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Isolation of probiotic bacteria from gastrointestinal tract of pacific white shrimp *Litopenaeus vannamei* and antibacterial activity of probiotic bacteria against *Vibrio* spp.

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Abstract
The research work was carried out at Fisheries Research Station, Junagadh Agril. University, Okha port, Gujarat during May 2016 to October 2016 with the aim to isolate potential probiotic bacterial strains from the digestive tract of white leg shrimp (*Litopenaeus vannamei*), and to carry out *in vitro* anti-vibrio activity of the isolates and *Lactobacillus plantarum* and *Bacillus subtilis*. Total ten putative bacterial strains were isolated and partially identified as *Lactobacillus* spp. based on microscopic observation and morphological characters. Among the isolates two strains showed comparatively higher anti-vibrio activity while *L. plantarum* and *B. subtilis* showed strong antibacterial activity against *Vibrio harveyi, V. parahaemolyticus* and *V. vulnificus*. Gut isolate PRT3 created maximum inhibition zone of 11.33 ± 1.53 mm against *V. harveyi* and *V. parahaemolyticus*, PRT7 created maximum inhibition zone of 10.67 ± 1.53 mm against *V. parahaemolyticus* followed by 10.33 ± 1.53 mm against *V. harveyi*. *L. plantarum* created highest inhibition zone of 14.67 ± 1.16 mm and 14.67 ± 0.58 mm against *V. parahaemolyticus* and *V. vulnificus* respectively. *B. subtilis* created maximum inhibition zone of 13.00 ± 1.00 mm against *V. harveyi* and *V. parahaemolyticus*. *L. plantarum* have better potential for use as a probiotic in aquaculture as an anti-*Vibrio* agent.

Keywords: Probiotic, antagonist, antibacterial, shrimp, isolation, *Vibrio*, lactobacillus, bacillus

1. Introduction
The disease outbreaks are constraint to aquaculture production system thereby its control has been achieved by following different methods using traditional ways, synthetic chemicals and antibiotics [1]. However, the use of such expensive chemotherapeutant for controlling diseases has been widely criticized for their negative impacts like accumulation of residues, development of drug resistance; immunosuppressant and reduced consumer preference for aqua products treated with antibiotics [1]. As an alternate of it probiotic bacteria has attracted the interest of the shrimp farming industry. Gatesoupe defined probiotics as "microbial cells administered in a certain way, which reaches the gastrointestinal tract and remain alive with the aim of improving health" [2]. Microbial outbreak of disease often arises in the early stages of shrimp.

In recent years, the use of probiotic bacteria has attracted the interest of the marine shrimp farming industry. Bacteria isolated from the host is expected to give a better effect because it can adhere to the host's gut and adapt well to compete with the pathogens in getting nutrients [3]. Therefore, probiotics isolation from the gastrointestinal tract of the host is the best approach to obtain the probiotic candidates. *Pseudomonas* 1-2 strain identified as *P. aeruginosa* isolated from estuarine water produced inhibitory compounds against *V. harveyii, V. fluvialis, V. parahaemolyticus, V. damsela* and *V. vulnificus* in *in vitro* experiments [4]. Gopalakannan studied the effect of various strains of lactic acid bacteria, isolated from marine fish, on *P. monodon* larvae to control *V. anguillarum* infection. Among the isolated strain seven bacterial strains showed antagonistic effect against *V. anguillarum* [5]. A total of 80 bacterial strains were isolated from hepatopancreas of healthy wild shrimp. *In vitro* probiotic effect was evaluated using agar diffusion techniques. Three strains identified as *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 showed inhibitory effects against *V. harveyi* [6]. Bacterial strain PPP 13 isolated from intestine of black tiger shrimp (*P. monodon*) was found to have antagonistic properties against three target pathogenic bacterial strains of *Vibrio alginolyticus, V. harveyi, V.*

vulnificus [7]. *Bacillus* spp. tested against pathogenic strains of *Vibrio* and recorded antagonistic effect and the bacterial strain was not harmful to the larvae [8]. The feasibility of shrimp gut probions with Anti-*Vibrio* in penaeid culture was studied and a total of 12 gut isolates were isolated from healthy adult shrimp *Penaeus monodon*. Among these 12 isolates, the probionts *Bacillus* sp. AVP03 and AVP07 rendered maximum activities in both *in vitro* and *in vivo* tests [9]. *Lactobacillus pentosus* isolated from digestive tract of white shrimp *Litopenaeus vannamei* exhibited antagonistic activity against shrimp pathogens like *Vibrio vulnificus, V. rotiferianus* and *V. campbellii* [10].

The present study was formulated to isolate the putative bacterial strains from gastrointestinal tract of white leg shrimp *Litopenaeus vannamei*, anti-*Vibrio* activity of the gut isolates and other probiotic strains like *Lactobacillus plantarum* and *Bacillus subtilis.*

2. Materials and methods

2.1 Collection of shrimp for gut samples

Litopenaeus vannamei shrimps weighing more than 10 g were collected form the private shrimp farms at Chorwad, Gujarat. Total four sampling was done at an interval of fifteen days from private shrimp farm and two samplings were done form the RCC pond reared shrimps (shrimps were reared from Post Larvae-14 with the probiotics free feed) in the month of May to July 2017 and transported to the laboratory at Fisheries Research Station, Junagadh Agricultural University, Okhaport, Gujarat (Latitude: 220 28' North, Longitude: 690 05' East). During transportation to the laboratory, samples were stored in dark and kept cool using ice box. Immediately after reaching the laboratory the shrimps were aseptically dissected for collection of the gastrointestinal tract sample. Digestive tracts, together with the contents, were sampled for weight at least one gram.

2.2 Isolation of bacterial strains

Collected 1 g gastrointestinal tract homogenized in a sterilized blender with sterile saline (0.9% NaCl, pH 7.0) on ice plate. The homogenates were serially diluted (1/10) in test tubes with sterile saline, and 1 ml aliquots were inoculated onto plates of de Man, Rogosa and Sharpe (MRS) Agar supplied by HIMEDIA Labs. The plates incubated at 32 ^{0}C for 24 h to 48 h Colonies with different morphological characteristics were marked under an optical microscope. The colonies with different colour and shapes were separated and re-cultured onto MRS Agar. The colonies were purified several times until singular and uniform colonies were obtained.

From six batches of sampling ten colony forming bacteria were isolated and purified. These were named as PRT1, PRT2, PRT3, PRT4… and PRT10. Following purification on MRS Agar, the bacterial strains were stored in glycerol saline (12% glycerol, 0.9% NaCl) at −80 ℃ until use.

2.3 Procurement of bacterial strains

The probiotic and pathogenic bacterial strains as listed below (table 1) were purchased from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

Table 1: List of bacterial strains purchased from MTCC.

S. No.	Name	Code
1	*Lactobacillus plantarum*	MTCC 9511
2	*Bacillus subtilis*	MTCC 10402
3	*Vibrio harveyi*	MTCC 7954
4	*V. parahaemolyticus*	MTCC 451
5	*V. vulnificus*	MTCC 1145

2.4 Preparation of bacterial strains

Laminar air flow system was used for carrying out the microbial work aseptically.

2.4.1 *Lactobacillus plantarum*

The pure strain of *L. plantarum* (MTCC 9511) was transferred aseptically from the vial to MRS broth and incubated at 37°C for 24 h. The CFU/ml was determined by spread plate method on agar plates. The strain thus cultured was stored in agar slants in test tubes at 4°C in refrigerator for subculture [11].

2.4.2 *Bacillus subtilis*

The pure strain of *B. subtilis* (MTCC 10402) was transferred aseptically from the vial to Nutrient broth and incubated at 37°C for 24 h. The CFU/ml was determined by spread plate method on agar plates. The strain thus cultured was stored in agar slants in test tubes at 4°C in refrigerator for subculture [11].

2.4.3 *Vibrio* spp.

The pure strain of *Vibrio* spp. were transferred aseptically from the vial to TCBS broth and incubated at 37°C for 24 h. The CFU/ml was determined by spread plate method on agar plates. The strain thus cultured was stored in agar slants in test tubes at 4°C in refrigerator for subculture [11].

2.5 *In vitro* anti-*Vibrio* activity of probiotics

In vitro antimicrobial activity of the isolated intestinal bacteria (PRT1 to PRT10), *L. plantarum* and *B. subtilis* was performed using agar well diffusion method [12] in the laboratory at Fisheries Research Station, Okhaport during the month of October 2017. The potential culture of these isolated bacteria and *L. plantarum* were grown in 50 ml MRS broth and *B. subtilis* was grown in 50 ml Nutrient broth and incubated for 24 h at 30 °C. After the incubation period it was centrifuged at 10,000 rpm for 10 min and the obtained supernatant was passed through a 0.25 µm syringe driven filter and neutralized (pH 7.0) with 2 N NaOH.

Mueller-Hinton agar plates were prepared and swabbed with fresh culture of three pathogenic bacteria of the *Vibrio* spp. in separate plates. Then, a 6 mm diameter hole was punched aseptically with sterile cork borer and the well was filled with 40 µL of filtered supernatant from each culture. In control plates sterile distilled water was filled. Three replicates of the each treatment were arranged. Then the agar plates were incubated for 24 hours incubation at 30 °C. The bacteria diffuses in the agar medium and inhibits the growth of the pathogenic bacteria. After the incubation period the diameter of the clear zone including the well was measured [12].

2.6 Statistical analysis

Analyses of the data to test for significant differences between treatments was conducted using one way ANOVA of

Complete Randomized Design and Post Hoc Duncan test for multiple comparisons was conducted using IBM SPSS Statistics data editor.

3. Results and discussion

Ten bacterial strains were isolated from the gastrointestinal tract of *L. vannamei* and named as PRT1, PRT2….and PRT10. They were partially identified as *Lactobacillus* spp. based on growth characteristic and microscopic observation. These ten bacterial strains, *L. plantarum* and *B. subtilis* were used to check the antagonist effect against *Vibrio harveyi*, *V. parahaemolyticus* and *V. vulnificus*. The observation was recorded by measuring the inhibitory zone including well. The results are depicted in Table 2. The data recorded were statistically analyzed using Post Hoc Duncan test in IBM SPSS Statistics data editor.

Among 10 isolates two (PRT3 and PRT7) had good antagonist activity (Plate 1) against *Vibrio* spp. Another probiotic bacteria *L. plantarum* and *B. subtilis* showed strong antagonist activity against the *Vibrio* spp. The zone of inhibition created by PRT3, PRT7, *L. plantarum* and *B. subtilis* was significantly ($p<0.05$) higher than the control and all other putative bacterial strains. *L. plantarum* created greater inhibition zone against *V. harveyi* (13.67 $\pm$ 0.58 mm) and *V. parahaemolyticus* (14.67 $\pm$ 1.16 mm) which was significantly higher than the control and all isolated bacterial strains. While it's antibacterial activity against *V. vulnificus* (14.67 $\pm$ 0.58 mm) was significantly higher than all other bacterial strains. *Bacillus subtilis* created significantly greater inhibition zone as compared to all isolated bacterial strains. Its antibacterial activity against *V. vulnificus* was significantly lower than *L. plantarum* and it showed non-significant difference with antibacterial activity against *V. harveyi* and *V. parahaemolyticus.* PRT2, PRT5 and PRT9 did not show any antibacterial activity against any of the *Vibrio* spp. while all other strains showed antibacterial activity. PRT3 and PRT7 showed significantly higher antibacterial activity against all three *Vibrio* spp. PRT3 and PRT7 showed almost same antibacterial activity and it was lower than *L. plantarum* and *B. subtilis*.

Many studies have shown that various compounds or bacteriocins produced by bacteria could be used to inhibit bacterial pathogens in aquaculture [13]. *L. plantarum* is antagonistic to Gram negative, Gram positive bacteria and yeast [14]. The *Lactobacillus* sp. (AMET1506) strain showed strongest antagonistic activity against different seafood bacterial pathogens such as, *E. coli*, *V. cholerae*, *V. parahaemolyticus, Salmonella sp*. and *Shigella* sp. [15]. In another experiment checking its antibacterial activity against *Vibrio harveyi* the maximum inhibition zone (18mm) was observed [16].

Many studies have reported that different species of the genus *Lactobacillus* produce bactericidal proteins [17, 18] which exhibit strong antimicrobial activity against many pathogenic microorganisms [19, 20]. *L. acidophilus* effectively inhibits the growth of *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* with maximum inhibition zone of 16 mm against *V. alginolyticus* while rest two showed average inhibition zone between 10 -16 mm [21]. Balcazar and Rojas reported the same inhibitory effect of *Vibrio* species against *Bacillus* [22]. Several studies have reported that *Lactobacillus* produces peptide antibiotics like bacteriocins, which are active against a wide range of Gram-positive and Gram-negative bacteria [23]. Fourteen strains of LAB isolated from gut of *Mugil cephalus* were *in vitro* tested against various pathogens including *V. harveyi* and *V. parahaemolyticus*. Most of isolates showed inhibition zone of 10-15 mm against these two *Vibrio* spp. [24]. Various *Lactobacillus* strains isolated from the shrimp intestine used for the *in vitro* antagonistic effect on various pathogen including *V. harveyi* and recorded 16 mm inhibition zone by two isolates while others showed inhibition zone in the range of 8-13 mm [25]. In present study, PRT3 and PRT7 bacterial strains isolated from shrimp gut showed respectively 11.33 and 10.33 mm average inhibition zone against *V. harveyi*, 11.33 and 10.67 mm average inhibition zone against *V. parahaemolyticus* and 10.67 and 10.00 mm average inhibition zone against *V. vulnificus. L. plantarum* also showed strong antibacterial activity against *Vibrio* spp. in present study. The inhibition zone created was 13.67 mm against *V. harveyi* and 14.67 mm against *V. parahaemolyticus* and *V. vulnificus.* The results agrees with the results obtained by Sivakumar *et al.* [21]. The results are also resembles with the antibacterial activity of LAB against *V. harveyi* and *V. parahaemolyticus* recorded by Ghosh *et al.* [24]. Vieira and associates [25] also obtained similar results of antibacterial activity of shrimp gut isolated various *Lactobacillus* strains against *V. harveyi* and other pathogenic bacteria. A similar pathogen inhibition *in vitro* was reported for potential probiotics isolated from aquatic organisms, such as *Lactobacillus* sp. isolated from *Salmo salar* [26].

Many authors have reported the antagonistic effect of *Bacillus* species against Gram negative microorganisms like *Vibrio* spp., *Coryneforms*, *Enterobacteriaceae*, *Pseudomonas* spp., *Moraxella* spp. and *Flavobacterium* spp. [27, 28, 29, 30]. *Bacillus* spp. isolated from *P. monodon* gut inhibited the growth of *V. harveyi* VSH5 [9]. They also used *B. subtilis* as reference strain of probiotic and recorded 16.9 mm inhibition while isolates showed highest inhibition zone of 19 mm and rest isolates created inhibition zone in the range of 13.4 to 16.9 mm. Most of the studies have suggested that the inhibitory effects of *Bacillus* sp. might be due to alteration of pH in the medium, utilization of essential nutrients and production of volatile compounds [6, 31]. In addition, several studies have also reported that the *Bacillus* spp. produces polypeptide antibiotics such as bacitracin, gramicidin S, polymyxin and tyrotricidin [32]. Sugita and associates recorded 41 mm inhibition zone by *Bacillus subtilis* against *V. vulnificus* [30]. *B. subtilis* showed 6 mm inhibition zone against *V. harveyi* [33]. Three different strains of the *Bacillus* were used by Janarthanam and associates for *in vitro* antagonist test and found to create 10 mm inhibition zone. In present study, *B. subtilis* showed strong antagonist activity against *Vibrio* spp. [34] and created 13 mm inhibition zone against *Vibrio harveyi*, the results are comparable with the results obtained by Ramesh *et al.* [9] in the same probiotic bacteria and pathogenic bacteria. They obtained 13.4 to 19 mm inhibition zone. The higher antibacterial activity recorded by them may be due to change in probiotic strain. The antibacterial activity of the isolates agrees with the results obtained by Janarthanam *et al.* [34] but in present study higher antibacterial activity recorded by *B. subtilis* and it is very lower than the results obtained by Sugita *et al.* [30].

Table 2: Inhibition zone (mm) of probiotic bacterial strain against *Vibrio* spp. (mean ± SD, n=3).

Treatment	*V. harveyi*	*V. parahaemolyticus*	*V. vulnificus*
PRT1	07.33 ± 0.58	07.67 ± 0.58	07.00 ± 0.00
PRT2	06.00 ± 0.00	06.00 ± 0.00	06.00 ± 0.00
PRT3	11.33^{bc} ± 1.53	11.33^{bc} ± 1.53	10.67^{c} ± 1.16
PRT4	08.00 ± 1.00	08.00 ± 0.00	08.00 ± 0.00
PRT5	06.00 ± 0.00	06.00 ± 0.00	06.00 ± 0.00
PRT6	07.67 ± 0.58	07.00 ± 1.00	06.67 ± 0.58
PRT7	10.33^{c} ± 1.53	10.67^{c} ± 1.53	10.00^{c} ± 1.00
PRT8	06.33 ± 0.58	06.33 ± 0.58	06.33 ± 0.58
PRT9	06.00 ± 0.00	06.00 ± 0.00	06.00 ± 0.00
PRT10	06.67 ± 0.58	07.00 ± 1.00	06.33 ± 0.58
L. plantarum	13.67^{a} ± 0.58	14.67^{a} ± 1.16	14.67^{a} ± 0.58
B. subtilis	13.00^{ab} ± 1.00	13.00^{ab} ± 1.00	12.67^{b} ± 0.58
Control	06.00 ± 0.00	06.00 ± 0.00	06.00 ± 0.00

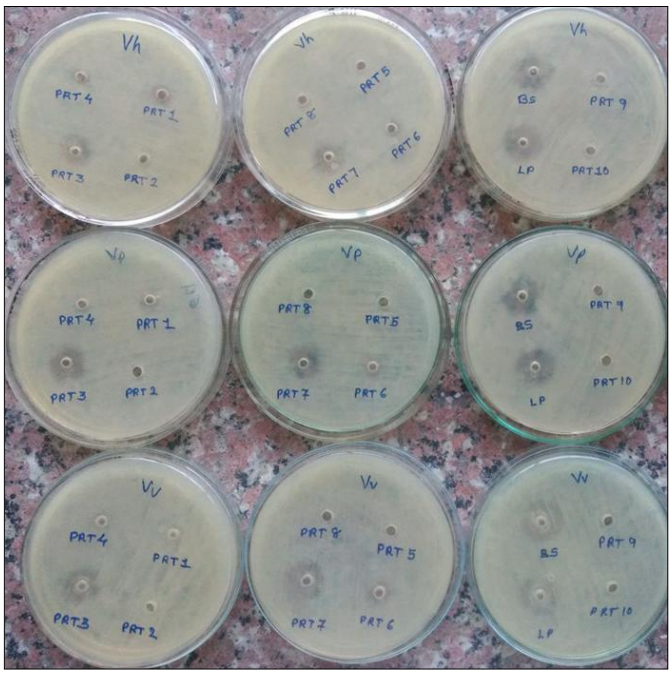

Plate 1: Anti-*Vibrio* activity of bacterial strains. (Vh = *Vibrio harveyi*, Vp = *V. parahaemolyticus*, Vv = *V. vulnificus*, BS = *B. tubtilis* and LP = *L. plantarum*)

4. Conclusion

Probiotic bacterial strains can be isolated from gastrointestinal tract of white leg shrimp *L. vannamei* and shows good antibacterial activity against *Vibrio* spp. *In vitro* experiment proved that gut isolated bacteria, *L. plantarum* and *B. subtilis* are antagonist to *V. harveyi*, *V. parahaemolyticus* and *V. vunlificus.* After confirming *in vivo* effect these bacterial strains can be used as probiotics in shrimp farming and shrimp hatchery to combat with *Vibrio* influenced diseases.

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