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The Use of Synbiotics to Prevent IMNV and *Vibrio harveyi* Co-Infection in *Litopenaeus vannamei*

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This study evaluated the effects on viral immune responses and bacterial co-infection, of different feeding frequencies of a synbiotic supplemented diet given to Pacific white shrimp (*L. vannamei*). A synbiotic-supplemented diet was formulated from probiotic *Vibrio alginolitycus* SKT-b^R and prebiotics from sweet potato (*Ipomoea batatas* L.) oligosaccharide. Pacific white shrimp were fed with synbiotic diet at different frequencies, i.e. daily (P1), twice a week (P2), and once a week (P3) for a 30 day pre-challenge test. After the 30 day feeding period, the shrimps were challenged by intramuscular injection of Infectious Myonecrosis Virus (IMNV) and *Vibrio harveyi*. The results showed that shrimp treated with a synbiotic-supplemented diet showed significantly higher growth performance than control groups (P<0.05). Shrimp treated under regime P1 showed the highest values for phenoloxidase (PO) and respiratory burst (RB) parameters compared to shrimp given with other treatments. Following the challenge test, higher survival rate were seen in the P1 treatment group, in comparison to the positive control, and the P1 treatment group showed the highest values in total haemocyte count (THC), PO, and RB.

Key words: synbiotic, IMNV, *Vibrio harveyi*, co-infection, *Litopenaeus vannamei*

INTRODUCTION

The Pacific white shrimp (*L. vannamei*) is the most cultivated shrimp species in the world (Teixeira-Lopes *et al*. 2011). Shrimp production in Indonesia has significantly increased in the past three years and the Government of Indonesia is promoting even greater rates of shrimp production in 2014. However, the current system of shrimp cultivation in Indonesia face several challenges, such as high rates of infectious disease.

The diseases that most often impact intensive shrimp cultivation are bacterial, viral, and co-infectious diseases (Teixeira-Lopes *et al*. 2011). According to Poulos *et al*. (2006), one of the most lethal and problematic viral diseases found in white shrimp culture is the Infectious Myonecrosis Virus (IMNV). IMNV can cause massive mortality of up to 70% in shrimp populations (Hasan 2011) and IMNV spread to Indonesia in 2006 (Senapin *et al*. 2007). Shrimp production is also hampered by *Vibrio* spp., a bacterial infectious disease often acting as a cause of secondary infection (Liu & Chen 2004). Teixeira-Lopes *et al*. (2011) reported that, in a farm of northeast brazil, white shrimp may naturally be infected by two different kinds of viruses, the *Infectious Hypodermal and Hematopoietic Virus* (IHHNV) and IMNV. Phuoc *et al*. (2009) reported in shrimp pond, shrimp body that infected already by *White Spot Syndrome Virus* (WSSV) could be easily infected by *Vibrio* spp. bacteria.

Infection by several pathogens at the same time (co-infections) can accelerate and increase mortality (Hasan 2011). Therefore, a specific method is needed to prevent these co-infections. According to Li *et al.* (2009), synbiotics represent an alternative method that can be used to decrease disease incidence, since they have been proven to increase the shrimp immune response and resistance to disease. Synbiotics are usually comprised of a balanced combination of probiotics and prebiotics for supporting the survival and growth of beneficial bacteria in the digestive tract.

Probiotics are usually live microorganisms which, when administered in adequate amounts, confer health benefits to their host (Nayak 2010). A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Ringo *et al*. 2010). Some studies have shown the benefits of using probiotics (Panigrahi *et al*. 2005; Watson *et al*. 2008; Nayak 2010) and prebiotics (Soleimani *et al*. 2012) separately for addressing disease in shrimp aquaculture. However, Merrifield

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et al. (2010) has reported that the application of synbiotics is better than the separate application of probiotics and prebiotics for reducing disease rates in shrimp aquaculture. Results of some studies indicate that administration of synbiotic through feed can effectively increase growth performance (Geraylou *et al*. 2013), increase immunity in fish and shrimp, and increase host resistance to pathogenic infection (Li *et al*. 2009; Ai *et al*. 2011; Lin *et al.* 2012).

IMNV and *V. harveyi* co-infection can cause a higher mortality rate in shrimp cultivation than other types of infection or than infection by either pathogen alone. Therefore, an application of synbiotics is suggested as an alternative method to conventional antibiotic treatment, for preventing the co-infection. The conventional antibiotic treatment considered to be bad due to antibiotic resistance, pollution, health or cost concern. Duration of supplementation is an important factor in increasing effectiveness of supplement application in cultured organisms at the commercial level (Merrifield *et al*. 2010). In addition, work by Nayak (2010) indicated that the frequency of synbiotic administration is an important factor which can affect the establishment and subsequent induction of immune responses in the host. This study aimed to evaluate the effect of different frequencies of synbiotic dietary supplementation given to Pacific white shrimp (*L. vannamei*) to boost viral immune response and prevent bacterial co-infection.

MATERIALS AND METHODS

Experimental Design. This study was conducted for six weeks in the Fish Health Laboratory, the Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. The experiments used pasific white shrimp *L. vannamei* in post-larval stadia (PL) 15 which were obtained from a commercial hatchery in Carita, Banten. Prior to the study, the post-larvae were acclimatized to laboratory conditions for 14 days. Then the shrimp were weighed (0.3 g $\pm$ 0.03 mean initial weight), and randomly distributed to five experimental groups, each of which had three replications. Each replication contained 15 shrimp, reared with the experimental diet for 30 days. The experimental diets were provided at amounts equal to 10-12% body weight. Shrimp were fed to apparent satiation four times daily. Water quality during the experiment was maintained by siphoning out shrimp faeces and exchanging culture media at a rate of 10% daily. Water quality during the experiment was kept at the following parameters: temperature 28-29 ^{o}C, salinity 29-32 ppt, dissolved oxygen 4.5-6.5 mg/L, pH 7.4-7.5, and ammonia-nitrogen 0.005-0.016 mg/L.

Virus and Bacteria Stock. The virus used in this study was *Infectious Myonecrosis Virus* (IMNV). The virus stock was obtained from IMNV-infected White shrimp from Situbondo Brackish Water Aquaculture Development Center (BBAP) Indonesia. The virus was extracted and stored -80 ^{o}C of temperature according to Rodriguez *et al*. (2007). The bacteria stock was *Vibrio harveyi* (MR 5339) which has been tagged with rifampicin-resistant gene sequences. The *V. harveyi* isolates were obtained from the Fish Health Laboratory, the Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University.

Probiotic and Prebiotic Preparation. The probiotic bacteria used in our experimental supplement was *Vibrio alginolitycus* SKT-b; its isolates had been tagged with rifampicin-resistant gene sequences (SKT-b^{R}) according to Widanarni *et al*. (2003), and re-characterized. Culturing of SKT-b^{R} probiotic bacteria was performed in Sea Water Complete (SWC-broth) (5 g bactopeptone, 1 g yeast extract, 3 mL glycerol, 750 mL sea water, and 250 mL aquades) that incubated in a waterbath shaker at a temperature of 29-30 ^{o}C, 160 rpm for18 h. We obtained cell bacterial pellets which were then washed twice in Phosphate Buffer Saline (PBS) solution.

The preparation of the prebiotic was conducted in several stages according to the method described by Marlis (2008). Production of probiotic was started with production of sweet potato starch, extraction of oligosaccharide using ethanol 70%, and measurement of total dissolved solids. Then the type and content of oligosaccharides were analyzed using High Performance Liquid Chromatography (HPLC) with the following results: 1.115% inulin, fructooligosaccharides (FOS) 1.015%, and galactooligosaccharides (GOS) 1.488%.

Experimental Diet Preparation. The experimental diet was prepared by adding 1 dose (1% probiotic and 2% prebiotic of feed weight) of synbiotic into commercial pellets contained 40% crude protein (Nurhayati *et al*. 2014). Combining of feed and synbiotic was carried out by adding 2% egg white as binder; the feed for control groups contained only the 2% egg white without synbiotics. The shrimp larvae were fed this diet for 30 days using a Completely Randomized Design consisting of five treatments in triplicate: control (-) (untreated diet without challenge test); control (+) (untreated diet with challenge test),