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Efficacy of Dietary Immunostimulants to Enhance the Immunological Responses and Vibriosis Resistance of Juvenile *Penaeus monodon*

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ABSTRACT

Immunoprophylaxis is widely acknowledged as a potential approach in the control of disease outbreaks in shrimp aquaculture. The present study evaluates the efficacy of dietary immunostimulants such as *Fucus vesiculosus* fucoidan (FCD), *Eucheuma cottonii* κ-carrageenan (CAR), heat-killed *Vibrio harveyi* cells (VHK), *Vibrio harveyi* lipopolysaccharide (VLP) and yeast β-glucan (BGN) in enhancing the immunological response and the resistance of juvenile *Penaeus monodon* against *Vibrio harveyi* infection. Experimental diets were incorporated with the test immunostimulants at 2.0 g kg^{-1} and a two-week feeding trial was conducted. Following the feeding trial, the experimental shrimp were challenged with pathogenic *Vibrio harveyi* through intramuscular injection and immunological responses were measured. Results showed significant enhancement of shrimp survival after *Vibrio harveyi* infection in the FCD (82.2%), BGN (84.4%) and VLP (84.4%) dietary treatments as compared to the control (51.1%), CAR (55.6%) and VHK (42.2%) treatments. The high survival against *Vibrio harveyi* infection in these treatments is associated with the prominent enhancement of hemocyte phagocytosis (VLP: 118.63±4.42; BGN: 120.01±0.60; FCD: 127.41±1.52) and serum antibacterial activities (VLP: 9.02±0.21; BGN: 9.19±0.012; FCD: 9.61±0.31) as compared to the hemocyte phagocytosis (CAR: 103.10±0.59; VHK: 108.01±2.02; Control: 79.170±1.06) and serum antibacterial activities(CAR: 7.61±0.01; VHK: 7.90±0.16; Control: 5.01±0.09) of the control and the other treatments. These results suggest that dietary supplementation of VLP, BGN and FCD at a dose of 2.0 g kg^{-1} can be used to boost the immunological response and enhance the resistance of *P. monodon* against *Vibrio harveyi* infection.

Key words: Immunostimulants, *Penaeus monodon*, *Vibrio harveyi*, lipopolysaccharide, fucoidan, β-glucan

INTRODUCTION

At present, outbreaks of microbiological diseases including viral, fungal and bacterial infections that threaten the sustainability and profitability of crustacean aquaculture have become an issue of global concern (Raa *et al.*, 1992; Afsharnasab *et al.*, 2009; Fouzi *et al.*, 2012). Luminous Vibriosis, caused by *Vibrio harveyi* is a serious disease problem in *Penaeus monodon* aquaculture. This disease has been implicated in considerable financial losses in shrimp farming activities of several countries (Pizzutto and Hirst, 1995; Robertson *et al.*, 1998; Pasharawipas *et al.*, 2011) including the Philippines (Lavilla-Pitogo *et al.*, 1990). The Philippine isolate of *Vibrio harveyi* is

highly pathogenic, can infect shrimp at various life stages and can cause up to 100% mortality even at bacterial cell densities as low as 10^2 colony forming units (CFU) mL^{-1} (Lavilla-Pitogo *et al.*, 1990). Disease symptoms are characterized by lethargy, anorexia, muscle opacity, atrophied hepatopancreatic tubules and luminescence of the host (Jiravanichpaisal *et al.*, 1994; Lavilla-Pitogo *et al.*, 1998).

The use of antibiotics was found to be a possible means of control (Baticados and Paclibare, 1992) but its use was eventually restricted due to the increased risk of developing antibiotic resistant microbial strains and being an environmental hazard (Karunasagar *et al.*, 1996). Consequently, alternative approach including the use of probiotics (Soundarapandian and Sankar, 2008), bacteriophage (Srinivasan *et al.*, 2007) and the use of immunostimulants have been advocated (Anderson, 1992; Raa *et al.*, 1992). To date, a number of substances have been reported as effective immunostimulants for different species of cultured shrimp. Inactivated microbes and their cellular components, seaweed polysaccharides, herbal extract and synthetic chemicals are some of the many substances reported to enhance disease resistance of cultured aquatic animals (Sakai, 1999; John *et al.*, 2011; Balasundaram *et al.*, 2012). In spite of these promising results, reports of substances that can improve resistance of *P. monodon* against *V. harveyi* have been relatively few. In addition, the differences in experimental animals (species, stage), purity, dose of immunostimulant and administration methods used in previous studies make selection of an appropriate immunostimulant for *P. monodon* difficult.

It is evident that different immunostimulants activate different aspects of the immune system and their efficacies vary with different host species, delivery methods and types of pathogens (Sakai, 1999; Smith *et al.*, 2003; Agrawal *et al.*, 2010; Agouz and Anwer, 2011). Hence, the present work was conducted to evaluate the efficacy of dietary administered immunostimulants such as, *Eucheuma cottonii* κ-carrageenan (CAR), *Focus vesiculosus* fucoidan (FCD), *V. harveyi* lipopolysaccharide (VLP), yeast β-glucan (BGN) and heat-killed *V. harveyi* cells (VHK) to strengthen the immune response of juvenile *P. monodon* and to enhance its survival against *V. harveyi* infection. These compounds have been known as potent activators of the non-specific immune response of terrestrial animals, fish and some crustacean species (Fujiki *et al.*, 1997; Alabi *et al.*, 1999; Crosbie and Nowak, 2004; Yuan and Song, 2005; Qin *et al.*, 2007; Cheng *et al.*, 2008; Parmentier *et al.*, 2010) but their efficacy on *P. monodon* by dietary administration have not been fully evaluated.

Dietary supplementation was used because it has been suggested as the most economical and feasible way of delivery in large-scale culture systems (Siwicki *et al.*, 1994; Sakai, 1999; Singh *et al.*, 2011). Furthermore, in order to understand the aspect of the immune system acted on by these substances, immune indices representing both the humoral and cellular immune responses were also evaluated.

MATERIALS AND METHODS

Immunostimulants: Fucoidan from *Fucus vesiculosus*, κ-carrageenan from *Eucheuma cottonii* and yeast β-glucan were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Pathogenic *V. harveyi* strain PN9801 isolated from diseased *P. monodon* was obtained from the bacterial collection of SEAFDEC Aquaculture Department in the Philippines. Heat-killed bacteria were prepared by growing *V. harveyi* on trypticase soy broth (BBL) supplemented with 2% NaCl for 24 h at 28°C with mild shaking. Bacterial cells were collected by centrifugation at 10, 000 rpm for 20 min, washed three times with phosphate buffered saline (PBS; pH 7.2), heat-killed at 80°C for

30 min, lyophilized and stored at 4°C until use. Sterility was confirmed by inoculating the heat-killed bacteria in thiosulfate citrate bile salt sucrose agar (TCBS, Difco) media, incubated at 28°C for 3 days and no bacterial growth was observed. Crude lipopolysaccharide (LPS) was isolated from log-phase culture of *V. harveyi* by hot phenol extraction following the procedure described by Schille *et al.* (1989).

Test diets: The basal diet was prepared following the formulation of Deshimaru *et al.* (1984), containing 46.7% protein, 8.2% fat, 15.2% ash 12% moisture and has been demonstrated to be nutritionally adequate for the optimal growth of juvenile *P. monodon*. The basal diet was used as control diet and the test immunostimulants were separately incorporated to the basal diet at 2 g kg^{-1} to formulate the other five experimental diets. This concentration was based on a previous study showing that immunostimulant supplementation at 2 g kg^{-1} provided optimal immune enhancing effects in *P. monodon* (Chang *et al.*, 1999, 2003; Lopez *et al.*, 2003).

Experimental animals and feeding: This study was conducted in 2004 (January to April) at the research facilities of the Institute of Aquaculture, College of Fisheries and Ocean Sciences University of the Philippines in Visayas. Juvenile *Penaeus monodon* were obtained from a population reared in 10 ton-capacity outdoor tanks and apparently healthy shrimp weighing 6.4±1.2 g were selected, acclimatized in laboratory conditions and fed with control diet for 10 days. Following acclimatization, shrimp were randomly distributed into eighteen 35 L aquaria at a density of 25 shrimp $aquarium^{-1}$, constituting the six dietary treatments including a control, all in triplicate.

Treatment groups were fed the test diets at 5% of shrimp biomass given twice daily at 08:00 and 16:00 h for a period of two weeks. Removal of wastes and replenishment of 40% rearing water were done daily to maintain optimum water quality. During the feeding trial, seawater temperature ranged from 27-28°C, pH from 7.8-8.2, salinity from 32-34$^{0}/_{00}$ and dissolved oxygen from 6.5-7.1 mg L^{-1}. At the termination of the feeding trial, surviving shrimp on each tank were counted, weighed and growth was calculated as:

$$\text{Weight gain (\%)} = \frac{\text{Final weight-Initial weight}}{\text{Initial weight}} \times 100$$

Challenge test: After the feeding trial, 45 shrimp from each dietary group were separated, maintained in separate aquaria and used for the challenge test. Individual shrimp were intramuscularly injected with 30 µL of *V. harveyi* suspension in Phosphate Buffered Saline (PBS) at a dose of 1×10^{6} CFU shrimp. This challenge dose was previously standardized to effect a 50-60% mortality in shrimp fed the control diet. Non-infected shrimp group, injected with PBS were also included. Mortalities were recorded daily for 12 days and the cause of death was verified by re-isolation of the bacteria from the hepatopancreas of moribund shrimp.

Immune assays

Extraction of haemolymph and total hemocyte counting: Haemolymph were collected from the ventral sinus cavity of shrimp using 1 mL tuberculin syringe (26 gauge) containing chilled (4°C) anticoagulant solution (10 mM $EDTA\text{-}Na_2$, 45 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3)

at a proportion of one part hemolymph to three parts anticoagulant solution. Haemolymph was centrifuged at 3 000 rpm for 10 min at 4°C, supernatant was discarded, hemocyte pellet were rinsed twice and re-suspended in shrimp salt solution (45 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3) (Vargas-Albores *et al.*, 1993). Hemocytes were counted using a hemacytometer and viability was assessed by trypan blue exclusion (Weeks-Perkins *et al.*, 1995). Serum was prepared by collecting haemolymph without anticoagulant, allowed to clot and subjected to a freeze thaw cycle 5 times to induce cell lysis and serum collected by centrifugation at 10 000 rpm for 10 min at 4°C (Sritunyalucksana *et al.*, 1999). Seven shrimp from each treatment were used for the assessment of each immunological parameter. All assays were conducted in triplicate.

Hemocyte phagocytosis activity: Phagocytic activity was measured following the method described previously (Weeks-Perkins *et al.*, 1995). Hemocyte suspension (100 µL) was allowed to adhere on a microscope slide, added with 50 µL of phloxine red-stained yeast cell suspension and incubated in a humidified chamber for 30 min at 25°C. The cells were fixed with 2.5% glutaraldehyde, stained with Wright stain and phagocytic hemocytes were observed using an epifluorescence microscope (Hund Wetzlar, Germany). Phagocytic hemocytes and number of ingested yeast cells were counted from 200 hemocytes and Phagocytic Index (PI) was calculated as described by Itami *et al.* (1994).

Hemocyte respiratory burst activity (NBT reductase assay): The reduction of nitro blue tetrazolium (NBT) to formazan was quantified as a measure of hemocyte superoxide anion production (Song and Hsieh, 1994). Hemocyte suspension (100 µL) was deposited in a 96-well microtiter plates to promote cell adhesion, washed with Leibovitz L-15 medium (Sigma) and stained with 100 µL of 0.1% Nitro blue tetrazolium (NBT) and 0.01% Phorbol Myristate Acetate (PMA) in Leibovitz L-15 at 25°C for 30 min. The supernatant was removed, hemocytes fixed with 70% methanol and air-dried. The formazan formed was dissolved in 120 µL of 2 M KOH and 140 µL of DMSO and quantified by reading the optical density at 630 nm. Control reactions were conducted without the stimulation of hemocytes with PMA. The ratio of the optical density reading of the stimulated hemocytes to the optical density of the control hemocytes was expressed as relative respiratory burst activity (Campa-Cordova *et al.*, 2000).

Serum antibacterial activity: One hundred microliter of *V. harveyi* suspension (1×10^5 CFU mL^{-1}) in PBS was added with equal volume of shrimp serum and incubated with shaking at 28°C for 1 h. Then, 50 µL of the sample samples was removed, serially diluted with sterile saline and a 50 µL aliquot of each dilution was spread on TCBS agar plates. Plates were incubated at 28°C for 24 h and bacterial colonies counted. Bacterial suspension added with PBS instead of serum served as the control. Serum antibacterial activity was calculated as described by Alabi *et al.* (2000). One unit of serum antibacterial activity was defined as a difference of 0.01 between R (control) and R (treatment), where R represents the ratio of change in bacterial counts from the beginning and end of the reactions and calculated as:

$$R=\frac{A_t-A_0}{A_0}$$

where, A_0 and A_t are the bacterial counts at the beginning and end of the reactions, respectively.

Serum hemolytic activity: Serum hemolytic assays were performed following a modification of the method described by Li *et al.* (2006). Shrimp serum (100 µL) was added to 900 µL of 3% chicken erythrocytes in saline (0.15 M NaCl), incubated at 25°C for 1 h, centrifuged at 500 rpm for 1 min and the released hemoglobin were collected and optical density was read at 540 nm. Control reactions were conducted using saline instead of serum. The 100% hemolytic reaction was carried out by adding deionized water to erythrocytes instead of saline. The results are expressed as percentage of hemolysis (Guzman *et al.*, 1993).

Statistical analyses: Chi-square test (χ^2) was used to compare the protective efficacy of the test substances. Data on growth and immunological responses were analyzed by One way analysis of variance (ANOVA) and Tukey's honest significant difference test was used to compare differences among treatment means (SPSS 10, SPSS, Inc., USA). All probability values were set at 0.05 level of significance.

RESULTS

Growth: At the end of the two-week feeding trial, all dietary treatments promoted positive growth but were not statistically different. Highest growth value was observed in VLP treatment with 33.50±0.8% followed by VHK with 33.23±1.7%. The control treatment exhibited a 32.97±1.3% growth, CAR with 32.77±1.1%, BGN with 32.14±0.5% and fucoidan treatment has the lowest growth value of 32.06±1.1% (Fig. 1). This indicates that the test immunostimulants were acceptable, not toxic and did not promote stress to the test animals at the concentration tested. Further, no mortalities were observed among the treatments throughout the experimental period.

Challenge test: Protective effects of the test compounds were evaluated by challenge test against *V. harveyi* and surviving shrimp were recorded until 12 days post-challenge (Table 1). No

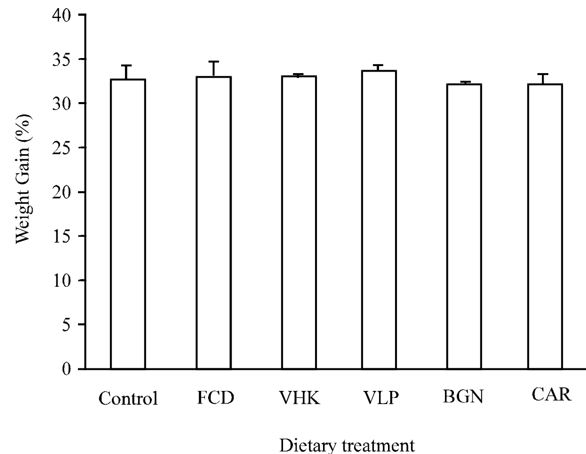

Fig. 1: Growth of shrimp fed diets supplemented with the test compounds for 14 days. Value are Mean±SE of three replicate groups per treatment. Control, diet without immunostimulant (32.97±1.3); VHK, heat-killed *Vibrio harveyi* cells (33.23±1.7); CAR, κ-carrageenan (32.77±1.1); VLP, Crude *Vibrio harveyi* lipopolysaccharide (33.50±0.8); BGN, β-glucan (32.14±0.5); FCD, fucoidan (32.06±1.1)

Table 1: Survival of shrimp in different treatment groups 12 days after the challenge test with *V. harveyi*

Treatments	No. alive shrimps	No. dead shrimps	Survival (%)
Control	23	22	51.1
VHK	19	26	42.2
CAR	25	20	55.6
VLP	38	7	84.4*
FCD	37	8	82.2*
BGN	38	7	84.4*
Buffer control	45	0	100*

*Significant difference from the control treatment (χ^2 test; $p<0.05$). Control, diet without immunostimulant; VHK, heat-killed *Vibrio harveyi*; CAR, κ-carrageenan; VLP, crude *Vibrio harveyi* lipopolysaccharide; FCD, fucoidan; BGN, β-glucan; Buffer control, shrimps injected with phosphate buffered saline instead of *V. harveyi*, n = 45

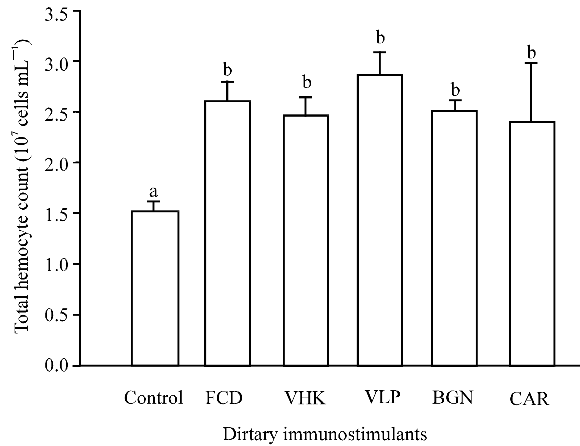

Fig. 2: Total hemocyte count of shrimp fed diets supplemented with the test compounds for 14 days. Value are Mean±SE of triplicate assays per shrimp (N = 7 shrimp) on each treatment group. Bars bearing similar superscript letters are not statistically different ($p<0.05$). Control, diet without immunostimulant ($1.50\pm0.12\times10^7$); VHK, heat-killed *Vibrio harveyi* ($2.46\pm0.19\times10^7$); CAR, κ-carrageenan ($2.40\pm0.58\times10^7$); VLP, Crude *Vibrio harveyi* lipopolysaccharide ($2.86\pm0.23\times10^7$); BGN, β-glucan ($2.50\pm0.12\times10^7$); FCD, fucoidan ($2.60\pm0.20\times10^7$)

mortalities were observed in PBS-injected control group. Significantly higher survival values were recorded in BGN (84.4%), FCD (82.2%) and VLP (84.4%) treatments in comparison with CAR (55.6%), VHK (42.2%) and control treatments (51.1%). Percentage survival of shrimp in CAR and VHK treatments were not significantly different from that of the control group. Luminescent colonies of *Vibrio harveyi* were isolated from the hepatopancreas of deceased shrimp.

Total hemocyte count: Shrimp in treatments receiving the test compounds exhibited a significantly higher total hemocyte counts as compared to the control (Fig. 2). However, no significant differences were observed in the total hemocyte counts of shrimp receiving the different immunostimulants. Highest value of total hemocyte count was observed in VLP ($2.86\pm0.23\times10^7$),

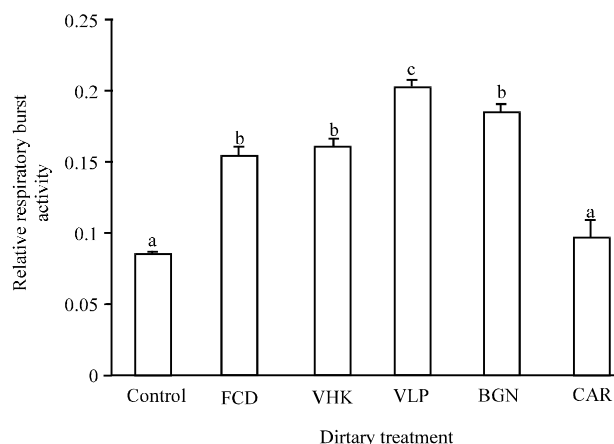

Fig. 3: Hemocyte respiratory burst activity of shrimp fed diets supplemented with the test compounds for 14 days. Values are Mean±SE of triplicate assays per shrimp (N= 7 shrimp) on each treatment group. Bars bearing similar superscript letters are not statistically different ($p<0.05$). Control, diet without immunostimulant (0.08±0.002); VHK, heat-killed *Vibrio harveyi* (0.16±0.006); CAR, κ-carrageenan (0.10±0.012); VLP, Crude *Vibrio harveyi* lipopolysaccharide (0.20±0.006); BGN, β-glucan (0.18±0.007); FCD, fucoidan (0.15±0.007)

followed by FCD ($2.60\pm0.20\times10^7$), BGN ($2.50\pm0.12\times10^7$), VHK ($2.46\pm0.19\times10^7$) and CAR ($2.40\pm0.58\times10^7$), respectively. The control treatment ($1.50\pm0.12\times10^7$) has the lowest total hemocyte count.

Hemocyte respiratory burst activity: Respiratory burst activity of shrimp in VLP treatment (0.20±0.006) was significantly higher than those of other treatments (Fig. 3). While respiratory burst activities of shrimp in BGN (0.18±0.007), VHK (0.16±0.006) and FCD (0.15±0.007) treatments were not significantly different but were significantly higher than those of CAR (0.10±0.012) and the control treatment (0.08±0.002).

Hemocyte phagocytosis activity (PI): All treatments receiving the test compounds elicited a significant enhancement of the hemocyte phagocytic activity as compared to the control (Fig. 4). Among the substances tested, FCD treatment elicited the highest PI (127.41±1.52) but not significantly different from BGN (120.01±0.60) and VLP (118.63±4.42) treatments. CAR (103.10±0.59) and VHK (108.01±2.02) treatments elicited a moderate response, which were significantly higher than the control but lower compared to FCD, BGN and VLP treatments. The control group (79.170±1.06) showed the lowest phagocytic response.

Serum antibacterial activity: FCD treatment (9.61±0.31) exhibited the highest serum antibacterial activity followed by BGN (9.19±0.012) and VLP (9.02±0.21) treatments. No significant differences in serum antibacterial activity were noted among these treatments. Groups receiving supplemented CAR (7.61±0.01) and VHK (7.90±0.16) showed significantly higher serum

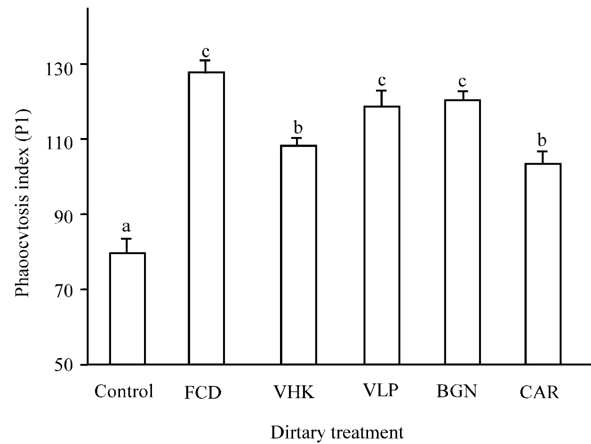

Fig. 4: Hemocyte phagocytosis activity of shrimp fed diets supplemented with the test compounds for 14 days. Values are Man±SE of triplicate assays per shrimp (N = 7 shrimp) on each treatment group. Bars bearing similar superscript letters are not statistically different ($p<0.05$). Control, diet without immunostimulant (79.170±1.06); VHK, heat-killed *Vibrio harveyi* (108.01±2.02); CAR, κ-carrageenan (103.10±0.59); VLP, Crude *Vibrio harveyi* lipopolysaccharide (118.63±4.42); BGN, β-glucan (120.01±0.60); FCD, fucoidan (127.41±1.52)

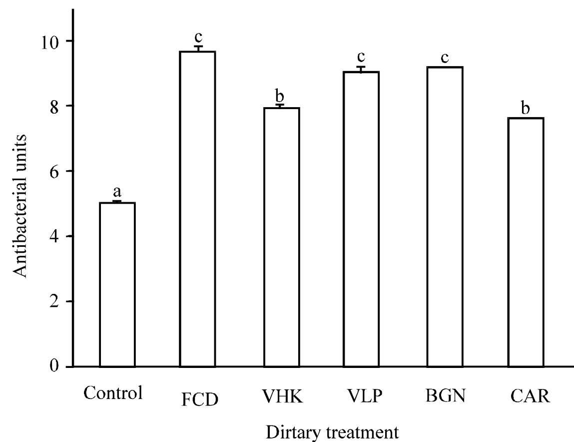

Fig. 5: Serum antibacterial activity of shrimp fed diets supplemented with the test compounds for 14 days. Values are Mean±SE of triplicate assays per shrimp (N = 7 shrimp) on each treatment group. Bars bearing similar superscript letters are not statistically different. Control, diet without immunostimulant (5.01±0.09); VHK, heat-killed *Vibrio harveyi* (7.90±0.16); CAR, κ-carrageenan (7.61±0.01); VLP, Crude *Vibrio harveyi* lipopolysaccharide (9.02±0.21); BGN, β-glucan (9.19±0.012); FCD, fucoidan (9.61±0.31)

dietary antibacterial activities than groups fed the control diet, but significantly lower compared to those of shrimp fed diets supplemented with FCD, BGN and VLP. The control group (5.01±0.09) showed the lowest serum antibacterial activity (Fig. 5).

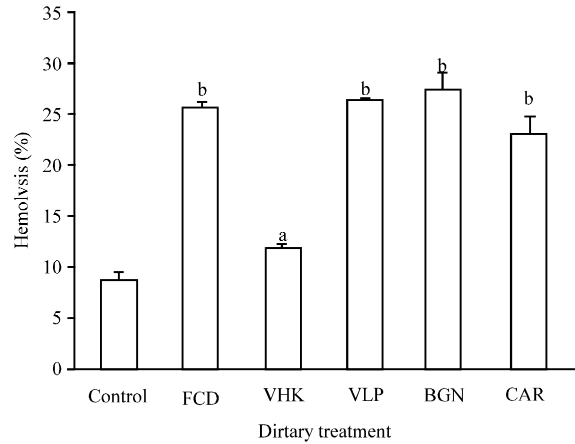

Fig. 6: Serum hemolytic activity of shrimp fed diets supplemented with the test compounds for 14 days. Values are Mean±SE of triplicate assays per shrimp (N = 7 shrimp) on each treatment group. Bars bearing similar superscript letters are not statistically different. Control, diet without immunostimulant (8.73_}0.93); VHK, heat-killed *Vibrio harveyi* (11.68±0.59); CAR, κ-carrageenan (11.68±0.59); VLP, Crude *Vibrio harveyi* lipopolysaccharide (25.98±0.17); BGN, β-glucan (27.23±1.52); FCD, fucoidan (25.50±0.55)

Serum hemolytic activity: Hemolytic activity was the highest in shrimp fed BGN (27.23±1.52) supplemented diets but not significantly different from those fed with FCD (25.50±0.55), CAR (11.68±0.59) and LPS (25.98±0.17) supplemented diets. The control (8.73±0.93) and VHK (11.68±0.59) groups exhibited significantly lower levels of serum hemolytic activity (Fig. 6).

DISCUSSION

The efficacy of dietary supplemented BGN, FCD, VLP, VHK and CAR to promote resistance against *V. harveyi* infection in *P. monodon* were evaluated in this study. Among these substances, BGN, FCD and VLP were found effective in promoting a protective immune response as measured in terms of percentage survival following a challenge test. CAR and VHK treatments exhibited lower protective effects that were comparable with the control treatment. In this study, all treatment groups exhibited a statistically similar growth response and no mortalities occurred throughout the experimental period. This suggests that the immunostimulants were acceptable and probably not inhibitory to the overall metabolism of the test animals at the given dose and duration of the experiment.

The enhanced protection conferred by BGN is associated with the enhancement of both the humoral and cellular immune responses. Relative to the control, hemocyte count, hemolytic, phagocytosis, respiratory burst and serum antibacterial activities were significantly elevated in BGN treatment, indicating the importance of these responses in resistance against *V. harveyi* infection. The present results corroborate with previous *in- vitro* and *in-vivo* findings, elucidating the potency of BGN in activating crustacean humoral and cellular immune responses (Sritunyalucksana *et al.*, 1999; Vitvicka and Sima, 2004). *In-vitro* incubation of crustacean hemocyte with soluble β-glucan resulted to increased hemocyte phagocyte activity (Smith and

Soderhall, 1983) and increased hemocyte respiratory burst activity of *P. vannamei* (Campa-Cordova *et al.*, 2000). Also in *P. monodon* β-glucan treatment was reported to activate the production of hemolymph antibacterial proteins (Sritunyalucksana *et al.*, 1999). Moreover, similar to the present findings Sung *et al.* (1994) reported enhanced survival of *P. monodon* post-larvae immersed in β-glucan and challenged with *Vibrio vulnificus*. Immersion with β-glucan was also documented to increase survival of *Macrobrachium rosenbergii* post larvae exposed to pathogenic *Vibrio alginolyticus* (Misra *et al.*, 2004) Enhanced protection against vibrio strain NU1 infection was also documented in *P. japonicus* fed with diets supplemented with β-glucan (Itami *et al.*, 1994). Additionally, dietary administration of β-glucan in juvenile *P. monodon* promoted increased resistance and improves survival against white spot syndrome virus infection (Chang *et al.*, 2003). All of these earlier findings indicate that the improvement of shrimp survival against microbial infection due to β-glucan treatment could be attributed to the enhancement of elevated immune responses similar to that observed in the present study.

Lipopolysaccharide (LPS) constitutes a part of the cell membrane of gram-negative bacteria and has been used as an important tool in defining vertebrate and crustacean immune functions (Sritunyalucksana *et al.*, 2002; Van de Braak *et al.*, 2002; Pakzad *et al.*, 2010). In spite of this, studies regarding the practical application of LPS as an immunostimulant for shrimp have been limited. In the present work, dietary supplementation of VLP resulted in higher protection against *V. harveyi* infection due to the up-regulation of immune responses. These results are consistent with earlier reports elucidating the role of bacterial LPS in immune activation. Injection of LPS has been shown to promote proliferation of hemocytes in *P. monodon* (Van de Braak *et al.*, 2002) and in *M. japonicus* (Sequira *et al.*, 1996), up-regulates gene transcription of antimicrobial peptides in *P. vannamei* (Okumura, 2007) and *in-vitro* incubation with hemocytes activated prophenol oxidase cascade (Cardenas *et al.*, 2004). Similar to the present findings, enhancement of hemocyte phagocytic activity and activation of hemolymph virus neutralizing factor resulting in improved survival against penaeid acute viremia virus was also demonstrated in *M. japonicus* fed diets supplemented with *Pantoea agglomerans* LPS (Takahashi *et al.*, 2000). Furthermore, it has been documented that the mechanism by which LPS promotes shrimp resistance against microbial infection is due to the activation of an anti-LPS peptide (Nagoshi *et al.*, 2006). This peptide has been characterized as having, a strong affinity to bacterial LPS, broad spectrum antibacterial, anti-fungal and antiviral activities (Somboonwiwat *et al.*, 2005; Liu *et al.*, 2006; De la Vega *et al.*, 2008). Activation of this peptide could probably be involved in the enhancement of *P. monodon* resistance against *V. harveyi* infection as observed in the present study.

Fucoidan is an acid-soluble polysaccharide of sulfated L-fucose mainly produced by brown algae (Eluvakkal *et al.*, 2010). This compound has been known to be a potent activator of phagocytosis and inducer of cytokine secretion in vertebrate immune cells (Heinzelmann *et al.*, 1998) but information on its effects on shrimp has been scarce. The present findings show that dietary fucoidan supplementation significantly elevated phagocytosis and serum antibacterial activities resulting to increased survival of *P. monodon* juveniles against *V. harveyi* infection and is the first time to be documented in shrimp. The enhanced resistance seen in FCD treatment could be attributed to the significant enhancement of immunological responses, specifically the hemocyte phagocytic and serum antibacterial activities. Although, detailed studies on the effects of fucoidan on crustacean immune functions are limited, but its effects on vertebrate immune system in promoting phagocytosis and release of cytokines are well documented (Heinzelmann *et al.*, 1998). Similar effect has been documented in *P. monodon* wherein fucoidan injection induced the

synthesis of a phagocytosis activating protein, known to activate hemocyte phagocytic response that eliminates invading viral or microbial pathogens (Deachamag *et al*., 2006). Dietary supplemented fucoidan has also been shown to increase the resistance of *M. japonicus* (Takahashi *et al*., 1998) and *P. monodon* (Chotigeat *et al*., 2004) against white spot syndrome virus infection. In these reports, significant enhancement of hemocyte phagocytic activity were also observed, suggesting a strong functionality of fucoidan towards the activation of cellular immune response in a manner similar to that observed in the present study and in the activation of macrophages in vertebrate organisms.

The low percentage survival observed in VHK and CAR treatments was associated with lower immunological responses in comparison with BGN, FCD and VLP supplementation. This suggests that the magnitude of immunological responses elicited by CAR and VHK was inadequate to render an effective protection against *V. harveyi*. The low immunological responses observed in these treatments could have been caused by the digestive degradation of the functional components of these substances, rendering it unrecognizable by the immune system. Decrease in vaccine efficacy due to digestive degradation has also been documented in fish (Jenkins *et al*., 1994). Similar results indicating the ineffectiveness of Vibrio bacterin supplementation in eliciting a protective immune response were also reported in *Penaeus indicus* larvae (Alabi *et al*., 1999). Also, the present findings is in agreement with the results of Goarant and Boglio (2000) in *Litopenaeus stylirostris* fed diets supplemented with Vibrio bacterin that did not improve the immunological responses of this shrimp. In contrast to the present findings, Heidarieh *et al*. (2010) reported enhanced survival of *P. vannamei* post larvae challenged with *V. harveyi*. The larvae were maintained with diets supplemented with a bacterin consisting of a mixture of different Vibrio species (Vibriomax) and an algin based immunostimulant (Ergosan). The combination of mixed Vibrio bacterin and alginate immunostimulant may account for the difference in the infection survival response observed in that study.

Contrary to the present findings, carrageenan administered by intramuscular injection has been reported to enhance the non-specific immune response and increase disease resistance of *Cyprinus carpio* (Fujiki *et al*., 1997) and *Epinephelus coioides* (Cheng *et al*., 2008). However, the differences in immune physiology between shrimp and fishes and the different route of administration may account for these contrasting results.

Overall, the present results suggest that dietary supplementation of BGN, FCD and VLP at 2 g kg^{-1} diet could enhance the immunological responses and improve resistance of juvenile *P. monodon* against *V. harveyi*. The potential use of these substances for practical application in *P. monodon* is promising but concerns regarding their effects on shrimp metabolism and growth on a long-term administration await further confirmations.

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