Effects of dietary mannan oligosaccharide (MOS) and β-glucan on growth, immune response and survival against white spot syndrome virus (WSSV) infection of juvenile tiger shrimp *Penaeus monodon*

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Abstract. Effects of dietary administration of mannan oligosaccharide (MOS) and β-glucan on growth, total haemocyte count (THC), respiratory burst activity and survival against White Spot Syndrome Virus (WSSV) challenge infection of tiger shrimp *Penaeus monodon* juveniles were investigated. Basal diet for tiger shrimp was supplemented with MOS and β-glucan formula (BZT® PRE-GE) at 0% (control), 0.1%, 0.2% and 0.5% kg^{-1} of feed. The results revealed that shrimps fed with MOS and β-glucan supplemented diet have significantly higher percent weight gain (%WG) and better feed conversion ratio (FCR) than those fed the control diet after a 60-day feeding period. However, survival of shrimp after growth trial was in the range of 64-81% and was not significantly different among treatments. In the WSSV challenge test, highest survival was at 0.2% MOS and β-glucan. Total haemocyte count (THC) was significantly higher in shrimp fed diets containing 0.2% MOS and β-glucan than the rest of the treatments. Respiratory burst activity of shrimps in all diets containing MOS and β-glucan was significantly higher than those fed the control diet. These results suggest that dietary administration of MOS and β-glucan at 0.2% have beneficial effects in enhancing growth, feed utilization, improved immune response and survival of shrimp against WSSV infection.
Key Words: MOS, β-glucan, immunostimulant, shrimp, feeding, total haemocyte count, respiratory burst activity.

Introduction. Aquaculture of penaeid crustaceans is an economically significant activity but limited by endemic and epidemic infectious diseases in different parts of the world (Bachere et al 1995). The world production of farm-raised shrimp has been restricted by significant losses caused by the outbreak of infectious diseases, specifically due to viruses and bacteria, these diseases have affected the profitability, sustainability and progress of the shrimp industry (Rattanachai et al 2005; Rodriguez & Le Moullac 2000).

White spot syndrome virus infection, with its associated mortality, is emerging as one of the most challenging problems for global shrimp industry, especially in Asia and South-east Asia and has already become established in the local marine environment and in wild populations of shrimps in the Philippines (Rajendran et al 1999; de la Peña et al 2007). Moreover, aquaculture practices themselves may further aggravate the problem because stock animals are often kept under stressful conditions of overcrowding, high food levels, elevated water temperature and poor water quality. To some degree, good husbandry practices and control measures which include farming technologies such as "greenwater" technology, use of reservoir and chlorination of ponds during culture period are beneficial in controlling disease outbreaks but additional forms of protection are essential to prevent epidemics (Corre et al 2000; Smith et al 2003). Also, traditional control strategies for the disease are being employed such as the use of antibiotics and chemical disinfectants. These are no longer recommended due to the development of

antibiotic resistance in microorganisms and to the growing concerns over environmental impact and wildlife protection (Montero-Rocha et al 2006). In keeping with developments in aquaculture, considerable effort is directed towards preventing the onset of disease caused by pathogens. One approach to prevention is the use of immunostimulants which are naturally occurring compounds that modulate the immune system by increasing the host's defence to pathogens which is a more environmental friendly approach to disease management (Chen et al 2007; Raa 1996; Smith et al 2003).

Immunostimulants include structural elements of bacteria, β-1,3-glucan products from bacteria and mycelial fungi, β-1,3/1,6-glucans from the cell wall of baker's yeast, complex carbohydrate structures or polysaccharides from various biological sources including seaweed, animal or plant extracts, nucleotides, nutritional factors, cytokines and other synthetic products (Raa 2000; Sakai 1999).

Different yeast preparations have been found to have immunostimulatory effects in fish and shellfish (Robertsen 1999; Sakai 1999; Raa 2000). Mannan oligosaccharide (MOS) is a yeast cell wall derived feed ingredient working locally in the gut. It has been shown to improve digestion and gut health in animals by pathogen adsorption and immune modulation. MOS may furthermore function as a prebiotic, favouring growth of beneficial bacteria in the gut (Staykov et al 2007; Refstie et al 2010).

Beta-glucan is another cell wall component of microorganisms. It is routinely used as a vaccine adjuvant, immunostimulatory feed ingredient and has been successfully used to enhance resistance of fish and crustaceans against bacterial and viral infections (Refstie et al 2010; Chang et al 2003). Furthermore, it has been shown that β-glucan may improve health, growth and general performance of many different animal groups including farmed shrimp and fish (Raa 2000).

Most studies focus on the use of a single immunostimulant. A few studies however, have revealed the positive effects of combined supplementation of immunostimulant in enhancing the growth performance and immune response of certain aquatic animals (Ye et al 2011; Zhang et al 2011; Gu et al 2011).

Penaeus monodon is one of the most commercially important shrimp species, endemic and grown in the Philippines. The present study aims to investigate whether supplementation of dietary MOS + β-glucan could improve the growth, feed conversion ratio (FCR) and enhance immune response and resistance of shrimp against White Spot Syndrome Virus (WSSV).

Material and Method

Experimental animals and acclimation period. The experiment was conducted on January 2012 at the Institute of Aquaculture-Brackish Water Aquaculture Center, College of Fisheries and Ocean Sciences, University of the Philippines Visayas. Healthy, *P. monodon* post larvae (PL-18) were obtained from South East Asian Fisheries and Development Center- Aquaculture Department (SEAFDEC-AQD), Tigbauan, Iloilo maintained and reared indoor in 1 ton-capacity fiber glass tanks at the Brackish Water Aquaculture Center, University of the Philippines Visayas, Leganes, Iloilo. The shrimps were acclimated for 2 weeks, continuous aeration was provided and shrimps were maintained with formulated shrimp diet (control diet, without immunostimulants) before the growth trial (final ABW of 0.2-0.3 g). Prior to the experiment, the shrimps were randomly selected and screened by PCR for WSSV infection. PCR assays were conducted at the University of the Philippines Visayas- National Institute of Molecular Biology and Biotechnology (UPV-NIMBB), Miag-ao, Iloilo.

Growth trial: rearing conditions. Water quality was maintained by chlorinating the culture water with 100 ppm sodium hypochlorite and dechlorinated by vigorous aeration for 3 days. Water was recirculated from the reservoir to the experimental tanks with biological and mechanical filtrations applied e.g. fiber fill, sand, pebbles, charcoal. Water quality parameters viz., salinity ($22\text{-}26^0/_{00}$), temperature (24-28°C), pH (7.8-8.0) and dissolved oxygen (6-7 ppm) were recorded daily and NH_3–N and NO_2–N maintained at optimal levels. Each experimental unit was provided with adequate aeration, 20% water

change was done in each tank daily and 100% water change was done in the reservoir every 5-7 days to maintain good water quality. No bottom substrate was used, however shelters were installed to prevent cannibalism. During the experiment biosecurity measures were strictly followed.

Growth trial: experimental design. Following acclimation, shrimps were randomly divided into 16 plastic containers of 50 L capacity with 15 shrimps to each container at a density of one individual L^{-1}. These constituted three dietary treatments and a control, all in four replicates, in a completely randomized design.

Ingredients of the basal ration were purchased and prepared at SEAFDEC-AQD. The formulated basal diet (Table 1) for tiger shrimp was supplemented with MOS and β-glucan formula (BZT® PRE-GE) at 0%, 0.1%, 0.2% and 0.5% kg^{-1} of feed. Inclusion levels were based on the manufacturer's indication. Each diet was fed to the shrimps of each treatment group at 8% body weight three times daily at 8:00, 12:00, 16:00 h for 60 days. Shrimps were weighed every 15 days and the daily feed allocation was adjusted accordingly. Growth was measured as percent weight gain (%WG), feed conversion ratio (FCR) and survival.

Table 1

Composition of test diets containing various concentrations of MOS + β-glucan (%)

Ingredients	0%	0.1%	0.2%	0.5%
Sardine Fish Meal	36.00	36.00	36.00	36.00
Squid Meal	10.00	10.00	10.00	10.00
Soybean Meal	20.00	20.00	20.00	20.00
Cellulose	7.48	7.38	7.28	6.98
Vit. Mix	2.00	2.00	2.00	2.00
Mineral Mix	1.00	1.00	1.00	1.00
BHT	0.02	0.02	0.02	0.02
Lecithin	0.50	0.50	0.50	0.50
Danish Fish Oil	5.00	5.00	5.00	5.00
Starch	18.00	18.00	18.00	18.00
MOS+β-glucan	0.00	0.10	0.20	0.50
Total	100.00	100.00	100.00	100.00

Immune response and disease resistance trial. After the 60 d growth trial the immune response and disease resistance trial followed. Same batch of shrimps obtained from SEAFDEC-AQD were used. The rearing conditions and experimental design were the same as that of the growth trial except that the shrimps were fed to satiation only up to 14 days with MOS and β-glucan at 0%, 0.1%, 0.2% and 0.5%. Shrimps were then subjected to WSSV infection challenge and immune analyses.

WSSV infection challenge. The test animals for the WSSV infection challenge were placed in 30 L plastic containers that were situated inside the challenge room. The set-up was a static water system but adequate aeration was provided. WSSV-infected shrimp was obtained from wild population of infected *P. monodon* and was activated through passage by feeding the infected tissues (shrimp flesh) to the batch of uninfected shrimps; transmittance of the virus was done three times. The test animals were then challenged through oral administration i.e., by feeding infected tissue at the rate of 1 g shrimp^{-1} (Joseph & Philip 2007). Infected shrimp tissues were pooled, minced thoroughly, homogenized and were equally distributed to the experimental animals and fed at least three times at 8:00, 12:00, and 16:00 h in one day to make sure that all animals were infected. A blank control (unchallenged control) was also carried out. Survival was monitored daily. The dead animals were removed promptly. Mortality by WSSV infection was confirmed by checking the characteristic white spots on the carapace as well as polymerase chain reaction (PCR) assay. Proper disposal of infected water was implemented. The infected water was disinfected by placing 100 ppm sodium

hypochlorite and allowed to stay in the reservoir (fiber glass tanks) for a period of 4 weeks (no disposal of infected water during challenge).

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^{-1} (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) and respiratory burst activity.

Immune assays

Total haemocyte count (THC). Total haemocyte count was performed using a Neubaeur'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^{-1} haemolymph (Joseph & Philip 2007).

Superoxide anion (O_2^-) production. Respiratory burst activity of haemocytes was quantified using reduction of nitroblue tetrazolum (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 DMSO (dimethyl sulfoxide). The optical density was read in a microplate reader at 620 nm and activity expressed as O.D. 100 μL^{-1} haemolymph (Joseph & Philip 2007). Blank control reactions were performed using 120 μL of KOH and 140 μL of DMSO.

Statistical analysis. Statistical analyses were carried out using the software SPSS 16.0. Data obtained from the growth trial, immune assay and survival rate following a WSSV challenge test were analyzed by one way (ANOVA) and Duncan's multiple comparisons of means. All probability values were set at significance level of 0.05.

Results and Discussion

Growth trial. Dietary supplementation of MOS and β-glucan to juvenile tiger shrimp *P. monodon* significantly enhanced the shrimp's growth performance in terms of percent weight gain and feed conversion ratio (FCR) in the present study.

Shrimps fed with MOS and β-glucan-supplemented diets have significantly higher % weight gain than those fed the control diet (Figure 1). Survival of shrimp after the growth trial was in the range of 64%-81% and was not significantly different among treatments (Figure 2). During the 60-day feeding trial, the MOS + β-glucan-supplemented diets were substantially better utilized by the test animals. There was a significant difference in the FCR between the control group and the MOS + β-glucan fed group (Figure 3).

Single dietary administration of MOS has been reported to enhance growth performance and feed conversion ratio of the shrimp *Penaeus semisulcatus* (Genc et al 2007) and freshwater crayfish *Astacus leptodactylus* (Mazlum et al 2011). MOS also improved the growth performance, survival, physiological condition, gut health and immune response to bacterial infection of tropical juvenile spiny lobster, *Panulirus ornatus* (Sang & Fotedar 2010). It also demonstrated to enhance growth of several fish species including rainbow trout *Oncorhynchus mykiss* (Staykov et al 2007), European sea bass *Dicentrarchus labrax* (Torrecillas et al 2007) and Japanese flounder *Paralichthys*

olivaceus (Ye et al 2011). In addition, the study of Torrecillas et al (2011) also revealed that dietary incorporation of MOS enhances sea bass FCR. On the other hand, growth promotion by sole administration of β-glucan were reported in fish species including carp *Labeo rohita* (Misra et al 2006) and snapper *Pagrus auratus* (Cook et al 2003). Feeding of shrimp with β-1,3/1,6-glucan also resulted in faster growth, reduced mortality and better feed utilization (Sung et al 1994). However, a few studies have shown that combination of two-differently acting immunostimulants may be better than only one. Apines-Amar et al (2014) have reported that combined supplementation of peptidoglycan and MOS improved % weight gain of tiger shrimp *P. monodon*. β-glucan and Vitamin C given permanently or combined with control diet also enhanced growth rate of *Litopenaeus vannamei* (Lòpez et al 2003). Combined supplementation of β-glucan and MOS in sea cucumber *Apostichopus japonicus* resulted in significantly higher growth compared to the unsupplemented control or sole supplementation of β-glucan or MOS (Gu et al 2011).

MOS and β-glucan in combination resulted to have significant effects in % weight gain and FCR of shrimp in the present study. MOS might have contributed to better gut integrity of the shrimp thus resulted to significantly increased growth and better FCR. MOS are believed to act as prebiotics and act on the intestinal microbiota (Staykov et al 2007). It beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria, which can improve the host health. In addition, β-glucan might also have a growth promoting effect. Probably β-glucan was degraded in the digestive gland by glucanases to produce energy, permitting the use of more proteins for growth (Lòpez et al 2003). The results presented here reveal that all diets supplemented with MOS and β-glucan resulted in significantly better %WG and FCR than that of the control.
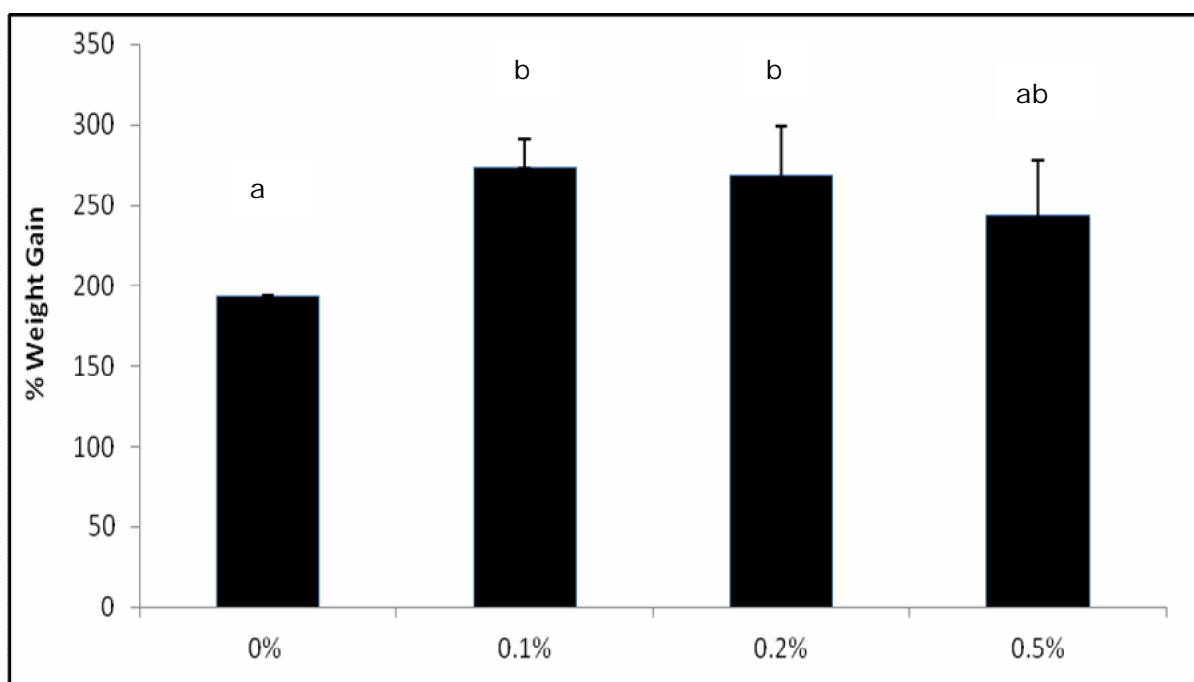

Figure 1. Percent weight gain of *P. monodon* fed different levels of MOS and β-glucan after 60-day growth trial. Values are expressed as Mean±SEM of four replicates (n = 15) on each treatment diet.
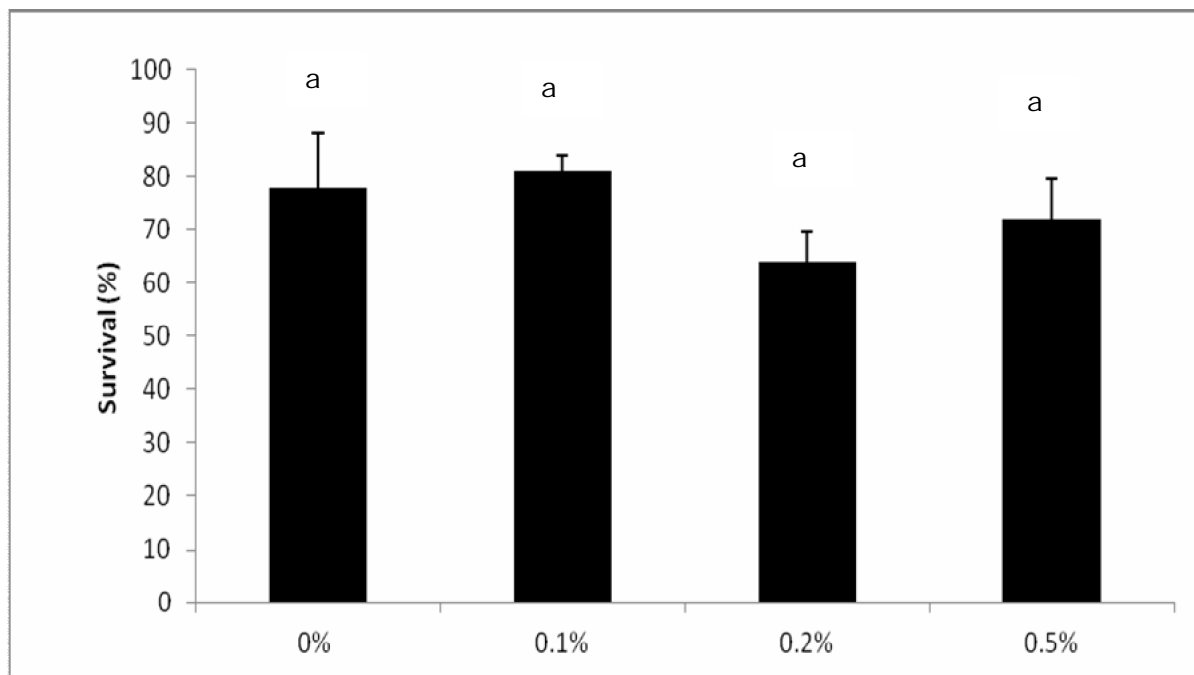
Figure 2. Survival (%) of *P. monodon* fed different levels of MOS and β-glucan after 60-day growth trial. Values are expressed as Mean±SEM of four replicates (n = 15) on each treatment diet.
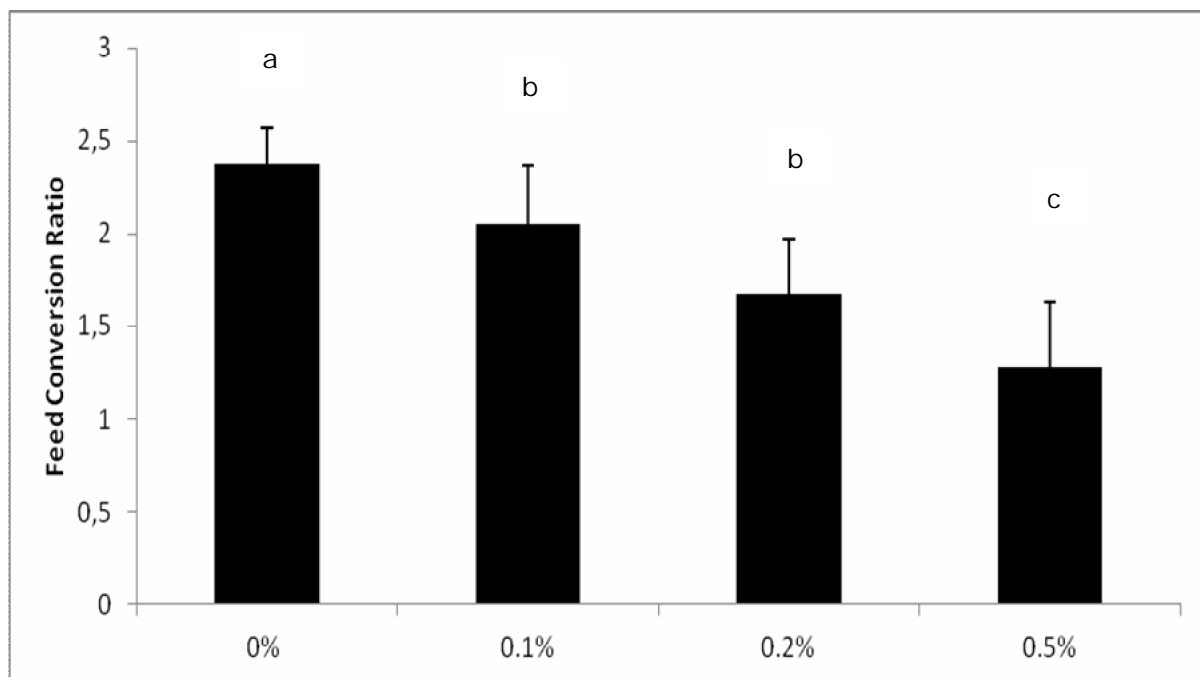

Figure 3. Feed Conversion Ratio of *P. monodon* fed different levels of MOS and β-glucan after 60-day growth trial. Values are expressed as Mean±SEM of four replicates (n = 15) on each treatment diet.

WSSV challenge and immune assay. The survival of shrimps fed the control and test diets are shown in Figures 4 and 5. The unchallenged shrimps (blank control) showed 100% survival. Percentage survival rates of *P. monodon* fed 0.2% and 0.5% MOS and β-glucan supplemented diets were significantly higher during the tenth day of post challenge than that of the shrimps fed the control diet. Least survival rate was observed in shrimps fed diet devoid of MOS and β-glucan as well as in 0.1% MOS and β-glucan which succumbed to death (100%) within the tenth day of challenge. Shrimps fed diets containing 0.2% MOS+ β-glucan showed the highest survival.
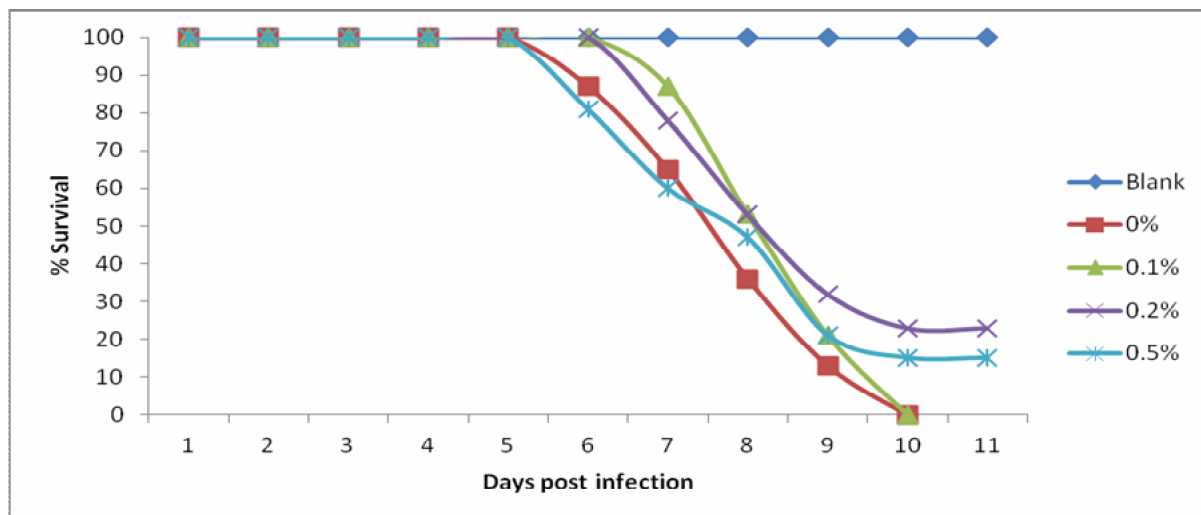
Figure 4. Percent survival of *P. monodon* fed different levels of MOS and β-glucan after WSSV challenge infection. Values are expressed as Mean±SEM of four replicates (n = 10) on each treatment diet.
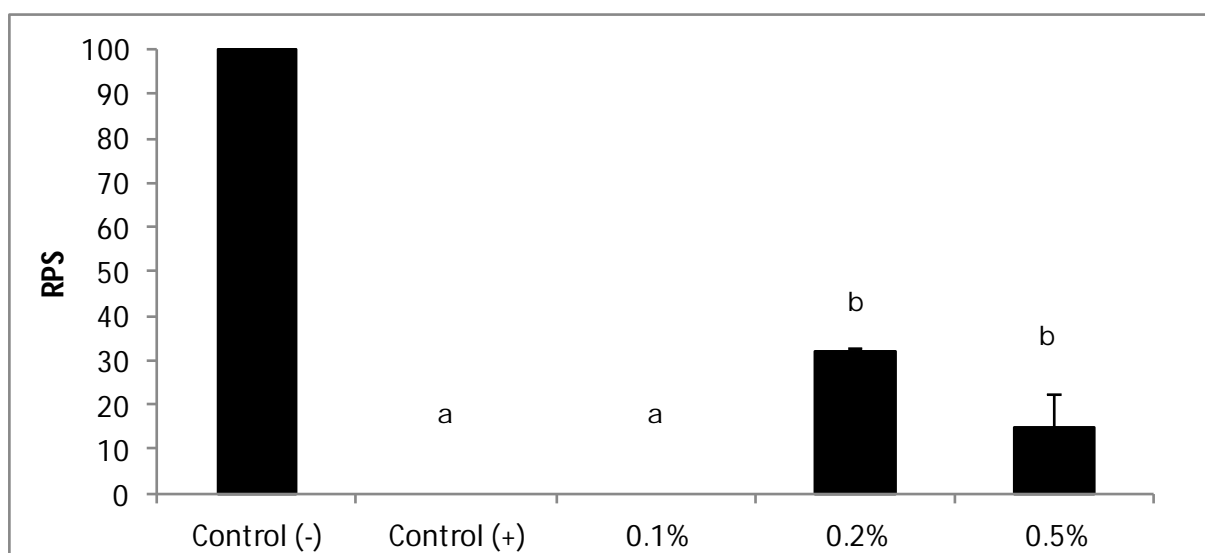

Figure 5. Relative Percent Survival of *P. monodon* at 10th day post challenge infection fed different levels of MOS and β-glucan after WSSV challenge infection. Values are expressed as Mean±SEM of four replicates (n = 10) on each treatment diet.

Survival after challenge with certain pathogens is considered a measure of disease resistance. The viral challenge was done through oral administration that is by feeding WSSV-infected shrimp tissues to the experimental animals. In the natural environment WSSV is transmitted with water as a vector or through ingestion or consumption of WSSV-infected animals thus in the present study feeding the experimental animals with WSSV-infected tissue reflects or mimics the natural route of infection. The present results revealed that 0.2% MOS and β –glucan-supplemented diets significantly enhanced survival of juvenile *P. monodon* against WSSV infection as compared with shrimps devoid of MOS and β– glucan. β– glucan mode of action might have played a major role in increasing shrimp's resistance against WSSV in the present study. β–glucan can activate macrophages thus increasing their capacity to kill pathogens. They have also been shown to reinforce other nonspecific immune factors such as lysozyme and complement activities (Misra et al 2006). β-1,3/1,6-glucans bind specifically to a "receptor molecule" on the surface of phagocytes, the cells then become more active in engulfing, killing and digesting microbes and at the same time secrete signal molecules (cytokines) which stimulate the formation of new white blood cells (Raa 2000).

Dietary administration of β–glucan have been reported to increase resistance of shrimp *Penaeus japonicus* against vibriosis (Itami et al 1994), further studies using *P. monodon* showed protection against vibriosis, white spot syndrome virus and *Vibrio damsela* (Su et al 1995; Song et al 1997) and also enhancement of survival and immunity during brood-stock rearing (Chang et al 2000). Supplementation of β–glucan could also enhance immunity and survival against bacterial pathogens of *L. rohita* fingerlings (Misra et al 2006).

Moreover, inclusion of MOS and β-glucan has shown positive effects on the immune indices of shrimp such as respiratory burst activity and THC in the present study. Decapod crustaceans have three major categories of blood cells (haemocytes) such as hyaline cells, semi-granular cells and granular cells. Each has distinctive morphological features and physiological functions. Haemocytes are responsible for clotting, exoskeleton hardening and elimination of foreign materials (Song & Hsieh 1994; Johansson et al 2000). When microorganisms are engulfed by haemocytes, a series of antimicrobial substances are generated. These substances include highly reactive oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxide ions (OH^-) and singlet oxygen (O_2). The superoxide anion O_2^- is the first product released from a respiratory burst (Campa-Cordova et al 2002).

Enhanced THC and respiratory burst activity was observed in *P. monodon* fed with combined supplementation of peptidoglycan and MOS (Apines-Amar et al 2014). Dietary β-glucan, MOS and their combinations significantly increased total coelomocytes count (TCC), phagocytosis, superoxide anion production and superoxide dismutase (SOD) activity of sea cucumbers *A. japonicus* (Gu et al 2011). Furthermore, immunostimulation with β-glucan and sulphated polysaccharide was found to be capable of generating an increase in the respiratory burst of *L. vannamei* (Campa-Cordova et al 2002). Similarly, *P. monodon* brooders showed enhanced haemocyte phagocytic activity, cell adhesion and superoxide anion production when glucan was administered in their diets (Chang et al 2000). Misra et al (2006) also reported that superoxide anion production in fish fed with β-glucan was always higher than the control fish; β-glucan also maintained the activation of phagocytic cells throughout the experimental period and was instrumental in achieving disease resistance and survival against bacterial pathogens. In the present study, total haemocyte count became significantly high when shrimps were supplemented with diet containing 0.2% MOS and β-glucan (Figure 6).
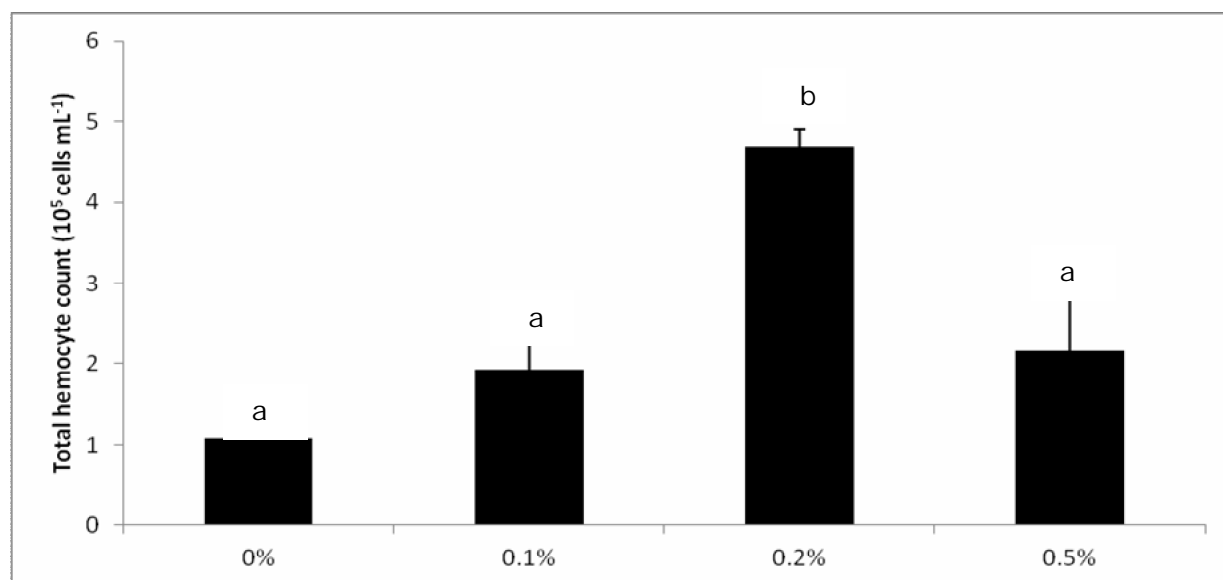

Figure 6. Total haemocyte count of shrimp fed different levels of MOS and β-glucan-supplemented diet for 14 days. Values are expressed as Mean±SEM of four replicates (n = 5) on each treatment diet.

Though at 0.5% inclusion of β-glucan and MOS a decrease in THC was observed. According to Cheng & Chen (2001) molting, development of an organ, reproductive

status, nutritional condition and occurrence of infection and even season have shown to influence haemocyte abundance thus in the present study these factors might have contributed to the lower THC of the experimental animals in this treatment group even though it was supplemented with β-glucan and MOS.

Respiratory burst responses of shrimps fed the MOS and β-glucan-supplemented diets were significantly higher than those fed the control diet (Figure 7). However, the MOS and β-glucan fed groups were not significantly different from each other.
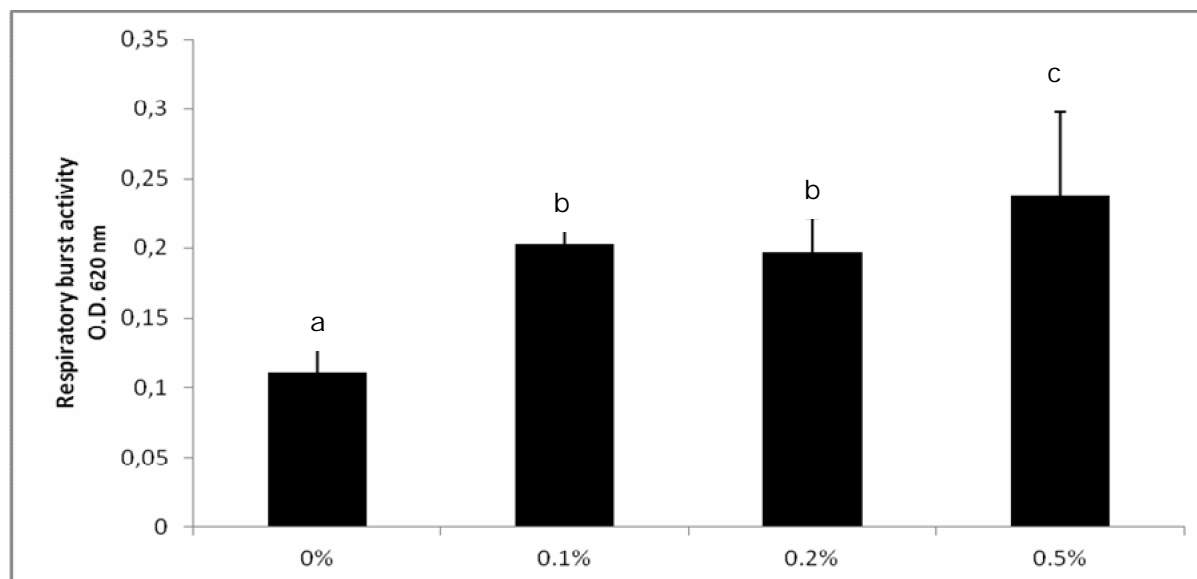
Figure 7. Respiratory burst activity of shrimp fed different levels of MOS + β-glucan-supplemented diet for 14 days. Values are expressed as Mean±SEM of four replicates (n = 5) on each treatment diet.

Conclusions. In summary, the present study revealed that among the levels of MOS and β-glucan-supplemented diets tested 0.2% inclusion in the diet was the optimum. Immunological indices such as THC and respiratory burst activity were enhanced in shrimp fed MOS and β-glucan. Furthermore, % weight gain and FCR have significantly improved in the MOS and β-glucan fed group. The results showed that MOS and β-glucan supplementation in the diet had the capacity to improve growth, feed utilization and enhance shrimp's immune response and survival against WSSV infection.

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