

Evaluation of probiotic bacteria *Leucosinostoc mesenteroides* against White Gut Disease in shrimp aquaculture**A.Sathyapriya*****Assistant Professor, Department of Biotechnology, Rathinam College of Arts and Science, Coimbatore*****Corresponding author email ID – sathyapriya.biotech@rathinam.in****Abstract**

With increasing demand for environment friendly aquaculture, use of beneficial bacteria to displace pathogens by competitive processes is being used in the animal industry as a better remedy and is now gaining acceptance for pathogen control in aquaculture. With this concern, the present study was designed for isolation of suitable probiotic bacteria from natural sources, application in an effective dose in the rearing environment is expected to control the blowout of White Gut Disease in aquaculture systems. The interaction of pathogenic *Vibrio anguillarum* and the inner surface of the digestive tract of *P. monodon*, with a specific focus on their in-situ morphology, aggregation and attachment characteristics presented with pathogenic bacterial species and under the control of probiotic bacteria were analyzed through. The percentage of granular cells of treated group was higher than the control. Histological studies revealed that the treated group has optimistic effect which helps in the reduction of tissue damage and decrease the mortality rate than the infected shrimps. *Leuconostoc* sp., can survive in the saline condition rather than other *Lactobacillus* sp., and its tolerance of acidic environment of the shrimp intestine and their adherence level at the intestine will progressively replace the *V. anguillarum* from the infected shrimps and commendably control the White Gut Disease.

Key words: *Penaeus Monodon*, *Vibrio anguillarum*, *Leuconostoc mesenteroides*, Histopathology, White gut disease, Probiotics.

Introduction:

One of the most profitable and fastest developing segments of the global seafood industry is the shrimp aquaculture market. While at least 50 countries around the world have been cultivating shrimp globally for several decades, the entire industry is highly concentrated in two major regions, Asia and the Americas. Asia (China, Thailand, Vietnam, Indonesia, Malaysia, Philippines, India and Bangladesh) accounted for 81% in 2017 (Mungkung, 2005; Tacon, 2013). The last three decades, the global aquaculture of Penaeid shrimps has expanded steadily (Ma, 2015). For eg, Penaeid shrimps belonging to the genus *Penaeus* expand to a greater size, tiger prawns reach a total length of 300 mm, *P. monodon* (Nandakumar and Maheswarudu, 2003). Improper agriculture, lack of technical resources, lack of high quality shrimp seeds, lack of infrastructure facilities, bad weather conditions, tension among local people and risk of disease are main impediments of shrimp aquaculture. In order to track and maintain their health, marine animals need more care relative to terrestrial animals and plants. In aquatic environments, diseases occur because of the complex interaction of three components: the farmed animal (host), the disease-causing organisms (pathogens) and the ecosystem (Sivasankar *et al.*, 2017). Although outbreaks of viral disease can be avoided with the provision of SPF-PL and proper water treatment, (Alfiansah, 2019) although outbreaks of lethal bacterial disease do occur regularly and often devastate shrimp farming.

In general, *Vibrio* sp., is particularly harmful among the aquatic pathogens and the potential routes of infection in shrimp are eating, gill, hepato pancreas, etc. Major *Vibrio* sp. viz. *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. splendidus*, *V. campbellii*, *V. fischeri*, *V. mimicus*, and *Streptococcus* sp. are typically associated with bacterial shrimp diseases (Alfiansah, 2019; Chatterjee and Haldar, 2012). Antibiotics and other medicinal agents are commonly used as popular therapies in post-infection practice in the marine shrimp community. Their indiscriminate application, though, has contributed to the appearance of antibiotic-resistant strains, thus contributing to shrimp meat and the ecosystem being polluted. The use of antibiotics has since been banned by many countries (Vieira *et al.*, 2010). Aqua scientists have selected beneficial microorganisms called Probiotic to displace pathogens by competitive processes and are now gaining recognition for the management of pathogens in aquaculture by finding a healthy and lasting solution for the prevention of pathogenic diseases (Raja *et al.*, 2015). Probiotics are live microorganisms that offer health benefits to the host and can be isolated from different sources. Fermented vegetables have historically been produced by using the natural microbiota associated with plant material (Orgeron *et al.*, 2016).

Fermenting vegetables helps in the development of Lactic Acid Bacteria (LAB) organic acids and a variety of antimicrobial compounds. Fermentation takes place over several weeks at temperatures between 15 and 20°C and is carried out in a microbial succession, characterized by two stages: a hetero fermentative stage dominated by *Leuconostoc mesenteroides*, followed by a homo fermentative stage dominated by *Lactobacillus plantarum* (Touret *et al.*, 2018).

Materials and method**Survey/Study area**

An extensive field survey was carried out in the shrimp aquaculture farms at Chidambaram Taluk, Cuddalore District during 2016–2017 for screening the presence of disease outbreak.

Sampling method

Sampling of infected shrimps (*Penaeus Monodon*) with pond soils were collected from farms situated in the coastal regions of Thiruvasaladi, Killai, Paragipettai Villages of Chidambaram Taluk, Cuddalore District, Tamil Nadu.

Isolation of pathogen

One gram of infected shrimp tissue was homogenized in PBS (1X), serially diluted and inoculated in Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar medium (Janarthnam *et al.*, 2012). Cultures obtained in TCBS agar plates were purified, stored in TCBS with 10% glycerol at 4°C until further use.

Isolation of lactic acid bacteria (LAB) from fermentation

To prepare sauerkraut, self-fermentations of cabbage (organically farmed) by natural microbes were conducted in glass jar (Suzanne *et al.*, 2007). For the isolation of LAB, samples from brine during fermentation (after 4 days) were plated onto MRS agar, incubated in anaerobic vessels at 30°C for 4 days (Tamminen *et al.*, 2004).

Efficacy of LAB against diseased *Peneaus monodon* via challenge trial

Preparation of experimental probiotic feed

The lyophilized *L. mesenteroides*, was formulated as probiotic feed (till the end of experiment) in sterilized calcite (10^{10} CFU/g) powder and allowed to dry for 12 hrs (Kumar *et al.*, 2014).

Culture of pathogenic bacteria

The isolated bacterial pathogen *V. anguillarum* density was adjusted to 1×10^9 CFU ml⁻¹ and used to infect *P. monodon* respectively.

Experimental design

Feeding material

For the probiotic supplemented diet, 5 g of powdered *L. mesenteroides* was mixed with Kg of commercial feed (Arun kumar, 2014).

Experimental animals

The experimental work was carried out for 120 days during the period of March - June, 2018 in Vaishale Prawn Hatchery, Kanchipuram District, Tamil Nadu. Healthy shrimps (*P. monodon*) at post larvae (PL 30) stage was taken up from the hatchery. The experiment was performed in tanks with 30 L capacity at 28 ± 5 °C with marine water was used throughout the study period.

Challenge trail

Before the experimentation, PL 30 was allowed to acclimatize in tanks of 30 L filled with marine water having salinity of 20- 25 ppt for five days. PL 30 was randomly distributed into six distinct experimental groups. For each group, duplicates were maintained. The tanks were stocked with the density of PL 30 in each tank with a body weight of 2 ± 0.2 g each (Raja *et al.*, 2017). The challenged shrimp groups were observed regularly for any overt signs of diseases including behavioural abnormalities for 15 days. After the infection, the treatments were set up as follows.

- T1 – Control (*P. monodon* fed with commercial feed alone)
- T2 – Probiotic control (*P. monodon* fed with mixed commercial and probiotic feed)
- T3 – Negative control (infected *P. monodon* fed with commercial feed alone)
- T4 – Treated (infected *P. monodon* fed with fed with mixed commercial and probiotic feed)
- T5 – Positive control (*P. monodon* with pathogen with antibiotics)

Water quality parameters

Water quality parameters such as temperature, pH, salinity, alkalinity, hardness, total dissolved solids (TDS), ammonia-nitrogen (NH₃-N), chloride, sulphate, sulphide, nitrate-nitrogen (NO₃-N), nitrite nitrogen (NO₂-N), phosphate-phosphorus (PO₄-P), fluoride, residual chlorine, iron in experimental tanks were performed periodically (APHA, 1998).

Total bacterial load

Total plate count (TPC) and total *Vibrio anguillarum* count (TVC) in water, HP and gut tissue were estimated (Raja *et al.*, 2017).

Growth performance and survival rate

Growth performance of shrimp experimental groups were measured based on mean individual weight at harvest, total weight gain, growth rate, feed conversion ratio and survival rate. The above were calculate as per formula of Suriya *et al.*, (2016).

Microscopic identification of white gut disease

The shrimp were examined for the clinical signs including external and internal lesions through microscopic techniques.

Histopathology evaluation

Histopathology screening was done based on the characteristic of internal lesions and the presence of intranuclear, perinuclear or intracytoplasmic inclusions for white gut disease. Mid-line sections of the gill and abdominal segments were removed and processed routinely with hematoxylin and eosin staining, mounted and observed under the light microscopy (Leica DM 750, Germany) (Lightner, 1996).

Hematology studies

To evaluate the blood physiological parameters, feeding were ceased for 24 hrs prior to sampling. Total haemocyte count (THC) as well as granular haemocyte (GH) and nongranular haemocyte (NGH) counts were calculated (Sritunyalucksana *et al.*, 2005).

Results and Discussion

Sample collection, bacterial pathogen isolation

Infected shrimp tissue with pond soil were collected from the shrimp growing areas of Thiruvasaladi, Killai, Parangipettai Villages, Chidambaram Taluk, Cuddalore District (Fig 1). Isolation of *Vibrio* sp., from infected shrimp and pond water using TCBS plates at 37°C for 48 hrs of incubation resulted in *Vibrio* sp., isolates with respect to colour, shape and size of the colony (Plate 1). The total prevalence of vibriosis was (64.4%) among the examined Indian prawn, *Peneaus monodon*.

Green and Yellow colonies presumptive as *Vibrio* sp., observed on TCBS plate containing the dilution of 10^{-2} were re streaked on TCBS plate for getting the pure culture. Similarly, Durai *et al.*, (2015) reported white gut and white feces disease in *Litopenaeus Vannamei* shrimp at Kodakaramulai, Sirkali Taluk, Nagai District. Gunalan *et al.*, (2014) reported Black Gill Disease, White Muscle Disease, White Gut Disease and Muscle Cramp Disease in Tamil Nadu and Andhra Pradesh. Whereas Jayasree *et al.*, (2006) reported the disease caused by *Vibrio* sp. in *Penaeus Monodon* as: tail necrosis, shell disease, red disease, Loose Shell Syndrome (LSS) and White Gut Disease (WGD). Similarly, Kumaran and Citarasu, (2016) isolated three major species namely *V. harveyi*, *V. anguillarum* and *V. parahaemolyticus* from infected *Artemia Franciscana* shrimps at Marakkanam, Kancheepuram District, Tamil Nadu. In the year 2015, Behura *et al.*, isolated four strains of *V. anguillarum* from gill, HP, hemolymph and carapace of diseased fresh water prawn and they were characterized based on the biochemical and serological tests.

Figure 1: Sampling site at Parangipettai Village, Cuddalore District



Plate 1 Isolated *Vibrio* sp., from sampling areas



Isolation and identification of LAB

The sliced cabbages were salted with 2 – 2.5 % (w/v) of NaCl solution for sauerkraut fermentation, incubated in the air tight container. After 4 days, brine samples were plated onto MRS agar and incubated. The viscous colonies presumed to be *Leuconostoc* sp., were picked up randomly from MRS agar plates, purified and stored at 4°C as glycerol stock for further identification. Based on Bergey's Manual of Systematic Bacteriology, the isolated natural microbiota were identified as *Leuconostoc mesenteroides* (Plate 2). The voucher specimen was sent to MCC, Pune for further confirmation and the specimen was deposited for public access (MCC 3276). Likewise, Touret *et al.*, (2018) and Barrangou *et al.*, (2002) isolated and identified *Leuconostoc* sp. and *Leuconostoc fallax* from industrial sauerkraut fermentation. In contrast Abekhti *et al.*, (2014), isolated and identify dominant osmophilic *Leuconostoc* strains from traditional date product (Btana). *Leuconostoc mesenteroides* from Algerian raw camel milk and sauce-type Kimichi

Plate 2 Isolation of probiotic microorganism from fermented vegetable



Challenge trail

The experiment was carried out for 120 days during the period of March - June, 2018 in Vaishale Prawn Hatchery, Kanchipuram District, Tamil Nadu. Healthy shrimps (*P. monodon*) at post larvae (PL 20) stage were challenged with *V. anguillarum* followed by treated with *L. mesenteroides* and antibiotics as positive control. During course of trial water quality, microbial load in water and tissue, growth and mortality rate, haematological and histopathological changes were measured periodically.

Water quality analysis

The physical parameters of water play vital role in the culture systems and maintenance of water quality is essential for optimum growth and survival of shrimp. The optimal water quality parameters for maintaining suitable culture is pH (7.0-8.5), Ammonia (100 ppm) respectively. In comparison of normal values with all the treatments, the negative control (T3) showed slight variations in quality parameter like pH (8.7), Ammonia (4.0 ppm), Carbonate (44 mg/L), Total Alkalinity (260 mg/L) and Iron (1.2 ppm), which may be of an microbial (infection) load (Table 4.15). Further water quality parameters of treatment 4 (*L. mesenteroides*) was found to be equal with treatment (1 and 2) by the same time which was superior as compared with Treatment 3 (Tab 1). Maintaining water quality during the larval rearing phase is very critical due to the sensitivity of the larvae to fluctuating water parameters (Santosh and Singh, 2007). The worse environmental condition is responsible for weakening of shrimp quality as well as rising of many shrimp diseases. Further in shrimp, the excess feed, fecal matter and metabolites will exert terrific influence on the water quality of shrimp farm (Soundarapandian and Gunalan, 2008).

Treatments	Normal Value	Days after treatment							
		Treatment 1				Treatment 2			
		30	60	90	120	30	60	90	120
pH	7.0-8.5	7.0	7.6	7.2	8.0	7.2	7.8	8.1	8.3
Temperature	25 - 27	26	26	25	26	27	26	25	27
Ammonia (ppm)	<1.00	0.5	0.3	0.6	0.7	0.2	0.4	0.7	0.8
Salinity (ppm)	5-30	23	20	15	21	21	23	24	28
Total hardness (mg/L)	Variable	5200	4800	3900	5000	5000	5350	5650	6250
Carbonate (mg/L)	0-40	10	15	23	35	12	17	24	32
Bicarbonate (mg/L)	20-200	110	150	120	180	140	120	130	140
Total Alkalinity (mg/L)	20-250	120	130	140	150	140	180	210	220
Iron (ppm)	<1.0	0	0.3	0.5	0	0.2	0.3	0.6	0.5
Calcium (ppm)	>100	300	340	320	310	290	340	367	290

Table 1: Analysis of water quality parameters in tank

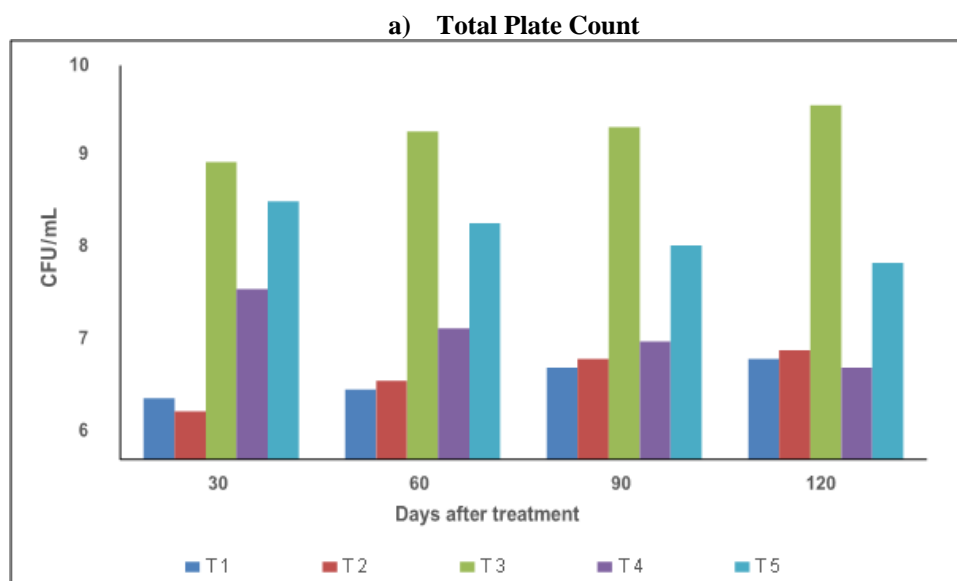
Treatments	Normal Value	Days after treatment							
		Treatment 3				Treatment 4			
		30	60	90	120	30	60	90	120
pH	7.0-8.5	7.6	8.3	8.7	8.5	7.3	7.5	7.4	8.0
Temperature	25 - 27	26	26	25	26	27	26	25	27
Ammonia (ppm)	<1.00	2.0	2.5	3.9	4.0	0.5	0.6	0.4	0.7
Salinity (ppm)	5-30	25	28	29	30	23	19	25	26
Total hardness (mg/L)	Variable	5850	6250	6450	6850	5300	4550	5850	600
Carbonate (mg/L)	0-40	37	43	44	40	21	27	33	35
Bicarbonate (mg/L)	20-200	180	189	195	198	110	130	125	130
Total Alkalinity (mg/L)	20-250	250	255	260	250	110	145	200	210
Iron (ppm)	<1.0	0.9	1.1	1.2	1.0	0.1	0.4	0.5	0.6
Calcium (ppm)	>100	280	250	220	270	230	260	320	350

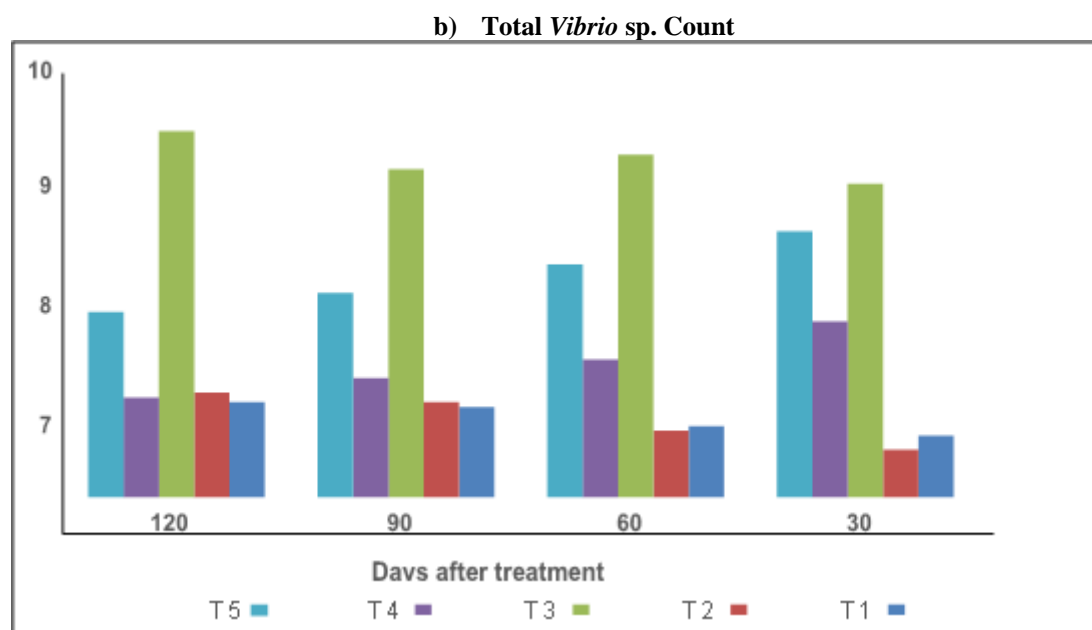
Treatments	Normal Value	Days after treatment			
		Treatment 5			
		30	60	90	120
pH	7.0-8.5	7.6	7.9	7.5	8.4
Temperature	25 – 27	26	26	25	26
Ammonia (ppm)	<1.00	0.4	0.5	0.7	0.6
Salinity (ppm)	5-30	18	17	24	25
Total hardness (mg/L)	Variable	4250	4100	5650	5850
Carbonate (mg/L)	0-40	24	27	32	31
Bicarbonate (mg/L)	20-200	130	140	170	180
Total Alkalinity (mg/L)	20-250	170	230	250	320
Iron (ppm)	<1.0	0	0.2	0.5	0.8
Calcium (ppm)	>100	280	290	330	370

Microbial load in tank water

The total bacterial count (TPC) and total *Vibrio* sp., count (TVC) of tank water was observed in five different groups with regular intervals (30, 60, 90, and 120). The maximum bacterial and *vibrio* sp., load was observed with increasing cell count from 30 to 120 days (7.8 to 9.1×10^6 CFU/mL and 7.6 - 8.7×10^6 CFU/mL) in the treatment (T3) groups. In treatment T4 groups, there were drastic reduction (30 to 120 days) in the TPC (4.9 to 3.1×10^6 CFU/mL) and TVC (4.7 to 3.1×10^6 CFU/mL) when compared with T3 groups. As well in T5 treatment group, there were minor reduction in TPC (6.9 to 5.5×10^6 CFU/mL) and TVC (6.6 to 4.9×10^6 CFU/mL), but not up to level of T4 group. In both the control (control diet and probiotic diet supplemented) T1 and T2 group, a least cell count of TPC (2.4 to 3.3×10^6 CFU/mL and 2.1 to 3.5×10^6 CFU/mL) and TVC (2.3 to 3.0×10^6 CFU/mL) and (2.2 to 3.2×10^6 CFU/mL) were observed and that could not produce any appreciable changes in the growth of the shrimps (Table 4.16; Fig. 4.5). From the results, the study concluded that after challenging with *V. anguillarum*, total bacteria and *Vibrio* sp., count was comparatively low in treated groups (T3) this could be due to adhesion of *L. mesenteroides* to digestive track wall which prevent colonization of pathogens by competitive inhibition (Fig1). In same line, Sivakumar *et al.*, (2012) observed the probiotic effect *Lactobacillus* sp., against *V. alginolyticus* from experimental tank water with two experiments (1 and 2). There were significance differences observed in total *Lactobacillus* sp., in both experiments from 15 to 30 days. Then after challenging with *V. alginolyticus*, high level of total bacterial load was observed in control tank water than the experimental tank water. As well as the total *Lactobacillus* sp., count was higher in the probiotic supplemented feed groups than the control feed groups. Similar such observations were recorded by the previous authors who reported probiotic bacterial feed supplemented changes in microbial load in the culture tank of *P. monodon* (Vieira *et al.*, 2008 & 2007; Vaseeharan and Ramasamy, 2003; Rengpipat *et al.*, 2000).

Figure 2: Microbial density in experimental tank water

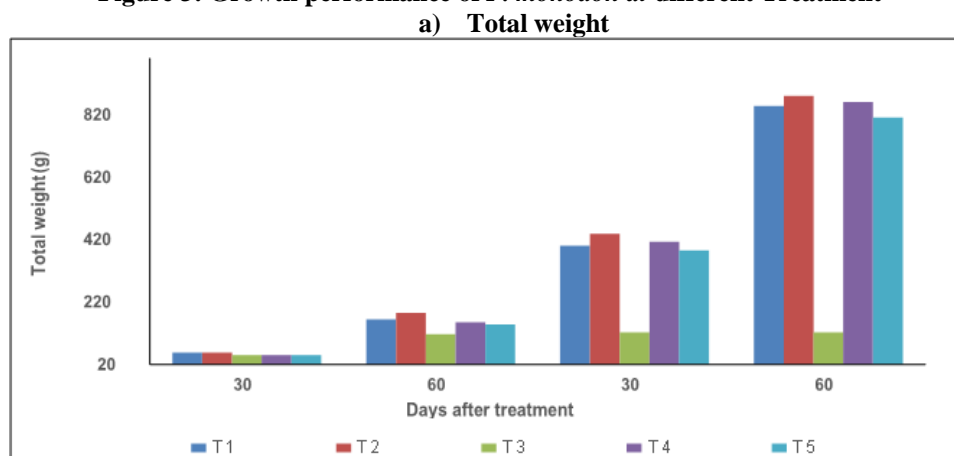


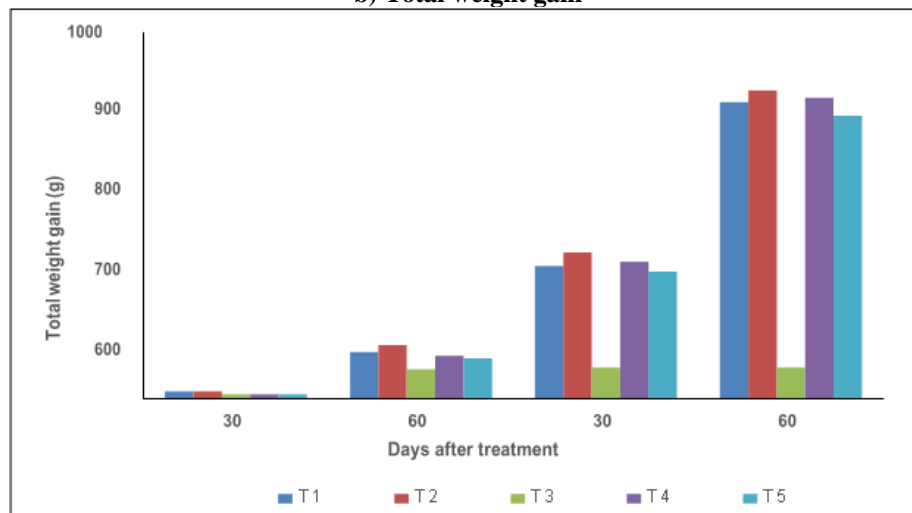
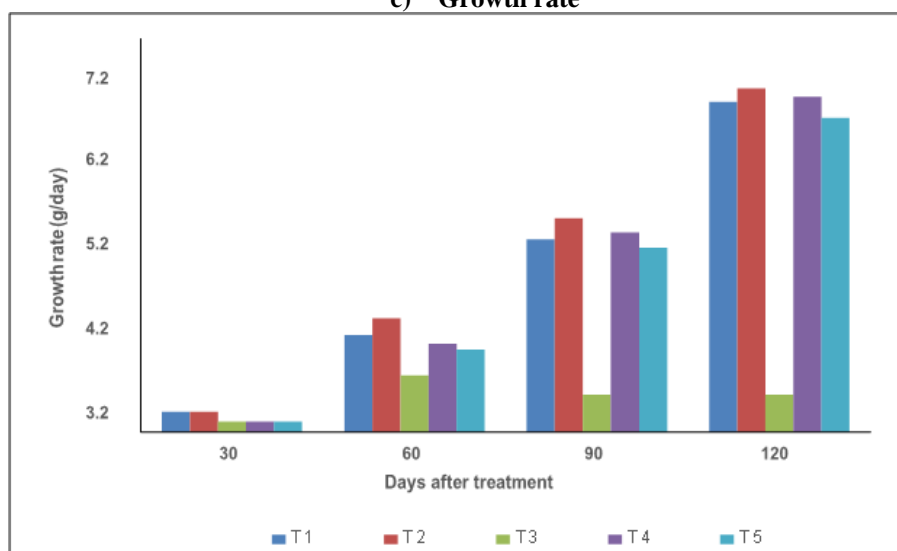


Growth index of *P. monodon*

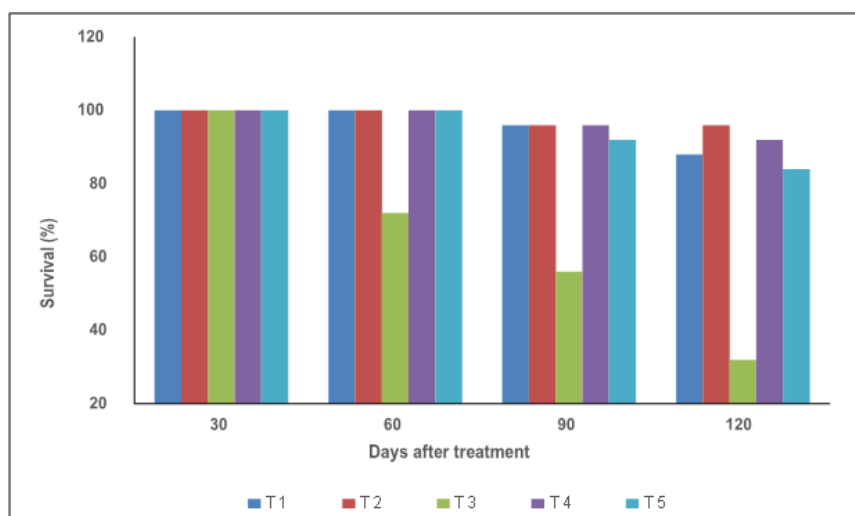
Currently, the use of probiotics in aquaculture might represent a valuable mechanism to increase shrimp growth and survival rate. In this study, among five treatment groups of PL 20, the artificial infection was given for treatment (T3, T4 and T5) groups before 10 days of first treatment and other groups (T1 and T2) acted as control (normal feed) and probiotic (as supplement) control. The initial weight of PL 20 is $1.5\text{g} \pm 0.2$ were taken for all treatment groups. The total weight gain in terms of growth and survival was checked periodically (30, 60, 90, 120 days) for all the experimental groups. Comparing to all treatment groups, T2 shows better growth and development by increasing weight gain periodically ($57.7\text{ g} \rightarrow 880\text{ g}$). In T3 groups, the severity was progressed day by day which mediated the shunted growth ($50\text{ g} \rightarrow 122.5\text{ g}$) and after 60 days the mortality was occurred. Simultaneously, in T4 group, fed with probiotic the rate of infection was suppressed and enhancement in the growth ($50\text{ g} \rightarrow 860\text{ g}$) was observed which was higher ($57.5\text{ g} \rightarrow 847.5\text{ g}$) than control group (T1). In T5 group, the infection rate was suppressed and prompt weight gain was observed periodically but not equal to probiotic fed group. In concern with growth and survival rate, all treatment groups showed 100% survival upto 60 days except T3 group because of severe infection it started decline. After 60 days of T3 group, diseased shrimps showed reduced feed intake, swimming lethargic at the edges of tank, finally 32% survival rate at the time of harvest was observed. Concurrently, in T4 group showed 92% survival rate at the time of harvest which was higher than T5 where the percentage was 84% only. Among all treated group, shrimps fed with probiotic supplemented feed (T2) showed 96% survival rate might be related to an immune reactive effect of probiotics on the host immune system (Fig 2), further the production of extracellular compounds which stimulate the nonspecific immune response in vertebrates. The potential strain *L. mesenteroides* has proven its probiotic effectiveness in *P. monodon* shrimp culture at laboratory scale experiments.

Figure 3: Growth performance of *P. monodon* at different Treatment



b) Total weight gain**c) Growth rate**

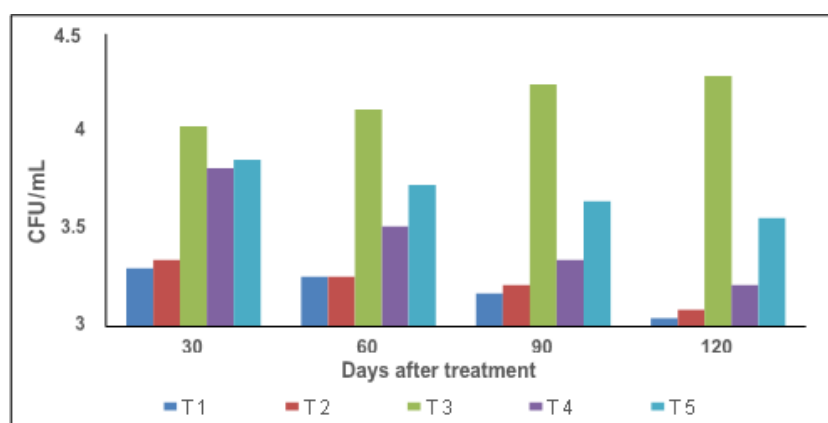
d) survival rate

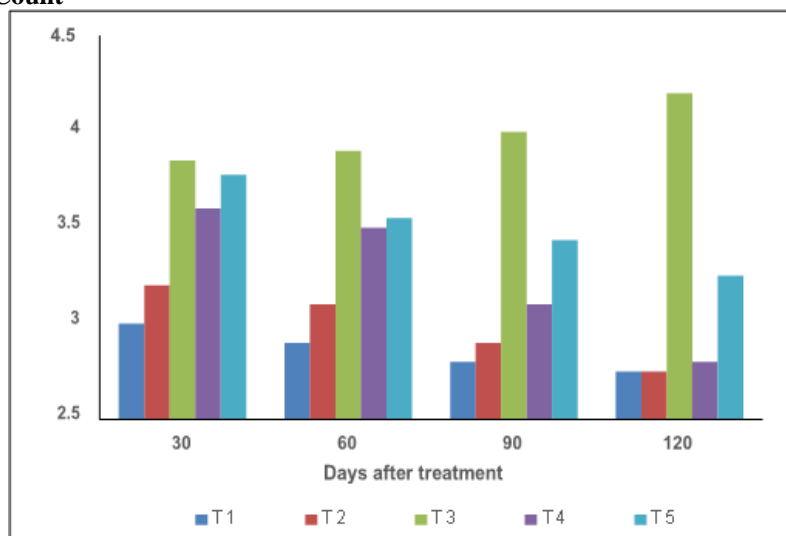
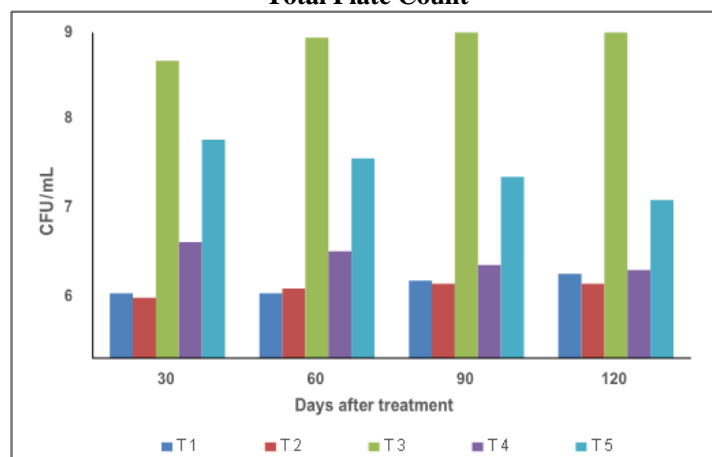


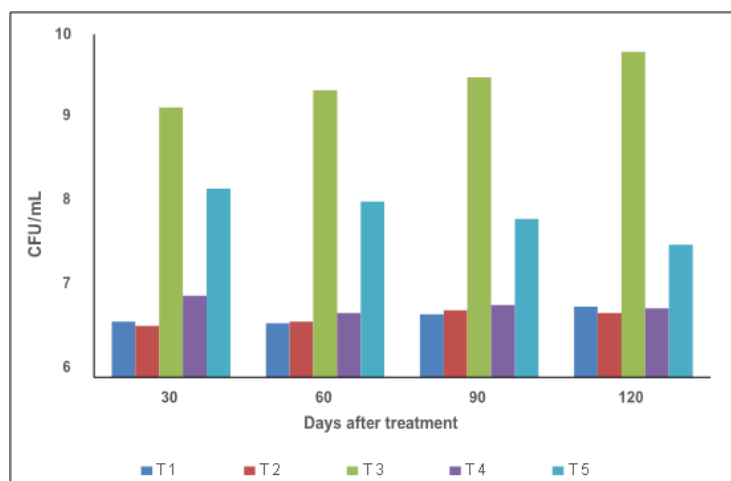
Microbial load in Haemolymph and Gill of *P. monodon*

The microbial load (TPC and TVC) of *P. monodon* tissue of Haemolymph and Gill were observed in five treatment groups with regular intervals of days (30, 60, 90 and 120). The highest bacterial load in TPC (3.4 to 4.0×10^6 CFU/mL) and TVC (3.2 to 3.9×10^6 CFU/mL) were observed in treatment T3 group. Concurrently, in treatment T4 group the TPC (2.9 to 1.5×10^6 CFU/mL) and TVC (2.7 to 1.1×10^6 CFU/mL) were reduced continuously from 30 to 120 days of interval which indicates the decrease level of infection may mediated by probiotic feed. On the other hand, T5 group TPC (3.0 to 2.0×10^6 CFU/mL) and TVC were also observed decrease level but not near to the level of T4 groups. In both control groups T1 and T2, TPC (1.7 to 1.1×10^6 CFU/mL and 1.8 to 1.2×10^6 CFU/mL) and TVC (1.5 to 1.0×10^6 CFU/mL and 1.9 to 1.0×10^6 CFU/mL) were observed as constant (Fig 3). With reference to the gill, the maximum bacterial and *Vibrio* sp., load were observed with increasing cell count from 30 to 120 days (3.2 to 3.9×10^6 CFU/mL and 3.1 to 3.8×10^6 CFU/mL) in the treatment (T3) groups. Whereas in treatment T4 groups, there were drastic reduction in the TPC (2.6 to 1.9×10^6 CFU/mL) and TVC (2.3 to 1.5×10^6 CFU/mL) when compared with T3 groups during the experimental period (30 to 120 days).

As well in T5 treatment group, there were minor reduction in TPC (2.9 to 2.5×10^6 CFU/mL) and TVC (3.3 to 2.0×10^6 CFU/mL), but not up to level of T4 group. In both the control T1 and T2 group, a least cell count of TPC (1.5 to 1.01×10^6 CFU/mL and 1.4 to 1.1×10^6 CFU/mL) and TVC (1.1 to 1.01×10^6 CFU/mL) and (1.5 to 1.06×10^6 CFU/mL) were observed (Fig 3) and that won't made any changes in the growth of the shrimps. Whereas analysing the microbial load in *P. monodon* and *L. Vannamei* culture groups (control and infected), bacterial count observed in the shrimp intestine was higher and it was decreased in *Lactobacillus* sp., AMET1506 and *L. acidophilus* treated shrimp was reported by Karthik *et al.*, (2015; 2014) and Sivakumar *et al.*, (2012). Similar such observations by the previous authors who reported about the effect of lactic acid bacteria on the inhibition of *V. harveyi* in *invitro* (Vieira *et al.*, 2007; Vaseeharan and Ramasamy, 2003).

Figure 4 a): Microbial density in Haemolymph of *P. monodon*

Total Plate Count**Total *Vibrio* sp. Count****b) Microbial density in Gill of *P. monodon*****Total Plate Count****Total *Vibrio* sp. Count**

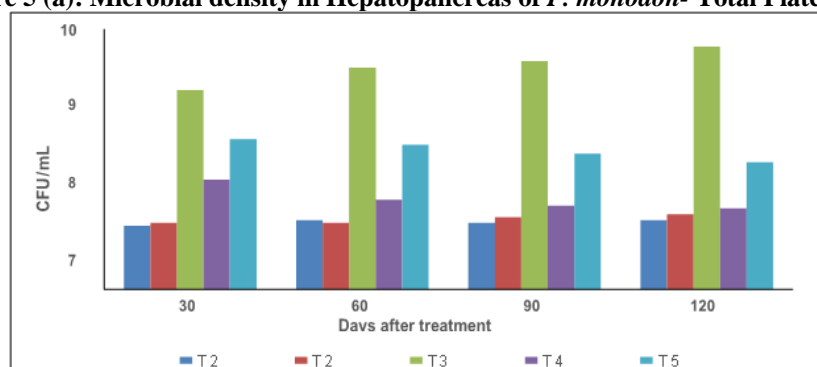


Microbial load in Hepatopancreas and Gut of *P. monodon*

At different time intervals microbial load (TPC and TVC) of *P. monodon* tissues of HP and Gut were observed in five treatment groups and the results were interrupted. The uppermost bacterial load of TPC (7.9 to 9.4×10^6 CFU/mL) and TVC (7.7 to 9.3×10^6 CFU/mL) were observed in treatment T3 group which confirms the severity (Fig 4). Alongside, in treatment T4 group the TPC (4.8 to 3.8×10^6 CFU/mL) and TVC (4.3 to 3.4×10^6 CFU/mL) were reduced constantly from 30 to 120 days of interval which indicates the step by step decrease in progress of infection. On the other hand, T5 group of TPC (6.2 to 5.4×10^6 CFU/mL) and TVC (6.0 to 5.06×10^6 CFU/mL) were also observed in decrease cell count but not close to the level of T4 groups. In both control groups T1 and T2 of TPC (3.2 to 3.4×10^6 CFU/mL and 3.3 to 3.6×10^6 CFU/mL) and TVC (3.0 to 3.5×10^6 CFU/mL and 3.1 to 3.3×10^6 CFU/mL) were observed with minimal colony forming units. In Gut, extreme bacterial and *Vibrio* sp., load were observed with high increasing cell count from 30 to 120 days (8.4 to 9.8×10^6 CFU/mL and 8.3 to 9.6×10^6 CFU/mL) in the treatment (T3) groups. Conversely in treatment T4 groups, there were drastic reduction (30 to 120 days) in the TPC (4.5 to 3.9×10^6 CFU/mL) and TVC (3.9 to 3.61×10^6 CFU/mL) when compared with T3 groups. As well in T5 treatment group, there were negligible reduction in TPC (6.7 to 5.4×10^6 CFU/mL) and TVC (6.4 to 5.1×10^6 CFU/mL), but not up to level of T4 group. In both the control T1 and T2 group, a least cell count of TPC (3.4 to 3.81×10^6 CFU/mL and 3.3 to 3.6×10^6 CFU/mL) and TVC (3.3 to 3.65×10^6 CFU/mL) and (3.2 to 3.5×10^6 CFU/mL) were observed.

Over all, the bacterial loads (TPC and TVC) in treatment groups were in increasing scores as Gut > HP > Hemolymph > Gill. In T3 treatment groups, TVC and TPC were observed at higher level which confirms the severe infection. Simultaneously, in probiotic treated group (T4), TVC was observed at lower level than the TPC may be the presence of probiotic bacteria. From the results, the study concluded that the *L. mesenteroides* strain will be helpful to manage the pathogenic luminous bacteria *V. anguillarum* in specific and other pathogenic bacteria.

Figure 5 (a): Microbial density in Hepatopancreas of *P. monodon*- Total Plate Count



Total *Vibrio* sp. Count

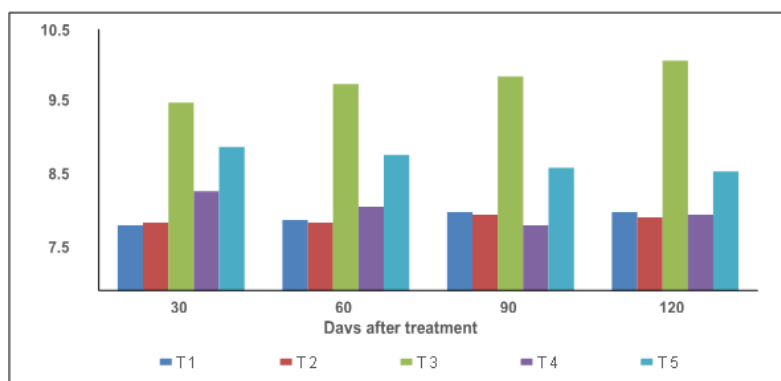
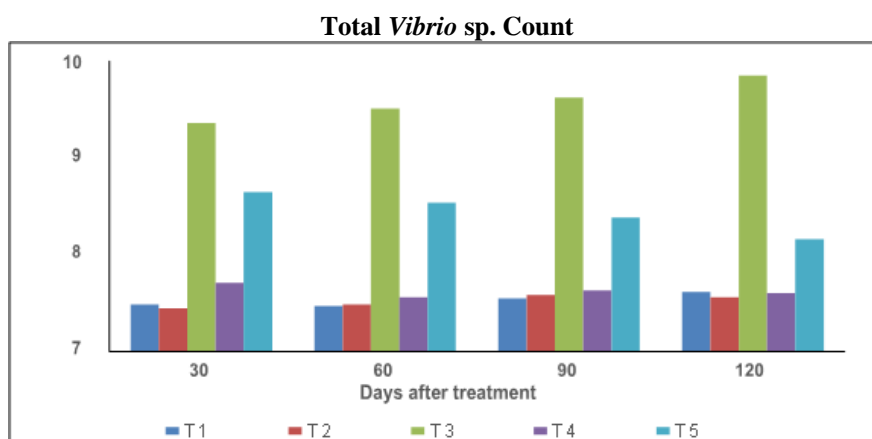
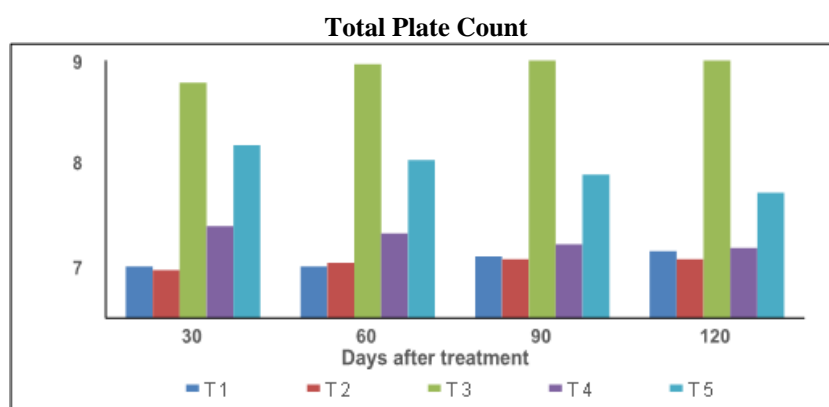


Figure 5(b): Microbial density in Gill of *P. monodon*



Haematological parameters

Haemocyte cell analysis was made almost near to the end of trail. The shrimp induced by the pathogen *V. anguillarum* (T3) showed the total haemocyte count were ranged $1.12-1.04 \times 10^6$ which was lower than all other groups. In control group (T1), expressed normal count of $3.1-3.04 \times 10^6$ cell/mL, whereas in treated group (T4) the total haemocytes count were ranged $5.6-5.68 \times 10^6$ cell/mL which showed drastic decreased when compared to T2 control group ($8.0-8.3 \times 10^6$ cell/mL). As well in T5 group, the count $2.0 - 2.48 \times 10^6$ cell/mL was increased but not up to the level of T4 group. Finally, this result confirmed that, in treated group (T4) immunostimulating effect was observed in terms of haemocyte cells and its constituents such as HC, semi-granular and granular would decrease the infection level. Rengipet *et al.*, (2000), analyzed immune response of *P. monodon* with probiotic feed *Bacillus* sp., S11 in two different challenge tests (I and II). In challenge I the total hemocyte was increased in probiotic treated group than the control group. In challenge II (after infection of *V. harveyi*) the probiotic treated group was increased but not upto the level of challenge I treated group. According to Raja *et al.*,

(2017), haemtopoiesis was completely affected by *V. harveyi* which showed drastic reduction in Total and Differential hemocyte cell count at different intervals of time. In contrast Saptiani *et al.*, (2020), demonstrated the stimulated immune response of *P. monodon* against *V. harveyi* induced from *Xylocarpus granatum* leaves extract.

Treatments	Total Haemocyte Count (Cell/mL) 10 ⁶ /mL	Differential haemocytes count*		
		HC	SGH	LGH
Treatment 1	(1.2) 3.1	89%	07%	04%
	(7.6) 3.04	83%	13%	04%
Treatment 2	(2) 8.0	83%	12%	05%
	(2) 8.3	86%	12%	05%
Treatment 3	(2.8) 1.12	91%	06%	03%
	(2.6) 1.04	86%	09%	05%
Treatment 4	(1.4) 5.6	90%	07%	03%
	(14.2) 5.68	78%	14%	08%
Treatment 5	(5) 2.0	82%	10%	08%
	(6.2) 2.48	80%	11%	09%

Table 2: Time dependent haematological parameters in relation with *P. monodon*

* HC- hyalinocytes; SGC - Small-granular hemocytes, LGH - Large-granular hemocytes

Histopathology evaluation of *P. monodon*

Histopathology of the hepatopancreas

A,B,C, hepatopancreas of control group of *P. monodon* showed well-organized glandular tubular structure normally observed in the shrimp. Four kinds of cells were dominated the hepatopancreas tubules, namely E (embryonalzellen or embryonic) cells, R (restzellen) cells, F (fibrillenzellen or fibrous) cells and B (blasenzellen) cells. Among the above four cells, hepatopancreas showed more number of R cells (High lipid storage) than the others. In infected group, granulomatous lesions in hepatopancreas tubular epithelial cells with surrounding severe haemocytic congestion and inside granulomas, basophilic bacterial masses were observed. In treatment group, the size of R cells becomes almost normal, reduced haemocytic congestion when compared with infected group was observed.

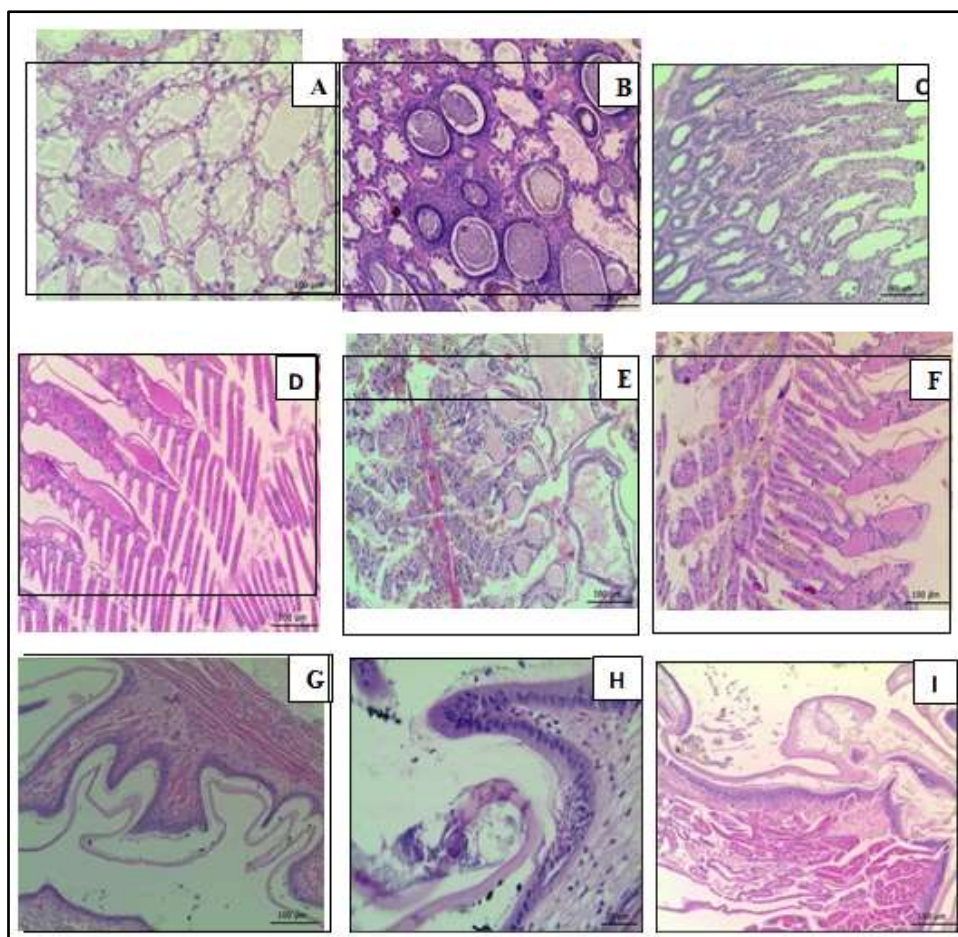
Histopathology of the gill

The cellular structure of gill of *P. monodon* in control group exhibited well organized structure showing primary gill filament branched from central axis. The secondary gill lamella appeared as finger type structures attached to primary gill lamella which embedded to supportive rays. The gills structure of *P. monodon* in infected groups exhibited vaculation and fusion of gill lamella. It created more space and inflamed with heavy load of organic accumulation. In case of treated group, the fusion of gill lamella improved to cellular structure of primary gill and branchial arch gill rays. Also inflammation reduced than the infected group. The treated group exhibited almost similar cellular structure of the gill of *P. monodon* of the control group

Histopathology of the intestine

In the control group the epithelial cells and cellular structure of villi are columnar in shape, showed normal intestinal crypts, villi and lamina propria. In infected group, it damages the intestinal villi layer. The cuticular layers with basophilic bacterial masses and degeneration of epithelial layers with spongy muscle tissue of the intestine may influence the severe haemocytic inflammation. Whereas in treated group, damages in intestinal villi layer was improved also reduced haemocytic inflammation was observed against infected group. Raja *et al.*, (2015) observed the cross segment of normal hepatopancreas with clear hepatopancreas tubules, vacuolated B-cells and haemal space but in the case of LSS infected shrimps, the hepatopancreas tubules were ruined and haemal space was enlarged. The shrimp histopathological studies of Lokha *et al.*, (2012) have showed condensation of hepatopancreatic (HP) tubules, melanised granulomas, vacuolation of HP cells and haemocytic infiltration. In addition, hepatopancreatic tubules tended to be highly necrotic with a thickened tubular sheath and an expanded inter-tubular area. Similarly, Alavandi *et al.*, (2008) also observed inflammation of the hepatopancreatic tubules with intertubular space enlargement and hemocytic infiltration of the affected shrimp tissue parts of the LSS. They also suggested the presence of fully sloughed HP tubules and extreme HP necrosis in the affected shrimp LSS.

Figure 6: Organization of the varies tissues of *P. monodon* under different treatments



Tissue sectioning of

A - Normal HP; B - Severe infection in HP; C- Probiotic treated in HP D - Normal gill; E - Severe infection in gill; F- Probiotic treated in gill
G - Normal intestine; H- Severe infection in intestine; I - Probiotic treated in intestine

Conclusion

In conclusion, the strain of Lactic Acid Bacteria, *Leuconostoc mesenteroides*, isolated from sauerkraut, might stand better adaptive to survival and colonization in the intestinal tract. Further *L. mesenteroides* played a vital role in growth, survival and disease resistance of aquatic *Penaeus monodon* by maintaining good water quality parameters throughout the culture period. Capacity to reduce the viable *V. anguillarum* cells in the tissues, as evident from reduced pathogen load in tissues (HP, Gut and Intestine) by competitive inhibition also increase the clearance efficiency of haemolymph. Histological observations confirmed that the probiotic bacteria had adverse effect on the health of the host. The above observation indicated that the isolate, *Leuconostoc mesenteroides*, could be used as the perspectival strain for probiotics in marine aquaculture. Introducing such specifically screened strains favors the wellbeing of farmed organisms, shrimp farmers and environment/surrounding.

References

1. Alavandi, S. V., Babu, T. D., Abhilash, K. S., Vijayan, K. K., Kalaimani, N., & Santiago, T. C. (2008). Loose shell syndrome of farmed *Penaeus monodon* in India is caused by a filterable agent. *Diseases of aquatic organisms*, 81(2), 163-171
2. Alfiansah, Y. R. (2019). Microbiome in shrimp *Litopenaeus vannamei* aquaculture: dynamic changes and bacterial lifestyles. Doctoral dissertation, University of Bremen.
3. American Public Health Association (APHA), American Water Works Association, Water Pollution Control Federation, & Water Environment Federation. (1915). *Standard methods for the examination of water and wastewater*, 2
4. Barrangou, R., Yoon, S. S., Breidt Jr, F., Fleming, H. P., & Klaenhammer, T. R. (2002). Identification and characterization of *Leuconostoc fallax* strains isolated from an industrial sauerkraut fermentation. *Appl. Environ. Microb.*, 68(6), 2877-2884.

5. Chatterjee, S., & Haldar, S. (2012). *Vibrio* related diseases in aquaculture and development of rapid and accurate identification methods. J Marine Sci. Res. Devp, 1(1), 1-7.
6. Durai, V., Gunalan, B., Johnson, P. M., Maheswaran, M. L., & Pravinkumar, M. (2015). Effect on white gut and white feces disease in semi intensive *Litopenaeus vannamei* shrimp culture system in south Indian state of Tamilnadu. Int. J. Marine Sci, 5(14), 1-5.
7. Far, H. Z., Saad, C. R. B., Daud, H. M., Harmin, S. A., & Shakibazadeh, S. (2009). Effect of *Bacillus subtilis* on the growth and survival rate of shrimp (*Litopenaeus vannamei*). African Journal of Biotechnology, 8(14), 3369-3376
8. Gunalan, B., Soundarapandian, P., Anand, T., Kotiya, A. S., & Simon, N. T. (2014). Disease occurrence in *Litopenaeus vannamei* shrimp culture systems in different geographical regions of India. Int. J. Aqua, 4(4) 24-28.
9. Janarthnam, K., George, M. R., John, K. R., & Jeyaseelan, M. J. (2012). In vitro and in vivo biocontrol of *Vibrio harveyi* using indigenous bacterium, *Bacillus* spp. Ind. J Geo-marine Sci, 4(1), 83-89.
10. Jayasree, L., Janakiram, P., & Madhavi, R. (2006). Characterization of *Vibrio* sp. associated with diseased shrimp from culture ponds of Andhra Pradesh (India). Journal of the world aquaculture society, 37(4), 523-532.
11. Jha, A. K. (2014). Probiotics: an effective feed supplement in Shrimp culture. J Bang. Acad. Sci, 38(2), 127-130.
12. Karthik, R., Hussain, A. J., & Muthezhilan, R. (2014). Effectiveness of *Lactobacillus* sp (AMET1506) as probiotic against *Vibriosis* in *Penaeus monodon* and *Litopenaeus vannamei* shrimp aquaculture. Biosci. Biotechnol. Res. Asia, 11, 297-305. •
13. Karthik, R., Pushpam, A. C., Chelvan, Y., & Vanitha, M. C. (2015). Efficacy of probiotic and nitrifier bacterial consortium for the enhancement of *Litopenaeus vannamei* aquaculture. Int. J. Vet. Sci. Res, 1(1), 29-34.
14. Kumar, A., Suresh Babu, P. P., Roy, S. D., Razvi, S. S., & Charan, R. (2014). Synergistic effects of two probiotic bacteria on growth, biochemical, and immunological responses of *Litopenaeus vannamei* (Boone, 1931). The Israeli J Aqua-Bamidgah, 66, 1009- 1016.
15. Kumaran, T., & Citarasu, T. (2016). Isolation and characterization of *Vibrio* species from shrimp and *Artemia* culture and evaluation of the potential virulence factor. Intel. Prop. Rights, 4(1).1-5.
16. Lightner, D. V. (1996). A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society.
17. Loka, J., Janakiram, P., Geetha, G. K., Sivaprasad, B., & Veerendrakumar, M. (2012). Loose shell syndrome (LSS) of cultured *Penaeus monodon*-microbiological and histopathological investigations. Indian Journal of Fisheries, 59(3), 117-12
18. Mungkung, R. (2005). Shrimp aquaculture in Thailand: application of life cycle assessment to support sustainable development, Doctoral Dissertation, University of Surrey (United Kingdom).
19. Nandakumar, G., & Maheswarudu, G. (2003). Penaeid shrimps. Status of Exploited Marine Fishery and Resource of India. 176-182.
20. Orgeron II, R. P., Corbin, A., & Scott, B. (2016). Sauerkraut: A Probiotic Super food. Func. Foods Health Dis, 6(8), 536-543.
21. Raja, R. A., Sridhar, R., Balachandran, C., Palanisammi, A., Ramesh, S., & Nagarajan, K. (2017). Pathogenicity profile of *Vibrio parahaemolyticus* in farmed Pacific white shrimp, *Penaeus vannamei*. Fish Shellfish Imm, 67, 368-381.
22. Raja, S., Nandhini, E., Sahana, K., & Dhanakkodi, B. (2015). Beneficial and destructive effects of probiotics in aquaculture systems-A Review. Int. J Fish. Aqua. Stu, 2(3), 153-159.
23. Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., & Menasaveta, P. (2000). Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture, 191(4), 271-288.
24. Santosh B and N.P Singh (2007) Guideline for Water Quality Management for Fish Culture in Tripura. I.C.A.R research complex Tripura for NEH region Tripura centre. 29.
25. Saptiani, G., Sidik, A. S., Ardhani, F., & Hardi, E. H. (2019). Response of hemocytes profile in the black tiger shrimp (*Penaeus monodon*) against *Vibrio harveyi* induced by *Xylocarpus granatum* leaves extract. Veterinary world, 13(4), 751-757.
26. Sivasankar, P., John, K. R., George, M. R., Anushalini, S. V., Kaviarasu, D., & Petchimuthu, M. (2017). Prophylactics in shrimp aquaculture health management: A Review. J. Ento. Zoo. Stud, 5(4), 1049-1055.
27. Soundarapandian, P., & Gunalan, B. (2008). Recent technology for the survival and production of giant tiger shrimp *Penaeus monodon* along south east coast of India. Int. J. Zool. Res, 4(1), 21-27.
28. Sritunyalucksana, K., Gangnonngiw, W., Archakunakorn, S., Fegan, D., & Flegel, T. W. (2005).

- Bacterial clearance rate and a new differential hemocyte staining method to assess immunostimulant activity in shrimp. *Dis. Aqua. Org*, 63(1), 89-94.
29. Suriya, M., Shanmugasundaram, S., & Mayavu, P. (2016). Stocking density, survival rate and growth performance of *Litopenaeus vannamei* (Boon, 1931) in different cultured shrimp farms. *Int. J Curr. Res. Biol Med*, 1(5), 26-32.
 30. Suzanne Johanningsmeier. Roger, Mcfeeters, F. Henry, P. Fleming and Roger, L. (2007). Thompson effects of *Leuconostoc mesenteroides* starter culture on fermentation of cabbage with reduced salt concentrations. *Journal Food Science*. 72 (5):166 -172.
 31. Tacon, A. G., Jory, D., & Nunes, A. (2013). Shrimp feed management: issues and perspectives. *On-farm feeding and feed management in aquaculture*, 583, 481-488.
 32. Tamminen, M. Joutsjoki, T. Sjoblom, M. Joutsen, M. Palva, A. Ryhanen, E.L. and Joutsjoki, V. (2004). Screening of lactic acid bacteria from fermented vegetables by carbohydrate profiling and PCR-ELISA. *Letter of Applied Microbiology*. 39: 439-444.
 33. Touret, T., Oliveira, M., & Semedo-Lemsaddek, T. (2018). Putative probiotic lactic acid bacteria isolated from sauerkraut fermentations. *PLoS One*, 13(9), 1-16.
 34. Vaseeharan, B., & Ramasamy, P. (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Lett. Appl. Microb*, 36(2), 83-87.
 35. Venkat, H. K., Sahu, N. P., & Jain, K. K. (2004). Effect of feeding *Lactobacillus*- based probiotics on the gut microflora, growth and survival of postlarvae of *Macrobrachium rosenbergii* (de Man). *Aquaculture Research*, 35(5), 501-507.
 36. Vieira, F. D. N., Buglione Neto, C. C., Mouriño, J. L. P., Jatobá, A., Ramirez, C., Martins, M. L., & Vinatea, L. A. (2008). Time-related action of *Lactobacillus plantarum* in the bacterial microbiota of shrimp digestive tract and its action as immunostimulant. *Pesquisa Agropecuária Brasileira*, 43(6), 763-769.
 37. Vieira, F. D. N., Pedrotti, F. S., Buglione Neto, C. C., Mouriño, J. L. P., Beltrame, E., Martins, M. L., & Arana, L. A. V. (2007). Lactic-acid bacteria increase the survival of marine shrimp, *Litopenaeus vannamei*, after infection with *Vibrio harveyi*. *Brazilian Journal of Oceanography*, 55(4), 251-255.
 38. Vieira, F. N., Buglione, C. C., Mourino, J. P. L., Jatobá, A., Martins, M. L., Schleder, D. D., & Vinatea, L. A. (2010). Effect of probiotic supplemented diet on marine shrimp survival after challenge with *Vibrio harveyi*. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 62(3), 631-638.