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SHORT COMMUNICATION

Supplementation of combined mannan oligosaccharide and β-glucan immunostimulants improves immunological responses and enhances resistance of Pacific Whiteleg Shrimp, *Penaeus vannamei*, against *Vibrio parahaemolyticus* infection

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Abstract A feeding trial was conducted to evaluate the combined effects of mannan oligosaccharide (MOS) + β-glucan (BZT® PRE-GE) supplementation administered at different frequencies on the immune responses and resistance of *Penaeus vannamei* against *Vibrio parahaemolyticus,* the causative agent of Early Mortality Syndrome (EMS) disease. Four experimental treatments were run with shrimps fed with a control basal diet (TCo), fed daily with diet containing 0.2% MOS+ β-glucan (Td), fed every 3 days with diet containing 0.2% MOS+ β-glucan (Td3) and fed every 7 days (Td7) with diet containing 0.2% MOS+ β-glucan. Following the 30-day feeding trial, shrimp immunological responses were quantified. Infection challenge test was also conducted to evaluate the influence of the treatments on the resistance of *P. vannamei* against *V. parahaemolyticus* infection. Results indicate that the immunostimulant application frequency did not affect shrimp survival and overall growth performance. Immunostimulant application every 3 days was found optimum to enhance the immunological responses including total hemocyte count, prophenol oxidase and serum antibacterial activities. Moreover, significant improvement in survival following the infection challenge with *V. parahaemolyticus* was observed in treatment fed with the immunostimulant every 3 days. Overall these findings suggest that 0.2% MOS+ β-glucan supplementation applied every 3 days could enhance the immune responses and improve the resistance of *P. vannamei* against *Vibrio parahaemolyticus* infection. Administration of 0.2% MOS+ β-glucan once every 3 days could be a practical immunoprophylactic strategy to manage and prevent outbreaks of mass mortalities caused by *V. parahaemolyticus* infection in *P. vannamei* farming.

Keywords Mannan oligosaccharide . β-glucan . *Penaeus vannamei* . *Vibrio parahaemolyticus* . Immunostimulant . Dose/frequency

Introduction

Vibriosis disease outbreaks have been associated with massive economic losses in the global shrimp farming industry (Karunasagar et al. 1994; Kumar et al. 2014). Vibrios are ubiquitous in nature and their ability to efficiently utilize organic nutrients, multiply rapidly, and dominate the culture system have been linked to disease outbreaks in shrimp farming (Kumar et al. 2014). Recently, the sustainability and economic viability of the South East Asian shrimp industry has been threatened by outbreaks of pathogenic *Vibrio parahaemolyticus*. This pathogenic Vibrio has been reported to affect post larvae of both *Penaeus vannamei and Penaeus monodon,* inflicting heavy mortalities at the first 35 days of culture (Kondo et al. 2014; Kumar et al. 2014; Dabu et al. 2015).

Shrimps, specifically the larval and early juvenile stages, are prone to Vibrio infections due to their lack of adaptive immunity and immature immune system. However it has been shown that the innate immunity of shrimp larvae can be primed with immune-active polysaccharide to enhance their resistance against Vibrio infections (Traifalgar et al. 2012; Hamsah et al. 2019). To date several compounds isolated

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from cellular components of microbes including glucans, mannose, lipopolysaccharide and peptidoglycan have been documented to elicit potent immunostimulatory effects on shrimp. Also, mannan oligosaccharide (MOS) incorporated in the diet at 12 mg L^{-1} and β-1, 3/1, 6-glucans was reported to improve the immune responses and disease resistance of *cultured P. vannamei* (Rodriguez et al. 2007; Hamsah et al. 2019). Heat-killed *Vibrio anguillarium* applied through immersion at 10^6 colony forming units (CFU) ml^{-1} was also documented to improve growth and enhance the immune responses of *P. monodon* (Azad et al. 2005). However most of these earlier works were focused to elucidate the functionality of these compounds on shrimp immunity but the optimization of application frequency on early juvenile shrimp has not been fully evaluated to date. Also, information on immunostimulating compounds that could protect juvenile shrimps against *V. parahaemolyticus* infection has been limited.

Moreover, earlier reports suggest the immunosuppressive effects of excessive immunostimulant application (beyond the optimum dose) in aquatic animals indicating the need to optimize dosage with application frequency (Sajeevan et al. 2009; Genio et al. 2015; Ojerio et al. 2017). Proper administration of the immunostimulant would lessen the risk of immunological fatigue that could decrease the resistance of juvenile shrimp against Vibrio infections.

The present study determines the optimum frequency application of combined mannan oligosaccharide and β-glucan immunostimulants that would improve the immunological responses and enhance the resistance of the *P. vannamei* against *V. parahaemolyticus* infection.

Materials and methods

Experimental animals and acclimation period

Apparently healthy juvenile *P. vannamei* were purchased from a commercial shrimp hatchery (Oversea Corporation, Cebu, Philippines) and transported to the experimental rearing facilities of the Institute of Aquaculture, University of the Philippines Visayas. Prior to the transport to the University facilities, shrimp were analyzed by molecular means and ensured to be free from pathogens. Polymerase chain reaction to detect white spot syndrome virus (WSSV) was done following the protocols described by Kimura et al. (1996). Further the presence of acute hepatopancreatic necrosis disease (AHPND) causing *V. parahaemolyticus* in the shrimp was evaluated following the method developed by Tinwongger et al. (2014).

The experimental shrimp were acclimatized in laboratory conditions and maintained with commercial shrimp feeds for 10 days. Following the acclimatization, the shrimp were weighed (≈ 0.23 g) and were randomly stocked in twelve 60-L tank at a density of 15 shrimps per container. The individual tank was provided with mild aeration and received a flow through water at an exchange rate of 300% a day. Throughout the experiment water parameters were maintained to the optimum requirement of *P. vannamei* (Temp: 28-30 °C; pH: 7.9-8.0; salinity: 33-35 g L^{-1}).

Experimental diet

A basal diet (control) containing fish and soybean meal as the main protein sources was formulated to satisfy the optimum nutrient requirements of *P. vannamei.* The ingredients and biochemical composition of the basal diet are presented in Table 1. The experimental diet was prepared by the inclusion of a mixture of MOS + β-glucan (BZT®PRE-GE, United-tech Inc., Tulsa USA) at 2 g kg^{-1} basal diet with the adjustment of cellulose to balance the dietary nutrients. The inclusion level of this immunostimulant mixture was previously optimized for shrimp done in our laboratory (Andrino et al. 2014; Solidum et al. 2016). All major dietary ingredients were obtained from the feed laboratory, Aquaculture Department, South East Asian Fisheries Development Center, Philippines. Components of the micro-nutrients including minerals and vitamin mix were obtained from Merck-Sigma Aldrich Inc., Darmstadt, Germany. The diets were prepared by the addition of the oil containing oil soluble vitamins to the dry ingredients and were thoroughly mixed. The immunostimulant was dissolved in an adequate amount of distilled water and added to the mixture. To the moist mash, agar solution as a binder was added and mixed in industrial food mixer (Hobart Inc., Ohio, USA) for 3 times. The formed dough was then pelletized using a food pelletizer and the pellets were dried in a mechanical convection oven (Thermo Fisher Scientific, USA) at 55 °C for 5 hr. The dried pellets

Table 1 Composition and proximate analysis of experimental diets containing 0 and 0.2% MOS + β-glucan (BZT® PRE-GE)

Ingredients	**Basal diet (g 100 g^{-1})**	**Experimental diet (g 100 g^{-1})**
Danish fish meal	46.00	46.00
Acetes meal	8.00	8.00
Soybean meal	21.00	21.00
Cellulose	4.50	4.30
Vitamin mix[1]	2.00	2.00
Mineral mix[2]	1.00	1.00
Lecithin	0.50	0.50
Cod liver oil	4.00	4.00
Starch	13.00	13.00
BZT® PRE-GE	0.00	0.20
TOTAL	100.00	100.00
Proximate Analysis	(g 100 g^{-1} Dry matter)	
Crude protein	47.30	
Crude fat	7.53	
Nitrogen free extract (NFE)	26.15	
Crude fiber	7.30	
Ash	11.72	
Gross energy[3] (kJ g^{-1} Dry Matter)	18.56	

[1] Vitamin premix composition: B-carotene, 1.8 g kg^{-1}; cholecalciferol, 0.02 g kg^{-1}; thiamine, 3.60 g kg^{-1}; riboflavin, 7.20 g kg^{-1}; pyridoxine 6.60 g kg^{-1}; cyanocobalamine, 0.02 g kg^{-1}; α-tocopherol, 16.50 g kg^{-1}; menadione, 2.40 g kg^{-1}; niacin, 14.40 g kg^{-1}; pantothenic acid, 4.00 g kg^{-1}; biotin, 0.02 g kg^{-1}; folic acid, 1.20 g kg^{-1}; inositol, 30.00 g kg^{-1}; stay C, 100.00 g kg^{-1}.

[2] Mineral premix composition: P, 12.0 %; 12.0 %; Mg, 1.5 %; Fe, 0.15 %; Zn, 0.42 %; Cu, 0.21 %; K, 7.50 %; Co, 0.011 %; Mn, 0.160 %; Se, 0.001 %; Mo, 0.0005 %; Al, 0.0025 %, I, 0.04 %.

[3]Gross Energy was calculated based on the estimated energy value of carbohydrate at 17.20 kJ g^{-1}; lipid at 39.8 kJ g^{-1} and protein at 23.4 kJ g^{-1} (Cho et al. 1982).

were cut to appropriate size (2 mm) and stored at 8 °C until used. Proximate composition analysis of the experimental diet was conducted following the methods outlined in AOAC (2000) for total protein, total lipid, carbohydrate as nitrogen free extract (NFE), crude fiber, and ash.

Feeding trial

The experimental diets were fed to each treatment groups, run in triplicate, for 30 days. Each treatment group were fed the experimental diets at a rate of 8% body weight per day, subdivided into three equal feedings at 9:00, 13:00 and 17:00. The control treatment (TCo) received the basal diet without the added immunostimulant while the 3 treatment groups received immunostimulant supplemented diet at different frequencies, daily (Td), once every 3 days (Td3) and once every 7 days (Td7) respectively. During the scheduled feeding with diets containing the immunostimulants, full dietary allocation subdivided into 3 feeding rations was given to the experimental animals. Experimental shrimp were fed the control diets during the period that they are not fed with the treated diet. Uneaten feeds and fecal wastes were collected daily and a 12 h light: 12 h dark photoperiod was maintained throughout the trial. Culture tank clean up and weighing of the experimental shrimp were conducted every 10 days to record the biomass and adjustment of the daily feed allocation. After the feeding trial shrimp on each experimental tank were bulk weighed and growth performance indices were measured. Weight gain (WG%), feed conversion ratio (FCR), and survival rate (S%) were calculated as follows:

$$\text{Weight Gain (\%)} = \frac{\text{Final weight - Initial weight}}{\text{Initial weight}} x\,100$$

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{Feed intake}}{\text{Weight Gain}}$$

$$\text{Survival (\%)} = \frac{\text{Final no. of shrimp}}{\text{Initial no of shrimp}} \text{ x } 100$$

Vibrio parahaemolyticus infection challenge test

After the feeding trial, 25 shrimps from each treatment were collected and subjected to a pathogen infection challenge test. Prior to the test lethal dose concentration (LD_{50}) of the *V. parahaemolyticus* was determined at 10^6 colony forming units (CFU) g^{-1} shrimp. The pathogenic strain of *V. parahaemolyticus* was delivered from University of Santo Tomas Philippines. The bacterial isolate was maintained in a cryofreezer at -80 °C and were grown in nutrient broth (NB, Pronadisa, Spain) supplemented with 2% NaCl prior to use. Prior to the infection challenge, the pathogencity of the *V. parahaemolyticus* was activated by in *vivo* passage. This was done by injecting live shrimp with the lethal dose of the pathogen at 10^5 CFU g^{-1} shrimp. Following the infection, moribund shrimp were collected and *V. parahaemolyticus* was collected from the heart of the shrimp. This process was repeated 3 times to guarantee the activation of bacterial virulence.

After the in *vivo* passage, colonies of virulent *V. parahaemolyticus* were collected and used in the infection challenge test. The infection challenge was conducted by injecting the shrimp at the base of the 3rd abdominal segment with 30 µL of *V. parahaemolyticus* at a dose of 10^5 CFU g^{-1} body weight. Another experimental group was added in this trial to serve as the negative control group, (-) control. In this group, shrimp were injected with sterile saline solution without the pathogen to ensure that saline solution injection alone could not cause mortalities. Mortalities were recorded daily until the mortality curve stabilized or have already plateaued. Confirmation of the cause of death was verified by the isolation of *V. parahaemolyticus* from the hepatopancreas and heart of the moribund shrimp. Re-isolation and confirmation of *V. parahaeemolyticus* was done using a HiCrome™ Vibrio Agar (Fluka Analytical, USA), a selective media for *V. parahaemolyticus* isolation.

Immune assays

Hemolymph extraction. Shrimp hemolymph was drawn from the ventral sinus cavity using1-mL tuberculin syringe (26-gauge) with anticoagulant solution (10 mM EDTA-Na2, 45 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3). To prevent clotting the hemolymph was collected at a ratio of one part hemolymph to three parts anticoagulant solution (Vargas-Albores et al. 1996). Twenty (20) µL of the collected hemolymph was used in counting total hemocytes (THC) and the remaining hemolymph was used for the measurement of prophenoloxidase (PO) activity.

Total haemocyte count (THC). To quantify the total hemocytes, a drop of hemolymph was placed in a Neubauer hemacytometer and the hemocytes were counted under the microscope. Values were expressed as cells ml^{-1} of hemolymph.

Prophenoloxidase (PO) activity. Prophenoloxidase activity was analysed spectrophotometrically through the quantification of dopachrome formed through the action of phenoloxidase on L-hydroxyphenylalanine (L-DOPA) as substrate (Hernandez-Lopez et al. 1996). In brief, hemolymph was centrifuged at 5000 rpm for 10 minutes at 4 ºC and the hemocyte pellets were collected. Two hundred µL of shrimp salt solution (450 mM NaCl, 10 mM KCl, 10 mM HEPES) was added to the collected hemocytes and the solution homogenized and centrifuged at 10000 rpm for 10 minutes at 4 ºC. Following the centrifugation, the supernatant was collected and the precipitated cell debris was discarded. Twenty five µL of the hemocytes supernatant was loaded in a 96-well microtiter plate together with 25 µL of 0.1 % trypsin in shrimp salt solution (SSS) and incubated at room temperature for 30 minutes. After the incubation, 25 µL of 0.3 % L-DOPA was added in each well and incubated for 3 minutes to allow color development. Optical density was read at 490 nm and PO unit activity expressed as change in absorbance $min^{-1}100$ uL^{-1} hemolymph (Joseph and Philip 2007).

Serum antibacterial activity. Hemolymph serum was used in the antibacterial activity assay. Serum was prepared by collecting the hemolymph without the anticoagulant. The hemolymph was allowed to clot and

was subjected to a freeze–thaw cycle for 5 times to induce cell lysis. Serum was collected by centrifugation at 7900 × g for 10 min at 4 °C. Serum antibacterial activity was carried out following the method of Ellof (1998) with slight modifications. Twenty microlitre of the serum was loaded in a 96-well microtiter plate together with 20 uL of overnight culture of *V. parahaemolyticus* and incubated for 3 hours at room temperature. After incubation, 20 uL of Lennox broth was then added and incubated for 2 hours. Then, 40 µL of INT (iodonitrotetrazolium) was added, incubated for 30 minutes and absorbance read at 490 nm. Wells without extract served as blank control (Buwa and van Staden 2006). Serum antibacterial activity is expressed as unit activity and computed as:

$$\text{Serum antibacterial activity} = \left(1 - \frac{\text{treatment absorbance at 490 nm}}{\text{control absorbance at 490 nm}}\right) x100$$

Statistical analysis

Statistical analysis was done using SPSS version 16.0. Data obtained from the growth experiment were subjected to one-way analysis of variance (ANOVA) with Duncan multiple range tests used to determine the significant differences between the means. Data on the pathogen challenge test was subjected to a Chi-square test to differentiate the differences in response among the treatment groups. All statistical tests were run at a significance level of α=0.05.

Results and discussion

In recent years the shrimp industry in Southeast Asia has suffered significant economic losses due to the Early Mortality Syndrome (EMS) disease, also known as APHND, caused by a virulent strain of *V. parahaemolyticus* (Kondo et al. 2014; Dabu et al. 2015). This disease problem persists until the present, slowing the recovery and growth of the shrimp industry in the Southeast Asian region. EMS/APHND disease is a serious threat affecting the *P. vannamei* aquaculture industry and to the best of our knowledge the present work is the first to document the optimized application of combined dietary immunostimulant MOS and β-glucan to improve the immune responses and enhance the resistance of *P. vannamei* against *V. parahaemolyticus* infection.

Our results suggest that dietary supplementation of 0.2 % MOS + β-glucan at different feeding frequencies was found not inhibitory to the overall shrimp growth and survival. No significant differences in WG %, FCR, and S % were observed in shrimps fed with the immunostimulant supplemented diets as compared to the control (Table 2). Similar results was reported by Solidum et al. (2016) showing that MOS + β-glucan dietary supplementation at 0.2%, 0.4% and 0.8% has no effect on the specific growth rate, feed conversion efficiency and survival of *P. vannamei.*

Also, feeding *P. vannamei* diets supplemented with yeast cell wall, rich in mannan oligosaccharide (MOS), and β-glucan was shown not to influence weight gain, FCR, specific growth rate and feed intake (Chotikachinda et al. 2008). In addition, a mixed dietary immunostimulant comprising yeast and lactic acid bacteria, containing mannose and β-glucan, was also reported not to affect *P. vannamei* growth rate but resistance against white spot syndrome virus (WSSV) was found significantly enhanced (Fierro-Coronado et al. 2019). These earlier reports concur with our present findings that MOS and β-glucan as immunostimulant does not influence growth performance of *P. vannamei* but are potent enhancers of immunological defences. In contrast it was reported that dietary supplementation of MOS + β-glucan significantly enhanced FCR and WG of juvenile *P. monodon* (Andrino et al. 2014). Also, MOS, β-glucan combinations as dietary supplement was found to improve the growth of the sea cucumber *Apostichopus japonicus* in comparison to the control group (Gu et al. 2011). These contrasting results could be due to species, metabolic differences, application dose, capacity to digest the immunostimulants and experimental conditions.

Early Mortality Syndrome disease caused by *Vibrio parahaemolyticus* has been associated with significant production losses in the aquaculture of *P. vannamei* in the tropical and subtropical regions (Kondo et al. 2014; Dabu et al. 2015). Until the present there has been no practical solution in the management and control of this bacterial disease. However, earlier works indicated that application of dietary immunostimulant could enhance shrimp immune responses and improve resistance against Vibrio infections. Further

application of dietary immunostimulants has been shown as an effective means of immunoprophylaxis to prevent Vibriosis in shrimp aquaculture (Huang et al. 2006; Traifalgar et al. 2013; Elshopakey et al. 2018). Results of the present study indicate that all treatments receiving the immunostimulant supplemented diets exhibited significant protection against *V. parahaemolyticus* infection as compared to the control (Figure 1). Optimum application frequency that elicited enhanced survival against *V. parahaemolyticus* infection was observed in treatment groups receiving the immunostimulant every 3 days. The immunoprotective effects of combined MOS and β–glucan applied at an optimum frequency to increase resistance against *V. parahaemolyticus* infection, to the best of our knowledge is unprecedented in *P. vannamei*. In addition the present results suggest that application of these immunostimulant combinations every 3 days is as effective as the daily application. This indicates that the immunological enhancement effects of these combined immunostimulants persist for 3 days. Shrimp has no specific immunity and for the shrimp innate immunity to remain active a continuous immunostimulation is needed. The low survival and lower immunological responses in experimental group receiving the immunostimulant every 7 days indicate ineffective and suboptimal stimulation of the shrimp immune system.

Recently, there has been a surge of interest in the determination of optimum application dose of immunostimulants in shrimp since excess and under dosage have been documented to be detrimental in protecting the shrimp against pathogenic infections (Sajeevan et al. 2009; Bai et al. 2010; Genio et al. 2015). Application of lipopolysaccharide immunostimulant every two days was documented to improve the survival of *P. monodon* against WSSV infection (Genio et al. 2015). Moreover, application frequency of mixed microbial immunostimulants done every 3 days was found optimum to enhance the survival of *P. vannamei* against WSSV infection (Fierro-Coronado et al. 2019). In contrast glucan immunostimulant at a dose of 0.2% in the diet applied every 7 days was found optimum to protect *P. indicus* against WSSV infection. Added to, enhanced survival of *P. chinensis* zoea larvae was reported by feeding daily with diets containing 0.25% glucan (Wang and Qi 2010). Collectively these reports suggest that optimum dose and frequency of application may vary depending on the type of immunostimulants and the species of shrimp being cultured. These reports also highlight the need for application frequency determination to achieve optimum immunoprotective effects of the immunostimulants against a specific pathogen affecting the cultured shrimp. Moreover, application of immunostimulants to enhance disease resistance adds additional cost in feed production. Thus, the present findings indicating the optimum application of immunostimulant every 3 days may lessen the amount of applied immunostimulant which could practically lower the production costs contributed by these feed additives in shrimp culture.

The high survival following the infection challenge against the pathogenic *V. parahaemolyticus* in the present study could be attributed to the synergistic immune activating properties of both β-glucan and MOS. The present results agree with earlier report of Andrino et al. (2014) highlighting the enhanced survival of *P. monodon*, fed diets supplemented with 0.2% MOS and β -glucan, against white spot syndrome virus (WSSV) infection. A combination of peptidoglycan and MOS was also shown to enhance survival of juvenile *P. monodon* exposed to WSSV (Apines-Amar et al. 2014). Dietary β-glucan administered at 0.2% of the diet every 7 days was documented to improve the survival of *F. indicus* challenged with WSSV (Sajeevan et al. 2009). Dietary supplementation of mannan oligosaccharide was also shown to increase the resistance of *Astacus leptodactylus* against *Aeromonas hydrophila* (Safari et al. 2014) while survival of *P. vannamei* after

Table 2 Growth performance indices of *P. vannamei* fed diets containing 0.2% MOS+β-glucan at administered at different frequencies. Values are expressed as mean ± SEM. Means in a row having similar superscripts are not significantly different (P >0.05)

Growth performance indices	**Experimental treatments**			
	TCo	**Td**	**Td3**	**Td7**
Initial weight (g)	0.23±0.01[a]	0.23±0.00[a]	0.23±0.01[a]	0.23±0.01[a]
Final weight (g)	1.19±0.12[a]	1.14±0.04[a]	1.07±0.08[a]	1.28±0.05[a]
Weight gain (%)	413.91±38.43[a]	386.73±13.61[a]	371.29±20.98[a]	464.44±30.67[a]
FCR	1.14±0.11[a]	1.28±0.04[a]	1.23±0.06[a]	1.12±0.06[a]
Survival (%)	86.66±6.67[a]	80.00±3.85[a]	71.11±2.22[a]	93.33±6.67[a]

challenge with pathogenic *V. harveyi* were increased when fed with copra-derived mannan oligosaccharides at 10 g kg^{-1} (Cuong et al. 2013). In sea cucumber, *Apostichopus japonicus* dietary application of MOS and β -glucan was shown to synergistically enhance the resistance against *V. splendidus* infection (Gu et al. 2011). These earlier reports support our present findings suggesting that dietary application of MOS+ β-glucan enhances the resistance of shrimp against bacterial pathogens.

The significant enhancement of resistance of *P. vannamei* against *V. parahaemolyticus* infection in the present study is associated with significant enhancement of the shrimp immune responses. Total hemocyte count, prophenoloxidase activity and serum antibacterial activity was significantly higher in shrimps fed diets supplemented with MOS + β-glucan than the control group (Table 3). Application of the immunostimulants every 3 days was found optimum in the enhancement of the shrimp immunological responses. The significant enhancement in the immunological responses of *P. vannamei* observed in the present study could be attributed to the potent biological activity of both β-glucan and MOS. β -glucan has been known to activate and initiate immune cascades in shrimp through binding with β-glucan binding proteins (BGBP) in the hemolymph. This ligand binding activates the prophenoloxidase system which induces plasma antimicrobial activity, enhances phagocytosis, cell adhesion and superoxide production in hemocytes (Vargas-Albores et al. 1996; Chang et al. 2000). In addition, MOS is known to be a potent activator of shrimp C-type lectin that exhibits strong binding and agglutinating activity of bacterial and viral pathogens (Denis et al. 2016; Gao et al. 2020).

The present results concur with the results on feeding MOS + β-glucan (Andrino et al. 2014) and combined peptidoglycan + MOS (Apines-Amar et al. 2014) to *P. monodon* that resulted to significant increase in THC, enhanced PO activity and efficient Vibrio clearance activity. Moreover, MOS, fructooligosaccharide and combinations of both increased the THC of *Astacus leptodactylus* (Safari et al. 2014). MOS, β-glucan and their combination enhanced the total coelomocytes count of *Apostichopus japonicus* (Gu et al. 2011) alongside with the enhancement of phagocytosis activity, superoxide and nitric oxide production. Similar to the present findings, THC and PO activity of *P. vannamei* was enhanced when fed diets supplemented with mushroom β-glucan (Yang et al. 2014). Further it was also shown that MOS supplementation at 2.0-4.0 g kg^{-1} enhanced the PO activity and improved the overall response of *P. vannamei* against ammonia stress (Zhang et al. 2014). These earlier works indicate the potent immune activating properties of β-glucan and MOS supplementation in aquatic invertebrates and may explain the enhanced resistance of *P. vannamei* against *V. parahaemolyticus* infection observed in the present study.

Conclusion

Collectively the present results suggest that combined dietary immunostimulants (MOS+ β –glucan) at a

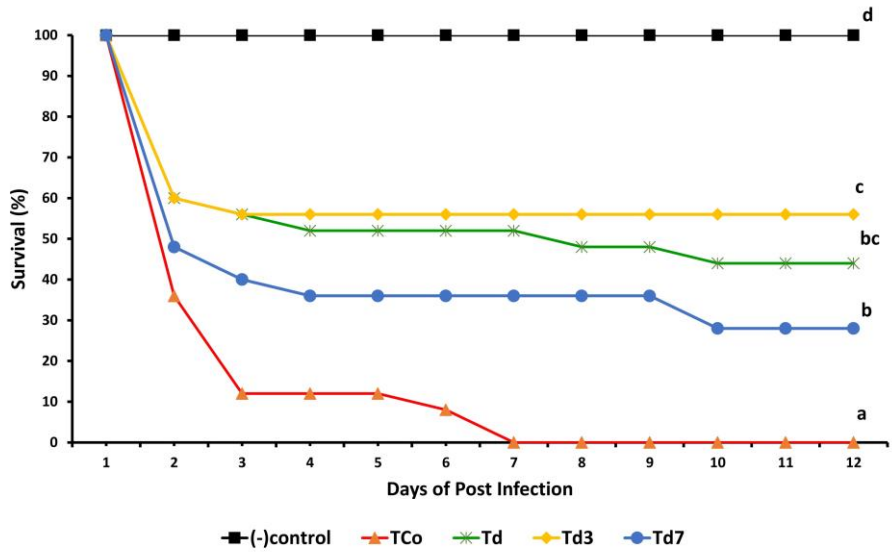

Fig. 1 Survival (%) of *P. vannamei* fed the control and experimental diets containing 0.2% MOS + β-glucan given at different frequencies and challenged with pathogenic *V. parahaemolyticus.* Treatments having similar superscripts are not significantly different (P >0.05)

Table 3 Immunological indices of *P. vannamei* fed the control and diets containing 0.2% MOS + β-glucan given at different frequencies. Values are expressed as mean ± SEM. Means in a row having similar superscripts are not significantly different (P >0.05)

Immunological indices	Experimental treatments			
	TCo	Td	Td3	Td7
Total hemocyte count (10^5 cells mL^{-1})	5.46±0.10^a	22.73±0.22^c	14.87±0.24^b	12.51±0.15^b
Prophenoloxidase activity (Units)	29.33±7.53^a	77.33±4.83^b	72.83±3.86^b	25.83±5.74^a
Serum antibacterial activity (Units)	51.97±1.86^a	78.91±9.49^b	74.72±5.65^b	53.24±3.09^a

dose of 0.2% diet applied every 3 days could enhance immune responses and increase the resistance of *P. vannamei* against *V. parahaemolyticus* infection. Application of this immunostimulant combination could be a practical approach to mitigate and prevent the occurrence of Early Mortality Syndrome disease in *P. vannamei* culture.

Conflict of interest The authors declare that they have no conflict of interest.

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