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Bacterial microflora associated with farmed freshwater prawn *Macrobrachium rosenbergii* (de Man) and the aquaculture environment

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Abstract

The initial counts of bacteria associated with farmed giant freshwater prawn (*Macrobrachium rosenbergii* de Man) as well as with the water and sediment from two farms located at Kottayam district in Kerala (India) were determined. A total of 367 randomly selected isolates were characterized and identified. Prawn samples yielded mean microbiological counts of 4.92 log CFU g^{-1} of shell with muscle and 7.78 log CFU g^{-1} of intestine at 30 °C. Bacterial numbers in the intestine of prawn were much higher than those in the pond water. Motile and non-motile aerobic Gram-negative bacteria together with Enterobacteriaceae accounted for 60–70% of the mesophiles isolated from water and prawn. However, the microorganisms isolated in significant numbers from shell with muscle and intestine of prawn were not recovered from the growing waters. Among Gram-positive bacteria, *Micrococcus*, *Bacillus*, coryneforms, and *Arthrobacter* were found. Faecal coliforms and enterococci were found in significant numbers in *M. rosenbergii*. The rearing practices such as feeding and pond fertilization could have influenced the microflora in prawn. The commensal microflora of freshwater prawn included opportunistic pathogens such as *Aeromonas* spp. and *Streptococcus* spp. Storage of prawn at elevated temperatures can permit their survival and growth leading to quality loss. Care should be exercised during handling and processing to prevent contamination of edible meat.

Keywords: farmed freshwater prawn, *Macrobrachium rosenbergii*, aquaculture environment, microflora, indicator bacteria

Introduction

The aquaculture production of giant freshwater prawn (*Macrobrachium rosenbergii* de Man) in India increased from 753 tonnes in 1997 to 1507 tonnes in 1998, with an estimated production of 7140 tonnes during 1999–2000 (Marine Products Export Development Authority (MPEDA) 2001). Exports of frozen *M. rosenbergii* (commercially known as scampi) increased from 1796 tonnes in 1994 to 1909 tonnes in 1998. The culture of giant freshwater prawn is now a very important commercial activity in several parts of the country. Despite this, very little attention has been paid to the bacterial flora associated with cultured *M. rosenbergii* (Miyamoto, Brock, Nakamura, Nakagawa, Shimojo, Sato & Akita 1983; Anderson, Shamsudin & Nash 1989) and its influence on the initial quality of this prawn species. Horsely (1973) emphasizes the influence of the environment and feeding on the composition and level of the microorganisms associated with Atlantic salmon. For this reason, the monitoring of certain microbial parameters in water and sediments of prawn farms is needed to evaluate the microbiological quality of farmed prawn.

Types and levels of bacterial populations associated with farmed *M. rosenbergii* are useful indicators of quality and safety of prawns. In addition, most diseases in *M. rosenbergii* are caused by opportunistic pathogens which are prevalent in the rearing environment (Lombardi & Labao 1991; Brock 1993; Tonguthai 1993; Jayasree, Janaki Ram & Madhavi 1999). It is essential to investigate the bacterial flora associated with prawn grow-out culture in order to develop safe farm management practices for the

production of prawn safe for human consumption and for prevention of prawn diseases (Reilly & Käferstein 1997). The purpose of this study was to determine the heterotrophic bacterial population associated with farmed freshwater prawns and their rearing environment. The information obtained should allow a better control of the bacteriological parameters in the aquacultured freshwater prawn and also a better control of processing to reduce the possible microbial risks.

Materials and methods

Sampling

Prawns were collected from two freshwater scampi farms of 0.8–1.2 and 1.7 ha respectively, with water depth of 90–150 cm located in Kerala within the district of Kottayam (India). Water was supplied from the Muvattupuzha river through a canal and the supply was controlled by pumping daily. All the ponds were fertilized with organic manure (cow dung) before stocking. Prawns were fed on animal protein sources such as clams and waste from slaughter house. Feed was supplied once daily. Specimens of the freshwater prawns weighed 60–90 g.

Prawns from each system were sampled twice, in June 1998 and in July 1999. Farmed *M. rosenbergii* were harvested by cast nets. Twenty-five animals were collected in sterile polyethene bags and transported to the laboratory on ice. Samples were aseptically analyzed within 2–4 h of collection. Water samples were collected from water inlet (feeder canal) and also from different locations of each farm by inverting sterile 1-L polystyrene bottles into the water to about 30–40 cm below the surface. Farm sediment samples were scooped out from water inlet and different locations on the farms, put in sterile polyethene bags and transported to the laboratory. Farm water and sediment samples collected from different locations were pooled before analysis and tests were initiated within 2–3 h.

Physico-chemical analysis

pH, dissolved oxygen, temperature and salinity of farm water were determined according to standard methods for the examination of water and waste water (APHA 1998). Turbidity was determined using the secchi disc (Boyd 1992; Brock 1993).

Bacteriological analysis

From each prawn, 10 g of shell with muscle portions were cut from two or three different parts of the surface. In addition, gut was excised, weighed and placed in sterile bags containing enough saline solution to make 1:10 dilutions and blended for 2 min in a Stomacher 400 lab blender (Seward Medical, UK).

Samples of sediment (25 g) were homogenized in 225 mL of saline solution using a Stomacher. Prawn and sediment homogenates and water samples were serially diluted and plated on Tryptone Soya Agar (TSA, Oxoid, UK) for total aerobic plate counts (TPC) (Austin & Al-Zahrani 1988) at 37 °C, 30 °C (2–3 days) and 22 °C (3–5 days). Psychrotrophs were determined on TSA at 7 °C for 10 days.

Coliforms, faecal coliforms and *Escherichia coli* counts were determined by a 3 replicate tube most probable number (MPN) procedure. Coliform numbers were determined using Mac Conkey broth incubated at 37 °C for 48 h and subculturing all positive tubes in brilliant green lactose bile (BGLB) broth, incubated at 37 °C for 24 h. Faecal coliforms were determined by subculturing all positive BGLB tubes in *Escherichia coli* (EC) and Tryptone broths incubated at 44.5 °C for 24 h. The EC tubes were examined for growth and gas (APHA 1998). Positive EC tubes were confirmed by streaking on to Eosine Methylene Blue Agar (EMB Agar, Difco, Detroit, Michigan, USA) and incubated at 37 °C for 24 h. Characteristic *E. coli* colonies were isolated and confirmed by biochemical tests as described by APHA (1998).

Enterococci numbers were estimated by using Dextrose azide broth (Oxoid, UK) and the MPN method for three tube series (West 1989). Positive tubes were confirmed by streaking on KF streptococcal Agar (Difco, Detroit, Michigan). Typical *Enterococcus* colonies were identified by checking growth at 45 ± 1 °C and growth in Dextrose azide broth containing 6.5% sodium chloride incubated at 35 ± 2 °C and confirmed by biochemical tests as described by APHA (1998). *Staphylococcus aureus* counts were estimated on Baird Parker Agar (BP, Oxoid, UK) at 37 °C for 2 days and typical colonies were confirmed by coagulase test (Food and Drug Administration (FDA) 1995). Total *Vibrio* counts were performed on Thiosulphate Citrate Bile Sucrose Agar (TCBS, Oxoid, UK) incubated at 37 °C for 24 h (FDA 1995) and characteristic colonies were confirmed by biochemical tests according to Alsina & Blanch (1994).

A total of 367 strains were randomly selected and isolated from water, sediment and prawn samples.

The bacterial strains picked from TSA plates incubated at 30 °C were tested for Gram reaction, cell morphology, catalase and oxidase reactions, motility, oxidation/fermentation test and presence of spores. They were then grouped according to the taxonomic schemes of Bergey's Manual of Systematic Bacteriology (Krieg & Holt 1984; Sneath, Mair, Sharpe & Holt 1986), further tested for the most relevant characteristics of each group and identified using the above schemes and key schemes proposed by several authors for identification (Allen, Austin & Colwell 1983; Austin 1988; Andrew & Mitchell 1997; Kirov 1997).

Statistical analysis

TPCs were expressed as CFU g^{-1} for sediment and prawn and as CFU mL^{-1} for water. TPCs were transformed into $\log_{10}$ values before statistical analysis. ANOVA was performed using the statistical tool package of Microsoft Excel 97 software. Student's *t*-test analysis was used to evaluate the significance of differences between means of microbial counts performed in water, sediment and prawn samples. $P < 0.05$ was considered statistically significant.

Results

Physico-chemical characteristics of water

Water physico-chemical parameters were similar in both farms and within the acceptable range for rearing *M. rosenbergii*. Temperatures were 28 ± 2 °C and pH values were between 6.8 and 7.0. Dissolved oxygen (mg L^{-1}) values ranged from 5.0 to 6.0. Salinity (ppt) levels ranged from 0.75 to 0.8 and turbidity (secchi disc, Brock 1993) was in the range of 35–50 cm.

Bacterial counts

Bacterial levels for water, sediment and prawn samples are given in Table 1. Aerobic mesophilic counts at 37, 30 and 22 °C for water, sediment and prawn did not differ significantly ($P > 0.05$). However, counts at 7 °C were significantly lower than those at 30 °C ($P < 0.05$).

Mesophilic counts (mean log CFU g^{-1} at 30 °C) were significantly higher in sediment samples than in water ($P < 0.01$) in the freshwater prawn grow-out cultures. Mesophilic bacterial counts at 30 °C were lower in sediments and water from the water inlet (feeder canal) than that from farms ($P < 0.01$). The bacterial numbers in the intestine samples were 7.78 log CFU g^{-1}, a value much higher than that of the prawn surface and the surrounding waters ($P < 0.01$).

Total coliforms, faecal coliforms, *E. coli* and enterococci were recovered from all samples (Table 2). The mean log counts (MPN g^{-1}) of faecal coliforms in the intestine of prawn and water were 3.78 and 3.22 respectively. Numbers of faecal coliforms and enterococci were significantly different between prawn surface and intestine samples ($P < 0.01$). There were also significant differences in numbers of *E. coli* and enterococci between intestine and water ($P < 0.01$). *Staphylococcus aureus* was not detected in any sample. *Vibrio* count (mean log CFU g^{-1}) on the surface of prawns was 3.83. Typical colonies were isolated. Among 10 *Vibrio* isolates, *V. furnissi* (5), *V. cholerae* (2) and *Photobacterium damselae* (3) were found. *V. cholerae* isolates did not belong to serogroup O1.

Identification of bacteria

On the basis of phenotypic traits, the selected 367 isolates belonged to 19 genera (Table 3). The main

Table 1 Mean microbiological counts ($\log_{10}$ CFU g^{-1}) of water, sediment and prawn from freshwater farms

	$\log_{10}$ CFU g^{-1} ± SD*					
					Macrobrachium rosenbergii	
Counts	Farm water†	Feeder canal water	Farm sediment	Feeder canal sediment	Shell with muscle	Intestine
Total 37 °C	3.66 ± 0.60	3.52 ± 0.43	6.05 ± 0.33	5.50 ± 0.39	4.83 ± 0.23	7.65 ± 0.89
30 °C	3.81 ± 0.47	3.62 ± 0.38	6.06 ± 0.35	5.79 ± 0.25	4.92 ± 0.21	7.78 ± 0.82
22 °C	3.66 ± 0.31	3.11 ± 0.70	5.57 ± 0.37	5.20 ± 0.18	4.74 ± 0.41	6.88 ± 0.25
7 °C	2.04 ± 0.03	1.15 ± 0.15	4.13 ± 0.47	2.20 ± 0.19	3.25 ± 0.26	5.16 ± 0.35
Vibrio	2.38 ± 0.68	2.02 ± 0.43	3.35 ± 0.25	2.41 ± 0.34	3.83 ± 0.60	4.34 ± 0.58

*SD, Standard deviation.
†For water CFU mL^{-1}

Table 2 Mean counts of indicator organisms ($\log_{10}$ MPN g^{-1}) in water sediment and prawn from freshwater farms

	$\log_{10}$ MPN $g^{-1} \pm$ SD*					
					Macrobrachium rosenbergii	
Indicator bacteria	**Farm water†**	**Feeder canal water**	**Farm sediment**	**Feeder canal sediment**	**Shell with muscle**	**Intestine**
Total coliforms	3.22 ± 0.72	2.48 ± 0.14	1.98 ± 0.52	1.78 ± 0.56	3.28 ± 0.32	3.78 ± 0.22
Faecal coliforms	3.22 ± 0.37	2.14 ± 0.66	1.51 ± 0.41	1.14 ± 0.60	2.91 ± 0.22	3.78 ± 0.22
Escherichia coli	2.19 ± 0.14	1.08 ± 0.37	1.07 ± 0.38	0.78 ± 0.53	2.91 ± 0.22	3.61 ± 0.37
Enterococci	1.50 ± 0.61	1.25 ± 0.79	1.93 ± 0.26	1.74 ± 0.39	2.83 ± 0.16	3.82 ± 0.37

*SD, Standard deviation.
†For water MPN 100 mL^{-1}.

Table 3 Percentage distribution of the main bacterial groups and genera associated with giant freshwater prawn and farm environment

		Water		**Sediment**		***Macrobrachium rosenbergii***	
		F*	**Fc**	**F**	**Fc**	**Shell with muscle**	**Intestine**
Bacterial group	**Genera**						
Enterobacteriaceae	*Enterobacter cloacae*	9.0				7.1	10.0
	Enterobacter aerogenes					5.3	1.7
	Enterobacter sakazakii					3.6	1.6
	Citrobacter freundii	9.0				5.3	8.4
	Klebsiella pneumoniae					12.5	5.1
	Kluvyera spp.		3.6			1.8	
Aeromonadaceae	*Aeromonas hydrophila*	16.2	15.0	11.8	11.3	10.7	8.4
	Aeromonas schubertii					1.8	5.0
	Aeromonas sobria					3.6	1.6
Niesseriaceae	*Chromobacterium violaceum*	5.4	7.4	6.1	5.6	1.8	3.4
Gram-negative non-motile aerobic rods	*Moraxella* spp.	1.8	3.5	2.1	7.6	1.8	1.7
	Acinetobacter calcoaceticus	5.4	5.6	5.9	5.7	7.1	6.7
Gram-negative motile aerobic rods	*Shewanella putrefaciens*	3.5		2.0		1.8	3.3
	Pseudomonas fluorescens	3.5	9.3	6.1	5.7	5.4	5.0
	Cytophaga spp.	8.8	9.3	7.9	11.4		5.0
	Flavobacterium spp.	3.5	2.0		3.7	1.8	1.6
Gram-positive cocci	*Kocuria varians*	5.3	9.4	9.9	7.5	5.3	3.4
	Enterococcus spp.	3.6	5.5	6.1	7.6	5.4	8.3
Gram-positive rods	*Bacillus* spp.	10.6	9.3	18.0	9.3	7.1	6.7
	Corynebacterium spp.	7.2	5.5	9.9	9.5	5.4	8.3
	Arthrobacter simplex	3.6	9.1	5.9	5.7	3.6	3.3
	Nocardia spp.	1.8		4.1	3.8		
	Kurthia gibsonii	1.8	5.5	4.2	5.6	1.8	1.5

*F, Farm; Fc, feeder canal.

bacterial groups were (1) motile Gram-negative aerobic rods (*Pseudomonas, Shewanella, Flavobacterium* and *Cytophaga*), (2) non-motile Gram-negative aerobic rods/coccobacilli (*Acinetobacter, Moraxella*), (3) Neisseriaceae (*Chromobacterium*), (4) Aeromonadaceae (*A. hydrophila, A. veronii* biovar *sobria* and *A. schubertii*), (5) Enterobacteriaceae (*Citrobacter, Enterobacter, Klebsiella*), (6) Gram-positive cocci (*Kocuria, Streptococcus*) and (7) Gram-positive rods (*Bacillus, Corynebacterium, Arthrobacter, Nocardia* and *Kurthia*).

The predominant bacterial groups on the surface as well as in the intestine of prawn were groups 1, 4, 5, 6 and 7. The percentage distribution of these bacterial groups in prawn and farm environment are given in Table 3. Gram-positive cocci and Gram-positive rods accounted for nearly 58% of the microflora in

sediments. Motile and non-motile aerobic Gram-negative bacteria together with Enterobacteriaceae accounted for 60–70% of the mesophiles isolated from water and prawn.

Gram-negative motile rods (*Pseudomonas, Shewanella* and *Flavobacterium*) were found in significant numbers in prawn as well as in the surrounding water in this study. *Aeromonas* represented nearly 16% of the prawn surface and intestine isolates and *A. hydrophila, A. veronii* biovar *sobria* and *A. schubertii* were recovered. Gram-negative bacteria belonging to Enterobacteriaceae (*Enterobacter, Citrobacter* and *Klebsiella*) accounted for 32–39% of the *M. rosenbergii* isolates. In the present study, microorganisms present in significant numbers (*Enterobacte*r spp. *Klebsiella* and *Aeromonas* spp.) in the microflora of prawn surface and intestine were not recovered from the growing waters.

Discussion

There have been few studies of bacteria associated with farmed giant freshwater prawn. The TPC on the surface of farmed freshwater prawn was within the range of 4–5 log CFU g^{-1} and samples yield mean aerobic counts of 4.9. The TPC values observed in the present study were comparable to that reported for farmed freshwater prawn and brackish water shrimp in India (Nayyarahamed, Karunasagar & Karunasagar 1995; Surendran, Thampuran & Gopakumar 1995; Lalitha & Gopakumar 1996; Surendran, Thampuran & Narayanan Nambiar 2000). The bacterial numbers in the intestine of prawn were much higher (>3.5 log) than those in the growing waters. The composition and level of the microorganisms associated with the intestinal tract is related to the environment as well as to the food consumed by fish (Horsely 1973; Austin & Allen-Austin 1985; González, Lòpez, García, Prieto & Otero 1999). It has also been reported that fish intestine provides a favourable ecological niche for some bacteria (Huss 1995; González *et al.* 1999).

The physico-chemical parameters of water fell within the range recommended for the grow-out culture of scampi (Brock 1993).

In the present study, faecal coliforms levels in *M. rosenbergii* were high as previously reported for tiger prawn farms in India (Surendran *et al.* 1995) and Philippines (Reilly, Bernate & Dangala 1984). This is in contrast with the initial microflora of marine shrimp where Enterobacteriaceae are rarely isolated as part of the initial flora. This microbial group is important in foods as indicator of hygienic quality of foods and also as spoilage flora (Gennari, Tomaselli & Cotrona 1999). Since influent water does not contain high numbers of these organisms, incidence of such high numbers of these organisms in prawn may be attributed to the feed or animal manure commonly used to fertilize ponds.

The numbers of enterococci were significantly different between prawn surface and intestine samples and also between water and intestine in the present study. Enterococci were identified as *Enterococcus faecalis* and *E. faecium*. Enterococci are known to cause bactraemias, neonatal and respiratory infections in humans on occasions (Andrew & Mitchell 1997). The presence of enterococci in significant numbers in prawn indicate that rearing practices such as feeding and pond fertilzation could have influenced the microflora. The influence of rearing practices on the microorganisms associated with the intestinal tract of farmed fish has been reported earlier (González *et al.* 1999).

The composition of the bacterial microflora found in *M. rosenbergii* farms is typical of freshwater environments and, as generally recognized, is dominated by Gram-negative bacteria (Austin & Allen-Austin 1985). Motile Gram-negative rods (*Pseudomonas* and *Shewanella*, aeromonads), the most prolific organisms during the ice storage of fish were found in significant numbers. The role of *Pseudomonas* and *Aeromonas* in the spoilage of prawn has been established (Reilly & Dangla 1986; International Commission on Microbiological Specifications for Foods 1998). Motile aeromonads are well-established as a component of the spoilage flora of freshwater fish at refrigeration temperatures (González, Santos, Garcia-Lòpez, González & Otero 2001) and as agents of gastroenteritis and extraintestinal infections (Kirov 1997). Members of the genera *Aeromonas* and *Vibrio* are found associated with black spot necrosis in juveniles and adults of *Macrobrachium* sp. (Lombardi & Labao 1991; Brady & Lasso de la Vega 1992). The presence of *Aeromonas* in *M. rosenbergii* indicates a potential risk to humans and prawn.

In conclusion, the study confirms that farmed freshwater prawn, *M. rosenbergii*, carry significant numbers of faecal coliforms and enterococci. However, influent water does not contain high numbers of these organisms. The rearing practices such as feeding and pond fertilization could have influenced the microflora in prawn. The study reveals the presence of pathogenic bacteria as part of the natural microflora of pond reared prawn. As these organisms

are mesophilic, storage at elevated temperature can permit their survival and growth. The need is thus felt to regulate the bacterial load in the freshwater prawn aquaculture system by adopting farm management practices like regular water exchange and feed regulation in order to safeguard against infectious agents. Farmed *M. rosenbergii* should be washed with clean iced water, maintained at a temperature below 5 °C during sorting and grading and transported with minimum delay to the processing plant to eliminate quality losses during primary handling.

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