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Surveillance and quantitative analyses of common gut bacteria in freshwater prawn *Macrobrachium rosenbergii* fed with Spirulina platensis, *Chlorella vulgaris* and *Azolla pinnata* incorporated feed

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Abstract
The present study was conducted to assess the surveillance of gut bacterial flora in formulated feed fed *Macrobrachium rosenbergii* postlarvae. The feed was prepared by 50% fishmeal replaced with *Spirulina platensis*, *Chlorella vulgaris* and *Azolla pinnata* and these were served as experimental feed. The fishmeal feed served as control. The experiment was prolonged for 90 days. The final day of experiment, the prawns were subjected to analyses the surveillance of bacterial flora in gut environment system. The culture water contain bacterial flora also identified. There were six genera of common gut microflora, *Bacillus* sp. *Pseudomonas* sp. *Escherichia coli*, *Streptococcus* sp. *Proteus* sp. *Salmonella* sp. were identified in culture water and feed fedprawn group, whereas the *Salmonella* sp. was not detected in experimental feed fed prawn groups. There were six genus of common gut microflora (*Bacillus* sp. *Pseudomonas* sp. *Escherichia coli*, *Streptococcus* sp. *Proteus* sp. *Salmonella* sp.) were identified in control feed fed PL, whereas the *Salmonella* sp. was not identified in experimental feed fed PL groups. The present results revealed that, the pathogenic bacteria, *Salmonella* sp. colony could have been eradicated by feeding *C. vulgaris, S. platensis* and *A. pinnata* incorporated feed. The present result suggested that *S. platensis*. *C. vulgaris* and *A. pinnata* can be used as alternative feed ingredients for maintain the healthy gut micro flora system of *Macrobrachium rosenbergii* postlarvae.

Keywords: microalgae, azolla, *Macrobrachium rosenbergii*, gut micro flora

Introduction
Giant freshwater prawn (*Macrobrachium rosenbergii*isan important commercial species due to property as food supply as well as a valuable export product. In India, giant freshwater prawn distributes mainly in the Southern region where environmental conditions are most favorable for the Giant freshwater prawn *M.rosenbergii* is an important commercial species due to property as food supply as well as a growth of scampi. Increasing demand of this species for domestic consumption and export markets has increased remarkably scampi cultured systems with large scale, high stocking density and intensive feeding [1].

The gut serves as the natural habitat for innumerable bacteria, some are beneficial to the host and others are harmful. The primary function of the gut is to take up water and nutrients. The specific role of resident colonic micro biota in digestion is to ferment the substances in the diet (e.g. dietary fiber) which cannot be digested by host in the small intestine. The gut microbiota of aquatic invertebrates highlights the questions and processes that merit acquisition of an understanding the role of gut microbes in the host invertebrates physiology and nutrient dynamics of aquatic systems [2].

The biodiversity and *insitu* abundance of the gut microbiota of abalone (*Haliotis discushannai*) as determined by culture-independent techniques have been investigated [3-4].The gut microbiota composition and the non-specific humoral and cellular immune responses in rainbow trout *Oncorhynchus mykiss* have been studied [5]. The latter category includes Clostridia, sulphate reducers and proteolytic bacteria, which are responsible for toxins production, which cause diarrhea, mucosal invasion and carcinogenesis. *Saccharolytic* species, primarily Benifid bacteria and *Lactobacilli*, are the main health promoting bacteria and are thought to be important barriers to disease [6]. The amylase-producing ability of intestinal bacteria in mangrove crab and seven fish species has been determined [7]. *Bacillus*, *Coryneforms*, *Enterobacteriaceae*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Vibrio* spp., were found in the intestinal tracts of Japanese flounder [8]. Fresh water prawn culture is rapidly expanding and *M. rosenbergii*is an important species cultured in many countries. Production of healthy and quality seeds has been a major obstacle in the expansion of the culture of *M. rosenbergii*. A complex mix of environmental factors, microbiological profiles and management practices influence the success of the production cycle [1].The aim of this study was to determine the surveillance of gut bacteria in *M. rosenbergii* PL fed with *Spirulinaplatensis*, *Chlorella vulgaris* and *Azolla pinnata* incorporated feeds.

Materials and Methods
Feeding experiment
Macrobrachium rosenbergii (PL-30) with the length and weight range of 1.56±0.29cm and 0.22± 0.039grespectively were used for feeding experiment. Thirty PL for each diet was maintained in plastic tanks with 40 L water. One group fed with fish meal diet, it was served as control. The experimental groups were fed with the respective concentration of 50% of fishmeal (FM) replaced with 50% of *S. platensis*, *C. vulgaris* and *A. pinnata* incorporated diets (Table 1). The feeding was adjusted to two times a day (6:00 am and 6:00 pm). The daily

ration was given at the rate of 10% of the body weight of PL with two equal half throughout the experimental period. The feeding experiment was prolonged for 90 days; mild aeration was given continuously in order to maintain the optimal oxygen level.

Table 1: Ingredients and proximate composition of experimental diets.

Ingredients (g/100g)	Control (BI+FM)	Diet-1 *S. platensis*	Diet-2 *C. vulgaris*	Diet-3 *A. pinnata*
Fishmeal	25	12.5	12.5	12.5
Groundnut oil cake	25	25	25	25
Soybean meal	25	25	25	25
Wheat bran	10	10	10	10
Egg albumin	7	7	7	7
Tapioca flour	5	5	5	5
Sunflower oil	2	2	2	2
Vitamin mix*	1	1	1	1
Spirulina/ Chlorella/ Azolla	0	12.5	12.5	12.5
Total	100	100	100	100
Proximate composition				
Protein	42.02	41.38	40.93	37.48
Carbohydrate	20.48	21.30	20.88	23.64
Lipid	13.70	13.46	13.41	12.30
Ash	11.86	13.30	13.00	12.40
Moisture	9.93	8.63	8.73	8.46
Gross energy (k.cal/kg-1)	2713.09	2850.46	3187.71	2781.77

BI – Basal ingredients; FM – Fishmeal

*BECOSULES CAPSULES (Each capsule contains):

Thiamine Mononitrate (IP):10mg; Riboflavin (IP):10mg; Pyridoxine Hydrochloride (IP): 3mg; Vitamin B_{12} (as tablets 1:100) (IP): 15mcg; Niacinamide (IP):100mg; Calcium pantothenate (IP): 50mg; Folic acid (IP): 1.5mg; Biotin USP (IP): 100mcg; Ascorbic acid (IP): (150mg)

Isolation of Gut Microbes andIdentification the colonies

At the end of feeding experiment, the prawns were anaesthetized in an ice bath for 5-10 min and the surface of each prawn was sterilized by immersion for 30 seconds in 70% ethanol. The prawn's intestine was dissected out carefully by incision on the dorsal side of the abdominal portion. The collected intestine samples were stored individually in sterile double strength phosphate buffered saline (PBS) solution (Disodium phosphate (2.3% w/v); sodium phosphate (0.6% w/v) and sodium chloride (1.2% w/v). The intestine samples were individually homogenized by gentle tapping with mortar and pestle upto 2 min. Further the homogenates were subjected to microbiological culture techniques in Bergey's manual [9].The isolated bacterial strains were identified by the following tests: Simple staining, Gram's staining, Spore staining, Motility test, Indole production test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Urea hydrolysis, Kovac's oxidase test, Cysteine desulfurase (H_2S production) test, Carbohydrate fermentation test, Nitrate reduction test, Starch hydrolysis test, Casein hydrolysis test, Gelatin hydrolysis test and Catalase test[9].

Enumeration of bacterial load in the intestine of experimental prawn

Each group of experiment feed fed *M. rosenbergii* PL were collected kept for freezing at -20ºC for 10 min to deactivate. The freeze and deactivate prawnsouter surface were sterilized by using 50 ppm formalin for 30 sec in order to remove the external microflora. The sterilized prawns digestive tracts were dissected out individually and homogenized with phosphate buffered saline (pH 7.2) under aseptic condition. The homogenate were serially diluted up to 10^{-5} dilution individually. From each dilutes 0.5 ml of aliquots were taken and seeded over the surface of freshly prepared nutrient agar plates. The plates were incubated at 37ºC for 24 h.

$$\text{Bacterial count (CFU/g)} = \frac{\text{Number of colonies} \times \text{dilution factor}}{\text{Volume of sample (g)}}$$

Results

The presences of common gut microflora in PL fed with 50% FM replaced experimental feeds are presented in Table 2. There were six genera of common gut microflora (*Bacillus* sp. *Pseudomonas* sp. *Escherichia coli*, *Streptococcus* sp. *Proteus* sp. *Salmonella* sp.) were identified in control feed fed PL (12×10^{-5}), whereas the *Salmonella* sp. was not identified in experimental feed fed PL groups (10×10^{-5}). But the culture water contained this genus of bacteria (7.6×10^{-5}). The percentage composition of isolated bacteria in experimental PL gut in 29.43 to 35.34% of *Bacillus* sp., 9.00-13.68% of *Pseudomonas* sp., 11.22-19.11% of *Escherichia coli*, 5.45-13.45% of *Streptococcus* sp.and 12.14-18.71% of *Proteus* sp. were identified. The *Salmonella* sp.was identified in the gut of control feed fed group showed 15.21%. Also, the culture water contained *Bacillus* sp.(20.45%), *Pseudomonas*sp. (11.00%), *Escherichia coli*, (24.25%), *Streptococcus* sp.(15.44%), *Proteus* sp. (10.21%)and *Salmonella* sp.(15.21%) were identified. The isolated bacterial genera were identified by the various biochemical methods (Table 3 & 4).

Table 2: Percentage composition and total viable count of bacterial flora in culture water, and gut of formulated feed fed *M. rosenbergii* PL (Dilution 10^{-5})

Sl. No	Genera	Percentage composition of Bacteria				
		Culture water	**Control PL**	***S. platensis* PL**	***C. vulgaris* PL**	***A. pinnata* PL**
1.	*Bacillus* sp	20.45	29.43	32.35	35.34	30.55
2.	*Pseudomonas*sp	11.00	13.68	10.42	9.00	11.25
3.	*Escherichia coli*	24.25	18.25	15.21	19.11	11.22
4.	*Streptococcus* sp	15.44	13.45	5.45	8.21	8.20
5.	*Proteus* sp	10.21	15.11	20.21	12.14	18.71
6.	*Salmonella* sp	15.21	5.68	NF	NF	NF
Total %		95.60%	89.60%	83.64%	83.80%	79.93%
Total Viable count CFU/g		7.6×10^{-5}	12×10^{-5}	10×10^{-5}	9×10^{-5}	10×10^{-5}

NF – Not found

Table 3: Biochemical characterization of control water

S. No	Tests	***Bacillus* sp.**	***Pseudomonas* sp.**	***E. coli***	***Streptococcus* sp.**	***Proteus* sp.**	***Salmonella* sp.**
1.	Gram's staining	+	-	-	+	-	+
2.	Motility test	+	+	+	+	+	+
3.	Indole test	-	-	+	-	-	-
4.	Methyl red test	-	-	+	-	+	-
5.	VP test	-	+	-	+	-	+
6.	Citrate Utilization test	+	+	-	+	+	+
7.	Starch hydrolases	+	-	+	+	-	+
8.	Gelatin Hydrolases	+	+	+	+	+	+
9.	Nitrate reduction test	+	-	+	+	+	+
10.	Oxidase test	-	+	+	-	-	-
11.	Catalase test	+	+	-	-	-	-
12.	Glucose test	A	A	A	A	A	A
13.	Lactose test	A	NA	A	A	NA	A
14.	Sucrose Test	A	A	A	A	A	A
15.	Manitol test	A	A	A	A	NA	A

+ Positive; - Negative; A-Acid Production; NA-No Gas production; W- Weak; G-Gas production.

Table 4: Biochemical characterization of experiment feed fed *M. rosenbergii* PL gut.

Tests	***Bacillus* sp.**				***Pseudomonas* sp**				***E. coli***				***Streptococcus* sp.**				***Proteus* sp.**				***Salmonella* sp.**			
	Groups				Groups				Groups				Groups				Groups				Groups			
	C	**SP**	**CV**	**AP**	**C**	**SP**	**CV**	**AP**	**C**	**SP**	**CV**	**AP**	**C**	**SP**	**CV**	**AP**	**C**	**SP**	**CV**	**AP**	**C**	**SP**	**CV**	**AP**
Gram's staining	+	+	+	+	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
Motility test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Indole test	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Methyl red test	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-
VP test	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
Citrate Utilization test	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-
Starch hydrolases	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-
Gelatin Hydrolases	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Nitrate reduction test	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Oxidase test	-	-	-	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Glucose test	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	-	-	-
Lactose test	A	A	A	A	NA	NA	NA	NA	A	A	A	A	A	A	A	A	NA	NA	NA	NA	NA	-	-	-
Sucrose Test	A	A	A	A	A	A	A	A	A		A	A	A	A	A	A	A	A	A	A	NA	-	-	-
Manitol test	A		A	A	A	A	A	A	A		A	A	A	A	A	A	NA	NA	NA	NA	A	-	-	-

Groups; C- Control; SP- *Spirulina platensis*; CV – *Chlorella vulgaris*; AP – *Azolla pinnata*

+ Positive; - Negative; A-Acid Production; NA-No Gas production; W- Weak; G-Gas production.

Discussion

Micro algae have been shown to positive effect son fish larvae during first feeding, and this has been attributed improvement of the nutritional quality of the live feed [10], and to microbial factors [11]. Addition of algae to the water in fish tanks alters the composition of the bacterial flora associated with larvae [12] and bacterial growth and composition of the flora in the water depends on both the algal species and state of growth of the algae [13]. How microalgae affect the bacterial flora of live feed animals has so far not been studied. The earlier hypothesized that the microalgae contribute to a change in the bacterial composition of live feed organisms, and also reduce

numbers, by expelling the gut contents which act as a substrate for bacterial proliferation and algae can possibly affect the bacterial community in the live feed by production of antibacterial substances as reported for *Tetraselmis* [14-17]. A comprehensive knowledge of the qualitative and quantitative aspects of the micro flora in water and those associated with larvae is a prerequisite for developing a strategy for microbial control [18].

In the present study, genus *Bacillus* sp. were identified in all PL gut and culture water. The experimental feeds fed groups especially microalgae *S. platensis* and *C. vulgaris* incorporated feed fed group showed higher percentage of composition in *Bacillus* sp. The Gram-positive bacteria, particularly members of the genus *Bacillus*, do secrete a wide range of exo-enzymes [19]. The presence of the probiont may in some way stimulate endogenous enzymes produced by the shrimp [20]. The *Bacillus* genus has not been reported as pathogens of the aquatic organisms [19], its application has been promoted and more widely accepted within the aquaculture industry [21]. *Bacillus* sp. are able to produce antibiotics, amino acids and enzymes [22]. Consequently, *Bacillus* probiotics may have positive nutritional effects on fish [23]. Therefore, administered *Bacillus* gave rise to the fry resistance to pathogens and enhanced survival by producing inhibitory substances to other microorganisms. The *Bacillus* species produce proteases (for example, subtilin), which helps in digestion [22]. They are also said to produce vitamin K and B_{12} [24]. Gram-positive bacteria, including members of the genus *Bacillus* might have supplied digestive enzymes and certain essential nutrients to promote better growth. *Bacillus subtilis* and *B. leicheniformis* can break down proteins and carbohydrates [25]. So it can be suggested that administration of *Bacillus* bacteria to trout fry results in enhanced digestion of food and improved growth, including low food conversion ratio (FCR), and high specific growth rate (SGR). High protein efficiency ratio (PER) as well as greater protein values of carcass in probiotic treatments may be due to proteins secreted by members of genus *Bacillus* [24].

In the present study, few common bacterial colonies also determined in the culture water and gut of experimental feed fed prawns. There are Pseudomonassp. *Escherichia coli* and *Proteus* sp. Similarly, *Pseudomonas* sp., and *E. coli* were reported in hatchery cultured *M. rosenbergii* [1]. The presentation of genera *Pseudomonas* was previously reported in *M. rosenbergii* [26-27] in *P. indicus* [28] larval cultures. Genes *Pseudomonas, E. coli* and *Proteus* bacteria were also isolated in the gut of *M. rosenbergii* and culture system [29]. Some dominant diverse bacterial flora like, *Aeromonas, Plesiomonas, Photo bacterium, Pseudo alteromonas, Pseudomonas* and *Vibrio* presentation were reported in wild and cultured prawns *F. Merguiensis* [30]. The similarity existing in the intestine bacterial flora of cultured prawns suggests the host specificity of intestinal microbial colonization. An understanding of the host intestinal bacterial floral interactions is of much significance for the development of a healthy cultivation environment and also to optimize the potential species growth [31].

In the present study, *Salmonella* sp. was isolated in the culture water and control PL gut. *Salmonella* sp. is a pathogenic bacteria and itis an indicator microorganism of adulteration and fecal pollution which are not indigenous to the aquatic environments [32-34]. It is commonly found in food products and water samples [35]. Products such as fish meal, meat, bone meal, maize and soy products may be contaminated with this bacterium at high prevalence [36]. In the present study, the *Salmonella* sp. was eradicate in the fishmeal replacement with *S. platensis*, *C. vulgaris* and *A. pinnata* incorporated feed fed PL gut.A heterotrophically grown, spray-dried unicellular algae, *Tetrasel missuecica*, has been used as a feed for penaeids and as a feed-additive for salmonids with data revealing a reduction in the level of bacterial diseases. It was suggested that the mode of action may have reflected the presence of unspecified antimicrobial compounds in the algal cells [16-17].

Conclusion

There were six genus of common gut micro flora (*Bacillus* sp. *Pseudomonas* sp. *Escherichia coli*, *Streptococcus* sp. *Proteus* sp. *Salmonella* sp.) were identified in control feed fed PL, whereas the *Salmonella* sp. was not identified in experimental feed fed PL groups. Therefore, it is presumed that, the pathogenic bacteria, *Salmonella* sp. colony could have been eradicated by feeding *C. vulgaris, S. platensis* and *A. pinnata*. Hence, the harmful bacterial load was reduced in the gut of experimental PL groups. The revealed result was suggesting that *S. platensis*. *C. vulgaris* and *A. pinnata* can be used to feed formulation for *Macrobrachium* culture. It can be beneficial to maintain the gut micro flora system of *M. rosenbergii* post larvae.

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