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Dietary effect of *Sargassum wightii* fucoidan to enhance growth, prophenoloxidase gene expression of *Penaeus monodon* and immune resistance to *Vibrio parahaemolyticus*

Madasamy Sivagnanavelmurugan, Bergmans Jude Thaddaeus, Arunachalam Palavesam, Grasian Immanuel*

Marine Biotechnology Division, Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam 629 502, Kanyakumari District, Tamilnadu, India

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ABSTRACT

The polysaccharide fucoidan from brown seaweed *Sargassum wightii* was extracted and it was incorporated with pellet diets at three concentrations (0.1, 0.2 & 0.3%). The fucoidan incorporated diets were fed to shrimp *Penaeus monodon* for 60 days and the growth performance was assessed. The weight gain and SGR of control group was 6.83 g and 9.72%, respectively, but the weight gain and SGR of various concentrations (0.1–0.3%) of fucoidan incorporated diets fed groups of shrimp was increased from 7.30 to 8.20 g and 9.83 to 10.03%, respectively. After 60 days of feeding experiment, the relative quantification of prophenoloxidase gene of experimental groups over control group was analysed by RT-PCR and it was ranged between 2.13 and 7.95 fold increase within 33.52–34.61 threshold cycles, respectively at 0.1–0.3% concentrations of fucoidan. After 60 days of feeding experiment, the *P. monodon* were challenged with shrimp pathogen *Vibrio parahaemolyticus* and the mortality percentage was recorded daily up to 21 days. The reduction in mortality percentage of experimental groups over control group was recorded from 44.56 to 72.79%, respectively in 0.1–0.3% of fucoidan incorporated diets fed groups. During challenge experiment, all the immunological parameters such as THC, prophenoloxidase activity, respiratory burst activity, superoxide dismutase activity, phagocytic activity, bactericidal activity and bacterial clearance ability of experimental groups were significantly ($P < 0.05$) increased than control group. The *V. parahaemolyticus* load was enumerated from the infected shrimp at every 10 days intervals during challenge experiment. In control group, the *Vibrio* load was increased in hepatopancreas and muscle tissues from 10th to 21st days of challenge test. But in the experimental groups, the *Vibrio* load in both the tissues decreased positively from 10th to 21st days of challenge duration. It is concluded that the *S. wightii* fucoidan had enhanced the innate immunity and increased resistance to *V. parahaemolyticus* infection in *P. monodon*.

1. Introduction

Shrimp farming has suffered great losses due to disease outbreaks associated with viral disease like WSSV, and bacterial diseases caused by *Vibrio* species [1–3]. The Vibriosis is an important disease known to affect juvenile and adult shrimp in grow-out culture system [4] and mostly caused by *Vibrio harveyi*, *Vibrio penaeicida*, *Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio campbellii* and other *Vibrio* spp and they act as primary pathogens in pond waters with increased their populations [5,6]. The disease prevention methods include disinfection and pond drying [7,8], removal of vectors and carriers [9,10], stocking specific pathogen-free (SPF) postlarvae [3,11], water filtering [12,13], etc. However, all these control strategies will not be worked out always. Therefore, the search for novel, effective immunostimulating agent against bacterial diseases is much essential.

The infectious diseases can be prevented in aquatic species by enhancing the host's nonspecific defence mechanism by immunostimulants with or without vaccines [14,15]. Many studies have been conducted to test the efficacy of feeding shrimp with diet containing immunostimulants to enhance their immune activity

* Corresponding author. Tel.: +91 4652 253078.
E-mail address: gimmas@gmail.com (G. Immanuel).

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and resistance against diseases. The most commonly used method for shrimp immunostimulants is oral administration. For example, hot-water extract of *Gracilaria tenuistipitata* [16], *Sargassum fusiforme* polysaccharide [17], saponin [18], β-1,3-glucan [19], sodium alginate [20], and various polysaccharides [21–23] incorporated in diets have been reported to enhanced the immune activity and resistance against diseases in shrimp. Some polysaccharides derived from microbes (β-glucan, lipopolysaccharide (LPS) & peptidoglycan) as well as from seaweeds (alginate, carrageenan, fucoidan & laminarin) are considered as immunostimulanting agents. Their capabilities to increase the innate immune response of teleost and shrimp have been studied *in vitro* and *in vivo* [14,15,23–25]. The crude fucoidan of *Sargassum polycystum* has been reported for the inhibition of shrimp pathogens like *V. harveyi*, *Staphylococcus aureus* and *Escherichia coli* [26].

Liu et al. [20] have reported the effect of commercial sodium alginate on the immune response and immune gene expression in tiger shrimp *Penaeus monodon* through dietary administration. Liu et al. [27] again studied the efficacy of *Panax ginseng* polysaccharide extract administered via diet on the immune responses and immune gene expression in white shrimp, *Litopenaeus vannamei*. Okumura [28] reported the efficacy of *E. coli* lipopolysaccharide on prophenoloxidase gene expression in haemocytes of *L. vannamei*. Wang et al. [19] observed the immune-related genes expression in *L. vannamei* at different time intervals in response to dietary administration of β-1,3-glucan of *Schizophyllum commune*. In view of the above findings, the present investigation was carried out to study the efficacy of *Sargassum wightii* fucoidan on growth performance and prophenoloxidase gene expression in shrimp *P. monodon* and also to determine the immune resistance against shrimp pathogen *V. parahaemolyticus*.

2. Materials and methods

2.1. Isolation of fucoidan

The polysaccharide fucoidan of seaweed *S. wightii* was isolated and characterized by the method described in Immanuel et al. [29].

2.2. Preparation of pellet diets

The pellet diet was prepared that contained 56% fish meal, 21 and 20.7% groundnut oil cake for control and experimental diets, 11% soybean powder, 6% wheat bran, 2% vitamins and mineral mix, 2% cod liver oil and 2% binder. The *S. wightii* fucoidan was incorporated individually to the test diets at different concentrations such as 0.1, 0.2 and 0.3% with a corresponding decrease in the amount of cellulose. The diet preparation process was described in Immanuel et al. [29].

2.3. Feeding trial

The shrimp *P. monodon* postlarvae (PL20) were collected from Matsya Federation Hatchery of Quilon, Kerala. The collected shrimp postlarvae were acclimatized for 10 days with the appropriate laboratory conditions and also fed with *Artemia franciscana* nauplii. After acclimation, uniform sizes of PL 30 (initial weight: 0.020 ± 0.004 g) stage of *P. monodon* were transferred into the individual experimental tanks (Fucoidan free diet served as control and 0.1, 0.2 & 0.3% concentrations of fucoidan incorporated diets served as experimental). The individual tank having 750 l of filter sterilized seawater in one tone capacity FRP tanks at ambient maintenance of salinity 35 ppt and temperature 28 ± 1 °C. The *P. monodon* postlarvae at the rate of 1/5 l (n = 150 no's in each experimental tank) were stoked in the control and experimental tanks. Simultaneously triplicate tanks were maintained in each group. In all tanks, oxygen level was maintained below 6 ppm through aeration. The individual group of shrimp was fed with the respective diets thrice a day (6th, 14th and 18th h) at the rate of 30, 30, and 40%, respectively. During feeding trial, the unfed and fecal materials were removed daily through exchange of 50% water. The feeding experiment was conducted for 60 days.

2.4. Growth performance

At the end of feeding experiment (60th day), the weight gain of shrimp was determined by deducting the initial weight of shrimp from final weight. The percentage weight gain of shrimp was calculated. The Specific growth rate (SGR) was determined through the formula described in Immanuel et al. [30].

2.5. Prophenoloxidase gene expression analysis of P. monodon through RT-PCR

Immediately after 60 days of feeding experiment, the prophenoloxidase gene expression was determined in the haemolymph samples of the individual group of shrimp by following the standard methodology.

2.5.1. Collection of haemocyte

Haemolymph (0.50 ml) was withdrawn individually from the ventral sinus cavity of each group of shrimp into a 1 ml sterile syringe (25-gauge needle) containing 0.5 ml of precooled (4 °C) anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, at pH 7.5 and with an osmolality of 780 mOsm kg^{-1}). The diluted haemolymph was centrifuged (500 × g) at 4 °C for 20 min, and the haemocyte pellet was washed once with cacodylate buffer (10 mM sodium cacodylate, 0.45 M sodium chloride, 20 mM calcium chloride; pH 7.0). The resulting haemocyte pellet was then used for the total RNA isolation.

2.5.2. Total RNA isolation and reverse transcription (RT)

Total RNA was extracted and purified by guanidinium thiocyanate method described by Chomczynski and Sacchi [31]. First-strand cDNA synthesis was done by reverse transcription (RT) method, proposed by Lai et al. [32].

2.5.3. Primer designing

The specific primer pairs was designed for prophenoloxidase gene sequence of mRNA [Forward primer: 5′-CGACTCCTGGATGCCATACAT-3′; Reverse primer: 5′-CATCGCGAAGAGGAACTTTGT-3′ (Accession no.: AF521948)] by using primer express software (Applied Biosystems, Foster City, CA, USA).

2.5.4. Quantification of prophenoloxidase (immune) gene expression by real-time (RT)-PCR

The relative mRNA expression of prophenoloxidase gene of shrimp, that was individually fed on control and experimental diets incorporated with different concentrations (0.1–0.3%) of *S. wightii* fucoidan for 60 days were analysed by real-time PCR. The cDNA was used for the assay of real-time PCR. The amplification was carried out in a 96-well plate in a 25 μl reaction volume containing 12.5 μl of 2× SYBR Green Master Mix (PerkineElmer Applied Biosystems), 2.5 μl each of the forward and reverse primers (10 μM), 1 μl of template (1 μg cDNA), and 9 μl of DEPC-water. The thermal profile for the SYBR green real-time RT-PCR was 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. In a 96-well plate, each sample was run in duplicate. DEPC-water replaced the template as the negative control. Data analysis

of the RT-PCR was performed with SDS software version 2.0. Relative quantification of prophenoloxidase gene expression was performed according to the manufacturer's instructions.

2.6. V. parahaemolyticus challenge experiment

2.6.1. Preparation of V. parahaemolyticus stock culture

The shrimp pathogen *V. parahaemolyticus* (MTCC-451) was cultured on the tryptic soy agar plates (TSA incorporated with 2.5% NaCl) at 25 °C for 24 h. The culture was transferred to 10 ml of tryptic soy broth (TSB incorporated with 2% NaCl) and the broth was incubated at 25 °C for 24 h. The broth culture was centrifuged at 7155 ×*g* at 4 °C for 15 min. From this, the *V. parahaemolyticus* pellet was separated and it was diluted in saline solution at 1×10^7 CFU ml^{-1} as stock bacterial suspension for challenge experiment.

2.6.2. V. parahaemolyticus challenge experiment

The challenge test was conducted by the injection of 20 μl *V. parahaemolyticus* suspension (1×10^7 CFU ml^{-1} $shrimp^{-1}$) in to the ventral sinus of the cephalothorax. The shrimp received no fucoidan and received *V. parahaemolyticus* suspension performed as the challenged control. The shrimp received no fucoidan and received only saline (20 μl) performed as the unchallenged control. During challenge experiment, the shrimp were fed with their respective control and different concentrations (0.1–0.3%) of *S. wightii* fucoidan incorporated experimental diets. After injection of *V. parahaemolyticus*, the survival rate of *P. monodon* was recorded daily for 21 days. The cumulative mortality index (CMI) and percentage reduction in mortality was determined by the method described in Immanuel et al. [29,33] and Sivagnanavelmurugan et al. [34].

2.7. Analysis of immunological parameters

During *V. parahaemolyticus* challenge experiment, the immunological parameters such as THC, prophenoloxidase activity, superoxide anion activity, superoxide dismutase activity and phagocytic activity were evaluated in the haemolymph of shrimp during 0th, 10th and 21st days by following the standard methodologies [29]. Simultaneously, the other immunological parameters such as the bactericidal activity and bacterial clearance ability were also analysed from the haemolymph samples of shrimp by following appropriate methodologies given below.

For bactericidal activity, *V. parahaemolyticus* was cultivated overnight on tryptic soy broth with 2.0% NaCl at 25 °C. The pellet of *V. parahaemolyticus* was collected by centrifugation and washed once in 2% sterile saline, then diluted with saline to obtain the bacterial suspension at optical density 0.1 (540 nm). After feeding experiment, the haemolymph was withdrawn from shrimp and centrifuged at 9700 rpm for 20 min with 2.5% L-Cysteine (as anticoagulant). Then 100 μl of bacterial suspension was incubated with 100 μl of cell free haemolymph. Samples were incubated in sterile microtube for 3 h at 25 °C. Aliquotes of 100 μl were taken from each microtube and spreaded on to thiosulphate citrate bile salts agar (TCBS) plates in order to count the colony forming units (CFU) [35]. In positive control, the bacteria were suspended in saline and incubated in K-199 with 2.5% L-Cysteine.

$$\text{Percentage inhibition}\,(\%) = (\text{Positive control CFU} - \text{Sample CFU})/\text{Positive control CFU}/100\,\mu\text{l}$$

After challenge test with *V. parahaemolyticus*, 100 μl of haemolymph samples were withdrawn from the ventral sinus of each group of shrimp to determine the bacterial clearance ability. Immediately after withdrawn, the hemolymph samples were individually mixed with 1.9 ml of ice cold sterile Van Harrevald's salt solution (VHS) [36]. Haemolymph (100 μl) in VHS were spreaded on to TCBS agar plates for enumeration of CFU. Total number of *V. parahaemolyticus* colonies in haemolymph on TCBS plates was counted after incubated at 37 °C for 18 h.

2.8. Enumeration of V. parahaemolyticus

After challenge experiment, the dead shrimp *P. monodon* were collected every 10 days intervals and were immersed in 50 mg/l formalin solution individually for 5 min (to remove the external bacteria present on the shrimp) and washed thoroughly using sterilized distilled water for 30 s to remove the remaining surface bacteria and disinfectant. From this, hepatopancreas and muscle tissues were removed aseptically and homogenized individually with 5 ml of 85% sterilized seawater and serially diluted the samples up to 10^{-5} dilution. From the samples, 0.5 ml each was taken and inoculated individually in the TCBS agar medium and incubated at 37 °C for 24 h. Simultaneously, triplicates were maintained in each treatment. After 24 h of incubation, the *V. parahaemolyticus* colonies were counted using a digital colony counter and the total bacterial count was calculated by the given formula.

$$V.\ parahaemolyticus\ \text{count}\,(\text{CFU/g}) = \text{Number of colonies} \times \text{dilution factor}/\text{Weight of the sample}\,(\text{g})$$

2.9. Statistical analysis

The data obtained in the present study were expressed as Mean ± SD and were analysed using ANOVA test at 5% level of significance. Further a multiple comparison test (Tukey's test) was conducted to compare the significant differences among the parameters using computer software STATISTICA 06 (Statosoft, Bedford, UK).

3. Results

3.1. Growth performance

The initial weight of shrimp was 0.020 ± 0.004 g. After 60 days of feeding experiment, the shrimp fed with control diet (fucoidan free diet) displayed the weight gain and SGR of 6.83 g (3315%) and 9.72%, respectively, but the growth performance was significantly ($P < 0.05$) increased in fucoidan incorporated diets fed experimental groups with respect to the variation in the concentrations. The lowest concentration (0.1%) of fucoidan incorporated diet fed group exhibited the weight gain and SGR of 7.30 g (3550%) and 9.83%, respectively, whereas in the highest concentrations (0.2 and 0.3%) of fucoidan incorporated diets fed shrimp displayed the weight gain and SGR of 7.65 g (3725%) & 8.20 g (4000%) and 9.91 & 10.03%, respectively (Table 1).

3.2. Relative quantification of prophenoloxidase gene expression

After 60 days of feeding experiment, the expression rate of prophenoloxidase gene was analysed through RT-PCR. The Prophenoloxidase mRNA expression of experimental groups of shrimp was significantly ($P < 0.05$) higher than the control group of shrimp. It was 2.13, 6.54 and 7.95 fold increase than control within 33.52, 34.0 and 34.61 threshold cycles respectively in 0.1, 0.2 and 0.3% fucoidan incorporated diets fed groups (Fig. 1).

Table 1
Growth performance of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets for 60 days.

Conc. of fucoidan (%)	Weight gain (g)	SGR (%)
Control	6.83 ± 0.20^{a} (3315%)	9.72 ± 0.23
0.1	7.30 ± 0.16^{b} (3550%)	9.83 ± 0.20
0.2	7.65 ± 0.30^{bc} (3725%)	9.91 ± 0.26
0.3	8.20 ± 0.18^{d} (4000%)	10.03 ± 0.24

Values in parenthesis indicated the weight gain [weight gain (%) = final weight − initial weight/initial weight × 100].
Each value is a Mean ± SD of three replicate analysis; within each column, means with different superscript letters are statistically significant from each other (one way ANOVA test; $P < 0.05$ and subsequent *post hoc* multiple comparison with the Tukey's test).

3.3. Shrimp pathogen *V. parahaemolyticus* challenge study

3.3.1. Cumulative mortality percentage

The shrimp were succumbed to start death from 2nd day of *V. parahaemolyticus* challenging experiment. In challenged control group, 3% mortality was occurred in 2nd day, at the same time, in the experimental groups, no mortality was recorded. At the lowest concentration of 0.1% fucoidan incorporated diet fed group, the mortality recorded was 3% during 3rd day of challenge test. But at the highest concentrations of 0.2 and 0.3% fucoidan incorporated diets fed groups, the percentage mortality recorded was 1% during 4th day of challenge experiment. When the duration of challenge experiment prolonged, the cumulative mortality percentage was also increased gradually. Finally within 21 days, 78% mortality was observed in challenged control group, whereas only 48, 31 and 23% mortality was recorded respectively in 0.1, 0.2 and 0.3% fucoidan incorporated diets fed groups. But in the unchallenged control group, only 7% mortality was noticed within 21 days (Fig. 2).

3.3.2. Cumulative mortality index (CMI) and reduction in mortality

The CMI in the challenged control group of *P. monodon* challenged with *V. parahaemolyticus* was 12,403, which was considerably reduced to 44.56, 61.26 and 72.79%, respectively in 0.1, 0.2 and 0.3% fucoidan incorporated diets fed groups (Table 2).

3.4. Analysis of immunological parameters

3.4.1. Total haemocyte count (THC)

After feeding experiment (60 days), THC recorded in the control group was 52.9×10^5 cells ml^{-1}, whereas it increased with increasing concentrations of fucoidan incorporated diets fed shrimp. For instance at 0.1% concentration of fucoidan, the haemocyte count observed was 69.8×10^5 cells ml^{-1}, but it was 75.6 and 85.9×10^5 cells ml^{-1}, respectively in 0.2 and 0.3% fucoidan incorporated diets fed groups. After challenge experiment with *V. parahaemolyticus*, the THC was decreased in challenged control group, i.e after 10 days of challenge experiment, the THC recorded in the control group was 49.1×10^5 cells ml^{-1}, at the same time in the experimental groups, the THC was significantly increased from 73.4 to 87.1×10^5 cells ml^{-1} in 0.1–0.3% fucoidan incorporated diets fed groups. However, at the end of the challenge study, in both control as well as experimental groups, the total haemocyte count was reduced to 47.7, 71.8, 76.6 and 86.8×10^5 cells ml^{-1}, in control and in all the experimental groups of 0.1, 0.2 and 0.3% concentrations of fucoidan incorporated diets fed shrimp, respectively. Invariably, there was no changes occurred in the unchallenged control group (Fig. 3).

3.4.2. Prophenoloxidase activity (proPO)

At the beginning of the challenge experiment (0 day), the proPO activity of control group recorded was 0.1438 (OD), whereas in experimental groups fed on 0.1–0.3% concentrations of fucoidan incorporated diets, the proPO activity was increased from 0.1702 to 1812 OD. Further the duration of challenge experiment increased, the proPO activity was also increased positively in both the control and experimental groups. For instance on 10th day of *V. parahaemolyticus* challenge study, the proPO activity increased between 0.1762 and 0.1864 OD in 0.1–0.3% fucoidan incorporated diets fed groups, whereas, it was only 0.1475 OD in challenged control group. On final day of the challenge study (21st day), again the proPO activity gradually increased (0.1789, 0.1814 and 0.1892 OD in 0.1, 0.2 and 0.3%) in the experimental groups. But there was no changes occurred in the unchallenged control group (Fig. 4).

3.4.3. Respiratory burst activity (NBT assay)

In control group, the respiratory burst activity recorded at the beginning of the challenge experiment was 0.0412 OD, whereas it increased to 0.0488, 0.0552 and 0.0681 OD, respectively in 0.1, 0.2 and 0.3% of fucoidan incorporated diets fed shrimp. When the duration of the challenging days increased, the respiratory burst

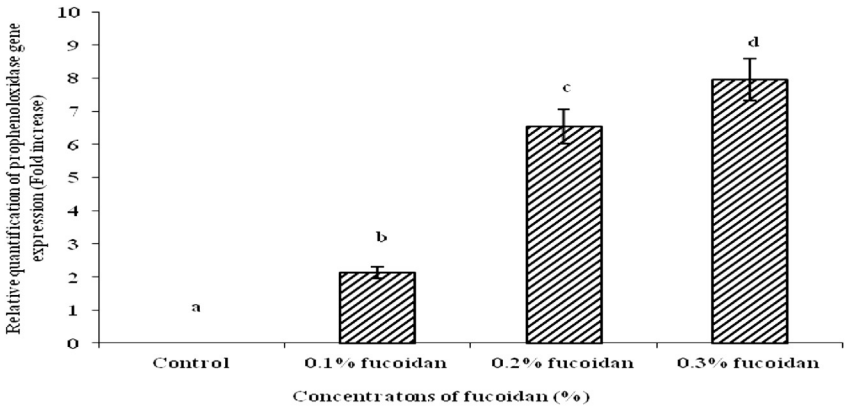

Fig. 1. Relative quantification of prophenoloxidase gene expression of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets after feeding experiment for 60 days. Each value is a Mean ± SD of three replicate analysis; bars with different superscript letters are statistically significant from each other (One way ANOVA test, $P < 0.05$ and subsequently *post hoc* multiple comparison with Tukey's test).

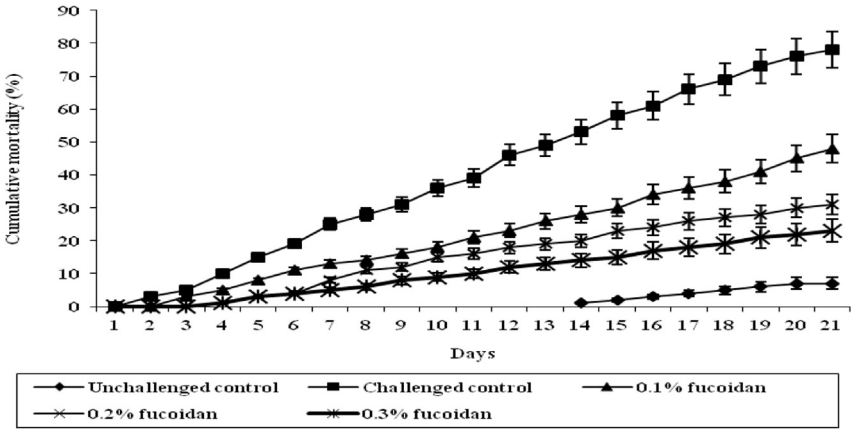

Fig. 2. Cumulative mortality percentage (%) of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets after challenged with *Vibrio parahaemolyticus* in 21 days interval. Each value is a Mean ± SD of three replicate analysis.

activity of experimental groups was also correspondingly increased. For instance it was increased from 0.0485 to 0.0704 OD and 0.0510 to 0.0755 OD, respectively in 0.1–0.3% fucoidan incorporated diets fed shrimp during 10th and 21st day's interval of challenging experiment. Invariably, in the challenged control group, the respiratory burst activity decreased gradually when the experimental duration prolonged to 10th (0.0372 OD) and 21st (0.0398 OD) day's (Fig. 5).

3.4.4. Superoxide dismutase activity (SOD)

The SOD activity in the control group was 36.75 Unit/ml at the beginning of challenge test, whereas in the experimental groups, the SOD activity was increased (54.96–59.88 Unit/ml) with increasing concentrations (0.1–0.3%) of fucoidan. Further the duration of the challenge experiment prolonged, the SOD activity decreased (35.20 and 33.92 Unit/ml during 10th and 21st days of challenge period) in challenged control group. But it was increased in the experimental groups, i.e. 57.14–60.94 Unit/ml during 10th day and 58.89–62.10 Unit/ml during 21st day in 0.1–0.3% fucoidan incorporated diets fed groups, respectively (Fig. 6).

3.4.5. Phagocytic activity

In experimental groups of shrimp, the phagocytic activity was more (6.73–7.14% in 0.1–0.3% fucoidan incorporated diets fed shrimp) than the control group of shrimp (5.70%) at the beginning of the challenge experiment. Subsequently, when the challenge duration increased, the phagocytic activity decreased in both challenged control and experimental groups. During 10th day, the phagocytic activity recorded in control group was only 4.38%, whereas it was 6.51–6.89% in the experimental groups fed with 0.1–0.3% fucoidan incorporated diets. Similarly at the end of the experiment (21st day), the phagocytic activity still decreased to 3.22% in control group and 6.02–6.58% in experimental groups of shrimp fed on 0.1–0.3% fucoidan incorporated diets, respectively (Fig. 7).

Table 2
Cumulative mortality index (CMI) and percentage reduction in mortality of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets after challenged with *V. parahaemolyticus* against control.

Conc. of fucoidan (%)	CMI	Reduction in mortality (%)
Challenged control	12,403 ± 197.12[a]	0.00 ± 0.000
Unchallenged control	651 ± 12.32[b]	94.751 ± 0.685
0.1	6876 ± 153.21[c]	44.56 ± 0.210
0.2	4804 ± 113.12[d]	61.26 ± 0.280
0.3	3374 ± 98.26[e]	72.79 ± 0.320

Each value is a Mean ± SD of three replicate analysis; within each column, means with different superscript letters are statistically significant from each other (one way ANOVA test, $P < 0.05$ and subsequently *post hoc* multiple comparison with Tukey's test).

3.4.6. Bactericidal activity

The result on bactericidal activity of control and experimental groups of shrimp tested against *V. parahaemolyticus* after feeding experiment for 60 days is given in Table 3. The control group showed 176 *V. parahaemolyticus* colonies. At the same time in the experimental groups, the *V. parahaemolyticus* load reduced considerably to 95, 44 and 10 numbers, with the percentage reduction of 46.02, 75 and 94.3% bactericidal activity in 0.1, 0.2 and 0.3% fucoidan incorporated diets fed shrimp, respectively than control.

3.4.7. Bacterial clearance ability

The control group showed no bacterial clearance ability after challenge with *V. parahaemolyticus* throughout the experiment. But in the experimental groups, the bacterial clearance ability was varied much, on 10th day 18.43–50.83% of bacterial clearance ability was noticed in 0.1–0.3% fucoidan incorporated diets fed groups. When the challenge duration increased (21st day), the bacterial clearance ability was also correspondingly increased from 58.94 to 83.50% in 0.1–0.3% fucoidan incorporated diets fed groups, respectively (Table 4).

3.5. V. parahaemolyticus load in hepatopancreas and muscle tissues of P. monodon

3.5.1. In hepatopancreas tissue

The *V. parahaemolyticus* load in hepatopancreas tissue samples of *P. monodon* was determined on 10th and 21st days of challenge period (Fig. 8). The shrimp reared on control diet displayed the *V. parahaemolyticus* load of 83 CFU/100 mg × 10^{-3} on 10th day, but it increased to 96 CFU/100 mg × 10^{-3} on 21st day. However, the experimental groups displayed the *V. parahaemolyticus* load of 65, 49 and 38 CFU/100 mg × 10^{-3} on 10th day respectively in 0.1, 0.2 and 0.3% fucoidan incorporated diets fed shrimp. When the challenge duration prolonged for 21 days, the *Vibrio* load again drastically decreased to 53, 41 and 35 CFU/100 mg × 10^{-3}, in 0.1, 0.2 and 0.3% fucoidan incorporated diets fed groups of shrimp, respectively.

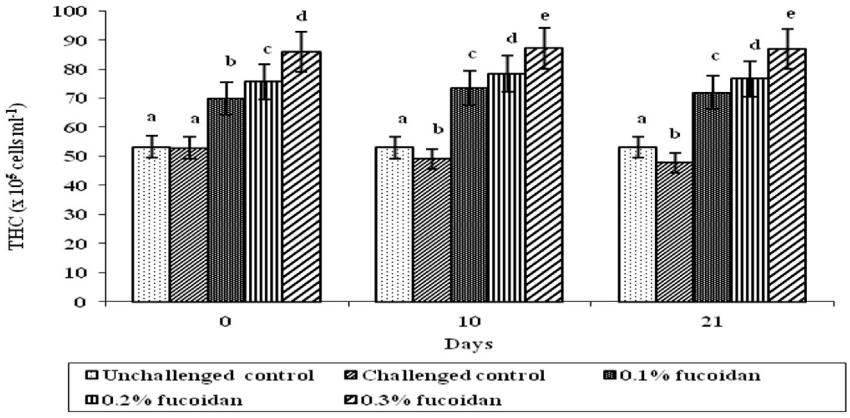

Fig. 3. Total Haemocyte count (THC) of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets during challenge experiment with *V. parahaemolyticus* in 21 days interval. See Fig. 1 for statistical information.

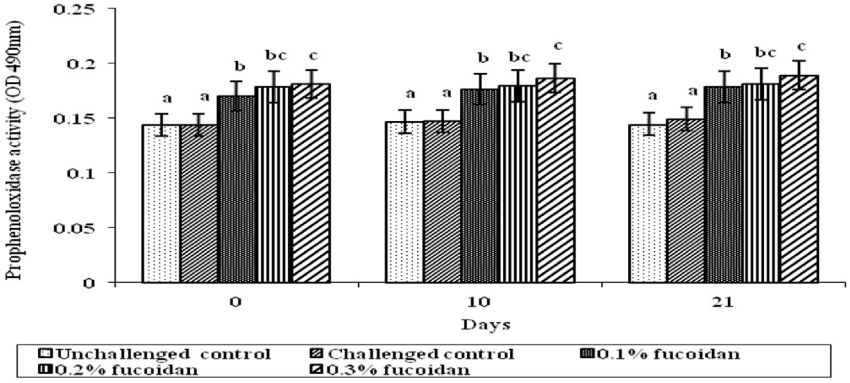

Fig. 4. Prophenoloxidase activity of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets during challenge experiment with *V. parahaemolyticus* in 21 days interval. See Fig. 1 for statistical information (bc: 0.1% vs 0.2%- nonsignificant; c: 0.2% vs 0.3%- nonsignificant).

3.5.2. In muscle tissues

During the challenge experiment, the *V. parahaemolyticus* load in the muscle tissue samples of *P. monodon* was also enumerated. In control group, the *V. parahaemolyticus* load recorded in the muscle tissue was 68 CFU/100 mg $\times$ 10^{-3} on 10th day and it increased to 75 CFU/100 mg $\times$ 10^{-3} on 21st day. Invariably in the experimental groups, during 10th day, the *V. parahaemolyticus* load recorded was 43, 37 and 22 CFU/100 mg $\times$ 10^{-3} in 0.1, 0.2 and 0.3% fucoidan incorporated diets fed groups, respectively. On 21st day of challenge experiment, still the *Vibrio* load in the muscle tissue was decreased to 38, 26 and 18 CFU/100 mg $\times$ 10^{-3} in 0.1, 0.2 and 0.3% fucoidan incorporated diets fed experimental groups, respectively (Fig. 9).

4. Discussion

Fucoidan is a complex sulphated polysaccharide, derived from marine brown seaweeds, usually containing large proportions of L-

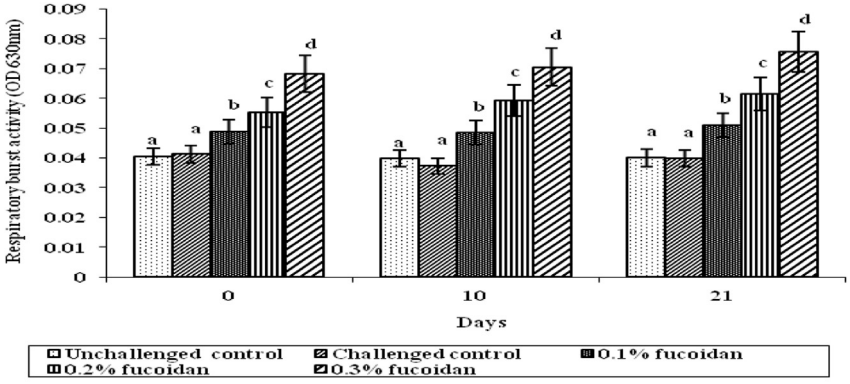

Fig. 5. Respiratory burst activity of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets during challenge experiment with *V. parahaemolyticus* in 21 days interval. See Fig. 1 for statistical information.

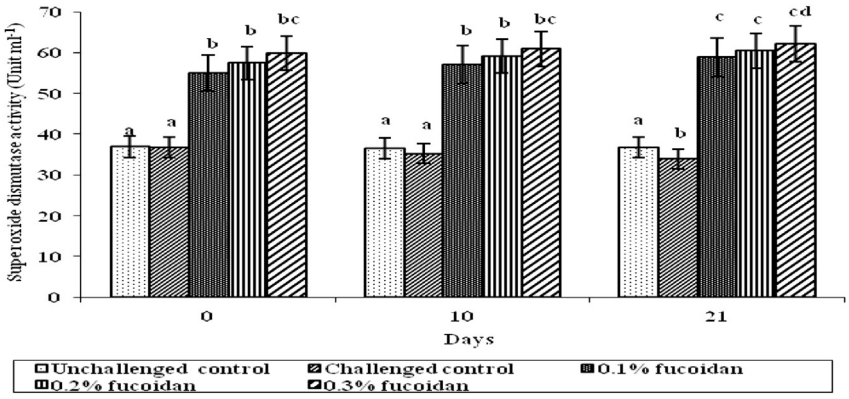

Fig. 6. Superoxide dismutase activity of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets during challenge experiment with *V. parahaemolyticus* in 21 days interval. See Fig. 1 for statistical information.

fucose and sulphate [37,38]. In the present study, fucoidan extracted from *S. wightii* was incorporated with pellet diets at three different concentrations (0.1–0.3%) and were fed to shrimp *P. mondon* for 60 days. At the end of feeding experiment, the growth performance of control and experimental groups of shrimp showed variation and it was found to be depending on the concentrations of fucoidan in the diet. The weight gain and SGR of shrimp fed with the highest concentrations of fucoidan (0.3%) were 4000% and 9.72% and it was significantly ($P < 0.05$) high when compared with control group. In consistence with this result, Trifalgar et al. [39] reported that the weight gain (899.1–1049.7%) and SGR (8–7.90%/day) of shrimp *P. monodon* fed with fucoidan extracted from seaweed *Undaria pinnatifida* were significantly high ($P < 0.05$; *t* test) at higher level of supplementation with the optimum range of 500–2000 mg fucoidan/kg diet. They also inferred that, the weight gain and SGR of control diet fed shrimp and also in shrimp fed with the lowest concentration of fucoidan (100 mg/kg diet) showed insignificant ($P > 0.05$) variation. Traifalgar et al. [40] have also studied the dietary supplementation of *U. pinnatifida* fucoidan on growth performance of juvenile shrimp *Marsupenaeus japonicas*. In this study, they reported that the highest weight gain (202.8 and 215.1%) and SGR (1.9 and 2.06%/day) were registered in shrimp fed on 500 and 1000 mg/kg fucoidan supplemented diets when compared with the values displayed by the shrimp fed on control diet with no fucoidan supplementation (149.5% and 1.6%/day). Enhancement of nutrient digestibility, resulting in efficient protein utilization and improvement of growth rate has also been reported in juvenile *L. vannamei* fed diets supplemented with polysaccharide from *Macrocystis pyrifera* [41]. The growth enhancement effects of dietary seaweed polysaccharides might be attributed to the efficient nutrient digestion and assimilation caused by the activation of fixed phagocytes in the hepatopancreas that secrete hydrolytic enzymes in the digestive gland [42].

Activation of the prophenoloxidase gene system is through recognition molecules in the hemolymph of invertebrates [43]. The proPO activation system is a non-self-recognition system in invertebrates that is able to recognize and respond to intruders via lipopolysaccharides or peptidoglycan from bacteria and β-1,3-glucans from fungi [43]. In the present study, the expression of proPO gene was analysed in shrimp after 60 days of feeding experiment. The relative quantification of prophenoloxidase gene of experimental groups fed on different concentrations (0.1–0.3%) of fucoidan showed 2.13–7.95 fold increase when compared with control group. Cerenius et al. [44] reported that the level of proPO mRNA showed an increase in *Astacus astacus* injected with laminarin, but the levels of actin or PE transcripts remained unchanged. They further inferred that the increase in proPO mRNA in *A. astacus* resulted from both an increase in granular haemocytes (GCs) as well as increased expression of this transcript in semi-granular haemocytes (SGCs). Bae et al. [45] have also reported a concentration dependent expression of proPO gene in β 1,3-glucan (BG) and rutin (RT) fed fleshy shrimp *Fennerropenaeus chinensis* and it was maximum in 1 g kg^{-1} BG (90%) and RT (132%) fed group after 10 days of experimentation.

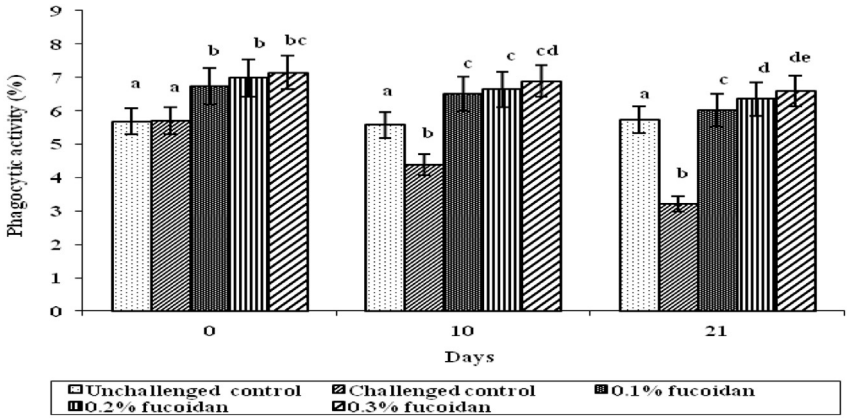

Fig. 7. Phagocytic activity of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets during challenge experiment with *V. parahaemolyticus* in 21 days interval. See Fig. 1 for statistical information.

Table 3
Bactericidal (*V. paraahaemolyticus*) activity of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets for 60 days.

Conc. of fucoidan (%)	Bactericidal activity (no. of colonies)	Bactericidal activity (%)
Control	176.0 ± 4[a]	0.0 ± 0.00
0.1	95.0 ± 2[b]	46.0 ± 0.400
0.2	44.0 ± 1[c]	75.0 ± 0.650
0.3	10.0 ± 0.5[d]	94.3 ± 0.790

Each value is a Mean ± SD of three replicate analysis; within each column, means with different superscript letters are statistically significant from each other (One way ANOVA test, $P < 0.05$ and subsequently *post hoc* multiple comparison with Tukey's test).

In the present study, *V. parahaemolyticus* challenge study was performed in shrimp *P. monodon* after 60 days of feeding experiment. The low mortality percentage (44.56–72.79%) was noticed respectively in 0.1–0.3% of fucoidan incorporated diets fed groups and it was inversely proportional with increasing concentrations of fucoidan in the diet. It further inferred that the lower concentration (0.1%) of fucoidan incorporated diet fed shrimp showed lower inhibitory activity against *V. parahaemolyticus*, and it was vice versa in higher concentration (0.3%) of fucoidan incorporated diet fed shrimp. In accordance with this study, Traifalgar et al. [39] have reported the effect of *U. pinnatifida* fucoidan against *V. harveyi* in shrimp *P. monodon* postlarvae. In this study, the survival of all the treatment groups fed diets containing fucoidan was higher than in the control group. Survival was lower in the control group and it was increased with increasing dietary levels (100–500 mg/kg) of fucoidan supplementation. Traifalgar et al. [40] also investigated the efficacy of *U. pinnatifida* fucoidan supplementation on survival of *Penaeus japonicus* against *V. harveyi*. They observed the post-challenge survival of *P. japonicus* larvae increased with increasing level of dietary fucoidan supplementation. Highest survival was observed in dietary treatment with 1000 mg/kg fucoidan supplementation, followed by the treatment with 500 mg/kg supplementation. Huang et al. [17] reported that the cumulative mortality of shrimp *F. chinensis* challenged with Vibriosis, but fed with 1% *S. fusiforme* polysaccharide extracts (SFPSE) was significantly ($P < 0.05$) lower when compared to control group after 24, 30 and 60 h of infection. Yeh and Chen [46] have also studied the effect of carrageenan (type I to V) on white shrimp *L. vannamei* against *V. alginolyticus* and reported that the survival was high (43.3, 40.0, 30.0, 56.7 and 43.3%) in experimental groups after 120 h of challenge experiment when compared with control. Immanuel et al. [30] reported the extract from seaweeds *Ulva lactuca* and *S. wightii* reduced the infection of shrimp pathogen *V. parahaemolyticus* in *Penaeus indicus* juveniles after 30 days of feeding bioencapsulated *Artemia* nauplii and here the experimental shrimp displayed 51.10 and 45.55% survival against the lowest (24.44%) survival exhibited by control group. The authors attributed these protective effects to the enhancement of haemocyte phagocytic activity and to the inhibition of pathogen adsorption to the host. Recent evidence suggests that shrimp larvae are capable of producing antibacterial peptides in their haemocytes as a response to the presence of infectious and immune stimulating agents [41]. The mode of action of fucoidan attribute that it may enhances shrimp immune response by the activation of a phagocytosis activating protein, known to initiate and enhance haemocyte phagocytic activity [26,47].

The crustaceans have an innate immune system instead of acquired immunity, which include an activation of the prophenoloxidase system (proPO system), clotting process, phagocytosis, encapsulation of foreign material, antimicrobial action and cell agglutination [48]. In the present study, the immunological parameters were analysed in shrimp during challenge experiment with *V. parahaemolyticus*. The haemocytes plays a major role in the cellular immune response in crustaceans [49]. The total haemocyte count recorded in the present study in experimental groups of shrimp challenged with *V. parahaemolyticus* was significantly ($P < 0.05$) high when compared with control group. Similarly, the influence of hot water extract of seaweed *Gracillaria tenuistipitata* on THC of white leg shrimp *L. vannamei* challenged with *V. alginolyticus* was studied by Hou and Chen [50]. They reported that the THC of *L. vannamei*, which received 4 and 6 μg/g of hot water extract was significantly higher than that of shrimp received saline and the control shrimp on 4th day, and further increase in challenge duration displayed no significant difference on THC among the control and experimental groups. Huang et al. [17] studied the effect of *S. fusiforme* polysaccharide extract on THC of *F. chinensis* after challenged with *V. harveyi*. They reported that the THC of the shrimp was progressively elevated with increase in dietary supplementation of SFPSE from 0.0% to 2%, and the THC of the 2% treatment group was significantly ($P < 0.01$) higher than that of the control.

The proPO system produce one of the protein called proPO, it plays a critical role in an important defence immune reaction in crustaceans [43,51]. In this mechanism, the proPO will be converted into PO by the enzyme serine protease (ppAE) [52]. In the present study, the prophenoloxidase activity of experimental shrimp showed a significant increase ($P < 0.05$) on 21st day of the challenge experiment with *V. parahaemolyticus* when compared with control group. Similarly, the effect of sodium alginate on proPO activity of *L. vannamei* after challenged with *V. alginolyticus* was studied by Cheng et al. [53]. They reported that the shrimp that received 20 and 50 μg/g of sodium alginate displayed higher proPO activity than control shrimp up to 4th day of challenge duration, beyond this challenge period, no significant ($P > 0.05$) difference in proPO activity was observed among the experimental and control groups. Huang et al. [17] evaluated the effect of *S. fusiforme* polysaccharide extract (SFPSE) on proPO activity of *F. chinensis* after challenged

Table 4
Bacterial clearance of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets during challenge experiment with *V. parahaemolyticus* in 21 days interval.

Conc. of fucoidan (%)	Bacterial clearance (no. of colonies)					
	0 day		10th day		21st day	
	No. of colonies	Bacterial clearance ability (%)	No. of colonies	Bacterial clearance ability (%)	No. of colonies	Bacterial clearance ability (%)
Unchallenged control	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0
Challenged control	0 ± 0.0	0 ± 0.0	179 ± 5.0[a]	0 ± 0.0	285 ± 6.0[a]	0 ± 0
0.1	0 ± 0.0	0 ± 0.0	146 ± 4.0[b]	18.43 ± 0.56	117 ± 5.0[b]	58.94 ± 0.62
0.2	0 ± 0.0	0 ± 0.0	108 ± 3.0[c]	39.66 ± 0.42	85 ± 2.0[c]	70.17 ± 0.72
0.3	0 ± 0.0	0 ± 0.0	88 ± 2.0[d]	50.83 ± 0.52	47 ± 1.0[d]	83.50 ± 0.86

Each value is a Mean ± SD of three replicate analysis; within each column, means with different superscript letters are statistically significant from each other (One way ANOVA test, $P < 0.05$ and subsequently *post hoc* multiple comparison with Tukey's test).

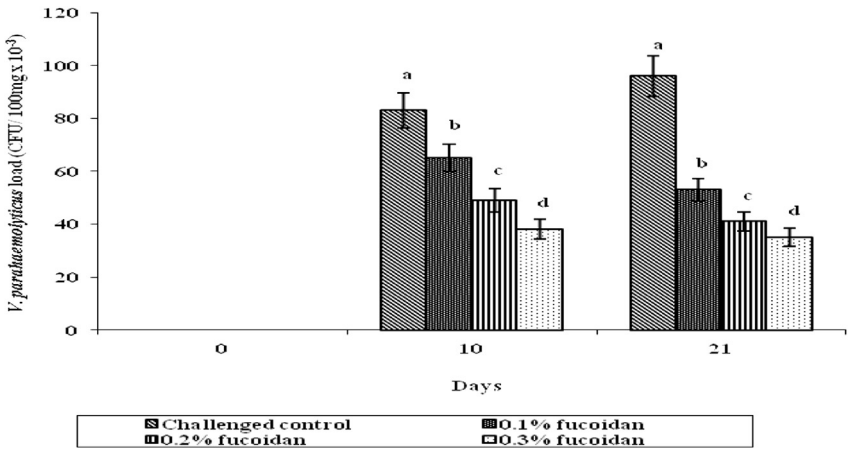

Fig. 8. *Vibrio parahaemolyticus* load (CFU/100 mg × 10^{-3}) in hepatopancreas tissue of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets during challenge experiment with *V. parahaemolyticus* in 21 days interval. See Fig. 1 for statistical information.

with *V. harveyi*. They pointed out that the proPO activity of 0.5% SFPSE group was significantly ($P < 0.05$) higher than that of the control. However, the proPO activity was significantly lower in 1 and 2% SFPSE group when compared to control.

During respiratory bursts of phagocytosis, reactive oxygen intermediates (ROIs) are released, which act as a defence mechanism against microbial infection [54]. In the present study, the respiratory burst activity of experimental groups was significantly ($P < 0.05$) high when compared with control group after challenge experiment and it was found to be influenced by the concentrations of fucoidan in the diet. Cheng et al. [53] reported that the respiratory burst activity of shrimp that received different concentrations (10–50 μg/g) of sodium alginate was significantly higher than that of control shrimp up to 6th days of challenge with *V. alginolyticus*. Further increase in duration indicated no significant difference on respiratory burst activity among the control and experimental treatments. Likewise, Hou and Chen [50] have studied the effect of hot water extract of *G. tenuistipitata* on respiratory burst activity of *L. vannamei* challenged with *V. alginolyticus*. They found that the experimental groups received 4 and 6 μg/g of hot water extract recorded a higher respiratory burst activity than control shrimp up to 6th day of challenge duration.

Superoxide dismutase (SOD) is one of the antioxidative enzymes that scavenge superoxide anions (O_2^-) in crustaceans [55]. In the present study, the SOD activity of experimental groups of shrimp was significantly ($P < 0.05$) high when compared to control group after challenged with *V. parahaemolyticus*. In consistence with the present study, Huang et al. [17] reported that during challenge study with *V. harveyi*, the SOD activity of *F. chinensis* fed with *S. fusiforme* polysaccharide extract showed minimum increase when compared with control group; whereas the variation between experimental shrimp was not significant ($P > 0.05$). In accordance with these, Cheng et al. [53] also stated that there was no significant change on SOD activity among control and different concentrations of sodium alginate treated experimental groups of shrimp *L. vannamei* after challenged with *V. alginolyticus*. Similarly Hou and Chen [50] have studied the effect of hot water extract of *G. tenuistipitata* on SOD activity of *L. vannamei* challenged with *V. alginolyticus* and they reported that the shrimp which received 4 and 6 μg/g of hot water extract have significantly higher SOD activity than that of control shrimp up to 6 days of challenge period, further the challenge period extended, the SOD activity was low both in control and experimental groups of shrimp.

Phagocytosis is an important cellular defence mechanism performed by haemocytes, the lymphoid organs, and the hepatopancreas in crustaceans [56,57]. In the present study, the phagocytic activity of experimental groups of shrimp was significantly ($P < 0.05$) higher than the challenged control group after

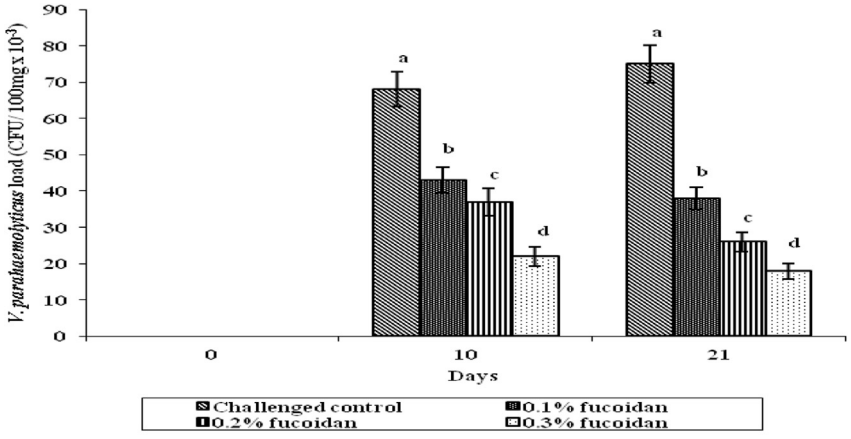

Fig. 9. *Vibrio parahaemolyticus* load (CFU/100 mg × 10^{-3}) in muscle tissue of shrimp *P. monodon* fed on different concentrations of fucoidan incorporated diets during challenge experiment with *V. parahaemolyticus* in 21 days interval. See Fig. 1 for statistical information.

challenged with *V. parahaemolyticus*. At the beginning of the challenge experiment, the phagocytic activity of experimental groups was more (6.73–7.14%) in 0.1–0.3% fucoidan incorporated diets fed shrimp when compared with the control group (5.70%). In correlation with this result, Cheng et al. [53] reported that the phagocytic activity of *L. vannamei* was found to be influenced by the dietary administration of sodium alginate, and further inferred that on 6th day of the challenge test with *V. alginolyticus*, the phagocytic activity of shrimp received 50 μg/g of sodium alginate was significantly ($P < 0.05$) high when compared with control group. Hou and Chen [50] have also reported that the phagocytic activity of *L. vannamei* was significantly ($P < 0.05$) high (53–56%) in those groups received higher concentrations (4 and 6 μg/g) of hot water extract of *G. tenuistipitata* when compared with control on 6th day of challenge test with *V. alginolyticus*.

In the present study, the bactericidal activity and bacterial clearance ability of experimental groups of shrimp were significantly ($P < 0.05$) high when compared with control group after challenge experiment with *V. parahaemolyticus*. In bactericidal activity, the percentage reduction in *Vibrio* load of the experimental groups (0.1–0.3%) of shrimp over control group was 46.02–94.3%. On 10th day, the amount of bacterial clearance was recorded with 18.43–50.83% respectively in 0.1–0.3% fucoidan incorporated diets fed groups. When the duration of the challenge test increased further, the bacterial clearance ability was also increased. Similarly, Cheng et al. [53] reported the effect of sodium alginate on bacterial clearance ability in *L. vannamei* after challenged with *V. alginolyticus*. They pointed out that the bacterial clearance efficiency was significantly higher (57.2–70.8%) for the shrimp that received 10–50 μg/g sodium alginate than that of the control shrimp on 2nd day. Further on 6th day of experiment, the bacterial clearance efficiency was significantly increased only for the shrimp that received higher (50 μg/g) concentration of sodium alginate. Hou and Chen [50] have demonstrated the effect of hot water extract of *G. tenuistipitata* on bacterial clearance ability of *L. vannamei* challenged with *V. alginolyticus*. They suggested that the clearance efficiency was significantly higher for the shrimp that received 4 and 6 μg/g extract than that of control shrimp up to 6 days of challenge period.

The immunostimulants such as β-glucan, laminarin, Lipopolysaccharides, peptidoglycan and zymosan could activate the prophenoloxidase system and stimulate the superoxide anion production in shrimp [58–60]. In the present study, the fucoidan could activate proPO-activating system and increases the PO activity as well as RB activity, which indicated that fucoidan could trigger innate immunity of shrimp. Similarly, Huynh et al. [61] reported that β-glucan and the extract of *Sargassum hemiphyllum* var. *chinense* could increase PO activity by triggering proPO activation system and increase RB *in vitro* indicated that both the powder and the extract could trigger innate immunity of shrimp. In penaeid shrimp, lipopolysaccharide-β-glucan binding protein (LGBP) and β-glucan binding protein (βGBP) contain a glucanase motif, two polysaccharide recognition motifs (polysaccharide- binding motif and β-glucan recognition motif), and two integrin-binding motifs, RGD and RGD [62]. In *P. monodon*, βGBP has the ability to bind curdlan, zymosan and lipopolysaccharide (LPS) [63]. The binding mixture of βGBP with curdlan, laminarin, and LPS activated the proPO activating system [64].

In the present study, the *V. parahaemolyticus* load was enumerated from the infected shrimp during challenge experiment. In control group, the *Vibrio* load in hepatopancreas and muscle tissues of shrimp showed increase when the challenging days progressed from 10 to 21 days. On the other hand in the experimental groups, the *Vibrio* load in hepatopancreas and muscle tissues of shrimp decreased considerably in all the tested groups (0.1–0.3%) of fucoidan incorporated diets fed shrimp during the progress of challenge experiment. Similarly, Immanuel et al. [30] have reported the *Vibrio* load in hepatopancreas and muscle tissues of *P. indicus* fed with seaweed extracts of *U. lactuca* and *S. wightii* and challenged with *V. parahaemolyticus*. They suggested that the bacterial load in hepatopancreas and muscle tissues had maximum in control group and low in experimental groups fed with seaweed extracts enriched *Artemia*. The algibind diet fed Atlantic salmon (*Salmo salar*) group got improved survival, growth and reduction in bacterial load against *Aeromonas salmonicida* [65].

In conclusion, this study suggests that increasing growth performance, prophenoloxidase gene expression and the enhancement of immunological parameters such as THC, proPO, respiratiry burst, SOD, phagocytic, bacterial clearance and bactericidal activities in *S. wightii* fucoidan incorporated diets fed shrimp seem to act as a promoter of the shrimp immune system against *V. parahaemolyticus* infection.

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