AACL BIOFLUX

Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

Dietary administration of crude lipopolysaccharide from *Vibrio harveyi* enhanced resistance of tiger shrimp, *Penaeus monodon* post larvae against white spot syndrome virus infection

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Abstract. This study evaluated the effects of *Vibrio harveyi* lipopoysaccharide (LPS) as immunostimulant to increase the resistance of post larvae *Penaeus monodon* against white spot syndrome virus (WSSV) infection. Shrimp were fed diets containing graded levels of *V. harveyi* LPS at 10, 25, 50, and 100 mg kg^{-1} diet, including a control, formulated without immunostimulant supplementation. A month-long feeding trial was conducted and resulted in 90-95% survival with no significant difference among treatments. Weight gain (WG), and specific growth rate (SGR) of shrimp were not affected by the test compound. Feed conversion ratio (FCR) was also not influenced by the levels of LPS in the diets. Infection challenge with WSSV by immersion showed that shrimp fed with 50 and 100 mg LPS kg^{-1} diet elicited significantly enhanced ($p < 0.05$) survival from the rest of the treatment groups. Survival rate was highest in shrimp fed with 50 mg immunostimulant (72%), followed by 100 mg LPS (60%) and 25 mg LPS (4%). Treatments receiving no dietary immunostimulant and those receiving 10 mg LPS exhibited 100% mortalities during the challenge. The present results suggest that *V. harveyi* LPS at a dose of 50 mg kg^{-1} diet is optimum to enhance resistance of post larvae *Penaeus monodon* against WSSV infection.
Key Words: immunostimulant, weight gain, specific growth rate, food conversion ratio.

Introduction. Among many microbial diseases known to infect farmed shrimp specifically *Penaeus monodon*, white spot disease (WSD) is considered the most detrimental, affecting almost all aspects of shrimp farming, including hatcheries and growout ponds around Asia (Chou et al 1995). The causative agent of the WSD is an envelope, non-occluded and rod-shaped baculovirus, white spot syndrome virus (WSSV) (Wang et al 1995). This virus has also been documented in shrimp larvae (Otta et al 1999).

A number of works have reported enhanced resistance of shrimp against various pathogens when fed with dietary immunostimulants. However, most of these studies dealt with adult and juvenile shrimp. Reports on the immunostimulation of shrimp larvae are rather scarce. Several earlier studies indicate that WSSV can infect and elicit significant mortalities at early post larval stages of shrimp (Venegas et al 1999; Manivannan et al 2002; Yoganandhan et al 2003). Stocking of post larvae in ponds at the early start of culture is one of the most critical phases in *P. monodon* farming. Post larvae are exposed to different environmental stressors associated with grow-out culture conditions that could weaken their immunological capacities, making them vulnerable to viral infections. Immunostimulants are eco-prophylactic agents which enhance the innate (non-specific) immune defenses of fish and crustaceans to provide resistance against potentially invasive and disease-causing organisms (Felix 2005; Citarasu et al 2006) and compounds of bacterial cells have been proven to be active immunostimulants in crustaceans (Boonyaraptalin & Boonyaraptalin 1995; Takahashi et al 2000). Few studies reported positive effects of immunostimulants on the antiviral defense of larval shrimp. Chang et al (1999) reported enhanced resistance of post larvae *P. monodon* against

WSSV following oral administration of β-1, 3-glucan. In another study, enhanced survival (72%) against WSSV infection was observed in *Penaeus indicus* post larvae fed with Indian medicinal herb *Phyllanthus niruri* in comparison with the control group, shrimp receiving no immunostimulant in the diet (Jayanthi et al 2013).

Gram-negative bacterium is a rich source of lipopolysaccharide (LPS) that can be found at the bacterial cell surface. It has been reported that this compound is involved in the activation of immune defenses of shrimp such as total haemocyte count, prophenol oxidase, phagocytosis, apoptosis, as well as activation of antiviral genes (Little et al 2005). Further, Cominetti et al (2002) reported that shrimp elicit different immune responses depending on the source of LPS, suggesting that there might be a putative role in microorganism recognition and affinity to the experimental animal. Similar results have been documented by Lorenzon et al (2002) when *Palaemon elegans* or rockpool prawn elucidated different responses when given LPS of *Salmonella enteritidis*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Escherichia coli*. These studies indicate that sources of LPS may elicit varying responses in shrimp.

Vibrio harveyi, a known pathogen of penaeids, is a rich source of LPS that can be found at the bacterial cell surface. To date, no studies have been documented regarding the use of *V. harveyi* lipopolysaccharide as immunostimulant on post larvae black tiger shrimp to enhance its resistance against WSSV infection. The main objective of this study is to evaluate the optimum dietary inclusion dose of *V. harveyi* LPS that would promote resistance of post larvae *P. monodon* against WSSV without affecting the overall growth performance of the test animals.

Material and Method

Experimental animals and design. The growth trial experiment was conducted from November to December 2011. *P. monodon* larvae (PL 12) were obtained from a commercial hatchery in Tigbauan, Iloilo, Philippines. Shrimp with size ranging from 7-8 mg (±0.5) were selected, acclimatized under laboratory conditions for one week, and maintained with a commercial diet. Following acclimation, shrimp were randomly distributed to fifteen 25-L aquaria at a density of one individual L^{-1}. These composed the 4 treatment groups (diet containing LPS at 10, 25, 50 and 100 mg kg^{-1} diet) and a control (diet without immunostimulant), all in three replicates, in a completely randomized design. Each tank was provided with sufficient aeration and salinity maintained at 30 ppt. Removal of excess feeds, fecal matters and replenishment of 40% of the rearing water were done daily to maintain good water quality. Ammonia and nitrite concentration were monitored and kept to minimum.

Treatment groups were fed the test diets at 10% of shrimp biomass given twice daily at 09:00 h and 17:00 h for a period of 30 days. At the end of the feeding trial, growth indices including weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were measured using the following formulas:

$$SGR(\%) = \frac{\ln(final\,weight) - \ln(initial\,weight) \times 100}{days\,of\,feeding}$$

$$WG(\%) = \frac{(final\,weight) - (initial\,weight) \times 100}{initial\,weight}$$

$$FCR = \frac{(feed\,given\,in\,dry\,weight)}{final\,weight - initial\,weight}$$

Extraction of LPS. The luminous pathogenic *V. harveyi* PN 9801 (de la Peña et al 2001) strain was obtained from the bacterial collection at Southeast Asian Fisheries Development Center (SEAFDEC) in Tigbauan, Iloilo, Philippines. The bacteria were inoculated in nutrient agar medium containing 1.5% NaCl by streaking and subcultured into fresh medium after 18-20 hours of incubation. Remaining cells were collected by scraping the agar surface using sterile spatula. Harvested cells were stored in -80°C until

use. Subsequent cultures were carried out using large agar plates for two weeks until desired amount of bacterial cells was obtained.

Crude LPS was isolated from this *Vibrio* species by hot chloroform-phenol extraction method described by Schill et al (1989) with slight modifications. In brief, 15 g of *V. harveyi* cells were concentrated and collected by centrifugation at 3000 x *g* for 30 min. Collected bacterial cells were washed by adding phosphate buffered saline (PBS), centrifuged at 1000 x *g* for 10 min and supernatant removed. The collected pellets were resuspended in 45 mL distilled water heated to 68°C. An equal volume (45 mL) of preheated 90% phenol was added. Bacterial cells were maintained at this temperature for 20 min under continuous stirring. The phenol-bacteria solution was collected by centrifugation at 3000 x *g* for 15 min and supernatant (LPS-saturated layer) was collected and dialyzed against running tap water for 24 hours. The dialyzed sample was then freeze dried and dissolved in a mixture of phenol, chloroform and petroleum ether at a ratio of 2:5:8 in a volume equal to that used in hot phenol extraction. These organic solvents were removed by rotary evaporation and LPSs were precipitated from the remaining solution by dropwise addition of distilled water. Precipitate was recovered and cleansed by centrifugation at 7000 x *g* for 30 min. The final LPS precipitate was collected, lyophilized, and stored at –20°C freezer until use. A dry weight yield of 500 mg of crude LPS was obtained.

Test diet formulation. The basal diet was formulated following from the formulation of Deshimaru et al (1984) containing 50.53% protein and 8.62% lipid and has been demonstrated to promote optimum growth in *P. monodon* (Table 1). The experimental bacterial LPS was incorporated into the basal diet at 10, 25, 50, and 100 mg kg^{-1} diet in a weight-to-weight basis. Cellulose was used to balance the nutrient levels of the test diets.

All the dry ingredients were mixed and ground in a mill, sieved to pass through a 120 micron sieve. In a separate container, all wet ingredients, including the test immunostimulant dissolved in lipid, were mixed and added with lecithin. The mixture was then added to the mixed dry ingredients. Feeds were pounded and hand-mixed until dough. Pelletizer with 2 mm die was used to produce spaghetti-like pellets and then oven-dried at 60°C. Diets were stored at -20°C until use. Prior to feeding to shrimp larvae, diets were pounded using mortar and pestle and passed through 200-micron sieve.

Table 1

Basal feed composition

Ingredients	Control	10 mg kg^{-1}	25 mg kg^{-1}	50 mg kg^{-1}	100 mg kg^{-1}
	Amount (g 100g^{-1})				
Danish fish meal	25.0	25.0	25.0	25.0	25.0
Shrimp meal	35.0	35.0	35.0	35.0	35.0
Gelatin	15.0	15.0	15.0	15.0	15.0
Cellulose	0.2	0.19	0.175	0.15	0.10
Immunostimulant	0.0	0.01	0.025	0.05	0.10
Wheat Flour	17.3	17.3	17.3	17.3	17.3
Vitamin mix	3.5	3.5	3.5	3.5	3.5
Danish Fish oil	3.0	3.0	3.0	3.0	3.0
Lecithin	1.0	1.0	1.0	1.0	1.0
Total	100.0	100.0	100.0	100.0	100.0

WSSV infection challenge test. After 30-day feeding trial, shrimp were subjected to disease resistance trial on January 2012. WSSV-infected shrimp were obtained from the collection of Southeast Asian Fisheries Development Center Aquaculture Department (SEAFDEC-AQD), Tigbauan, Iloilo, Philippines. The viral stock solution was prepared following the method of Chang et al (2003). Gills, lymphoid organs, and attached epidermis of the WSSV-infected shrimp sufficient to make 30% biomass were homogenized in 0.9% saline solution to make (1:9 w/v) viral stock solution. This viral concentration caused 50% mortality in challenged shrimp in 5 days in a preliminary study. The filtered supernatant was used as the viral stock solution.

The virulence of the virus prepared from this procedure was tested in shrimp fed with commercial diet. WSSV test challenge was carried out by immersion method described by Chotigeat et al (2004) with slight modifications. Five mL of viral stock solution was inoculated in 30-L tank holding the test animals. Following immersion in water containing WSSV inoculum for 2 hours, shrimp were transferred in clean tanks stocked with fresh, UV-filtered water. Fifty percent (50%) mortality was obtained in 5 days. The same procedure was carried out in all treatment groups with. The same batch of WSSV-infected shrimp from SEAFDEC-AQD was used for viral stock preparation. An unchallenged control was carried out in a separate tank fed with basal diet. Challenged shrimp were fed with their corresponding treatment diets throughout the challenge period. Removal of wastes and 30% water change were done daily. Test animals were monitored for 12 days after viral exposure and checked daily for mortalities. Dead and moribund shrimp were subjected for PCR analysis for WSSV confirmation at SEAFDEC, Tigbauan, Iloilo, Philippines. Using a primer for WSSV, electrophoresis of PCR products revealed heavy bands in shrimp infected with WSSV.

Statistical analysis. One-way ANOVA, through SPSS 16.0, was used to check significant difference in the growth performance of *P. monodon* as an effect of different treatments. WSSV challenge results were processed using Chi-Squared Test to compare the efficacy of the test compound in eliciting protection against WSSV infection.

Results and Discussions. Final average body weight of shrimp was in the range of 0.21–0.27 g. Shrimp receiving 10 mg LPS had the highest gain in weight but were not significantly different from the other treatments (Figure 1). Similar trend can be observed in the SGR of the test animals (Figure 2). Survival of shrimp after the month-long feeding trial was 96-100%, indicating no toxicity effects of the compound in shrimp. No significant difference was observed in the feed conversion ratio of all test groups (Figure 3). FCR values ranged from 1.72–1.79. WSSV challenge results showed that mass mortality commenced on the 7th day of post-challenge test in the control group (Figure 4). One hundred percent mortality was finally recorded on the 10th and 11th day for the control and 10 mg treatment group, respectively. No more mortalities were recorded after day 10 in shrimp fed with 50 and 100 mg LPS. Survival at the end of the experiment was highest in the group receiving 50 mg LPS (72%) with no significant difference from group fed with 100 mg LPS (60%). All dead and moribund shrimp from the treatment groups were one-step positive (982 bp) to WSSV by PCR analysis. Unchallenged shrimp were negative for WSSV. The viral test challenge was terminated on the 12th day because no more deaths were reported, indicating a stable survival in the treatment groups.
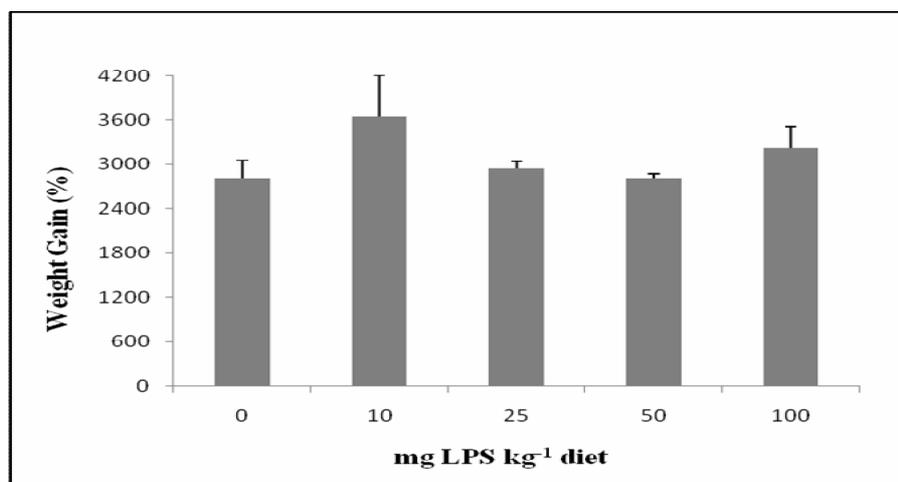

Figure 1. Weight gain (% of the initial weight) of *P. monodon* fed varying levels of dietary *V. harveyi* lipopolysaccharide for 30 days. Bars bearing similar superscripts are not significantly different ($a = 0.05$).

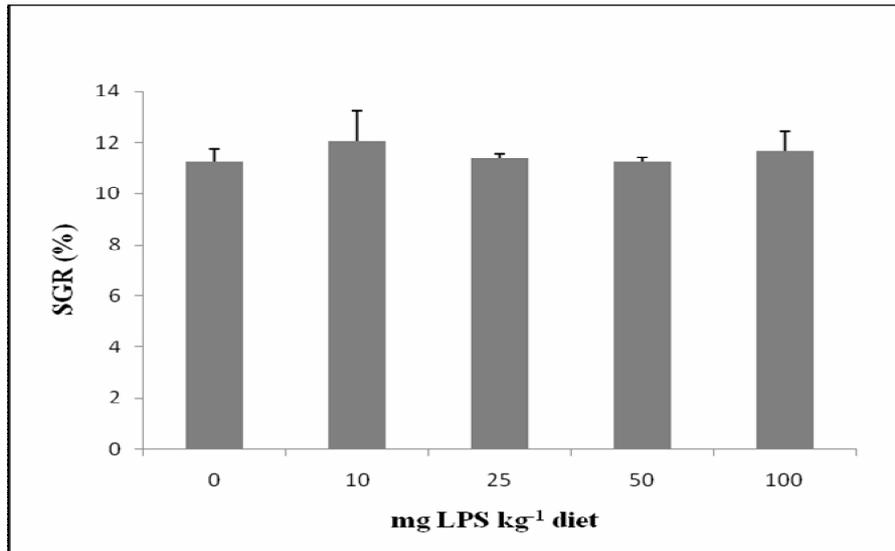Figure 2. Specific growth rate (% day^{-1}) of *P. monodon* fed varying levels of dietary *V. harveyi* lipopolysaccharide for 30 days. Bars bearing similar superscript are not significantly different (a = 0.05).
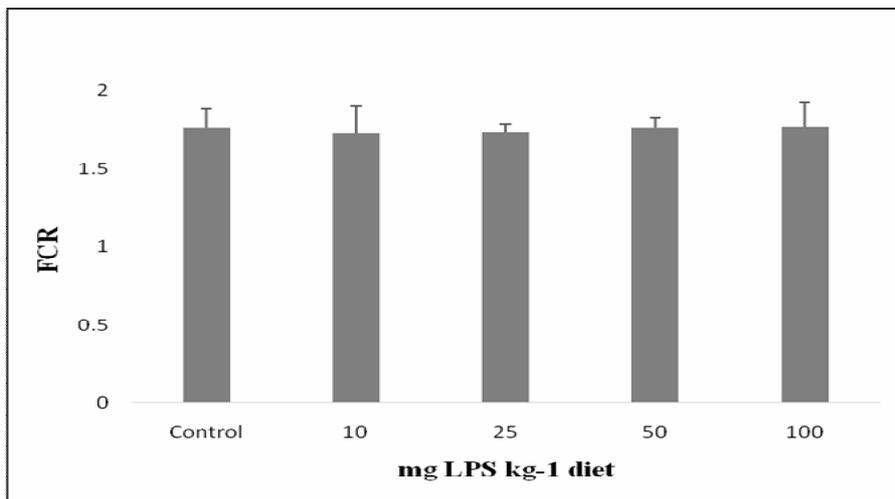

Figure 3. Feed conversion ratio (FCR) of *P. monodon* fed varying levels of dietary *V. harveyi* lipopolysaccharide for 30 days. Bars bearing similar superscript are not significantly different (a = 0.05).
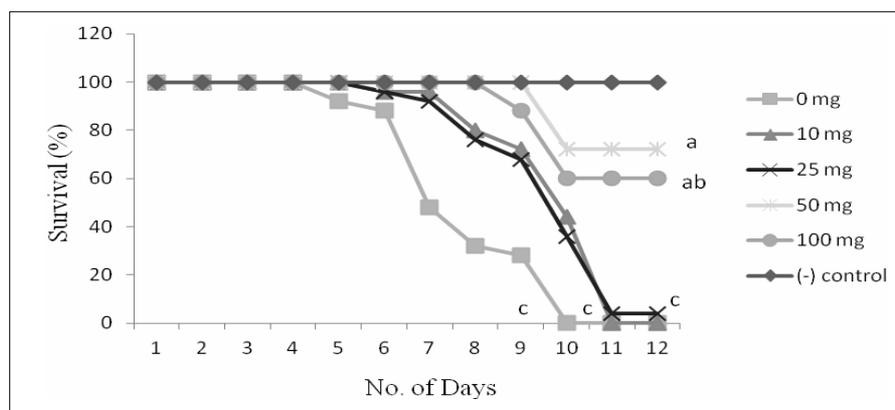

Figure 4. Survival (%) of *P. monodon* challenged with WSSV infection for 12 days. Unchallenged group served as negative (-) control. Lines bearing similar superscript are not significantly different (a = 0.05).

To date, several researches involving immunostimulant-enriched diets use to increase immune resistance against diseases in crustaceans, have been well-documented. These compounds are proven to elicit immunostimulation in crustaceans, primarily in shrimp (Newman 1999). These immunostimulants are known to boost the immune defense system of the organism by acting on the cellular and humoral pathways that direct certain actions in eliminating invading pathogens. The present study evaluated the effects of dietary *V. harveyi* LPS as an immunostimulant on the disease resistance of post larvae *P. monodon* against WSSV infection.

After the feeding trial, growth patterns of experimental animals receiving the test immunostimulant are not statistically different from the control, suggesting that this compound is not toxic. Similar growth results have been documented by Azad et al (2005) on the immunostimulation of heat-killed *Vibrio anguillarum* on post larvae *P. monodon*. FCR values in the present study did not differ statistically in all test groups, indicating that the test immunostimulant did not hamper nutrient utilization of the diet. Survival of shrimp after the growth trial was also high (96-100%) and not affected by LPS supplementation. These findings suggest that the test compound at all levels was not inhibitory to the growth and overall metabolism of shrimp.

WSSV challenge test showed that highest survival (72%) was observed in the group receiving the 50 mg LPS-enriched diet. The control group had the highest mortalities at the shortest time. The positive effects of dietary LPS against microbial infections have been documented in earlier studies and dosage of administration was found to be a critical factor in achieving optimum results. Newman (2000) administered *E. coli* LPS for juvenile penaeid shrimp at 20, 40 and 100 mg kg^{-1} body weight and later challenged with WSSV. Highest survival was reported at 20 mg (75%) while survival rates of shrimp fed with 40 and 100 mg LPS were recorded at 64.7% and 52.9%, respectively. Ulvan extract incorporated at 1000 mg kg^{-1} diet reported prolonged survival of *P. monodon* against WSSV infection and significantly enhanced cellular immunity of animals (Declarador et al 2014). An improved survival (75% higher than the control) against penaeid acute viremia virus was evident in adult *Marsupenaeus japonicus* fed diet containing *Pantoea agglomerans* LPS at a dose of 20 μg kg^{-1} body weight (Takahashi et al 2000). A similar study by Felix (2005) reported that at optimum of dose of 30 mg *E. coli* LPS kg^{-1} diet was enough to protect juvenile *P. monodon* against *Vibrio parahaemolyticus* infection with a reported survival of 75%. However, when LPS was administered at a higher dose, lower survival was obtained. This result suggests that higher concentration of LPS on feed beyond the assumed optimum level would not have any significance. In the present study, shrimp fed with 50 mg dietary LPS elicited the highest protection against WSSV infection with 72% survival. At a higher dose of 100 mg, survival fell to 60%. The disparities on the optimum dose reported in these studies may be accounted to the varying sources of LPS and the life stages and species of the test animals. Moreover, most of the previous studies were done based on dosage in terms of body weight of shrimp which limits the practical applications of this earlier finding in the actual field settings. In the present study, the test compound was incorporated in the diet on a percent basis. The differences in results obtained with the previous findings may have been due to the differences in dietary inclusion of this immunostimulant.

Expression of immune-related genes has been evident in the early stages of larval and post larval *P. monodon* (Jiravanichpaisal et al 2007). Hemocyanin and ferritin genes were up-regulated following infection of *V. harveyi* in post larvae black tiger shrimp (Nayak et al 2010). Haemocyanin activates prophenoloxidase activity by partial proteolysis and sometimes, can function as phenoloxidase enzyme by itself under favorable conditions (Decker & Rimke 1998). Phenoloxidase was reported to be an important defense molecule against WSSV (Zhang et al 2004). In the study of Misra et al (2004), immunostimulation of *Macrobrachium rosenbergii* post larvae by β-glucan displayed significantly enhanced lysozyme activity that eventually induced protection against *Vibrio* infection. Lysozyme is key protein component of crustacean humoral defense system produced by activated haemocytes. The improved WSSV resistance of shrimp fed with LPS in the present work suggests active stimulation of immunological responses in shrimp larvae.

In the present study, the high survival rate of shrimp receiving LPS-supplemented diets against WSSV infection could be attributed to the protective effects of the test immunostimulant that may have induced certain immune responses in shrimp. However, these immune indices were not measured.

Conclusions. Dietary administration of *V. harveyi* LPS at a dose of 50 mg kg^{-1} diet provided the optimum protection of post larvae *P. monodon* against WSSV infection. This indicates the potential of the test immunostimulant in improving shrimp farming. Although these results are encouraging, further studies are needed to evaluate the effects of *V. harveyi* LPS on the immune responses of shrimp in order to fully elucidate and understand the mechanisms involved in the viral resistance of the test animals receiving the immunostimulant. Frequency and length of feeding of the optimum dose should also merit consideration.

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How to cite this article:
Genio J. E. J., Traifalgar R. F. M., Corre Jr. V. L., 2014 Dietary administration of crude lipopolysaccharide from *Vibrio harveyi* enhanced resistance of tiger shrimp, *Penaeus monodon* post larvae against white spot syndrome virus infection. AACL Bioflux 7(5):342-350.