Commercial probiotics as controlling agents of bacterial micro flora in semi-intensive shrimp culture ponds

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SUMMARY

This study examines the possible use of probiotics as controlling agents of pathogenic *Vibrio* (yellow and green colonies) in the culture ponds of *Penaeus monodon*. The total heterotrophic bacteria (THB) and *Vibrio* (yellow and green colonies) were monitored through 30, 60, 90 and 120 days of culture in three successive summer crops (C_1, C_2 and C_3) in control (CP) and probiotic treated (WFB, FPB and WFPB) ponds. THB count increased significantly ($P<0.05$) in control as well as probiotic treated pond water with increase of culture duration. *Vibrio* (yellow and green colonies) count was significantly ($P<0.01$) higher in control (CP) than in probiotic treated ponds. FPB and WFPB treated pond waters showed no *vibrio* colonies at 90 days of culture in C_2. The application of probiotics obviously reduced *Vibrio* population through competitive exclusion was discussed.

Key words : *P. monodon*, Probiotics, THB, *Vibrio*, Semi-intensive culture

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Consecutive outbreaks of viral and bacterial diseases have devastated shrimp culture in many Asian countries during the last few years resulting in huge economic losses (Moriarty, 1998; Sung *et al.*, 2001). In India alone losses due to shrimp disease outbreaks were put at US $ 400 million between 1993 and 1995 (MPEDA, 2000). *Vibrio* species represent a large proportion of bacterial population of marine environment and act as pathogenic primary and secondary invaders of shrimp in culture systems (Lightner, 1996; Sung *et al.*, 2001). As a result farmers started using antimicrobial compounds and antibiotics as prophylactics in large quantities even when pathogens are not evident. This has led to an increase in *Vibrio* and presumably other bacteria, having multiple antibiotic resistance and to an increase in more virulent pathogens (Moriarty, 1998).

Bioremediation with probiotics is a potent biotechnological approach in the degradation of hazardous organic wastes to substances that are environmentally safe by induction of microorganisms like *Pseudomonas, Bacillus, Nitrosomonas*, and *Nitrobacter* spp. which have the ability to degrade organic substances (Mohan Kumar *et al.*, 2005) in aquatic environment.

Microorganisms present in the bottom of the pond are known to play an important role in nutrient cycling and decomposition (Anderson, 1987; Coleman and Edwards, 1987; Rheinheimer, 1992) by degradation of organic matter in pond waters (Avnimelech *et al.*, 1995) and thereby improving the water quality. Organic matter is degraded by a wide variety of microorganisms. For instance, heterotrophic microorganisms oxidize organic matter consuming oxygen and releasing carbon dioxide while autotrophic nitrifying and sulphur bacteria consume oxygen and carbon dioxide in the process of oxidizing ammonia, nitrite and sulphide.

Although studies have been carried out on the role of heterotrophic bacterial populations in shrimp culture (Ruanpam *et al.*, 1995; Anand *et al.*, 1996) much attention has not been paid to understand the role of probiotics (beneficial bacteria) in nutrient recycling. Thus an attempt was made in the present study to understand the role of probiotic bacteria in controlling and minimizing the pathogenic bacterial load in the pond environment.

MATERIALS AND METHODS

The present work was carried out in a private shrimp farm (Sharat Sea Foods Industries Ltd.) near Venkannapalem village (14°.2'E; 80°.5'N) of Nellore District, Andhra Pradesh, India during three summer crops (March to July, 2004; Feb. to June, 2005; March to June, 2006). Semi-intensive shrimp culture ponds (~ 1ha) adopted for this work during each summer were divided into four groups of three each. The first group of ponds treated with no probiotics forms the control ponds (CP); the second group treated with commercial probiotic, Wunapuo-15 @ 30 kgha^{-1} every 15d upto 110 d of culture is designated experimental-I (WPB); the third group where the shrimp were fed on feed containing the commercial feed probiotic, Aqualact @ 5 mgkg^{-1} feed every alternate day starting from the day of culture till harvest constitutes experimental-II (FPB): and the third group treated both with water and feed probiotics as per the schedules followed for WPB and FPB respectively forms experimental-III (WPB + FPB). Culture ponds adopted for this study were uniformly prepared, following usual culture practices. Culture ponds were simultaneously stocked with *P. monodon* post larvae (PL20) obtained from Sharat Shrimp Hatchery (SSF industries Ltd.) after PCR screening for White Spot Syndrome Virus (WSSV) @ 12 PLm^{-2}.

After stocking, post larvae were fed with CP shrimp feed (CP Aquaculture India Ltd, Chennai, India) @ 1 to 1.5 kg/day/one lakh seed, increased @ 400-500 g/d upto 30 days of culture. After that feed consumption is regularly monitored through check tray observation and also adjusted according to average body weight (ABW) of shrimps measured every 7-10 days by random sampling.

Estimation of total heterotrophic bacteria (THB):

Total heterotrophic bacteria (THB) were estimated following the method of Harris *et al.* (1986). To enumerate THB population, 1 ml sample from the desired dilution was pipetted out into a sterile petridish into which 15-20 ml melted and cooled Zobell's Marine Agar (ZMA) medium (Hi-media Pvt. Ltd., Mumbai, India) was poured, mixed thoroughly and incubated in an inverted position at room temperature (28°±2°C) for three to four days. Viable bacterial colonies in petri plates were counted using bacterial cell colony counter and expressed as colony forming units per ml (cfu/ml).

Estimation of total vibrio count (TVC):

Total *Vibrio* count (TVC) was determined following the procedure of Dalmin *et al.* (2001). TCBS (Thiosulphate citrate bile salts sucrose) agar medium was used for isolating *Vibrios* from the water samples. Spread plate method was used to inoculate bacteria from the samples into agar petriplates. The petriplates were incubated in an inverted position at 36°C for 20-24 hr. Bacterial colonies could be seen after 18 h. TVC was expressed as colony forming units / ml (cfumL^{-1}). Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were employed to know the statistical significance between treatment means (SPSS : 14.0 version).

RESULTS AND DISCUSSION

Results obtained on total heterotrophic bacteria (THB) and *Vibrio* (yellow and green colonies) counts on 30, 60, 90 and 120 days of culture in control (CP) and probiotic treated (WPB, FPB, WFPB), *P. monodon* culture ponds during three successive summer crops (C_1 : Crop-I, C_2 : Crop-2, C_3 : Crop-3) are presented in figures 1 to 3. Log values of THB count obtained from control and probiotic treated ponds of C_1, C_2 and C_3 are presented in Fig. 1. The results clearly show that the THB count increased significantly ($P<0.05$) in control as well as probiotic treated pond waters with increase in culture duration. The results also show that the THB count was significantly higher ($P<0.05$) in the waters of probiotic treated ponds than in control ponds at different time intervals of culture except at 120 d in C1.

Log values of total *Vibrio* (yellow colonies) count obtained from control and probiotic treated pond waters of C_1, C_2 and C_3 are presented in Fig. 2. It is clear from the results that the *Vibrio* (yellow colonies) count was not only significantly higher ($P<0.01$) in control ponds than in probiotic treated ponds at all time intervals of culture but also increased with increase in culture duration. On the other hand *Vibrio* count in probiotic treated ponds remained almost unchanged through out the culture duration.

Log values of *Vibrio* (green colonies) count from control and probiotic treated pond waters of C_1, C_2 and C_3 are presented in Fig. 3. It is evident from the results that *Vibrio* (green colonies) count in control ponds was not only significantly higher ($P<0.01$) than in probiotic treated ponds at all time intervals of culture but also increased with increase in culture duration. On the contrary *Vibrio* count in probiotic treated ponds decreased rapidly and highly significantly by the end of 120 d culture or, some times, it was even found that the *Vibrio* count in FPB and WFPB treated ponds of C_1 and in WFPB treated ponds of C_3 dropped down to zero level at the end of 60 d culture. It was also found that the *Vibrio* count in FPB and WFPB treated ponds of C_2 dropped down to zero level at the end of 90 d culture.

Total heterotrophic bacteria are a group of organisms which require organic matter as a source of carbon. THB, which include both beneficial and pathogenic bacteria, always dominate the other groups of bacteria in the natural pond environment. Left over feed particles, shedded shrimp shells and metabolic waste contribute relatively higher to bacterial load in culture ponds (Sharmila *et al.*, 1996). Generally *Bacillus*

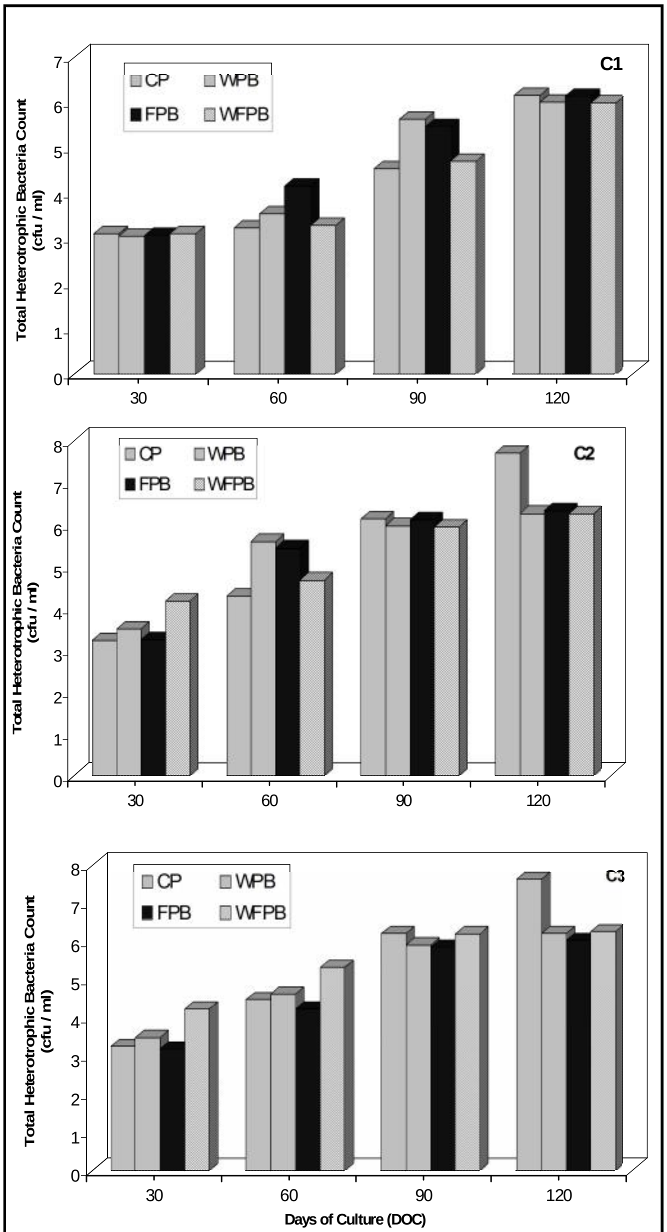

Fig.1 : Mean (ï SD; n=6) log values of total heterotropic bacteria (THB) count (cfu/ml) in the waters of control and probiotic treated *P. monodon* culture ponds (CP: control pond; WPB: water probiotic treated pond; FPB: feed probiotic treated pond; WFPB: water and feed probiotic treated pond; C1, C2 and C3: three successive summer crops)

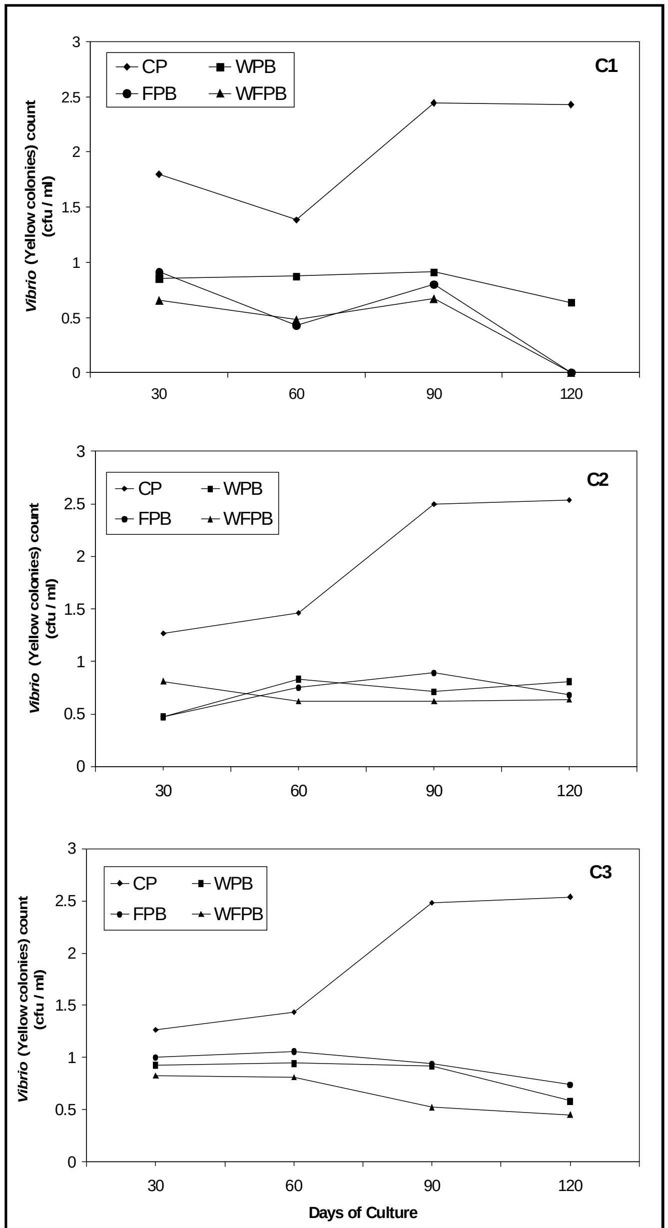

Fig. 2: Mean (ï SD; n=6) log values of total *Vibrio* (yellow colonies) count (cfu/ml) in the waters of control and probiotic treated *P. monodon* culture ponds (CP: control pond; WPB: water probiotic treated pond; FPB: feed probiotic treated pond; WFPB: water and feed probiotic treated pond; C1, C2 and C3: three successive summer crops)

sp. contribute around 20% and *Vibrio* sp. around 40% to THB in coastal ecosystem (Sharmila *et al.*, 1996). The results presented in Fig. 1 demonstrate that the THB count increased significantly ($P<0.01$) in control as well as probiotic treated pond waters with increase in culture duration. While an increase in THB in control ponds indicates multiplication of enteric pathogenic microorganisms, an increase in probiotic treated ponds indicate multiplication of beneficial microorganisms, a process facilitated by competitive exclusion (Fuller and Turvey, 1971; Moriarty, 1998). Another important observation was that the THB count was significantly higher ($P<0.05$) in the waters of probiotic treated ponds than in those of control ponds at different time intervals of culture. This again suggests that the probiotic bacteria might have outnumbered other bacteria by rapid multiplication and competitive exclusion process as suggested by Moriarty (1999). An increase in THB

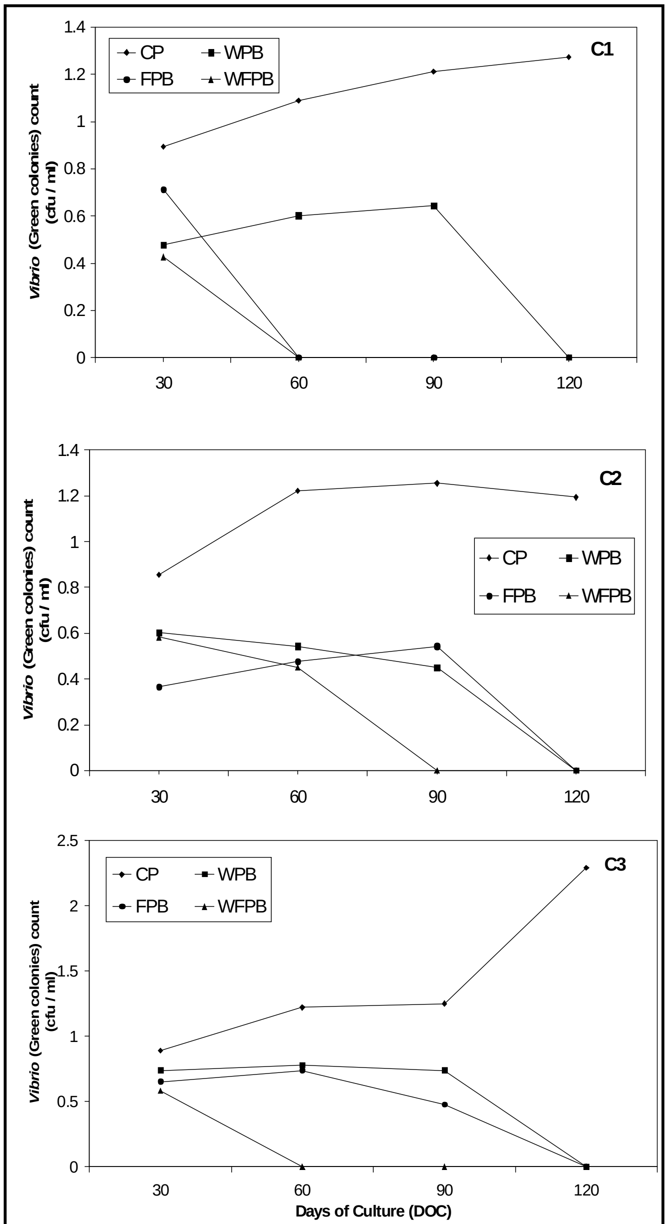

Fig. 3: Mean (ï SD; n=6) log values of total *Vibrio* (green colonies) count (cfu/ml) in the waters of control and probiotic treated *P. monodon* culture ponds (CP: control pond; WPB: water probiotic treated pond; FPB: feed probiotic treated pond; WFPB: water and feed probiotic treated pond; C1, C2 and C3: three successive summer crops)

population in shrimp culture ponds and sediments has also been reported by Dalmin *et al.* (2001); Ravi *et al.* (1998); Kumar *et al.* (2005) and Wang *et al.* (2005).

Vibrios are by far the most numerous of the reported bacterial agents of penaeid shrimp and are reported to constitute majority of bacteria present in the normal microflora of cultured and wild penaeid shrimp (Dempsey *et al.*,1989; Hameed, 1993; Leano *et al.*, 1998; Singh *et al.*, 1998). Infections due to *Vibrios* are characterized by massive colonization of the appendages and foregut followed by infection of the midgut, hepatopancreas and a terminal *septicaemia* (Ajitha et al. 2004). Although the initial *Vibrio* count (yellow as well as green colonies) was significantly lower (P<0.05) in probiotic treated ponds than in control ponds, it kept decreasing further with increase in culture duration (Fig. 1 and 2) unlike in control ponds where the count recorded an increase with increase in culture duration. Especially the decrease in green colonies was most rapid in probiotic treated ponds with the count reaching almost zero level either at 60 or 90 d of culture. This suggests that the application of probiotics might have reduced *Vibrio* population through competitive exclusion process (Matias *et al.,* 2002). Moriarty (1999) also obtained similar results and reported that the routine use of commercial probiotics in a shrimp farm in West Java, resulted in reduced incidence of *Vibriosis* and other viral outbreaks. In addition, Moriarty (1998) found that the application of probiotics could prevent luminescent *Vibrio* infestation either by lowering or completely eliminating luminous *Vibrios* in pond water and the sediment.

Further, the works of Rengpipat *et al.* (1998), Dalmin *et al.* (2001), Sambasivam *et al.* (2003) and Vaseeharan *et al.* (2004) showed similar results reflecting higher survival of shrimp. It has been shown that the alimentary tract of penaeids provides a congenial environment for *Vibrios* to multiply and, activation of any stress factors in the culture system may make the animal susceptible to the invasion of pathogenic strains of the genus (Singh *et al.*, 1998). Therefore, the concept of altering this microbial flora dominated by *Vibrios* through the application of probiotics sounds promising and the results of this study exemplify this view.

The present investigation demonstrates that the application of commercial probiotics in shrimp culture ponds improved water quality, reduced toxic metabolites in pond water and reduced the pathogenic bacterial load and increased total heterotrophic bacteria (THB) there by improving the survival rates and growth of shrimp in natural field conditions. It has also been shown that the commercial probiotics are better agents in controlling harmful pathogenic bacteria in culture ponds as they basically settle onto the bottom sediment and act from there to limit or eliminate the pathogenic bacteria through antagonistic properties.

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