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.

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 probiotics 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 acclimatized 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 (10⁷, 10⁸ and 10⁹ 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 cm², 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) °C, 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) °C] 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[11]. The commercial shrimp feed (Charoen Pokpood Aquaculture India Pvt. Ltd., Chennai, India) was used for the preparation of dietary probiotic. The probiotics were diluted in normal saline for expected concentrations of 10⁴, 10⁵, and 10⁶ 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 °C. The sterile gelatin water free from bacterial cells added to the commercial diet served as the control. Probiot 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[15]. 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 °C), 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 10⁴ CFU/mL, (ii) group II shrimps fed with probiotic diet at a concentration of 10⁵ CFU/mL, (iii) group III shrimps fed with probiotic diet at a concentration of 10⁶ 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 × 10⁴ CFU/mL) and *Vibrio parahaemolyticus* (*V. parahaemolyticus*) (5 × 10⁵ 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[11]. 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].

\[
\text{Mortality} \% = \frac{\text{Number of dead shrimps} \times 100}{\text{Total number of shrimps} \times A} \times \text{A}
\]

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]:

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 salt sucrose agar. After incubation at 29 °C 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, scalpels (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 °C 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 probiotics 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 aerated brackish water (25 °C) and fed with respective shrimp diets. The feeding trial was continued for a period of 21 days. During the treatment phase, on day of culture-22, shrimps in the treatment were sampled randomly to collect haemolymph. In apparently healthy shrimps at intermoult stage from each 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 °C). 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[9]. At the end of feeding trial, shrimps were sedated and haemolymph samples were collected as previously described. The freshly collected haemolymph (1000 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 haemolymph 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-Grnwald-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.

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. paraalginolyticus 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 (~10⁶) 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 microplate and was successively incubated at 20 °C 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 Grnwald-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 rpm at 4 °C for 20 min. The pelletted cells (haemocytes) were rinsed, re-suspended gently in chilled 1:10 cadoclate buffer (sodium cadoclate 0.01 mol/L, sodium chloride 0.45 mol/L, sodium citrate 0.10 mol/L, pH 7.0) and re-centrifuged at 10 000 rpm at 4 °C for 15 min. Subsequently, the pellet was re-suspended with 200 µL cadoclate buffer (sodium cadoclate 0.01 mol/L, sodium chloride 0.45 mol/L, calcium chloride 0.01 mol/L, magnesium chloride 0.26 mol/L, kalium chloride 2.7 mol/L) and equal volume of cadoclate buffer to the test tube and stored in a refrigerator for further studies.

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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 probions 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.

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 experiment. The probiotic incorporated feeds were well accepted

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 appenced 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 depicted in Figures 1–6. Among the MAPS15 probiont treated groups, mortality rate of the shrimps that received probiotic diet at a concentration of $10^5$ 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 60% 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

| Group Treatment | V. harveyi Infection (CFU/mL) | Mortality | V. parahaemolyticus Infection (CFU/mL) | Mortality |
|----------------|-------------------------------|-----------|----------------------------------------|-----------|
| I $10^4$       | 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 $10^5$     | 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                          | 0 0 0 0                                    |

Figure 1. Cumulative mortality of probiotic treated (10^5 CFU/mL) with respective Vibrio spp. Mean ± SD, n = 10.
3.4. Effect of probiotics 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 $(7.2 \pm 2.5) \times 10^2$ CFU/mL was noticed in control shrimps. The TVC of medicated shrimps was $(6.2 \pm 0.5) \times 10^2$ 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 depicted 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 $10^3$ CFU/mL was slightly higher. The THC observed for those shrimps was $28 \times 10^3$ cells/mL.

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 probiotics, shrimps that received saline, and control shrimps after a 28 days of treatment (Figure 8).

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

![Graph](image_url)  
**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.

| Table 2 | Percent survival index of probiotic treated shrimps. |
|---------|-----------------------------------------------------|
| Concentration (CFU/mL) | *V. harveyi* | *V. parahaemolyticus* |
| $10^2$ | 18 | 17 |
| $10^3$ | 36 | 31 |
| $10^4$ | 69 | 73 |

![Graph](image_url)  
**Figure 3.** Cumulative mortality of probiotic treated (10$^2$ CFU/mL) and non-treated *P. monodon* challenged with *V. harveyi*.  

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

![Graph](image_url)  
**Figure 5.** Cumulative mortality of probiotic treated (10$^3$ CFU/mL) and non-treated *P. monodon* challenged with *V. parahaemolyticus*.  
*para*: *V. parahaemolyticus*.

![Graph](image_url)  
**Figure 6.** Cumulative mortality of probiotic treated (10$^3$ CFU/mL) and non-treated *P. monodon* challenged with *V. parahaemolyticus*.  
*para*: *V. parahaemolyticus*.  

![Graph](image_url)  
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\* 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 probiotics in aquaculture has a ginormous scope and the application of probiotics in aquaculture for the disease management has a splendidous 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 10^7, 10^8 and 10^9 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 animals 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 immunity-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 untreated 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 early stages of life. PO is a recognized defence enzyme, and serves as a potential immuno-stimulant in shrimp diet.

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Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Manilal A, Selvin J, George S. In vivo therapeutic potentiality of red seaweed, Asparagopsis (Bonnemaisonales, Rhodophyta) in the treatment of vibriosis in Penaeus monodon Fabrictius. Saudi J Biol Sci 2012; 19(2): 165-75.
[2] Sugathan S, Manilal A, Geznu T, Merdekios B, Selvin J, Tsalla T, et al. Evaluating the antibacterial potential of Streptomyces sp. Transl Biomed 2015; 6(1): 1-7.
[3] Pham D, Ansquer D, Chevalier A, Dauga C, Peyramale A, Wabete N, et al. Selection and characterization of potential probiotic bacteria for Litopenaeus stylirostris shrimp hatcheries in New Caledonia. Aquaculture 2014; 432: 475-82.
[4] Puririvirojuk W. Application of probiotic bacteria for controlling pathogenic bacteria in fairy shrimp Branchinella thailandensis culture. Turk J Fish Aquat Sci 2013; 13(1): 187-96.
[5] Manilal A, Selvin J, Sugathan S. Immuno-modulatory efficacy of Indian red algae Asparagopsis taxiformis, in Penaeus monodon. J Appl Aquac 2013; 25(1): 81-93.
[6] Sujith S. Development of novel probiotics from marine bacteria for proactive shrimp health management [dissertation]. Tiruchirapalli: Bharathidasan University; 2012.
[7] Manilal A, Selvin J, Sugathan S, Panikkar MVN. Evaluation of therapeutic efficacy of Indian green alga, Acrosiphonia orientalis (J. Agardh) in the treatment of vibriosis in Penaeus monodon. Thalassas 2012; 28(1): 33-46.
[8] Söderhäll K, Hall L. Lipopolysaccharide-induced activation of prophenoloxidase activating system in crayfish haemocyte lysate. Biochim Biophys Acta 1984; 797(1): 99-104.
[9] Sung HH, Yang YL, Song YL. Enhancement of microbialicidal activity in the tiger shrimp Penaeus monodon via immunostimulation. J Crustacean Biol 1996; 16(2): 278-84.
[10] Rengpipat S, Rukpratanporn S, Piyatiratitivorakul S, Menasaveta P. Immunity enhancement in black tiger shrimp (Penaeus monodon) by a probiotic bacterium (Bacillus S11). Aquaculture 2000; 191(4): 271-88.
[11] Ninawe A5, Selvin J. Probiotics in shrimp aquaculture: avenues and challenges. Crit Rev Microbiol 2009; 35(1): 43-66.
[12] Balczúzar JL, Rojas-Luna T, Cunningham DP. Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (Litopenaeus vannamei) following immersion challenge with Vibrio paraaeremotylus. J Invertebr Pathol 2007; 96(2): 147-50.
[13] Das S, Ward LR, Burke C. Screening of marine Streptomyces spp. for potential use as probiotics in aquaculture. Aquaculture 2010; 305(1-4): 32-41.
[14] Liu KF, Chiu CH, Shiu YL, Cheng W, Liu CH. Effects of the probiotic, Bacillus subtilis E20, on the survival, development, stress tolerance and immune status of white shrimp, Litopenaeus vannamei larvae. Fish Shellfish Immunol 2010; 28(5-6): 837-44.
[15] Pattukumar V, Kannani P, Kumar RS, Yuvraj N, Paari A, Arul V. Enhancement of innate immune system, survival and yield in Penaeus monodon reared in ponds using Streptococcus phocae PI80. Aquac Nutr 2014; 20(5): 505-13.
[16] Karthik R, Jaffar Hussain A, Muthezhilani R. Effectiveness of Lactobacillus sp (AMET1506) as probiotic against vibriosis in Penaeus monodon and Litopenaeus vannamei shrimp aquaculture. Biosci Biotechnol Res Asia 2014; 11(1): 297-305.