Molecular characterization of indigenous and exotic probiotic strain and its effective treatment on bacterial diseases in aquaculture

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Submitted by

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CONTENTS

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3. UGC REFERENCE NO. AND DATE: **F. No 36.5/2008(SR) 24.03.09**

4. DATE OF IMPLEMENTATION : **01.05.2009**

5. TENURE OF THE PROJECT : **3 years**

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9. TITLE OF THE PROJECT : **Molecular characterization of indigenous and exotic probiotic strains and it effective treatment on bacterial diseases in aquaculture.**

10. **OBJECTIVES OF THE PROJECT**

- Molecular characterization of native strains from wild aquatic animals and exotic probiotic strains from commercial brands.

- To elucidate the antibacterial activity and study the complete mode of action in resident and exotic probiotic strain against pathogenic strains.
- To compare the effectiveness of native and exotic strains on water quality parameters especially on toxic substances viz., Total ammonia, Nitrite, H_2S, TPC, etc.
- To compare the tolerance level of exotic and native strains on various temperature and salinity to find out the growth performance.
- Compare the Long residential time of native strain and exotic strain in gut and culture environment.

11. WHETHER OBJECTIVES WERE ACHIEVED

Yes. Objectives of the project were achieved.

The details of the objectives achieved are as follows

Procurement of equipment, selection of project fellow, collection and isolation of native strain from various aquatic sources and animal parts. Maintenance of the collected strains for further experiments. Collection of commercially available exotic probiotic strains. Molecular characterization and confirmation of bacterial isolates by 16S rDNA typing and PCR. Total of probiotic stains (50 numbers) and pathogenic *Vibrio* (30 numbers) with morphologically different stains evaluated for antimicrobial activity. DNA extractions, RAPD analysis by random primers were carried out from the native and exotic strains. The isolates showed better antibacterial activities against pathogenic bacteria were stored in aseptic conditions and used for further studies. Bacterial strains were identified by 16S rDNA technology. The sequences were submitted in NCBI Gene bank.

12. ACHIEVEMENTS FROM THE PROJECT

(See **Annexure I** for detailed methodology and results)

- Sample Sea water, Mangroove water, Animal tissue, collection was made from east coast area of Tamil Nadu (Kanayakumari, Tuticorin, Mandabam, Chidambaram and Chennai). Bacteria with strong inhibitory activity were enumerated from Pond sediments, Seawaters and animal organs.

- Cell-free extracts of *Bacillus* spp. and other strains from aquaculture sources were tested and showed inhibitory activity against the pathogenic *Vibrio* spp. which was isolated from infected *Penaeus monodon.*
- Ten strains with promising antagonistic activity (against pathogenic *Vibrio* spp) were characterized. The stains belong to the genus *Bacillus* identified by 16S rDNA technology and the sequences were submitted in National Centre for Biotechnological Information (NCBI) Nucleotide database.
- The Randomly amplified polymorphic DNA (RAPD) markers revealed that each *Bacillus* spp. isolate from different locations in the east coast of India produced a unique RAPD profile and suggests that isolates are genetically heterogenous.
- An effective probiotic strain *B. licheniformis* isolated from shrimp culture environments which was identified by 16S DNA sequence (NCBI genbank accession no: - HM235407). Cell-free extracts of the *B. licheniformis* strain DHAB1(B10DAHB1), showed the greatest inhibitory effects out of seven *B. licheniformis* strains tested against the growth of Vibrios determined by agar well diffusion assay.
- Effect of indigenous and exotic probiotic strain were tested for better water parameters like Temperature, salinity, dissolved oxygen (DO), pH, total alkalinity, and transparency were measured

13. SUMMARY OF THE FINDINGS

Probiotic bacteria *Bacillus* spp. from both pond sediments and shrimp native microflora were isolated and screened against the pathogenic *Vibrio* sp. Four strains from pond sediment showed antagonistic activity against 15 tested *Vibrio* sp. We have compared the anagonistic effect of *Bacillus* isolates from animal flora (shrimp) and two commercial probiotic *Bacillus* sp. against standard *Vibrio* cultures. *In vitro* Vibriostatic effects of probiotic strains from both wild and commercial sources against aquaculture important *Vibrio* spp. was evaluated. *B. licheniformis* isolated from both wild and commercial sources were monitored for antagonistic effects against the pathogenic *Vibrio harveyi*, *V. anguillarum*, *V. vulnificus* and *Photobacterium damsela* sp. *damsela* isolated from shrimp culture hatcheries and farms. Cell-free extracts of *B. licheniformis* strain Dhab1, showed greater inhibitory effects out of seven *B. licheniformis* strains tested against the growth of Vibrios determined by agar antagonism assays as well as co-culture experiments. The genetic status of these seven strains were analysed through RAPD analysis

using eighteen RAPD primers. Co-culture experiments showed that, among the seven isolates, *B. licheniformis* Dahb1 effectively controlled the growth of *Vibrio harveyi, V. anguillarum, V. vulnificus* and Photobacterium *damsela* ssp. d*amsela*. Among the 18 primers studied, only six generated repeatable polymorphic DNA bands with sizes ranging from 250 to 1000 bp in all seven isolates of *B. licheniformis.* The UPGMA generated dendrogram of RAPD profiles showed two major branches (Groups I–II) with three clusters. *B. licheniformis* Dahb1 can be used to control Vibrios infections in shrimp culture system. Also, RAPD can be used to discriminate between wild type *B. licheniformis* and commercial strains. Probiotic *B. licheniformis* Dahb1 can be used as an effective substitute for antibiotic usage in shrimp culture practices. This could benefit the aquaculture industry by controlling opportunistic *Vibrio* pathogens that cause severe mortalities and economic loss.

14. SUMMARY OF THE FINDINGS

In the present project study, the usage of native probiotic bacteria treatment improves the production of aquatic animals and maintain the water quality of the culture ponds. The outcome of the project would be helpful to the aqua farmers to treat the pathogenic bacteria by probiotic native isolates of *Bacillus* spp. World health organization emphasis the disease management should be on prevention which is likely to be more cost effective than cure and this will lead to less reliance on the use of chemical. Aquaculture is one of the fastest-growing food production sectors, disease outbreak are being increasingly constraint on aquaculture production and trade affecting the economic development. Use of disinfectants and antimicrobial drugs has limited success in the prevention or cure of aquatic diseases. The interaction between the microbiota, including probiotic, and the host is not limited to the intestinal tract. Probiotic bacteria could also be active on the gills or the skin of the host but also in its ambient environment. The aim of this study is to isolate the native strains of beneficial probiotic against pathogenic organism to control the disease in aquaculture. After field trails this strain can be used in very effective in aquaculture system than that of the exotic probiotic strains. Through this we can improve the production of aquatic animals.

15. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT

- Yes (one Ph. D candidate enrolled)

- Name of the Candidate: **B. David Jayaseelan**
- Title: **Identification and molecular characterization of probiotic bacteria for the Possible treatment of aquatic diseases in crustaceans.**

- **Year of Enrolment: - September - 2009**
- **Guide: Dr. B. Vaseeharan**

16. 16SrDNA sequences NCBI -14

14 NCBI Nucleotide database submissions of 16S rDNA was done for probiotic as well as pathogenic vibrios. (**Annexure –II**)

Publications (**Annexure -III**)

1. David Jayaseelan Baranabas., Vaseeharan Baskaralingam, Maharajan Athisuyambulingam, Shanthi Sathappan, Gopalakrishnan Vinoj. (2012). Vibriostatic effects of probiotic *Bacillus licheniformis* Dahb1 and its molecular phylogeny resolved through RAPD markers. *Annals in Microbiology (Under review)*

2. Jeyachandran Sivakamavalli, Perumal Rajakumaran and Baskaralingam Vaseeharan. (2012) Prophenoloxidase and Immune Indices of Indian White Shrimp *Fenneropenaeus indicus*. *Aquaculture Research & Development* 3,6; 148.

3. Vaseeharan B, Ramasamy P, Srinivasan P, Manikandan R, Arulvasu C., Prabhu N.M. (2010). Isolation and characterization of pharmaceutically important Fungal Microflora from *P. monodon* culture system. *Inventi Rapid: pharm Tech Vol. 1, Issue 3*.

1.1. INTRODUCTION

Aquaculture has become an important economic activity in many countries. In large-scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses. Prevention and control of diseases have led during recent decades to a substantial increase in the use of veterinary medicines. However, the utility of antimicrobial agents as a preventive measure has been questioned, given extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria. Antibiotics have been used in

the biosphere during an antibiotic era. These amounts of antibiotics have exerted a very strong selection pressure towards resistance among bacteria, which have adapted to this situation; mainly by a promiscuous flow of resistance genes several bacterial pathogens can develop plasmid-mediated resistance. Plasmids carrying genes for resistance to antibiotics have been found in marine *Vibrio* species and they could be In addition, other evidence of the transmission of resistance between aquaculture ecosystems and human has been demonstrate, when a novel chloramphenicol resistance gene, in *Salmonella typhimurium*, which also confers resistance to chloramphenicol, is almost identical by molecular sequence to the resistance gene first described in Photo bacterium damsela, bacterium found in fish.

The use of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment the term probiotics is generally used to denote bacteria that promote the health of other organisms. described them as substances secreted by one microorganism, which stimulated the growth of another Generally, probiotic strains have been isolated from indigenous and exogenous microbiota of aquatic animals. It is important to note that the population of endogenous microbiota may depend on genetic, nutritional and environment factors. However, microorganisms present in the immediate environment of aquatic species have a much larger influence on the health status than with terrestrial animals or humans. The gut microbiota of aquatic animals is probably constituted by the indigenous microbiota jointly with artificially high levels of microorganisms so maintained by their constant ingestion from the surrounding water. As the point denotes, we studied the antibacterial effect of indigenous and exogenous microbiota and its beneficial effect towards the cultured black shrimp *Penaeus monodon*

Annexure – I

1.1.1. METHODOLOGY

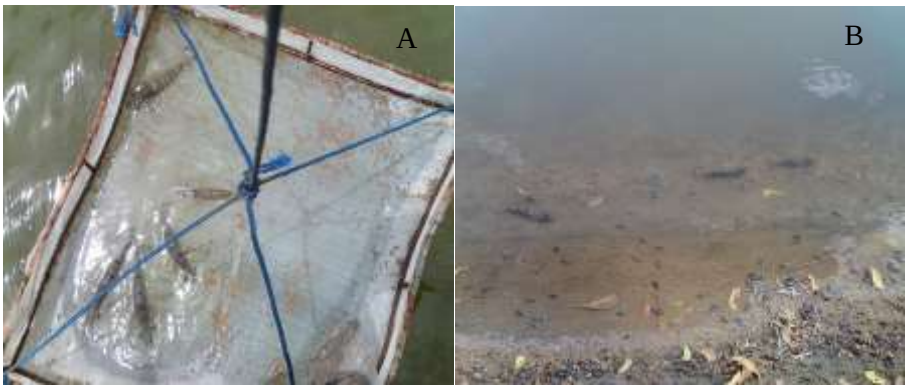
Sample collection

Samples were periodically collected from east coast area of Tamil Nadu (Kanayakumari, Tuticorin, Mandabam, Chidambaram and Chennai) (Fig-1). Seawater, soil sediment, animals from aquaculture system sites were collected from Mandapam (Fig-2) Chidambaram (Fig-3) for bacteria enumeration.

Fig-1. *Sample collection area along the east coast of Tamil Nadu, India for the enumeration of probiotic bacteria.*

Fig- 2. *Sampling site and sources of probiotic bacteria isolated from sea water (A), Mangrove (B)*

Fig -3. *Sampling site and sources of probiotic bacteria isolated from shrimp culture pond from Chidambaram.*



1.1.2. Enumeration of Bacteria

Nutrient agar**,** Thiosulfate Citrate Bile Salts Sucrose agar (TCBS), MRS agar, Hicrome agar, King'sB agar and Potato dextrose agar (Himedia Bombay) was used for the isolation of bacteria (520 isolates) from the collected samples. Sea water, soil sediments were homogenized under aseptic conditions by using sterile distilled water. Animal parts like gills, stomach, hepatopancreas were aseptically dissected out and homogenized with sterile distilled water. Colony forming units (CFU) were countered using 1ml portion of diluted samples poured in specific media plates, incubated at 28^{O}C to 37ºC for 24-36 hours. Isolated species were maintained in (NA) and (MRS) agar slants for further identification.

1.1.3. DNA Isolation from *Bacillus* and *Vibrio* spp.

Total DNA extraction was carried out using over night culture of *Bacillus* spp and *Vibrio* spp. culture were centrifuged at 5000 rpm for 15 min. *Bacillus* spp pellets (40 to 50 mg) were washed in water, re-suspended in 600 µl of lysis buffer (20 mmol l^{-1} Tris-HCl, 2 mmol l^{-1} EDTA, pH 8·0, 0·5% SDS, 10 g proteinase K) at 28°C for 1 h followed by the addition of 100 µl of 5 mmol l^{-1} NaCl and 80 µl of 10% w/v CTAB and incubated at 65^{O}C for 10 min. Total DNA was purified by chloroform extraction, precipitated with isopropanol, quantified using Bio photometer plus (Eppendorf-Germany) and then used in RAPD and PCR reactions.

1.1.4. In Vitro Antagonism Assay (Agar well diffusion assay)

The initial antibacterial activity of selected Bacillus strain against *Vibrio* spp. was studied by the agar well diffusion plate assay method. The isolated strains were identified with an API 50 CHB kit (BioMkrieux) and confirmed by 16S rRNA typing methods. The pathogenic *Vibrio* spp (*Vibrio harveyi*, *V. anguillarum*, *V. vulnificus,* and *P. damsela* subsp. d*amsela)* used in the study were identified and reported in our previous publications (Vaseeharan and Ramasamy 2003).

Bacillus spp. were streaked in nutrient agar plate and incubated at 28 ºC over night to check its efficiency for inhibitory activity. *Bacillus* culture was centrifuged at 9,600 rev min^{-1} for 15 min and the supernatant fluid filtered through a 0·22-µm membrane filter to obtain cell-free extracts (culture supernatant fluid). 100 µl of *Bacillus* cell-free extracts was introduced into the wells of

the agar plates and incubated for a period of 24–48 h at 28 ºC. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the well.

1.1.5. *Vibrio* growth pattern with *Bacillus* culture (Broth Dilution Assay)

Vibrio spp. were pre cultured separately in LB broth media at 28 ºC for 12 hrs. 100µl of 12 hrs *Vibrio* culture was inoculated into LB broth at an initial cell density of approx. 10^3 cfu ml^{-1} *Bacillus* strain of different concentration were introduced into the tube to check the efficiency of the isolated *Bacillus* in liquid condition and the growth curve of the pathogenic *Vibrio* was determined using OD values at 600 nm by spectrophotometer for every 6 hrs interval up to 32 hrs.

1.1.6. 16S rRNA Gene Amplification

The 16S ribosomal gene (rRNA) of the DNA extracted from each bacterial strain was amplified by PCR. The reagent mixture was prepared with the universal 16S rRNA Fp5'-AGATGATCCTGGCTCAG-3' and 16S rRNA Rp 5' – ACGGCTACCTTGTTACGACTT-3'. (Hughes et al., 2000). Samples were amplified by PCR in standard buffer (2.5µl), dNTPs (0.5 µl), forward and reverse primers each (1.0 µl), Taq (0.2 µl) and template DNA (1.0 µl). The PCR consisted of 40 cycles of 95 ºC (5min), 55 ºC (1 min), and 72 ºC for (2min), with final 72 ºC for 10 min for the elongation process. This was performed on all the Bacterial strain concentrated in this study yielding positive amplification for all DNA tested, as determined by visualization on agarose gel electrophoresis.

1.1.7. Phylogenetic Analysis

Searches in the GenBank with the BLAST program were performed to determine the closest known relatives of the 16S rDNA sequences of *Bacillus* and for the *Vibrio* spp obtained. The 3' end of the 16S rDNA for the species were sequenced in this study. The sequences were aligned using the ClustalW program. (Thompson *et al.*, 1994) and the most parsimonious phylogenetic trees were constructed using the DNAPARS program of the PHYLIP package, version 3.6a2 (Felsenstein, 1989). The order of the input sequences was randomized by DNAPARS. Stability of the groupings was estimated by bootstrap analysis on 100 trees using SEQBOOT in the same package. Trees were visualized using TREEVIEW software, version 1.6.1.

1.1.8. Randomly amplified Polymorphic DNA analysis (RAPD-PCR)

RAPD analysis was carried out in 25-µl reactions, using 100 ng of genomic DNA. Eighteen DNA primers (DAHMRA01 to DAHMRA018) (Table-1), were artificially synthesised by Automated Solid phase synthesis containing 10-base oligonucleotides were selected based on G+C content ranging from 60 to 70% (Table-1). Amplification reactions were performed in 25 µl volume PCR reaction mixtures containing 80 mM $MgCl_2$, PCR buffer, 3.75 mM dNTP mix (MBI Fermentas), 10 picomoles of each RAPD primers, 100 ng of template and 1Unit of Taq DNA polymerase (MBI Fermentas). Amplifications were carried out by a thermal cycler (Eppendorf - Germany) programmed for 1 min at 94 ^oC followed by 30 cycles, each consisting of 1 min at 94 ^oC, 1 min at 36 ^oC and 2 min at 72 ^oC and a final extension period of 7 min at 72 ^oC. Ten microlitres of amplified PCR products were separated by electrophoresis through 1.4% agarose gels in 1 X TBE buffer for 2.30 h at 65 V. The gels were stained with 0.5µl µg ml^{-1} of ethidium bromide and the patterns were analyzed using gel documentation system (Bio-Rad). The size of RAPD bands was determined by comparison with λ-DNA double digest (Hind III and EcoRI) and 100-bp DNA ladder. The DNA from each isolate was subjected to the RAPD assay at least three times to ensure reproducibility of the results.

Table- 1. Random primers used for PCR RAPD analysis.

S. No	Primer name	Sequence
1	DAHMRM01	5’ TGC CGA GCT G 3’
2	DAHMRM02	5’ AGT CAG CCA C 3’
3	DAHMRM03	5’ AAT CGG GCT G 3’
4	DAHMRM04	5’ GAA ACG GGT G 3’
5	DAHMRM05	5’ GTG ACG TAG G 3’
6	DAHMRM06	5’ GTG ATC GCA G 3’
7	DAHMRM07	5’ CAA TCG CCG T 3’
8	DAHMRM08	5’ CAG CAC CCA C 3’
9	DAHMRM09	5’ TCT GTG CTG G 3’
10	DAHMRM10	5’ CTT GAG TGG A 3’
11	DAHMRM011	5’ TCC TCA AGA C 3’
12	DAHMRM012	5’ GAG ATG ACG A 3’
13	DAHMRM013	5’ TTC CCC GTC G 3’
14	DAHMRM014	5’ GTA TTG CCC T 3’

15	DAHMRM015	5’ TTC CCC GAC C 3’
16	DAHMRM016	5’TAC GAT GAC G 3’
17	DAHMRM017	5’ TCC CTC GTG C 3’
18	DAHMRM018	5’TAC AAC GTC G 3’

1.1.9. RAPD Data Analysis

The fingerprint was analysed both by visual inspection and computer-aided methods. The RAPD analysis was based on the manual scoring for the presence or absence of bands, as observed for the RAPD banding profile obtained. Only the clear, prominent and reproducible bands from repeats of the experiment at least three times were given consideration showing the true polymorphism. Bands which appeared in the RAPD primer amplification are denoted by 1 while the absence of a band is denoted by 0. An input matrix was produced by entering the data regarding presence (1) or absence (0) of an amplified fragment for Jaccards’s pair wise genetic distance analysis and to generate the neighbour-joining tree calculated by the unweighted pair group method with arithmetic averages. The genetic relationships between the isolates are represented graphically by the dendrogram.

1.1.10. Protein profile of *Bacillus* spp. and *Vibiros* spp.

For Protein isolation *Bacillus* spp. and *Vibrio* spp. cells were separately pelleted by centrifugation at 10,000 rpm (10 min, 4 ^{o}C) and the supernatant was discarded. Protein was prepared by suspending the pellet of 10 ml cell culture in 1ml lysis buffer (100 mM NaCl and 25mM Tris HCl, pH 8.0) and incubated on ice for 20 minutes. The mixture was lysed with an ultrasonicator (Riveria) for 10 minutes and centrifuged at 13,000 rpm for 5 minutes at 4 ^{o}C. Supernatant was recovered and stored at -20 ^{o}C for future use. Protein concentration was determined by the method of Lowry using bovine serum albumin as a standard.

1.1.11. SDS-PAGE

12% SDS-PAGE (Laemmli, 1970) was performed using vertical slab gel apparatus (ProGen Biotech) with the stacking gel containing 4.5% acrylamide and the resolving gel with 11% acryamide. Briefly, the sonicated samples were diluted with reducing sampl buffer ((20% glycerol, 1% SDS, 1.125M Tris/HCl, 2% ß-mercaptoethanol and 0.5% bromophenol blue),

heated for 5 minutes at 100 ^{o}C. The mixture was loaded into a single well and subjected to electrophoresis. The gel was calibrated using molecular weight markers (ProGen and Fermentas) and stained either with coomassie brilliant blue or silver nitrate.

1.1.12. Biofilm formation assay

Biofilm formation was tested over glass slide, *Vibrio* isolates were grown in a 24-well polystyrene tissue culture plate in 1000 µl of (LB) medium at 37 ^{o}C. The cultures were grown for three days to attain mature bioflim. Different concentration of Bacillus cell free extract was introduced in to the wells and kept for overnight incubation. After the slides were removed from the well and washed with phosphate buffer saline to remove unwanted cell debris attached to the slide, all the slides were stained using acridine orange and washed with deionizer water to remove excess strains from the glass slide and imaged using Confocal laser scanning microscope.

1.2.1Animal collection

Animals were collected from Chidamberam maintained in 500-L capacity FRP tanks in the laboratory for experiment (Fig.13). Tanks were filled with filtered seawater with 30 ppt salinity and aerated till the end of the experiment. Shrimp, *Penaeus monodon* with average body weight 12 g were used in the trail, animals were allowed for acclomation, after a week of conditioning 10 shrimps per tanks and fed with commercially available feed. The FRP tanks were covered with net to prevent from shrimp jumping out from the trail tanks.

1.2.2. Experimental design

The experiment was conducted in two treatments trails standard diet and diet supplemented with probiotic. Each group consists of 3 replicates, 1 as control tank and 2 were trail tank receiving probiotic diet. Feeding with probiotic started on (day 0) one week after stocking the tanks with the shrimps. Previously all the trail tanks had the same standard commercial diet. Shrimps were sampled weekly in the trail tank and in control tank, trail were made in test pond A and B, each pond was sampled once in the course of the trial. Each pond sample consisted of 2 groups of 50 shrimps. The first group was sampled one hour before the meal and the second group sampled two hours after the meal. The animals were captured and the samples were immediately placed in sterile containers of 5 shrimps each, packed on ice in a cooler and brought to the laboratory

within 30 min. At the end of the trial sampling for every two hours from both trail tanks, 2 shrimps were collected for each treatment.

1.2.3. Microbial analysis before challenge with pathogenic microbes

Sampling of shrimp and water was done before the application of microbial products in the water and feeds. Water and shrimp were sampled 2, 12, and 24 hrs before introducing microbes in water and feeding every 24 hrs until the trail. Gut and hepatopancreas of shrimp and water were aseptically pooled for bacteriological examination. Dissolved oxygen, water, temperature, salinity was monitored, water pH was monitored using pH meter.

1.2.4. Farm site and study

The study took place in two ponds of a semi-intensive farm located in Mudacaloddai, Chidambaram, TamilNadu, India. The experiment was conducted over 10 weeks after standrizating farm parameters, before the usual period for outbreaks of this disease. The water temperature during the trial was within the thermal condsion of *Vibrio* spp infection except when the temperature elevates above the upper limit of this pond. During the course of experiment trail the temperature of the pond was maintained between 22.2 ^{o}C and 34.8 ^{o}C

1.2.5. In *Vitro* experiments

The cell free supernatant was filtered using sterilized filters paper with pore size of 0.2mm and pH was adjusted to 6.8. Five ml of nutrient broth were individually supplemented with *B. licheniformis* strain supernatants tubes were then inoculated with freshly grown culture of *V. alginolyticus* were incubated at 28°C for 48 hrs and growth was recorded by measuring the optical density at 540nm. Control tubes were comprised of nutrient broth inoculated with *V. alginolyticus* alone.

1.2.6. In vivo challenge experiments

After four weeks of feeding, *B. licheniformis* was used as probiotic strain in the trail, 5 shrimps from each of the treatments were injected with 0.1 ml of an aliquot of *V. alginolyticus* containing 10^4 cells per ml. All the infected shrimps were maintained in segregation but under the same experimental conditions being fed with a probiotic added feed every day. Animals were observed for a week for signs of vibriosis and morbidity. At the end of trail surviving animals of each

inoculated group were sacrificed and hepatopancreas and muscle were dissected out under aseptic conditions, organs were grained using phosphate buffer saline. Serial dilutions were carried out and plated onto Thiosulfate-citrate-bile salts-sucrose agar plates and incubated at 28°C.

1.2.7. Adherence of Probiotics on gut

Probiotics strain establishes in the gut believed to execute their functions including immune stimulatory activity this system of the gut is referred to as gut associated interaction. The interaction of endogenus and exotic probiotics with gut differs from the dominant group of probiotics that are used in this tank culture trail belong to Gram positive lactic acid bacteria *B. licheniformis* on the other trail. Gram negative *Pseudomonas Fluorescens* and *Enterobacteria lactis* species were also used as probiotics in this trail. All these bacteria differ greatly in colonization effect in the gut of the trail animals, each strain has unique properties and the probiotic effects of a specific strain must not be extrapolated to other strains.

1.2.8. Residential time of Probiotic viability

The mechanisms by which probiotics losses viability are due to the factors such as adhesion properties, attachment site, stress factors, diet and environmental conditions. The colonization of probiotics in the gut of the animal showed to be specific to strain, the origin and source of probiotics viability dose and duration of supplementation help to regulate their activities. There is no doubt that probiotics can stimulate immune system but inappropriate dose and duration of probiotics supplementation can cause undesirable results therefore, the type of probiotics, and method of administration with respect to trail animals are critical factors that can regulate the colonization of the probiotic strain to the trail animals. However, when different probiotics supplemented in combined form should complement each other and acquire difference within the gut micro flora environment. Nevertheless, the probiotic sources and their relatedness can also affect the synergitic effects in combined form. Multispecies probiotics may be more effective than single probiotics strains in our study.

1.2.9. Probiotic in gut and culture environment

The study revealed that concentration of the probiotics could be important factor for beneficial effects. The optimum concentration of probiotics is not only required for establishment and subsequent proliferation in gut. Same strain that should have better anti-bacterial activity *in vitro* study has to given in more quantity in case of *in vivo* studies. It is also found that the concentration of probiotics 10^6cfu/ml cells at which they should have better effect in in-*vitro* has to be given as 10^8cfu/ml in case of *in vivo* study to get least mortality in the trail tank. In this study it is clearly observed that salinity and pH play a major role for the colonization of the probiotic strain in gut of the animal.

1.2.10. Haemolymph

After rinsing the shrimps with sterile seawater, Haemolymph was collected from the ventral-sinus cavity using 1 ml sterile needle and syringe. Samples were then plated on Marine agar with 2% glycerol, on which Vibrio spp. produce pigmented colonies. Plates were incubated for 72 hrs to 96 hrs at 29 ^{o}C. The number of putative *Vibrio* colonies were counted and recorded for each animal. Prevalence and load of the *Vibrio* colonies were determined weekly for shrimps' samples in the ponds.

1.2.11. Gut microbiota

Shrimps were dissected using sterilized surgical scissors to remove mid gut and hind gut. To avoid possible external contamination while removing organs, the surface of each shrimp was previously cleaned using 70% ethanol. The hind-gut of sample shrimps was placed in a sterile tube containing 1 ml of sterile artificial seawater and weighed before homogenization. Bacteriological determination was made using serial dilution in sterile saline solution followed by plating on zobell Marine agar and Thiosulfate citrate bile salt agar (TCBS) for vibrio and for determination of probiotic microbes plated in (MRS), were used in order to determine total cultivable heterotrophic bacteria.

1.2.12. Water quality analysis

Temperature, salinity, dissolved oxygen, pH and transparency were measured in the field using disk. Water samples were collected from the center and sides of each pond approximately once

Every 21 days of culture period, water samples were collected in sterile bottles and transported aseptically to the laboratory and processed immediately for analysis. The concentrations of inorganic nitrogen compounds, dissolved reactive phosphorus, chemical oxygen demand (COD), and carbon dioxide, (CO_2) in water were measured according to the procedures. Water samples were suitably diluted using physiological saline (0.9% NaCl) before inoculation. Estimation of total plate count was maid in (TPC) for *Vibrio* count and (MRS) agar was used for *Bacillus spp*.

Result

2.1.1. Enumeration of Bacteria

Bacillus spp. and *Vibrios* spp. (520 isolates) were isolated by the specific species selective medias. All *Bacillus* spp were tested for antibacterial activity against the *Vibrio* spp. The strains that showed effective inhibitory activity were taken and streak in selective media to obtain pure culture. The selected *Bacillus* spp. and *Vibrio* spp. morphological features were listed in the (Table 2a & 2b).

(Fig - A) Gram straining was done for identification the bacteria and view under Nikon inverted microscope

Fig - A Nikon inverted microscope

Table-2a. Morphological features of Vibrio spp. and source of collection.

Culture	Media	Source	Location	Colour	Shape	Surface	Elevation	Consisten
V1	TCBS	Shrimp	MANDABAM	Pale yellow	Flat	smooth	Flat	Pinpoint
V2	TCBS	Shrimp	CHENNAI	Yellow	Round	Smooth	Convex	Mucoid
V3	TCBS	Shrimp	CHIDAMBARAM	Yellow	Regular	Smooth	Convex	Mucoid raised
V4	TCBS	Shrimp	CHIDAMBARAM	Dark yellow	Regular	Smooth	Convex	Mucoid pinpoint
V5	TCBS	Shrimp	CHIDAMBARAM	Greenish	Regular	Smooth	Convex	Mucoid pinpoint
V6	TCBS	Shrimp	MANDABAM	Greenish	Regular	Smooth	Convex	Mucoid
V7	TCBS	FISH WILD	MANDABAM	Greenish	Irregular	Smooth	Convex	Mucoid
V8	TCBS	Shrimp	KANYAKUMARI	Greenish	Round	Smooth	Convex	Mucoid
V9	TCBS	Shrimp	CHIDAMBARAM	Yellow	Round	Rough	Convex	Spread
V10	TCBS	Shrimp	MANDABAM	Yellow	Round	Rough	Convex	Spread
V11	TCBS	Wild FISH	CHENNAI	Pale yellow	Irregular	Rough	Flat	Mucoid spread
V12	TCBS	FISH	CHENNAI	Yellow	Round	Rough	Flat	Pin point
V13	TCBS	FISH	KANYAKUMARI	Yellow	Round	Smooth	Convex	Mucoid
V14	TCBS	Shrimp	CHIDAMBARAM	Yellow	Smooth	Smooth	Convex	Mucoid
V15	TCBS	FISH	CHENNAI	Black	Round	Smooth	Convex	spreaded

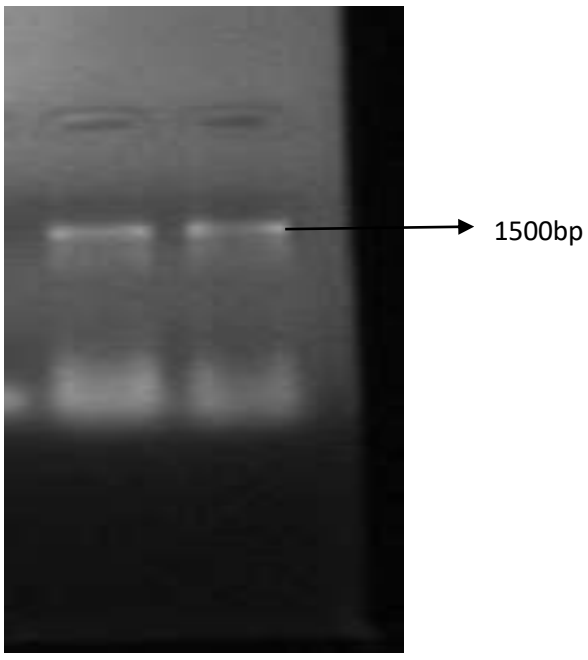
Table- 2 b. Morphological features of Bacillus spp. and source of collection

Culture	Media	Source	Location	Colour	Shape	Surface	Elevation	Consistenc
B 1	MRS	Shrimp	CHIDAMBARAM	Wight	Flat	smooth	Umbonate	Spreaded pinpoint
B 2	MRS	Shrimp	CHIDAMBARAM	Wight	Round	Smooth	Umbonate	Mucoid
B 3	MRS	Shrimp	CHIDAMBARAM	Wight	Round	Smooth	Umbonate	Mucoid
B 4	MRS	Shrimp	CHIDAMBARAM	Wight	Round	Smooth	Umbonate	Mucoid
B 5	MRS	Shrimp	CHIDAMBARAM	Wight	Round	Smooth	Umbonate	Mucoid pinpoint
B 6	MRS	Shrimp	MANDABAM	Wight	Round	Smooth	Convex	Dry
B 7	MRS	Shrimp	CHENNAI	Wight	Round	Smooth	Umbonate	Spreaded pinpoint
B 8	MRS	Shrimp	CHENNAI	Wight	Round	Smooth	Convex	Dry
B 9	MRS	Shrimp	MANDABAM	Wight	Round	Smooth	Umbonate	Mucoid pinpoint
B 10	MRS	Shrimp	CHIDAMBARAM	Wight	Round	Smooth	Convex	Mucoid
B 11	MRS	Shrimp	MANDABAM	Wight	Round	Smooth	Flat	Powdery
B 12	MRS	Shrimp	MANDABAM	Wight	Round	Smooth	Umbonate	Glistering, Mucoid pinpoint
B 13	MRS	Shrimp	KANYAKUMARI	Wight	Round	Smooth	Umbonate	Mucoid
B14	MRS	Shrimp	KANYAKUMARI	Wight	Round	Smooth	Convex	Mucoid pinpoint
B15	MRS	Shrimp	CHENNAI	Wight	Round	Smooth	Convex	Mucoid

2.1.2. 16S rDNA Gene Amplification

The 16S ribosomal gene (rDNA) of the DNA (Fig-4) extracted from each bacterial strain was amplified by PCR. The reagent mixture was prepared with the universal 16S rDNA Fp 5'-AGATGATCCTGGCTCAG-3' and 16S rDNA Rp 5' – ACGGCTACCTTGTTACGACTT-3' (Hughes et al., 2000). Samples were amplified by PCR in standard buffer (2.5µl), dNTPs (0.5 µl), forward and reverse primers (each 1.0 µl), Taq (0.2 µl) and template DNA (1.0 µl). The PCR consisted of 40 cycles of 95^{o}C (5min), 55 ^{o}C (1 min), and 72 ^{o}C for (2 min), with final 72 ^{o}C for 10 min for the elongation process. This was performed on all the seven strains, yielding positive amplification for all DNA tested as determined by visualization on agarose gel electrophoresis. The amplification products were purified using Real genomics kit, according to the specifications of the manufacturer (LifeTech, India).

Fig-.4 16S rDNA amplification from bacteria showed 1500bp specific band.



2.1.3. Cloning and Sequencing Methods.

The amplified DNA was cloned into a pCRII-TOPO cloning vector using the TOPO TA cloning kit (Invitrogen), following the manufacturer's instructions. Transformants were selected on LB agar plates containing kanamycin (50 µg ml^{-1}), X-Gal (40 µg ml^{-1}) and IPTG (0·5 mM). The

recombinant plasmids were isolated using the alkaline-lysis method, and visualized on agarose gels to confirm the presence of the inserted fragment. The Dye-termination method (Applied Biosystems, USA) using the near-infrared fluorescence automated DNA sequencer (LI-COR model 4200), was used to sequence the DNA fragments. The sequence was deposited to NCBI genbank database with accession number 14 different bacteria(Table- 2C) were identified and the sequence were deposited in the NCBI Gene bank **(Annexure-II)**

Table 2C- Bacteria species identification and accession Numbers

s.no	**Bacteria**	**Accession number**
1	*Proteus sp*	HQ116442
2	*Proteus vulgaris*	HQ116441
3	*Proteus sp*	HQ009354
4	*Bacillus licheniformis*	HM235407
5	*Enterococcus faecalis*	HQ693279
6	*Bacillus thuringiensis*	HQ693278
7	*Pseudomonas sp*	HQ693277
8	*Pseudomonas aeruginosa*	HQ693274
9	*Bacillus pumilus*	HQ693273
10	*Pseudomonas aeruginosa*	HQ693272
11	*Bacillus sp*	HQ116443
12	*Vibrio sp*	HQ693276
13	*Vibrio parahaemolyticus*	HQ693275
14	*Pseudomonas aeruginosa*	HQ400663

2.1.4. RAPD Analysis

The generation and counts of RAPD band patterns of different *Vibrio* spp. with 18 primers are given in Table 3, 4, 5. The agarose gel electrophoresis photographs of RAPD band pattern are given in Fig-5 A, B, C, D, E, F. Out of 18 primer used only 6 primers produced 1 to 16 repeatable band patterns. The molecular size ranging from 100 bp to 6000 bp size. Among the isolates of *B. licheniformis* collected from shrimp culture environments, which shows good inhibitory activity were subjected to RAPD fingerprinting, 3 to 16 repeatable and clear loci fragments in the size range of 250–1000 kbp with DAHMRM01-DAHMRMO3 primer (Fig. 5) Primer DAHMRM04 showed maximum number of bands than others five primers. The primer DAHMRM06 showed only two bands in all the seven isolates. Of the 18 primers used for RAPD analyses, only six (DAHMRM01, DAHMRM02 DAHMRM06, DAHMRM09, DAHMRM012, DAHMRM018) generated inferable results and yielded 110 polymorphic bands, which were used to construct a dendrogram. The results explained that each *B. licheniformis* isolate from different locations in the east coast of India produced a unique RAPD profile and suggests that *Bacillus* isolates are genetically heterogeneous. Three major clusters (with two major branches) were resolved through UGPMA analysis of *B. licheniformis* DNA fragments obtained with six RAPD primers. Moreover, *B. licheniforms* isolated from commercial probiotic brands were clearly differentiated into separate branches than the other species (Groups I–II). Maximum genetic distance was found between BT10DAHB3 and BT10DAHB1, whereas the minimum was found between BT10DAHB6 and BT10DAHB7 (Table-6, 7) (Fig -6). In conclusion the present study reports an effective probiotic strain *B. licheniformis* isolated from shrimp culture environments which was identified by 16S DNA sequence (NCBI genbank accession number HM235407).

Table-3 RAPD result of Vibrio spp sample with random primers

Primers	**V1**	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**	**V9**	**V10**	**V11**	**V12**	**V13**	**V14**	**V15**
Primer 1	+	+	+		+				+	+	+	+	+	+	
Primer 2	+	+	+		+			+	+	+	+		+	+	
Primer 3	+		+	+				+	+	+	+		+	+	+
Primer 4	+	+	+					+	+	+	+	+	+		+
Primer 5	+	+			+		+		+	+	+	+	+		+
Primer 6	Not Working														
Primer 7	+	+	+		+		+		+	+	+		+		+
Primer 8	Not Working														
Primer 9															
Primer10	+	+	+				+	+	+	+	+		+		+
Primer11	+	+	-		-			-	+	+	-			+	-
Primer12	Not Working														
Primer13	+	+	+	+	+		+	+	+	+				+	
Primer14	+		+				+	+	+	+	+		+	+	+
Primer15	+	+	+	+	+	+	+	+	+	+	+	+	+		+
Primer16	+	+	+		+			+	+	+		+	+		+
Primer17	+	+			+		+	+	+	+	+		+		+
Primer18	+	+	-		+			-	+	-	+		+	+	

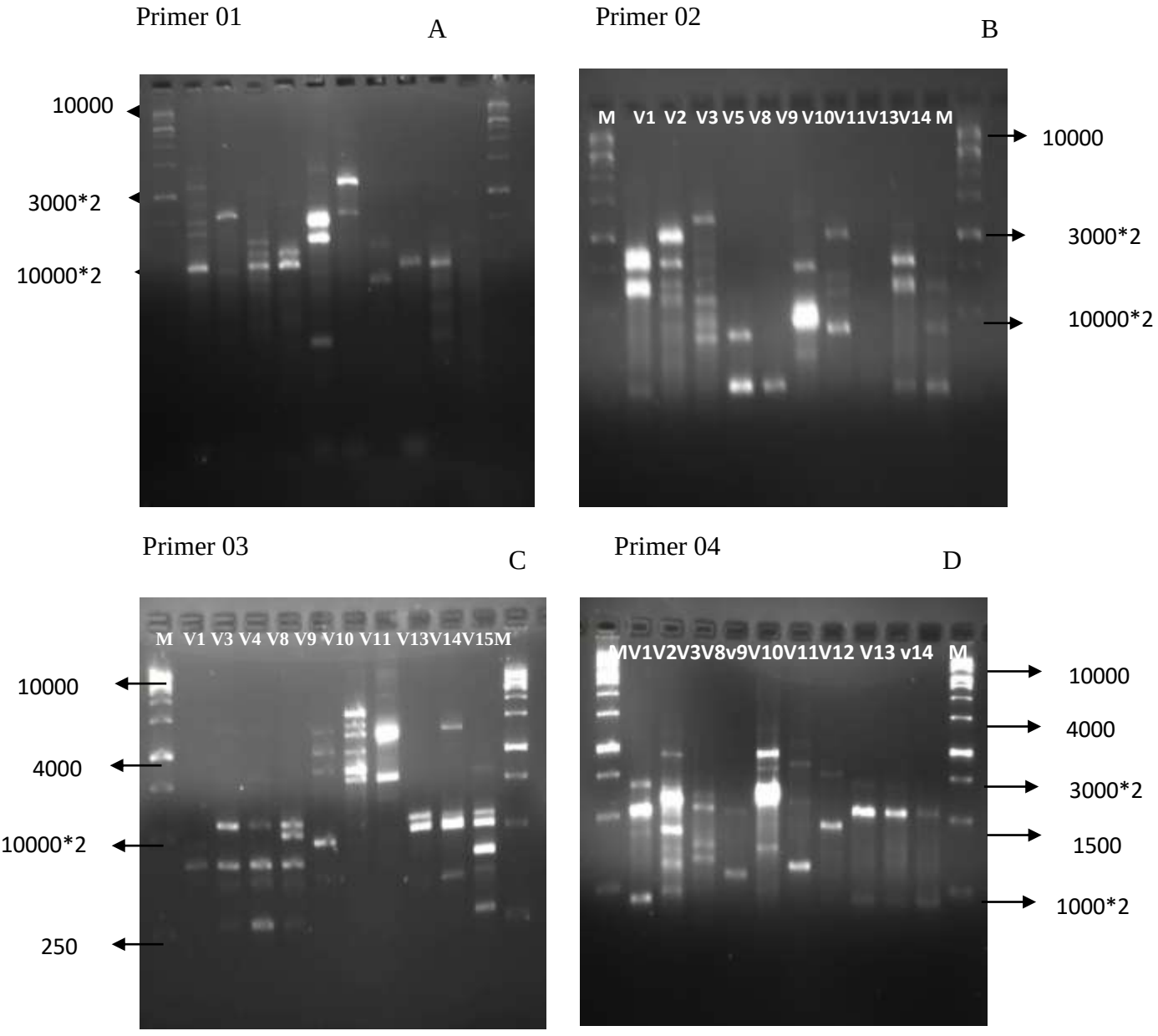
Table- 4. Comparison of Amplification with the Primers and V1-V15 DNA

Primers	DNA	Results
Primer 1	V1 – v15	+
Primer 2	V1 – v15	+
Primer 3	V1 – v15	+
Primer 4	(Except v5 & v7)	+
Primer5	(Except v3&v11)	+
Primer6	V1 – v15	-
Primer7	(Except v7)	+
Primer8	V1 – v15	-
Primer9	V1 – v15	-
Primer10	(Except V5 & v13)	+
Primer11	(Except v3, v5, v7, v11, v13, v14)	+
Primer12	V1 – v15	+
Primer13	(Except v8)	+
Primer14	(Except v12)	+
Primer15	V1 – v15	+
Primer16	(Except v7, v8, v11, v14)	+
Primer17	(Except v3, v8, v9, v11, v12)	+
Primer18	(Except v3, v7, v8, v9, v12)	+

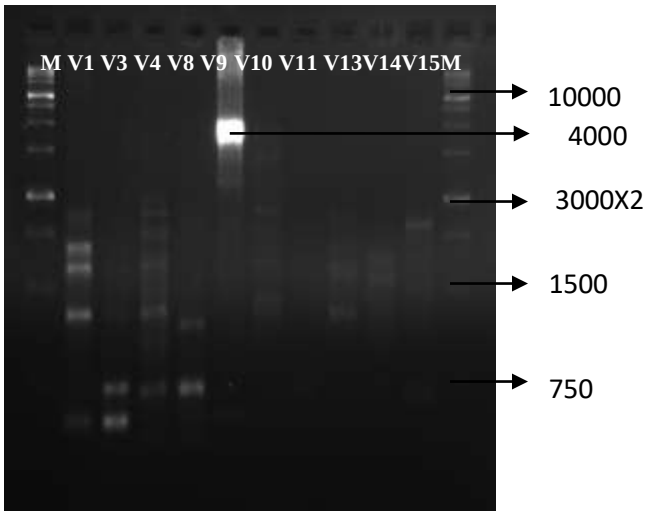
Table -5. RAPD band pattens of Vibrio spp.

Primers	**V1**	**V2**	**V3**	**V5**	**V7**	**V8**	**V9**	**V10**	**V11**	**V12**	**V13**	**V14**	**V15**
Primer 1	5	2	1	1	1	1	3	5	2	1	5	6	6
Primer 2	3	1	3	2	1	1	3	3	1	4	6	4	2
Primer 3	3	1	3	2	1	1	5	3	1	1	5	2	2
Primer 4	3	1	4	-		1	2	1	2	2	4	4	3
Primer5	6	1	-	11	2	-	1	2	-	1	2	1	2
Primer6	-	-	-	-	-	-	-	-	-	-	-	-	-
Primer7	4	1	3	3	-	5	7	2	4	2	3	4	3
Primer8	-	-	-	-	-	-	-	-	-	-	-	-	-
Primer9	-	-	-	-	-	-	-	-	-	-	-	-	-
Primer10	2	1	1	-	1	1	3	3	1	1	-	1	2
Primer11	1	1	1	-	-	1	3	1	-	-	-	-	2
Primer12	3	2	1	1	1	1	4	2	2	3	7	1	1
Primer13	5	5	1	1	4	-	3	3	4	1	6	1	3
Primer14	4	4	1	1	5	1	2	2	2	-	2	1	2
Primer15	3	1	5	1	1	1	2	2	1	1	3	1	1
Primer16	3	1	6	2	-	-	3	2	-	1	2	-	1
Primer17	1	2	-	3	1	-	-	1	-	-	1	1	1
Primer18	4	1	-	3	1	-	-	1	4	-	2	1	1

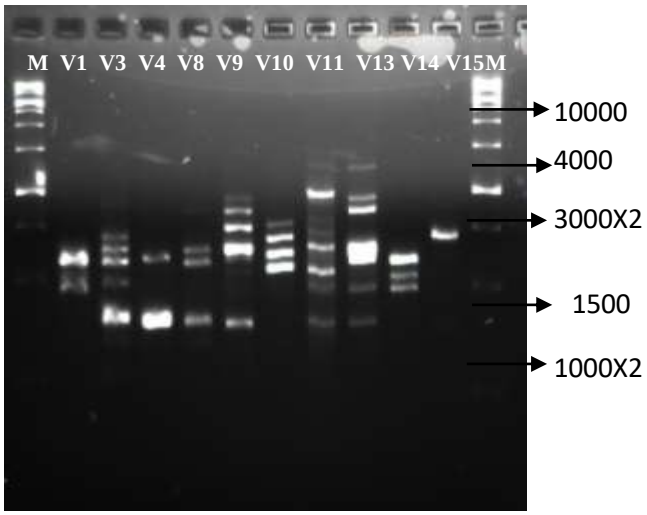
Fig-5 A, B, C, D, E, F. Randomly amplified polymorphic DNA (RAPD) banding profiles of Vibrio spp.



Primer 05 E



Primer 07 F



RAPD band patterns of different *Bacillus* spp. with 15 primers are given in (Table 6, 7). The Agarose gel electrophoresis photo graphs of RAPD band pattern are given in (Fig-6). Out of 18 primers used only, six primers showed the one to 16 repeatable band patterns. The molecular size ranging from 100 bp to 6000 bp size

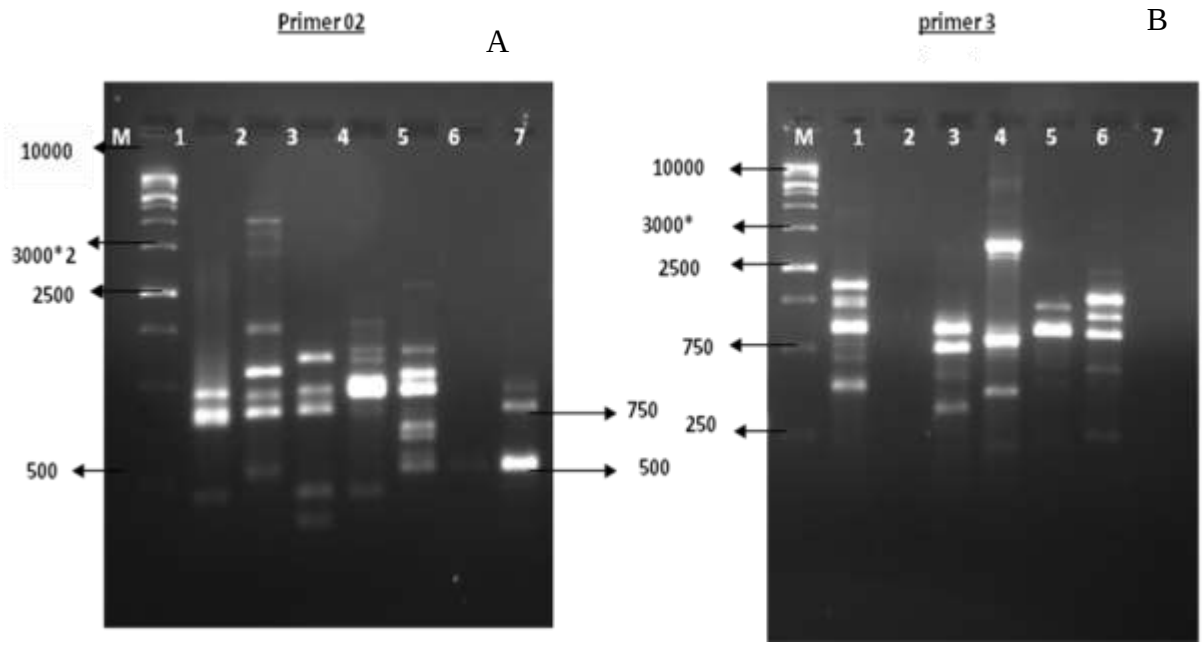
Table- 6. Randomly amplified polymorphic DNA (RAPD) banding profiles of Bacillus spp

Primers	**B3**	**B5**	**B6**	**B7**	**B10**	**B14**	**B15**
Primer 02	+	+	+	+	+	+	+
Primer 03	+	-	+	+	+	+	-
Primer 07	+	+	+	+	+	+	+
Primer 10	+	+	+	+	+	+	+
Primer 13	+	+	+	+	+	+	+
Primer 15	+	+	+	+	+	+	+

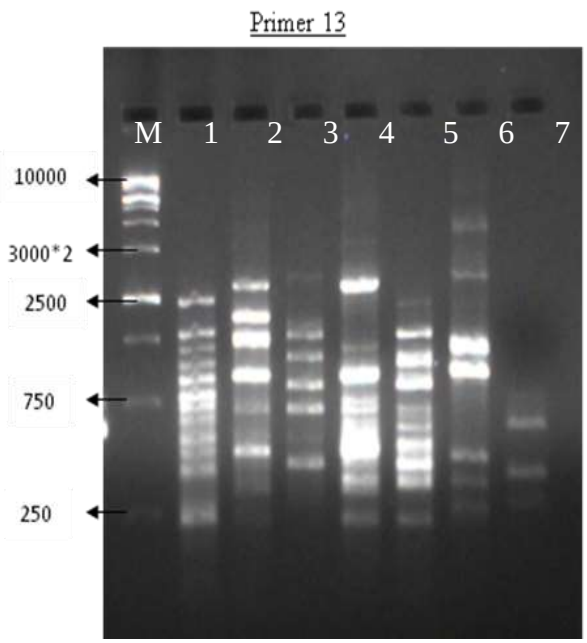
Table -7. BAND patterns of RAPD results of Bacillus samples with random primers

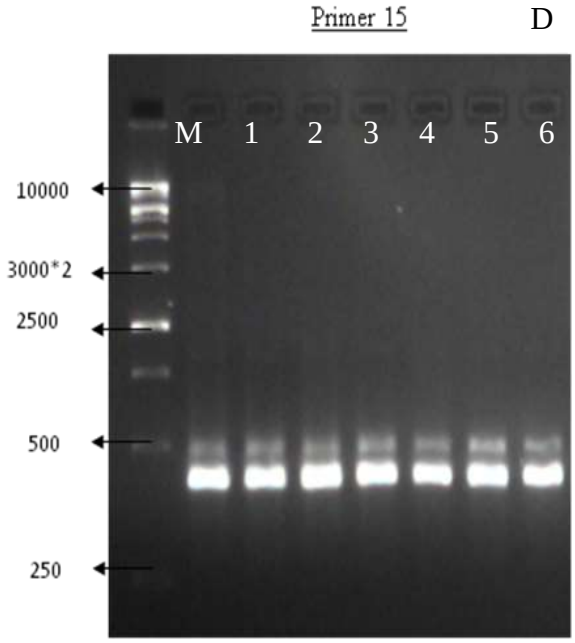
Primers	B3	B5	B6	B7	B10	B14	B15
Primer 02	3	8	5	7	8	1	3
Primer 03	6	-	4	6	5	7	-
Primer 07	5	5	4	4	2	2	10
Primer 10	1	3	5	3	3	4	9
Primer 13	12	6	7	10	10	7	3
Primer 15	2	2	2	2	2	2	2

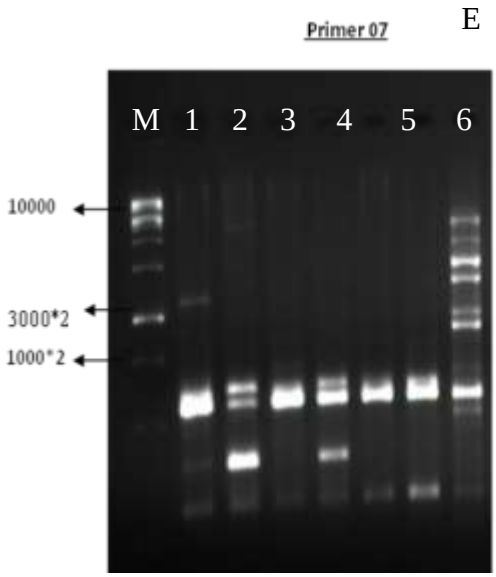
Fig- 6. A-F Randomly amplified polymorphic DNA (RAPD) banding profiles of Bacillus spp. with different primers

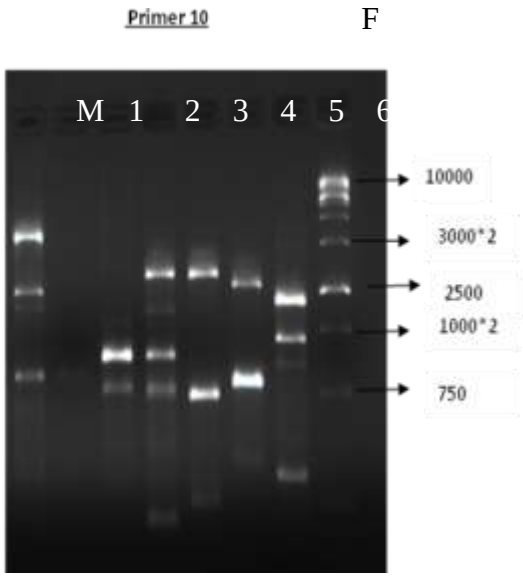


C









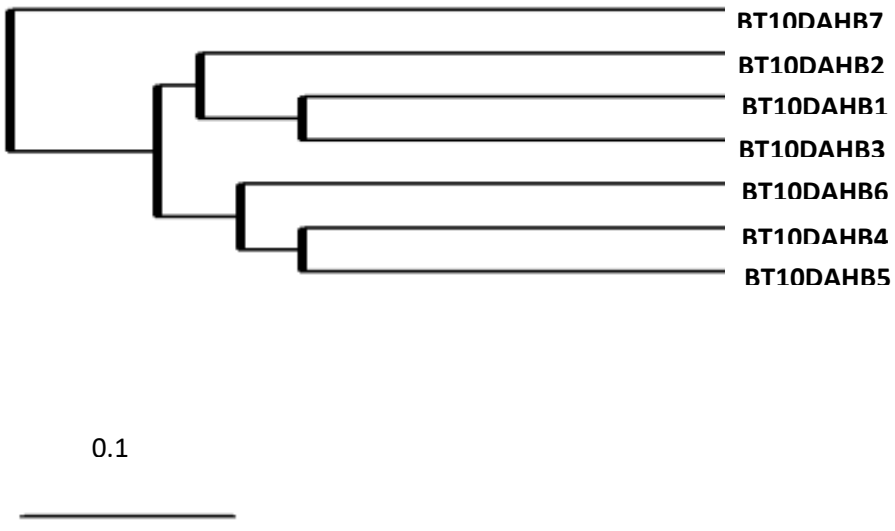
The Dendrogram (Fig-7) results explained that each *B. licheniformis* isolate from different locations in the East coast of India produced a unique RAPD profile and suggests that *B. licheniformis* isolates are genetically heterogeneous. Three major clusters (with two major branches) were resolved through UGPMA analysis of *B. licheniformis.* DNA fragments obtained

with six RAPD primers. Moreover, *B. licheniforms* isolated from commercial probiotic brands were clearly differentiated into separate branches than the other species (Groups I–II). Maximum genetic distance was found between BT10DAHB3 and BT10DAHB1, whereas the minimum was found between BT10DAHB6 and BT10DAHB7

Table -8. Genetic distance of few Bacillus strains was studied revealed through six RAPD 10 mer primers

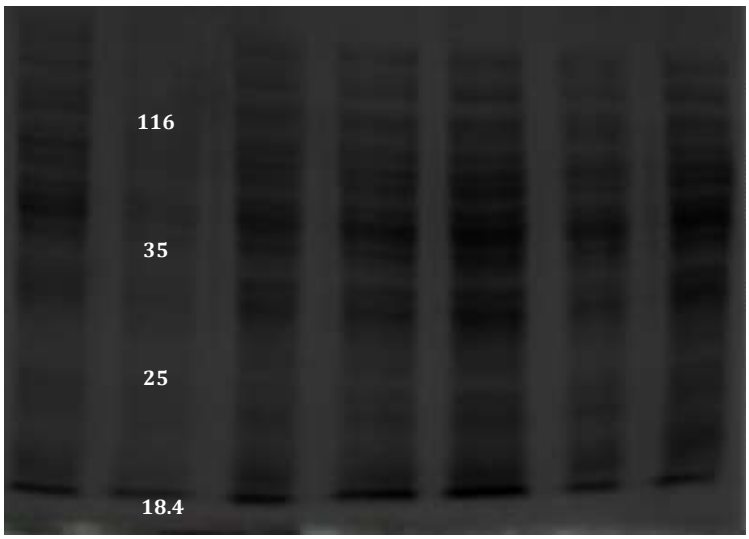
	BT10DAHB1	**BT10DAHB2**	**BT10DAHB3**	**BT10DAHB4**	**BT10DAHB5**	**BT10DAHB6**	**BT10DAHB7**
BT10DAHB1	0.00000						
BT10DAHB2	0.53171	0.00000					
BT10DAHB3	0.58377	0.46111	0.00000				
BT10DAHB4	0.45966	0.44394	0.48640	0.00000			
BT10DAHB5	0.54048	0.43537	0.57756	0.57756	0.00000		
BT10DAHB6	0.42460	0.41019	0.50463	0.50463	0.53433	0.00000	
BT10DAHB7	0.36775	0.31944	0.34459	0.34459	0.30253	0.27197	0.00000

Fig -7. Dendrogram of genetic relationship between seven isolates of Bacillus obtained with six RAPD primers. The scale below indicates the similarity index.



2.2.5. SDS-PAGE: The *Bacillus* spp. and *Vibrio* spp. protein profile were isolated to evaluate and differentiate in band patterns**.** The result showed that the band size of about 120 kda to 15kda (Fig-8). The protein profile explained that there is no dissimilarity among the isolates used. The gel was calibrated using molecular weight markers (ProGen and Fermentas) and stained either with comassive brilliant blue or silver nitrate. The bands that showed similarity were identified

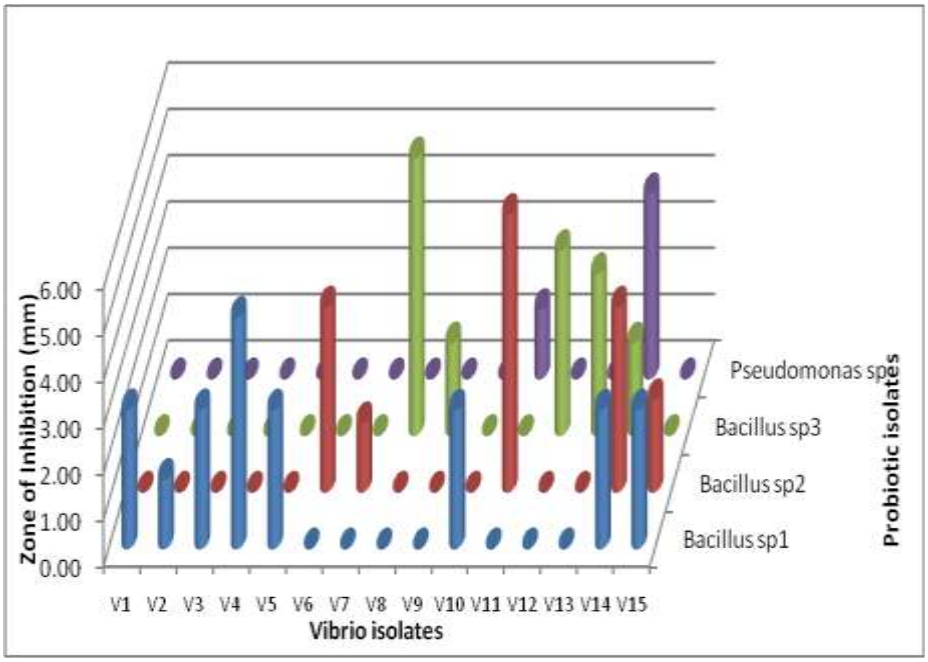
Fig- 8. Lane 1: Bacillus S2 (10µl), Lane 3: Bacillus S4 (10µl), Lane 4: Bacillus B6 (10µl), Lane 2: Protein Molecular Weight Marker (5µl), Lane 5: Bacillus B10 (10µl), Lane 6: Bacillus B12 (10µl), Lane 7: Vibrio V8 (10µl)



2.2.6. In vitro Antagonism Assay

Bacillus spp showed higher zone of inhibition were taken separately. *Bacillus* showed higher activity after 24 hrs and at the pH of (7 to 8). Bacterial viability was checked by heating at different temperature (40 ^{0}C-120 ^{0}C) and the best activity was shown at 45 ^{0} C to 50^{0}C. Based on the broad spectrum of antagonistic effect recorded, the selected isolate was used for further studies. Antagonistic activity of probiotic *Bacillus spp* and *Pseudomonas sp* against Vibrio (v1-v15) are represented in Fig- 9.

Fig- 9. Antagonistic activity of probiotic isolates Bacillus spp. from pond sediment samples against pathogenic Vibrio isolates (Well diffusion assay of cell free extracts)



2.2.7. In vitro Antagonism Assay of Commerical and wild *Bacillus* strains against *Vibrio* spp.

The cell-free extract of seven strains of *B. licheniformis* (B10DAHB1 to B10DAHB7), showed inhibitory activity against the tested Vibrios. Of these, *B. licheniformis* Dahb1 (Table-9) showed a higher inhibitory activity than the other six *B. licheniformis* tested. The diameters of the inhibitory zones around the growth of *Vibrio* spp. were about 6 -12 mm (Fig. 10). Furthermore, *B. licheniformis* Dahb1 showed inhibitory activity against 162 *Vibrio* spp. *V. parahaemolyticus*, (53 isolates), obtained from *P. monodon* culture ponds and from wild source. Based on the broad

spectrum of antagonistic effect recorded, this strain was used for further studies (16S rDNA typing and co-culture experiments). Among tested probiotic strains, *Bacillus* Dahb1 showed significant difference in *Vibrio* antagonism than any other strains ($p<0.05$). The growth of selected pathogenic *Vibrio* sp. *V. parahaemolyticus,* were inhibited by *B. licheniformis* Dahb1 (BT10DAHB1) culture inoculated at an initial level of 10^5–10^8 cfu ml^{-1} (Fig. 11). Lower concentrations of *B. licheniformis* Dahb1 (10^5 and 10^6 cfu ml^{-1}) allowed initial growth of these *Vibrio* spp, but CFU densities never reached the level of the control. High concentrations (10^8 cfu ml^{-1}) of *B. licheniformis* Dahb1 allowed an initial increase of the *Vibrio* spp followed by a decrease in the total viable counts (Fig-11). Co-culture experiment results showed that, when the concentration of *B. licheniformis* Dahb1 increased, the growth of *Vibrio* sp. *V.parahaemolyticus* were controlled under *in vitro* conditions.

Table- 9. Initial screening of Bacillus Dahb1-7 isolates against Vibrio spp.

S. No	Strain Name	Source of isolation	Inhibitions Zone against *Vibrio* spp (mm)	
			Vibrio spp	*V.parahaemolyticus*
1	*Bacillus licheniformis* Dahb1(BT10DAHB1)	Culture pond	13 ± 2^{a}	12 ± 2.5^{b}
2	*Bacillus* Dahb2(BT10DAHB2)	Culture pond	9 ± 2^{b}	7 ± 1.5^{b}
3	*Bacillus* Dahb3(BT10DAHB3)	Culture pond	7 ± 1.5^{b}	9 ± 2^{b}
4	*Bacillus* Dahb4(BT10DAHB4)	Culture pond	7 ± 1^{b}	10 ± 2^{b}
5	*Bacillus* Dahb5(BT10DAHB5)	Culture pond	10 ± 2^{b}	9 ± 1.5^{b}
6	*Bacillus* Dahb6(BT10DAHB6)	Epicin-Commercial brand	6 ± 1.5^{b}	7 ± 1.5^{b}
7	*Bacillus* Dahb7(BT10DAHB7)	Epicin-Commercial brand	8 ± 2^{b}	7 ± 1.5^{b}

Fig -10. Inhibitory activity of Bacillus licheniformis (A), (C) control and (i, ii, iii) Different concentration of bacillus cell free extract against the growth of pathogenic Vibrio spp isolated from Penaeus monodon culture ponds

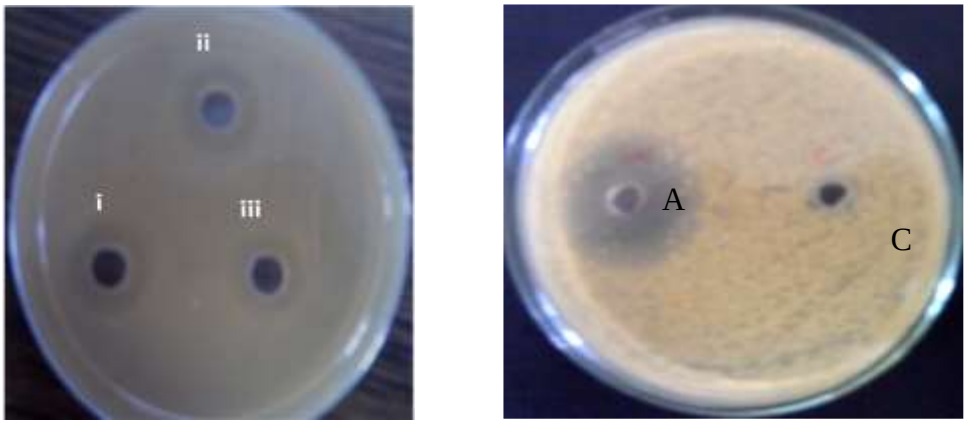
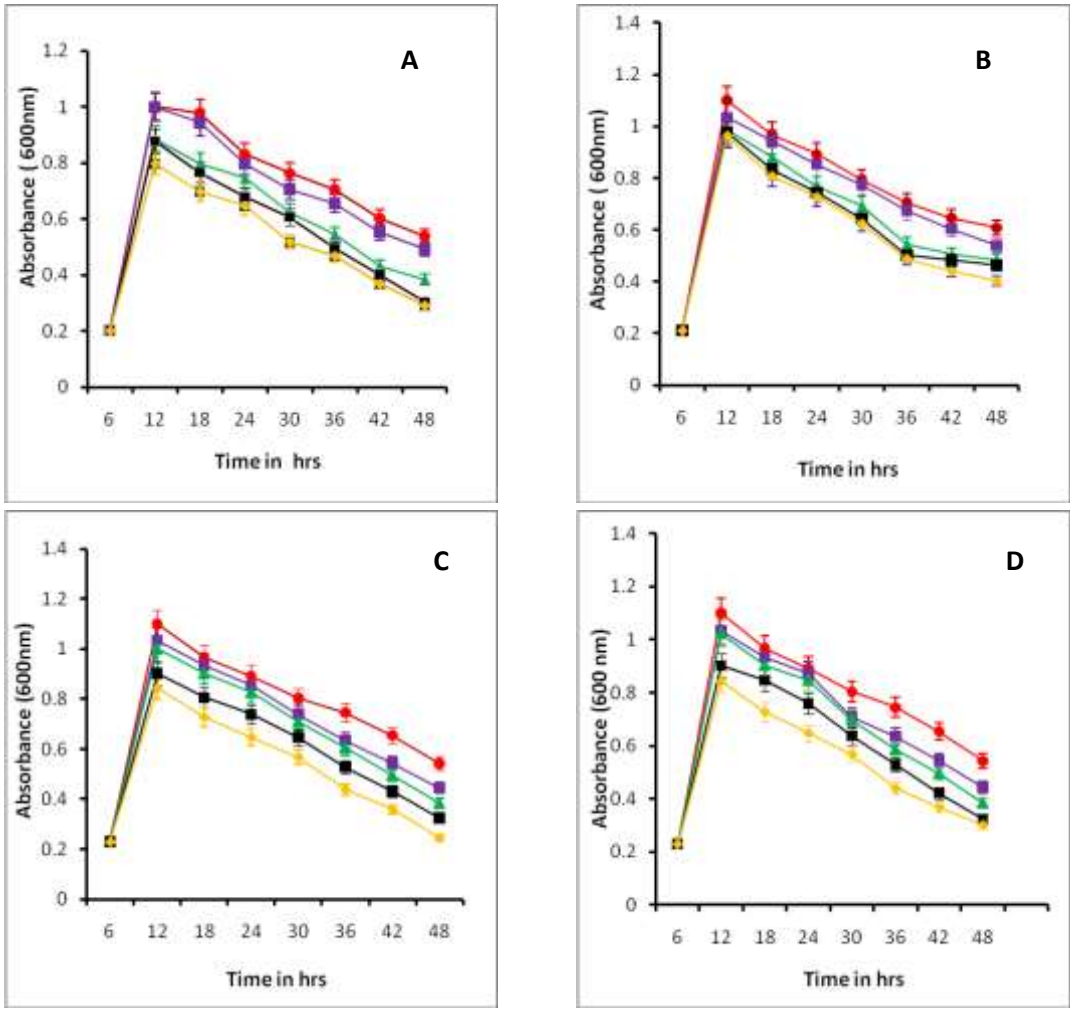


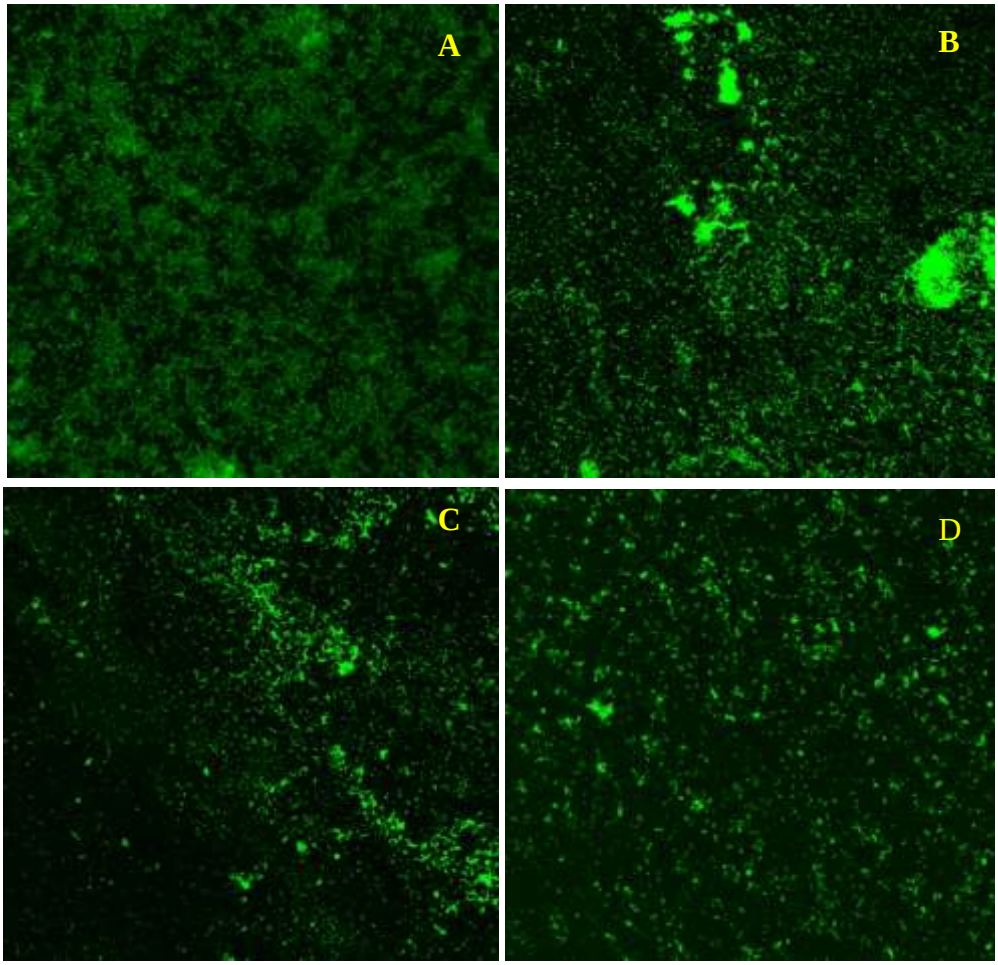
Fig -11. Inhibitory activity of Bacillus Dahb1-Dahb7 against the growth of pathogenic Vibrio spp isolated from Penaeus monodon culture ponds



2.2.8. Bioflim formation assay

Inhibitory activity of *Bacillus* Dahb1 cell free extract over bioflim (Fig; 12). (A) Control (B) cell free extract 50µl (C) Bacillus cell free extract 100µl (D) *Bacillus* cell free extract 150µl. Concentration of bacillus cell free extract was supplemented with the tested organisms and this cell free extract have should a promising activity over *Vibrio* bioflim formation. *Vibrio* bioflim were reduced at low concentration of the bacillus cell free extract and higher concentration of extract result in complete damage to bioflim formed by *Vibrio* spp. This showed that more of cell free extract concentration will prevent bioflim formation and there by improve the healthy environment in aquaculture environment.

Fig-12 (A) Control of Vibrio bio film (B) Treatment with bacillus cell free extract 50µl (C) Treatment with bacillus cell free extract 100µl (D) Treatment with bacillus cell free extract 150µl



2.2.9. Animal maintenance

All the FRP tanks were washed with disinfectant, continuous aeration was provided to maintain water temperature, pH and oxygen for maintenance of shrimp (Fig -13). One week after stocking the tank with the shrimps had received the same standard commercial diet before transferred in to the trail tank. The entire tank was covered with thin net to avoid jumping of shrimp to other tanks it all so protect the tank from dust.

Fig-13 Animal maintenance tank

2.2.10. In Vitro experiments

The cell free supernatant was obtained from over night grown culture by centrifuge at 5000 rpm for 15 min with pH 7 showed better antibacterial activity (Table-10). Epicin commercial used strain was consider as exotic probiotic strain, same method was followed to obtain cell free supernatant. When compared with *B. licheniformis*, Epicin showed less activity and its effect in liquid culture should less viability time.

Table -10 Inhibitory activitys of endogenous and exotic probiotic strain

Vibrio sp 10^4 dilution	Efficiency of probiotics in time (hrs/days)	Antagonistic activity of endogenus *strain B. licheniformis*	Antagonistic activity of exotic probiotic strain Epicin-Commercial brand
V.alginolyticus	2 hrs	+	+
V.alginolyticus	6 hrs	+++	++
V.alginolyticus	12 hrs	+++	++
V.alginolyticus	16 hrs	+++	++
V.alginolyticus	20 hrs	+++	++
V.alginolyticus	24 hrs	+++	+++
V.alginolyticus	32 hrs	++	++
V.alginolyticus	38 hrs	++	+
V.alginolyticus	42 hrs	++	-
V.alginolyticus	4 day	+	-
V.alginolyticus	6 day	+	-
V.alginolyticus	End of the trail	+	-

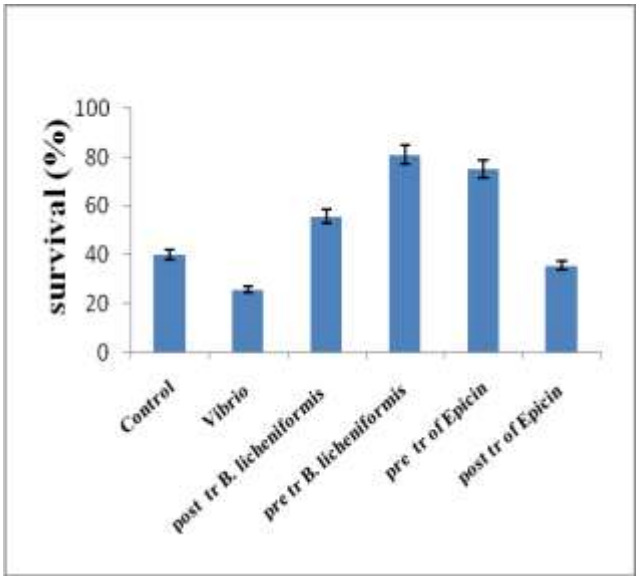
2.2.11. In Vivo challenge experiments

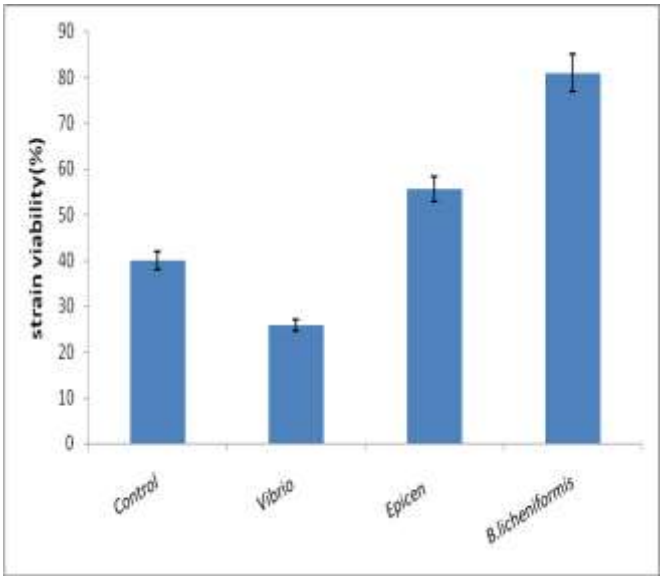
B. licheniformis used as probiotic strain in the trail, shrimps infected with *V. alginolyticus* showed high rate of mortality, where the pre and post treatment of *B. licheniformis* and *Epicin*

probiotics should a desirable variation from each of the treatments. The percentage of survival of shrimp with the exotic and endogenous probiotics (Fig-14) differs towards 3×10^4 cells per ml of *V. alginolyticus*. This infective dose was selected based on the results got earlier in our lab shrimp trail. All infected shrimp were maintained in segregation but under the same experimental conditions being fed with a compounded feed every day. Animals were observed for a week for signs of vibriosis and morbidity. At the end of trail surviving animals of each inoculated group were sacrificed hepatopancreas, muscle was dissected out under aseptic conditions, that were serially diluted and were plated onto TCBS Agar plates and incubated at 28 ± 2°C

Fig -14. Survival of shrimp in trail both with post and pre-treatment with exotic and endogenous Probiotics strain

Fig-15. Strain viability of exotic and endogenous





2.2.12. Water quality analysis

The temperature of shrimp ponds varied with the weather and solar intensity. Water temperatures were nearly the same in the ponds on each sampling, and there were no differences between treated and control ponds. The temperature of the ponds varied between 22.2^{o}C and 34.8^{o}C. The salinity which varied between 30 to 40 ppt and there was no significance in treated ponds compared with control ponds (Fig-17). The results of pH in the treated ponds with the probiotics and control ponds (Fig-16). The pH levels in the control ponds fluctuated considerably and in probiotics treated ponds were often at levels that would have no harmful to shrimp with the minimum and maximum values of 7.0 and 9.0, respectively. The value for transparency was an important index changed as pH increases.

Fig- 16. With and without probiotic treatment pH of water

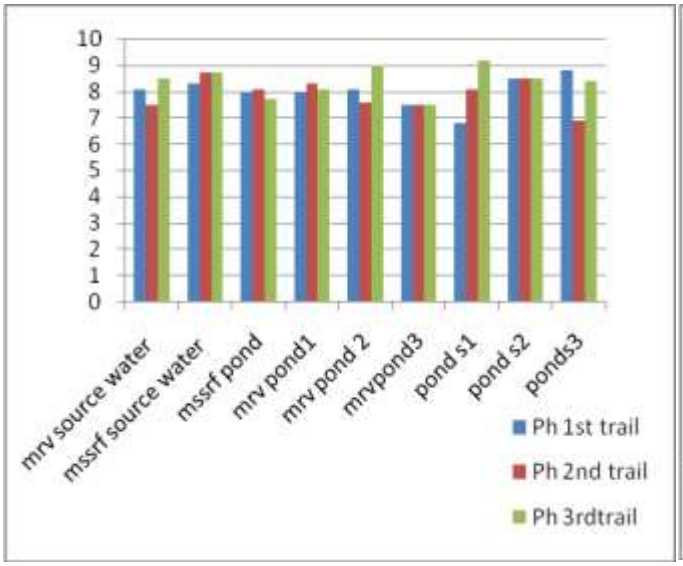
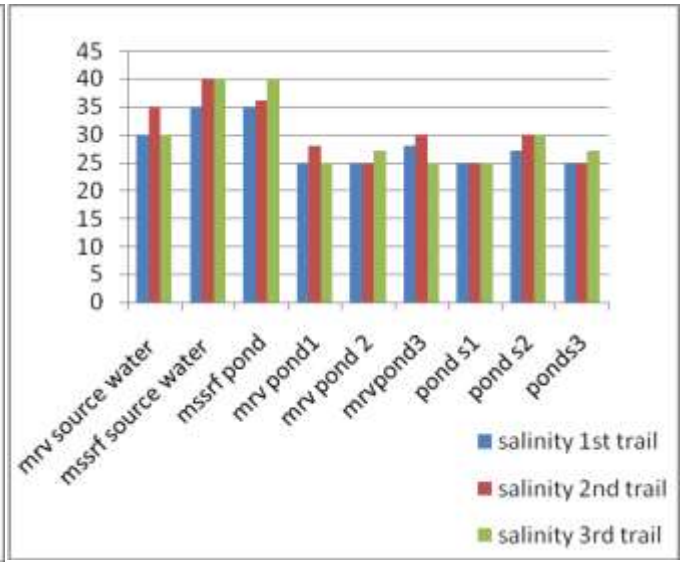


Fig -17. Salinity of water throughout the trail



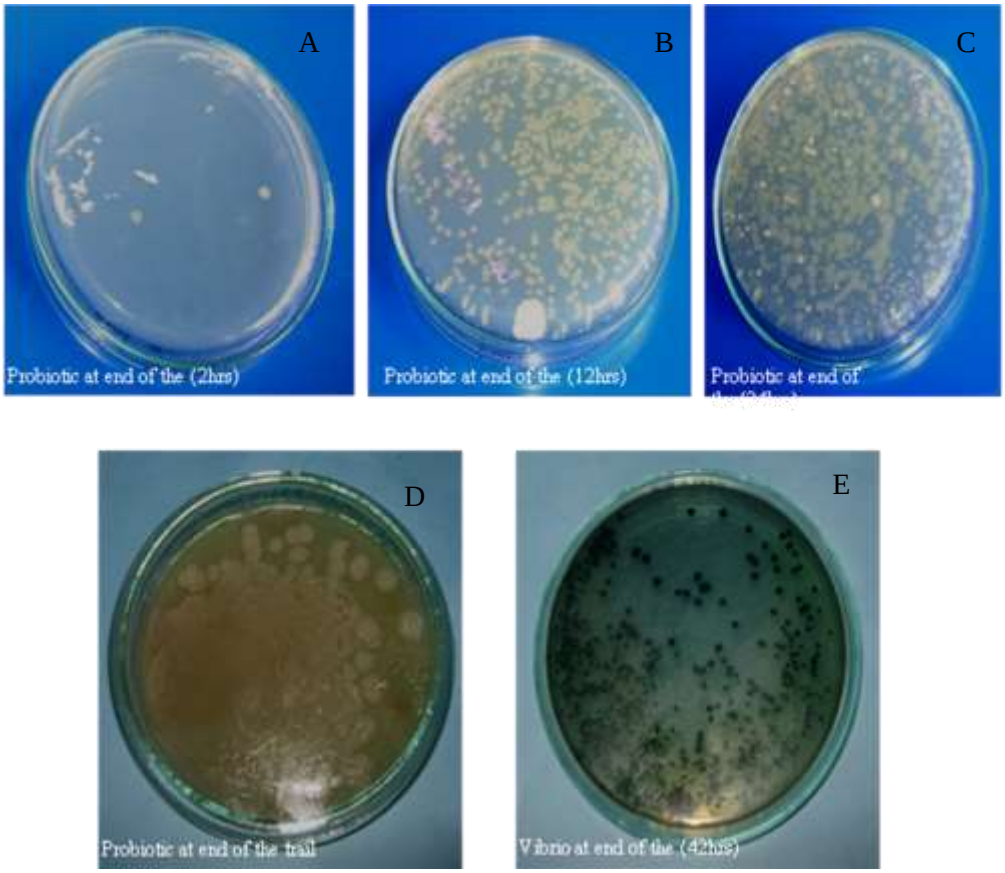
2.2.13. Haemolymph

Haemolymph samples plated on Marine Agar with 2% glycerol has showed a greater number of probotics bacterial count. *Vibrio* sp colonies produce a pigment in control pond sample. The number of putative *Vibrio* colonies counted was recorded for each animal for treated and in control pond differs greatly the number of colonies per infected animals were sampling weekly for shrimps in the ponds.

2.2.14. Gut microbiota

B. licheniformis was never detected in control pond animals. There were significant differences for the concentration of *B. licheniformis* recorded in the intestine before and after the feeding with *B. licheniformis*, but it was found successively on three weeks (Fig-18 A, B) with concentrations close to 10^2cful. Two hours after feeding, the concentrations of *B. licheniformis* reached mean value of 1.3×10^4cfu/ml throughout the experiment (Fig-18 C). Subsequently the probiotic concentration in the shrimp gut decreased to reach values in case of exotic, this strain needs more periodical adding of exotic probiotic strain. The average counts of heterotrophic bacteria including *Bacillus* Sp. (Fig-18D) and ammonifying bacteria were found to be significantly higher in treated ponds compared to control ponds with the exception of total plate count. The average counts of total plate count were 1.95 $x10^5$cfu/ml in water of treated ponds and 1.55 $x10^5$cfu/ml in water of control ponds. An increase in presumptive *Vibrio* count was observed in the water during the period of culture and the average density was up to 2.9 $x10^3$ cfu/ml.

Fig- 18. Colony at 2hrs for Bacillus (A) Colony at 12hr for Bacillus (B) Colony at 24hr for Bacillus (C) Heterotrophic bacteria Colony at end of trail (D) Colony of Vibrio at end of the trail (E)



Annexure-II

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ORGANISM Proteus sp. Dahp2
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Proteus.
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AUTHORS Vaseeharan,B., Prabhu,N.M., David Jayaseelan,B., Vinoj,G., Chen,J.C. and Lin,Y.C.
TITLE Antibacterial activity of silver nanoparticles against bacteria isolated from aquaculture systems
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1431)
AUTHORS Vaseeharan,B., Prabhu,N.M., David Jayaseelan,B., Vinoj,G., Chen,J.C. and Lin,Y.C.
TITLE Direct Submission
JOURNAL Submitted (09-AUG-2010) Animal Health and Management, Alagappa University, Alagapapuram, Karaikudi, Tamilnadu 630 003, India
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  AUTHORS   Vaseeharan,B., David Jayaseelan,B., Vinoj,G. and Chen,J.C.
  TITLE     Antibacterial activity of silver nanoparticles against bacteria
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1433)
  AUTHORS   Vaseeharan,B., David Jayaseelan,B., Vinoj,G. and Chen,J.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-AUG-2010) Animal Health and Management, Alagappa
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AUTHORS Vaseeharan,B. and Vinoj,G.
TITLE Screening of probiotic bacteria against pathogenic Vibrio strains isolated from Vibriosis-infected post-larval stages of Feneropenaeus indicus
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1377)
AUTHORS Vaseeharan,B. and Vinoj,G.
TITLE Direct Submission
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  AUTHORS   Vaseeharan,B. and Vinoj,G.
  TITLE     Screening of probiotic bacteria against pathogenic Vibrio
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            larval stages of Feneropenaeus indicus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1445)
  AUTHORS   Vaseeharan,B. and Vinoj,G.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-MAY-2010) Animal Health and Management, Alagappa
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AUTHORS David Jayaseelan,B., Esakkirajan,M., Vaseeharan,B. and Chen,J.C.
TITLE Isolation of Probiotic Bacteria from Aquaculture Environments
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1415)
AUTHORS David Jayaseelan,B., Esakkirajan,M., Vaseeharan,B. and Chen,J.C.
TITLE Direct Submission
JOURNAL Submitted (19-NOV-2010) Animal Health and Management, Alagappa University, Algappapuram, Karaikudi, Tamilnadu 630001, India
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      961 tttgaccact ctagagatag agctttccct tcggggacaa agtgacaggt ggtgcatggt
     1021 tgtcgtcagc tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc aacccttatt
     1081 gttagttgcc atcatttagt tgggcactct agcgagactg ccggtgacaa accggaggaa
     1141 ggtggggatg acgtcaaatc atcatgcccc ttatgacctg ggctacacac gtgctacaat
     1201 gggaagtaca acgagtcgct agaccgcgag gtcatgcaaa tctcttaaag cttctctcag
     1261 ttcggattgc aggctgcaac tcgcctgcat gaagccggaa tcgctagtaa tcgcggatca
     1321 gcacgccgcg gtgaatacgt tcccgggcct tgtacacacc gcccgtcaca ccacgagagt
     1381 ttgtaacacc cgaagtcggt gaggtaacct tttgg
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LOCUS HQ693278 1456 bp DNA linear BCT 08-JAN-2011
DEFINITION Bacillus thuringiensis strain Dahb4 16S ribosomal RNA gene, partial sequence.
ACCESSION HQ693278
VERSION HQ693278.1 GI:315932747
KEYWORDS .
SOURCE Bacillus thuringiensis
ORGANISM Bacillus thuringiensis
Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.
REFERENCE 1 (bases 1 to 1456)
AUTHORS Vaseeharan,B., David Jayaseelan,B., Rajakumaran,P. and Manju,S.
TITLE Isolation of probiotic bacteria from aquaculture environments
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1456)
AUTHORS Vaseeharan,B., David Jayaseelan,B., Rajakumaran,P. and Manju,S.
TITLE Direct Submission
JOURNAL Submitted (19-NOV-2010) Animal Health and Management, Alagappa University, Algappapuram, Karaikudi, Tamilnadu 630001, India
FEATURES Location/Qualifiers
source 1..1456
/organism="Bacillus thuringiensis"
/mol_type="genomic DNA"
/strain="Dahb4"
/db_xref="taxon:1428"
rRNA <1..>1456
/product="16S ribosomal RNA"
ORIGIN

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      121 tttgaaccgc atggttcgaa attgaaaggc ggcttcggct gtcacttatg gatggacccg
      181 cgtcgcatta gctagttggt gaggtaacgg ctcaccaagg caacgatgcg tagccgacct
      241 gagagggtga tcggccacac tgggactgag acacggccca gactcctacg ggaggcagca
      301 gtagggaatc ttccgcaatg gacgaaagtc tgacggagca acgccgcgtg agtgatgaag
      361 gctttcgggt cgtaaaactc tgttgttagg gaagaacaag tgctagttga ataagctggc
      421 accttgacgg tacctaacca gaaagccacg gctaactacg tgccagcagc cgcggtaata
      481 cgtaggtggc aagcgttatc cggaattatt gggcgtaaag cgcgcgcagg tggtttctta
      541 agtctgatgt gaaagcccac ggctcaaccg tggagggtca ttggaaactg ggagacttga
      601 gtgcggaaga ggaaagtgga attccatgtg tagcggtgaa atgcgtagag atatggagga
      661 acaccagtgg cgaaggcgac tttctggtct gtaactgaca ctgaggcgcg aaagcgtggg
      721 gagcaaacag gattagatac cctggtagtc cacgccgtaa acgatgagtg ctaagtgtta
      781 gagggtttcc gcccctttagt gctgaagtta acgcattaag cactccgcct ggggagtacg
      841 gccgcaaggc tgaaactcaa aggaattgac gggggcccgc acaagcggtg gagcatgtgg
      901 tttaattcga agcaacgcga agaaccttac caggtcttga catcctctga aaaccctaga
      961 gatagggctt ctccttcggg agcagagtga caggtggtgc atggttgtcg tcagctcgtg
     1021 tcgtgagatg ttgggttaag tcccgcaacg agcgcaaccc ttgatcttag ttgccatcat
     1081 taagttgggc actctaaggt gactgccggt gacaaaccgg aggaaggtgg ggatgacgtc
     1141 aaatcatcat gccccttatg acctgggcta cacacgtgct acaatggacg gtacaaagag
     1201 ctgcaagacc gcgaggtgga gctaatctca taaaaccgtt ctcagttcgg attgtaggct
     1261 gcaactcgcc tacatgaagc tggaatcgct agtaatcgcg gatcagcatg ccgcggtgaa
     1321 tacgttcccg ggccttgtac acaccgcccg tcacaccacg agagtttgta accacccgaa
     1381 gtcggtgggg taacctttt ggagccagcc gcctaaggtg ggacagatga ttggggtgaa
     1441 gtcgtaacaa ggtagc
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LOCUS       HQ693277                320 bp    DNA     linear   BCT 08-JAN-
2011
DEFINITION  Pseudomonas sp. Dahp4 16S ribosomal RNA gene, partial sequence.
ACCESSION   HQ693277
VERSION     HQ693277.1  GI:315932746
KEYWORDS    .
SOURCE      Pseudomonas sp. Dahp4
  ORGANISM  Pseudomonas sp. Dahp4
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 320)
  AUTHORS   Vinoj,G., Vaseeharan,B. and Prabhu,N.M.
  TITLE     Isolation and characterisation of bacterial strains from
            aquaculture environments
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 320)
  AUTHORS   Vinoj,G., Vaseeharan,B. and Prabhu,N.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-DEC-2010) Animal Health and Management, Alagappa
            University, Algappapuram, Karaikudi, Tamilnadu 630001, India
FEATURES             Location/Qualifiers
     source          1..320
                     /organism="Pseudomonas sp. Dahp4"
                     /mol_type="genomic DNA"
                     /strain="Dahp4"
                     /db_xref="taxon:937851"
     rRNA            <1..>320
                     /product="16S ribosomal RNA"
ORIGIN
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       61 aaagcagggg accttcgggc cttgcgctat cagatgagcc taggtcggat tagctagttg
      121 gtggggtaaa ggcctaccaa ggcgacgatc cgtaactggt ctgagaggat gatcagtcac
      181 actggaactg agacacggtc cagactccta cgggaggcag cagtggggaa tattggacaa
      241 tgggcgaaag cctgatccag ccatgccgcg tgtgtgaaga aggtcttcgg attgtaaagc
      301 actttaagtt gggaggaagg
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LOCUS       HQ693274                399 bp    DNA     linear   BCT 08-JAN-
2011
DEFINITION  Pseudomonas aeruginosa strain Dahp2 16S ribosomal RNA gene,
partial
            sequence.
ACCESSION   HQ693274
VERSION     HQ693274.1  GI:315932743
KEYWORDS    .
SOURCE      Pseudomonas aeruginosa
  ORGANISM  Pseudomonas aeruginosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 399)
  AUTHORS   Vinoj,G., Vaseeharan,B., David Jayaseelan,B., Latha,M. and
            Prabhu,N.M.
  TITLE     Isolation and screening of bacterial strains from aquaculture
            environments
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 399)
  AUTHORS   Vinoj,G., Vaseeharan,B., David Jayaseelan,B., Latha,M. and
            Prabhu,N.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-DEC-2010) Animal Health and Management, Alagappa
            University, Algappapuram, Karaikudi, Tamilnadu 630001, India
FEATURES             Location/Qualifiers
     source          1..399
                     /organism="Pseudomonas aeruginosa"
                     /mol_type="genomic DNA"
                     /strain="Dahp2"
                     /db_xref="taxon:287"
     rRNA            <1..>399
                     /product="16S ribosomal RNA"
ORIGIN
        1 gcatggctgt cgtcagctcg tgtcgtgaga tgttgggtta agtcccgtaa cgagcgcaac
       61 ccttgtcctt agttaccagc acctcgggtg ggcactctaa ggagactgcc ggtgacaaac
      121 cggaggaagg tggggatgac gtcaagtcat catggccctt acggccaggg ctacacacgt
      181 gctacaatgg tcggtacaaa gggttgccaa gccgcgaggt ggagctaatc ccataaaacc
      241 gatcgtagtc cggatcgcag tctgcaactc gactgcgtga agtcggaatc gctagtaatc
      301 gtgaatcaga atgtcacggt gaatacgttc ccgggccttg tacacaccgc ccgtcacacc
      361 atgggagtgg gttgctccag aagtagctag tctaaccgc
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LOCUS       HQ693273                347 bp    DNA     linear   BCT 08-JAN-
2011
DEFINITION  Bacillus pumilus strain Dahb3 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   HQ693273
VERSION     HQ693273.1  GI:315932742
KEYWORDS    .
SOURCE      Bacillus pumilus
  ORGANISM  Bacillus pumilus
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 347)
  AUTHORS   Vaseeharan,B., Vinoj,G., Clara,G.S. and Manju,S.
  TITLE     Isolation and chracterization of microbes from aquaculture
            environment
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 347)
  AUTHORS   Vaseeharan,B., Vinoj,G., Clara,G.S. and Manju,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-DEC-2010) Animal Health and Management, Alagappa
            University, Algappapuram, Karaikudi, Tamilnadu 630001, India
FEATURES             Location/Qualifiers
     source          1..347
                     /organism="Bacillus pumilus"
                     /mol_type="genomic DNA"
                     /strain="Dahb3"
                     /db_xref="taxon:1408"
     rRNA            <1..>347
                     /product="16S ribosomal RNA"
ORIGIN
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      121 atcatgcccc ttatgacctg ggctacacac gtgctacaat gggcagaaca aagggcagcg
      181 aagccgcgag gctaagccaa tcccacaaat ctgttctcag ttcggatcgc agtctgcaac
      241 tcgactgcgt gaagctggaa tcgctagtaa tcgcggatca gcatgccgcg gtgaatacgt
      301 tcccgggcct tgtacacacc gcccgtcaca ccacgagagt ttgtaac
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LOCUS       HQ693272                331 bp    DNA     linear   BCT 08-JAN-
2011
DEFINITION  Pseudomonas aeruginosa strain Dahp3 16S ribosomal RNA gene,
partial
            sequence.
ACCESSION   HQ693272
VERSION     HQ693272.1  GI:315932741
KEYWORDS    .
SOURCE      Pseudomonas aeruginosa
  ORGANISM  Pseudomonas aeruginosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 331)
  AUTHORS   Vaseeharan,B., Vinoj,G. and Esakkirajan,M.
  TITLE     Isolation and characterization of bacterial strains from
            aquaculture environments
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 331)
  AUTHORS   Vaseeharan,B., Vinoj,G. and Esakkirajan,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-DEC-2010) Animal Health and Management, Alagappa
            University, Algappapuram, Karaikudi, Tamilnadu 630001, India
FEATURES             Location/Qualifiers
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                     /organism="Pseudomonas aeruginosa"
                     /mol_type="genomic DNA"
                     /strain="Dahp3"
                     /db_xref="taxon:287"
     rRNA            <1..>331
                     /product="16S ribosomal RNA"
ORIGIN
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      121 acaatggtcg gtacaaaggg ttgccaagcc gcgaggtgga gctaatccca taaaaccgat
      181 cgtagtccgg atcgcagtct gcaactcgac tgcgtgaagt cggaatcgct agtaatcgtg
      241 aatcagaatg tcacggtgaa tacgttcccg ggccttgtac acaccgcccg tcacaccatg
      301 ggagtgggtt gctccagaag tagctagtct a
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LOCUS HQ116443 1427 bp DNA linear BCT 13-SEP-2010
DEFINITION Bacillus sp. DahB2 16S ribosomal RNA gene, partial sequence.
ACCESSION HQ116443
VERSION HQ116443.1 GI:306441136
KEYWORDS .
SOURCE Bacillus sp. DahB2
ORGANISM Bacillus sp. DahB2
Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE 1 (bases 1 to 1427)
AUTHORS Vaseeharan,B., Manikandan,R., David Jayaseelan,B., Vinoj,G., Lin,Y.C. and Chen,J.C.
TITLE Antibacterial activity of silver nanoparticles against bacteria isolated from aquaculture systems
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1427)
AUTHORS Vaseeharan,B., Manikandan,R., David Jayaseelan,B., Vinoj,G., Lin,Y.C. and Chen,J.C.
TITLE Direct Submission
JOURNAL Submitted (09-AUG-2010) Animal Health and Management, Alagappa University, Alagapapuram, Karaikudi, Tamilnadu 630 003, India
FEATURES Location/Qualifiers
source 1..1427
/organism="Bacillus sp. DahB2"
/mol_type="genomic DNA"
/strain="DahB2"
/db_xref="taxon:882681"
rRNA <1..>1427
/product="16S ribosomal RNA"
ORIGIN

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        1 gctatacatg caagtcgagc ggacagatgg gagcttgctc cctgatgtta gcggcggacg
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      121 aataccggat gcttgtttga accgcatggt tcaaacataa aaggtggctt cggctaccac
      181 ttacagatgg acccgcggcg cattagctag ttggtgaggt aatggctcac caaggcgacg
      241 atgcgtagcc gacctgagag ggtgatcggc cacactggga ctgagacacg gcccagactc
      301 ctacgggagg cagcagtagg gaatcttccg caatggacga aagtctgacg gagcaacgcc
      361 gcgtgagtga tgaaggtttt cggatcgtaa agctctgttg ttagggaaga acaagtaccg
      421 ttcgaatagg gcggtacctt gacggtacct aaccagaaag ccacggctaa ctacgtgcca
      481 gcagccgcgg taatacgtag gtggcaagcg ttgtccggaa ttattgggcg taaagggctc
      541 gcaggcggtt tcttaagtct gatgtgaaag cccccggctc aaccggggag ggtcattgga
      601 aactggggaa cttgagtgca gaagaggaga gtggaattcc acgtgtagcg gtgaaatgcg
      661 tagagatgtg gaggaacacc agtggcgaag gcgactctct ggtctgtaac tgacgctgag
      721 gagcgaaagc gtggggagcg aacaggatta gataccctgg tagtccacgc cgtaaacgat
      781 gagtgctaag tgttaggggg tttccgcccc ttagtgctgc agctaacgca ttaagcactc
      841 cgcctgggga gtacggtcgc aagactgaaa ctcaaaggaa ttgacggggg cccgcacaag
      901 cggtggagca tgtggtttaa ttcgaagcaa cgcgaagaac cttaccaggt cttgacatcc
      961 tctgacaatc ctagagatag gacgtcccct tcgggggcag agtgacaggt ggtgcatggt
     1021 tgtcgtcagc tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc aacccttgat
     1081 cttagttgcc agcattcagt tgggcactct aaggtgactg ccggtgacaa accggaggaa
     1141 ggtggggatg acgtcaaatc atcatgcccc ttatgacctg ggctacacac gtgctacaat
     1201 ggacagaaca aagggcagcg aaaccgcgag gttaagccaa tcccacaaat ctgttctcag
     1261 ttcggatcgc agtctgcaac tcgactgcgt gaagctggaa tcgctagtaa tcgcggatca
     1321 gcatgccgcg gtgaatacgt tcccgggcct tgtacacacc gcccgtcaca ccacgagagt
     1381 ttgtaacacc cgaagtcggt gaggtaacct ttaggagcca gccgccg
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LOCUS HQ693276 437 bp DNA linear BCT 08-JAN-2011
DEFINITION Vibrio sp. Dahv3 16S ribosomal RNA gene, partial sequence.
ACCESSION HQ693276
VERSION HQ693276.1 GI:315932745
KEYWORDS .
SOURCE Vibrio sp. Dahv3
ORGANISM Vibrio sp. Dahv3
Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 437)
AUTHORS Vaseeharan,B. and Vinoj,G.
TITLE Isolation and characterization of bacterial strains from aquaculture environment
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 437)
AUTHORS Vaseeharan,B. and Vinoj,G.
TITLE Direct Submission
JOURNAL Submitted (07-DEC-2010) Animal Health and Management, Alagappa University, Algappapuram, Karaikudi, Tamilnadu 630001, India
FEATURES Location/Qualifiers
source 1..437
/organism="Vibrio sp. Dahv3"
/mol_type="genomic DNA"
/strain="Dahv3"
/db_xref="taxon:937852"
rRNA <1..>437
/product="16S ribosomal RNA"
ORIGIN

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       61 ataccgcat gatgcctacg ggccaaagag ggggaccttc gggcctctcg cgtcaggata
      121 tgcctaggtg ggattagcta gttggtgagg taagggctca ccaaggcgac gatccctagc
      181 tggtctgaga ggatgatcag ccacactgga actgagacac ggtccagact cctacgggag
      241 gcagcagtgg ggaatattgc acaatgggcg caagcctgat gcagccatgc cgcgtgtgtg
      301 aagaaggcct tcgggttgta aagcactttc agtcgtgagg aaggtggtgt agttaatagc
      361 tgcattattt gacgttagcg acagaagaag caccggctaa ctccgtgcca gcagccgcgg
      421 taatacggag ggtgcga
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LOCUS HQ693275 366 bp DNA linear BCT 08-JAN-2011
DEFINITION Vibrio parahaemolyticus strain Dahv2 16S ribosomal RNA gene, partial sequence.
ACCESSION HQ693275
VERSION HQ693275.1 GI:315932744
KEYWORDS .
SOURCE Vibrio parahaemolyticus
ORGANISM Vibrio parahaemolyticus
Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 366)
AUTHORS Vaseeharan,B. and Vinoj,G.
TITLE Isolation and characterization of bacterial strains from aquaculture environment
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 366)
AUTHORS Vaseeharan,B. and Vinoj,G.
TITLE Direct Submission
JOURNAL Submitted (07-DEC-2010) Animal Health and Management, Alagappa University, Algappapuram, Karaikudi, Tamilnadu 630001, India
FEATURES Location/Qualifiers
source 1..366
/organism="Vibrio parahaemolyticus"
/mol_type="genomic DNA"
/strain="Dahv2"
/db_xref="taxon:670"
rRNA <1..>366
/product="16S ribosomal RNA"
ORIGIN
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61 tcccgcaacg agcgcaaccc ttatccttgt ttgccagcga gtaatgtcgg gaactccagg
121 gagactgccg gtgataaacc ggaggaaggt ggggacgacg tcaagtcatc atggcccttа
181 cgagtagggc tacacacgtg ctacaatggc gcatacagag ggctgccaac ttgcgaaagt
241 gagcgaatcc caaaaagtgc gtcgtagtcc ggattggagt ctgcaactcg actccatgaa
301 gtcggaatcg ctagtaatcg tggatcagaa tgccacggtg aatcacgttc ccgggccttg
361 taccac
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LOCUS HQ400663 1397 bp DNA linear BCT 13-NOV-2010
DEFINITION Pseudomonas aeruginosa strain Dahp1 16S ribosomal RNA gene, partial
sequence.
ACCESSION HQ400663
VERSION HQ400663.1 GI:311335043
KEYWORDS .
SOURCE Pseudomonas aeruginosa
ORGANISM Pseudomonas aeruginosa
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas.
REFERENCE 1 (bases 1 to 1397)
AUTHORS Vaseeharan,B. and Vinoj,G.
TITLE Screening of probiotic bacteria against pathogenic Vibrio strains isolated from Vibriosis infected post larval stages of Fenneropenaeus indicus
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1397)
AUTHORS Vaseeharan,B. and Vinoj,G.
TITLE Direct Submission
JOURNAL Submitted (13-OCT-2010) Animal Health and Management, Alagappa University, Alagappapuram, Karaikudi, Tamil Nadu 630003, India
FEATURES Location/Qualifiers
source 1..1397
/organism="Pseudomonas aeruginosa"
/mol_type="genomic DNA"
/strain="Dahp1"
/db_xref="taxon:287"
rRNA <1..>1397
/product="16S ribosomal RNA"
ORIGIN
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121 ccgcatacgt cctgagggag aaagtggggg atcttcagac ctcacgctat cacatgagcc
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301 cagtggggaa tattggacaa tgggcgaaag cctgatccag ccatgccgcg tgtgtgaaga
361 aggtcttcgg attgtaaagc actttaagtt gggaggaagg gcagtaagtt aataccttgc
421 tgttttgacg ttaccaacag aataagcacc ggctaacttc gtgccagcag ccgcggtaat
481 actaagggtg caagcgttaa tcggaattac tgggcgtaaa gcgcgcgtag gtggttcagc

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 541 aagttggatg tgaaatcccc gggctcaacc tgggaactgc atccaaacta ctgagctaag
 601 tacggtagag ggtggtggaa tttcctgtgt agcggtgaaa tgcgtagata taggaaggaa
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 901 aattcgaagc aacgcgaaga accttacctg gccttgacat gctgagaact ttccagaaat
 961 ggattggtgc cttcgggaac tcagacacag gtgctgcatg gctgtcgtca gctcgtgtcg
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1081 gggtgggcac tctaaggaga ctgccggtga caaaccggag gaaggtgggg atgacgtcaa
1141 gtcatcatgg cccttacggc cagggctaca cacgtgctac aatggtcggt acaaagggtt
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1261 aactcgactg cgtgaagtcg gaatcgctag taatcgtgaa tcagaatgtc acggtgaata
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