

**EVALUATION OF *BACILLUS LICHENIFORMIS* AS
PROBIOTIC TO GIANT FRESHWATER PRAWN,
MACROBRACHIUM ROSENBERGII (DE MAN)**

Dissertation submitted in partial fulfillment
of the requirements
for the degree of

M. F. Sc. (Fish Pathology and Microbiology)

by

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DECLARATION

I, hereby, declare that the thesis entitled “**EVALUATION OF *BACILLUS LICHENIFORMIS* AS PROBIOTIC TO GIANT FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII* (DE MAN)**” is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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ABSTRACT

An attempt has been made to assess the probiotic potential of *Bacillus licheniformis*, a Gram-positive, aerobic, spore forming bacterium in *Macrobrachium rosenbergii* juveniles. Six hundred and forty five numbers of inter-moult juveniles of *M. rosenbergii* were randomly distributed into five distinct experimental groups with three replicates each for three experimental trials. A total of five experimental diets were prepared, with four probiotics enriched diets having different concentrations of *B. licheniformis*, and a control. The diets were as follows: control (Basal diet), T₁ (1.0×10^6 cfu g⁻¹), T₂ (1.0×10^7 cfu g⁻¹), T₃ (1.0×10^8 cfu g⁻¹) and T₄ (1.0×10^9 cfu g⁻¹). The gastro-intestinal colonization by *B. licheniformis* was monitored fortnightly. The higher colonization was observed in T₂, T₃ and T₄ respectively. *B. licheniformis* administered to *M. rosenbergii* through diets inhibited pathogenic *Vibrio* sp., Enterobacteriaceae, *Aeromonas* and *Pseudomonas* by means of competitive exclusion. Effect of *B. licheniformis* on immunological responses was studied. There was significant increase in Total Haemocyte Count (THC), Phenol oxidase (PO) activity, Superoxide dismutase (SOD) activity, Phosphatase activities, Total proteins, Glucose, Antibacterial and Bacteriolytic activities, Bacterial agglutination titre and Urea content in haemolymph, while there was no significant difference in Respiratory burst activity of haemolymph after 45 days of feeding. After challenge with *Vibrio alginolyticus*, the lowest cumulative mortality was recorded in T₄ fed group, whereas the highest mortality was recorded in control. There was significant increase in the clearance efficiency of haemolymph of *M. rosenbergii* fed with probiotic diet when challenged with *V. alginolyticus*. Histological studies regarding hepatopancreas of *M. rosenbergii* after feeding with *B. licheniformis* confirmed that probiotic bacteria had no adverse effect on the health of host. When infected with *V. alginolyticus*, histopathological studies revealed that *M. rosenbergii* fed with probiotic diet had less tissue level changes compared to control diet fed uninfected prawns. Weight gain percentage, specific growth rate, feed conversion ratio and feed efficiency ratio were observed better in the *B. licheniformis* supplemented groups than control. The survival was significantly high ($p < 0.05$) in all *B. licheniformis* treated groups compared to the control. Based on the result of the present study, it was found that inclusion of *B. licheniformis* in the diets of *M. rosenbergii* helped in its better colonization in the gut, enhanced the immune responses and provided higher protection against bacterial infection.

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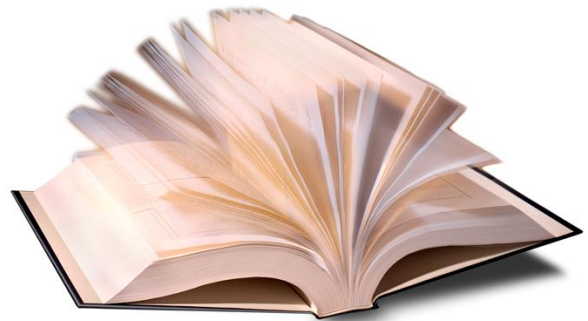
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Introduction

1. INTRODUCTION

Aquaculture has been the fastest growing food production system during the last three decades. Its significance and contribution towards agricultural and national economies, livelihood and nutritional security, employment generation and foreign exchange earnings have been enormous. The sector has increasingly been recognized as a powerful tool for poverty reduction through creating sustainable rural livelihoods. Their significance and contribution towards agricultural (4.6 Per cent GDP) and national economy (1.3 per cent GDP), livelihood and nutritional security, employment generation (11 million people) and foreign exchange earnings (Rs.7, 245 crores) have been enormous though understated so far.

India is currently the third largest fish producer and second largest aquaculture producer in the world. Crustaceans contribute substantially to the total aquaculture production. Large number of commercially important finfish and shellfish are under culture practice in freshwater, brackishwater and seawater. Among the freshwater crustacean species, farming of freshwater prawns is getting immense popularity world over. Commercially important prawns come under genus *Macrobrachium*, which comprises of more than 150 species (Brown, 1991). About 25 species of *Macrobrachium* are listed from the inland waters (Dwivedi *et al.*, 1984). Among them, *M. rosenbergii* and *M. malcomsonii* are suitable for freshwater aquaculture.

The giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879) is an omnivorous crustacean of the family Palaemonidae and it is a natural inhabitant of rivers and estuaries throughout the Indo-Pacific region. It has been one of the most desirable candidate species for freshwater aquaculture in different parts of tropical and subtropical belts and has got tremendous cultural importance due to its fast growth, high market demand and tolerance to environmental condition (Ranjeet and Kurup 2002; New, 1995). Giant freshwater prawn farming is a major contributor to global aquaculture, both in terms of quantity and value. Way back in 1993, since when its cultivation picked up, its overall production was 17,164 tonnes, worth US\$ 113,999,000 which in 2007 zoomed to 213,274 tonnes with a net value of

US\$ 943,612,000 (FAO, 2008). In the last decade, the average *M. rosenbergii* production increased by 9.0 to 35.48% in quantity, and 19.68 to 24.5% in value (Wowor and Ng, 2007). Among freshwater prawns, *M. rosenbergii* is the most economically important species and it is cultured in different regions of India. India is the third largest producer of freshwater prawn in world (New, 2005). The total annual production of freshwater prawn (*M. rosenbergii* and *M. malcomsonii*) in India increased from 7,140 MT to 42,780 MT (5.9 fold increase) and an increase in the corresponding culture area from 12,022 ha to 34,630 ha (3.6 fold increase) from 1999-2000 to 2005-2006 period, respectively (MPEDA, 2006). The area devoted to its culture in India has increased tremendously due to the high prevalence of various disease problems in penaeid shrimp culture. India has the potential to produce 50,000 MT of freshwater prawn per year by 2010 (Selvraj and Kumar, 2003).

Presently, the biggest problem faced by the aquaculture industry worldwide, is diseases and epizootics caused due to various biotic and abiotic factors. The outbreak of disease involves a series of interactions of host, pathogen and environment (Moriarty, 1996). When faced with disease problems, the common response of most aqua-farmers has been to turn towards antimicrobial drugs. Consequently, the livestock and aquaculture industries have faced widespread use (and abuse) of antibiotics and other antimicrobial drugs. The massive use of antibiotics and other antimicrobial compounds for disease control in animals increases the selective pressure exerted on the microbial environment and led to the development of antibiotic resistant bacteria (W.H.O., 2002). There are several environmental concerns with regard to use of antibiotics such as residual toxicity leading to bioaccumulation and lower rate of biodegradation and persistence in the environment for long periods.

The emphasis in disease management should be on prevention, which is likely to be more cost effective than cure. Alternative strategies such as vaccination, use of probiotics and immunostimulants have been proposed and applied very successfully in aquaculture. Vaccination is a useful prophylaxis for infectious disease of fish and is already commercially available for bacterial infections such as vibriosis, redmouth disease, and furunculosis. Enhancing the non specific immune responses of the host by immunostimulants is another promising approach

(Raa, 1996). In recent years, increasing attention has been given to the use of probiotics in the aquaculture industry. Contrary to vaccination and immunostimulants, “Probiotics” enhances the gastro-intestinal microflora as well as influences the immune system of animals. The role of gastro-intestinal microflora in disease resistance has been already established in human and veterinary sciences, which has lead the foundation of the concept of manipulating gastro-intestinal microflora for better health management.

Probiotics can be defined as “ a live microbial adjuvant 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” (Gatesoupe, 1999). Probiotics secure optimal use of the feed by aiding in its digestion (Ghose *et al.*, 2002, 2004), improve water quality (Boyd and Gross, 1998) and stimulate the immune system (Chiu *et al.*, 2007). They may stimulate appetite and improve nutrition by the production of vitamins, detoxification of compounds in the diet and by breakdown of indigestible components. Probiotics provide health benefits to the animals beyond their inherent nutritional capacity when administered in adequate levels.

Probiotic bacteria have high relevance in juvenile production, where opportunistic bacteria are the cause of disease outbreak and high larval mortality. Supplementing these bacteria through feed may provide beneficial effect on the larvae and juveniles by eliminating opportunistic pathogenic microflora. Considering the recent successes of these alternative approaches of microbe supplemented feed in aquaculture, the F.A.O. considered use of probiotics and bio-augmentation for the improvement of aquatic environmental quality as major area for further research in aquaculture (Subasinghe, 1997).

A variety of microorganisms have been tried in human and animal husbandry practices as probiotic agents, some of the most commonly used microbes are *Lactobacillus* spp., *Enterococcus faecium*, *L. sporogenes*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, *B. licheniformis*, *Streptococcus* etc. In humans and in terrestrial animals, it is easy to minimize the existing microflora by applying antibiotics

and then administrating desired probiotic species. However, in aquaculture systems, as the aquatic animals are exposed to fluctuating and varying environmental conditions, several kinds of bacterial flora may enter into the gut via water, food, gills and body surface. Therefore, the gut of aquatic organisms gets flushed with a range of new bacteria which may act as competitor to probiotic flora.

Bacillus spores have been widely used as probiotics for human and animal consumption owing to their immunostimulatory properties on the gastro-intestinal system (Casula and Cutting, 2002). *Bacillus* secretes many enzymes that degrade slime and biofilms and allow their antibiotics to penetrate slime layer around Gram negative bacteria (Moriarty, 1998). Sugita *et al.* (1998) isolated a strain of *Bacillus* sp. that was antagonistic to 63% of isolates of fish intestine. *Bacillus* sp. have been shown to possess adhesion abilities, produce bacteriocins (antimicrobial peptides) and provide immunostimulation (Cherif *et al.*, 2001; Cladera-Olivera *et al.*, 2004; Brahmachari and Raman, 2007; Kumar *et al.*, 2008). Moriarty (1998) noted an increase of prawn survival in ponds, where some strains of *Bacillus* spp. were introduced. Rengpipat *et al.*, 2000 reported that *Peneaus monodon* receiving *Bacillus* S11 supplemented in feed had better survival after bacterial challenge tests and showed a heightened immune response. Li *et al.* (2007) reported that when *Bacillus licheniformis* was administered to white shrimp, *Litopenaeus vannamei*, there was a gradual decrease of pathogenic bacteria in the gut and increase in immune response when fed with the probiotic diet.

In the present experiment, an attempt has been made to study the following objectives:

- To evaluate the microbiological count from the gut microenvironment of scampi, *M. rosenbergii*.
- To evaluate the immune response of giant freshwater prawn to probiotic bacterium (*B. licheniformis*).
- To study possible protective effects of *B. licheniformis* on survival and disease protection in scampi challenged by bacterial infection.
- To standardize the dose of *B. licheniformis* for *M. rosenbergii*.



Review of Literature

2. REVIEW OF LITERATURE

The giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879) is the largest known palaemonid in the world. *M. rosenbergii* culture is widely practised in many countries in Asia-pacific such as India, China, Bangladesh, Malaysia, Philippines, Vietnam and Thailand. Between 1999 and 2001, the production of *M. rosenbergii* increased at an annual rate of 86% in India (New, 2005). An earlier phase of the boom in freshwater prawn farming in India, mainly in the coastal provinces of Tamil Nadu and Andhra Pradesh, was described by Fegan and Sriram (2001). The farming of *M. rosenbergii* has gained tremendous momentum both in India and abroad because of very high demand for the species in foreign markets, and its great cultural traits such as disease resistance, ready acceptance of both plant and animal based diets, ability to withstand wide fluctuations in water quality and fast growth rate (Smith *et al.*, 1982). Despite scampi farming being a very successful aquaculture activity in several parts of the world, very little attention has been paid to the bacterial flora associated with cultured *M. rosenbergii* (Miyamoto *et al.*, 1983; Anderson *et al.*, 1989, Jaiswal, 2002) and its influence on the intestinal microflora of the prawn species.

Both human and animals need a normal bacterial flora in the digestive tract, for protection against disease causing bacteria and production of compounds that are necessary for a well functioning digestive system. These bacteria in general can be used for improving the health and to control harmful bacterial flora in the intestine of animal and water. The application of probiotics in aquaculture systems seems relatively recent (Kozasa, 1986) but the interest in such environment friendly microbes is increasing rapidly in aquaculture.

2.1 Probiotics and Antibiotics

Probiotics meaning 'for life', is exactly opposite to antibiotics that are 'against life'. Growing concern over uses and abuses of antibiotics and other antimicrobial drugs as disease controlling agents in human medicine, agriculture and aquaculture have drawn significant attention of scientific communities regarding development of resistant strains of bacteria (Weaton, 1996). Off late aquaculturists

the world over have heavily relied on the use of antibiotics, synthetic drugs and chemotherapeutants to combat the disease problems facing the aquaculture industry. Farmers have also resorted to reckless use of antibiotics in fish ponds. Due to this, large numbers of cases of antibiotic resistant strains of bacteria have come to light (Aoki, 1992; Raman and Kumar, 1996; Mishra *et al.*, 1996). Germs are winning the war against diseases. Fish farmers are facing the challenge of drug resistant bacterial pathogens, which have stopped responding to most of the conventional antibiotics (Miranda and Zemelman, 2002). Besides, there is the problem of bioaccumulation of antibiotics in the fish body. Off late, several consignments of seafood mostly cultured shrimps exported from India have been rejected by many EU countries due to the presence of antibiotic residues in them resulting in considerable economic losses to the seafood exporters. Moreover treatment with antibiotics or other sources of chemotherapy involves very high cost to the aqua-farmers and many times cost of medicine is higher than the cost of the fish to be saved.

Bacterial resistance can be readily transferred to other non resistant strains of bacteria either by alteration in the existing genome or by transfer of genetic material between the cells through plasmid or bacteriophage (Towner, 1995). In this way, not only resistant strains of bacteria proliferate but also, they transfer resistance to bacteria that have never been exposed to antibiotic drugs (Verschuere *et al.*, 2000a). Bohnoff *et al.* (1954) and Freter (1955, 1956) showed that antibiotics being used as growth promoters could kill beneficial gut microflora. Later Tomoda *et al.* (1983) observed that *Lactobacillus acidophilus* feeding to the animal could restore growth suppressed by antibiotic treatment. The emphasis in disease management should be on prevention which is likely to be more cost effective than cure. This may lead to less reliance on the use of chemicals (antimicrobials, disinfectants, and pesticides), which largely treat the symptoms of the problem and not the cause (Planas *et al.*, 1999). Therefore, more attention should be paid to reduce the over and inappropriate use of antimicrobial drugs and chemicals in aquaculture.

Microbial interference therapy *i.e.*, the use of non-pathogenic microorganisms to eliminate pathogens, has been advocated as an alternative, where possible, to antibiotics in an effort to reduce the growing problem of

antibiotic- resistant bacteria (Bengmark, 2000). The rationale of microbial treatment is to promote health by altering the ecological balance of the microbial flora through the ingestion of probiotics or dietary compounds which favour growth of probiotic bacteria (probiotics) or both (synbiotics) (Collins and Gibson, 1999; Gorbach, 2000).

2.2 Concept of Probiotics

The use of foods derived from microbial activity goes back to the dawn of human civilization and fermented milks were probably the first foods to contain active microorganisms. Long before their discovery, microbes have been unwarily used to preserve food, and these empirical methods contributed to improve human health (Bengmark, 1998). The term “probiotics” was coined by Lilly and Stillwell (1965), literally meaning “for life”. They defined probiotics as “substances produced by one protozoan which stimulates the growth of another”. Early in the century, Metchnikoffs (1907), proposed to implant lactic acid bacteria into the human intestine, with a view to suppressing the detrimental activity of others microbes. He defined probiotics as “microbes ingested with the aim of promoting good health”. Parker (1974) modified the definition as “organism and substance which contribute to the intestinal microbial balance”. Later Fuller (1989) redefined probiotics as a “live microbial feed supplement that beneficially affect the host animal by improving its intestinal microbial balance”. Tannock (1997) noted that the effect on the “intestinal microbial balance” has not been demonstrated in most cases, and he proposed to speak of “living microbial cells administered as dietary supplements with the aim of improving health”. The latest definition stands out as “a viable mono or mixed culture of organism which when applied to the animal or man beneficially affects the host by improving the properties of indigenous flora” (Gatesoupe, 1999). Thus, probiotics can be 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.

Several microorganisms, under the name of ‘probiotics’, have been proposed and used in a wide range of clinical trials, ranging from diarrhoeal disease to cancer prevention (Fuller, 1994; Kaur *et al.*, 2001). Considerable efforts are still being made to develop alternative or supplementary methods to improve fish health.

Among such methods, the prophylactic uses of immunostimulants and probiotics have attracted special interest (Raa, 1996; Ringo and Gatesoupe, 1998). One obvious reason for this is that such approaches can be implemented at larval and early fry stages where vaccines cannot be used. At this stage mortality can be high due to proliferation of opportunistic pathogens (Muroga *et al.*, 1987). In such situation minor improvement of the non specific immune responses or the intestinal microflora may reduce the mortality significantly.

2.3 Probiotic Strains

Ideally, microbial probiotics should have a beneficial effect and not to cause any harm to host. Typically, safety evaluation of a proposed or accepted probiotic strains includes:

- Ability of cells to produce metabolites and enzymes,
- Colonization (or adhesion) properties,
- Factors that influence the strain survival, and
- Interactions with host, particularly in terms of pathogenicity.

It has been established that several species of Lactic acid bacteria (LAB) are part of the natural intestinal microflora of healthy fish (Ringo and Gatesoupe, 1998), and it is well known that LAB often produces bacteriocins which may inhibit the growth of Gram negative fish pathogens.

In addition, use of other bacterial groups such as *Bacillus* sp. (Casula and Cutting, 2002; Chang and Liu, 2002; Ghosh *et al.*, 2002; 2004), *Lactobacillus* (Venkat *et al.*, 2004) and *Bifidobacterium* sp. (Gill *et al.*, 2001a), for probiotics have also been recognised and accepted. In fish farming, there is a need to seek alternative probiotics that are bile-tolerant and stable even at low pH (Joborn, 1997). New probiotic candidates that are acid resistant would be ideal for this approach which may include *Vibrio alginolyticus* and *Carnobacterium* spp. (Austin *et al.*, 1995; Robertson *et al.*, 2000).

Characteristics of potential probiotic strains

As per Mercenier *et al.*, 2002, the important characteristics of a probiotic strain should be as follows:

- Non toxic and non pathogenic
- Accurate taxonomic position
- Normal inhabitant of the targeted species
- Capable of survival, proliferation and metabolic activity in the target site, which implies:
 - resistance to gastric acid and bile
 - ability to persist, albeit for short periods, in the gastro-intestinal tract
 - adherence potential preferred
 - ability to compete with the resident flora
- Production of antimicrobial substances
- Antagonism (in vivo) towards pathogenic bacteria
- Ability to modulate immune responses
- Ability to exert at least one clinically documented health benefit
- Genetically stable
- Amenability of the strain and stability of the desired characteristics during processing, storage and delivery
- Viability at high populations
- Desirable organoleptic and technological properties when included in fermentation processes

2.4 Importance of Gut Microflora

The establishment of normal gut microbiota may be regarded as complementary to the establishment of a healthy digestive system, and under normal conditions, it serves as a barrier against invading pathogens. Larval forms are directly hatched into the aquatic environment, therefore, gut microflora play an important role in fish and shellfish health (Hanson and Goodwin, 1977). The gut is sterile until hatching but soon after hatching different kinds of microbes colonize the

gut (Muroga *et al.*, 1987; Munro *et al.*, 1994). Larvae may ingest substantial amount of bacteria by grazing on suspended particles and egg debris (Beveridge *et al.*, 1989). Digestive tract of fish contains a much higher number of bacteria than the surrounding water, as many as 10^8 cells per gram (Ringo *et al.*, 1995). In fish of temperate water the number of bacteria in most cases is below the minimum limits of 10^7 cells per gram of digestive tract (Lesel, 1991).

2.5 Probiotics Type

2.5.1 Feed probiotics

The incorporation of bacterial spores into the aquaculture feed preparation has been designed and adopted from animal husbandry practices (Gatesoupe, 1999). Feed probiotics act in a different way in which bacteria grow and multiply in large numbers in the digestive tract of the host and compete with pathogenic bacteria (Lipton, 1998). They inhibit multiplication of pathogenic bacteria by producing organic acids and antibiotic substances. Uma (1995) observed effect of commercially available probiotics containing *Lactobacillus*, *Streptococcus* and *Saccharomyces* species on survival, growth and immune response of white shrimp (*Penaeus indicus*). Sandhyarani (1996) reported the effect of commercial probiotics on the disease prevention in prawn farms. Suralikar (1997) observed the enhanced growth rate of non-penaeid shrimps when fed with feed containing *Lactococcus lactis* sub species *cremoris*. Sharma (1999) also reported enhanced growth rate, survival, feed conversion and increased immunity in fishes through probiotics supplemented diet.

2.5.2 Water probiotics

Water probiotics are applied directly to water surface as a part of water quality management (Moriarty, 1998; Sharma, 1999). Water probiotics are of unique importance, as the larvae of aquatic organisms are directly released into the water where they are exposed to various kinds of microbes (Campbell and Buswell, 1983; Timmermans, 1987; Muroga *et al.*, 1987; Ringo *et al.*, 1996). In the aquaculture ponds, dissolved and particulate organic carbon can be minimized by using higher levels of these gram positive bacteria (Scura, 1995). Perfittini and Bianchi (1990)

reported that inoculation of frozen probiotics bacterial cells in closed seawater culture system resulted in shortened nitrification time by about 30%. The application of water probiotics in aquaculture ponds has opened a new vista in sustainable aquaculture, variously referred to as 'bioremediation'.

2.6 Mode of Action

Considering the possible probiotic effect *in vivo*, one has to make a distinction between the intrinsic ability of microbial strain to positively influence the host and its ability to reach and maintain itself in the location where the effect is to be exerted. Similarly, if the candidate probiotic is not capable of efficient proliferation in the gut after being ingested, it is improbable that it will exert strong effects unless it is added regularly through the diet. Hence, the possible modes of action require implicitly that the candidate probiotics be able to reach the location where their effect is required (Fuller, 1989; Verschuere *et al.*, 2000b).

2.6.1 Suppression of Viable Count

2.6.1.1 Production of antimicrobial compounds

The presence of bacteria producing antimicrobial substances in the intestine of the host, on its surface, or in its culture medium is thought to constitute a barrier against the proliferation of (opportunistic) pathogens. The antibacterial effect of bacteria is due to the following factors, either singly or in combination: production of antibiotics (Williams and Vickers, 1986), siderophores, lysosomes, proteases, hydrogen peroxide, alteration of pH values by the production of organic acids (Sugita *et al.*, 1997) and production of bacteriocins (Vandenbergh, 1993; Pybus *et al.*, 1994).

2.6.1.2 Competition for nutrients

Competitive exclusion is primarily based on the principle to reduce intestinal colonization by enteric pathogens sharing the common environment (Barrow, 1992). This competition may be for nutrients and energy (Fredrickson and Stephanopaulus, 1981). Verschuere *et al.* (2000a) reported that aquatic environment is dominated by heterotrophs which compete for organic substrate, carbon, and

energy sources. He also described the protective action of selected bacteria by competing with the pathogen for chemicals and available energy.

Verschuere *et al.* (1999) selected several strains with a positive effect on the survival and growth of *Artemia* juveniles and suggested that the selected bacteria exerted their protective action by competing with the pathogen for chemicals and available energy (Verschuere *et al.*, 2000b). Reid *et al.* (1993) effectively correlated the ability of siderophores to scavenge iron from the environment and reduction of the pathogenic *Vibrio* count in salmon. The requirement for iron is high for many pathogens, including *V. anguillarum*. In a challenge test with this bacterium, salmon mortality increased linearly with dietary iron content (Gatesoupe *et al.*, 1997; Rorvik *et al.*, 1991). Smith and Davey (1993) reported that fluorescent *Pseudomonas* F 19/3 is capable of inhibiting the growth of *Aeromonas salmonicida* in culture media due to competition for iron.

2.6.1.3 Competition for adhesion site

One possible mechanism for preventing colonization by pathogens is competition for adhesion sites in the gut or other tissue surfaces. Ability to adhere on the enteric mucus and wall surface is necessary for bacteria to establish in fish intestine (Onarthein, 1990; Westerdahl *et al.*, 1991; Olsson *et al.*, 1992). Since bacterial adhesion to tissue surface is important during the initial stages of pathogenic infection (Krovacek *et al.*, 1987), competition for adhesion receptors with pathogens might be the first probiotic effect (Montes and Pugh, 1993).

Adhesion of the probiotic bacteria can be nonspecific based on physico-chemical factors involving adhesion molecule on the surface of adherent bacteria and receptor molecule on epithelial cell (Salminen and Isolauri., 1996a). Nurmi and Rantala (1973) studied the ability of probiotic bacteria to establish in the gut of chickens. Salminen and Wright (1993) also described that probiotic effect may be due to adhesion of the bacteria to epithelial surface of the gut wall. Adhesion capacity and growth on or in intestinal or external mucus has been demonstrated for fish pathogens like *V. anguillarum* and *Aeromonas hydrophila in vitro* (Krovacek *et al.*, 1987; De la Bonda *et al.*, 1992; Garcia *et al.*, 1997) and for candidate probiotics such as *Carnobacterium* strain K1 (Joborn *et al.*, 1997) and

several isolates inhibitory to *V. anguillarum* (Olsson *et al.*, 1992). Tannock (1990) also described that extracellular substances like polysaccharides, proteins, lipotechoic acid, lipids and lactin interaction with glycoproteins or glycolipids important factors governing the adherence of lactic acid bacteria to the intestinal mucosa.

2.6.2 Alteration of microbial metabolism

Alteration of microbial metabolism may occur due to increased or decreased enzyme activity (Fuller, 1989). The causes of enzyme deficiency can be congenital or due to a variety of clinical conditions (Dahliquist and Asp, 1975). Lactose intolerance can be alleviated using probiotic bacteria secreting useful enzymes e.g., β -galactosidase (Fuller, 1989). *L. acidophilus* in humans could suppress the activity of β -glucuronidase, nitroreductase and azoreductase (Goldin and Gorbach, 1984).

Nitrate reductase convert the nitrate into nitrite which is highly toxic to aquatic life as it combines with haemoglobin to form methaemoglobin which is unable to transport oxygen to the tissues causing hypoxia in the affected fish, therefore, decreased number of nitrate reductase producing bacterial flora is desirable and can be achieved using probiotic bacteria (De Bruin, 1976). Microbial metabolism can also be affected by pH changes due to production of lactic acid by bacteria, which may further affect enzyme activity (Ford, 1974). Vitamin B and A synthesized by Lactic acid bacteria may also compensate its requirement by the host to some extent (Pivnyak and Konykhin, 1973).

2.6.4 Stimulation of immunity

Immunostimulants are chemical compounds that activate the immune system of animals and renders them more resistant to infections by viruses, bacteria, fungi and parasites (Raa, 1996). In larval stages of fish and prawn, immune system is reported to be less developed than adults, and is dependent primarily on nonspecific immune responses for their resistance to infection (Soderhall and Cerenius, 1998). Observations obtained in experiments with warm-blooded animals indicate that probiotics (lactic acid bacteria) administered orally may provide increased resistance

to enteric infections (Holzapfel *et al.*, 1998). It is also reported that bacterial compounds act as immunostimulants in fish and shrimp (Sakai, 1999). Norqvist *et al.* (1989) claimed that vaccination of rainbow trout with attenuated *V. anguillarum* induce protection against *A. salmonicida*. Sakai *et al.* (1995) reported that rainbow trout immersed in *V. anguillarum* bacterial solution showed increased protection.

2.6.5 Improvement of water quality

In several studies, impact on the pond water quality has been monitored during the addition of the probiotics, especially *Bacillus* spp. The rationale is that the gram-positive *Bacillus* spp. are generally more efficient in converting organic matter back to carbon dioxide than are gram-negative bacteria, which would convert a greater percentage of organic carbon to bacterial biomass or slime (Stanier *et al.*, 1963). It has been found that the introduction of *Bacillus* spp. in proximity to pond aerators reduced chemical demand (Porubean, 1991b). Nitrifying cultures could also be added to the ponds or to the tanks when an incidental increase of ammonia or nitrite levels is observed (Lewis and Morris, 1986).

2.6.6 Enzymatic activity

The enzymes liberated by probionts help in increasing the digestive utilization of feed or detoxify metabolites liberated by the pathogenic bacteria. The alteration of microbial metabolism is affected either by increased or decreased enzymatic activity (Nayak, 2002). Tovar *et al.* (2002) reported increase in amylase and trypsin secretion in sea bass (*Dicentrarchus labrax*) larvae by feeding live yeast *Debaryomyces hansenii*. Moreover, Yanbo and Zirong (2006) noted elevated level of digestive enzyme (protease, amylase and lipase) activities in common carp by feeding *Bacillus* sp. and mixture of probiotic bacteria. The *Bacillus* sp. isolated from *Cyprinus carpio* having considerable extracellular amylolytic, cellulolytic, proteolytic and lipolytic activities (Bairagi *et al.*, 2002; Ghosh *et al.*, 2002, 2004).

2.7 Probiotics in Animal Husbandry

Metchnikoff (1907) studied the effect of sour milk on human being using *Lactobacillus bulgaricus*. Even now most of the commercial probiotic preparation

contains lactic acid bacteria such as *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. delbruki*, *L. helveticus*, *L. lactis*, *L. plantarum* and *Streptococcus thermophilus* (Fuller, 1992). The use of probiotics for stimulation of immunity, disease resistance, and nutritional value improvement has been reviewed by (Chassy, 1986; Vanbelle *et al.*, 1989; Fuller, 1992; and Havenar *et al.*, 1992). Dilworth and Day, (1978) has reported the effects of probiotics supplementation on growth and egg production in chickens. Baird (1977) noticed increased daily weight gain and improved feed conversion rate with growing and finished pigs separately using *Lactobacillus*. Han *et al.* (1984) also observed significant improved weight gain and improved feed conversion in chickens and pigs fed with *Lactobacillus sporogens* and *Clostridium butvricum*. In ruminants, *Streptococcus faecicum*, *L. acidophilus* (Johnson and Olsson, 1985; Lee and Botts, 1988) are commonly used probiotics. Adams *et al.* (1981) and Huber *et al.* (1985) also reported the use of yeast as a probiotic in cattle.

2.8 Probiotics in Aquaculture

Aquaculture aims at maximum production in a limited culture area. Intensified aquaculture practices also increases the chances of disease causing agents to attack (Ringo and Gatesoupe, 1998). Probiotics are having high demand in aquafarms worldwide particularly when ecofriendly aquaculture is getting much importance (Nayak and Savan, 1999).

2.8.1 Impact on early developmental stages of fish

The development of fish farming on a commercial scale is limited for many species due to difficulties in producing sufficient quantities of larvae. High mortalities can occur during the early stages of larval rearing, especially the transition from yolk sac to the first feeding stage of development (Ringo and Birkbeck, 1999). The beneficial effects of probiotics in larval rearing have been demonstrated in fishes by several investigators (Charlon and Bergot, 1984; Dabrowski and Kaushik, 1985; Bergot *et al.*, 1986; Alami-Durante *et al.*, 1991). A relatively dense non-pathogenic and diverse adherent microbiota is reported to be present on the fish egg, which probably act as effective barrier against colony formation by pathogen on the egg (Hansen and Olafsen, 1989; Olafsen, 1998).

Improved survival rate and resistance of turbot larvae to pathogenic *Vibrio* was observed by daily addition of Lactic acid bacteria through live food organism (Gatesoupe, 1997). De la Bonda *et al.* (1992) also noticed increased survival in turbot larvae fed with Lactic acid bacteria up to 66% compared to 34% survival in the control group. *Lactobacillus* use decreased mortality in turbot larvae when challenged with pathogenic *Vibrio* species (Gatesoupe, 1997). Kennedy *et al.* (1998) used *Bacillus* 48 to enhance the survival of common snook, *Centropomus undecimalis* (Bloch) larvae, increased food absorption by enhancing protease levels and gave better growth.

2.8.2 Impact on fish juveniles and adults

An enhanced disease resistance was observed in Atlantic cod fry when fed with feed containing *Carnobacterium divergence* (Gildberg *et al.*, 1997). Metallier and Hollocou (1993) observed improved growth rate in European sea bass (*Dicentrarchus labrax*) fed with three different probiotic supplemented diets. Swain *et al.* (1994) also reported significant higher growth and nutrient utilization through probiotic supplement diet. Using *Lactobacillus* as feed additive for flounder and tilapia, respectively, Gildberg *et al.* (1995) did not find any improvement in growth by using *Lactobacillus* bacteria isolated from salmon intestine.

2.8.3 Impact on crustaceans

Maeda and Liao (1992) reported the use of soil bacterial strain PM-4 and showed *in vitro* inhibition of *Vibrio anguillarum* strain along with enhanced growth rate of *Penaeus monodon* nauplii. Nagomi and Maeda (1992) isolated a bacterial strain that was found to improve the growth of crab (*Portunus trituberculatus*) and repress the growth of pathogenic *Vibrio* sp. They also suggested that bacterium might improve the physiological rate of crab larvae by serving as nutrient source during its growth. Morairty (1998) also concluded that the use of several *Bacillus* as probiotics in penaeid culture ponds completed the culture period of 160 days without any problem, compared to almost failure of culture ponds having no *Bacillus* used. Gatesoupe *et al.* (1989) reported that Lactic acid bacteria showed improvement in the dietary value of rotifers. Uma (1995) also reported enhanced growth rate and survival of *Penaeus indicus* through the addition of *L. plantarum* in the water. *Bacillus*

S11 used in *Penaeus monodon* culture with diet showed mean weight increase and survival of larvae and postlarvae, decreased mortality after challenge with the pathogen *V. harveyi* D331 (Rengpipat, 1998). Harzevili *et al.* (1998) used *Lactococcus lactis* AR21, which stimulated the growth of rotifers and inhibited the growth of *V. anguillarum*. Hirata *et al.* (1998) used mixed cultures consisting mainly of *Bacillus* species to improve performance of rotifer *Brachionus plicatilis* in water.

2.8.4 Microflora associated with *Macrobrachium rosenbergii* (de Man)

With the development of modern prawn farming techniques, bacteriological surveys are receiving more attention because some species of bacteria associated with the prawns cause diseases; while other bacteria seem to be useful food for prawn larvae. Several workers have reported the results of quantitative and qualitative investigations on bacterial population in fish guts (Liston, 1957; Colwell, 1962; Yoshimizu *et al.*, 1976), however, there have been no proper and systematic studies done on the bacteriological flora associated with the gut of prawn (Rengpipat, 1998).

Microorganisms have been implicated in many disease conditions such as bacterial necrosis (AQUACOP, 1997) and larval-mid cycle disease (MCD) (Brock, 1983). Anderson *et al.* (1989) identified *Alcaligenes* sp. and *Enterobacter* sp. from *M. rosenbergii* larvae with MCD. The occurrence of viral (Anderson *et al.*, 1990; Tung *et al.*; 1999) and fungal diseases (Anderson, 1989; New, 1995) also have been reported. The luminescent bacterial disease caused by *Vibrio* sp. (Tonguthai, 1995) has been reported to cause serious mortalities in *M. rosenbergii* hatcheries. Lavilla-Pitogo *et al.* (1990) and Karunasagar *et al.* (1994) reported luminescent vibriosis to be a major problem causing significant mortalities in systems employing saline water such as *Penaeus monodon* hatcheries. Most of the bacteria are part of the commensal flora and only some of them may be opportunistic pathogens.

Miyamoto *et al.* (1983) reported 13 genera of bacteria from *M. rosenbergii* larvae including *Aeromonas*, *Pseudomonas* and *Vibrio* as the dominant flora while Anderson *et al.* (1989) studied the aerobic heterotrophic

microflora in *M. rosenbergii* hatcheries in Malaysia and reported *Alcaligenes* sp. and *Vibrio* sp. to be most frequently encountered genera.

2.8.5 Impact on bivalve and mollusc

Several studies have focused on the nutritional contribution of probiotics to mollusc larvae. Douillet and Langdon (1994) observed enhanced growth of *Crassostrea gigas* larvae when supplemented with bacterial strain CA2 as a feed supplement. A bacterium *Alteromonas* sp. was used effectively in Pacific oyster (*Crassostrea gigas*) larvae culture and these larvae were noticed to enhance survival by 21-22% and increased growth by 16-21% compared to control larvae (Douillet and Langdon, 1993). Axenic bacterial cultures have also been used successfully in bivalve (*Crassostrea gigas*) larval rearing (Besse *et al.*, 1989). He suggested that bacteria provide some essential nutrients for bivalve growth. A bacterial strain isolated from the gonads of Chilean scallop (*Argopecten purputatus*) broodstock and characterized as *Aeromonas haloplanctis* displayed *in vitro* inhibitory activity against the pathogen *V. ordalii*, *V. parahaemolyticus*, *V. anguillarum* and *A. hydrophila* (Riquelme *et al.*, 1996).

2.8.6 Probiotics in human and terrestrial Animals

In contrast to aquaculture, probiotics for use in humans and terrestrial animals have centered on use of Lactic acid bacteria, particularly, representatives of *Bifidobacterium*, *Lactobacillus* and *Streptococcus* (Fuller, 1989). Prophylactic, probiotic microbes are now being used widely for treatment of poultry, swine and other land animals to protect against pathogenic microbes (Fox, 1988; Fuller, 1989, 1999; Holzapfel *et al.*, 1998). Indeed, a common concept of a probiotic is of a beneficial lactic acid bacterium, which is suited for survival in the digestive tract because of tolerance to acidity and bile salts (Fuller, 1989, Smoragiewicz *et al.*, 1993).

2.9 *Bacillus* as a Probiotics

The use of *Bacillus* sp. as probiotics have been widely recognized (Adami and Cavazzoni, 1999; Casula and Cutting, 2002; Kumar *et al.*, 2008). *Bacillus* spores are being used as probiotics for human and animal consumption due to their

immunostimulatory properties on the gastro-intestinal system (Casula and Cutting, 2002). It has been found that *B. subtilis* possesses antibiotic activity against pathogenic *Vibrio* sp. and effectively improves pond water quality and enhances the survival of black tiger prawn (Vaseeharan and Ramasamy, 2003) and fish (Kumar *et al.*, 2008). Research concerning oral administration of *Bacillus* sp. to cultured fish species has merely consisted of challenge experiments. *B. toyoi* was evaluated for its capacity to reduce Edwardsiellosis in the European eel (Chang and Liu, 2002), whereas a mixture of *B. subtilis* and *B. licheniformis* added to the diet of 120 day old rainbow trout for 42 days increased survival of fish against *Yersinia ruckeri* infection (Raida *et al.*, 2003). *B. subtilis* and *L. delbrueckii* were evaluated by Salinas *et al.* (2005) and found to have stimulating effects on the gilthead seabream cellular innate immune parameters such as increased cytotoxic activity and phagocytic activity.

The strains of *Bacillus* sp. used as probiotics for terrestrial livestock have telluric origins and they are not autochthonous in the gastro-intestinal tract, but they may be active during intestinal transit (Gournier-Chataeu *et al.*, 1994). There are many reports of isolation of *Bacillus* strains from fish (Hamid *et al.*, 1978; Strom and Olafsen, 1990; Sadhukhan *et al.*, 1997; Sugita *et al.*, 1998; Ghosh *et al.*, 2002; Mandal *et al.*, 2005), crustaceans (Austin and Allen, 1982; Sharmila *et al.*, 1996; Sugita *et al.*, 1996a) and bivalves (Sugita *et al.*, 1981). Ghosh *et al.* (2002) isolated *Bacillus circulans* Lr 1.1 from the intestine of Indian major carp, *Labeo rohita* (Ham.). Mandal *et al.* (2005) isolated *Bacillus licheniformis* from the intestine of fresh water fish, *Labeo rohita*.

Moriarty (1998) noted an increase in prawn survival in culture ponds, wherein some strains of *Bacillus* sp. were introduced. This treatment decreased the proportions of pathogenic *Vibrio* sp. in the sediments, and to lesser extent in the water. Rengpipat *et al.* (2000) reported that *Penaeus monodon* receiving *Bacillus* S11 supplemented in feed had better survival after bacterial challenge tests and showed a heightened immune response. Spores of *Bacillus* sp. isolated from soil reduced the mortality of Japanese eel which were infected by *Edwardsiella* sp. (Kozosa, 1986). The same feed additive increased the growth rate of yellow tail (Kozasa, 1986).

Bacillus secretes many enzymes that degrade slime and biofilms and allow their antibiotics to penetrate slime layer around Gram negative bacteria. Furthermore, *Bacillus* competes for nutrients and thus inhibits other bacteria from growing rapidly (Moriarty, 1990). Many *Bacillus* sp. produce antibiotics, especially in relation to the sporulation process (Brock, 1974), and some antibiotics may be produced by proteolysis of the vegetative cells of *Bacillus licheniformis* (Vitkovic and Sadoff, 1977). Gatesoupe (1999) reported that when rotifers were fed with spores of *Bacillus* spp., a significant decrease of Vibrionaceae population in the rotifers were observed, which might be due to the release of antibiotics from the cells of *Bacillus* sp.

Sugita *et al.* (1998) isolated a strain of *Bacillus* sp. that was antagonistic to 63% of the isolates from fish intestine. In most of the *in vitro* tests, Pathogenic strains of *Vibrio* or *Aeromonas* have been targeted, but some other fish pathogens have been also tested such as *E. tarda* and *Y. ruckeri* (Dopazo *et al.*, 1988; Gibson *et al.*, 1998). Some *Bacillus* are also antagonistic to viruses (Direkbusarakom *et al.*, 1998) and as such, they may be effective in the biocontrol of viral diseases (Maeda *et al.*, 1997).

The spores of *Bacillus* sp. are especially easy to introduce in dry food, and as such, it is an additional advantage of these promising candidate probiotics (Moriarty, 1998; Sugita *et al.*, 1998). Vaseeharan and Ramaswamy (2003) reported, cell free extract of *Bacillus subtilis* BT23 inhibitory against the growth of *Vibrio harveyi*. *B. subtilis*, in particular, is now being used for oral bacteriotherapy (Green *et al.*, 1999), ingestion of appropriate quantities of *B. subtilis* has been found to revive the normal microflora after the antibiotic use.

2.10 Immune parameters

The sustainability of aquaculture industry depends largely on disease control and health status of shrimp (Rodriguez *et al.*, 2000). Immune system is a tool to assess shrimp health (Bachere *et al.*, 1995a). The innate immune system is a host defence mechanism present in both vertebrate and invertebrate animals, and is mainly involved in the recognition and control of early stage of infection. Invertebrates in particular lack immunoglobulins, and instead they possess an efficient innate

system to protect themselves against invading foreign microorganisms (Hoffmann *et al.*, 1999). This defence system of invertebrates is based on both cellular and humoral immune responses (Smith and Soderhall, 1986). The cellular mechanism is mediated mostly by circulating blood cells, haemocytes (Ratcliffe *et al.*, 1985). The humoral mechanism employs constitutive and inducible extracellular molecules that can bind to and lyse invading microorganisms (Hoffmann *et al.*, 1999; Roch, 1999). These include a number of antimicrobial proteins (Kawano *et al.*, 1990; Schnapp *et al.*, 1996), the clotting system of arthropods (Iwanaga *et al.*, 1998; Hall *et al.*, 1999) and the prophenol oxidase (proPO) activating system (Soderhall and Cerenius, 1998).

2.10.1 Hemogram counts

Haemocytes play central role in crustacean immune defence. Firstly, they remove foreign particles in the hemocoel by phagocytosis, encapsulation and nodular aggregation (Soderhall and Cerenius, 1992). Secondly, haemocytes take part in wound healing by cellular clumping and initiation of coagulation processes through the release of factors required for plasma gelation (Johansson and Soderhall, 1989; Omori *et al.*, 1989; Vargas-Albores *et al.*, 1998), and carriage and release of the prophenoloxidase (proPO) system (Johansson and Soderhall, 1989; Hernandez-Lopez *et al.*, 1996). They are also involved in the synthesis and discharge in the haemolymph of important molecules, such as alpha2-macroglobulin (Rodriguez *et al.*, 1995; Armstrong *et al.*, 1990), agglutinins (Rodriguez *et al.*, 1995), and antibacterial peptides (Destoumieux *et al.*, 1997; Schnapp *et al.*, 1996; Lester *et al.*, 1997). The haemogram consists of the total haemocyte count (THC) and the differential haemocyte count (DHC). For the DHC, most researchers agree with the identification of three cell types in penaeid shrimp: large granule haemocytes (LGH), small granule haemocytes (SGH) and agranular haemocytes or hyaline cells (HC) (Tsing *et al.*, 1989; Martin and graves, 1985; Rodriguez *et al.*, 1995; Van de Braak *et al.*, 1996).

In Giant freshwater prawn *Macrobrachium rosenbergii*, haemocytes are classified differently like fusiform, rounded and large ovoid haemocytes (Sierra *et al.*, 2001). The large ovoid and rounded haemocytes possibly carry out the

function of the proPO activating system, like small granular haemocytes (SGH) and granular haemocytes (GH) in other crustaceans (Johansson and Soderhall, 1989).

Total haemocyte count (THC) can be easily determined using a haemocytometer; where as determination of DHC requires more complex haemocyte identification. DHC can be determined by the use of morphological criteria such as size and shape of cells and the difference of haemocyte refractivity using a phase contrast microscope (Tsing *et al.*, 1989; Martin and Graves, 1985; Le Moullac *et al.*, 1997). Although this technique is rapid, it has been observed that when using this technique large variations in results were seen, which may be possibly due to interpretation errors.

Different haemocyte types can be determined using cytochemical studies of enzyme activity detection or specific stains. In *S. ingentis*, Hose *et al.* (1987) reported that acid phosphatase activity was more abundant in SGH, while haemocytes were distinctively stained with Sudan black. In *P. japonicus*, Sequeira *et al.* (1995) performed cytochemical staining of haemocyte subpopulations separated by flow cytometry, and reported positive peroxidase activity only in large granular haemocytes (LGH).

2.10.2 Measurement of reactive oxygen intermediates (ROIs)

Phagocytosis is the most common reaction of cellular defence. During phagocytosis, particles or microorganisms are internalized into the cell which later forms a digestive vacuole called the phagosome. The elimination of phagocytosed particles involves the release of degradative enzymes into the phagosome and the generation of ROIs. This last process is known as the respiratory burst. The first ROI generated during this process is the superoxide anion (O_2^-). Subsequent reactions produce other ROIs, such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^\cdot) and singlet oxygen (1O_2). Hydrogen peroxide can be converted to hypochlorous acid (HOCL) via the myeloperoxidase (MPO)- H_2O_2 -Cl system, forming a potent antibacterial system (Bayne, 1990; Anderson, 1996).

In penaeid shrimp most studies concerning phagocytosis have been performed through observations of clearance processes of injected bacteria or particulate materials (Fontaine and Lightner, 1974; Tsing, 1987; Martin *et al.*, 1993)

but this procedure is inappropriate to quantify phagocytosis. In invertebrates, most of the studies regarding ROI generation have been performed on molluscs (Dikeboom *et al.*, 1985; Bachere *et al.*, 1991; Pipe, 1992; Noel *et al.*, 1993; Anderson, 1994) and some quantitative procedures have been applied for shrimp research, as the nitro blue tetrazolium (NBT) reduction technique for the measurement of intracellular O_2^- and the reduction of ferricitochrome C for extracellular O_2^- . The determination of H_2O_2 is performed by horseradish peroxidase (HRP)-dependent oxidation of phenol red, while chemiluminescence (CL) is used for the measurement of light emission from ROIs.

The first evidence that crustacean haemocytes produce ROIs was given by Bell and Smith (1993) in the shore crab *Carcinus maenas*. They showed O_2^- generation from hyaline cells using phorbol myristate acetate (PMA) as elicitor. Song and Hsieh (1994) described for the first time the oxidative metabolism in penaeid shrimp (*P. monodon*). They measured O_2^- using the NBT reduction technique and H_2O_2 by (HRP) dependent oxidation of phenol red and detected an MPO-like enzyme activity. Bachere *et al.* (1995b) demonstrated the existence of respiratory burst in *P. japonicus* induced by PMA and Zymosan, measuring the CL using a scintillation counter. In *P. vannamei*, Munoz *et al.* (2000) worked on a simplified procedure to measure intracellular O_2^- by the NBT reduction assay in microtiter plates. The measurement of the activity in unstimulated haemocytes (base activity) allows the detection of previous excitation states of haemocytes that could indicate the existence of an inflammatory process. The assay was found to be specific, obtaining a decrease on O_2^- by SOD and an inhibition by N-ethyl-maleimide (NEM).

Despite the limited number of studies focusing on respiratory burst in penaeid shrimp, the results are quite interesting in view of their value as biomarker of environmental disturbances (Le Moullac and Haffner, 2000). Furthermore, the importance of respiratory burst as a microbicidal mechanism in penaeid shrimp is strongly suggested by the fact that pathogenic bacteria of shrimp have developed ways of circumventing this mechanism. In *P. vannamei*, O_2^- generation is not produced when virulent *Vibrio vulnificus* is used as elicitor, as opposed to strong stimulation generated by *V. alginolyticus* and other bacteria, such as *Escherichia coli* (Munoz *et al.*, 2000)

2.10.3 Measurement of prophenoloxidase (proPO) and phenoloxidase (PO)

The PO is responsible for the melanization process in arthropods. The PO enzyme results from the activation of the proPO enzyme. The proPO activating system has been very well studied in crustaceans, especially in crayfish (Soderhall and Cerenius, 1998; Soderhall *et al.*, 1996). Melanin and its reactive intermediates have shown to be fungistatic (Soderhall and Ajaxon, 1982; Persson *et al.*, 1987).

In penaeid shrimp, the first work on melanin formation was focussed on histochemical observations of its presence in inflammation sites with haemocytic activity (Lightner and Redman, 1977). Cytochemical staining of shrimp haemocytes showed that the proPO system was confined to LGH and SGH (Hose *et al.*, 1987; Tsing *et al.*, 1989; Sequeira *et al.*, 1995). The process of activation of the proPO system has been studied in several penaeid shrimp (Hernandez-Lopez *et al.*, 1996; Vargas-Albacores *et al.*, 1997; Perazzolo and Barraco, 1997; Sung *et al.*, 1998). The release of the proPO system is amplified by peroxinection, a 76-kDa protein identified in haemocytes. This protein has cell adhesion, degranulation, opsonic and peroxidase activity (Johansson *et al.*, 1995). The proPO has been cloned and sequenced in crayfish (Aspan *et al.*, 1995) and *P. monodon* (Sritunylucksana *et al.*, 1999).

The PO activity is measured spectrophotometrically by recording the formation of dopachrome from L-dihydrophenylalanine (L-DOPA) at 490 nm (Leonard *et al.*, 1985). PO can be obtained in different ways. The proPO is released from haemocytes by incubating them with laminarin or zymosan as elicitor in presence of Ca^{2+} (Vargas-Albacores *et al.*, 1993a; Le Moullac *et al.*, 1997). PO can also be obtained from cellular lysates containing inactivated proPO system, trypsin is used to activate the proPO to PO (Smith and Soderhall, 1991). The procedure of PO activity assay has been simplified, the reaction carried out completely in microtiter plates (Hernandez-Lopez *et al.*, 1996).

Using these different approaches, the function of the proPO system can be better understood in relation to the health status of shrimp. Some studies have

shown that proPO could be used as health status of shrimp. Some studies have been shown that proPO could be used as environment markers because changes are correlated with infectious state and environmental variations (Le Moullac and Haffner, 2000).

2.10.4 Antibacterial activity quantification

Antibacterial peptides and proteins have been well studied in arthropods, mainly in insects and chelicerata (Hetru *et al.*, 1994; Iwanaga *et al.*, 1998), where the families of antimicrobial molecules have been isolated and characterized. In crustaceans, some studies have shown the ability of crustacean haemolymph to inhibit bacterial growth (Adams, 1991; Chisholm and Smith, 1992; Noga *et al.*, 1994, 1996). Several antibacterial proteins, active *in vitro* against Gram-positive and Gram-negative bacteria, were found in *C. maenas* (Schnapp *et al.*, 1996; Smith, 1997). Lester *et al.* (1997) found a small peptide named callinectin, which was reported to be responsible for the majority of antibacterial activity observed in the haemolymph of blue crab *Callinectes sapidus*. Destoumieux *et al.* (1997) characterized three members of a new family of antimicrobial peptides, named penaeidins which are the first antimicrobial molecules found in penaeid shrimp.

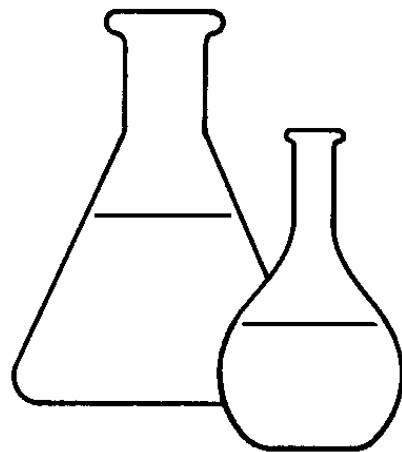
Antibacterial activity can be considered as an environmental marker (Le Moullac and Haffner, 2000). Therefore, many quantitative antibacterial assays have been developed based on inhibition of bacterial growth on agar plate (Zone inhibition assay and colony-forming units(CFU) inhibition assay) or in liquid medium on microtitre plates (turbidometric assay), to detect the antibacterial ability in crustacean haemolymph. Using the CFU inhibition technique, antibacterial activity has been found in granular haemocytes of the shore crab (*C. maenas*) and in other crustacean species (Chisholm and Smith, 1992; 1995). Noga *et al.* (1994) reported a potent antibacterial activity in the serum of *C. sapidus*, using the zone inhibition assay and turbidimetric test. Adams (1991) has described antibacterial activity against *Vibrio* spp. in the haemolymph of *P. monodon*. In *P. vannamei*, using turbidimetric assay strong antibacterial activity in the plasma against different marine bacteria, has been observed (Rodriguez *et al.*, 2000). Both techniques (using agar

plates or liquid medium) allow the detection of antibacterial activity in crustacean haemolymph.

2.10.5 Measurement of protein concentration

Crustaceans have an open circulatory system in which the haemolymph carries out several physiological functions. One of these functions is the transport of the respiratory protein (haemocyanin) which is the most abundant molecule of the haemolymph (60% to 95% of total protein), followed by the clotting protein and other humoral components (Djangmah, 1970). The measurement of plasma protein concentration is based on classic methods, such as Lowry method (Lowry *et al.*, 1951).

Evidence has been given regarding the physiological importance of plasma protein concentration and its susceptibility to environmental or physiological changes in the animal. Chisholm and Smith (1994) found a relation between the protein concentration and water temperature, showing low plasma concentrations when temperatures are in lowest and highest in the year. The concentrations of total proteins are also reported in moult cycle of shrimp. In *P. japonicus*, Chen and Cheng (1993) have reported lower levels of protein concentration during post moult stage as opposed to higher levels found in early premoult. Engel *et al.*, 1993) reported a negative effect of low levels of dissolved oxygen on haemocyanin concentration in serum of the blue crab. On the other hand, several immune molecules have been identified and purified in crustaceans such as LPS-binding protein (Vargas-Albores *et al.*, 1993b), Beta-glucan-binding protein (BGBP) (Vargas-Albores *et al.*, 1996), and clotting protein (CP) (Hall *et al.*, 1995; Montano-Perez *et al.*, 1998). In crayfish, some of these proteins are characterised at gene level; BGBP (Cerenius *et al.*, 1994) and CP (Hall *et al.*, 1999) are very similar to their homologues in shrimp. Specific antibodies would allow the use of ELISA technique to clinically estimate the presence of these proteins in shrimp plasma. Monoclonal antibodies are available against the clotting factor, alpha2M and agglutinin of *P. japonicus* (Rodriguez *et al.*, 1995) as well as polyclonal antibodies against Penaeid BGBP (Yepiz-Plascencia *et al.*, 1998).



Materials and Methods

3. MATERIAL AND METHODS

3.1 Experimental Animals

The species used for the present experiment was giant freshwater prawn, *Macrobrachium rosenbergii*, the systematic position of which is as follows:

Phylum	: Arthropoda
Class	: Crustacea
Order	: Decapoda
Family	: Palaemonidae
Genus	: <i>Macrobrachium</i>
Species	: <i>rosenbergii</i>

The juveniles of wild giant freshwater prawn, *M. rosenbergii* were collected from Kalu river, near Kalyan, in the state of Maharashtra with help of local fishermen. On reaching the wet laboratory, the prawns were disinfected by dipping in 50 ppm formalin and carefully transferred to a circular FRP tank (3000 L). Prawns were kept separately in aerated tanks provided with tiles for hiding and acclimatize to captive conditions for a period of 15 days and were fed twice a day with a commercial prawn feed.

3.2 Site of the Experiment

The experimental trials were conducted at the Central Institute of Fisheries Education (CIFE), Mumbai. The water quality, microbial, immunological and histological studies were carried out in the laboratories of Fish Pathology and Microbiology Department at CIFE, Mumbai.

3.3 Experimental Procedures

3.3.1 Maintenance of animals

The experimental animals were maintained in rectangular fibre tubs (100 L capacity) covered with perforated lids and the water used for rearing is drawn from bore well. The tubs were initially washed and filled with potassium permanganate solution (4 mg/L) and left overnight. The tubs were flushed out on the following day and were thoroughly washed with clean water and allowed to dry under the sunlight. Round the clock aeration was provided in all the experimental units from a centralized aeration unit to maintain the optimum level of dissolved oxygen as reported (Boyd and Zimmermann, 2000).

3.3.2 Experimental design

Experiment I: Influence of *B. licheniformis* on microbiological and immunological parameters of *M. rosenbergii*.

Two hundred and twenty five of inter-moult juveniles of *M. rosenbergii* were randomly distributed into five distinct experimental groups. Each group was having three replicates designed following a completely randomized design (CRD). Fifteen prawns with a weight of 4-6 g were stocked in each tub. Prawns in five groups were used to evaluate the effect of *B. licheniformis* on microbial and immunological parameters. The trial was conducted for a period of 45 days. Prawns were sampled on 0, 15, 30 and 45th day for microbial analysis and on 45th day for immunological parameters.

Experiment II: Cumulative mortality against bacterial infection.

Two hundred and seventy prawns of inter-moult juveniles of *M. rosenbergii* were randomly distributed into six distinct experimental groups. Each group was having three replicates designed following a completely randomized design (CRD). Fifteen prawns with a weight of 4-6 g were stocked in each tub. Prawns from six groups used to evaluate cumulative mortality, clearance efficiency and histopathological changes against *Vibrio alginolyticus* infection. After 45 days of

feeding all the treatment groups were challenged with *V. alginolyticus*. The experimental trial was conducted for a total period of 49 days.

Experiment III: Influence of *B. licheniformis* on growth and survival in *M. rosenbergii*.

One hundred and fifty of inter-moult juveniles of *M. rosenbergii* were randomly distributed into five distinct experimental groups. Ten prawns with an average weight of 3.61 ± 0.32 g (Mean \pm SE) were stocked in each tub. The experimental trial was conducted for a period of 60 days. Prawns were anesthetized with clove oil and the total average body weights were taken for every 20 day intervals to assess the growth. The prawns were starved overnight before taking the weight.

Treatment groups T₁, T₂, T₃ and T₄ were fed with four different concentrations of *B. licheniformis* and control (C) were fed with feed without *B. licheniformis*.

The experimental groups were as follows:

- C : Control (without *B. licheniformis*)
- T₁ : 1.0×10^6 cfu g⁻¹ of diet
- T₂ : 1.0×10^7 cfu g⁻¹ of diet
- T₃ : 1.0×10^8 cfu g⁻¹ of diet
- T₄ : 1.0×10^9 cfu g⁻¹ of diet

3.3.3 Experimental set-up

Experiment I: The trial was done for a period of 45 days in the hatchery complex of Aquaculture Division of CIFE, Mumbai. The set up consisted of 15 tubs (100 L capacity) covered with perforated lids. Two hundred and twenty five of inter-moult juveniles of *M. rosenbergii* were randomly distributed into five distinct experimental groups, with each group having three tubs following a completely randomized design (CRD) and each tub with fifteen prawns. The total volume of the water in each tub was maintained at 75 L throughout the experimental period. Round

the clock aeration was provided. The aeration pipe in each tub was provided with an air stone and a plastic regulator to control the air pressure uniformly in all the tubs. The experimental conditions were kept same throughout the experiment.

Experiment II: The trial was done for a period of 49 days in the hatchery complex of Aquaculture Division. The set up consisted of 18 tubs (100 L capacity) covered with perforated lids. Two hundred and seventy of inter-moult juveniles of *M. rosenbergii* were randomly distributed in to six distinct experimental groups, with each group having three tubs following a completely randomized design (CRD) and each tub with fifteen prawns.

Experiment III: The trial was done for a period of 60 days in the hatchery complex of Aquaculture division. One hundred and fifty Inter-moult juveniles of *M. rosenbergii* were randomly divided into five groups with 3 tubs per treatment and each tub with ten prawns.

3.4 Chemicals and Glasswares

The glasswares used throughout the experiment were neutral glass of Borosil make. Enzyme kinetic studies and the absorbance of the solutions was measured using single beam spectrometer (Libra S32 PC, Biochrom Ltd., England). Chemicals used were of various companies viz. Sigma, HiMedia and Qualigens Fine.

3.5 Cleaning and Siphoning

The experimental tubs were cleaned manually and siphoning was done every day in order to remove the excess feed pellets and remaining faecal matter. An equal volume of clean fresh water replaced the siphoned water. This was carried out throughout the experimental period.

3.6 Preparation of *B. licheniformis* Culture

The pure bacterial isolate of *B. licheniformis* was received from Microbial Type Culture Collection (MTCC), Chandigarh and was maintained at 4 °C. Subsequently, the bacteria were inoculated into conical flask containing Brain Heart

Infusion (BHI) broth (HiMedia) and kept in BOD incubator for 24 h at 30 °C. After which a loopful of bacterial culture was streaked on Tryptone Soya Agar (TSA) (HiMedia) plate.

The bacterial colonies, which grew on the TSA, after 24 h of incubation at 30 °C, were irregular, wrinkled or mucoid in appearance. The colonies were confirmed as pure isolate of *B. licheniformis* by performing the essential biochemical tests for confirmation and the culture was used for mass culture for subsequent use in the experiment.

3.6.1 Quantification of *B. licheniformis* for inoculating into feed

To determine the concentrations of the bacterial inoculums to be added into the feed for the experiments, bacteria was streaked on TSA plates and incubated for 12 h at 30 °C. One colony was picked and transferred into 50 ml of BHI broth, and incubated under the same conditions for 4 h. A third transfer was carried out into 100 ml, under same conditions. Then optical density (O.D) of the bacterial samples was recorded at 600 nm. Simultaneously serial dilutions were performed for each hour. The dilutions were plated onto the TSA agar by spread plate technique. After 12 h of incubation at 28 °C colonies were counted. The data were related in graphs, obtaining the relationship cfu vs. OD₆₀₀ vs. time. Based on this the bacteria was added to the feed at different concentrations.

3.6.2 Mass culture of *B. licheniformis*

The pure culture of *B. licheniformis* was inoculated into BHI broth in a 500 ml conical flask, and incubated at 30° C for 24 h in a shaking incubator. The culture was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was discarded, while the pellet was re-suspended in phosphate buffer saline (pH 7.2). The bacterial pellets were washed and centrifuged similarly and then mixed in phosphate buffer saline at different concentrations as required and added to 100 g of feed.

3.7 Formulation and Preparation of Experimental Diets

Commercial ingredients such as fish meal, soya bean flour, wheat flour, casein, sunflower oil, vitamin-mineral mixture, cholesterol, vitamin C, butylated hydroxy toluene (BHT), cellulose, and cod liver oil were taken for feed formulation (Table-1). A total of five experimental diets were prepared, with four probiotics enriched diets having different concentrations of *B. licheniformis*, and a control. The diets were control (Basal diet), T₁ (1.0×10^6 cfu g⁻¹), T₂ (1.0×10^7 cfu g⁻¹), T₃ (1.0×10^8 cfu g⁻¹), and T₄ (1.0×10^9 cfu g⁻¹). All the experimental diets were prepared regularly at fortnight intervals *i.e.* once in a week.

All the ingredients were weighed properly as per the requirement and kept in a big plastic container. The required mixed ingredients were then mixed to form dough with the addition of necessary quantity of water. When the dough was formed, the calculated concentration of the oils were added and mixed well. The dough was then transferred to an aluminium container, which was then placed in a pressure cooker for cooking. The cooking was done for half an hour. The pressure cooker was then removed from the flame and kept aside for cooling. The steamed dough was taken out and was cooled further. When the steamed dough was completely cooled, vitamins and mineral mixture along with the *B. licheniformis* were added so as to prevent their loss. After incorporation of these elements, the dough was mixed properly and pressed through a hand pelletizer to get uniform sized pellets. These pellets were spread on a sheet of paper and initially fan dried. After that the feed was transferred to trays and kept in sunlight for complete drying. After drying, pellets were packed in polythene bags, sealed airtight and labelled according to the treatments and kept at 4 °C until use.

3.8 Feeding

Feeding was done @ 3 % of the body weight. The daily ration was divided into two equal parts and was fed at 0900 h in the morning and 1800 h in the evening. The unconsumed feed and faecal matter was removed twice a day by siphoning.

Table No- 1: Composition of experimental diets

INGREDIENTS	Experimental diets
	Amount per Kg
Fish meal	250g
Soya bean flour	310g
Wheat flour	300g
Caesin ¹	30g
Sunflower oil	60ml
Vitamin-Mineral mix ³	20g
Cholestrol ¹	5g
Cod liver oil	20ml
Vitamin-C ²	2g
BHT ¹	3g
Cellulose ¹	20g

¹ Casein Vitamin free (84% CP), Cholesterol (5-Cholesten-3B-ol), Cellulose powder, and Butylated Hydroxy Toluene (BHT) - 2,6-Di-tert-Butyl-p-cresol, Betaine - Make of HIMEDIA Laboratories pvt.Ltd, Mumbai.

² Rovimix Stay-C 35 (Roche), L-Ascorbate Triphosphate - Make of S.D-Fine Chemicals, Mumbai

³ Composition of Vitamin-Mineral mix (Agrimin) (quantity /kg1) Vitamin A-6,25,000 IU; Vitamin D3-62,500 IU; Vitamin E-250 mg; Nicotinamide-1 g; Cu-312 mg; Co-45 mg; Mg-6 g; Fe-1.5 g; Zn-2.13 g; I-156 mg; Se-10 mg; Mn-1.2 g; Ca-247.34 g; P-114.68; S-12.2 g; S-12.2 g; Na-5.8mg; K-48.05 mg.

3.9 Physico-chemical Parameters of Water

Water quality parameters viz. Temperature, pH, dissolved oxygen and free carbon dioxide were monitored following the standard methods outlined in APHA (Anon, 1998).

3.10 Growth and Survival

The growth parameters of the prawn juveniles were assessed by taking their average body weight at 20 days intervals. The prawns were kept starved overnight before measurement of body weight. The weight was taken with an electric balance (Sartorius, Germany). The growth performance was assessed using the following formulae

3.10.1 Percentage weight gain

$$\text{Percentage weight gain} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

3.10.2 Specific growth rate (SGR)

$$\text{Specific growth rate} = \frac{\ln(\text{Final weight}) - \ln(\text{Initial weight})}{\text{Experimental periods in days}} \times 100$$

3.10.3 Food conversion ratio (FCR)

$$\text{Food conversion ratio} = \frac{\text{Feed given (dry weight)}}{\text{Body weight gain (wet weight)}}$$

3.10.4 Feed efficiency ratio (FER)

$$\text{Feed efficiency ratio} = \frac{\text{Net weight gain (wet weight)}}{\text{Feed given (dry weight)}}$$

3.10.5 Survival rate

Survival rate was calculated at the end of the experiment by counting the number of prawns in each tub and is calculated as follows

$$\text{Survival (\%)} = \frac{\text{Total number of animal harvested}}{\text{Total number stocked}} \times 100$$

3.11 Survivability of Added Probiotics in Feed

Cultivable count of probiotic micro-organisms in feed was done by total aerobic plate count method using spread plate techniques. *Bacillus* medium was used for counting of *B. licheniformis*. 1 g of feed was taken along with 9 ml of physiological saline in a sterile glass tube and this mixture is plated on *Bacillus* medium. The plating was done in duplicate for six different dilutions. Colonies were counted after 24 h at 28 °C and expressed in cfu g⁻¹ of feed. Survival was checked on 0th, 15th and 30th day after feed preparation.

3.12 Analysis of Gastro-intestinal Microflora

The gastro-intestinal microflora analysis was done on 0th and on 15th, 30th and 45th day of post feeding. Nine prawns were randomly selected from each treatment (i.e. three prawns from each replicate group) and collected in sterile plastic bags. The prawns were starved overnight before the sampling.

3.12.1 Sample preparation

The surfaces of prawns were sterilized by the application of alcohol and standard aseptic procedures were used to make the biopsy. Gut (Intestine + Hepatopancreas) was aseptically taken out from each prawn. The gut of all three prawns in each replicate of a treatment was weighed equally to make 1.0 g of sample and homogenized. This was then transferred to tubes containing 9.0 ml sterile 0.85% saline. The homogenates were serially diluted to 10⁻⁶ in 9 ml volumes of sterile 0.85% saline.

3.12.2 Media and enumeration procedures

Media for bacterial analysis were obtained from HiMedia. The solid media was employed and the bacteria enumerated were as follows: Nutrient agar (total bacterial count), *Bacillus* medium (for *Bacillus* count), TCBS agar (for selective isolation of *Vibrio*), EMB agar (for members of family Enterobacteriaceae), *Aeromonas* isolation medium base (for selective isolation of *Aeromonas*), *Pseudomonas* isolation media (for selective isolation of *Pseudomonas*).

The isolation was made by serial dilution and spread plate technique. 0.1 ml of diluted sample was plated on the appropriate enriched medium. The plates were incubated at 28 °C for 24-48 h in BOD incubator. After the required incubation period the plates were taken out and reported as an average of duplicate plates. The plates with 30-300 colonies were taken for total bacterial count and for the remaining bacteria the plates having colonies from lowest dilution were taken for enumeration. Counts were expressed as cfu g⁻¹ and transformed into log₁₀ values before statistical analysis.

3.12.3 Isolation and confirmation

The isolation was made by picking different individual colonies from plates. These were then streaked on nutrient agar plates. The plates were incubated at 28 °C for 24-48 h. The confirmation was done by morphological and biochemical tests, which was obtained from the Bergy's Manual of Systemic Bacteriology (Bergy, 1986).

3.13. Analysis of Immunological Parameters

3.13.1 Sampling procedure

At the end of the experimental trials, prawns were anesthetized using 300 mg/l clove oil (Coyle *et al.*, 2005). Immediately after the sedation, the haemolymph was collected and the prawns were killed by giving hypothermic shock. The haemolymph of prawns from the same tub were pooled and used for sample preparation.

3.13.2 Collection of haemolymph

Immediately after anesthetizing prawn, the haemolymph was drawn directly from the heart using 2 ml syringe with 26 G needle. Then haemolymph was transferred into a eppendorf tube containing 100 μ l anticoagulant (10 mM Tris-HCl, 250 mM Sucrose, 100 mM Sodium citrate, pH 7.6) to prevent clotting. The haemolymph from the prawns of same tub were pooled together. More amount of anticoagulant was added to make an equal volume ratio of haemolymph to anticoagulant. A volume of 50 μ l anticoagulated haemolymph was fixed with an equal volume of neutral buffered formalin (10%) for 30 min to measure the total haemocyte count (THC). A volume of 50 μ l was used for respiratory burst test. The remaining anticoagulated haemolymph was centrifuged at 2,650 X g for 10 min and the supernatants were used for the analysis of immunological parameters (Li *et al.*, 2007).

3.13.3 Total haemocyte count (THC)

Fixed haemolymph was diluted 2, 4, 6, 8, 16 and 32 times with ice-cold phosphate buffer saline (PBS, pH 7.2). Total haemocytes were counted using an improved Neubauer Haemocytometer and light microscope (Luica, Japan) at 40 X. Haemocytometer counts were made for 5/25 squares (volume of each square = $0.2 \times 0.2 \times 0.1 \text{ mm}^3$) to calculate THC ml^{-1} haemolymph. THC was calculated by the formula given by Sritunyalucksana *et al.* (2005).

$$\text{THC} = 5 \times \text{count} \times 10^4 \times \text{dilution factor}$$

3.13.4 Phenol oxidase (PO) activity

PO activity was quantified by following the method described by Tanner *et al.* (2006). The PO activity was measured by monitoring the rate of formation of dopachrome from L-3,4-dihydroxyphenylalanine (L-DOPA) in a colorimetric assay. In a 1 ml cuvette, 100 μ l of sample was combined with 25 μ l 10 % SDS and allowed to incubate for 15 min at room temperature. Then, 875 μ l of the L-DOPA solution (1 mg of L-DOPA/ml in 100 mM sodium phosphate, at the test pH) was added to the cuvette. The cuvette was immediately capped, inverted, blanked on

its own absorbance and monitored for changes in optical density at 475 nm (OD_{475}) in a spectrophotometer over 5 min. PO activity was recorded as the maximum change in absorbance over any one min interval (OD_{475}) during the first 5 min of the assay.

3.13.5 Superoxide dismutase (SOD) activity

Superoxide dismutase was assayed according to the method described (Misra and Fridovich, 1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme. 100 μ l of sample was taken into the cuvette and add 1.5 ml (0.1 M) Carbonate-bicarbonate buffer containing 57 mg/dl EDTA (pH 10.2) and 0.5 ml epinephrine (3 mM) and mixed well and read the change in optical density at 480 nm immediately for 2 min in a spectrophotometer. One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation.

3.13.6 Alkaline phosphatase (ALP)

The haemolymph alkaline phosphatase activity was quantified by using alkaline phosphatase kit (Qualigens Fine Chemicals, Mumbai, India). The amount of ALP was expressed in IU/L.

3.13.7 Acid phosphatase (ACP)

The haemolymph acid phosphatase activity was quantified by using acid phosphatase kit (Qualigens Fine Chemicals, Mumbai, India). The amount of ACP was expressed in KA units.

3.13.8 Haemolymph total proteins

Total protein was estimated using total protein kit (Biuret and BCG dye binding method, Qualigens Fine Chemicals, Mumbai, India).

3.13.9 Haemolymph glucose

Glucose in haemolymph was estimated by glucose oxidase/ peroxidase method using glucose kit (GOD/POD method, Qualigens Fine Chemicals, Mumbai,

India). Glucose present in haemolymph is oxidised by enzyme glucose oxidase to give D-gluconic acid and hydrogen peroxide. The hydrogen peroxide in the presence of enzyme peroxidase oxidizes phenol which combines with 4-aminoantipyrine to produce a red coloured quinoneimine dye. The intensity of the colour developed is proportional to glucose concentration. The absorbance was recorded at 505 nm against blank. The amount of glucose was expressed in mg%.

3.13.10 Haemolymph urea level

The haemolymph urea was quantified by using urea kit (Qualigens Fine Chemicals, Mumbai, India). The amount of urea was expressed as mg/dl.

3.13.11 Nitroblue tetrazolium (NBT) assay

Nitroblue tetrazolium assay was done by the method described by Stasiack and Baumann (1996). Fifty microlitres of haemolymph was placed into the wells of 'U' bottom microtitre plates and incubated at 37⁰ C for 1 h to facilitate adhesion of cells. Then the supernatant was removed and the loaded wells were washed three times in phosphate buffer saline (PBS). After washing, 50 µl of 0.2 % NBT was added and plate was incubated for further 1 h. The cells were then fixed with 100 % methanol for 2-3 min and again washed thrice with 70 % methanol. The plates were then air dried. Then 120 µl of 2 N potassium hydroxide (KOH) and 140 µl of dimethyl sulphoxide (DMSO) were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue coloured solution was then read in ELISA reader at 620 nm.

3.13.12 Antibacterial and bacteriolytic activities of haemolymph

Bacteriolytic activity was measured using *E. coli* and antibacterial activity was measured with *V. alginolyticus*, according to method of Hultmark *et al.* (1980). The bacteria were cultured in BHI broth for 18-20 h at 30⁰ C was harvested and washed twice in PBS and suspended in the PBS to O.D₅₇₀ = 0.3. Each bacterial suspension (3 ml) was mixed with 50 µl haemolymph in tubes in ice water (0⁰ C) and the optical density at 570 nm (A₅₇₀) was measured. The tubes were transferred to a water bath at 37⁰ C for 30 min, then returned to ice water (0⁰ C) for

10 min to stop the reaction and the optical density at 570 nm (A) was measured again.

The antibacterial activity was calculated as follows

$$U_a^2 = (A_0 - A) / A$$

The bacteriolytic activity was calculated as follows

$$U_b = (A_0 - A) / A$$

3.13.13 Bacterial agglutination titre

The bacterial agglutination titre was performed following Plumb and Areechon (1990). *V. alginolyticus* was grown BHI (supplemented with 1.5% NaCl) broth for 24 h at 30° C was harvested and washed twice in PBS. Bacteria was killed with 1% formalin, washed twice in PBS and checked for sterility by streaking on Tryptone Soya Agar. The bacteria were then diluted to 2×10^6 cells ml⁻¹.

Haemolymph sample was serially diluted in two fold steps with PBS using 5 µl of sample and PBS in microtiter plate. An equal volume of formalin killed *V. alginolyticus* was added to each well. Then the plates were incubated overnight at room temperature. The agglutination titre was determined at the highest dilution of haemolymph sample at which visible agglutination occurred and expressed as the reciprocal of that dilution.

3.14 Susceptibility of *M. rosenbergii* to *V. alginolyticus*

The pure bacterial isolate of pathogenic *V. alginolyticus* strain was procured from MTCC, Chandigarh and was maintained at 4 °C. Subsequently, the bacteria were inoculated into conical flask containing BHI (supplemented with 1.5% NaCl) broth (HiMedia) and kept in BOD incubator for 24 h at 30 °C. After which a loopful of bacterial culture was streaked on Tryptone Soya Agar, (HiMedia) supplemented with 1.5% NaCl) before being transferred to 100 ml BHI broth (supplemented with 1.5% NaCl), where it remained for 24 h at 28 °C. The broth culture was centrifuged at 1000 rpm for 10 min at 4 °C. The supernatant fluid was

discarded and the bacterial pellet was re-suspended in a saline solution at 1×10^6 cfu ml⁻¹ for susceptibility study.

Challenge trials were carried out in *M. rosenbergii* juveniles following 45 days of being fed *B. licheniformis* containing diets and control diet. On 46th day the anesthetized prawns in all treatments were injected with 20 µl of bacterial solution containing 1×10^6 cfu ml⁻¹ into the 2nd abdominal segment. Prawns fed with the control diet and then injected with same quantity of saline (20µl) served as unchallenged control. Total 10 prawns in each tub were injected, and each treatment used 30 prawns. The water was renewed daily, and the post infection experiment lasted for 96 h (4 days). Shrimp was fed twice daily with the experimental diets after challenge. The daily percent mortality was recorded every day. The cause of mortality was further confirmed by re-isolating the organism from moribund prawn.

3.15 Clearance Efficiency of *M. rosenbergii* to *V. alginolyticus*

Twenty microliter of bacterial suspension (1×10^4 cfu ml⁻¹) was injected into the prawn for the clearance efficiency study. After injection, the prawns were kept for 1 h in a separate tub. Then the haemolymph was collected from the heart with anticoagulant. Test was carried out on two prawns in each tub, and each treatment used 6 prawns.

Clearance efficiency was measured following the method of Adams (1991). The haemolymph with anticoagulant was further diluted with 20 ml of saline. 50 µl of this diluted haemolymph sample was spread plated on tryptone soya agar and incubated at 28 °C for 24 h before colonies were counted using a colony counter. The number of colonies from prawn that received saline was control values, and the number of colonies from the prawn that received experimental diets was the test values. Clearance efficiency to *V. alginolyticus*, defined as percentage inhibition (PI), was calculated as:

$$PI = 100 - \frac{\text{cfu in test group}}{\text{cfu in saline group}} \times 100$$

3.16 Samples for Histological Analysis

The gills and hepatopancreas were fixed in Davidson's fixative (330 ml 95% ethyl alcohol, 220 ml 100% formalin, 115 ml glacial acetic acid and made up to 1000 ml using distilled water) for 48 h and then transferred to 50% ethanol and 70% ethanol and finally stored in 70% ethanol until blocks were prepared. Hepatopancreas after 45 days of feeding was taken for histological studies and compared with the control group. For histopathological studies gills and hepatopancreas of 2nd and 4th day post infection of all experimental groups were taken and compared with that of healthy prawn.

3.16.1 Tissue processing, block making and sectioning of samples

Preserved samples of *M. rosenbergii* were dehydrated in increasing concentrations (70 and 90%) of alcohol for an hour followed by absolute alcohol (thrice) for 45 min each. The samples were transferred into alcohol acetone mixture (1:1), acetone and finally into acetone xylene (1:1) for 45 min each. Then cleared in xylene (twice) for 30 min and infiltration is done in hot paraffin 60 °C (thrice) for 45 minutes. The samples were then blocked and were allowed to cool at 4 °C for a day. Blocks were sectioned on a rotatory microtome at 5 µm and mounted on slide coated with egg albumin.

3.16.2 H & E staining

The sections were deparaffinized in xylene and rehydrated serially in alcohol (absolute, 90%, 70%, 50%) and finally in distilled water. The sections were stained in Haematoxylin for 3 minutes, and washed in tap water. In case of excess staining, excess stain was removed by dipping in 1 % acid alcohol and washed in tap water, followed by Scott's tap water. The section was serially dehydrated through 50%, 70%, 90% alcohol and allowed to stain in Eosin for 30 S and again dehydrated by dipping in absolute alcohol for 1 minute. Then stained sections were kept in xylene for 1 minute each and mounted with DPX. Prepared slides were examined under a light microscope (10 X and 40 X magnifications) and photographed. The histological observations were carried out as per Bell & Lightner (1988).

3.17. Statistical Analysis

All the data, thus generated were analysed by running one way ANOVA using SPSS V. 16 software (SPSS Inc., Chicago, Illinois, USA). The means were compared using Duncan multiple range test to find the difference at 5% ($P < 0.005$) level.



Results

4. RESULTS

4.1 Physico-chemical Parameters of Water

There was no much variation in the average value of water quality parameters in different experimental units during the entire period of study. The water temperature, throughout the experimental period, was maintained around 26 °C to 28 °C. Dissolved oxygen ranged from 6.2 to 7.1 mg/L, while pH varied between 7.18 to 7.89 respectively. The free CO₂ was not detectable during the entire experimental period.

4.2 Growth Parameters

4.2.1 Average body weight

Data pertaining to the weight of the experimental groups recorded at 20 days intervals are shown in Table-2. Initial body weight among the experimental groups varied from 3.31±0.01 (C) to 3.84±0.02 (T₃). The final body weight ranged from 5.44±0.07 (C) to 7.63±0.11 (T₄).

4.2.2 Percentage weight gain

Data pertaining to the body weight gain is expressed in percentage to nullify the initial variation in body weight and is given in Table-3 and graphically presented in Fig. 1. The weight gain (%) was found to be significantly different (P<0.05) among the various experimental groups. The highest weight gain (%) was recorded in T₄ (112.25±3.76) group and the lowest weight gain% was recorded in control group (64.53±2.41).

4.2.3 Specific growth rate (SGR)

Data pertaining to the specific growth rate is presented in the Table-3 and Fig. 1. There was a significant difference (P<0.05) in the specific growth rate among the different stock groups. The lowest SGR was found in control group (0.36±0.01), while the highest was found in T₄ (0.54±0.01).

4.2.4 Feed conversion ratio (FCR)

The FCR values are given in Table-3 and Fig. 1. FCR of different groups varied significantly ($P < 0.05$). The lowest FCR was recorded in T_4 (2.14 ± 0.05), which was significantly different from the other groups. The highest FCR was observed in control group (3.10 ± 0.10).

4.2.5 Feed efficiency ratio (FER)

The FER values of the experimental groups are given in Table-3 and Fig. 1. The mean FER values among the experimental groups varied significantly ($P < 0.05$). The highest FER was observed in T_4 (0.47 ± 0.02) group. The FER was lowest in control (0.32 ± 0.02) group.

4.2.6 Survival rate (%)

The survival rates of the prawn juveniles at the end of the experiment are presented in Table-4 and Fig. 2. The survival rate ranges from 60 ± 0.00 (C) to 83.33 ± 3.3 % (T_4). There was significant difference in survival rate among the treatments.

Table-2: Average body weight (g) of *M. rosenbergii* juveniles of different experimental groups at 20 days intervals

Treatments	Periods (Days)			
	Initial	0-20	0-40	0-60
C	3.31 ^a ±0.01	3.48 ^a ±0.04	4.22 ^a ±0.06	5.44 ^a ±0.07
T₁	3.59 ^a ±0.01	4.13 ^a ±0.08	5.23 ^a ±0.08	6.47 ^a ±0.08
T₂	3.71 ^a ±0.12	4.32 ^a ±0.09	5.63 ^a ±0.09	6.86 ^a ±0.10
T₃	3.84 ^a ±0.02	4.53 ^a ±0.11	5.97 ^a ±0.11	7.37 ^a ±0.10
T₄	3.60 ^a ±0.08	4.51 ^a ±0.12	6.25 ^a ±0.12	7.63 ^a ±0.11

Mean Values (n= 3) in rows bearing different superscript vary significantly (p < 0.05). Data expressed as Mean±S.E.

Table-3: Weight gain (%), Specific growth rate (SGR), Feed conversion ratio (FCR), and Feed efficiency ratio (FER) of *M. rosenbergii* juveniles fed with different experimental diets

Treatments	Weight gain (%)	SGR	FCR	FER
C	64.53 ^a ±2.41	0.36 ^a ±0.01	3.10 ^d ±0.10	0.32 ^a ±0.02
T₁	80.23 ^b ±1.95	0.43 ^b ±0.01	2.70 ^c ±0.03	0.37 ^b ±0.01
T₂	84.98 ^{bc} ±3.40	0.44 ^{bc} ±0.01	2.61 ^{bc} ±0.08	0.38 ^{bc} ±0.02
T₃	92.09 ^c ±1.87	0.47 ^c ±0.01	2.44 ^b ±0.02	0.41 ^c ±0.01
T₄	112.25 ^d ±3.76	0.54 ^d ±0.01	2.14 ^a ±0.05	0.47 ^d ±0.02

Mean Values (n= 3) in rows bearing different superscript vary significantly (p < 0.05). SGR - Specific Growth Rate, FCR - Feed Conversion ratio, FCE - Feed Conversion Efficiency

Fig. 1: Weight gain (%), Specific growth rate (SGR), Feed conversion ratio (FCR), and Feed efficiency ratio (FER) of *M. rosenbergii* juveniles fed with different experimental diets

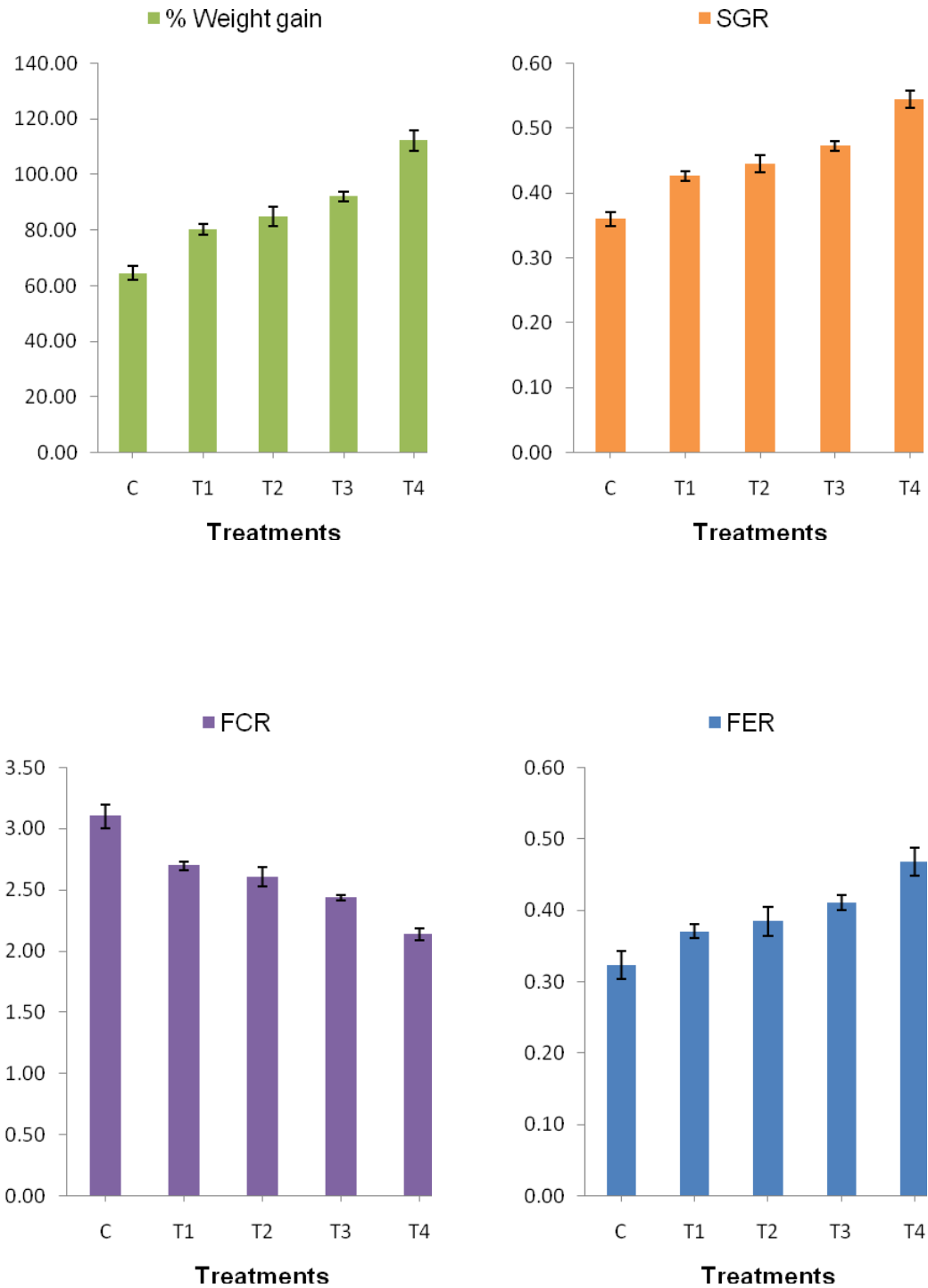
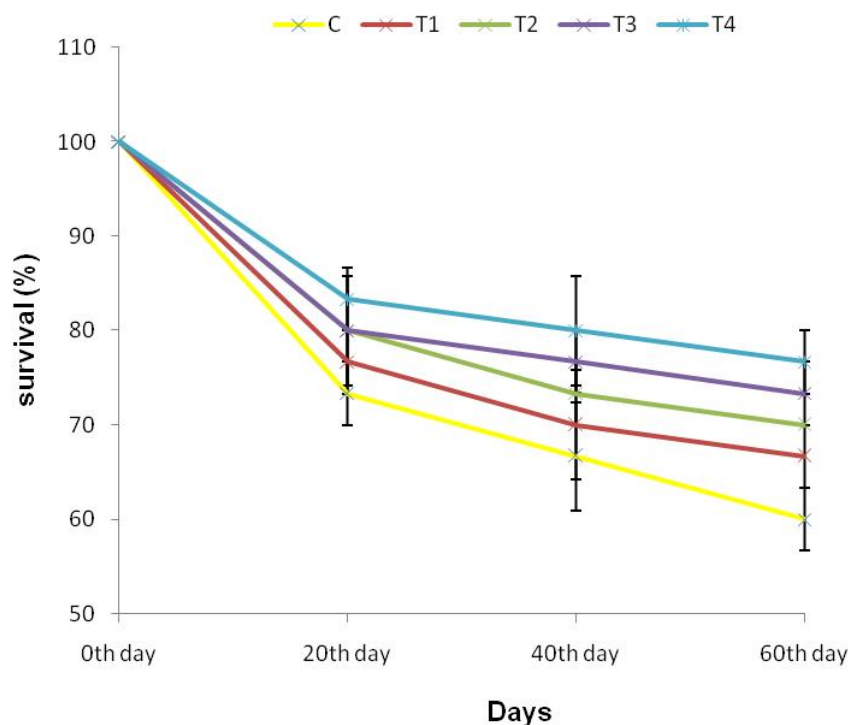


Table-4: Percentage survival of prawn (% Survival \pm SE) at 20 days interval of experimental study

Treatments	20 days	40 days	60 days
C	73.33 ^a \pm 3.3	66.67 ^a \pm 3.3	60.00 ^a \pm 0.0
T₁	76.67 ^a \pm 3.3	70.00 ^a \pm 5.8	66.67 ^{ab} \pm 3.3
T₂	80.00 ^a \pm 5.8	73.33 ^a \pm 3.3	70.00 ^{bc} \pm 0.0
T₃	80.00 ^a \pm 5.8	76.67 ^a \pm 3.3	73.33 ^{bc} \pm 3.3
T₄	83.33 ^a \pm 3.3	80.00 ^a \pm 5.8	76.67 ^c \pm 3.3

Mean Values (n= 3) in rows bearing different superscript vary significantly (p < 0.05). Data expressed as Mean \pm S.E.

Fig. 2: Percentage survival of prawn at 20 days interval during experimental Study



4.3 Survivability of added probiotics in feed

Different experimental diets were assessed for viability of *B. licheniformis* cells on 0th, 15th and 30th days which are shown in Table-5 and Figures 3 and 4. It was observed that on 15th day, the highest survival of *B. licheniformis* cells was found in T₃ (77%), while the lowest survival was recorded in T₁ (64%). On the 30th day of experimentation, the highest count was observed in T₁ (38%) while the lowest survival was noticed in T₂ (32%) respectively.

Table-5: No. of added probiotic (*Bacillus licheniformis*) cells survived in different experimental diets

Probiotic	C	T ₁	T ₂	T ₃	T ₄
0 th day cfu	0	1x10 ⁶	1x10 ⁷	1x10 ⁸	1x10 ⁹
% survival	0	100	100	100	100
15 th day cfu	0	6.4x10 ⁵	6.5x10 ⁶	7.7x10 ⁷	7.5x10 ⁸
% survival	0	64	65	77	75
30 th day cfu	0	3.8x10 ⁵	3.2x10 ⁶	3.7x10 ⁷	3.4x10 ⁸
% survival	0	38	32	37	34

Unit: cfu g⁻¹ of feed

Fig. 3: Survival of *B. licheniformis* added in feed at different levels observed at 15 days interval

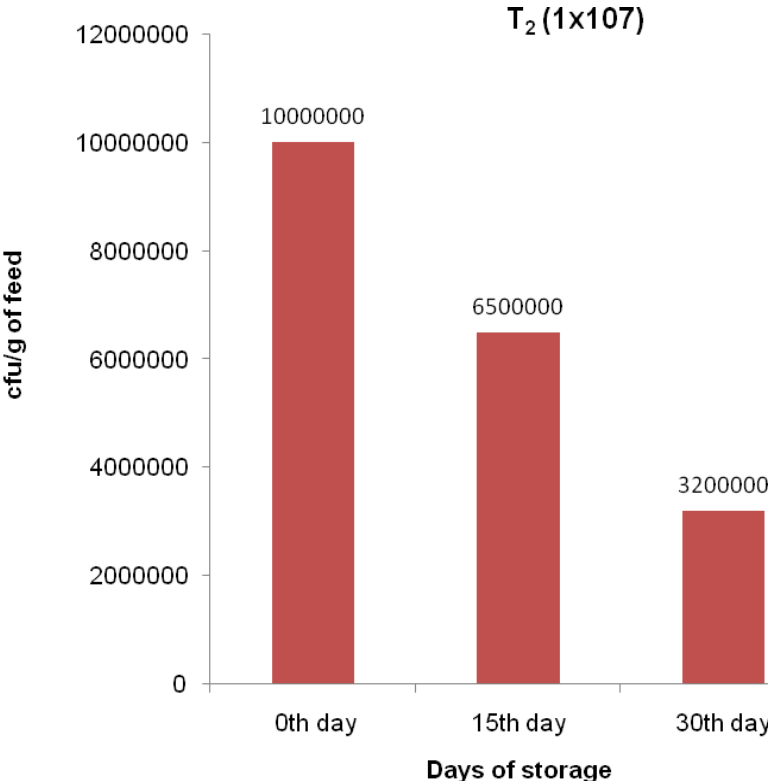
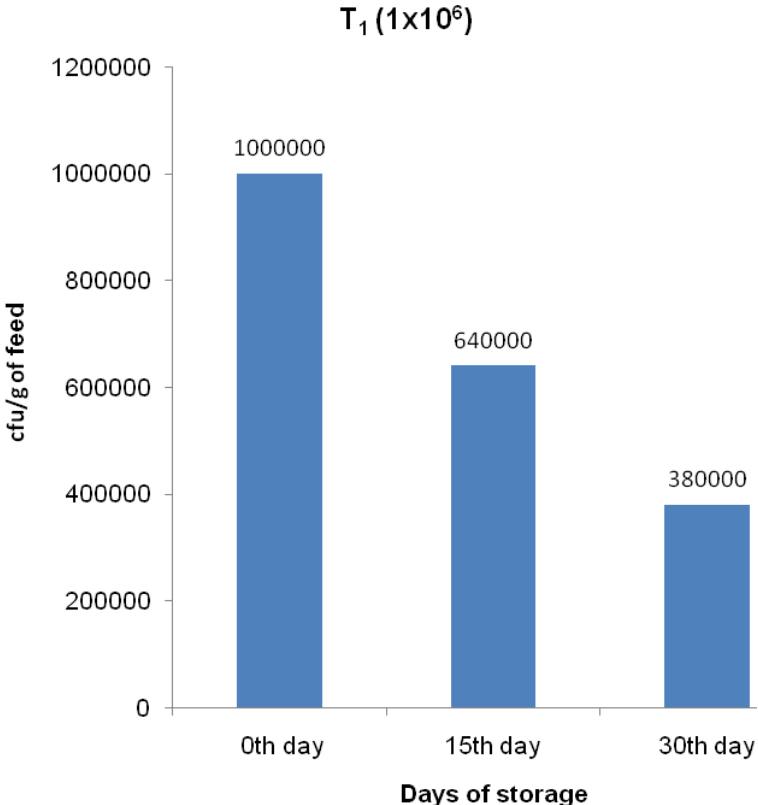
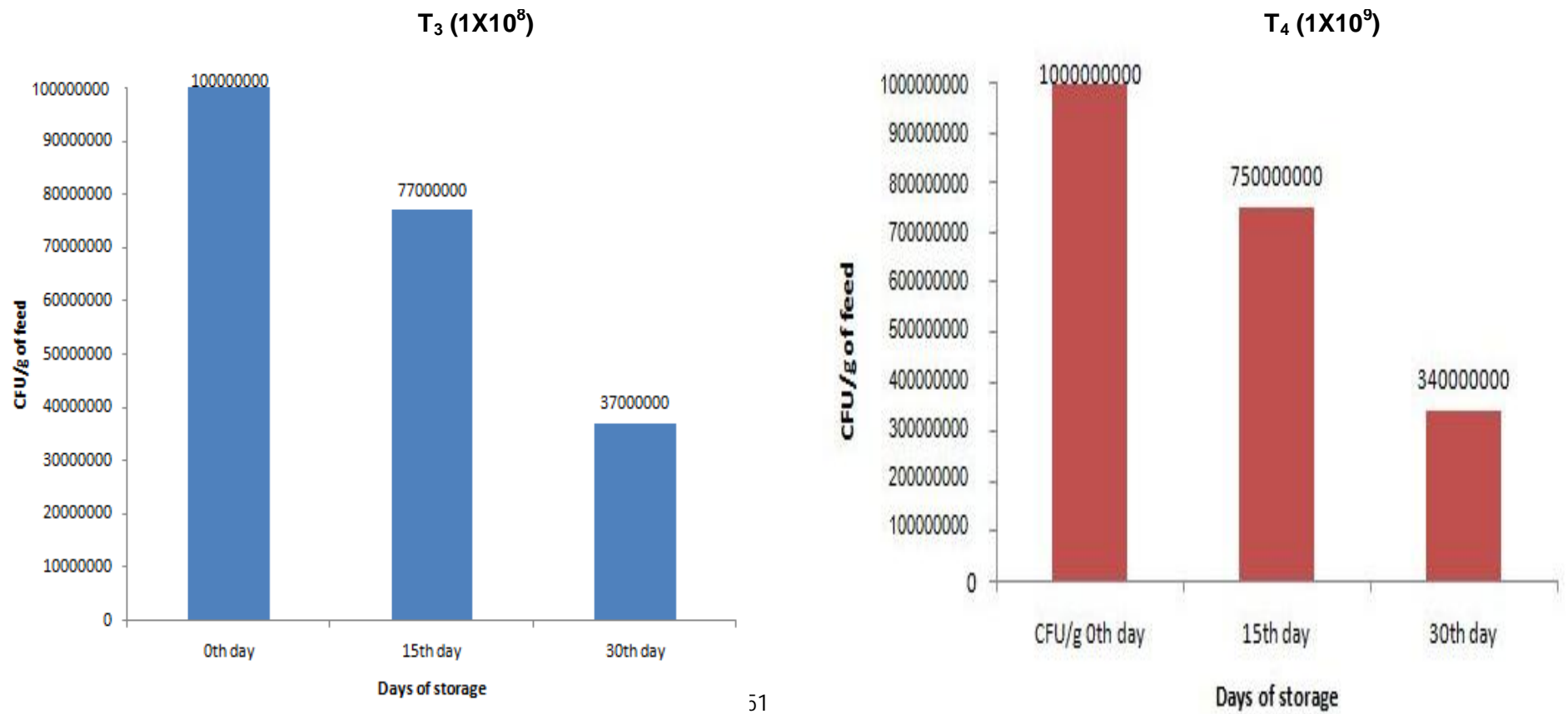


Fig. 4: Survival of *B. licheniformis* added in feed at different levels observed at 15 days interval



4.4 Effect of *B. licheniformis* on the gut microflora of *M. rosenbergii*

Data pertaining to different bacterial counts from the gut of *M. rosenbergii* on various sampling days are presented in Tables 6-9 and in Fig. 5-10. It was found that total bacterial counts in all treatment groups increased gradually in the final sampling when compared to that of initial sampling. Initially the total bacterial count was higher in T₃ (\log_{10} cfu g⁻¹ 6.78±0.58), but on 45th day the total bacterial count was found to be higher in T₄ (\log_{10} cfu g⁻¹ 8.59±0.57) group. *Bacillus* count initially was high in control group (\log_{10} cfu g⁻¹ 3.14±0.37), but finally higher count was observed in T₄ group (\log_{10} cfu g⁻¹ 4.57±0.33). *Vibrio* and Enterobacteriaceae counts do not differ much during initial and final sampling days, but there was decreased counts observed in final sampling. Besides, there was also decrease in counts of *Aeromonas* and *Pseudomonas* in final sampling compared to initial sampling in all treatments. No significant difference was observed for all bacterial groups among the various treatments on different sampling days.

In the first sampling, Enterobacteriaceae was found to be the dominating group in control as well as in treatments. Similarly, the second dominating genus in all the treatment groups was observed to be *Vibrio* in all the treatments. But at final sampling, T₂, T₃ and T₄ showed higher colonization of *Bacillus*. Though the table indicate the highest genera were Enterobacteriaceae members and *Vibrio* but it was reduced as compared to the first sampling. Also the *Aeromonas* and *Pseudomonas* counts decreased significantly.

Biochemical tests done to confirm the bacteria from the gut of *M. rosenbergii* are presented in Table-10.

Table-6: Effect of *B. licheniformis* on mean microbiological counts (\log_{10} cfu g^{-1}) on 0th day in the gut of *M. rosenbergii* fed with different experimental diets

Treatments	Total bacteria	<i>Bacillus</i>	<i>Vibrio</i>	Enterobacteriaceae	<i>Aeromonas</i>	<i>Pseudomonas</i>
C	6.76 ^a ±0.57	3.14 ^a ±0.37	4.45 ^a ±0.34	4.49 ^a ±0.34	3.76 ^a ±0.32	3.61 ^a ±0.33
T₁	6.77 ^a ±0.57	3.06 ^a ±0.34	4.44 ^a ±0.34	4.49 ^a ±0.34	3.79 ^a ±0.33	3.63 ^a ±0.33
T₂	6.60 ^a ±0.63	3.06 ^a ±0.37	4.46 ^a ±0.34	4.49 ^a ±0.34	3.81 ^a ±0.34	3.65 ^a ±0.33
T₃	6.78 ^a ±0.58	3.11 ^a ±0.35	4.49 ^a ±0.34	4.51 ^a ±0.34	3.82 ^a ±0.33	3.67 ^a ±0.33
T₄	6.71 ^a ±0.55	3.11 ^a ±0.38	4.50 ^a ±0.34	4.49 ^a ±0.34	3.84 ^a ±0.34	3.65 ^a ±0.34

Mean Values (n= 3) in rows bearing different superscript vary significantly (p < 0.05).
Data expressed as Mean±S.E.

Table-7: Effect of *B. licheniformis* on mean microbiological counts (\log_{10} cfu g^{-1}) on 15th day in the gut of *M. rosenbergii* fed with different experimental diets

Treatments	Total bacteria	<i>Bacillus</i>	<i>Vibrio</i>	Enterobacteriaceae	<i>Aeromonas</i>	<i>Pseudomonas</i>
C	7.04 ^a ±0.57	3.25 ^a ±0.38	4.46 ^a ±0.34	4.47 ^a ±0.34	3.70 ^a ±0.34	3.64 ^a ±0.31
T₁	7.10 ^a ±0.56	3.56 ^a ±0.32	4.40 ^a ±0.34	4.42 ^a ±0.34	3.62 ^a ±0.34	3.47 ^a ±0.31
T₂	7.20 ^a ±0.57	3.86 ^a ±0.34	4.43 ^a ±0.34	4.45 ^a ±0.34	3.60 ^a ±0.30	3.50 ^a ±0.31
T₃	7.36 ^a ±0.58	4.06 ^a ±0.32	4.45 ^a ±0.34	4.48 ^a ±0.34	3.67 ^a ±0.34	3.57 ^a ±0.30
T₄	7.80 ^a ±0.57	4.12 ^a ±0.35	4.48 ^a ±0.34	4.45 ^a ±0.34	3.69 ^a ±0.34	3.53 ^a ±0.32

Mean Values (n= 3) in rows bearing different superscript vary significantly (p < 0.05).
Data expressed as Mean±S.E.

Table-8: Effect of *B. licheniformis* on mean microbiological counts (\log_{10} cfu g^{-1}) on 30th day in the gut of *M. rosenbergii* fed with different experimental diets

Treatments	Total bacteria	<i>Bacillus</i>	<i>Vibrio</i>	Enterobacteriaceae	<i>Aeromonas</i>	<i>Pseudomonas</i>
C	7.35 ^a ±0.57	3.39 ^a ±0.34	4.47 ^a ±0.34	4.49 ^a ±0.35	3.74 ^a ±0.34	3.64 ^a ±0.40
T₁	7.43 ^a ±0.57	4.02 ^a ±0.35	4.37 ^a ±0.34	4.38 ^a ±0.34	3.48 ^a ±0.34	3.22 ^a ±0.39
T₂	7.79 ^a ±0.57	4.16 ^a ±0.35	4.38 ^a ±0.33	4.41 ^a ±0.34	3.51 ^a ±0.34	3.27 ^a ±0.39
T₃	8.18 ^a ±0.57	4.26 ^a ±0.37	4.42 ^a ±0.34	4.44 ^a ±0.34	3.53 ^a ±0.34	3.38 ^a ±0.38
T₄	8.34 ^a ±0.58	4.42 ^a ±0.34	4.41 ^a ±0.34	4.40 ^a ±0.33	3.56 ^a ±0.34	3.13 ^a ±0.36

Mean Values (n= 3) in rows bearing different superscript vary significantly ($p < 0.05$).
Data expressed as Mean±S.E.

Table-9: Effect of *B. licheniformis* on mean microbiological counts (\log_{10} cfu g^{-1}) on 45th day in the gut of *M. rosenbergii* fed with different experimental diets

Treatments	Total bacteria	<i>Bacillus</i>	<i>Vibrio</i>	Enterobacteriaceae	<i>Aeromonas</i>	<i>Pseudomonas</i>
C	7.92 ^a ±0.59	3.56 ^a ±0.34	4.49 ^a ±0.34	4.52 ^a ±0.34	3.69 ^a ±0.32	3.60 ^a ±0.37
T₁	8.14 ^a ±0.57	4.19 ^a ±0.32	4.32 ^a ±0.34	4.32 ^a ±0.34	3.34 ^a ±0.32	2.85 ^a ±0.31
T₂	8.23 ^a ±0.57	4.31 ^a ±0.30	4.32 ^a ±0.35	4.35 ^a ±0.34	3.36 ^a ±0.29	2.98 ^a ±0.33
T₃	8.37 ^a ±0.58	4.34 ^a ±0.32	4.32 ^a ±0.35	4.34 ^a ±0.35	3.34 ^a ±0.32	2.96 ^a ±0.40
T₄	8.59 ^a ±0.57	4.57 ^a ±0.33	4.33 ^a ±0.34	4.32 ^a ±0.34	3.34 ^a ±0.32	2.87 ^a ±0.34

Mean Values (n= 3) in rows bearing different superscript vary significantly (p < 0.05)
Data expressed as Mean±S.E.

Fig. 5: Mean Total bacterial counts (\log_{10} cfug $^{-1}$) in the gut of *M. rosenbergii*

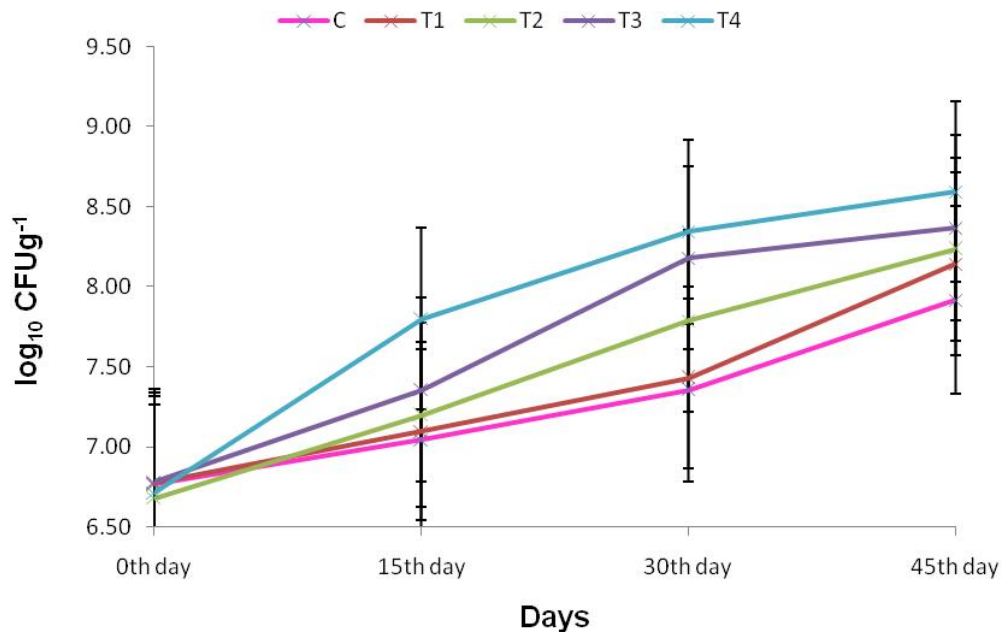


Fig. 6: Mean *Bacillus* counts (\log_{10} cfug $^{-1}$) in the gut of *M. rosenbergii*

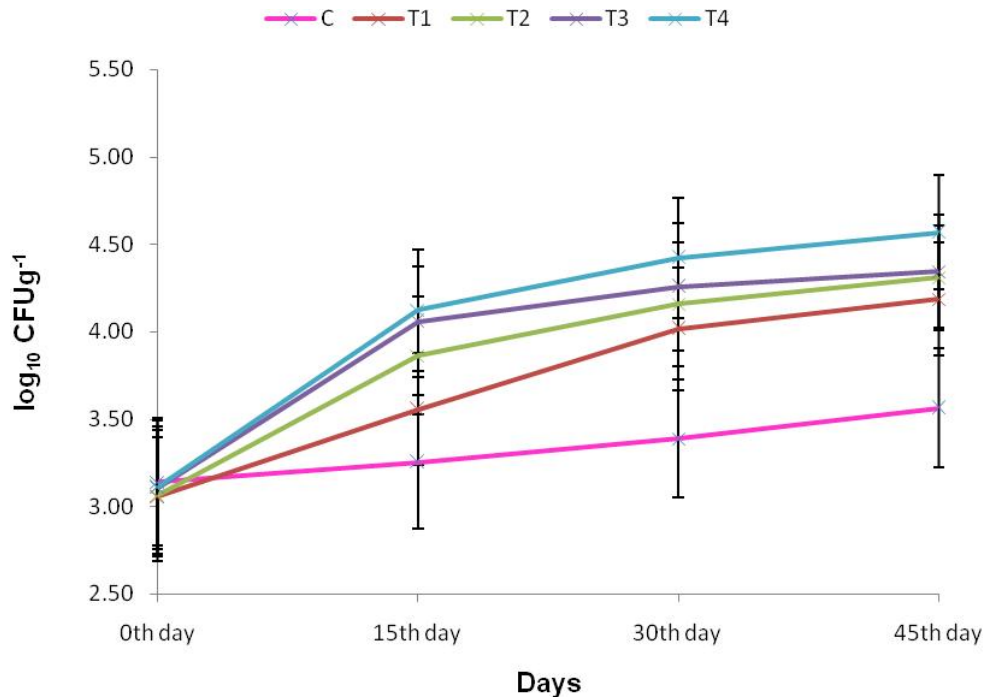


Fig. 7: Mean *Vibrio* counts (\log_{10} cfug⁻¹) in the gut of *M. rosenbergii*

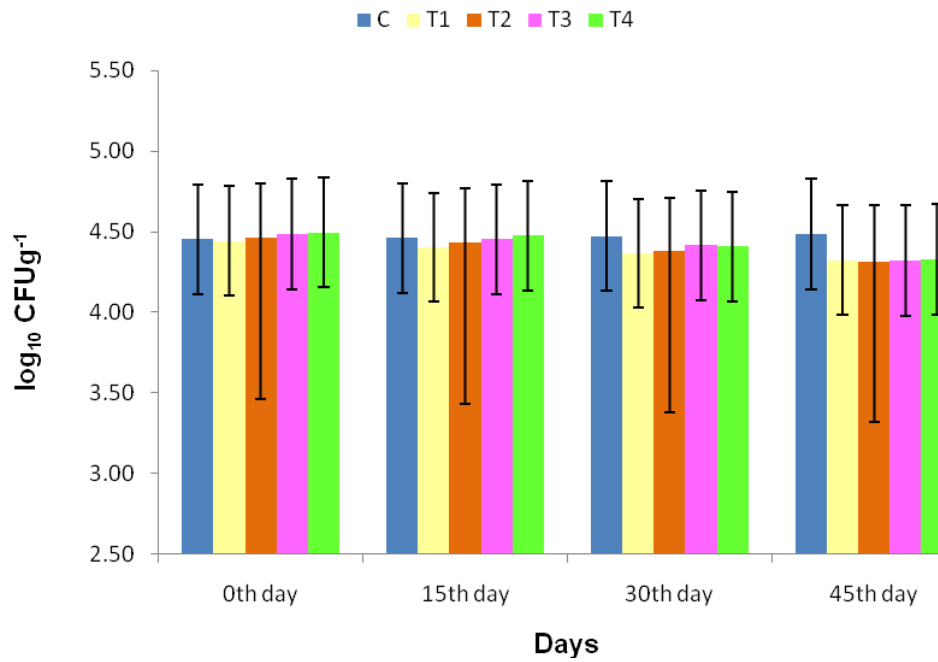


Fig. 8: Mean Enterobacteriaceae counts (\log_{10} cfug⁻¹) in the gut of *M. rosenbergii*

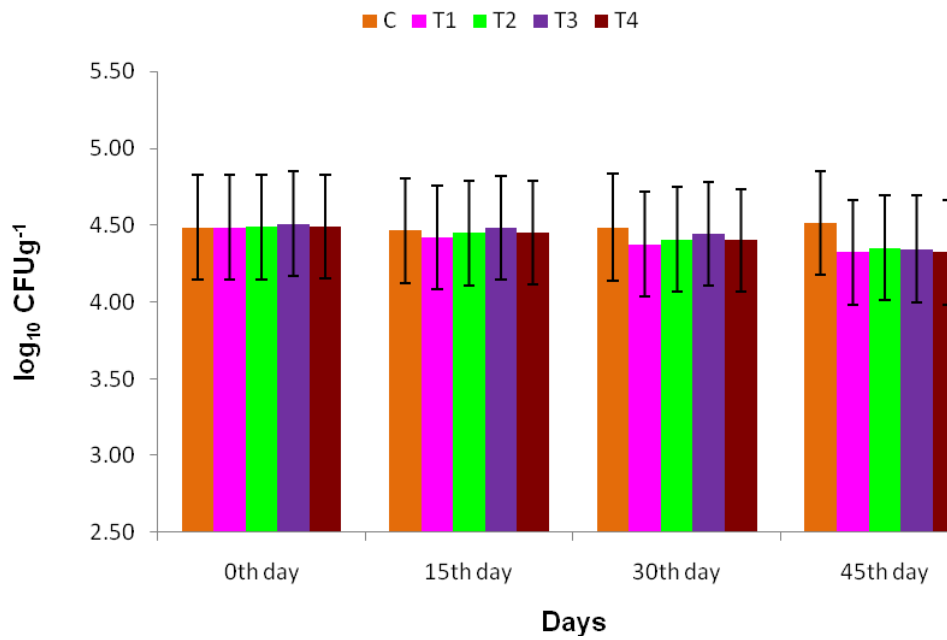


Fig. 9: Mean *Aeromonas* counts (\log_{10} cfug $^{-1}$) in the gut of *M. rosenbergii*

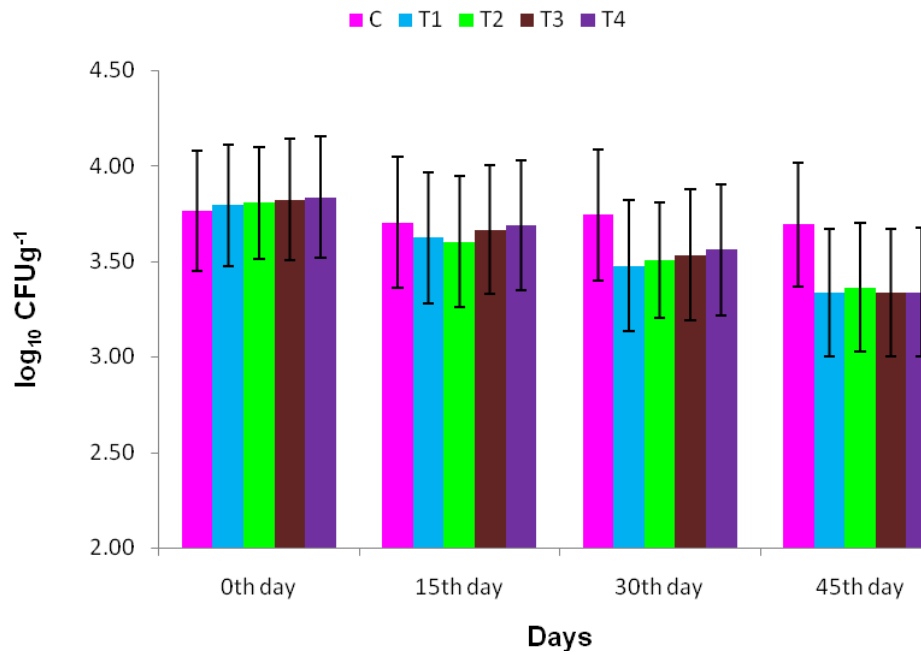


Fig. 10: Mean *Pseudomonas* counts (\log_{10} cfug $^{-1}$) in the gut of *M. rosenbergii*

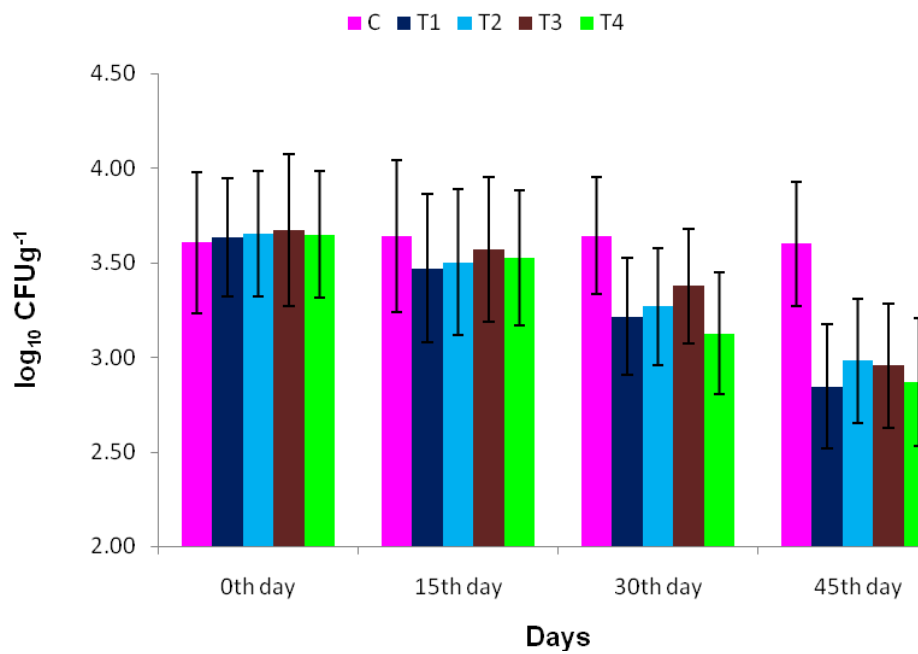


Table-10: Biochemical tests done to confirm bacteria isolated from the intestine of different experimental groups

Tests	<i>B. licheniformis</i>	<i>V. alginolyticus</i>	<i>Bacillus</i>	<i>Vibrio</i>	Enterobacteriaceae	<i>Aeromonas</i>	<i>Pseudomonas</i>
Gram staining	+	-	+	-	-	-	-
Motility	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Indole	-	+	-	v	-	+	-
Methyl red	-	+	-	+	v	v	-
Voges-Proskauer	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	-	+
H ₂ S Production on TSI	+	-	+	-	-	+	-
ONPG	+	-	v	-	v	-	-
Gas in D- glucose	+	-	+	-	+	+	+
Acid produced from							
Glucose	+	+	+	+	+	+	v
Sucrose	+	+	-	-	+	+	+
Arabinose	+	-	+	-	+	+	v
Mannitol	+	+	+	+	+	+	+

(+)= Positive reaction
v = Variable reaction

(-) = Negative reaction

4.5. Immunological Parameters

4.5.1 Total haemocyte count (THC)

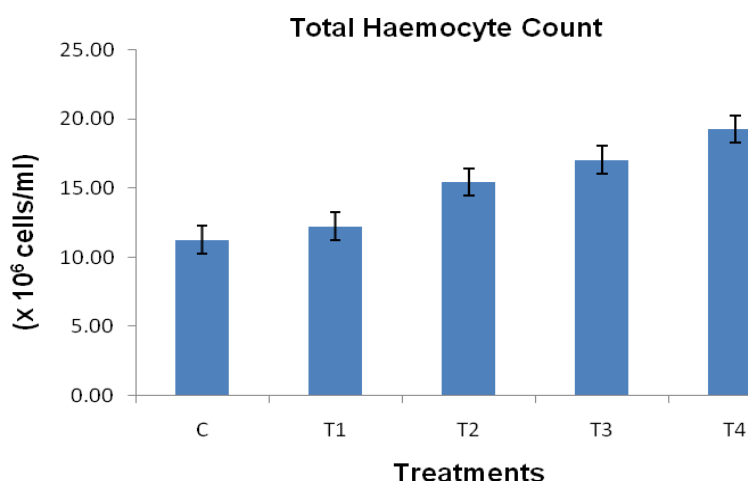
Data on THC of haemolymph are depicted in Table-11 and Fig.11. There was significant difference among various groups. The highest count (19.27 ± 0.70) was found in T₄ group while the lowest (11.27 ± 0.56) was found in control group.

Table-11: Effect of *B. licheniformis* on Total haemocyte count ($\times 10^6$ cells ml⁻¹) in haemolymph of *M. rosenbergii*

Treatments	THC ($\times 10^6$ cells ml ⁻¹)
C	$11.27^a \pm 0.56$
T ₁	$12.23^a \pm 0.58$
T ₂	$15.43^b \pm 0.60$
T ₃	$17.07^b \pm 0.87$
T ₄	$19.27^c \pm 0.70$

Mean Values (n= 3) in rows bearing different superscript vary significantly ($p < 0.05$). Data expressed as Mean \pm S.E.

Fig.11: Total haemocyte count ($\times 10^6$ cells ml⁻¹) in haemolymph of *M. rosenbergii*



4.5.2 Phenol oxidase (PO) activity

Data on PO activity of haemolymph are depicted in Table-12 and Fig.12. There were significant differences among various treatment groups. The higher activity (0.47 ± 0.00) was noticed in T₄ group and lowest (0.32 ± 0.00) was observed in control group.

4.5.3 Superoxide dismutase activity

Data on SOD activity of haemolymph are depicted in Table-12 and Fig.12. There were significant differences among various treatment groups. The higher activity (6.58 ± 0.24) was noticed in T₄ group and lowest (3.14 ± 0.10) was observed in control group.

4.5.4 Alkaline phosphatase (ALP)

Data on ALP activity of haemolymph are depicted in Table-12 and Fig.12. There were significant differences among various treatment groups. The higher activity (380.67 ± 5.93) was noticed in T₄ group and lowest (250.73 ± 4.71) was observed in control group.

4.5.5 Acid phosphatase (ACP)

Data on ACP activity of haemolymph are depicted in Table-12 and Fig. 12. There were significant differences among various treatment groups. The higher activity (3.91 ± 0.14) was noticed in T₄ group and lowest (1.82 ± 0.11) was observed in T₁ group.

Table-12: Effect of *B. licheniformis* on Phenol oxidase (PO), Superoxide dismutase (SOD), Alkaline phosphatase (ALP), and Acid phosphatase (ACP) activity in haemolymph of different experimental groups

Treatments	PO ¹	SOD ²	ALP ³	ACP ⁴
C	0.32 ^a ±0.00	3.14 ^a ±0.10	250.73 ^a ±4.71	2.08 ^a ±0.14
T₁	0.37 ^b ±0.01	4.89 ^b ±0.08	275.82 ^b ±5.06	1.82 ^a ±0.11
T₂	0.42 ^c ±0.00	5.39 ^c ±0.13	293.43 ^c ±4.32	3.26 ^b ±0.11
T₃	0.44 ^d ±0.01	6.06 ^d ±0.17	331.45 ^d ±2.53	3.55 ^{bc} ±0.11
T₄	0.47 ^e ±0.00	6.58 ^e ±0.24	380.67 ^e ±5.93	3.91 ^c ±0.14

Mean Values (n= 3) in rows bearing different superscript vary significantly (p < 0.05).

Data expressed as Mean±S.E.

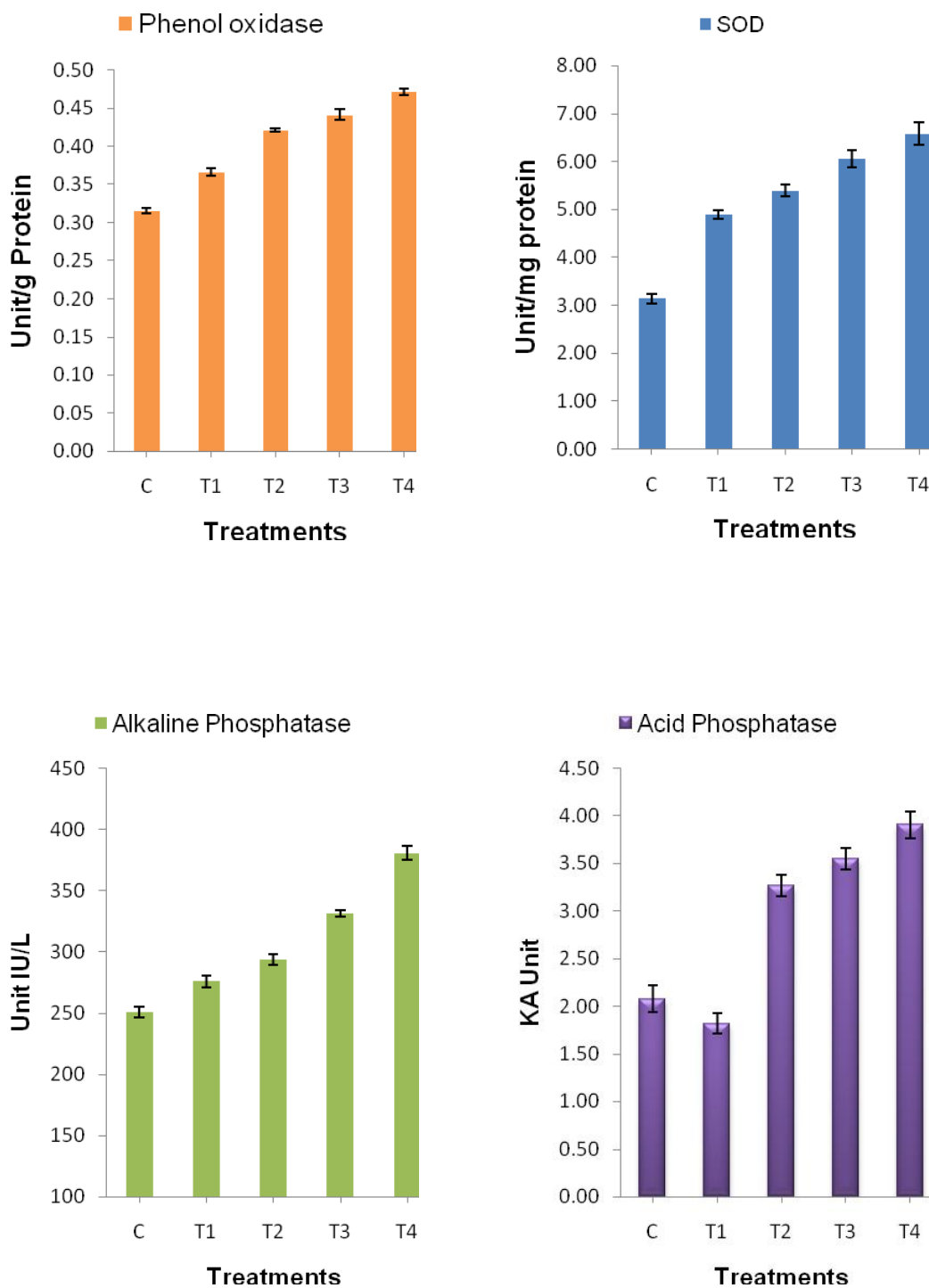
¹ Activity of Phenol oxidase is expressed as Unit/ g protein

² The activity of SOD is expressed Unit/mg protein

³ Activity of ALP is expressed in IU/L

⁴ The activity of ACP is expressed in KA units

Fig-12: Phenol oxidase (PO), Superoxide dismutase (SOD), Alkaline phosphatase (ALP) and Acid phosphatase (ACP) activity in the haemolymph of *M. rosenbergii*



4.5.6 Haemolymph total proteins

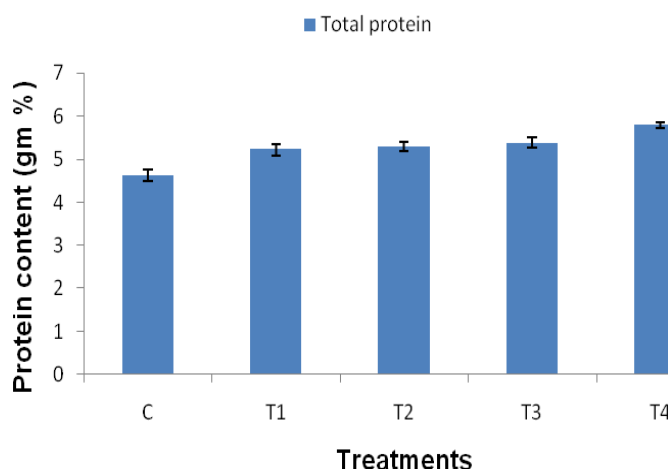
Data on total protein content in haemolymph of *M. rosenbergii* in different treatment groups are depicted in Table-13 and Fig.13. Significant ($P < 0.05$) change was noticed in total protein content among experimental groups. The higher total protein (5.79 ± 0.07) content was observed in T₄ group and lowest total protein (4.62 ± 0.14) was observed in control group.

Table-13: Effect of *B. licheniformis* on haemolymph total protein content of different experimental groups.

Treatments	Total protein (gm %)
C	$4.62^a \pm 0.14$
T1	$5.23^b \pm 0.13$
T2	$5.30^b \pm 0.11$
T3	$5.38^b \pm 0.12$
T4	$5.79^c \pm 0.07$

Mean Values ($n = 3$) in rows bearing different superscript vary significantly ($p < 0.05$). Data expressed as Mean \pm S.E.

Fig.13 Haemolymph Total protein of different experimental groups



4.5.7 Haemolymph glucose

Data on glucose content in haemolymph of *M. rosenbergii* are depicted in Table-14 and Fig.14. There were significant differences among various treatment groups in the glucose level. The higher glucose content (24.66 ± 0.31) was noticed in T₄ group while the lowest (18.39 ± 0.35) was observed in control group.

4.5.8 Haemolymph urea level

Data on urea level in haemolymph of *M. rosenbergii* are depicted in Table-14 and Fig.15. There was no significant difference among various treatment groups in the urea level. The higher level (30.97 ± 2.57) was noticed in T₄ group while the lowest (24.80 ± 1.22) was observed in control group.

4.5.9 Nitroblue tetrazolium (NBT) assay

Respiratory burst activity (NBT reduction) of haemocytes of *M. rosenbergii* juveniles in the different experimental groups are presented in Table-14 and Fig. 16. No significant ($P < 0.05$) difference could be noticed among different treatment groups.

Table-14: Effect of *B. licheniformis* on haemolymph glucose level, urea level and NBT *i.e.* respiratory burst activity (OD₆₂₀), of different experimental groups

Treatments	Glucose (mg %)	Urea (mg/dl)	NBT
C	18.39 ^a ±0.35	24.80 ^a ±1.22	0.45 ^a ±0.022
T ₁	21.53 ^b ±0.32	27.58 ^a ±2.59	0.43 ^a ±0.013
T ₂	22.63 ^c ±0.12	28.67 ^a ±2.58	0.43 ^a ±0.014
T ₃	23.44 ^c ±0.17	29.47 ^a ±2.53	0.45 ^a ±0.017
T ₄	24.66 ^d ±0.31	30.97 ^a ±2.57	0.45 ^a ±0.025

Mean Values (n= 3) in rows bearing different superscript vary significantly (p < 0.05). Data expressed as Mean±S.E.

Fig.14 Haemolymph glucose level of different experimental groups

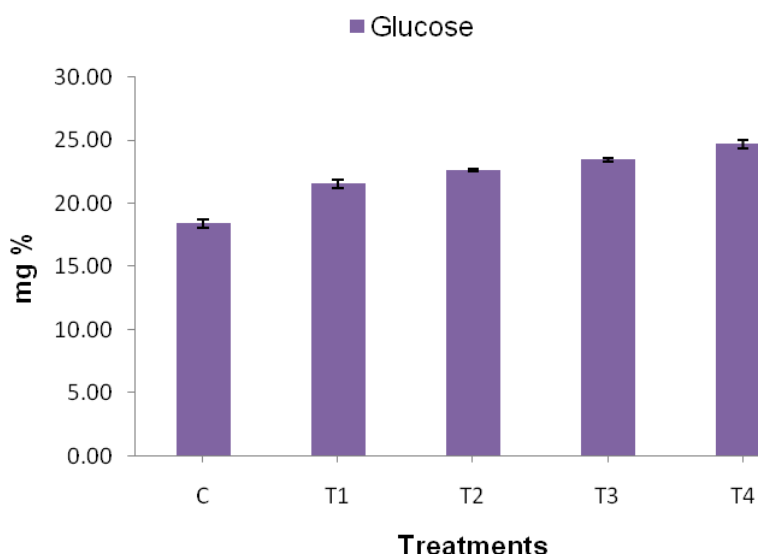


Fig.15 Haemolymph urea level of different experimental groups

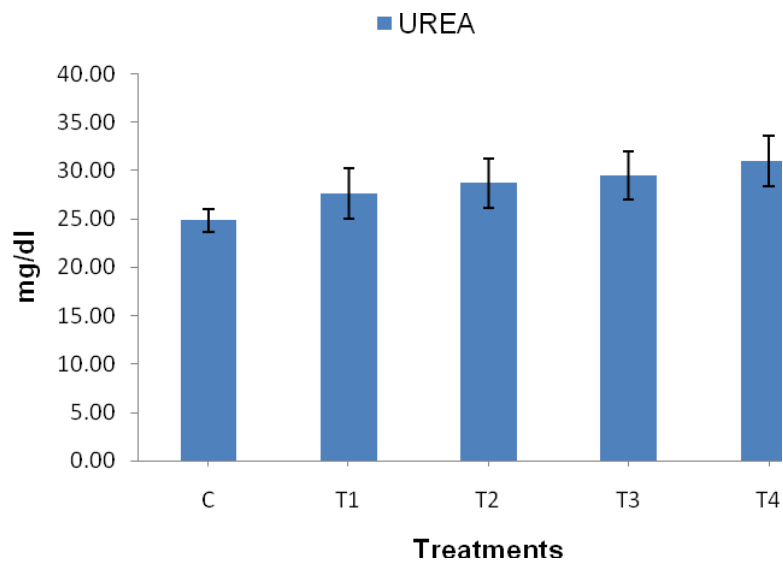
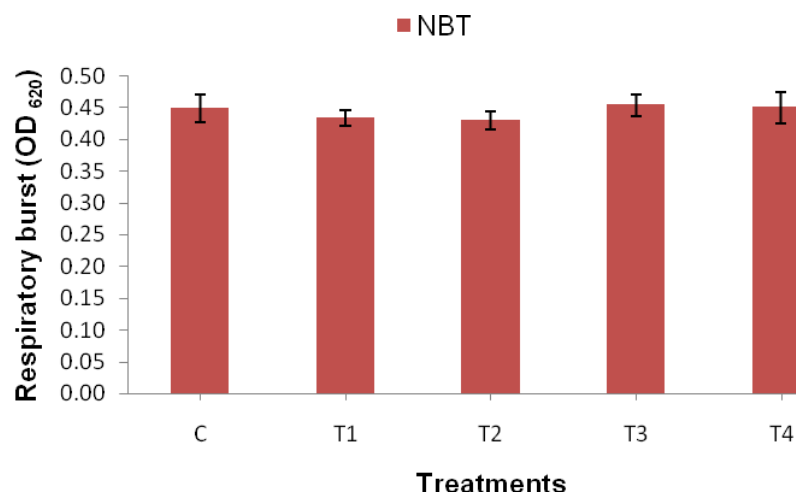


Fig.16 Haemolymph NBT *i.e.* respiratory burst activity (OD_{620}), of different experimental groups



4.5.10 Antibacterial and bacteriolytic activities of haemolymph

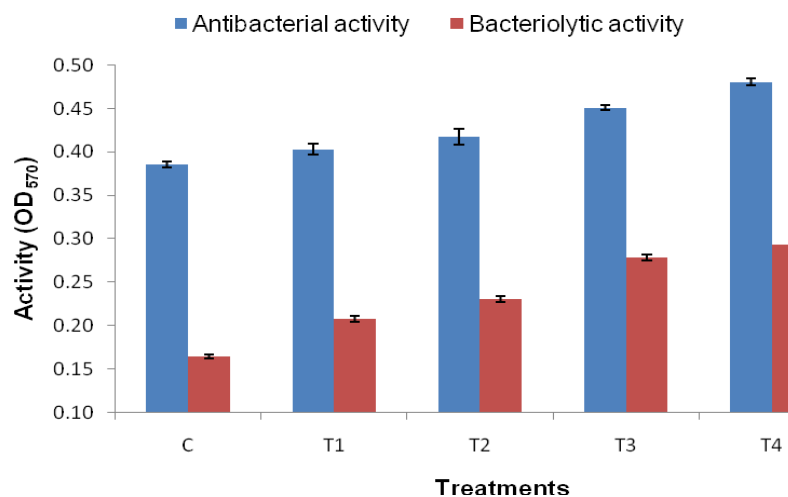
Data on antibacterial and bacteriolytic activities of haemolymph are depicted in Table-15 and Fig.17. There was significant difference among various groups. The higher activities (0.48 ± 0.00), (0.29 ± 0.01) were noticed in T₄ group and lower activities (0.39 ± 0.00), (0.16 ± 0.00) were observed in control group respectively.

Table-15: Effect of *B. licheniformis* on antibacterial and bacteriolytic activities (OD₅₇₀) of haemolymph

Treatments	Antibacterial activity	Bacteriolytic activity
C	$0.39^a \pm 0.00$	$0.16^a \pm 0.00$
T ₁	$0.40^{ab} \pm 0.01$	$0.21^b \pm 0.00$
T ₂	$0.42^b \pm 0.01$	$0.23^c \pm 0.00$
T ₃	$0.45^c \pm 0.00$	$0.28^d \pm 0.00$
T ₄	$0.48^d \pm 0.00$	$0.29^e \pm 0.01$

Mean Values (n= 3) in rows bearing different superscript vary significantly ($p < 0.05$). Data expressed as Mean \pm S.E.

Fig.17 Antibacterial and bacteriolytic activities (OD₅₇₀) of haemolymph



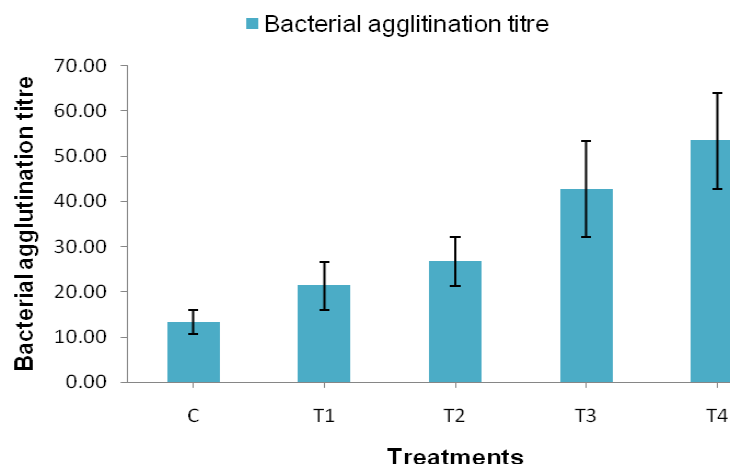
4.5.11 Bacterial agglutination titre

The bacterial agglutination titre against formalin killed *V. alginolyticus*, are presented in Table-16 and Fig.18. The agglutination titre showed significant differences among different treatment groups.

Table-16: Effect of *B. licheniformis* on bacterial agglutination titre of haemolymph

Treatment	Replicates			Mean± SE
	1	2	3	
C	16	16	8	13.33±27
T ₁	16	16	32	21.33±5.33
T ₂	16	32	32	26.67±5.33
T ₃	32	32	64	42.67±10.67
T ₄	32	64	32	53.33±10.67

Fig.18 Bacterial agglutination titre of haemolymph



4.6 Susceptibility of *M. rosenbergii* to *V. alginolyticus*

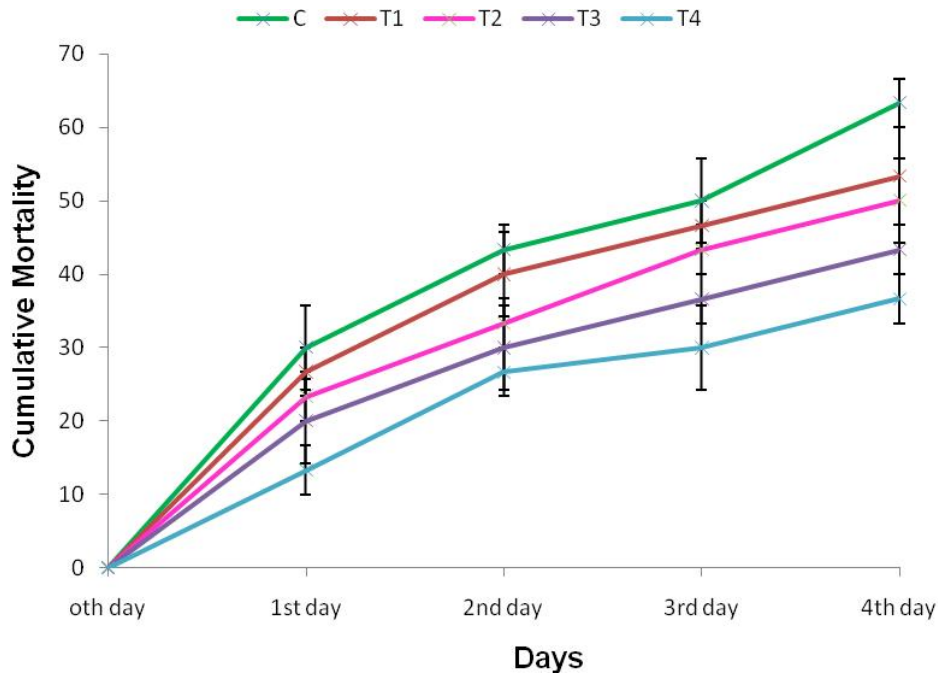
Data pertaining to cumulative mortality of *M. rosenbergii* against *V. alginolyticus* infection are depicted in Table-17 and Fig.19. All the unchallenged prawns in the negative control injected only with PBS survived. After one day post challenge, the mortality of prawns fed with T₄ diet was significantly lower than that of the prawns fed with other diets. After 4 days of challenge, the cumulative mortality of prawns fed with probiotic bacteria inoculated diets was significantly lower ranging from 36% in T₄ to 53% in T₁ than that of the prawns fed with control diet which showed higher mortalities reaching upto 63%.

Table-17: Cumulative mortality of *M. rosenbergii* challenged with *V. alginolyticus* fed with different experimental diets

Bacterial dose (CFU ml ⁻¹)	Treatments	Cumulative mortality (%), time after challenge (h)			
		24	48	72	96
Saline	Control	100	100	100	100
1x10 ⁶	C	30.00 ^b ±5.8	43.33 ^b ±3.3	50.00 ^b ±5.8	63.33 ^c ±3.3
1x10 ⁶	T ₁	26.67 ^{ab} ±3.3	40.00 ^{ab} ±5.8	46.67 ^{ab} ±3.3	53.33 ^{bc} ±6.7
1x10 ⁶	T ₂	23.33 ^{ab} ±3.3	33.33 ^{ab} ±3.3	43.33 ^{ab} ±3.3	50.00 ^{bc} ±5.8
1x10 ⁶	T ₃	20.00 ^{ab} ±5.8	30.00 ^{ab} ±5.8	36.67 ^{ab} ±3.3	43.33 ^{ab} ±3.3
1x10 ⁶	T ₄	13.33 ^a ±3.3	26.67 ^a ±3.3	30.00 ^a ±5.8	36.67 ^a ±3.3

Mean Values (n= 3) in rows bearing different superscript vary significantly (p < 0.05).
Data expressed as Mean±S.E

Fig.19 Cumulative mortality of *M. rosenbergii* against *V. alginolyticus* infection during experimental Study



4.7 Clearance efficiency of *M. rosenbergii* to *V. alginolyticus*

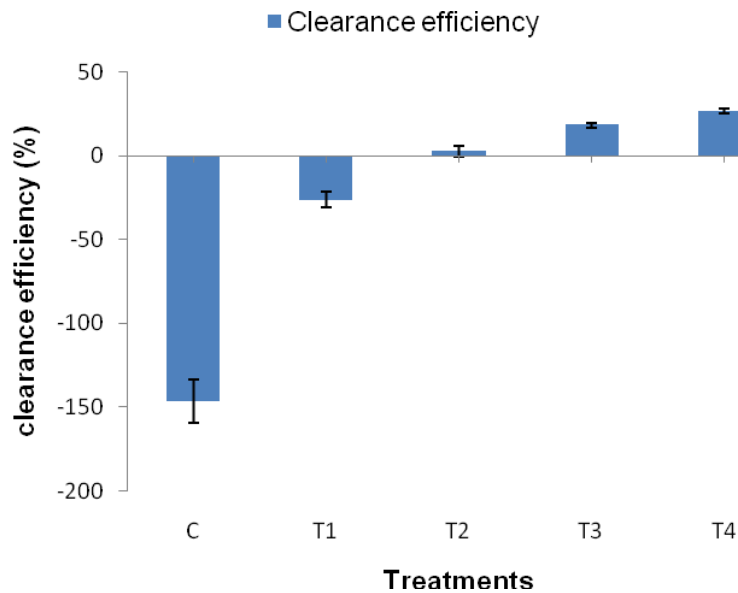
Data on clearance efficiency (%) of hemolymph against *V. alginolyticus* are depicted in Table-18 and Fig. 20. There were significant differences among various treatment groups. After 1 h, clearance efficiency of the haemolymph decreased by 146% and 26% in control and T₁ respectively as compared to that of prawn that received saline (PBS). The results showed significant increase in the clearance efficiency in the probiotic treated groups of T₂, T₃, and T₄.

Table-18: Effect of *B. licheniformis* on Clearance efficiency of *M. rosenbergii* to *V. alginolyticus*

Treatments	Clearance efficiency (%)
C	-146.33 ^a ±13.12
T1	-26.3333 ^b ±4.70
T2	2.6667 ^c ±3.48
T3	18.3333 ^{cd} ±1.45
T4	26.6667 ^d ±1.45

Mean Values (n= 3) in rows bearing different superscript vary significantly (p < 0.05). Data expressed as Mean±S.E.

Fig. 20 Clearance efficiency of *M. rosenbergii* to *V. alginolyticus*



4.8 Histological analysis

Histological sections of hepatopancreas of *M. rosenbergii* of different treatments fed with probiotic appeared to be normal compared to control. Administration of *B. licheniformis* incorporated feeds to *M. rosenbergii* juveniles failed to elicit any structural variations in the hepatopancreas after 45 days of feeding. The hepatopancreas showed normal structure for all groups fed with control and probiotic diets (Plates 1- 4).

On the other hand *M. rosenbergii* juveniles infected with *V. alginolyticus* showed lesions characteristic of prawn with bacterial infection. The tubules were collapsed and contain intraluminal haemocytes. Histological sections (plates 9 and 10) revealed hepatopancreatic necrosis and tubules were found severely damaged. Many tubules were found walled off by haemocytes around the thickened basal lamina. The hepatopancreatic cells were found separated from basal lamina. Compared to the control, necrosis and degeneration of the hepatopancreatic tubules in probiotics-fed prawns was observed to be less on 2nd (Plates 7 and 8) and 4th day (Plates 11 and 12) respectively.

Gills of *M. rosenbergii* in control plates 13 and 14) showed uniform arrangement of lamellae with uniform interlamellar space and normal haemocoelic space with optimum number of haemocytes.

Structural changes were observed in the gills of prawn as a result of *V. alginolyticus* infection. These changes include the accumulation of haemocytes in the haemocoelic space, fusion of lamellae and abnormal gill tips (plates 17 and 18). The affected gills on 2nd day showed excessive proliferation of gill lamellar tissue, fusion of lamellae and abnormal gill tips (plates 15 and 16).

Histopathological changes in hepatopancreas and gills of *M. rosenbergii* were more prominent on 4th day compared to that of 2nd day after infection with *V. alginolyticus*.

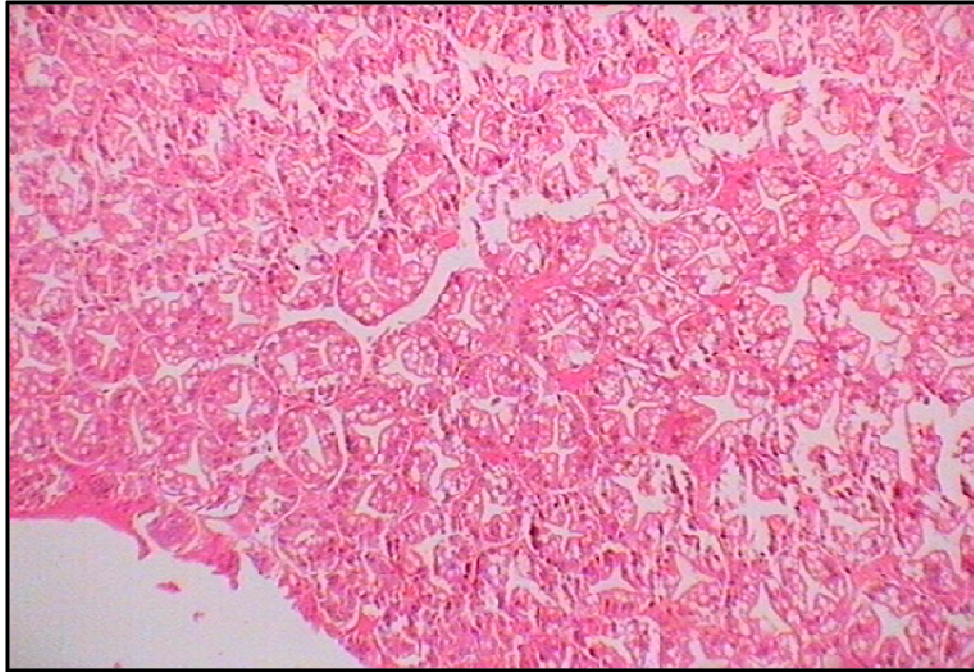


Plate 1: Hepatopancreas of *M. rosenbergii* of control group showing normal appearance (H & E, 10X)

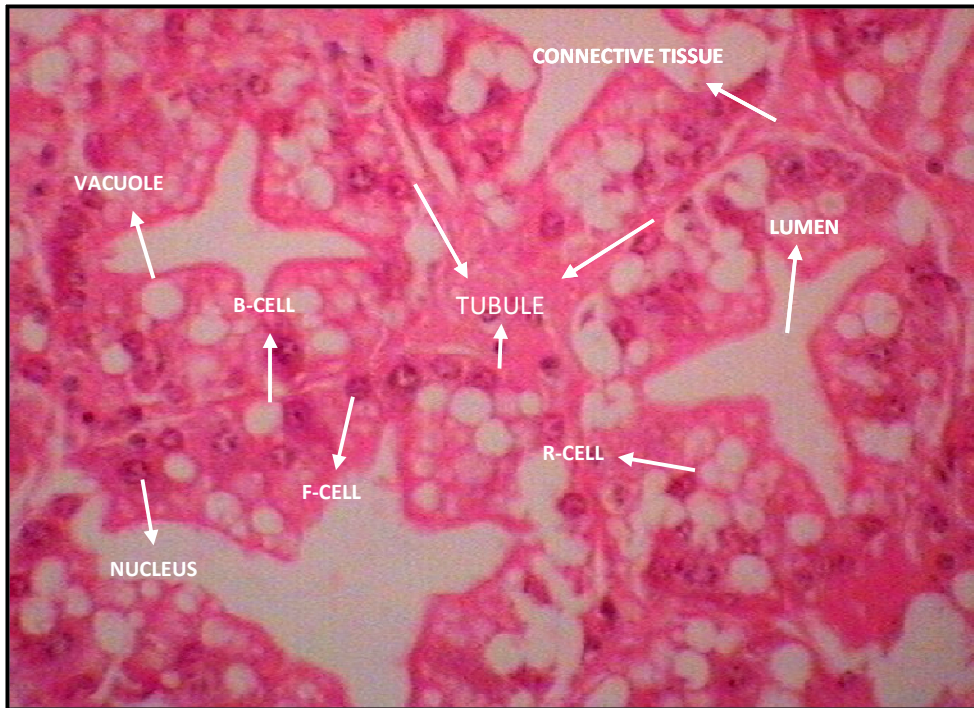


Plate 2: Hepatopancreas of *M. rosenbergii* of control group showing normal appearance (H & E, 40X)

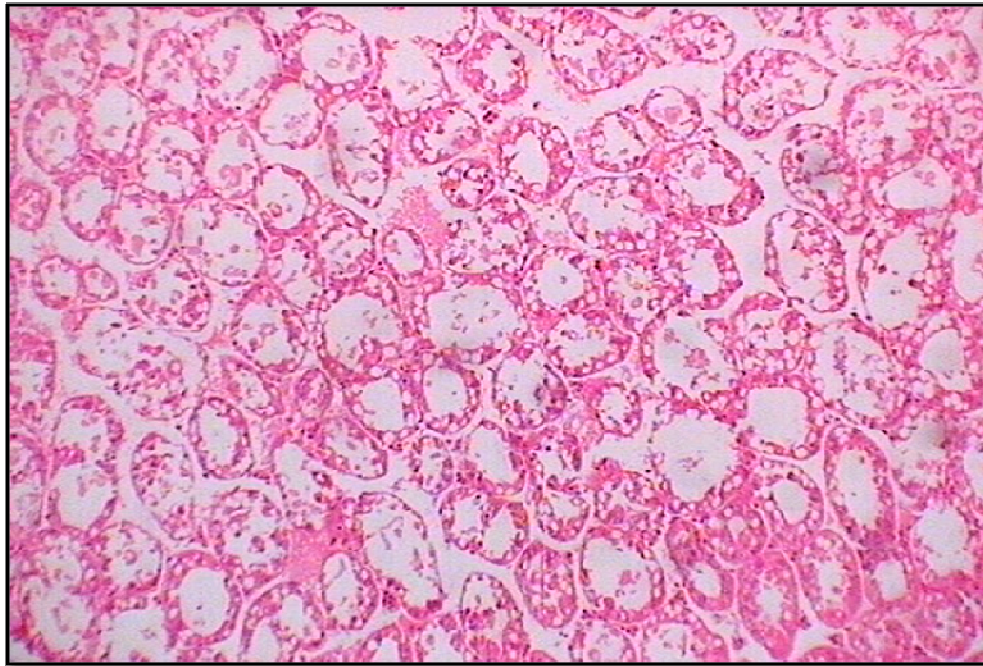


Plate 3: Hepatopancreas of *M. rosenbergii* of probiotic fed group showing normal appearance (H & E, 10X)

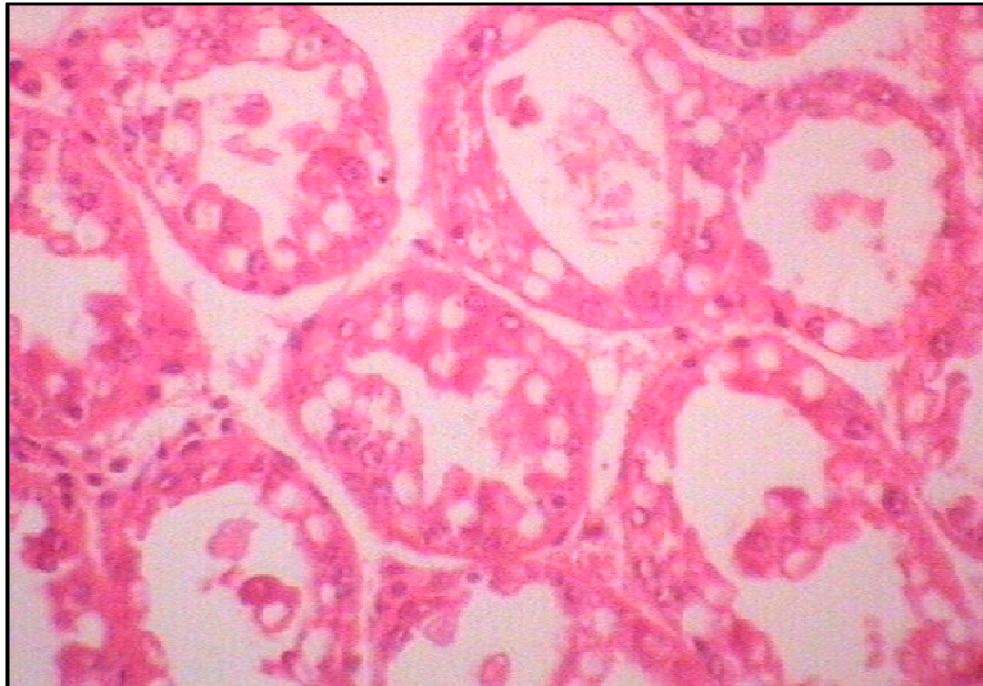


Plate 4: Hepatopancreas of *M. rosenbergii* of probiotic fed group showing normal appearance (H & E, 40X)

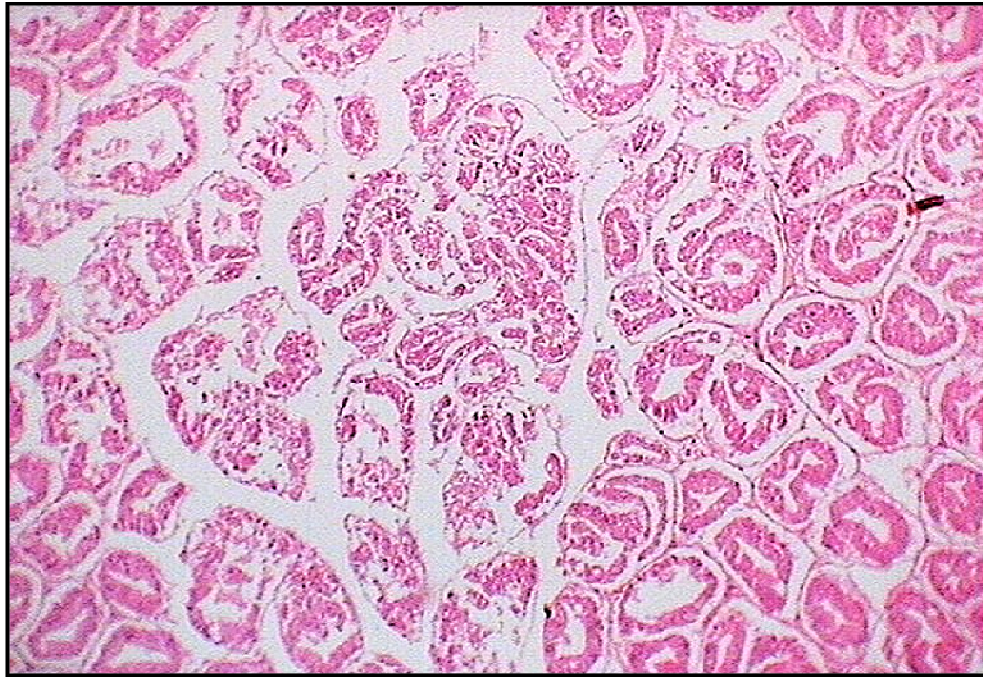


Plate 5: 2nd Day infected hepatopancreas of *M. rosenbergii* of control group showing collapsed tubules (H & E, 10X)

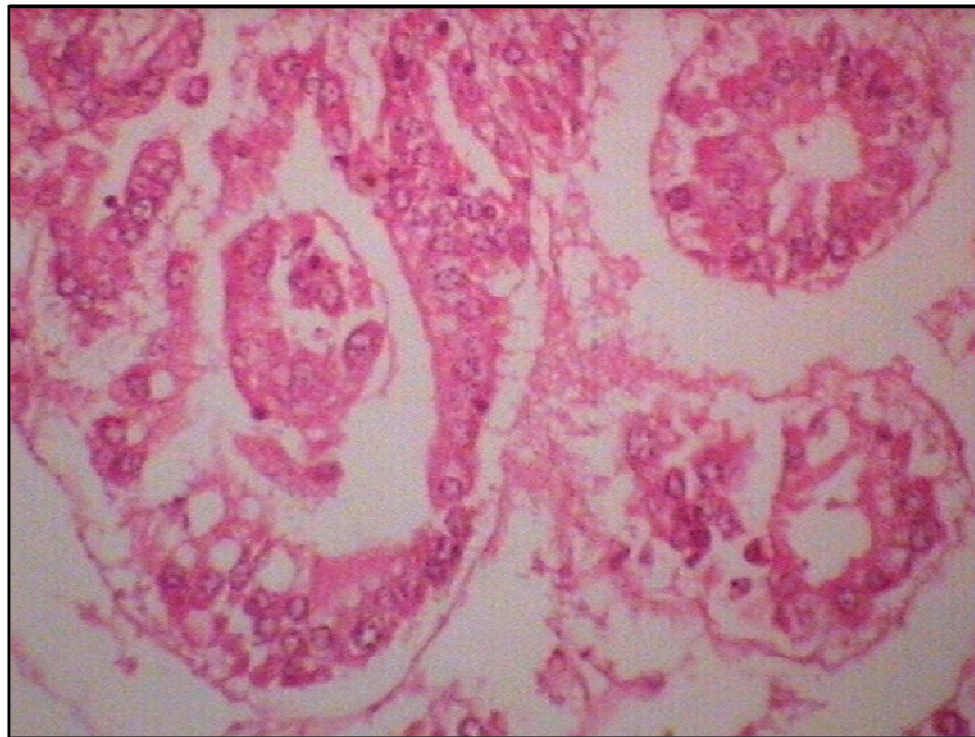


Plate 6: 2nd Day infected hepatopancreas of *M. rosenbergii* of control group showing collapsed tubule (H & E, 40X)

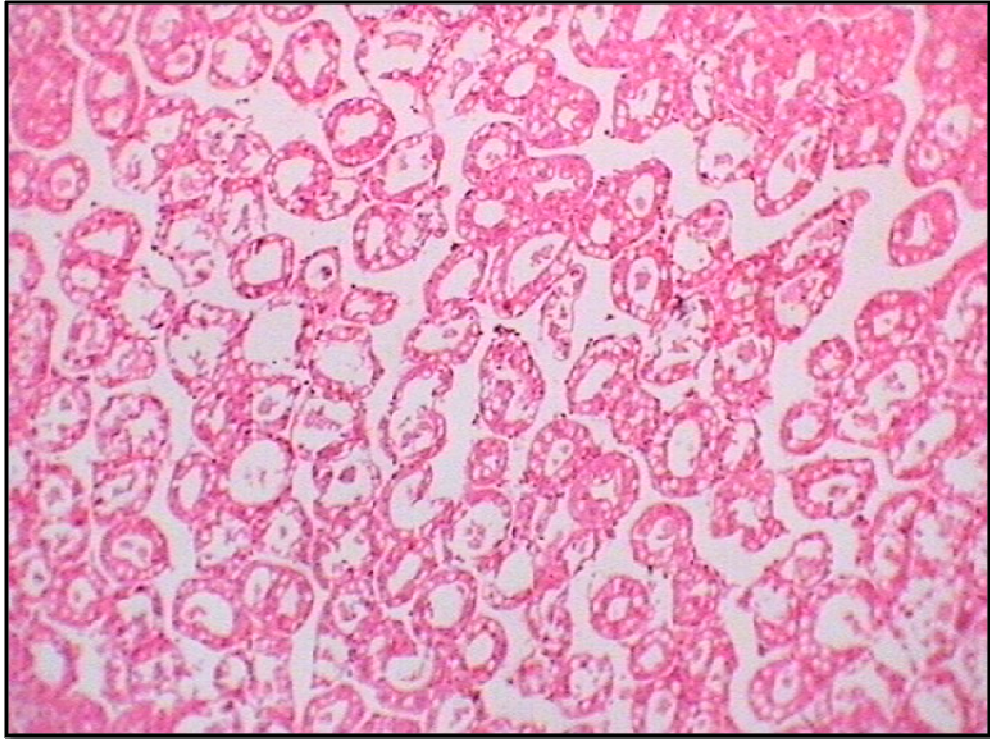


Plate 7: 2nd Day infected hepatopancreas of *M. rosenbergii* of probiotic fed group showing collapsed tubules. (H & E, 10X)

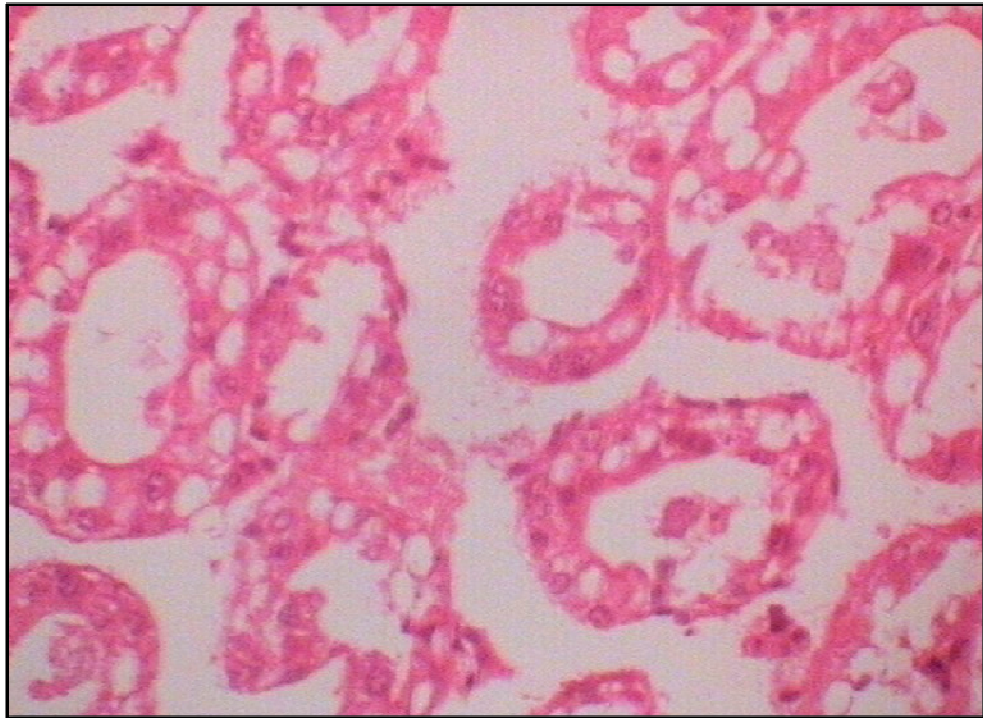


Plate 8: 2nd Day infected hepatopancreas of *M. rosenbergii* of probiotic fed group showing collapsed tubules. (H & E, 40X)



Plate 9: 4th Day infected hepatopancreas of *M. rosenbergii* of control group showing degenerated tubules (H & E, 10X)

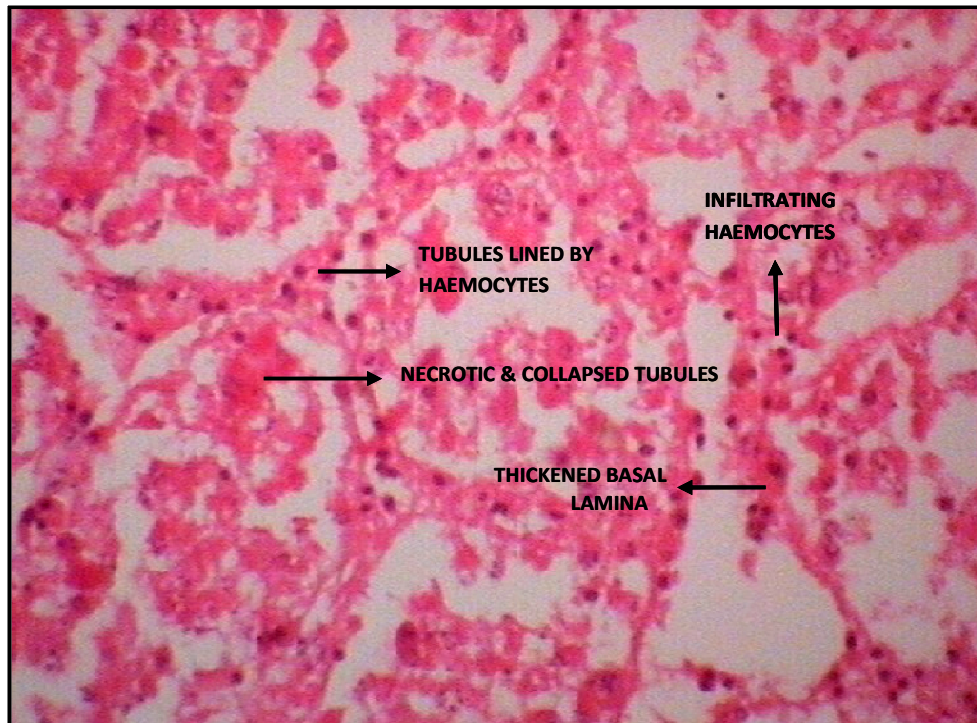


Plate 10: 4th Day infected hepatopancreas of *M. rosenbergii* of control group showing degenerated tubules (H & E, 40X)

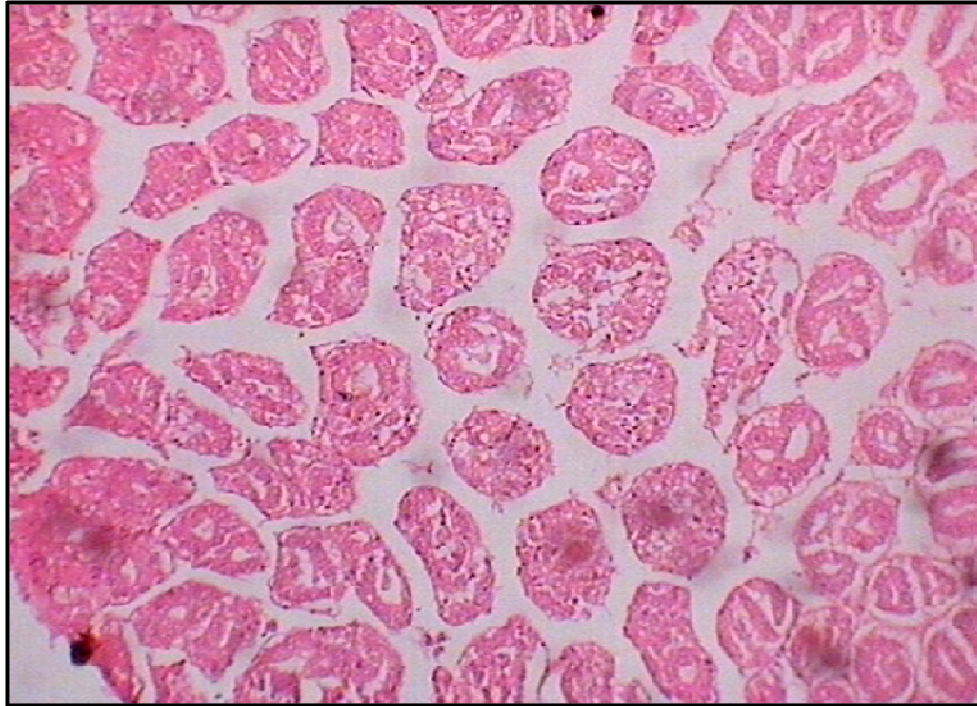


Plate 11: 4th Day infected hepatopancreas of *M. rosenbergii* of probiotic fed group showing collapsed tubules (H & E, 10 X)

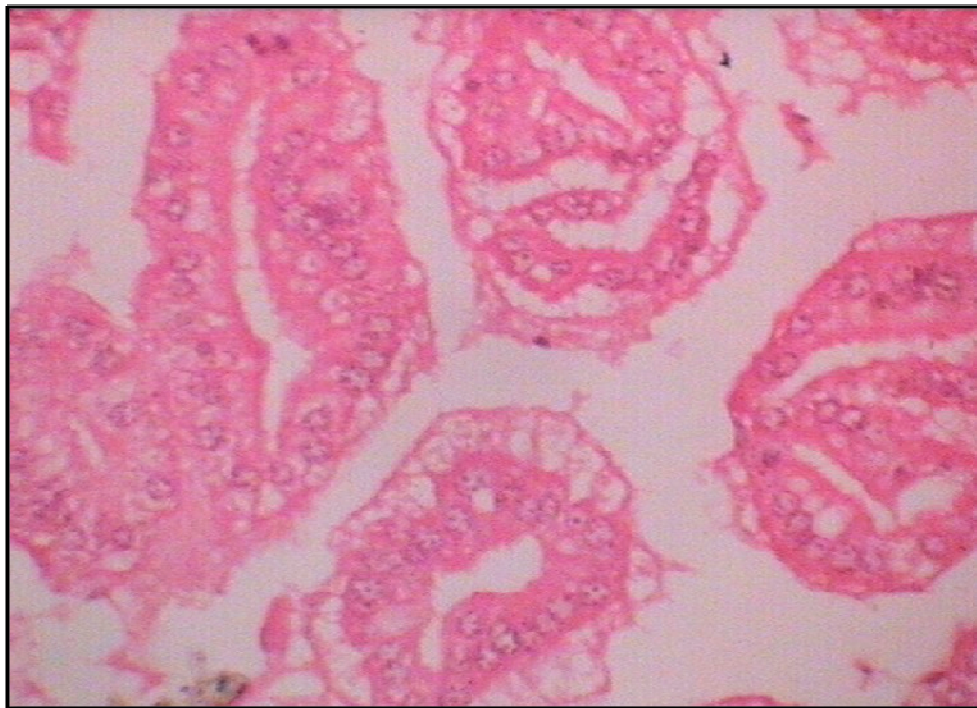


Plate 12: 4th Day infected Hepatopancreas of *M. rosenbergii* of probiotic fed group showing collapsed tubules (H & E, 40 X)

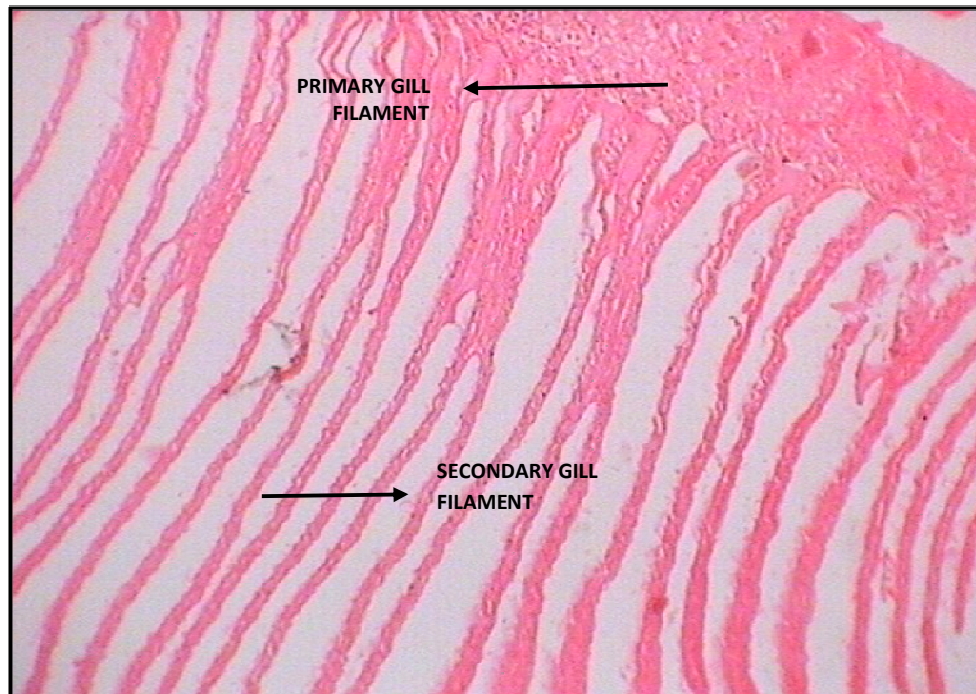


Plate 13: Gills of *M. rosenbergii* of control group showing normal appearance (H & E, 10X)

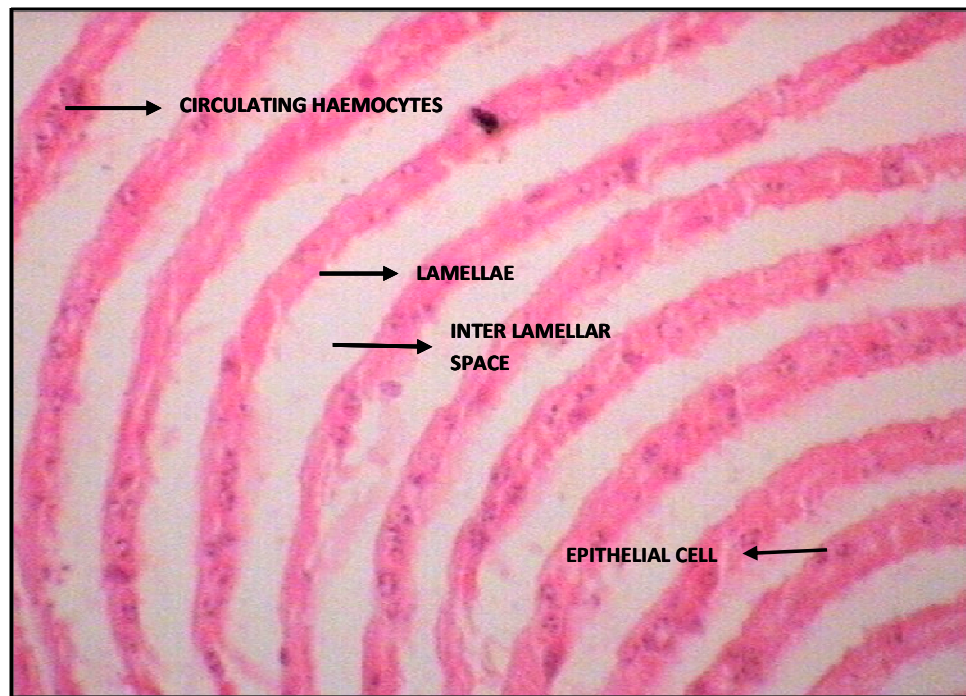


Plate 14: Gills of *M. rosenbergii* of control group showing normal appearance (H & E, 40X)

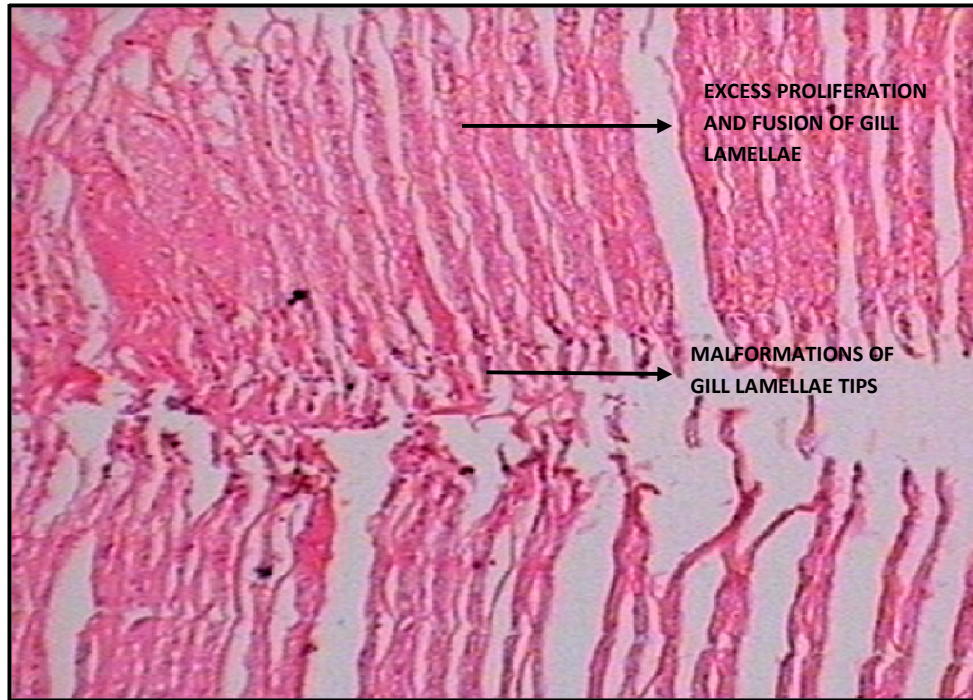


Plate 15: 2nd day infected gills of *M. rosenbergii* showing proliferation and fusion of filaments (H & E, 10X)



Plate 16: 2nd day infected gills of *M. rosenbergii* showing proliferation and fusion of filaments (H & E, 40X)



Plate 17: 4th day infected gills of *M. rosenbergii* showing proliferation and fusion of filaments (H & E, 10X)

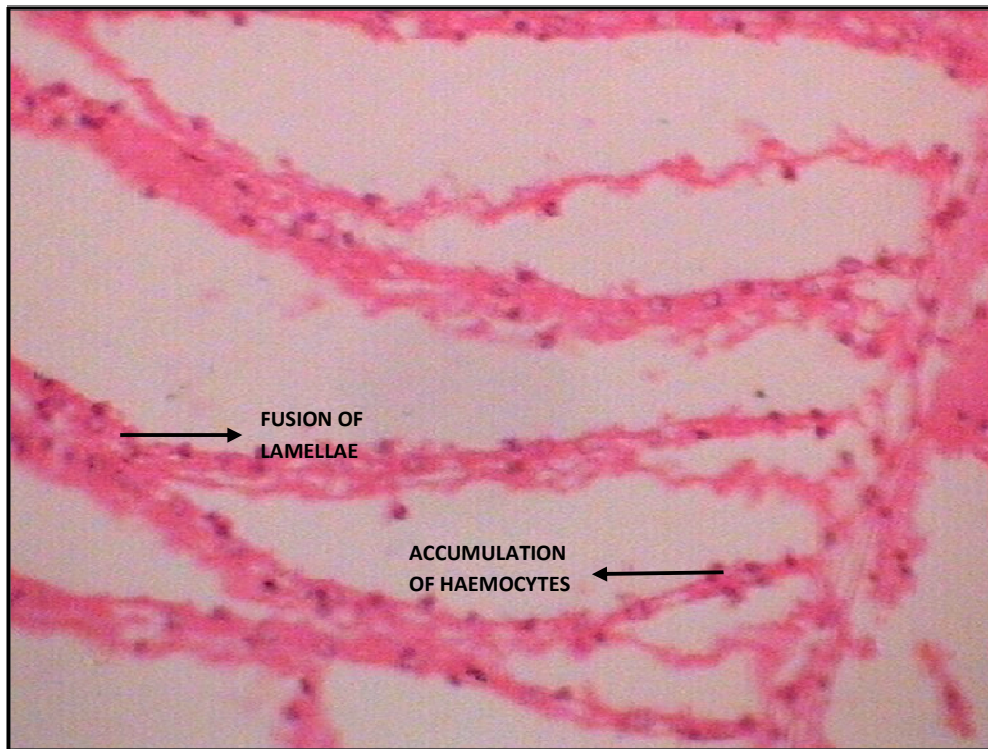


Plate 18: 4th day infected gills of *M. rosenbergii* showing proliferation and fusion of filaments (H & E, 40X)



Discussion

5. DISCUSSION

The use of probiotics as feed additives has been preferred over that of antibiotics as they do not exhibit any of the undesirable effects associated in the use of antibiotics *viz.* toxicity, allergy, residues in food, bacterial drug resistance and indiscriminate suppression of intestinal microflora. Gatesoupe (1999) defined probiotics as “live microbial adjuvant which has 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”. Diseases that may result from pathogenic bacteria are prevented or reduced by the use of probiotics (Olsson *et al.*, 1992, Tannock, 1983). Probiotics sometimes also have direct effect by involvement in nutrient uptake, or by providing nutrients or vitamins (Ringo and Gatesoupe, 1998). Besides, probiotics may enhance growth (Noh *et al.*, 1994; Ghosh *et al.*, 2004) and immune system (Orunto *et al.*, 2002; Nikoskelainen *et al.*, 2003; Salinas *et al.*, 2005). The importance of application of probiotics in finfish and shellfish aquaculture systems have been discussed by several authors (Parker, 1974; Staley and Stanley, 1986; Baticados, *et al.*, 1990; Gatesoupe, 1991, 1998; Strom and Ringo, 1993; Mohanty *et al.*, 1993, 1996; Swain *et al.*, 1996; Riuz *et al.*, 1996, Sugita, *et al.*, 1996a, 1996b, 1998; Boyd and Gross, 1998; Querroz and Boyd, 1998; Sugita and Hirose, 1998; Veerschuere *et al.*, 2000; Ghosh *et al.*, 2002; Venkat *et al.*, 2004; Kim and Austin, 2006; Brahmachari and Raman, 2007; Chiu *et al.*, 2007; Kumar *et al.*, 2008; Li *et al.*, 2009)

In the present study, an attempt has been made to evaluate the gastro-intestinal colonization and immunomodulatory effect of *B. licheniformis* and disease resistance of *M. rosenbergii* to bacterial infection. Following studies have been conducted during the course of experimentation, *viz.* growth parameters like percentage weight gain, SGR, FCR and FER, survival, analysis of gastro-intestinal microflora and analysis of immunological parameters such as Total haemocyte count (THC), Phenol oxidase (PO) activity, Superoxide dismutase (SOD) activity, Alkaline phosphatase (ALP), Acid phosphatase (ACP), Haemolymph total proteins, Haemolymph glucose, Haemolymph urea, Nitroblue tetrazolium (NBT) assay,

Antibacterial and Bacteriolytic activities of haemolymph and Bacterial agglutination titre. Besides, susceptibility and clearance efficiency of *M. rosenbergii* to *V. alginolyticus* and histopathological studies were also performed to evaluate *B. licheniformis* as an effective probiotic for giant freshwater prawn, *M. rosenbergii*.

5.1 Physio-chemical Parameters of Water

In aquatic animals, physical and chemical parameters of water play an important role in regulation of metabolic activities. There has been no much variation in the average value of water quality parameters during the experimental periods. All water quality parameters recorded were within optimum range for freshwater prawn (Boyd and Zimmermann, 2000). Among water quality parameters, temperature is a key factor in determining the physiological activity of prawn. In present study, temperature was maintained around 26 °C to 28 °C. Research has shown that *M. rosenbergii* can exist in water temperature range between 15 °C and 35 °C, but optimum growth occurs when the animals are reared in a temperature range of 27 °C to 29 °C (Smith, 1981).

The pH of water in all the experimental groups ranged from 7.18 to 7.89, which is within the acceptable range (6.5-9.0) as suggested by Swingle (1969). The dissolved oxygen content of water varies due to a large numbers of factors such as temperature, metabolic rate, biomass density etc. In the present experiment, the dissolved oxygen level in different experimental tubs was recorded within the range of 6.2 to 7.1 mg/L. The free carbon dioxide concentration was not detected, and as such did not have any effect on the survival and performance of the experimental animals. This may be due to the low biomass, continuous aeration and frequent water exchange during the experimental period.

5.2 Growth and Survival

In the present experiment, a significant growth increase and feed utilization was observed in the prawns fed with *B. licheniformis* diets, compared with the prawns fed with control diet. The initial and final average body weights of experimental animals were influenced positively by addition of *B. licheniformis* in the feed. T₄ group showed highest average body weight as compared to the control.

Maedo and Liao (1992) have reported that the soil bacterial strain, PM-4, promoted the growth of *P. monodon nauplii*. Regpipat *et al.* (1998) have reported similar result in *P. monodon* using *Bacillus* S11 as probiotic in the feed. After feeding for 100 days, they found significant growth differences among the probiotic treatments and control. Similarly, Garriques and Arevalo (1995), using *V. alginolyticus* in commercial *P. vannamei* hatcheries in Ecuador, have reported less growth in the control group than in the probiotic treated group. Fuller (1989) has reported that probiotics improve digestive activity by synthesis of vitamins, cofactors or improved enzymatic activity (Gatesoupe, 1999). These properties could be the cause of the weight increase, improved digestion or nutrient absorption (Gullian *et al.*, 2004).

In the present study, weight gain % and SGR was found higher in T₄ group, while lower weight gain % was observed in control group. The SGR followed similar pattern as that of weight gain in the experimental animals. This result is in agreement with those of Suralikar (1997) and Suralikar and Sahu (2001). Suralikar (1997) reported highest SGR in *M. rosenbergii* post larval group fed with *Artemia* encapsulated *Lactobacillus sporogenes*. Suralikar & Sahu (2001) have also observed higher SGR in the post larvae of *M. rosenbergii*, when *Lactobacillus cremoris* was inoculated in diet.

The lowest feed conversion ratio (FCR) was observed in T₄ group and the highest in control group and vice versa for feed efficiency ratio (FER). The better FCR and FER values observed with *B. licheniformis* supplemented diets showed improved feed utilization in the probiotic treated animals. Uma *et al.* (1999) observed positive effect on addition of probiotic feed supplement 'Lactosac' on the growth of shrimp and found significantly lower FCR than the control group. Similarly, they observed a significant improvement on FCR and FER of shrimp larvae when fed with *L. plantarum* bio-encapsulated in *Artemia*. Similar results have also been reported in the post larvae of *M. rosenbergii* fed with probiotic *L. cremoris* diets (Suralikar and Sahu, 2001).

The survival rate varied significantly among the experimental groups. The highest survival was recorded in T₄ group (76.67±3.30) compared to control which recorded the lowest survival (60±00). Mortality was not found significantly high in any of the experimental groups fed with *B. licheniformis* inoculated diets compared

to that of control group, indicating no adverse effect of administration of probiotics through diet on survival of *M. rosenbergii* juveniles.

5.3 Survivability of Added Probiotics in Feed

In the present study, different concentrations of *B. licheniformis* such as 0, 1×10^6 , 1×10^7 , 1×10^8 and 1×10^9 cfu were added per gram of feed and were designated as C, T₁, T₂, T₃, and T₄ respectively. It was important to find out if the numbers of cells added were still surviving in the same number or it was varying after addition in feed. As such, all the feeds were assessed for viability of *B. licheniformis* on 0th, 15th and 30th day.

In the present experimentation, on the 0th day *i.e.* at the time of starting the experiments, the viability count of probiotic bacteria (*B. licheniformis*) was 100% in all the diets which a fortnight later *i.e.* on 15th day got reduced, ranging between 64% to 77% in different diets. On the 30th day, the viability count of probiotic bacteria ranged between 32% to 38% only. This clearly shows the reduced survival of probiotic bacteria upon storage, in different treatment diets. In other words, the bacterial count in the feed was reduced to roughly about two-third of the total bacteria in the diets on 15th day, which was further reduced to only about one-third of the total count on the 30th day. The reduced viability of probiotic bacteria in the diets, upon storage, due to subsequent passing of time, might be due to different factors such as environmental temperature, moisture content and nutrient status of the feeds etc. Thus, in the management planning of the application of probiotic added feed in any culture operation should take into account the reduced viability count of probiotic bacteria in the diets upon storage. Thus, it is advisable to use freshly prepared probiotics feeds for application in culture systems.

5.4 Analysis of Gastro-intestinal Microflora

The colonization potential of probiotics is very important criteria to evaluate the efficiency and efficacy of a probiotic species (Gournier-Chatau *et al.*, 1994). As such, it is essential to evaluate the persistence of probiotics in the gut. Hence, the present study was conducted to find out the presence of supplemented microbial organism (*B. licheniformis*) and its effect on native microflora of the

gastro-intestinal tract. The gastro-intestinal microfloral analysis was done on 0th day and on 15th, 30th and 45th day of post feeding with *B. licheniformis*.

The *Bacillus* genus has not been associated with aquatic organism pathologies. For this reason, its use has been promoted (Moriarty, 1998) and more widely accepted in the industry (Gullian *et al.*, 2004). In the present study, T₄ group ($4.57 \pm 0.33 \log_{10} \text{ cfu g}^{-1}$) showed higher *Bacillus* sp. in the gut than T₁ group ($4.19 \pm 0.32 \log_{10} \text{ cfu g}^{-1}$). This clearly shows the *Bacillus* colonization in gastro-intestinal flora of treated groups.

According to Conway (1996), a microbe is able to colonise the gastro-intestinal tract when it persists for long time, it may be due to higher multiplication rate than the expulsion rate. In the present study, the bacteria incorporated feed was fed twice a day to the animals, that's why the expected intake of bacteria is more than the expulsion resulting in higher colonization of *B. licheniformis* in the gut. This finding is in agreement with that of Gournier-Chetau *et al.* (1994), who observed that the probiotic bacteria LAB colonise very early in the gastrointestinal tract of terrestrial animals upon treatment. However, occurrence of *Bacillus* could also be noticed in the control group which was not fed with *B. licheniformis*, this can be construed as the natural flora of the gut of *M. rosenbergii*.

There are several ways by which probiotic bacteria can induce bacterial antagonism: by producing antimicrobial agents such as antibiotics, antimicrobial peptides, or siderophore substances (Sugita *et al.*, 1998). *Bacillus* has been linked to polymyxin, bacitracin and gramicidin antibiotic production (Rhodehamel and Harmon, 1998). Moriarty (1998) reported that bacterial antagonism also can be provoked by competition between different bacterial microflora to obtain nutrients. Skjermo and Vadstein (1999) pointed out that bacterial colonization depends on several factors such as adhesion properties, bacterial attachment site, stress factors, diet and environmental factors.

In the present study, it was observed that the total count of *Vibrio* sp. in the gut of *M. rosenbergii* decreased significantly with probiotic treatment from $4.50 \pm 0.34 \log_{10} \text{ cfu g}^{-1}$ on 0th day to about $4.33 \pm 0.34 \log_{10} \text{ cfu g}^{-1}$ on 45th day. This

finding is in agreement with that of Dempsey *et al.* (1989), who also observed similar results in the digestive tract of penaeid shrimp. This clearly shows that *B. licheniformis* played a significant role as a competitive exclusion agent, because its administration into the host through feed changed the bacterial population scenario of the its digestive tract and led to the decrease in the population of *Vibrio* sp. which is an important group widely associated with shrimp pathology (Mohney *et al.*, 1994).

Similar decrease was also found in case of Enterobacteriace counts (from $4.49 \pm 0.34 \log_{10} \text{ cfu g}^{-1}$ on 0th day to about $4.32 \pm 0.34 \log_{10} \text{ cfu g}^{-1}$ on 45th day) from the gut of *M. rosenbergii* in the different treatment groups. Besides, *Aeromonas* counts (from $3.84 \pm 0.34 \log_{10} \text{ cfu g}^{-1}$ on 0th day to around $3.34 \pm 0.32 \log_{10} \text{ cfu g}^{-1}$ on 45th day) and *Pseudomonas* (from $3.65 \pm 0.34 \log_{10} \text{ cfu g}^{-1}$ on 0th day to around $2.85 \pm 0.31 \log_{10} \text{ cfu g}^{-1}$ on 45th day) in the gut also decreased respectively. This further shows the exclusion of harmful bacteria by the probiotic bacteria from the digestive tract. It shows that *B. licheniformis* was able to out-compete other bacteria for nutrients and space and could exclude them through the production of antibiotics (Moriarty, 1998; Verschuere *et al.*, 2000a).

In contrast, in the control group, there was significant increase in counts of *Vibrio* sp. ranging from $4.45 \pm 0.34 \log_{10} \text{ cfu g}^{-1}$ on 0th day to about $4.49 \pm 0.34 \log_{10} \text{ cfu g}^{-1}$ on 45th day, and Enterobacteriaceae from $4.49 \pm 0.34 \log_{10} \text{ cfu g}^{-1}$ on 0th day to about $4.52 \pm 0.34 \log_{10} \text{ cfu g}^{-1}$ on 45th day from the gut of *M. rosenbergii* respectively. However, no significant difference could be observed in *Aeromonas* and *Pseudomonas* counts which shows that there was no competitive exclusion of these bacteria from the gut of *M. rosenbergii* in control group.

In the present study, overall total bacterial counts in the gastro-intestinal tract of *M. rosenbergii* increased in all the treatment groups ranging from the initial sampling (6.76 ± 0.57 (C) $\log_{10} \text{ cfu g}^{-1}$ on 0th day to 8.59 ± 0.57 (T₄) $\log_{10} \text{ cfu g}^{-1}$ on 45th day) on final sampling. Sugita *et al.* (2002) reported that the intestinal microflora of Japanese flounder ranged from 10^5 - 10^7 cfu g^{-1} . Prawns, being a bottom dwelling detritus feeder, often have higher bacterial population than finfish. This shows that

these supplemented non-indigenous microbes can survive in the gut of target animals.

5.5 Analysis of Immunological Parameters

5.5.1 Total Haemocyte Count (THC)

The life cycle, food intake, disease outbreak, pollutants, and environmental stresses affect the quantity of circulating haemocyte counts of crustaceans (Persson *et al.*, 1987; Soderhall *et al.*, 1988; Smith and Johnston, 1992; Le Molluac *et al.*, 1997; Le Molluac and Haffner, 2000; Cheng and Chen, 2001). Circulating haemocytes are also affected by extrinsic factors such as temperature, pH, salinity, and dissolved oxygen, which have been reported to affect THCs in several species of decapods crustaceans. In freshwater prawn, *Macrobrachium rosenbergii*, haemocytes are classified differently like fusiform, rounded and large ovoid haemocytes (Sierra *et al.*, 2001) which are important for their association with the recognition and removal of foreign material.

In crustaceans, an increase in the THC is considered to enhance the immune capability during periods of stress (Truscott and White, 1990) leading to disease resistance (Le Moullac *et al.*, 1998). In addition, haemocytes not only play an important role in immune defence but are also involved in physiological functions including carbohydrate metabolism, transport and storage of proteins and aminoacids (Ratcliffe *et al.*, 1985; Martin *et al.*, 1991).

In the present study, it was observed that the circulating haemocytes of *M. rosenbergii* significantly increased in all the probiotics treated groups, with the highest number noticed in T₄ group (19.27±0.70) as compared to that of control (11.27±0.56). Further, it was also observed that THC increased with increasing concentration of the probiotic bacteria in the gut of treated prawns. This phenomenon may be due to the fact that increased numbers of this probiotic bacteria (*B. licheniformis*) in the digestive tract of the host prawns, increased the proliferating activity of the haematopoetic tissue of the prawns, which was probably helped by better digestive function leading to the better growth and health status of the probiotics fed prawns. Similar observations have been made by Tsing (1987) and

Van de Braak *et al.* (2002) who also pointed out that an increase in circulation of haemocytes might be an indicator of an intense proliferating activity of haematopoietic tissue. *B. licheniformis* surface antigens or their metabolites might act as immunogens to *M. rosenbergii*. Rengipipat *et al.* (2000) showed increase in THC in *P. monodon* by *Bacillus* S11 after feeding for a period of 90 days. Gullian *et al.* (2004) also reported an increase in THC in *P. vannamei* by different selected probiotics after feeding for 25 days. Significant increase in THC was reported by Li *et al.* (2007) in *P. vannamei* by addition of *Bacillus licheniformis* to water for a period of 40 days.

5.5.2 Phenol oxidase (PO) activity

Phenol oxidase (PO) enzyme plays a vital role in immune system of crustaceans. Haemocytes are associated with the prophenoloxidase (proPO) system involved in encapsulation and melanization and which functions as a non-self recognition system (Smith and Soderhall, 1983). Soderhall *et al.* (1990) reported that phenoloxidase was produced from the activation of the prophenoloxidase (proPO) as a cascade phenomenon. In the present study, PO activity was influenced by *B. licheniformis* incorporation in diets compared to that of control diet. For prawns receiving *B. licheniformis*, the PO activity was significantly higher in T₄ group compared to the control group, which corresponds to an increased THC in treated groups. Similar increase in PO activity was also reported in *Penaeus vannamei* (Gullian *et al.*, 2004) and in white shrimp, *Litopenaeus vannamei* (Li *et al.*, 2007).

5.5.3 Superoxide dismutase (SOD) activity

Aerobic organisms continuously produce endogenous reactive oxygen species (ROS) in the process of metabolism, which could affect macromolecules, such as proteins, carbohydrates, nucleic acids and lipids, in a process of oxidative damage or oxidative stress (Di Giulio *et al.* 1989). Therefore, mechanisms are in place to remove excessive ROS by an antioxidant defence system comprising an enzymatic system, such as superoxide dismutase (SOD).

SOD activity is related to the immunity of aquatic organisms being related to the defensive ability of the phagocytes and the whole immune response

(Mou *et al.*, 1999; Liu and Li., 1998). In the present study, it was observed that prawns in the T₄ group had a significantly higher SOD activity compared to the control group and there was significant difference among the treatments. The possible reason is that the dose of bacteria is important in stimulating immune response causing an increase in activity of SOD (Li *et al.*, 2007). Increase in SOD activity after probiotic treatment has also been reported in white shrimp, *Litopenaeus vannamei* (Li *et al.*, 2007; Chiu *et al.*, 2007).

5.5.4 Phosphatase activities in haemolymph

Acid Phosphatase (ACP) activity in haemolymph showed increasing trend with the increasing administration of different doses of *B. licheniformis* in the experimental diets. In the present experiment, *M. rosenbergii* fed with T₄ showed the highest ACP activity while the prawns fed with control diet had the lowest ACP activity.

Further, the prawns fed with *B. licheniformis* exhibited significantly higher Alkaline Phosphatase (ALP) activity in the haemolymph than the prawns fed with control diet. ALP activity showed increasing tendency with the increasing administration doses of *B. licheniformis* (P<0.05). Present findings are in agreement with that of Li *et al.* (2009) who also reported increase in ACP and ALP activities in haemolymph of *P. vannamei* fed with *Bacillus* OJ.

5.5.5 Haemolymph total proteins

The total protein concentration has been used as one of the tools to measure the immunity status of animals. Evidence has been given regarding the physiological importance of the protein concentration and its susceptibility to environmental and physiological changes in crustaceans (Rodriquez and Moullac, 2000). In the present study, significant increase in protein levels was observed in all treatment groups fed with *B. licheniformis* diets. Similar observations have been made by Gullian *et al.* (2004) who also reported increase in protein content in *P. vannamei* fed with different selected probiotics and Li *et al.* (2007) who showed similar results in *L. vannamei* supplemented with different levels of *B. licheniformis* added in water.

The increase of haemolymph protein may result in good growth performance with protein accumulation. Shrimp immune system has a strong protein base (Pascual *et al.* 2006). The protein increase in haemolymph could have a good consequence for immune capacity and growth. Lopez *et al.* (2003) reported β -1,3-glucan (BG) and vitamin C given permanently or combined with the control diet enhanced growth rate, and found that haemolymph protein were higher in shrimp fed with BG and vitamin C than in shrimp fed with a control diet.

5.5.6 Haemolymph glucose

Several metabolic variables, for example glucose, lactate, haemolymph proteins, cholesterol and haemocyanin can be used to evaluate the physiological condition of shrimp (Yu *et al.*, 2008). The type of food is a dominant factor affecting shrimp haemolymph metabolites (Pascual *et al.* 2003).

In the present study, significant difference was noticed in the hemolymph glucose levels among the different treatment groups which showed increasing trend in the treated prawns with increasing concentration of the probiotic bacteria (*B. licheniformis*). The higher glucose level was observed in T₄ group. The increase of these components in probiotic supplemented prawns indicates that the probiotic supplements enhanced the digestive and assimilative ability of the shrimp. Rosas *et al.* (2002) reported that once glucose is produced in digestive gland, it may be directed to glucose-6-P, which is then delivered to the haemolymph to be used as a source of energy in tissues. Similar increase in glucose levels has also been reported by Yu *et al.* (2008) in *L. vannamei* fed with *Bacillus*.

5.5.7 Hemolymph urea

There was no significant difference in urea levels of haemolymph. This indicates similar immune status in all experimental groups. The supplementation of *B. licheniformis* does not have any effect on urea levels in different treatments.

5.5.8 Nitroblue tetrazolium (NBT) assay

Nitroblue tetrazolium (NBT) assay has been used for both qualitative and quantitative analyses of O₂⁻ generated by haemocytes which is the first product

of respiratory bursts (Holmblad and Soderhall, 1999). There was no significant variation in respiratory burst activity in haemolymph of *M. rosenbergii*. This compares favourably with immunostimulation studies *in vivo* performed by Sung *et al.* (1996) in *P. monodon* using *V. vulnificus* antigen. Similar result was also shown by Gullian *et al.* (2004). A probable explanation could be the expression of antioxidants such as super oxidase dismutase, glutathione peroxidase or catalase that neutralises reactive oxygen metabolites to avoid self-damages (Smith *et al.*, 2003). This further indicates similar immune status in all experimental groups.

5.5.9 Antibacterial and bacteriolytic activities of haemolymph

In most of the crustacean species, the antibacterial activity has been located in the haemolymph and/or in the haemocytes (Haug *et al.*, 2002). Antibacterial activity in the haemolymph has been described in a wide range of crustacean species (Jayasankar *et al.* (1999); Chisholm *et al.* (1995); Sritunyalucksana *et al.*, 1999). In the present study, antibacterial and bacteriolytic activities of haemolymph varied significantly among different treatment groups. The higher activity was noticed in T₄ group compared to that of control group. The reason for this may be that the antibacterial and bacteriolytic activities may be elicited when they are exposed to foreign bacteria. Prawns may need some time to adapt to or respond immunologically to their changing environment and to produce antibacterial substances. Similar increase of antibacterial activity of the haemolymph has also been reported by Rengpipat *et al.* (2000).

5.5.10 Bacterial agglutination titre

Agglutinins in several crustaceans have been characterized as heat-labile and calcium dependent substances (Acharya *et al.*, 2004). It has been seen that agglutinins in the sera of prawns and crab selectively agglutinate Gram negative bacteria (Fragkiadakis *et al.*, 1997). Sritunyalicksana *et al.* (1999) observed a considerable difference in antibacterial and agglutination activity in *P. monodon*. Vazquez *et al.* (1996) showed bacterial agglutinin in *M. rosenbergii* to agglutinate *B. cereus* and *Aeromonas* sp. Similarly, *P. indicus* was also shown to contain bacterial agglutinin that acted on *Vibrio* sp. and *Psuedomonas* sp. (Jayasree, 2001). Agglutination activity has also been detected in haemolymph of *M. rosenbergii* by

Chand *et al.* (2006). The observed bacterial agglutination titre against *V. alginolyticus* in the present study was higher in all treatments fed with *B. licheniformis* as compared to control. It indicates better immune status in all the probiotics treated prawns as compared to control. Similar observation was also been reported in rainbow trout (*Onchorhynchus mykiss*) fed with *Lactobacillus rhamnosus* (Nikoskelainen *et al.*, 2003).

5.6 Susceptibility of *M. rosenbergii* to *V. alginolyticus*

In the present study, *M. rosenbergii* receiving *B. licheniformis* containing diets exhibited immune modulation resulting in increased resistance to *V. alginolyticus* infection. Similar results have been reported with the administration of probiotics by many authors (Kim *et al.*, 2006; Irantio and Austin., 2002; Villamil *et al.*, 2002). After being fed for 100 days with *Bacillus* strain S11-supplemented feed, *P. monodon* post larvae were challenged with a pathogenic *V. harveyi* strain, D331, by immersion of shrimp. After 10 days, all groups treated with *Bacillus* S11 strain showed 100% survival, whereas control group had only 26% (Rengpipat *et al.*, 1998). In the present study, *M. rosenbergii*, following feeding with *B. licheniformis* diets for 45 days and challenged with *V. alginolyticus*, showed reduced mortalities from 36.67% to 13.33 % in T₄ group compared to control group (63.33% to 30.00%) indicating that *B. licheniformis* has the potential as a probiotic to improve *M. rosenbergii* resistance against *V. alginolyticus* infection. This may be due to increase in immune resistance provided by *B. licheniformis*. Similar results has also been reported by Chiu *et al.* (2007) who showed decrease in cumulative mortality in shrimp (*L. vannamei*) following feeding different levels of *L. plantarum* containing diets and then challenging with *V. alginolyticus*. Griffith (1995) and Gomezgil (1995) have also observed the effect of LAB in increasing disease resistance to *Vibrio* pathogens.

5.7 Clearance efficiency of *M. rosenbergii* to *V. alginolyticus*

Clearance efficiency is an important humoral defence mechanism in crustaceans (Ratcliffe *et al.*, 1985). In the present study, clearance efficiency significantly increased in *M. rosenbergii* following feeding of *B. licheniformis* diets, suggesting that *B. licheniformis* was beneficial to prawn in terms of enhancing

the immune response. Similar observations have also been made by Regpipat *et al.* (2000) who reported an increase in clearance efficiency in the haemolymph of *L. vannamei* (Chiu *et al.*, 2007). Although injection challenge probably does not represent the normal pathway by which prawn become infected in natural environment, Alday-Sanz *et al.* (2002) showed that bacterial challenge by injection, oral and incubation results in common clearance pathways.

5.8 Histological analysis

In the present study, *M. rosenbergii* fed on diet with or without different levels of *B. licheniformis* showed similar and healthy histological structures and none of the dietary levels of *B. licheniformis* resulted in any detrimental effect on the hepatopancreatic tissue. Similar result has been reported by Genc *et al.* (2007) in *Penaeus semisulcatus* fed on mannan oligosaccharides. Gullian *et al.* (2004) has also reported that *Penaeus vannamei* fed with selected probiotic bacteria showed no pathogenic effect on the host.

In the present study, marked inflammatory response was detected in hepatopancreas and gill tissues of *M. rosenbergii* challenged with *V. alginolyticus*. Infected hepatopancreas appeared poorly vacuolated indicating low lipid and glycogen reserves (Anderson *et al.*, 1988). In the hepatopancreatic lesions, the thickening of the basal laminae of the tubules was an early defensive reaction because bacteria were confined in the lumen. Similar changes in infected hepatopancreas have also been reported by Jiravanichpaisal *et al.* (1994) in *Penaeus monodon* challenged with *Vibrio harveyi*.

The infected prawns had notable structural alterations of the gill lamellae including accumulation of haemocyte, fusion of gill lamellae and the formation of disorganised mass of disrupted gill lamellae. These observed inflammatory changes may be viewed as a protective mechanism since the vulnerable surface of the gill was decreased in order to maintain the osmoregulatory functions. Similar observations have also been reported by Sharshar *et al.* (2008) in freshwater prawn *M. rosenbergii* infected with *V. vulnificus*.



Summary

6. SUMMARY

The giant freshwater prawn, *Macrobrachium rosenbergii* is the economically important species, cultured in most tropical and subtropical regions of world, which contributes substantially to the global aquaculture production. In India although 14 species of freshwater prawns are available under genus *Macrobrachium*, *M. rosenbergii* is the dominant species in freshwater prawn farming due to its fast growth, disease resistance, high market demand and adaptation to varied environmental conditions. In spite of these advantages, there are some constraints in *M. rosenbergii* culture like cannibalism and differential growth, low stocking rate etc. Recently, prawn farming encountered problems like poor growth, survival, fecundity, susceptibility to various pathogens etc.

A growing concern about the high consumption of antibiotics in aquaculture has initiated a research for alternative methods of disease control and growth promotion. Improved resistance against infectious can be achieved by the use of probiotics. Probiotics are live preparations of microbial cells that, when ingested in high enough concentration, beneficially affect the host's health and growth by improving the intestinal microbial balance. Probiotic protection can be due to different mechanisms such as nutritional competition or production of antibacterial substances.

Probiotics render animals more resistant to infectious disease and reduce the risk of disease outbreak if administered prior to situations known to result in stress and impaired general performance. Keeping the importance of probiotics in aquaculture, the present study was aimed to evaluate the probiotic potential of *B. licheniformis* in the diets of *M. rosenbergii* juveniles for gastrointestinal colonization and optimum response. Moreover, the studies were conducted to assess the influence of *B. licheniformis* on the survival of prawn after challenging with *Vibrio alginolyticus*. Histology and histopathological studies of *B. licheniformes* fed prawns and infected prawns was also conducted. In addition, growth and survival of *M. rosenbergii* feeding with *B. licheniformis* was also assessed.

In the present study, three experimental trials were designed with first one to evaluate the influence of *B. licheniformis* on microbiological and immunological parameters of *M. rosenbergii* for a period of 45 days, second one to evaluate cumulative mortality against *V. alginolyticus* infection for a total period of 49 days and third one to evaluate influence of *B. licheniformis* on growth and survival in *M. rosenbergii* for a period of 60 days respectively. Totally six hundred and forty five of inter-moult juveniles of *M. rosenbergii* were randomly distributed into five distinct experimental groups with three replicates each. Five diets were prepared with *B. licheniformis* in different concentration, except the control. The diets were control (Basal diet), T₁ (1.0×10^6 cfu g⁻¹), T₂ (1.0×10^7 cfu g⁻¹), T₃ (1.0×10^8 cfu g⁻¹), and T₄ (1.0×10^9 cfu g⁻¹).

Throughout the experimental period the water quality was maintained at optimal level. Monitoring of the gastrointestinal colonization by *B. licheniformis* was carried out on 15th, 30th and 45th day, immune parameters on 45th day and growth at 20 day interval. After fed with probiotic feed for 45 days the prawns were challenged intramuscularly with a virulent strain of *V. alginolyticus* (1×10^6 cfu ml⁻¹) for susceptibility study for 4 days.

Growth parameters like weight gain percentage, specific growth rate, feed conversion ratio and feed efficiency ratio were observed better in the *B. licheniformis* supplemented groups than control. The survival was significantly high ($p < 0.05$) in all *B. licheniformis* treated groups compared to the control.

The present study revealed that *B. licheniformis* administered to *M. rosenbergii* through diets inhibited pathogenic *Vibrio* sp., Enterobacteriaceae, *Aeromonas* and *Pseudomonas*. This shows that *B. licheniformis* may have played a role as a competitive exclusion agent because its administration changed the bacterial population of the digestive tract. However, there was no competitive exclusion of these bacteria from the gut in control group.

The present study also showed improved prawn immunity by increasing their THC, Phenol oxidase (PO) activity, Superoxide dismutase (SOD) activity, Phosphatase activities, total proteins, glucose, antibacterial and bacteriolytic activities and bacterial agglutination titre of haemolymph. There was no significant

difference in urea and respiratory burst activity of haemolymph. This shows that *B. licheniformis* administration provided both cellular and humoral immune responses. These responses were elicited by presence of *B. licheniformis* in the prawns gut.

The present study also showed that the resident *B. licheniformis* in gut of *M. rosenbergii* presumably further protected the treated prawns against pathogenic bacterial infection by competitive exclusion. *B. licheniformis* have the capacity to reduce the viable bacterial cells, as evident from increase in the clearance efficiency of haemolymph of *M. rosenbergii* to *V. alginolyticus* infection.

Histological studies regarding hepatopancreas and gut of *M. rosenbergii* after feeding with *B. licheniformis* confirmed that probiotic bacteria had no adverse effect on the health of host. Histopathological studies reveal that *M. rosenbergii* fed with probiotic diet had less histopathological changes as compared to control diet fed prawns when infected with *V. alginolyticus*.

Based on the results of present study following conclusions are made:

- Supplementation of *Bacillus licheniformis* in diet increased the growth of *M. rosenbergii* juveniles.
- The *B. licheniformis* supplemented group showed better survival than non-supplemented group.
- *Bacillus licheniformis* is an adherent strain with the ability to colonize the gut.
- The immune parameters increased significantly in *B. licheniformis* supplemented group than that in non-supplemented group. Among supplemented group the 1×10^9 cfu g⁻¹ (T₄) showed better immunity.
- The *B. licheniformis* supplemented group showed lesser cumulative mortality than non-supplemented group.
- Histopathological changes were less in the *B. licheniformis* supplemented group than in non-supplemented group.
- *Bacillus licheniformis* can be used for oral bacteriotherapy in aquaculture industry.

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7. REFERENCES

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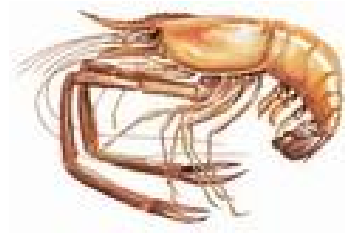
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*Not seen in original.



Appendices

APPENDIX-1: REAGENTS AND SOLUTIONS

I. Phosphate Buffer Saline (PBS) pH 7.2

NaCl	8.0 g
NaH ₂ PO ₄	1.44 g
KH ₂ PO ₄	0.2 g
KCl	0.2 g

Added to 800 ml of distilled water. Then pH was adjusted to 7.2, final volume was made to 1000 ml, autoclaved and stored at room temperature.

II. Physiological Saline

NaCl	0.85 g
Distilled water	100 ml

Autoclaved and stored at room temperature.

III. Anticoagulant Solution pH 7.6

Tris-HCl	10 mM
Sucrose	250 mM
Sodium citrate	100 mM

Added to required quantity of distilled water. Then pH was adjusted to 7.6, make up to final volume, autoclaved and stored at room temperature.

IV. L-DOPA solution

L-DOPA	1 mg
Sodium phosphate	100 mM

1 mg of L-DOPA was added to per ml of 100 mM Sodium phosphate.

V. Carbonate-bicarbonate buffer

Na ₂ CO ₃	0.1 M
NaHCO ₃	0.1 M

VI. Davidson's fixative

95% ethyl alcohol	330 ml
100% formalin	220 ml
Glacial acetic acid	115 ml
Distilled water	335 ml

VII. Acid alcohol

HCl	1 ml
Alcohol (70%)	99 ml

VII. Scott's tap water

NaHCO ₃	2 g
MgSO ₄	20 g
Distilled water	1000ml

APPENDIX-2: ABBREVIATIONS

%	percentage
@	at the rate
µl	Micro litre
A/G	Albumin/ Globulin ratio
ACP	Acid phosphatase
ALP	Alkaline phosphatase
APHA	American Public Health Association
B cells	Blasenzellen cells
BCG	Bromocresol Green
BHI	Brain Heart Infusion
BHT	Butylated Hydroxy Toluene
BOD	Biological Oxygen Demand
C	Control
cfu g ⁻¹	Colony Forming Unit per Gram
CO ₂	Carbon dioxide
CRD	Completely Randomized Design
DMSO	Dimethyl Sulphoxide
EDTA	Ethylene Diamine Tetraacetic acid
ELISA	Enzyme Linked Immuno-sorbent assay
EMB	Eosine Methylene Blue agar
F cells	Fibrillen-zellen cells
F.A.O.	Food and Agricultural Organization
FCR	Food conversion ratio
FER	Feed efficiency ratio
FRP	Fibre Reinforced Plastic
g%	Gram Percentage
g	Gram
G	Guage
GOD/POD	Glucose oxidase/ Peroxidase method
H ₂ S	Hydrogen Sulphide
Ha	Hectare
H	Hour
H & E	Haematoxylin & Eosin Staining

IU/L	International units per Litre
KA	King Armstrong
Kg	Kilogram
KOH	Potassium Hydroxide
L-DOPA	L-3,4-dihydroxyphenylalanine
L	Litre
Ln	Natural Log value
mg%	Milligram Percentage
mg/dl	Milligram per Decilitre
mg/L	Milligram Per Litre
min	Minute
ml	Millilitre
mm ³	Milli Meter Cube
mM	Milli Molar
M	Molar
MPEDA	Marine Product Export Development Authority
MTCC	Microbial Type Culture Collection
MT	Metric Tonnes
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NBT	Nitroblue tetrazolium
Nm	Nanometre
N	Normality
°C	Degree Celsius
O.D	Optical Density
ONPG	o-Nitrophenyl-β-D-Galactopyranoside
PBS	Phosphate Buffer Saline
PI	Percentage Inhibition
PO	Phenol oxidase
ppm	Parts Per Million
R cells	Restzellen cells
rpm	Revolutions Per Minute
S	Second
SDS	Sodium Dodecyl Sulphate

SGR	Specific growth rate
SOD	Superoxide dismutase
sp.	Species
T ₁	Treatment 1
T ₂	Treatment 2
T ₃	Treatment 3
T ₄	Treatment 4
TCBS	Thiosulphate Citrate Bile salt Sucrose agar
THC	Total Haemocyte Count
Tris-HCl	Tris-Hydrochloride
TSA	Tryptone Soya Agar
TSI	Triple sugar Iron
US\$	United States Dollar
W.H.O.	World Health Organization