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Feasibility of Shrimp Gut Probiotics with Anti-vibrio and Anti-QS in Penaeid Culture

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ABSTRACT

A total of 12 gut isolates were isolated from healthy adult shrimp *Penaeus monodon*. Consecutive screening approaches were done to evaluate the anti-vibrio and anti-quorum sensing potential towards *Vibrio harveyi* VSH5 and *Chromobacterium violaceum* MTCC2656. Among the 12 isolates, the probiotics *Bacillus* sp. AVP03 & AVP07 rendered maximum activities in both *in-vitro* and *in-vivo* tests. The study also suggests that the isolated anti-vibrio probiotics possessed anti-QS (quorum quenching) activity which significantly reduced the mortality when compared to the animals treated with the pathogen in post larvae and juvenile challenge study. Both probiotics were reached their stationary phase within 6 hrs in tryptic soy broth (TSB) and hence it was considered as best medium for their mass culturing. Studies regarding haemolytic activity of the isolated probiotics revealed that non-toxic in nature and safety for use. Thus they can be effectively used as probiotics in aquaculture either separately or in combination.

Keywords: Probiotics, Anti-vibrio, Anti-QS, *Penaeus monodon*

1. Introduction

The emergence of antibiotic-resistant pathogens with the threat of gene transfer to human pathogens and environmental issues led to the development of an alternative biological tool to manage diseases in aquaculture. As a control measure to Vibriosis, a bacterial disease caused by *Vibrio* pathogens, probiotic treatment has been preferentially conducted in aquaculture as the use of probiotics does not lead to serious damage to either marine products or the environment when compared to other treatments such as antibiotics [1]. For the last few years, probiotics have been successfully used to control disease and improve nutrition in aquaculture following the development of disease resistant strains. Probiotics in contrast to antibiotics can be a safer ecological alternative tool for sustainable aquaculture. Earlier the probiotics were defined as organisms delivered through feed which improved the health and survival of the hosts [2]. However, the definition was expanded to include the organisms also that created a favorable environment for the growth and well-being of the host animals by their transience through the gastrointestinal tract or merely by their presence in the water [3, 4]. In this context, manipulation of microbial balance in the larval rearing tanks by introducing non-pathogenic probiotics isolated from the native environment became a promising technology [5, 6, 7]. Many studies have reported the effects of probiotic treatments in aquaculture [8, 9, 10] and available probiotics have generally been used successfully on shrimp farms to reduce mortality and improve crop yields [11, 10].

Disruption of quorum sensing was suggested as a new anti-infective strategy to control pathogenic bacteria without interfering with their growth [12, 13] and it may be a particularly useful method in aquaculture [14]. Interest in the application of probiotics in aquaculture is fairly recent and the application of quorum quenching to select the new probiont gives an alternative to antibiotic to control bacterial infections in aquaculture [6]. *Bacillus* spp. has been extensively used as probiotic bacteria in shrimp aquaculture from last few decades [4, 15]. However, most of these *Bacillus* spp. strains have not been exploited for disease control because they usually do not produce antibiotics that are effective against bacteria. The main purpose of this study was to isolate, screen and characterize the intestinal bacteria (gut probiotics) of healthy tiger shrimp (*Penaeus monodon*) for potential use as probiotics with anti-vibrio and anti-QS.

2. Materials and methods

2.1 Isolation of anti-vibrio probiotics from shrimp gut

Four live healthy shrimps (*P. monodon*) were collected from a commercial shrimp hatchery located in Tuticorin, South India. The gut portion of the shrimp was dissected out using dissection kit and homogenized under aseptic condition in sterile saline (0.85% NaCl) and then serially diluted upto 10^{-5} . From the serially diluted sample 0.1 ml of aliquote was spreaded over on Zobell marine agar and incubated at 30°C for 24 hrs. The shrimp pathogenic bacteria, *V. harveyi* VSH5 from the stock maintained in Microbial Biotechnology Laboratory of Manonmaniam Sundaranar University, Tirunelveli was used as the target pathogen throughout the study. Around twelve colonies (probiotic isolates) on marine agar were randomly selected based on their colony morphology and labeled as AVP01 to AVP12. Each probiotic was streaked centrally on TSA plates containing 1.5% NaCl as single line and incubated at 30°C until heavy growth occurred. *V. harveyi* VSH5 was streaked perpendicularly to the centre streaking without touching but very close to the. The plates were again incubated at 30°C for 24 hrs to check the absence of *V. harveyi* VSH5 growth nearer to the probiotics (anti-vibrio activity). Cell-free supernatant (CFS) of each probiotics were separated by centrifugation and checked for anti-vibrio activity by agar well diffusion.

2.2 Screening of anti-QS (quorum quenching) activity

Chromobacterium violaceum quorum sensing system was used as bio-monitor for this study. Quorum sensing in this wild type bacterium is correspondingly known to control the pigment production (violacein, a purple pigment) due to the production and in response to autoinducer such as C6-acyl homoserine lactones and C4-acyl homoserine lactones. The bio-monitor *C. violaceum* MTCC 2656 was obtained from MTCC, Chandigarh, India and maintained in appropriate agar slant at 4°C throughout the study. Anti-QS activities of the above said twelve isolates were screened by the method described by parallel arrow streaking. Probiotics were streaked as aero line onto TSA (with 1.5% NaCl) plates (one isolate per plate) and incubated overnight at room temperature. *C. violaceum* MTCC 2656 was streaked 0.5 cm away from each isolate and incubated overnight at room temperature. If an isolate produced a quorum quenching enzyme or other molecule that interfered with this quorum sensing system, *C. violaceum* MTCC 2656 colonies exhibited the natural white color. Plates which indicated inhibition of *C. violaceum* MTCC 2656 pigments were photographed and that isolates were considered as anti-QS probiotics. Anti-QS of CFS of selected probiotics were also done by agar well diffusion.

2.3 Identification of potent probiotics

The biochemical characterization of the test cultures were carried out for genus identification according to Bergey's Manual of Systematic Bacteriology. Further identification of the potent probiotics was achieved by 16S rRNA sequencing and NCBI-BLAST search. The DNA was isolated by HiPurA bacterial DNA isolation and purification kit (Hi-media, Mumbai) and used as template. PCR amplification of the 16S rRNA was done by using master mix kit, MEDOX-MIX (Medox, India) as per user manual. The primers and PCR conditions were adapted from previous report [16]. The PCR product was subsequently sequenced and

the sequence information of the isolate was compared with the existing sequences available in the GenBank of NCBI using BLAST search. The sequence was aligned using ClustalW program within BioEdit version 7.0.5.3 and phylogenetic tree was constructed by Neighbor-Joining method.

2.4 Haemolytic activity by plate assay

Hemolytic activities of the probiotics were detected by streaking (continuous streak) the isolates on TSA plates supplemented with 1% defibrinated sheep blood. The plates were covered with parafilm and incubated at 30°C for 48 hrs to check the haemolytic patterns.

2.5 Vibrio challenge study with post larvae (PL)

The experimental setup for post larvae rearing comprised ten litres cylindrical fiber glass tanks containing five litre of filtered seawater at 20 ppt salinity. Shrimp (*P. monodon*) post larvae (PL 20) were introduced into five sets of tanks at a stocking rate of 10 animals per litre. Three replicates were used for a total of 150 PL per treatment. Each experimental unit was separated to avoid cross contamination. The larvae were acclimatized for 24 hrs and fed with freshly hatched *Artemia* nauplii. Continuous aeration was provided by portable air pump. Treatment tanks were inoculated with harvested cells of probiotics at a rate of greater than 10^6 CFU/ml in rearing medium to facilitate attachment / colonization on the larvae for 24 hrs. After one day, they were then exposed to test pathogen (*V. harveyi* VSH5) at 10^5 CFU/ml for 1 hr. Survival in each treatment was recorded for four days at 24 hrs intervals and no water exchange was done during that period. Total plate count (TPC) and presumptive vibrio count (PVC) were assessed on Zobell marine agar and TCBS agar at 24hrs interval by the serial dilution method.

2.6 Vibrio challenge study with juveniles

The experimental tanks for juveniles rearing encompass ten litre plastic tank containing six litre of filtered sea water (30 ppt). About eight healthy tiger shrimp juveniles with each an average length: 6.5 ± 0.5 cm and average weight 1.5 ± 0.3 g were maintained in five sets of tanks. The experimental setup included treatments in duplicate and kept in such a way to avoid cross contamination. The shrimps were individually netted out and about 2 mm of a small wound (hereafter wounded shrimp-WS) was made through the cuticle and into the muscle of the third abdominal segment by pushing a scalpel until it penetrated [17]. The pathogen (*V. harveyi* VSH5) and probiotics (AVP03 and AVP07, each separately) were grown in 150ml marine broth and 200ml of TSB with 1.5% NaCl respectively. The bacterial cells were harvested by centrifugation at 6000 rpm and added to the experimental tank water as PL treatment. About 20% of water in all the treatments was replaced with filtered dechlorinated seawater containing equal concentration of bacterial cells as in the initial stage. The animals were fed with *ad libitum* commercial feed and aerated continuously. Total plate count (TPC) and presumptive vibrio count (PVC) in each experimental set up was estimated. The survival percentage and the bacterial count were examined for four days. Infected animals were confirmed by its luminescence under dark.

2.7 Selection of medium for probiotics by growth curve analysis: Growth curves were determined using three different

medium for the isolated probiotics. The Probiotic bacterium was precultured in five ml nutrient broth (with 1.5% NaCl) at 37°C in an Orbital shaker for 18 hrs. About 100 µl of this inoculum was inoculated into 250 ml of different broths such as Nutrient broth (NB), Zobell marine broth (ZMB) and Tryptic soy broth (TSB) and incubated at 37°C in an Orbital shaker at 100 rpm. Bacterial growth was monitored at 0, 2, 4, 6, 8, 16, 20, and 24 hrs by taking OD at 600 nm [18]. Respective uninoculated broth was considered as the blank. Growth curve was plotted as OD 600 readings against time and different phases of growth were hence analyzed.

2.8 Effect of probiotics CFS on *Vibrio* grown in liquid culture mode

About 10 ml aliquots of probiotics CFS prepared from 24 hrs culture (TSB broth) were transferred to separate conical flasks containing 50ml of marine broth and inoculated *V. harveyi* VSH5. The cultures were incubated at 30°C on an orbital shaker at 100 rpm. Samples were taken for every two hrs for measuring the OD at 600 nm. An OD value of more than one was diluted tenfold and assays were performed in duplicates. *Vibrio* without CFS acted as the control.

3. Results & discussion

Around 12 bacteria were obtained from the gut homogenates based on colony morphology on Zobell marine agar and labelled as AVP01 to AVP12 (AVP abbreviate Anti *Vibrio* Probiotic). Among the 12 isolates, four (AVP03, AVP04, AVP06 and AVP11) had strong antagonistic activity (Plate: 1) and they were partially identified as the genus *Bacillus* sp. based on the growth characteristics and microscopic observation. Many studies have shown that compounds produced by bacteria from various sources could be used to inhibit bacterial pathogens in aquaculture [19, 20]. The use of such compounds rather than antibiotics is now prompt acceptance in shrimp farming to control pathogens as it can be a better and more cost effective alternative [4, 19]. In the present study the *Bacillus* spp. (AVP03, AVP04, AVP07 and AVP11) isolated from the shrimp gut inhibited the growth of *V. harveyi* VSH5. Most of the studies have suggested that the inhibitory effects of *Bacillus* sp. might be due to alteration of pH in the medium, utilization of essential nutrients and production of volatile compounds [21, 22, 23]. In addition, several studies have also reported that the *Bacillus* spp. produces polypeptide antibiotics such as bacitracin, gramicidin S, polymyxin and

tyrotricidin [24, 25].

Table 1 indicates that 70 µl of the CFS from anti-vibrio probiotics inhibited the growth of *V. harveyi* VSH5 with clear zones in a size range of 13.43 ± 0.69 to 19.02 ± 0.13 mm. Based on the anti-vibrio efficacy, two potent probiotics were identified as AVP07 and AVP03 and AVP11 whereas AVP04 as less effective against the tested pathogen. The reference strain, BS6710 also rendered a highest inhibition rather than AVP04 and AVP11. Among the three antibiotics tested, tetracycline rendered a maximum inhibitory zone of 24.26 ± 0.80 mm followed by Oxolinic acid and norfloxacin. The results of quorum quenching (anti-QS) screening indicated that the two isolates namely AVP03 and AVP07 were observed to be possible quorum quenchers which did not cause blue pigmentation in *C. violaceum* MTCC 2656 (Plate: 2). The results of well diffusion quorum quenching demonstrate that the higher concentrations such as 40 and 50 µl of both CFS had considerable activity (Table: 2). To confirm the quorum quenching, the zone of colourless growth around the well was swabbed using sterile cotton buds and streaked onto fresh agar surface to differentiate growth inhibition from QS inhibition [26].

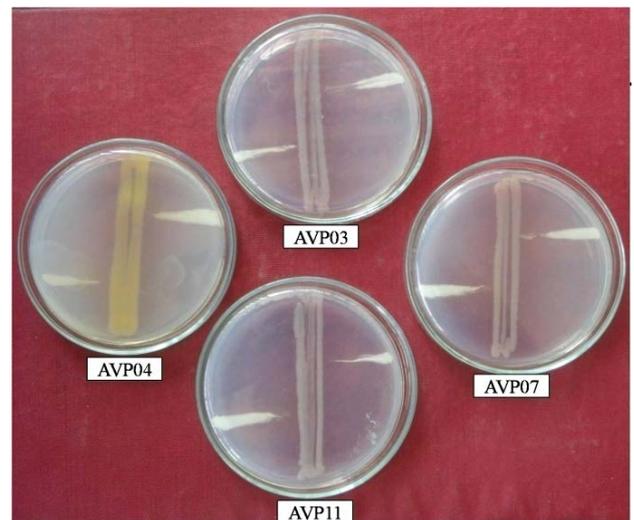


Plate 1: Positive anti-vibrio activity of four shrimp gut isolates by cross streak method

Table 1: Inhibitory effect of anti-vibrio probiotics against *V. harveyi* VSH5

Zone of inhibition (mm) against VSH5							
AVP03	AVP04	AVP07	AVP11	BS6710	Nx	T	Oa
16.2 ± 0.6	13.4 ± 0.6	19.0 ± 0.1	14.3 ± 0.8	16.9 ± 0.6	22.3 ± 0.7	24.1 ± 0.7	19.9 ± 0.9

Results are expressed as mean \pm standard deviation from six independent replicates (n=6), Nx = norfloxacin (10 µg), T = tetracycline (30 µg), Oa = oxolinic acid (2 µg), AVP03, AVP04, AVP07 & AVP11 = anti-vibrio probiotics (70 µl), BS6710 = reference strain of *B. subtilis* (70 µl).

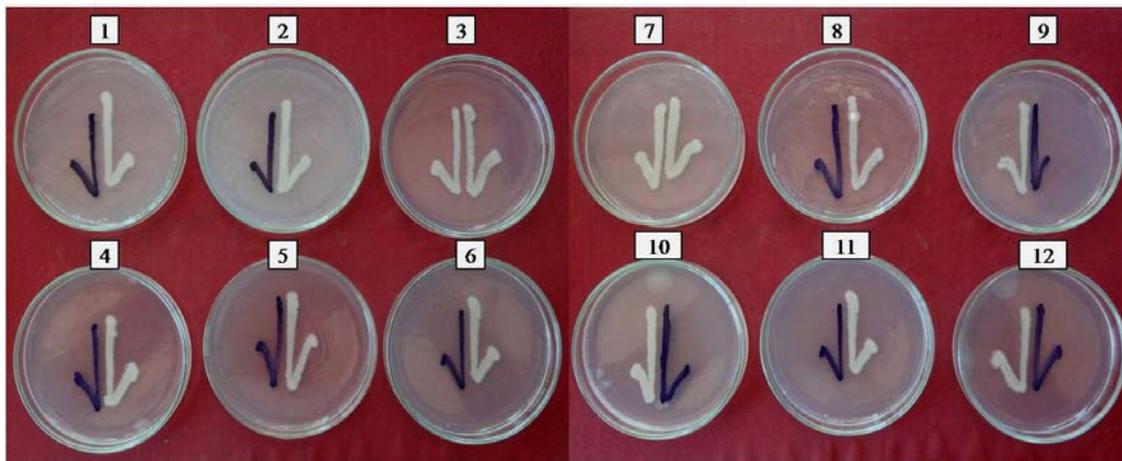
Plate 2: Anti-QS activity of shrimp gut isolates by parallel arrow streak method

Plate 1 to 12 indicates the isolates AVP01 to AVP12. The isolates AVP03 and AVP07 shows positive quorum quenching activities.

Table 2: Anti-QS activity of different concentrations of CFS by well diffusion method

Probiotics	Anti-QS activity of CFS (in mm)			
	20 μ l	30 μ l	40 μ l	50 μ l
AVP03	-	-	12.4 \pm 0.1	13.2 \pm 0.1
AVP07	-	-	10.1 \pm 0.1	12.7 \pm 0.1

Results are expressed as mean \pm standard deviation from three replicates (n=3), - : no considerable activity

To date, research has shown anti-QS activity in only a few higher plants and seaweed [27, 28, 29]. Marine microbes are known to be the most potential natural resource of important compounds [30, 31, 32]. However, very little attention has been paid in their anti-QS properties. To this context, the present study confers the evidence of two such quorum quenching bacteria from marine source (shrimp gut). The ability to interfere with AHL quorum sensing by degrading the signal molecules seems to be widely distributed in the bacterial kingdom [33]. *Bacillus* species were amongst the first bacteria reported to degrade AHLs by producing lactonase enzymes which inactivate AHLs by opening the lactone ring [34, 35]. The isolates (AVP03 and AVP07) screened out in our study also belonged to *Bacillus* sp. In aquaculture, the use of *Bacillus* species as probiotics is expanding rapidly and a number of commercial biocontrol products are composed of mixtures of *Bacillus* spores [36]. Hence the anti-vibro with quorum quenching nature of our isolates might remain an interesting novel biocontrol agent for use in aquaculture to protect shrimp health.

In anti-vibro assay, maximum activity was found in AVP07 followed by AVP03 whereas in anti-QS activity the results obtained reversibly. Hence both the bacteria were subjected to biochemical and molecular identification and used for further study. The results of biochemical tests for AVP03 and AVP07 indicated that both the isolates were Gram-positive, motile, spore forming, rod shaped aerobic bacteria. They were positive for oxidase, catalase, and citrate utilization and had the ability to hydrolyse the starch, casein and gelatin by producing extracellular enzymes. Both of them produced acid end product by fermenting glucose, arabinose, glycogen and

xylose. Acetoin production, nitrate reduction, mannose fermentation and growth at 40°C were positive for AVP03 but negative for AVP07. At the same time AVP07 revealed positive in fermenting mannitol whereas it was negative for AVP03. Based on biochemical properties both the isolates remained close resemblance to *Bacillus* sp. even if, they illustrate variations in few tests.

The genomic DNA of AVP03 and AVP07 were isolated and checked on agarose gel and was found to be of high molecular weight and intact. The DNA was subjected to 16S rRNA gene amplification for identification. PCR product of the length 1494 bp (AVP03) and 1338 bp (AVP07) were purified and commercially sequenced in Acme Progen Biotech (India) Pvt. Ltd. The 16S rRNA sequence of the probiotics were subjected to BLAST using mega blast tool of GenBank and confirmed the identity of the bacterial isolates as *Bacillus* sp. AVP03 and AVP07. A phylogenetic tree was constructed by aligning 16S rRNA partial gene sequence of 12 closest neighbouring strains of different *Bacillus* species taken from GenBank, NCBI and sequence of the probiotic isolates of this study (Fig: 1). The phylogenetic tree was developed based on Neighbor-Joining method and the percentage difference in the genetic relationship between the allied strains of *Bacillus*. It showed the isolate AVP03 was related to *Bacillus* sp. and shared 66% similarity with *B. cereus* Probio-32 whereas AVP07 shared 70% with other *Bacillus* species. The 16S rRNA gene sequences of these strains have been deposited in the GenBank under the A.No. KC261415 (*Bacillus* sp. AVP03) and KC261416 (*Bacillus* sp. AVP07).

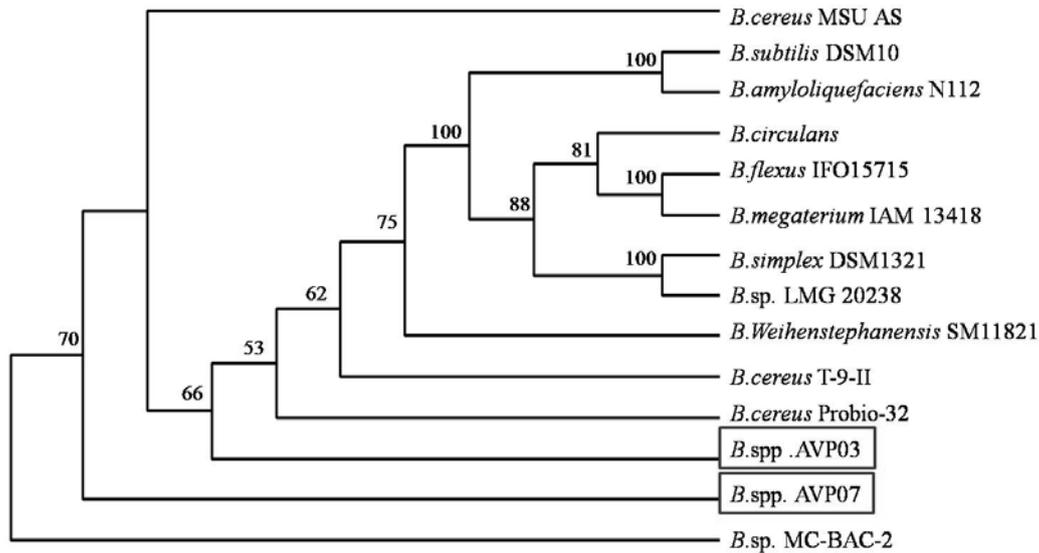


Fig 1: Phylogenetic tree based on the Neighbor-Joining method using partial 16S rRNA sequences of AVP03 and AVP07. The inferred relationship based on partial 16S rRNA sequences of *Bacillus* sp. AVP03 and *Bacillus* sp. AVP07 (within the box) to other bacteria. The tree was rooted with *Bacillus* sp. MC-BAC-2 as an out group. Bootstrap values are expressed as percentages of 1000 replications. Only bootstrap values above 50% are illustrated at nodes.

Haemolytic activity of the isolated probionts, *Bacillus* sp. AVP03 and AVP07 were examined against sheep blood by plate assay and the result showed no occurrences of such haemolysis in both the organisms. The same experiment was carried out in triplicates to conclude the results. The results do clearly report that the probionts are non-haemolytic, avirulent in nature and do not show any adverse effect on animals and are very safe to use. *Bacillus* strains are most commonly used as probiotics against bacterial diseases in shrimp aquaculture because they render many antibacterial substances [37]. There is less evidence that *Bacillus* strains exert harmful effects on shrimp or the environment [38]. However, though the *B. subtilis* is known as a probiotic they sometimes produce haemolysin [39]. The result of haemolytic activity of our study includes *Bacillus* sp. AVP03 and AVP07 as non-haemolysin producers and hence could effectively be used to control bacterial diseases in aquaculture.

The percentage survival of *P. monodon* post larvae reared in the experimental tanks was recorded up to four days (Fig. 2). However, less survival (28%) was observed in the negative control (PL treated with *Vibrio*) at fourth day whereas 100% survival was observed in the positive control (PL only) at the end of the first day followed by 98.6% in the combined treatment of probiotics (PL treated with vibrio, *Bacillus* sp. AVP03 and *Bacillus* sp. AVP07). Low survival in negative control is due to the virulent effects of *V. harveyi* VSH5. At the end of the fourth day of the commencement of the experiment larval survival significantly differed between the control and the treated groups (Fig. 2). About 72% survival was achieved when the larvae was treated with *Bacillus* sp. AVP03 and AVP07 individually against *Vibrio* but the

combined treatment recorded 82% survival at the end of the experiment. The larvae treated with probionts either individually or in combination reduced the mortality from first day onwards than the control. Thus, anti-vibrio activity of probiotics effectively controlled the pathogen under *in vivo* and yielded positive result in terms of PL survival. The *in vivo* result of this study is similar to the findings of Gram *et al.*, who observed significant reduction in mortality to 25% after long term and short term probiotic (*P. fluorescens* AH2) treatment [40]. Similarly many other available reports also states the good potential probiotic activity of *Bacillus* sp. against *V. harveyi* [41, 42, 43, 44, 37].

TPC and PVC were enumerated from PL tank water during four day study and tabulated (Table: 4). In the positive control (T₁) both the bacterial counts (*Vibrio* and *Bacillus* sp.) were increased day by day, but in the experimental tanks (T₃, T₄ and T₅), the *Vibrio* count was found to be decreased while the *Bacillus* count increased. However, continuous decrease in the count of TPC and increase in PVC was observed in the negative control (T₂). This result may be due to the *in vivo* antagonistic activity between the probionts and *V. harveyi* VSH5 [45]. The TPC count at the end of day 1 in combined treatment (T₅) was found to be 9.0×10^7 CFU/ml. This increased 7.1×10^8 in day 2, 7.4×10^8 in day 3 and 1.2×10^9 CFU/ml in day 4. But *Vibrio* counts decreased drastically at different intervals. Fluctuation in TPC was observed in T₃ (AVP03 treated) at day 3, it recorded 4.3×10^8 CFU/ml but it was 6.5×10^8 and 7.3×10^8 CFU/ml in day 2 and day 4 respectively.

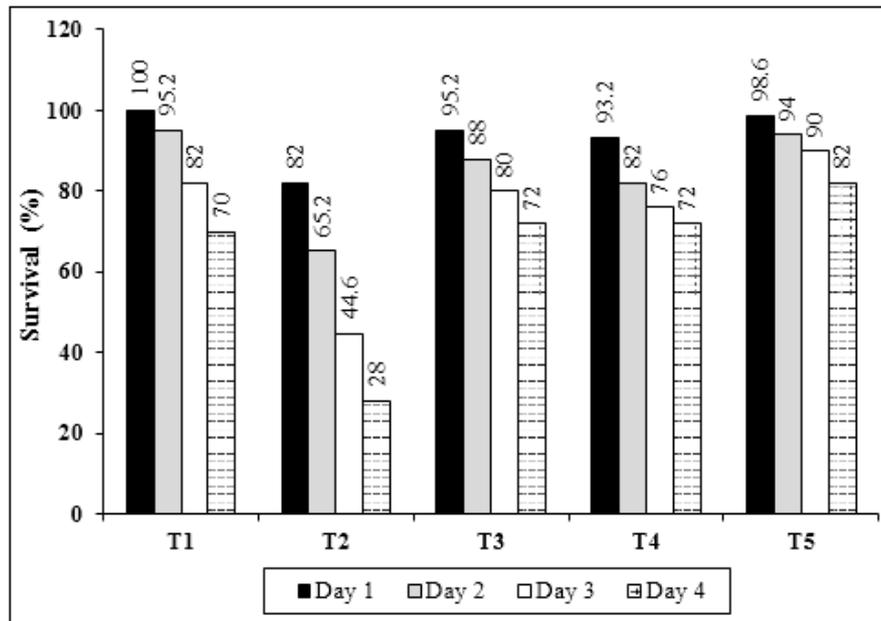


Fig 2: Percentage survival of PL on control and probiotics treated

T₁ = PL alone (+ve control), T₂ = PL+Vh (-ve control) T₃ = PL+AVP03+Vh, T₄ = PL+AVP07+Vh, T₅ = PL+AVP03+AVP07+Vh

Table 3: Enumeration of TPC and PVC from experimental tank of PL larvae

Treatment	Day 1		Day 2		Day 3		Day 4	
	TPC	PVC	TPC	PVC	TPC	PVC	TPC	PVC
T ₁	1.2×10 ⁵	1.0×10 ¹	1.4×10 ⁵	1.1×10 ⁴	4.3×10 ⁵	6.3×10 ⁴	8.2×10 ⁵	1.2×10 ⁵
T ₂	5.5×10 ⁸	2.2×10 ⁷	3.3×10 ⁸	5.5×10 ⁷	1.2×10 ⁸	2.8×10 ⁸	2.1×10 ⁷	3.6×10 ⁸
T ₃	1.2×10 ⁸	5.8×10 ⁵	6.5×10 ⁸	5.7×10 ⁵	4.3×10 ⁸	3.5×10 ⁵	7.3×10 ⁸	3.1×10 ⁵
T ₄	1.3×10 ⁸	1.0×10 ⁶	5.8×10 ⁸	5.1×10 ⁵	6.3×10 ⁸	3.6×10 ⁵	6.4×10 ⁸	3.4×10 ⁵
T ₅	9.0×10 ⁷	1.1×10 ⁶	7.1×10 ⁸	3.5×10 ⁵	7.4×10 ⁸	5.4×10 ⁴	1.2×10 ⁹	5.0×10 ⁴

T₁ = PL alone (+ve control), T₂ = PL+Vh (-ve control) T₃ = PL+AVP03+Vh, T₄ = PL+AVP07+Vh, T₅ = PL+AVP03+AVP07+Vh

The result of survival percentage of juvenile treatment is shown in Fig: 3. Less survival was observed in the negative control (WS treated with *Vibrio*) from 2nd day onwards whereas 100% survival was observed in positive control (WS only) in all days. Though the negative control recorded 100% survival in initial, the animals rendered heavy luminescence under dark which indirectly indicates the rapid bio-film forming and infective nature of *V. harveyi* VSH5. The animals in positive controls and probiotics treated groups did not show any luminescence even at the end of the experiment. Hence this study concludes that the probiotics either in individual or in combination of treatment prevented the occurrence of vibriosis in juveniles also. This finding coincides with Moriarty, who found that *Bacillus* sp. eliminate luminescent bacterial disease in shrimp culture condition [42]. About 75% and 66.6% survival were achieved in the larvae treated with *Bacillus* sp. AVP03 and AVP07 respectively but the combined treatment showed 87.5% survival at the end of the experiment. The animals could survive even with the wounds in the abdominal segment which normally make the shrimps susceptible to attack by opportunistic pathogens. The survival

of the shrimps in the experiments could be due to the prevention of colonization ability of *V. harveyi* VSH5 in both the gut and wound surfaces [17].

TPC and PVC of juveniles were enumerated for every day (Table: 4) using marine agar and TCBS agar. Similar to PL study, bacterial (*Bacillus* and *Vibrio*) count increased continuously in positive control while in probiotics treated groups (T₃, T₄ and T₅) *Bacillus* load only increased but *Vibrio* count decreased constantly during the experimental days. Negative control showed decreased plate count and increased *Vibrio* count. The TPC count at the end of day 1 in combined treatment (T₅) was found to be 1.0×10⁸ CFU/ml. This was found to be increased to 8.1×10⁸ in day 2, 9.3×10⁸ in day 3 and 1.7×10⁹ CFU/ml in day 4. But considerable *Vibrio* count was observed in different intervals for the same treatment. Deviation in TPC was found in T₄ (AVP03 treated) at third day as 6.9×10⁸, but at second and fourth day it showed 8.6×10⁸ and 9.4×10⁸ respectively.

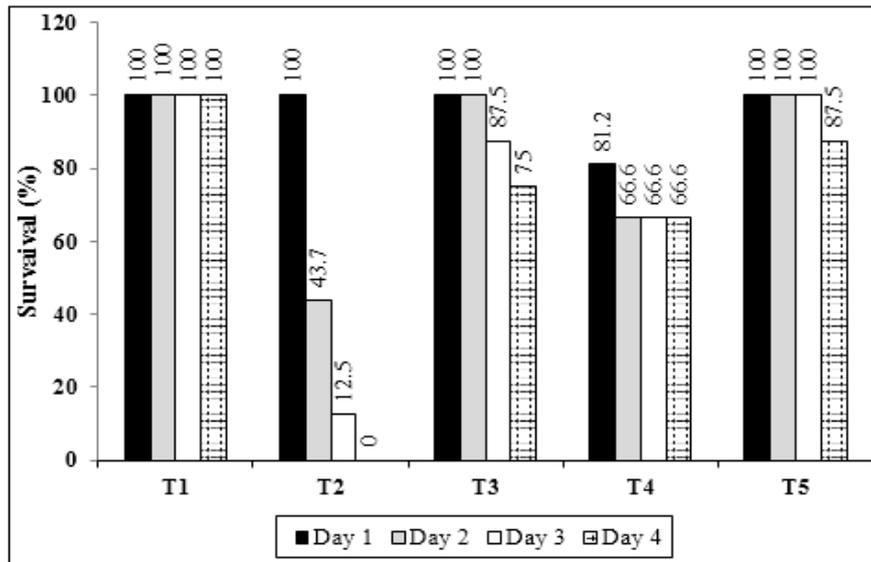


Fig 3: Percentage survival of Juveniles on control and probiotics treated
T₁ = WS alone (+ve control), **T₂** = WS+Vh (-ve control) **T₃** = WS+AVP03+Vh,
T₄ = WS+AVP07+Vh, **T₅** = WS+AVP03+AVP07+Vh

Table 4: Enumeration of TPC and PVC from experimental tank of juveniles

Treatment	Day 1		Day 2		Day 3		Day 4	
	TPC	PVC	TPC	PVC	TPC	PVC	TPC	PVC
T₁	1.8×10^5	1.2×10^1	2.1×10^5	1.8×10^4	4.8×10^5	6.8×10^4	9.3×10^5	1.8×10^5
T₂	6.4×10^8	2.9×10^7	4.6×10^8	6.8×10^7	1.7×10^8	4.0×10^8	3.1×10^7	4.7×10^8
T₃	1.9×10^8	6.6×10^5	7.7×10^8	7.8×10^5	8.4×10^8	5.5×10^5	1.2×10^9	9.3×10^5
T₄	1.6×10^8	2.7×10^6	8.6×10^8	6.6×10^5	6.9×10^8	4.0×10^5	9.4×10^8	4.5×10^5
T₅	1.0×10^8	2.4×10^6	8.1×10^8	6.4×10^5	9.3×10^8	7.2×10^4	1.7×10^9	4.1×10^4

T₁ = WS alone (+ve control), **T₂** = WS+Vh (-ve control) **T₃** = WS+AVP03+Vh,
T₄ = WS+AVP07+Vh, **T₅** = WS+AVP03+AVP07+Vh

Growth characteristics of probiotics (*Bacillus* sp. AVP03 and AVP07) in different medium is shown in Fig: 4(A & B). Both bacteria reached their stationary phase after 6 hrs in NB (nutrient broth) whereas MB (marine broth) required 8 hrs to reach stationary phase. The lowest time for bacterial growth was observed in TSB. This medium allowed the bacteria to reach their stationary phase after 6 hrs and hence TSB was

considered as best medium for culturing *Bacillus* sp. AVP03 and AVP07. This observation is in correlation to the study conducted by Nair *et al.*, who found TSB as the best medium for culturing *B. cereus* TC-2 isolated from coconut retting effluent [18].

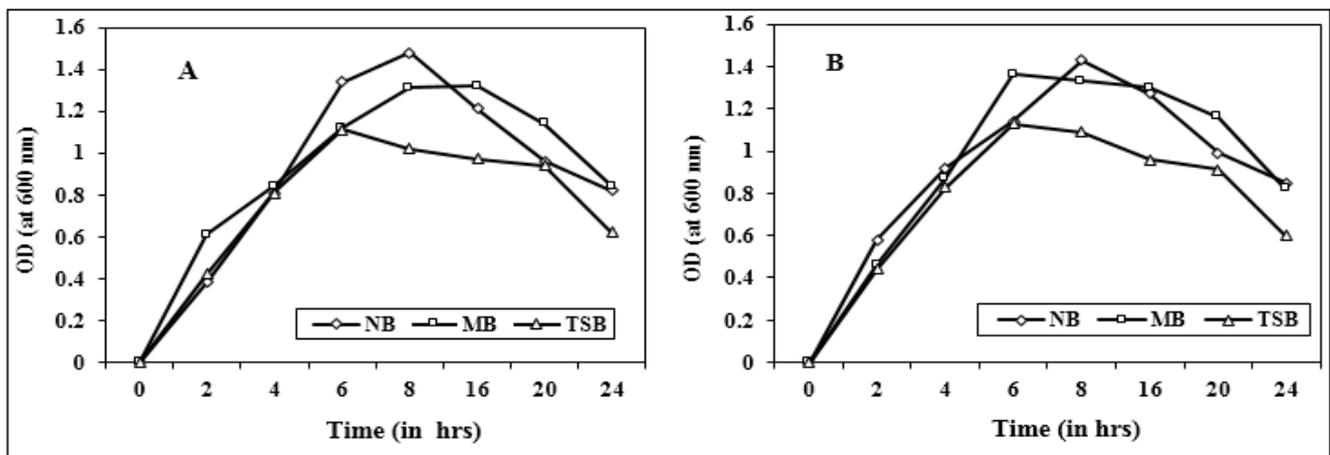


Fig 4: Growth characteristics of probionts grown in different medium

Growth characteristics of probionts (A) *Bacillus* sp. AVP03 and (B) *Bacillus* sp. AVP07 in different growth medium at different time intervals. NB - nutrient broth, MB – marine broth, TSB - tryptic soy broth.

The effect of probiotics CFS in the liquid culture model on *V. harveyi* VSH5 was conducted and presented in Fig: 5. Based on the comparison with control (*Vibrio* alone) both bacterial supernatant reduced the growth of *V. harveyi* VSH5. This finding indirectly explained the CFS rendering anti-vibrio compounds that may act on the growth of *Vibrio*.

Nakayama *et al.*, found out that the supernatant of *B. subtilis* showed reduced growth on the three *V. harveyi* species (VT, VP and VL), while *B. megaterium* supernatant reduced VP only [1]. But in the present study, both of the bacterial CFS did reduce the growth which might be due to anti-vibrio or quorum quenching ability of our probiotics.

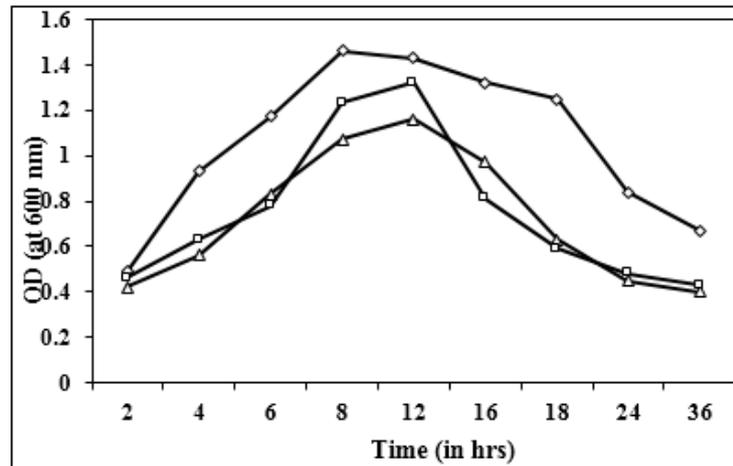


Fig 5: Growth inhibition of *V. harveyi* VSH5 by Probiot's CFS

Growth inhibition test of *V. harveyi* VSH5 by the addition of *Bacillus* sp. AVP03 and *Bacillus* sp. AVP07 CFS in liquid culture. (◆) *V. harveyi* VSH5 alone (control). (■) *Vibrio* treated with CFS of AVP03. (▲) *Vibrio* treated with CFS of AVP07.

The overall results of the present investigation thus evinced that the isolated probiotics *Bacillus* sp. AVP03 and AVP07 inhibited the pathogenic *V. harveyi* VSH5 both *in vitro* and *in vivo* methods. It appeared to be an effective way of controlling the pathogen against Penaeid shrimp which could substitute the negative impacts of antibiotics used in aquaculture. The commercial level *in vivo* experiments will be an area for future research along with elucidation of the mechanism of antagonistic action between probiotics and pathogen.

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