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ALTERATIONS IN THE MICROBIAL LOAD AT CERTAIN NON-SPECIFIC IMMUNE SITES OF *MACROBRACHIUM ROSENBERGII* SUPPLEMENTED WITH *CENTELLA ASIATICA*

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ABSTRACT
Macrobrachium rosenbergii has been the focus of research in India in the past few years. As a negative impact to the success of aquaculture due to intensification lead to higher disease outbreaks. The bacterial diseases are the most common due to intensification. In the present study the *Macrobrachium rosenbergii* fed with diet containing medicinal plant, *Centella asiatica* to test the antimicrobial activity. The prawns were fed with diet containing *C.asiatica* (0.2%). After 4 months both control and the experimental prawns were examined for microbial flora. Isolation and identification were also done. The investigation showed a significant reduction in the pathogenic bacteria and also found an improvement in the probiotic bacteria in prawns fed with experimental diet containing *C.asiatica* than the control diet. The % survival is 75% in medicated diet, but it is only 40% in control diet. The medicated diet also showed improved growth parameters. The total microbial load at non-specific immune sites, such as gill, gut and exoskeleton of both control and medicated feed were examined after the experiment. The total microbial load at gill, gut,exoskeleton were 41.00×10^5,50.00×10^6,30.00×10^6 respectively in control feed, but total microbial load at gill, gut, exoskeleton were 34.00×10^5, 28.00×10^6, 22.00×10^6 respectively in medicated feed. Major pathogenic bacteria found in culture were *Vibrio type I, Staphylococcus type III, Micrococcus type I, Strepto coccus I, Acinetobacter type I, Acinetobacter typeIII, Arthrobacter type I, Enterobacteriacea, Flavobacterium Vibrio type II, Strepto coccus II, Pseudomonas*. After treating with medicated diet, diversity and intensity of microbial flora get reduced and culture of medicated diet also showed presence of probiotic bacteria such as *Bacillus*.

INTRODUCTION

Crustaceans continue to constitute a major component of the global shellfish culture where shrimp farming has been the focus of the aquaculture industry over the years. Concomitant with the high production in farming systems unpresedent diseases outbreaks has had a limiting effect on aquaculture production worldwide. Therefore, sustainable disease management practices have to be integral part of all farming systems. (Fred and Meyer, 1991) .To combat these diseases, widespread use of broad- spectrum chemotherapeutics had led to drug resistance and biomagnifications problems in aquaculture sector. Therefore understanding factors that control pathogen virulence and how management of environmental parameters can influence them may make it possible to develop alternative disease control measures (Romero *et al.,* 2012).

Aeromonas salmonicid, Vibrio anguillarum Saprolegnia spp V. salmonicida, Renibacterium salmoninarum, are some of the bacterial pathogens causing serious threat to aquaculture (Torgersen and Hastein, 1995).

Substances that allow an animal to react in a non- specific manner against a variety of pathogens are being commercialized for use in aquaculture .These substances generally called as immunostimualnts are obtained from diverse natural sources and many are chemically synthesized (Sakai,1999).

Immunostimulants and non-specific immune- enhancers are being incorporated into diets to provide added protection to the animals, even though our knowledge of shrimp immunity is limited at present, The large number of commercial "immunostimulants" available on the market reflects the interest of the industry in broadening the scope of tools available to manage shrimp diseases. The cell wall preparations that are made from bacteria, fungus and yeast include polysaccharides, lipopolysaccharides and lipopeptides. Phorbol 12-myristate 13-acetate (PMA), a surface-active agent, is used as an immunostimulant. Dietary components such as astaxanthin, ascorbic acid-polyphosphate and HUFA are also used as immuno stimulants. Information is inadequate on the extract mode of these compounds (Jan Raa. 1996)

Understanding antiviral, antimicrobial, immunomodulatory efficacies of plant Antiviral research using plant extracts has gained momentum since a couple of decades. Scores of medicinal herbs have already been tested and used with good results in the control of viral and bacterial diseases in shrimp and fish (Citarasu *et al.,* 2006). Probiotics are defined as a live microbial adjunct which has a beneficial effect on the host by modifying the host- associated or ambient microbial community, by ensuring improved use of the feed or enhancing

its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment (Verschuere, 2000). Bacterial species such as Bacillus considered as good probiotic in aquaculture. In a work done by Nejad *et al.,* 2006, probiotic was administered during both the hatchery stages ($Nauplius_{1-2}$ through PL_{30}) and the farming stages, the feed conversion ratio, specific growth rate, and final production were slightly, but significantly ($P < 0.05$), higher in shrimp receiving the probiotic than in control shrimp which had received no probiotic. Because these improvements in growth parameters in postlarval shrimp were significant only in shrimp that had received the probiotic both during hatchery stages and during farming stages, it appears to be important for the shrimp to receive the probiotic in all ontogenetic stages in order for these improvements to be realized. Probiotics, as 'bio-friendly agents' such as lactic acid bacteria and *Bacillus spp*., can be introduced into the culture environment to control and compete with pathogenic bacteria as well as to promote the growth of the cultured organisms. In addition, probiotics are nonpathogenic and nontoxic microorganisms without undesirable side-effects when administered to aquatic organisms. These strains of bacteria have many other positive effects (Farzanfar A, 2006).

Natural plant product from *Ocimum sanctum* (Tulsi), *Phyllanthus emblica* (Amla), *Azadirachta indica* (Neem), *Solanum trilobatum* (Purple Fruited Pea Eggplant*), Eclipta alba* (Bhringraj), *A. hydrophila, Zingiber officinale* (Ginger), *Echinacea* (purple coneflowers) and *Allium sativum* (garlic) promote various activities such as Antistress, Growth promotion, Appetite stimulation, Immunostimulation, Aphrodisiac and Antimicrobial properties due to the active principles such as alkaloids, flavanoids pigments, phenolics, terpenoids, steroids and essential oils (Bairwa, 2012)

In the present study *M, rosenbergii* is treated with diet incorporated with extract of medicinal plant *Centella asiatica* and intended to test its antimicrobial potential. The microbial load and characterization of microbes at the gut, gill and exoskeleton were studied. Growth parameters were also studied.

MATERIALS AND METHODS

Preparation of crude methanolic extraction of *Centella aisatica*

The crude methanol extract of *Centella aisatica* was obtained by extracting 50g of dried plant powder in 100mL 80% methanol and kept on a rotary shaker for 24h. The extract was vacuum filtered and dried in an oven at 40 ºC. The extract was stored at 4ºC in airtight bottles (Thomas *et al.,* 1994).

Feed preparation and incorporation with methanolic extract

The powered ingredients were mixed well into dough with 100mL water. This was steamed for 10 minutes in an autoclave and palletized using a Pelletiser. Pellets were dried in an oven at 50ºC for 18h. The pellets were broken into pieces. The medicated feeds were prepared by are incorporating methanolic extract of *Centella asiatica* at 0.2% concentration. The feed is also checked for water stability. Feeds were stored in airtight polythene bags at 20ºC in freezer for further use.

Experimental protocol

Prawns were purchased from ADAK (Agency for Development of Aquaculture in Kerala, Varkala.) and acclimated in lab conditions for 3 weeks. Proper water quality and aeration were provided. Experiments were carried out in glass tanks of 1000L capacity with proper aeration. 50% of water and feacal matter removed daily. Two sets of experiment were done in triplicates with 15 prawns per each tank. The prawns were fed twice daily with medicated and control feeds at the rate of 10 % of the body weight. After 2 and 4 months the total microbial load and genus diversity at certain non-specific immune sites-gut, gill, exoskeleton of prawns of both control and medicated culture were done (Kannan, 2002, Buck *et al*., 1963; Akagawa-Matsushita *et al.,* 1992; Holtt *et al.,* 1994; Webster, 1995). Growth (Uma, 1999) and water quality (Trivedi and Goel, 1984) parameters were also done.

RESULTS

The results obtained as per the experimental design given is presented in Tables1-4.The total body weight gain of prawns fed with control feed is 0.86g and it in medicated feed is about 1.13g. The total length gain in control feed is 2.17cm and that in the medicated feed is about 2.74cm. The control prawns showed a SGR of 1.80% while the same for treated prawns has 2.28%.

The total microbial load of the water in culture tanks which has been considerably varied during the 2^{nd} and 4^{th} month sampling. The values (CFU) of control tank being 50.00×10^5 and 64.00×10^5, the same for treatment tanks was 38.00×10^5 and 42.00×10^5 for 2^{nd} and 4^{th} months respectively.

The microbial load at the gill, gut and exoskeleton of *M.rosenbergii* were examined at the two terminations. After two months, the Total microbial load at gill is 32.00×10^5

CFU in control feed and 29.00×10^5 CFU in medicated diet. The Total microbial load at gut is 30.00×10^6 CFU in control feed and 18.00×10^6

CFU in medicated diet. The Total microbial load at exoskeleton is 18.00×10^6 CFU in control feed and 15.00×10^6CFU in medicated diet. After four months, the Total microbial load at gill is 41.00×10^5CFU in control feed and 34.00×10^5

CFU in medicated diet. The Total microbial load at gut is 50.00×10^6

CFU in control feed and 28.00×10^6 CFU in medicated diet. The Total microbial load at exoskeleton is 30.00×10^6CFU in control feed and 22.00×10^6CFU in medicated diet. Among these tissues, gut microbial load expressed (CFU/gm) a sharp fall from

30.00×10^6 to 18.00×10^6 and from 50.00×10^6 to 28.00×10^6 at 2^{nd} and 4^{th} terminations respectively.

The major bacterial diversity at the gut, gill and exoskeleton of *Macrobrachium rosenbergii* were examined at the final termination. Marked alterations were observed among the control and treated experiments.

Enterobacteriacea, Bacillus type I, Vibrio type I, Staphylococcustype III, Micrococcus type I, Strepto coccus I, Acinetobacter type I, Acinetobacter typeIII, Arthrobacter

type I, Flavobacterium, Bacillus tupe III, Vibrio type II, Strepto coccus II, Pseudomonas are the major bacterial flora found in the culture. among these The *Acinetobacter typeI*, is varied from 20 to 66 in gill, 80 to 44 in gut and 200 to 157 in the exoskeleton of control and treated tissue samples respectively. The same for Pseudomonas species was from 2 to 3 in gill and 3 to 4 in gut and 4 to 5 in exoskeleton of the control and treated tissue samples respectively. *Vibriop type I* and *type II* recorded a remarkable drop. The same was for *Flavobacterium, Streptococcus I, II, Arthrobacter type I, Micrococcus I*.

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Table 1: The growth parameters of *Macrobrachium rosenbergii* over the treatment schedule

Sample	% Survival	Weight gain(g)	Length gain(cm)	SGR (%)
1. Control feed	40	0.86	2.17	1.8
2. Medicated feed	75	1.13	2.74	2.28

Table 2: Total microbial load of water in culture tanks during the course of time

Sample	Total Plate count(CFU/mL)	
	2 months	4 months
1.Control	50.00×10^5	64.00×10^5
2.Medicated	38.00×10^5	42.00×10^5

Table 3: Total microbial load at non-specific immune sites such as gut, gill and exoskeleton of *Macrobrachium rosenbergii*

sample	Termination time	Total Plate count(CFU/gm)	
		Control	Treatment.
1.Gill		32.00×10^5	29.00×10^5
2.Gut	2 months	30.00×10^6	18.00×10^6
3.Exoskeleton		18.00×10^6	15.00×10^6
1.Gill		41.00×10^5	34.00×10^5
2.Gut	4 months	50.00×10^6	28.00×10^6
3.Exoskeleton		30.00×10^6	22.00×10^6

DISCUSSION

In *Macrobrahium rosenbergii* the main bacterial flora associated is gram-negative, comprising more than 75% of the total isolated strains. *Aeromonas, Alcaligenes* and *Pseudomonas* were the most frequently encounter genera in water. whereas *Alcaligenes, Enterobacteriacea, Pesudomonas* and *Streptococcus* were the most abundant strains associated with larvae were detected in eggs and water but were conspicuously absent in larvae.

Uddin and Al-Harbi, 2005 done Quantitative and qualitative analyses of bacterial flora associated with the digestive tract of the giant freshwater prawn *Macrobrachium rosenbergii* cultured in earthen ponds of Saudi Arabia were carried out. The bacterial flora was predominantly gram-negative, accounting for 80% of total isolated strains. Altogether, 21 bacterial species of 16 genera were identified. *Aeromonas hydrophila*, *Shewanella putrefaciens*, other *Aeromonas* spp., *Vibrio* spp. and *Enterococcus* spp. were the most abundant bacterial species (prevalencee"10%) in pond water; *S. putrefaciens*, *A. hydrophila*, *Vibrio* spp., *Enterococcus* spp. and *Aeromonas* spp. were the most abundant on the prawn carapace and *A. hydrophila*, *S. putrefaciens*, *Vibrio* spp., *Enterococcus* spp. and *Pasteurella* spp. were the most abundant in the digestive tracts. In every population studied, *Aeromonas* spp., *S. putrefaciens*, *Enterococcus* spp., *Vibrio* spp., *Pasteurella* spp., *Chryseomonas* spp. and *Pseudomonas* spp. were present.

Probiotic bacteria offer a number of benefits to the host produce digestive enzymes and essential growth nutrients such as vitamins and aminoacids, which are beneficial for enhancing better growth, also they could benefit to their invertebrates host by competitive exclusion against pathogens. (Balasubramanian *et al.,* 2006).

Probiotic in aquaculture industry were shown to improve intestinal microbial balance and also to improve feed absorption, thus leading to increased growth rate(Parker,1974) and also reduced feed conversion ratio(FCR) during the cultural period (Wang, 2005).

An experiment was carried out to study the antimicrobial activity of petroleum ether, ethanol and water extract of

Table 4: Major bacterial diversity at non-specific immune sites such as gut, gill and exoskeleton of *Macrobrachium rosenbergii*

Isolates(gm/tissue)	Gill		Gut		Exoskeleton	
	control	Medicated	control	Medicated	control	medicated
Bacillus type I	1	1	2	-	-	-
Vibrio type I	4	2	3	-	-	-
Staphylococcustype III	6	2	18	2	-	-
Micrococcus type I	10	2	20	7	-	-
Strepto coccus I	11	1	18	-	19	-
Acinetobacter type I	20	66	80	44	200	157
Acinetobacter typeIII	1	-	1	-	2	-
Arthrobacter type I	8	-	10	2	15	-
Flavobacterium	11	-	20	1	42	-
Bacillus tupe III	-	-	-	-	-	18
Vibrio type II	10	-	10	-	12	1
Strepto coccus II	10	-	11	-	41	2
Pseudomonas	2	3	3	4	4	5
Enterobacteriacea	3	-	12	4	1	-

Centella asiatica plant by agar diffusion method. Zone of inhibition produced by petroleum ether, ethanol and water extract in dose of 62.5, 125, 250, 500 and 1000 μg/mL against some selected strains was measured and compared with standard antibiotics ciprofloxacin (10μg/mL). In a work (Wei, 2008) , *C. asiatica* whole plant methanol extract was found inhibited *Streptococcus* sp, *V. alginolyticus* and *V. vulnificus*, while its aqueousextract showed effect against *C. freundii*, *V. alginolyticus*, *V. cholerae*, *V. harveyi* and *V. parahaemolyticus*. The present study demonstrated that the methanolic extract of Centella asiatica has higher antimicrobial activity than petroleum ether and water extract (Jagtap, 2009).

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