vibrio* in European lobster larvae supported by prebiotic mannan-oligosaccharide (MOS) (Daniels *et al.,* 2010). Recent research

also reported that shrimp fed the probiotic-supplemented diet significantly reduced the abundance of *Vibrio* spp. and increase the abundance of lactic acid bacteria found in the intestinal tract of shrimp (Vieira *et al*., 2016).

After treatment, synbiotic produces the best growth performances compared to other treatments. This result indicates that there might be probiotic *P. piscicida* 1Ub can utilize prebiotic MOS with aid of enzymatic activity enhanced the digestive system of shrimp larvae to more easily absorb the nutrition for their growth, so that the growth performance is increasing. Wang *et al*. (2019) reported that higher enzyme activites in the digestive tract enhance digestive capabilities and growth performance of the host. The digestive enzyme is a useful comparative indicator for food utilization, digestive capacity, and growth performance of the host (Cerezuela *et al.,* 2011). The previous study has shown that probiotic *P. piscicida* 1Ub was able to produce protease, lipase, amylase, and mannanase enzymes (Hamsah *et al.,* 2017b). Several studies have shown that probiotics with exo-enzyme activities could significantly improve the growth performance of the Tilapia (Liu *et al.,* 2017; Han *et al.,* 2015), so this might be related to the production of digestive enzymes in Pacific white shrimp larvae. Zhang *et al*. (2010) reported that dietary supplementation of *B. subtilis* (10^7 CFU/g) and 0.25% fructooligosaccharide (FOS) significantly increased SGR and disease resistance of sea cucumber against *Vibrio splendidus* infection. The results of this study also showed that synbiotic could increase the survival rate after synbiotic administration. It is thought that prebiotics are utilized by targeted probiotic in intestinal and some metabolites are released such as chain fatty acids (SCFAs), amino acids, or polyamines that may boost the health of host as well as it will increase the survival rate of the host (Hyunh *et al.,* 2017).

Before the challenge test, the high value of THC, activity of PO and RB indicated defense activity by shrimp larvae against the invasive pathogen. After the challenge test, the high value of THC demonstrated the proliferation and movement of haemocyte cells in the tissues infected by *Vibrio harveyi* MR5339. Maftuch *et al.,* (2013) reported that the open haemocyte circulation system could distribute haemocytes in both the vascular system and tissues. This study showed that the administration of synbiotic could increase immune responses (THC, PO and RB activity) better than other treatments. Similarly, Nurhayati *et al.* (2015) also reported that dietary supplementation of synbiotic through the feed for 30 days could increase THC, PO and RB activity of Pacific white shrimp. Hyunh *et al.* (2017) reported that synbiotics can trigger encapsulation and phagocytosis processes in shrimp. Furthermore, the high PO activity demonstrated the enhanced capability of the shrimp in distinguishing foreign particles. Wongsasak *et al.* (2014) reported that synbiotic-supplemented feed also increased PO activity of Pacific white shrimp. Respiratory burst (RB) defined the release of foreign particles by phagocytes involving degradative enzyme released to phagosome (oxygen-dependent killing). Rodriguez and Le Moullac (2000) explained that increased RB activity was associated with higher phagocytosis activity in the host. Zhang *et al.* (2011) reported that the conjoining of isomalto-oligosaccharide (IMO) and *Bacillus* (*B. lichenformis* and *B. subtilis*) promoted to enhance RB activity on *Penaeus japonicas.*

The mode of synbiotic actions against invaders has demonstrated by Hyunh *et al.* (2017). Cerenius *et al.* (2004) reported that the cell wall components of probiotic bacteria such as β-glucan and lipopolysaccharides contribute to immunostimulatory effects through pattern-recognition proteins (PRPs) that are recognized and bound the foreign molecules that have pathogen-associated molecular patterns (PAMPs). Hamsah *et al.* (2019) reported that the administration of fresh culture *P. piscicida* 1Ub and prebiotic MOS in Pacific white shrimp produces the gene expression of lipopolysaccharide and β-glucan-binding protein (LGBP) higher than control. Lipoposacharide and β-glucan-binding protein (LGBP) is pattern recognition proteins that play an important role in innate immunity of crustaceans such as activation of the proPO system to recognize and bound foreign molecules and pathogens (Amparyup *et al.,* 2013). Additionally, Arockiaraj *et al.* (2015) reported that mannose-binding lectin (MBL) that mediates cellular recognition has also been reported. MBL is a class of protein with specific carbohydrate recognition such as sugar and plays an important role in the immune system (Drickamer *et al*., 1988; Medzhitov *et al*., 2002). Moreover, MOS can stimulate mannose receptors and MBL by liver secretion triggering a complete cascade stimulating the immune system of rainbow trout *Oncorhynchus mykiss* (Rodriguez-Estrada *et al.,* 2009). The mode of action of several kinds of research indicates that the increase of shrimp

immune responses is related to the performance of the shrimp immune system which is triggered by the synergistic of probiotic and prebiotic action.

The high survival rate in synbiotic treatment after the challenge test demonstrated the increased immune responses of Pacific white shrimp larvae. This also might be due to by reduction of total bacterial *Vibrio* and *V. harveyi* MR5339 Rf^R in Pacific white shrimp larvae. The ability of *P. piscicda* 1Ub in utilizing MOS for their growth also contributed to the competition of selecting appropriate sites for attachment and colonization in the digestive tract of the Pacific white shrimp larvae, thus reducing the growth of *Vibrio* and *V. harveyi* MR5339 Rf^R. Zhang *et al.* (2011) reported that dietary administration of synbiotic (consisting of isomalto-oligosaccharide (IMO) and *Bacillus* (*B. lichenformis* and *B. subtilis*) could reduce the population of *Vibrio* on shrimp *Penaeus japonicas.* Moreover, it is similar to recent research (Hyunh *et al.,* 2019) that the administration of synbiotics (*Lactobacillus plantarum* and galactooligosaccharides) able to reduce the *Vibrio* species as well as GOS supported the selected probiotic and non-endemic pathogenic bacteria in the digestive tract of shrimp. Russo *et al.* (2012) also reported that probiotic bacteria can protect the host from pathogens due to competitive exclusion for adhesion sites.

In conclusion, microencapsulation technology can produce dry products with the viability of probiotic bacteria that stable during 4 months and can protect probiotic bacteria from the process of making and storing, as well as applying to shrimp through *Artemia* sp. Besides, this technology also supports provides beneficial effects on probiotic bacteria to be able to utilize the prebiotic optimally in the digestive tract of Pacific white shrimp larvae. The administration of probiotic *P. piscicida* 1Ub Rf^R, prebiotic MOS, and synbiotic through the enrichment of *Artemia* sp. demonstrated beneficial effects on the bacterial population, growth performances, immune responses, and disease resistance of Pacific white shrimp larvae against *V. harveyi* MR5339 Rf^R. This present study concluded that the best result was synbiotic treatment.

ACKNOWLEDGMENT

We would like to thank the Laboratory of Fish Health Management at the Department of Aquaculture, Faculty of Fisheries and Marine Science and Food Processing Pilot Plant of SEAFAST (South East Asian Food and Agricultural Science and Technology) Center that have provided research facilities.

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