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Dietary Mannan Oligosaccharides Positively Affect the Growth, Digestive Enzyme Activity, Immunity and Resistance Against *Vibrio harveyi* of Pacific White Shrimp (*Litopenaeus vannamei*) Larvae

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Abstract

This study aimed to determine the effect of dietary mannan oligosaccharides (MOS) delivered through *Artemia* sp. at different concentrations (i.e. 0, 3, 6 and 12 mg L^{-1}) on the survival, growth, immunity, digestive enzymes and resistance against *Vibrio harveyi* of Pacific white shrimp larvae in four treatment groups (in triplicates), i.e. control, MOS3, MOS6 and MOS12. Larvae were reared in tank with a dimension of 25 cm × 20 cm × 30 cm containing 4 L of disinfected seawater at a stocking density of 30 larvae L^{-1}. The larvae were maintained until they reached post larval stage for 15 days and were subsequently challenged with *V. harveyi* (10^7 CFU mL^{-1}) for 5 days. The survival and growth of the shrimp in MOS treatments were significantly higher (P<0.05) than control. Following challenge test with *V. harveyi*, the shrimp in MOS treatments demonstrated higher survival, total haemocyte count (THC), phagocytic activity, phenoloxidase and respiratory burst activities than those of the positive control (P<0.05). The digestive enzyme activities in shrimp larvae fed with MOS were higher than those in the control. The increase of digestive enzyme activity was positively correlated with the increase of MOS concentrations with correlation coefficients for protease, lipase and amylase were 0.96, 0.95 and 0.91, respectively (P<0.05). In conclusion, the present study demonstrated that dietary MOS supplementation through *Artemia* enrichment at the concentration up to 12 mg MOS L^{-1} could significantly improve Pacific white shrimp post larval digestive enzymes activities, growth, survival and resistance against *V. harveyi* infection.

Keywords: *Litopenaeus vannamei*, mannan oligosaccaharides, prebiotics, immune response, *Vibrio harveyi*

Introduction

Shrimp has been recognized as the second most traded aquaculture products for decades. During the recent years, however, shrimp production in major producing countries has declined, mostly due to disease problem (FAO, 2016). Stentiford, Sritunyalucksana, Flegel, Wiliam, Withyachumnarnkul, Itsathitphaisarn, and Bass (2017) recently reported that infectious diseases has caused shrimp industry to suffer devastating economics and social impacts with total losses exceeding 40% of global capacity. Amongst the efforts have been done to alleviate this problem, avoidance of disease outbreaks by management of pond and animal microbiomes remains to be the most feasible approach to mitigate losses rather than attempting to eliminate the presence of pathogens (Bossier, De

Schryver, Defoirdt, Ruwandeepika, Natrah, Ekasari, Toi, Nhan, Tinh, Pande, Karunasagar, & Van Stappen, 2016; Stentiford et al., 2017). In this regard, the strategies to address disease problems in shrimp production have been directed toward improving the culture environment (De Scrhyver, Defoirdt, & Sorgeloos, 2014) and enhancing the cultured animals strength and defence against disease infection (Stentiford et al., 2017). The later include improvement of the animal's health status by supplying sufficient nutrients to support good growth and robustness (Pohlenz & Gatlin, 2014), applying vaccination and immunostimulation to improve immunity and conditioning the animal's microbiome by probiotics and prebiotics application (Vadstein, Bergh, Gatesoupe, Galindo-Villegas, Mulero, Picchietti, Scapigliati, Makridis, Olsen, Dierckens, Defoirdt, Boon, De Schryver, & Bossier, 2013). Prebiotics are defined as non-digestible carbohydrates that cannot be digested by the host, but can be metabolized by normal gastrointestinal (GIT) microflora (Manning & Gibson, 2004; Ringø, Olsen, Gifstad, Dalmo, Amlund, Hemre, & Bakke, 2010). The administration of prebiotics has been shown to facilitate the growth of beneficial GIT microbes, which have demonstrated some positive effects on the host health and growth, i.e. by decreasing the presence of intestinal pathogens and/or changing the production of health related bacterial metabolites (Sealey, Conley, & Bensley, 2015). Mannan oligosaccharides (MOS) are one of the prebiotics groups that have been gaining attention for the application in aquaculture, which are glucomannoprotein complexes derived from the cell wall of yeast, *Saccharomyces cereviceae* (Ringø et al., 2010; Torrecillas, Montero, & Izquierdo, 2014). Some beneficial effects of MOS have been recorded so far include enhancing health and growth performance (Sang & Fotedar, 2010a; Sang, Fotedar, & Filler, 2011a), improving gut morphology and microbiota (Daniels, Merrifeild, Boothroyd, Davies, Factor, & Arnold 2010; Zhang, Liu, Tian, Yang, Liang, & Xu, 2012), increasing immunity against disease (Rungrassamee, Kingcha, Srimurat, Maibunkaew, Karoonuthaisiri, & Visessanguan, 2014; Safari, Shahsayani, Paolucci, & Atash, 2014; Sang, Ky, & Fotedar, 2009) and robustness against stress (Liu, Xu, Ge, Xie, Xu, Zhou, Pan, & Zhang, 2013). The applications of MOS as a prebiotic have been reported in some aquaculture species with varying levels of success, including in crustacean such as Pacific white shrimp (Zhang et al., 2012; Rungrassamee et al., 2014), European lobster (Daniels et al., 2010; Daniels, Merrifield, Ringo, & Davies, 2013), spiny lobster (Sang & Fotedar, 2010b; Do Huu & Jones, 2014) and freshwater crayfish (Sang et al., 2011; Mazlum, Yilmaz, Genc, & Guner, 2011), marron (Sang & Fotedar, 2009; Sang & Fotedar, 2010a; Sang, Fotedar, & Filer, 2011b), narrow clawed crayfish (Safari et al., 2014) and tiger shrimp (Sang, Kien, & Thanh Thuy, 2014).

Despite the growing evidence of prebiotic beneficial effects on the growth, survival and immune response of various aquaculture species, there are only few studies have been done to explore the effects of prebiotic encapsulation in live food on the larviculture of aquaculture species. On the other hand, the quality of larvae, usually represented by their growth, survival and robustness, is of important aspects in aquaculture; as it may influence not only the performance of hatchery production, but also the performance of grow out production. Prebiotics application at earlier life stage, i.e. in larviculture, has been suggested as a potential strategy to enhance the microbial contribution to larval nutrition, physiology and immunity by steering the microbial community composition and activity in both the larval culture environment and intestinal tract (Vadstein et al., 2013). In this context, the present study demonstrated the effect of MOS bioencapsulated in *Artemia* nauplii on Pacific white shrimp (*Litopenaeus vannamei* Boone) post larvae growth performance and immunity. This study used *Artemia* to facilitate the delivery of MOS to the larvae, as it is the most common live food used in shrimp hatchery, which has a compatible size and nutritionally adequate for the larvae. In addition, we also demonstrated the contribution of dietary MOS supplementation on the digestive enzymes activities and RNA/DNA ratios that represented the larvae

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digestive capacity and growth potential.

Materials and Methods

Experimental Design

The present study compared the growth performance and immune response of Pacific whiteleg shrimp larvae fed with *Artemia* naupli enriched with MOS at different concentrations, i.e. 0, 3, 6 and 12 mg L^{-1}. In this regard, four groups of treatments in triplicates were applied in the experiment, i.e. control, MOS3, MOS6 and MOS12.

Shrimp Rearing

The experiment used 12 units of glass aquarium with a dimension of 25 cm × 20 cm × 30 cm which were placed inside a fibre tank (2 m × 1 m × 0,5 m) previously filled with 100 L of freshwater. This arrangement was done to attain stable water temperature in each replicate tank. Water temperature throughout the experiment was maintained at a range of 30 to 32 ^{o}C by using five units of thermostat. Aeration was provided to each experimental aquarium through an aeration unit connected to an air blower.

Specific pathogen free Pacific white shrimp larvae at mysis 1 stage were obtained from a local hatchery (PT. Suri Tani Pemuka Labuan, Banten Province, Indonesia) and acclimatized at laboratory condition until they reached mysis 3 stage. During this stage, shrimp larvae were fed with *Artemia* nauplii at a range of 3 - 4 nauplii per larvae per feeding. Thereafter, the larvae (0.25 ± 0.01 mg) were randomly distributed into each experimental tank at a density of 30 shrimp L^{-1}.

Feeding was performed using *Artemia* nauplii previously enriched with MOS at different concentrations. Feed quantity was gradually increased following the developmental stage of the shrimp at a level ranging from 8 to 10 *Artemia* nauplii per larva per feeding (Nimrat, Boonthai, & Vuthiphandchai, 2011). Feeding was offered 6 times a day at 02.00, 06.00, 10.00, 14.00, 18.00, and 22.00 for 15 days of culture.

Artemia Enrichment

Artemia enrichment was performed following the procedures described by Daniels et al. (2010) with some modifications. *Artemia* naupli was obtained by hatching 2 g of *Artemia* cyst (Supreme Plus, US) in 1 L of seawater. Enrichment was performed by adding MOS (BIOMOS, Altech Inc., KY USA) into newly hatched *Artemia* nauplii culture medium (seawater at a salinity of 30 gL^{-1}) at a concentration depending on the treatment. *Artemia* nauplii were maintained in prebiotic suspension at a density of 100 individual mL^{-1} for 4 h. Similar procedure was applied to *Artemia* nauplii provided for the shrimp in control treatment, except there was no prebiotic added to the *Artemia* culture medium. The nauplii were subsequently collected using a plankton net, rinsed with fresh seawater and directly transferred to the shrimp larvae culture tank or kept in a refrigerator (4^{o}C) for later feeding time that day.

Zoo Technical Parameters

Shrimp survival, growth, digestive enzymes activities, bacterial count, immune parameters were determined at the final day of experimentation. Shrimp survival was the proportion of survived shrimp at the end of the experiment over the initial number of shrimp at the beginning of the experiment. Specific growth rate was calculated using the following formula:

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$$\text{SGR} = \left[\sqrt[t]{\frac{Wt}{Wo}} - 1\right] \times 100$$

Where:

SGR = specific growth rate (% day^{-1})

Wo= mean initial body weight (mg)

Wt= mean final body weight (mg)

t = culture period (day)

Absolute growth of length was calculated by subtracting the mean final shrimp body length (mm) by the mean of initial shrimp body length (mm).

Digestive enzymes activities measured in the present study were amylase, protease, and lipase activities by pooling about 25 to 30 shrimps (0.5 g) per replicate tank. Protease and amylase activities were determined following the procedures described in Worthington (1993), whereas lipase activity was determined according to the method described in Borlongan (1990). RNA/DNA ratio in shrimp larvae whole body was measured to evaluate the effect of prebiotic administration on the shrimp growth potential (Tanaka, Gwak, Tanaka, Sawada, Okada, Miyashita, & Kumai, 2013). Three shrimps were collected from each replicate tank and pooled together for RNA and DNA extractions, which were performed using extraction kits ISOGEN (Nippon Gene, Japan) and Puregene (Qiagen), for RNA and DNA isolation, respectively. The concentrations were subsequently measured using DNA/RNA Quant.

Challenge Test

Upon the completion of the feeding experiment, challenge test was performed using pathogenic *Vibrio harveyi* MR5339 isolated from shrimp infected with luminescent vibriosis (a collection of the Fish Health Laboratory of the Department of Aquaculture, Bogor Agricultural University). Challenge test was carried out on 10 shrimp from each replicate tank placed 15 units of glass container previously filled with 1 L of disinfected seawater with the procedure previously described in Merchie, Kontara, Lavens, Robles, Kurmaly, and Sorgeloos (1998) and Widanarni, Noermala, and Sukenda (2014). The bacterial suspension was added to each container at an initial density of 10^7 CFU mL^{-1}. On the following day, *V. harveyi* suspension was occasionally added to replace loss due to water replacements, which were done on day 1 and 3 of the challenge test period. Feeding during challenge test was done using non-enriched *Artemia* nauplii, which was offered five times a day. Negative control using the shrimp from the control treatment was applied in this test with similar treatment, except no *V. harveyi* suspension was added to the container. Challenge test was performed for 5 days.

Immune Parameters

Immune parameters were measured after feeding experiment (prior to challenge test) and after the completion of challenge test. Body fluid collection was conducted following the procedures previously described in Tampangallo, Pakidi, and Rantetondok (2012) with some modifications. Briefly, about 0.3 g of larvae (five to six post larvae) were collected from each replicate tank, placed in a pestle and added with 900 µL of precooled anticoagulant solution (30mM trisodium citrate, 0.34 M sodium chloride, 10mM EDTA and 0.12 M glucose, pH of 7.55) (Liu & Chen, 2004). Thereafter, each larva was slightly compressed to let the haemolymph flowing out

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the shrimp's body and got mixed with anticoagulant solution. The solids were subsequently removed and haemolymph mixture was transferred into new microtube for further analyses. Total haemocyte count (THC) was measured following the procedures described by Yeh and Chen (2009), whereas phagocytic index was measured according to Anderson and Siwicki (1995). Phenoloxidase activity was measured according to Liu and Chen (2004) and Martín, Castillo, Arenal, Rodriguez, Franco, Santiesteban, Sotolongo, Forrellat, Espinosa, Carrilo, and Cabrera (2012) by measuring the formation of dopachrome at an optical density (OD) of 490nm. Phenoloxidaseactivity unit was determined as the formation of dopachrome per 50 µL of homogenates. Respiratory burst was performed according to the procedure described in Song and Hsieh (1994) and Martín et al. (2012), which were based on the formation of formazan. Respiratory burst was determined as the formation of blue formazan at an OD of 630 nm per 10 µL of homogenates.

Water Quality

Water temperature in each tank was monitored daily, whereas dissolved oxygen (DO) concentration, pH and salinity were measured weekly. All water quality parameters were in normal range for shrimp larviculture, with the ranges for temperature, DO, pH and salinity were 30 – 32 ^{o}C, 3.1 – 5.4 mg L^{-1}, 7.8 – 8.0 and 28 – 31.8 g L^{-1}, respectively.

Data Analyses

All data was represented in mean values and standar deviation. The distribution and the variance homogeneity of the data were assessed by Kolmogorov-Smirnov test and Levene's test, respectively, and all data were homoscedastic and normally distributed. All data was subsequently subjected to analyses of variance (ANOVA). Significant differences were determined by Duncan post hoc test, whereas coefficient correlations were determined using regression analyses. Statistical analyses were performed using statistical software SPSS version 23.

Results

Larvae Growth Performance

The highest shrimp survival was observed in MOS12 treatment, which was significantly different from other treatment groups (P<0.05). MOS administrations on shrimp larvae resulted in higher growth as indicated by the significantly higher SGR and shrimp final length (Table 1, P<0.05). The highest growth was observed in shrimp in MOS12 treatment.

Digestive Enzyme Activities and RNA/DNA Ratio in Shrimp Larvae

The digestive enzyme activities in shrimp larvae fed with MOS were higher than those in the control group (Table 2). Furthermore, the increase of digestive enzyme activity was positively correlated with the increase of MOS concentrations with correlation coefficients for protease, lipase and amylase were 0.96, 0.95 and 0.91, respectively (P<0.05). The ratio of RNA/DNA in shrimp with treatment MOS6 and MOS12 were significantly higher than those with the control and MOS3 treatment (P<0.05) (Figure 1).

Immune Parameters and Post Challenged Survival

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After feeding experiment, the immune parameters in treatments with MOS were evidently improved, with the levels increased with the increase of MOS concentrations (Table 3). After challenge test, decreases in all immune parameters were observed compared to those before challenge test. However, the shrimp immune parameters before and after challenge test showed similar trends, where shrimp fed with MOS-encapsulated *Artemia* nauplii showed higher levels of immune responses (Table 4). This was confirmed by the higher number of shrimp survivors in MOS treatments than that of the positive control following challenge test against *V. harveyi* (Figure 2).

Discussion

To date, most of MOS or other prebiotics studies were performed in juvenile or adult (grow out) stage and only limited information is available on their application in larvae production of aquaculture species. Thereby more research is needed to fully comprehend the efficacy of dietary prebiotics in crustacean larvae production. The application of MOS on crustacean larvae production has been reported so far was done by Daniels et al. (2010 and 2013) who demonstrated the positive effects of MOS-enriched *Artemia* on the growth and immunity of European lobster postlarvae.

The present study demonstrated that MOS encapsulation using *Artemia* nauplii positively affected the growth performance of shrimp larvae. Although the differences between MOS3 and MOS6 treatments were not significant, it was generally shown that the increase of prebiotic concentrations was concomitant with the increase of shrimp specific growth rate and final total length. Furthermore, the differences in growth potential of the shrimp post larvae amongst the treatments were also demonstrated by the levels of RNA/DNA ratio as an indicator of protein synthesis (Li, Haga, Masuda, Takahashi, Ohta, Ishida, & Satoh, 2013). The positive effects of MOS in improving growth performance were consistent with previous study by Zhang et al. (2012) on Pacific white shrimp juveniles.

The underlying modes of actions of dietary MOS on the animal's growth have been reported to this point mostly relates to: 1) the improvement of gut morphology and functionality, i.e. increase of microvilli length and density, higher number of goblet cells, higher production of intestinal mucus, which may facilitate higher capacity for nutrient digestions and absorptions in the animal's intestine (Sang et al., 2011a; Zhang et al., 2014), 2) the facilitation of beneficial microbiota in the animal's gastrointestinal tract (GIT) that may contribute to the excretion of digestive enzymes and to the alteration in host's nutrients metabolism; 3) the improvement of feed intake by contributing in satiation and appetite signals and regulations (Torrecillas et al., 2014). Indeed, our study demonstrated that dietary supplementation of MOS could improve the activity of main digestive enzymes, i.e. protease, lipase and amylase, which may contribute to the increase capacity of nutrient digestion and ultimately resulted in better growth of the shrimp postlarvae. Previous studies showed that the effect of MOS on digestive enzymes might be different between species or between developmental stages (Torrecillas et al., 2014). For instance, Akter, Sutriana, Talpur, and Hashim (2016) reported that dietary MOS supplementation could increase the activity of digestive enzymes in juvenile striped catfish, and attributed this positive effects to the modulation of the animals gut morphology. Sang et al. (2011) showed that MOS could improve the digestive enzyme activity in hepatopancreas and midgut of yabbies (*Cherax destructor*), thus increasing the bioavailability of nutrients in the diet. However, further study is needed to elucidate the role of prebiotics on the digestive enzyme activity.

The enhanced shrimp survival in MOS treatments might be explained by the enhanced shrimp robustness against

stress and immunity against possible disease infection. Indeed, our results showed that following 15 days of dietary MOS treatments, the shrimp total haemocyte count, phagocytic and PO activities and respiratory burst were significantly enhanced. These results confirmed previous study showing that dietary administration of MOS on Pacific white shrimp juvenile resulted in enhanced the shrimp antioxidative capacity and immunocompetence as well as its robustness against ammonia exposure stress (Zhang et al., 2014). The elevating immunocompetence and robustness against stress of the shrimp may explain the higher resistance of the shrimp PLs *V. harveyi* challenge, as observed in this study. It is even interesting to note that the shrimp survival in treatment MOS12 was not significantly different to that of the negative control and that the immune parameters in the after challenge test were still significantly higher in MOS treatments than those of the control indicating the higher defense capacity of the animals against the disease.

Along with the immune system stimulating effects, MOS may also play an important role in blocking pathogen colonization in the animal's GIT. Torrecillas et al. (2014) suggested that MOS protection against disease outbreaks maybe associated to the promotion of gut pre-epithelial defense mechanism, i.e. by increasing intestinal goblet cells density and mucus production in the intestinal tract. It has been suggested that mannose could be used as inhibitors of pathogen adhesion to intestinal cells. Pathogenic bacteria may recognize and adhere to mannan that mimics the specific carbohydrates groups of intestinal cells. As MOS is not digested, it will be removed with the feces and therefore reducing the incidence of the potential disease. In addition, MOS as immune modulator maybe related to the activation of pattern recognition receptors (PRRS) and proteins (PRPs), which further activate the innate immune system (Torrecillas et al., 2014). The macrophages and endothelial cell are equipped with mannose receptor that recognizes both self-glycoprotein and microbial glycan ligands. The mannose-containing ligands subsequently induce intracellular signalling cascades that would activate the production of proinflamatory cytokines (Song, Beck, Kim, Park, Kim, Kim, & Ringo, 2014).

In conclusion, the present study demonstrated that MOS administration through bioencapsulation in *Artemia* nauplii at the concentration up to 12 mg MOS L^{-1} could improve the digestive capacity, growth, survival and immunity of Pacific white shrimp post larvae against *V. harveyi* infection.

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Table 1. Survival, specific growth rate (SGR) and final length of Pacific white shrimp larvae fed with *Artemia* naupli enriched with MOS at different concentration after 15 days of experimentation

Treatment	Survival (%)	SGR (%/day)	Final body weight (mg)	Final total length (mm)
Control	67.78±3.94[a]	25.08±1.79[a]	8.82±1.67[a]	7.30±0.33[a]
MOS3	71.39±2.93[a]	31.44±1.25[b]	16.63±2.20[b]	10.42±0.51[b]
MOS6	76.11±5.09[a]	33.06±1.27[b]	18.65±2.44[b]	10.63±0.58[b]
MOS12	91.67±7.95[b]	37.19±2.34[c]	20.44±5.13[b]	12.50±0.88[c]

*Different superscript letters following mean values (± standard deviation) indicate significant differences (P<0.05)

Table 2. Protease, lipase and amylase activity in Pacific white shrimp larvae fed with *Artemia* enriched with MOS at different density after 15 days of feeding experiment

Treatment	Enzyme activity

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	Protease (mU mL $^{-1}$ min^{-1})	Lipase (mU mL $^{-1}$ min^{-1})	Amylase (mU mL $^{-1}$ min^{-1})
Control	33.7±0.3^{a}	88±1.5^{a}	0.84±0.004^{a}
MOS3	37.7±0.2^{b}	104±1.5^{b}	1.12±0.002^{b}
MOS6	43.9±0.6^{c}	108±0.5^{c}	1.13±0.006^{b}
MOS12	47.2±0.2^{d}	119±0.5^{d}	1.30±0.006^{c}

*Different superscript letters following mean values (± standard deviation) indicate significant differences (P<0.05).

Table 3. Total haemocyte count (THC), phagocytic activity, phenoloxidase (PO) activity and respiratory burst (RB) in Pacific white shrimp larvae fed with *Artemia* enriched with MOS at different density after 15 days of feeding experiment.

Treatment	THC (×10^{6} cell mL^{-1})	Phagocytic activity (%)	PO activity (OD 490 nm)	RB (OD 630 nm)
Control	2.90±0.23^{a}	32.93±2.55^{a}	0.40±0.11^{a}	0.79±0.04^{a}
MOS3	4.49±0.80^{b}	44.00±11.14^{ab}	0.53±0.05^{ab}	0.94±0.25^{ab}
MOS6	5.41±0.88^{b}	50.67±9.45^{b}	0.55±0.03^{b}	1.04±0.01^{ab}
MOS12	5.66±0.75^{b}	52.64±1.18^{b}	0.52±0.05^{ab}	1.12±0.04^{b}

*Different superscript letters following mean values (± standard deviation) indicate significant differences (P<0.05)

Table 4. Total haemocyte count (THC), phagocytic activity, phenoloxidase (PO) activity and respiratory burst (RB) in Pacific white shrimp larvae fed with *Artemia* enriched with MOS at different density after challenged with *V. harveyi*

Treatment	THC (×10^{6} cells mL^{-1})	Phagocytic activity (%)	PO (OD 490 nm)	RB (OD 630 nm)
Control (-)	4.15±0.38^{c}	37.33±3.89^{b}	0.61±0.01^{e}	1.00±0.02^{e}
Control (+)	1.20±0.14^{a}	19.95±4.44^{a}	0.19±0.00^{a}	0.21±0.02^{a}
MOS3	3.24±0.11^{b}	35.35±2.56^{b}	0.35±0.03^{b}	0.72±0.01^{c}
MOS6	4.06±0.23^{c}	38.38±4.11^{b}	0.39±0.01^{c}	0.63±0.04^{b}
MOS12	4.86±0.09^{d}	45.04±1.68^{c}	0.42±0.01^{d}	0.86±0.03^{d}

*Different superscript letters following mean values (± standard deviation) indicate significant differences (P<0.05)

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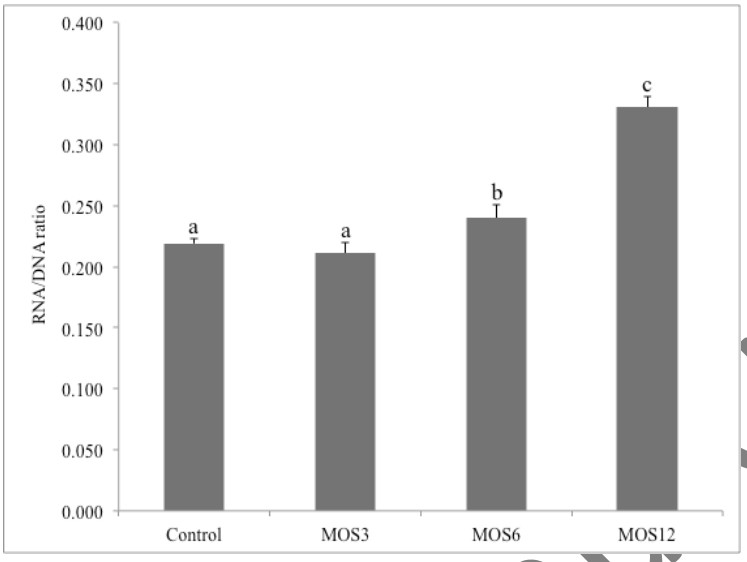

Figure 1. RNA/DNA ratio of Pacific white shrimp fed with *Artemia* naupli enriched with MOS at different concentration after 15 days of experimentation. Different letters above different bars indicate significant differences (P<0.05)

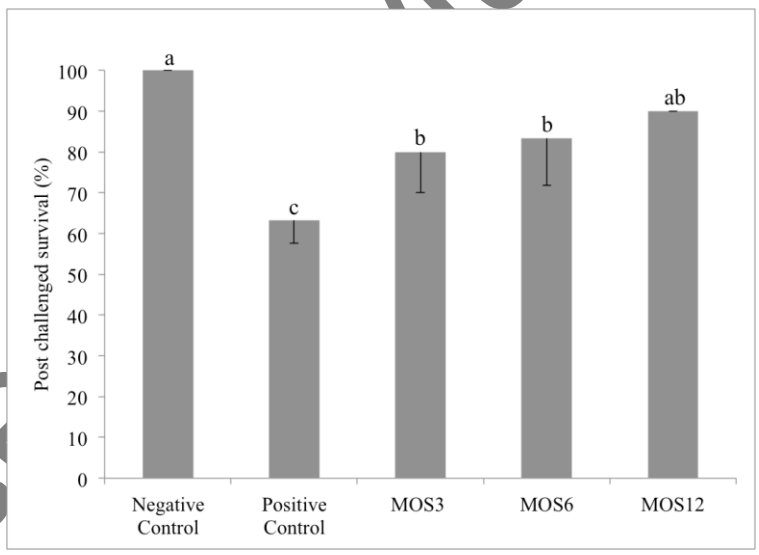

Figure 2. The survival of Pacific white shrimp following challenge test with *Vibrio harveyi*. Different letters above different bars indicate significant differences (P<0.05)