Invitro Antagonistic Activity of a Probiotic Bacteria *Enterococcus Faecalis* Against White Fecal Disease Induced by *Vibrio Alginolyticus* of Shrimp *Peneaus Monodon*

Mrs.B. MohanaPriya [1]*, Assistant Professor, Department of Biotechnology, Rathinam college of Arts and Science, Coimbatore, mohanapriya.bio@rathinam.in

Dr.A. SathyaPriya[2], Assistant Professor, Department of Biotechnology, Rathinam college of Arts and Science, Coimbatore, sathyapriya.biotech@rathinam.in

Dr.S. Aravindh[3], Assistant Professor, Department of Biotechnology, Rathinam college of Arts and Science, Coimbatore, aravindh.bio@rathinam.in

Dr.C. Kanagaraj[4], Assistant Professor, Department of Biotechnology, Rathinam college of Arts and Science, Coimbatore, hod.biotechnology@rathinam.in

ABSTRACT

Shrimp aquaculture is a normal practice in developing nations throughout Asia and the tropical world, offering non-urban communities with a means of survival and, as a consequence, poverty reduction. The white feces syndrome has caused significant economic damage in the cultured shrimp industry in China, Indonesia, Malaysia, Thailand, Vietnam and other countries in Southeast Asia. Thus, more attention should be paid to the pathogenesis of this disease. Probiotics, or beneficial bacteria that control pathogens through a variety of mechanisms, are increasingly being viewed as an alternative to antibiotic treatment. The present study was aimed to determine the probiotic activity of Enterococcus faecalis against Vibrio alginolyticus., in shrimp aquaculture. Traditional bacterial identification depended on phenotypic characteristics. Dual culture, cross streak method, and SEM analysis were used to evaluate in vitro antagonist activities. The study concludes that invitro antagonistic assay of dual culture test with Enterococcus faecalis clearly exhibited the growth inhibition of V. alginolyticus. In co-culture experiment, growth of pathogenic V. alginolyticus was inhibited by Enterococcus faecalis culture inoculated at equal ratio even in low ratio was also controlled V. alginolyticus the under invitro condition. Morphological analysis by SEM revealed that V. alginolyticus treated with Enterococcus faecalis showed major structural disruption in the cell envelope as well as a preponderance as irregular rods forms than the controls.

Keywords: *Aquaculture, Probiotics, Enterococcus faecalis, Vibrio alginolyticus, SEM analysis*

INTRODUCTION:

Shrimp aquaculture has seen tremendous growth, and the farming of commercially important shrimp species has a large business sector. Shrimp aquaculture has remained an important economic resource for many developing countries, as well as a source of food and livelihood for people all over the world. It contributes to over 50% of worldwide shrimp production and is regarded as the most valued aquaculture business [1]. Viruses, bacteria, fungus, and even protozoans can infect marine creatures because they swim in potential pathogens.

Shrimp's white feces infections are caused by opportunistic bacterial pathogens such as *Vibrio vulnificus, Vibrio fluvialis, Vibrio parahaemolyticus* and *Vibrio alginolyticus*, that leads to huge losses in the *P. monodon* shrimp farmed industry. White feces diseases severely infect shrimp's hepatopancreas and gut, and turn it to white and pale in colour [2]. In severe cases, white feaces syndrome (WFS) could

lead to farm losses due to decreased survival, retarded growth increased feed conversion ratios referred to as WFS [3]. Incidences of WFS are also associated with high stocking densities, poor pond bottom, high plankton blooms, and poor feed management, as well as high pollution in pond water. The affected shrimp have a loose exoskeleton and a protozoan fouling infestation, which causes dark gill coloration [2].

The use of probiotics during the early larval stages has emerged as a viable alternative to antibiotic therapy. Several studies on the use of probiotics in aquaculture have yielded promising results. Probiotics improve shrimp health by inhibiting pathogen colonization through competition, releasing metabolites that inhibit pathogen growth, and thus increasing shrimp resistance to diseases. To date, only a few Enterococcus species have been studied for their probiotic properties.[1]. These include *E. faecalis, E. faecium, E. lactis, E. hirae, E. durans* which were shown to be effective against pathogenic bacteria [4]. With this concern the objectives of study were designed to isolate and identify the pathogenic Vibrio sp., and probiotic bacteria, and finally to find out the *invitro* antagonist activity of probiotics against pathogen.

MATERIALS AND METHODS:

Study area:

An extensive field survey was carried out in the shrimp aquaculture farms at Chidambaram Taluk, Cuddalore District during 2020–2021 for screening the presence of bacterial diseases. The diseased shrimps showed symptoms of stunted growth and opaque white gut visible through the transparent cuticle as a white streak were collected and concentrated for further studies.

Isolation of Pathogen

Infected shrimp *(Penaeus Monodon)* specimens were collected from shrimp farms situated along the coast of Tamil Nadu in the region of Parangipettai, Chidambaram TK, Cuddalore District. The specimens were brought to the laboratory in ice stored conditions. One gram of infected shrimp tissue sample was taken and inoculated in to Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar medium and incubated at 37ºC for 24 – 48 hrs respectively [5]. Presumptive yellow colonies in TCBS agar plates were (*Vibrio alginolyticus*) collected at random and purified further. Isolated culture was sub cultured in TCBS and stock was maintained at 4ºC in 10% glycerol for further use.

Isolation of Probiotic bacteria

For the isolation of probiotic bacteria, Chinese sauerkraut fermentation was prepared, from the brine were sampled after 5 days of single laboratory fermentation and plated onto MRS agar, incubated aerobically at 30°C for 5 days [6]. Two uncrowded viscous colonies presumed to be *Enterococcus* sp., were picked up randomly from MRS agar plates and purified by streaking on MRS plate. The isolated pure culture was sub cultured and stored at 4°C in 10% glycerol for further use.

Phenotypic identification of isolated pathogen and probiotic organism

An array of biochemical test for determining the phenotypic profile of the isolated pathogen and probiotic organism as per Bergey's Manual of Systematic Bacteriology will be carried out [7].

In vitro challenge test

To evaluate the antagonistic activity of the prospective bacteria against the pathogen were done by dual culture method [8]. *In vitro* antimicrobial activity of the isolated pathogen against probiotic bacteria was performed using agar well diffusion method. The isolated *E. faecalis* were grown in 50 ml MRS 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 pathogenic bacteria *V. alginolyticus.* 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. Control plates were filled with sterile distilled water. Three replicates of each treatment were arranged. Then the agar plates were incubated for 24 hours incubation at 30 °C. The bacteria diffuse 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 [9].

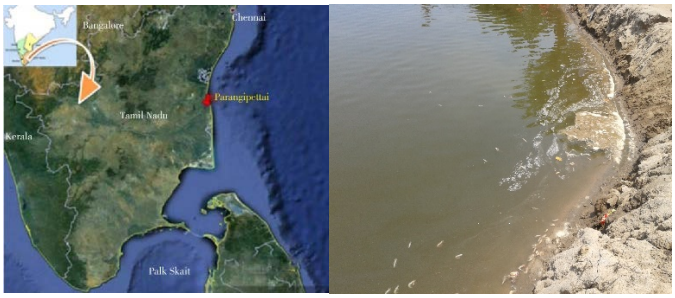
Morphology analysis by Scanning Electron Microscopy

To understand the morphological changes, colonies grown on TCBS plates, both treated and untreated (control), were examined using a scanning electron microscope (FEI Quanta SEM 200., Netherlands). The colonies were transferred into sterile Eppendorf containing phosphate buffered saline (pH 7.4). The colonies were washed twice with PBS and fixed with 4% paraformaldehyde and allowed for 30mins. After fixation the cells were again washed twice in PBS and then resuspended in sterilized distilled water to avoid salts crystallization during drying process and SEM measurements. Finally, 100 µl of culture was dripped onto the copper plates and air dried. The samples were stored at 4ºC before measurements. The samples in copper plates were treated for gold coating before placing for magnification. The magnifications were performed at 30,000x and photographs were detained [10].

RESULTS AND DISCUSSION:

The survey undertaken during 2020–2021 at the area Cuddalore District revealed the occurrence of white feces disease in shrimp, designated, based on external symptoms, Dark discoloration of the gills, Hepatopancreas and gut become white and pale in color, Floating white feces strings, Infected shrimps show loose shell. Mass mortalities occurred in the ponds affected by white feces disease. The following account describes the external symptoms of various diseased shrimp as well as the bacteria involved in disease manifestation. Likewise, several earlier studies have reported that the *L. vannamei* majorly affected by white gut and white feces diseases which reduced feed intake, growth and survival of shrimp culture [11, 1]).

Fig 1-Map Showing Study Area

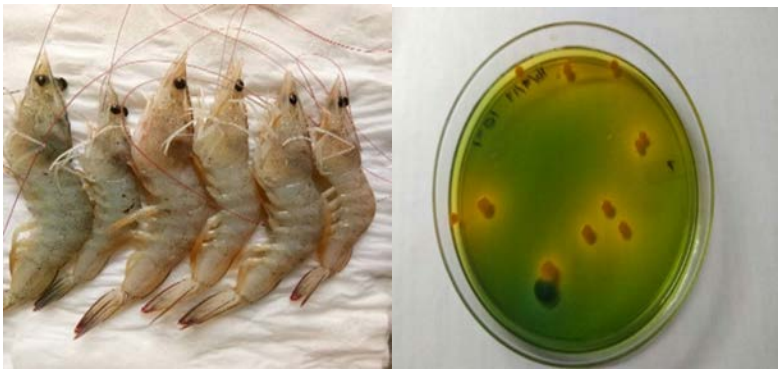


Isolation and identification of pathogen

Infected shrimp tissue was collected from the shrimp growing area of Parangipettai, Cuddalore District was plated onto TCBS plates, incubated for 48 hrs at 37ºC. Yellowish colonies presumptive as *Vibrio alginolyticus* was observed on TCBS plate containing the dilution of 10-2. Various biochemical

tests were performed to ascertain the genus of the isolate as per Bergey's Manual of Systematic Bacteriology and the isolated pathogen was identified as *Vibrio alginolyticus* (Table 1). According to Mastan *et al*., 2015, five species of bacteria namely *Vibrio parahaemolyticus, V. fluvialis, V.mimicus, V.alginolyticus* and Vibrio sp. were isolated from WFS affected shrimps. *V. parahaemolyticus* and *V. alginolyticus* predominated among the five species in all diseased shrimp samples. The same symptoms were also reported by number of workers [13].

Fig 2 – Isolation of Pathogen



Isolation of probiotics

For the isolation of *Enterococcus* sp, samples taken from brine during the course of fermentation (after 4 days) and plated onto MRS agar and incubated. The viscous colonies presumed to be *Enterococcus* sp., were picked up randomly from MRS agar plates, purified by streaking on MRS plate and stored at 4ºC as glycerol stock. Based on the biochemical tests as per Bergey's Manual of Systematic Bacteriology the isolated natural microbiota was identified as *Enterococcus faecalis* (Table 1). Simultaneously, the bacterial strain Enterococcus sp (*Enterococcus faecium* DUTYH_16120012) proved a promising probiotic candidate and that also can be used to remove lead from food or feed were isolated from Chinese sauerkraut fermentation. [6].

Fig 3- Isolation of Probiotic Bacteria

Table 1- Phenotypic identification of isolated pathogen and probiotic organism

S.no	Biochemical Tests	Pathogenic bacteria	Probiotic bacteria

1	Shape	Curved Rod	Cocci
2	Gram Staining	- Ve	+ Ve
3	Spore Staining	Non-Spore	Non-Spore
4	Motility	Non-Motile	Non-Motile
5	Capsule	Non-Capsulated	Non-Capsulated
6	Flagella	Flagellated	Non-Flagellated
7	Indole	+ Ve	- Ve
8	MR	+ Ve	- Ve
9	VP	+ Ve	+ Ve
10	Citrate	+ Ve	- Ve
11	H_2S production	- Ve	- Ve
12	Urease production	- Ve	- Ve
13	Gelatin hydrolysis	+ Ve	Variable
14	Nitrate reduction	+ Ve	+ Ve
15	Oxidase test	+Ve	- Ve
16	Glucose	+ Ve	+ Ve
17	Lactose	- Ve	+ Ve
18	DNase	+ Ve	+ Ve
19	Sucrose	+ Ve	+ Ve
20	Catalase	+ Ve	- Ve
21	Casein	- Ve	+ Ve
22	Starch	Variable	+ Ve
23	Fructose	- Ve	+ Ve
24	Dextrose	- Ve	+ Ve
25	Galactose	+ Ve	+ Ve
26	Xylose	- Ve	+ Ve
		V. aliginolytics	*E. faecalis*

In vitro challenge test

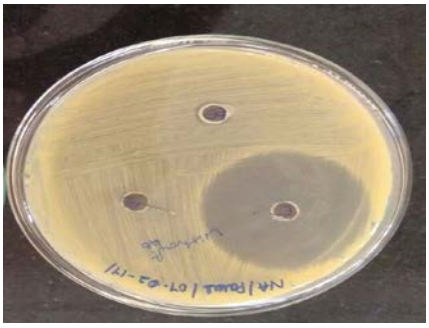
Two methods were performed for the analysis of *invitro* inhibition of *V. alginolyticus.* In dual culture method, the isolates *E. faecalis* and *V. alginolyticus* was streaked in the individual plates and incubated at 37ºC. The diameter of the colonies and the zone of inhibition was measured. The routine observation (5 days of incubation) showed that the pathogen growth was seen in 1-3 days of incubation. During 4-5 days of incubation the growth was inhibited and further no colony development was observed (Plate 1). The highest zone of inhibition of 3.2 cm was observed in probiotic bacteria (*E. faecalis*) (Tab 2). The growth suppression pattern of pathogen indicated that the compound which is produced by the *E. faecalis* may be responsible for its decreased growth. In the same line, Janarthanam *et al.*, [5] was described that the probiotic organism inhibited the growth of pathogen. of *B. subtilis* BT2 (antagonist) was required to inhibit *V. harveyi* in *invitro* conditions. The bacterial strain, *E. faecalis* were used to check the antagonist effect against *V. alginolyticus*, the observation was recorded by measuring the inhibitory zone including well. *E. faecalis* created significantly greater inhibition zone as compared to *V. alginolyticus* and its proved antibacterial activity against *V. alginolyticus.*

Tab 2- *Invitro* antagonistic activity of., *E. faecalis* against *V. alginolyticus* – Dual culture method

E. faecalis* against *V. alginolyticus

Days of Incubation	Growth in diameter (cm)		Inhibition zone (cm)
	E. faecalis (Probiotic)	*V. alginolyticus* (Pathogen)	
Day 1	0.5	0.1	5.3
Day 2	1.4	0.4	4.6
Day 3	2.5	0.6	3.0
Day 4	2.9	0.7	3.1
Day 5	3.6	0.8	3.2

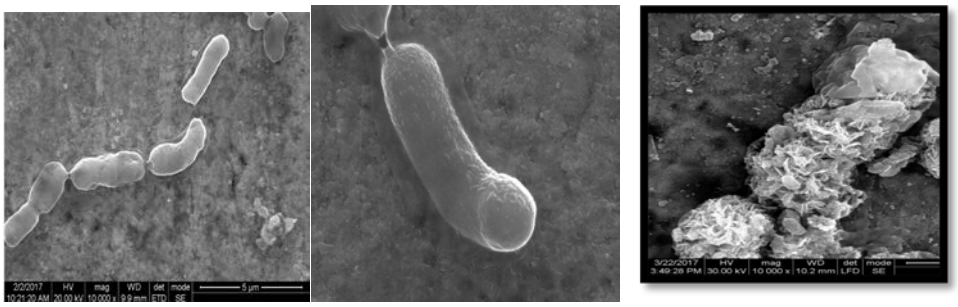
Fig 4: Inhibition zone (mm) of probiotic bacterial strain *E. faecalis* against *V. alginolyticus*



Morphological analysis by Scanning Electron Microscopy

The morphological changes of target bacteria were examined by SEM analysis. As seen in the scanning electron micrographs, target bacterial cells *V. alginolyticus* treated for 6 h showed major structural disruption in the cell envelope as well as a preponderance of irregular rod forms with wrinkled surfaces. The present findings supported by Ma *et al.*, [14]. Likewise, Prabhurajeshwar and Chandrakanth, [15], analyzed the cell morphology of test pathogen treated with supernatant of Lactobacillus sp., resulted in alteration or rupture in the cell morphology. A similar pattern was observed and it has been reported that high concentrations of natural/artificial chemicals confer toxic effects to cells due to disruption of membrane components, leading to cell death [16,17]. In this regard data obtained from SEM analyses on target pathogenic bacteria were consistent with cellular responses to chemicals found in other bacteria.

Fig 5- Morphological analysis by Scanning Electron Microscopy



ACKNOWLEDGEMENT

Authors are gratefully thankful to staff members for their valuable suggestions and Head of the Department of Biotechnology, Rathinam college of Arts and Science College, Coimbatore for providing facilities to carry out this research work successfully.

CONCLUSION:

The present study revealed the potential probiotic effects of *E. faecalis* against pathogenic bacteria *V. alginolyticus* and its associated health-promoting effect on *P.monodon*. *In vitro* assays showed that *E. faecalis* resulted in decreased growth of the pathogenic bacteria. Moreover, *E. faecalis* supplemented diet enhanced the growth performance, and immunity of *P.monodon*. Although *in vivo* assays, histological features of before and after probiotic treatment were not determined in the current study, they may be of interest in future investigations.

REFERENCES

1. *Jamal, M. T., Abdulrahman, I. A., Al Harbi, M., & Chithambaran, S. (2019). Probiotics as alternative control measures in shrimp aquaculture: A review. Journal of Applied Biology and Biotechnology, 7(3), 6-7.*
2. *Mastan, S. A. (2015). Incidences of white feces syndrome (WFS) in farm-reared shrimp, Litopenaeus vannamei, Andhra Pradesh. Indo American journal of pharmaceutical research, 5(9), 3044-3047*
3. *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. International Journal of Marine Science, 5.*
4. *Baccouri, O., Boukerb, A. M., Farhat, L. B., Zébré, A., Zimmermann, K., Domann, E., ... & Connil, N. (2019). Probiotic potential and safety evaluation of Enterococcus faecalis OB14 and OB15, isolated from traditional tunisian testouri cheese and rigouta, using physiological and genomic analysis. Frontiers in Microbiology, 10, 881.*
5. *Janarthanam, K., George, M. R., John, K. R., & Jeyaseelan, M. J. (2012). In vitro and In vivo biocontrol of Vibrio harveyi using indigenous bacterium, Bacillus sp.*
6. *Yang, Y., & Pei, J. (2020). Isolation and characterization of an Enterococcus strain from Chinese sauerkraut with potential for lead removal. European Food Research and Technology, 246(10), 2055-2064.*

7. *Baumann, P. S., & Schubert, R. H. W. (1984). The family Vibrionaceae veron. Bergey's Manual of Systematic Bacteriology, 1, 515-538.*
8. *Sathyapriya.A, & Dr. A. Anitha Evaluation of Leuconostoc mesenteroides., against Bacterial white spot disease induced by Vibrio cholerae in shrimps Indian Journal of Applied Research 2018 8(9) 249-555X 57-60.*
9. *Tank, P. R., Vadher, K. H., & Patel, M. P. (2018). Isolation of probiotic bacteria from gastrointestinal tract of pacific white shrimp Litopenaeus vannamei and antibacterial activity of probiotic bacteria against Vibrio spp. Journal of Entomology and Zoology Studies, 6, 974-78.*
10. *Chae-Woo., Yun-Seok., and Kye-Heon Oh.(2009). Removal of pathogeneic bacteria and nitrogens by Lactobacillus sp. JK-8 and JK-11. Aquaculture. 287, 266.*
11. *Biju V.N., and Gunalan B., 2016, Prevalence of white faeces syndrome in Penaeus (Litopenaeus) vannamei farms in Nagapattinam district, Int. J. Curr. Sci. Res., 2(12): 1019-1025.*
12. *Kumara, K. R. P. S., & Hettiarachchi, M. A. N. G. A. L. I. K. A. (2017). White faeces syndrome caused by Vibrio alginolyticus and Vibrio fluvialis in shrimp, Penaeus monodon (Fabricius 1798)-multimodal strategy to control the syndrome in Sri Lankan grow-out ponds. Asian Fish Sci, 30, 245-261.*
13. *Sriurairatana, S., Boonyawiwat, V., Gangnonngiw, W., Laosutthipong, C., Hiranchan, J., & Flegel, T. W. (2014). White feces syndrome of shrimp arises from transformation, sloughing and aggregation of hepatopancreatic microvilli into vermiform bodies superficially resembling gregarines. PloS one, 9(6), e99170*
14. *Ma, C. W., Cho, Y. S., & Oh, K. H. (2009). Removal of pathogenic bacteria and nitrogens by Lactobacillus spp. JK-8 and JK-11. Aquaculture, 287(3-4), 266-270.*
15. *Prabhurajeshwar, C., & Chandrakanth, R. K. (2017). Probiotic potential of Lactobacilli with antagonistic activity against pathogenic strains: An in vitro validation for the production of inhibitory substances. Biomedical Journal, 40(5), 270-283*
16. *Cho, Y. S., Schiller, N. L., Kahng, H. Y., & Oh, K. H. (2007). Cellular responses and proteomic analysis of Escherichia coli exposed to green tea polyphenols. Current microbiology, 55(6), 501-506.*
17. *Ho, E. M., Chang, H. W., Kim, S. I., Kahng, H. Y., & Oh, K. H. (2004). Analysis of TNT (2, 4, 6-trinitrotoluene)-inducible cellular responses and stress shock proteome in Stenotrophomonas sp. OK-5. Curr. Microb, 49(5), 346-352*