

Effects of Dietary Probiotic *Bacillus* sp. D2.2 and Prebiotic Sweet Potato Extract on Growth Performance and Resistance to *Vibrio harveyi* in Pacific white shrimp, *Litopenaeus vannamei*

Esti Harpeni^{1*}, Limin Santoso², Supono², Wardiyanto², Ari Widodo², and Laksmi Yolanda²

¹ Marine Science Study Program, University of Lampung, Bandar Lampung, Indonesia

² Aquaculture Study Program, University of Lampung, Bandar Lampung, Indonesia

Email: esti.harpeni@fp.unila.ac.id^{*}, limin.sentiko@gmail.com, supono_unila@yahoo.com, wardibdifp@gmail.com, ariwido216@gmail.com, and laksmiyolanda23@gmail.com

Abstract

Esti Harpeni, Limin Santoso, Supono, Wardiyanto, Ari Widodo, and Laksmi Yolanda. 2017. Effects of Dietary Probiotic *Bacillus* sp. D2.2 and Prebiotic Sweet Potato Extract on Growth Performance and Resistance to *Vibrio harveyi* in Pacific white shrimp, *Litopenaeus vannamei*. *Aquacultura Indonesiana*, 18 (2): 55-61. In this study, the effects of oral administration of probiotic *Bacillus* sp. D2.2 and prebiotic from sweet potato extract on growth performance and resistance against *Vibrio harveyi* in Pacific white shrimp (*Litopenaeus vannamei*) were investigated. During 32-day feeding experiment, 360 individuals of Pacific white shrimp (PL15) with initial weight of 0.02 ± 0.002 g were fed with basal diet as control (A); supplemented with 6% probiotic and 0% prebiotic (B); 6% probiotic and 2% prebiotic (C); 6% probiotic and 4% prebiotic (D). At the end of feeding trial, weight gain (WG), average daily growth (ADG), feed conversion ratio (FCR), and survival rate (SR) were assessed. WG, ADG and FCR of the shrimp were significantly better in treatment D than those of the shrimp in other treatments. Control and treatment D as the best feeding trial were selected for challenge test with infectious *V. harveyi*. Survival rate and mean time to death (MTD) of the shrimp fed the supplemented diet were not significantly different ($P > 0.05$) to the control. Infection levels in shrimp were evaluated using morphological scoring methods. Infection levels of *V. harveyi* in shrimp fed the diet were lower compared to the control.

Keywords: Disease resistance; Growth; Prebiotic; Probiotic *Bacillus* sp. D2.2

Introduction

The demand for environment-friendly in shrimp culture has increased mostly due to negative side effects of antibiotic-resistant bacteria (Wright, 2010). Therefore, the use of antibiotics in aquafeed has been restricted, such as in Europe (EC Regulation 1831/2003) and USA (U.S. Food and Drug Administration, 2008). As an alternative, the uses of probiotics or prebiotics have heightened attention. Probiotic are live microbial feed supplement that contributes to intestinal microbial balance and maintains the organism's health (Soccol *et al.*, 2010). In recent years, several researchers have demonstrated that probiotics can enhance the disease resistance of shrimp by suppressing the pathogens, enhancing immunity or improving water quality (Verschuere *et al.*, 2000). Thus, usage of probiotics has been considered as one of the most promising preventive methods in aquaculture. Quite a few microorganisms from the genus *Bacillus* have been used widely as

putative probiotics. Correspondingly, a number of researchers have demonstrated that *Bacillus* can enhance the nutritional and health benefits of shrimp (Rengpipat *et al.*, 1998; 2000; 2003; Li *et al.*, 2009). *Bacillus* sp. D2.2 that used in this experiment was non-pathogenic bacteria (Hardiyani *et al.*, 2016) and able to inhibit the *in vitro* growth of *Vibrio harveyi* (Setyawan *et al.*, 2014). Meanwhile, prebiotics can increase probiotics performance since prebiotics as non-digestible food ingredients benefit to selectively stimulate the growth and/or activity of bacteria in the host's intestinal tract (Gibson *et al.*, 2004). In addition, nutritional and health benefits of prebiotics oligosaccharides have been demonstrated in shrimp (Li *et al.*, 2007; Zhou *et al.*, 2007; Li *et al.*, 2009).

Findings from the latest research show that many diseases in aquatic animals are associated with the changes in intestinal microbiota (Xiong *et al.*, 2015). One of the most promising methods for controlling shrimp diseases in aquaculture is to enhance their non-specific defense and adjust

the structure of intestinal flora via combination of probiotic and prebiotic, such as in lobster (Daniels *et al.*, 2010), sea cucumber (Sun *et al.*, 2012), and shrimp (Partida-Aranguere *et al.*, 2013). Synbiotic, a combination of probiotics and prebiotics beneficially affects the host and improves host welfare by improving the survival and colonization of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria (Gibson and Roberfroid, 1995). Synbiotic has also been considered to replace antibiotic as the result of the negative effects such as the appearance of antibiotic-resistant pathogens and concerns over the dispersal of antibiotic-resistant genes brought by using antibiotic (Bengmark, 2005).

A few studies have been revealed the effects of combined supplementation of probiotic *Vibrio alginolyticus* SKT-b and sweet potato in enhancing the growth performance and immune response (Azhar, 2013; Oktaviana *et al.*, 2014; Nurhayati *et al.*, 2015). The aim of this study is to evaluate the effects of oral administration of probiotics *Bacillus* sp. D2.2 together with prebiotic of sweet potato extract on the growth and disease resistance of shrimp, *Litopenaeus vannamei*.

Materials and Methods

Probiotic and prebiotic preparation

The experiment used *Bacillus* sp. D2.2 as the probiotic bacteria. It was isolated from traditional tiger shrimp farm in East Lampung (Setyawan, *et al.*, 2014). The concentration of *Bacillus* sp. D2.2 was approximately 10^6 colony-forming unit (CFU) ml⁻¹. Probiotic bacteria was prepared by culturing on seawater complete-agar (SWC, 5 g bacto peptone (Oxoid), 1 g yeast extract (Oxoid), 3 ml glycerol, 15 g bacto agar (Himedia), 750 ml seawater, and 250 ml distilled water) and then transferred to SWC-broth (without agar) with rotary shaker at 140 rpm for 24 h at 30°C.

Production of prebiotic was started with the production of sweet potato starch (Harpeni *et al.*, 2016). Extraction of oligosaccharide was done by soaking the potato in boiling water. Five grams of sweet potato starch was mixed with 40 ml boiled water and then stirred for 10 minutes in 85±2°C (Sukenda *et al.*, 2015). Two types of oligosaccharides, sucrose and raffinose, were

analyzed by using High Performance Liquid Chromatography (HPLC). The concentrations were 2.59% w/v (sucrose) and 0.04% w/v (raffinose).

Experimental diet preparation

The basal diet was commercial pellets that contained approximately 30% w/w crude protein and 5% w/w crude lipid which were suitable for the growth of the shrimp. Four treatments were used as the experimental diets were: Diet A (basal diet used as the control), Diet B (basal diet supplemented with 6% probiotic *Bacillus* sp. D2.2 w/w), Diet C (basal diet supplemented with 2% prebiotic and 6% probiotic *Bacillus* sp. D2.2 w/w), and Diet D (basal diet supplemented with 4% prebiotic and 6% probiotic *Bacillus* sp. D2.2 w/w). All supplements were thoroughly mixed with 2% egg yolk as a binder (Sukenda *et al.*, 2015). Subsequently, the pellets were air dried at room temperature and stored in the plastic bags until used.

Culture condition

This study was conducted in the Aquaculture Laboratory, Department of Fisheries and Marine Sciences, Faculty of Agriculture, University of Lampung. The experiments used Pacific white shrimp *L. vannamei* in post-larval stadia (PL) 15. These shrimps were obtained from a commercial hatchery in Kalianda, South Lampung. The shrimp were weighed (0.02 g ± 0.002 mean initial weight), and randomly distributed to four experimental groups, and replicated in three times. The rearing tanks were 50x40x40 cm in 40 litres of volume. Each replication contained 30 shrimp, fed on experimental diets at the dose of 8-10% body weight and reared for 32 days. Shrimp were fed to apparent satiation three times daily. Water quality during the experiment was maintained by siphoning out shrimp feces and exchanging culture media at a rate of 10% daily. Water quality during the experiment was kept at the following parameters: temperature 27-28 °C, salinity 29-32 ppt, dissolved oxygen >3.5 mg/L, and pH 7.5-8.5.

Growth trial parameters

After 32 days experiment, the total numbers of shrimp were counted and weighed. Weight gain (WG), average daily growth (ADG),

feed conversion ratio (FCR) as well as survival rate (SR) was calculated using the following equations:

$$WG = (\text{final weight} - \text{initial weight}) / \text{initial weight}$$

$$ADG = \text{weight gain} / \text{days}$$

$$FCR = \text{total dry feed intake (g)} / \text{wet weight gain (g)}$$

$$SR = (\text{final number of shrimp} / \text{initial number of shrimp}) \times 100$$

The test aimed to study the performance of the best experimental diet during growth trial in increasing *V. harveyi* resistance. The pure culture of *Vibrio harveyi* was obtained from the Fish Health Laboratory, Center for Marine Aquaculture, Lampung. Two hundred and forty shrimps (PL 25) were distributed into two treatments (basal diet as control and treatment D as the best experimental diet) and replicate four tanks. Shrimps were reared in different tanks. All shrimps were fed for 7 days, and were then infected with 10^6 colony-forming unit (CFU)/mL of *V. harveyi* by immersion. Seven days after infection, shrimp were then observed. The resistance parameters including SR, relative percent survival/RPS (Khimmakthong *et al.*, 2011) and mean time to death/MTD (Nitimulyo *et al.*, 2005) were calculated.

$$RPS = 1 - \left\{ \frac{\% \text{ of mortality (treated)}}{\% \text{ of mortality (control)}} \right\} \times 100\%$$

$$MTD = \frac{\sum_{i=1}^n a_i b_i}{\sum_{i=1}^n b_i}$$

Note: a = mortality time (hours)

b = number of dead shrimp

The clinical signs and hepatopancreatic histological examination were also observed. Shrimps were immediately fixed in Davidson's fixative (Bell and Lightner, 1988; Joshi *et al.*, 2014) until processing. After fixating, the tissues were embedded in paraffin wax, sectioned, stained with hematoxylin and eosin (H&E) and then examined under light microscopy (at 400x magnification).

Data analysis

The growth trial data were analyzed using one-way ANOVA followed by LSD's multiple range test. Histological observation on the hepatopancreatic damages i.e. percentage necrosis, vacuolation and degeneration were calculated. Relative percent of survival and mean time to death were analyzed using one sample t-Test (IBM SPSS version 22). Clinical signs were scored based on the infection levels of shrimp. Score 1 (light infection): lose appetite and balance, score 2 (mild infection): reddened body and tail, score 3 (heavy infection): gill damages, and score 4 (very heavy infection): hepatopancreatic damages until dead.

Result

Growth performance

The significant differences in weight gain and ADG were observed among treatments. The better percentage of prebiotic resulted in better weight gain and ADG as well as FCR. Based on growth performance, the best treatment was diet D and was used for the challenged test. The higher percentage of prebiotic results in the higher weight gain and ADG. Similarly, average FCR among treatments was significantly different. The higher percentage of prebiotic results in the lower FCR. The FCR of shrimp fed with Diet A (Control) was 3.70 ± 0.40 , the highest FCR among other treatments. Survival of shrimp was high for all treatments ranging from 71 to 90%. No significant difference was found between diet A (control) and diet B (supplemented with 6% probiotic and 0% prebiotic, w/w); and also between diet C (supplemented with 6% probiotic and 2% prebiotic, w/w) and diet D (6% probiotic and 4% prebiotic, w/w) (Table 1). Based on the growth performance, the best experimental diet was diet D (supplemented with 6% probiotic and 4% prebiotic, w/w) and used in the challenged test.

Table 1. Growth, diet utilization and survival rate of *L. vannamei* fed on different experimental diets for 32 days. Means in a column with different letters were

Treatments	Initial weight (g)	Final weight (g)	WG (g)	ADG (mg)	FCR (%)	SR (%)
A	0.02 ± 0.002	0.52 ± 0.07	0.50 ± 0.07^a	15.7 ± 2.3^a	3.70 ± 0.40^a	71.11 ± 6.94^a
B		0.83 ± 0.07	0.81 ± 0.07^b	25.4 ± 2.2^b	2.90 ± 0.20^b	73.33 ± 6.67^b
C		1.40 ± 0.01	1.38 ± 0.01^c	43.1 ± 2.4^c	1.52 ± 0.06^c	83.33 ± 3.33^c
D		1.90 ± 0.04	1.88 ± 0.04^d	58.6 ± 1.3^d	1.12 ± 0.04^d	90.00 ± 3.33^{cd}

Resistance Parameters

Survival rate between control and Diet D (supplemented with 6% probiotic and 4% prebiotic) was not significantly different (87 ± 7.37 and $95\% \pm 1.96$ respectively). RPS for Diet D was 53.48%. Shrimp fed with Diet A (control) had the slower average time of death compared to shrimp fed with experimental diet (108 ± 27.3 and 75 ± 35.1 hours respectively) (Figure 1), however, MTD was not significantly different.

Numbers of shrimp suffered from various

infection levels of *V. harveyi* were basically lower in shrimp fed with treatment diet. Mostly the shrimp suffered from mild infection (score 2) indicated with redness of tail (Figure 2).

After seven days of the challenged test, more than 90% shrimps indicated the prominent degenerative hepatopancreas, 36-52% of necrosis appeared in hepatocytes and 37-43% of vacuolation was noticeable. In contrast, hepatopancreas of shrimp fed with probiotic and prebiotic in diet D had less damaged than those fed with Diet A (Control) (Figure 3 and 4).

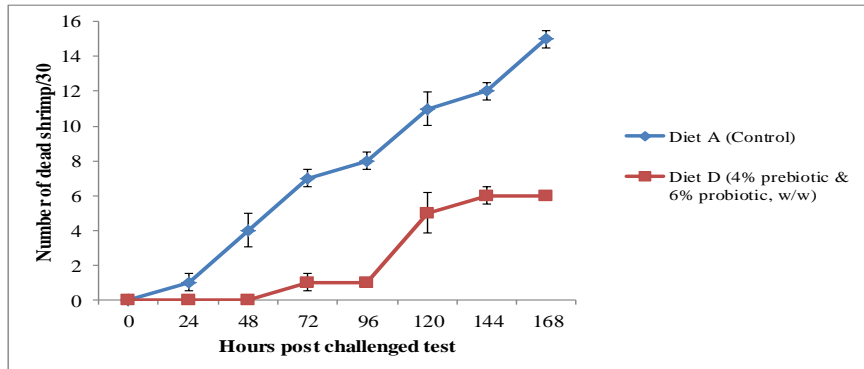


Figure 1. Mortality of shrimp after challenged test of two diets, i.e. basal diet and experimental diet (basal diet supplemented with 4% prebiotic and 6% probiotic *Bacillus* sp. D2.2)

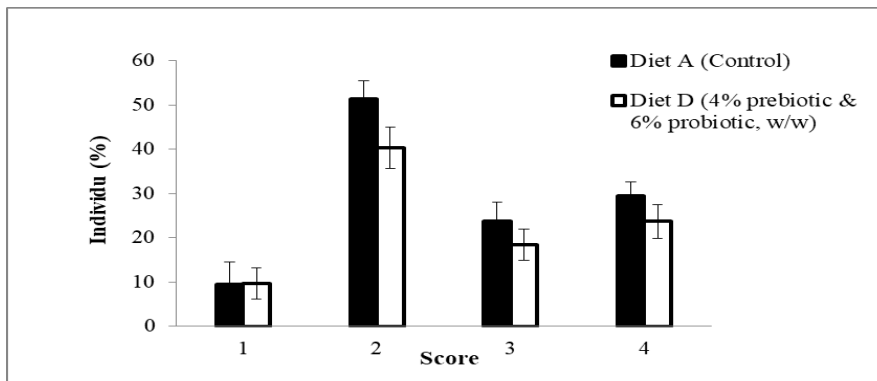


Figure 2. Scoring infection level of shrimps fed on Diet A (Control) and Diet D (4% prebiotic and 6% probiotic, w/w) after the challenged test. Data are expressed as Mean \pm SD. Score 1 to 4 indicated light, mild, heavy and very heavy infections, respectively.

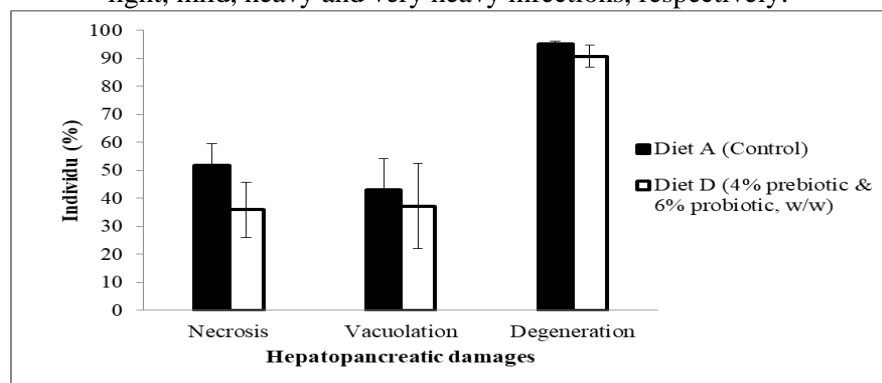


Figure 3. Percentage shrimps suffered from three parameters of hepatopancreatic damages fed on Diet A (Control) and Diet D (4% prebiotic and 6% probiotic, w/w) after seven days challenged test. Data are expressed as Mean \pm SD.

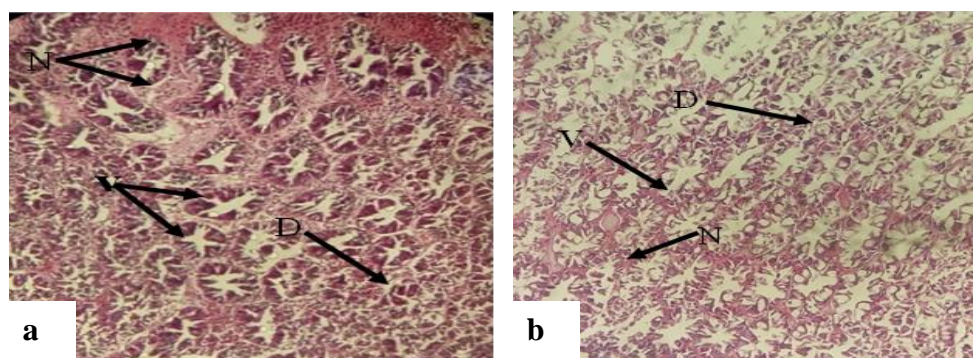


Figure 4. Comparative hepatopancreatic conditions of shrimps fed on Diet A, control (a) and Diet D 4% prebiotic and 6% probiotic, w/w (b), histologically. Necrosis (N), vacuolation (V), and degenerative hepatopancreas (D).

Discussion

The shrimp which were fed with experimental diets containing probiotic and or prebiotic supplements showed better growth performance compared to those in the control group. After 32 days of culture, weight gain and ADG of shrimp has improved while FCR has decreased. The highest growth was measured in the Diet D treatment group. Other research has suggested the similar condition; the dietary administration of synbiotics (application of probiotics together with prebiotics) can influence the growth performance of shrimp (Nurhayati *et al.*, 2015). Administration bacteria such as *Bacillus subtilis* (Zokaefar *et al.*, 2012) and *Pediococcus pentosaceus* (Adel *et al.*, 2017) via dietary supplements in white shrimp may activate the shrimps' digestive enzyme. The improvement in digestive enzyme activities allows the host to digest and absorb more nutrients (Cerezuola *et al.*, 2011). According to Ai *et al.* (2011), gastrointestinal bacteria take a part in the decomposition of nutrients, providing the host organism with physiologically active materials such as enzymes, amino acids, and vitamins thus enhance food utilization and digestion. The increase in growth and FCR as a result of dietary supplementation with synbiotics has been credited to physiological and biological changes in the gastrointestinal environment (Daniels *et al.*, 2010).

The changes are suggested to increase absorptive surface area and improve microvilli structure (Dimitroglou *et al.*, 2009). Another study indicates that the application of synbiotics allows for the more efficient conversion of ingested food into structural protein, with subsequent improved growth (Hai and Fotedar, 2009). The Previous study showed that prebiotic extracted

from sweet potatoes could support the growth of probiotic bacteria such as *Vibrio alginolyticus* SKT-b (Nurhayati *et al.*, 2015) and *Bacillus* sp. D2.2 (Harpeni *et al.*, 2016). In shrimp, Ringo *et al.* (2010) suggested that prebiotic can selectively support the growth of specific species of bacteria in their digestive tract.

Although survival rate of the shrimp fed with synbiotic supplementation was not significantly increase compared with control diet, this experimental diet (Diet D) could protect the shrimp with RPS value 53.48%. Other research reported RPS values of *L. vannamei* were 45.4 – 51.9 % while supplemented with probiotics and infected by *V. harveyi* (Liu *et al.*, 2014). Arisa (2011) reported that synbiotic may enhance the resistance of white shrimp to *V. harveyi*. Further study also revealed that combination of probiotic and prebiotic from sweet potato in shrimp diets can significantly improve disease resistance by reducing mortality and stimulating immunity of the shrimp (Nurhayati *et al.*, 2015). Meantime to death of the shrimp fed with Diet D could be longer than those consumed control diet. However, it seems that the Diet D only protect shrimp from bacterial attack. If the shrimp in Diet D still can be infected by *V. harveyi*, the using of Diet D will be not significantly different to the development of bacterial infection. Shrimps tend to survive longer in Diet D then those in control indicated that the diet was capable to block the bacterial attack at the beginning. However, the further development of bacterial infection has not been supported by significance of MTD. Therefore, mean time to death of the Die D was not significantly different than of the control diet. Mild infection and mostly degenerative hepatopancreas occurred in shrimp after the challenged test. Diet D has created less damage of hepatopancreas in shrimp. (Figure 3).

In conclusion, this study showed that probiotic *Bacillus* sp. D2.2 with prebiotic from sweet potato in pacific white shrimp (*Litopenaeus vannamei*) diets can significantly improve growth performance and could protect the shrimp from bacterial infection by presumably enhancing immunity and modulating microflora in the digestive tract of shrimp. Based on the result of growth performance (Table 1), longer time of MTD (Figure 1), lower level of infection and less of hepatopancreatic damages (Figure 2 and 3), the best dietary synbiotic in this study was basal diet supplemented with 6% probiotic and 4% prebiotic, w/w.

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