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Crude fucoidan from *Sargassum polycystum* stimulates growth and immune response of *Macrobrachium rosenbergii* against white spot syndrome virus (WSSV)

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Abstract. *Macrobrachium rosenbergii* is an economically important species of shrimp that is cultured for commercial farming. Infection of White Spot Syndrome Virus (WSSV) causes high mortality in this species resulting in a decline in production. *Sargassum polycystum* collected from Calatagan, Batangas was used to extract crude fucoidan through ethanolic extraction. Crude fucoidan is seen as a possible prevention for the occurrence of WSSV infection and as a possible growth enhancer for normal shrimps. The extract was supplemented in shrimp feeds in three concentrations: 100 mg kg^{-1}, 300 mg kg^{-1} and 500 mg kg^{-1}. Three setups were done for the study: Each for 1) growth trial (measured as percentage weigth gain and feed conversion ratio after 28 days of feeding), 2) immune response where the prophylactic (feeding for 28 days followed by WSSV infection) effects were measured thru total haemocyte count (THC) and prophenoloxydase (PO) activity; and 3) mortality, recorded everyday during the duration of the study. Results showed that 500 mg kg^{-1} crude fucoidan supplemented feeds had the highest increase in growth. Increased immune response was observed in experimental groups with the 100 mg kg^{-1} concentration yielding the highest THC, PO and percentage survival. Therefore, crude fucoidan from *S. polycystum* demonstrated growth enhancing and immunostimulating effects on *M. rosenbergii*

Key Words: crude fucoidan, phenoloxidase activity, total haemocyte count, white spot syndrome virus, growth, immunostimulation.

Introduction. Shrimp aquaculture is a major source of livelihood for Filipinos living in rural areas near coastal shores. This industry has brought about vast growth in the economy because of its high international trade value (Manilal et al 2009). According to the Food and Agriculture Organization (FAO) of the United Nations, shrimps became top marine export products from the Philippines, generating $300,000,000 in 1992. This success, however, has suffered a huge decline in 1995 because of disease outbreaks caused by bacteria and viruses that affected shrimp farms in the country (FAO 2011). It has since become a constraint for the recovery and sustainable increase of shrimp production. Due to this major loss, alternative shrimp cultures have been the immediate concern of the industry. Aquaculturists and local shrimp farmers alike have turned their attention to freshwater prawn culture (Wowor & Ng 2007). *Macrobrachium rosenbergii*, locally known as ulang, is one of the most economically important species of freshwater shrimp. Being the largest species in its genus and because of its ability to survive in turbid water conditions, it is most favored for freshwater shrimp farming purposes (Nhan et al 2009).

However, viral pathogens still remain a constant threat in freshwater farming where several diseases cause low survival rates in hatchery of these shrimps. One of the most dreadful pathogens is the White Spot Syndrome Virus or WSSV. It is an enveloped, double stranded DNA virus that has an ovoid to bacilliform shaped virion with a rod-shaped nucleocapsid (van Hulten et al 2000). WSSV is extremely virulent and possesses a broad range of host specificity and targets various tissues. Within ten days of infection, it may cause a mortality rate of up to 100% which results in major losses to the shrimp farming industry (Flegel 2006).

The use of immunostimulants in preventing and controlling the occurrence of WSSV is now largely considered. Since immunostimulants can increase disease resistance in shrimps, fucoidan, a known immunostimulant, is seen to be a possible feed supplement in aquaculture since it is comprised of bioactive compounds necessary for stimulation and activation of shrimp immune response (Selvin et al 2004). It is found in brown macro-algae like *Sargassum polycystum*, which is abundant in the Philippine coastal areas. The utilization of this macro-algae and its efficiency in promoting growth and preventing diseases in *M. rosenbergii* is not yet widely studied. This study aims to evaluate the efficiency of crude fucoidan from *S. polycystum* in promoting growth and enhancing the immune response of *M. rosenbergii* against WSSV.

Material and Method

S. polycystum was collected along the coast of Barangay Poblacion Dos, Calatagan, Batangas. After thorough washing, the macroalgae was left in the sun to dry. The extraction of crude fucoidan from *S. polycystum* was performed as previously described by Immanuel et al (2012). The percentage yield of crude fucoidan extracted was 6% for every 5 g dried biomass. Preparation of fucoidan supplemented feeds was done by mixing the crude fucoidan in different concentrations (100, 300 and 500 mg kg^{-1} shrimp feeds), in this proportion: 1 kg shrimp feeds, 20 g starch (as binder) and 500 mL distilled water. After mixing all the dry ingredients, 500 mL distilled water was added to produce dough; the dough was pelletized using a molder producing pelletized feeds driend in an oven at 60^{o}C overnight. Pellets were stored in plastic containers at room temperature until use.

A total of 80 postlarvae (PL-40) and 240 *M. rosenbergii* juvenile shrimps were collected from Central Luzon State University in Nueva Ecija. Upon arrival in the laboratory facilities of Thomas Aquinas Research Complex (TARC) in the University of Santo Tomas, Manila, the shrimps were separately stocked and acclimated in communal tanks for 7 days. Shrimps were fed with commercial feeds prior to all experimental procedures.

Experimental design. For the growth experiment, the 80 PL-40 shrimps were utilized from the acclimated stock, transferred to 4 previously decontaminated 50 L capacity tanks resulting in 20 shrimps for each tank. The tanks were maintained at room temperature and were provided with ample aeration. The tanks were then assigned to four treatments: 1 - Control, 2 - 100 mg fucoidan kg^{-1} shrimp feed, 3 - 300 mg fucoidan kg^{-1} shrimp feed, and 4 - 500 mg fucoidan kg^{-1} shrimp feed. Prior to feeding, the average weight (in grams) of the shrimps was measured and it served as the baseline for the analysis of growth parameters. The control and experimental groups were then fed with commercial shrimp feeds and fucoidan supplemented feeds, twice a day (9:00 AM and 6:00 PM) for 28 days. The daily allocation of feeds was 10% of the shrimp's initial body weight. Shrimps were weighed in bulk from each tank and their average body weight was recorded weekly. Growth measured as percentage weight gain (% WG) and feed conversion ratio (FCR) was calculated as previously described by Saad et al (2009).

For the immune response experiment, 120 juvenile *M. rosenbergii* shrimps, mean weight of 1 g each, were utilized from the acclimated stock and were transferred to 4 previously decontaminated 50 L capacity tanks with each tank containing 24 shrimps in each tank. An additional tank containing 20 shrimps was assigned as the control group

and was not subjected to any experimental procedures. The tanks were maintained at room temperature and were provided with ample aeration.

Similar to the growth trial, the tanks were assigned to four treatments. Triplicates were assigned each for the 1 - control, 2 - 100 mg kg^{-1}, 3 - 300 mg kg^{-1}, and 4 - 500 mg kg^{-1} fucoidan supplemented feeds groups.

Shrimps were then fed twice a day (9:00 AM and 6:00 PM) for 28 days with a daily allocation of 10% the shrimp's initial body weight. Sampling was done prior to feeding, hemolymph samples were collected from triplicates of the control and experimental groups for analysis of total hemocyte count (THC) and phenoloxidase (PO) activity. Triplicates of haemolymph from both control and experimental groups was collected at different sampling points (Days 0, 7, 14, 21, 28, 0, 35 and 42) and used for THC and PO activity. After 28 days of feeding experiment, the control and experimental groups were challenged with WSSV by injection of the virus solution at the third dorsal abdominal segment at a dose of 10 µL per shrimp. Three hours after infection (Day 0), triplicates from the control and experimental groups were collected for hemolymph collection, THC and PO analysis. Feeding of assigned feeds was continued for 14 days. On the 7th and last day of feeding, hemolymph samples were collected for analysis of immunological parameters. Consequently, mortality was observed and recorded.

Similarly the mortality experiment consisted of 120, mean weight of 1 g, juvenile *M. rosenbergii* shrimps. Four setups similar to the immune response experiment were fed with their assigned feeds for 28 days, infected at the 28^{th} day and fed continuously up to 14 days post-infection. An additional tank containing 20 shrimps was assigned as the control group, fed with commercial feeds and left uninfected. Daily cumulative mortality was observed and recorded.

Analysis of immunological parameters. The analysis of immunological parameters was performed according to the procedures described by Maningas et al (2013) with some modifications. Hemolymph was drawn from the ventral sinus of each shrimp in to a 3 mL sterile syringe containing 2.7 mL precooled (4°C) anticoagulation solution (trisodium citrate 30 mM, sodium chloride 0.34 M, EDTA 10 mM, pH 7.55 and with an osmolality of 780 mOsm kg^{-1}).

Total hemocyte count (THC). A 100 µL hemolymph sample was mixed with 14 µL of trypan blue. Then 10 µL of the solution was placed into a hemocytometer. The average cells per sample were then computed as follows:

$$\text{Average viable cell count} = \frac{\text{total number of viable cells in 4 squares}}{4}$$

$$\text{Viable cells/mL} = \text{Average viable cell count x } 104$$

$$\text{Total viable cells per sample} = \text{Viable cells/mL x Original volume of sample fluid}$$

Phenoloxidase (PO) activity. A 300 µL hemolymph sample was centrifuged at 8000 rpm at 4°C for 3 minutes. The supernatant was discarded and the pellet was resuspended in 300 µL cacodylate-citrate buffer and the solution was centrifuged again. The supernatant was then discarded and the pellet was resuspended in 60 µL cacodylate buffer. 30 µL of the cell suspension was then moved in a separate microcentrifuge tube. The solution was then incubated at room temperature for 10 minutes before adding 15 µL of zymosan into the suspension. After 5 minutes, 15 µL of L-DOPA and 240 µL of cacodylate buffer were added. 200 µL of the solution was then transferred in a microtray and the absorbance at 490 nm was measured using the Elisa reader.

Statistical analysis. All data were analyzed using the GraphPad Prism version 6.0 software. The data for the measurement of growth parameters (% WG and FCR) were subjected to one-way analysis of variance (ANOVA) at a significant level of 0.05 and

Tukey's post hoc honestly significant difference (HSD) test to determine significant differences between groups.

Data for measurement of immunological parameters (THC and PO activity) were subjected to one-tailed paired t-test at a significant level of 0.05 to determine significant differences among treatments. Cumulative survival percentage of shrimp groups was also computed and analyzed using one-way ANOVA at a significant level of 0.05 and Tukey's post hoc HSD test.

Results and Discussion. FCR is the measure of the efficiency of an organism in converting its food to increase its body weight (Saad et al 2009). A low FCR is an indication that the organism has utilized the nutrients present in the feeds effectively resulting to weight gain (Pholdaeng & Pongsatmart 2010). Higher percentage weight gain (% WG) was evident in shrimps fed with crude fucoidan supplemented diet as compared to the shrimps fed with commercial feeds with the optimum response at the 500 mg kg^{-1} supplementation (Figure 1).

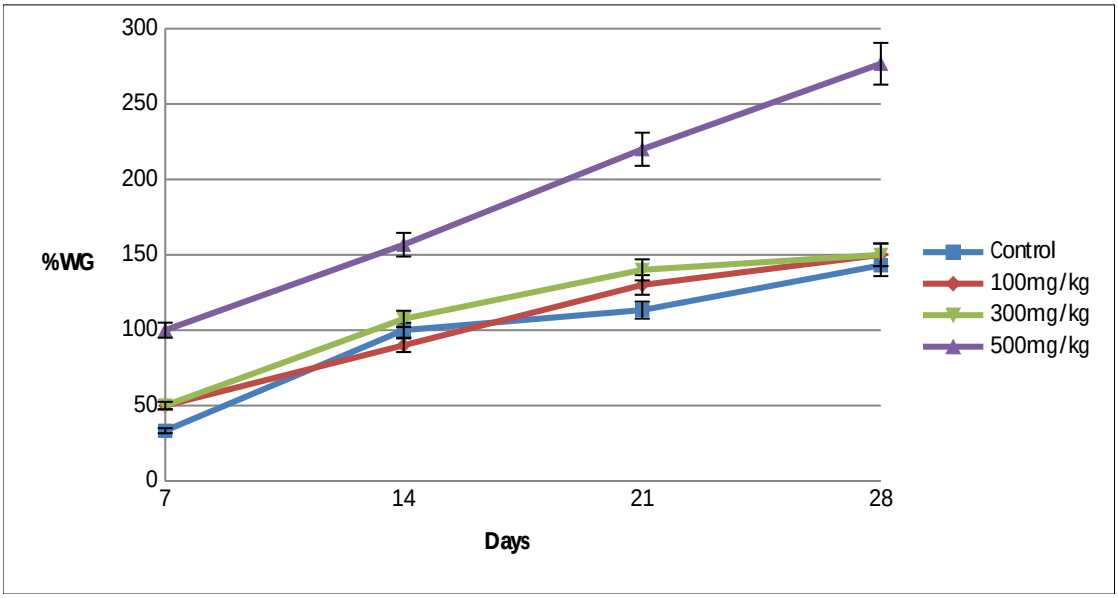

Figure 1. Percentage weight gain (% WG) of *M. rosenbergii* fed with commercial feeds (control) and crude fucoidan supplemented diets (in 100 mg kg^{-1}, 300 mg kg^{-1} and 500 mg kg^{-1} concentration) for 28 days.

Conversely, a lower feed conversion ratio (FCR) was calculated for the experimental groups as compared to the control group. The 500 mg kg^{-1} concentration recorded the lowest FCR value while the other two concentrations (100 mg kg^{-1} and 300 mg kg^{-1}) followed with values not far from the first one (Figure 2). One-way ANOVA showed a significant difference ($p<0.05$) between groups and Tukey's post hoc HSD test determined the significant difference in %WG and FCR in shrimps fed with the 500 mg kg^{-1} concentration.

Immunostimulants such as fucoidan activate phagocytes in the hepatopancreas that in turn produce lytic enzymes upon stimulation. This increase in production of enzymes enhances food digestion and nutrient absorption (Azad et al 2005). Thus, the shrimps fed with crude fucoidan supplemented diet recorded a lower FCR and in turn a higher % WG than those fed with commercial feeds. Studies made by Traifalgar et al (2009) on the growth effects of fucoidan from *Undaria pinnatifida* on *Penaeus monodon* also showed that the concentration of 500 mg kg^{-1} fucoidan diet significantly increased % WG and enhanced FCR as compared to higher concentrations of 1000 mg kg^{-1} and 2000 mg kg^{-1}. This indicates that 500 mg kg^{-1} fucoidan supplementation is the adequate concentration that promotes growth in prawns.

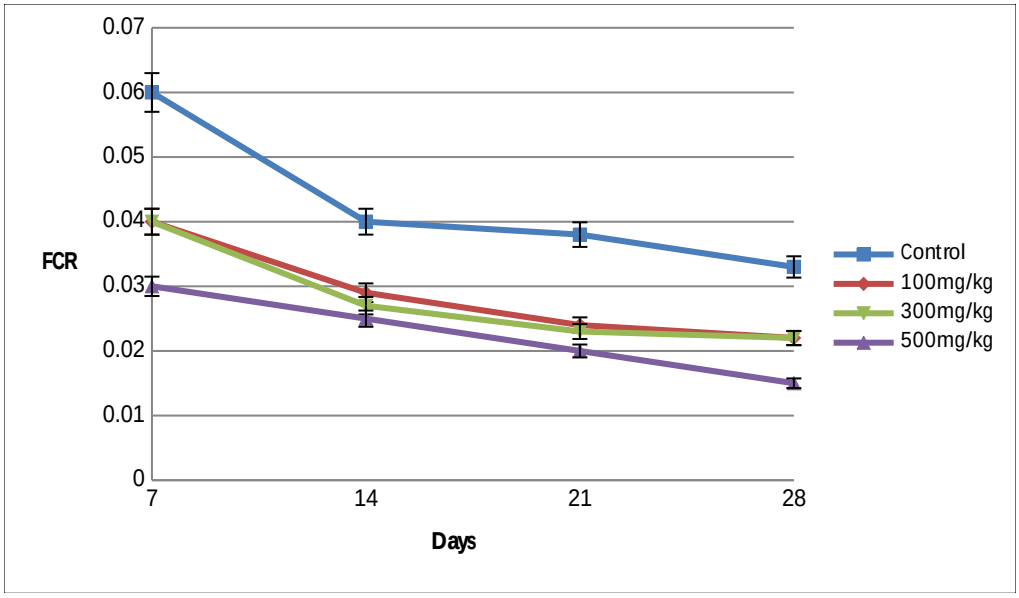

Figure 2. Feed conversion ratio (FCR) of *M. rosenbergii* fed with commercial feeds (control) and crude fucoidan supplemented diets (in 100 mg kg^{-1}, 300 mg kg^{-1} and 500 mg kg^{-1} concentration) for 28 days.

The immunostimulating property of crude fucoidan from *S. polycystum* was confirmed by the increase in total hemocyte count (THC) and phenoloxidase (PO) activity of shrimps from the experimental groups before and after infection of WSSV. One-tailed paired t-test showed a significant increase ($p<0.05$) in THC for the experimental groups compared to the control group with the 100 mg kg^{-1} supplementation recording the highest count. The THC in the experimental groups is higher compared to the control group prior to infection and on the seventh day of infection of WSSV, a rapid increase in THC was observed in all groups. The THC then continued to increase until the 14th day (Figure 3).

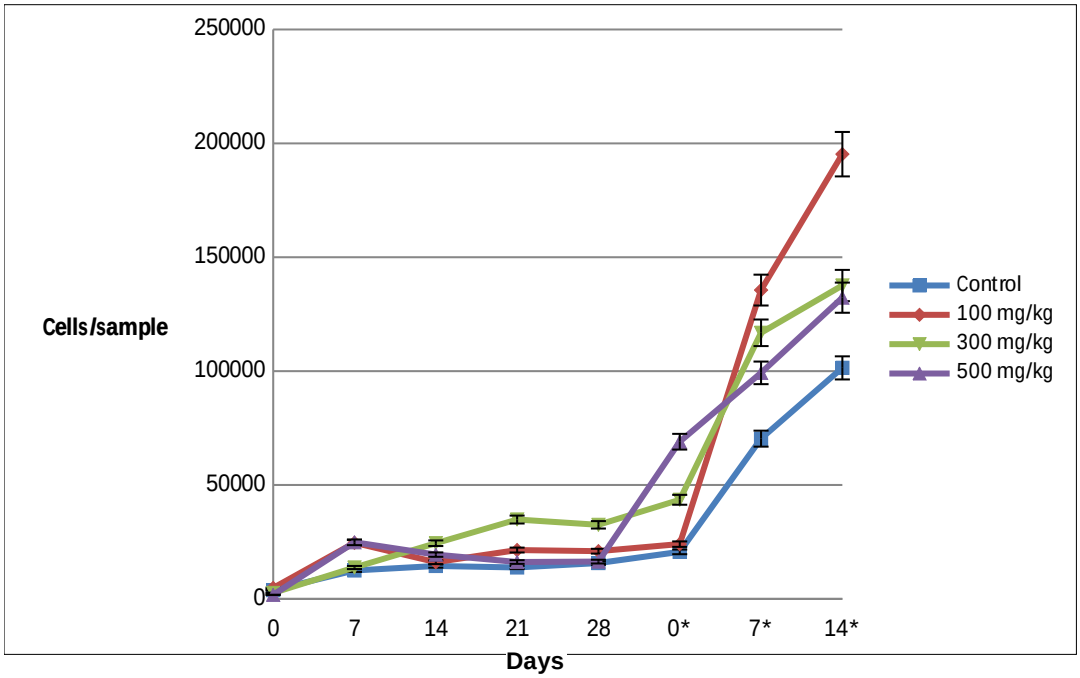

Figure 3. Average total hemocyte count (THC) of triplicate samples of *M. rosenbergii* fed with commercial shrimp feeds (control) and crude fucoidan supplemented diets (100 mg kg^{-1}, 300 mg kg^{-1}, and 500 mg kg^{-1}) for 28 days before WSSV infection and 14 days after infection. The red line indicates WSSV infection. *Days post-infection.

Hemocytes play an important role in the innate immune system of shrimps. They function for cell-to-cell recognition, phagocytosis, melanin production, encapsulation and production of cytotoxic materials (Pholdaeng & Pongsatmart 2010). High hemocyte count is associated with increased resistance against foreign invaders (Huynh et al 2011). The increase in THC of the experimental groups may indicate that the crude fucoidan have stimulated the shrimp's immune system to have an increased pathogenic response even if they are not really infected. Our results are in accordance with a previous study conducted by Immanuel et al (2012), where THC have significantly increased on *P. monodon* fed with fucoidan extracted from *S. wightii* as compared with the control group after infection with WSSV.

Crude fucoidan from *Sargassum* species is considered as a sulfated fucogalactan. The sulfate content in crude fucoidan may have caused the increase in THC of the experimental groups since it was reported that sulfate contents of fucoidan causes cell proliferation in crustaceans (Haroun-Bouhedja et al 2000).

A vital part of the shrimp's immune system is the prophenoloxidase (proPO) cascade. It has been shown to be localized in the hemocytes (Ai et al 2009) and is a major component of the shrimp humoral response. The proPO system is triggered by very low amounts of bacterial cell wall components such as peptidoglycans, lipopolysaccharides and β-glucans and polysaccharides like fucose, galactan and mannuronic acid (Fagutao et al 2011; Manilal et al 2009). Upon activation of the proPO cascade, the inactive enzyme precursor, proPO, is converted into phenoloxidase (PO). PO eventually catalyzes the oxidation of tyrosine to produce toxic quinone substances that leads to the formation of melanin. Melanin binds to the surface of foreign pathogens, thus accelerating their removal by nodule formation (Cerenius et al 2008).

One-tailed paired t-test also showed a significant increase ($p<0.05$) in the PO activity of shrimps fed with crude fucoidan supplemented diets as compared to the shrimps fed with commercial feeds. The shrimps fed with 100 mg kg^{-1} concentration of crude fucoidan supplemented diet yielded the highest PO activity (Figure 4).

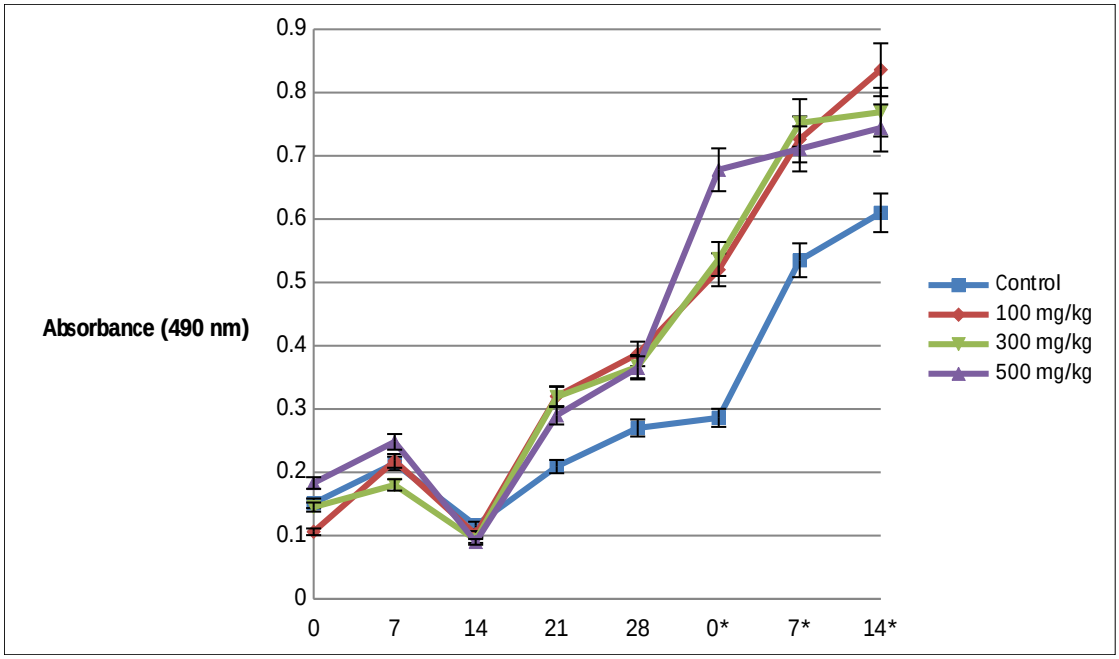

Figure 4. Average phenoloxidase (PO) activity of triplicate samples of *M. rosenbergii* fed with commercial shrimp feeds (control) and crude fucoidan supplemented diets (100 mg kg^{-1}, 300 mg kg^{-1}, and 500 mg kg^{-1}) for 28 days before WSSV infection and 14 days after infection. The red line indicates WSSV infection. *Days post-infection

All groups have constantly rising PO activity after Day 14 pre-infection but the PO activity of the control group is lower compared to the experimental groups fed with fucoidan supplemented diets. This may be due to the presence of the virus in the shrimps' system.

A significant increase in PO activity after WSSV infection of *P. monodon* fed with fucoidan from *S. wightii* was also observed in a study conducted by Immanuel et al (2012).

HPLC analysis conducted by Kasetsart (2010) showed that the polysaccharide present in crude fucoidan extracted from *S. polycystum* by ethanolic extraction include fucose in large percent composition and mannose and glucose in minimal concentrations. These polysaccharides present in crude fucoidan mimics the process of infection of foreign pathogens in shrimps, thus resulting in an increased immune response. In effect, the shrimps fed with crude fucoidan supplemented diet recorded a higher THC and PO activity compared to the control group even before infection of WSSV. This increase in THC and PO activity of experimental groups prior to infection may have conditioned the shrimps to have elevated immune response upon infection of WSSV which in turn resulted into a higher percentage survival of these shrimp groups (Figure 5).

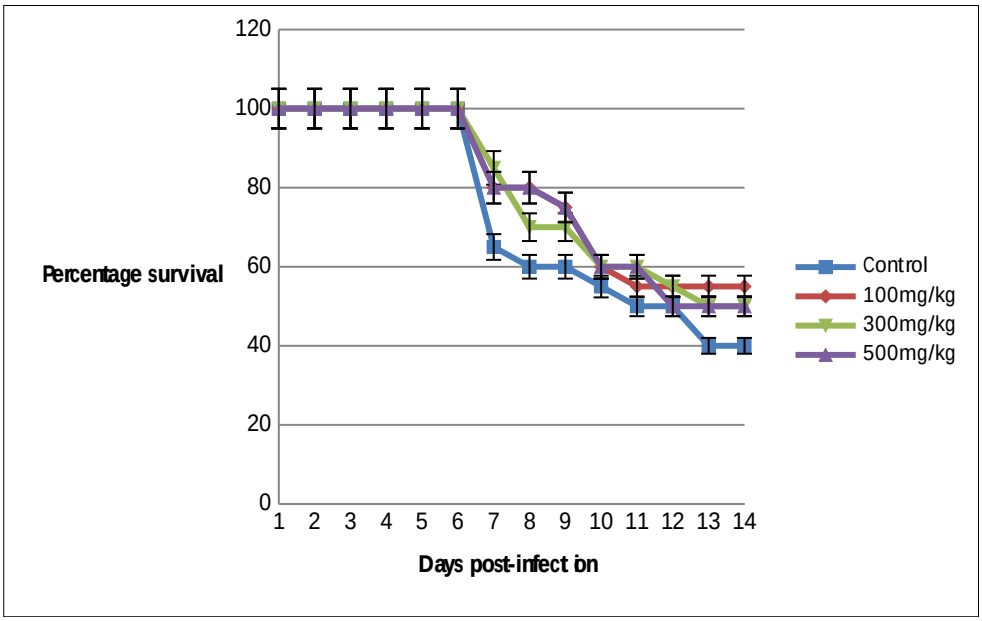

Figure 5. Percentage survival of *M. rosenbergii* fed with commercial feeds (control) and crude fucoidan supplemented diets (in 100 mg kg^{-1}, 300 mg kg^{-1}, and 500 mg kg^{-1} concentration) 14 days after infection of WSSV.

Mortality in shrimps started at the seventh day after infection with WSSV and the control group had the lowest survival rate. Consequently, one way ANOVA showed a significant difference ($p<0.05$) between shrimp groups and Tukey's post hoc HSD test determined that the percentage survival was significantly increased in shrimps fed with crude fucoidan in 100 mg kg^{-1} concentration. This concentration also gave the highest percentage survival of WSSV-infected shrimps.

These results are similar to previous studies made by Chotigeat et al (2004) and Takahashi et al (1998) that showed an increased survival rate of *P. monodon* and *P. japonicus* shrimps against WSSV after incorporating crude fucoidan and partially purified fucoidan from *S. polycystum* and *Cladosiphon okamuranus*, respectively.

Conclusions. Crude fucoidan from *S. polycystum* in 500 mg kg^{-1} shrimp feed concentration significantly increased the nutrient utilization and growth performance of *M. rosenbergii* shrimps. In addition to growth effects, crude fucoidan from *S. polycystum* also stimulated and enhanced the immune response of *M. rosenbergii* which in effect increased the shrimp's resistance against WSSV. The 100 mg kg^{-1} concentration yielded the highest values for THC and PO and significantly increased survival at the end of the challenge test.

Our results showed that crude fucoidan from *S. polycystum* has both growth enhancing and immunostimulating properties and can be used as an alternative feed additive that can prevent the occurrence of WSSV infection in *M. rosenbergii*.

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