Use of *Streptomyces fradiae* and *Bacillus megaterium* as probiotics in the experimental culture of tiger shrimp *Penaeus monodon* (Crustacea, Penaeidae)

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Abstract. Shrimp hatcheries often beset with diseases, mainly the bacterial infection and antibiotics are widely used for prevention of disease. Presently, beneficial bacteria (probiotics) are used to prevent diseases instead of antibiotics and increasing the production. In the present study, the two new microbial strains *Bacillus megaterium* and *Streptomyces fradiae* isolated from mangrove sediments were applied (experimental culture) for the post larval rearing of *Penaeus monodon* which is compared with control culture tanks (without probiotics). The water quality condition such as temperature (27-29°C), salinity (26-28%) and dissolved oxygen (4.7-5.0 mg L⁻¹) of both control and experimental culture were more or less similar. Concentration of ammonia and pH were significantly different (p<0.05) between the control and experimental culture during the study period. The feed assimilation efficiency is higher (above 80%) in experimental culture tank when compared to control tank (74.76%). The growth rate was higher - 1.70 and 1.67 in *S. fradiae*, 1.66 and 1.63 in *B. megaterium* - through feed and water, respectively, while in control tank it was 1.4. The FCR values were 2.06 and 2.12 in *S. fradiae* treated tanks through feed and water, respectively, while 2.51 and 2.55 were observed in *B. megaterium* treated through feed and water respectively. The FCR value was found higher (4.02) in the control tank. The average total heterotrophic bacteria (THB) and total presumptive *Vibrio* bacteria both in culture water and post larvae were lower during experimental culture in compared to control culture. The present study indicates that the probiotic treatment using two new microbial strains such as *B. megaterium* and *S. fradiae* would help in better aquaculture production.

**Key Words:** *Penaeus monodon*, post larvae, *Streptomyces fradiae*, *Bacillus megaterium*, probiotics.

Introduction. Shrimp farms are growing throughout the world to meet the demand for shrimp which is one of the most popular types of seafood in the world and Bangladesh is no exception. The tiger shrimp, *Penaeus monodon* is one of the largest shrimp among penaeids and it is most suitable for aquaculture in Bangladesh (Aftabuddin et al 2005). Shrimp cultivation and export in Bangladesh have undergone rapid expansion over the last two decades. During 2008-2009 fiscal year, export of shrimp and fish products was 117.31 million Lbs earning 454.53 million US dollar. Shrimp alone contributed about 78% of total export earning from fishery product (BFFEA 2009). With the rapid expansion of shrimp growout farm, the hatchery industry has progressed rapidly during the last ten years. There are 50 shrimp hatcheries (at various stages of operation and management) in Bangladesh and they produced nearly 5 billion post larvae in 2004 (Aftabuddin & Kader 2006) which was sufficient to supply the entire shrimp culture industry in Bangladesh. However, many of these hatcheries had to stop production in the last three years due to invasion of different pathogens in their system (Aftabuddin et al 2008).

Bacteria are the most common biological agents in the aquaculture sector and it is known that marine crustaceans can be infected by one or more type of bacteria. In many shrimp producing countries, *Vibrio* and *Aeromonas* are considered as the most common
and significant infectious pathogens (Lightner 1996; Moriarty 1997; Vaseeharan et al 2005; Aftabuddin et al 2008). Bacterial diseases are the major problem affecting the shrimp hatcheries and most of the mass mortalities reported in shrimp (P. monodon) hatcheries are associated with luminous bacteria (Lavilla-Pítogo et al 1990; Jiravanichpaisal et al 1994; Karunasagar et al 1994). Vibrio spp. are by far the major bacterial pathogens and can cause severe mortalities, particularly in hatcheries. Vibriosis is caused by a number of Vibrio species including: V. harveyi, V. vulnificus, V. parahaemolyticus, V. alginolyticus, V. penaeicida (Brock & Lightner 1990; Ishimaru et al 1995). Different antibiotics i.e. Oxytetracycline, Chloramphenicol, Erythromycin, Furazolidone, Neomycin sulphate, Kenamycin sulphate, Ciprocin and others are used in P. monodon shrimp hatcheries in Bangladesh (Aftabuddin & Kader 2006). Prolonged exposure of a bacterial population to a given antibiotic can result in development of pathogens that are resistant to those antibiotics. This resistance can occur either by mutation within the target bacterial population or by inadvertent selection of a minor percentage of the original population that is innately resistant (Chamberlain 1988). On the other hand, the use of beneficial bacteria (probiotics) to displace pathogens by competitive processes is being used in the animal industry as a better remedy than administering antibiotics and is now gaining acceptance for the control of pathogens in aquaculture (Havenaar et al 1992). The probiotics are live microbial feed supplements, used for environment-friendly aquaculture. The probiotics are usually applied through water or feed, they act beneficially in improving the water quality, increasing growth of the shrimp and making the animals active and healthy (Gatesoupe 1999). The probiotics includes Vibrios, Pseudomonades, lactic acid bacteria, bacilli and yeasts. Among them Bacillus spp. and yeasts are commonly studied probiotics (Cherif et al 2001; Vaseeharan & Ramasamy 2003; Dahan et al 2003; Duc et al 2004; Soundararapandian & Babu 2010).

Marine environment contains a wide range of distinct microorganisms that are not present in the terrestrial environment (Ramesh & Mathivanan 2009). Many promising bioactive compounds including antimicrobial, antitumour, immunosuppressive agents and enzymes are being discovered from marine actinomycetes (Tanaka & Omura 1990). Marine mangrove ecosystems are a great source for isolation of new microbes that are potentially rich to produce the important bioactive metabolites (Mangamuri et al 2012).

The environment of the mangrove ecosystem is saline, and highly rich in organic matter due to its various microbial enzymatic and metabolic activities (Kizhekkedathu & Parukuttyamma 2005). Approximately 70% of thousands of naturally occurring antibiotics have been isolated from marine actinomycetes (Takizawa et al 1993) and the majorities were isolated from the genus Streptomyces (Zheng et al 2000; Atta & Ahmad 2009; Reddy et al 2011). Marine actinomycetes, especially Streptomyces, have been considered in antibiotic production for chemical factories (Okazaki & Okami 1972; Ellaiah & Reddy 1987). However, in addition, little information was found on the application of Streptomyces as probiotics in aquaculture (Das et al 2006).

On the other hand, Bacillus megaterium is a gram positive, spore producing bacteria. It is found in diverse environments from rice paddies to dried food, seawater, sediments, fish, normal flora, and even in bee honey (Vary 1994). Taxonomically, B. megaterium is positioned into the B. subtilis group of Bacilli (Priest 1993; Vary 1994).

In contrast to other bacilli strains B. megaterium has an advantage, that no alkaline protease is present (Vary et al 2007). This fact enables excellent production and secretion of foreign proteins without degradation (Meinhardt et al 1989; Rygus & Hillen 1991). In compare to gram negative organisms like Escherichia coli, it does not produce endotoxins associated with the outer membrane (Vary et al 2007). These features make B. megaterium well applicable in food and even in pharmaceutical industry. B. megaterium has economic importance because of its commercially important enzymes such as penicillin amidase and steroid hydrolases (Vary et al 2007).

Presently, probiotics used in shrimp culture industry in Bangladesh is entirely import oriented. It is also not clear about the source of beneficial microorganisms used in the preparation of imported probiotics. In recent years, the increasing consumer concern about the residues of antibiotics and the danger of development of antibiotic resistant...
bacterial strains have led to the use of probiotic feed additives in the aquaculture industry. Therefore, the present study was undertaken to examine the effects of *B. megaterium* and *S. fradiae* as probiotics when treated through feed and culture medium of *P. monodon*. Water quality parameters of cultured environment, net growth efficiency, assimilation, food conversion ratio (FCR), growth rate, total heterotrophic bacteria (THB) and total *Vibrio* bacteria were also studied, with a view to develop a potent microbial supplement to ensure better aquaculture production maintaining the environment friendly.

**Material and Method.** In the present study, strains of *B. megaterium* and *S. fradiae* were isolated from mangrove sediments of Chokaria mangrove area, Cox’s Bazar, Bangladesh (Figure 1). This study was carried out from January 2011 to December 2011 in Prime Shrimp Hatchery Limited, Cox’s Bazar, where adequate research facilities for the study of hatchery operation and management were readily available and Shrimp & Fish Disease Diagnosis Laboratory, Institute of Marine Sciences and Fisheries, University of Chittagong, Bangladesh.

![Figure 1. Sediment sampling area of the present investigation.](image-url)
Isolation and identification of Bacillus megaterium. The sediment samples were collected by inserting a grab sampler at 1-2 meter depth. The sampler was sterilized with 70% alcohol before sampling. The sample was then transferred to a sterilized polyethylene bag. Sediment was dried overnight in a laminar flow hood and when clumping occurred, was grinded with mortar and pestle. Ten gram of sediment sample was suspended in 100 ml distilled water in a 200 ml conical flask and serial dilutions were made. From the serial dilutions 100 µl of soil suspension at different dilutions were drawn and poured onto the Blood Agar medium and incubated at 35 to 37°C for 24-48 hours (HPA 2007). Bacterial cells from typical colonies were observed with a light microscope (400X) and purified on nutrient agar medium. Colonies were white, round smooth and shiny. During the microscopic examination all the isolates were found gram positive rods. The isolated colonies were identified as *B. megaterium* using standard morphological, physiological and biochemical plate and tube tests (Holt et al 1994). All strains were stored in Luria-Bertani (LB) broth cultures with sterile glycerol (15% v/v).

Isolation and identification of Streptomyces fradiae. The collected sediment samples were air-dried aseptically. All the marine sediment samples were subjected to pre-heating treatment prior to serial dilution. Pre-heating treatment was performed by incubating the sediment samples in a water bath at 50°C for 60 minutes (Takizawa et al 1993). Ten grams of sediment samples were suspended in 95 ml of sterile aged seawater (7 day old seawater) and these suspensions were considered as 10⁻¹ dilution. Starch casein agar (SCA) medium was used for isolation of *Streptomyces* spp. Serial dilutions were done and overlaid on the surface of SCA. The medium consisted of 10 g soluble starch, 0.3g casein, 2g KNO₃, 2g KH₂PO₄, 0.05g MgSO₄·7H₂O, 2g NaCl, 0.02g CaCO₃, 0.01g FeSO₄·7H₂O, 20g Agar and natural aged sea water 1000ml. The final pH of the medium was adjusted to 7.0 ±2 with 0.1 N NaOH before sterilization (Ramesh & Mathivanan 2009). The medium was supplemented with 50 mg L⁻¹ and 20 mg L⁻¹ of tetracycline and nystatin respectively as antibacterial and antifungal agents to inhibit the bacterial and fungal contamination (Reddy et al 2011). The plates were incubated at room temperature 28±2°C for one week.

Purification. The appearance and growth of marine actinomycetes were observed everyday on SCA plates. Again, the colonies have been observed using a light microscope for their filamentous nature, spores, width of hyphae and spiral sporophores. Individual colonies were picked up, and subcultured on SCA and on International Streptomyces Project medium 2 (ISP2) i.e. Yeast extract-malt extract agar (Pridham et al 1956) by repeated streaking to ascertain their purity. The medium consisted of Bacto Yeast Extract (Difco, USA) 4g, Bacto Malt Extract (Difco, USA) 10g, Bacto Dextrose (Difco, USA) 4g, distilled water 1 liter, Bacto agar 20g and adjusted pH 7.3. The pure cultures of marine actinomycetes were sub-cultured in SCA slants, incubated at room temperature for 5–7 days to achieve good sporulation, and then preserved in 20% glycerol vials at -80°C (Williams & Cross 1971). The inocula used in all the experiments were seven-day-old cultures, unless otherwise stated.

Characterization of the Streptomyces isolates. The microorganisms were characterized by acid-fast staining and Gram's staining techniques. The morphological, physiological and biochemical characterization of the isolated was performed according to the method of International Streptomyces Project (ISP) (Shirling & Gottlieb 1966; Kokare et al 2004). Hydrolysis of starch was performed by using the media of Gordon et al (1974) Liquefaction of gelatine was done by the method of Waksman (1961). Growth characteristic was also observed using tyrosine agar medium (ISP-7) (Shinobu 1958). Carbon source utilization (CSU) tests have been used for the classification and identification of bacteria for many years in the form of simple tests such as citrate utilization, D-glucose, sucrose, inositol etc. Biochemical tests and carbon and nitrogen utilization were performed according to standard methods described by Shirling & Gottlieb (1966) and Tresner et al (1968).
Experimental set up. Healthy and active post larvae (PLS<sub>20-22</sub>) were acclimatized to the laboratory conditions for 5 days. One hundred PL were used for each treatment tank of 100 liters capacity. The tank was filled with 90 liters of filtered seawater and was aerated using air-stones. Salinity was maintained between 22 and 25 ppt. The experimental animals were fed with formulated feed (Brine Shrimp flakes, Higashimaru 3, Singapore) at 10% body weight of the PL.

The experiment was done in two ways using the cultures of <em>B. megaterium</em> and <em>S. fradiae</em>. One way was that the cultures with a density of 10<sup>8</sup> cells per ml and total 10 ml were inoculated into the tank having the 90 liters of seawater considering that this small amount of culture media would not be affected the experiment. Another way was that the cultures were mixed thoroughly with feed at 10<sup>9</sup> cells per gram of the feed. The treated feed was given two times a day at 7.00 a.m. and 7.00 p.m. All the treatments (water and feed) were done at an interval of every 5 days of the total 60 days of culture. In control tanks, no microbial treatment either through water or through feed was made. Water exchange was made for 35% of the culture water every morning. Ten tanks were used (five for <em>Bacillus</em> spp. and five for <em>Streptomyces</em> spp.) for conducting the experiment. ANOVA test was done to see the treatment effects of the new probiotics used.

Water parameters analysis. The water quality parameters of the probiotics (<em>B. megaterium</em> and <em>S. fradiae</em>) treated and control tanks were regularly monitored. Water quality parameters such as salinity, temperature, pH, dissolved oxygen, and ammonia were estimated daily in the morning hours. The water salinity was measured by using a hand refractometer (ATAGO, Japan). The pH of the water was measured by using electronic pen pH meter manufactured by Hanna Instrumental Company (Singapore). Water temperature was measured by using a digital thermometer (DT801, China). Dissolved oxygen (mg L<sup>-1</sup>) and ammonia (mg L<sup>-1</sup>) were measured by HANNA instruments products, Singapore, Model no. HI 3810 and HI 3826, respectively.

Microbial evaluation. For enumeration of total heterotrophic bacteria (THB) in water and post larvae the pour plate method was used with sterile Zobell’s marine agar medium (Hi-media, Mumbai, India). For presumptive <em>Vibrio</em> count TCBS and TSA medium (Oxoid, England) were used. This work was carried out for 10 days interval i.e. 10<sup>th</sup>, 20<sup>th</sup>, 30<sup>th</sup>, 40<sup>th</sup>, 50<sup>th</sup> and 60<sup>th</sup> days of culture period.

Analysis of shrimp growth. The shrimp growth in terms of length and weight was measured after 60 days of culture. Total length was measured with the help of a plastic graduated scale. Fresh weight was taken by weighing the live shrimps on an electronic balance. From the data collected, the food consumption, mean production weight, assimilation, metabolism, growth rate, relative growth rate, digestibility, gross conversion efficiency, net conversion efficiency, food conversion ratio (FCR) and food-coefficient were calculated by Crisp (1971), Qasim & Easterson (1974) and Easterson (1987). Before feeding and water exchange, unutilized feed and faecal matter were collected separately by siphoning out the water using a 2 cm (dia) plastic hosepipe to determine the feed assimilation rate. The collected unutilized feed and faecal matter were placed separately on a pre-weighted filter paper (WHATMAN<sup>TM</sup> No. 1), cleaned with distilled water, dried in an oven at 60°C for 24-h and then weighted.

Results and Discussion. The results shown in Table 1 demonstrate the physiological and biochemical characteristics of <em>B. megaterium</em> and <em>S. fradiae</em>. The enumerated total heterotrophic bacterial (THB) population was higher in treated tanks than in the control tank. In control tank, the total heterotrophic bacterial populations in water were recorded 63.80±1.1x10<sup>3</sup> to 15.10±0.72x10<sup>4</sup> cfu ml<sup>-1</sup> while in post larvae, the recorded THB was 5.4 ±1.70x10<sup>2</sup> to 2.38±0.78x10<sup>4</sup> cfu g<sup>-1</sup>. In the <em>S. fradiae</em> treated tank, the value ranged from 65.07±1.0x10<sup>3</sup> to 44.30±0.5x10<sup>3</sup> cfu ml<sup>-1</sup> for feed experiment and from 69.56±1.2x10<sup>2</sup> to 43.10±0.60x10<sup>5</sup> cfu ml<sup>-1</sup> for water experiment. In the <em>B. megaterium</em> treated tank, the recorded values varied from 62.21 x 10<sup>3</sup> to 42.90 x 10<sup>3</sup> cfu ml<sup>-1</sup> and 8.4±0.58x10<sup>3</sup> to 2.18±0.72x10<sup>4</sup> cfu g<sup>-1</sup> in water and post larvae respectively while
treated with food and from $59.83 \times 10^3$ to $39.22 \times 10^6$ cfu ml$^{-1}$ and $6.1 \pm 1.1 \times 10^2$ to $1.99 \pm 0.35 \times 10^4$ cfu g$^{-1}$ in water and post larvae respectively while treated with water. In the *S. fradiae* treated tank’s post larvae, the THB ranged from $3.48 \pm 1.24 \times 10^3$ to $3.45 \pm 0.82 \times 10^4$ in feed experiment and from $8.8 \pm 2.3 \times 10^2$ to $6.1 \pm 1.2 \times 10^3$ cfu g$^{-1}$ in water experiment. The THB values are much different between the control and treated tanks (Figure 2). The observed total *Vibrio* bacteria were lower in *S. fradiae* and *B. megaterium* treated tank compared to control tank (Figure 3) and the values were significantly different ($p<0.05$). The average THB and *Vibrio* bacteria in water and post larvae are shown in Table 2.

### Table 1

**Different physiological and biochemical characteristics of *B. megaterium* and *S. fradiae***

<table>
<thead>
<tr>
<th>Tests</th>
<th><em>B. megaterium (n=10)</em></th>
<th><em>S. fradiae (n=10)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reduction of nitrate</td>
<td>-(d)</td>
<td>ND</td>
</tr>
<tr>
<td>Liquefaction of Gelatin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Citrate test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Egg yolk reaction</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Anaerobic utilization of glucose</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Voges proskauer reaction</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 45°C</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Growth at NaCl 7% (w/v)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Melanin Pigment on Peptone yeast extract</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosin decomposed (ISP-7)</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Lysozyme-resistant</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid produced from Manitol</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetylglucosamine</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Arbutin</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>D-fructose</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>D-trehalose</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Rhamose</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Carbon utilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Inositol</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>ND</td>
<td>+</td>
</tr>
</tbody>
</table>

"+" = 90-100% strains positive; "-" = 90-100% strains are negative; ND = Not done; "/-" = 50-50% strains are positive; "-(d)" = most strains are negative.

Microbes are unavoidable in the culture system and they have beneficial as well as detrimental effects (Das et al 2006). In aquaculture systems, total heterotrophic bacteria play a significant role through mineralization and decomposition of wastes and provide supplementary feed for shrimp larvae (Sunilkumar 1996) whereas *Vibrio* can cause diseases and mass mortality (Karunasagar et al 1994; Lightner 1993). In the present study, total heterotrophic bacteria increased in the experimental tanks due to the addition of *S. fradiae* and *B. megaterium* cells but decreased in the control tank. In contrast, the *Vibrio* count decreased with the introduction of probiotics and increased in...

Figure 2. Total heterotrophic bacteria (cfu ml\(^{-1}\)) observed in control and treated tanks water.

![Graph showing total heterotrophic bacteria (cfu ml\(^{-1}\))](image)

Figure 3. No. of *Vibrio* bacteria cfu ml\(^{-1}\) (10\(^2\)) in treated and control tanks water.

![Graph showing no. of Vibrio bacteria cfu ml\(^{-1}\) (10\(^2\))](image)

Water quality plays an important role in *P. monodon* post larvae production. Managing of water quality during the larval rearing phase is very important due to the sensitivity of larvae to the fluctuation of water parameters (Aftabuddin et al 2009). The quality of water during the production period will deteriorate mainly due to the accumulation of metabolic wastes of living organisms, decomposition of unutilized feed and decay of biotic materials. Efficient removal of imbalances, which cause impairment in water quality, is difficult. However, addition of some commercial preparations such as chemicals, antibiotics and probiotics are reported to effectively deal with these substances and in this way they are helpful in maintaining water quality parameters and improving growth
rate and survival rate (Soundarapandian & Babu 2010). The average water quality parameters of experimental and control tanks were shown in Table 2.

### Water quality parameters and total heterotrophic bacteria (THB) and Vibrio (TVC) counts in P. monodon culture tanks treated with S. fradiae and B. megaterium

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>B. megaterium treated with feed</th>
<th>S. fradiae treated with feed</th>
<th>B. megaterium treated with water</th>
<th>S. fradiae treated with water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>29</td>
<td>27.6</td>
<td>27.2</td>
<td>27.4</td>
<td>27</td>
</tr>
<tr>
<td>Salinity (ppt)</td>
<td>28</td>
<td>27</td>
<td>27</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Dissolved oxygen (mg L⁻¹)</td>
<td>4.8</td>
<td>4.7</td>
<td>5.0</td>
<td>4.8</td>
<td>4.9</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
<td>7.5</td>
<td>7.7</td>
<td>7.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Ammonia (NH₃ – N) (mg L⁻¹)</td>
<td>0.45</td>
<td>0.025</td>
<td>0.018</td>
<td>0.022</td>
<td>0.020</td>
</tr>
<tr>
<td>THB (cfu ml⁻¹) in water</td>
<td>10.33±0.5x10⁴</td>
<td>21.54±1.2x10⁴</td>
<td>22.98±0.8x10⁴</td>
<td>19.56±0.6x10⁴</td>
<td>22.52±1.1x10⁴</td>
</tr>
<tr>
<td>THB (cfu g⁻¹) in post larvae</td>
<td>2.3±0.3x10³</td>
<td>11.2±1.5x10³</td>
<td>18.5±0.9x10³</td>
<td>4.14±0.7x10¹</td>
<td>6.42±0.9x10³</td>
</tr>
<tr>
<td>Vibrios (cfu ml⁻¹) in water</td>
<td>469.66±1.2x10²</td>
<td>63.33±0.9x10²</td>
<td>60.66±1.0x10²</td>
<td>72.83±0.72 x10²</td>
<td>62.33±1.3x10²</td>
</tr>
<tr>
<td>Vibrios (cfu ml⁻¹) in post larvae</td>
<td>233.11±2.3x10¹</td>
<td>10.12±0.5x10¹</td>
<td>9.36±0.3x10¹</td>
<td>13.38±0.45x10¹</td>
<td>14.12±0.65x10¹</td>
</tr>
</tbody>
</table>

NB: Values are average of 60 days of analysis for temperature, salinity, DO and pH; THB (total heterotrophic bacteria) and Vibrios are averages of 6 days of analysis (10⁶, 20⁶, 30⁶, 40⁶, 50⁶ and 60⁶ days of culture time).

The optimum range of temperature for the black tiger shrimp larval rearing is between 28 to 32°C (Kannupandi et al 2002). The temperature varied from 27 to 29°C in both control and experimental tanks during the study period. There were no marked differences in temperature between control and experimental tanks of the present study.

P. monodon can tolerate a wide range of salinity. The range of salinity with which the normal growth of P. monodon can be maintained is between 15 and 30 ppt (Chen 1976; Chen 1985). In P. monodon, the optimum salinity range for growth may change during their life history with 10 ppt for early post larvae (Valencia 1976), 15-20 ppt for late post larvae (Chen 1976) and 15-25 ppt for juvenile (Chen 1984). In the present study, the salinity ranged between 26 and 28 ppt on all the tanks. There were slight differences between control and treated tanks (S. fradiae and B. megaterium). Salinity was slightly higher in S. fradiae and B. megaterium treated tanks mixed with feed than mixed in water. For a shrimp hatchery, the recommended salinity range is 28-35 ppt (Kannupandi et al 2002).

The average DO level ranged between 4.7 and 5.0 mg L⁻¹ in the experimental and control tanks during the study and there was no significant difference (p>0.05) noticed between the control and treated tanks. Liao & Murai (1986) reported that the respiration rate of P. monodon remained constant at dissolved oxygen (DO) concentrations above 3.0 to 4.0 ml L⁻¹ at salinities 4-45 ppt and temperatures of 20-30°C.

The average pH was observed from 6.8 to 7.7 in both control and treated tanks. There was a significant difference (p<0.05) between the control and treated tanks. Law (1988) reported that P. monodon could tolerate pH down to 6.0 to 6.5. High mortality was observed at pH below 6.0. In the present study the pH level was less in the control tank (6.8) and considerably high in experimental tank (7.7). The results pointed that probiotics (B. megaterium and S. fradiae) present in the experimental tanks were helpful in maintaining the pH at desired level.

Ammonia exists in water in both ionized (NH₄⁺) and unionized (NH₃) forms. Unionized ammonia is considered the more toxic form of ammonia due to its ability to
diffuse readily across cell membrane (Fromm & Gillette 1968; Emerson et al 1975). The reaction of \( \text{NH}_3 \) depends on pH, temperature and to a lesser extent on salinity (Bower & Bidwell 1978). As pH or temperature rises, \( \text{NH}_3 \) increases relative to \( \text{NH}_4^+ \), and the toxicity of ammonia to animals increases (Chien 1992). The average ammonia level ranged between 0.018 to 0.45 mg L\(^{-1}\) in the experimental and control tanks during the study. There was a significant difference \((p<0.05)\) between the control and treated tanks. Chien (1992) reported that the safe levels of ammonia for various stages of \( P. \text{monodon} \) are 0.15, 0.1 and 0.08 mg L\(^{-1}\) for PL\(_{30-50}\), juvenile and adolescent respectively.

**Shrimp growth.** The results obtained from the experiment are presented in Table 3a. Growth (in terms of wet weight gain) was significantly higher \((p<0.05)\) in the treated than in the control tanks. The percent of increment was 100.10 and 98.09 in \( B. \text{megaterium} \) treated through feed and water respectively. The percent increment was 102.33 and 100.44 in \( S. \text{fradiae} \) treated through feed and water respectively while in the control group it was 89.32 percent (Table 3b).

### Table 3a

Weight gain of \( P. \text{monodon} \) post larvae after 60 days culture treated with new probiotics \( S. \text{fradiae} \) and \( B. \text{megaterium} \) (mixed with feed and water)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial weight ( w_1 )</th>
<th>Final weight ( w_2 )</th>
<th>Weighed means ( W = w_1 + w_2/2 )</th>
<th># Feed consumed ( C ) ( \text{g} ) (feed given-unutilized feed)</th>
<th>Faecal output ( F ) ( \text{g} )</th>
<th>Assimilation ( A = C - F ) ( \text{g} )</th>
<th>Production Growth ( P = w_2 - w_1 ) ( \text{g} )</th>
<th>Metabolism ( R = C - (P + F) ) ( \text{g} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control tank</td>
<td>0.34</td>
<td>2.32</td>
<td>1.33</td>
<td>5.35</td>
<td>1.35</td>
<td>4.00</td>
<td>1.98</td>
<td>2.02</td>
</tr>
<tr>
<td>( S. \text{fradiae} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mixed with feed)</td>
<td>0.34</td>
<td>4.28</td>
<td>2.31</td>
<td>4.78</td>
<td>0.75</td>
<td>4.03</td>
<td>3.94</td>
<td>0.09</td>
</tr>
<tr>
<td>( S. \text{fradiae} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mixed with water)</td>
<td>0.35</td>
<td>3.90</td>
<td>2.12</td>
<td>4.50</td>
<td>0.85</td>
<td>3.65</td>
<td>3.55</td>
<td>0.10</td>
</tr>
<tr>
<td>( B. \text{megaterium} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mixed with feed)</td>
<td>0.33</td>
<td>3.60</td>
<td>1.96</td>
<td>4.92</td>
<td>0.90</td>
<td>4.02</td>
<td>3.27</td>
<td>0.75</td>
</tr>
<tr>
<td>( B. \text{megaterium} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mixed with water)</td>
<td>0.35</td>
<td>3.44</td>
<td>1.89</td>
<td>4.85</td>
<td>0.88</td>
<td>3.97</td>
<td>3.09</td>
<td>0.88</td>
</tr>
</tbody>
</table>

The relative growth rate in shrimp was higher in treated tanks than in control. The best relative growth rates 1.70 and 1.67 were observed in the tank inoculated with \( S. \text{fradiae} \) respectively through feed and water. The growth rates were 1.66 and 1.63 respectively in the \( B. \text{megaterium} \) treated through feed and water. However, the lowest growth rate 1.48 was recorded in the control tank (Table 3b).

The shrimp showed high assimilation efficiency (digestibility) of 84.30 and 81.85% in \( S. \text{fradiae} \) and \( B. \text{megaterium} \) treated through feed and water respectively. Assimilation efficiency was also observed at 81.70 and 81.11% in \( B. \text{megaterium} \) and \( S. \text{fradiae} \) treated through feed and water respectively (Table 3b). Qasim & Easterson (1974) observed the assimilation efficiency ranged from 89 to 97% in the shrimp, \( Metapenaeus \text{monoceros} \) in India.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Assimilation efficiency or digestibility = A/C (%)</th>
<th>Gross growth (conversion) efficiency = k1 = P/C (%)</th>
<th>Net growth (conversion) efficiency = k2 = P/A (%)</th>
<th>Food Conversion Ratio = Total food consumed/Total live weight increased</th>
<th>Food co-efficient C/P</th>
<th>Relative Growth Rate = P/W x 1</th>
<th>Growth Rate = Fw - Iw/W x 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control tank</td>
<td>74.76</td>
<td>37.00</td>
<td>49.50</td>
<td>4.02</td>
<td>2.70</td>
<td>1.49</td>
<td>89.32</td>
</tr>
<tr>
<td><em>S. fradiae</em> (mixed with feed)</td>
<td>84.30</td>
<td>82.42</td>
<td>97.76</td>
<td>2.06</td>
<td>1.21</td>
<td>1.70</td>
<td>102.33</td>
</tr>
<tr>
<td><em>S. fradiae</em> (mixed with water)</td>
<td>81.11</td>
<td>78.88</td>
<td>97.26</td>
<td>2.12</td>
<td>1.26</td>
<td>1.67</td>
<td>100.44</td>
</tr>
<tr>
<td><em>B. megaterium</em> (mixed with feed)</td>
<td>81.70</td>
<td>66.46</td>
<td>81.34</td>
<td>2.51</td>
<td>1.50</td>
<td>1.66</td>
<td>100.10</td>
</tr>
<tr>
<td><em>B. megaterium</em> (mixed with water)</td>
<td>81.85</td>
<td>63.71</td>
<td>77.83</td>
<td>2.55</td>
<td>1.56</td>
<td>1.63</td>
<td>98.09</td>
</tr>
</tbody>
</table>

The gross growth (conversion) efficiency in the shrimps was 82.42 and 78.88% through feed and water respectively in *S. fradiae* treated tank. Gross growth (conversion) efficiency were 66.46 and 63.71% in *B. megaterium* treated through feed and water respectively (Table 3b). The gross conversion efficiency was found very high in *S. fradiae* treated tanks in comparison to the control and *B. megaterium* treated tanks. In crustaceans, the reported gross growth (conversion) efficiency was 4-68% (Qasim & Easterson 1974).

The highest value of net conversion efficiency in shrimps was recorded as 97.76 and 97.26% in *S. fradiae* treated through feed and water respectively. The net conversion efficiency was 81.34 and 77.83 in *B. megaterium* treated through feed and water respectively. Net conversion efficiency in the control tank was 49.50 (Table 3b). Qasim & Easterson (1974) reported that net conversion efficiency was 24 to 71% in crustaceans. The maximum food coefficient value was 2.70 recorded in animals of the control tank, followed by the values of 1.56 and 1.50 in *B. megaterium* and 1.26 and 1.21 in *S. fradiae* treated tanks through feed and water, respectively.

The food conversion ratio (FCR) was lower in treated tanks than in control tank. The FCR values were 2.06 and 2.12 in *S. fradiae* treated tank through feed and water respectively while 2.51 and 2.55 were observed in *B. megaterium* treated through feed and water, respectively. The FCR value is higher 4.02 in the control tank.

The present study has demonstrated the positive effect of beneficial microorganisms such as *S. fradiae* and *B. megaterium* on the production of the *P. monodon* shrimp post larvae. The microbial supplements improve the growth and survival of the tiger shrimp, besides biomass production, food conversion efficiency, food conversion ratio and assimilation efficiency (Tables 3a and 3b). This can be attributed to the better water quality ensured by the microbial supplements. This may also be due to activation of immune system induced by the microbes through the production of enzymes and antibiotics produced by the treated microbes that activate the defense system of the shrimps (Itami et al 1998). However, the precise mechanism of action of microbial supplements in improving the disease resistance of shrimp is not known. This is perhaps because shrimps possess a less developed immune system and are relatively more dependant on the non-specific immune processes. Porubcan (1991) has recorded that the microbial strain isolated from
the tiger shrimp pond improves water quality growth and productions of *P. monodon*. The beneficial effect is attributed to the efficiency of bacteria in antagonisms to pathogen, gut colonization with possible adhesion to intestinal mucus (Evelyn 1996) and to increase resistance of the host to pathogens.

The actinomycetes i.e. *Steptomyces* sp. are well known for their production of antibiotics, growth-promoting substances. Therefore, it is probably that the *S. fradiae* used in the present study has improved the growth of shrimps through growth promoting substances produced and in making the shrimps healthy by reducing the load of pathogenic vibrios in the culture medium through antibiotics formed. It is therefore, necessary to characterize the growth promoting substances and antibiotics produced by the actinomycetes to develop them as microbial production for better aquaculture practices.

Moriarty (1998) has noted an increase of prawn survival in the pond water inoculated with bacillus species. This is attributed to the water quality improved by the bacterial inoculations because of degradation of organic matter. This treatment also has decreased the pathogenic luminoous vibrio species and other bacteria (Maeda 1994). The antagonistic activities of *Bacillus* species may be mediated by many inhibitory substances, such as organic acids, hydrogen peroxide (Rings & Gatesoupe 1998). The strains of bacillus are also known to compete for nutrients and for space for pathogenic bacteria. These mechanisms of bacillus may even prevent the emergence of resistant strains of pathogenic bacteria, a well-known risk of antibiotics treatments (Moriarty 1998).

**Conclusion.** The two microbial species namely *B. megaterium* and *S. fradiae* have been proved to have potential for their use as probiotics in shrimp aquaculture to improve water quality, balance microbial population and to reduce pathogenic bacteria. This practice is a new approach, deserving more attention. There is a great scope for application of marine actinomycetes as probiotics for a glorious future of aquaculture.

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