

A Study on the Probiotic Role of Bacteria Isolated from Wild Marine Shrimp, *Penaeus Monodon* (Fabricius) to Control Pathogenic Bacteria

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Abstract:

Aquaculture is crucial to global food security and economic stability, garnering substantial research investment. Shrimp farming significantly contributes to the global economy; however, persistent disease outbreaks pose a considerable threat. Probiotics emerge as a viable and promising solution for managing pathogenic diseases in shrimp, fostering a more sustainable aquaculture industry. The aim of the work was to isolate and identify the probiotic bacteria from the naturally collected wild healthy shrimp and to control the shrimp pathogens isolated from the diseased shrimp. The healthy live tiger shrimp, *Penaeus monodon* of body weight 150.54 ± 10.60 were collected from the fisherman and brought to the laboratory. In the laboratory, the bacteria present in gut, gill and slime were isolated by serial dilution method and identified up to genus level by Bio-chemical tests. The isolated bacteria were tested against the pathogens. Eight bacterial isolates were taken from the wild healthy shrimp. They are *Bacillus sp.*, *Acetobacterium sp.*, *Pseudomonas sp.*, *Enterobacter sp.*, *Alteromonas sp.*, *Klesella sp.*, *Proteus sp.*, and *Halobacter sp.* Among the eight isolated bacteria, the *Bacillus sp.*, *Acetobacterium sp.* and *Pseudomonas sp.*, were selected as probiotic bacteria based on their beneficial effect on shrimp and their antagonistic effect on shrimp pathogens. The probiotic bacteria isolated from the gut of shrimp controlled the colonization of pathogens on gut, gill and slime of shrimp. This study highlights potentials of shrimp gut isolated bacteria in controlling the pathogens causing disease in shrimp and providing a significant step forward in antibiotic-free disease management.

Keywords: Probiotics, Shrimp, Antagonistic effect, Aquaculture, Gut Probiotics and Tiger Shrimp.

1. Introduction:

The use of beneficial bacteria probiotics to displace pathogens by competitive exclusion 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. R et al 1992). The term "probiotic" has been defined as: "probiotic is a mono- or mixed culture of live microorganisms that, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora" (Havenaar. R et al 1992). In extending their definition to aquaculture, David J. W. Moriarty 1999 suggest that it also applies to the addition of live, naturally-occurring bacteria to tanks and ponds in which the animals live, because these bacteria modify the bacterial composition of the water and sediment, The health of animals is thus improved by the removal, or decrease in population density, of pathogens and by improving water quality through the more rapid degradation of waste organic matter.

Probiotics such as the Gram - positive *Bacillus* offer an alternative to antibiotic therapy for sustainable aquaculture. *Bacillus* species are commonly found in marine sediments and therefore are naturally ingested by animals such as shrimps that feed in or on the sediment. An advantage of using *Bacillus* species is that they are unlikely to use genes for antibiotic resistance or virulence from the vibrios or related Gram - negative bacteria. There are barriers at the transcriptional and translational levels to the expression of genes from plasmid, phage and chromosomal DNA of *E. coli* in *B. subtilis* (Rabinowitz *et al.*, 1986). Bacterial species composition in shrimp ponds, which are large water bodies up to a hectare or more in size, hatchery tanks and shrimp guts can easily be changed and thus result in an improvement in shrimp production. In particular, luminous vibrio can be controlled in this manner. The mode of action of bacterial inocula is claimed to be the enhancement of natural processes such as organic matter degradation, nitrification, ammonia removal, denitrification, sulfide oxidation, and degradation of toxic pollutants. Some vendors state that by increasing the abundance of useful bacteria, competitive exclusion of undesirable species occurs. They even declare that the bacterial inocula can reduce the incidence of fish and shrimp disease in ponds. Ponds have a natural microbial flora capable of conducting the entire range of organic matter and nutrient transformation that occur in natural ecosystems.

Probiotic bacteria directly take up or decompose the organic matter and improve the water quality of an aquatic ecosystem. Beneficial microbial cultures produce a variety of exoenzymes like amylase, protease, lipase, etc., which help to degrade the unconsumed feed and feces in the pond, in addition to the possible role of these enzymes in the nutrition of the animals by improving feed digestibility and feed utilization. Among all the microbial interventions to augment the production, use of probiotics is in the central dogma. The modes of action of probiotics include the inhibition of a pathogen through the production of bacteriocin-like compounds, competition for attachment sites, competition for nutrients (particularly iron in marine microbes), alteration of enzymatic activity of pathogens, immunostimulatory functions, and nutritional benefits such as improving feed digestibility and feed utilization (Fuller 1G8G; Kesarcodi-Watson *et al.*, 2008).

2. Materials and Methods:

2.1 Isolation and Identification of Probiotic Bacteria from shrimp gut:

The healthy wild shrimp were collected from the fisherman in live condition. The collected shrimp were transferred to the laboratory under aseptic conditions for further studies. The shrimp pathogenic bacteria were obtained from the Microbiology laboratory of CMST, Marine Science, Rajakkamangalam, Tamil Nadu, India.

From the collected infected shrimp, the gut, gill and slime were removed from the animal in aseptic condition. Then the collected body parts were washed with sterile distilled water and smashed with the help of mortar and pestle. These samples were serially diluted with 9ml sterile distilled water. They are plated on nutrient agar and TCBS plates and Zobell Marine Agar plates using spread plate technique and incubated at 37°C for 24 to 48 hours. Then, the isolated colonies were identified up to genus level by biochemical test.

Determination Probiotic Bacteria:

Desirable characteristics for the selection of potential probiotics include (**Verschuere et al., 2000; Kesarcodi-Watson et al., 2008**).

i) no harm to the host; ii) acceptance by the host through ingestion, and colonization and proliferation within the host; iii) ability to reach target organs where they can work; and iv) no virulent resistance or antibacterial resistance

2.2 Collection and maintenance of shrimp:

The shrimp were collected from a shrimp farm and kept in quarantine tank with 0.5 ppm antibiotics (Chloramphenicol) to remove intestinal and surface bacteria. Ten shrimps were kept in plastic troughs each was filled with four litres of sea water of 30 ± 2 ppt. The water exchange was done daily to remove faecal matter and unused food materials. Aerators were provided for aerating the shrimp culture. Thirteen animals were reared in each trough and four replicates were also maintained. The temperature and dissolved oxygen were 30 ± 2 and 5.64 ± 1.60 respectively.

2.3 Experimental Set Up:

The experiment was carried out in an indoor culture system of 100 litre capacity of sterilized FRP tanks for a duration of 20 days. Ten numbers of two days starved adult shrimp were released gradually into the tank containing saline water (20 ppt). To begin with the experiment, the shrimp were fed on respective control and experimental diet. During the experimental period water quality parameters were also maintained regularly by giving 25 -30% of water exchange daily with proper aeration throughout the culture period.

2.4 Feeding and collection of unfed:

During the experimental period, the shrimp were fed twice daily i.e. 9.00 a.m. and 5.00 p.m. at ad libitum by check tray method. The unfed remains were collected from the check tray. The influence of the probiotic and pathogens on the growth responses of *P.monodon* was assessed during the 20th day of experimental duration.

2.5 Haemolymph sampling:

Haemolymph was obtained from the ventral part of the haemocoel of the second abdominal segment using a 25 gauge needle and an 1 ml syringe filled with 0.2 ml cold modified Alsever's solution (AS; 19.3 mM Na citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA (ethylene diaminetetra-acetic acid); pH 7.2) as an anticoagulant. Thus, prevented melanisation and kept haemocytes in a quiescent state (Rodriguez et al., 1995). The puncture procedure prevented to extract tissue particle during the haemolymph sampling. Directly after sampling, two haemolymph smears per animal were made and airdried. Remaining haemolymph was stored in 5 ml Eppendorf cups and kept on ice till analysing within 1 h after sampling.

2.6 Haemolymph sampling for bacterial count:

The haemolymph samples were collected after ten minutes of injection of pathogens and drawn the lymph was ten minutes interval, in cold modified Alsever's solution (AS). The uninjected control animals were

sampled at 1 h. The haemolymph was serially diluted in 15% NaCl solution, plated on BHI agar and incubated for 24 hr. at 28°C. The colony forming units (CFU) were counted and the concentration of live bacteria in the haemolymph was determined at the different sampling items.

$$\text{No. of bacterial population/ml} = \frac{\text{Average no. of colonies}}{\text{Vol. of inoculum added} \times \text{Dil. Factor}}$$

3. Results:

3.1 Identification of Bacteria:

Ten isolates which were identified as probiotics to shrimp were tested for antagonistic activity against pathogens. Among these only pathogenic bacteria were identified to control the pathogenic bacteria. These three probiotic bacteria were identified using bio-chemical test. The pathogenic bacteria were identified as *Aeromonas sp.*, *Enterococcus sp.*, *Vibrio sp.*, and *Vibrio sp.* The non-pathogenic bacteria were identified as *Bacillus sp.*, *Acetobacterium sp.*, and *Pseudomonas sp.*

3.2 Antagonistic activity of probiotics against pathogens:

Eight different bacteria were tested against the four pathogenic bacteria. Among the eight bacteria, three bacteria only controlled the pathogens. Among the three bacteria, *Bacillus sp.*, and *Acetobacterium sp.*, controlled all the pathogens but *Pseudomonas sp.*, controlled only one pathogen. *Acetobacterium sp.*, produce more zone of Inhibition (9.24mm) against *Aeromonas sp.*, and followed by *Bacillus sp.*, (8.60mm). But very low zone formation was found in *Pseudomonas sp.* (6.24mm). The *Acetobacterium sp.*, produced more zone of inhibition (4.30mm) against *Enterococcus sp.*, followed by *Bacillus sp.*, (3.68mm). No inhibitory zone formation was seen in *Pseudomonas sp.*, against *Enterococcus sp.* Antagonistic activity of probiotics *Acetobacterium sp.*, and *Bacillus sp.*, produced a zone size of 10.25mm and 10.60mm respectively against *Vibrio harveyi*. But no inhibition zone was formed in *Pseudomonas sp.* The *Bacillus sp.*, produced the highest zone of inhibition (5 ± 0.09) against *Vibrio parahaemolyticus* and followed by *Acetobacterium sp.*, (4 ± 0.09). No inhibitory zone was formed in *Pseudomonas sp.* (Table 1).

Table 1. Antagonistic activity against pathogenic bacterium:

Sl.NO	Name of the Probiotics	Name of the Pathogens			
		AE (mm)	EC (mm)	VH (mm)	VP (mm)
1.	<i>Bacillus sp</i>	8.60 ± 0.007	3.68 ± 0.0089	10.25 ± 0.020	5 ± 0.09
2.	<i>Acetobacterium sp</i>	9.24 ± 0.005	4.30 ± 0.0015	10.60 ± 0.018	4 ± 0.09
3	<i>Pseudomonas sp</i>	6.24 ± 0.005	-	-	-

AE: *Aeromonas sp.*; EC: *Enterococcus sp.*; VH: *Vibrio harveyi*; VP: *Vibrio parahaemolyticus*.

3.3 Survival rate of shrimp treated with bacterial probiotics and challenged with pathogen:

100 mg of lyophilized bacteria were mixed with 100 gm of feed in the concentration of 1.26×10^5 cfu/gm and were prepared separately for each probiotic and pathogens. The bacteria mixed feed was fed for 10 days, during this time survival rate and growth were recorded. No mortality was recorded in probiotic bacteria fed shrimp. But in pathogenic bacteria mixed feed like *Aeromonas sp.*, there was no mortality observed till fourth day and after that mortality was observed. Its survival rate was 96.67 to 33.34 from fifth day to tenth day. In *Enterococcus sp.*, no mortality was found in the first six days, then mortality observed from seventh day. Its survival rate was 96.67 to 83.34 in seventh day to tenth day. In *Vibrio*

harveyi no mortality stated for first three days, then mortality started from fourth day onwards. Their survival rates decreased from 96.67 to 13.34 from fourth day to tenth day. No mortality was observed in *Vibrio parahaemolyticus* for first three days, then mortality was observed on fourth day onwards. Its survival rate was 96.67 to 26.67 from fourth day to tenth day. (Table 2).

Table 2. Survival Rate of Shrimp challenged with probiotics and pathogens:

Sl.NO	No.of.Days	Survival rate of shrimp challenged with probiotics			Survival rate of shrimp challenged with pathogens			
		BA	AB	PM	AE	EC	VH	VP
1.	1.	100	100	100	100	100	100	100
2.	2.	100	100	100	100	100	100	100
3.	3.	100	100	100	100	100	100	100
4	4.	100	100	100	100	100	96.67± 4.715	96.67± 4.715
5	5.	100	100	100	96.67± 4.715	100	83.34± 9.429	91 ±8.165
6	6.	100	100	100	93.34± 4.715	100	76.67± 4.72	83.34± 9.429
7	7.	100	100	100	83.34± 9.42	96.67± 4.71	63.34± 4.71	73.34± 9.429
8	8.	100	100	100	66.67± 4.47	96.67± 4.71	43.34± 9.42	61± 8.16
9	9.	100	100	100	53.34± 12.47	93.34± 4.71	23.34± 9.42	43.34± 4.71
10	10.	100	100	100	33.34± 9.42	83.34± 9.42	13.34± 4.71	26.67± 4.71

AE: *Aeromonas sp*; EC: *Enterococcus sp*; VH: *Vibrio harveyi*; VP: *Vibrio parahaemolyticus*

BA: *Bacillus sp*; AB: *Acetobacterium sp*; PM: *Pseudomonas sp*.

3.4 Cell counting of Pathogen in the haemolymph of shrimp by plate count assay:

The pathogens were injected into the haemolymph of shrimp, and then the haemolymph were taken out in different time interval from 10 min to 60 min. The haemolymph obtained from different pathogens sensitized shrimps taken in different time intervals were plated in suitable selective media. The data showed that the number of pathogens increased when time duration increased. The data revealed that the OD value of *Aeromonas sp.*, sensitized shrimp were increased 0.91 and 0.32 between 10 min to 60 min of administration. In *Vibrio parahaemolyticus* the OD values were 0.251 and 0.401 increased between from 10 min to 60 min after injection. In *Vibrio Harveyi* the OD values were observed 0.18 and 0.41 with the time interval from 10 to 60 min. But no growth was observed in control (Table 3).

Table 3. Pathogenic count in the haemolymph of shrimp after administrating pathogenic bacteria:

SI.NO	Time duration (min)	Pathogens count				
		VP (x10 ²)	EC (x10 ²)	VH (x10 ²)	AE (x10 ²)	Control
1	10	0.251±0.05	0.211±0.10	0.181±0.10	0.91±0.21	0
2	20	0.277±0.06	0.217±0.24	0.231±0.09	0.111±0.16	0
3	30	0.301±0.05	0.226±0.15	0.298±0.12	0.171±0.20	0
4	40	0.331±0.11	0.257±0.18	0.336±0.10	0.221±0.25	0
5	50	0.37±0.11	0.277±0.25	0.383±0.15	0.283±0.20	0
6	60	0.401±0.06	0.297±0.25	0.411±0.25	0.328±0.10	0

Table 4. Clotting time of haemolymph, after the injection of pathogenic bacterium

SI.NO	Time duration (min)	Pathogens				
		AE (seconds)	EC (seconds)	VH (seconds)	VP (seconds)	Control (seconds)
1	10	64.1	63.5	67.0	64.6	61.42
2	20	68.4	68.4	77.4	75.1	61.20
3	30	71.1	70.0	85.1	83.4	60.45
4	40	73.19	71.05	93.52	91.18	61.20
5	50	77.16	74.42	101.42	96.40	60.32
6	60	83.45	78.47	111.06	105.44	61.42

AE: *Aeromonas sp*; EC: *Enterococcus sp*; VH: *Vibrio harveyi*; VP: *Vibrio parahaemolyticus*.

4. Discussion:

In the present study, probiotic bacteria were isolated from the healthy shrimp collected from the sea. Among the eight non-pathogenic bacteria tested for antagonistic activity against the pathogenic bacteria, three bacteria only had the antagonistic effect to control the pathogenic bacteria and therefore the three bacteria were only used as a probiotic in further study. The three probiotic bacteria identified were *Bacillus sp.*, *Acetobacterium sp.* and *Pseudomonas sp.*, according to their biochemical and cultural characteristics. Studies conducted by Laster and Giese, 1954; Fang and Mann, 1980 reported that the possibility of a symbiotic gut flora in several marine invertebrates such as sea urchin (Laster and Giese, 1954), Copepods (Sochard et al, 1979) and penaeid shrimp (Hood et al., 1971; Dempsey, 1989) *Pseudomonas* like bacteria (*Pseudomonas anthems*, *Altermonas sp.*) were identified in shrimp as well as its environment.

The four pathogens and three probiotic bacteria were challenged in shrimp culture and studied the survival of shrimp and bacterial load in different parts of shrimp. Although all the four pathogens decreased the survival rate, the pathogens like *Vibrio harveyi* and *Vibrio parahaemolyticus* effected more mortality when compared to the other pathogen like *Aeromonas sp.*, and *Enterococcus sp.*, This study confirmed that the *Vibrio harveyi* is the highly virulent pathogen than the other pathogens in shrimp. But non-pathogenic bacteria treated shrimp culture showed no mortality. The non-pathogenic bacterium like *Bacillus sp.*, *Acetobacterium sp.*, and *Pseudomonas sp.*, were found to be the beneficial normal flora of shrimp when compared to the pathogenic bacterium.

Rabinowitz JC and Roberts, M in 1986 stated that Probiotics such as the gram-positive *Bacillus* offer an alternative to antibiotic therapy for sustainable aquaculture. *Bacillus* species are commonly found in marine sediments and therefore are naturally ingested by animals such as shrimps that feed in or on the sediment. An advantage of using *Bacillus* species is that they are unlikely to use genes for antibiotic resistance or virulence from the vibrios or related Gram-negative bacteria. In our work, *Bacillus sp.*, *Acetobacterium*, *Pseudomonas sp.*, bacteria were isolated from the gut of a healthy shrimp and also used as probiotics against *Vibrio sp.*, *Aeromonas sp.*, and *Enterococcus sp.*

After challenging study, the bacterial colonization ability in the gut, gill and slime was also tested. The colonization ability of the probiotic strain was more in gut than pathogens. When compared among the probiotic bacteria *Acetobacterium sp.*, forms more colonies than *Bacillus sp.*, and *Pseudomonas sp.*, colonization in the slime was also higher than the pathogens. This data revealed that the probiotic bacteria only having more colonization ability in gut, gill and slime of shrimp than the pathogens.

In earlier studies, Moriarty, DJW in 1998 reported when the probiotic bacteria were used in a shrimp pond, no disease was experienced and indeed survival was very high (80-100%), even in the presence of luminous *Vibrio* species. In our study shrimp were reared with bacterial probiotics after challenged with pathogens the survival rate was very high in *Bacillus sp.*, followed by *Acetobacterium sp.*, and *Pseudomonas sp.*, applied experimental cultures.

The OD value in haemolymph collected from shrimp revealed that the prophenol oxidase activity of pathogenic *Vibrio harveyi* administered had high value of prophenol oxidase activity and followed by *Vibrio parahaemolyticus sp.*, *Aeromonas sp.*, *Enterococcus sp.*, and control. It might be expected that showing of higher prophenol oxidase activity would be a signal for the higher pathogenic power of the pathogen. When compared to the prophenol oxidase assay of all pathogen the *Vibrio harveyi* evoked very high prophenol oxidase activity assay, which proves the higher virulence capacity of *Vibrio harveyi* than the other shrimp pathogens.

In most *Vibrios* produce many heat and toxic label and haemolycins factors which have been demonstrated by Rodrigues et al. in 1993. In our research the selected pathogens were administered into the haemolymph of shrimp and collected the haemolymph after thirty mins of sensitization. Among the sensitized haemolymph collected from the treated animals, the pathogen *Vibrio sp.*, required more time duration when compared to the other pathogens and control. So, it is concluded that the *Vibrio harveyi*, was the virulent pathogen in shrimp.

The overall data of different parameters of the present study revealed that a higher virulence was found in *Vibrio harveyi* and *Vibrio parahaemolyticus* in shrimp than the other pathogens *Aeromonas sp.*, and *Enterococcus sp.* In the plate count assay, the cell count of *Vibrio harveyi* was more than in number than other pathogens and therefore *Vibrio harveyi* is the more virulent pathogen and it infect the shrimp and found to be the important pathogen

5. Conclusion

The present study successfully isolated and identified probiotic bacteria from healthy shrimp and evaluated their ability to control bacterial pathogens associated with diseased shrimp. A total of eight bacterial isolates were obtained from the gut, gill, and slime of healthy shrimp, among which *Bacillus sp.*, *Acetobacterium sp.*, and *Pseudomonas sp.*, were identified as potential probiotics based on their beneficial effects and strong antagonistic activity against shrimp pathogens. Pathogenic bacteria *Aeromonas sp.*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Enterococcus sp.*, in which *Vibrio harveyi* identified as the

most virulent pathogen. The results demonstrated that probiotic bacteria from the shrimp gut effectively inhibited pathogen colonization in the gut, gill, and slime, highlighting their potential role in controlling bacterial diseases in shrimp culture without altering the natural microbial balance.

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