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Antagonistic Activity of the Gut Microflora Isolated from Farmed Tiger Shrimp (*Penaeus monodon)*

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Abstract

The antagonistic action of the gut microflora of the tiger shrimp (*Penaeus monodon*) from three different farms (A, B and C) was tested against four bacterial shrimp/fish pathogens viz., *Aeromonas hydrophila, A. sobria, Vibrio vulnificus* and *V. fischeri* using the double agar overlay method. The total bacterial load of gut microflora ranged from 10^6-10^8 cfu.g^{-1}. The bacterial composition of shrimp gut predominantly consisted of *Vibrio* sp. (30-52%) followed by *Bacillus* sp., *Pseudomonas* sp., *Photobacterium* sp., *Plesiomonas* sp. Of the 185 isolates tested, 26.27% showed antagonistic action against fish/shrimp pathogens. The gut microflora, *Pseudomonas* sp. and *Photobacterium* sp. of farm A; and *Bacillus* sp. of farm C inhibited the fish pathogen, *A. sobria,* while *Vibrio* sp. and *Corynebacterium* sp. of farm C inhibited *A. hydrophila.* Only a single strain of *Vibrio* sp. (C2) from farm C was found to show antagonistic action against the shrimp pathogen, *V. vulnificus*. The intestinal gut microflora of shrimp shall therefore be evolved as putative probiotic bacteria to counteract disease problems.

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Introduction

Animals in the aquatic environment carry bacterial flora, which is a reflection of the flora in the environment (Chandrasekaran 1985). Intestinal bacteria, such as *Aeromonas* sp. and *Vibrio* sp. often cause opportunistic infections (Asfie et al. 2000). Gut microflora plays an important role in the digestive process, growth and disease susceptibility of marine deposit feeders (Fenchel and Kofoes 1976; Yingst 1976). However, some bacteria which possess the ability to tolerate the low pH in gastric juices, resist the action of bile acids and lysozyme secreted in intestines and the immune responses, and adhere to the mucus and/or enteric wall surface, could persist for a relatively long time and eventually make intestinal microflora specific to each host animal (Olsson et al. 1992). Bacteria producing anti-bacterial substances were recently isolated from marine fish intestines (Westerdahl et al. 1991; Sugita et al .1996; Sugita et al. 1997; Sugita et al. 1998; Sugita et al. 2002; Onarheim and Raa 1990), but not from shrimp.

Presence of these antagonistic bacteria helps to prevent the diseases caused by the invading bacteria. In India, shrimp culture is a booming industry but outbreaks of diseases have been experienced since 1995. The farmers are administering several antibiotic and probiotic feeds to counter-act the disease problems. Commercially used probiotic feed mostly harbors *Bacillus* sp. and *Lactobacillus* sp., which are effective in terrestial animals and their effect on aquatic animals was not clearly elucidated. Shrimp harbours a large number of bacteria in its gut from water, sediment and feed and a study of their antagonistic action against shrimp/ fish pathogens can help to evolve effective putative bacteria to counteract disease problems. The present study was therefore undertaken to examine the antibacterial ability of intestinal bacteria of tiger shrimp isolated from different farms.

Materials and Methods

Shrimp

Tiger shrimp (*Penaeus monodon*) were collected from three different shrimp farms located in Tamil Nadu, Southern State of India and were designated as Farm “A”, “B” and “C”. Farms ‘A’ and ‘B’ are private shrimp farms located in Chennai (North Tamil Nadu) and Pazhayakayal

(South Tamil Nadu), respectively. Farm 'C' is a raceway shrimp farm of our Institution. At the farm sites, shrimps were aseptically beheaded, peeled and gut region removed using sterile sharp knife and forceps. The gut was then dispensed in pre-weighed sterile 1% peptone water taken in a screw cap test tube. Samples were then brought to the laboratory in insulated containers for further analysis.

Bacteriological sampling

In the laboratory, gut was transferred to a sterile homogeniser, homogenized with an appropriate volume of sterile 1% peptone water and serial dilutions made with the same diluent. The diluted suspensions were plated simultaneously onto trypticase soy agar (TSA) with 1% NaCl following the spread plate method (APHA 1976). The plates were then incubated at 30^0C for 24-48 h. The colonies developed on the plates were counted and expressed as cfu/g.

Representative colonies of bacteria from shrimps of each farm (A = 82, B = 16 and C = 87) were picked up from TSA + 1 % NaCl plates and purified. The purified colonies were then identified to generic levels following standard biochemical tests described in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbsons 1982). Various biochemical tests performed include gram's reaction, catalase, oxidase, oxidative – fermentative, motility, sensitivity to O/129, indole, methyl red (MR) test, sugar fermentation, citrate utilization test, decarboxylase test, voges-proskaeur (VP) test and penicillin sensitivity. All the bacteriological media and reagents were obtained from Hi-Media, Mumbai, India.

Assay for antibacterial activity

The antagonistic activity of the different gut microflora against four selected shrimp/fish pathogens were studied following the double agar layer method (Dopazo et al. 1988). They were *Vibrio vulnificus* (MTCC 1146), *Vibrio fischeri* (MTCC 1738), *Aeromonas sobria* (MTCC 1608) and *Aeromonas hydrophila* (MTCC 646) obtained from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. They were referred to as test organisms and maintained in TSA slants. The *Vibrio sp.* was maintained in TSA + 3% NaCl medium.

Producer organisms (gut microflora) were grown in trypticase soy broth (TSB) with 1% NaCl at 37^0C for 18 h. Macrocolonies of the pro-

ducer organisms were developed on TSA + 1% NaCl plates by inoculating 5µl of the broth culture with a micropipette. After incubation at 37^0C for 48h, the colonies were killed by exposure to chloroform vapour for 15-20min. The test organisms were grown in suitable broth at 37^0C for 18h. Ten µl of this broth culture was thoroughly suspended in 8 ml of soft agar (TSB broth + 0.7% agar) maintained at 45-50^0C. The soft agar was immediately poured over the macrocolonies of producer organisms on agar plate. The plates were incubated for 24 h at 37^0C and observed for a definite zone of clearance around the macrocolony of the producer organism. Control plates without the macrocolonies of the producer organisms were also used to evaluate the possible effect of chloroform on the growth of the test organism. The assay was carried out in triplicate and the mean of the inhibition zone was calculated.

Results and Discussion

Microflora

Total bacterial load in the gut of tiger shrimp procured from three different farms are presented in figure 1. The average total bacterial population ranged from 10^6 – 10^8 cfu.g^{-1}. Higher counts were recorded in shrimps obtained from farm 'B', which lower counts were recorded in shrimps from farm – 'C'. Sugita et al. (2002) reported that the intestinal microflora of Japanese flounder ranged from 10^5 – 10^7 cfu.g^{-1}. Shrimps, being a bottom dwelling detritus feeder, often have higher bacterial population than finfish. Among the shrimps collected from the three farms, the gut microflora of shrimps in farm -'C' were lower (10^6 cfu.g^{-1}), as they were harvested from the raceway farm of our Institution with regular water exchange and aeration, whereas farms 'A' and 'B' are extensive shrimp culture farms.

Gut microflora of the shrimps identified to generic level along with their percentage incidence are given in table 1. Of the 82 isolates from the shrimps gut of farm 'A', *Vibrio* sp. was dominant (52.4%) followed by *Pseudomonas* sp. (18.2%), *Plesiomonas* sp. (15.8%), *Bacillus* sp. (7.3%) and *Photobacterium* sp. (8.0%). Bacteria isolated from gut of shrimps from farm 'B' consisted of only two genera, *Vibrio* sp. (50%) and *Plesiomonas* sp. (50%). The shrimps from farm 'C' harboured in their gut region diversified flora consisting of *Bacillus* sp. (31%), *Vibrio* sp. (29.8%), *Staphylococcus* sp. (16%), *Hafnia* sp. (13.7%) and *Corynebacterium* sp. (9.1%).

The *Vibrio* sp. was found to be the dominant intestinal microflora (>50%) and the shrimps from farms 'A' and 'B', as it is the native microflora of shrimp. Sugita et al. (2002) reported that the isolates from the intestinal tracts of Japanese flounder belonged to *Bacillus*, Coryneforms, Enterobacteriaceae, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Vibrio* sp. It has also been reported by several workers, that the fish of marine origin harbor *Vibrio* sp. as the predominant intestinal microflora (Cahill 1990; Sugita et al. 1991). A high incidence of 59% *Vibrio* sp. was also isolated by Asfie et al. (2000) from Japanese flounder. The dominance of *Bacillus* sp. in gut of shrimps from farm 'C' was probably because of the incorporation of probiotic feed, "AQUALACT" in the raceway culture systems. Presence of *Bacillus* sp. was also noticed in the shrimps from farm 'A'. Use of probiotic feed in shrimp farms was found to alter the native bacterial composition of gut microflora.

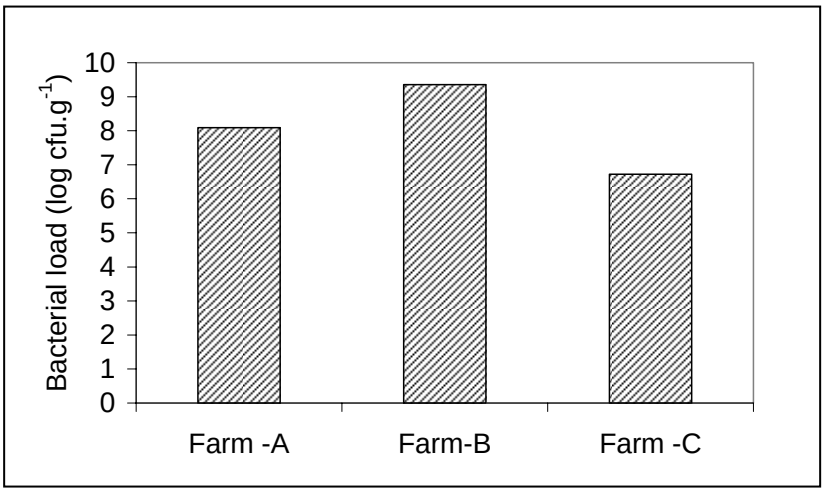

Figure 1. The total bacterial load (cfu.g^{-1}) of the gut microflora of tiger shrimp from different farms in Tamil Nadu, India

Antagonistic action of gut microflora

The results of the antagonistic action of the gut microflora against the shrimp/fish bacterial pathogens are given in table 2. In total, 26.7% of the gut microflora isolated from shrimps showed antagonistic activity against the shrimp/fish pathogens. Earlier reports showed that only about 1 – 10% of intestinal bacteria from both freshwater and marine fish possessed inhibitory action against fish bacterial pathogens (Sugita et al. 1998; Onarheim and Raa 1990). Sugita et al. (2002) have reported that more than

10% of the isolates from intestinal tracts of Japanese flounder exhibited antagonistic action. Among the three farms, the gut microflora of shrimps from farms A and C only showed antagonistic action at 36.59% and 43.67% levels, respectively, while microflora from farm B did not exhibit any antagonistic action.

Table 1. Identification of the gut microflora isolated from farmed tiger shrimps (*Peneaus monodon*)

Source	Microorganisms	Incidence (%)
Farm - A	*Vibrio* sp. (n = 43)	52.4
	Pseudomonas sp. (n = 15)	18.2
	Plesiomonas sp. (n = 13)	15.8
	Bacillus sp. (n = 6)	7.3
	Photobacterium sp. (n = 5)	6.0
Farm – B	*Vibrio* sp. (n = 8)	50.0
	Plesiomonas sp. (n = 8)	50.0
Farm - C	*Bacillus* sp. (n = 27)	31.0
	Vibrio sp. (n = 26)	29.8
	Staphylococcus sp. (n = 14)	16.0
	Hafnia sp. (n = 12)	13.7
	Corynebacterium sp. (n = 8)	9.1

Three strains of *Vibrio* sp. (A1, A2 and A3) were identified in shrimps from farm A, and the strain A1 alone showed antagonistic action against *Aeromonas hydrophila.* The isolates of *Photobacterium* sp. and *Pseudomonas* sp. showed greater inhibitory action against *A. sobria*. Smith and Davey (1993) reported that *Pseudomonas fluorescences* reduced disease caused by *A. salmonicida*. None of the gut microflora of shrimps from farm A inhibited the shrimp pathogen, *V. vulnificus* and *V. fischeri*. Two strains of *Bacillus* sp. (C1 and C2) were identified in shrimps from farm C, and the strain C2 showed inhibitory action against *A. sobria*. Another isolate of *Corynebacterium* sp. showed antagonistic action against *A. hydrophila.* A strain of *Vibrio* sp. (C2) inhibited the growth of *V. vulnificus*. Sugita et al. (1997) have reported that a strain of *Vibrio* isolated from fish intestine exhibited a wide antibacterial spectrum against *V. vulnificus*, *Pasteurella piscicida*, *Escherchia coli* and *Edwardsiella seriolicida*. Similarly, Moriarty (1998) reported that *Bacillus* strains showed antibiotic activity against luminescent *Vibrio* sp.

Table 2. Inhibitory action of the gut microflora isolated from farmed tiger shrimp against shrimp/fish pathogens

Farm – A

Isolates	Zone of Inhibition (mm)			
	A. hydrophila	*A. sobria*	*V.vulnificus*	*V.fischeri*
Vibrio –A1 (n = 10)	16 (12.19)	-	-	-
Vibrio –A2 (n = 8)	-	-	-	-
Vibrio – A3 (n = 25)	-	-	-	-
Pseudomonas (n = 15)	-	28 (18.29)	-	-
Plesiomonas (n = 13)	-	-	-	-
Photobacterium (n = 5)	-	32 (6.03)	-	-
Bacillus (n = 6)	-	-	-	-

Farm – B

Isolates	Zone of inhibition (mm)			
	A.hydrophila	*A. sobria*	*V.vulnificus*	*V.fischeri*
Vibrio – B1 (n = 5)	-	-	-	-
Vibrio – B2 (n = 2)	-	-	-	-
Vibrio – B3 (n = 1)	-	-	-	-
Plesiomonas–B1 (n=6)	-	-	-	-
Plesiomonas–B2 (n=2)	-	-	-	-

Farm – C

Isolates	Zone of inhibition (mm)			
	A. hydrophila	*A. sobria*	*V.vulnificus*	*V.fischeri*
Bacillus – C1 (n = 17)	-	-	-	-
Bacillus – C2 (n = 10)	-	32 (11.49)	-	-
Vibrio – C1 (n = 6)	-	-	-	-
Vibrio – C2 (n = 20)	-		12 (13.79)	-
Staphylococcus (n= 14)	-	-	-	-
Hafinia (n = 12)	-	-	-	-
Corynebacterium (n=8)	17 (9.19)	-	-	-

Percentage given in parenthesis

The gut microflora of shrimps that showed wide antibacterial activity belonged to the genera, *Pseudomonas* sp., *Photobacterium* sp. and *Bacillus* sp. with a zone of inhibition of 28-32 mm against *A. sobria.* The fish pathogen, *A. hydrophila* was inhibited by a strain of *Vibrio* sp. and *Corynebacterium* sp. Similarly the shrimp pathogen, *V. vulnificus* was inhibited by a strain of *Vibrio* sp. alone. Further studies are required to isolate effective gut microflora showing antagonistic action against the shrimp/fish pathogens so as to formulate a putative bacterial package to prevent the outbreak of infectious diseases in farmed shrimps.

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References

APHA, 1976. Compendium of methods for the microbiological examination of foods. M.L. Speck (ed), American Public Health Association, Washington DC.

Asfie, M., T. Ishigaki, R. Okano, N. Akiyama and H. Sugita. 2000. Antibacterial abilities of microflora in the Japanese flounder rearing aquaria. Suisanzoshoku 48: 227-231.

Buchanan, R.E. and N. E. Gibbons. 1982. Bergey's Manual of Determinative Bacteriology (8^{th} ed.), Baltimore, USA.

Cahill, M.M. 1990. Bacterial flora of fishes: A review. Microbial Ecology 19: 21 –41.

Chandrasekaran, M. 1985. Studies on the microbial spoilage of *Penaues indicus.* Ph.D. Thesis, Cochin University, Kerala.

Dopazo, C. P., M. L. Lemos, C. Loderiors, J. Bolinches, J. L. Barja and A. E. Toranzo. 1988. Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. Journal of Applied Bacteriology 65: 97 –101.

Fenchel, T., L. H. Kofoes. 1976. Evidence for exploitative interspecific competition in mud snails. Oikos 27: 367 – 376.

Moriarty, D. J. W. 1998. Control of luminous *Vibrio* species in Penaeid aquaculture ponds. Aquaculture 164: 351 – 358.

Olsson JC., A. Westerdhal, PL. Conway and S. Kjelleberg. 1992. Intestinal colonization potential of turbot (*Scophthalmus maximus)*- and dab (*Limanda limanda)*- associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied Environmental Microbiology 58: 551 –556.

Onarheim, AM. and J. Raa. 1990. Characterization and possible biological significance of autochthonous flora in the intestinal mucosa of sea water fish. In:Lesel R(ed.). Microbiology in Poecilotherms. Elsevier. Amsterdam. pp. 197 – 201.

Smith, P. and S. Davey. 1993. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. Journal of Fish Diseases 16:521-524.

Sugita, H., C. Miyajima and Y. Deguchi. 1991. The vitamin B_{12} – Producing ability of the intestinal microflora of freshwater fish. Aquaculture 92: 267 – 276.

Sugita, H., N. Matsuo, K. Shibuya and Y. Deguchi. 1996. Production of antibacterial substances by intestinal bacteria isolated from coastal crab and fish species. Journal of Marine Biotechnology 4: 220 – 223.

Sugita, H., N. Matsuo, Y. Hirose, M. Iwato and Y. Deguchi. 1997. *Vibrio* sp. strain NM 10 with an inhibitory effect against *Pasteurella piscicida* from the intestine of Japanese coastal fish. Applied Environmental Microbiology 63: 4986 – 4989.

Sugita, H., T. Ishigaki, D. Iwai Y. Suzuki, R. Okano, S. Matsuura, M. Asfie, E. Aono and Y. Deguchi. 1998. Antibacterial abilities of intestinal bacteria from three coastal fishes. Suisanzoshoku 46: 563 – 568.

Sugita, H., R. Okano, Y. Suzuki, D. Iwai, M. Mizukami, N. Akiyama and S. Matsuura. 2002. Antibacterial abilities of intestinal bacteria from larval and juvenile Japanese flounder against fish pathogens. Fisheries Science 68: 1004-1011.

Westerdahl, A., LC. Olsson, S. Kijelleberg and P. Conway. 1991. Isolation and characterization of turbot (*Scophthalmus maximus)*- associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied Environmental Microbiology 57: 2223 – 2228.

Yingst, J. 1976. The utilization of organic matter in shallow marine sediments by an epibenthic deposit feeding holothurian. Journal of Experimental Marine Biology and Ecology 21: 53-59.