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EVALUTION OF *LEUCONOSTOC MESENTERIODES.*, AGAINST BACTERIAL WHITE SPOT DISEASE INDUCED BY *VIBRIO CHOLERAE IN SHRIMPS*

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ABSTRACT The shrimp disease in culture pond mainly accounted as a *Vibriosis* caused by *Vibrio* sp. Probiotic supplementation of live microorganism in aquaculture aids in preventing disease, thereby increasing production and decreasing economic loss. The use of probiotics or beneficial bacteria, which control pathogen through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment. The present study was aimed to determine the probiotic activity of *Leuconostoc mensenteroides* (MCC NO 3276) against *Vibrio cholerae*., in aquaculture. Traditional bacterial identification has been based on different phenotypic characteristics. *Invitro* antagonist activities were tested by dual culture, cross streak method, SEM analysis. The study concludes that *invitro* antagonistic assay of dual culture test with *L. mensenteroides* clearly exhibited the growth inhibition of *V. cholerae*. In co-culture experiment, growth of pathogenic *V.cholerae* was inhibited by *L. mensenteroides* culture inoculated at equal ratio even in low ratio was also controlled the *V. cholerae* under *invitro* condition. Morphological analysis by SEM revealed that *V.cholerae* treated with *L. mensenteroides* showed major structural disruption in the cell envelope as well as a preponderance as irregular rods forms than the controls.

KEYWORDS : Aquaculture, Probiotics, *Leuconostoc mensenteroides, Vibrio cholerae,* SEM analysis.

1.0 INTRODUCTION

Aquaculture is one of the world's fastest growing food producing sectors. The field has emerged as an industry in all over the world to supply protein rich foods [1]. A significant challenge in the production is the loss of stock because of the disease. Diseases especially bacterial infections remain primary constraints to its continued expansion [2]. *Vibrios* are gram negative, curved rod shaped motile organisms. Vibriosis is caused by infection of *Vibrio* sp., is one of the most predominant disease in fishes and other aqua culture organisms. In general, among the aquatic pathogens *Vibrio* sp., are highly dangerous and it will have detached with shrimp epithelium and affect highly by eliminating the two layers which protects the shrimp from the infections and finally ended with mortality [3].

So far, to prevent the establishment of pathogens, conventional approaches such as antibiotics and antimicrobial drugs are indiscriminately being used in Indian farms in Andhra Pradesh, Tamil Nadu, Kerala and Karnataka. The use of antibiotics has resulted in development of multiple antibiotic resistant in the pathogens [4]. With increasing demand for environment friendly aquaculture, the use of alternatives to antibiotics in aqua foods nutrition is now widely accepted. The search of safe and permanent solution for preventing pathogenic disease led aqua scientist to choose beneficial microorganisms called probiotics to displace pathogens by competitive processes, and is now gaining acceptance for the control of pathogens in aquaculture [5]. Enhancement of feed utilization and digestion in host species through the production of exoenzymes has observed [6]. This property may increase the weight of the shrimps and that will be beneficial for the farmers. With this concern the objectives of study was 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.

2.0 MATERIALS AND METHOD

Study area

An extensive field survey was carried out in the shrimp farms at Chidambaram Taluk during 2016–2017 for screening the presence of bacterial diseases. The disease shrimp showing external symptoms (white spots) were subjected for isolation and identification of pathogen involved.

Sample collection and isolation of pathogen

Infected shrimp (*Penaeus Monodon*) specimens were collected from shrimp farms situated along the coast of Tamil Nadu in the region of Thiruvasaladi, 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 [7]. Presumptive yellow colonies in TCBS agar plates were (*Vibrio cholerae*) 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. An array of biochemical test for determining the phenotypic profile of the isolated pathogen was carried as per Bergey's Manual of Systematic Bacteriology [8] and the results were interpreted.

Modest identification of Vibrio cholerae

For the modest identification of *Vibrio cholerae*, the following two methods arginine hydrolysis and lysine & ornithine decarboxylase assay were performed [9].

Isolation of probiotic organism

To prepare sauerkraut, fresh cabbage (organically farmed) was purchased from Sreevatsa Organic Farm Products, Coimbatore, Tamil Nadu, India, during 2016. Laboratory fermentations of cultivars of cabbage were conducted in glass jar. Cabbage was prepared by removing the outer leaves and core, cut in to 1/16 inch slicing disk. The shredded cabbage was salted with 2 – 2.5 % (w/v) of NaCl solution (brining solution i.e. rough sea salt) and all together was pressed tightly and covered with a plastic film. The jars were incubated at room temperature for fermentation by natural micro-biota associated with cabbage [10].

Isolation of Lactic acid bacteria

For the isolation of LAB, samples taken from brine during the course of fermentation (after 4 days) and plated onto MRS agar, incubated in anaerobic vessels at 30ºC for 4 days. Two uncrowded viscous colonies presumed to be *Leuconostoc* sp., were picked up randomly from MRS agar plates and purified by streaking on MRS plate. Purified cultures from cabbage fermentations were identified as per Bergey's Manual of Systematic Bacteriology [8]. The identified culture was stored at 4ºC in 10% glycerol for further use [11].

Time dependent invitro growth of probiotic bacteria and pathogen

Bacterial growth curve analysis was performed to determine the mid log phase of the *V. cholerae* and *L. mesenteroides.,* to perform the antagonistic assay. 100 µl of the overnight culture from nutrient broth was taken up and inoculated in fresh nutrient broth and incubated at 37ºC at 100 rpm. An aliquot was taken at every 30 mins at read 600nm to determine the growth. The analysis was performed thrice and the results were interpreted.

Invitro antagonism of bacterial isolate against V. cholerae

Two methods were carried out to evaluate the antagonistic activity of the prospective bacteria against the pathogen: the cross streak method and the co-culture method. In cross streak method was performed and recorded [7]. The co-culture method was performed to observe the antagonistic potential of the bacterial strain when grown along with the target pathogen (*V. cholerae*) concurrently [12].

Morphology analysis by Scanning Electron Microscopy

Colonies grown TCBS plates, both treated and non-treated (control) were examined by Scanning Electron Microscope (FEI Quanta SEM 200., Netherlands) to understands the morphological changes. 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 [13].

3.0 RESULTS

The survey undertaken during 2016–2017 at the area Cuddalore District revealed the occurrence of white spot disease in shrimp, designated, based on external symptoms, epithelial tissue pigmentation and as white spots in gill. Mass mortalities occurred in the ponds affected by white spot disease. The following account furnishes details of external symptoms of the various diseased shrimp and the bacteria involved in the manifestation of the disease.

Isolation and identification of pathogen

Infected shrimp tissue was collected from the shrimp growing area of Thiruvasaladi, Cuddalore District was plated onto TCBS plates, incubated for 48 hrs at 37ºC. Yellowish colonies presumptive as *Vibrio cholerae* 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 cholerae* (Table 1).

Modest identification of Vibrio cholerae

For the modest identification of *Vibrio cholerae*, the following two methods were performed. After 24 hrs incubation of isolate in LB broth containing 1% L- arginine with phenol indicator, no color change was observed. Further in Moeller decarboxylase broth base medium with 1% amino acid**,** dark purple color was observed in the tubes containing Lysine and Ornithine compared to the color obtained with the base medium without an amino acid. The results further confirm that the isolated pathogen is solely belongs to *Vibrio cholerae.*

Isolation of probiotics

For the isolation of LAB, 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 *Leuconostoc* 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 *Leuconostoc mesenteroides* (Table 1). The identified isolates *L. mesenteroides* was confirmed by Microbial Culture Collection, Maharashtra (MCC NO 3276).

TABLES:

TABLE 1. Morphological and Biochemical identification of isolated organisms

Tests	Results for Pathogen	Results for Probiotic
Shape	Curved Rod	Round
Gram Staining	- Ve	+ Ve
Spore Staining	Non Spore forming	Non Spore forming
Motility	Motile	Motile
Capsule	Non Capsulated	Non Capsulated
Flagella	Flagellated	Non Flagellated
Indole	+ Ve	- Ve
MR	- Ve	+Ve
VP	Variable	- Ve
Citrate	+ Ve	- Ve
H2S production	- Ve	-ve
Urease production	- Ve	- Ve
Gelatin hydrolysis	+ Ve	- Ve
Nitrate reduction	+ Ve	+ Ve
Oxidase test	+ Ve	- Ve
Glucose	+ Ve	+ Ve
Lactose	Variable	+ Ve
DNase	+ Ve	+ Ve
Sucrose	+ Ve	+ Ve
Catalase	- Ve	- Ve
Casein	- Ve	- Ve
Starch	- Ve	- Ve
Fructose	- Ve	+ Ve
Dextrose	- Ve	+ Ve
Galactose	+ Ve	+ Ve
Xylose	- Ve	+ Ve

Time dependent invitro growth of probiotic bacteria and pathogen

Bacterial Growth analysis was performed for both *L. mesenteroides*., and *V. cholerae* to determine the log and stationary phase for the analysis of antagonistic activity. For *L. mesenteroides.,* the log phase was obtained at the period of 5th h of incubation and the organism entered stationary phase at 21st h, then the phase was prolonged up to 96 hrs. Later, the organism started to reach decline phase. From this the growth period of *L. mesenteroides.,* was confirmed as 5-21 hrs. For *V. cholerae,* the log phase was obtained at the period of 7th h of incubation and the stationary phase was started from 26th h and prolonged to 90 hrs. Further the growth of the *V. cholerae* was not observed. Growth period of *V. cholerae.,* was confirmed as 7-26 hrs of incubation (Table-2).

Table 2. *Invitro* antagonistic activity of *Leuconostoc* sp., against *Vibrio cholerae* - Cross streak method

Days of Incubation	Growth in diameter (cm)		Inhibition zone (cm)
	(Probiotic)	(Pathogen)	
Day 1	0.5	0.3	4.3
Day 2	1.5	0.5	3.1
Day 3	3.0	0.7	1.4
Day 4	3.8	0.8	0.5
Day 5	4.2	0.8	0.1

Invitro antagonism of bacterial isolate against V. cholerae

Two methods were performed for the analysis of *invitro* inhibition of *V. cholerae*, In cross streak method the *L. mesenteroides.,* and *V. cholerae* was streaked in the same plate and incubated at 37ºC. The routine observation (5days of incubation) shown that the pathogen growth was seen in 1-3 days of incubation. During 4-5 days of incubation the growth was inhibited and no further colony development was observed. Whereas in case of *L. mesenteroides.,* rapid growth was observed up to the end of the experiment. The diameter of the colonies and the zone of inhibition was measured and tabulated in Table 3. The growth suppression pattern in pathogen indicated that the compound which is produced by the probiotic organism may be responsible for its decreased growth.

Based on the cross streak results, the *invitro* co culture method was performed as per the standard procedure. The *V. cholerae* was treated with different concentration of *L. mesenteroides.,* and plated onto TCBS plates along with non-treated one. The colony forming units were observed randomly in all the treated plates (Table 3). The growth of pathogenic *V. cholerae* was inhibited by *L. mesenteroides.,* culture inoculated at the ratio of 5:5 and. Lower concentrations of *L. mesenteroides.,* (1:4) allowed initial growth of *V. cholerae*, but CFU densities never reached the level of the control. High concentrations (4:1) of *L. mesenteroides.,* allowed an initial increase of *V. cholerae* followed by a decrease in the total viable counts. Co-culture experiment results showed that, when the concentration of *L. mesenteroides.,* increased, the growth of *V. cholerae* was controlled under *invitro* conditions.

Table 3. *Invitro* antagonistic activity of *L. mesenteroides*., against *Vibrio cholerae* – Co culture method

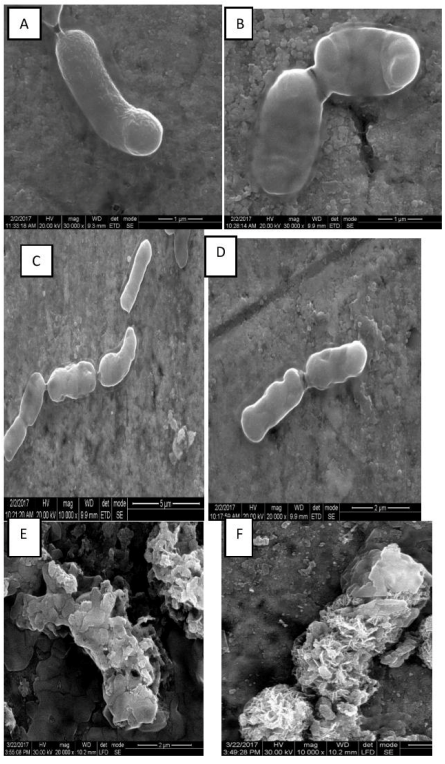
TCBS Plate	No. of Colonies
A (Control)	-
B (*Leuconostoc* sp.,)	No growth in TCBS
C (*V. cholerae*)	900+

D (400:100)	800+
E (300:200)	400+
F (200:300)	300+
G (100:400)	250+
H (500:500)	120+

Morphological analysis by Scanning Electron Microscopy

Colonies grown TCBS plates were pre-fixed and examined by scanning electron microscope to understand the morphological changes and the results of treated and non-treated samples were shown in Fig-1. As seen in the scanning electron micrographs, target bacterial cells *V. cholerae* grown in TCBS medium exhibit a typical rod shape with smooth surface. However, cells treated for 6 h with *L. mesenteroides.,* showed major structural disruption in the cell envelope as well as a preponderance of irregular rod forms with wrinkled surfaces. From the results it has been concluded that high concentrations of *L. mesenteroides.,* confer toxic effects to cells due to disruption of membrane components, leading to cell death.

Fig-3 Morphological analysis by Scanning Electron Microscopy



A & B *L. mesenteroides*; C & D - *V. cholerae* (Control), E & F. - *V. cholerae* (Treated with Probiotics).

4.0 DISCUSSION

The survey undertaken during 2016–2017 at the area of Cuddalore District revealed the occurrence of bacterial white spot disease in shrimp. Similarly a new bacterial white spot syndrome (BWSS) in cultured tiger shrimp *Penaeus monodon* was observed by [14]. The affected shrimp showed white spots similar to those caused by white spot syndrome virus (WSSV). The diseases caused by *Vibrio* sp*.,* in *Penaeus monodon* as: tail necrosis, shell disease, red disease, loose shell syndrome (LSS), and white gut disease (WGD). Among these, LSS, WGD, and red disease caused mass mortalities in shrimp culture ponds.

The pathogen was isolated from the infected shrimp tissue and identified as *Vibrio cholerae.* Generally, the *Vibrio* sp., are more abundant in aquatic conditions, the water samples have been used to isolate the pathogen. Similarly the work done by [15], isolated the *Vibrio* sp., from the gill of the infected shrimp and further characterized. As per the biochemical identification, the appearance of red color in arginine hydrolysis and dark purple color in lysine and ornithine decarboxylase assays indicating the presence of *Vibrio cholerae.* The above results as supported by [9]. The simple identification methods arginine hydrolysis, lysine and decarboxylase assays were carried out to confirm the *Vibrio cholerae.*

The probiotics like *Lactobacillus* sp., *Arthobacter* sp., and *Bacillus* sp., has shown their effective response towards the pathogens [3]. Probiotic organism was isolated in the MRS plates inoculated with the serially diluted fermentation brain. The isolated colonies where further analyzed and characterized as *L. mesenteroides*. Similarly the isolation of *L. mesenteroides* was carried out from the natural fermented medium, probiotic potential and its survival under the bile salt conditions has been reported by [16]. *L. mesenteroides*., to check their effective response towards the pathogen inducing bacterial white spot disease in shrimps. The results shown the use of *L. mesenteroides*., can survive in the salinity conditions and effectively replace the pathogen from the shrimps and thereby controls the bacterial white spot disease. The use of probiotics controls the pathogens in the aqua culture had been reported as beneficial, cost effective and no chance of evaluating resistance strains among the pathogens [2].

The *invitro* dual culture method showed that the no change in the pathogen colony size but the probiotic growth (Zone of inhibition) was continued to increase in the plates after 5 days of incubation. Likewise, *invitro* dual culture method carried out by [7] was also described that the probiotic organism inhibited the growth of pathogen.

Invitro antagonistic assay, results in the damages in the cell membrane and the changes in the shape and size was observed clearly in Scanning Electron Microscope indicating the inhibition of pathogen. Similar co culture method and SEM analysis was performed by [12] stated that the morphological changes occur in the pathogen when the probiotic is treated.

5.0 CONCLUSION

The study concluded that the *L. mesenteroides*., isolate will be helpful in the management of *V. cholerae* related bacterial disease in shrimp *P. monodon*. *L.mesenteroides*, 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. cholerae* from the infected shrimps and commendably control the Bacterial White Spot Disease. Introducing such specifically screened strains bound to favor the farmer's in turn of high yield in shrimp production. The future works embraces the molecular identification, characterization of antibacterial peptide, colonization ability and *in vivo* studies.

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7.0 REFERENCES

1. Vijaya Baskar, P., and Kannan, S. (2009). Marine bacteria as probiotics to control pathogenic vibrio on infected shrimp. SB Academic Review XVI (1 & 2). 77-85.
2. Aditiya Kesarcodi Watson., Heinrich Kaspar., Josie Lategan., and Lewis Gibson.(2008). Probiotics in aquaculture: The need, Principles and mechanism of action and screening processes. Science Direct. 274, 1-14.
3. Karthick, R., Angelin C Pushpam., and Vanitha, M.C. (2015). Development of Marine derived probiotic bacterial consortium for the sustainable management of Litopenaeus vannamei culture. International Journal Advanced Research in Engineering and Technology.6, 62-75.
4. Talpur, A.D., Memon, A.J., Khan, V., Ikhwanuddin, M., Danish, M.M., Daniel., and Abol Munafi, A.B.(2012).Inhibition of pathogens by Lactic acid bacteria and applications as water additive multi isolates in early stages larviculture of P. pelagicus (Linnaeus). The Journal of Animal & Plant Sciences. 22(1), 5-64.
5. Raja, S., Nandhini, E., Sahana, K., and Dhanakkodi, B. (2015). Beneficial and destructive effects of probiotics in aquaculture systems. International Journal Fisheries and Aquatic Studies. 2 (3), 153-159.
6. Madhura Mukadam.(2015).Probiotics in aquaculture. International Journal of Advanced Research in Biological Sciences. 2(6),45-47.
7. Janarthanam, K., Rosalind George, M., Riji John., and Prince Heyaseelan, M.J.(2012). In vitro and in vivo biocontrol of Vibrio harvevi using indigenous bacterium Bacillus sp. Indian Journal Geo-Marine Science.41(1) , 83-89.
8. Baumann, P., and Schubert, R.H. (1984). Vibrio naceae Veron. Bergey's manual of systematic bacteriology. 516-550.
9. Nipa Choopun., Valerie Lousis., Anwar Hug., and Rita Colwell, R. (2001). Simple procedure for rapid identification of Vibrio cholerae from the aquatic environment. Applied and Environmental Microbiology. 68(2),995-998.
10. Suzanne Johanningsmeier., Roger, F., Mcfeeters Henry., Fleming, P., and Roger, L. (2007). Thompson effects of Leuconostoc mesenteroides starter culture on fermentation of cabbage with reduced salt concentrations. Journal of Food Science. 72 (5), 166-172.
11. 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. Lett. Applied Microbiology.39, 439-449.
12. Vaseeharan, B., and Ramasamy, P. (2003). Control of pathogenic Vibrio spp. by Bacillus subtilis BT23, a possible probiotic treatment for black tiger shrimp Penaeus monodon. Lett. Applied Microbiology. 36, 83-87.
13. 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.
14. Wang, Y.G., Lee, K.L., Najiah, M., Shariff, M., and Hassan, M.D. (2000). A new bacterial white spot syndrome (BWSS) in cultured tiger shrimp Penaeus monodon and

its comparison with white spot syndrome (WSS) caused by virus. Diseases of Aquatic Organisms. 41, 9-18.
15. Mohammad Jalil Zorriehzahra., and Reza Banaederakhshan.(2015). Early Mortality Syndrome as new Emerging Threat in Shrimp Industry. Adv. Animal and Veterinary Sciences. 3(2), 64-72.
16. Rishikesh Shukla., Ilia Iliev., and Arun Goyal. (2014). Leuconostoc mesenteroides NRRL B-1149 as probiotic and its dextran with anticancer properties. Journal Bioscience. Biotech. 3(1), 79-87.