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Article

Antagonistic effect of *Lactobacillus* spp. on experimentally *Vibrio* spp. infected *Penaeus monodon*

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Abstract: The research was aimed to determine the present status of probiotics (*Lactobacillus* spp.*)* and pathogenic bacteria (*Vibrio* spp.) of culture pond as well as to determine whether the isolated probiotic *Lactobacillus* spp. can act as a controlling agent on experimentally pathogenic *Vibrio* spp. infected *Penaeus monodon.* In *In-vitro* test of gills and intestinal tracts, the potential antagonistic activity of *Lactobacillus* spp. toward *Vibrio* spp. was gradually observed at 4^{th} hour of probiotic treatment. At 12^{th} hour the viable count of *Vibrio* spp. was drastically reduced in gill from 2.33×10^5 to 1.03×10^3 ($CFUg^{-1}$) and in intestinal tract 2.35×10^5 to 6.43×10^2 ($CFUg^{-1}$). While, in *In-vivo* test, in case of muscle, gills and intestinal tract antagonistic activity of *Lactobacillus* spp. toward *Vibrio* spp. was noticed after 9 hours, 21 hours and 27 hours respectably of probiotic injected shrimps. However, only the result of in-vitro challenge test revealed that, *Lactobacillus* spp. significantly reduced the *Vibrio* spp. viable count of the gills of the selected samples (P=0.037). The investigation showed antagonistic effect of probiotic (*Lactobacillus* spp.) on experimentally *Vibrio* spp. infected shrimp.

Keywords: *Lactobacillus* spp.; *Vibrio* spp.; shrimp; In-vitro; In-vivo

1. Introduction

Shrimp culture is one of the fastest-growing animal food-producing sector which play a vital role to fulfill the demand of protein and economic development. In spite of this great potentiality, this sector is facing major economic losses due to a wide range of bacterial diseases. Among various diseases, Vibriosis is a potentially serious illness caused by a group of bacteria called *Vibrio* in shrimp worldwide. Though, both prophylactic and therapeutic chemicals as well as antibiotics are widely used to combat these pathogens in shrimp hatcheries and culture site, antibiotic resistance has become a critical threat to aquaculture as well as to global health. However, keeping mind of the above mentioned issues, several efforts has been taken current years to develop strategies for microbial control in aquaculture sector in alternative of the application of therapeutic chemicals and antibiotics. The application of probiotics is the prominent one. The prolegomenon of probiotics to control pathogens in aquaculture is well documented and crucial for the future of environment friendly aquaculture (Noh et al., 1994; Gomezgil, 1995; Bogut et al., 1998; Nowroozi et al., 2004; Nayak, 2010; Austin and Austin, 2012).

Therefore, the present study was aimed to observe the present scenario of probiotic bacteria (*Lactobacillus* spp.) and pathogenic bacteria (*Vibrio* spp.) of culture ponds by isolating from collected shrimps as well as to perform both In-vitro and In-vivo challenge test to observe antagonistic effect of probiotics *Lactobacillus* spp. towards experimentally *Vibrio* spp. infected shrimps.

2. Materials and Methods

2.1. Sample collection

Shrimp samples of average weight and length of 15±3 g and 12±2 cm respectively were collected by using random sampling technique from different culture ponds located at Dumuria upazilla of Khulna District, Bangladesh. Live samples were immediately carried to the laboratory of Fisheries Molecular Pathology, Fisheries and Marine Resource Technology (FMRT) Discipline of Khulna University by using separate oxygenated polythene bags. The shrimps were aseptically dissected and the organ samples of gill and intestinal tract were removed gently to isolate and enumeration of *Lactobacillus* spp. and *Vibrio* spp. (Hameed et al., 1998). Fifty live shrimp samples weighting from 26.80 g to 60.20 g (15.60 cm to 19.50 cm) were collected for experimental infection and In-vivo analysis.

2.2. Isolation and enumeration technique of *Lactobacillus* spp. and *Vibrio* spp. from collected samples

Here, gills and intestinal tracts of the collected shrimp samples were used to as sample organs for isolating both *Lactobacillus* spp. and *Vibrio* spp. We followed the method described by Nowroozi et al. (2004) as well as Hirsch (1960) for the isolating *Lactobacillus* spp. and preparing stock solution from sample organs respectively. While ISO procedure (ISO/TS 21872-1, 2007) was followed to isolate *Vibrio* spp. from sample organs of the collected samples. Finally, the number of colonies was enumerated after incubation.

2.3. In-vitro challenge test of the isolated probiotic towards the *Vibrio* spp. collected from sample

At first to prepare stock solution of organs of each shrimp were aseptically dissected. Then organs were taken into eppendorf tube with peptone water and homogenized by tissue homogenizer. After that homogenized solutions were centrifuged at 3000 rpm for 3 minutes. The upper liquid portion was collected with a micropipette and taken into eppendorf tube after centrifugation (Hameed et al. 1998). After that 0.5 ml isolated probiotic solution and 0.5 ml of test solution were mixed and kept for 4 hours for proper mixing. That duration was given for finding the antagonistic effect of lactobacillus against *Vibrio* spp. Then, TCBS agar media was used for inoculation of 0.1 ml mixer solution and this procedure was repeated at intervals of 4 hours up to 12 hours. Same procedure also applied for test solution of each sample without probiotic. Finally, inoculated TCBS agar plates were subjected to incubation (37^0 C, 24 ± 3 hours). Lastly, standard plate count was conducted (BBSOP0019-ss04, 2015).

2.4. In-vivo challenge test of the isolated probiotic on the experimental shrimps

The day before starting the in-vivo test, 50 shrimps were collected and transferred in aquariums. Shrimps were cultured in three subsequent aquariums. Aquarium-1 (negative control) contained 18 samples. The rest of the two aquariums contained 16 shrimps. 2 shrimps were sampled from negative control aquarium to enumerate the initial *Vibrio* spp. load of in-vivo challenge test. Water volume, salinity, dissolve O_2 and temperature were 100±3 liters, 5±0.32 ppt, 4.5±0.46 mg/L and $26±1.2^0$C respectively.

1 loop full *Lactobacillus* spp. was stirred with 1ml peptone water while 1 loop full *Vibrio* spp. was with 1 ml ASPW properly in different sterilized test tubes as well as further used for administration. After that, sterilized insulin syringe was used for administration of *Lactobacillus* spp. and *Vibrio* spp. solution.

Samples of aquarium-1 were injected with alkaline saline peptone water (mock infection) and denoted as negative control. Whereas a group of shrimps (32) were artificially injected with *Vibrio* spp. (0.1 ml solution) and kept in aquarium-2 as positive control. Half of those shrimps (16 individuals) were again injected with *Lactobacillus* spp. (0.1 ml solution) and kept in aquarium-3 as treatment. The injection was administered at 45° angels with the vertical axis of each shrimp body in the ventral portion of 2^{nd} abdominal segment, no anesthesia was used during injecting process. After that their muscles and intestinal tracts were collected for further experiment.

Before injection only two (02) samples were taken from 1^{st} aquarium (negative control) to enumerate the total *Vibrio* spp. load. Then samples were collected at 03, 09, 15, 21, 27, 33, 39 and 45 hours after injection.

Finally, marking and preservation of samples in deep freeze (-40°C) were conducted for further laboratory analysis. Then the isolation and enumeration of *Lactobacillus* and *Vibrio* spp. load in gill and gut were accomplished. Every time, two shrimps were sacrificed from each group of experiment. For bacterial colony count, each sample was spread on three different petri dished as replication.

2.5. Statistical analysis

The data collected during experiment were recorded. Data were analyzed using MS excel and the statistical package SPSS (16). One-way ANOVA was performed to observe the degree of difference between the treatments at the 5% level of significance.

3. Results

3.1. Enumeration of bacterial load of various organs of the collected samples (*P. monodon*)

The average *Lactobacillus* spp. and *Vibrio* spp. count in sample organs of collected shrimps are presented in Table 1.

3.2. In-vitro challenge test

In-vitro challenge test showed a reduction of *Vibrio* spp. load in gills after 4 hours of probiotic application. But a drastic reduction in the load of *Vibrio* spp. obtained at 12^{th} hour of probiotic application. Similarly, in case of intestinal tracts the load of *Vibrio* spp. was lowest after 12 hours of probiotic application (Table 2).

3.3. In-vivo challenge test

There was no presence of *Vibrio* spp. in the muscle of the samples taken from the negative control aquarium in each sampling interval. In *in-vivo* challenge test, significant reduction of *Vibrio* spp. load in the experimental muscle had been obtained from 21^{st} hour to rest of the time intervals of probiotic application (Table 3). Whereas, significant reduction of *Vibrio* spp. in intestine noticed after 27 hours of probiotic treatment.

Table 1. Total *Lactobacillus* spp. and *Vibrio* spp. count in selected organs of collected shrimp.

Pond No.	Gill		Intestinal tract	
	Average *Lactobacillus* load ($CFUg^{-1}$)	Average *Vibrio* spp. load ($CFUg^{-1}$)	Average *Lactobacillus* load ($CFUg^{-1}$)	Average *Vibrio* spp. load ($CFUg^{-1}$)
01	1.54×10^5	4.03×10^3	6.45×10^3	7.23×10^2
02	4.50×10^4	2.02×10^3	2.56×10^3	4.57×10^2
03	2.37×10^4	1.12×10^3	1.74×10^3	3.64×10^2
04	1.49×10^5	1.35×10^4	1.81×10^4	1.04×10^3
05	2.10×10^5	6.37×10^3	3.08×10^4	1.22×10^3
06	3.24×10^4	2.30×10^3	2.61×10^4	1.72×10^3

Table 2. In-vitro challenge test and enumeration of Vibrio spp. load in gills and intestinal tracts with and without probiotics.

Interval (Hours)	Gills		Intestinal tracts	
	Without probiotics Average *Vibrio* spp. load ($CFUg^{-1}$)	With probiotics Average *Vibrio* spp. load ($CFUg^{-1}$)	Without probiotics Average *Vibrio* spp. load ($CFUg^{-1}$)	With Probiotics Average *Vibrio* spp. load ($CFUg^{-1}$)
00	7.625×10^3	0	5.645×10^3	0
04	5.5590×10^4	9.35×10^2	2.9800×10^4	1.0810×10^4
08	1.23850×10^5	4.840×10^3	1.22300×10^5	2.455×10^3
12	2.33000×10^5	1.030×10^3	2.35100×10^5	6.43×10^2

* P = 0.037 in Gills and P = 0.069 in Intestinal Tracts

Table 3. In-vivo challenge test of muscle, gills and intestinal tract with probiotics and without probiotics.

Interval (Hours)	Average *Vibrio* spp. load ($CFUg^{-1}$) in muscle		Average *Vibrio* spp. load ($CFUg^{-1}$) in gills		Average *Vibrio* spp. load ($CFUg^{-1}$) in intestinal tract	
	Without probiotics	With probiotics	Without probiotics	With probiotics	Without probiotics	With probiotics
00	0	0	0	0	00	00
03	6.10×10^4	1.31×10^5	0	0	00	00
09	4.53×10^6	1.59×10^6	1.75×10^3	4.49×10^3	00	00
15	6.65×10^6	2.74×10^6	6.48×10^4	6.80×10^4	3.83×10^3	2.88×10^3
21	3.52×10^7	3.30×10^6	1.36×10^6	3.68×10^5	8.36×10^3	6.72×10^3

27	5.82×10^{7}	4.83×10^{6}	2.90×10^{6}	1.09×10^{5}	7.04×10^{4}	7.90×10^{3}
33	7.00×10^{7}	7.05×10^{6}	3.71×10^{6}	2.26×10^{5}	1.08×10^{5}	8.38×10^{3}
39	5.38×10^{8}	3.13×10^{6}	5.93×10^{7}	4.01×10^{5}	3.12×10^{5}	6.85×10^{4}
45	5.73×10^{9}	3.42×10^{7}	4.30×10^{7}	1.02×10^{5}	1.98×10^{5}	2.06×10^{4}

* P = 0.469 in Muscle; P = 0.510 in Gills and P = 0.687 in Intestinal Tract;

4. Discussion

The results of the present experiment revealed that both bacterial count (*Vibrio* spp. and *Lactobacillus* spp.) were much more in gills than that of intestinal tract in shrimp samples. That's because gills are external organ and play a vital role in respiration. Hence, opportunistic pathogens from the environment could taken up restlessly by different processes like respiration, osmoregulation as well as by feeding. On the contrary, intestinal tract is an internal organ and might not provide suitable and permanent adherence to the *Lactobacillus* spp. due to presence of different types of enzyme, digestive juice and acids. Moreover, it is well established findings that, *Lactobacillus* spp. are less prominent in piscine intestinal microbiome. Few strains of *Lactobacillus* spp. are more sensitive to incubation period and nutrient medium due to their slow growth rate, hence they need special nutrients enrich habitat. Therefore, the probability of high microbial abundance is to a greater extent on the external organs of an aquatic animal than in internal organ, which is supported by findings of different researchers (Moriarty, 1990; Ringo and Gatesoup, 1998; Hansen and Olafsen, 1999).

As we know that only effectual probiotic strains could able to colonize in intestinal mucosa through rendering the attachment site of pathogenic microbial strain by adhesion properties and could propagate chemical substances, metabolites and enzymes which are ultimately toxic or restrictive to pathogenic microbial strain; could execute immunostimulatory activity.

In-vitro testing result of the present study also unveiled a remarkable finding that, inoculated *Lactobacillus* spp. successfully reduced the *Vibrio* spp. viable count of the gills and intestinal tract of the selected samples in which marked reduction observed at 8th and 12th hour. This finding was also supported by Koga et al. (1998), Farzanfar (2006), Ramesh and Umamaheswari (2011) and Ariole and Nyeche (2013).

Whereas *In-vivo* diagnostic test consequence of the present study showed that, *Lactobacillus* spp. successfully impede the *Vibrio* spp. load of the muscle, gills and intestinal tract in probiotic injected shrimps gradually after 9, 21 and 27 hours of probiotic injection respectively. The load of *Vibrio* spp. in muscle and gills was found more in *Vibrio*-probiotic injected shrimps than only *Vibrio* injected shrimps respectably at 3 and 9 hours of post inoculation. Direct administration of *Vibrio* spp. in muscle and stress might be responsible for this situation. As far we know not only every individual has its own defense mechanism but also every bacterium has its own latent period, could able to colonize in the gastrointestinal tract when it could persist in that environment for a long time by possessing a multiplication rate that is higher than its expulsion rate. *Lactobacillus* need more time to activate and multiply than *Vibrio* spp. (Brock and Madigan, 1991; Ringo and Gatesoupe, 1998). *In-vivo* is related with live organisms and many physiological factors are involved in this. This might be a reason which reduces the rate of multiplication of *Lactobacillus* spp. than *Vibrio* spp. However, a few in vivo experiment were conducted on the antagonistic activity of *Lactobacillus* on *Vibrio* spp. in crustacean (Kesarcodi-Watson et al., 2008), though this finding was supported by Griffith (1995).

5. Conclusions

The present investigation showed an excellent antagonistic effect of *Lactobacillus* spp. on experimentally *Vibrio* spp. infected shrimp. Therefore, *Lactobacillus* spp. could be used as an effective agent to control the shrimp diseases caused by pathogenic *Vibrio* spp. in culture system.

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Conflict of interest

None to declare.

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