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ORIGINAL RESEARCH

Sargassum polycystum polysaccharide extract improved immunological responses and enhanced resistance of *Penaeus monodon* against *Vibrio harveyi* infection

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Abstract The present study evaluated the influence of acidic polysaccharide extracted from *Sargassum polycystum* (SAPE) on growth, immunological responses and resistance to *Vibrio harveyi* infection of juvenile *Penaeus monodon*. Five experimental diets supplemented with 100, 500, 1000 and 1500 mg/kg SAPE including a control (0 mg/kg) were formulated and fed to juvenile *P. monodon* for 50 days. Following the feeding trial growth indices including weight gain and specific growth rate were significantly enhanced, ($P < 0.05$), in treatment groups fed with diets containing 2000 mg/kg SAPE. Highest survival (46.67±11.54%, $P < 0.05$) following the infection challenge test with the pathogenic *V. harveyi* was observed in the treatment group receiving the 2000 mg/kg SAPE supplementation while all shrimp died in the group receiving the control diet. The enhanced resistance to bacterial infection of SAPE treatment groups was further supported by improved immune responses ($P < 0.05$) including total hemocyte counts and serum antibacterial activities. The present study indicates that dietary supplementation of acidic polysaccharide extracted from *Sargassum polycystum* at a dose of 2000 mg/kg could enhance growth performance, elevate immune responses and significantly improve resistance of juvenile *P. monodon* against *V. harveyi* infection.

Keywords *Penaeus monodon* . *Sargassum polycystum* . Immunostimulant . *Vibrio harveyi*

Introduction

Vibriosis disease caused by *Vibrio harveyi* is a persistent and recurring disease that threatens the sustainability and economic viability of global penaeid shrimp aquaculture (Abraham and Sasmal 2009; Soto-Rodriguez et al. 2010; Zhou et al. 2012). Outbreak of this disease is implicated in severe losses at any stages of shrimp culture including the hatchery, nursery and grow-out culture operations (Jiravanichpaisal et al. 1994; Lavilla-Pitogo et al. 1998; Soto-Rodriguez et al. 2010). Production losses in China on farmed *P. vannamei* have been attributed to a highly virulent strain of non-luminous *V. harveyi* (Zhou et al. 2012). While in Mexico bright red disease caused by *V. harveyi* has also been implicated in production losses of farmed *P. vannamei* (Soto-Rodriguez et al. 2010). In the Philippines, luminous Vibriosis disease in hatcheries and pond grow-out systems is considered a significant hindrance in the expansion of the industry (de la Peña et al. 2003). The risks associated with Vibriosis disease occurrence limit the investments and capital inflow for the shrimp aquaculture business.

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After decades of intensive research, the cumulative information attained has led to the better understanding of the mechanics on how the shrimp immune system works. Recently bioactive polysaccharides received much attention as these compounds were found to modulate the non-specific shrimp immune system. Immunomodulatory polysaccharides elicit their effects by binding and activating cell receptors that could result to immune activation and pathogen removal (Abdala-Diaz et al. 2010; Patel 2012). Earlier reports highlighted the use of bacterial, viral and fungal cell membrane components and polysaccharides including glucan, lipopolysaccharide and peptidoglycan as potent immunological activators for shrimp (Traifalgar et al. 2013; Kono et al. 2014; Genio et al. 2015; Pan et al. 2015; Mameloco and Traifalgar 2020). Although these compounds are highly immunomodulatory and effective in eliciting enhanced disease resistance in shrimp but the cost, storage stability and limited amount of pure material are the obstacles limiting the practical application of these compounds in the shrimp production systems.

Other than the microbes, brown algae are also known as a source of cell wall-derived polysaccharides that are known to be immunostimulatory, anti-inflammatory and available in bulk quantities. Unique to the brown algae are its content of acidic and sulfate containing polysaccharides including fucans, laminarin and algins that are potent activators of immune cells. The immunomodulatory properties of these algal-derived polysaccharides on farmed aquatic animals have been well-documented (Vatsos and Rebours 2014; Marudhupandi and Inbakandan 2015; Akbary and Aminikhoei 2018; Liu et al. 2020).

The brown seaweed *Sargassum polycstum* is widely distributed in almost all tropical coastal areas. Like the other species of brown algae, polysaccharides extracted from the different species of Sargassum have been reported to exhibit anti-tumor, antibacterial and antiviral properties (Zhuang et al. 1995; Chotigeat et al. 2004; Huang et al. 2006; Mohsen et al. 2007; Sivagnanavelmurugan et al. 2014). Although there have been studies on the bioactivities of solvent extracted low molecular weight compounds from *Sargassum* species (Patel 2012; Vatsos and Rebours 2014; Kim et al. 2014; Puspita et al. 2017) but the immunoactive effects of polysaccharides from *Sargassum polycystum* on shrimp has not been fully investigated to date. In the Philippines there is not much utility for this seaweed and the drifted decomposing biomass in shorelines are considered as waste biological resource.

As an attempt to develop utility for this marine biomass resource the present study was conducted to evaluate the bioactivity of crude acidic polysaccharide extracted from *Sargassum polycystum* on its influence on growth performance, immune responses and resistance to *Vibrio harveyi* infection of juvenile *Penaeus monodon.*

Materials and methods

Acidic polysaccharide extraction

Acidic polysaccharide extraction was done following the method described by Koo et al. (1995) with slight modifications. The sun-dried algal biomass of *Sargassum polycystum* was obtained from the local fishermen of Antique Philippines. The seaweed was dried in a convection oven at 60°C until the moisture content of the biomass is about 4%. Five kilogram of the oven dried seaweed was pulverized and sieved to pass 100µm mesh. One kilogram of the seaweed powder was defatted by soaking in 4 liters of methanol for 24 hours. This step is a modification form the original method as describe by Koo et al. (1995) that only soaked the seaweed in alcohol for 2 hours. The alcohol from the seaweed was removed by filtration through an 80µm nylon mesh and the remaining seaweed powder was collected and dried in an oven 60°C until the moisture reached 4%. This drying method is also modified since in the original method, the crude polysaccharide was freeze dried. Three hundred grams of the defatted seaweed powder was collected, added with 600 ml of distilled water at pH 2 and heated to 100°C for an hour. The pH of the water was adjusted by the gradual addition of 0.2M HCl until pH 2 was attained. The boiled biomass slurry was filtered in nylon mesh (80µm) on a sintered glass funnel and the resulting supernatant was collected and brought to neutral pH with 0.2M NaOH. To remove the alginate, 3 volumes of a 2 % $CaCl_2$ solution were gradually added to the collected supernatant. Then the solution was centrifuged at 5,000 rpm for 15 min and the precipitated alginate was discarded. The resulting supernatant was collected and dialyzed against distilled water overnight. After the dialysis, three volumes of ethanol were added to the solution and the precipitate formed collected after centrifugation at 5,000 rpm for 15 min. The collected polysaccharide was dried, stored at -30

Table 1 Basal diet and proximate composition

Ingredients	g 100^{-}g diet
Peruvian Fish Meal*	46.00
Squid Meal*	8.00
Soybean Meal	21.00
Cellulose*	5.00
Vitamin Mix[1a]	1.00
Mineral Mix[1b]	1.00
Lecithin[1]	1.00
Cod Liver Oil[1]	4.00
Starch (wheat)[1]	13.00
Sargassum Polysaccharide extract	0.00
Total	100.00
Proximate Composition	
Dry Matter	91.01%
Crude Protein	53.15%
Crude Lipid	3.75%
Crude Fiber	1.63%
Ash	14.81%

*Sourced from Southeast Asian Fisheries Development Center Feed Mill Laboratory; Peruvian fish meal composition: total protein 68.0 %, total fat 5.9 % ash 17.3%; Soy bean meal composition: : total protein 36.0 %, total fat 19.8 % ash 5.6%; Squid meal composition total protein 74.0 %, total fat 7.1 % ash 8.0%.

[1] Chemicals were obtained from Merck-Sigma Aldrich Inc., Darmstadt, Germany.

[a]Vitamin mix composition: Vitamin mix contribution 100 g^{-1} of feed: β-carotene, 5.4 mg; Cholecalciferol, 0.45 mg; Thiamine, 10.8 mg; Riboflavin, 21.6 mg; Pyridoxine, 19.8 mg; Cyanocobalamin, 0.06 mg; α-tocopherol, 49.5 mg; Menadione, 7.2 mg; Niacin, 43.2 mg; Pantothenic acid, 12 mg; Biotin, 0.06 mg; Folic acid, 3.6 mg; Inositol, 90 mg, Stay C, 300 mg.

[b]Mineral mix composition: Mineral mix contribution 100 g^{-1} of feed: Phosphorus, 480 mg; Calcium, 480 mg; Magnesium, 60 mg; Iron, 6 mg; Zinc, 16.8 mg; Copper, 8.4 mg; Potassium, 300 mg; Cobalt, 4.4 mg; Manganese, 6.4 mg; Selenium, 0.004 mg; Molybdenum, 0.002 mg; Aluminum, 0.1 mg; Iodine, 1.6 mg.

^{o}C and used as the crude acidic polysaccharide extract. Carbohydrate analysis was performed using phenol-sulfuric acid method as described by Masuko et al. (2005) and soluble protein was quantified following the method of Bradford (1976).

Test diets

Composition of the basal diet used in the study is shown in Table 1. The diet is a fish meal based diet containing 53.15% crude protein and with 3.75% crude fat. Crude acidic polysaccharide extracted from *Sargassum polycystum* (carbohydrate: 98%; protein: 0.5%; ash: 1.3%) was incorporated to the test diets, based on carbohydrate content, at 100, 500, 1000 and 2000 mg/kg diet. Cellulose in the experimental diets was adjusted to balance the other nutrients. A control group without polysaccharide supplementation was also included in the treatment. The diets were prepared by sieving all dry ingredients in a 100 µm mesh. The sieved dry ingredients were added with oil containing oil soluble vitamins and were thoroughly mixed. The seaweed polysaccharide was dissolved in an adequate amount of distilled water and was added to the ingredient mixture. To the moist mash, cooked gelatinized wheat starch was added as a binder and the ingredients were mixed in industrial food mixer (Hobart Inc., Ohio, USA) for 15 minutes. The formed dough was extruded in a 2 mm die laboratory pelletizer. The feed pellets were collected and were oven-dried (Thermo Fisher Scientific, USA) at 55 °C for 5 hr. The dried feed pellets were cut to appropriate size and stored at 8 °C until used. Proximate composition analysis of the experimental diet, only the basal diet, was conducted following AOAC (2000) for total protein, total lipid, carbohydrate as nitrogen free extract (NFE), crude fiber, and ash.

Feeding trial

Feeding trial was conducted at the Multi-species hatchery of the Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines in the Visayas. *Penaeus monodon* post larvae PL13 were obtained from a commercial hatchery in Tigbauan, Iloilo, Philippines. Shrimp were acclimatized in individual experiment rearing containers for one week and maintained with the control diet before the start

of the feeding trial. The rearing tank is a 60L-capacity polypropylene container with a length of 60 cm, width 40 cm and a height of 25 cm. The experiment was run following complete randomized design with 5 treatments run in triplicate. Twenty shrimp (individual weight: 0.085± 0.001) was reared in each treatment filled with 50L of chlorinated UV filtered seawater. Each tank was provided with ample aeration that provided dissolved oxygen above 5.0±1.0mg L^{-1}. Recirculating system was employed in the experiment at a flow rate of 8.6L min^{-1}. Water temperature, pH and salinity were maintained at 28.0±1.0°C, 7.3±0.6 and 25ppt respectively. The shrimp were fed the experimental diet daily at 10% of their body weight that was subdivided into 3 feeding allocations given at 9:00, 13:00, and 17:00. Fecal wastes and uneaten feeds were siphoned out every morning just before the first feeding. Complete water change was done weekly to maintain good water quality. A 12h light: 12h dark photoperiod was maintained throughout the trial. The feeding experiment was run for 50 days. Each treatment group was run in triplicate.

Disease resistance trial

Following the feeding trial, shrimp were subjected to bacterial challenge experiment with the pathogenic strain of luminous *Vibrio harveyi* PN 9801 (de la Peña et al. 2003) obtained from the bacterial collection of SEAFDEC Aquaculture Department, Philippines. Prior to the disease resistance trial, shrimp from each treatment group were sampled and analyzed for the presence of luminous *V. harveyi* using the thiosulfate citrate bile salt sucrose agar media (TCBS). The shrimp used in this test was confirmed to be free of this luminous Vibrio pathogen. Four hundred fifty shrimps, were transferred to 15 new tank (20L capacity) representing the five experimental treatments in triplicate. Each of the experimental tanks contained 10 shrimp. Another group of 3 tanks were used to hold the experimental shrimp without bacteria, serving as the negative control. The shrimp used in the negative control group were obtained from a population of shrimp (same batch, size and age of shrimp used in the experiment) maintained in the rearing facilities of the university.

The virulent strain of *Vibrio harveyi* PN 9801 (de la Peña et al. 2003) used in this test was obtained from the bacterial collection of SEAFDEC Aquaculture Department, Philippines. This pathogen was maintained in an ultralow freezer at -80 °C until used. Prior to the test, the pathogenic *V. harveyi* and were grown in nutrient broth (NB, Pronadisa, Spain) supplemented with 2% NaCl. The virulence of the *V. harveyi* was activated by in vivo passage for 3 times. This was done by injecting a live shrimp with the lethal dose of the pathogen at 10^7 CFU g^{-1} shrimp (Traifalgar et al. 2009). A day after the infection, moribund shrimp were collected from the rearing tank, dissected and luminous *V. harveyi* was isolated from the hepatopancrease. Pure colonies of the virulent *V. harveyi* collected from the moribund shrimp were grown in nutrient broth (NB, Pronadisa, Spain) supplemented with 2% NaCl and used in the disease resistance test.

The disease resistance trial was performed by immersion of the experimental shrimp in tanks containing 10^7cfu ml^{-1} *V. harveyi* PN 9801 for 3 hours. This concentration has been previously optimized as the lethal dose concentration (LD50) of this pathogen in *P. monodon* (Traifalgar et al. 2009). Bacterial concentration was measured using McFarland Standards. Following the immersion period, shrimp were transferred to clean rearing units containing clean, UV sterilized and filtered seawater. A static system was employed in the study and the shrimp were fed at 3.0% of their body weight that was given in 3 equal portions at 9:00, 13:00, and 17:00 during the disease resistance trial. During this test the water parameters were maintained based on the optimum requirement of *P. monodon* (Temp: 28-30 °C; pH: 7.9-8.0; Dissolve Oxygen: 5-7 mg L^{-1} ;salinity: 33-35 g L^{-1}).

Mortalities were monitored daily until 15 days post-challenge. To verify the cause of death, bacteria from the heart of moribund shrimp were isolated and cultured on thiosulfate citrate bile salt sucrose agar media (TCBS). Luminous Vibrio was isolated, identified and confirmed as *V. harveyi* by biochemical characterization using the API 20E strips (bio-Mérieux Vitek Inc. France) following the manufacturer instructions.

Immunological analysis

Hemolymph extraction and total hemocyte count

Hemolymph was withdrawn from the ventral sinus using tuberculin syringe (26 gauge) with pre-cooled anticoagulant (450 mM NaCl, 10 mM KCl, 10 mM HEPES and 10 mM EDTA, pH 7.3, 850 mOsm/kg) (Hernandez-Lopez et al. 1996). A total of 9 shrimp were used per dietary treatment and individual shrimp was used in the analysis of the Total Hemocyte Count. Thirty microlitre of hemolymph was used for the quantification of the total hemocytes in individual shrimp and the rest of the collected hemolymph was used in the analysis of prophenoloxidase activity. Haemocytes were counted using Neubauer hemocytometer viewed under a microscope. Values were expressed as number of haemocytes per mL haemolymph (Joseph and Philip 2007).

Prohenoloxidase activity assay

Prophenoloxidase activity was measured spectrophotometrically through the formation of dopachrome by the oxidation of L-dihydroxyphenylalanin (L-DOPA) as described by Subramanian et al. (2014). Hemolymph withdrawn from the test animals were subjected to centrifugation at 4000 x g for 15 min at 4°C. The supernatant was discarded and the concentrated haemocytes were homogenized and added with Shrimp Salt Solution buffer at (1:4,v/v). Twenty five µl of this solution was placed into a flat-bottom 96 well microtiter plates and incubated with equal volume of 0.1% trypsin in SSS (Shrimp Salt Solution) for 30 minutes. Shrimp Salt Solution loaded into the wells were used as a blank control. After incubation, 25µl of 0.3% L- DOPA (L-3, 4-dihydroxyphenylalanine) was added to the wells and the formation of dopachrome was monitored every minute for four minutes. Optical density was measured at 490 nm using Ledetect 96 microplate reader. Enzyme unit activity was expressed as the change of 0.001in absorbance min^{-1} μg^{-1} haemolymph protein (Traifalgar et al. 2010).

Serum antibacterial activity assay

Serum antibacterial activity was determined based on the microplate method of Eloff, (1998) with slight modifications. The modifications involved the use of shrimp serum rather than plant extract. The test sample and the test bacterial volume used was 20ul each while in the original method it is in the range of 25-100ul. Also in the present test the bacterial tester used was *V. harveyi* while in the original method several tester bacteria were used including , *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, or *Pseudomonas aeruginosa*. Briefly, collected haemolymph were allowed to clot in 1ml Eppendorf tube and were subjected to freeze thaw cycle for five times. The collected haemolymp, obtained from 3 shrimp per replicate tank, were pooled and centrifuged at 5000rpm for 15 minutes and the cell-free supernatant (serum) was collected. Twenty microliters of an overnight marine broth culture of shrimp pathogenic *Vibrio harveyi* was incubated with equal volume of the collected serum in 96-well microtiter plates for 3 hours. Following the incubation, 20µL of Lennox broth were added to each well and further incubated for another two hours. As an indicator of bacterial growth, 40µL of iodonitrotetrazolium (INT) chloride dissolved in distilled water at 0.6mg/ml were added in each well and incubated at room temperature for 30 minutes (Buwa and van Staden 2006). The plate was then read at 490nm in a microplate reader. The bacterial growth in the media was measured as the optical density at 490nm (O.D. 490). Serum antibacterial unit activity was measured using the following formula (Mamelocp and Traifalgar 2020):

$$Serum\ Antibacterial\ Activity = \left[1 - \left\{\left(\frac{O.D.490\ Treated}{O.D.490\ Control}\right) X\ 100\right\}\right]$$

Statistical analysis

Data were analyzed using statistical packages for social sciences (SPSS) version 20.0 Levene's test was used to test the homogeneity of variance. One way analysis of variance was used to analyze significant difference among treatment means. Differences among the treatment means were further resolved through post hoc analysis using Tukey's honestly significant difference test (HSD) at $P < 0.05$.

Table 2 Percent survival, weight gain and specific growth rate of juvenile *P. monodon* fed diets supplemented with graded levels of *Sargassum* polysaccharide extract for 50 days.

	SAPE dietary inclusion levels (mg/kg)				
	0	100	500	1000	2000
Survival (%)	81 ± 5	85 ± 5	85 ± 10	84 ± 4	87 ± 10
Initial weight (g)	0.085± 0.001	0.085± 0.001	0.085± 0.001	0.085± 0.001	0.085± 0.001
Final weight (g)	1.11 ± 0.01^{a}	1.01 ±0.01^{a}	1.01 ± 0.01^{a}	1.09 ± 0.03^{a}	1.30 ± 0.02^{b}
WG (%)	1206 ± 29^{a}	1083± 10^{a}	1083± 25^{a}	1189 ± 56^{a}	1433± 51^{b}
SGR (%/day)	5.71 ± 0.05^{a}	5.49 ± 0.17^{a}	5.48 ± 0.49^{a}	5.67 ± 0.93^{a}	6.06 ± 0.73^{b}
Feed Intake (g)	2.08 ± 0.04	1.93 ±0.05	1.87 ± 0.06	2.05 ± 0.06	2.34 ± 0.12
FCR	2.03 ± 0.04	2.10 ±0.06	2.03 ± 0.03	2.03 ± 0.04	1.92 ± 0.06

Values are means ± SEM of three replicates. Mean values having similar superscript within a row is not significantly different (P > 0.05).

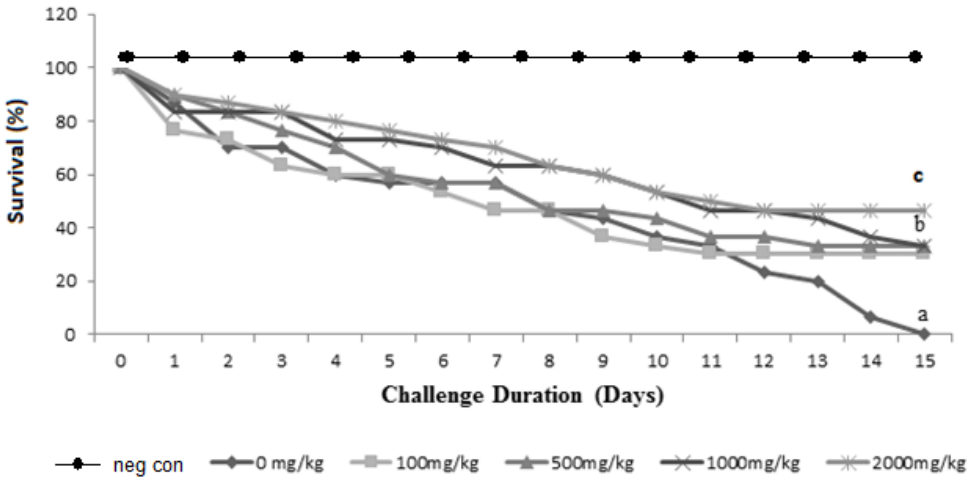

Fig. 1 Survival of *Penaeus monodon* fed diets supplemented with graded levels of Sargassum polysaccharide extract upon challenged with pathogenic *Vibrio harveyi*. Data points with different superscript letters are statistically different (P < 0.05).

Results

Growth trial

Table 2 summarizes data on percent weight gain, specific growth rate and shrimp survival after 45 days of feeding with diets supplemented with graded levels of *Sargassum* polysaccharide extract. At the end of the feeding trial, shrimp survival was high in the range of 81 ± 5 to 87 ± 10 and no negative effects of the dietary supplementation were observed. Weight gain and specific growth rate were found better (P < 0.05) in treatment receiving diet containing 2000mg//kg SAPE. Weight Gain and SGR of experimental groups maintained with diets containing 100, 500 and 1000 mg/kg SAPE were found similar to that of the control group. Feed intake and FCR were found similar in all the dietary treatments.

Disease resistance trial

Infection trial with the pathogenic *Vibrio harveyi* indicates a significant influence of dietary *Sargassum* polysaccharide extract on the shrimp resistance against this bacterial pathogen. Mortality of the treatment groups due to infection were gradual exhibiting a typical bacterial infection mortality curve (Fig. 1). Treatment groups receiving diets supplemented with SAPE has a mean survival of 30±11.54%, 33.33±10.00%, 33.33±11.54 and 46.67±11.54% for 100mg/kg, 500mg/kg, 1000mg/kg and 2000mg/kg diet respectively (Fig. 1). Survival in all the treatment groups fed diets containing polysaccharide extract was significantly higher (P < 0.05) than the control group. All shrimp in the positive control group died due to luminous bacterial infection. Highest survival was obtained in shrimp fed with 2000mg/kg polysaccharide. All experimental shrimp in the control group succumb to infection and died.

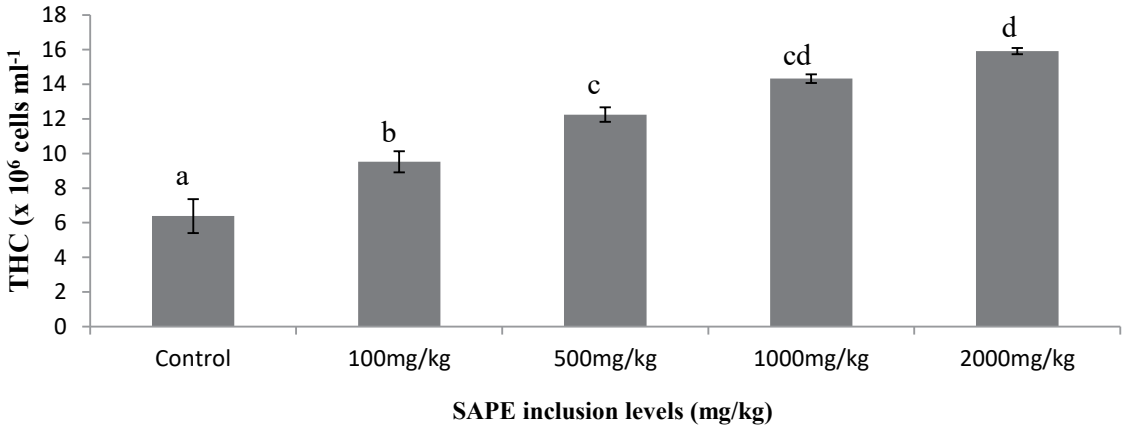

Fig. 2 Total hemocyte count (THC) of shrimp *P. monodon* fed diets containing graded levels of *Sargassum* polysaccharide extracts. Bars are means ± SEM of three replicates. Bars having similar superscript letters are not statistically different ($P < 0.05$).

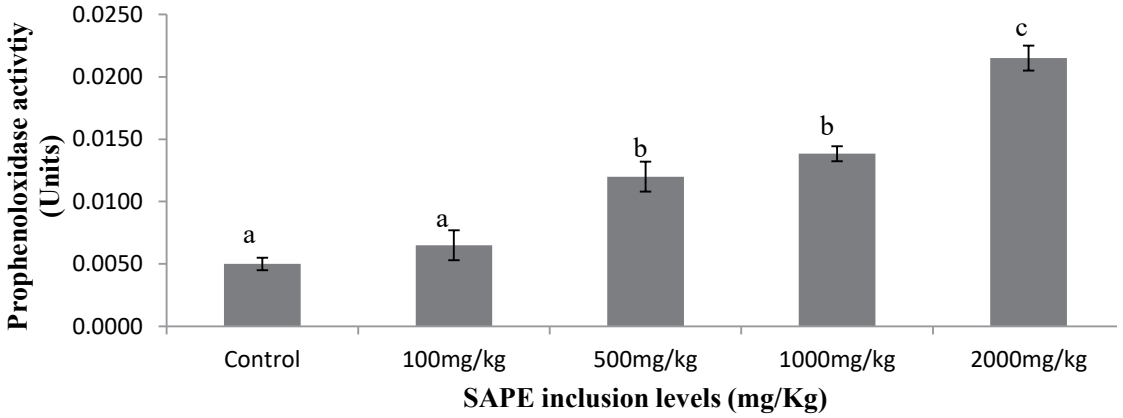

Fig. 3 Prophenoloxidase activity of shrimp *P. monodon* fed diets containing graded levels of *Sargassum* polysaccharide extracts. Bars are means ± SEM of three replicates. Bars having similar superscript letters are not statistically different ($P < 0.05$).

Immunological analysis

Total haemocyte count (THC)

Total haemocytes count increased following the increasing dose of the dietary immunostimulant. All treatment groups exhibited significantly higher values of THC as compared to the control group which exhibited the lowest value of THC. Highest haemocytes counts were recorded in treatment group's receiving the 1000-2000 mg/kg of SAPE supplementation.

Prophenoloxidase (PO) activity

Prophenol oxidase activity were found to increase with increasing dietary inclusion levels of SAPE following a dose response curve pattern. Apparently, lowest PO activity was recorded on the control group that is not significantly different from those of the treatment receiving the lowest concentration 100 mg/kg (Fig. 3) of the test compound. Highest activity was exhibited in treatment groups receiving 2000 mg/kg SAPE.

Serum antibacterial activity

Supplementation of graded levels of *Sargassum* polysaccharide extracts significantly enhanced shrimp serum antibacterial activity against *Vibrio harveyi*. All treatment groups receiving dietary SAPE exhibited significantly higher serum antibacterial activity as compared to the control group. Highest serum antibac-

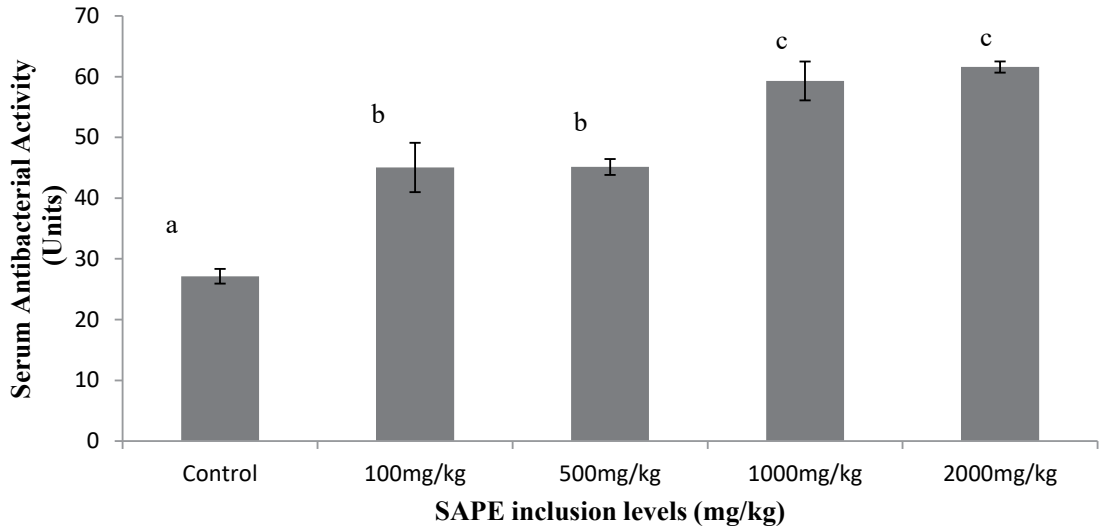

Fig. 4 Serum antibacterial activity of shrimp *P. monodon* fed diets containing graded levels of *Sargassum* polysaccharide extracts. Bars are means ± SEM of three replicates. Bars having similar superscript letters are not statistically different ($P < 0.05$).

terial activity was exhibited in treatment receiving 1000 and 2000 mg/kg dietary SAPE. The control group exhibited the lowest serum antibacterial activity.

Discussion

For decades it has been recognized that glycosaminoglycan's (GAGs) or bioactive polysaccharides can modulate cellular physiological responses including immune cell activation, cell death, differentiation and recruitment. These compounds are also known to exhibit potent antimicrobial activities (Farrokhi et al. 2012; Marudhupandi and Inbakandan 2015). Further, the discovery that algae polysaccharides exhibit unique molecular structure and mimic the functionalities of GAGs has attracted much attention on the practical application of these compounds in the animal growing industries including aquaculture. Sulfated acidic polysaccharides extracted from the family Phaeophyta (brown algae) have been known to exhibit potent immune modulatory, anticancer and anti-microbial activities (Patel 2012; Vatsos and Rebours 2014). The present study evaluates the potential of *Sargassum polycystum* polysaccharide extract as a dietary immunostimulant for juvenile *P. monodon*. Results of the present study suggest that dietary supplementation of *Sargassum polycystum* polysaccharide extract (SAPE) at a concentration of 2000 mg/kg diet can improve growth performance and enhance resistance of juvenile *P. monodon* against the luminous Vibriosis disease. Shrimp survival was recorded high after the feeding trial and was not affected by the dietary inclusion levels of SAPE suggesting that this compound is not toxic to the experimental animals. Our present findings on the significant improvement in growth of *P. monodon* as an effect of SAPE supplementation at a dose of 2000 mg/kg diet is in the range of dietary *Sargassum wightii* fucoidan supplementation at a dose of 1000- 3000 mg/kg diet that was reported to promote the growth of juvenile *P. monodon* (Sivagnanavelmurugan et al. 2014). Also, significant improvement of nutrient retention and growth was reported in *P. japonicus* fed diets containing *U. pinnatifida* polysaccharide extracts at a dose of 1000 and 2000 mg/kg diet (Traifalgar et al. 2010). Dietary supplementation of *S. polycystum* fucoidan at a dose of 500 mg/kg diet was also documented to enhance the growth of juvenile *Macrobrachium rosenbergii* (Arizo et al. 2015). In addition a dietary dose of 500 mg/kg diet of semi-purified *U. pinnatifida* fucoidan was also reported to promote growth of juvenile *P. monodon* (Traifalgar et al. 2009). The differences in the optimum dietary supplementation of this brown seaweed polysaccharide to promote growth of cultured shrimp could be due to the degree of purity of the polysaccharide extracts, the different shrimp species and the species of seaweeds used in these studies.

Earlier reports suggest that *S. polycytum* extracts exhibits potent antibiotic properties (Chotigeat et al. 2004) and growth promoting effects of antibiotics on farmed terrestrial animals are well documented

(Reda et al. 2013). In livestock it has been well-documented that antibiotics application improves overall growth performance by suppressing gut inflammatory processes caused by bacterial infections thus resulting to efficient feed nutrient absorption and assimilation (Graham et al. 2007). Growth promoting effects of dietary immunostimulant has also been reported in shrimp as an effect of the activation of hepatopancreas absorbing cells and enhancement of digestive protease activities resulting to efficient nutrient assimilation (Pan et al. 2015). In addition, muscle growth promoting effects of fucoidan polysaccharide from *Undaria* in Asian sea bass has been attributed to its capacity to inhibit the biological action of myostatin that is a negative regulator of muscle growth (Tuller et al. 2014).

All treatments receiving the SAPE polysaccharide supplementation exhibited higher resistance against *V. harveyi* infection as compared to the control group but the treatment at 2000 mg/kg diet exhibited the highest response. The present findings are comparable to the results reported by Sivagnanavelmurugan et al. (2014) showing that dietary supplementation dose of 1000-3000 mg/kg of *S. wightii* sulfated polysaccharide could enhanced resistance of juvenile *P. monodon* against *V. parahaemolyticus* infection. Likewise, Huang et al. (2006) showed significant enhancement of disease resistance of juvenile *Fenneropenaeus chinensis* against luminous *V. harveyi* when feed with *Sargasuum fusiforme* extract at a dose of 500 -1000mg/kg diet. Further enhancement of WSSV viral infection resistance in *P. monodon* fed with diets containing *S. wightii* (Immanuel et al. 2012) and *S. polycystum* extracted polysaccharides (Chotigeat et al. 2004) have been reported. All of these earlier reports support the present findings indicating the potent activity of *Sargassum* polysaccharides in enhancing disease resistance of cultured shrimp.

In the present study, the enhanced survival of the treatment group receiving the SAPE supplemented diets following the infection challenged test with the pathogenic *V. harveyi* could be attributed to elevated levels of immune responses. Significantly higher values of THC and serum antibacterial activities were found in all treatment groups receiving the SAPE supplemented diet. Activation of immune cells by sulfated polysaccharides from seaweed has been well documented. Sulfated polysaccharide from *U. rigida* was documented to induce and promote murine macrophage proliferation, chemokine, nitric oxide and interleukin productions (Leiro et al. 2007). In *P. vannamei* evidence suggest that in-vitro tests on isolated haemocytes revealed that fucoidan can activate haemocyte degranulation to release antibacterial proteins, promotes hematopoietic cell replication that increases the number of circulating haemocytes (Kitikiew et al. 2013.). Similar observations were reported by Immanuel et al. (2012) and Sivagnanavelmurugan et al. (2014) showing that increased THC of *P. monodon* is correlated with dietary dosage of fucoidan supplementation and shrimp survival against WSSV infection. Huang et al. (2006) also reported that THC of *Fenneropenaeus chinensis* increases with increased dietary supplementation of *S. fusiforme* polysaccharide extract and is associated with enhanced protection against pathogenic bacterial infection. In the present study significant improvement in resistance against *V. harveyi* infection in treatments receiving feeds with SAPE supplementation could be attributed to the high total haemocytes counts similar to that observed in previous studies.

Also, phenol oxidase activity and serum antibacterial activities were found elevated, as compared to the control group, in all SAPE supplemented treatments except in treatment with 100 mg/kg supplementation. Phenol oxidase activity is an important component of the shrimp immune response that is directly related to the number of circulating haemocytes (Soderhall et al. 1998). Activation of haemocytes could result to induction of phenol oxidase activity and the elevated levels of phenol oxidase activity in the present study could be attributed to the activation of haemocytes by dietary SAPE. Our findings corroborate with the report of Sivagnanavelmurugan et al. (2014) showing that enhanced resistance of *P. vannamei* against *V. parahaemolyticus* infection could be attributed to the heightened phenol oxidase activity as a physiological response to immunostimulation with dietary *Sargassum wightii* polysaccharides. Also, in juvenile *P. monodon* maintained with diets containing unprocessed *Sargassum wightii* biomass, heightened phenol oxidase activity was observed in treatments with the highest supplementation dose. In addition the supplementation of *Sargassum wightii* has significantly increased the survival of the experimental shrimp when artificially challenged with the pathogenic *Vibrio parahemolyticus.* (Felix et al. 2004). Enhancement of resistance against WSSV infection attributed to heightened phenol oxidase activity has also been documented in juvenile *P. monodon* maintained with diets supplemented *Sargassum wightii* fucoidan (Immanuel et al. 2012). In addition the higher resistance against *V. harveyi* infection of the experimental shrimp receiving 2000 mg/kg dietary SAPE in the present study is associated with a higher serum antibacterial activity.

Studies in crustaceans indicate the importance of humoral antibacterial responses including the secretion of lysozyme, lectins and antibacterial peptide for an organism to survive microbial infections and seaweed polysaccharides are known to be potent elicitors of humoral antibacterial immune responses. In *F. indicus* it has been reported that immersion in dissolved polysaccharide extracted from *S. glaucescens* resulted to the enhancement of vibrio clearance as a consequence of heightened serum antibacterial activity (Ghaednia et al. 2011). Secretion of a potent phagocyte activator protein as a consequence of dietary supplementation of *Sargassum polycsytum* fucoidan has also been reported in juvenile *P. monodon* (Deachamag et al. 2006). Activation of serum antibacterial activity resulting to enhanced resistance against *V. harveyi* infection was also documented in *P. monodon* fed with *F. vesiculusos* fucoidan (Traifalgar et al. 2013). Similar findings were also reported in *P. monodon* highlighting enhanced resistance against *P. damseale* infection as a result of elevated serum antibacterial activity elicited by dietary immunostimulation with acidic polysaccharide extracted from *S. glaucescens* (Chang et al. 2013). These earlier reports conform with the findings of the present study elucidating the immunoactive functionality of dietary SAPE resulting to the enhancement of resistance of juvenile *P. monodon* against *V. harveyi* infection.

Efficient feed assimilation, improved growth rate and disease resistance are production criteria for shrimp that are highly wanted by shrimp growers. The use of *S. polycystum* acidic polysaccharide as dietary supplement at a dose of 2000 mg/kg is a practical approach in improving growth performance and enhancement of disease resistance of cultured *P. monodon*.

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

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