MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.12, No.3, September 2018, p 92-98

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.12.3.4

The Administration of *Pseudoalteromonas piscisida* 1UB through *Artemia* sp. to Enhance Growth Performance, Immune Response and Resistance of White Shrimp (*Litopenaeus vannamei*) Larvae against *Vibrio harveyi*

WIDANARNI[1], SAVNI RETALIA SABABALAT[1], MUNTI YUHANA[1], AND DIAH AYU SATYARI UTAMI[2]

[1]*Department of Aquaculture, Faculty of Fisheries and Marine Science, Institut Pertanian Bogor, Dramaga Campus, Bogor 16680, West Java, Indonesia;*
[2]*Department of Aquaculture, Politeknik Kelautan dan Perikanan, Jembrana, Desa Pengambengan, Kecamatan Negara, Jembrana 82218, Bali, Indonesia.*

This study aimed to evaluate the effectiveness of the supplementation of *Pseudoalteromonas piscisida* 1UB through *Artemia* sp. to enhance the growth performance, immune response and the resistance of white shrimp (*Litopenaeus vannamei*) larvae to the infection of *Vibrio harveyi*. The natural feed given to the white shrimp larvae was *Artemia* sp. enriched with *P. piscisida* 1UB^{R} at concentrations of 10^{6}, 10^{7}, 10^{8} CFU mL^{-1}, and a control (*Artemia* sp. without any enrichment). The experimental shrimps (0.25±0.02 mg shrimp^{-1}) were reared in the aquarium (25 × 20 × 30 cm) containing 4 L sea water with a stocking density of 30 shrimps L^{-1}. The experimental shrimps were fed the experimental feed from mysis 3 to PL12, and after that they were challenged with *V. harveyi* (10^{7} CFU mL^{-1})through an immersion method. The results of this study revealed that the administration of *Artemia* sp. enriched with *P. piscisida* 1UB could improve the survival, daily growth rate and absolute growth of length of white shrimp larvae. The activities of protease, lipase, and amylase of white shrimp larvae treated with probiotic were higher ($p<0.05$) than those of the control. After the challenge test, white shrimp larvae treated with probiotic also had better survival and immune response (total hemocyte count, phagocytic activity, phenoloxidase activity and respiratory burst activity) than those of the the positive control. The best results were obtained in the probiotic application with a concentration of 10^{8} CFU mL^{-1}.

Key words: daily growth rate, hemocyte cell, mysis, post-larvae, probiotic, survival

Penelitian ini bertujuan untuk mengetahui efektivitas dari suplementasi *Pseudoalteromonas piscisida* 1UB melalui *Artemia* sp. untuk meningkatkan kinerja pertumbuhan, respons imun, dan resistensi larva udang vaname (*Litopenaeus vannamei*) terhadap infeksi *Vibrio harveyi*. Pakan alami yang diberikan pada larva udang vaname yaitu *Artemia* sp. yang diperkaya dengan *P. piscisida* 1UB^{R} pada konsentrasi 10^{6}, 10^{7}, 10^{8} CFU mL^{-1}, dan kontrol (*Artemia* sp. tanpa pengayaan). Udang uji (0,25±0,02 mg ekor^{-1}) dipelihara di dalam akuarium (25 × 20 × 30 cm) yang berisi 4 L air laut dengan padat tebar 30 ekor L^{-1}. Udang uji diberi pakan uji dari mysis 3 hingga PL12, setelah itu diuji tantang dengan *V. harveyi* (10^{7} CFU mL^{-1}) melalui metode perendaman. Hasil penelitian ini membuktikan bahwa pemberian *Artemia* sp. yang diperkaya dengan *P. piscisida* 1UB dapat meningkatkan kelangsungan hidup, laju pertumbuhan harian dan panjang mutlak larva udang vaname. Aktivitas protease, lipase, dan amilase larva udang vaname yang diberi perlakuan probiotik lebih tinggi ($p<0,05$) dibanding kontrol. Setelah uji tantang, larva udang vaname yang diberi probiotik juga memiliki kelangsungan hidup dan respons imun yang lebih baik (total hemosit, aktivitas fagositik, aktivitas phenoloxidase, dan aktivitas *respiratory burst*) dibanding kontrol positif. Hasil terbaik diperoleh pada aplikasi probiotik dengan konsentrasi 10^{8} CFU mL^{-1}.

Kata kunci: kelangsungan hidup, laju pertumbuhan harian, mysis, post-larva, probiotik, sel hemosit

White shrimp (*Litopenaeus vannamei*) is one of the most widely cultivated fishery commodities both in Indonesia and in the world. Indonesia is one of the largest white shrimp exporters in the world besides Ecuador, Thailand, Vietnam, China, India, and Malaysia (FAO 2013). Production of white shrimp must be supported by a sustainable supply of high quality shrimp larvae. However, various problems, especially diseases, still become the main obstacles in the white shrimp larvae production businesses, causing a low survival and growth. One of the diseases that attacks white shrimp is vibriosis, caused by *Vibrio harveyi* (Phuoc *et al.* 2009). It causes the high mortality of the shrimp larvae in hatcheries (Chrisolite *et al.* 2008) at all stadia, from the nauplius, zoea, mysis, and post-larvae to adult shrimp in grow-out ponds (Saulnier *et al.* 2000).

Various efforts have been undergone to control these diseases, for example by using antibiotics, vaccines, immunostimulants, and probiotics. Disease control using antibiotics has been restricted, because it

*Corresponding author: Phone: 62-8129357404; Email: widanarni@yahoo.com

causes the pathogens to be resistant to the antibiotics. Currently, many safe and effective biological control methods have been developed, and one of them is the application of probiotics. Probiotics are beneficial for cultivated organisms, because they can modify microbial communities, improve nutritional values, improve the host's response to a disease, improve the environmental quality (Verschuere *et al.* 2000), and improve immune response (Nayak 2010). The results of several previous studies have proven the probiotic success in increasing the shrimp's growth, survival, immune response and resistance (Chiu *et al.* 2007; Nimrat *et al.* 2012; Zokaeifar *et al.* 2012; Nurhayati *et al.* 2015; Widanarni *et al.* 2015). In this study, the probiotic used was *Pseudoalteromonas piscisida* 1UB which had been tested and had been proven to be able to inhibit the growth of *V. harveyi* through *in vitro* tests, and its application through immersion method could increase the survival of giant tiger shrimp larvae (Widanarni *et al.* 2009). The administration of a probiotic to shrimp larvae could be done through the enrichment of *Artemia*, the main natural feed for shrimp larvae, due to its ideal size for the larvae, its high nutritional value, and digestibility. This study aimed to evaluate the effectiveness of the administration of *P. piscisida* 1UB through *Artemia* sp. to enhance the growth performance, immune response and resistance of white shrimp larvae infected by *V. harveyi.*

MATERIALS AND METHODS

Probiotic Preparation. The probiotic used was *Pseudoalteromonas piscisida* 1UB (a wild type isolate) that had been marked with the antibiotic rifampicin (*P. piscisida* 1UB^{R}) as a molecular marker (Widanarni *et al.* 2003). This method aimed to ensure that probiotic isolate used in this study was *P. piscisida* 1UB which was a collection of the Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, Institut Pertanian Bogor, Indonesia, or was not an isolate from other sources. This method also aimed to distinguish *P. piscisida* 1UB isolate with other bacterial isolates which could contaminate sample or medium used in this study. The *P. piscisida* 1UB^{R} was cultured in sea water complete (SWC) slant agar medium and incubated at room temperature (28-30 ^{o}C) for 24 hours. Then, probiotic cells were harvested and were inoculated into a SWC broth medium and those were incubated in a waterbath shaker at a temperature of 28-29 ^{o}C (140 rpm; 16 h).

Experimental Design. The present study compared the growth performance and the immune response of white shrimp larvae fed *Artemia* sp. nauplii enriched with *P. piscisida* 1UB^{R} at different concentrations, i.e. 10^{6}, 10^{7}, 10^{8} CFU mL^{-1} and without any probiotic (control). Four groups of treatments in triplicates were applied in this experiment, i.e. 1UB6, 1UB7, 1UB8 and control.

White Shrimp Rearing. This experiment used 12 units of glass aquarium with a dimension of 25 cm × 20 cm × 30 cm filled with 4 L of disinfected seawater. Water temperature throughout the experiment was maintained at a range of 30 to 32 ^{o}C by using thermostat. Aeration was provided to each experimental aquarium through an aeration unit connected to an air blower.

Specific pathogen free white shrimp larvae at mysis 1 stage were obtained from a local hatchery (PT. Suri Tani Pemuka, Labuan, Banten Province, Indonesia) and acclimatized at laboratory condition until they reached mysis 3 stage. During this stage, shrimp larvae were fed *Artemia* sp. nauplii at a range of 3-4 nauplii per larvae per feeding. The larvae (0.25±0.02 mg shrimp^{-1}) were randomly distributed into each experimental tank at a density of 30 white shrimps L^{-1}.

Feeding was performed using *Artemia* sp. nauplii previously enriched with *P. piscisida* 1UB^{R} at different concentrations. Feed quantity was gradually increased following the developmental stage of the shrimp at a level ranging from 8 to 10 *Artemia* sp. nauplii per larvae per feeding (Nimrat *et al.* 2011). Feeding was offered 6 times a day at 02.00, 06.00, 10.00, 14.00, 18.00, and 22.00 for 15 d of culture.

Enrichment of *Artemia* sp. *Artemia* sp. enrichment was performed following the procedures described by Daniels *et al.* (2010) with some modifications. *Artemia* sp. nauplii was obtained by hatching 2 g of *Artemia* sp. cyst (Supreme Plus, US) in 1 L of seawater. Enrichment was performed by adding *P. piscisida* 1UB^{R} suspension into newly hatched *Artemia* sp. nauplii culture medium (seawater at a salinity of 30 g L^{-1}) at a concentration depending on the treatment. *Artemia* sp. nauplii were maintained in the probiotic suspension at a density of 100 individual mL^{-1} for 4 hours. Similar procedure was applied to *Artemia* sp. nauplii provided for the shrimp in control treatment, except there was no probiotic added to the *Artemia* sp. culture medium. The nauplii were collected using a plankton net, rinsed with fresh seawater and directly transferred to the shrimp larvae culture tank or kept in a

refrigerator with a temperature of 4 °C for later feeding time in the same day.

Growth Performance Parameters. White shrimp survival, growth (daily growth rate and absolute growth of length), digestive enzymes activities, and bacterial count were determined at the final day of experiment. Digestive enzymes activities measured in the present study were amylase, protease, and lipase activities by pooling 25 to 30 shrimps (0.5 g) per replicate tank. Protease and amylase activities were determined following the procedures described in Worthington (1993), while lipase activity was determined according to the method described in Borlongan (1990). The measurement of amylase, protease, and lipase activities aimed to evaluate the ability of probiotic isolate used in this study to secrete several exogenous enzymes such as amylase, protease, and lipase to promote the growth performance of white shrimp larvae. RNA:DNA ratio in the whole body of shrimp larvae was measured to evaluate the effect of probiotic administration on the shrimp growth potential (Tanaka *et al.* 2007; Zehra and Khan 2013). Three shrimps were collected from each tank and pooled together for RNA and DNA extractions, performed using extraction kits ISOGEN (Nippon Gene, Japan) and Puregene (Qiagen), respectively. The concentrations were subsequently measured using DNA/RNA Quant.

Challenge Test. After the completion of the feeding experiment, challenge test was performed using pathogenic *Vibrio harveyi* MR5339 isolated from shrimp infected with luminescent vibriosis (a collection of the Fish Health Laboratory, Department of Aquaculture, Bogor Agricultural University, Indonesia). Challenge test was carried out on 15 shrimps from each tank placed in 15 units of glass container previously filled with 1 L of disinfected seawater with the procedure previously described in Merchie *et al.* (1998) and Widanarni *et al.* (2014). The bacterial suspension was added to each container at an initial density of 10^7 CFU mL^{-1}. On the following day, *V. harveyi* suspension was occasionally added to replace loss due to water replacement, that were done on day 1 and 3 of the challenge test period. Feeding during challenge test was done using non-enriched *Artemia* nauplii, which was offered five times a day. Negative control used the shrimp from the control treatment applied in this test with similar treatment, except there was no *V. harveyi* suspension added to the container. Challenge test was performed for 5 days.

Immune Parameters. Immune parameters were measured after feeding experiment (prior to challenge test) and after the completion of the challenge test. Body fluid collection was conducted following the procedures previously described in Tampangallo *et al.* (2012) with some modifications. Briefly, five to six post-larvae (0.3 g) were collected from each tank, placed in a pestle and added with 900 µL of precooled anticoagulant solution (30mM trisodium citrate, 0.34 M sodium chloride, 10mM EDTA and 0.12 M glucose, pH of 7.55) (Liu and Chen 2004). Each larvae was slightly compressed to let the hemolymph flowing out the shrimp's body and mixed with anticoagulant solution. The solids were subsequently removed and hemolymph mixture was transferred into a new microtube for further analysis. Total hemocyte count (THC) was measured following the procedures described by Yeh and Chen (2009), while phagocytic index was measured according to Anderson and Siwicki (1995). Phenoloxidase activity was measured according to Liu and Chen (2004) and Martín *et al.* (2012) by measuring the formation of dopachrome at an optical density (OD) of 490 nm. Respiratory burst was performed according to the procedure described in Song and Hsieh (1994) and Martín *et al.* (2012), that were based on the formation of formazan. Respiratory burst was determined as the formation of blue formazan at an OD of 630 nm per 10 µL of homogenates.

Water Quality. Water temperature in each tank was monitored daily, while dissolved oxygen (DO) concentration, pH and salinity were measured weekly. All water quality parameters were in normal range for shrimp larviculture, with the ranges for temperature, DO, pH and salinity were 31-32 °C, 3.8-4.5 mg L^{-1}, 7.3-8.0 and 28-32 g L^{-1}, respectively.

Statistical Analysis. All data was represented in mean and standar deviation. The data, except bacterial count data, were subsequently subjected to analysis of variance (ANOVA). Significant differences were determined by Duncan post hoc test. Statistical analysis was performed using statistical software SPSS version 23.

RESULTS

The shrimp larvae's survival in all probiotic treatments were significantly different ($p<0.05$) from the control (63.06±5.50%). Probiotic administrations on white shrimp larvae resulted in higher growth as indicated by the significantly higher DGR and absolute growth of length ($p<0.05$). The highest growth was

Table 1 Growth performance of white shrimp larvae (*Litopenaeus vannamei*) administered P*seudoalteromonas piscisida* 1UB with different concentrations through *Artemia* sp.

Parameter	Probiotic concentration			
	Control	1UB6	1UB7	1UB8
Survival (%)	63.06±5.50 [a]	79.44±2.39 [b]	89.72±4.37 [c]	92.78±6.32 [c]
DGR (% day $^{-1}$)	29.54±0.93 [a]	31.95±0.09 [b]	33.78±1.20 [b]	36.41±0.81 [c]
AL (mm)	7.54±0.15 [a]	9.32±0.39 [b]	9.83±0.14 [b]	11.48±0.24 [c]
RNA:DNA (ng µL $^{-1}$)	0.249 ±0.015 [a]	0.345±0.029 [b]	0.381±0.024 [bc]	0.393±0.007 [c]

Abbreviations are as follows: DGR = daily growth rate; AL; absolute length; RNA:DNA = RNA:DNA ratio. Values are presented as means±standard deviations. Different uppercase letters in the same row indicate significant differences ($p<0.05$) among treatment groups.

Table 2 The digestive enzymes activity in white shrimp (*Litopenaeus vannamei*) larvae administered *Pseudoalteromonas piscisida* 1UB^R with different concentrations through *Artemia* sp.

Treatments	Digestive enzyme activity (U mL^{-1} minute^{-1})		
	Protease	Lipase	Amylase
Control	0.0280±0.0012^a	0.083±0.004^a	0.629±0.004^a
1UB6	0.0440±0.0002^b	0.108±0.001^b	1.103±0.006^b
1UB7	0.0490±0.0008^c	0.109±0.001^b	1.318±0.012^c
1UB8	0.0540±0.0003^d	0.112±0.001^b	1.418±0.004^d

Values are presented as means±standard deviations. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) among treatment groups.

observed in white shrimp larvae in 1UB8 (36.41±0.81 % day^{-1}; 11.48±0.24 mm). The ratios of RNA:DNA in white shrimp larvae administered probiotic were significantly higher ($p<0.05$) than those in the control (0.249±0.015 ng µL^{-1}) (Table 1). The digestive enzyme activities in white shrimp larvae fed probiotic were higher ($p<0.05$) than those in control group (Table 2).

Total bacterial counts at the beginning of the treatment were relatively similar, 1.60-3.76 x 10^6 CFU larvae^{-1}, while at the end of the treatment (PL12), total bacterial counts increased with the highest value obtained in 1UB8 (2.86 x 10^8 CFU larvae^{-1}), the lowest total bacterial count was found in control (2.13 x 10^7 CFU larvae^{-1}). The *P. piscisida* 1UB^R at the end of the rearing period was found in 1UB6, 1UB7, and 1UB8. The highest total *P. piscisida* IUB^R was found in 1UB7 (2.40 x 10^5CFU larvae^{-1}) (Table 3).

After feeding experiment, the immune parameters in white shrimp larvae supplemented with probiotic were better than those of the control groups. The similar trends were observed in immune parameters before and after challenge test, that showed white shrimp fed with probiotic-encapsulated *Artemia* sp. nauplii showed higher ($p<0.05$) levels of immune responses than those of the positive control. This was confirmed by the higher white shrimp survival in probiotic treatments than that of the positive control following challenge test against *V. harveyi* (Table 4).

DISCUSSION

The results of the study revealed that the administration of *P. piscisida* 1UB^R produced better results in growth performance parameters, including survival, DGR, and absolute growth of length than those of the control. The high survival in all probiotic treatments is suspected to be caused by the ability of *P. piscisida* 1UB^R to improve the white shrimp larvae's fitness through the improvement of the microbial community in the white shrimp larvae's body. Some studies demonstrated that probiotics could increase the survival of aquatic organisms. The enrichment of *Artemia* naupli with *Lactobacillus sporogenes* could increase *Macrobrachium rosenbergii* post-larvae survival (Seenivasan *et al.* 2012). The administered probiotic bacteria had a function as a source of macro and micro nutrients (Verschuere *et al.* 2000), improving the nutritional value of the *Artemia* sp. fed to the white shrimp larvae, that will then lead to a better white shrimp growth. The better growth was also supported by the higher RNA:DNA ratios, total bacterial counts, protease activities, and amylase activities in probiotic groups at the end of the feeding experiment than those of the control. The highest results were found in 1UB8. Hamsah *et al.* (2018) reported that the administration of *P. piscisida* 1UB at a dose of 10^6 CFU mL^{-1} through bio-encapsulation of

Table 3 The bacterial population in white shrimp (*Litopenaeus vannamei*) larvae administered *Pseudoalteromonas piscisida* 1UB^R with different concentrations through *Artemia* sp.

Treatments	Total bacteria		Total *Pseudoalteromonas piscisida* 1UB^R bacteria	
	Mysis 3 (CFU larvae $^{-1}$)	PL12 (CFU larvae $^{-1}$)	Mysis 3 (CFU larvae $^{-1}$)	PL12 (CFU larvae $^{-1}$)
Control	3.76 x 10^6	2.13 x 10^7	n.d.	n.d.
1UB6	1.72 x 10^6	2.24 x 10^7	n.d.	1.94 x 10^5
1UB7	1.60 x 10^6	2.20 x 10^7	n.d.	2.40 x 10^5
1UB8	1.76 x 10^6	2.86 x 10^8	n.d.	9.56 x 10^4

n.d. = not detected any colonies of *Pseudoalteromonas piscisida* 1UB^R grown on the test medium.

Table 4 The immune response and the resistance of white shrimp (*Litopenaeus vannamei*) larvae administered *Pseudoalteromonas piscisida* 1UB at different concentrations through *Artemia* sp. before and after the challenge test with *Vibrio harveyi*

Parameters	Probiotic concentration				
	Control (+)	Control (-)	1UB6	1UB7	1UB8
THC (x10^6)					
Pre-challenge test	14.26±0.91^a	14.82±0.91^a	16.24±1.71^{bc}	16.79±1.44^{bc}	19.60±2.55^c
Post-challenge test	9.08±0.22^a	17.10±0.12^b	20.55±0.56^c	21.50±0.11^{cd}	24.10±0.73^d
PA (%)					
Pre-challenge test	17.00±1.63^a	17.70±1.25^a	22.00±1.63^b	23.50±0.41^b	28.30±0.47^c
Post-challenge test	15.00±3.56^a	28.00±0.82^b	36.67±1.25^c	45.67±2.49^d	54.00±0.82^e
PO (O.D. 490 nm)					
Pre-challenge test	0.54±0.02^a	0.53±0.01^a	0.50±0.01^a	0.66±0.03^b	0.53±0.01^a
Post-challenge test	0.350±0.007^a	0.670±0.002^b	0.680±0.014^b	0.750±0.017^b	0.810±0.035^b
RB (O.D. 630 nm)					
Pre-challenge test	0.76±0.04^a	0.77±0.07^a	0.95±0.02^{ab}	1.03±0.09^b	1.08±0.20^b
Post-challenge test	0.28±0.08^a	1.04±0.03^c	1.10±0.01^c	0.75±0.02^b	1.40±0.02^c
Survival (%)					
Post-challenge test	66.67±9.43^a	100.00±0.00^c	84.44±8.31^b	86.67±5.44^b	93.33±5.44^b

Abbreviations are as follows: THC = total hemocyte count; PA = phagocytic activity; PO = phenoloxidase activity; RB = respiratory burst activity. Values are presented as means±standard deviations. Different uppercase letters in the same parameter and observation period indicate significant differences ($p< 0.05$) among treatment groups.

Artemia sp. resulted higher growth performance and survival of white shrimp than those of the control. The *P. piscisida* 1UB^R was found in all probiotic treatments, but it was not found in the control. It showed that probiotic used in this study could colonize in white shrimp's larvae body. Probiotic commonly could improve nutritional status, modulate other microbes, colonize, and produce several exogenous enzymes (Widanarni *et al.* 2009; Labh 2015). The *P. piscisida* 1UB can secrete amylase protease, lipase, and mannase (Hamsah *et al.* 2017). The *P. piscisida* 1UB can be combined with a prebiotic and work as synbiotic to reach a better effect to the host. Hamsah *et al.* (2018) reported that the administration of synbiotic (a combination of *P. piscisida* 1UB and mannan oligosaccharide) through bio-encapsulation of *Artemia* sp. resulted better growth performance and survival of white shrimp larvae than those of control, probiotic, and prebiotic treatments.

The white shrimp's survival after the challenge test in probiotic groups, was higher than that of the positive control. This demonstrated that the administration of *P. piscisida* 1UB^R had a positive effect in the resistance of the shrimp against the infection of *V. harveyi*. This was supported by higher total hemocyte counts, phagocytic activities, PO activities, and RB activities in probiotic groups after the challenge test than those of positive control. Probiotic is an immunogenic material (Aly *et al.* 2008), which has β-glucan, lipopolysaccharides, and peptidoglycans in its cell wall which have an

immunostimulatory effect (Smith *et al.* 2003; Gullian *et al.* 2004). This would also increase the phagocytic activity of hemocyte cells and PO activity. The administration of the probiotic could increase total hemocyte count, that would lead to an immune response improvement during infections by pathogens in shrimp (Chiu *et al.* 2007). The increasing of the total hemocyte count caused the increasing of the RB activity, because all RB activities occur inside hemocyte cells, which conduct foreign particle elimination activities in the phagocytosis process (Rodriguez and Le Muollac 2000).

In summary, the administration of *P. piscisida* 1UB through *Artemia* sp. could effectively improve the white shrimp larvae's growth performance. It also improved immune response and the resistance of white shrimp larvae to the infection of *V. harveyi* with the best results obtained in 1UB8. The results of this study can be a new strategy to enhance the production of white shrimp larvae through the application of probiotic and a protocol to create standard operational procedure of the application of probiotic through bio-encapsulation of *Artemia* sp. in a shrimp hatchery.

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