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 perlakuan 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, penicillin, rifampicin, chloramphenicol, 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 arabinofuranosyl-oligosaccharide (AXOS), fructo-oligosaccharide (FOS), galactooligosaccharides (GOS), mannan-oligosaccharide (MOS), xylo-oligosaccharides (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; Zubaida 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). 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 thermostaker 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) 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 thermostaker 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 cm³; 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 RP (10⁶ 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, presumptive *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 KH₂PO₄, 0.15 g Na₂HPO₄, 0.02 g KCl, 100 mL distilled water).

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**Note:** The text provided is a natural representation of the document's content, maintaining a coherent narrative and formatting consistent with standard academic writing practices.
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$, TCBS medium to count total Vibrio and TCBS+Rif medium to count $V. harveyi$ MR5339 Rf$.

**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 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 $7.38 \times 10^5$ 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 ($4.26 \times 10^8$ CFU/larvae) and probiotic $P. piscicida$ 1Ub Rf ($1.51 \times 10^5$ CFU/larvae). Meanwhile, the highest presumptive
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 symbiotic 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 symbiotic treatment (31.00 ± 0.5%) 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). Symbiotic 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 symbiotic (4.80 ± 1.04 × 10^8 cell/mL) and significantly different with control (3.47 ± 12 × 10^5 cell/mL), probiotic (2.30 ± 0.92 × 10^7 cell/mL), and prebiotic (3.00 ± 0.00 × 10^7 cell/mL). Whereas the other treatments there was not significantly different (P>0.05). THC has increased after being challenged with V. harveyi MR5339 RfR. The highest THC after challenged was symbiotic (10.83 ± 1.27 × 10^8 cell/mL) and significantly different (P<0.05) with negative (4.30 ± 1.25 × 10^8 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), symbiotic (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), symbiotic (probiotic P. piscicida 1Ub 10 g/L + prebiotic MOS 12 mg/L) (C) through enrichment Artemia sp.
The present study revealed that the administration of symbiotic showed a lower population of *Vibrio* compared to control (C+ and C−), indicating that symbiotic treatment could inhibit the development of *Vibrio*. The high total bacteria and probiotic *P. piscicida* 1Ub in symbiotic 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 symbiotic administration, prebiotics that has been incorporated into symbiotics 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, symbiotic 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 activities 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 symbiotic could increase the survival rate after symbiotic 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 symbiotic could increase immune responses (THC, PO and RB activity) better than other treatments. Similarly, Nurhayati et al. (2015) also reported that dietary supplementation of symbiotic through the feed for 30 days could increase THC, PO and RB activity of Pacific white shrimp. Hyunh et al. (2017) reported that symbiotics 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 symbiotic-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. licheniformis and B. subtilis) promoted to enhance RB activity on Penaeus japonicas.

The mode of symbiotic 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. Lipopolysaccharide 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).
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 symbiotic 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® in Pacific white shrimp larvae. The ability of *P. piscicida* 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®. 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®, 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®. This present study concluded that the best result was symbiotic 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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