



# Immune responses and resistance to vibriosis of juvenile Pacific whiteleg shrimp *Penaeus vannamei* fed with high dose mannan oligosaccharide and $\beta$ -glucan

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**Abstract.** A 45-day feeding trial was conducted to evaluate the effects of mannan oligosaccharide (MOS) and  $\beta$ -glucan supplementation in Pacific whiteleg shrimp, *Penaeus vannamei* juvenile. Shrimps (0.6 g ABW) were fed diets supplemented with different levels of mannan oligosaccharide (MOS) and  $\beta$ -glucan (BZT<sup>®</sup> PRE-GE) as immunostimulants. Experimental diets were formulated to contain 0.2, 0.4 and 0.8% and a control. The feeding trial was conducted in 50-L capacity rectangular plastic container stocked with 20 shrimps each in triplicates. Growth, survival, resistance to *Vibrio parahaemolyticus*, total haemocyte count (THC), respiratory burst activity, phenoloxidase activity, and clearance efficiency were evaluated. Results showed that growth and survival were not affected by supplementation of immunostimulant. On the other hand, bacterial challenge showed 100% mortality in 0.8% MOS +  $\beta$ -glucan fed group though not significant with the control. Total haemocyte count, respiratory burst and phenoloxidase activity were significantly enhanced in the group supplemented with 0.4% and 0.2% of MOS +  $\beta$ -glucan. Immune responses of the group fed with the highest concentration (0.8%) were significantly suppressed. The same trend was obtained for the clearance efficiency. The present results demonstrated that using MOS +  $\beta$ -glucan less than or equal to 0.4% activate immune responses and resistance against vibriosis, otherwise overstimulation of the immune indices could cause immunosuppression.

**Key Words:** immunostimulant, *Vibrio parahaemolyticus*, *Penaeus vannamei*, immunosuppression.

**Introduction.** Aquaculture production of penaeid shrimps has been considered highly profitable. However, development in shrimp aquaculture is at risk due to the different constraints including environmental and pathological problems (Bachere et al 1995; Jory 2014). The epidemics of viruses and diseases with bacterial etiology, particularly *Vibrio* species, have inflicted the shrimp farming industry worldwide especially in Asian countries (Chiu et al 2007; Jory 2014; Loy 2011; Magbanua et al 2000; de la Peña et al 2003).

Vibriosis, a disease caused by gram-negative bacteria is one of the major disease problems in the aquaculture of shellfish and finfish (Adams 1991; Chen et al 2000; Lavilla-Pitogo et al 1996; Lavilla-Pitogo et al 1998; Lightner et al 1992; Lightner & Lewis 1975). Some vibrio species identified to cause vibriosis include *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. penaeicida* (Brock & Lightner 1990; Ishimaru et al 1995). These bacteria are part of the natural microflora of wild and cultured shrimps. They become opportunistic pathogens once the environment becomes favorable for their growth such as poor water quality, crowding, low dissolved oxygen (DO), high water temperature, low water exchange and suppressed natural defense mechanisms of the animal (Brock & Lightner 1990; Lewis 1973; Lightner & Lewis 1975; Sizemore & Davis 1985).

In 2009, a new disease emerged in Southeast Asia. It started in China and spread to Vietnam, Malaysia and Thailand affecting both *P. monodon* and *P. vannamei* farms

(Lightner 2012; Zorriehzahra & Banaederakhshan 2015). The disease was named early mortality syndrome (EMS) due to mass mortality few days after stocking of shrimp post larvae. Gross signs include: (1) atrophy of hepatopancreas; (2) pale, yellowish or white within hepatopancreas connective tissue capsule; (3) black spots or streaks sometimes visible; and (4) hepatopancreas does not squash easily between thumb and finger. EMS has two distinct phases. One is an acute phase in which significant acute sloughing of hepatopancreatic tubules epithelial cells can be observed. Second and the terminal phase ends with the destruction of hepatopancreas. EMS was also named acute hepatopancreatic necrosis disease (AHPND) (Lightner 2012).

Early 2013 when researchers of the Aquaculture Pathology Laboratory at the University of Arizona identified the causative agent of EMS/AHPND to be a phage virus-infected *V. parahaemolyticus* which releases a potent toxin ([www.thefishsite.com](http://www.thefishsite.com)).

Traditional remedies like antibiotics and chemical disinfectants were extensively used in shrimp farms to treat or prevent microbial infections and diseases. However, these are no longer recommended due to its impacts on the environment and human health (Bray et al 2006). It could also lead to the development of antibiotic resistance in microorganisms (Smith et al 2003). Consequently, alternative approach of preventing the onset of disease caused by pathogens is the focus of the recent studies. One of these is the use of immunostimulants (Anderson 1992; Gatesoupe 1999; Montero-Rocha et al 2006; Raa et al 1996) which are chemical compounds that activate the defense system of the animal (Raa 2000). These include structural components of bacteria, fungi and viruses, biological extracts of plants like seaweeds and some synthetic compounds (Raa 2000).

Recent studies on the immunostimulatory effects of different extracts from seaweeds (Traifalgar et al 2012, 2013; Declarador et al 2014; Serrano & Declarador 2014), microalgae (Seraspe et al 2014) and bacteria (Apines-Amar et al 2014; Andrino et al 2014; Genio et al 2014; Pan et al 2015) have shown positive results in penaeid shrimps. Also, several types of yeast preparations such as  $\beta$ -glucans have already been well studied (Sakai 1999).  $\beta$ -1,3/1,6 glucans, prepared from *Saccharomyces cerevisiae* have been evaluated as a protection against disease. These types of glucans under experimental conditions proved to enhance the biological activity of haemocytes and improve growth, survival rate as well as feed conversion efficacy of shrimps (Raa 2000).

Mannan oligosaccharide (MOS) is another cell wall component of yeast which works locally in the gut. MOS have shown to improve digestion and gut health in animals. It may also function as a prebiotic, which favors the growth of beneficial bacteria in the gut (Staykov et al 2007; Refstie et al 2010).

However, most of the studies focus on the use of a single immunostimulant and dose optimization of these compounds. There are only few studies on the use of combined supplementation and revealed positive effects in enhancing the growth performance and immune responses of a certain cultured aquatic animal (Apines-Amar et al 2014; Gu et al 2011). However, effects of overstimulation of the defense system of the cultured animal have not been well investigated.

*Penaeus vannamei* or the Pacific whiteleg shrimp is one of the shrimp species being intensively cultured in the Philippines. The present study aims to determine the effects on growth performance and immune responses of MOS +  $\beta$ -glucan supplementation given at above optimum dose to *P. vannamei* juvenile.

## Material and Method

**Experimental animals and acclimation period.** The experiment was conducted at the Institute of Aquaculture Multispecies Hatchery, College of Fisheries and Ocean Sciences, University of the Philippines Visayas. Healthy *P. vannamei* juvenile were obtained from a commercial hatchery in Minglanilla, Cebu. The shrimps were maintained and reared in a 10 ton-capacity tank provided with continuous aeration. They were acclimated and fed with commercial diet (Table 1) for 2 weeks.

Table 1

## Composition of the commercial diet

<i>Ingredients</i>
Fish meal
Soybean meal
Squid liver meal
Shrimp meal
Fish soluble
Wheat flour
Fish oil
Binders
Vitamins
Minerals
Lecithin
Mold Inhibitor

Crude Protein-38.0%, Crude Fat- 4.0%, Crude Fiber- 4.0%, Moisture- 12.0%, Ash- 16.0%.

**Experimental design.** Following acclimation, shrimps were randomly divided into 12 plastic containers of 50 L capacity. Each container had 20 shrimps. These constituted three dietary treatments and a control, all in three replicates.

The formulated basal diet (Table 2) was supplemented with MOS and  $\beta$ -glucan formula (BZT® PRE-GE) at 0%, 0.2%, 0.4% and 0.8%  $\text{kg}^{-1}$  diet. Each diet was fed to the shrimps of their assigned treatment group at 10% body weight three times daily at 8:00, 12:00, 16:00 h for 45 days. Test animals were weighed every 15 days and feed ration was adjusted accordingly.

Table 2

Composition of test diets containing various concentrations of MOS +  $\beta$ -glucan (%)

<i>Ingredients</i>	0%	0.2%	0.4%	0.8%
Danish fish meal	46.00	46.00	46.00	46.00
Acetes meal	8.00	8.00	8.00	8.00
Soybean meal	21.00	21.00	21.00	21.00
Cellulose	5.00	4.80	4.60	4.20
Vitamin mix	2.00	2.00	2.00	2.00
Mineral mix	1.00	1.00	1.00	1.00
Lecithin	0.50	0.50	0.50	0.50
Cod liver oil	4.00	4.00	4.00	4.00
Starch	13.00	13.00	13.00	13.00
MOS + $\beta$ -glucan	0.00	0.20	0.40	0.80
Total	100.00	100.00	100.00	100.00

**Immune response and disease resistance trial.** Same batch of shrimps were used for the immune response and disease resistance trial. Rearing condition was the same as that of the growth trial except that the shrimp was fed to satiation up to 14 days.

**Susceptibility of shrimp to *V. parahaemolyticus*.** After the feeding trial, the shrimps were challenged with pathogenic *V. parahaemolyticus* by injection using a 26-gauge syringe (1 mL). The bacteria were obtained from University of Santo Tomas in Manila, Philippines.  $\text{LC}_{50}$  was determined to be  $10^4$  cells  $\text{mL}^{-1}$ . Following the method of Pattukumar et al (2012), the bacterium was inoculated into tryptic soy agar (TSA) with 2% NaCl (w/v), incubated for 24 hours and suspended in a sterile saline solution (0.85% NaCl). The shrimps were placed in a 20-L container with a static UV-treated seawater (25 ppt) and fed twice daily with their respective test diets. Mortality by *V. parahaemolyticus*

infection was confirmed by isolation of the inoculum using a selective media HiCrome™ Vibrio Agar (Fluka Analytical).

**Clearance efficiency of shrimp to *V. parahaemolyticus*.** Shrimps of each treatment were injected in the ventral sinus with bacterial suspension of 10 µL containing 10<sup>4</sup> cells mL<sup>-1</sup> in 0.85% NaCl. After injection, the shrimps were held in separate tanks of the same water condition for 3 h before collecting 200 µL volume of haemolymph from the ventral sinus and mixed with 200 µL sterile anticoagulant. The mixture was used to measure clearance efficiency following the methods of Adams (1991). The colonies were counted after 12 h, 18 h and 24 h using a colony counter. Clearance efficiency, defined as percentage inhibition (PI) of *V. parahaemolyticus* was calculated as:

$$PI = 100 - \left\{ \frac{\text{cfu in test group}}{\text{cfu in positive control group}} \right\} \times 100$$

**Haemolymph extraction.** Anticoagulant for haemolymph extraction was prepared using 450 mM NaCl, 10 mM KCl, 10 mM HEPES and 10 mM EDTA at pH 7.3, 850 mOsm kg<sup>-1</sup> (Hernandez-Lopez et al 1996). Using a 1-mL tuberculine syringe with 26 gauge needle rinsed with pre-cooled anticoagulant, haemolymph was extracted from the ventral sinus of the first abdominal segment of each test animal. The collected haemolymph from each shrimp was divided for total haemocyte count (THC) respiratory burst activity and phenoloxidase activity.

#### **Immune assays**

**Total haemocyte count (THC).** Total haemocyte count was performed using a Neubauer's haemocytometer. Haemolymph was withdrawn using tuberculin syringe with anticoagulant, transferred into a microcentrifuge tube and fixed using 10% formalin in 0.45 M NaCl. A 20 µL aliquot was then stained using 1.2% Rose bengal in 50% ethanol and allowed to stain for 20 min. Haemocytes were counted and values were expressed as THC mL<sup>-1</sup> haemolymph (Joseph & Philip 2007).

**Superoxide anion (O<sub>2</sub><sup>-</sup>) production.** Respiratory burst activity of haemocytes was quantified using reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion production (Muñoz et al 2000). One hundred microliter of the sample was placed in each well of a microtiter plate containing 200 µL anticoagulant and was incubated at room temperature for 2 hours. The supernatant was discarded and replaced with 50 µL MHBSS (Modified Hank's Balanced Salt Solution) medium. One hundred microliter NBT-PMA (Nitroblue tetrazolium-phorbol myristate acetate) solution was added and incubated for 30 min. Supernatants were removed and the haemocytes were fixed by adding 200 µL absolute methanol for 10 min and washed twice with 70% methanol, and then dried. The formazan deposits were then solubilized in 120 µL 2M KOH and 140 µL dimethyl sulfoxide (DMSO). The optical density was read in a microplate reader at 620 nm and activity expressed as O.D. 100 µL<sup>-1</sup> haemolymph (Joseph & Philip 2007). Blank control reactions were performed using 120 µL of KOH and 140 µL of DMSO.

**Phenoloxidase assay.** Formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) was measured spectrophotometrically (Hernandez-Lopez et al 1996) to assay the phenoloxidase activity.

In a sterile 1.5 mL microcentrifuge tubes, anticoagulant-free haemolymph was placed and subjected to a freeze-thaw cycle five times in order to induce the lysis and degranulation of the cell. The haemocyte supernatant was collected after samples were vortexed and centrifuged at 13,000 x g for 15 minutes at 4°C. In order to measure PO, haemocyte supernatant with a volume of 25 µL was placed in 96-well microtitre plates and incubated for 30 minutes with 25 µL 0.1% trypsin in shrimp salt solution (SSS). Twenty five microliter of 0.3% L-DOPA was added and incubated for 10 minutes. With the use of microplate reader, optical density was measured at 490 nm. Enzyme activity was expressed as the change in absorbance min<sup>-1</sup> 100 µL<sup>-1</sup> haemolymph (Joseph & Philip 2007).

**Statistical analysis.** Statistical analyses were performed using the software SPSS version 16.0. Data obtained from growth trial, immune assays, survival rate following *V. parahaemolyticus* challenge test and clearance efficiency were analyzed by one-way ANOVA at  $P < 0.05$  level of significance.

## Results and Discussion

**Growth trial.** Specific growth rate (SGR), feed conversion efficiency (FCE) and % survival of shrimps after 45 days of feeding were not significantly different in all treatments (Table 3). This suggests that it was not affected by the inclusion of MOS +  $\beta$ -glucan over the test period. Hence, there could be a possibility of obtaining different results if the period of administration is longer.

Table 3  
Growth performance of *Penaeus vannamei* fed different levels of MOS +  $\beta$ -glucan

Parameter	0%	0.2%	0.4%	0.8%
SGR	3.95 ± 0.344 <sup>a</sup>	4.03 ± 0.416 <sup>a</sup>	3.59 ± 0.392 <sup>a</sup>	3.48 ± 0.214 <sup>a</sup>
FCE	0.33 ± 0.049 <sup>a</sup>	0.40 ± 0.019 <sup>a</sup>	0.38 ± 0.048 <sup>a</sup>	0.37 ± 0.014 <sup>a</sup>
% Survival	48.3 ± 4.410 <sup>a</sup>	55.00 ± 5.000 <sup>a</sup>	48.33 ± 6.009 <sup>a</sup>	61.67 ± 4.410 <sup>a</sup>

SGR = specific growth rate (% day<sup>-1</sup>), FCE = feed conversion efficiency.

**Susceptibility of shrimp to *V. parahaemolyticus*.** The present study revealed disease resistance of *P. vannamei* against *V. parahaemolyticus* in groups fed diets containing 0.2% and 0.4% MOS +  $\beta$ -glucan having 39.74% and 43.59% survival respectively. Shrimps fed control diet had 8.01% survival but has no significant difference with the group fed 0.8% MOS +  $\beta$ -glucan which succumbed to death 3 days after injection. Mortality was observed one day after infection and 100% was observed on the 3<sup>rd</sup> day of post challenge (Figure 1).

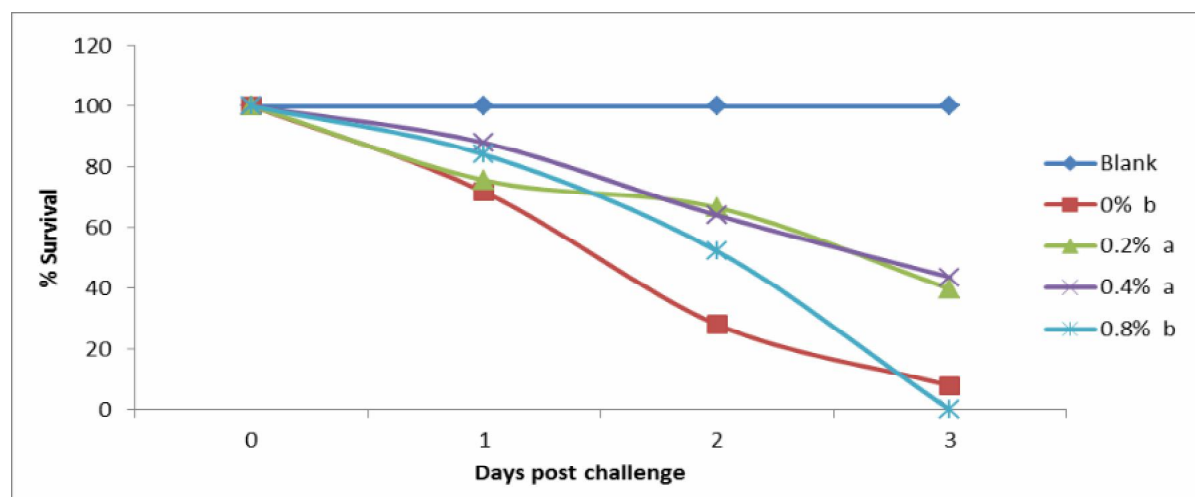


Figure 1. Survival (%) of shrimp *Penaeus vannamei* fed different levels of MOS +  $\beta$ -glucan after challenged with *Vibrio parahaemolyticus*. Values are means ± SEM (n=25).

Survival of the shrimp challenged with microbial pathogen is generally used to evaluate the disease resistance. Results in the present study revealed that 0.2% and 0.4% MOS +  $\beta$ -glucan-supplemented diets significantly enhanced survival of *P. vannamei* juvenile against *V. parahaemolyticus* infection as compared with shrimps fed the 0.8% supplemented and control diets.

$\beta$ -glucan supplementation was reported to increase resistance of *P. japonicus* against vibriosis (Itami et al 1994), and *P. monodon* against vibriosis, white spot syndrome virus and *Vibrio damsela* (Su et al 1995; Song et 1997; Chang et al 2003).

Supplementation of a combination of MOS and peptidoglycan (Apines-Amar et al 2014) and MOS +  $\beta$ -glucan (Andrino et al 2014) also enhanced immunity of *P. monodon* against white spot syndrome virus. However, high dose supplementation of these compounds could result in immunosuppression. It was reported that effects of immunostimulants were not directly dose-dependent as high doses may not enhance and may inhibit the immune system of shrimp. Moreover, immunostimulants often show a distinct maximum at a certain intermediate concentration and even a complete absence of effect at higher concentrations (Sakai 1999). This was supported by the data obtained by Sajeevan et al (2009) in *P. indicus*. Lower survival was observed in shrimp fed higher concentrations of glucan as a result of continuous overproduction of superoxide anions and free radicals of the phagocytes and excessive degranulation of haemocytes causing the release of prophenoloxidase (Sajeevan et al 2006; Sajeevan et al 2009). These could support the results of the present study wherein 100% mortality was observed in the highest dose of MOS +  $\beta$ -glucan supplementation.

**Immune assay.** Positive effects on the immune indices of shrimp such as THC, respiratory burst activity, and phenoloxidase activity after inclusion of MOS and  $\beta$ -glucan were observed at a level of up to 0.4% in the present study. Haemocytes of shrimps are involved in defense processes like phagocytosis, encapsulation, melanization and coagulation (Johansson et al 2000) and also in the production of reactive oxygen metabolites and release of microbicidal proteins (Smith et al 2003). The count of haemocytes is used to measure the cellular immunological response which is a good indicator of the health status of shrimp (Bachere 2000; Sritunyalucksana et al 2005).

Combined supplementation of MOS +  $\beta$ -glucan (Andrino et al 2014) and peptidoglycan and MOS (Apines-Amar et al 2014) showed enhanced THC and respiratory burst activity in *P. monodon*. Furthermore, increase in respiratory burst of *L. vannamei* was observed by Campa-Cordova et al (2002) using  $\beta$ -glucan and sulphated polysaccharide as immunostimulants. In the present study, total haemocyte count was found to be significantly enhanced in shrimps fed diets containing 0.2% and 0.4% MOS and  $\beta$ -glucan (Figure 2).

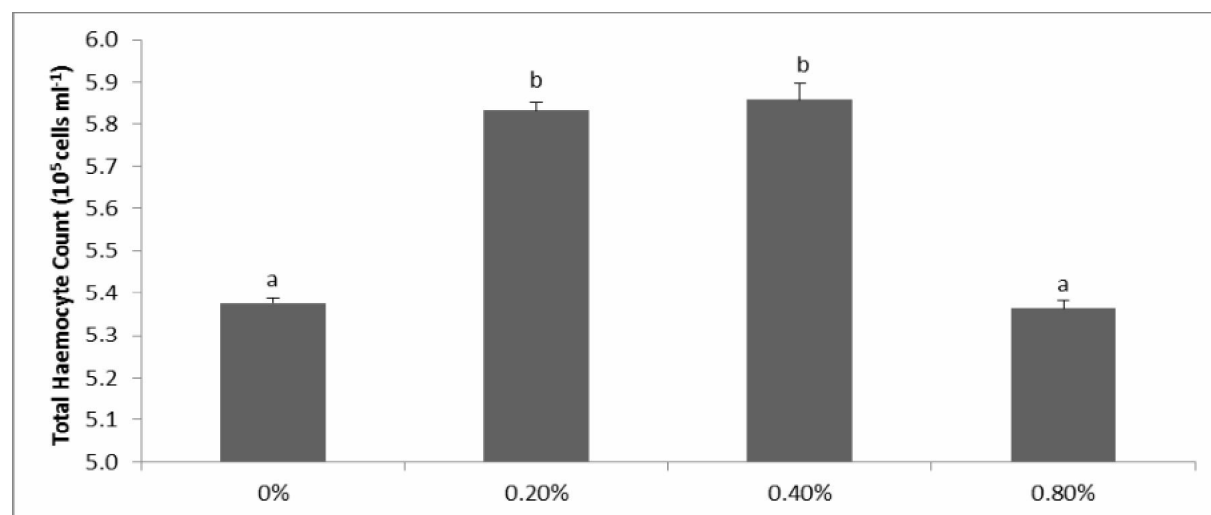


Figure 2. Total haemocyte count (THC) of *Penaeus vannamei* fed different levels of MOS +  $\beta$ -glucan. Values are expressed as Means  $\pm$  SEM (n=4).

Higher number of haemocytes in shrimps fed 0.4% MOS +  $\beta$ -glucan might have been the results of enhanced oxygen uptake and transfer. On the other hand, the significant decrease in THC of those fed 0.8% MOS +  $\beta$ -glucan might be the result of the increase in numbers of ROIs which in return could cause immunosuppression as mentioned previously.

Respiratory burst of shrimp supplemented with 0.2% MOS +  $\beta$ -glucan was enhanced but not significantly different with those fed 0.4% in the present study (Figure

3). Superoxide anion ( $O_2^-$ ) production was enhanced in *P. monodon* after feeding with immunostimulant (Chang et al 2003; Citarasu et al 2006; Joseph & Philip 2007). Similar results were obtained by Andrino et al (2014) and Apines-Amar et al (2014).

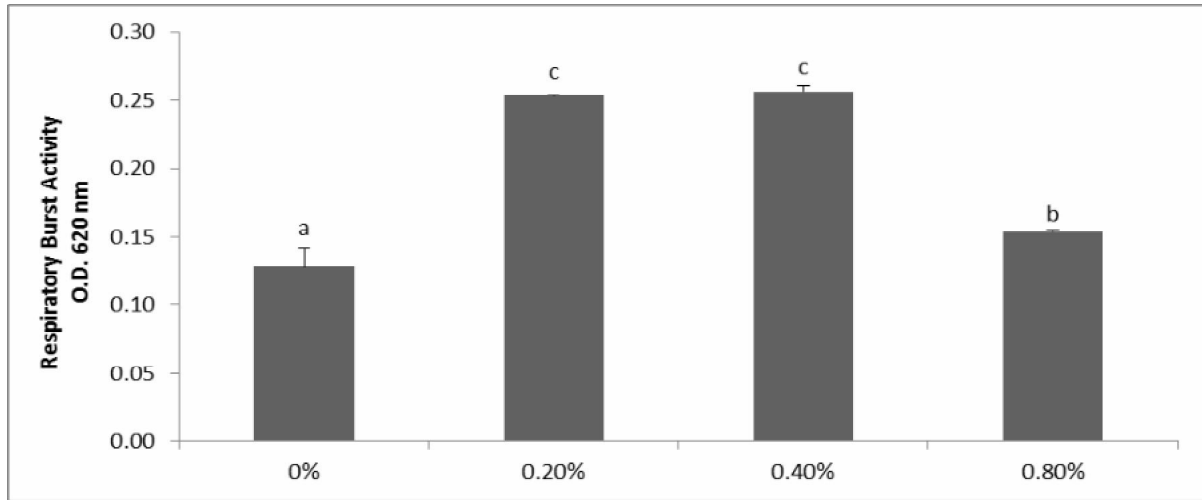


Figure 3. Respiratory burst activity of *Penaeus vannamei* fed different levels of MOS +  $\beta$ -glucan. Values are expressed as Means  $\pm$  SEM (n=4).

Enhanced PO activity was found in the haemocyte of the shrimp fed diet supplemented with 0.4% MOS +  $\beta$ -glucan while the lowest was observed in the group fed 0.8% MOS +  $\beta$ -glucan and control (Figure 4). Significant increase in THC and NBT reduction among these groups might have also affected the PO activity.

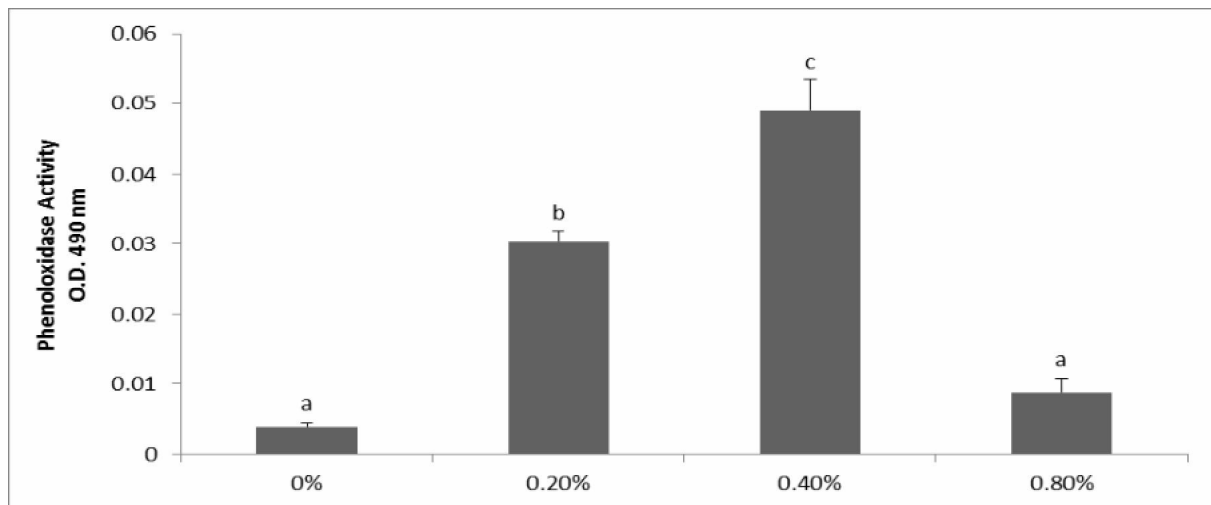


Figure 4. Phenoloxidase (PO) values of *Penaeus vannamei* fed different levels of MOS +  $\beta$ -glucan. Values are expressed as Means  $\pm$  SEM (n=4).

Clearance efficiency of shrimp to *V. parahaemolyticus* showed a continuous significant decrease after 12 h, 18 h and 24 h of incubation though 0.2% MOS +  $\beta$ -glucan fed group remained high (Figure 5). These results were comparable to that of Sritunyalucksana et al (2005) using yeast extract-coated feeds in shrimp and Chiu et al (2007) in *L. vannamei* fed with *Lactobacillus plantarum* containing diets. Moreover, a significant increase in clearance efficiency to *V. alginolyticus* in whiteleg shrimp that received *Petalonia binghamiae* extract was also observed (Chen et al 2014).

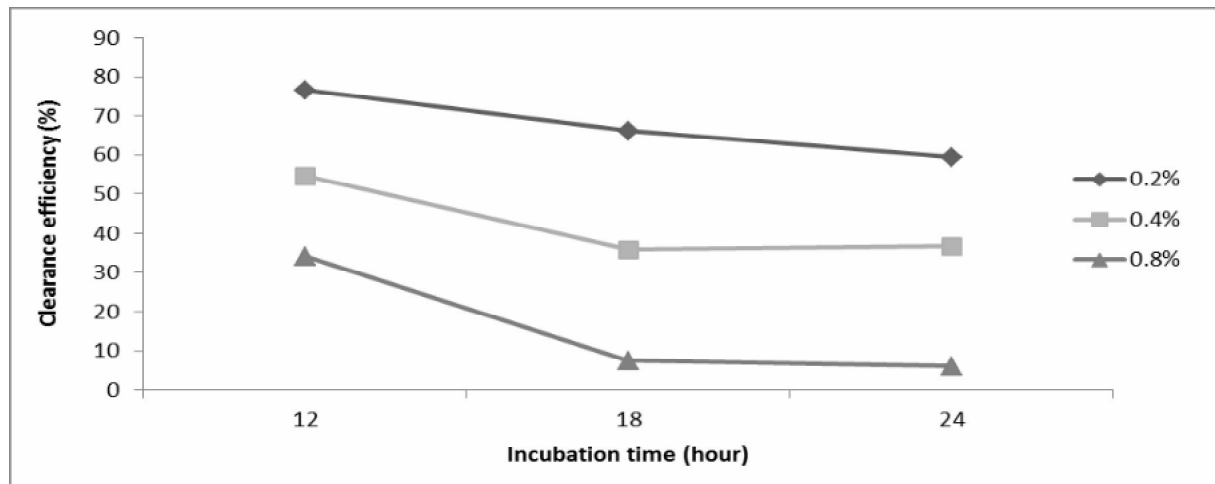


Figure 5. Clearance efficiency (%) values of *Penaeus vannamei* fed different levels of MOS +  $\beta$ -glucan. Values are expressed as Means  $\pm$  SEM (n=3).

**Conclusions.** In summary, the present study revealed that supplementation of MOS +  $\beta$ -glucan in the diet did not affect the growth performance of *P. vannamei*. Also, immunological indices such as THC, respiratory burst activity and phenoloxidase activity were suppressed in the 0.8% MOS +  $\beta$ -glucan fed group. The results showed MOS +  $\beta$ -glucan supplementation of less than or equal to 0.4% had the capacity to enhance shrimp's immune responses, survival against *V. parahaemolyticus* infection and clearance efficiency, otherwise it could overstimulate the immune responses of shrimp resulting in the damage of cells rendering less protection and high susceptibility of the animal to infections.

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