CHAPTER – X1

ISOLATION, CHARACTERISATION AND MASS CULTURE OF PUTATIVE PROBIOTIC BACTERIA- EFFECTS OF INOCULATION IN LARVAL TANKS

The realization of fact that use of antibiotics could promote the development of antibiotic-resistant bacteria in fish and the environment (Alderman and Hastings, 1998; Smith *et al.*, 1994; Pedersen *et al.*, 1995; Hameed and Balasubramanian, 2000), has led to several alternative strategies in disease control. The techniques used in order to increase larval survival, include the use of vaccines (Itami *et al.*, 1989; Bechteler and Holler, 1996; Pereira, 1996; Teunissen *et al.*, 1998), immunostimulants for the enhancement of the nonspecific defense mechanisms of the host (Raa, 1996; Sakai, 1999), and the use of probiotic bacteria (Garriques and Arevalo. 1995). Thus the emphasis in disease management is now on prevention, which is likely to be more cost-effective than cure.

Probiotics include microbial adjuncts that prevent pathogens from proliferating in the intestinal tract, on the superficial structures, and in the culture environment of the cultured species. A probiotic may secure optimal use of the feed by aiding in its digestion, that improve water quality, or that stimulate the immune system of the host. The use of probiotics has been studied extensively in terrestrial organisms (Conway, 1989). However, these methods have been studied only recently in marine environment as antagonists to disease in the culture of fish (Westerdahl *et al.*, 1991; Bergh, 1995), molluscs (Douillet and Langdon, 1994; Gibson *et al.*, 1998; Riquelme *et al.*, 1996; Avendano and Riquelme, 1999), live feed (Gatesoupe, 1994; Harzevelli *et al.*, 1998;

Gomez-Gil *et al.*, 1998) and in crustaceans (Maeda and Liao, 1992; Garriques and Arevalo, 1995; Rengpipat *et al.*, 1998a,b, 2000). Bacterial genus namely, *Aeromonas, Bacillus, Pseudomonas*, *Vibrio,* and *Cornybacterium* have been reported for their probiotic activity against pathogenic *Vibrio* (Gatesoupe, 1994; Moriarty and Body, 1995; Queiroz and Boyd, 1998; Gram *et al.*, 1999; Chythanya *et al.*, 2002).

In the hatcheries, it is assumed that uncontrolled development of the microbial communities is one of the major reasons for the unpredictable and often variable results. After maturation, spawners usually spawn axenic eggs in the water, whose surface then gets colonized by ambient bacteria. Furthermore, freshly hatched larvae do not have a fully developed gut and have no microbial community in the intestinal tract or on the exoskeleton. Thus the early stages of larvae depend for their primary microbiota partly on the water in which they are reared (Cahill, 1990; Ringo and Birkbeck, 1999).

Even after filtration, both pathogenic and non-pathogenic bacteria manage to enter the rearing system. In this case, instead of allowing spontaneous primary colonisation of the rearing water by bacteria accidentally present, the water could be preemptively colonized by the addition of probiotic bacteria, since it is generally recognized that preemptive colonisation may extend the reign of pioneer organisms (Atlas and Bartha, 1998). Microbial control of culture through preemptive colonisation of probiotic bacteria has been previously reported (Garriques and Arevalo, 1995; Verschuere *et al.*, 1999). Commensal *Vibrio* with inhibitory activity against pathogens have been isolated and used successfully in culture (Westerdahl *et al.*, 1991; Olafsen, 1998, Riquelme *et al.*, 1997; Sugita *et al.*, 1997).

In this study, putative probiotic bacteria were isolated from rearing water of well performing tanks. The bacteria were identified and analysed for its colonisation potential and for control of deleterious *Vibrio* spp. associated with culture through preemptive colonisation of the larviculture medium.

MATERIALS AND METHODS

In the first stage of this study, bacterial isolate was selected based on its positive effect on the larviculture under monoxenic conditions. In the second stage, xenic cultures were performed in media preemptively colonized by the selected bacterial strain, and their effect on the microbial community and larval performance were assessed.

Isolation and characterization of putative probiotic bacteria

The putative probiotic bacteria of *Vibrio* genus were isolated from the rearing water of well-performing larval rearing tanks. Numerically dominant yellow colonies in TCBS (Plate 6), associated with well performing tanks were initially segregated. Morphologically similar colonies were isolated and transferred to individual test tubes with sterile seawater and shaken for 5 minutes to homogenize the colonies which were then replated in TCBS agar to ascertain the purity of the colonies. The colonies were also plated on TSA agar with 2.5% NaCl and incubated for 24 hours at 28 °C.

Pure cultures of the bacterial strains were subjected to standard morphological, physiological and biochemical plate and tube tests. Since the focus was on the *Vibrio* genus, gram character (Buck, 1982), oxidase test, morphology, motility, oxidative-fermentative metabolism of glucose, sensitivity to the vibriostatic agent O/129 (2, 4-diamino-6, 7-diisopropylpteridine phosphate; Oxoid), and swarming in solid media

PLATE - 6

Bacterial culture of well-performed larval tank water in TCBS, exhibiting dominance of yellow colonies and rare green colonies

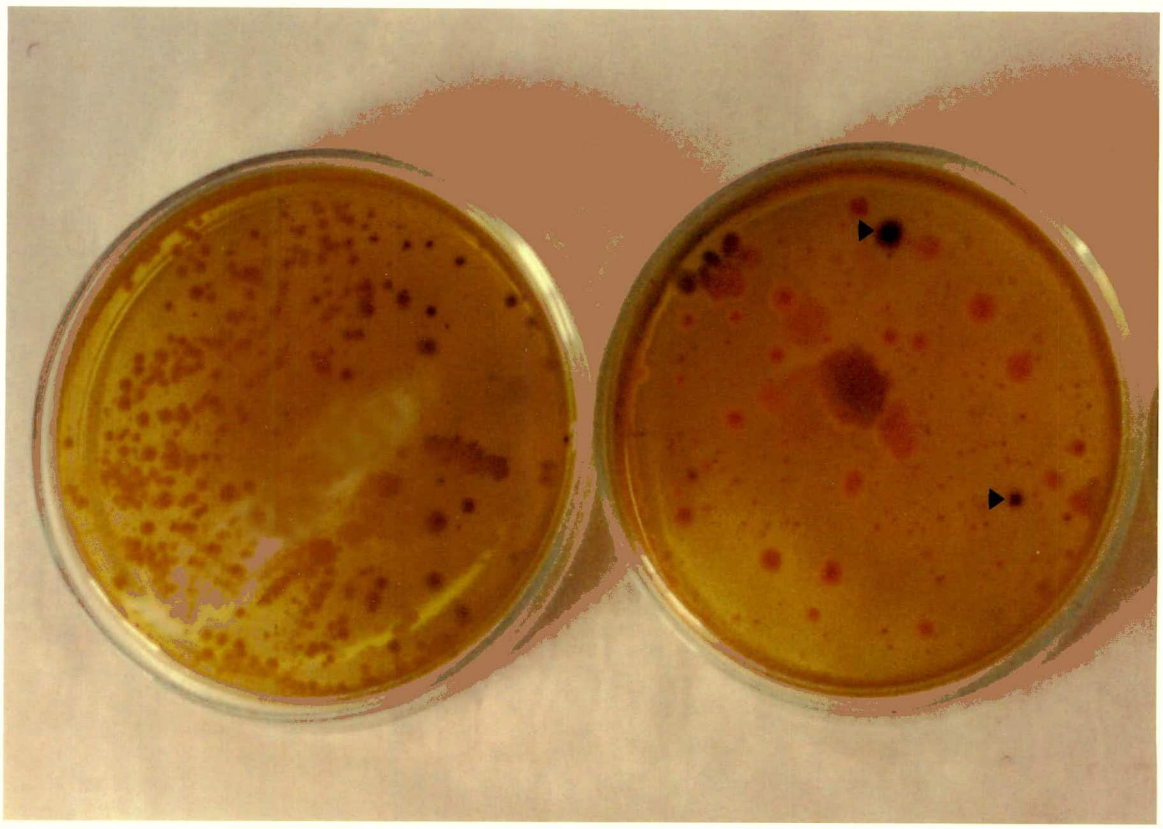


PLATE - 6

and growth on TCBS were the tests used to identify the isolates that are members of this genus. Identification of species was achieved following the scheme of Alsina and Blanch (1994) and Bergey's Manual of Systematic Bacteriology (Baumann and Schaubert, 1984) (Table 38).

Pathogenicity test under monoxenic conditions

This was done to examine the action of added microbiota on larvae and avoid interference by other microorganisms, About 40,000, nauplii 2 obtained from single spawner were thoroughly rinsed in sterile seawater on a, harvesting bucket (with 150 µm mesh), disinfected with 10 µg/ml of active iodine for 15 min and flushed using filtered seawater. About, 100 nauplii were macerated and plated on TCBS agar to test the disinfection. Roughly 10,000 nauplii were placed in each of four, 5 litre tanks filled with UV-treated seawater and kept at 30 °C with gentle filtered aeration and continuous illumination. One randomly purified colony of each *Vibrio* isolate was transferred to 10 ml of a 1% Trypticase peptone broth with 2.5% NaCl and incubated for 8 hours to achieve a 0.5 Mac Farland standard ($\pm 1 \times 10^6$ cells/ml).

The bacterial culture in the tubes were added to three of the 10 litre tanks to achieve an initial inoculation of approximately 3.5×10^3 cells/ml. One of the tank was kept as a control. After 24 hours 5×10^4 cells/ml of *Chaetoceros muelleri* from an axenic stock was inoculated to favour the feeding of first feeding stage, namely zoea. Samples of 100 animals were removed every 24 hours till the 72 hours. The larvae were checked for phototactic response, then analysed under the microscope for health status. After 72 hours, the test was terminated and the percentage survival was determined. The axenic condition of the control cultures was assessed regularly by inoculating 100 µl of the culture water on TSA agar.

Similar pathogenic test was also conducted with pathogenic luminescent *Vibrio harveyi* (strain PLB 3) at a concentration of 10^3 cfu/ml. The pathogen had been isolated from infected postlarvae.

Mass culture system for bacterial isolate

After identification of the putative bacterial strain, a mass culture system for bacterial isolate was devised to provide enough inoculum for the production tanks. A batch system similar to microalgae culture was used. Pure colonies from agar plates were suspended in test tubes containing 10 ml of sterile 2.5% peptone water with 2.5% NaCl and allowed to grow at 35°C for 8 hours. The 10 ml tubes with culture were diluted in 200 ml flasks then to one litre flask and finally to 4l flask. Each step required 8 hour culture time in sterile peptone water in 2% NaCl. The final batch was inoculated into a 500l fiberglass tank filled with UV-treated, 1 μ filtered seawater. In 500 l tanks, 1 kg of sucrose (table sugar), 10 g of $NaNo_3$ and 300 mg of $Na_2 PO_4$ were dissolved and bacterial culture was inoculated from one 4 l flask. The mixture was then gently aerated and incubated for 24 hours at 35 °C. After incubation, this culture was used as inoculum and was added to the selected spawning, larval rearing and algal tanks.

To prepare bacterial inoculum 10 ml of a fresh bacterial culture was centrifuged at 5,000 rpm for 10 min at 12 °C, the supernatant was then discarded, and the sediment was suspended in sterile saline solution. This process was repeated again. The cell concentration in the suspension was adjusted to an optical density (OD) of 1.00 at 610 nm in a spectrophotometer. Thus the bacterial concentration was standardized. To estimate the bacterial concentration achieved at the fixed OD level, the suspension was serially diluted in sterile saline and spread plated in TSA (Difco).

***In vivo* testing of the bacterial isolate in larval rearing**

The larval rearing experiments were carried out to evaluate the following (1) the effect of inoculating probiotic bacteria on the growth and survival of larvae and (2) influence of probiotic bacteria on the pathogenic *Vibrio* population.

UV-treated water filtered up to 5 micron was filled up to 60% in the 5 ton larviculture tanks. The water was treated with EDTA at 5 ppm level and aerated. The bacterial culture was inoculated in the culture tanks at a concentration of 5×10^3 cells/ml (day 0, V_a treatment). After cross checking the bacterial density, the microalgae (*Chaetoceros muelleri*) was pumped to the larval rearing tanks to a density of 1×10^5 cells/ml. Nauplii from the hatching tanks were flushed in the harvesting bucket with filtered water of similar temperature and transferred after sampling and acclimation. *Artemia* was fed from mysis 1 stage. Water exchange regime included filling up to total capacity till zoea 3 stage, 30% exchange at mysis 1 and 50% till postlarva 1. Second bacterial inoculation was not done as both water and larval samples indicated the dominance of probiotic bacteria.

Bacterial cultures were made from samples of live animals (Z_1 100 numbers, M_1 75 numbers and PL_1 50 numbers), which were rinsed with distilled water and macerated in 0.25 ml sterile seawater. This macerate was diluted to 10 ml with sterile seawater and 0.1 ml was plated on TCBS agar, and then incubated at 28°C for 24 hours Counts of yellow and green colonies were recorded. At harvest, random samples of 75 postlarvae from each tank were blotted on paper towel and weighed on a analytical balance.

In another set of tanks, after twenty-four hours of nauplii stocking and inoculation of probiotic bacteria (at 10^5 cfu/ml concentration), the larval culture waters were experimentally inoculated with *V. harveyi* at a concentration of 10^3 cfu/ml (V_a + V_h). Other treatments included control tanks with no bacterial inoculation and tanks infected only with *V. harveyi* PLB3 at a concentration of 10^3cfu/ml (V_h). The larval samples and rearing water were analysed and survival till PL_1 stage was recorded. All treatments were carried out in triplicates.

Colonisation capacity of selected bacterial strains and the pathogen

The colonisation of zoeae by the selected bacterial strain was quantified 24 h after the inoculation of the culture water. Therefore, 25 zoeae were cultured in the water preemptively colonized by the selected strain (10^4 cfu/ml) and were harvested one day after. The zoeae were rinsed, put on a sterile 150-µm-pore-size nylon filter and rinsed with sterile seawater. The sample was homogenized for 5 min and used for bacterial culture. The same culture procedure was carried out simultaneously with PLB3 also (at an initial concentration of 10^4 cfu/ml).

Pathogen challenge test

Postlarvae 3 from both control and experimental tanks were transferred to four, 5 liter tanks at a density of 60/l. Immediately after the transfer of postlarvae, the culture water was inoculated with pathogenic luminescent bacterial strain (*Vibrio harveyi* PLB3). The challenge test was conducted at a concentration of 2.5×10^3 cfu/ml. In control tanks, also postlarvae were exposed to pathogen under similar concentration. The survival of the PL was recorded two days after the inoculation.

RESULTS

Bacterial characterization

The putative bacteria were confirmed as *Vibrio* genus, as they were gram-negative short rods, oxidase positive, and motile, had fermentative metabolism of glucose, and were sensitive to the vibriostatic agent O/129. The *Vibrio* isolate was able to utilize sucrose, D-mannitol, and L-leucine, but, did not utilize cellobiose, lactose, L-arabinose, D-elibiose, L-rhamnose, β-hydroxybutyric acid or γ-aminobutyric acid (Table 38). Moreover, the isolate was negative for β-galactosidase (O-nitrophenyl-β-D-galactosidase (ONPG) and positive for nitrate reduction, and gelatinase. The isolates swarmed in solid media and they were non-pigmented colonies in TSA, while in TCBS, they formed big smooth yellow colonies. The isolate required Na+ to grow and were positive for oxidase, catalase, indole and Voges–Proskauer. With these characteristics, it was identified as *Vibrio alginolyticus*. At an optical density 1 at 610 nm wave length, the isolate gave a concentration of 3.42×10^7 cfu/ml.

Pathogenicity test

The 72 hour pathogenicity tests by rearing of nauplii with *V. alginolyticus* strain gave larval survival of 88% and 91% with the control and probiotic bacteria inoculated tanks respectively. At the end of experiment, the larvae were active and showed a good phototactic response. With *V. harveyi*, there was 100% larval mortality at the end of 72 hours.

Colonisation potential

The colonisation potential of both probiotic and pathogenic bacteria on larvae after 24 hours of exposure was estimated. The probiotic bacteria showed higher colonisation

Table 38. Main biochemical characteristics of the strain of *V. alginolyticus* isolated from rearing water of well performed tanks.

Characteristics	*V. alginolyticus*
Gram	–
Motility	+
Oxidase	+
Catalase	+
TCBS	+ (yellow colony)
Swarming on solid media	+
Arginine dihydrolase	–
Ornithine decarboxylase	+
Lysine decarboxylase	+
O/F glucose	+/+
Citrate	+
Indole	+
Voges– Proskauer	+
ONPG	–
H2S production	–
Na+ required for growth	+
Gelatinase	+
Urease	–
Growth at (°C):	
4	–
15	+
37	+
Acid from:	
Glucose	+
Mannitol	+
Inositol	–
Sorbitol	–
Rhamnose	–
Sucrose	+
Melibiose	–

recording a value of 8.41 (± 2.38) x 10^2 cfu/larvae and the pathogen had a lesser colonisation potential with the larvae recording 3.18 (± 1.19) x 10^2 cfu/larvae.

Larval rearing experiments

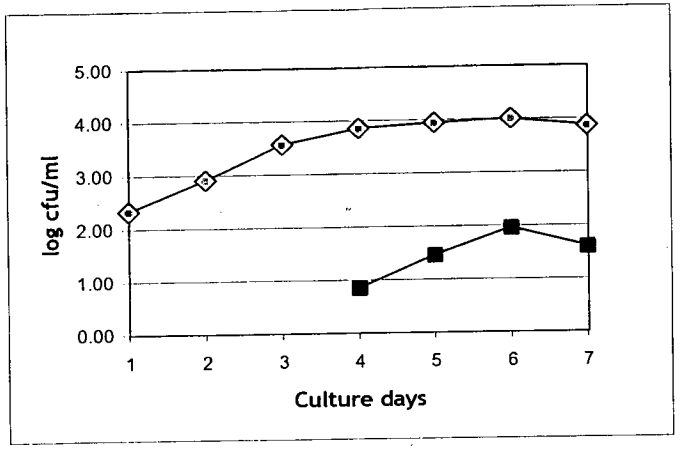
The results of the experiments are presented in Fig. 32-33. In the rearing water samples, even in control tanks where no bacteria was inoculated also exhibited the dominance of yellow colonies (cross checked for *V. alginolyticus*). The appearance of green colonies coincided with the introduction of *Artemia*, indicating the possibility of the latter serving as a vector. Despite, continued *Artemia* feeding green colonies could not dominate, but were present in water till the end of the experiment. Yellow colonies kept increasing in concentration and attained its asymptotic at 10^3 levels.

The larval tanks with inoculation of probiotic bacteria were prominent by the faster multiplication of yellow colonies, reaching 10^4 levels on day 2 itself and attained the maximum concentration 3.3 x 10^5 cfu/ml in the mysis 3 tank. The green colonies were not detected even in *Artemia*-fed tanks.

In the V_a + V_h treatments, the *V. harveyi* colonies were detected till the fifth day (but in meager quantities on the last day) and then disappeared. The growth of V_a colonies was slightly slower in the initial days and then picked up. Tanks with V_h inoculation indicated pure dominance of the same through out the experimental period. The pathogenic concentration of 10^3 level was noticed on the 3rd day, resulting in total collapse of the tank on 6th day.

In larval samples, generally the bacterial counts were higher than the respective water samples. The dominance of colonies showed similar pattern as in water. However, there was a clear indication of the dominance of yellow colonies when it comes

Fig. 32. The *Vibrio* count (both yellow and green colonies) of larval rearing water samples from (A) control, (B) tank inoculated with probiotic bacteria alone, (C) tank inoculated with probiotic and pathogenic bacteria and (D) tank inoculated with pathogenic bacteria alone.



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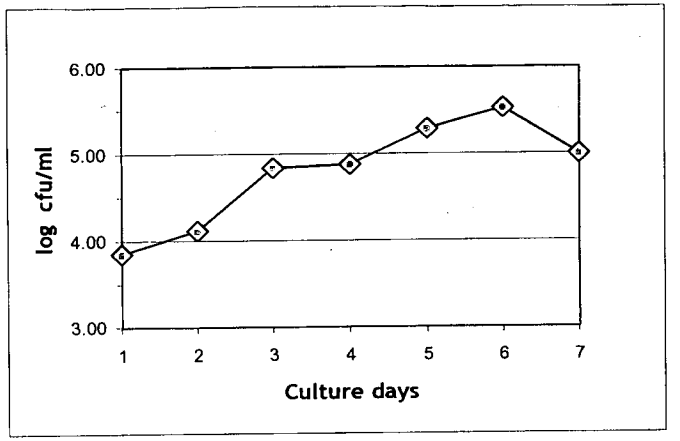

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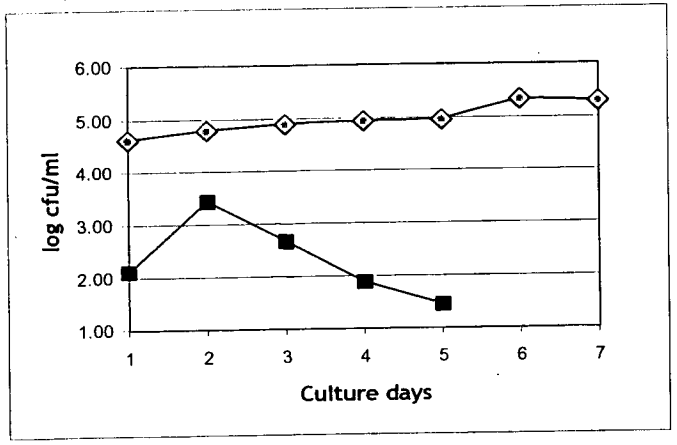

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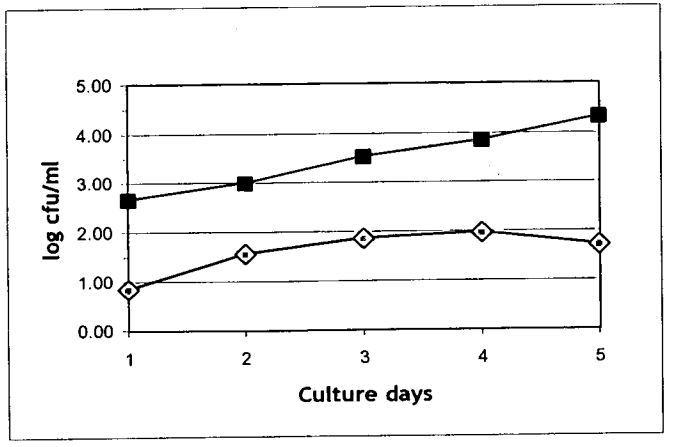

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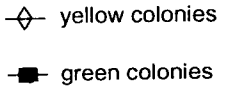
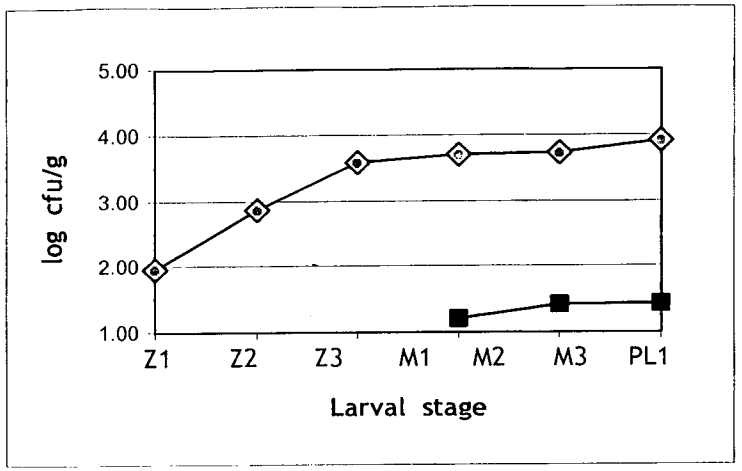


Fig. 33. The *Vibrio* count (both yellow and green colonies) of larval samples from (A) control, (B) tank inoculated with probiotic bacteria alone. (C) tank inoculated with probiotic and pathogenic bacteria and (D) tank inoculated with pathogenic bacteria



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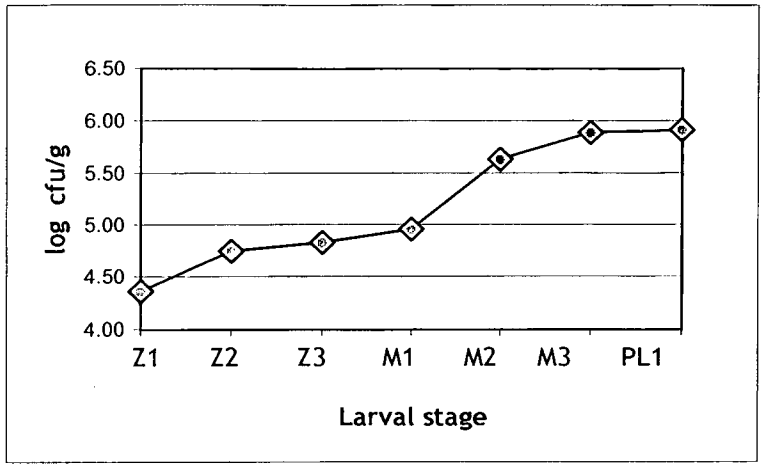

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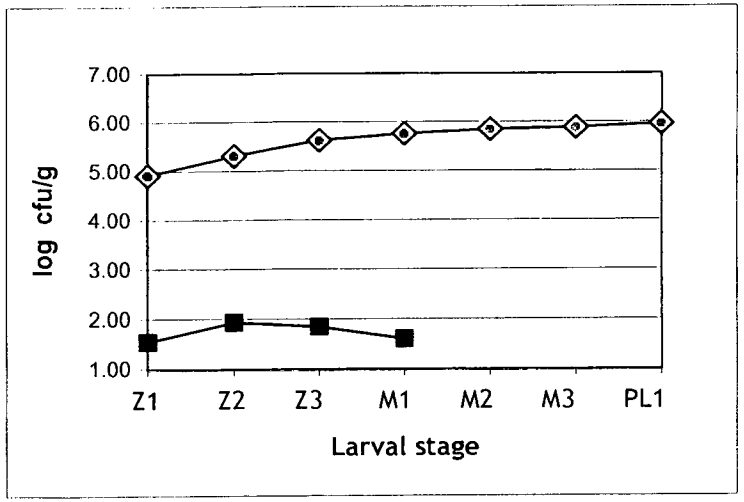

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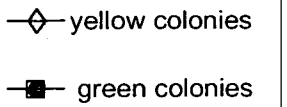

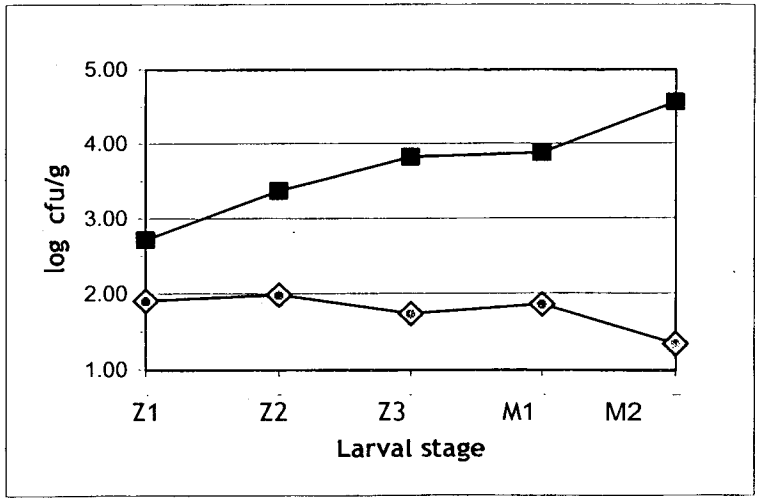

D

to colonizing the animal. V_a + V_h treatment tanks though indicated high number of V_h colonies in water samples, the larval samples gave only 10^1 level counts. In the V_h inoculated tanks, the pathogen dominated the microflora resulting in total collapse of the tank on the sixth day. The weak, moribund larvae recorded green colonies in the range of 1.7 and 4.9 x 10^4 cfu/g.

Table 39. Survival and wet weight of postlarvae from control tanks and tanks inoculated with probiotic bacteria (V_a) , tanks inoculated both with probiotic and pathogenic bacteria (V_a + V_h).

Treatment	Survival (%)	Wet weight (mg)	Challenge test survival (%)
Control	63.67 ± 2.87 [a]	2.42 ± 0.063[a]	34.7 ± 2.53[a]
V_a	74.00 ± 1.91[b]	2.54 ± 0.059 [b]	62.3 ± 2.95[b]
V_a+V_h	56.00 ± 3.66[a]	2.38 ± 0.061[c]	56.7 ± 2.80[b]

* No V_h treatment in the table as all the larvae died before reaching PL stage.

The survival and wet weight of postlarvae from the treatments are given in table 39. The survival value of V_a treatment was significantly higher than both control and V_a + V_h treatments ($P<0.05$). The V_a + V_h treatment though recorded the lowest survival value was not significantly lesser than the control ($P>0.05$). With the PL_1 wet weight, all the treatments differed significantly ($P<0.05$). The probiotic bacteria alone added tanks gave the highest value followed by control and V_a + V_h. The 48 hours pathogen challenge test recorded higher PL survival (at PL_3 stage) in tanks that were inoculated with probiotic bacteria. The treatments with inoculation of both probiotic and pathogenic bacteria recorded survival value slightly lesser than the V_a treatment but the values were not significantly different ($P<0.05$).

DISCUSSION

In hatcheries, vibriosis has been mostly caused by dominance of *V. harveyi*. Vandenberge *et al.* (1999) reported that in 60% of the vibriosis outbreaks in *Litopenaeus vannamei* postlarval stages, *V. harveyi* was isolated as the dominant bacterium. Occurrence of nauplii, larval and postlarval mortality caused by luminescent *V. harveyi* has been reported in commercial hatcheries (Sunaryanto and Mariam, 1986; Tansutapanit and Ruangpan, 1987; Lavilla-Pitogo *et al.*, 1990, Baticados *et al.*, 1990; Karunasagar *et al.*, 1994). The authors proved pathogenicity of the bacteria in all three larval stages (zoea, mysis and postlarva). Studies have also reported the biological control of *V. harveyi* using probiotic bacteria especially with *Artemia* (Patra and Mohammed, 2003) and shrimp (Rengpipat *et al.*, 1998a; Chythanya *et al.*, 2002).

The appearance of green colonies (*V. harveyi*) in hatchery larval rearing water was usually associated with lower survival or total collapse of the tank. It is for this reason that the probiotic bacteria were tested against the particular pathogen. Previous studies have reported the usage of *Vibrio* spp. including *V. alginolyticus* as probiotic in aquaculture (Garriques and Arevalo, 1995; Gomez-Gil *et al.*, 1998; Olafsen, 1998 ; Riquelme *et al.*, 1997).

Results from the present experiment clearly indicated that *V. alginolyticus* was able to restrict the growth of *V. harveyi* PLB3, especially when the former was present in higher initial density (10^4 cfu/ml) in the rearing water. Still higher initial densities of probiotic bacteria might still bring down the period the green colonies were detected in rearing water. The dominance of yellow colonies (numerically dominant ones were found to be *V. alginolyticus*) 24 hours after stocking itself (10^2 cfu/ml) suggest the

possible inoculation of same by microalgae added to the tank. The initial inoculation in higher numbers would have resulted in unhindered colonisation of external and internal surfaces by the probiotic bacteria, especially the microflora-free gut. The culture of a potentially probiotic *V. alginolyticus* C7b strain with *Chaetoceros muelleri* has been reported (Gomez - Gil *et al.*, 2002).

The appearance of green colonies coincided with *Artemia* feeding and it is possible that the live feed served as a vector for the pathogen. Though, *Artemia* cysts were disinfected and nauplii were flushed well before feeding, the contamination was still there. The bacterial recolonisation of disinfected *Artemia* cysts has been reported by Sorgeloos *et al.* (2001). The appearance of green colonies in the control tanks and the failure of same with V_a and V_a+V_h treatments clearly indicate the inhibitory effect of probiotic bacteria.

It was also striking to note that the colonisation capacity of *V. alginolyticus* was slightly higher than that of *V. harveyi* PLB3. The colonisation capacity is related to the competition for adhesion sites on or in the larvae. The same may also be a consequence of a more efficient use of resources like nutrients and available energy present in the ambient environment. A positive correlation between the colonisation potential and the protective ability of the selected probiotic strains for *Artemia* has been reported (Verscheure *et al.*, 2000). So, it could be assumed that if inoculated at similar densities the probiotic one will dominate the culture after certain period. Nevertheless, the explosive growth capacity of pathogen (in the presence of probiotic bacteria at lower density level 10 cfu/ml) in the V_h treatment even at lower densities explains the virulence of the pathogen, causing mortality of the infected larvae. Decrease in the density of

luminescent *Vibrio* in shrimp ponds has been achieved with the addition of probiotic *Bacillus* sp. to the culture water (Moriarty, 1998).

Comparison of larval and rearing water counts from the control samples depicted higher counts of green colonies in rearing water than in larvae. This well suggests lesser ability of the pathogen to proliferate in the gut or attach to external surface. Interestingly, the bacterial counts were usually higher with larval samples than in rearing water. The lower *Vibrio* count in the rearing water compared to larvae might be due to the fact that larval surface provide a suitable micro-environment for bacterial growth (Stevenson, 1978).

There was an increase in density of bacteria in rearing water as well as in larvae with increase in larval stages and culture days. It might be that the bacteria developed faster during the later period due to the nutrient enrichment of the culture medium and the ingestion of the bacteria might have increased due to its adhesion to the food particles. The amount of feed fed to the larvae also increased with increase in culture days. Hameed (1993) reported an increase in heterotrophic bacterial count from egg to PL where *Vibrio* was dominant (25-32%).

The significantly higher survival obtained with *V. alginolyticus* addition, very much ascertain the positive effect of the probiotic bacteria. Other than being able to prevent the proliferation of opportunistic pathogens, the added probiotic bacteria might remove toxic metabolic substances that could adversely affect the growth and survival of the larvae especially under the suboptimal conditions. Bacteria are reported to contribute to the nutritional value of foods by being a major source of protein, amino acids and vitamins (Gorospe *et al.*, 1996). The results of Intriago and Jones (1993) suggested that

the bacteria also assisted in the digestion of the unicellular algae, although convincing evidence was not provided. Similarly, extracellular enzymes may be produced by the bacteria, helping in the breakdown of refractory compounds of the food (Hood *et al.*, 1971).

Interestingly, the closer, non significant PL_1 survival value reported between control and V_a+V_h treatments suggest lesser pathogenicity of *V. harveyi* in the presence of probiotic bacteria. The survival also seems to be more influenced by the bacterial density in larvae than the rearing water.

The higher survival reported with V_a treatment in challenge test explain the improved disease resistance capacity of the postlarvae due to probiotic bacteria. The lower survival in control tanks compared to other treatments must be due to lower density of probiotic bacteria in PL_1 and also due to the presence of green colonies at this stage. It is very much possible that green colonies with the lesser concentration of probiotic bacteria took domination during the challenge test period resulting in poor survival. The pathogenic concentration for *V. harveyi* for the challenge test was similar to the 10^3 cfu/ml concentration noticed by Prayitno and Latchford (1995) as pathogenic to *Penaeus monodon* larvae.

Rengpipat *et al.* (2000) reported that juveniles exposed to probiotic bacteria (Bacillus S11) when challenged with *V. harveyi* gave better survival. They also suggested that effective probiotic treatments, may provide broader-spectrum and greater non-specific disease protection as a result of both serological immunity enhancement and competitive exclusion in shrimp gut.

The positive results on larvae with usage of probiotic bacteria have been previously reported. The usage of different *V. alginolyticus* strains as probiotic bacteria has been reported in fish (Austin *et al.*, 1995; Gatesoupe *et al.*, 1997), *Artemia* (Gomez-Gil *et al.*, 1998) and shrimp (Garriques and Arevalo, 1995). *V. alginolyticus* was found to be a dominant *Vibrio* species associated with healthy larvae (Vandenberghe *et al.*, 1998). Garriques and Arevalo (1995) first reported the usage of probiotic *V. alginolyticus* in a commercial shrimp hatchery in Ecuador. The probiotic treated tank also did not show any appearance of pathogenic *V. parahaemolyticus*. Similarly, when another strain of *V. alginolyticus* C 14 was used as probiotic with *Artemia* there was a decrease in mortality of *Artemia* nauplii when challenged with *V. parahaemolyticus* (Gomez-Gil *et al.*, 1998). Austin *et al.* (1995) reported that probiont *V. alginolyticus* applied to salmon could reduce the diseases caused by *Aeromonas salmonicida, V. anguillarum*, and *V. ordalli.*

To summarize, this experiment has confirmed that the preemptive colonisation of the culture medium with *V. alginolyticus* strain led to a manipulation of the ambient and associated microbiota, resulting in higher survival and growth of the larvae. In the present study the probiotic strain arrested the proliferation of deleterious *V. harveyi.* So, the results from the present study added to the earlier positive results with similar strains very much advocate the use of V. *alginolyticus* with probiotic properties in the hatcheries.However, as *V. alginolyticus* strains have been isolated from diseased larvae and adult shrimp (Vandenberghe *et al.*, 1999; Lightner, 1988) and fish (Iwata *et al.*, 1978; Kasuda *et al.*, 1986), it would be prudent to use only genotypically well characterized strains and thus avoid the mistaken usage of pathogenic ones.