Laboratory Diagnosis of Pathogenic Bacteria *Vibrio harveyi* by PCR test and its bioremediation with indigenous Probiotic Bacteria *Lactobacillus* spp.

A Thesis

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Declaration

Diagnosis of Pathogenic Bacteria *Vibrio harveyi* by PCR test and its bioremediation with indigenous Probiotic Bacteria *Lactobacillus* spp.

The results submitted in this thesis are entirely of 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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Fatema Ahmmed
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Abstract

The present study was conducted to diagnose *Vibrio harveyi* using PCR with its bioremediation applying indigenous probiotic (*Lactobacillus* spp.) of prawn body. For these purposes, 30 specimens of prawn sample (28.6 ± 0.1g, 12 ± 1cm) were collected from local market, Khulna. *Vibrio harveyi* and *Lactobacillus* spp. which were isolated to check antagonistic effect through *in-vitro* and *in-vivo* test. In *in-vitro* challenge test, the potential and significant ($P<0.5$) antagonistic effect of *Lactobacillus* spp. against *Vibrio harveyi* was gradually reduced at 0, 4th, 8th and 12th hour of probiotic inoculation. In *in-vivo* challenge test, fifty (50) fingerlings of prawn (1±0.2g, 0.45±0.02cm) were stocked in earthen ponds (70m x 70m x 1m). Larvae were fed twice per day with probiotic mixed feed for treatment group while no probiotic were given for control groups. The result indicated slow reduction of *Vibrio harveyi* up to 40 days where as significant ($P<0.5$) reduction was obtained at 50th and 60th days of experiment. The result showed that treatment groups have shown better performance rather than control groups in terms of growth, digestibility and immunity. Probiotic treated prawn achieved higher growth are these the achievements of probiotic treated groups based on final weight (16.49 ± 0.54), percentage weight gain (549. 30 ± 40.02) and specific growth rate (3.11 ± 0.10) than their counter control group while final weight (9.01 ± 0.36), percentage weight gain (310.77 ± 18.68) and specific growth rate (2.35 ± 0.08). Absorbance of protease (2.74 mg/ml) and amylase (1.23 mg/ml) were significantly higher ($P<0.5$) than the protease (1.82 mg/ml) and amaylase (0.7 mg/ml) absorbance of control group. The result of this experiment indicated higher immunity of treatment group compared to control group. So, better growth, strong digestibility and powerful immunity of treatment group supports the use of probiotic mixed feed. The result also suggested that probiotic treatment can be an effective alternative to the use of antibiotics in treatments of bacterial diseases in prawn aquaculture.

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List of Abbreviations

Abbreviation	Elaboration
MRS	Man, Rogosa and Sharpes
TCBS	Thiosulfate Citrate Bile Salt
CFU/gm	Colony forming unit per gram
cm	Centimeter
g	Gram
°C	Degree celsius
%	Percentage
‰	Parts per thousands
et al.	et alli; Latin word meaning "and others"
FMRT	Fisheries Marine Resources Technology
ml	Mili-liter
mg/L	Miligram per liter
h	Hour
VP	Voges-Proskauer
KOH	Potassium hydroxide
NaCl	Sodium chloride
ASPW	Alkaline Saline Peptone Water
rpm	Revolutions per minute
spp.	Species

Chapter 1

Introduction

1. Introduction

Aquaculture is the farming of aquatic organisms, including fish, molluscs, crustaceans and seaweeds. It is important to note that aquaculture has a long tradition in the developing countries, which supplies 90 % of the world's aquaculture production. Global aquaculture sector is to maintain its current average growth rate of 8 to 10 percent per year to 2025 (FAO, 2011). Bangladesh is one of the resourceful countries with its wide range of marine aquatic bio-diversities. There are about 1093 marine aquatic organisms where 44.35% are finfish, 32.23% shellfish, 15.10% seaweeds and only 8.32% are other organisms including shrimps. In 2014-2015, the total production of fish is 37.03 lakh MT (Bangladesh economic review, 2015).

In 2014-2015, the fisheries sector including shrimp, contributes about 3.69% to the GNP and about 22.60% to the GDP earnings (Bangladesh economic review, 2015). Shrimp alone contributes about 93% of export earning and 4.99% of the national earning item in Bangladesh (DoF, 2007a).

Macrobrachium rosenbergii is a species of aquaculture importance owing to its high fecundity, rapid growth, wide range of salinity and temperature tolerance, disease resistance as well as its superior taste and high commercial value. Prawn culture has become most profitable industry in many parts of the world and has developed rapidly over the last four decades to become an important economic activity worldwide. Shrimp species harvested from our country are mainly black tiger shrimp (Bagda), brown shrimp (Horina), Indian white shrimp (Chaka) and giant freshwater shrimp (Golda).

The freshwater prawn, locally known as Golda (*Macrobrachium rosenbergii*), is one of the largest export commodities in the fisheries economy of Bangladesh aquaculture. It is the second largest export industries after garments from which Bangladesh earned as US$ 456 m in the year 2006 (BFFEA, 2008). Among shrimp producing countries, Bangladesh ranks fourth with respect to area of shrimp farming and sixth in volume of production. Bangladesh with its favorable resources, sub-tropical climatic condition and a vast area of fresh water bodies has a unique home for the production of giant water prawn (*Macrobrachium rosenbergii*,). About twenty-four species of freshwater prawns including 10 species of *Macrobrachium* spp. are found in Bangladesh (Karim, 1978), among them only *Macrobrachium rosenbergii* is

commercially cultured (Ahmed, 2001 and Muir, 2003). Over the last two decades, prawn farming has come to farmer's attention because of its easy operation, significant export potential and lower susceptibility to diseases compared to marine shrimp (*Penaus monodon*). Almost 95% or more of the total production of Golda is exported currently and the rest is catered to local market. In fiscal year 2010-11 Bangladesh earned about 3568.2 cores Tk. from exported sales of *Macrobrachium rosenbergii* (DoF, 2012). In fiscal year 2014-2015 (January, 2015) Bangladesh earned 3, 204.24 cores Tk. from exported sales of fish and fishery product at 0.52 lakh MT (Bangladesh economic review, 2015).

Although having a lot of positive sides, this industry is not completely free from diseases caused by biological and non-biological agents. Among the best known groups of microorganisms that cause serious losses in prawn culture, are the bacteria. In spite of the great potentiality, prawn culture of Bangladesh is affected with a wide range of microbial disease problems. In much of the world, however, the shrimp and prawn aquaculture industry is beset by disease, mostly due to bacteria (especially the luminous *Vibrio harveyi*) and viruses.

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). Vibriosis is ubiquitous throughout the world and all marine crustaceans, including prawn are susceptible to it. *Vibrio* species are widely distributed in culture facilitates throughout the world.

Vibriosis is a major problem in prawn culture, causing heavy mortality and severe economic loss. Though they are members of the normal microbiota of prawns, vibrio species often act as opportunistic pathogens or secondary invaders and many induce mortality even up to 100% in the affected population (Bell and Lightner, 1988; Rosemark and Fisher, 1988). Major species causing vibriosis in prawns are *Vibrio alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. anguillarum* (Bell and Lightner, 1988; Chanratchakool *et al.*, 1995). Bacterial septicemia is a common symptom of vibriosls (Mermoud *et al.*, 1998).

These moribund prawns display a wide spectrum of clinical signs, like disoriented swimming, letharry, weakness and abnormal colouration of body and appendages. Infections due to Vibrio species are prevalent in hatcheries and grow-out facilities, causing mass morta-lities and shell deformities (Lavilla-Pitogo *el al.*, 1990).

Disease outbreaks are being increasingly recognized as a significant constraint in prawn production and trade, affecting the economic development of the sector in our country. It is one of the limiting factors to prawn production. Bacterial diseases, mainly due to *Vibrio*, have been reported in penaeid shrimp and prawn culture systems implicating over a dozen of species and they are *Vibrio harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbelli, V. fischeri, V. damsella, V. pelagicus, V. orientalis, V. ordalii, V. mediterrani, V. logei* etc. Vibrios are among the most important bacterial pathogens of cultured shrimp responsible for a number of diseases, causing mortalities up to 100%, reported by Lightner, 1983. *Vibrio* species are part of the natural micro-flora of wild and cultured shrimps (Sinderman, 1990) and become opportunistic pathogens when natural defense mechanisms are suppressed (Brock and Lightner, 1990). They are usually associated with multiple etiological agents. These opportunistic pathogenic microbes apparently establish lethal infections as a result of other primary conditions that might include other infectious diseases, nutritional diseases, extreme environmental stress and wounds. However, some *Vibrio* species or strains of certain species have been identified as primary pathogens (Owens *et al.*, 1992; Pena *et al.*, 1995).

Infections by these bacteria display massive colonization on the appendages and foregut followed by infection of the mid gut, hepatopancreas and a terminal septicemia. Vibriosis is expressed by way of a number of syndromes. These include: oral and enteric vibriosis, appendage and cuticular vibriosis, localized vibriosis of wounds, shell disease, systemic vibriosis and septic hepatopancreatitis (Lightner, 1996).

A major cause of shrimp larval mortality in hatcheries is infection by the Gram-negative bacterium *V. harveyi* commonly known as luminescent bacteria due to the characteristic blue-green light it emits. The organism has been reported as an opportunistic pathogen to both juvenile and adult prawn during culture where it

seriously threatens economic feasibility by causing mass mortalities. *V. harveyi,* the causative agent of luminous bacterial disease is considered a serious pathogen of larval prawn in hatcheries (Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.,* 1994). Antibiotic medication is widely followed to control this pathogen and to improve the production of shrimp larvae (Baticados *et al.,* 1990).

Bacterial diseases are considered to be a menace in the intensive larval culture and production of aquaculture species owing to the large-scale mortality they cause in hatcheries and culture systems (Singh, 1990; Cheng and Chen, 1998). Although antibiotics have played a major role in combating many diseases of cultured aquatic organisms, their indiscriminate use in aquaculture has led to undesirable consequences such as development of antibiotic-resistant bacteria and persistence of antibiotic residues in farm-raised prawn, shrimp, fish etc. (Chaithanya, 1999).

Disinfectants and antimicrobial drugs (antibiotics) are very expensive and mainly used in the cases of semi-intensive or intensive culture farms run by the richer farmers. But most of the shrimp farmers in our country are very poor and practice traditional or improved traditional culture systems. They cannot afford such expenses due to lack of enough capital support. Furthermore, there is a growing concern about the use and particularly, the abuse of antimicrobial drugs not only in human medicine and agriculture but also in aquaculture.

Frequent use of chemotherapeutic agents, especially antibiotics, leads to the emergence of resistant strains bacteria (Akoki, 1975) pathogenic to the animals. If antibiotics or disinfectants are used to kill bacteria, some bacteria will survive, because they carry genes for resistance. These will then grow rapidly because their competitors are removed (Moriarty, 1999). Extensive use of antibiotics to control the incidence of diseases not only resulted in the emergence of multiple antibiotic resistant bacterial strains (Karunasagar *et al.*, 1994, Abraham *et al.*, 1997) but has also caused severe environmental and human health concerns leading to prohibition of their use.

So far, conventional approaches, such as the use of disinfectants and antimicrobial drugs (antibiotics) such as chloramphenicol, furazolidone, oxytetracycline and streptomycine, have had limited success in the prevention or cure of aquatic microbial disease (Subasinghe, 1997).

Increased concern about the emergence of antibiotic resistant microorganisms has led to alternative options for disease prevention, such as the use of non-pathogenic bacteria such as probiotic bio-control agents.There is a tendency to consider all micro-organisms as harmful; to equate bacteria with germs. But not all bacteria are harmful, some are beneficial. Recently microbes are enormously used for beneficial purposes. The term probiotics was first introduced in 1953 by Kollath (Hamilton-Miller, 2003). Probiotics, which has been widely used: A live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). Probiotics as "live microbial cells administered to cultured organisms to colonize the digestive tract and improve their immune response" (Gatesoupe, 1999). There is a tendency to consider all micro-organisms as harmful; to equate bacteria with germs. But not all bacteria are harmful, some are beneficial. Recently microbes are enormously used for beneficial purposes. The term probiotics was first introduced in 1953 by Kollath (Hamilton-Miller, 2003). Probiotics which has been widely used: A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989).

The World Health Organization has defined probiotic bacteria as "live microorganisms which when administrated in adequate amounts confer a health benefit on the host" (FAO/WHO, 2011). The use of probiotics in the culture of aquatic organisms is increasing with demand for more environment friendly aquaculture practices (Gatesoupe, 1999). The microorganisms used as probiotics, including *Lactobacillus, Bacilliusand* yeasts, have been reported in penaeids and fish (Capkin and Altinok, 2009; Al-Dohail *et al.*, 2009 and Boonthai *et al.*, 2011).

It acts as natural immune enhancers, which provoke the disease resistance in shrimp farm and to modify or manipulate the microbial communities in water and sediment, reduce or eliminate pathogens. The use of microbial probiotics in aquaculture is now widely accepted alternative disease management process (Gatesoupe 1999; Sharma and Bhukhar 2000; Verschuere *et al.*, 2000; Irianto and Austin, 2002 and Wang,

2007). Generally, probiotic strains have been isolated from indigenous and exogenous microbiota of aquatic animals. Appropriate probiotic applications were shown to improve intestinal microbial balance,thus leading to improved food absorption (Parker, 1974 and Fuller, 1989), digestive enzymes activities (Tovar *et al.*, 2004) and reduced pathogenic problems in the gastrointestinal tract (Pivnick *et al.*, 1981; Cole and Fuller, 1984). The application of probiotics in aquaculture as the environment friendly treatments arealso increasing rapidly (Gatesoupe, 1999) and some papers are associated with the effect of probiotics in fish (Mohanty *et al.*, 1993, 1996; Sharma and Bhukhar, 2000).

The modes of action of probiotic (Lactic acid bacteria) were as follows: production of inhibitory compounds; competition for chemicals or available energy; competition for adhesion sites; enhancement of the immune response; improvement of water quality; interaction with phytoplankton; source of macro- and micronutrients; enzymatic contribution to digestion (Verschuere *et al.*, 2000).

The effects of probiotics for freshwater prawn, *M. rosenbergii* have been reported by (Seenivasan *et al.*, 2012; Saad *et al.*, 2009; Keysami *et al.*, 2007; Venkat *et al.*, 2004 and Suralikar and Sahu, 2001).

In marine shrimp 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 (Holmstrom *et al.*, 2003). Therefore, several countries have banned the use of antibiotics such as chloramphenicol (FAO, 2002).

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 (Gatesoupe, 2008). Studies have demonstrated the beneficial effect of the addition of such bacteria in the culture of several aquatic species (Gatesoupe, 2008).

The term "probiotic" inevitably refers to Gram-positive bacteria. *Lactobacillus* are in general characterized as Gram-positive, nonspore- forming and non-flagellated rods or coccobacilli, technologically are regarded as non-pathogenic and safe

microorganisms (Soccol *et al.*, 2010). Lactic acid bacteria (LAB) convert lactose into lactic acid, thereby reducing the pH in the gastro intestinal environment and naturally preventing the colonization by many bacteria (Klewicki and Klewicka, 2004). *Lactobacillus* is distributed in various ecological niches throughout the gastrointestinal tracts and constitutes an important part of the indigenous microflora of aquatic animals (Ringo and Gatesoupe 1998). LAB has been used as probionts against shrimp pathogens (Gatesoupe, 1999; Ross *et al.*, 2005 and Senok *et al.*, 2005).

The most commonly used probiotics are the strains such as *Lactobacillus, Bifidobacterium* (Ross *et al.*, 2005; Gursoy and Kinik, 2006), *Streptococcus* (Shah, 2007), *Bacillus* (Barbosa *et al.*, 2005), and few non pathogenic *Vibrio* sp. They are also used for enhancement in inhibition of pathogenic bacteria (Huys *et al.*, 2001), enhancing enzyme activity (Kumar *et al.*, 2006 and Ghosh, *et al.*, 2008), better feed conversion, fecundity and fry production (Wang and Xu, 2006 and Ghosh *et al.*, 2007) as well as for immunity enhancement and disease resistance.

Hemato-immunological parameters, such as the hemotocyte, phenoloxidase (PO) activity, and agglutinin activity of serum have been employed to monitor marine shrimp health conditions (Gullian *et al.*, 2004). Enhancement of immune response and enzymatic contribution to digestion may also be the ways by which probiotics act (Balcazat *et al.*, 2006). Some studies were conducted on the use of probiotics in the diets of the fresh water prawn *Macrobrachium rosenbergii* (Suralikar and Sahu, 2001; Venkat *et al.*, 2004).

At the backdrop of the existing situation of prawn aquaculture problems, this study was undertaken *in-vitro* and *in-vibo* to find out the isolation of *Vibrio harveyi* using PCR test and its bioremediation with indigenous probiotic (*Lactobacillus* spp.) in prawn farms with the following objectives.

1) To isolate pathogenic bacteria (*Vibrio harveyi*) and beneficial bacteria specially (*Lactobacillus* spp.) from the prawn.
2) To find out the antagonistic property of probiotics bacteria (*Lactobacillus* spp.) against *Vibrio herveyi* on the infected prawn (*in-vitro* and *in-vivo* test).
3) To elucidate the effect of a probiotic (*Lactobacillus* spp.) supplemented diet on growth performance and digestive enzyme activity.

4) To illuminate some hemato-immunological parameter which enhance its immunity of a freshwater prawn (*Macrobrachium rosenbergii*) after challenge with *Vibrio harveyi*.

From the research work, the expected result will help to understand whether prawn body itself contain various types of beneficial bacteria which would offer ample scope as probionts showing antagonism towards *Vibrio* pathogens by in-*vitro* and in-*vivo* test. If the result of this experience is being positive, a new idea can generate where farmer can nurse the indigenous beneficial bacteria in prawn culture. It will be very much cost-effective and helpful. For this reason, I keenly interested to research on that topics.

Chapter 2

Literature Review

2. Review of Literature

Probiotics are bio-friendly agents which have been recently used in sustainable forms of aquaculture. In the present study, the efficacy of probiotics was assessed by counting both the beneficial and pathogenic bacteria in comparison with harvest outcomes. Probiotic supplementation of live microorganisms in aquaculture aids in preventing disease, thereby increasing production and decreasing economic loss. Application of probiotics bacteria in aquaculture systems plays significant role that determines the fate and success rate of culture. In the present study efforts have been made of consult a few available literatures on the use of probiotics against the pathogenic bacteria in aquaculture; the literatures consulted were on the line of probiotics organisms and their functions, their use results as antagonistic agents, effects on growth performance, digestive enzyme activity and immunity enhancement, use in white fish species, in shrimp, in prawn and their effects on water quality of culture farms.

Seenivasan, C., (2014) studied on the effect of *Lactobacillus sporogenes* on survival, growth, biochemical constituents and energy utilization of freshwater prawn *Macrobrachium rosenbergii* post larvae and result was that *L. sporogenes* incorporated diet fed PL produced better growth performance.

Kumar *et al.*, (2013) investigated the effect of *Bacillus subtilis* and *L. rhamnosus* incorporated diet on growth pattern and antioxidant enzymes in *Penaeus vannamei* and found significant result compare to control.

In the study by Giri *et al.*, (2013) *Lactobacillus plantarum* in three concentrations was added to *Labeo rohita* diet, daily weight growth (WG) and feed conversion ratio (FCR) showed a significant increase and SGR had a significant increase.

Shyne *et al.*, (2013) studied digestive enzyme activity in *Penaeus monodon* shrimp influenced by periphyton food supplement.

Zokaeifar *et al.*, (2012) studied on effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei* and reported that it can improve growth performance and disease resistance through an enhanced immune response in shrimp.

According to Seenivasan *et al.*, (2012b–e) it has also been reported that the growth rate performance and nutrient energy utilization were higher in *L. sporogenes*, *B. subtilis* and *S. cerevisiae* inclusion diet fed *M. rosenbergii* PL.

Soleimani *et al.*, (2012), studies digestive enzyme activity and Caspian roach growth performance in fry step and used FOS (Fructo-oligosaccharid) a food supplement that is a kind of probiotic in three levels 1%, 2% and 3% for 7 weeks and their study showed that the highest survival was associated with 3% group and higher growth performance and digestive enzyme activity was related to 2% and 3% groups. The results from the effect of using different levels of *lactobacillus casei* probiotic in *B. gryprus* on α-amylase enzymes activity, trypsin, chymotrypsin, alkaline phosphates and lipase showed a significant difference in the above mentioned enzymes, so that chymotrypsin was in (B) concentration on the 30th day.

According to Liu *et al.*, (2012) investigation the bacterial cells or cell components such as peptidoglycan, teichoic acid, and exopolysaccharide are having immunomodulatory activities.

Seenivasan *et al.*, (2011) investigated the effect of probiotics, BinifitTM on survival, growth, biochemical constituents and energy budget of the freshwater prawn *Macrobrachium rosenbergii* post larvae (PL) and reported that BinifitTM supplementation diet fed PL resulted in better growth performance.

Soccol *et al.*, (2010) reported that *Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus delbrueckii* ssp. *Bulgaricus, Lactobacillus fermentum Lactobacillus helveticus, Lactobacillus lactis, Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus reuteri* and *Lactobacillus salivarius* has been used as commercial probiotics.

From the study of Dhanaraja *et al.*, (2010) it was reported that probiotics *L. acidophilus* and yeast *S. cerevisiae* supplemented diets had improved the energy budget of Koi Carp, C. carpio.

From Vieira *et al.*, (2010) study it has been reported that the administration of a probiotic strains positively influenced the growth and survival of juveniles of shrimp and presented a protective effect against the pathogens *Vibrio harveyi.*

Vieira *et al.*, (2010) studied about the effect of a *Lactobacillus plantarum*-supplemented diet on shrimp growth, digestive tract bacterial microbiota, survival, and some hemato-immunological parameters after an experimental challenge with *Vibrio harveyi* and reported that there was a significant result compared to control.

From the research work of Ashim *et al.*, (2009) it has been also reported that the growth, SGR and weight gain were increased in bioencapsulated *Lactobacillus* spp. fed fish, *Carassius auratus* and *Xiphophorus helleri*.

Gatesoup, (2008) reported that high levels of gram-positive bacteria, particular heterotrophic bacteria, can be used to minimize the buildup of dissolved and suspended organic carbon during the production cycle. The end result is reduction in final black sludge buildup after harvest. It has been reported that use of *Bacillus sp.* improved water quality, reduced black sludge, improved survival and growth rates and increased the health status of juvenile *Penaeus monodon* and reduced the pathogenic vibrios .

Suzer, C. (2008) investigated that *Lactobacillus* spp. bacteria act as probiotics in gilthead sea bream (*Sparus aurata*) larvae: had positive effects on growth performance and digestive enzyme activities.

Shinde *et al.*, (2008) have reported the use of different commercial probiotic supplements administered through feeds to PL of *M. rosenbergii.*

According to Shinde *et al.* (2008) it was reported that different probiotics supplemented diets had improved growth rate, weight gain, FCR and FER of *M. rosenbergii* PL.

From the studied of Abdel-Tawwab *et al.*, (2008) it was reported that the growth rate and feed energy utilization were increased in *S. cerevisiae* supplemented diet fed Nile tilapia, *O. niloticus*.

According to Gatesoupe, (2008) lactic bacteria present in the digestive tract can also produce immunostimulating substances.

According to Suzer *et al.*, (2008) it is thought that probiotics influences digestive processes by enhancing the population of beneficial microorganisms, microbial enzyme activity, improving the intestinal microbial balance, consequently improving the digestibility and absorption of food and feed utilization.

According to the study of Hsieh *et al.*, (2008) in shrimp, the most important role of the circulation hemocytes is the protection of animals against invading microorganisms by participating in recognition, phagocytosis and melanization.

According to Delcenserie *et al.*, (2008) several species of LAB such as *Lactobacillus acidophilus*, *L. rhamnosus* and *Lactococcus lactis* are effectively induced phagocytic activity in several animals.

According to El-Haroun *et al.*, (2006) after transition through the stomach, the probiotics germinate in the intestine and use a large number of sugars (carbohydrates) for their growth and produce a range of relevant digestive enzymes (amylase, protease and lipase).

Ziaei-Nejad *et al.*, (2006) and Wang, (2007) described, all the probiotic-supplemented diet resulted in an increase of growth, SGR and survival, showing that the addition of probiotics increased the growth performance of shrimps *P. vannamei* and *F. indicus*.

According to Yanbo and Zirong, (2006) showed that probiotics (*Bacillus* spp.) increased the growth performances highly and digestive enzyme activities and decreased FCR for common carp (*Cyprinus carpio*).

From the study of Martin and Graves, (2005) the granulocytes of *Penaeus paulensis* were easily identified as SGH and LGH according to the descriptions of other decapods.

According to Klewicki and Klewicka, (2004) Lactic acid bacteria (LAB) convert lactose into lactic acid, thereby reducing the pH in the gastro intestinal environment and naturally preventing the colonization by many bacteria.

Li and Gatlin, (2004) suggested that functional additive, like probiotics, is a new concept on aquaculture where the additions of microorganisms on diets show a positive effect on growth caused by the best use of carbohydrates, protein and energy.

Gullian, (2004) demonstrated a significant growth increase in shrimp inoculated with *Bacillus* sp. Venkat *et al.*, (2004) it has been reported that the dietary supplementation of *Lactobacillus acidophillus* and *L. sporogenes* for *Macrobrachium rosenbergii* increased shrimp growth rate.

Nikoskelainen *et al.*, (2003) stated that the use of probiotics stimulates rainbow trout immunity by stimulating phagocytic activity, complement mediated bacterial killing and immunoglobulin (Ig) production.

Meunpol *et al.*, (2003) recorded the effects of ozone and probiotics on the survival of black tiger shrimp (*Penaeus monodon*). They investigated the effects of ozone with and without feeds supplemented with the probiotic Bacillus S11 on bacterial (*Vibrio harveyi*) growth and shrimp (*P. monodon*) survival. According to the results of their study, shrimp survival after probiotic treatment, coupled with ozonation, increased significantly compared with controls.

Lara-Flores *et al.*, (2003) reported for other fishes in which digestibility was shown to increase considerably with the use of a probiotic in the diet.

According to Immanuel *et al.*, (2003) it was reported that the probiotics Lactobacillus and yeast supplemented diets had improved the energy utilization of pearl spot *Etroplus suratensis*.

Irianto and Austin, (2002) evaluated Bacillus spp. as probiotics, with uses including the improvement of water quality by influencing the composition of water-borne microbial populations and reducing the number of pathogens in the vicinity of the farm species. Thus, the Bacilli are thought to antagonize potential pathogens in the aquatic environment.

According to the studied of Marteau *et al.*, (2002) Lactic acid bacteria are known to produce extracellular compounds that can stimulate the non-specific immune response in vertebrates.

According to Bomba *et al.*, (2002) Probiotic influences the digestive processes by enhancing the population of beneficial microorganisms, microbial enzyme activity; improving the intestinal microbial balance, consequently improving the digestibility and absorption of food and feed utilization.

From the study of Perazzolo *et al.*, (2002) hemocytes number sometimes used as an indicator of shrimp health status.

Rengpipat *et al.*, (2000) suggested that *Bacillus* administration also has been shown to increase shrimp survival by enhancing resistance to pathogens by activating both cellular and humoral immune defenses in shrimp.

Sirirat, (2000) studied on Immunity enhancement in black tiger shrimp *Penaeus monodon*/ by a probiont bacterium *Bacillus* S11. And reported that *Bacillus* S11 provided disease protection by activating both cellular and humoral immune defenses, as well as presumably providing competitive exclusion in the shrimp's gut.

Verschuere *et al.*, (2000) investigated that there are several mechanisms of probiotics action, including the production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, the enhancement of the immune response and improvement of water quality.

Probiotics were defined by Verschuere *et al.*, (2000b) 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.

Johansson *et al.*, (2000) studied about the defense mechanisms of Shrimp hemocytes such as phagocytosis, encapsulation, clot formation and melanization.

Moriarty, (1999) reported the abundance of luminous *Vibrio* strains decreased in ponds and tanks where specially selected, probiotic strains of *Bacillus* species were added.

Lee *et al.*, (1999) found that the virulence of *V. harveyi* recognized in a small but expanding list of cultured marine animals particularly in penaeids in Asia and Australia.

Gatesoupe, (1999) and Dimitroglou *et al.*, (2011) articulated their investigation that some of the most relevant effects of probiotics used in aquaculture include improved survival in the larval period, suppression of pathogen growth, immunological enhancement, stimulation of growth and improvement of stress tolerance, therefore involving economic benefits for fish farmers.

Thompson *et al.*, (1999) and Verschuere *et al.*, (2000) pointed out that one of the main modes of action and beneficial effects of probiotics in aquacultured organisms is

enhancement of nutrition of host species through the production of supplemental digestive enzymes and higher growth and feed efficiency, prevention of intestinal disorders and pre-digestion of antinutritional factors present in the ingredients.

Gatesoupe, (1999) reported that the bacteria could also have improved digestive activity via synthesis of vitamins and cofactors or via enzymatic improvement.

Rengpipat *et al.*, (1998a) reported the isolation of a bacterial probiont, *Bacillus* S11, from healthy *P. monodon*, which reduced *P. monodon* mortality when challenged with *V. harĺeyi*.

Olafsen, (1998) pointed out that the introduction of microbial control practices by means of probiotics may have a beneficial effect on the cultures.

Gildberg and Mikkelsen, (1998) reported that the addition of *Carnobacterium divergens*, showed a certain improvement in disease resistance in cod (*G. morhua*) fry after a challenge with *V. anguillarum*.

It has been observed that survival of halibut (*Hippoglossus hippoglossus*) larvae in the first 2 weeks after hatching is affected by incubation with indigenous bacteria isolated from fish (Olafsen, 1998). Larval survival in the presence of *Vibrio salmonicida*-like strains and *Lactobacillus plantarum* amounted to 95%; wheras *Vibrio iliopiscarius* reduced survival to 63% compared to the control group (81%).

Douilett, (1998) used a probiotic additive consisting of a blend of bacteria in a liquid suspension in intensive production systems. The addition of this blend to culture systems reduced the concentration of Vibrio strains and thus controlled diseases caused by Vibrio strains.

Olsson *et al.*, (1998) found that the growth of *V. anguillarum* in fecal extracts can perform an inhibitory activity against *Vibrio* spp. might be used to decrease the load of fish-pathogenic *Vibrio* spp. in turbot hatcheries.

Itami *et al.*, (1998) reported that *Bacillus* surface antigens or their metabolites act as immunogens for shrimp by stimulating phagocytic activity of granulocytes.

Moriarty, (1998) noted an increase of prawn survival in ponds where probiotics including some strains of *Bacillus* sp. were introduced.

Rengpipat *et al.*, (1998) also showed the effects of a probiotic bacterium on black tiger shrimp (*Penaeus monodon*) survival and had the similar results.

Gildberg *et al.*, (1997) had analyzed the effect of a probiotics of lactic acid bacteria in the feed of Atlantic cod fry (*Gadus morha*) on growth and survival rates. In their study, a dry feed containing lactic acid bacteria (*Carnobacterium divergens*) that had been isolated from adult intestines was given to cod fry. After 3 weeks of feeding the fry were exposed to a virulent strain of *Vibrio anguillarum*. The number of deaths was recorded during a further 3 weeks of feeding with feed supplemented with lactic acid bacteria. A certain improvement in disease resistance was obtained, and at the end of the experiment lactic acid bacteria dominated the intestinal flora in surviving fish given feed supplemented with lactic acid bacteria.

According to Moriarty, (1997) the adoption of better farm management practices by using probiotics can also increase considerably the harvests and profits as well as promoting sustainable shrimp farming systems.

According to Sung and Song, (1996) since shrimp possess a non-specific immune response vaccination or immunostimulation may provide only short-term protection against specific pathogens.

From Ueberschar, (1995) study the activity of digestive enzymes may be used as a comparative indicator of the rate of development of the fish larvae, food acceptance, digestive capacity, as well as of their further survival rate.

Noh *et al.*, (1994) and Bogut *et al.*, (1998) also proved that the commercial probiotics preparations of *Streptococcus faecium* improved the growth and feed efficiency of carp.

From Ueberschar, (1993) it was well known that digestive enzyme activity can be used as an indicator of larval food acceptance and to some extent serve as an indicator for the digestive capacity in relation to the type of feed offered.

Garcia de la Banda *et al.*, (1992) added lactic acid bacteria (*Streptococcus lactis* and *Lactobacillus bulgaricus*) to *Brachionus* and *Artemia* used in turbot larva feeding. In a

single experiment without replicates, 55% survival was found on day 17 when living lactic acid bacteria had been added and 66% survival was found with disabled ones, as opposed to 34% in the control group. Apparently, the bacterial cells, alive or disabled, provoked improved survival of the turbot larvae.

Ratanapo and Chulavatnatol, (1992) reported a higher production of lectins in the shrimp fed the probiotic-supplemented diet in response to the infection, which indicates a possible immune reactive effect of *L. plantarum*. *P. monodon* shrimp presented elevation of the agglutination activity in the hemolymph when infected with *V. vulnificus*.

From Smith and Chisholm, (1992) they have characterized invertebrate immune systems as belonging to two broad categories, including:1. humoral defenses such as antibacterial activity, agglutinins, cytokine-like factors, modulators, and clotting factors; 2. cellular defenses such as hemolymph clotting, phagocytosis, nodule formation, encapsulation, and prophenoloxidase system.

As reported by Lightner, (1992) the need to reduce the lethal and weakening effects of pathogens is stimulating a renewed interest in the defense mechanisms and the immune system of crustaceans.

Gatesoupe, (1991) and Rengpipat *et al.*, (1998) reported that probiotics bacteria have been used in live feed such as *Artemia* or rotifers and fed to aquatic animals to enhance their growth rate and protect them from pathogenic bacteria.

Lee, (1990) reported that *Bacillus subtilis* have been shown to produce digestive enzymes such as amylase, protease and lipase which enrich the concentration of intestinal digestive enzymes.

It is noteworthy from Hsu *et al.*, (1977) that better results are obtained when using crude extracts obtained from the digestive tract of fish rather than using combinations of commercially purified enzymes.

Chapter 3

Materials and Methods

3. Materials and methods

3.1. Selection of the study area and period

At first the prawn sample were collected from local market, Khulna. Probiotic bacteria *Lactobacillus* spp. and *Vibrio* spp. were isolated from the collected prawn samples. The laboratory work was performed in the Biochemistry and Molecular Genetics Lab, Khulna University, Khulna. The field work was conducted in the research pond complex of Fisheries and Marine Resource Technology Discipline at Khulna University, Khulna, Bangladesh. The prawn in ponds was fed with probiotic bacteria *Lactobacillus* spp for three months. Then prawn sample was collected from the experimental ponds for additional experiment. The whole study was conducted from January to November, 2014.

3.2. Sampling and sample size

At the first phase of the study, for counting normal bacterial load, isolating *Lactobacillus* spp. and *Vibrio* spp. and for *in-vitro* challenge test, 30 specimens of prawn, *Macrobrachium rosenbergii* were collected. The standard lengths of the specimens were between 12-14 cm and the weights were between 23.5-28.6g. In this phase the experimental organs used were intestinal tract. Afterwards the effect of *Lactobacillus* spp. was studied *in-vitro* on *Vibrio harveyi*. Then the effect of probiotic bacteria was studied *in-vivo* analyses with growth performance, digestive enzyme assay and immunity.

3.3. Water quality parameters

The following water quality parameters of the ghers were recorded during sampling.

Table -1: Water quality parameters of three experimental units of the study area (pond complex, Khulna University).

Water quality parameters	Treatment-1	Treatment-2	Treatment-3
Temperature	26.5°C	27°C	27°C
Dissolve O_2	5.2mg/L	4.9mg/L	4.8 mg/L
pH	7.5	7.8	8.0
Ammonia	0.02	0.04	0.05
Nitrate (NO_2)	0.01	0.02	0.03
Salinity	2‰	1‰	2‰

3.4. Preparation of stock solution of the target organs

The target organs of the samples were collected aseptically and weighted in electric balance. Weights of intestinal tracts were between 0.13-0.25g. Organs were taken into eppendorf tube with peptone water (James and Hirsch, 1960) for isolating *Lactobacillus* spp. and alkaline saline peptone water was used for isolating *Vibrio* spp. Then they were homogenized using tissue homogenizer. Then homogenized solutions were centrifuged at 3000 rpm for 3 minutes (James and Hirsch, 1960). After centrifugation the supernatant liquid portion was collected with a micropipette and taken into eppendorf tube and preserved in deep freeze.

3.5. Media preparation and sterilization of equipments

3.5.1. Preparation of MRS *Lactobacillus* Agar Media

The following steps were followed in the preparation of bacteriological MRS *Lactobacillus* Agar Media.

- Definite amount of MRS *Lactobacillus* Agar (33.57gm) was accurately weighed and taken in volumetric flask (500 ml) containing distilled water (half of the required volume).
- Then the final volume (500 ml) was adjusted by pouring additional distilled water.
- The volumetric flasks were kept in autoclave for sterilization. Autoclaving was done at a temperature of 121°C and at a pressure of 15 lbs/sq inch for 15 minutes.
- After autoclaving the agar media was kept under the bio-safety cabinet for 20-30min for cooling and then transferred in required number of petridishes by 25ml volume for preparing the plates.

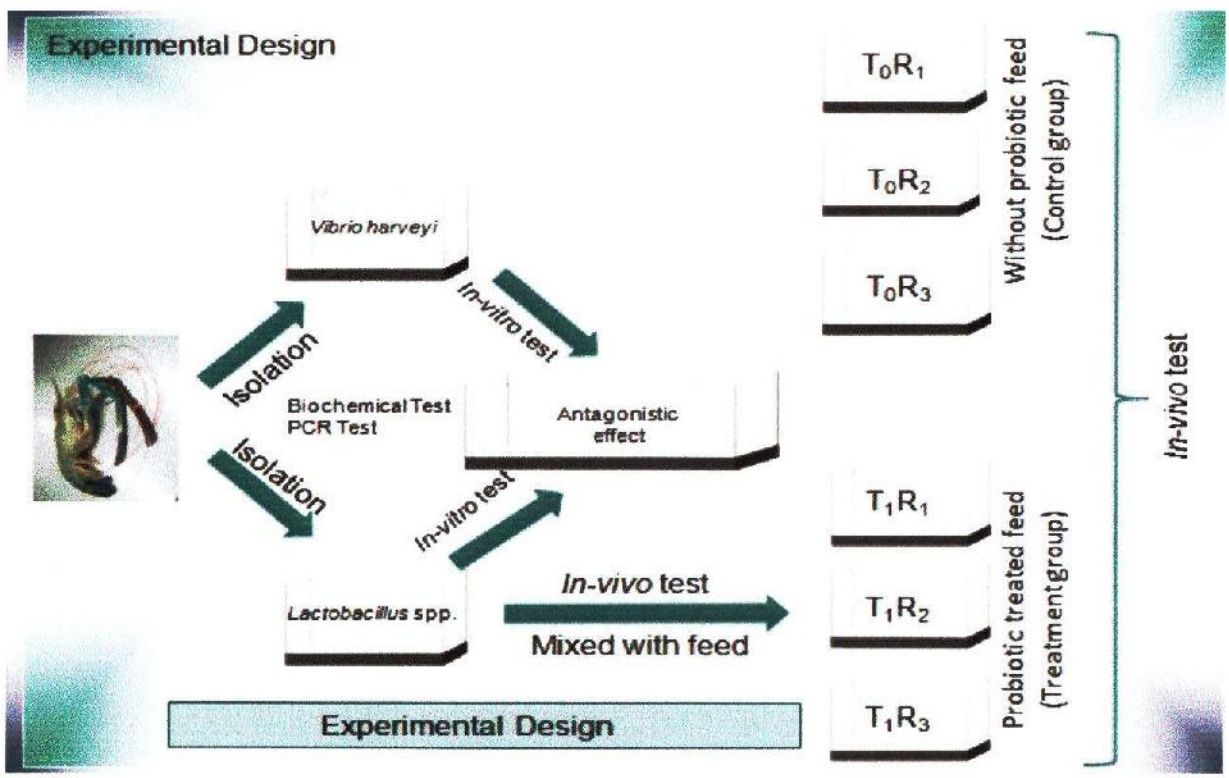
3.5.2. Preparation of Thiosulfate Citrate Bile Salt (TCBS) Agar Media.

- Definite amount of TCBS Agar (44.50 gm) was accurately weighed and taken in volumetric flask (500 ml) containing distilled water (half of the required volume).
- Then the final volume (500 ml) was adjusted by pouring additional distilled water.
- The volumetric flask containing TCBS Agar solution was kept on hotplate for dissolving fully at 100°C.
- After dissolving the agar media was kept under the bio-safety cabinet for 20-30 min for cooling and then transferred in required number of petridishes by 25 ml volume for preparing plates.

3.5.3. Sterilization of different equipments

Test tubes, eppendorf tubes, plastic tips, Petridishes, isolating loops, scissors and other glassware were sterilized by autoclaving at a temperature at 121°C and a pressure of 15 lbs/sq inch. for 15 minutes.

3.6. Experimental design



3.6.1. Isolation of probiotic bacteria (*Lactobacillus* spp.)

Lactobacillus spp. was isolated from intestinal tracts of the collected shrimp samples. The stock solution was diluted (tenfold dilution) with peptone water (James and Hirsch, 1960). 0.1 ml suitable dilution of each stock solution was inoculated in MRS *Lactobacillus* agar media and incubated at 37^{0}C temperature for 24-48 hours. After incubation at 37^{0}C temperature for 24–48 h, cream-colored colonies with yellow halos were collected and preserved for further experiment.

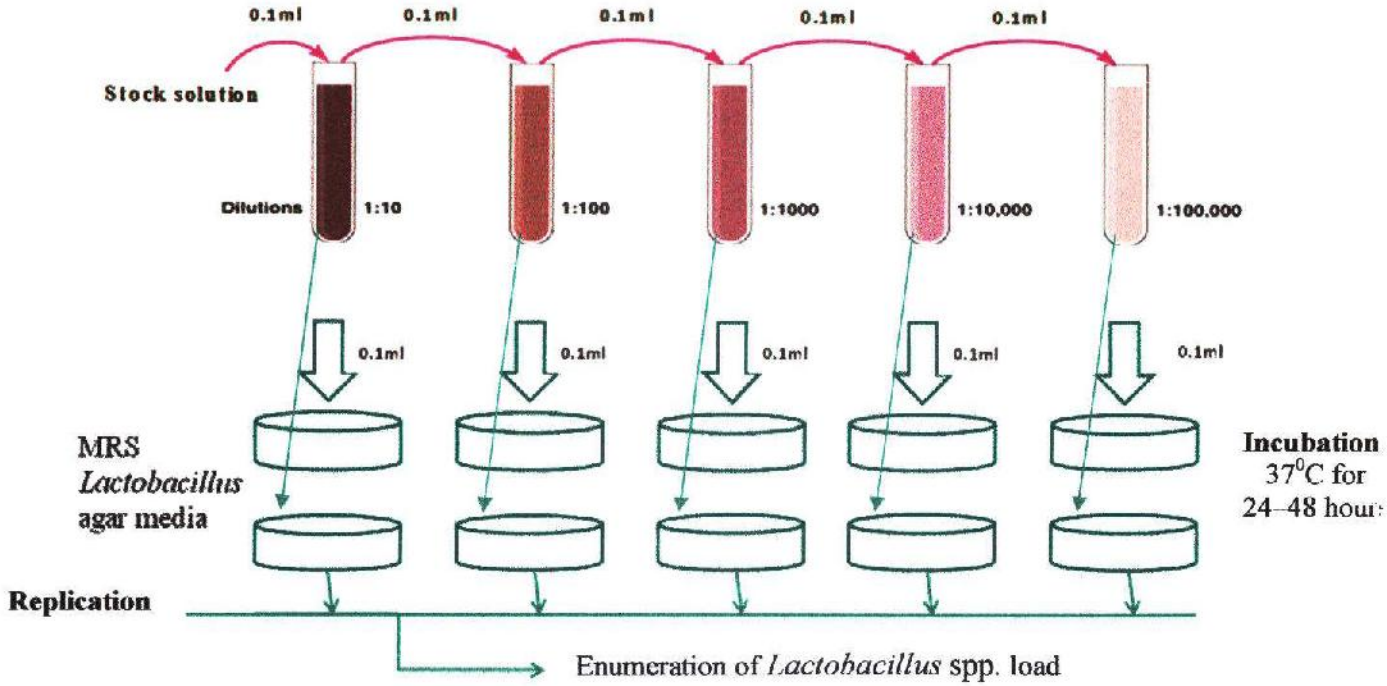


Figure-1: Isolation and enumeration of probiotic bacteria (*Lactobacillus* spp.) from intestinal tracts of prawn.

The colony was enumerated with the following formula,

$$\text{Colony forming unit (CFU/ g)} = \frac{\text{Number of colonies x dilution factor}}{\text{Volume of sample (g)}}$$

3.6.2. Isolation of *Vibrio* spp.

Vibrio spp. was isolated from the intestinal tracts of the collected shrimp samples from study area. ISO method was followed for isolating *Vibrio* spp. 0.1ml stock solution of each gill and intestinal tract was taken and mixed with 0.9ml alkaline saline peptone water

(ASPW) in sterilized test tubes. Then the mixture was incubated at 37 °C for 6 h ± 1. This was the first selective enrichment. After that, whole culture of each test tube obtained in first selective enrichment was taken and transferred into other test tubes each containing 10 ml ASPW. Then the solution was incubated at 41.5 °C for 18 h ± 1. This was the second selective enrichment. Then serial dilution (tenfold) was done and 0.1ml suitable dilution of each culture was inoculated in thiosulfate citrate bile and sucrose (TCBS) agar plates. Then the inoculated TCBS agar plates were incubated at 37 °C. After 24 h ± 3 h of incubation, the plates were examined for the presence of typical colonies of presumptive *Vibrio* spp. (ISO/TS 21872-1, 2007). The total process was done according to the following flowchart.

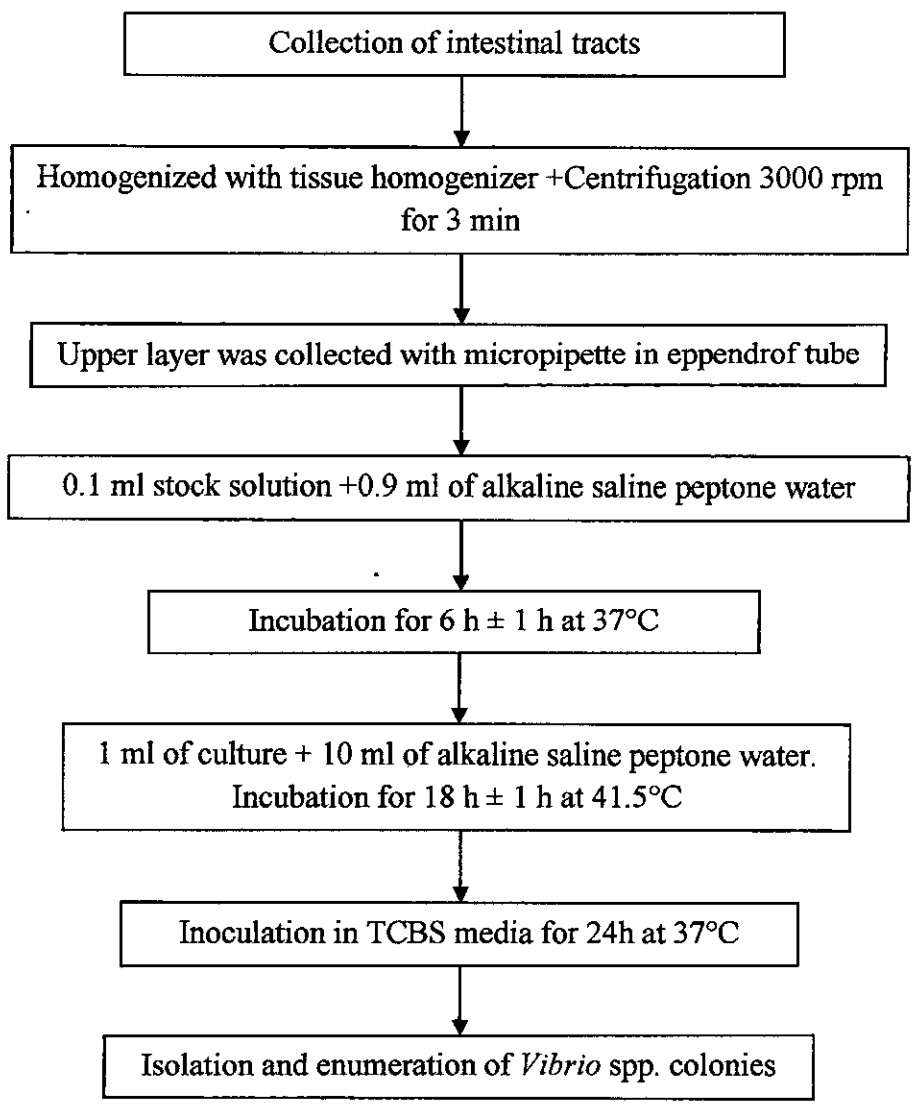


Figure-2: Flow chart of isolation and enumeration of *Vibrio* spp.

3.6.3. The identification of *Vibrio harveyi*

The identification of the *Vibrio harveyi* colonies was done by performing various biochemical tests viz, methyl red test, VP test, indole ring test, Salt tolerance test (0%, 1%, 3%, 5%, 7%, and 10%) and growth at (4°C, 28°C, 37°C, 55°C).

3.6.4. Biochemical tests

The isolates were identified at the species level with the use of biochemical key.

3.6.4.1. Gram Staining

Gram stain was done to identify the gram positive and gram negative bacteria (Cowan and Steel's, 1993).

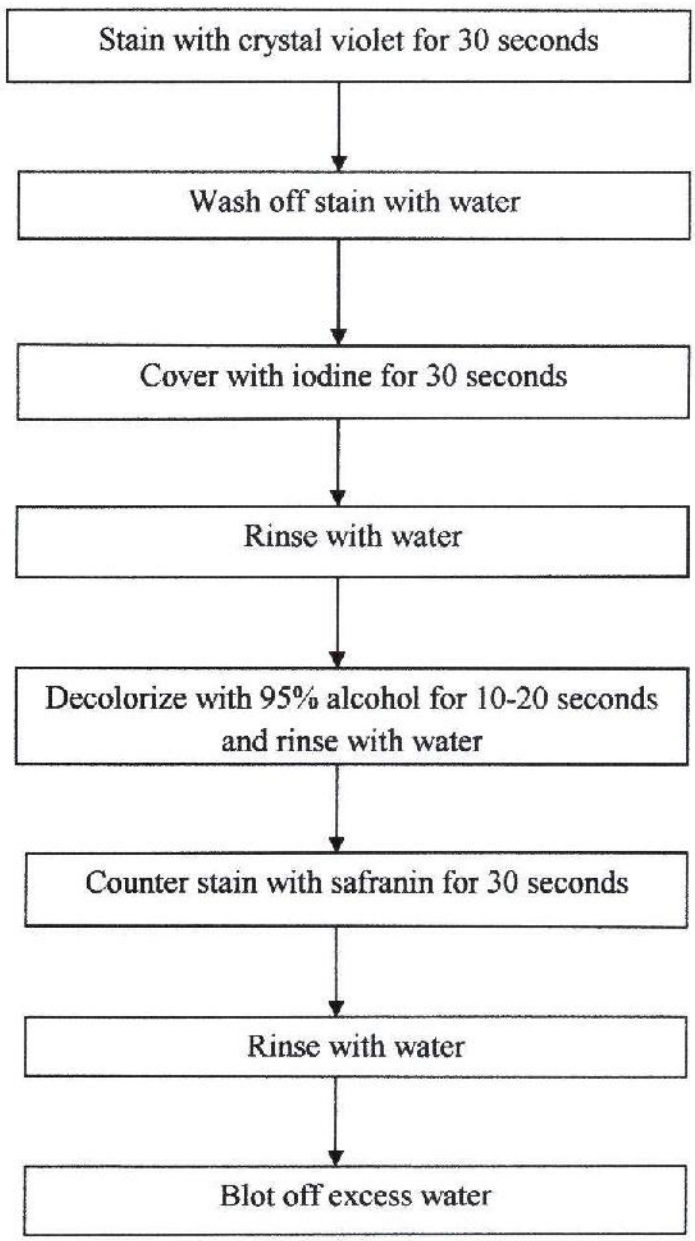


Figure-3: Flow chart of gram staining process.

3.6.4.2. Indole test

The Indole test was performed in a 48h culture in peptone water adding about 1ml ether; shake; run 0.5 ml Ehrlich's reagent down the side of the tube. A red color in the solvent indicates positive reaction (Cowan and Steel's, 1993).

3.6.4.3. MR test

This test detects acid production to a sufficient degree (below PH 4.5). The test organisms were inoculated in 2.5 ml of sterile MR-VP broth and incubated for 24 hours at 37°C. After incubation 5 drops of methyl red solution was added and recorded immediately. Development of red color indicates that pH has been reduced to 4.5 or less, means positive result. A yellow color indicates a negative reaction (Cheesbrough, 1985).

3.6.4.4. Voges-Proskauer (VP) test

This test detects the formation of acetyl methyl carbonol (acetone) from glucose. Single colony from the pure culture of the test organism was inoculated in 2.5 ml of sterile MR-VP broth and incubated for 24 hours at 37°C. Then 12 drops of Barrit's Reagent A and 4 drops of Barrit's Reagent B were added and carefully shacked for 30 second to 1 minute. The tubes were allowed to stand for 30 minutes to observe the color formation. According to Cheesbrough, 1985, a bright pink or eosine red colour indicates positive reaction.

3.6.4.5. Salt Tolerance Test

This test was done on nutrient agar media supplemented with varying amounts of NaCl (0%, 1%, 3%, 6%, 8%, and 10%). This was performed to study the salt tolerance range of the isolated species and the optimum concentration, once determined was supplemented in various media required to test their biochemical properties (Cowan and Steel's, 1993).

3.6.4.6. Growth at different temperature range

The isolated *Vibrio* spp. was kept in incubator after inoculation at subsequent interval at temperature 4^0C, 28^0C, 37^0C, 55^0C and checked for their survivability and colony formation obtained at 28^0C and 37^0C (Cowan and Steel's, 1993).

3.6.5. Biochemical tests to characterize lactic acid bacteria

3.6.5.1. Gram Staining

Gram stain was done to identify the gram positive and gram negative bacteria (Cowan and Steel's, 1993).

3.6.5.2. MR test

This test detects acid production to a sufficient degree (below PH 4.5). The test organisms were inoculated in 2.5 ml of sterile MR-VP broth and incubated for 24 hours at 37°C. After incubation 5 drops of methyl red solution was added and recorded immediately. Development of red color indicates that pH has been reduced to 4.5 or less, means positive result. A yellow color indicates a negative reaction (Cheesbrough, 1985).

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3.6.5.4. Catalase test

This test was used to check the production of enzyme catalase. For this test a clean microscopic slide was taken. A drop of 3% H_2O_2 was taken on the microscopic slide aseptically. A loop full of bacterial culture was taken and mixed with 3% H_2O_2 solution on the slide and the presence of the bubble production observed (Renuka *et al.*, 2012).

3.6.5.5. Lactose fermentation test

Lactose is a carbohydrate source. Fermentation of lactose is demonstrated by the production of acid and gas. For this study, each test tube was filled with 9 ml of lactose broth solution. Then Durham's tubes were inserted upside down in all test tubes, shaken gently to remove air and sterilized by autoclaving. Then 1 ml of 10 % lactose solution sterilized by petroleum ether was added in each test tube, inoculated and incubated for 24-48 h at 37°C. Acid production was indicated by the color change from greenish-blue to yellow and gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube, indicating positive result (Cheesbrough, 1985).

3.6.6. Conformation *Vibrio* spp. by PCR test

At the very beginning of extraction process, one colony from cultured sample was taken into PCR tube; samples were mixed with 50µl DW (distilled water) by spinning and boiled at 100^0 C for 10 minute. Then 2µl of stock sample which was stocked in glycerin.

3.6.6.1. Preparation of media and culture of organism

Nutrient (NB) broth was prepared by mixing 2.8 g NB powder in 100 ml water and then it was autoclaved (121^0 C, 20 min), then 5 ml NB broth was taken into test tube and one colony was taken into it shaking incubator at 37°C overnight. Then stock solution was prepared by adding 150 ml of cultured sample into 850 ml glycerin in a vial. From stock solution10 µl samples were was used for DNA extraction (Ausubel *et al.*, 1990).

3.6.6.2. DNA Extraction

According to the *In-vitro* gen manual for DNA zol Reagent, bacterial DNA was extracted from the cultured bacteria. DNA zoL Reagent is a complete and ready-to-use reagent for the isolation of genomic DNA isolation solid and liquid samples. The DNA zoL Reagent procedure is based on the use of a novel guanidine-detergent lysing solution which permits selective precipitation of DNA from a cell lysate. DNA zoL Reagent is an advanced DNA isolation reagent that combines both reliability and efficiency with simplicity of the isolation protocol. The DNA zoL Reagent protocol is fast and permits isolation of genomic DNA from a large number of samples of small or large volumes. During the isolation, a biological sample is lysed (or homogenized) in DNA zoL Reagent and the genomic DNA is precipitated from the lysate with ethanol. Following an ethanol wash, DNA is solubilized in 8 mM NaOH. The procedure can be completed in about 30 min with DNA recovery of 70-100%. The isolated DNA can be used without additional purification for applications polymerase chain reaction (PCR). (Invitrogen, Life Technologies, USA; Ausubel *et al.*, 1990)

3.6.6.3. Qualitative and quantitave determination of extracted DNA

Integrity of extracted DNA sample was studied by agarose gel electrophoresis as described by Seenivasan *et al.*, (2012). Briefly, electrophoresis was performed using a horizontal electrophoresis apparatus (Bioneer, Korea) at a constant volt of 100 with a 1% agarose gel containing 1.2 µl ethidium bromide in 120 ml 1x TAE buffer. Supply of power for electrophoresis was continued until DNA migrated near about the two-third of the gel

towards the positive electrode. DNA size was determined by comparison with a DNA size marker (100 bp DNA ladder, Bioneer, Korea). DNA bands were visualized by UV illumination and photographed using the High Performance UV Transilluminators, (Ultra-Violet Products Ltd., UK).

3.6.6.4. Description of Target genes and primers

Target genes: The target genes were chosen for this investigation included: virA gene for *Vibrio*.

Table-2: Specific gene for pathogenic bacteria detection.

Specie	**Specific Gene**	**Accession No.**	**References**
Vibrio	virA gene	D26468	Tarr*et al.*,2007

3.6.6.5. Oligonucleotide primers

The DNA fragment to be amplified is determined by selecting primers. Primers are short, artificial DNA strands that are complementary to the beginning and the end of the DNA fragment to be amplified. They anneal to the DNA template at these starting and ending points, where the DNA-polymerase binds and begins the synthesis of the new DNA strand. In this study, seven pairs of primers were used. Sequences of the seven PCR primer pairs and size of expected amplification products are shown in **Table 3**.

Table 3: Primers and expected size of PCR amplified gene targets of pathogenic bacteria.

Specie	Primers	Sequence (5' →3')	Product size (bp)	Temp
Vibrio	Vibr-F	CGGTGAAATGCGTAGAGAT	663	49.5
	Vibr-R	TTACTAGCGATTCCGAGTTC		

The final mixture for the PCR test was 25 µl. In the mixture, to prepare 1 unit *Taq* DNA polymerase, it was 3 times diluted with de-ionized water. The components of the mixture are shown in **Table 4**.

Table -4: The component of reaction mixture for the PCR

Component	**Final volume**
Di- ionized water	13 µl
10X Reaction Buffer (Tris; pH 9.0, 15 mM $MgCl_2$)	5 µl
10 mMdNTP mixture (each dNTP 2.5 mM)	2 µl
Template	2 µl
Primers	2 µl (Forward 1 µl + Reverse 1 µl)
A *Taq*Polymerase (5 units/µl; Top DNA Polymerase, Bioneer, Korea)	1 µl
Total volume	25µl

3.6.6.6. Top DNA polymerase enzyme

The enzyme was isolated room recombinant E. coli. Strain containing the DNA polymerase gene from *Thermusther mophillus*. It exhibits highest activity at pH 9.0 and 72°C (Bioneer, Korea). To prepare 1 unit Top DNA Polymerase from purchased 5 units, it was diluted with enzyme dilution buffer (50 mMTris-HCL, 0.1 mM EDTA, 1mM DTT, Stabilizers, 50% Glycerol, pH 8.2) at 4: 1 ratio (Buffer : Enzyme).

3.6.6.7. Deoxynucleotide Triphosphates (dNTPs) mixture

In the present experiment, 10 mMdNTP mixture (each dNTP 2.5 mM) was used (Bioneer, Korea).

3.6.6.8. Buffers used for PCR

10x Reaction buffer (Tris; pH 9.0, 15 mM $MgCl_2$) was used for PCR test.

3.6.6.9. DNA amplification by a thermal cycler

Desired DNA amplification and target gene amplification were conducted by PCR analysis. In this method, following DNA extraction from samples with forward and reverse primers and *Taq* polymerase. PCR amplification was performed in a Gene Cycler under the following conditions: In brief, heat denaturation at 94°C for 2 min followed by 35 cycles of heat denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and DNA extension at 72°C for 1 min. This is followed by incubation at 72°C for 10 min followed by cooling at 4°C (**Table 3**) and the PCR product was analyzed by agarose gel electrophoresis. Before PCR, PCR sample was made by the following way, in Brief, 3 µl DNA extract were directly used as template in each PCR-tube for the reaction. The reaction mixture consisted of 1 µl primer (Forward 0.5 µl + Reverse 0.5 µl), 2 µl 10x Reaction buffer, 2 µl dNTPs, 1 µl 1 unit Top DNA Polymerase enzyme as well. These all components were mixed in 13 µl distill water and thus a total of 20 µl PCR sample was prepared in a 0.2 ml PCR-tube to operate in a thermal cycler.

3.6.6.10. Evaluation of PCR products

After completion of the thermal cycling, a sample of 8 µl from each PCR products was analyzed electrophoretically by running on a 2% agarose gel and the amplified product size was determined by comparison with a 100 bp DNA size marker.

3.6.7. *In-vitro* challenge test and determination of antagonistic activity of the probiotics

After identification of *Vibrio harveyi* by biochemical test and PCR test one colony of *V. harveyi* was taken into eppendorf tube by isolating loop into 0.9ml peptone water. Prepared stock solution of *V. harveyi* (ISO/TS 21872-1, 2007). The stock solution was diluted (tenfold dilution) with peptone water (James and Hirsch, 1960) and prepared test solution of *V. harveyi*. Then 0.5 ml isolated probiotic solution was separately mixed with 0.5ml of test solution (*V. harveyi)*. 0.1ml suitable dilution of the mixer solution was inoculated in TCBS agar media after at subsequent intervals of 4 hour up to 12hour. This procedure was done for 2 times. Test solution of *V. harveyi* ; without probiotic was also inoculated in TCBS agar media at 0 hour, 4^{th} hour, 8^{th} hour and 12^{th} hour subsequently. All the inoculated TCBS agar plates were incubated at 37^0 C for 24± 3 hours. Standard plate count was done after incubation.

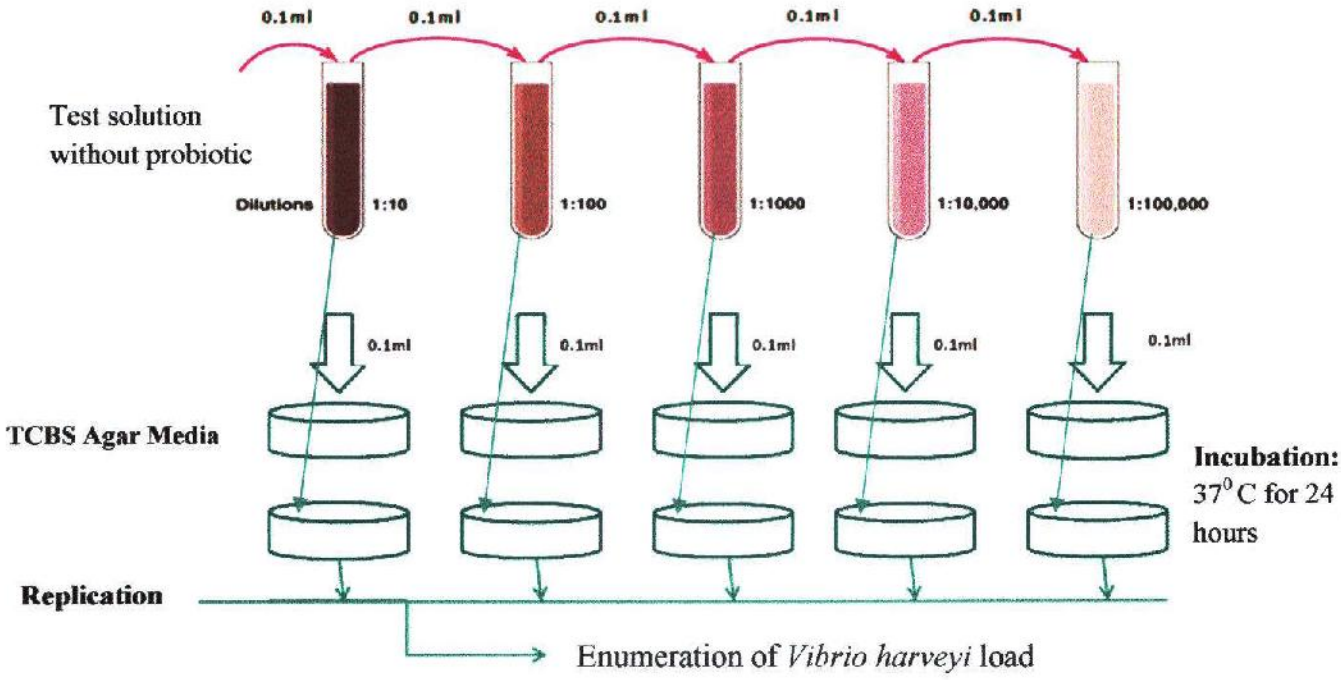


Figure-4: *In-vitro* challenge test and enumeration of *Vibrio harveyi* load without probiotic.

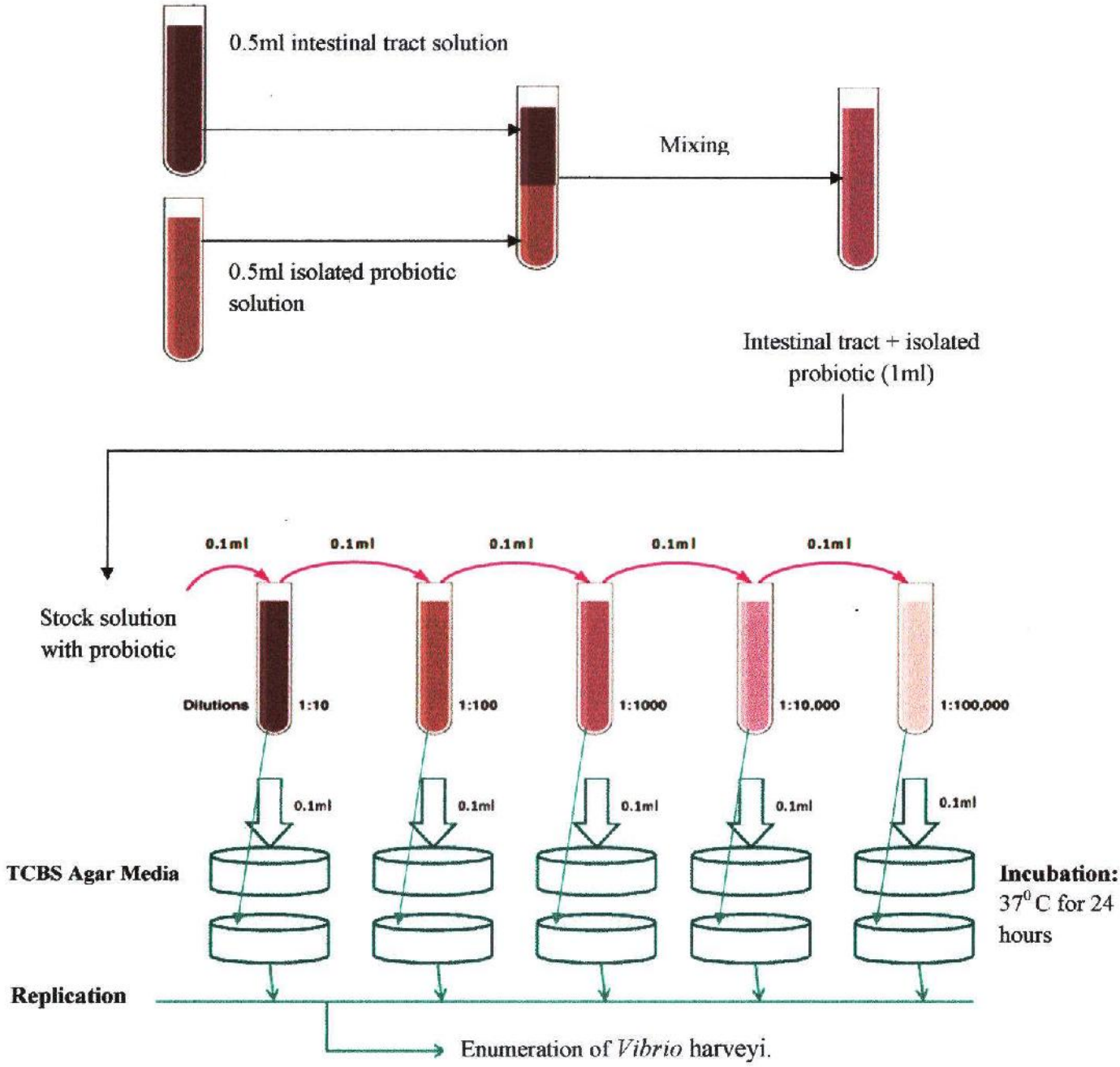


Figure-5: *In-vitro* challenge test and enumeration of *Vibrio harveyi.* load with probiotics.

3.6.8. *In-vivo* challenge test and determination of antagonistic activity of the probiotics

3.6.8.1. Experimental design

In-vivo test (Experimental Design)

	Control	Treatment
Study period	60 days	60 days
Stocking	50 PL/pond	50 PL/pond
Initial Weight (g)	2.19± 0.05 g	2.55± 0.18g
Pond size	70 × 70 × 1 m^3	70 × 70 × 1 m^3
Feed	**Without probiotic**	**With probiotic**

3.6.8.1.2. Pond preparation for fry rearing

The experiments were performed simultaneously at the Fisheries and Marine Resource Technology Discipline and Discipline's ponds, Khulna University, Khulna. Four earthern ponds were selected for the experiment. There were one control and one treatment with three replicate. Before starting the experiments the ponds were dewatered and sundried and agricultural lime ($CaCO_3$) at 2.5 kg/100 m^2 was applied. The total area of the ponds was fenced with nylon net so that undesirable organisms cannot enter into the pond from outside. In order to control predator and other undesirable fishes, amphibians, reptiles, insects, frogs' etc, Bleaching powder was used for controlling germ and harmful microbes. Bushes on the banks of the ponds were removed manually to destroy the habitat of other predators like otters, snakes, etc. The total area of the ponds was fenced so that undesirable organisms cannot enter into the pond from outside. Green or gray-blue color indicates the adequate primary productivity in the ponds. Within 3-4 days after fertilization the water of the experimental ponds turned green color indicating that adequate plankton had been produced. Besides some water was taken in a clean conical flask or bicar and then examined holding against the sun. So the ponds were ready for stocking.

3.6.8.1.3. PL Nursing and stocking

After pond preparation, prawn PL nursing was done in the pond to reach the PL into juvenile to reduce the mortality rate of prawn into grow out pond. The PL was nursed for 30 days from May 15 to June 15. After nursing the PL for 30 days, they were stocked in grow out pond on 16th June when the average individual weight was about 1g. Before releasing in the pond, acclimatization of fry in the new environment was done by keeping the pots in pond water for a while and then gradually mixing pond's water with fry containing water, which was very important to reduce mortality. The *in-vivo* test was carried out in earthen ponds (70m x 70m x 1m) with a stocking density of 50 fingerlings per treatment pond. Two different feeding treatment *i.e.* probiotic mixed feed and normal feed (T0=control) were applied with triplication at a feeding rate of 7% of their body weight.

3.6.8.1.4. Probiotic bacteria treatment

For preparing the probiotic incorporated feed, the stocked probiotic solution was cultured in nutrient broth media. Then the cells of probiotic bacteria after incubation were harvested by centrifugation at 3000×10 rpm for 10 min. The cells were thoroughly mixed with the commercial scampi feed to obtain 2×10^9 cfu g-1 in feed and the mixture was spread out and aseptically dried in an oven for 1-2h at 60°c (Rahiman *et al.*, 2010). Then the feed was applied in the research ponds at a rate of 7-11% of prawn body weight for the first month and from the next month 5-6% of body weight per day at a frequent like twice per day until harvested. Thus the prawn were cultured in the research ponds about 60 days (Ziaei-Nejad *et al.*, 2006). The quantity of bacteria means probiotic was measured by using expectophotometer. The espectophotometere read that one ml of cultured solution contains about 1.44×10^9 probiotic bacteria that mean *Lactobacillus* spp.

3.6.9. Digestive enzyme analysis

3.6.9.1. Determination of Growth performance

At the end of the experiment, the growth parameters such as weight gain (WG), and specific growth rate (SGR) of different treatments were determined by following equations according to (Seenivasan *et al.*, 2012 and Kumar *et al.*, 2013)

$$\text{Weight Gain (\%)} = \frac{\text{Final weight (g)} \times \text{Initial weight}}{\text{Initial weight (g)}} \times 100$$

$$\text{Specific growth rate (SGR day)} = \frac{\text{In final weight (g)} - \text{In initial weight (g)}}{\text{Duration (days)}} \times 100$$

3.6.10. Preparation of intestinal tract for Digestive Enzyme Assays

To measure digestive enzyme activity, samples of prawn were collected and then were washed with cold distilled water and immediately frozen at -80^{0}C until enzyme assays were conducted. As with monitoring of bacteria, digestive tract was dissected out and homogenized. Samples were homogenized in 9 volumes of 0.05 M Tris-HCl buffer, pH 7.8, in a Wise Tis Homoginizerin 700×10 rpm for five minutes, and were centrifuged at 4800×g for 60 min at 4^0 C according to the method outlined (Lovett and Felder, 1990). The supernatant of each sample was assayed in triplicate.

3.6.10.1. Amylase Activity Assay

The amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld 1995), using 1% soluble starch (Merck, product number 1257, Darmstadt, Germany) as substrate. The sample of 250 microliters was incubated for 30 min at 35°C with 260 µl Tris-HCl buffer and 40µl soluble starch. The reaction was stopped by addition of 100 µl DNS and heated in boiling water for 10 min. 3, 5-Dinitrosalicylic acid is a color reagent that the reducing groups released from starch by amylase action are measured by the reduction of 3, 5- dinitrosalicylic acid. The boiling water is for stopping the amylase activity and catalyzing the reaction between DNS and reducing groups of starch. Then absorbance was read at A_{540} (absorbance at 540 nm) after cooling in ice for 5 min. Enzyme activities were measured as the change in absorbance using a double beam spectrophotometer (HITACHI, U-2910, Japan). One unit of amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35°C. A standard curve of absorbance against the amount of maltose released was constructed to enable calculation of the amount of maltose released during amylase assays. Serial dilutions of maltose (Merck, Product Number 105911, Mr 360.32 mg mol-1) in the Tris-HCl buffer at pH 6.5 were made to give following range of concentrations of 2, 1, 0.5, 0.25, 0.125 mg ml^{-1}(**According to Appendices 8**).

3.6.10.2. Protease Activity Assay

Sigma's non-specific protease activity assay was used as a standardized procedure (Anson, 1938) to determine the proteases activity, using 0.65% weight/volume casein solution (Merck, product number 1257, Darmstadt, Germany) as substrate. To assay the protease activity two vials were used. One vial was used as a blank, and other was used to assay activity of the protease. In each vials added 5mls of our 0.65% casein solution, and submerged in a water bath at 37°C for 5 minutes. Then added 1ml of enzyme solution to the test sample, 1ml buffer in the blank. The solution was mixed by swirling and incubated for 37°C for ten minutes. After that in each tube added 5 mls of the TCA (Trichloroacetic acid) reagent to each tube to stop the reaction. Then the final solution was incubated at 37°C for 30 minutes. Then 5mls of sodium carbonate and 1 ml of Folin's reagent are added immediately. Sodium carbonate is added to regulate any pH drop created by the addition of the Folin's reagent. These solutions became cloudy after the addition of sodium carbonate and the Folin's reagent was reacted with free tyrosine. Then the solution was mixed by swirling and incubated at 37°C for 30 minutes. After this incubation, appreciable colour change was noticed. 2mls of these solutions were filtered using a 0.45 um poly ethersulfone syringe filter into suitable cuvettes. Then absorbance was read at A_{660}(absorbance at 660 nm) after cooling in ice for 5 min. Enzyme activities were measured as the change in absorbance using a double beam spectrophotometer (HITACHI, U-2910, Japan). One unit of protease activity was defined as the amount of enzyme required to produce 1 mg tyrosine in 30 min at 37°C. A standard curve of absorbance against the amount of tyrosine released was constructed to enable calculation of the amount of maltose released during protease assays. A standard curve of absorbance against the amount of tyrosine released was constructed to enable calculation of the amount of tyrosine released during protease assays. Serial dilutions of tyrosine were made to give following range of concentrations of 500 $\mu g\ ml^{-1}$, 1000 $\mu g\ ml^{-1}$ and 1500 $\mu g\ ml^{-1}$(**According to Appendices 9**).

3.7. Immunity Assay

3.7.1. Prawn Haemocyte Analysis

Use of an electric Microscope and clinical haemocytometer for differential counts of prawn *Macrobrachium rosenbergii* haemocytes following a well knowed method (Owens and ONeill's, 1997) with some modifications.

3.7.1.1. Haemolymph collection

Prawns were collected from the experimental ponds and carried out alive to the laboratory using bucket. The prawns were washed thoroughly with clean freshwater and kept in freshwater for around 15 minutes. Then it was pulled from water, gently wiped to minimize water content. After that, fresh haemolymph were extracted in an anti-coagulant pre-loaded syringe. The syringe are previously contained with anticoagulant solution, since crustacean haemocytes change shape or lyse almost spontaneously following removal from the body. The haemolymph was withdrawn from the pericardial sinus using a fine needle on a syringe. The cardiac puncture was performed with the 1.5, 2.5 gauge needle attached to a 1 ml syringe which were pre-loaded with the well-established crustacean anticoagulant i.e., 0.5 ml citrate-EDTA [EDTA 0.01 M; trisodium citrate 0.03 M; citric acid 0.026 M; NaCI 0.45 M; glucose 0.1 M, adjusted to pH 4.6 with 1 M HC 1(Soderhall and Smith, 1983)] and 0.5 m 1 of haemolymph was extracted.

3.7.1.2. Haemolymph preparation

Immediately after Haemolymph collection, a pre-chilled, 0.5mI sequestrene tubes or 1 ml WBC pipette (micropipette tube) containing 0.5 ml of prawn haemolymph were diluted in an equal amount of anticoagulants i.e. citrate- EDTA, practically anticoagulants needed to load first to avoid coagulation. Then these liquid substances were placed on a mechanical rotator and stirred well for around 2 minutes to ensure complete mixing. Thus, the samples were diluted 1:1 (volume/volume) to maintain cell integrity, thereby preventing clotting and clumping of cells in *vitro*. These samples were used to determine total and differential haemocyte counts using the 40X objective of a light microscope (Olympus CX-21).

3.7.1.3. Haemolymph slide preparation for Haemocyte count

After mixing with anticoagulants, a drop of the liquid was quickly poured on the corner of a slide and a spreader is utilized to first draw back the sample to spread it both left and right side and then push the sample to the entire front side of the slide at a single pressure in around 45° angles, and thus a monolayer smear was created. This monolayer smear of haemolymph was carefully fixed and stained as it represents the different cells information for Light microscopy. The slide was then kept for few minute to dehydrate. After drying, the Giemsa stain were used on the slide, the stain cautiously dispersed well on the slide so that it cover the entire slide and wait for 1-2 minute, then distilled water (around 2 times of

stain) were utilized with a dropper (for protecting the slide from scorching). Then again the stained slide was kept for 3-5 minutes to air. After that, it was gently washed and kept again for drying. Finally the slide were improved its quality by gently giving a smear of liquid paraffin. This slide is ready for Haemocyte count under light microscope. It was observed that, any delay of mixing with anticoagulant or any gap in the Sequential procedure lead to form an unsuccessful slide, where no or little blood cell was found. This type of slide was discarded during this study.

3.7.1.4. Cell Counting from Slide

The slide was fixed well on the mechanical stage of the Olympus CX21 Microscope. The CX21 LED had an illumination system using LED light to acquire clear images. The slide was placed before a 10X lens for adjustment of the cell image. Then it was observed in 40X lens. The light is adjusted as per requirement of the image clearness. The cells were counted and placed the data in the DLC (Differential Count) Chart for further analysis.

3.7.1.5. Cell counting using Haemocytometer

Total haemocyte counts (THC) were performed with an improved Neubauer haemocytometer using a modification of the method described by (Bain *et al.*, 2001) for analysis of human blood samples. First, a special cover slip was taken on the improved Neubauer chamber. The WBC pipette containing anti-coagulated 0.05 ml blood sample were mixed well with 0.95m1 WBC fluid and a drops were discarded outside first, then one drop sample was taken in the edge of the special cover slip and allowed to run under the cover slip throughout the Neubauer chamber. When it dispersed over the chamber, the haemocyte cells were visualized under light microscope. The Neubauer haemocytometer had four large corners of 16 squares. So it possess a total 4×16-64 squares (volume of each large corner square =1mm×1mm×0.lmm or 10 mm 3). To calculate total haemocyte count (THC) of haemolymph, the number of haemocyte cell found in these 64 square was multiplied with 50 (as per calculating formula) and that was the total number of haemocyte in per cubic millimeter (Rahman, 2008).

3.7.1.6. Calculating Formula

Total haemocyte count was determined from the volume represented by the chamber counted and the blood dilution factor according to the formula

$$\text{Total Haemocyte count/cubic millimeter} = \frac{\text{cell counted x blood dilution x chamber}}{\text{Area of chamber counted}}$$

3.7.1.7. Determining Blood dilution

Since, 0.05 ml (50 µL, 0.5 marked) blood mixed with preloaded 0.95ml (950 µL, 11 marked) anticoagulants and made lml of sample. Thus the dilution of blood sample was 1 in 20. At that point, 10.5µL anti-coagulant (instead of WBC fluid) and 0.5 µL haemolymph was mixed in 1: 20 ration.

3.7.1.8. Determining chamber volume

Determining volume of each large corner Square =1mm×1mm×0.1mm=1mm×1mm× 1/10mm==1/10(10×10×1) mm^3 = 1/10×100 mm^3= 1/10×100 mm^3 = 10mm^3

THC/cmm = (cell courted) × (20) × (10) / 4

THC/cmm = (cell counted) × 50

3.7.1.9. Sequential Procedure of Total Haemocyte Count

1. A special cover-slip and haemocytometer were ensured as grease-free and clean (alcohol used to clean).

2. Moistened (with water or exhaled breath) and affixed cover-slip to the haemocytometer.

3. Verifying that the cover slip was properly adhered via suction to the haemocytometer and "Newton's Rings" appeared. Newton's refraction rings are seen as rainbow-like rings under the cover-slip.

4. The WBC pipette was used to take well-mixed blood cell suspension (where 0.5 ml blood sample stained with equal volumes of Giemsa were mixed gently with 0.95ml anticoagulants e.g. mix 0.5 ml Giemsa stained cell sample with 0.95ml anticoagulants).

5. Then fisrt 1/2 drops of sample were discarded outside and one drops were pipetted (approximately 10µl) at the edge of the cover-slip and allowed to run under the cover slip to disperse throughout the Neubauer chamber.

6. The haemocytometer grid were visualized under the microscope, where the cells within the square and cells touching middle line of top and left were counted but the cell touching middle line of bottom and right sides were excluded.

7. The cells were counted in one or more large corner squares and cell counts were recorded.

8. To obtain an accurate cell count result, more than one large corner square of the haemocytometer was counted.

9. To calculate cell concentration per millimetre the following formula followed,

Total haemocyte count/cubic millimetre= (cell counted x blood dilution x chamber volume) / Area of chamber counted

In this traditional using system, the improved Neubauer chamber blood Dillution factor was 20, chamber Volume was 10 mm^3, Area of chamber counted was 4. To attain the result per litre, the result of per cubic millimeter to be multiplied by 10^6 as per arithmetic rule.

3.7.1.10. Correlation between counts from light microscopy and Haemocytometer

The experienced pathologist repeatedly observed and found that the focusing slide area appeared under 40X lens is equally relevant to the each smallest cell area of the Haemocytometer. So, the total number of cells in an area encompassing under 40X lens is equivalent to the each smallest cell area of the haemocytometer. Thus the light microscopic cell count under 40X lens of Olympus CX21. Microscope is equally correlated with each cell counting data of haemocytometer.

3.8. Statistical analysis

Data are reported as arithmetic mean ± standard deviation (SD). After appropriate transformation (arcsine or logarithm), **Independent sample T test** was applied on the data to assess the treatment effect. All the statistical analyses were done on a computer using statistical software package SPSS (version 7.5).

Chapter 4

Results

4. Results

For the enumeration of bacterial loads, intestine was aseptically taken out from the samples. The organs were taken into eppendorf tubes with peptone water (James and Hirsch, 1960) for isolating *Lactobacillus* spp. and alkaline saline peptone water was used for isolating *Vibrio* spp. (ISO/TS 21872-1, 2007). Then the colonies of *Lactobacillus* spp. and *Vibrio* spp. were counted. The result revealed that the load of *Lactobacillus* spp. were more (2.58×10^6, 2.30×10^6, 1.85×10^6 CFU/g than *Vibrio* spp. (2.45×10^5, 2.87×10^5, 1.80×10^5 CFU/g in intestines; Depending on the biochemical test the isolates were identified up to species level and it was also confirmed by PCR test. It was found that almost all are *Vibrio harveyi* (**Table-7**) and then *Lactobacillus* spp. were employed *in-vitro* and *in-vivo* to determine antibacterial effects on *V. harveyi*.

The average load of *Lactobacillus* spp. in the intestinal tracts of the prawn samples collected from local markets were 2.58×10^6, 2.30×10^6, 1.85×10^6 CFU/g respectively which is shown in **Table-5**.

Table-5: Load of *Lactobacillus* spp. in intestinal tracts of collected prawn.

Sample number	Replication number	Load of *Lactobacillus* spp. in intestinal tracts (CFU/g)	Average load in intestinal tracts (CFU/g)
1.	1.	2.30×10^6	**2.58×10^6**
	2.	2.83×10^6	
	3.	2.60×10^6	
2.	1.	2.30×10^6	**2.30×10^6**
	2.	2.10×10^6	
	3.	2.52×10^6	
3.	1.	2.12×10^6	**1.85×10^6**
	2.	1.80×10^6	
	3.	1.63×10^6	

The average load of *Vibrio* spp. in the intestinal tracts of the prawn samples collected from 3 markets were 2.45×10^5, 2.87×10^5, 1.80×10^5 CFU/g respectively which is shown in **Table-6.**

Table-6: Load of *Vibrio* spp. in intestinal tracts of collected prawn.

Sample number	Replication number	Load of *Vibrio* spp. intestinal tracts (CFU/g)	Average load in intestinal tracts (CFU/g)
1.	1.	2.48×10^5	**2.45×10^5**
	2.	2.74×10^5	
	3.	2.13×10^5	
2.	1.	2.35×10^5	**2.87×10^5**
	2.	2.85×10^5	
	3.	3.40×10^5	
3.	1.	2.10×10^5	**1.80×10^5**
	2.	1.80×10^5	
	3.	1.60×10^5	

Eight biochemical tests were performed (**Table-7**) to identify *V. harveyi* from the isolated colonies of *Vibrio* spp. The isolated colonies were inoculated in different biochemical media, supplemented with 1% NaCl to provide the optimum condition for growth, and incubated overnight at 30°C. The dendrogram based on the tests of Gram stain, Motility test, Indole ring, VP test, Methyl red test (MR test), Growth in NaCl at (0%, 1%, 3%, 5%, 7%, 10%) and Growth at (4°C, 28°C, 37°C, 55°C), Colony color on TCBS test for the identification of *V. harveyi*, as proposed by Cowan and Steel's (1993) was followed for primary identification.

The Gram stain result was observed under total magnification by using a light microscope and showed all *V. harveyi* isolates to be Gram negative short rods. All *V. harveyi* isolates showed positive results to the Motility test, Indole ring, MR test. Majority of *V. harveyi* isolates (96.7%) were able to grow in salt tolerance test at 0% to 5%. These bacterial isolates showed inhibition at temperatures of 4°C and 55°C but were able to grow well at temperatures of 28°C and 37°C. The green and yellow colonies were observed on TCBS agar plates.

Table-7: Biochemical test to identify *Vibrio harveyi.*

Biochemical test	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	Commends
Gram stain	-	-	-	-	-	-	-	-	-	-	Isolated colony are gram negative bacteria
Motility	+	+	+	-	N/D	+	-	+	N/D	+	Motile
Indole formation	+	+	+	-	-	+	-	+	-	+	60% positive
VP test	-	-	-	-	+	-	-	-	+	-	80% negative
MR test	+	+	+	+	-	+	N/D	+	-	+	70% positive
Growth in NaCl at											90% isolates grow at (0 - 5% NaCl)
0%	+	+	+	+	+	+	+	+		+	
1%	+	+	+	+	+	+	+	+	N/D	+	
3%	+	+	+	+	+	+	+	+		+	
5%	+	+	+	+	+	+	+	+		+	
7%	-	-	-	-	+	-	-	-		-	
10%	-	-	-	-	-	-	-	-		-	
Growth at											80% isolates grow
4°C	-	-	-	-	-	-	-	-		-	at(28°C
28°C	+	+	+	+	N/D	+	+	+	N/D	+	-37°C)
37°C	+	+	+	+	+	+	+	+		+	
55°C	-	-	-	-	-	-	-	-		-	
Colony color on TCBS	G	G	G	Y	G	G	G	G	N/D	G	80% isolates are green in color
Identification	Vh	Vh	Vh	Vs	Vs	Vh	Vs	Vh	N/D	Vh	60% isolates are *Vibrio harveyi*

N.B: Vh = *Vibrio harveyi*, + = positive, - = negative, G = Green, Y = Yellow, Vs = *Vibrio* Spp.

The biochemical test was done with 10 colonies of *Vibrio* spp. From the conformation test it was found that 60% was *V. harveyi* among 10 samples. After that the isolated *V. harveyi* colonies were stocked for further confirmation by PCR test and also *in-vitro* tested to determine the antagonistic effect of *Lactobacillus* spp. on *V. harveyi.*

Three bands of target genes were found on electrophoresis gel at 663 bp position for *Vibrio* sp. which indicated the presence of pathogenic *Vibrio* in prawn sample. (**Figure-6**) showed the presence of pathogenic bacteria in prawn.

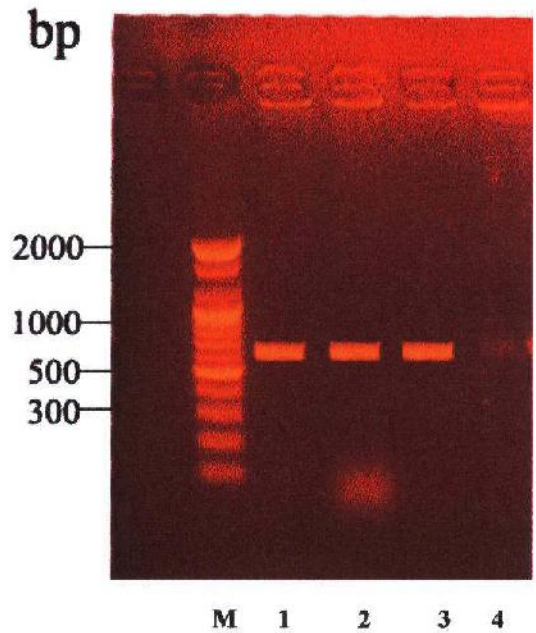


Figure-6: Electrophoretic analysis of PCR-amplified target genes from *Vibrio* sp. obtained under optimal conditions of PCR of isolates from biochemical test. Mobilities of the different target genes are indicated on the right. Lane M, 100-bp DNA ladder (size marker); lane 1-2, gene for stock sample amplimers; lane 3, gene for isolated colony amplimers and lane 4, gene for control group amplimers.

To characterize *Lactobacillus* spp., 16 presumptive lactic acid producing strains were isolated from 8 different samples. Colonies were observed on the surface of MRS agar Petri dish. More than one type of colony was observed on surface of MRS agar Petri plate. Small and large two types of colonies were observed. Most of colonies were creamy to white. The cultural and morphological characteristics were further resolved on the basis of microscopic examination. Majority of the microorganisms were Gram positive rods shaped bacteria. Almost (81.25%) bacterial isolates showed inhibition at temperatures of 10°C and 45°C but were able to grow well at temperatures of 15°C and 37°C.

After characterization, the isolates were referred to genus *Lactobacillus*. The strains were phenotypically characterized on the basis of their morphological, cultural, and biochemical characteristics. Gram's staining of the bacterial culture showed they were gram positive and their cell morphology was rod. *Lactobacillus* spp. isolates showed positive results to the MR

test and negative results to VP test. Catalase test showed that the only twelve isolates out of 16 isolates were not able to produce bubbling when mixed with 3% H_2O_2. This showed that there was absence of catalase enzyme. The absence of catalase enzyme showed that identified bacteria were from *Lactobacillus* species. The bacteria use peroxidase to detoxify H_2O_2, an enzyme that does not evolve O_2.

Table-8: Showing the biochemical characteristics of Isolates (*Lactobacillus spp.*).

S/N	Isolate	Types of colony	Gram staining	Microscopic morphology	Colony color on MRS media	Growth at			MR test	VP test	Catalase test	Sugar fermentation test (Lactose fermentation test)
						10 °C	35 °C	45 °C				
01	S1	Small	+	Rods	cream	- ve	+ ve	- ve	+ ve	- ve	- ve	+ ve, H
	L1	Large	+	Rods	cream	- ve	+ ve	- ve	+ ve	- ve	- ve	+ ve, H
02	S1	Small	+	Cocci	cream	- ve	+ ve	- ve	+ ve	N/D	+ ve	+ ve
	L1	Large	+	Rods	cream	- ve	+ ve	- ve	- ve	- ve	- ve	+ ve, H
03	S1	Small	+	Round	Whitis h	- ve	+ ve	- ve	- ve	N/D	+ ve	N/D
	L1	Large	+	Rods	cream	- ve	+ ve	- ve	+ ve	- ve	- ve	+ ve, H
04	S1	Small	+	Rods	cream	- ve	+ ve	- ve	- ve	- ve	- ve	+ ve, H
	L1	Large	+	Cocci	cream	- ve	N/D	- ve	N/D	-ve	N/D	N/D
05	S1	Small	+	Rods	cream	- ve	+ ve	- ve	+ ve	- ve	- ve	+ ve, H
	L1	Large	+	Round	cream	- ve	+ ve	- ve	- ve	- ve	- ve	+ ve, H
06	S1	Small	+	Rods	Whitis h	- ve	+ ve	- ve	+ ve	- ve	- ve	+ ve, H
	L1	Large	+	Cocci	cream	- ve	N/D	- ve	N/D	N/D	+ ve	N/D
07	S1	Small	+	Round	cream	- ve	+ ve	- ve	- ve	- ve	- ve	+ ve, H
	L1	Large	+	Rods	cream	- ve	N/D	- ve	+ ve	N/D	+ ve	+ ve
08	S1	Small	+	Round	Whitis h	- ve	+ ve	- ve	+ ve	- ve	- ve	+ ve, H
	L1	Large	+	Round	cream	- ve	+ ve	- ve	- ve	- ve	N/D	- ve

Legend: **75% isolates were *Lactobacillus* spp.** and the rest were Lactic acid bacteria, H= homofermentation (acid production not gas), N/D= Not detected.

Further, if these bacterial cultures were used for sugar fermentation, it showed that the bacteria were homo fermentative. The microorganism fermented glucose to acid which was evident by changing colour of medium from red to yellow. There was no gas production in the Durham's tube.

In-vitro challenge tests were performed to investigate the antibacterial effect of the isolated probiotics (*Lactobacillus* spp.) on the *Vibrio harveyi* of infected prawn. When *in-vitro* challenge tests were run it was found that the average load of *V. harveyi.* in zero (0) hour was 1.87×10^5 CFU/g and it was 2.37×10^5, 2.57×10^5 and 3.18×10^5 at 4th, 8th and 12th hour respectively. However, after treatment with the isolated probiotics, the average load of *V. harveyi* at 4th, 8th and 12th hour were reduced to 1.88×10^5, 1.82×10^4 and 1.87×10^3 respectively. The present study showed a slight reduction of *V. harveyi* load at the 4th hour of probiotic application where as a drastic and significant reduction in the load of *V. harveyi* had been obtained at the 8th and 12th hour of probiotic application. The result of the *in-vitro* challenge test with and without probiotics had been presented in **Table-9**.

Table-9: *In-vitro* challenge test and enumeration of *V. harveyi* load with and without probiotics.

Time Interval	Sample No.	Without Probiotics		With Probiotics	
		Vibrio harveyi load (CFU/g)	Average load (CFU/g)	*Vibrio harveyi* load (CFU/g)	Average load (CFU/g)
0 hour	01.	1.95×10^5	**1.87×10^5**	1.95×10^5	**1.87×10^5**
	02.	2.04×10^5		2.04×10^5	
	03.	1.62×10^5		1.62×10^5	
4th hour	01.	2.33×10^5	**2.37×10^5**	1.67×10^5	**1.88×10^5**
	02.	2.50×10^5		2.04×10^5	
	03.	2.29×10^5		1.92×10^5	
8th hour	01.	2.37×10^5	**2.57×10^5**	1.87×10^4	**1.82×10^4**
	02.	2.63×10^5		1.63×10^4	
	03.	2.71×10^5		1.96×10^4	
12th hour	01.	2.92×10^5	**3.18×10^5**	1.38×10^3	**1.87×10^3**
	02.	3.42×10^5		2.04×10^3	
	03.	3.21×10^5		2.21×10^3	

In *in-vivo* challenge test the average load of *Vibrio harveyii* in the intestine at 10th, 20th, 30th, 40th, 50th and 60th day of control group infection was 1.61×10^5, 2.03×10^6, 2.45×10^6, 3.36×10^6, 3.98×10^6, and 2.69×10^7 respectively. After application of probiotic treated feed in treatment group the average load of *Vibrio harveyii* at 10th, 20th, 30th, 40th, 50th and 60th day became 1.29×10^5, 1.25×10^6, 1.81×10^6, 3.06×10^5, 2.90×10^5, and 1.24×10^4 respectively. The present study also showed that there was slight reduction of *Vibrio harveyii* load in the experimental treatment was found at 10th to 40th day of probiotic treated feed application where as a drastic and significant reduction in the load of *V. harveyi* had been obtained at the 50th and 60th day of probiotic treated feed application.The result of the *in-vivo* challenge test of the experiment with and without probiotics treated feed was presented in **Table-10**.

Table-10: *In-vivo* challenge test and enumeration of *V. harveyi* load with and without probiotics treated feed.

Time Interval	Sample No.	Without Probiotics (Control)		With Probiotics (Treatment)	
		Vibrio harveyi load (CFU/g)	Average load (CFU/g)	*Vibrio harveyi* load (CFU/g)	Average load (CFU/g)
10 days	01.	1.61×10^5	**1.61×10^5**	1.28×10^5	**1.29×10^5**
	02.	1.83×10^5		1.17×10^5	
	03.	1.39×10^5		1.44×10^5	
20 days	01.	2.00×10^6	**2.03×10^6**	1.04×10^6	**1.25×10^6**
	02.	2.27×10^6		1.45×10^6	
	03.	1.82×10^6		1.27×10^6	
30 days	01.	2.36×10^6	**2.45×10^6**	1.81×10^6	**1.81×10^6**
	02.	2.55×10^6		1.77×10^6	
	03.	2.45×10^6		1.86×10^6	
40 days	01.	3.36×10^6	**3.36×10^6**	3.09×10^6	**3.06×10^5**
	02.	3.18×10^6		3.18×10^5	
	03.	3.54×10^6		2.90×10^5	
50 days	01.	3.86×10^6	**3.98×10^6**	2.90×10^5	**2.90×10^5**
	02.	4.09×10^6		2.95×10^5	
	03.	4.00×10^6		2.86×10^5	
60 days	01	2.27×10^7	**2.69×10^7**	1.36×10^5	**1.24×10^4**
	02	2.86×10^7		1.27×10^5	
	03	2.95×10^7		1.09×10^5	

The effect of diets containing *Lactobacillus* spp., on growth performance of *Macrobrachium rosenbergii* was also established in this study. For this experiment two types of feed were used, one was probiotic incorporated feed in different treatments (T1, T2 and T3) and another was normal feed in control. Analyzed data on growth performances and feed utilization including initial mean weight (g), final mean weight, weight gain (%), specific growth rate (SGR) and survival rate (%) for control and treatment groups were reported in **Appendix A4**. Among them, the weight gain (WG) and specific growth rate (SGR) of treatment was significantly (P<0.05) greater (**Appendix 5 and Appendix 6**) than control. *Lactobacillus* spp. treated groups showed significant increase in WG % of (549. 30 ± 40.02) followed by control group (310.77 ± 18.68). Similar trend was observed with SGR that probiotics treated groups showed significant increase (3.12 ± 0.10) followed by control group (2. 35 ± 0.08). *Lactobacillus* spp. supplement diet appeared to provide beneficial effects on the growth of *M. rosenbergii* PL. (**Figure-7 and Figure-8**).

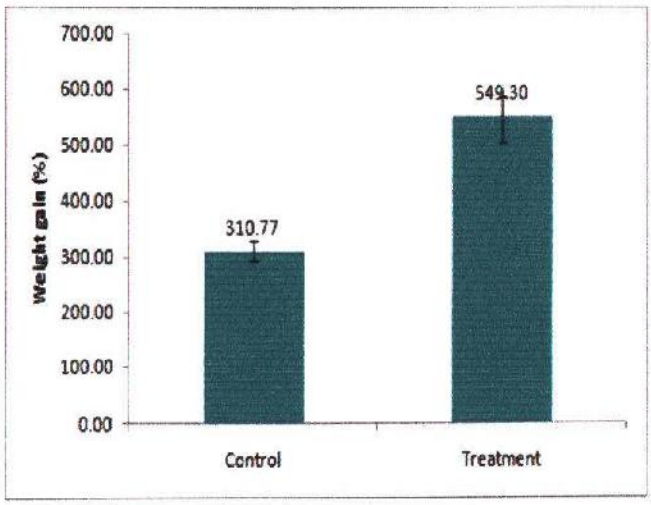


Figure-7: Comparison of percentage weight gain

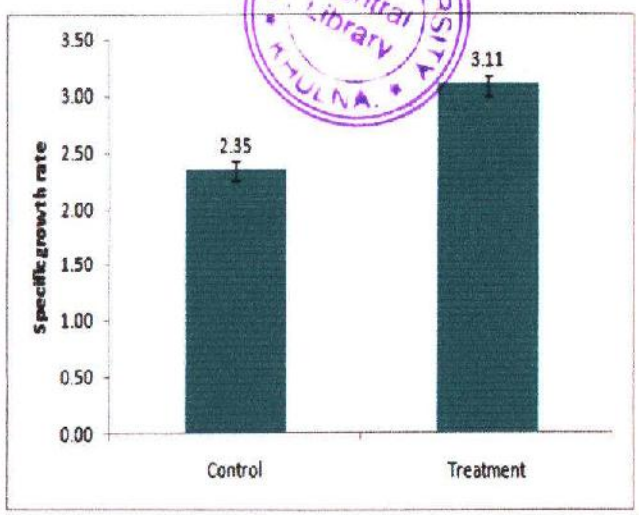


Figure-8: Comparison of specific growth rate

Independent Sample T test
N= 15
P= 0.000, <0.05
df=4
Sd
Control=13.12
Treatment=9.98

Independent Sample T test
N= 15
P= 0.002, <0.05
df=4
Sd
Control=0.07
Treatment=16

After 60 days, mean digestive enzyme activities of all probiotics treatment groups (trials 1–3) were significantly different (P<0.05) with that of the control (**Figure-9 and Figure- 10**). Amylase assays showed significant activity in treatment (P<0.05) as compared to the control. Amylase assays in treatment (1.23mg/ml) revealed significantly higher activity as compared to control (0.7 mg/ml). As for protease activity was remarkably higher (P<0.05) in treatment (2.74 mg/ml protein) compared with control (1.82 mg/ml protein). Clearly *Lactobacillus* spp. showed their ability to increase digestive enzyme activities (**Appendix A7 and Appendix A8**) and growth performance.

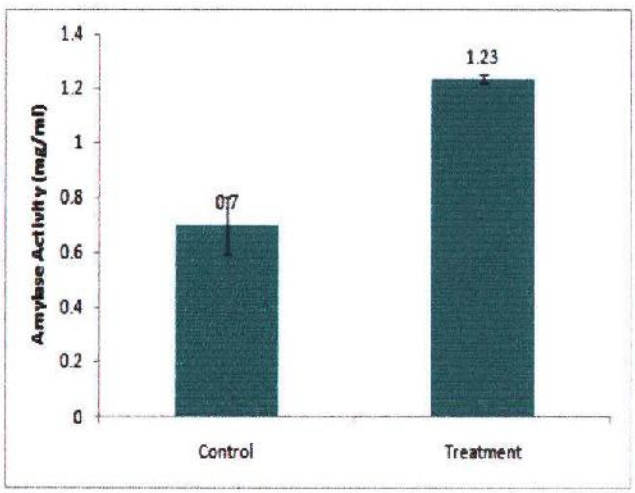


Figure-9: Amylase activity

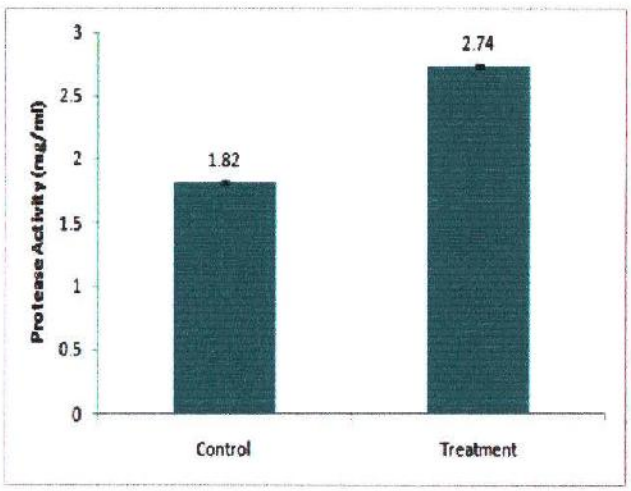


Figure-10: Protease activity

Independent sample T test
N= 3
P= 0.001, <0.05
df=4
Sd
Control=1.0
Treatment=0.02

Independent sample T test
N= 3
P= 0.000, <0.05
df=4
Sd
Control=0.02
Treatment=0.01

In this study, the heterogeneous prawn haemocyte population of the prawn haemolymph was presented mainly based on the presence of cytoplasmic granular deposit, size of granules and its resemblance with human leucocytes. The three distinct categories of haemocyte including non granular haemocyte (NGH), small granular haemocyte (SGH) and large granular haemocyte (LGH) were identified in the present study. The total haemocytes counts (THC) were found about $(5.2 \pm 0.73) \times 10^6$ in control group without any probiotic and $(5.4 \pm 0.53) \times 10^6$ in probiotic treated group with probiotic *Lactobacillus spp.* respectively (**Appendix A10**). In control group non granular haemocyte represented the highest percentage of haemocyte (75.33 ± 15.93%) followed by small granular haemocyte (22.24 ± 9.13%) and large granular haemocyte (2.43 ± 2.2%). In case of probiotic treated group about 76.27 ± 17.33% of haemocyte were NGH where as only about 21.36 ±10.32% SGH and 2.37 ± 1.9% LGH respectively (**Figure-11 and Appendix A10**).

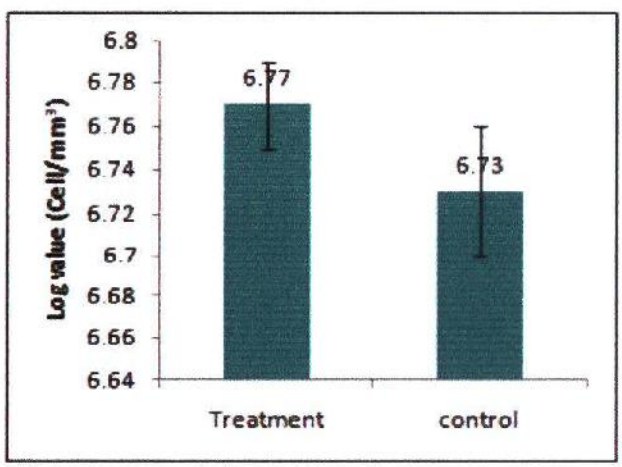


Figure-11: Total Haemocyte count

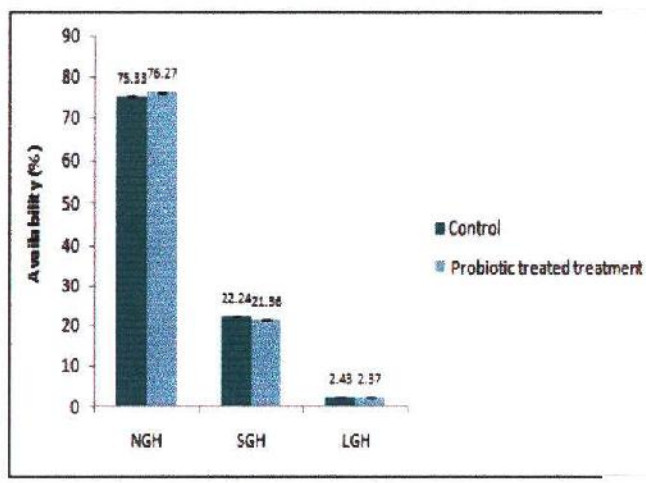


Figure-12: Differential Haemocyte count in prawn haemolymph

Independent sample test
N= 3
P= 0.163 > 0.05
df= 4
Sd
Control=0.03
Treatment=0.02

N= 3
Sd
Control=0.05
Treatment=0.09

Chapter 5

Discussions

5. Discussion

The purpose of this study was to diagnose indigenous probiotic bacteria especially *Lactobacillus* spp. and the pathogenic ones *Vibrio* spp. viz., *Vibrio harveyi* from the collected prawn by PCR test. For this purpose, *in-vitro* and *in-vivo* challenge test were done due to find out the antagonistic probiotics effects (*Lactobacillus* spp.) on *V. harveyi.* After that to investigate the optimization of probiotic, *Lactobacillus* spp. on growth, digestive enzyme activities, immunity and survival of the freshwater prawn *Macrobrachium rosenbergii* in grow-out condition in the farm.

The present study revealed that *Lactobacillus* spp. loads were higher than *Vibrio* spp. in intestine **(Table-5 and Table-6, Appendix 1)** *Lactobacillus* spp. is a beneficial bacteria and the presence of higher amount of *Lactobacillus* spp. in intestine is a good sign. Because this *Lactobacillus* spp., acts as natural probiotics in infected prawn in grow-out condition. It would be noted that due to the probiotic treated feed the greater load of *Lactobacillus* spp. was found.

These results also agreed with the findings of two other separate experiments Lee *et al.,* (2010) and Vine *et al.,* (2011) who stated that successful probiotics bacteria are usually able to colonize the intestine, at least temporarily, by adhering to the intestinal mucosa. The adhesive probiotics bacteria could prevent the attachment of pathogens, such as coliform bacteria and clostridia, and stimulate their removal from the infected intestinal tract. Both of these experiments revealed the temporary colonization and adherence of probiotic bacteria.

From the biochemical test it was found that most of the *Vibrio* spp. were *Vibrio harveyi* (**Table-7**) and also confirmed that the isolates were as *Vibrio harveyi* by PCR test (**Figure-6**). From another biochemical test it was found that 75% isolates were *Lactobacillus* spp. and the rest were Lactic acid bacteria (**Table-8**). Prawn gut seems to be a good source of LAB. These results contracted with Nair and Surendran, (2009) isolated lactic acid bacteria from various samples of fresh and frozen fish and prawn. They stated that the growth of gram-negative bacteria may have been inhibited by the bacteriocin produced by LAB. Our result also similar to Johnson *et al.,* (1998) reported the inhibition of a gram-negative bacteria *A. hydrophilla* by bacteriocin produced by LAB. Strom (2008) from his research work also expressed the same result that growth of fish pathogen *V. anguillarum* was strongly inhibited by both culture filtrates and viable bacteria of LAB isolated from salmon

intestines. According to Piard and Desmazeaud, (1992) though there are many reports about the antibacterial activity of LAB, most are against gram positive bacteria are similar to this study. Lewus *et al.*, (1999) investigation agrees with this result that the activity against *A. hydrophila* of 19 LAB strains including *Carnobacterium piscicola* and *L. plantarum.* These studies support the present study on antagonistic effects of lactic acid bacteria on *Vibrio* spp.

The effect of LAB in increasing disease resistance to *Vibrio* pathogens was tested experimentally in challenge studies by Griffith, (1995) who reported on the beneficial effects of nutritional probiotics in developing shrimp of high immunity. The studies of Garriques and Arevelo, (1995) also agree with these observations and recommend the use of probiotics in increasing the disease resistance of animals.

Although Ringo and Gatesoup, (1998) found that lactic acid bacteria are not dominant in the normal intestinal microbiota of fish, at variance with homeotherms, but some strains can colonize the gut. It is, however, possible to maintain artificially the lactic acid bacterial population at a high level by regular intake with food.

In *in-vitro* challenge test, the potential antagonistic effects of *Lactobacillus* spp. against *V. harveyi* was gradually obtained at 4^{th}, 8^{th} and 12^{th} hour of probiotics treatment. At 8^{th} and 12^{th} hour the load of *V. harveyi* was significantly reduced (**Table-9 and Appendix 2**).

From *in-vitro* test it was proved that *Lactobacillus* spp. have antibacterial effects on other bacteria. According to Balcazar, (2002) bacterial antagonism is a common phenomenon in nature. It is well known that the microbiota in the body of aquatic animals can be modified, for example by ingestion of other microorganism; therefore, microbial manipulation constitutes a viable tool to reduce or eliminate the incidence of opportunist pathogens.

Gatesoupe, (1999) also works on effect of probiotics on shrimp, their findings was also related with the findings of this study. They demonstrated that Lactic acid bacteria have been tested as probiotics in warm-blooded animals and attempts have been made to use lactic acid bacteria as antagonists (probiotics) of shrimp pathogens.

The result of this research work also similar to Austin and Brunt, (2008) also mentioned that the probiotics actively interfere with the colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients, oxygen and/or space, by alteration of microbial metabolism, and/or by the stimulation of the innate immune

response, including enhancement of phagocytic and respiratory burst activities and lysozyme reduction. The result of the present study matched with several findings of other works. Smith, (2004) reported that *Pseudomonas fluorescens* reduced diseases caused by *Aeromonas solmonicida* in fish. Rengipipat *et al.*, (1998a) got the same result that inoculation of *Bacillus* S11, a saprophytic strain, resulted in greater survival of the post-larval *P. monodon* that were challenged by pathogenic luminescent bacterial culture.

Balcazar, (2002) also works on effect of probiotics on shrimp, his findings also relate with the findings of the present study. He demonstrated that the administration of a mixture of bacterial strains (*Bacillus* spp. and *Vibrio* spp.) positively influenced the growth and survival of juveniles of white shrimp and presented a protective effect against the pathogens *Vibrio harveyi* and white spot syndrome virus. This protection was due to a stimulation of the immune system, by increasing phagocytosis and antibacterial activity.

In *in-vivo* challenge test of potential antagonistic effect of *Lactobacillus* spp. against *Vibrio harveyi* was gradually obtained after 10 to 60 days because of probiotic treated fed (**Table-10 and Appendix 3**). The present study showed the effective antagonistic activity in treatment compared with control. And significant antibacterial effect was found in 40 days to 60 days. This might cause of probiotic treated fed in treatment and probiotic bacteria are able to out-compete other bacteria for nutrients and space and can exclude other bacteria through the production of antibiotics.

This result also matched with Gomez-Gil, (2000) that the inhibition of bacterial proliferation by probiotic supplemented-diet is usually related to the production of inhibitory compounds or to exclusive competition. Jiravani *et al.*, (1997) reported the use of *Lactobacillus sp.* as the probiotic bacteria in the giant tiger shrimp (*P. monodon* Fabricius). They designed to investigate an effective treatment of *Lactobacillus sp.* against vibriosis and white spot diseases in *P. monodon*. They investigated the growth of some probiotic bacteria, and their survival in the 20 ppt sea water for at least 7 days. Inhibiting activity of two *Lactobacillus* sp. against *Vibrio sp.*, *E. coli*, *Staphylococcus sp.* and *Bacillus subtilis* was determined.

Lactobacillus spp. supplement diet appeared to provide beneficial effects on the growth of *M. rosenbergii* PL. The results indicated that the prawn which had access to diet containing *Lactobacillus* spp., exhibited greater growth than that of the control diet (**Figure-7 and**

Figure-8). There were significant differences in growth increment ($P < 0.05$) between experimental and control groups. Final body weight gain, specific growth rate and survival ratio in PL offered with *Lactobacillus* spp. incorporated diets were superior to those of experimental groups as well as control (**Appendix A4, A5, A6**). Kumer et al., (2013) conducted his study for three weeks in three circular tanks and got similar results on significant improvement in weight gain (ranged from 93 to 125%) and in specific growth rate (ranged from 1.11 to 1.25 %) by *Bacillus subtilis* and *Lactobacillus rhamnosus* incorporated probiotic diet.

Venkat *et al.,* (2004) conducted his study for 60 days in a circular cement tank and got similar results on significant improvement in weight gain (ranged from 99.48 to 132.52%) by bio-encapsulated of *L. acidophilus* and *L. sporogenes* supplemented diets fed *M. rosenbergii* PL.

The result of the study also matched with Lara-Flores *et al.,* (2003) reported that probioitics *S. faecium* and *L. acidophilus* (0.1%), and the yeast *S. cerevisiae* (0.1%) incorporated diets had improved the weight gain of Nile tilapia, *Oreochromis niloticus.*

From Venkat *et al.,* (2004) study a similar result on significant improvement in specific growth rate (1.14 to 1.41%) was recorded by *L. acidophillus* and *L. sporogenes* supplemented diets fed *M. rosenbergii* PL. Likewise, Hernandez *et al.* (2009) reported that *Poecilopsis gracilis* fed with bio-encapsulated *L. casei* (0.7x 108 cfu ml^{-1}) has significantly increased specific growth rate (2.57 and 2.64%).

The results of survival revealed that prawn in ponds treated with probiotics exhibited fairly higher survival than that of control pond. Similar results on significant improvement in growth, weight gain and SGR in *M. rosenbergii* fed with bio-encapsulated *L. sporogenes* were reported by Seenivasan *et al.*, (2012a). It has been reported that the growth performance, SGR and weight gain were higher in *L. sporogenes*, *B. subtilis* and *S. cerevisiae* supplemented diet fed *M. rosenbergii* PL (Seenivasan *et al.*, 2012c). It has been also reported from Ashim *et al.*, (2009) study that the growth, SGR and weight gain were increased in bio-encapsulated *Lactobacillus spp.* fed fish, *Carassius auratus* and *Xiphophorus helleri.*

According to Shinde *et al.*, (2008), different probiotics supplemented diets had improved growth rate, weight gain, FCR and FER of *M. rosenbergii* PL. Therefore, in the present study, improvement of growth and food conversion may be due to *L. sporogenes.*

From the experiment of Jafarian *et al.*, (2011) reported a significant improvement in growth factor by mixing experimental diets with three mixture of bacterial probiotic including *Lactobacillus*, commercial *bacillus* and isolated *bacillus* from Iranian sturgeon (*Acipencer perscius*) intestine and *Huso Huso* in three levels to trout larval food for 32 days.

Probiotics are micro-organisms or their products that ensures health benefit to the host which are used in aquaculture as a means of diseases control, improve immunity of cultured animals to pathogenic microorganisms and positively affects the growth.

Similar results were obtained by Ziaei-Nejad *et al.*, (2006) in which the *Bacillus* probionts showed positive effect on digestive enzyme activity, survival and growth in the shrimp *Fenneropenaeus indicus.*

In the present study, all probiotic- supplemented diets resulted in higher growth in prawn than the control diet, suggesting that the addition of probiotics enhance the growth performance and feed utilization.

Similar results were obtained by Gullian, *et al.*, (2004) demonstrated a significant growth increase in shrimp inoculated with *Bacillus* sp. The observed increase in specific activities of the digestive enzymes in probiotic treatment may have led to enhanced digestion and increased absorption of food, which in turn contributed to the improved survival and growth in *Fennerpenaeus indicus,* including improved feed conversion ratio (FCR) and specific growth rate (SGR).

And also, several researchers are reported by Parthasarathy and Ravi, (2011) that probiotics *L. plantarum* and *B. megaterium* supplemented diets had improved the growth performance in *Catla catla* and by Dhanaraj *et al.*, (2010) in Koi Carp fed with *L. acidophilus* and yeast *S. cervisiae.*

In this study, the higher level of total digestive enzyme activity was recorded in prawn fed *Lactobacillus* spp. supplemented diets where the better growth performances were observed compared to control (**Figure-9 and Figure-10**). Similar results have been reported by Ziaei-Nejad *et al.*, (2006) who observed a higher digestive enzyme activity in shrimp (*Fenneropenaeus indicus*) treated with Bacillus spp. than the controls. In addition, it is important to mention that a better appetite was observed in shrimp fed diets containing *Lactobacillus* spp. than the control group during the feeding period. A better feed digestion may be related to an increase of the digestive enzyme activity and subsequently increased

the appetite in treated shrimp. Therefore by taking into account of the enzyme activity and appetite stimulation, together with the colonization of *Lactobacillus* spp. of prawn fed diets, healthier prawn and higher survival rate of 89% were resulted, respectively, compared to control with 74% (**Appendix-A7**).

Sixty days (60) after the start of the feeding period, shrimp were challenged with pathogenic bacterium *V. harveyi*. As (**Figure-9 and Figure-10**) show, a higher digestive enzyme activity was observed in prawn fed diets containing *Lactobacillus* spp. A significantly lower mortality was recorded in prawn fed *Lactobacillus* spp. incorporated diets compared to the control group.

The result of the present study matched with Ueberschar, (1993) that digestive enzyme activity can be used as an indicator of larval food acceptance and to some extent serve as an indicator for the digestive capacity in relation to the type of feed offered. Moreover, the assessment of the presence and level of activity of digestive enzymes may be used as a comparative indicator of the rate of development of the fish larvae, food acceptance, digestive capacity, as well as of their further survival rate. It was also similar to Moriarty, (1998) study that treatment of the Lactobacillus bacteria as probiotics to larvae resulted in an increase in the specific activity of all measured digestive enzymes in the digestive tract of larvae. The digestive system of Sparids is activated particularly in the early stages of larval development where the probiotics would have the greatest effect and because gram-positive bacteria, particularly members of the genus Lactobacillus, do secrete a wide range of exoenzymes.

The results of the immunity study suggest that hemocytes in *M. rosenbergii* comprise three major groups, small granule cells (SGC), large granule cells (LGC) and non granular haemocyte (NGH) depend on cell and granules size. These categories were looked respectively as like as of lymphocytes, neutrophil and eosinophil shaped. The heterogeneous prawn haemocyte population of the prawn haemolymph was presented mainly based on the presence of cytoplasmic granular deposit, size granules and its resemblance with human leucocytes. Similar output got from Kondo *et al.*, (1998), the hemocytes of the shrimp (Penaeidae) are classified into three types, and hyaline cells (HC or NGH), small granular cells (SGC) and large granular cells (LGC) associated with the presence of cytoplasmic deposit and size of granules.

The nongranular haemocyte (NGH), small granular haemocyte (SGH), large granular haemocyte (LGH) and total haemocytes counts (THC) were little bit higher in probiotic treated treatment than control (**Figure-11 and Appendix 10**). Thus both in the control and treatment most of the haernocytes were belong to non granular haemocytes where as a very few were belonged to large granular haemocyte.

This output was similar to Vazquez *et al.*, (1997) that morphologically three types of haemocytes were observed in the freshwater prawn of which hyaline haemocyte, granular haemocyte, undifferentiated haemocytes which were 70%, 20% and 10%. Also similar to Hose *et al.*, (1992) recorded that the HC comprises 50–60% of the circulating hemocytes, whereas the SGH and LGH represented 30% and 10%, respectively.

So to say, the ponds with probiotic revealed higher THC than the ponds without probiotic. It clarifies the fact that probiotic are capable to increase THC in the prawn haemolymph which enhances its immunity. 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 prawn guts. This suggests that probiotic treatment is an effective alternative for enhancing prawn health because probiotic acts as immunostimulant.

It was well-known from Smith and Chisholm, (1992) characterized invertebrate immune systems as belonging to two broad categories, including: 1. humoral defenses such as antibacterial activity, agglutinins, cytokine-like factors, modulators, and clotting factors; and 2. cellular defenses such as hemolymph clotting, phagocytosis, nodule formation, encapsulation, and prophenoloxidase system.

It also matched with Gargioni and Barracco, (1998) stated that the SGH and LGH were actively phagocytic when examined on blood cell monolayers incubated with the yeast *Saccharomyces cerevisiae.*

Similarly Kondo, (2003) believed that these cells are similar to SGC but has large granules and PPO (prophenoloxidase). Therefore, he believed that in *Penaeus japonicus*, semigranulocytes are the immature LGC. Granulocytes involved the immune system as well as hyaline cells. However it seems hyaline cells or nongranular haemocyte (NGH) start some reaction such as clot formation.

In Crustacean haemocytes play important roles in their immune response including recognition, phagocytosis, melanization, cytotoxicity and cell-cell communication.

Similarly Marteau *et al.*, (2002) reported that lactic acid bacteria are known to produce extracellular compounds that can stimulate the non-specific immune response in vertebrates. From Jiravani *et al.*, (2007) it is well known that hemocytes play important roles in the host immune responses. They are responsible for cellular defense mechanisms and also releasing humoral defense molecules in order to protect the body toward microbial intruders.

From the overall results of the present study indicated that probiotics treatment offers a promising alternative to the use of antibiotics in prawn aquaculture. These works strongly suggest the effective control of microflora in fish and shellfish in culture environments by antibiotic-producing bacteria. The relative importance of these defenses is still unknown, but they collectively conferred bacterial disease protection. It also appears that the use of this probiont was most effective when it was provided at an early age and continued during culture.

We found that supplementation of probiotic to live food (rotifer and Artemia), followed by feeding was an effective means by which to deliver the probiotic to the S. aurata larvae. Similarly, as a result of Ziaei-Nejad *et al.*, (2006) and Wang, (2007) administration of probiotic to directly tank water could not significantly enhance growth parameters and digestive enzyme activities and therefore, labor and expense involved in this manner of administration by this method would not be cost effective as indicated by some studies.

The administration of *Lactobacillus* spp. improved the growth performances, digestive enzyme activity and immune response against the pathogenic bacterium, *V. harveyi*. In addition, a better growth and survival rate was obtained in shrimp fed probiotic diets after challenge with *V. harveyi*.

Therefore, *Lactobacillus* spp. can be supplemented in formulated diets for the maintenance of healthy *M. rosenbergii* at nursery and grows out in ponds. Also, *M. rosenbergii* can be used in farm management at a small scale level, and consequently, inland aquaculture of *M. rosenbergii* may be promoted in a sustainable manner.

Chapter 6

Conclusion

6. Conclusion

The probiotic bacteria produce inhibitory substances that inhibit the bacterial pathogens in aquaculture systems. The use of such bacteria to inhibit pathogens by release of antimicrobial substances in now gaining importance in fish and prawn farming as a better and more effective alternative than administering antibiotics to manage the health of fish and prawn. The use of beneficial bacteria (probiotics) to displace pathogens by competitive processes is being used in the aquaculture as a better remedy than administering antibiotics and is now gaining acceptance for the control of pathogens. The role of beneficial bacteria to limit and to control environmental pathogens will become particularly important in future aquaculture, especially with regard to the emergence increasing number of antibiotic resistant strains of bacteria, the convulsion of governmental and environmental regulations of treatments, and the cost-effectiveness of the process.

It could be concluded that the addition of probiotics (*Lactobacillus* spp.) in prawn diets improved growth performances, feed utilization and digestive enzyme activities and immune response against the pathogenic bacterium, *V. harveyi*. In addition, a better survival rate was obtained in shrimp fed probiotic diets after challenge with *V. harveyi*.

Therefore, *Lactobacillus* spp. can be supplemented in formulated diets for the maintenance of healthy *M. rosenbergii* at nursery and grows out in ponds. Also, *M. rosenbergii* can be used in farm management at a small scale level, and consequently, inland aquaculture of *Macrobrachium* may be promoted in a sustainable manner.

On the basis of the results obtained from *in-vitro* and *in-vivo* test, it could be concluded that probiotics, as 'bio-friendly agents' such as *Lactobacillus* spp., could be introduced into the culture environment to control and compete with pathogenic bacteria as well as to promote the growth of the cultured organisms.

Chapter 7

References

7. References

Abdel-Tawwab, M.; Abdel-Rahman, A.M. and Ismael, N.E.M. 2008. Evaluation of commercial live baker's yeast, *Saccharomyces cerevisiae* as a growth and immunity promoter for fry Nile tilapia, *Oreochromis niloticus* challenged in situ with *Aeromonas hydrophila*. *Journal of Aquaculture,* 280: pp. 185–189.

Abraham, T.J.; Manley, R.; Palaniappan, R. and Dhevendaran, K. 1997. Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. *Journal of Aquaculture in the Tropics,* 12: pp. 1-8.

Ahmed, N. 2001. Socio-economic aspects of freshwater prawn culture development in Bangladesh. PhD Thesis, Institute of Aquaculture, University of stirling, Scotland, UK.

Akoki, T. 1975. Effects of chemotherapeutics on bacterial ecology in the ponds and the intestinal tracts of cultured fish. *Journal of Microbilogy*, 19: pp. 7-12

Akoki, T.; Takami, K. and Kotao, T. 1990. Drug resistance in a non-haemolytic *Streptococcus* sp. isolated from cultured yellow tail *Seriolaquinque radiata. Journal of Disease of Aquatic Organisms,* 8: pp. 171-177.

Alapide-Tendencia, E.V. and Dureza, L.A. 1997. Isolation *Vibrio* spp. from *Penaeus monodon* (Fabricius) with red leg disease syndrome. *Journal of Aquaculture research*, 154: pp. 107-114.

Al-Dohail, A.M.; Hashim, R. and Aliyu-Paiko, M. 2009. Effects of the probiotic, *Lactobacillus acidophilus*, on the growth performance, haematology parameters and immunoglobulin concentration in African Catfish (*Clarias gariepinus*, Burchell 1822) fingerling. *Journal of Aquaculture research*, 40: pp. 1642-1652.

Amal S.; Saad, I. and Madlen, M. 2009. Growth Response of the Freshwater Prawn, *Macrobrachium rosenbergii* (De Man), to diets having different levels of biogen. *Journal of World Applied Sciences,* 6 (4): pp. 550-556.

Anderson, D.P. 1992. Immunostimulants, adjuvants and vaccine carriers in fish: application to aquaculture. Annual Review of Fish Diseases, 2: pp. 281–307.

Anson, M. L. 1938. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *Journal of Genetics and Physiology*, 22: pp. 79–89.

Ashim, N.S.; Rout, S.K.; Dasgupta, A. and Abraham, J. 2009. Water quality characteristics in controlled production of ornamental fishes as influenced by feeding a probiotic bacterium, *Lactobacillus* spp. Bio-encapsulated in *Artemia* sp. *Indian Journal of Fisheries* 56: pp. 283–286.

Austin, B. and Brunt, J. 2008. Probiotics and disease control in aquaculture. *Journal of the World Aquaculture Society*, 6: pp.45.

Ausubel, F. M.; Brent, R.; Kingston, R. E.; Scidrnarn, J. C. and Struhl, K. 1990. Preparation of genomic DNA from bacteria: bacterial genomic DNA. John Wiley and Sons, lnc. New York, *In Current Protocols in Molecular Biology*, 2: pp. 1-5.

Balcazar, J.L. 2002. Use of probiotics in aquaculture: general aspect. Zaragoza, Spain, pp. 877-881.

Balcazat, J.L.; Blas, I.D.; Ruiz-Zaruela, I.; and Muzquiz, T.L. 2006. The role of prcbiotics m aquaculture. *Journal of Veterinary Microbiology*, 114: pp. 173-186.

Bangladesh economic review, 2015.

Barbosa T.M.; Serra C.R.; La Ragione R.M. and Henriques, A.O. 2005. Screening for Bacillus isolatres in the broiler gastrointestinal tract. *Journal of Applied Environmental Microbiology*, 71: pp. 968–978.

Baticados, M.C.L.; Lavilla-Pitogo, C.R.; Cruz-Lacierda, E.R.L.D.; Pena, D. L. and Sunaz, M.A. 1990. Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. *Journal of Disease of Aquatic Organisms*, 9: pp. 133-139.

Bell, T.A. and Lightner, D.V. 1988. A Handbook of Normal Penaeid Shrimp Histology.1st Edn, Word Aquaculture Society, Baton Rouge, Louisiana, ISBN 0-9358118-37-2.

BFFEA, 2008. Shrimp and Fish News : News Letter of Bangladesh Frozen Foods Exporters Association (BFFEA), April-June, 2008, BFFEA, Dhaka, Bangladesh. pp. 2-41.

Bogut, K.; Mitul, S. and Amol, G. 1998. Effect of commercial probiotics in aquaculture. *Journal of Applied Bacteriology* 65: pp. 261-268.

Bomba, A.; Nemcoal, R.; Guba, P. and Mudron, D. 2002. Improvement of the probiotic effect of microorganisms by their combination with maltodextrins, fructo-oligo - saccharides and polyunsaturated fatty acids. *British Journal of Nutrition,* 88: pp. 95- 99.

Boonthai, T.; Vuthhiphandchai, V. and Nimrat, S. 2011. Probiotic bacteria effects on growth and bacterial composition of black tiger shrimp (*Penaus monodon*). *Journal of Aquaculture Nutrition*, 17: pp. 634-644.

Brock, J.A. and Lightner, D.V. 1990. Chapter 3: *Diseases of Crustacea. Journal of Diseases of Marine Animals,* 3: pp. 245-424.

Capkin, E. and Altinok, I. 2009. Effects of dietary probiotic supplementations on prevention/treatment of yersiniosis disease.*Journal of Applied Microbiology*, 106: pp. 1147–1153.

Chaithanya R., Nayak D.K. and Venugopal, M.N. 1999. Antibacterial resistance in aquaculture. *Journal of Fish Biology*, 6: pp. 30-32.

Chamatchakool P, M.; Pearson, C. and Roberts, R. J. 1995.Oxytetracyclinesensivity of *vibrio* species isolated ftom diseased black tiger shrimp *Penaeus monodon* Fabricius. *Journal of Fish Diseases*, 18: pp. 79-82.

Cheesbrough M. 1985. Medical laboratory manual for tropical countries. Vol. II. Microbiology. 400-480.

Cheng, W. and Chen, J.C. 1998. Isolation and characterization of *Enterococcus* like bacterium causing muscle necrosis and mortality with *Macrobrachium rosenbergii* in Taiwan. *Journal of Diseases of Aquatic Organisms*, 34: pp. 93-101.

Cole, C.B. and Fuller, R.; 1984. A note on the effect of host specific fermented milk on the coliform population of the neonatal rat gut. *Journal of Appllied Bacteriology,* 56: pp. 495–498.

Cowan and Steel's, 1993. Manual for the Identification of Medical Bacteria.pp. 201-233.

Delcenserie, V.; Martel, D.; Boutin, Y. and Roy, D. 2008. Immunomodulatory effects of probiotics in the intestinal tract. *Journal of Molicular Biology*, 10: pp. 37–54.

Department of Fisheries, 2007a.

Department of Fisheries, 2012; Ministry of Fisheries and Livestock; Government of Peoples Republic of Bangladesh, 2013.

Dhanaraja, M.; Haniffaa, M.A.; Singha, S.V., Seetharamana, S. and Arthimanjua, R. 2010. Effect of probiotics on growth performance of koi carp (*Cyprinus carpio*). *Journal of Applied Aquaculture*, 22: pp. 202–209.

Dimitroglou, A.; Merrifield, D.L. and Carnevali, O. 2011. Microbial manipulations to improve fish health and production- a Mediterranean perspective. *Journal of Fish and Shellfish Immunity*, 30: pp. 1–16.

Douillet, P.A. and Langdon, C. J. 1998. Use of a probiotic for the culture of larvae of the pacific oyster (*Crassostreagigas*). *Journal of Aquaculture,* 119: pp. 25-40.

El-Haroun, E.R.; Goda, A. and Chowdury, M.A.K. 2006.Effect of dietary probiotic Biogen supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *Oreochromis niloticus* (L.). *Journal of Aquaculture Research*, 37: pp. 1473–1480.

FAO, 2002. *The state of world fisheries and aquaculture*, Rome: SOFIA, pp. 150.

FAO, 2011. Demand and supply of feed ingredients for farmed fish and crustaceans: Trends and prospects. In: Fisheries and Aquaculture Technical Paper – 564. Ed. Tacon, A.G.J.; Hasan, M.R.; Metian, M.; Food and Agriculture Organization of the United Nations, Rome, pp. 88.

Fuller, R. 1989. Probiotic in man and animals. *Journal of Appllied Bacteriology*, 66: pp. 365–378.

Fuller, R. 1992. History and development of probiotics. *Journal of Aquaculture*, pp. 1-18.

Garcia, D.L.B.; Amol, K. and Xu, M. 1992. Effects of LAB in aquaculture. *Journal of Appllied Bacteriology*, 6: pp. 36–48.

Gargioni, R. and Barracco, M. A. 1998. Hemocytes of the palaemonids *Macrobrachium rosenbergii* and *M. acanthurus* and of the penaeid *Penaeus paulensis*. *Journal of Morphology*, 236: pp. 209-221.

Garriques, D. and Arevalo, G. 1995. An evaluation of the production and use off five bacterial isolates to manipulate production of *P. vannamei* post larvae in Eucador. *World Aquaculture meeting*, pp. 1-14.

Gatesoupe, F.J. 1991. The effect of three strains of lactic bacteria on the production rate of rotifers, *Brachionus plicatilis*, and their dietary value for larval turbot, *Scophthalmus maximus*. *Journal of Aquaculture*, 96: pp. 335–342.

Gatesoupe, F.J. 2008. Updating the importance of lactic acid bacteria in fish farming: natural occurrence and probiotic treatments. *Journal of Molecular Biology and Biotechnology*, 14: pp.107-114.

Gatesoupe, F.J.1999. The use of probiotics in aquaculture. *Journal of Aquaculture*, 180: pp.147-165.

Ghosh, S.; Sinha, A. and Sahu, C. 2007. Select of probiotic on reproductive performance in female live bearing ornamental fish. *Journal of Aquaculture Research*, 38: pp. 518-526.

Gildberg, A. and Mikkelsen, H. 1998. Effect of supplementing the feed of Atlantic cod (Gadus morhua) fry with lactic acid bacteria and immune stimulating peptides during a challenge trial with *Vibrio anguillarum*. *Journal of Aquaculture*, 167: pp. 103–113.

Gildberg, A.; Mikkelsen, H.; Sandaker, E. and Ringo, E.1997. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod, *Gadus morhua* Hydrobiologia, 352: pp. 279–285.

Giri, S.S.; Sukumaran, V. and Oviya, M. 2013. Potential probiotic *Lactobacillus plantarum* VSG3 improves the growth, immunity, and disease resistance of tropical freshwater fish, *Labeorohita*. *Journal of Fish and Shellfish Immunology*, 34(2): pp. 660-666.

Gomez-Gil, B. 2000. The use of bacteria as probiotics in shrimp larviculture. Larvi 95, fish and shellfish larva culture symposium, Belgium. pp. 479-480.

Griffith, D.R.W. 1995. Microbiology and role of the probiotics in Eucadorian shrimp hatcheries. *Journal of Fish and shellfish larva culture Symposium*, pp. 478-481.

Gullian, M.; Thomposon, F. and Rodriguez, J. 2004. Selection of probiotic bacteria and study of their immune stimulatory effect in *Penaeus vannamei*. *Journal of Aquaculture*, 233: pp. 1-14.

Gursoy, O. and Kinik, O. 2006. Probiotics: A new popular for option for cancer inhibition. *International Journal of Dairy Sci*ence, 1: pp. 100-103.

Hamilton-Miller, J.M. 2003. The role of probiotics in the treatment and prevention of Helicobacter pylori infection. *International Journal of Antimicrobial Agents,* 4: *pp.* 360–366.

Hernandez, H.H.L.; Barrera M.J.C. and Dosta, M. 2009. Effects of the commercial probiotic *Lactobacillus casei* on the growth, protein content of skin mucus and strees resistance of juveniles of the Porthole live bearer *Poecilopsis gracilis*. *Journal of Aquaculture Nutrition*, 3: pp. 45-67.

Holmstrom, K.; Gräslund, S. and Wahström, A. 2003. Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International Journal of Food Science Technology*, 38: pp. 255-266.

Hose, J. E.; Martin, G. G.; Tiu, S. and Mckrell, N., 1992. Patterns of hemocyte production and release throughout the molt cycle in the penaeid shrimp *Sicyonia ingentis*. *Journal of Biological Bulletin*, 183: pp. 185–199.

Hsieh, S. L.; Ruan, Y. H.; Hu, C. H. and KUO, C. M. 2008. Immune and physiologicalresponses in Pacific white shrimp (*Penaeusvannamei*) to *Vibrio alginolyticus*. *Journal of Aquaculture*, 275: pp. 335-341.

Hsu, H. W.; Vavak, D.L. Satterlee, L.D. and Miller, G.A. 1 977. A multi enzyme technique for estimating protein digestibility. *Journal of Food Science*, 42: pp. 1269-1273.

Huys, M.; Mikun, O. and Alimon, A.R. 2001. Diseases of cultured Penaeid shrimps. *Journal of Fish Diseases*, 7: pp. 293–320.

Immanuel, G.; Beena, S. and Palavesam, A. 2003.Effect of probionts on the growth, food utilization and biochemical changesin pearl spot Etroplussuratensis (Bloch). *Indian Journal of Fish*, 50: pp. 273–278.

Irianto, A. and Austin, B. 2002. Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases*, 25: pp. 333–342.

ISO/TS 21872-1, 2007. Microbiology of food and animal feeding stuffs — Horizontal method for the detection of potentially entero-pathogenic *Vibrio* spp. — Part 1: Detection of *Vibrio parahaemolyticus* and *Vibrio cholerae*.

Itami, T.; Asano, M.; Tokushige, K.; Kubono, K.; Maeda, M. and Kondo, M. 1998. Enhancement of disease resistance of Kuruma shrimp, *Penaeus japonicus*, after oral administration of peptide glycan derived from *Bifidobacterium thermophilum*. *Journal of Aquaculture*, 164: pp. 277– 288.

Jafarian, M.; Shahab, Z.S.H. and Fadaee, F.F. 2011. Studying the effect of different amounts of Protoxin probiotic in weight growth, survival ratio and food conversion ratio of rainbow trout. Journal of Aquatic Research, 3: pp. 33-40.

James and Hirsch, 1960. Comparative bacterial activity of blood serum and plasma serum. *Journal of Biology*, 66: pp. 391-398.

Jiravani C.; Puanglarp, P.; Petkon, N. and Donnuea, S. 2007. Expression of immune-related genes in larval stages of the giant tiger shrimp, *Penaeus monodon*. Fish Shellfish and Immunology, 23: pp. 15-24.

Jiravani, C.P.; Chuaychuwong, P. and Menasveta, P. 1997. The use of *Lactobacillus* sp. as the probiotic bacteria in the giant tiger shrimp, *Penaeusmonodon*. Proceedings of the poster session of the 2nd Asia-Pacific marine biotechnology conference and 3rd Asia-pacific conference on algal biotechnology, 2: pp. 16.

Johansson, M. W.; Keyser, P.; Sritunyalucksana, K. and Soderhall, K. 2000. Crustacean hemocytes and hematopoiesis. *Journal of Aquaculture*, 191: pp.45-52.

Johnson, M.C.; Bhunia, A.K. and Ray, B. 1998. Characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactili*. *Journal of Applied Bacteriology* 65: pp. 261-268.

Karim, M. 1978. Status and potential of Bangladesh fisheries.*Ministry of Fisheries and Livestock.Govt. of Bangladesh, Dhaka*, 125.

Karunasagar, L. P.; Pai, G.R.; Malathi and Karunasagar, L. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. *Journal of Aquaculture*, 128: pp. 203-209.

Keysami, M.A.; Saad, C.R.; Sijam, K.; Daud, H.M. and Alimon, A.R. 2007. Effect of *Bacillus subtilis*on growth development and survival of postlarvae *Macrobrachium rosenbergii* (de Man). *Journal of Aquaculture Nutrition*, 13: pp.131-136.

Klewicki, R. and Klewicka, E. 2004. Antagonistic activity of lactic acid bacteria as probiotics against selected bacteria of the *Enterobaceriacae* family in the presence of polyols and their galactosyl derivatives. *Journal of Biotechnology,* 26: pp. 317–320.

Kondo, M. 2003. Experiments of body defense mechanisms in Crustacean. *Institute of Applied Aquabiology*, National Fisheries University, pp.1-13.

Kondo, M.; Itami, T.; Fujii, R. and Tomonaga, S. 1998. Ultrastructural and cytochemical characteristics of phagocytes in Kuruma prawn. *Journal of Fish Pathology*, 33: pp. 421-427.

Kumar R.; Mukherjee, S.C.; Prasad, K.P. and Pal, A.K. 2006. Evaluation of Bacillus subtilis as a probiotic to Indian major carp Labeorohita (Ham.). *Journal of Aquaculture Research,* 37: pp. 1215-1221.

Kumar, P.J.; Jyothsna, S. and Sreevani, S. 2013. Effect of *Bacillus subtilis* and *Lactobacillus rhamnosus* incorporated probiotic diet on growth pattern and enzymes in *penaeus vannamei. International journal of Life Science and Pharma Research*, 3: pp. 125-130.

Lara-Flores, M.; Olvera-Novoa, M.A. and Guzman-Mendez, B.E. 2003. Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae*as growth promoters in Nile tilapia (*Oreochromis niloticus*). *Journal of Aquaculture* 216, pp. 193–201.

Lavilla-Pitogo, C.R.; Baticados, M.C.L.; Cruz-Lacierda, E.R. and Pena, L.D. 1990. Occurence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. *Journal of Aquaculture,* 91: pp. 1-13.

Lee, K.K.; Chen, Y.L. and Liu, P.C. 1999. Hemostasis of tiger prawn *Penaeus monodon* affected by *Vibrio harveyi*, extracellular product and a toxic cysteine protease. Blood Cells, Molecules and Diseases, 25: pp. 180-192.

Lee, S.Y. and Lee, B.H. 1990. Esterlytic and lipolytic activities of *Lactobacillus* spp. *Journal of Food Science*, 55: pp. 119.

Lee, Y.K; Lim, W.L; Tuomola, E.M. and Salminen, S. 2010. Quantitative approach in the study of adhesion of lactic acid bacteria to intestinal cells and their competition with enterobacteria. *Journal of Applied Environmental Microbiology,* 66:pp. 3692–3697.

Lewus, C.B.; Kaiser, A. and Montville, T.J. 1999. Inhibition of food –borne bacterial pathogens by bacterial pathogens by bacteriocincins from lactic acid bacteria isolated from meat. *Journal of Applied Environmental Microbiology* 57: pp. 1683-1688.

Li, P. and Gatlin, D. M. 2004. Dietary brewer's yeast and the probiotic Grobiotic TM AE influence growth performance, immune responses and resistance of hybrid striped bass (*MonronechrypsopsX M. saxatilis*) to *Streptococcus inane* infection. *Journal of Aquaculture*, 231: pp. 445-456.

Lightner, D. V. 1992. Shrimp virus diseases: Diagnosis, distribution and management. Proceedings of the Special Session on Shrimp Farming, Baton Rouge: Orlando: *Journal of World Aquaculture Society*, pp. 238–253.

Lightner, D.V. 1983. Diseases of cultured Penaeid shrimps. *Journal of Fish Diseases*, 7: pp. 289–320.

Lightner, D.V. 1996. Handbook of shrimp pathology and diseases of cultured penaeid shrimp. *Journal of World Aquaculture Society*, 3: pp.78-85.

Liu, K.X.; Zhu, Y.G.; Wang, X.D. and Qu, J.M. 2012. Probiotics' effects on the incidence of nosocomial pneumonia in critically ill patients: a systematic review and meta-analysis. *Critical Care*, 16: pp. 109.

Lovett, D. L. and Felder, D. L. 1990. Ontogeny change in digestive enzyme activity of larval and post larval white shrimp *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). *Journal of Biological Bulletin,* 178: pp. 144– 159.

Marteau, P.; Seksik, P. and Jian, R. 2002. Probiotics and intestinal health effects: a clinical perspective, *British Journal of Nutrition,* 88: pp. 51-57.

Martin G. G. and Graves, L. 2005. Fine structure and classification of shrimp hemocytes. *Journal of Morphology*.185:339-348.

Mermoud, I.; Ferre, R.; Goarant, C. and Haffner, P. 1998. Svndrome 93 in new Caledonian outdoor rearing ponds of *Penaeus stylirostris*: Historv and description of the three major outbreaks Aquaculture, 161: pp. 323-335.

Meunpol, O.; Loponyosiri, K. and Menasveta, P. 2003. The effects of ozone and probiotics on the survival of black tiger shrimp, *Penaeus monodon. Journal of Aquaculture,* 220: pp. 437–448.

Mohanty, S.N.; Swain, S.K. and Tripathi, S.D. 1996. Rearing of catla (*Catlacatla*Ham) spawn on formulated diets. *Journal of Aquaculture in Tropical Region,* 11: pp. 253–258.

Mohanty, S.N.; Swain, S.K.; and Tripathi, S.D. 1993. Growth and survival of rohu spawn fed on a liver based diet. I*nternational Journal of Inland Fisheries*, 25: (2) pp. 41–45.

Moriarty, D.J.W. 1997. The role of microorganisms in aquaculture ponds. *Journal of Aquaculture*, 151: pp. 333-349.

Moriarty, D.J.W. 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Journal of Aquaculture* 164: pp. 351–358.

Moriarty, D.J.W. 1999. Disease control in shrimp aquaculture with probiotic bacteria. Proceedings of the 8th International Symposium on Microbial Ecology, Atlantic Canada Society for Microbial Ecology, Halifax, Canada.pp. 237–243.

Muir, J.F. 2003. The future for fisheries: economic performance. Fisheries sector Review and Future Development Study. *Commissioned with the association of the* World Bank, DANIDA, USAID, FAO, DFID with the cooperation *of the Bangladesh Ministry of Fisheries and Livestock and the Department of Fisheries, Dhaka*, 172.

Nair, P.S. and Surendran, P.K. 2009. Biochemical characterization of lactic acid bacteria isolated from fish and prawn. *Journal of Culture Collection*, pp. 448-452.

Nikoskelainen, S.; Ouwehand, A.C.; Bylund, G.; Salminen, S. and Lilius, E. 2003. Immune enhancement in rainbow trout (Oncorhynchus mykiss) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Journal of Fish and Fish Immun*ology, 15: pp. 443-452.

Noh, G.; Xu, K. and Moor, N. 1994. Uses of commercial probiotics. *Journal of Aquaculture*, 11: pp.1–12.

Olafsen, J. A. 1998. Interactions between hosts and bacteria aquaculture. In: US-EC Workshop on Marine Microorganisms: Research Issues For Biotechnology, Brussels. Proceedings... Brussels: European Commission, pp. 127-145.

Olsson, J.C.; Joborn, A.; Kjelleberg, S. and Conway, P. L. 1998. Survival, persistence and proliferation of *Vibrio anguillarum* in juvenile turbot, *Scophthalmus maximus*, in intestine and faeces. *Journal of Fish Disease,* 21: pp. 1–9.

Owens, L. and O'Neill, A. 1997. Use of clinical cell flow cytometer for differential counts of prawn *Penaeus monodon* haemocytes. *Journal of Disease of aquatic organisms*, 31: pp. 147-153.

Owens, L.; Muir, P.; Sutton, D. and Wingfield, M. 1992. Diseases in Asian Aquaculture 1.Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 165-172.

Parker, R.B. 1974. Probiotics, the other half of the antibiotics story. *Journal of Animal Nutrition and Health,* 29: pp. 4–8.

Parthasarathy, R. and Ravi, D. 2011. Probiotic bacteria as growth promoter and biocontrol agent against *Aeromonas hydrophila* in *Catla catla* (Hamilton, 1822). *Indian Journal of Fisheries*, 58: pp. 87-93.

Pena, L.D.; Tamaki, T.; Momoyama, K.; Nakai.and Muroga, K. 1995. Characteristics of the causative bacterium for vibriosis in the kuruma prawn *(Penaeus japonocus). Journal of Aquaculture*, 115: pp.1–12.

Perazzolo, L.M.; Gargioni, R.;Ogliari, P. and Barracco, M.A.A. 2002. Evaluation of some hemato immunological parameters in the shrimp Farfante *penaeus paulensis* submitted to environmental and physiological stress. *Journal of Aquaculture*, 214:19–33.

Petlu, M.; Jeevan, K.; Sangeetha, M. J. and Sreemanthula S. 2013. Effect of *Bacillus Subtilis* and *Lactobacillus Rhamnosus* incorporated probiotic diet on growth pattern and enzymes in *Penaeus vannamei. International Journal of life science and pharma research,* 3: pp. 32-43.

Piard, J.C. and Desmazeaud, M. 1992. Inhibiting factor produced by lactic acid bacteria. Bacteriocins and other antibacterial substances. Lactic acid bacteria, 72: pp. 113-142.

Pivnick, H.; Blanchfield, B. and D'Anst, J.Y. 1981. Prevention of *Salmonella* infection in chicks by treatment with fecal cultures from mature chickens Nurmi cultures. *Journal of* Food *Protect*, 44: pp. 909–916.

Rahiman, M.; Jesmim, K.M.Y, Thomas, A.P. and Hatha, A.M.M. 2010. "Probiotic effect of Bacillus NL110 and Vibrio NE17 on the survival, growth performance and immune response of *Macrobrachium rosenbergii* (de Man)."*Journal of Aquaculture Research,* 41: pp. 120-134.

Rahman, M.A. 2008. A handbook on clinical pathology.4th edition, Capital Book Centre, Nilkhet, Dhaka, pp. 75-78.

Ratanapo, S. and Chulavatnatol, M. 1992. Monodin-induced agglutination of *Vibrio vulnificus*, a major infective bacterium in black tiger prawn (*Penaeus monodon*). *Journal of Comparative Biochemestry and Physiology*, 102B: pp. 855-859.

Rengpipat, S.; Phianphak, W.; Piyatiratitivorakul, S. and Menasveta, P. 1998a. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Journal of Aquaculture*, 167: pp. 301–313.

Rengpipat, S.; Rukpratanporn, S.; Piyatiratitivorakul, S. and Menasaveta, P. 2000. Immunity enhancement on black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (Bacillus S11). *Journal of Aquaculture,* 191: pp. 271–288.

Renuka, G.; Dhingra, H.; Bajpai, P. and Joshi. N. 2012. "Characterization of the *Lactobacillus* isolated from different curd samples." *African Journal of Biotechnology,* 11: pp. 14448-14452.

Ringo, E. and Gatesoupe, F.J. 1998. Lactic acid bacteria in fish: a review. *Journal of Aquaculture*, 160: pp. 177–203.

Rosenrark, R. and Fisher, W. S. 1988. Vibriosis of Lobsrer. In: Disease l) Diagnosis and control Noth American Marine Aquaculture, Sindermann, C J. and D.V. Lishtner (Eds). Development in Aquaculture and Fishedes Science Publishing, New York, USA. pp. 240-242.

Ross, R.P.; Desmond, C.; Fitzgerald, G.F. and Stanton, C. 2005. Overcoming the technological hurdles in the development of probiotic foods. *Journal of Applied Microbiology,* 98: pp. 1410–1417.

Saad, S.A.; Habashy, M.M. and Sharshar, M.K. 2009. Growth response of the freshwater prawn, *Macrobrachium rosenbergii* (De Man), to diets having different levels of Biogen®. *Journal of World Applied Sciences*, 6: pp. 550-556.

Schulze, A.D.; Alabi, A.O. and Tattersall-Sheldrak, A.R. 2006. Bacterial diversity in a marine hatchery: balance between pathogenic and potentially probiotic bacterial strains. *Journal of Aquaculture*, 256: pp. 50-73.

Seenivasan, C.; Bhavan, P.S.; Radhakrishnan, S. and Shanthi, R. 2012a. Enrichment of *Artemia* nauplii with *Lactobacillus sporogenes* for enhancing the survival, growth and levels of biochemical constituents in the post-larvae of the freshwater prawn, *Macrobrachium rosenbergii*. *Journal of Fisheries Science*, 12: pp. 23-31.

Seenivasan, C.; Muralisankar, T. and Saravana, B.P. 2012b-e. *Bacillus subtilis* on survival, growth, biochemical constituents and energy utilization of the freshwater prawn *Macrobrachium rosenbergii* post larvae. *Journal of Egyptian Aquatic Research*, 38: pp. 195–203.

Seenivasan, C.; Radhakrishnan, S.; Shanthi, R.; Muralisankar, T. and Saravana, B.P. 2014. Effect of *Lactobacillus sporogenes* on survival, growth, biochemical constituents and energy utilization of freshwater prawn *Macrobrachium rosenbergii* post larvae. *Journal of Basic and Applied Zoology*, 67: pp. 19–24.

Seenivasan, C.; Saravana Bhavan, P.; Radhakrishnan, S. and Muralisankar, T. 2012c. Effects of probiotics on survival, growth and biochemical constituents of freshwater prawn *Macrobrachium rosenbergii* post larvae. *Journal of Fisheries Aquatic Science*, 12: pp. 331–338.

Seenivasan, C.; Saravana, P.B. and Radhakrishnan, S. 2011. Effect of probiotics (BinifitTM) on survival, growth, biochemical constituents and energy budget of the freshwater prawn *Macrobrachium rosenbergii* post larvae. *Journal of Elixir Aquaculture*, 41: pp. 5919-5927.

Senok, A.C.; Ismaeel, A.Y. and Botta, G.A. 2005. Probiotics: facts and myths. *Clinical Microbiology Infestation*, 11 (12): pp. 958–966.

Shah, N.P. 2007. Functional cultures and health benefits. *Journal of International Dairy*, 17: pp. 1262-1277.

Sharma, O.P. and Bhukhar, S.K.S. 2000. Effect of Aquazyn-TM-1000, a probiotic on the water quality and growth of *Cyprinus carpio* var. communis (L.). *Indian Journal of Fisheries*, 47 (3): pp. 209–213.

Shinde, A.N.; Mulye, V.B.; Chogale, N.D.; Bhatkar, V.R.;Bondre, R.D. and Mohite, A.S. 2008. Effect of different probiotics *Macrobrachium rosenbergii* (De-Man) post larvae. *Journal of Aquaculture*, 9: pp. 7-12.

Shyne, A.; P.S.; Kohli, M.P.S.; Kumar, S. and Sukham, M.K. 2013. Effect of dietary supplementation of periphyton on growth performance and digestive enzyme activities in *Penaeus monodon*. *Journal of Aquaculture*, 392: pp.59-68.

Sindermann, C.J. 1990. Principal diseases of marine fish and shell fish. *Journal of Diseases of Marine Shell Fish*, 2: pp. 53– 59.

Singh, I.S.B. 1990. Bacterial flora of larvae and larval rearing systemof giant freshwater prawn (*M. rosenbergii*). In: Proceedings of 2nd Kerala Science Congress, Trivandrum, India (ed. by N. Balakrishnan Nair), pp. 190–194.

Sirirat, K.M. 2000. The prevention of harmful microorganisms in aquaculture ponds. *Journal of Aquaculture*, 151: pp. 333-349.

Skjermo, J. and Vadstein, O.1999. Techniques for microbial control in the intensive rearing of marine larvae. *Journal of Aquaculture*, 177: pp. 333-343.

Smith, P.; Hiney, M. P. and Samuelsen, O.B. 2004. Bacterial resistance to antimicrobial agents used in fish farming: a critical evaluation of method and meaning. *Journal of Fish Disease*, 4: pp. 273-313.

Smith, V.J. and Chisholm, J.R.S. 1992. Non-cellular immunity in crustaceans. *Journal of Fish Shellfish Immunology*, 2: pp. 1–31.

Soccol, C.R.; Vandenberghe, L.P.; Spier, M.R.; Yamaguishi, C.T.; Pandey, A. and Thomaz-Soccol, V. 2010. The potential of probiotics: a review. *Journal of Food Technology and Biotechnology*. 48(4): pp. 413–434.

Soderhall, K. and Smith, V.J. 1983. Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods and prophenoloxidase distribution. *Journal of Developmental and Comparative Immunology*, 7: pp. 229-239.

Soleimani, N.; Hoseinifar, S.H.; Barati, M. and Abadi, Z.H. 2012. Dietary supplementation of fructo-oligosaccharide (FOS) improves the innate immune response, stress resistance, digestive enzyme activities and growth performance of Caspian roach (*Rutilusrutilus*) fry. *Journal of Fish and shellfish immunology,* 32(2): pp. 316-321.

Strom, E. 2008. Melkesyrebakterier i fisketarm. Isolasjon karakterisering og egenskaper. MSc thesis. The Norwegian College of Fishery Science, pp. 88.

Subasinghe, R. 1997. Fish health and quarantine. Review of the state of the world aquaculture. FAO Fisheries circular no. 886. Food and Agriculture Organization of the United Nations. Italy, Rome. pp. 45–49.

Sung, K. and Song, N. 1996. Non-cellular immunity in crustaceans. *Journal of Fish Shellfish Immunology*, 4: pp. 20–45.

Suralikar, V. and Sahu, N.P. 2001. Effect of feeding probiotic (*Lactobacillus cremoris*) on growth and survival of *Macrobrachium rosenbergii* post larvae. *Journal of Applied Animal Research*, 20: pp. 117-124.

Suzer, C.; Coban, D.; Kamaci, H.O.; Saka, S. and Kucuksari, H. 2008. *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*) larvae: Effects on growth performance and digestive enzyme activities. *Journal of Aquaculture*, 280 (1-4): pp.140-145.

Suzer, C.; Okan, H.; Saka, S.; Firat, K; Otgucuoglu, O. and Kucuksari, H. 2008. *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: Effects on growth performance and digestive enzyme activities. *Journal of Aquaculture*, 280: pp. 140–145.

Thompson, F.L.; Abreu, P.C. and Cavalli, R. 1999. The use of microorganisms as food sourcefor *Penaeus paulensis* larvae. *Journal of Aquaculture,* 174: pp. 139–153.

Tovar-Ramrez, D.; Zambonino, I.J.; Cahu, C.; Gatesoupe, F.J. and V´azquez-Ju´arez, R. 2004. Influence of dietary live yeast on European sea bass (*Dicentrarchus labrax*) larval development. *Journal of Aquaculture,* 234: pp. 415–427.

Ueberschar, B. 1993. Measurement of proteolytic enzyme activity: significance and application in larval fish research. Physiological and biochemical aspects of fish development, pp. 112-125.

Ueberschar, B. 1995. The use of tryptic enzyme activity measurement as a nutritional condition index: laboratory calibration data and field application. *Journal of ICES Marine Science,* 201: pp. 119–129.

Vazquez, L.; Perez, A.; Millan, D.; Agundis, C. and Zenteno, E. 1997. Morphology of hemocytes from the freshwater prawn *Macrobrachium rosenbergii. Journal of Morphology*, 234: pp.147-153..

Venkat, H.K.; Sahu, N.P. and Kamal, K.J. 2004. Effect of feeding *Lactobacillus*- based probiotics on the gut microflora, growth and survival of post larvae of *Macrobrachium rosenbergii* (de Man). *Journal of Aquaculture Research*, 35: pp. 501-507.

Verschuere, L.; Heang, H.; Criel, G.; Sorgeloos, P. and Verstraete, W. 2000b. Protection of *Artemia* against the pathogenic effects of *Vibrio proteolyticus* CW8T2 by selected bacterial strains. *Journal of Applied and Environmental Microbiology*, 66: pp. 1139–1146.

Verschuere, L.; Rombaut, G.; Sorgeloos, P. and Verstraete, W. 2000. Probiotic bacteria as biological control agents in aquaculture. *Journal of Molicular Microbiology*, 64: pp. 655–671.

Vieira, F.N.; Buglione, C.C.; Martins, M.L.; Barraco, M.A. and Vinatea, L.A. 2010. Effect of probiotic supplemented diet on marine shrimp survival after challenge with *Vibrio harveyi. Journal of Aquaculture,* 62: pp. 631-638.

Vine, N.G.; Leukes, W.D. and Kaiser, H. 2006. Probiotics in marine larviculture. *FEMS Microbiology Review*, 30: pp. 404-427.

Vine, N.G.; Leukes, W.D.; Baxter, J. and Hecht, T. 2011. Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. *Journal of Fish Disease,* 27: pp. 319–326.

Wang, Y. 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei. Journal of Aquaculture*, 269: pp. 259-264.

Yanbo, W. and Zirong, X. 2006. Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance, feed utilization (feed conversion ratio, FCR) and digestive enzyme (protease, amylase and lipase) activities. *Journal of Animal Feed Science and Technology*, 127: pp. 283–292.

Ziaei-Nejad, S.; Rezaei, M.H.; Takami, G.A.; Lovett, D.L.; Mirvaghefi, A.R. and Shakouri, M. 2006. The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Journal of Aquaculture,* 252: pp. 516–524.

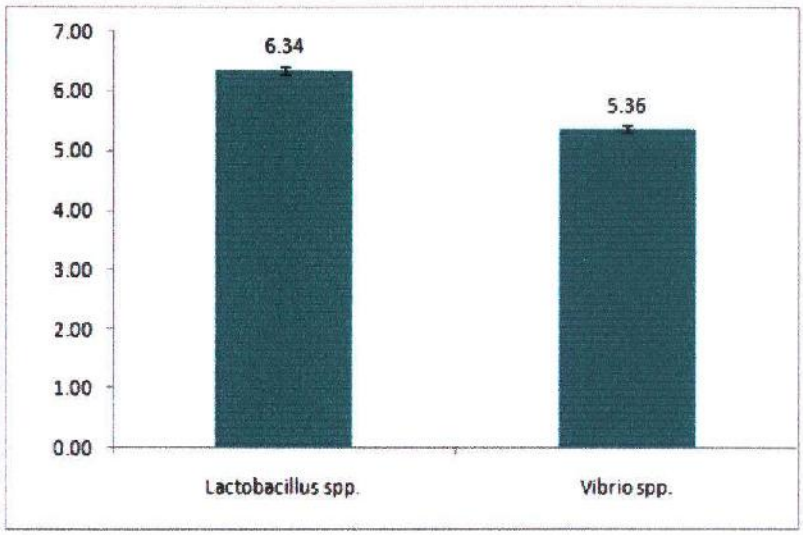
Zokaeifar, H.; Balcázar, J.S.; Saad, C. R.; Sijam, K.; and Nejat, N. 2012. Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*. *Journal of Fish and Shellfish Immunology,* 33: pp. 683-689.

Chapter 8

Appendices

8. Appendices

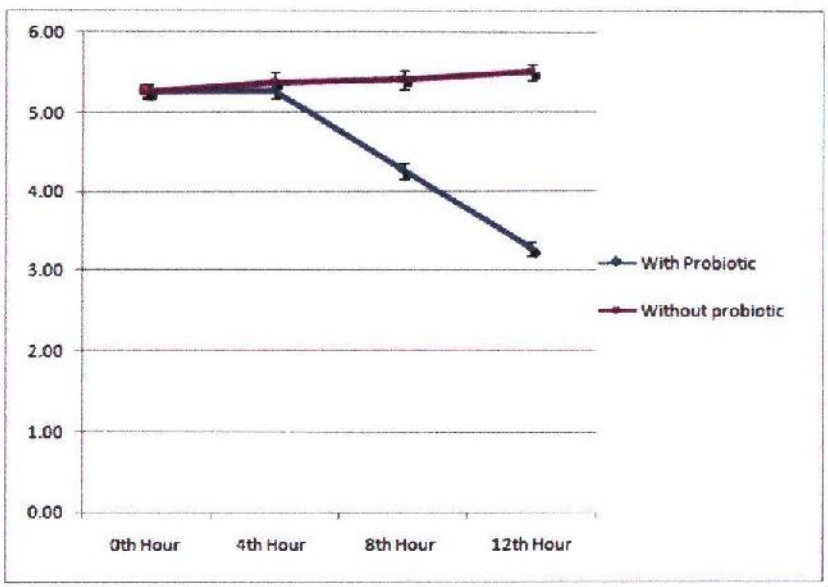
Appendix A1:



N= 3
Sd
Lactobacillus spp.=0.07
Vibrio spp.=0.06

Fig. : Comparison between load of *Lactobacillus* spp. and *Vibrio* spp. in intestine

Appendix A2:



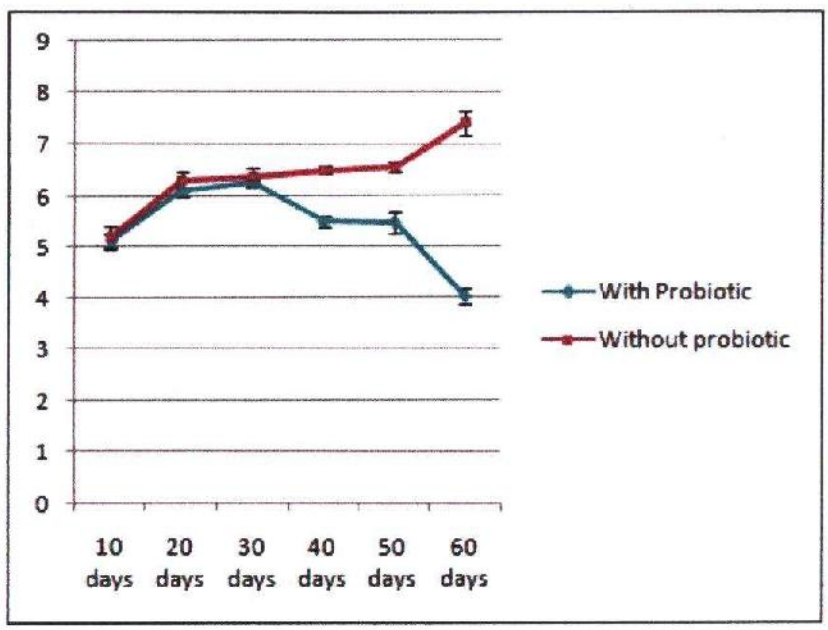
Independent Sample T Test		
	df	Sig. (2-tailed)
0 Hour	4	.725
4 Hour	4	.143
8 Hour	4	.002
12 Hour	4	.01
Sample Size, N=3 Significant level 5%		

Fig: Antagonistic effect of probiotic bacteria on pathogenic bacteria by *In-vitro* challenge test.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Hour_0	Equal variances assumed	1.276	.322	-.378	4	.725	-.03272	.08653	-.27298	.20754
	Equal variances not assumed			-.378	3.204	.729	-.03272	.08653	-.29845	.23301
Hour_4	Equal variances assumed	2.500	.189	1.821	4	.143	.18667	.10252	-.09798	.47132
	Equal variances not assumed			1.821	2.512	.184	.18667	.10252	-.17855	.55188
Hour_8	Equal variances assumed	5.917	.072	7.611	4	.002	1.10000	.14453	.69872	1.50128
	Equal variances not assumed			7.611	2.459	.009	1.10000	.14453	.57719	1.62281
Hour_12	Equal variances assumed	1.638	.270	4.520	4	.011	1.81333	.40114	.69960	2.92707
	Equal variances not assumed			4.520	3.132	.018	1.81333	.40114	.56664	3.06003

Appendix A3:



Independent Sample T Test		
	df	Sig. (2-tailed)
Day_10	4	.246
Day_20	4	.508
Day_30	4	.804
Day_40	4	.000
Day_50	4	.000
Day_60	4	.000
Sample Size, N=3		
Significant level 5%		

Fig : Antagonistic effect of probiotic bacteria on pathogenic bacteria by *In-vivo* challenge test.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Day_10	Equal variances assumed	.014	.912	1.357	4	.246	.05333	.03930	-.05578	.16245
	Equal variances not assumed			1.357	3.965	.247	.05333	.03930	-.05618	.16282
Day_20	Equal variances assumed	3.237	.146	.727	4	.508	.08333	.11465	-.23498	.40165
	Equal variances not assumed			.727	2.556	.528	.08333	.11465	-.32010	.48677
Day_30	Equal variances assumed	.068	.807	-.178	4	.868	-.01000	.05627	-.16624	.14624
	Equal variances not assumed			-.178	3.964	.868	-.01000	.05627	-.16679	.14679
Day_40	Equal variances assumed	1.370	.307	21.655	4	.000	.94667	.04372	.82529	1.06804
	Equal variances not assumed			21.655	3.080	.000	.94667	.04372	.80957	1.08377
Day_50	Equal variances assumed	.000	1.000	16.635	4	.000	1.07667	.06472	.89697	1.25636
	Equal variances not assumed			16.635	3.994	.000	1.07667	.06472	.89686	1.25647
Day_60	Equal variances assumed	2.969	.160	42.098	4	.000	2.44667	.05812	2.28530	2.60803
	Equal variances not assumed			42.098	2.386	.000	2.44667	.05812	2.23165	2.66168

Appendix A4:

Effect of Lactobacillus spp. incorporated diet on growth performance of *Macrobrachium rosenbergii*.

Variables	Control	Treatment
Initial mean weight (g)	2.19 ± 0.05	2.55 ± 0.18
Final mean weight (g)	9.01 ± 0.36	16.49 ± 0.54
Weight gain (%)	310.77 ± 18.68	549. 30 ± 40.02
Specific growth rate (SGR)	2. 35 ± 0.08	3.12 ± 0.10
Survival rate (%)	74%	89%

Appendix A5:

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
weight_gain	Equal variances assumed	.274	.628	-19.784	4	.000	-247.83667	12.52690	-282.61692	-213.05641
	Equal variances not assumed			-19.784	3.730	.000	-247.83667	12.52690	-283.63273	-212.04061

Appendix A6:

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Specific_growth_rate	Equal variances assumed	2.843	.167	-6.879	4	.002	-.72000	.10467	-1.01061	-.42939
	Equal variances not assumed			-6.879	2.825	.008	-.72000	.10467	-1.06511	-.37489

Appendix A7:

Parameters	Treatments	Controls
Amylase activity	1.23	0.7
Protease activity	2.74	1.82

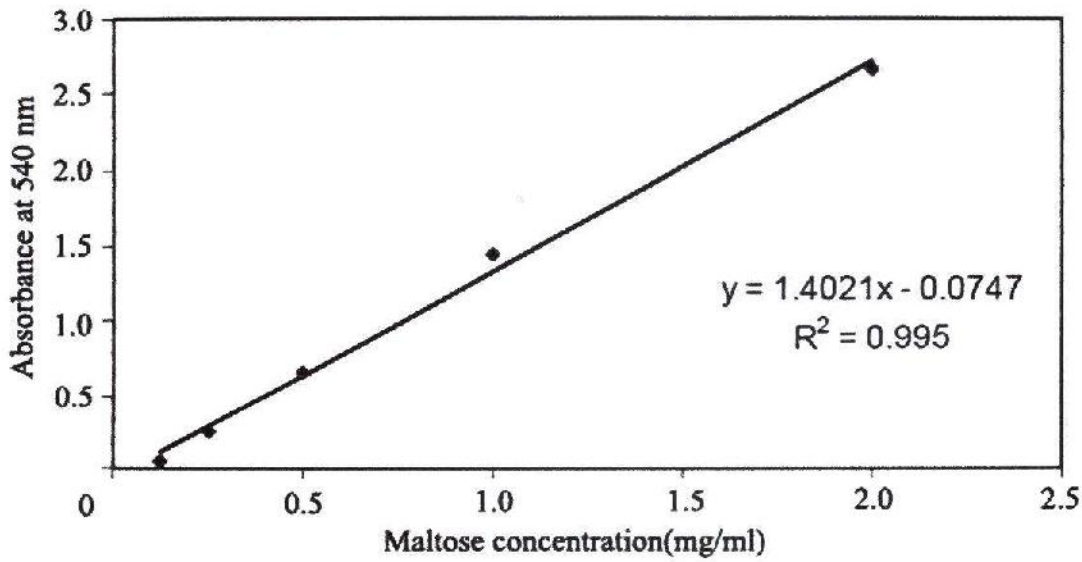
Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Amylase_activity	Equal variances assumed	2.738	.173	-9.132	4	.001	-.53333	.05840	-.69549	-.37118
	Equal variances not assumed			-9.132	2.093	.010	-.53333	.05840	-.77420	-.29247

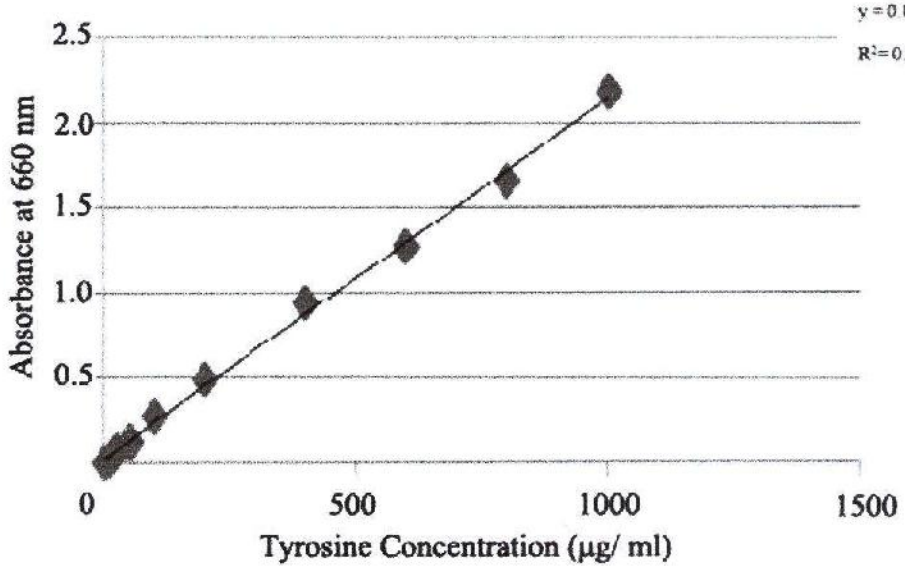
Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Protease_activity	Equal variances assumed	.727	.442	-87.595	4	.000	-.92333	.01054	-.95260	-.89407
	Equal variances not assumed			-87.595	3.448	.000	-.92333	.01054	-.95454	-.89212

Appendix A8:



Appendix A9:



Appendix A10:

Parameters	Control	Treatment
Nongranular haemocyte (NGH)	75.33	76.27
Small granular haemocyte (SGH)	22.24	21.36
Large granular haemocyte (LGH)	2.43	2.37
Total haemocyte count (THC)	$(5.2 \pm 0.73) \times 10^6$	$(5.4 \pm 0.53) \times 10^6$

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Total_Haemocyte_count	Equal variances assumed	.232	.655	-1.709	4	.163	-.05000	.02925	-.13121	.03121
	Equal variances not assumed			-1.709	3.723	.168	-.05000	.02925	-.13365	.03365

Chapter 9

Photo galleries

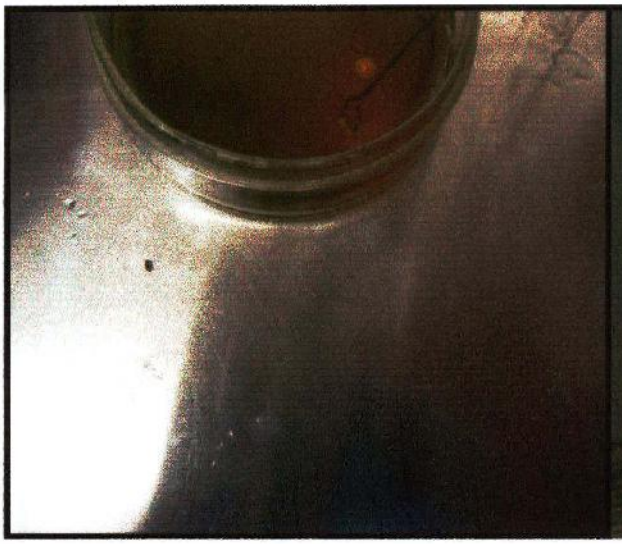


Photo-7: Taken out *Lactobacillus* spp. for biochemical test

Photo-8:Result of VP test

Photo-9: Result of Indole test

Photo-10: Result of MR test

Photo-11: PCR Test

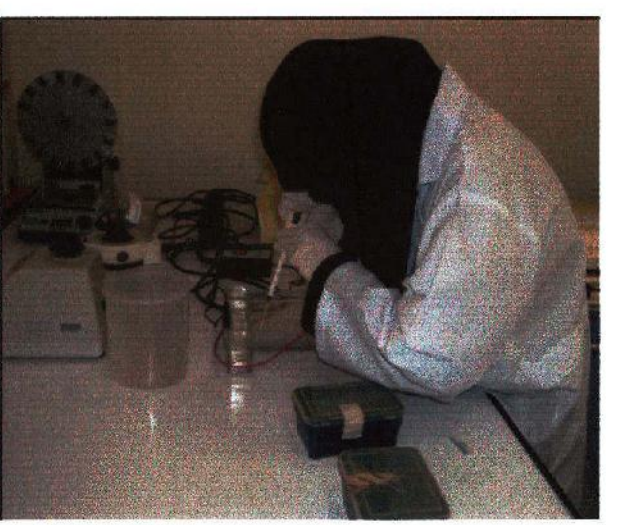


Photo-12: PCR Test

Photo-13: Sampling

Photo-14: Prawn after catching

Photo-15: Study area

Photo-16: Sampling

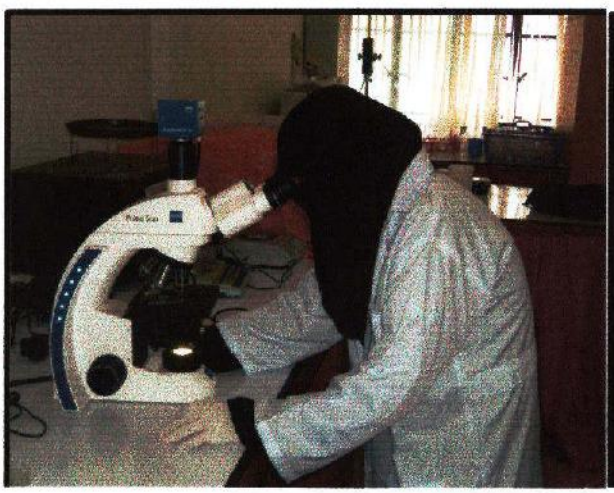


Photo-17: Haemocyte counting

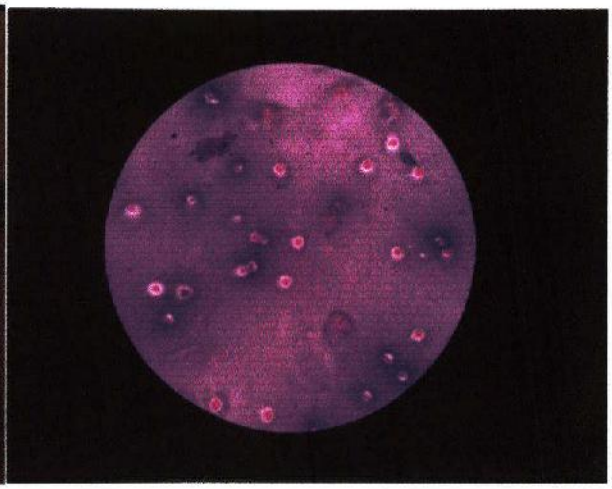


Photo-18: Haemocyte