Pro-PO Based Immunomodulatory Effect of Different Commercial Probiotics on Freshwater Prawns (*Macrobrachium rosenbergii*)

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A Thesis

By

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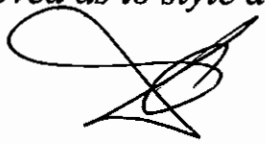

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DECLARATION

Pro-PO Based Immunomodulatory Effect of Different Commercial Probiotics on Freshwater Prawns (*Macrobrachium rosenbergii*)

The results submitted in this thesis are entirely the candidate's own investigations and no part of the results has been accepted for any degree, nor is it being concurrently submitted for any degree.

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Dedicated To

My Beloved Parents

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Ramisa Nowshin

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ABSTRACT

The effect of different commercial probiotic on Giant Freshwater Prawn (*Macrobrachium rosenbergii*) under different stocking density was studied. Three different types of probiotic were applied to see the effect of these probiotics on pro-PO concentration in prawn body. Nine treatments and one control group each having three replications of uniform dimensions ponds were selected in which culture of prawn (*Macrobrachium rosenbergii*) was done. After three months of experiment, *M. rosenbergii* fed with probiotic 3 represented a significantly ($p < 0.05$) greater growth rate ($T_7 = 22.39 \pm 0.05$, $T_8 = 14.08 \pm 1.65$, $T_9 = 9.74 \pm 1.16$) than did the control ($C = 5.96 \pm 1.67$). In addition, significant difference was also recorded in treatment and control in case of weight gain. A significant effect ($P<0.05$) of probiotics on the treatment groups was revealed after the overall analysis of the sample. The result of the analysis showed that the concentration of PO was higher in the treatments (T_1, 0.044 ± 0.002; T_4, 0.074 ± 0.003; T_7, 0.093 ± 0.004) compared to the control group (C, 0.0335 ± 0.004) during the first sampling and also for the second sampling (T_1, 0.2 ± 0.003; T_4, 0.187 ± 0.001; T_7, 0.2245 ± 0.014) comparing with the control group (C, 0.075 ± 0.008). Significant difference among the treatments was found but there was no significant difference found between T_1 ($P=0.098$) and control (C) group during the first sampling. Phenoloxidase concentration PO was higher in sampling 1 and 2 (T_7, 0.093 ± 0.004; T_7, 0.2245 ± 0.014) in shrimp fed with probiotic 3 diet and having a lower stocking density ($2m^{-2}$) in comparison with the shrimp fed with other two probiotics (Probiotic 1 and Probiotic 2) and having a relatively higher stocking density ($4,6\ m^{-2}$). The SPSS analysis of the treatments data of first sampling reveled that there is a significant effect of probiotic and stocking density combinedly on prawn's PO concentration. In the second sampling the SPSS analysis of the treatments data reveled that there was no significant effect of probiotic and stocking density combinedly on prawn's PO concentration.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

Abbreviation	Elaboration
µl	Microliter
ANOVA	Analysis of variance
C	Control
DANIDA	Danish International Development Agency
DFID	Department for International Development
DoF	Department of Fisheries
e. g.	For Example
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	And Others
etc.	Et cetera
FAO	Food and Agriculture Organization
Fig.	Figure
gm	Gram
i.e.	For Example
KU	Khulna University
L	Litre
L-DOPA	Dyhidrophenile alanine
M. Sc.	Master of Science
nm	Nanometer
PL	Post Larvae
PPAE	Prophenoloxidase activating enzyme
PPO	Polyphenoloxidase
ppt	Parts per thousand
pro-PO	Prophenoloxidase
spp.	Species

SPSS	Statistical Package for Social Studies
T	Treatment
USAID	United States Agency for International Development
MT	Metric Ton
LPS	Lipopolysaccharide
PG	Polygalacturonase
FRSS	Fisheries Resources Survey System

Chapter.....1

Introduction

1.1 Introduction

Bangladesh is considered as one of the most suitable countries in the world for giant freshwater prawn (*Macrobrachium rosenbergii* De Man 1879) farming, because of its favorable resources and agro-climatic conditions. A sub-tropical climate and a vast area of water bodies provide a unique opportunity for the production of *M. rosenbergii* culture in Bangladesh. Among twenty-four species of freshwater prawns 10 species of *Macrobrachium* are found in Bangladesh (Akand & Hasan 1992; Ahmed 2001; Muir 2003). Now this days freshwater prawn farming is currently contributing a lot in the national economy of the country (DoF, 2006). Though prawn culture has undergone rapid development, but sustained production is increasingly hampered by environmental pollution, poor management and epizootic diseases (Itami *et al.*, 1989). Recently, diseases in fresh water prawn culture are controlled by using antibiotics and chemicals (Karunasagar *et al.*, 1994). However, their indiscriminate use has led to the appearance of antibiotic-resistant strains (Skjermo and Vadstein, 1999) as well as contamination of prawn meat and the environment (Holmström *et al.*, 2003). Therefore, several countries have banned the use of antibiotics such as chloramphenicol (FAO, 2002). Recently, an alternative that has been widely employed in the aquaculture industry is the dietary supplementation with probiotic bacteria (Gatesoupe, 1999; Vine *et al.*, 2006). Gatesoupe (1999) defines probiotics as "live microbial cells administered to cultured organisms to colonize the digestive tract and improve their immune response".

Research in terms of immunity has received a high priority to control disease and to ensure long-term survival of shrimp culture. Invertebrates, including crustaceans, do not have acquired immunity, instead they have an innate immune system, which includes cellular events (e.g. phagocytosis, nodule formation and encapsulation) and humoral events (e.g. antimicrobial peptide synthesis, lectin and complement like factors, and melanization by activation of the prophenoloxidase activating system) (Soderhall, 1999). Melanosis is a process that is triggered by a biochemical mechanism consisting of oxidation of phenols to quinones by means of an enzymatic complex known as polyphenoloxidase (PPO). This is followed by nonenzymatic polymerization of the quinones, giving rise to pigments of high molecular weight and very dark or black colouring (Ogawa *et al.*, 1984). The pro-PO mechanism is one of the main defensive mechanisms as a non-self-recognition system (LeMoullac *et al.*, 1997). The pro-PO system acts both as recognition and effector component of the arthropod defense system, since it can specifically be enhanced by

polysaccharides from fungal or bacterial cell walls (Vargas-Albores, 1995). In prawns, pro-PO is activated by two steps: in the first step, degranualation occurs when haemocytes are stimulated by microbes, which enable the inactive form of pro-PO and prophenoloxidase activating enzyme (PPAE) to be released. The second step requires the participation of Ca^{3+} for the conversion of inactive PPAE to an active serine protease that in turn transform pro-PO to active PO (Vargas-Albores *et al.,* 1998). Thus, the commercial probiotics might be capable of stimulating prawn haemocytes to release cellular components for immune enhancement of prawn.

Various types of research work in the area of innate immunity in arthropods is rapidly progressing, whereas prawn immunity research has been a subject of minor interest compared to similar research performed on other crustaceans and insects. The present knowledge of the immune system of prawns is still limited. The giant fresh water prawn can be a good model to use mainly because they are short lived compared to many other crustaceans. This study describes the characterization of Pro-PO extracted from imperial fresh water prawn (*Macrobrachium rosenbergii*) and the possible effect of some commercial probiotics on Pro-PO activity.

1.2 Objectives

Objectives of the investigation was-

- To evaluate the effect of different commercial probiotics and stocking densities on the pro-Phenoloxidase enzyme activity in *Macrobrachium rosenbergii.*

Chapter.....2

Review of Literature

2. Literature review

2.1. Overview of aquaculture

For giant freshwater prawn farming Bangladesh is considered one of the most suitable countries in the world (*Macrobrachium rosenbergii* De Man 1879). Farming Freshwater prawns (*Macrobrachium rosenbergii,* locally called "golda") in ghers has become an important climate resilient livelihood option for small farmers, especially in south-western Bangladesh. This species is suitable for polyculture and is readily integrated with fish and rice cultivation. Prawn farming has grown in popularity since its introduction to Bangladesh in the early 1980s and is now widely practiced in coastal areas (Barmon *et al.*, 2007; Wahab *et al.*, 2008). Relatively high market values, fast growth, temperature tolerance and disease resistance have led increasing numbers of farmers to involve in freshwater prawn farming (Roustaian *et al.*,2001; Gupta *et al.*, 2007; Ahmed *et al*, 2010). The fisheries sector in Bangladesh has been earning a notable amount of foreign exchange. The sector earned BDT 4,287.64 crore by exporting almost 68.31 thousand MT of fish and fisheries products in the year 2017-2018 in which shrimp and prawn contributed BDT 3527.07 with a 30% contribution from prawn (FRSS, 2018). The economic and nutritional potential of freshwater prawn farming hinges in part on the availability of cost-effective quality feeds, seed availability and the management of pathogens (Wahab *et al.*, 2012).

Prawn aquaculture, as well as other industries, constantly requires new techniques in order to increase production yield. Modern technologies and other sciences such as biotechnology and microbiology are important tools that could lead to a higher quality and greater quantity of products (Fuller, 1992). Probiotics, as 'bio friendly agents' such as lactic acid bacteria and *Bacillus* spp., can be introduced into the culture environment to control and compete with pathogenic bacteria as well as to promote the growth of the cultured organisms. In addition, probiotics are nonpathogenic and nontoxic microorganisms without undesirable side-effects when administered to aquatic organisms (Ali, 2000).

This literature review will rely on Giant Fresh Water Prawn- their biology, life history, etc., probiotics and their importance as immunostimulator and some works that have been already done about the effects of probiotics on pro PO activities of crustaceans.

2.2. Giant Freshwater Prawn, (*Macrobrachium rosenbergii*)

2.2.1. Taxonomy

Domain: Eukaryota

Kingdom: Metazoa

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Subclass: Eumalacostraca

Order: Decapoda

Suborder: Natantia

Unknown: Palaeomonoidea

Family: Palaemonidae

Genus: *Macrobrachium*

Species: *Macrobrachium rosenbergii* (De Man, 1879)

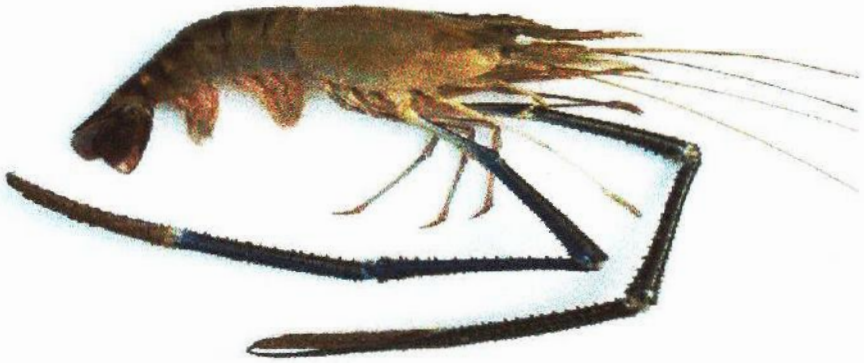

Fig 1: Giant Fresh Water Prawn (*Macrobrachium rosenbergii*) [Source: Ahmed, 2009]

2.2.2. Morphology

Macrobrachium rosenbergii is an invertebrate with exoskeleton or shell. The body of a prawn is composed of three parts: head, abdomen and tail (Nakagawa and Nagayama, 1981; Ogawa *et al.,* 1984). There are five pair of walking legs at the head part of which first pair is used in putting food into the mouth. The first 'walking leg' is not readily visible being very long and delicate in form but tightly folded up under the cephalothorax and functions as a feeding appendage with fine forcep-like chelae at their tips. The second pair is much larger than the others and ends in pronounced claws and is used for self-defense and catching food. In the males these walking legs can have a vibrant shade of blue and can also be twice the body length

(Simpson *et al.*, 1987). Holthuis (1980) found a distinctive feature in the adult male is that the moveable finger of the second walking leg or cheliped is covered in tightly packed long setae that give a velvety appearance to the appendage. The rostrum of this species is long reaching beyond the antennal scale and is slightly slender with dorsal and ventral rostrum teeth number 12 to 15 and 8-14 respectively. The tail part is composed of two uropods and one telson (Ali *et al.*, 1994). In contrast to the cephalothorax the segmentation of the abdomen is very distinct. This part possesses six segments where each segment bears a pair of ventral appendages called pleopods or swimmerets. The antennules and antennae of number of crustaceans are considered the most important sites of sensory reception (Chen *et al.*, 1991). Smooth rounded dorsal body surface present in *Macrobrachium* spp., (Dewitt, 1988; Taylor and Bush, 1986). Males can attain larger size than females and in dominant males the second walking legs are much longer and thicker. The abdomen of the male is narrower and the female, as well as having a wider abdomen, has longer pleura (the overlapping plates of cuticle extending from the exoskeleton) and these combined forms a chamber for incubating the eggs carried on the pleopods. The male genital openings are on the fifth walking legs and the female's genital pores are on the third walking leg (Ismael and New, 2000).

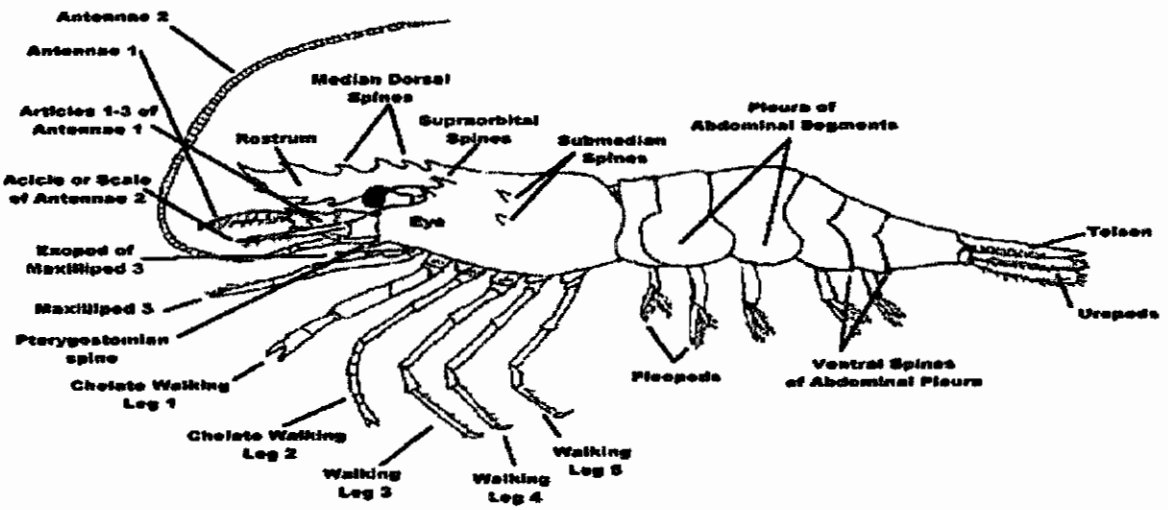

Fig 2: Morphology of Giant Fresh Water Prawn (*Macrobrachium rosenbergii*) [Source: New, 2002]

2.2.3. Coloration

Silva (2007) reported that prawns are able to change their colour (e.g. from greenish-grey) depending upon growth, background colouration and time of day. This colouration is due to

the colour of the chromatophores found in the prawn's skin beneath the external shell. In small individuals, delicate striping on the cephalothorax can be seen but these markings are not apparent in tank-reared specimens. The chelipeds of dominant males are bright blue but more yellowish in non-dominant males and females. The ventral side is pale and translucent (Graziani *et al.,* 2003).

2.2.4. Habitat and distribution

There are 150 species of *Macrobrachium* in the world of which 49 are commercial. Twenty-seven of the commercial species are found in Asia and the Pacific. Most live in freshwater a few species live in brackish water in the mouth of rivers. *M. rosenbergii* is found in the tropical and sub-tropical waters in the Indo-Pacific region in Bangladesh, Malaysia, Thailand, the Philippines, India, Sri Lanka, Myanmar, Indonesia and Vietnam. They are generally found in freshwater ponds, rivers, lakes, ditches, canals, depressions, low lying flood plains and river mouths. Prawns move upstream, entering lakes and even paddy fields, up to about 200km from the sea. This type of migration is observed not only in *M. rosenbergii*, but also in other species of *Macrobrachium.* (McEvily *et al.*, 1991; Otwell *et al.,* 1992; Slattery *et al.,* 1995).

Distribution of different life stages of this species have been reported from India (Rajyalakshmi and Maheswardu 1986, Rao, 1991) and from the river systems of Bangladesh (Patra, 1977; Hill and Kibria, 1979; Kibria, 1983). George (1969) and Ling (1969) reported that the adult *M. rosenbergii* are available throughout the year in both the freshwater and brackish water zones of Bangladesh, except the north- west region (Pabna to Dinajpur) and Chittagong Hill tracts. The berried females need a salinity range from 5 to 20 ppt and a temperature range from 26 to 33°C to breed. Distribution of larvae and post- larvae of *M. rosenbergii* were found to be strictly restricted only at the brackish water zone, with a few populations in the Dakatia, Chandpur and the Kapatakkha rivers during the dry and rainy seasons. Distribution of *M. rosenbergii* at a wide range of salinity (0.0 to 20 ppt) and temperature ranged from 25 to 33°C was also reported by Sebastian (1990) and Roa (1991). Bangladesh has a rich population of *M. rosenbergii* throughout her major river systems and the vast estuary.

Fig 3: Main producer countries of *M. rosenbergii* (FAO Fishery statistics, 2006)

2.2.5. Reproductive Behavior

The mating of adult *Macrobrachium* through deposition of a gelatinous mass of semen (referred to as a spermatophore) on the underside of the thoracic region of the female's body. Successful mating can only take place between ripe females, which have just completed their pre-mating moult (usually at night) and are therefore soft-shelled, and hard-shelled males. All of the various types of males are capable of fertilizing females but their behavior is different (Cornick and Stewart, 1968; Stew- art and Zwicker, 1972; Mori and Stewart, 1978).

In nature mating of prawn occurs throughout the year, although the peaks of activity related to environmental conditions. Within a few hours of copulation, eggs are extruded through the gonopores and guided by the ovipositing setae (stiff hairs), which are at the base of the walking legs, into the brood chamber. During this process the eggs are fertilized by the semen attached to the exterior of the female's body. Fertilized eggs are released into the sea, where they hatch. According to New and Singholka (1985), fecundity of *M. rosenbergii* varies considerably with the age, size and stage of maturity. Female prawns of *M. rosenbergii* are reported to lay from 80 000 to 100 000 eggs during one spawning when fully mature (Ling, 1969; Manus *et al.*, 2006).

The zoea are planktonic, swimming upside down within the water column and exhibit positive phototaxy. Larval development is temperature dependent. After metamorphosis, the PL assume a benthic lifestyle and begin to migrate upstream into freshwater (New and Singholka, 1985). *M. rosenbergii* larvae require brackish water for survival (Unestam and Soderhall, 1977; Soderhall, 1982; Smith and Soderhall, 1983; Hose *et al.*, 1990

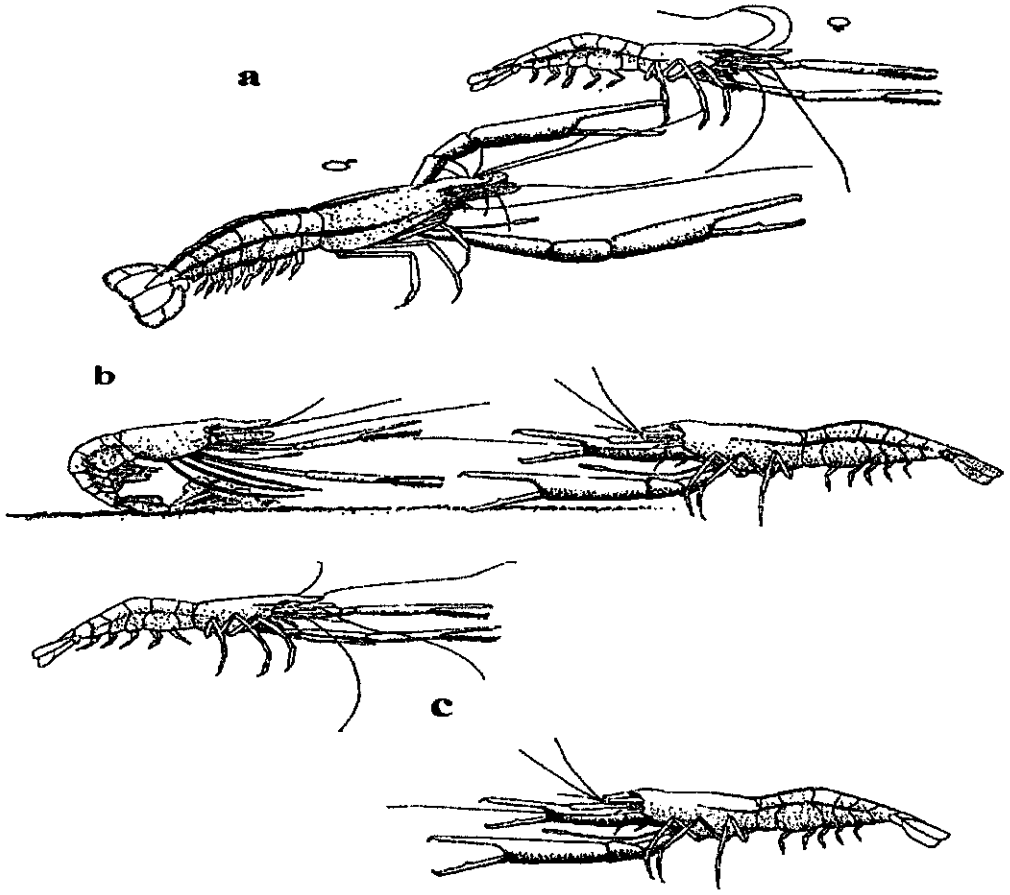

Fig 4: Mating behavioral patterns during natural mating in the interspecific crosses, between *M. rosenbergii* males and *M. rosenbergii* females. (a) Antennal and/or antennular recognition, (b) retreat of the female from the aggressive male, (c) both animals locate distantly from each other.

2.2.6. Feeding habit

The main food contents of prawns are vegetable matter, digested matter, detritus, crustaceans' matter and non-crustaceans matter (Marshal and Orr, 1960). Juvenile and adult prawns are omnivorous and feed frequently and voraciously on a wide variety of food items. According to Chopra (1939), the prawns eat all types of food living or dead what comes on their way. Prawns consume algae, planktonic organisms, small muscle pieces of their own kind or fish etc., which could be held in the chelae of legs are taken. Larvae are omnivorous, feeding mainly on zooplankton. In the absence of live food, they are capable of feeding on small particles of organic matter (Ling, 1969; New and Singholka, 1985). Post larvae and adult *M. rosenbergii* are omnivorous benthivores, and mainly feed on algae, aquatic plants, mollusks, oligochates,

aquatic insects and other crustaceans (Ling, 1969). P. Sivanandavel, P. Soundarapandian and T. Kannupandi (2007) suggest that food of animal origin is preferred over plant material.

2.2.7. Growth

The growth rate of prawn is fast, showing sexually dimorphic pattern. The males grow faster with heterogenous growth rate (Holthuis, 1980; Hartnoll, 1982). All freshwater prawns (like other crustaceans) have to regularly cast their 'exoskeleton' or shell in order to grow. This process is referred to as moulting and is accompanied by a sudden increase in size and weight. There are four distinct phases in the life cycle of the freshwater prawn, namely eggs, larvae, post larvae (PL) and adults. The time spent by each species of *Macrobrachium* in the different phases of its life cycle (and its growth rate and maximum size) varies, not only specifically but according to environmental conditions, mainly temperature (Ismael and New, 2000; Karplus *et al.*, 2000).

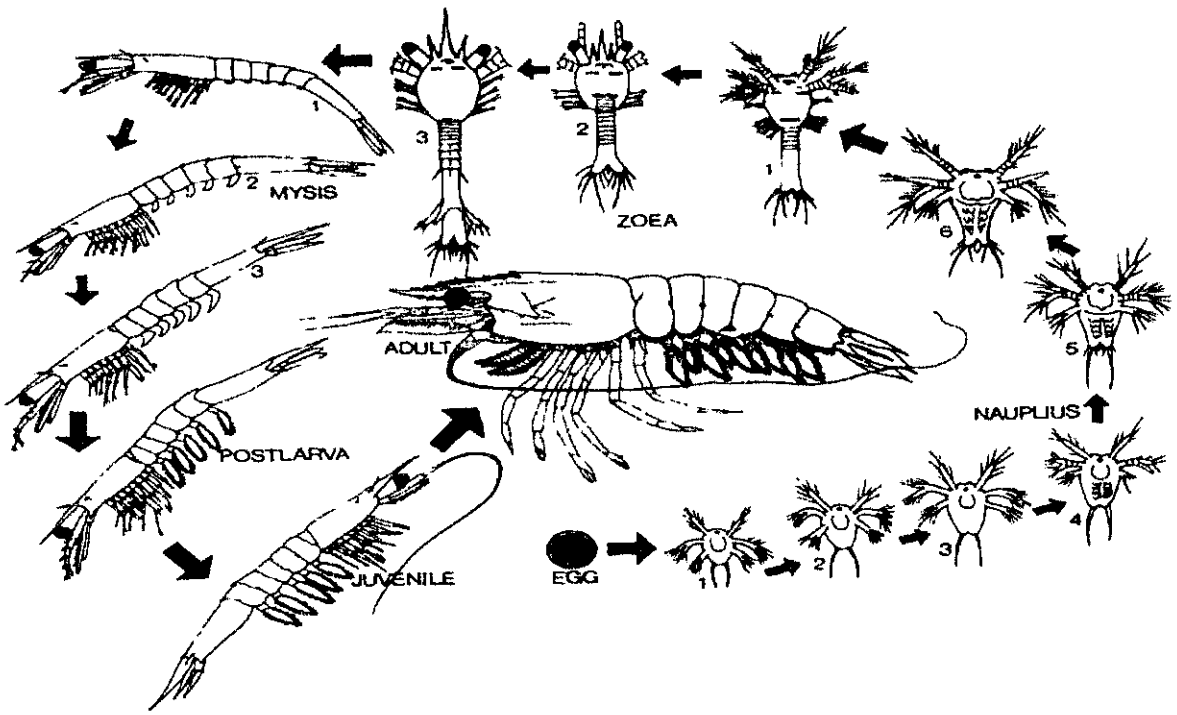

Fig 5: Life cycle of giant fresh water prawn (*Macrobrachium rosenbergii*)

2.3. Prawn Immunity

2.3.1. Immune mechanism of *M. rosenbergii*

The freshwater prawn *M. rosenbergii*, has a proven potential for use as an aquaculture species (Willis *et al.*, 1976; Sandifer and Smith, 1977; Hanson and Goodwin, 1977). Fortunately, it is

largely resistant to infectious disease, the major hazard to successful culture being black-spot disease (Sindermann, 1977a). In freshwater prawn the innate defense system – also known as natural or non-specific defense system includes both cellular and humoral components which work in jointly coordination for the elimination of all foreign organisms potentially hazardous for the host (Jiravanichpaisal *et al.,* 2006). Cellular defense components include all those reactions performed directly by hemocytes (phagocytosis, encapsulation, nodule formation). On the other hand, humoral components include the activation and release of molecules stored within hemocytes, such as anticoagulant proteins, agglutinins, phenoloxidase enzyme, antimicrobial peptides, protease inhibitors, etc. (Jiravanichpaisal *et al.,* 2006; Holmblad and Soderhall, 1999).

The phenoloxidase system has been recognized as an efficient defense mechanism against the non-self. This system is stored and produced by semi-granular and granular hemocytes, and it can be activated by a minimum presence of microbes. Activation of the prophenoloxidase system results in the production of melanin, a dark-brown pigment responsible among other processes for inactivating foreign particles, and preventing their spread throughout the host body, as well as for healing cuticle damages (Sritunyalucksana and Soderhall, 2000).

Agglutinins, bactericidins, and phagocytes have also been found in the serum of various *Macrobrachium* spp (Tripp, 1966; Bang, 1967; Rabin, 1969; Hildemann and Reddy, 1973; Paterson and Stewart, 1974; Gushing, 1975; Mori and Stewart, 1978). The agglutinins are capable of distinguishing red blood cells and bacterial species, but there is presently no evidence of antibody production similar to that of vertebrates. An understanding of *Macrobrachium* immune reactions will help to define crustacean resistance, and the extent to which it can be augmented

2.3.2. Recognition molecules

Invertebrates do not have immunoglobulins, the specific recognition molecules found in higher animals, although proteins containing immunoglobulin-like domains have been identified in many species (Lanz-Mendoza and Faye, 1999). Instead they have the so-called pattern recognition proteins PRPs to recognize and respond to microbial intruders by the presence of signature molecules on the surface of the intruders (Janeway, 1989). Several pattern recognition molecules have been isolated and characterized for example, b-1,3-glucan binding proteins BGBP (Ochiai and Ashida, 1988; Soderhall *et al.,* 1988; Duvic and Soderhall, 1990, 1993; Seki *et al.,* 1994) LPS-binding proteins (Sun *et al.,* 1990; Natori and Kubo, 1996; Lee *et*

al., 1996; Dimopoulos *et al.*, 1997) and Peptidoglycan-binding proteins (Yoshida *et al.*, 1996; Kang *et al.*, 1998; Ochiai and Ashida, 1999). A plausible hypothesis is that the BGBPs developed from a primitive glucanase and then evolved into proteins without glucanase activity, but instead bind glucans and after binding, operate as elicitors of defense responses. Recently, two molecules isolated and cloned from the coelomic fluid of the earthworm (Beschin *et al.*, 1998) and from the hemocytes of crayfish, (Lèe *et al.*, 2000) showed affinity to both b-1,3-glucans and LPS and both molecules have been shown to be involved in the activation of the proPO system. In shrimp, a 175-kDa LPS-binding protein from the hemolymph was isolated, but its function in immunity is still unclear (Vargas-Albores *et al.*, 1993). Peptidoglycan-recognition proteins, since PG can induce activation of the proPO system in shrimps, it may indicate that PGRP may be present in shrimp (Sritunyalucksana *et al.*, 1999)

2.3.3. Pro Phenoloxidase Mechanism

The prophenoloxidase (proPO) activating system is an important innate immune response against microbial infections in invertebrates. The prophenoloxidase (ProPO) system is the origin of melanin production and serves as an innate defense mechanism in several crustacean species, including prawn, lobster, crab, bivalves etc. (Coles and Pipe, 1994; Asokan *et al.*, 1997; Cong *et al.*, 2005; Aladaileh *et al.*, 2007; Hellio *et al*, 2007). In these species, proPO has been detected in both the haemolymph and in haemocytes (Ashida *et al.*, 1983; Saul *et al.*, 1987; Hernández-Lopez *et al.*, 1996). The proPO, an inactive proenzyme, can be activated by lipopolysaccharide (LPS), β-1,3-glucans and peptidoglycans (Söderhall and Hall, 1984) through a signal cascade mediated by serine proteases. Moreover, proPO expression can be modulated by pathogens such as bacteria and parasites (Hong *et al.*, 2006; Muñoz *et al.*, 2006; Bezemer *et al.*, 2006).

However, in crustaceans, several immune mechanisms have been described as well as some of the mechanisms which trigger them, and they are derived essentially from haemolymph cells (Soderhall and Smith, 1983; Hose and Martin, 1989; Hose *et al.*, 1992; Smith and Chisholm, 1992; Clare and Lumb, 1994; Destoumieux *et al.*, 1997). Three cell types: hyaline, semigranular and granular, each having a main function, are commonly recognized in crustaceans (Bauchau, 1981; Tsing *et al.*, 1989; Hose *et al.*, 1990) as the main functions are coagulation, phagocytosis and the production of melanin by the prophenoloxidase proPO system. Haemocytes are activated with the help of the plasmatic system-recognizing microorganisms (Vargas-Albores, 1995; Varga-Albores *et al.*, 1997) and are involved in phagocytosis which eliminates foreign particles (Hose and Martin, 1989; Bachere *et al.*, 1995).

The immune response involves the release of another important protein, the peroxinectin, from the blood cells (Johansson *et al.*, 1995) and has cell adhesion, degranulation, opsonic and peroxidase activity. The proPO system involved in melanization and limiting bacterial proliferation is contained in the granulocytes (Soderhall and Smith, 1983; Johansson and Soderhall, 1989). The proPO is activated in phenoloxidase PO by a serine protease (Soderhall, 1983) that is under the control of inhibitors (Hergenhahn and Soderhall, 1985).

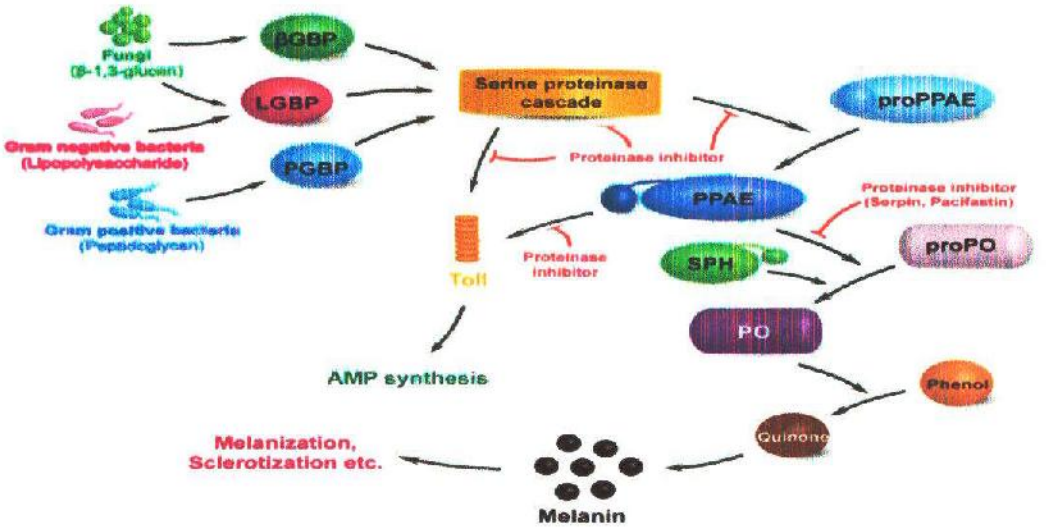

Fig 6: Diagram of the Pro-PO activation system in crustaceans [Source: Schulze, 2006]

2.3.4. Probiotics: an immunostimulator

The microbiota in the aquatic environment is usually in equilibrium and it is composed by bacteria that are either benefic or neutral to cultured animals, or by harmful obligate and opportunistic pathogenic bacteria (Schulze *et al.*, 2006). Such equilibrium can be disturbed by inadequate management and result in the proliferation of pathogenic bacteria (Karunasagar *et al.*, 1994).

In prawn culture, the control of bacterial diseases is done mainly by the use of antibiotics (Karunasagar *et al.*, 1994). However, their indiscriminate use has led to the appearance of antibiotic-resistant strains (Skjermo and Vadstein, 1999), further to the contamination of shrimp meat and also the environment (Holmström *et al.*, 2003). Therefore, several countries have banned the use of antibiotics such as chloramphenicol (FAO, 2002).

Recently, an alternative that has been widely employed in the aquaculture industry is the dietary supplementation with probiotic bacteria (Gatesoupe, 1999; Vine *et al.*, 2006). Gatesoupe (1999) defines probiotics as "live microbial cells administered to cultured organisms to

colonize the digestive tract and improve their immune response". The use of probiotics in the culture of aquatic organisms is increasing, with the demand for more environment-friendly aquaculture practices (Gatesoupe, 1999). Probiotics in aquaculture have been shown to have several modes of action: competitive exclusion of pathogenic bacteria through the production of inhibitory compounds; improvement in water quality; enhancement in the immune response of the host species to resist against infectious agents; and enhancement in the nutrition and growth of host species (Thompson *et al.*, 1999; Verschuere *et al.*, 2000). Bacteria that have been used successfully as probiotics include Bacillus, lactic acid bacteria, yeasts, *Pseudomonas* and *Vibrio* (Moriarty 1998; Rengpipat *et al.* 2000; Alavandi *et al.*, 2004; Wang *et al.*, 2008). Among the probiotic bacteria used in aquaculture, the lactic acid bacteria stand out for their easy multiplication, production of antimicrobial compounds (bacteriocins, hydrogen peroxide, organic and lactic acids), and the stimulation of the non-specific immune response of the host (Kohen & Nyska, 2002). Studies have demonstrated the beneficial effect of the addition of such bacteria in the culture of several aquatic species (Kohen and Nyska, 2002). Studies have shown that administration of the bacteria as probiotics in the shrimp *Litopenaeus monodon* and *Fenneropenaeus indicus* as well as in the *Labeo rohita* led to improved growth and survival rate were improved, as well as enhancement in immunity and disease resistance (Rengpipat *et al.*, 1998; Rengpipat *et.al.*, 2000; Saeed *et al.*, 2006; Nayak *et al.*,2007). Among these, *Bacillus* bacteria have been used widely as putative probiotics. Especially *B. subtilis*, a Gram positive nonpathogenic, spore-forming bacterium, is now being used for oral bacterial therapy, prophylaxis of gastrointestinal disorders, improving pond water quality and the survival of animals in aquaculture (Irene *et al.*, 2005). Latory immunostimulatory agent in a variety of diseases have been documented (Green *et al.*, 1999). A number of microbial cell components such as muramyl dipeptide, lipopolysaccharides, b-glucans and heat-killed bacterial preparations are reported to possess immunostimulatory properties (Sakai, 1999). Nevertheless, only a few studies have been conducted to assess the use of bacteria strains isolated from marine prawn. However, studies on the use of live bacterial cells as probiotics to improve the immune system and antioxidant capacity are scarce (Rengpipat *et al.*, 2000).

2.4. About the present study

In aquaculture, both farming practices and environmental conditions subject prawns to stressful situations that impact them strongly. These stressors (pollution, low level of oxygen, temperature variations, sorting and transfer, osmotic shock, high stocking density) can favor the development of opportunistic pathogens and mortal infections. Various factors affect the

release of pro-phenol oxidase enzyme in prawn body, which is responsible for the innate defense system. This pro-phenol oxidase enzyme causes the elimination of all foreign organisms potentially hazardous for the host (Jiravanichpaisal *et al.,* 2006). High density can negatively affect (Iguchi *et al.,* 2003), resulting in lowering their metabolic rate, weakening their immunity ability (Yin *et al.,* 1995; Ellis *et al.,* 2002; Sloman *et al.,* 2002; Trenzado *et al.,* 2006). In this study an attempt was taken to evaluate how different probiotics can affect the release of prophenoloxidase enzyme under different stocking density in *M. rosenbergii*.

Chapter.....3

Materials and Methods

3.4. Design of experiment

There were 10 experimental ponds. The experimental ponds used in this study were same sizes. While 9 ponds each were used for the three immunostimulant-incorporated feeds having three replications (T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8 and T_9), the remaining 1 pond was maintained for the control feed (C) also having three replications. In this experiment, three commercial probiotics (Table 1) were used to intensify *M. rosenbergii* culture. Probiotic application and stocking density were designed according to the following table (Table 2). The two samples of prawn were collected at an interval of 30 days from these ponds.

Table 1: Bacterial composition of the three probiotics

Commercial name of probiotics	Bacterial composition	Fungi	Other ingredients
Probiotic 1	*Rhodococcus, Rhodobacter, Nitrosomonus*		
Probiotic 2	*Rhodococcus, Rhodobacter, Nitrosomonus +Bacillus subtilis. Streptococcus faecalis, Bacillus mesentericus, Clostrodium butyricum*		Mutagen
Probiotic 3	*Bacillus mesentericus, Bacillus subtilis, Bacillus licheniformis, Lactobacillus acidophilus, Nitrobacter* sp., *Nitrosomonus* sp., *Saccharomyces cerevisiae*	*Aspergillus oryzae*	

Table 2: Layout of the Experiment

Treatments	Replication	Commercial Probiotics	Stocking density(/m²)
C	3	No	2
T_1		Probiotic-1	2
T_2		Probiotic-1	4
T_3		Probiotic-1	6
T_4		Probiotic-2	2
T_5		Probiotic-2	4
T_6		Probiotic-2	6
T_7		Probiotic-3	2
T_8		Probiotic-3	4
T_9		Probiotic-3	6

3.5. Apparatus

The apparatus which were used was 26-gauge needle, 1ml syringe (DISPOVAN), Eppendorf tube, Weight machine, Glass jar, Rotator, Pipet, Centrifuge machine, Incubator, and Spectrophotometer.

3.6. Reagents and solutions

The chemicals that were used was Trisodium citrate, NaCl, Glucose, EDTA, Zymosan, Anticoagulant, Cacodylated buffer and L-DOPA (Dyhidrophenile alanine). Because the Pro PO system can be activated by endotoxins, all glassware was washed with E-Toxa-Clean. All solutions were prepared with pyrogen free water for the same reason.

3.7. Sample collection

Samples were collected at 30 days interval from the ponds in order to determine the pro phenol oxidase activity. After collecting the samples from the pond, they were taken to the lab as soon as possible. To carry them ice boxes were used.

3.8. Pro-PO assay

3.8.1. Haemolymph collection

Haemolymph was collected from experimental prawns (after giving probiotics - incorporated prawn feed) using a 26-gauge needle of 1 ml syringe by inserting it into the ventral sinus located at the base of the first abdominal segment. Haemolymph was collected 1:1in anticoagulant to carry out pro-PO assay. The collected haemolymph was then transported to the laboratory in the chilled condition for enzyme assay.

3.8.2. Measurement of immune parameter

Phenoloxidase activity in haemocytes was measured spectrophotometrically by recording the formation of dopachrome from L-dihydrophenylalanine (L-DOPA). Zymosan (0·1%) was prepared in cacodylate buffer (sodium cacodylate 10 mM, $CaCl_2$ 100 mM, pH 7) centrifuged for 10 min at 2000 g and the supernatant used as an elicitor of the pro PO system. Haemolymph withdrawn was centrifuged for 10 min at 1000 g at 5^0 C. The supernatant was discarded, the pellet rinsed and suspended gently in cacodylate buffer, centrifuged again and finally taken again in 200 µl cacodylate buffer. Cell suspensions were incubated with an equal volume of zymosan for 1 h at room temperature. At the end of the incubation, the mixture was centrifuged for 5 min at 700 g and 180 µl of supernatant were placed in microtitration plate wells. A 75 µl aliquot of L-DOPA (4 mg ml^{-1} of cacodylate buffer) was then added. After 10 min, a further 900 µl of cacodylate buffer was added and the optical density at 490 nm measured in duplicate in a microplate reader.

3.8.3 Statistical analysis.

All analysis was carried out at 95 % confidence level. Data were analyzed using Microsoft Excel (2019) and SPSS 25.0 (IBM Corp., New York, USA). Before performing statistical test, the normal distribution of the data was checked by K-S test, Shapiro Wilk test and Q-Q plots. In this study one-way Anova was performed to compare the means between control and treatments. Two-way anova was performed to compare among the treatments.

Chapter.....4

Results

4. Results

4.1 Growth performance of *M. rosenbergii*

At the end of the experiment the growth parameter such as weight gain (WG) of *M. rosenbergii* from the nine treatment and one control pond was measured. After three months of experiment, *M. rosenbergii* fed with probiotic 3 represented a significantly ($p < 0.05$) greater growth rate ($T_7 = 22.39 \pm 0.05$, $T_8 = 14.08 \pm 1.65$, $T_9 = 9.74 \pm 1.16$) than did the control ($C = 5.96 \pm 1.67$) (Fig.8). In addition, significant difference was also recorded in treatments and control in case of weight gain. As for probiotic 1, T_1 having a stocking density of 2 m^{-2} showed better result than T_2 and T_3, which has a stocking density of 4,6 m^{-2}. Same result was found for probiotic 2 and probiotic 3 where treatments having a stocking density 2 m^{-2} showed better growth than the other two treatments. Among the three probiotics, probiotic 3 revealed better growth in T_7 ($T_7 = 22.39 \pm 0.05$) compared with the other treatments.

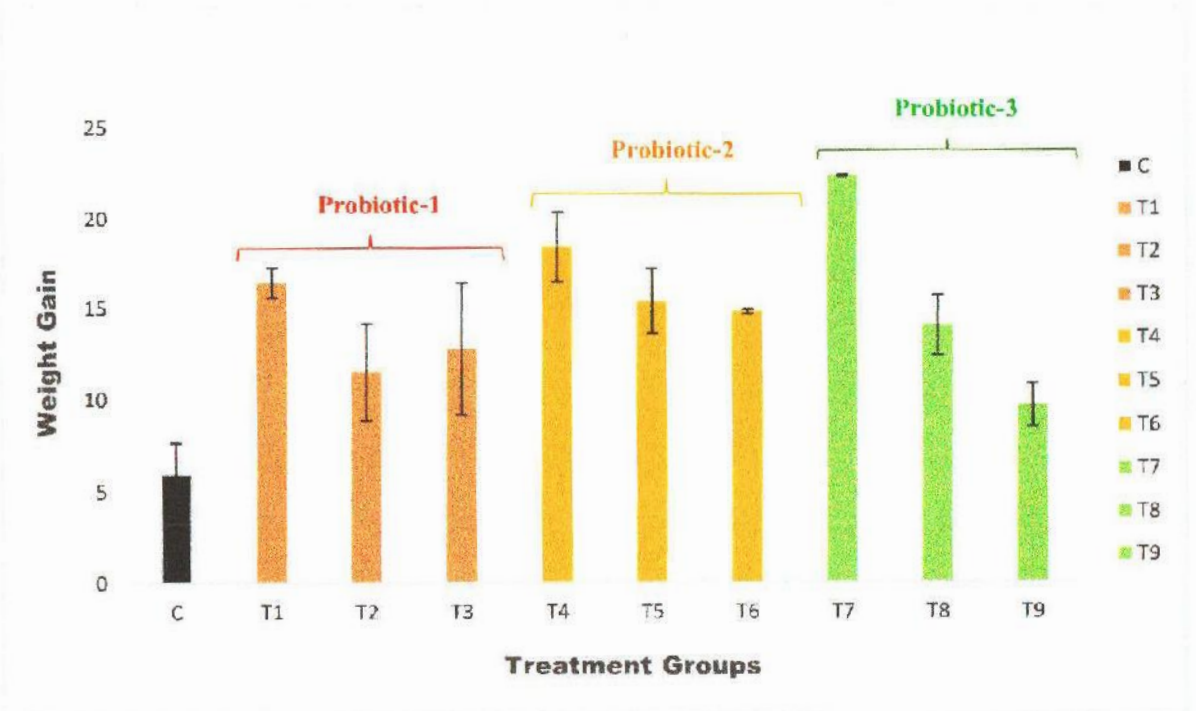

Fig 8: Weight gain of *M. rosenbergii* after five months of experiment.

4.2 Immunological parameters of *M. rosenbergii*

4.2.1 Assay 1

A significant effect ($P<0.05$) of probiotics on the treatment groups has been revealed after the overall analysis of the first sample. The result of the analysis showed that the concentration of

PO was higher in the treatments (T_1, 0.044 ± 0.002; T_4, 0.074 ± 0.003; T_7, 0.093 ± 0.004) compared to the control group (C, 0.0335 ± 0.004) (Fig 9). The post-hoc analysis has revealed that PO concentration of T_7 was higher than T_1 (P=0.001) and T_4 (P=0.011). Significant difference among the treatments was found but there was no significant difference found between T_1 (P=0.098) and control (C) group .

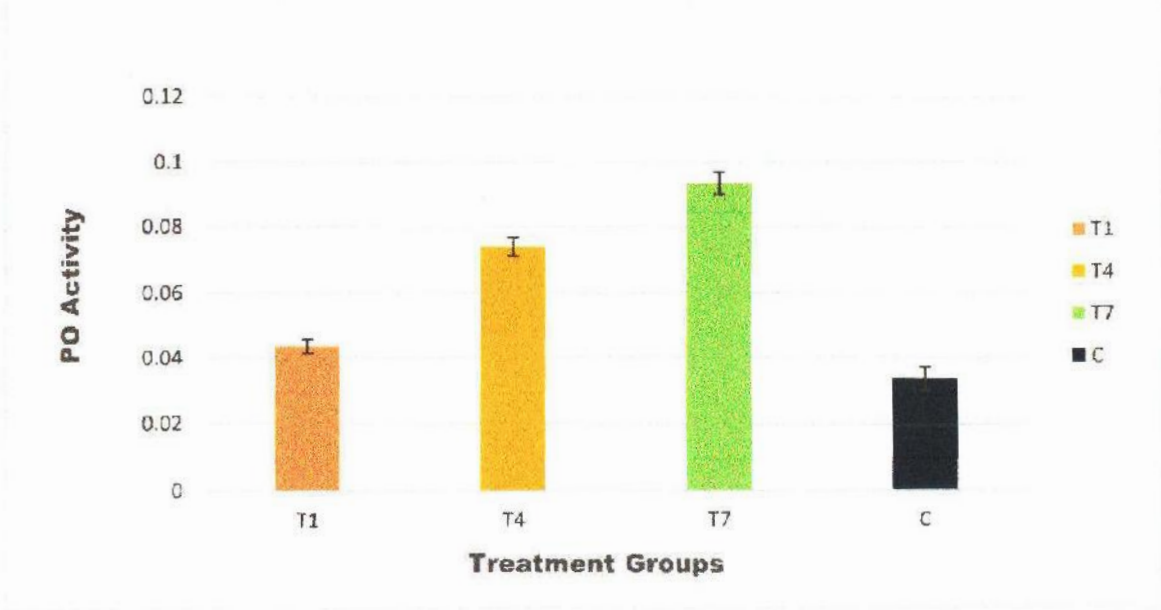

Fig 9: Comparison of the concentration of pro-phenoloxidase enzyme between control and treatments after the first sampling.

Phenoloxidase concentration PO was higher (T_7, 0.093 ± 0.004) in shrimp fed with probiotic 3 diet and having a lower stocking density (2 m^{-2}) in comparison with the shrimp fed with other two probiotics (Probiotic 1 and Probiotic 2) and having a relatively higher stocking density (4 and 6 m^{-2}). For probiotic 1 in different stocking density the phenoloxidase concentration ranged between T_1 = 0.044 ± 0.002, T_2 = 0.0435 ± 0.002, T_3 = 0.0425 ± 0.0007. For probiotic 2 it resulted T_4 = 0.074 ± 0.003, T_5 = 0.074 ± 0.003, T_6 = 0.07 ± 0.003 and for probiotic 3 the concentration was T_7 = 0.093 ± 0.004, T_8 = 0.061 ± 0.004, T_9 = 0.053 ± 0.0014 (Fig 10). These results explain that *M. rosenbergii* fed with probiotic 2 incorporated feed showed better results than probiotic 1 in different stocking density. The SPSS analysis of the treatments data reveled that there is a significant effect of probiotic and stocking density combinedly on prawn's PO concentration (Table 2).

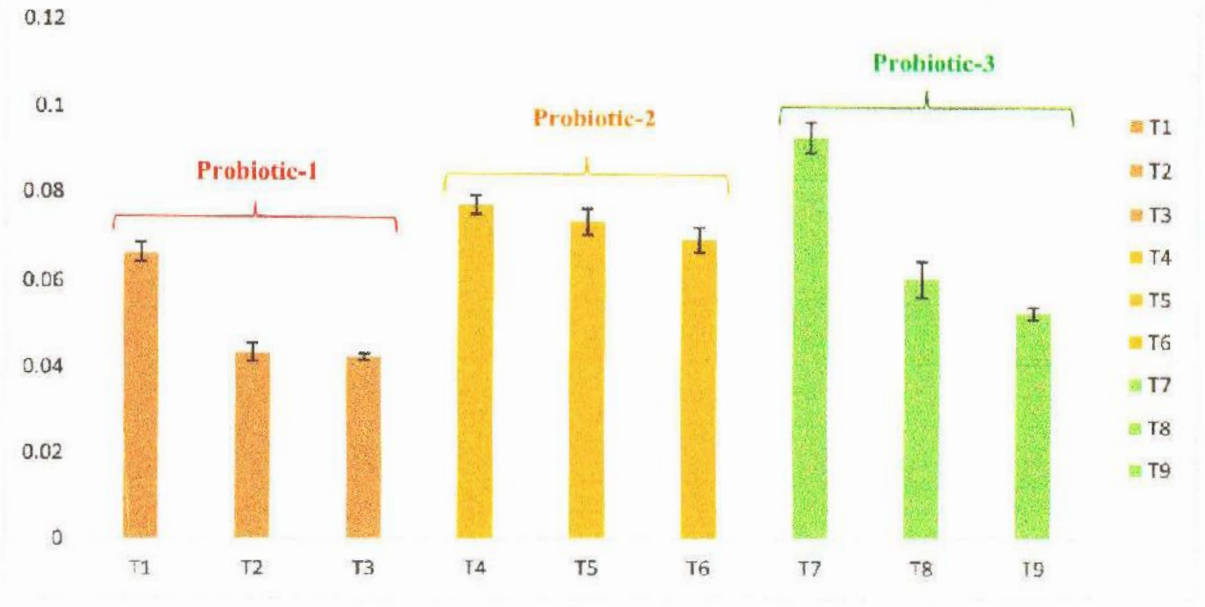

Fig 10: Comparison of the concentration of pro-phenoloxidase enzyme among the treatments after the first sampling.

Table 3. Evaluation of the combined effect of probiotics and stocking density in first sampling

Tests of Between-Subjects Effects

Dependent Variable: Absorbance

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.004[a]	8	.001	79.340	.000	.986
Intercept	.075	1	.075	10820.552	.000	.999
Probiotic	.002	2	.001	127.712	.000	.966
StockingDensity	.001	2	.000	58.664	.000	.929
Probiotic * StockingDensity	.002	4	.000	65.492	.000	.967
Error	6.250E-5	9	6.944E-6			
Total	.080	18				
Corrected Total	.004	17				

a. R Squared = .986 (Adjusted R Squared = .974)

4.2.2 Assay 2

After an interval of 30 days the second sampling was done. Again, a significant effect ($P<0.05$) of probiotics on the treatment groups has been revealed after the overall analysis of the sample. It showed PO concentration was higher in the treatments (T1, 0.2 ± 0.003; T4, 0.187 ± 0.001; T7, 0.2245 ± 0.014) compared to the control group (C, 0.075 ± 0.008) (Fig 11). The post-hoc analysis revealed the PO concentration of T7 was higher than T1 (P=0.015) and C (P=0.009).

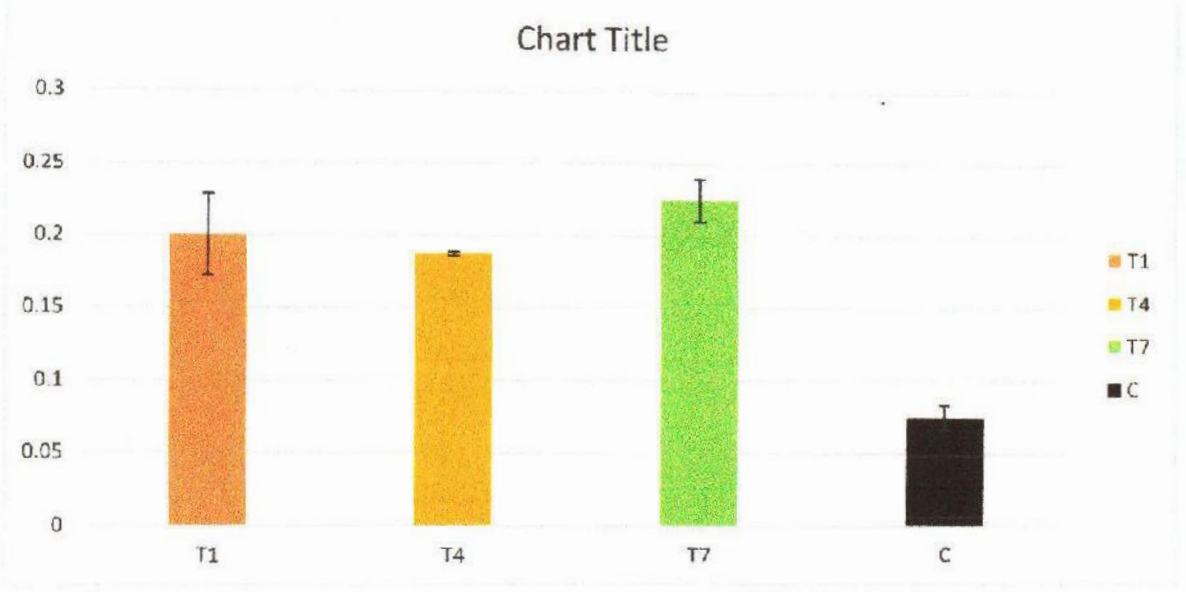

Fig 11: Comparison of the concentration of pro-phenoloxidase enzyme between control and treatments after the second sampling.

In the second sampling Phenoloxidase concentration PO was again higher (T_7, 0.2245 ± 0.014) in shrimp fed with probiotic 3 diet and having a lower stocking density (2 m^{-2}) in comparison with the shrimp fed with other two probiotics (Probiotic 1 and Probiotic 2) and having a relatively higher stocking density (4,6 m^{-2}). For probiotic 1 in different stocking density the phenoloxidase concentration ranged between T_1= 0.2 ± 0.003, T_2= 0.191± 0.007, T_3= 0.115 ± 0.031. For probiotic 2 it resulted T_4= 0.187 ± 0.001, T_5= 0.095 ± 0.021, T_6= 0.088 ± 0.01 and for probiotic 3 the concentration was T_7 = 0.224 ± 0.014, T_8 = 0.1755± 0.030, T_9 = 0.0795 ± 0.008 (Fig 12). The data shows for probiotic 2 results was better than probiotic 1 in different stocking density. The SPSS analysis of the treatments data reveled that there was no significant effect of probiotic and stocking density combinedly on prawn's PO concentration

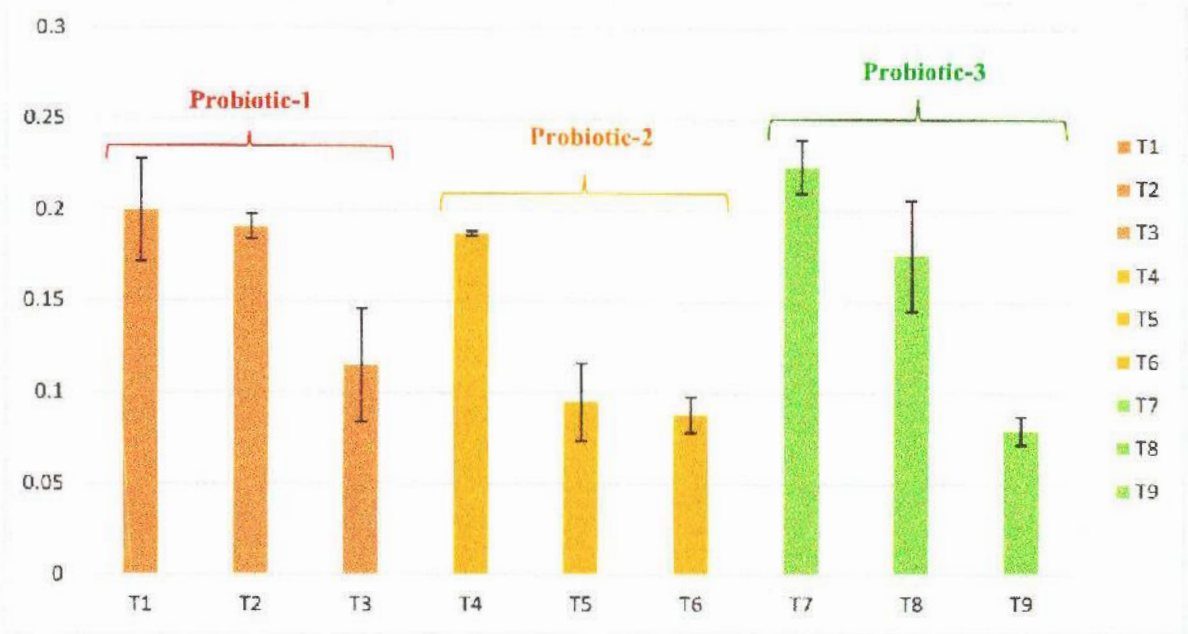

Fig 12: Comparison of the concentration of pro-phenoloxidase enzyme among the treatments after the second sampling.

Table 4. Evaluation of the combined effect of probiotics and stocking density in second sampling

Tests of Between-Subjects Effects

Dependent Variable: Absorbance

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.016[a]	8	.002	.490	.836	.304
Intercept	.408	1	.408	99.185	.000	.917
Probiotic	.002	2	.001	.220	.807	.047
Stocking	.002	2	.001	.271	.768	.057
Probiotic * Stocking	.012	4	.003	.735	.591	.246
Error	.037	9	.004			
Total	.462	18				
Corrected Total	.053	17				

a. R Squared = .304 (Adjusted R Squared = -.316)

Chapter.....5

Discussion

5. DISCUSSION

5.1 Effects on growth

Crustaceans, which lack adaptive immune system, are completely dependent on the innate immune system that respond against antigens on the surface of potential pathogens (Vazquez *et al.,* 2009). Haemocytes play an important role in cellular defense mechanisms such as phagocytosis, melanization, encapsulation and foreign cell lysis in crustaceans (Mats Johansson *et al.,* 2000). In general haemocytes are continuously released from the hematopoietic tissue and are known to release phenoloxidase and reactive oxygen species to kill the pathogens or as a defense against the diseases (Song and Hsieh, 1994; Sung *et al.,* 1998; Smith *et al.,* 2003). The dietary administration of probiotics to fish and crustaceans had found to enhance the immune system through increased haemocytes, phagocytosis, melanization, encapsulation, coagulation, plasma recognition proteins, SOD and PO activity (Moullacet *et al.,* 1998; Vargas-Albores *et al.,* 1998; Mats Johansson *et al.,* 2000).The focus of this work was to investigate the effect of different commercial probiotics in different stocking density in prawn (*Macrobrachium rosenbergii*). In this study, we attempted to study the effect of probiotics and stocking density on phenoloxidase activity in the haemolymph of prawn. This was intended to obtain the first record of an innate immune response of these prawns affected by probiotics and stocking density. Growth difference was found between treated and non-treated *Macrobrachium rosenbergii* in earlier studies (Rengpipat *et al.,* 1998) were more pronounced than during our present study. *Macrobrachium rosenbergii* growth was greater for prawns treated with probiotic bacteria in our present study ($T_7 = 22.39 \pm 0.05$, $T_8 = 14.08 \pm 1.65$, $T_9 = 9.74 \pm 1.16$, $C = 5.96 \pm 1.67$) (Fig.8). Since shrimp possess a non-specific immune response (Anderson, 1992), vaccination or immunostimulation may provide only short-term protection against specific pathogens (Sung and Song, 1996; Sung *et al.,* 1996). Effective probiotic treatments, on the other hand, may provide broader-spectrum and greater non-specific disease protection as a result of both serological immunity enhancement and competitive exclusion in prawns guts. This suggests that probiotic treatment is an effective alternative for enhancing prawn's health and thus promoting growth.

5.2 Effects on pro-phenoloxidase concentration

Use of probiotics for boosting the defense mechanism in crustaceans in general and shrimps in particular is a new and promising field (Sung *et al.,* 1994; Newman, 1996). Itami *et al.* (1989) demonstrated that the immunized shrimps (*P. japonicus*) were better protected against

challenge with pathogenic organisms. Previously, the dietary administration of chitin, chitosan and sodium alginate increased phenoloxidase and respiratory burst activities in *L. vannamei* (Wang and Chen, 2005; Cheng *et al.,* 2003). The oral administration of nucleotide and brewer's yeast were reported to increase proPO and respiratory burst activities in the freshwater prawn, *M. rosenbergii* (Shankar *et al.,* 2012; Parmar *et al.,* 2012). Additionally, the dietary administration of herbal immunostimulants also shown increased RB and proPO activities in *M. rosenbergii* (Kumari *et al.,* 2004) and *Penaeus monodon* (Citarasu *et al.,* 2006). Induction of resistance of *P. monodon* against challenge with Vibrio vulnificus after treatment with β-glucan was reported by Sung *et al.,* (1994) and recorded that the preferred route of delivery in aquaculture system would be the oral route. Newman (2000) administered LPS at different doses to penaeid shrimps viz., 20, 40 and 100 mg/kg and challenged with white spot syndrome virus (WSSV). In the present study, a significant effect ($P<0.05$) of probiotics on the treatment groups has been revealed. The result of the analysis showed that the concentration of PO was higher in the treatments (T_1, 0.044 ± 0.002; T_4, 0.074 ± 0.003; T_7, 0.093 ± 0.004) compared to the control group (C, 0.0335 ± 0.004) (Fig 9) in assay 1. The post-hoc analysis of assay 1 has revealed that PO concentration of T_7 was higher than T_1 ($P=0.001$) and T_4 ($P=0.011$). Significant difference among the treatments was found but there was no significant difference found between T_1 (P=0.098) and control (C) group. In assay 2 again signficant difference was found between treatment and control (T_1, 0.2 ± 0.003; T_4, 0.187 ± 0.001; T_7, 0.2245 ± 0.014; C, 0.075 ± 0.008) (Fig 11). The SPSS analysis of the treatments data reveled that there is a significant ($P<0.05$) effect of probiotic and stocking density combinedly on prawn's PO concentration (Fig: 10, 12). This is supported by the similar observations of Anas *et al.* (2005), where *M. rosenbergii* were fed with probiotic supplemented diet and showed high antimicrobial activity against 48 isolates of Vibrio species. Similarly, *M. rosenbergii* fed a diet containing bovine lactoferrin had shown enhanced resistance against *Aeromonas hydrophila* infection (Chand *et al.,* 2006).

In summary, the probiotic 3 provided better result among the three probiotics. It also appears that the use of this probiotic was most effective when it was used in lower stocking density. All the three probiotics resulted in enhanced growth rate than the control. The combined effect of probiotics and stocking density significantly affected the treatments in assay 1 but in assay 2 no significant effect was found. From the above discussion we can say that probiotic use is more desirable and environmentally benign compared to the use of antibiotics and chemicals.

When probiotics are used in appropriate amount and in appropriate stocking density it results in better immune system and better growth rate in prawns (*M. rosenbergii*).

Chapter.....6

Conclusion

6. Conclusion

Macrobrachium prawn is an interesting animal model to use for the study of defense mechanisms in crustaceans mainly because of its economic value, its short life-time, its ability to survive in microbe-rich environments without gross signs of disease and the high number of samples that can easily be collected from farms. This study demonstrated that the use of different commercial probiotics in different stocking density resulted in enhanced pro-PO activities in *Macrobrachium rosenbergii.* These commercial probiotics has been found very effective in promoting weight gain and immune response of *Macrobrachium rosenbergii.* Overall the result of the study indicates that the optimal incorporation of probiotics would serve as an immunostimulant in prawn diet and helpful in stimulating immune response of *Macrobrachium rosenbergii.*

Recommendations:

- ✓ This experiment should be repeated under more closed condition to get more acceptable data and make this work more authentic.

Chapter.....7

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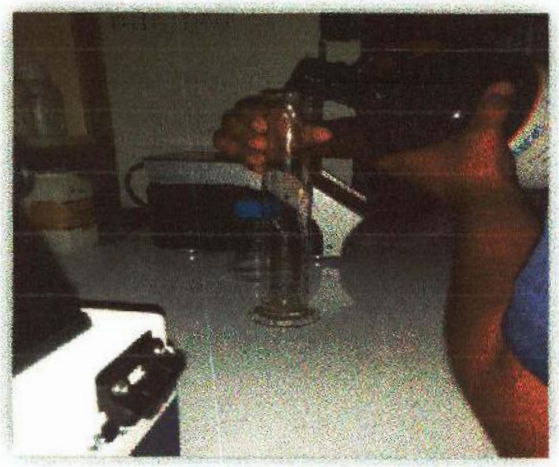
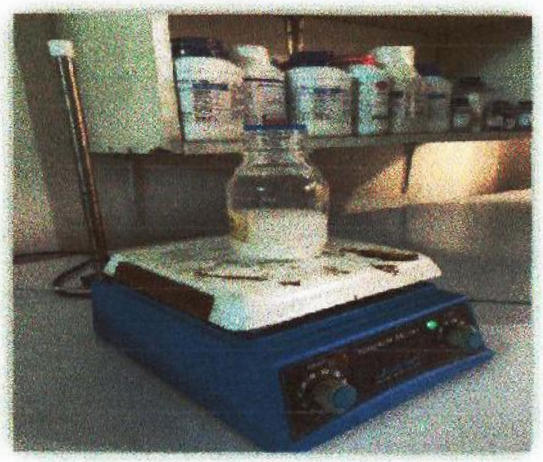
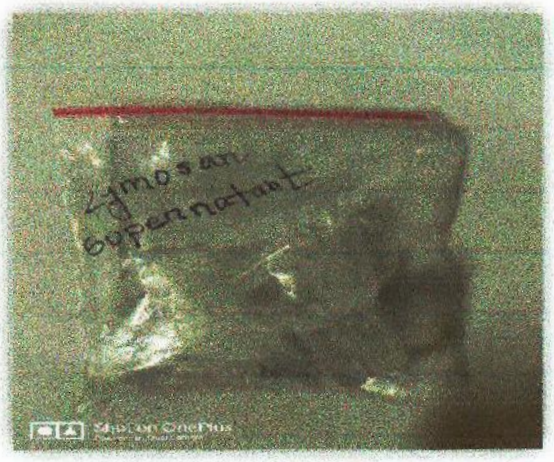
Appendices: Photo Gallery






Zymosan
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