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# An *in vivo* efficacy validation and immune-modulatory potential of *Streptomyces* sp.

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ABSTRACT

**Objective:** To investigate the *in vivo* efficacy and immune-modulatory potential of antagonistic strain, *Streptomyces* sp. MAPS15 isolated from marine sponge in *Penaeus monodon* (*P. monodon*).
**Methods:** In this study, culture of *Streptomyces* sp. was incorporated into a commercial feed. *P. monodon* was orally administered with MAPS15 diet for a period of 21 days followed by a challenge experiment and survival rate was calculated. In addition, the effect of MAPS15 diet on immunological parameters of the haemolymph of *P. monodon* was also assessed.
**Results:** The overall results of the study showed that survival performance was prominent in MAPS15 treated group when compared with un-treated control groups. That could pertain to the ability of MAPS15 to produce antibiotic compounds to suppress the growth of invading pathogens and thereby increase the disease resistance potency and survival rate. From the results of the immunological studies, it can be envisaged that the immune responses were generally more pronounced with MAPS15 diet treated group.
**Conclusions:** Based on the overall findings, it could be inferred that the health of *P. monodon* is improved when they are fed with MAPS15 diet for a period of 21 days.

## 1. Introduction

Infectious diseases are considered as a bane to the successful development and good continuation of shrimp culture as they limit production in terms of quality, quantity and regularity. Therefore, prevention of the diseases and the management has been considered as a priority for this industry[1]. Numerous types of zoo-techniques were practiced worldwide to dispel pathogenic microbes in aquaculture, *viz*., filtration of water, addition of sodium chloride, ozonation, use of ultraviolet light, synthetic antibiotics, herbal drugs, *etc*., but turn to be impractical due to cost, time consideration and associated deleterious effects. Broad-spectrum antimicrobials have been extensively used as control measure on many aquaculture facilities. Presently, the application of chemotherapeutics has proved to be unsustainable and environment unfriendly as they can lead to the emergence of bacterial resistance[1]. Albeit different types of vaccines being developed, and tested successfully, they cannot be utilized as a universal disease control strategy in aquaculture.

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The biological control such as the exercise of probiotics in aquaculture is becoming popular due to a mounting demand for environment-friendly aquaculture. Therefore, a rigorous exploration for novel and potent strains as probionts is essential to stave off emerged diseases. In contrast to the flora and fauna of terrestrial environment, marine entities such as free living and associated microorganisms are the ginormous source of natural products[2]. These organisms produce bioactive metabolites to cope and confront to the harsh environment where they thrive. Microorganisms possessing antimicrobial activity of inhibiting pathogens *in vitro* have been employed as probiotics by various investigators[3,4]. The sponges are well known to foster diverse types of endophytic/epiphytic microorganisms capable of producing lead compounds with bioactive potentials[2]. This clearly highlights the possible role of marine antagonistic bacteria associated with sponges in providing solution to the problem of *Vibrio*-prone diseases of shrimp.

The results of our previous studies envisaged that a sponge associated bacteria, *Streptomyces* sp. (strain MAPS15), was efficacious in repressing the growth of tested shrimp *Vibrio* pathogens *in vitro*[2], which clearly indicates the good possibility of developing a 'natural' or 'biological' method for controlling *Vibrio*-prone diseases of shrimp. Therefore, this antagonistic strain was selected for *in vivo* studies to develop a prospective bio-control agent. In this regard, the aim of the present study is to evaluate the *in vivo* efficacy of MAPS15 on the survival rate and immune status

of *Penaeus monodon* (*P. monodon*) under laboratory conditions.

## 2. Materials and methods

### 2.1. Bio-safety evaluation (pathogenicity or toxicity) of candidate probionts

Biological safety (non-pathogenicity) of the suspected probiotic candidate was evaluated by using pre-adult shrimps. For this purpose, apparently healthy 30-day-old shrimps (3.2 ± 1.0) g body weight were procured from the shrimp farm of Monroe Island and acclimated for one week in a 1 000 L high density plastic (HDP) tank. After acclimatization, shrimps were divided into 4 equal groups (3 replicates 10 in each) and challenged by using different doses of bacterial suspension ($10^2$, $10^4$ and $10^8$ CFU/mL). The bacterial suspension was delivered intramuscularly by using a 1 mL tuberculin syringe at ventral side between the second and third segments of healthy shrimps[5]. The control groups were injected with 0.1 mL of normal saline only. The shrimps were monitored for a period of one week for the mortality/infection. The bio-safety of probionts was evaluated by lack of the clinical sign, lesions and mortalities.

### 2.2. Mass production of prospective probiont strain

The mass production of probionts was performed according to the liquid state (sub-merged type) fermentation methodology described elsewhere[6]. Briefly, ZoBell marine broth was prepared in 500 mL Erlenmeyer flasks, and the prospective strain was aseptically inoculated and incubated for a period of 14 days. After the complete development of the biofilm mat (area: ~38.46 $cm^2$, wet weight: ~0.640 g), the strain was aseptically transferred to a 500 L glass aquaria filled with conditioned brackish water of average salinity 15‰, temperature at (26.0 ± 1.5) ℃, pH of 8, dissolved oxygen of 5.2 mg/mL and incubated for 6 weeks. After the completion of incubation period, the resultant probiotic mat was harvested manually, washed with saline and re-suspended, and cell dry weight was determined after the cells were dried in a hot air oven [(40.0 ± 0.5) ℃] for 4 days for constant weight. The completely dried mat was powdered in a coffee grinder and stored in air-tight plastic bags until used.

### 2.3. Preparation of probiotic incorporated experimental shrimp diet

Spray-drying method was used to prepare the probiotic diet[1]. The commercial shrimp feed (Charoen Pokhpond Aquaculture India Pvt. Ltd., Chennai, India) was used for the preparation of dietary probiotic. The probionts were diluted in normal saline for expected concentrations of $10^2$, $10^4$, and $10^8$ CFU/mL. The selected dilutions were incorporated into the feed by spraying appropriate biomass of bacterial cells dispensed/suspended in 50 mL of 6% gelatin water on the surface of the feed slowly by using a thin layer chromatographic sprayer. The sprayed probiotic diet was spread out and dried under sterile condition in an air-forced oven for 2–3 h at 37 ℃. The sterile gelatin water free from bacterial cells added to the commercial diet served as the control. Probiont incorporated feed was then hived away in air-tight plastic bags.

### 2.4. Exploratory experiment on the efficacy of different doses of probiotic diet

#### 2.4.1. Experimental system and animal maintenance

The process of acclimatization, rearing, conditioning and selection of shrimps for *in vivo* efficacy validation were done as per the methodology described elsewhere[1,5]. Briefly, *P. monodon* juveniles [Day of culture: (25 ± 2) days; average body length: (3.8 ± 0.5) cm] were sourced from a reputable farm located at Kollam Prefecture, Kerala, India. The specimens were randomly quarantined to the 1 000 L capacity fibre reinforced plastic tanks which were supplied with filtered and well aerated brackish water at a stocking density of 100 shrimps per tank for one week prior to the experimentation. Tanks were covered to reduce light intensity and shrimps were reared *ad libitum* three times a day regularly throughout the experiment. The shrimps were monitored for their normal behavior and growth rate to determine their health status. All the tanks were provided with continuous aeration and cleaned daily by siphoning. The brackish water was partially changed (about 30% per day) to maintain water quality. The water quality parameters such as temperature (25–28 ℃), dissolved oxygen (6 mg/L) and the salinity (14‰ to 16‰) were measured daily by using the Hach kit model (Hach Company, Loveland, CO).

#### 2.4.2. Feeding system design and regime

To study the *in vivo* efficacy of probiotic diet, shrimps in the treatment groups were orally fed with varying doses of probiotic inclusion for a period of 35 days. The clinically healthy shrimps (no evidence of disease signs) were selected for the *in vivo* experiment. The shrimps were randomly distributed into four experimental groups ($n$ = 60 for each) in a 1 000 L HDP tank. The experimental animals consisted of: (i) group I shrimps fed with experimental feed supplemented with probiotic preparation at a concentration of $10^2$ CFU/mL, (ii) group II shrimps fed with probiotic diet at a concentration of $10^4$ CFU/mL, (iii) group III shrimps fed with probiotic diet at a concentration of $10^8$ CFU/mL, (iv) control group which received normal feed throughout the trial (free of probiotics). An un-challenged, untreated group (group V) was used to verify the cause of natural mortality. Each probiotic diet was provided at rate of 3.5% of biomass daily, 7 days a week. Daily ration was divided into three equal portions supplied at 6, 12 and 24 h. That was estimated to be sufficient to satiate the shrimps with minimum feed wastage.

#### 2.4.3. Challenge procedures

At the end of the feeding experiment (the 22nd day), three duplicate groups each consisting of control and probiotic consumed shrimps ($n$ = 10 for each group) were challenged intramuscularly with higher dose of *Vibrio harveyi* (*V. harveyi*) ($5 \times 10^5$ CFU/mL) and *Vibrio parahaemolyticus* (*V. parahaemolyticus*) ($5 \times 10^5$ CFU/mL), and transferred to the 500 L glass aquaria. They were kept under observation for two weeks to record the mortality and external clinical symptoms[1]. After challenged, the animals were continuously fed with the respective probiotic diets for a further 14 days. Efficacy of each treatment was determined by comparing the mortality in treated shrimps with the control animals after the challenge experiment. The percentage of mortality rate was calculated two weeks after infection[7].

$$\text{Mortality (\%)} = \frac{\text{Number of dead shrimps - A}}{\text{Total number of shrimps - A}} \times 100$$

where, "A" represents the number of dead shrimps on the first day after challenge. This mortality rate could be considered as a result of handling stress.

The percentage of survival index (therapeutic efficacy) was calculated as described by Manilal *et al.*[7]:

$$\text{Survival index (\%)} = 1 - \frac{\text{\% of specific mortalities in treated group}}{\text{\% of specific mortalities in control group}} \times 100$$

### 2.5. Microbiological analysis

The microbiological analysis of the survivor shrimps was carried out after the completion of the experiment. *Vibrio* count was made by using serial dilution of dissected body parts of shrimps extracted

in phosphate buffer saline (PBS) followed by plating triplicates on thiosulfate citrate bile saltssucrose agar. After incubation at 29 ℃ for 24 h, colonies were enumerated and recorded.

### 2.6. Effect of probiotic diet on the gut micro-flora

This study was designed to determine the potential influence of probiotic diet on intestinal micro-flora of treated shrimps. Following the termination of probiotic treatment, the shrimps were fasted for 12 h to empty their gut contents. After starvation, specimens were first rinsed three times in Milli-Q water followed by 60% ethanol to reduce the abundance of adhering bacteria from the surface. Shrimps in each treatment were aseptically dissected by using sterilized micromanipulation scissors, scalpel (blade 4 mm length) and forceps to remove mid- and hind-gut under the stereo microscope and to characterize the microbial populations. The hind-gut of 5 shrimps were pooled, liquefied with PBS and weighed before homogenization. The dissected gut samples were blended with PBS to produce gut homogenate. Bacteriological determination was made by using serial dilution of gut extract in PBS followed by plating triplicates on nutrient agar. After incubation at 29 ℃ for 24 h, colonies were enumerated and recorded. All the results were presented as CFU/mL of fresh digestive tract. All dissection instruments were rinsed in acetone to avoid contamination.

### 2.7. Evaluation of immune status

Three separate experiments were conducted to investigate the effect of prospective probionts in the haemato-immunological parameter of treated *P. monodon*. For this experiment, 30-day-old shrimps at length of 4.5 cm with averaged fresh body weight of 2.8 g were obtained from a shrimp farm in Manroe Island, Kollam, South India and acclimatized for one week in 5 000 L HDP tanks under optimum hydrological conditions. During the acclimation period, shrimps were fed *ad libitum* with a standard shrimp feed (C.P. feed). After seven days of adaptation, experimental animals were divided into two treatments with three replicates. The group comprising of treatment (fed with effective dose of probiont incorporated shrimp diet) and control (fed with normal shrimp diet) was randomly stocked in 1 000 L fibre reinforced plastic tanks containing 800 L of aerated brackish water (25 ℃) and fed with respective shrimp diets. The feeding trial was continued for a period of 21 days. During the treatment phase, shrimps were fed three times daily at 3.5% body weight per day. The waste and fecal matter were siphoned out daily. The water quality parameters were monitored daily and maintained at an acceptable level [temperature: (25 ± 1) ℃, dissolved oxygen 4 mg/L and salinity 16‰] by regular renewal of 20% of the water. Both the treatments were replicated. At the conclusion of the treatment phase, on day of culture-22, shrimps in the treatment were subjected to haemato-immunological analysis.

#### 2.7.1. Haemolymph extraction

Apparently healthy shrimps at intermoult stage from each treatment were sampled randomly to collect haemolymph. In order to reduce the effect of stress before bleeding, shrimps were sedated by immersing in chilled brackish water (10 ℃). Before the collection of haemolymph, body part of the shrimps was washed with distilled water and wiped with 70% of ethanol. Haemolymph (ca. 100 μL per shrimp) was individually sampled from the ventral sinus of each shrimp by using a prechilled 1 mL sterilized syringe fitted with 25 gauge needle preloaded with 0.8 mL cold modified Alsever's solution (19.3 mmol/L sodium citrate, 239.8 mmol/L NaCl, 182.5 mmol/L glucose, 6.2 mmol/L ethylene diamine tetraacetic acid in pH 7.3) as an anticoagulant solution[5]. The haemolymph samples collected from the respective treatments (10 animals) were mixed together to reduce inter-individual variations. The mixed haemolymph samples were immediately poured to polypropylene tubes and stored in a refrigerator for further studies.

#### 2.7.2. Total haemocyte counts

Total haemocyte number in haemolymph was determined according to the methodology described elsewhere[5]. At the end of feeding trial, shrimps were sedated and haemolymph samples were collected as previously described. The freshly collected haemolymph (1 000 μL) was immediately diluted with ice-cold PBS (10 mmol/L PBS, pH = 7.4) in a micro-tube. After thorough mixing, a few drops of the haemocyte suspension were carefully loaded on a haemocytometer to measure the total haemocyte count (THC) by using a light microscope at 40× magnification. The haemocyte enumeration was performed in triplicate. The ratio of the THC of the treated shrimps to that of control shrimps were used as an index to compare the efficacy of different treatments on the haemocyte content. The results were explicated as relative haemocyte count.

#### 2.7.3. Differential haemocyte count

Differential haemocyte counts (DHCs) were performed after haemolymph smears were stained according to May-Grünwald-Giemsa[5]. A minimum of 200 cells were analyzed in each smear. Cells were identified on the basis of morphology, size and granular content as documented in the previous studies[5], by using light microscopy at a magnification of 100×. The relative percentage of the DHC was estimated by analyzing 200 cells of each sample through a light microscope.

$$\text{DHC (\%)} = \frac{\text{Number of specific type of cells counted}}{\text{Total number of cells counted}} \times 100$$

#### 2.7.4. Phagocytic assay

The *in vitro* phagocytic activity of haemocytes was detected by using formalin killed *Vibrio alginolyticus* (*V. alginolyticus*), *V. harveyi* and *V. parahaemolyticus* as target cells[5]. The bacteria were killed by the addition of 10% formalin. A total of 200 μL of whole haemolymph was transferred to a clean micro-tube and thoroughly mixed with 800 μL of anticoagulant. Subsequently, equal volume of respective suspension of bacteria killed by formalin (~$10^8$) was added and tube was placed in a shaker incubator for 10 min. An aliquot of sample suspension (100 μL) was smeared onto a sterile micro-plate and was successively incubated at 20 ℃ in sterile humidified chamber for 30 min. Following this, the micro-plates were washed 4 times with PBS to eliminate non-phagocytised bacterial cells. Shortly thereafter, the plates were air-dried and stained with 10% May Grunwald-Giemsa for 15 min. Finally, the plates were washed thrice with distilled water. A minimum of 200 haemocytes were observed under a light microscope (100× magnifications) and the number of haemocytes that had been phagocytised more than two bacterial cells were recorded and the phagocytic rate was calculated as:

$$\text{Phagocytic rate} = \frac{\text{Number of phagocytic haemocytes}}{\text{Total haemocytes observed}} \times 100$$

#### 2.7.5. Determination of phenoloxidase (PO) activity

The total PO activity was determined by using L-dihydroxyphenylalanine[5]. The initially pooled haemolymph was sedimented by centrifugation at 8 500 r/min at 4 ℃ for 20 min. The pelleted cells (haemocytes) were rinsed, re-suspended gently in chilled 1:10 cacodylate buffer (sodium cacodylate 0.01 mol/L, sodium chloride 0.45 mol/L, tri-sodium citrate 0.10 mol/L, pH 7.0) and re-centrifuged at 10 000 r/min at 4 ℃ for 15 min.

Subsequently, the pellet was re-suspended with 200 μL cacodylate buffer (sodium cacodylate 0.01 mol/L, sodium chloride 0.45 mol/L, calcium chloride 0.01 mol/L, magnesium chloride 0.26 mol/L,

pH 7.0). An aliquot of 100 µL cell suspension (enzyme source) was mixed with 50 µL of trypsin (1 mg/mL) and incubated for 10 min at 25–26 ˚C. Fifty microlitres of L-dihydroxyphenylalanine (substrate) was then added to the enzyme source followed by the addition of 850 µL of cacodylate buffer after 5 min. The optic density at a wave length of 490 nm was measured by using ultraviolet spectrophotometer (PG Instrument, England) (in triplicate). The enzyme activity was calculated from the increase of absorbance during the reading period (IU/min). The enzyme activity unit was equivalent to the increase of 0.001 in absorbance[8]. The average rate of enzyme activity of the stimulated shrimps to that of control shrimps was used as an index for comparing the effects of different medicated shrimps on PO activity[9].

$$\text{Average rate of PO activity} = \frac{\text{PO activity in treated shrimps with medicated feed}}{\text{PO activity in control group}}$$

### 2.8. Statistical analysis

Data were statistically processed for One-way ANOVA to find out any significant differences among the experimental groups. A *t*-test for equality of means was carried out to analyze the data at 95% confidence interval of the difference.

## 3. Results

In the present study, MAPS15 was subjected to an array of assays to investigate its use as a probiotic. The MAPS15 was incorporated into a commercial feed and shrimps were orally administered with MAPS15 diet for a period of 21 days followed by a challenge experiment. The probiotic incorporated feeds were well accepted during the experimental period. Water quality parameters of the test and control tanks were more or less similar. The overall results of the study showed that the survival rate of the probiotic fed shrimps increased as the probiotic inclusion level increased. In addition, the effect of probiotic diet on immunological parameters of the shrimp haemolymph was also assessed. The results showed that immune responses were generally more pronounced with probiotic diet treated groups. The overall results suggested that shrimp health was improved when they were fed with probiotic diet for a period of 21 days.

### 3.1. Bio-safety evaluation of MAPS15 probiont

One of the critical factors for the selection of a probiotic is to confirm that no pathogenic effects can occur in the host. Therefore, the host animal should be challenged with the MAPS15, under the normal or stress conditions. This can be accomplished by injection challenges, by bathing the host in a suspension of the candidate MAPS15, or by adding the MAPS15 to the culture.

The bio-safety of suspected MAPS15 candidate was examined by lack of the clinical sign, lesions and mortalities after *in vivo* challenge experiment. In the present study, *in vivo* toxicity assay revealed that MAPS15 probiont was innocuous to *P. monodon*. No clinical signs or mortalities were recorded following the *in vivo* challenge experiment.

### 3.2. Biomass production of prospective MAPS15 probionts

The mass culture of the MAPS15 probionts was carried out by using optimized culture medium and conditions. The 42-day incubation yielded a maximal quantity of ~312 g wet weight of microbial mat. The resultant wet mat was dried in an oven and powdered to yield 48 g of MAPS15 probiont.

The powdered MAPS15 probiont was stored in air-tight plastic cover for further use.

### 3.3. In vivo efficacy of probiotic diets on shrimps

The efficacy of different preparation of MAPS15 probiont diets on the mortality rate of shrimps challenged with *V. harveyi* and *V. parahaemolyticus* was appended in Table 1. In general, shrimps fed with all doses of MAPS15 probiont diets had a lower mortality rate compared with those received control diet. The time course of cumulative mortalities of *Vibrio* challenged shrimps with or without MAPS15 probiont treatment was depictured in Figures 1–6. Among the MAPS15 probiont treated groups, mortality rate of the shrimps that received probiotic diet at a concentration of $10^8$ CFU/mL (group III) was significantly lower than other groups. The mortality rate of group III shrimps challenged with *V. harveyi* and *V. parahaemolyticus* was 41% and 35%, showing the percent survival indexes of 69% and 73% respectively (Table 2). Mortality of group III shrimps challenged with *V. harveyi* and *V. parahaemolyticus* was observed within 48 h of post-infection. The cumulative percentage of mortality for control shrimps challenged with *V. harveyi* and *V. parahaemolyticus* was 74% and 93%. All the shrimps that died exhibited clinical symptoms such as 'shell necrosis' and 'black spots' on the shell. Similarly, the rate of infection was lower (40%–45%) in the group III shrimps whereas the shrimps in the other groups exhibited 60%–80% infection at the 48th hour of post-infection. However, 100% of the group IV shrimps (+ control) displayed clinical symptoms after 24 h of post-infection. No obvious signs of infection or mortalities were observed in the un-challenged group V shrimps.

**Table 1**

Infection and mortality (%) of treated and control shrimps challenged (ca. $10^5$ CFU/shrimp) with respective *Vibrio* spp. Mean ± SD, $n$ = 10.

| Group | Treatment (CFU/mL) | *V. harveyi* | | *V. parahaemolyticus* | |
|---|---|---|---|---|---|
| | | Infection | Mortality | Infection | Mortality |
| I | $10^2$ | 80.00 ± 3.21 | 77.00 ± 3.34 | 76.00 ± 2.23 | 72.00 ± 2.26 |
| II | $10^4$ | 60.00 ± 4.37 | 57.00 ± 3.28 | 63.00 ± 2.43 | 61.00 ± 3.24 |
| III | $10^8$ | 45.00 ± 2.19 | 41.00 ± 2.27 | 40.00 ± 1.81 | 35.00 ± 2.14 |
| IV | + Control | 100 | 74.00 ± 2.40 | 100 | 93.00 ± 2.45 |
| V | - Control | 0 | 0 | 0 | 0 |

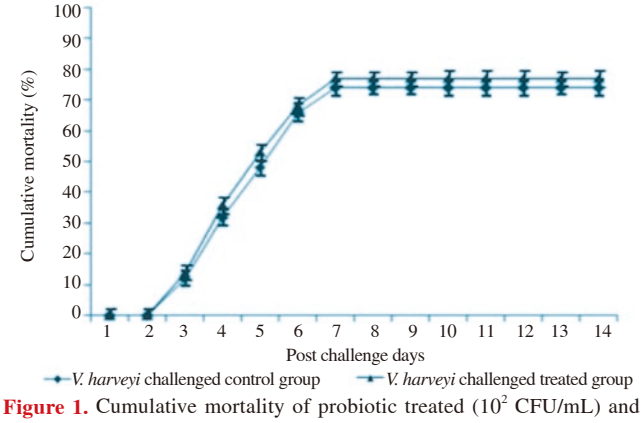


**Figure 1.** Cumulative mortality of probiotic treated ($10^2$ CFU/mL) and non-treated *P. monodon* challenged with *V. harveyi*.

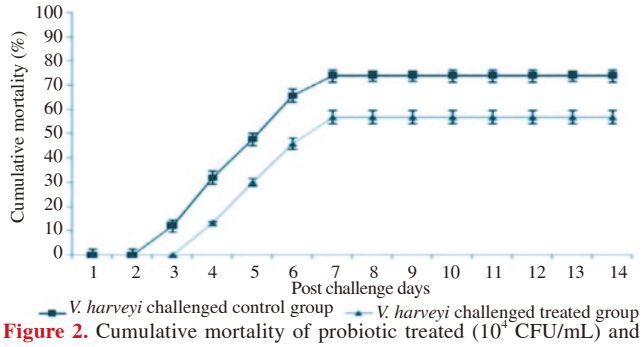


**Figure 2.** Cumulative mortality of probiotic treated ($10^4$ CFU/mL) and non-treated *P. monodon* challenged with *V. harveyi*.

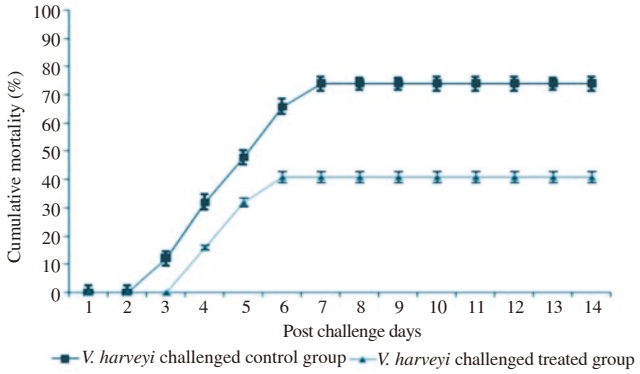


**Figure 3.** Cumulative mortality of probiotic treated ($10^8$ CFU/mL) and non-treated *P. monodon* challenged with *V. harveyi*.

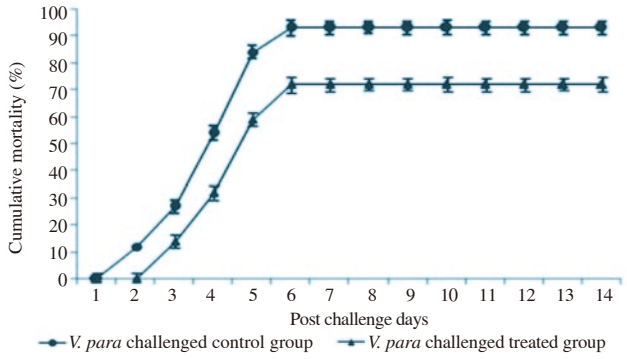


**Figure 4.** Cumulative mortality of probiotic treated ($10^2$ CFU/mL) and non-treated *P. monodon* challenged with *V. parahaemolyticus*.
*V. para*: *V. parahaemolyticus*.

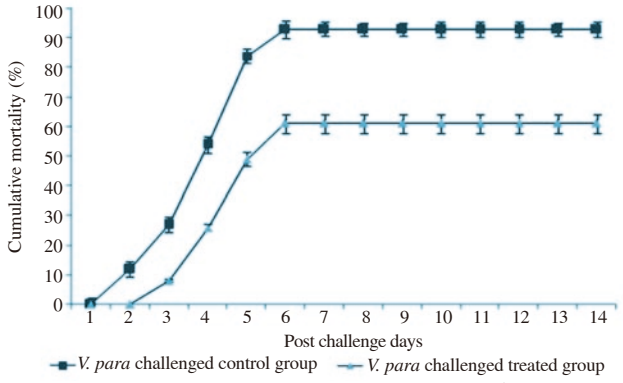


**Figure 5.** Cumulative mortality of probiotic treated ($10^4$ CFU/mL) and non-treated *P. monodon* challenged with *V. parahaemolyticus*.
*V. para*: *V. parahaemolyticus*.

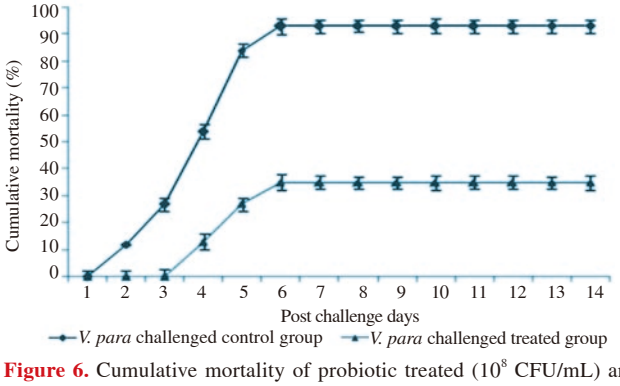


**Figure 6.** Cumulative mortality of probiotic treated ($10^8$ CFU/mL) and non-treated *P. monodon* challenged with *V. parahaemolyticus*.
*V. para*: *V. parahaemolyticus*.

**Table 2**
Percent survival index of probiotic treated shrimps.

| Concentration (CFU/mL) | *V. harveyi* | *V. parahaemolyticus* |
|---|---|---|
| $10^2$ | 18 | 17 |
| $10^4$ | 36 | 31 |
| $10^8$ | 69 | 73 |

### *3.4. Effect of probionts on the gut micro-flora of P. monodon (intestinal tract content)*

The gut microbial vegetation of the shrimp was analysed at the end of experiment. The total viable count (TVC) was similar in the intestinal samples of control shrimps and treated shrimps. The TVC value of (7.2 ± 2.5) × $10^4$ CFU/mL was noticed in control shrimps. The TVC of medicated shrimps was (6.2 ± 0.5) × $10^4$ CFU/mL. Results proved that the probiotic diet was harmless to the gut bacterial vegetation.

### *3.5. THC*

THCs in the haemolymph of control and probiotic fed shrimps were depictured in Figure 7. The THC of entire three groups of shrimps that received probiotic diets was significantly higher than that of shrimps that received saline as well as the control shrimps. Among the treated groups, THC of shrimps that received probiotic diet at a concentration of $10^8$ CFU/mL was slightly higher. The THC observed for those shrimps was 28 × $10^5$ cells/mL.

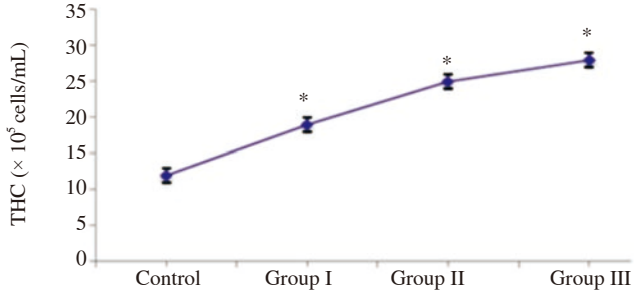


**Figure 7.** THC in the haemolymph of shrimp *P. monodon* treated with probiotic diets and normal feed ($n$ = 10).
*: Values are significantly different at $P < 0.05$.

### *3.6. DHC*

The impact of probiotic diets on the haematological profile of the shrimps was determined as the increase in the haemocyte count after feeding for a period of 21 days. In the present study, there was no marked variation in DHCs among shrimps that received dietary probionts, shrimps that received saline, and control shrimps after a 28 days of treatment (Figure 8).

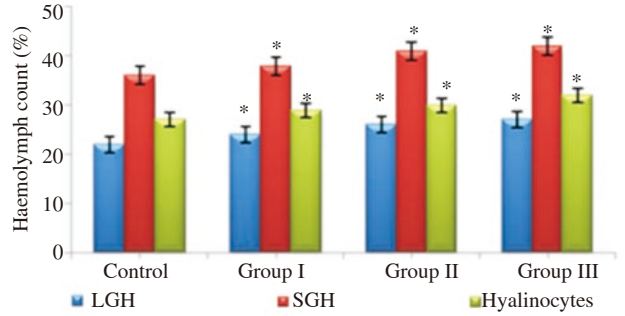


**Figure 8.** DHC in the haemolymph of shrimp *P. monodon* treated with probiotic diets and normal feed ($n$ = 10).
*: Values are significantly different at $P < 0.05$; LGH: Large granular haemocytes; SGH: Semi-granular haemocytes.

### 3.7. Phagocytic rate

In the present study, shrimps were fed with different formulations of probiotic diets for 28 days and the phagocytic rate was determined (Figure 9). Phagocytic activity of shrimps that received any types of probiotic diets was significantly higher than that of shrimps that received saline as well as the control shrimps. The phagocytic rate of Group III treated shrimps was in the range of 49%–51% while the phagocytic rate of control shrimps was 41%–42%. The highest phagocytic rate was exhibited by the shrimps that received probiotic diet at a concentration of $10^8$ CFU/mL. The phagocytic rate of this group was 49%, 52% and 51% respectively against *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus*.

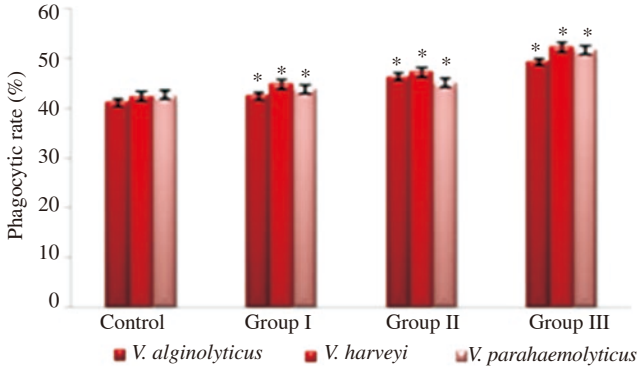


**Figure 9.** Phagocytic activity of haemocyte in treated and control shrimps. *: Values are significantly different at $P < 0.05$.

### 3.8. PO assay

The results of PO activity in the haemolymph supernatant of treated and control shrimps at the end of 21 days of culture were given in Figure 10. It was observed that PO activity of shrimps that received all formulations of the probiotic diets was significantly higher than that of the control shrimps. It was observed that PO activity of control shrimps did not show variations during the experiment. Within the treatment, shrimps that orally administered with probiotic diet at a concentration of $10^8$ CFU/mL showed a significantly more active PO activity than other groups. The PO activity of this group was 0.26.

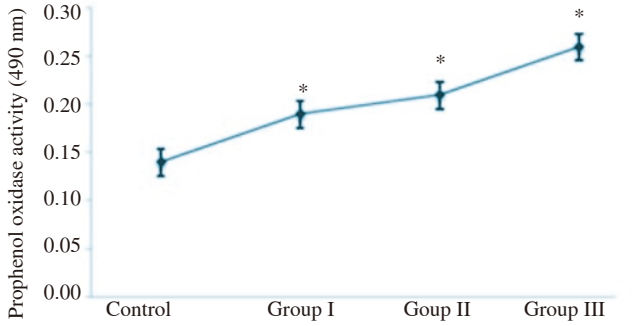


**Figure 10.** PO activity in the haemolymph of *P. monodon* treated with probiotic and normal diet for 21 days.
Data were expressed as mean ± SD. *: Values are significantly different at $P < 0.05$.

## 4. Discussion

### 4.1. Bio-safety evaluation

One of the paramount criteria for the selection of probiotic is knowledge about their pathogenic characteristics in the host. The examination of pathogenicity/susceptibility of probionts to the host has become a standard procedure for selecting a probiotic strain for aquaculture application. The results of the *in vivo* toxicity showed no mortality or clinical signs in the shrimps administered with MAPS15 intramuscularly. That could be highlighted because the sponge associated prospective probionts are preferred to have no pathogenic/hurtful effect on shrimps.

### 4.2. In vivo efficacy

The use of marine sponge associated probionts in aquaculture has a ginormous scope and the application of probiotics in aquaculture for the disease management has a splendiferous future. It is noted that dose, timing and duration of the administration of probiotics may be an important factor affecting its efficacy.

In the present study, shrimps were pre-fed with different formulations of probiotic diets at $10^4$, $10^8$ and $10^{12}$ CFU/mL of body weight/day before and after being challenged with *V. harveyi* and *V. parahaemolyticus*. Overall, probiotic treatment induced a clear reduction in mortality. It was observed that survival performance was prominent in probiotic treated groups when compared with un-treated control groups. This could pertain to the ability of MAPS15 to produce antibiotic compounds to suppress the growth of invading pathogens and thereby to increase the disease resistance potency and survival rate. In comparison to the control animals, the probiotic treated shrimps were less affected by the infection. At the end of the experiment, the lowest cumulative mortality of shrimps was observed with experimental diet enriched with MAPS15 and the highest cumulative mortality was found in control groups that received the normal diet. During the first three days after challenge experiment, a sharp increase in the mortality was observed in all groups. The higher mortality rate in the control group and the lower mortality rate in the treated groups could be attributed to the inclusion of effective dose of probiotics in the shrimp diets and non-inclusion in the control diet. The decreased mortality rate in treated shrimps may be due to exclusion of pathogenic *Vibrio* by the probiont. These findings are in corroboration with those reported by Rengpipat *et al.*[10], that *Bacillus* sp. renders disease protection to shrimps by activating both cellular and humoral immune defences. It was observed that the *Vibrio* count of probiotic treated groups consistently decreased after 14 days of post treatment. Similarly, in the treated groups, there was a marked decrease in the clinical signs after two weeks of probiotic administration which corresponds to the increase in survival rate. Therefore, these results clearly show that oral administration of probiotic bacteria at a dose level of $10^8$ CFU/mL for a period of 21 days is a promising alternative for the prevention of *Vibrio* spp. In addition, the presence of bacteria in the negative control group might be explained by the entrance of microorganisms from the water through the wound caused by the injection. Various types of probiotic strains have been used to increase the survivorship and improve resistance against diseases of shrimps[11]. For instance, Balcázar *et al.*[12], found a reduction in mortality of juveniles of *Litopenaeus vannamei* (*L. vannamei*) infected with *V. parahaemolyticus* after treatment with *B. subtilis* UTM126. Similarly, Das *et al.*[13], tested *Bacillus* sp. as probiotic for *P. monodon* post larvae before the infection by *V. harveyi*, resulting in lower mortality of shrimps and *Vibrio* spp. counts in the water.

### 4.3. Total and differential haemocyte count

Haemato-immunological indices, such as the haemogram, PO activity, and agglutinin activity in serum have been widely exercised to check the health status of shrimps[5]. In the present study, THC was highly variable in treated and un-treated groups. It was observed

that THC was higher in the shrimps treated with probiotic and the lowest in the control group. However, the results of the DHC showed no significant difference between the control and treated groups. The high variability observed in the THC of shrimps may be an indication that the probiotic treatment had a significant effect on the immune system of the treated shrimps which in turn reflected on the PO activity. Increase in the number of circulating haemocytes of probiotic fed shrimps over the control shrimp could be due to the quicker reposition of such cells by the hematopoietic tissue corresponding to the stimulation of the immune system. The high level of haemocytes suggests an improvement of the immunological status of probiotic fed groups. Therefore, MAPS15 can also be used as a potential immuno-stimulant in shrimp diet.

### 4.4. Phagocytic activity

Phagocytic assay was able to elucidate the effect of dietary probiotic on the immunity of shrimps. Results of the present study indicate that oral feeding of probiotic feed could enhance the phagocytic rate of shrimps. On the other hand, the phagocytic rate of control shrimps was lower. It could be attributed to the difference in the number of circulating haemocytes in treated and un-treated shrimps. In un-treated shrimps, the total number of haemocytes was lower whereas the treated shrimps possessed higher percentage of haemocytes which resulted in the higher phagocytic rate. In previous studies, it was reported that the administration of *B. subtilis* E20 to larvae of *L. vannamei* increased phagocytic activity, resulting in higher survival after shrimps were challenged with *V. alginolyticus*[14].

### 4.5. PO activity

Crustaceans possess a unique host defence system differing from vertebrates. Due to the lack of acquired immune response, the host defence against microbial infection in crustaceans solely depends on innate immune systems[14]. All animals reared under field conditions are prone to immunological stress, especially in the early stages of life. PO is a recognized defence enzyme, and serves as a non-self recognition system in the host defence reactions, and is usually an indicator of the immune response in crustaceans[5]. In the present study, PO activity of the haemolymph supernatant of shrimps fed with probiotic diet was significantly different from that of control group. The PO activity was significantly higher for treated shrimps than that for non-treated control group. The decreases in the PO activity of control group were consequence of decrease in the THC. Higher survival of shrimps fed with probiotic diet might pertain to an immune reactive effect of probiotics on the host. There are numerous reports on the effect of probiotics on the immune system of *P. monodon*[15,16]. It was reported that the administration of *B. subtilis* E20 to larvae of *L. vannamei* increased PO, resulting in higher survival after *L. vannamei* were challenged with *V. alginolyticus*[14]. The present study also revealed that in shrimps fed with probiotic incorporated diet for a period of 21 days, the immunological indexes such as THC, DHC, phagocytic rate and PO activity were significantly improved. Therefore, on the basis of immuno-stimulatory properties, this prospective probiont could be used as a veterinary grade probiotic in aquaculture after proper field evaluation studies.

## Conflict of interest statement

We declare that we have no conflict of interest.

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