Molecular Diagnosis of *Vibrio* spp. and it's Bioremediation with Indigenous Probiotic (*Streptococcus faecalis*) in Prawn Farm

A Thesis

By

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Examination Roll No: MS-140618

A thesis submitted to the Fisheries and Marine Resource Technology Discipline in partial fulfillment of the requirements for the degree of

Master of Science

in

Fish Genetics and Biotechnology

Fisheries and Marine Resource Technology Discipline

Khulna University, Khulna

Bangladesh.

November, 2015

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Declaration

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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

ACKNOWLEDGEMENT

At first all my admiration goes to the Supreme Creator of the universe **Allah**, whose mercy keeps me alive and enables me to pursue my education in Fisheries and Marine Resource Technology Discipline and to complete this thesis work.

I would like to express my sincere gratitude and profound appreciation to my supervisor, **Dr. Md. Ayaz Hasan Chisty**, Head and Professor, Fisheries and Marine Resource Technology Discipline, Khulna University, for his continuous guidance, suggestion and sustained ssupport throughout the experiment.

I would like to express my sincere gratitude and profound respect to my co-supervisor, **Dr. Muhammad Abdur Rouf**, **Professor,** Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna for his guidance, valuable suggestions and sustained support in preparing this thesis.

I would like to express my sincere gratitude to my respected and honorable teacher **Dr. Md. Golam Sarower**, Professor, Fisheries and Marine Resource Technology Discipline, Khulna University, for his continuous guidance, valuable suggestions and sustained support throughout the experiment.

I am highly grateful to my Friend, Md. Nazmul Hasan Zilani, Md. Saifuddin Sumon and Fatema Ahmmed for their helpful cooperation and encouragement during the study.

I would like to express my deepest gratitude to my beloved parents and my family for standing all the way by my side during the preparation of the thesis.

Sharmin Sultana Khushi

November, 2015

Abstract

Probiotics are gaining popularity as eco-friendly alternatives to antibiotics in improving prawn health and minimizing diseases. Present study was assessed to detect the pathogenic bacteria *Vibrio* spp. at molecular level and to examine the effect of indigenous probiotic, *Streptococcus faecalis* against *Vibrio* spp. on freshwater prawn *Macrobrachium rosenbergii.* Identification of pathogenic *Vibrio* spp. was confirmed by PCR, targeting the virA gene. An antagonistic effect of *Streptococcus faecalis* against *Vibrio* spp. was found significantly ($P<0.05$) at 8^{th} and 12^{th} hour of probiotic treatment in *in-vitro* and after 60 days of probiotic fortified diet treatment in *in-vivo* challenge tests. Dietary administration of probiotic significantly ($P<0.05$) increased the growth and digestive enzymes (protease and amylase) activity of *Macrobrachium rosenbergii*. This bacterium also improved immune system by augmenting total haemocyte count of freshwater prawn, although it was not significant ($P>0.05$) compared to control group. From the present study, it can be inferred that *Streptococcus faecalis* as a probiotic bacterium may be used as biological control agent against vibriosis in freshwater prawn aquaculture.

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List of Abbreviations

Abbreviation	Meaning
%	Percentage
et al.	Et alia
°C	Degree Celsius
spp.	Species
TCBS	Thiosulphate-citrate-bile salt sucrose
MR	Methyl Red
VP	Voges-Proskauer
bp	Base pair
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
CFU	Colony Forming Unit
SGR	Specific Growth Rate
rcf	Rotation Centrifugal Force
rpm	Rotation Per Minut
mL	millilitre
mg	milligram
g	gram

Chapter One

INTRODUCTION

Chapter 1. Introduction

1.1. Background of the study

Aquaculture, the fastest growing aquatic food-producing sector, is considered as an economically important industry in the world (Subasinghe *et al.*, 2009). Resembling many countries Bangladesh, the world's largest deltaic basin, has also achieved credibility in aquaculture because of its unique geographical location and amiable agro-climate condition. In national economy of Bangladesh the fishery sectors currently contribute about 3.69% of the Gross Domestic Production and 22.60% in agricultural production and 2.09% in export earnings (DoF, 2015). In 2013-2014 total fish production was 35.48 lakh mt. in where the most important division, prawn culture contributes about 223788 mt. ton in which 57.34% is from culture basis (DoF, 2015).

Macrobrachium rosenbergii has gained aquaculture importance owing to its high fecundity, rapid growth, and temperature tolerance, disease resistance, high commercial value (New, 1995; Roustaian *et al.*, 2001), omnivorous feeding habit, attractive size and better meat quality with good amount of protein (Ling, 1969). Farming of giant freshwater prawn, *M. rosenbergii* has been gained increased interest in recent years due to its high economic value in the world market (Radheyshyam, 2009). Due to favorable resources, availability in nutrition and agro-climatic conditions farming of *M. rosenbergii* has developed into one of the most emerging economic activities in southwest regions of Bangladesh.

The outbreak of new pathogens and infectious diseases has become one of the major obstacles in the rapid expansion and intensification of *M. rosenbergii* farming (Gatesoupe, 2008; Geng *et al.*, 2012). Generally most of the infectious diseases are caused by viruses, bacteria and parasites in where majority of bacterial diseases are by the families Pseudomonadaceae (*Pseudomonas*) and Vibrionaceae (*Vibrios*). Vibriosis caused by *Vibrios* that are motile, rod shape, non-spore formers, mesophilic, chemo-organotrophic, facultative fermentative, oxidase positive in character, is prevalent in the early life stages (eggs, larvae, and postlarvae) of *M. rosenbergii* and it may retard growth or destroy total production (Breed *et al.*, 1957; Bhat and Singh, 1999). Major species causing vibriosis in prawn are *Vibrio harveyi, V. alginolyticus, V. anguillarum* and *V. parahaemolyticus* (Chanratchakool *et al.*, 1995; Alapeda-Tendencia and Dareza, 1997). Among them *Vibrio harveyi* is the most detrimental to *M. rosenbergii* (Tonguthai, 1997; Pande *et al.*, 2013a). Though *M. rosenbergii*

is more resistant to viral diseases in comparison to *Penaeus monodon* (Wang *et al.*, 1998), former is more sensitive to bacterial diseases that cause large-scale mortality in hatcheries and culture systems (Grisez and Ollevier, 1995; Cheng and Chen, 1998).

Identification of bacteria based on clinical observation as well as detection of toxin in sample is essential for diagnosis and proper treatment of diseases in *M. rosenbergii*. But isolation as well as identification is frequently complicated by the presence of nontoxigenic strains that phenotypically and genetically resemble and exhibit a high relatedness with their toxigenic counterparts (Hatheway, 1992; Broda *et al.*, 1998). Among different conventional identification methods biochemical identification involves cultivation of bacteria followed by their biochemical tests. Accurate phenotypic identification of *Vibrio* species is problematic, largely because of the great variability in biochemical characteristics (Thompson *et al.*, 2004). Molecular methods that utilize the PCR and nucleotide sequence determination overcome many of the limitations of phenotypic methods. Due to laborious, time-consuming and possibility of false positive result in conventional phenotypic methods; in recent past molecular-based detection methods such as polymerase chain reaction (PCR) have been developed that are more rapid, sensitive and specific have been widely used (Ingianni *et al.*, 2001; Pathmanathan *et al.*, 2003; Thiem *et al.*, 2004; Lovseth *et al.*, 2004; Panicker *et al.*, 2005; Tarr *et al.*, 2007). In PCR, small, often undetectable, amounts of DNA can be amplified to produce detectable quantities of the target DNA. The presence or absence of a product following PCR may be sufficient to indicate whether a sample is infected by a certain pathogen. Among different PCR types the single step PCR is usually used for the specific detection of pathogenic *Vibrio spp.* strains. Early and accurate detection of these pathogens is a crucial factor affecting the success of aquaculture systems (Cunningham, 2002) and hatchery operations (Muroga, 2001). Conventional PCR assays have been developed to detect luminous *Vibrio* spp. in various organisms such as fish, shrimp and shellfish (Hernández and Olmos, 2004; Pang *et al.*, 2006; Thaithongnum *et al.*, 2006; Sun *et al.*, 2009; Cao *et al.*, 2010; Hwang *et al.*, 2010).

During the last decades, as traditional strategy for fish diseases management a wide range of antibiotics (oxytetracycline, ciprofloxacin, chloramphenicol etc.) are used to combat microbial infection in hatcheries and farms of freshwater prawns and marine shrimps (Karunasagar *et al.*, 1994; Hameed and Balasubramanian, 2000; Prasad *et al.*, 2012). But the use of antibiotics may cause water pollution in the aquaculture environment (Petersen *et al.*,

2002; Alcaide *et al.*, 2005), contamination of shrimp meat (Holmström *et al.*, 2003) and development of multiple antibiotic resistant bacteria (Skjermo and Vadstein, 1999; Kim *et al.*, 2004; Cabello, 2006). The antibiotic resistant pathogens may cause mass mortality in hatchery-reared larvae and post-larvae of *M. rosenbergii*. Also transmission of resistant bacteria from aquaculture products may be detrimental to human health by inhibiting or killing beneficial microbiota in the gastrointestinal (GI) ecosystem (Cabello, 2006). On the other hand these products are sometimes banned due to rejection of export consignments. For these reasons use of antibiotics as disease controllers and growth promoters is currently restricted or forbidden in many countries (Verstegen, 2001). To overcome these problems both consumer and manufacturer are looking for the alternative health management strategy, which can be accomplished by microbial intervention (Galindo, 2004; Panigrahi and Azad, 2007). The use of probiotics may be the light in the tunnel.

Probiotics are food supplement containing mono or mixed culture of live microorganism in sufficient number with or without byproducts, when consumed in adequate amounts antagonism against pathogens, help development of the immune system, provide nutritional benefits such as improving feed digestibility and feed utilization and assist the intestinal mucosal barrier to the host (Gatesoupe, 1999; Verschuere *et al.*, 2000; Vaughan *et al.*, 2002; Irianto and Austin, 2002). It ensures eco-friendly aquaculture, improves water quality and the immune potential of the animal by balancing bacterial flora in water and reducing pathogenic bacterial load (Verschuere *et al.*, 2000; Wang *et al.*, 2005; Vine *et al.*, 2006; Kesarcodi-Watson *et al.*, 2008). Thus, replacing drugs with effective and inexpensive probiotics was became increasingly evident and necessary to avoid resistance in fish farming sites and antibiotic residues in fish flesh destined for human consumption (Vershuere *et al.*, 2000; Balcazar *et al.*, 2006; Rengpipat *et al.*, 2008).

Probiotic agents exert their beneficial effects via a wide array of actions; these include competition for adhesion sites and resistance to colonization, competition for essential nutrients, production of antagonistic compounds against pathogens for competitive exclusion of pathogens (Gomez-Gil *et al.*, 2000; Vine *et al.*, 2004), improve the intestinal digestion by secreting enzymes (Ramirez and Dixon, 2003), enhancement of the immune response against pathogens (Rengpipat *et al.*, 2000; Irianto and Austin, 2002) and improve their environment (Moriarty, 1999). However, all of these proposed modes of action depend on the ability of the potential probiotic to reach the desired location where their effect is required and the

colonizing power in there (Verschuere *et al.*, 2000). The beneficial effect of probiotic as supplements include improved feed value, enzyme activity, enzymatic contribution to digestion, inhibition of pathogen, antimutagenic and anticarcinogenic activity, growth promoting factors, increase immune response, fish health and performance (Lara-Flores *et al.*, 2003; Wang *et al.*, 2005; Ziaei-Nejad *et al.*, 2006; Wang and Xu, 2006; Wang, 2007; Denev *et al.*, 2009). These are the causes that's why the use of probiotics to manipulate the microbiota in larval cultivation have become increasingly popular (Gomez-Gil *et al.*, 2000; Vine *et al.*, 2006; Silva *et al.*, 2011; Nimrat *et al.*, 2011, 2012) and is gaining interest as an environmentally safe alternative to antibiotics and chemotherapeutics (Song *et al.*, 1997). Though many probiotics have been developed for use in aquaculture (Brunt and Austin, 2005; De Schrijver and Ollevier, 2000; Robertson *et al.*, 2000), researchers are still attempting to isolate beneficial bacteria from various sources like soil, water and animal gut for better control of diseases causing pathogens in aquaculture systems (Gomez-Gil *et al.*, 2000; Ahmed *et al.*, 2005; Skrodenyte-Arbaciauskiene *et al.*, 2006; Kim *et al.*, 2007; Lakshmi *et al.*, 2013).

Most probiotics proposed as biological control agents in aquaculture belong to the lactic acid bacteria and several species of *Streptococcus*, *Bacillus* and *Pseudomonas* genus have also been studied. Lactic acid bacteria (LAB) produce lactic acid as the end product of sugar fermentation; have been recognized for their fermentative ability, health and nutritional benefits as well as strong antimicrobial activities. Antimicrobial activities of these bacteria are considered through antagonistic effect against fish pathogens (Ringo and Gatesoupe, 1998). The lactic acid bacterium (LAB) has been reported to improve water quality, nutrition and survival as a means to increase population growth and aquaculture output (Skjermo and Vadstein, 1999; Verschurere *et al.*, 2000; Planas *et al.*, 2004).

Streptococcus faecalis is a member of Gram positive bacteria belonging to the lactic acid bacteria group. *Streptococcus faecalis* have been reclassified and placed in the genus *Enterococcus* ; thus, *Streptococcus faecalis* is now known as *Enterococcus faecalis* (Schleifer and Kilpper-Balz, 1984). Many strains of *E. faecalis* produce lactic acid at the end of the sugar fermentation and bacteriocins like antimicrobial compound named enterocins, a family of safe (Belguesmia *et al.*, 2011) and ribosomally synthesized antimicrobial peptides (Driderand and Rebuffat, 2011) which are active against pathogens including *Staphylococcus aureus* and *Listeria monocytogenes* (Campos *et al.*, 2006). Immune

stimulation and adjuvant benefits have been reported when farmed animal diets were supplemented with *E. faecalis* (Shimada *et al.*, 2004; Rodriguez-Estrada *et al.*, 2009). *E. faecalis* is involved in the reduction or prevention of gastrointestinal tract infections (Franz *et al.*, 2011). It prevents the colonization of pathogenic bacteria in the body of its host by competing with the pathogens for binding sites and nutrients. They may also prime the immune system by inducing the production of low levels of antibodies against its own components which, in turn, makes the immune system more efficient (Pulugurtha, 2014). *Bacillius* sp., *Rodobacter* sp., *Streptococcus faecalis* containing probiotics are used to control virbiosis (Shamsuzzaman and Biswas, 2012). Studies have shown that dietary supplementation of *S. faecalis* and MOS (Mannan Oligosaccharide) in rainbow trout strongly influenced the immune system, growth performance and reduced cumulative mortality (40%) after challenge with *Aeromonas salmonicida* (Rodríguez-Estrada, 2013). Probiotic products containing *Streptococcus faecalis, B. mesentericus,* and *Clostridium butyricum* are capable of producing a wide range of exoenzymes such as protease and amylase that enhance digestive capacity (Nimrat and Vuthiphandchai, 2011). *Lactobacillus sporogenes, Bacillus subtilis* used as probiotic have enhanced the growth, survival, biochemical constituents and feed utilization performance in *M. rosenbergii* (Seenivasan *et al.,* 2012a, 2013). Several beneficial effects of probiotics in *M. rosenbergii* have been reported (Venkat *et al.*, 2004; Keysami *et al.*, 2007; Shinde *et al.*, 2008; Saad *et al.*, 2009; Seenivasan *et al.*, 2012). Probiotics can be administered to *M. roesenbrgii* either through feed or as bioencapsulated in *Artemia* (Venkat *et al.,* 2004).

It is essential to investigate the bacterial flora associated with prawn culture in order to develop safe farm management practices for the production of prawn safe for human consumption and for prevention of prawn diseases. The use of probiotic bacteria in aquaculture has tremendous scope and the study on application of probiotics in aquaculture has a glorious future. Probiotic treatment offers a promising alternative to the antibiotics for prawn aquaculture system, but peoples involved in aquaculture activities both farming and hatchery operations have not enough knowledge about probiotics and their beneficiary effects. The application of probiotics in Bangladesh is fewer and confined only to prawn and shrimp cultures and to some profit-making pisciculture. Isolation of suitable probiotic bacteria from natural sources, optimization of its antagonistic activity and finally, application in an effective dose in the rearing environment is expected to prevent the growth of undesirable pathogenic microorganisms to proliferate.

1.2. Objective of the Study

Based on detrimental effect of *Vibrio* spp. and beneficial effect of probiotics on aquaculture present study was undertaken to detect *Vibrio* spp. on molecular basis and elucidate the biological effect of *Streptococcus faecalis* as probiotic on *Macrobrachium rosenbergii* culture with the following objectives:

- To detect the pathogenic bacteria *Vibrio spp.* by PCR in prawn farm.
- To find out the antibacterial effect of probiotic, *Streptococcus faecalis* on *Vibrio* spp. infected *Macrobrachium rosenbergii.*
- To examine the effect of probiotic, *Streptococcus faecalis* on growth, digestive enzyme activity and immunity of *Macrobrachium rosenbergii.*

Chapter Two

Literature Review

Chapter 2. Literature Review

Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal balance (Fuller, 1989), improving the properties of the indigenous flora (Havenaar and Veld, 1992), and which when consumed in adequate amounts, confer a health effect on the host (Guarner and Schaafsma, 1998). Probiotics also confer antagonism against pathogens, help development of the immune system, provide nutritional benefits such as improving feed digestibility and feed utilization and assist the intestinal mucosal barrier (Vaughan *et al.*, 2002; Ibrahem, 2013).

In recent years, probiotics have been increasingly used in the biological control to prevent diseases in aquaculture, which will make aquaculture products more environmental friendly and acceptable to consumers (Gatesoupe, 1999; Verschuere *et al.*, 2000; Nayak, 2010; Ringø *et al.*, 2010). Many studies revealed the effects of probiotics to improve feed conversion, growth rates, weight gain, immune system and disease resistance of fish (Nayak, 2010; Dimitroglou *et al.*, 2011; Merrifield *et al.*, 2010). The beneficial effects of many probiotics have been reported on prawns such as *Lactobacillus* spp. and *Streptococcus* spp., improved the growth and survival of the green tiger shrimp, *Penaeus semisulcatus* when supplemented with clam meat and pellet feed, Irawan-300-Grower (Murugesan *et al.*, 2008).

Probiotic, *Enterococcus faecalis* belongs to the lactic acid bacteria (LAB) group. Immune stimulation and adjuvant benefits have been reported when farmed animal diets were supplemented with *E. faecalis* (Shimada *et al.*, 2004; Rodriguez-Estrada *et al.*, 2009). In this study few available literatures have been made on the effect of probiotics on growth performance, digestive enzyme activity and anti-bacterial activity on aquaculture.

Elumalai *et al.*, (2013) reported that administration of commercially available probiotics (*Streptococcus faecalis, Streptococcus faecium, Bacillus natto, Clostridium butyricum, Saccharomyces cerevivisiae*) in *Peneaus monodon* significantly improved growth and survival of shrimp, increased beneficial bacteria in shrimp culture and enhanced water quality.

Enterococci belong to the group of LAB, which is known to produce lactic acid as the end product of sugar fermentation, and antimicrobials, which are active against pathogens including *Staphylococcus aureus* and *Listeria monocytogenes* (Campos *et al.*, 2006).

Many strains of *E. faecalis* produce bacteriocins name denterocins, a family of safe (Belguesmiaeta, 2011), and ribosomally synthesized antimicrobial peptides (Driderand and Rebuffat, 2011). Al Atya *et al.*, (2015) performed a challenge tests, indicated that inhibition of *S. aureus* ATCC33862 by *E. faecalis* 28 and *E. faecalis* 93 was due to production of lactic acid and bacteriocin.

Elumalai and Raffi, (2013) reported that application of probiotics (*Streptococcus faecalis, Streptococcus faecium, Bacillus mesentericus, Bacillus subtilis, Bacillus natto, Clostridium butyricum* and *Saccharomyces cerevivisia)* has improved the growth and survival of *P. monodon* and which in turn paved way to reap better profit for the farmers.

Swain *et al.*, (2009) suggested that isolation of a bacterial probiont, *Streptococcus phocae* PI80, from healthy Indian white shrimp, *Fenneropenaeus indicus*, not only improved growth in *P. monodon* post larvae but also reduced mortality when challenged with luminescent *Vibrio harveyi* and *V. parahaemolyticus.*

Some study shown that shrimp farmers were found to use a range of probiotic (*Bacillius* sp., *Rodobacter* sp., *Streptococcus faecalis*) products to control virbiosis, lunimiscent bacteria, improve water and soil quality and control pH (Shamsuzzaman and Biswas, 2012).

Nimrat and Vuthiphandchai, (2011) reported that commercial probiotic products (*Streptococcus faecalis, Bacillus mesentericus, Clostridium butyricum*) used for marine shrimp cultivation in Thailand capable of producing a wide range of exoenzymes such as protease and amylase.

Allameh *et al.*, (2014) revealed that *Enterococcus faecalis* has potential probiotic properties. Improved survival of snakehead fish was observed when fed with *Enterococcus faecalis*-fortified diet in an in-vivo challenge test using *Aeromonas hydrophila.* This study suggested that *E. faecalis* is a safe alternative to antibiotics to inhibit *Aeromonas hydrophila* activity in snakehead culture.

Rodriguez-Estrada *et al.*, (2009) reported that feeding a synbiotic combination of mannan oligosaccharide and *Enterococcus faecalis* improved survival of rainbow trout challenged with *V. anguillarum*. Enterococci, particularly *Enterococcus faecalis* and *E. faecium* are involved in the reduction or prevention of gastrointestinal tract infections (Franz *et al.*, 2011).

Li *et al.*, (2010) reported that scallop (*Patinopecten yessoensis)* accumulated *E. faecalis* in larger numbers in the digestive tract and all scallops exposed to *E. faecalis* showed significantly higher trypsin and amylase activity.

Lara-Flores and Olvera-Novoa, (2013) isolated five lactic acid strains (*Enterococcus faecium, E. durans, Leuconostoc* sp., *Streptococcus* sp. I and *Streptococcus* sp. II) from intestinal tract of nile tilapia (*Oreochromis niloticus*). All native bacterial strains were improved animal growth and mitigated the effect of stress factors, such as the low protein level in diets.

Cebrián *et al.*, (2012) reported that *E. faecalis* S-48 produced enterocin AS-48 which exhibits bactericidal activity against a wide variety of gram-negative and gram-positive bacteria, including food spoilage and pathogenic bacteria such as *Bacillus cereus*, *Clostridium botulinum*, *C. difficile*, *C. perfringens*, *Staphylococcus aureus* and *L. monocytogenes*.

Gomes *et al.*, (2010) reported that *E. faecalis* strains are generally more active in proteolytic system to convert proteins to peptides and then to amino acids than other enterococci strains.

Franz *et al.*, (2011) reported that *E. faecalis* are involved in the reduction or prevention of gastro intestinal tract infections.

Rodriguez-Estrada *et al.*, (2009) reported that dietary incorporation of MOS (Mannan oligosaccharide) in a single or a combined inclusion with *E. faecalis*, enhances the rainbow trout growth and activates its immune system.

Rodríguez-Estrada, (2013) examined the effects of inactivated *Enterococcus faecalis* and mannan oligosaccharide and their combination on growth, immunity, and disease protection

in rainbow trout and demonstrated that dietary supplementation of *E. faecalis* and MOS (mannan oligosaccharide) in rainbow trout strongly influenced the immune system, growth performance and reduced cumulative mortality (40%) after challenge with *Aeromonas salmonicida.*

Nueno-Palop *et al.*, (2011) conducted an experiment on probiotic assessment of *Streptococcus faecalis* CP58 isolated from human gut. A total of seventy lactic acid bacteria (LAB) were isolated from the faeces of healthy humans and their identities were confirmed by sequencing of their 16S rDNA genes. *Streptococcus faecalis* showed the best resistance and it was the most adherent strain. These results suggest that the strain *Streptococcus faecalis* might be applied as probiotic for beneficial effect of digestion of other animal.

Cuív *et al.*, (2013) conducted an experiment on the draft genome sequence of *E. faecalis* PC1.1, a candidate probiotic strain isolated from human feces. *E. faecalis* is commonly isolated from the gastrointestinal tract of healthy infants and adults, where it contributes to host health and well-being.

Saad *et al.*, (2009) investigated the impact of adding probiotics (Biogen®) in the diet of prawn (*Macrobrachium rosenbergii)* during the post larval stage of growth. They suggested that probiotics were able to enhance the growth performance and feed utilization of *M. rosenbergii.*

Seenivasan *et al.*, (2014) reported that probiotic, *Lactobacillus sporogenes* incorporation had significantly improved survival, growth, specific growth rate, feed conversion rate, feed conversion efficiency and protein efficiency rate, biochemical constituents and energy utilization of *M. rosenbergii* post larvae.

Far *et al.*, (2009) isolated *Bacillus subtilis* from digestive tract of *Macrobrachium rosenbergii.* This isolates displayed antibacterial activity against *Vibrio* spp. and improved shrimp (*L. vannamei*) survival rate.

Rahiman *et al.*, (2010) reported that two probionts namely *Bacillus* NL110 and *Vibrio* NE17 improved water quality, growth, and survival, specific growth rate (SGR), feed conversion

ratio and immune parameters such as the total haemocyte count, phenoloxidase activity and respiratory burst of *Macrobrachium rosenbergii.*

Probiotics product Binifit™, *Lactobacillus sporogenes*, *Bacillus subtilis* and *Saccharomyces cerevisiae* showed better survival, growth, biochemical constituents and energy utilization in post larvae of *M. rosenbergii* when supplemented with feed (Seenivasan *et al.*, 2011, 2012a-c, 2014a-c).

El-Haroun *et al.*, (2006) reported that after the probiotic settlement in the intestine, it start to consume carbohydrates for self-growth and produce a range of digestive enzymes as amylase, protease and lipase which improve digestibility, in return a higher growth rates due to stimulation of a pre-digestion of secondary compounds and intestinal free disorders.

Ziaei-Nead *et al.*, (2006) examined the effects of *Bacillus* spp. on *Fenneropenaus indicus* at different shrimp stages and recorded a significant difference in the growth rate in comparison with control groups. Tested shrimp ponds showed significantly higher activity of amylase, total protease, and lipase with a significantly higher apparent digestibility.

Balcazar *et al.*, (2006) and Gatesoupe, F. J., (2008) reported that administration of probiotic modulate the nonspecific immune responses by increasing some cellular and humoral parameters thus, increase disease resistance during bacterial infections in aquatic animals.

Li *et al.*, (2014) reported that potential probiotics mixture species *B. subtilis* YB-1 and *B. cereus* YB-2 beneficial for enhancing sea cucumbers health and controlling skin ulceration disease caused by pathogenic *V. alginolyticus* by modulating the intestinal microflora and stimulating innate immune system.

Vand *et al.*, (2014) reported that dietary supplementation of food with probiotic *lactobacillus casei* at 5×10^7 CFU g-1 concentration in day 30 and at 5×10^8 CFU g-1 concentration in day 60 are suitable for enhancing the growth and digestive enzymes activity of *Barbus grypus.*

Zokaeifar *et al.*, (2012) reported that administration of probiotic, *B. subtilis* strains L10 and G1 improved the growth performances, survival rate, digestive enzyme activity and immune response of shrimp (*L. vannamei*) against the pathogenic bacterium, *V. harveyi.* A significant

reduction of ammonia, nitrite and nitrate ions and the expression of all immune-related genes was significantly up-regulated ($P < 0.05$) in probiotic treated group compared to the control groups (Zokaeifar *et al.*, 2014).

Pur *et al.*, (2015) examined the effects of dietary *Lactobacillus casei* (PTCC 1608) on some hematological and immunological parameters of rainbow trout (*Oncorhynchus mykiss*) and reported that probiotic *Lactobacillus casei* fed to the rainbow trout, with the quantity of 5×10^6 and 5×10^7 CFU g^{-1}, can improve the performance of the immune system.

Rengpipat *et al.,* (2000) reported that probiont bacterium *Bacillus* S11 provided disease protection by activating both cellular and humoral immune defenses, as well as presumably providing competitive exclusion of the pathogenic bacteria *V. harveyi* in the shrimps gut.

Vieira *et al.*, (2010) reported that probiotic *Lactobacillus plantarum*-supplemented diet modifies shrimp digestive tract bacterial microbiota, increasing survival, total hemocyte count, serum agglutination activity and resistance to *V. harveyi* infection.

Chiu *et al.*, (2007) reported *Lactobacillus plantarum* supplemented food increased the activities of superoxide dismutase (SOD), phenoloxidase (PO), respiratory burst as well as also inhibited *Vibrio alginolyticus* in white shrimp, *Litopenaeus vannamei.*

Jayanthi *et al.*, (2015) reported that 4% LactoBacil® plus (combination of *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium* longum, *Bifidobacterium bifidum* and *Saccharomyces boulardi*) incorporated diet fed prawns showed significantly ($P<0.05$) improved survival, growth, nutritional indices, activities of protease, amylase and lipase, concentrations of total protein, carbohydrate and lipid, level of essential amino acids and fatty acids.

There are numerous PCR-based methods which have been developed for identification of *Vibrio* species. Tarr *et al.*, (2007) had proposed a multiplex PCR using species-specific primers to amplify the *rpoB* gene regions in four species (*V. cholerae, V. parahaemolyticus, V. vulnificus,* and *V. mimicus*) followed by DNA sequence analysis.

Adhikari *et al.*, (2015) used a multiplex PCR system for the rapid detection of pathogenic bacteria *Vibrio sp.* in shrimp farms. They used virA gene to amplify internal fragments of

these genes by PCR to generate PCR products that could be analyzed and confirmed with relative ease by gel electrophoresis.

Paydar, (2013) identified *vibrio* species from seafood based on colony appearance on selective media such as thiosulfate-citrate-bile salts-sucrose (TCBS) agar, followed by conventional biochemical tests including methyl red (MR) and voges-proskauer (VP), and salt tolerance tests.

Tarr *et al.*, (2007) isolated and identified *Vibrio* spp. by conventional methods and confirmed the isolates by the PCR method targeting 16S rRNA gene. A multiplex PCR targeting *gyrB* and *pntA* gene to detect *Vibrio* species reported by Teh *et al.*, (2010).

Khalil *et al.*, (2014) investigated the presence of pathogenic *Vibrio* spp. using standard PCR. They used 16SrRNA gene to confirm the genus *Vibrio* and reported that all the isolates were positive for 16S rRNA gene fragment confirming the genus in all the isolates studied.

Chapter Three

MATERIALS & METHODS

Chapter 3. Materials and Methods

3.1. Study area and duration of the study

To know the present scenario of *Vibrio* spp. sample were collected from the local market of Khulna. After sampling; samples were analyzed in the Biochemistry and Molecular Genetics Lab, Khulna University, Khulna. Probiotic and *Vibrio* spp. were also isolated from the collected samples. The field work was conducted in the research pond complex of Fisheries and Marine Resource Technology Discipline at Khulna University, Khulna, Bangladesh. The study was conducted from January to November, 2014.

3.2. Sampling and sample size

At the first phase of the study, for isolating and counting pathogenic bacteria, *Vibrio* spp. and probiotic bacteria, *Streptococcus faecalis* load; nine (9) specimens of prawn, *Macrobranchium rosenbergii* were collected from the study area. The standard lengths of the specimens were between 17cm-20cm and the weights were between 26g-29g. In this phase intestinal tract were used as the experimental organs. These samples were used for detecting *Vibrio* spp. and *Streptococcus faecalis.* Afterwards *in-vitro* analyses were done. At the second phase of the study *in-vivo* analyses were conducted. In this phase the experimental organs were intestinal tract of prawn sample.

3.3. Preparation of stock solution of the target organs

The target organs of the prawn samples were collected aseptically and weighed in electric balance. Weights of intestinal tracts were between 0.20g-0.25g. Organs were taken into eppendorf tubes with peptone water (James and Hirsch, 1960) for isolating *Streptococcus faecalis* 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.

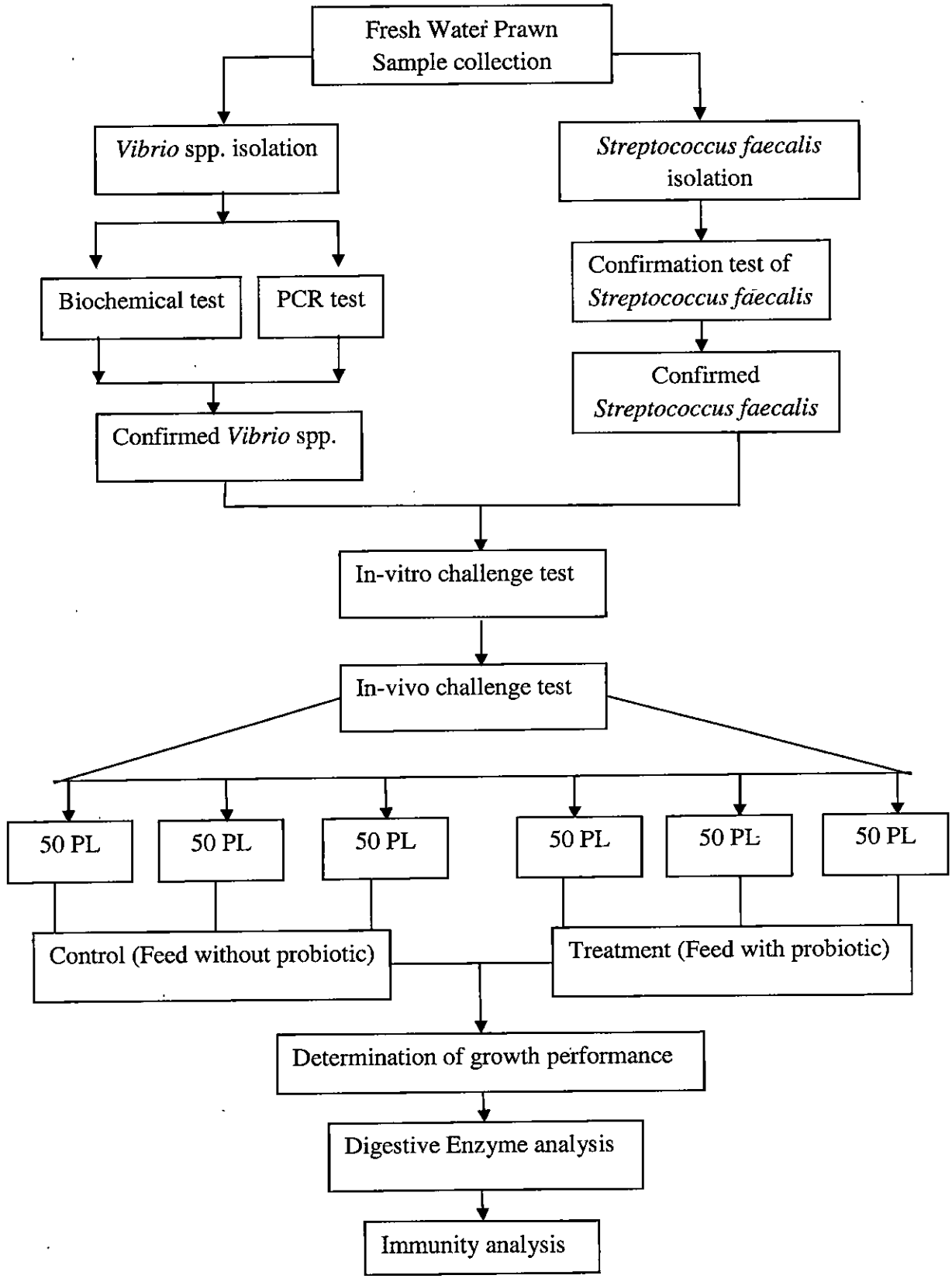

Figure 01: An outline of the steps and the experiments, conducted during the study

3.4. Media preparation and sterilization of equipments

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

Definite amount of TCBS Agar (44.50gm) was accurately weighed and taken in volumetric flask (500ml) containing distilled water (half of the required volume).Then the final volume (500ml) was adjusted by pouring additional distilled water. Then 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 20-30min for cooling and then transferred in required number of petridishes by 25ml volume for preparing plates.

3.4.2. Sterilization of different equipments

Test tubes, eppendorf tubes, plastic tips and other glassware were sterilized by autoclaving at a temperature of 121°C and a pressure of 15 lbs/sq inch. for 15 minutes. Petri dishes, isolating loops etc. were subjected to dry heat sterilization at 170°C for 1 hour in electric oven.

3.5. Isolation of pathogenic bacteria (*Vibrio* spp.)

3.5.1. Procedure for isolation and enumeration of *Vibrio* spp.

Vibrio spp. was isolated from the gills and intestinal tracts of the collected prawn 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 hour. 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 h. 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.

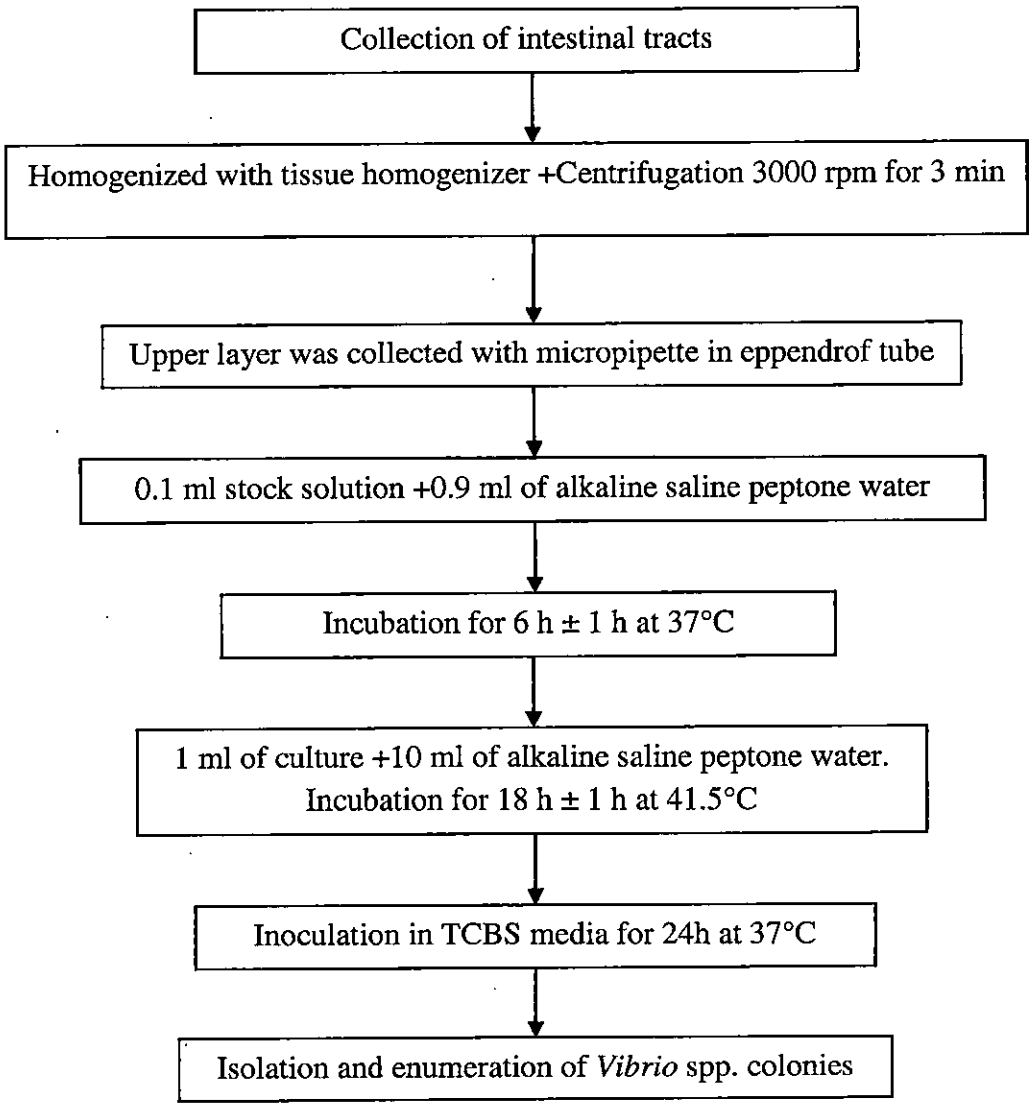

Figure 02: Flow chart of isolation and enumeration of *Vibrio* spp.

3.6. Confirmation of the isolates of *Vibrio* spp. by using PCR

The isolates, identified as *Vibrio* spp. by biochemical tests and PCR test were used to confirm the identification.

3.6.1. Preparation of media and culture of organism

Bacterial cultures on Nutrient broth (NB) were used to extract crude DNA, by the use of boiling cell extraction method. Nutrient broth (NB) was prepared by mixing 2.8g NB powder in 100 ml water and then it was autoclaved (121°C, 20 min), then 5 ml NB broth was taken into test tube. A single colony of bacterial cells was suspended in 50 µl of distilled water and vortexed shortly. The suspension was heated at 99°C for 5 min and then was chilled in ice for 10 min. The cell debris was pelleted by centrifugation at 13,400 rpm for 2 min and 10 µl

of clear supernatant was used as the DNA template in a PCR (Paydar, 2013). 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).

3.6.2. DNA Extraction

According to the Invitrogen 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. At the very beginning of extraction process, 2 ml (1 ml in each tube) cultured sample was centrifuged at 10000 rcf for 5 minutes with a centrifuge machine (Vision Micro High Speed Refrigerated Centrifuge, Model no: VS-15000 CFN II). At the end of centrifugation, clear supernatant layer was removed by a pipette (1000 µl) and deposited plate layer was kept for further procedure. 500 µl DNA zol reagents were added to each tube of a sample pair. The plate was dissolved completely by gentle pipetting and the dissolved sample in pair tube was mixed up to keep into a single tube. The dissolved cells were centrifuged for 10 min at 10,000 rcf at 4°C or room temperature. Following centrifugation, the resulting viscous supernatant was carefully transferred to a fresh tube. This step removes insoluble tissue fragments, RNA, and excess polysaccharides from the lysate or homogenate. This process was performed in order to minimize RNA carry-over into the DNA. DNA from the lysate was precipitated by the addition of 0.5 ml of 100% ethanol per 1 ml of DNA zoL Reagent used for the isolation. Samples were mixed by inversion and store them at room temperature for 1-3 minutes. Then it was centrifuged for 10 minutes at 10000 rcf in cool temperature. DNA should quickly become visible as a cloudy precipitate. Carefully the supernatant was decanted leaving the DNA pellet near the top of the tube. The tubes were placed upright for 1 min and was aspirated the remaining lysate from the bottom of the tube. The DNA precipitate was washed twice with 0.8-1.0 ml of 75% ethanol. At each wash, the DNA in ethanol suspended by inverting the tubes 3-6 times. The tubes were stored vertically for 0.5-1 min to allow the DNA to settle to the bottom of the tubes and remove ethanol by pipetting. At each wash, the DNA in ethanol was vortexed and was suspended by centrifugation for 10 minutes at 10000

rcf. After removing the ethanol, the DNA was air dried by storing in an open tube for 5-15 seconds. (If the DNA is exposed to air for more than a few seconds, it will be much more difficult to dissolve.). The DNA was dissolved in 8 mM NaOH by slowly passing the pellet through a pipette tip. Use of the 8 mM NaOH was assured full solubilization of the DNA precipitate. 100 µl of 8 mM NaOH was added to the DNA precipitate to dissolve. Then it was centrifuged for 3 minutes at 10000 rcf.

3.6.3. Qualitative determination of extracted DNA (Agarose gel electrophoresis)

Purity of extracted DNA sample was tested by agarose gel electrophoresis as described by Sambrook *et al.,* (1989). A horizontal electrophoresis apparatus (Bioneer, Korea) was used for the electrophoresis process. 1.5% agarose gel was used. The gel was prepared with 150 ml 1x TAE buffer, 2.25 g agarose powder and 2 µl ethidium bromide. A constant volt of 100 V was passed through the gel, which was merged in sufficient 1x TAE buffer. A 100 bp marker was loaded along with the sample for the comparison of the size of DNA. Supply of power for electrophoresis was continued until DNA migrated near about the two-third of the gel towards the positive electrode. DNA bands were visualized by UV illumination and photographed using the High Performance UV Transilluminators, (Ultra-Violet Products Ltd., UK).

3.6.4. Description of Target gene and primers

Target gene: The target gene was chosen for this investigation included: virA gene for *Vibrio*. One pair of specific primer was designed from above mentioned gene sequence (Table 01).

Table 01: Specific gene for pathogenic bacteria detection

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

3.6.5. 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, one pair of primers was used. Sequences of the one PCR primer pair and size of expected amplification products are shown in Table 02.

Table 02: Primers and expected size of PCR amplified gene targets of pathogenic bacteria

Specie	Primers	Sequence (5' →3')	Product size (bp)	Temperature (°C)
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 3.

Table 03: The component of reaction mixture for the PCR

Component	Final volume
De-Ionized Water	7.8 µl
10X Reaction Buffer (Tris; pH 9.0, 15 mM $MgCl_2$)	5 µl
10 mM dNTP mixture (each dNTP 2.5 mM)	2 µl
Template	2 µl
Primer	2 µl (Forward 1 µl + Reverse 1 µl)
A *Taq*Polymerase (5 units/µl; Top DNA Polymerase, Bioneer, Korea)	1 µl

3.6.6. Top DNA polymerase enzyme

The enzyme was isolated room recombinant *E. coli*. Strain containing the DNA polymerase gene from *Thermus thermophillus*. 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 mM Tris-HCL, 0.1 mM EDTA, 1mM DTT, Stabilizers, 50% Glycerol, pH 8.2) at 4: 1 ratio (Buffer : Enzyme).

3.6.7. Deoxynucleotide Triphosphates (dNTPs) mixture

In this experiment, 10 mM dNTP mixtures (each dNTP 2.5 mM) was used (Bioneer, Korea).

3.6.8. Buffers used for PCR

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

3.6.9. 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 49.5°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, 2 µl DNA extract were directly used as template in each PCR-tube for the reaction. The reaction mixture consisted of 2 µl primer (Forward 1 µl + Reverse 1 µl), 5 µl 10x Reaction buffer, 2 µl dNTPs, 1 µl 5 unit Top DNA Polymerase enzyme as well. These all components were mixed in 13 µl di-ionized water and thus a total of 25 µl PCR sample was prepared in a 0.2 ml PCR-tube (Eppendrof, Humburg, Germany) to operate in a thermal cycler. In PCR running, control reactions consisted of reaction mixtures with no template DNA.

3.6.10. Evaluation of PCR products

After completion of the thermal cycling, a sample of 10 µl from each PCR products was analyzed electrophoretically by running on a 1.5% agarose gel following the procedure described in the "Qualitative determination of extracted DNA (Agarose gel electrophoresis)" and the amplified product size was determined by comparison with a 100 bp DNA size marker.

3.7. The identification of *Vibrio harveyi*

The identification of the *Vibrio harveyi* colonies was done by performing various biochemical tests viz, motility test, 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).

3.7.1. Biochemical tests

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

3.7.1.1. Gram Staining

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

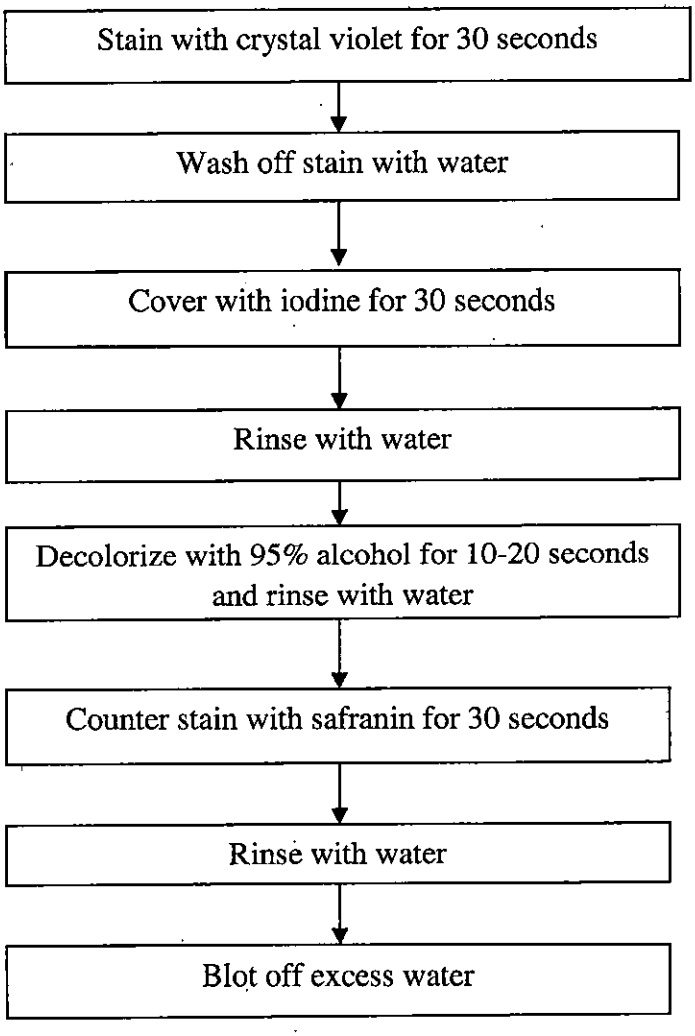

Figure 03: Flow chart of identification of gram positive and gram negative bacteria.

3.7.1.2. 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).

3.7.1.3 Voges-Proskauer (VP) test

The VP test was performed after completion of the methyl red test adding 0.6ml 5% α-naphthol solution and 0.2 ml 40% KOH aqueous solution; shake well, slope the tube, and examine after 15 min and 1 h. A positive reaction is indicated by a strong red color (Barritt, 1936).

3.7.1.4. Arginine hydrolysis

Arginine hydrolysis inoculated 5ml Arginine broth and after incubation for 24h adding 0.25ml Nessler's reagent. Arginine hydrolysis is indicated by the development of a brown color (Cowan and Steel's, 1993).

3.7.1.5. MR test

The MR test inoculated the isolated bacteria with buffered glucose broth and incubated at 37°C for 48h. After incubation adding a few drops of methyl red solution to the culture, read immediately. A red color represents a positive test (Cowan and Steel's, 1993).

3.7.1.6. Motility test

The motility test stabbed inoculates tubes of motility medium to a depth of about 5mm. Incubate at or below the optimum growth temperature. Motile organisms migrate throughout the medium, which becomes turbid; growth of non-motile organisms is confined to the stab inoculum (Cowan and Steel's, 1993).

3.7.1.7. 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.

3.7.1.8. Growth at different temperature range

The isolated *Vibrio* spp. was kept in incubator after inoculation at subsequent interval at temperature (4°C, 28°C, 37°C and 55°C).

3.8. Isolation of probiotic bacteria (*Streptococcus faecalis*)

3.8.1 Preparation of KF Streptococcal Agar Media

Definite amount of KF Streptococcal agar (7.64g) was accurately weighed and taken in volumetric flask (200ml) containing distilled water. Then the final volume (200ml) was adjusted by pouring additional distilled water. Then the volumetric flasks were kept in autoclave for sterilization. Autoclave was done at a temperature of 121°C and pressure of 15 lbs/sq inch for 15 minutes. After autoclave 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 plates.After completion of autoclaving, the media was kept aseptically (hygienically) in the bio-safety cabinet for 20-30 min for cooling at 45-50°C. Then added 2 ml of TTC 1 % supplement in 100 ml media. The TTC 1 % supplement is previously reconstituted in 5 ml of sterile distilled water. The following formula was followed for preparation of TTC 1%. Then the media was transferred in required number of Petri dishes by 25 ml volume for preparing the plates.

3.8.2. Procedure for isolation and enumeration of probiotic bacteria (*Streptococcus faecalis*)

Streptococcus faecalis was isolated from intestinal tracts of the collected prawn 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 KF Streptococcal agar media and incubated at 37°C temperature for 24-48 hours. After incubation at 37°C temperature for 24–48 h, red-colored colonies were collected and preserved for further experiment (Mario, R. C.1976 and Donnelly *et al.*, 1992). After the specified period of incubation the bacterial colonies (30 to 300) of each Petri dish were counted, using the colony counting equipment.

The colony was enumerated with the following formula,

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

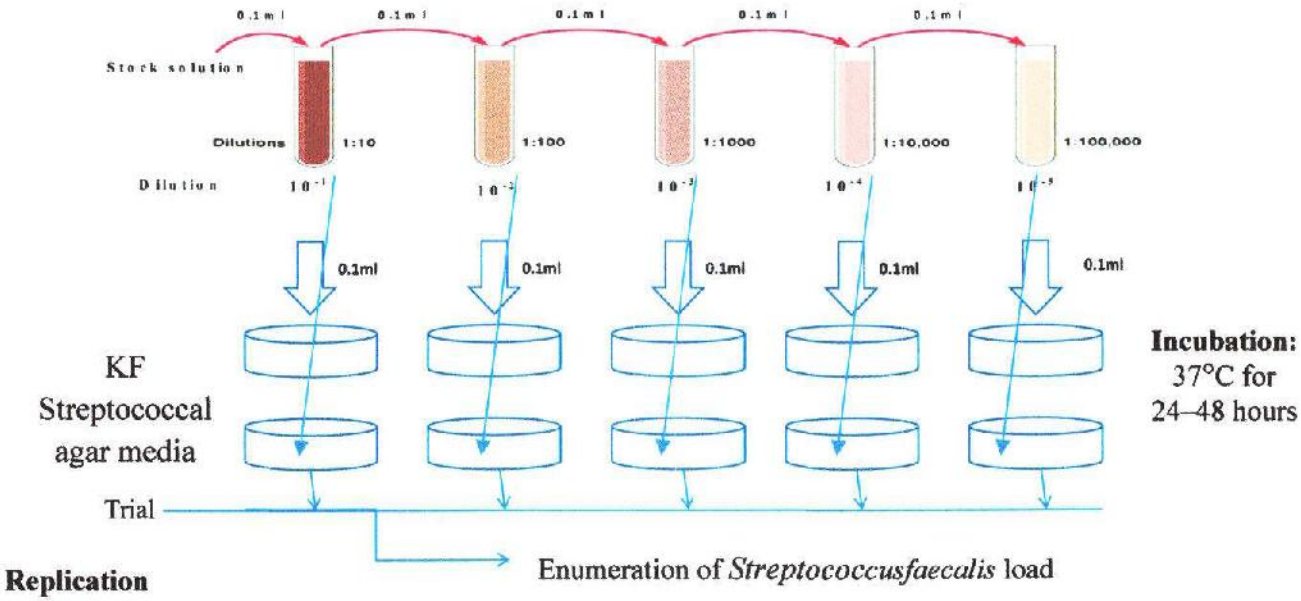

Figure 04: Isolation and enumeration of probiotic bacteria (*Streptococcus faecalis*) from intestinal tracts of selected samples.

3.8.3. Confirmation test of *Streptococcus Faecalis*

3.8.3.1. SF (*Streptococcus faecalis*) broth media preparation

SF (*Streptococcus faecalis*) broth was used for the confirmation test of *Streptococcus faecalis*. An amount of 3.6 g media was dissolved in 100 ml of distilled water. Then the media were mixed well and dissolved by frequent agitation. After that, the media was sterilized in autoclave at 121°C for 15 min. After completion of autoclaving, the media was aseptically made to utilize for test samples.

3.8.3.2. Procedure for the conformation test of *Streptococcus faecalis*

Conformation test of *Streptococcus faecalis* was performed by the inoculation of isolated bacteria with SF (*Streptococcus faecalis*) broth and incubated at 45-46°C for 18-48 h. A positive reaction was indicated by turbidity and a yellow-brown color due to the fermentation of dextrose (Hajna and Perry, 1943; Wehr and Frank, 2004; Eaton *et al.*, 2005; Downes and Ito, 2001).

3.9. *In-vitro* challenge test and determination of antagonistic activity of the probiotic (*Streptococcus faecalis*)

After identification of *Vibrio harveyi* by biochemical 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 an subsequent intervals of 4 hour up to 12 hour. This procedure was done for 2 times. Test solution of *V. harveyi*; without probiotic was also inoculated in TCBS agar media at 0 hour, 4th hour, 8th hour and 12th hour subsequently. All the inoculated TCBS agar plates were incubated at 37°C for 24± 3 hours. Standard plate count was done after incubation.

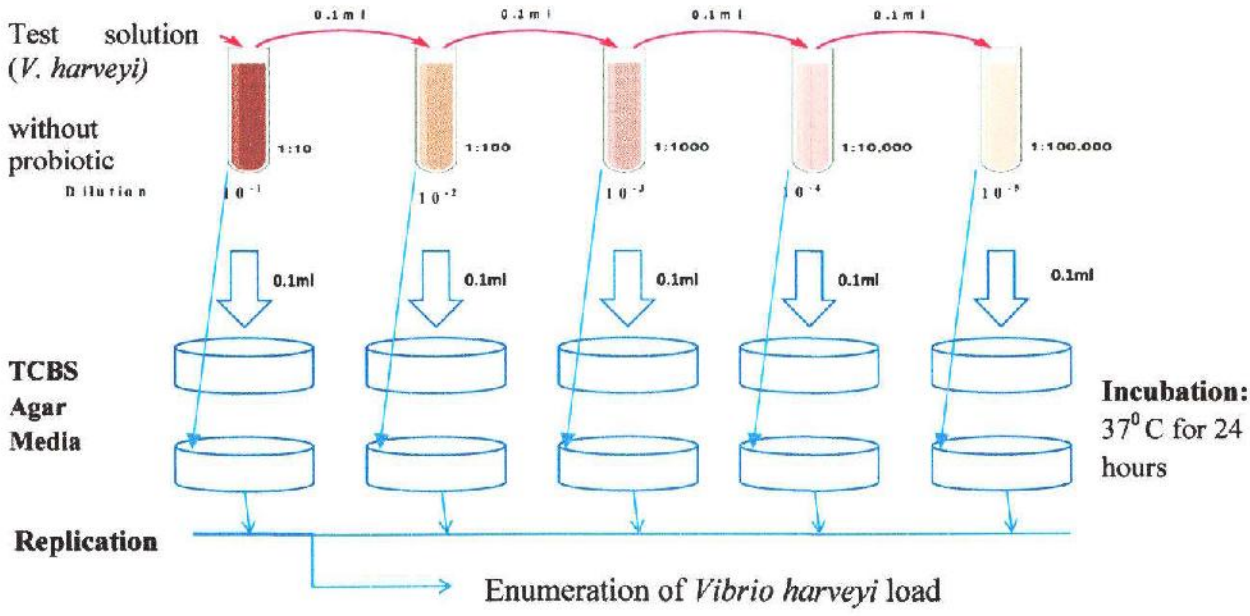

Figure 05: *In-vitro* challenge test and enumeration of *Vibrio harveyi* load without probiotics.

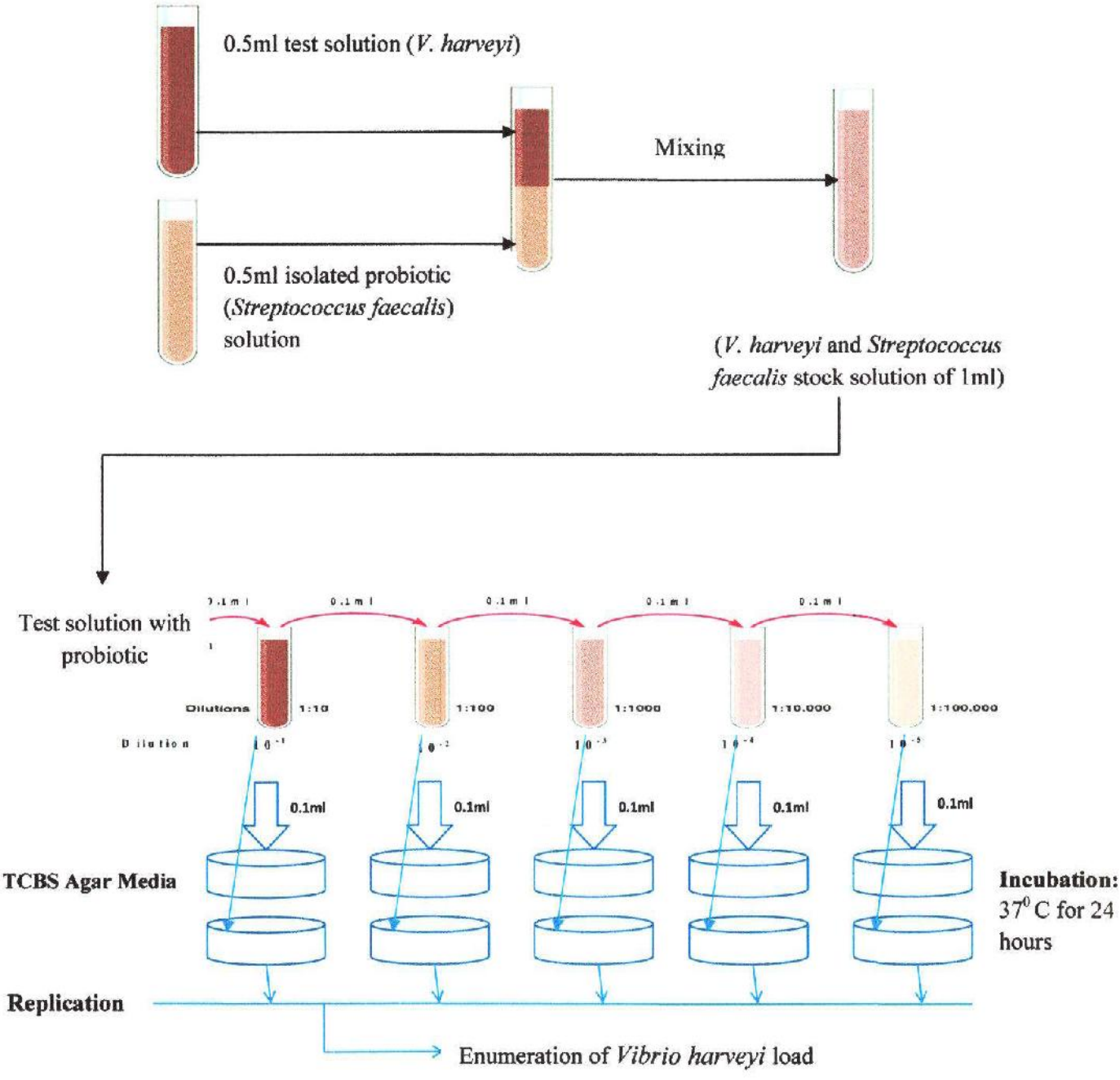

Figure 06: *In-vitro* challenge test and enumeration of *Vibrio harveyi* load with probiotics.

3.10. *In-vivo* challenge test and determination of antagonistic activity of the probiotic *Streptococcus faecalis* against *Vibrio* spp.

3.10.1. Experimental design

Four earthen ponds were selected for the experiment. There were one control and one treatment with three replicate. Fishes in control group were given commercial feed without

any probiotic and fishes of each replicate under the treatment were given feed mixed with probiotic for about 60 days.

3.10.2. 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. 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. It is also 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 and then examined holding against the sun and the ponds were ready for stocking.

3.10.3. 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 16thJune. 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 on-station experiment was carried out in 4 ponds of 70 m^2area and 1 m mean depth of each whereas each pond contains 50 fingerlings. Three of them (ponds) were given feed mixed with probiotic named pond 1, pond 2, pond 3 and the rest one (pond 4) is control where feed without any probiotic was given for a period of 60 days. After stocking, prawn feed at 7% of their body weight was used at the first time. The water quality was maintained in the acceptance level.

3.10.4. Probiotic bacteria treatment

For preparing the probiotic incorporated feed, 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 CFUg-1 in feed and the mixture was spread out and aseptically dried in an oven for 1-2h at 60^0 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 prawns were cultured in the research ponds about 60 days (Ziaei-Nejad *et al.*, 2005).

3.10.5. Enumeration of *Vibrio harveyi* load in intestine of prawn with and without *Streptococcus faecalis*

After 60 days of probiotic supplementation, *Vibrio harveyi* enrichment of stock solution of each sample was done. Then obtained solution was serially diluted and suitable dilution of the solution was cultured in TCBS agar media at 37°C for 24 h ± 3 hours and *Vibrio harveyi* enumeration was done.

3.11. 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); Kumar *et al.*, (2013); Elumalai *et al.*, (2013) and Ziaei-Nejad *et al.*, (2005):

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

$$\text{Specific growth rate (SGR, \%)} = \frac{(\ln W_t - \ln W_o)}{t} \times 100$$

Where t is the culture period in days, $\ln W_0$ is the natural logarithm of the weight of the prawn at beginning of the experiment, and $\ln W_t$ is the natural logarithm of the weight of the prawn at day t (W_0 and W_t are in gm).

3.12. Digestive enzyme analysis

3.12.1. 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 Homoginizer in 700×10 rpm for five minutes and were centrifuged at 4800×g for 60 min at 4^0 C according to the method outlined in Lovett and Felder, (1990). The supernatant of each sample was assayed in triplicate.

3.12.2. 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. 250 microliters of the sample was incubated for 30 min at 35°C with 260 µlTris-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 used to enable calculation of the amount of maltose released during amylase assays (Mehrabadi and Bandani, 2009).

3.12.3. 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 at 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 polyethersulfone 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 used to enable calculation of the amount of tyrosine released during protease assays (Prasad *et al.*, 2013).

3.13. Immunity assay

3.13.1. Prawn haemocyte analysis

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

3.13.2. 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 preloaded 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, 25 gauge needle attached to a 1 ml syringe which were preloaded 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; NaCl 0.45 M; glucose 0.1 M, adjusted to pH 4.6 with 1 M HCl (Soderhall and Smith, 1983)] and 0.5 ml of haemolymph was extracted.

3.13.3 Haemolymph preparation

Immediately after Haemolymph collection, a pre-chilled, 0.5ml 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).

3.13.4. 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.

3.13.5. 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 I OX 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.

3.13.6. 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).

3.13.7. Calculating Formula

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

Total Haemocyte count (cell/mm^3) = (cell counted x blood dilution x chamber volume) / Area of chamber counted

3.13.8. Determination of Blood dilution

Since, 0.05mI (50 µL, 0.5 marked) blood mixed with preloaded 0.95m1 (950 µL, ll 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.

3.13.9. Determination of chamber volume

Determining volume of each large corner square = 1mm×1mm×0.1mm = 1mm×1mm× 1/10mm= 1/10(10x10x1) mm^3 = 1/10x100mm^3 = 10mm^3

THC (cell/mm^3) = (cell courted) x (20) x (10)/4

THC (cell/mm^3) = (cell counted) x 50

3.13.10. 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 were used to take well-mixed blood cell suspension (where 0.5 ml blood sample stained with equal volumes of Giemsa and were mixed with 0.95 ml anticoagulants

5. Then first 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 millimeter = (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.

3.14. Data collection and analysis

During the experiment the data was recorded, accumulated, grouped and interpreted according to the objectives as well as parameters. All values were expressed as mean ± standard deviation. Data were analyzed using independent sample T-Test. A value of $P<0.05$ was considered statistically significant.

Chapter Four

RESULT

Chapter 4. Result

4.1.Bacterial load in the intestinal tracts of the collected prawn samples

4.1.1. Enumeration of *Streptococcus faecalis* and *Vibrio* spp. load in the intestinal tracts of prawn samples

The average load of *Streptococcus faecalis* in the intestinal tracts of the prawn samples were (4.23±0.03) × 10^4 CFU/g (Appendix-1). The average load of *Vibrio* spp. in the intestinal tracts of the prawn samples were (1.53±0.04) × 10^4 CFU/g (Appendix-2).

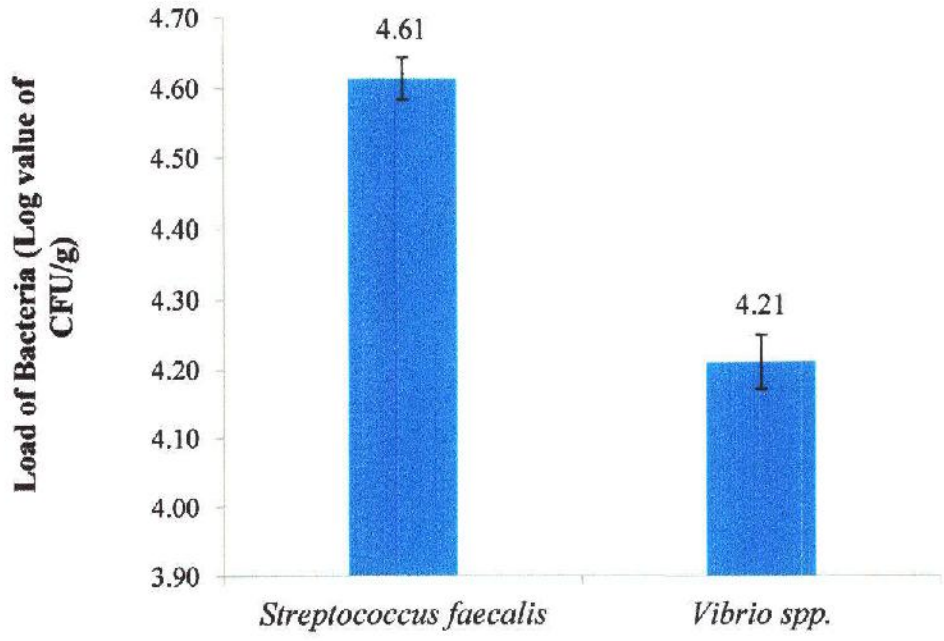

Figure 07: Average load of *Streptococcus faecalis* and *Vibrio* spp. in the intestinal tracts of collected prawn samples.

4.1.2. Conformation test of *Streptococcus faecalis*

Conformation test of *Streptococcus faecalis* was inoculated isolated bacteria with SF (*Streptococcus faecalis*) broth and incubated at 45-46°C for 18-48 h. After incubation it reflected yellow-brown colour that indicated the presence of *Streptococcus faecalis* in the collected samples.

4.2. Detection of pathogenic bacteria *Vibrio* spp. by using PCR

Three bands of target gene were found on electrophoresis gel at 663 bp position for *Vibrio* spp. which indicated the presence of pathogenic *Vibrio* spp. in prawn sample. The presence of pathogenic bacteria *Vibrio* spp. in prawn sample is shown in Figure 08.

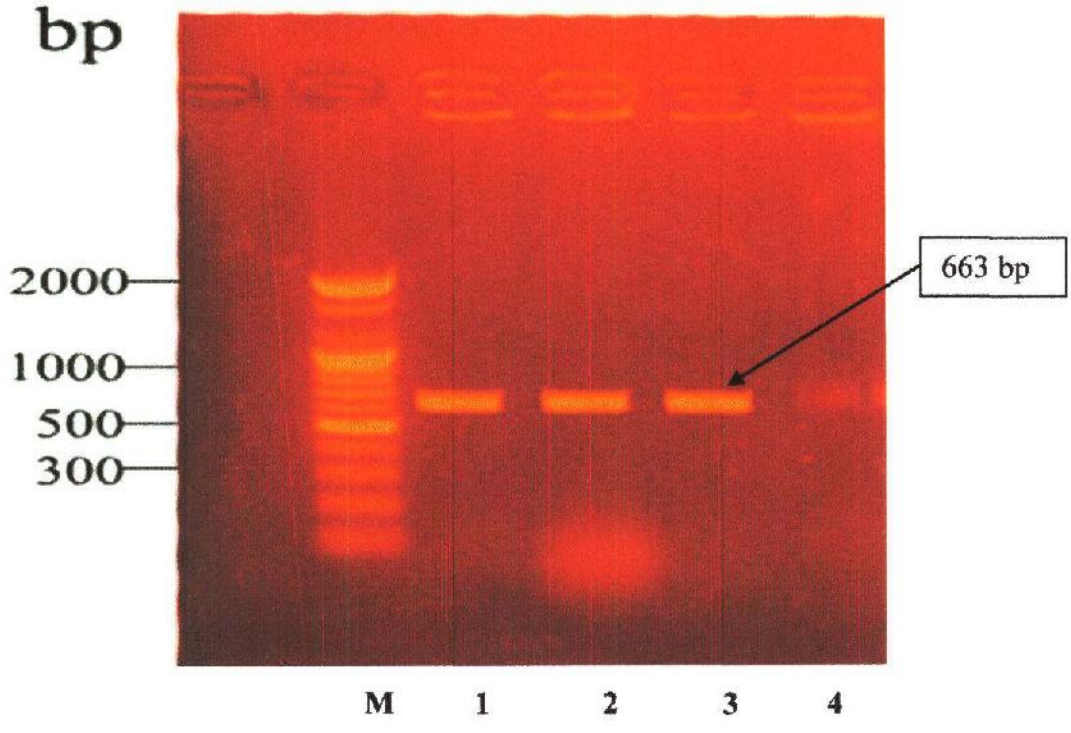

Figure 08: Electrophoretic analysis of PCR-amplified target gene from *Vibrio* spp. obtained under optimal conditions of PCR of isolates from biochemical test. Mobilities of the target gene are indicated on the right. Lane M, 100-bp DNA ladder (size marker); lane 1-2, gene for stock sample amplimers; lane 3, gene for isolated colony amplimers and lane 4, gene for control group amplimers.

4.3 Biochemical test

Nine biochemical tests were performed (Table 04) to identify *vibrio harveyi* from the isolated colonies of *Vibrio* sp. 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 dendogram 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 *Vibrio harveyi*, as proposed by Cowan and Steel's (1993) was followed for primary identification.

Table 04: Biochemical test to identify *Vibrio harveyi.*

Biochemical test	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Gram stain	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	N/D	+	-	+	N/D	+
Indole formation	+	+	+	-	-	+	-	+	-	+
VP test	-	-	-	-	+	-	-	-	+	-
MR test	+	+	+	+	-	+	N/D	+	-	+
Growth in NaCl at										
0%	+	+	+	+	+	+	+	+		+
1%	+	+	+	+	+	+	+	+		+
3%	+	+	+	+	+	+	+	+	N/D	+
5%	+	+	+	+	+	+	+	+		+
7%	-	-	-	-	+	-	-	-		-
10%	-	-	-	-	N/D	-	-	-		-
Growth at										
4°C	-	-	-	-	N/D	-	-	-	N/D	-
28°C	+	+	+	+		+	+	+		+
37°C	+	+	+	+		+	+	+		+
55°C	-	-	-	-		-	-	-		-
Colony color on TCBS	G	G	G	Y	G	G	G	G	Y	G
Identification	Vh	Vh	Vh	Vs	Vs	Vh	Vs	Vh	Vs	Vh

N.B: Vh = *Vibrio harveyi*, + = positive, - = negative, G = Green, Y = Yellow, Vs = *Vibrio* Spp.; ND = Different reactions given by lower taxa (genera, species, varieties); C1, 2....., 10 = Number of colonies.

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 grow to 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 plate. The biochemical test was done with 10 colonies of *Vibrio* spp. From the conformation test it was found that 80% was *Vibrio harveyi* among 10 *Vibrio* spp. After that with the isolated *Vibrio harveyi* colonies were further in-vitro tested to determine the antagonistic effect of *Streptococcus faecalis* on *Vibrio harveyi.*

4.4. *In-vitro* challenge test of intestinal tracts with and without probiotic (*Streptococcus faecalis*)

When *in-vitro* challenge test was performed, the average load of *Vibrio harveyi* in the intestinal tract of prawn in the control group at zero (0) hour was (4.69±0.29) × 10^3 CFU/g and it was (2.30±0.08) ×10^4, (2.36±0.24) ×10^5 and (4.24±0.12) ×10^5 CFU/g at 4th, 8th and 12th hour respectively. However, after treatment of isolated probiotics, *Streptococcus faecalis* the average load of *Vibrio harveyi* in the intestinal tract at 4th, 8th and 12th hour was reduced to (1.11±0.08) ×10^4, (7.34±0.17) ×10^3 and (1.05±0.24) ×10^3 CFU/g (Appendix-3) respectively. The present study showed a slight reduction of *Vibrio harveyi* load in the experimental intestinal tracts at the 4th hour of probiotic application whereas significant ($P<0.05$) reduction of *Vibrio harveyi* load was obtained at the 8th and 12th hour of probiotic application (Appendix-9, Figure 09).

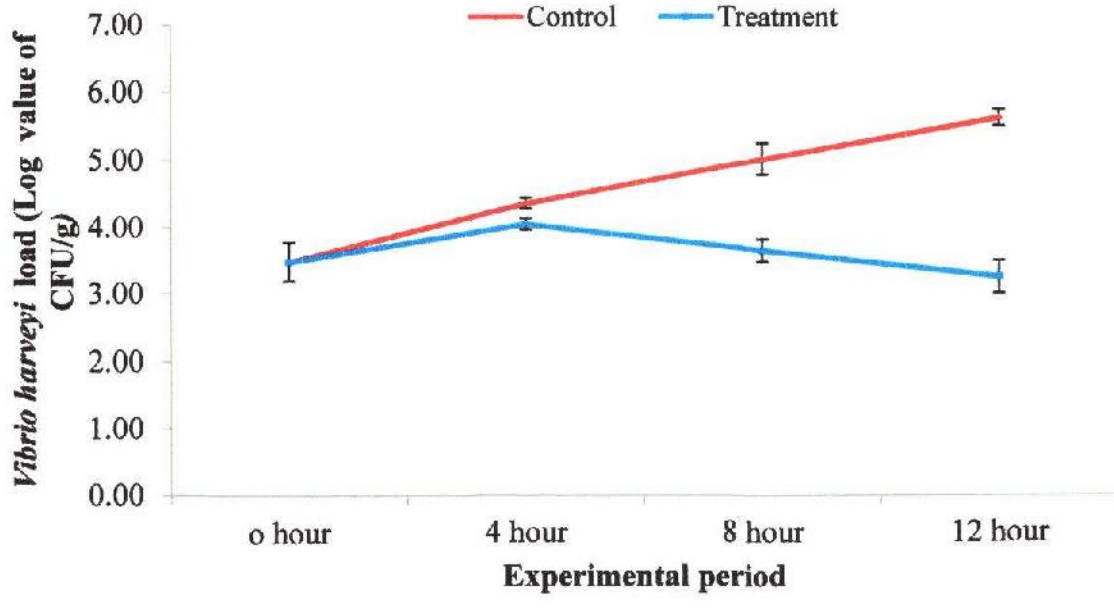

Figure 09: *In-vitro* challenge test and enumeration of *Vibrio harveyi* load with and without probiotics (*Streptococcus faecalis*). Each value is given as mean ± standard deviation.

4.5. *In-vivo* challenge test of intestinal tract with and without probiotic (*Streptococcus faecalis*)

In *in-vivo* challenge test the average load of *Vibrio harveyi* in the intestinal tract of prawn in the control group at 10 days, 20 days, 30 days, 40 days, 50 days and 60 days were $(2.37\pm0.06)\times10^5$, $(2.65\pm0.10)\times10^5$, $(1.49\pm0.09)\times10^6$, $(3.45\pm0.08)\times10^6$, $(5.74\pm0.07)\times10^6$ and $(6.85\pm0.08)\times10^6$ CFU/g respectively. After application of probiotic, *Streptococcus faecalis* the average load of *Vibrio harveyi* in the intestinal tract of prawn at 10 days, 20 days, 30 days, 40 days, 50 days and 60 days became $(2.31\pm0.08)\times10^5$, $(2.21\pm0.09)\times10^5$, $(1.42\pm0.08)\times10^5$, $(2.70\pm0.08)\times10^4$, $(1.44\pm0.09)\times10^4$ and $(1.03\pm0.10)\times10^4$ CFU/g respectively (Appendix-4). The present study showed no significant ($P>0.05$) reduction of *Vibrio harveyi* load in the experimental intestinal tract at 10 and 20 days of probiotic application whereas it was significant ($P<0.05$) at 30 days to rest of the time of probiotic application (Appendix-10, Figure 10).

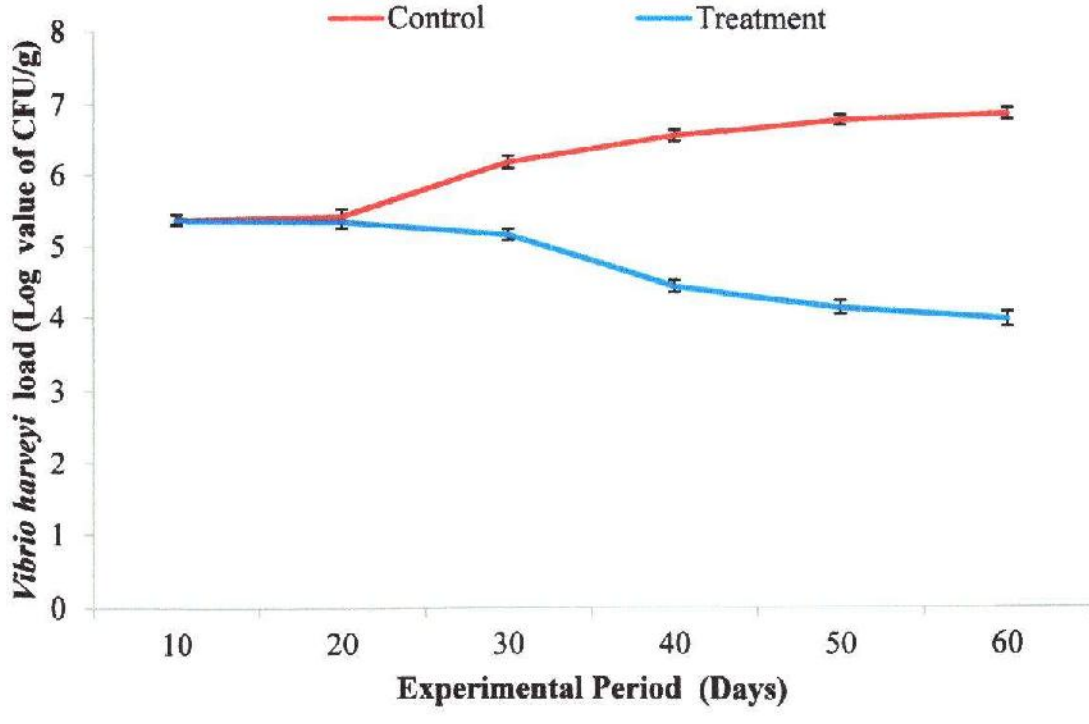

Figure 10: *In-vivo* challenge test and enumeration of *Vibrio harveyi* spp. load with and without probiotics (*Streptococcus faecalis*). Each value is given as mean ± standard deviation.

4.6. Growth performance

The effect of diet containing *Streptococcus faecalis*, on growth performance was established in this study. Analyzed data on the growth performance of freshwater prawn in treatment and control including initial weight, final weight, weight gain and specific growth rate are showed in (Appendix-5). At the end of the experiment, statistical analysis showed that prawn fed *Streptococcus faecalis* diet grew significantly faster than the control group. Final weight was recorded significantly different ($P<0.05$) between treatment (16.84 ± 0.24g) and control (10.34 ± 1.03g) group. The weight gain of the prawns fed *Streptococcus faecalis* incorporated diets were found to be significantly ($P<0.05$) increased (481.22 ± 36.61%) when compared with control (371.31 ± 43.95%) group (Appendix-5, 11; Figure 11). The better growth achieved in *Streptococcus faecalis* incorporated diet fed prawns indicates that probiotics have the characteristic ability of growth promotion in *M. rosenbergii*. The specific growth rate was significantly higher ($P<0.05$) in probiotic treated prawn (2.93 ± 0.14%) compared with control group prawn (2.60 ± 0.24%) after 60 days feeding experiment (Appendix-5, 12; Figure 12).

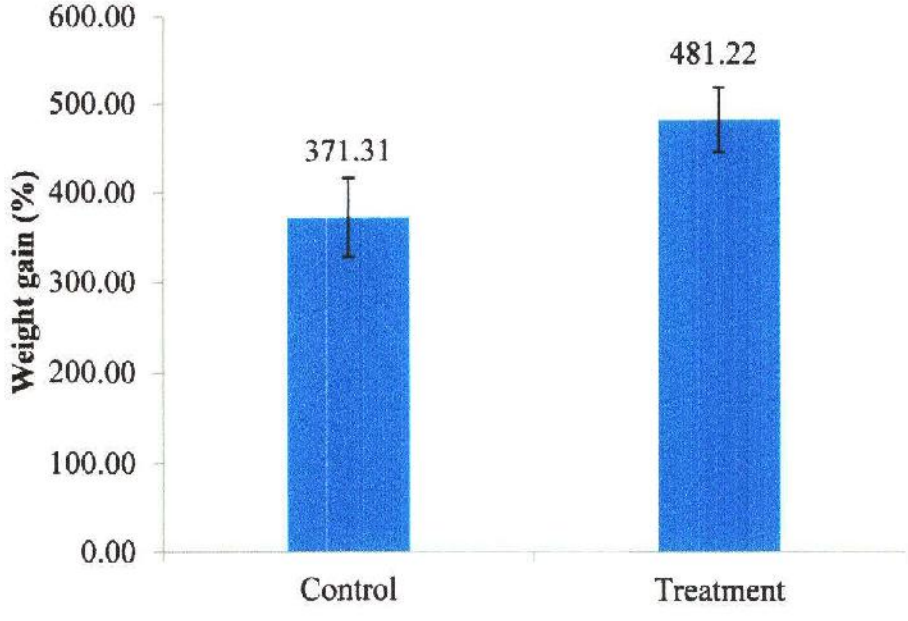

Figure 11: The weight gain of *M. rosenbergii* in treatment and control after 60 days fed with or without probiotic, *Streptococcus faecalis*. Each value is given as mean ± standard deviation. The mean differences are significant at ($P<0.05$).

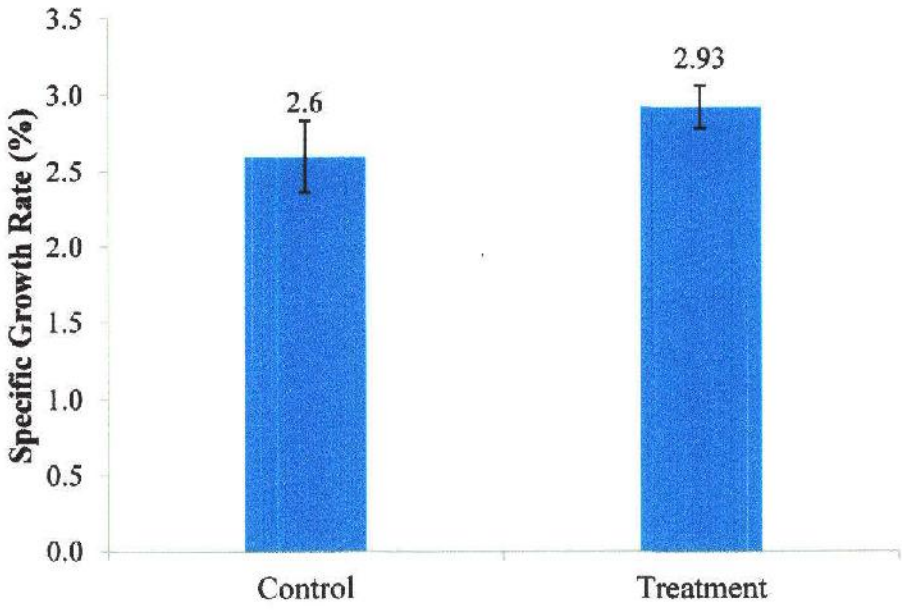

Figure 12: The specific growth rate of *M. rosenbergii* in treatment and control after 60 days fed with or without probiotic, *Streptococcus faecalis*. Each value is given as mean ± standard deviation. The mean differences are significant at (P<0.05).

4.7. Digestive Enzyme Activity

The specific amylase enzyme activities was significantly higher (P<0.05) in probiotic treated prawn (1.26 ± 0.08) Unit/mg intestine than in control group prawn (0.70 ± 0.03) Unit/mg intestine examined after 60 days of feeding experiment (Appendix- 6, 7, 13; Figure 13). The specific protease enzyme activities was significantly higher (P<0.05) in probiotic treated prawn (2.78 ± 0.02) Unit/mg intestine than in control group prawn (1.79 ± 0.02) Unit/mg intestine examined after 60 days of feeding experiment (Appendix-6, 8, 14; Figure 14). Clearly probiotic, *Streptococcus faecalis* showed their ability to increase digestive enzyme activities.

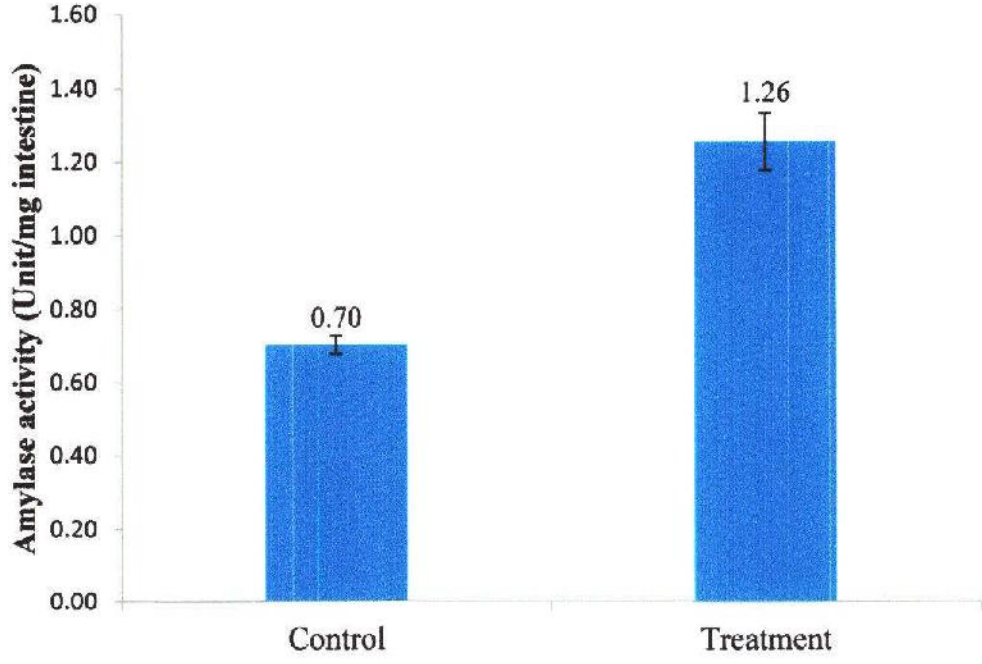

Figure 13: The specific amylase enzyme activity of *Macrobrachium rosenbergii* in treatment and control group after 60 days fed with or without probiotic, *Streptococcus faecalis*. Each value is given as mean ± standard deviation. The mean differences are significant at ($P<0.05$).

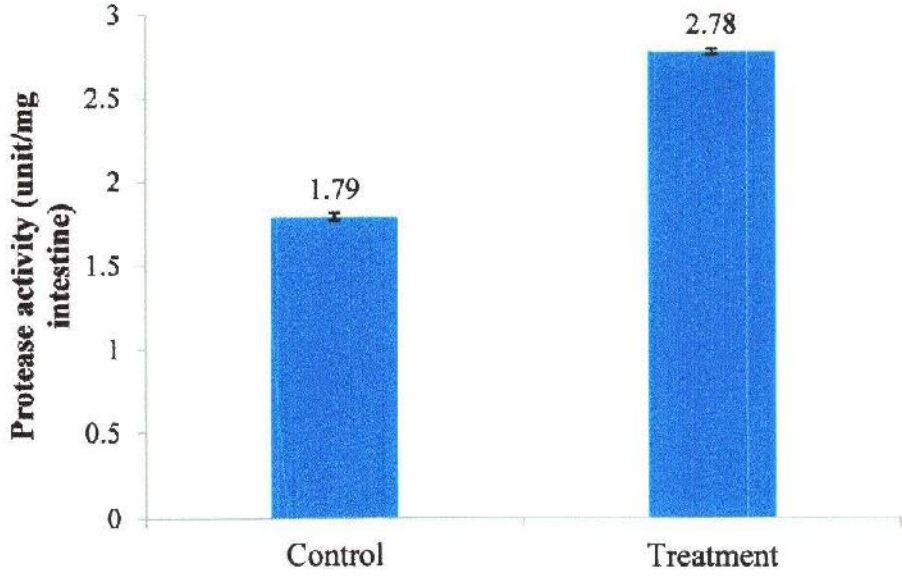

Figure 14: The specific protease enzyme activity of *Macrobrachium rosenbergii* in treatment and control after 60 days fed with or without probiotic, *Streptococcus faecalis*. Each value is given as mean ± standard deviation. The mean differences are significant at ($P<0.05$).

4.8. Prawn Haemocyte Analysis

Total haemocyte count (THC) of prawn fed probiotic, *Streptococcus faecalis* supplemented diet was found to be enhanced little bit in treatment group, although it was not significant ($P>0.05$) compared to control group. Total haemocyte count (THC) was higher (5.8 ± 0.17) $\times 10^6$ Cell/mm^3 in prawn receiving the probiotic than in control prawn (5.2 ± 0.31) $\times 10^6$ Cell/mm^{3}which had received no probiotic (Table 05). In this study, the prawn haemocyte population of the 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 nongranular haemocyte (NGH), small granular haemocyte (SGH) and large granular haemocyte (LGH) were identified in the present study. In control group nongranular haemocyte represented the highest percentage of haemocyte ($75.33 \pm 0.23\%$) followed by small granular haemocyte ($22.24 \pm 0.06\%$) and large granular haemocyte ($2.43 \pm 0.05\%$). In case of probiotic treated group about $76.42 \pm 0.11\%$ of haemocyte were NGH whereas only about $22.34 \pm 0.09\%$ and $2.24 \pm 0.09\%$ were SGH and LGH respectively (Figure 16). Thus both in the control and treatment group most of the haemocytes were belonged to nongranular haemocytes whereas a very few were belonged to large granular haemocyte.

Table 05: Total haemocyte count (THC) and differential haemocyte count (DHC) of *Macrobrachium rosenbergii* fed the control diet and probiotic, *Streptococcus faecalis*-supplemented diet after 60 days in the experiment. Each value is given as mean ± standard deviation.

Experimental group	THC (Cell/mm^3)	DHC (%)		
		Non granular	SGH	LGH
Control	$(5.2 \pm 0.31) \times 10^6$	75.33 ± 0.23	22.24 ± 0.06	2.43 ± 0.05
Treatment	$(5.8 \pm 0.17) \times 10^6$	76.42 ± 0.11	22.34 ± 0.09	2.24 ± 0.09

N.B: THC =Total haemocyte count, DHC = differential haemocyte count, SGH= Small granular haemocyte, LGH = Large granular haemocyte.

Chapter Five

Discussion

Chapter 5. Discussion

Manipulation of microbiota using probiotics has been reported as a worthy practice in aquaculture. Probiotic is used to improve the digestion process, nutrition level, growth performance, immune response and intestinal microbial balance (Verschuere *et al.*, 2000; Balcazar *et al.*, 2006; Perez *et al.*, 2010). It is also used to reduce pathogenic bacteria load in the host gastrointestinal tract by competitive exclusion (Holzapfel *et al.,* 1998; Rengpipat *et al.,* 1998; Uma *et al.,* 1999; Gomez-Gill *et al.,* 2000; Balcazar *et al.*, 2006) and to prevent antibiotic resistance, frequent outbreak of microbial disease that consequently ensure better health of aquatic animals.

Aquatic organisms often harbour a great number of bacteria in their intestinal tract, gills and body surface, which they acquire from water, sediment and food. Farmed freshwater prawn inhabits considerable populations of bacteria in their gastrointestinal tract (Lalitha *et al.*, 2010). The presence of different lactic acid bacteria namely *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae*, *Enterococcus durans*, and *Weissella hellenica* in the intestines of *Macrobrachium rosenbergii* has been reported in previous studies (De Man, 1879; Devriese *et al.*, 1987; Cai *et al.*, 1998).

A reliable diagnosis is the initial part of a successful monitoring and surveillance plan for vibrios investigations. Detection of the strains with high sensitivity and specificity is desirable. Laborious, expensive and time consuming methods are not appropriate for larger scales. There are lots of detection systems for bacteria like cultural and biochemical methods, PCR technology etc. In cultural method, detection of *Vibrio* spp. has consisted of enrichments steps and isolation steps on selective agar medium followed by biochemical testing. In PCR method, target specific primers are used. In this study both cultural and molecular method were used for the identification and characterization of *Vibrio* spp. in *Macrobrachium rosenbergii.*

TCBS selective medium was applied for colony appearance based preliminary identification of *Vibrio* species. Colony appearance on selective media was followed by conventional biochemical tests, including MR test, VP test, Indole test, argentine hydrolysis and salt tolerance tests for detection of *Vibrio* spp. The overall findings of these tests indicated that they are able to be used for detection of vibrios at genus level. Although the majority of the results obtained from the biochemical tests indentifies vibrios, PCR

based molecular method is also used for confirmation. The results of this research indicated higher efficiency of Polymerase Chain Reaction (PCR) for identification of *vibrio* spp.

PCR-based detection targets the specific region of DNA, for identification of bacterial strains. Adhikari *et al.*, (2015) used a multiplex PCR system for the rapid detection of pathogenic bacteria *Vibrio sp.* in shrimp farms by targeting virA gene. Polymerase Chain Reaction targeting *gyrB, pntA,* 16S rRNA, *toxR, dnaJ, rpoB* gene was developed to detect *Vibrio* species (Oakey *et al.*, 2003; Le Roux *et al.*, 2004; Tarr *et al.*, 2007; Nhung *et al.*, 2007a,b; Teh *et al.*, 2010). In the present study, all the isolates were analyzed for the presence of virulence associated genes. The vir*A* gene specific for the genus *Vibrio* was used to confirm the genus in all isolates. All isolates amplified the vir*A* gene fragment (663 bp). So combination of biochemical tests and PCR assist in the confirmation of identity of isolates suspected to be *Vibrio* species in this study. On the other hand in the present study presence of probiotic *Enterococcus faecalis* in the intestine of study samples of *Macrobrachium rosenbergii* was confirmed by yellow-brown color due to the fermentation of dextrose in SF (*Streptococcus faecalis*) broth medium.

Gut bacterial microbiota in aquatic animals is mainly composed of Gram-negative bacteria, being the genera *Vibrio*, *Pseudomonas*, and *Aeromonas* predominant (Vine *et al.*, 2006). Nevertheless, several studies have demonstrated that the effect of such microbiota can be modified by the addition of Gram-positive bacteria in the diet (Ziaei-Nejad *et al.*, 2006; Vieira *et al.*, 2008) or in the culture water (Rengpipat *et al.*, 2000). Such amendment can be achieved using antagonistic effect of an agent that attenuate negative effects of other agents. *Streptococcus faecalis*, gram-positive bacterium exerts beneficial effects through preventing adhesion in receptor binding site and colonization of pathogenic bacteria (Krovacek *et al.*, 1987; Montes and Pugh, 1993), competing for nutrients that favour their growth (Gatesoupe, 1999), stimulating host immune system (Reid *et al.*, 2001; Merrifield *et al.*, 2010a) and secreting inhibitory substances like lactic acid, bacteriocins and other antimicrobial compounds in the mucus layer of the host (Pulugurtha, 2014).

Bacterial adhesion to intestinal mucosal surface and subsequent colonization is important during the initial stage of pathogenic infection (Krovacek *et al.*, 1987). Higher affinity for adhesion receptors competitively excludes the pathogenic bacteria (Gatesoup, 2008), consequently provide protective barrier that might be the first possible mechanism of antagonistic effect of probiotic against pathogens (Montes and Pugh, 1993). Several

investigations have reported that probiotic strains of lactic acid bacteria positively influence the growth and survival of juveniles of shrimp providing protective barrier against pathogens *Vibrio harveyi* (Nikoskelainen *et al.*, 2001; Ajitha *et al.*, 2004; Ringø *et al.*, 2007a, 2007b; Vieira *et al.*, 2007; Vieira *et al.*, 2008; Salinas *et al.*, 2008b; Balc´azar *et al.*, 2009; Vieira *et al.*, 2010).

Probiotic microorganisms have the ability to release chemical substances with bactericidal or bacteriostatic effect on pathogenic bacteria that are in the intestine of the host, thus constituting a barrier against the proliferation of opportunistic pathogens (Verschuere, 2000). It has reported that lactic acid bacteria of *Streptococcus* and *Lactobacillus species,* in addition to lactic acid produce different chemical agents similar in activity as antibiotics (Mc Donald *et al.*, 2002; Klose *et al.*, 2010). *Streptococcus faecalis* produces inhibitory substances like lactic acid, peptide antibiotics like bacteriocins which has inhibitory action on the intestinal pathogens (Franz *et al.*, 2011; Bourouni *et al.*, 2012; Allameh *et al.*, 2014) such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Vibrio cholera, Vibrio harveyi* (Vaseeharan and Ramasamy, 2003; Campos *et al.*, 2006; Vieira *et al.*, 2007). Probiotic of lactic acid bacteria supplemented diet provide increased resistance to *V. harveyi* infection by producing antimicrobial agents such as hydrogen peroxide and different organic acids (Vieira *et al.*, 2010). Also, cytolysin, a two-peptide bacteriocin produced by *E. faecalis* possesses erythrocytes and prokaryotic cell lytic activity (Booth *et al.*, 1996). These characteristic properties help the host in providing a defense against an intestinal organisms, acting as a colonization factor and facilitating nutrient acquisition from prokaryotic or eukaryotic sources (Tyne *et al.*, 2013). Another inhibitory compound, enterocin AS-48 produced by *E. faecalis* S-48, a cyclic peptide exhibits bactericidal activity against a wide variety of Gram-negative and Gram-positive pathogenic bacteria such as *Bacillus cereus*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Staphylococcus aureus* and *Lactobacilious monocytogenes* (Cebrián *et al.*, 2012). Thus *Enterococcus* spp. is used as biological control agents in aquaculture (Calo-Mata *et al.*, 2007).

In the present study, pathogenic *V. harveyi* load was reduced and probiotic *Streptococcus faecalis* colony was significantly dominated in comparison to control. This result showed significant antagonistic effect of *Streptococcus faecalis* against *V. harveyi.* Adhesion receptor antagonism as well as inhibitory substance released by probiotic bacterium may be

the possible mechanism of antagonistic effect of *Streptococcus faecalis* in *M. rosenbergii* expled out in the present study.

Growth is an index of water quality and nutrients provided. As far as freshwater prawn culture is concerned, there are many factors relate the growth and feeding activity, which include a functional digestive system to efficiently utilize the nutrients present in the food offered and also physiological conditions and the rearing environment (Lee and Lawrence, 1997; New and Valenti, 2000; Anderson and De-Silva, 2003). In aquaculture, specific growth rate is a trait of great economic importance.

In the present study, prawn fed probiotic supplemented diet displayed a significantly better growth performance and higher specific growth rate than diet without probiotic. The specific growth rate in these study was significantly higher ($P < 0.05$) in probiotic treated prawn ($2.93 \pm 0.14\%$) compared with control group prawn ($2.60 \pm 0.24\%$) after 60 days feeding experiment. Likewise, Zokaeifar *et al.*, (2012) reported that administration of probiotic, *B. subtilis* strains has significantly increased specific growth rate ($2.97 \pm 0.14\%$) compared to control ($2.38 \pm 0.09\%$) after 55 days feeding experiment. These results also agreed with those of Elumalai *et al.*, (2013), who reported that administration of commercially available probiotics (*Streptococcus faecalis, Streptococcus faecium, Bacillus natto, Clostridium butyricum, Saccharomyces cerevivisiae*) in *Peneaus monodon* has significantly improved growth and survival of shrimp, increased beneficial bacteria and water quality in shrimp culture. Different probiotics supplemented diets such as Binifit™, *Lactobacillus sporogenes, Bacillus subtilis* and *Saccharomyces cerevisiae* have improved the growth, survival and energy utilization in PL of *M. rosenbergii* (Seenivasan *et al.*, 2011, 2012a-c, 2014a-c; Shinde *et al.*, 2008). In addition, positive effect of growth promotion in *M. rosenbergii* PL was observed using supplementary diet with *L. sporogenes* (Seenivasan *et al.*, 2014), *B. subtilis* (Keysami *et al.*, 2007), *L. acidophilus, L. sporogenes* (Venkat *et al.*, 2004) and *L. ceremoris* (Suralikar and Sahu, 2001). Similar results have been reported in *M. rosenbergii* fed with bio-encapsulated *Lactobacillus cremoris* (Suralikar and Sahu, 2001), *L. sporogenes, L. acidophilus* (Venkat *et al.*, 2004), Biogen® (Saad *et al.*, 2009), *Lactobacillus cremoris, L. sporogenes, L. acidophillus and S. cerevisiae* (Suralikar and Sahu, 2001; Venkat *et al.*, 2004; Seenivasan *et al.*, 2013). Dietary supplementation of *E. faecalis* and MOS (mannan oligosaccharide) in rainbow trout

strongly influenced growth performance and reduced cumulative mortality (Rodríguez-Estrada, 2013).

Streptococcus faecalis supplemented diet appeared to provide beneficial effects on the growth of *M. rosenbergii*. The findings of the present study revealed greater growth performance in *M. rosenbergii* fed *Streptococcus faecalis* supplemented diet. The overall growth response of *M. rosenbergii* may be due to increased digestibility (Shinde *et al.*, 2008) and use of vitamin B-complex produced by *E. faecalis* in the gut of *M. rosenbergii* (Goldin and Gorbach, 1992; Mondal *et al.*, 2003; Abraham *et al.*, 2007).

Digestion process consists of a series of enzymatic action that convert complex food particles into simple and absorbable compounds. The digestion processes of aquatic animals can be enhanced by addition of microorganisms that modulate digestion processes by producing supplementary extra-cellular digestive enzymes like amylase, proteases, lipases (Balcazar *et al.*, 2006; Vine *et al.*, 2006) and supplying fatty acids, vitamins and other nutritional components as growth factors (Suzer *et al.*, 2008; Avnimelech, 2009). Some of the microbes prevent gastrointestinal obstacles and anti-nutritional factors present in the ingested ingredients (Gatesoupe, 1999, 2007; Thompson *et al.*, 1999; Balcázar *et al.*, 2006). The probiotic microorganisms after transition through stomach, they attach in the intestine and use a large number of carbohydrates for their growth and produce a range of relevant digestive enzymes such as amylase, protease and lipase, that increase the digestibility of ingested foods (organic matter and protein), in return a higher growth rates due to stimulation of a pre-digestion of secondary compounds and also prevent intestinal disorders of host animal (Gatesoupe, 1999; El-Haroun *et al.*, 2006; Lara-Flores *et al.*, 2010).

The digestive enzyme activity in crustacean plays a central role in nutritional physiology and may directly or indirectly regulate growth (Lovett and Felder, 1990), moult cycle (Wormhoudt and Favrel, 1988) and dietary formulation (Le Moullac *et al.*, 1996). In the present study, *Macrobranchium rosenbergii* fed probiotic (*Streptococcus faecalis*) supplemented diet showed a significantly higher enzyme (amylase and protease) activities ($P < 0.05$) than control group.

Administration of the *Streptococcus faecalis* to *Macrobranchium rosenbergii* starts to consume carbohydrates for self-growth and produce a range of digestive enzymes such as

amylase and protease. The observed increases in specific activities of digestive enzymes in probiotic treatment may have led to increase digestion and enhanced absorption of food, which in turn contributed to the improved survival and growth in *Macrobrachium rosenbergii*. The specific amylase activity in these study was significantly higher ($P<0.05$) in probiotic treated prawn (1.26 ± 0.08) Unit/mg intestine compared with control group prawn (0.70 ± 0.03) Unit/mg intestine after 60 day feeding experiment. Likewise, Zokaeifar *et al.*, (2012) reported that administration of probiotic, *B. subtilis* strains has significantly increased amylase activity (1.19 ± 0.13) U mg^{-1} protein compared to control (0.68 ± 0.12) U mg^{-1} protein after 55 day feeding experiment. The specific protease activity was significantly higher ($P<0.05$) in probiotic treated prawn (2.78 ± 0.02) Unit/mg intestine than in control group prawn (1.79 ± 0.02) Unit/mg intestine 60 day feeding experiment. Likewise, Zokaeifar *et al.*, (2012) reported that administration of probiotic, *B. subtilis* strains has significantly increased protease activity (1.43 ± 0.20) U mg^{-1} protein compared to control (0.93 ± 0.03) U mg^{-1} protein after 55 day feeding experiment. Findings of our study are comparable to the previous studies that reported the increased protease and amylase activity after administration of probiotic products of *Streptococcus faecalis* in *M. rosenbergii* (Nimrat and Vuthiphandchai, 2011). Li *et al.*, (2010) reported that scallop (*Patinopecten yessoensis*) accumulated *E. faecalis* in larger numbers in the digestive tract and all scallops exposed to *E. faecalis* showed significantly higher trypsin and amylase activity. Similar improvement in activities of protease, amylase and lipase has been reported in *L. sporogenes*, *B. subtilis* and *S. cerevisiae* incorporated diet fed *M. rosenbergii* (Seenivasan *et al.*, 2014c).

Modulation of immune system is one of the most common benefits of the probiotics (Nayak, 2010). Prawn are invertebrates that lack adaptive immune responses (Sakai, 1999), which in higher animals are involved in antibody production and immunological memory. The key defence weapons of prawn are the non-specific immune systems in the hemocyte, which plays an important defense role against infections and injuries (Johansson *et al.*, 2000). They produce anti-microbial products of importance for defence and they carry out phagocytosis and remove foreign particles and infectious agents from tissues and body fluids (Jiravanichpaisal *et al.*, 2007).

The circulating haemocyte of decapods crustacean plays an important physiological functions including hardening of exoskeleton, wound repair, carbohydrate metabolism,

transport and storage of protein and amino acid and HL coagulation (Ratcliffe *et al.*, 1985; Martin *et al.*, 1991). In pathological concern, haemocytes play crucial role in nonspecific cellular immunity including the primary immune responses of recognition, phagocytosis, encapsulation, nodule formation, cytotoxic mediation and pro-phenol oxidase (PO) system activation (Anderson, 1992; Johansson *et al.*, 2000).

The present study showed that total haemocyte count (THC) of prawn fed probiotic, *Streptococcus faecalis*-supplemented diet was little bit increased than prawn from the control group. It indicates a little bit immunity enhancement of treatment group compared to the control group. Owens and O'Neill, (1997) found the similar result and concluded that there was no significant difference between the total cell counts in prawn body fed with probiotic mixed diet and without probiotic diet. Rengpipat *et al.*, (2000) also studied on immunity enhancement of probiotic treated feed and found that the total haemocyte number didn't vary significantly between treatment and control group. Rodriguez-Estrada *et al.*, (2013) reported that supplementation of inactivated *Enterococcus faecalis* cells enhanced immune parameters, including total hemocyte count, hematocrit value, phagocytic activity in rainbow trout. The immune stimulant capacity of *E. faecalis* shown in this study could be compared with those of other studies demonstrating that this probiotic was able to improve the non specific immune function of mice (Shimada *et al.*, 2009). In contrast, some finding was reported that dietary administration of probiotic increased total haemocyte count (THC) in shrimp (Vieira *et al.*, 2010), in *M. rosenbergii* (Cheng and Chen, 2001; Rahiman *et al.*, 2010) and in *M. malcomsonii* (Chand and Sahoo, 2006). It is predicted that this deviation might be due to the differences of methodology and species variation. This study confirmed a little bit increased of total haemocytes count in *M. rosenbergii* feed *E. faecalis* supplemented diet that is possibly a sign of stimulation of the immune response. Also the enhanced immune response may be responsible for reduction of pathogenic *V. harveyi* load.

The haemocyte in the prawn haemolymph was presented mainly based on the presence of cytoplasmic granular deposit, size of granules and its resemblance with human leucocytes. The present study showed that three types of haemocytes including non granular, small-granular, and large-granular haemocyte were found; they were looked respectively as like as of lymphocytes, monocytes, neutrophil, basophil and eosinophil shaped. Each cell type is active in defense reactions, for example; in crayfish, the nongranular hyaline cells are chiefly involved in phagocytosis, the semigranular cells are the cells active in encapsulation, while granular and semi granular cells can be cytotoxic and lyse foreign eukaryotic cells (Soderhall *et al.*, 1985).

Kakoolaki *et al.,* (2010) reported that total haemocyte count in farmed shrimp, *Fenneropenaeus indicus* was (5.3 ± 2.0) $\times 10^6$ cell/mm^3. Owens *et al.,* (1997) reported that in prawn, *Penaeus monodon*, non-granular, small-granular and large-granular haemocytes were 82.7 ± 14, 15.6 ±17, and 1.7 ± 2% respectively of the cell types. Vazquez *et al.,* (1997) reported that morphologically three main types of haemocytes were observed in the freshwater prawn of which hyaline haemocytes, granular haemocytes, undifferentiated haemocytes which were 70%, 20% and 10% respectively. In case of ridgeback penaeid shrimp *Sicyonia ingentis*, Hose *et al.,* (1992) recorded that the hyaline cell comprises 50-60% of the circulating haemocytes, whereas the small granular haemocyte and large granular haemocyte represented 30% and 10%, respectively. This result also agreed well with the findings of Hose *et al.*, (1990a and b) and Kondo *et al.*, (1998). They reported that, the hemocytes of the shrimp are classified into three types, hyaline cells (HC), small granular cells (SGC) and large granular cells (LG) associated with the presence of cytoplasmic deposit and size of granules. It is mentionable that, classification of crustacean hemocytes remains controversial due to different types of methodology and criteria adopted by different fisheries researchers (Hose *et al.*, 1990).

The hyaline hemocytes have phagocytic activity and attach and spread on surfaces of macrophages. The main function of the granular hemocytes is to store the pro-phenol oxidase enzyme that catalyses the oxidation of phenols to semi-quinones and quinones which have high antimicrobial activity and this system plays a key role in the defence mechanism of crustaceans. This hemocyte can be triggered to undergo exocytosis and release of pro-PO enzyme that may be activated by ß-1.3-glucans, peptido-glycans and lipo-polysaccharides (Barracco *et al.*, 1991; Söderhäll *et al.*, 1994). But activation by ß-1.3-glucan is very specific due to presence of ß-1.3-glucan receptor in this type of cell (Smith and Söderhäll, 1983; Johansson and Söderhäll, 1985). The finding of the present study revealed the possible probiotic potential of *Streptococcus faecalis* due to the increased number of total haemocyte count as well as individual haemocyte count of prawn fed probiotic, *Streptococcus faecalis*-supplemented diet.

These findings demonstrate that dietary supplementation of food with *Streptococcus faecalis* can improve growth performance, digestive enzymes activity and disease resistance through an enhanced immune response in prawn. It is concluded that the natural bacterial flora associated with the cultured fish and shellfish might have bacterial strains

with good probiotic potential, which may be screened and exploited for an environment-friendly and organic mode of aquaculture.

Chapter Six

Conclusion

Chapter 6. Conclusion

Vibriosis is a major problem in prawn culture, causing heavy mortality and severe economic loss. The use of antibiotics is a common to treat bacterial diseases but resulted in antibiotic-resistant strains of bacteria which are more harmful. 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.

The present study was conducted to detect *Vibrio* spp. and to determine the antagonistic effect of probiotic bacteria, *Streptococcus faecalis* on experimentally *Vibrio* spp. The findings of the present study reported that the molecular method PCR can assist in identification of *Vibrio* spp. in lieu of conventional methods. This molecular approach, in combination with other strategies for effective health management in aquaculture would result in good production through lower incidence of mortalities during the culture period. From *in-vitro* and *in-vivo* challenge test with *Streptococcus faecalis* it was found that it significantly reduced the *Vibrio spp.* load of the selected freshwater prawn samples. *Streptococcus faecalis* was proved as antibacterial agent with an excellent alternative for chemicals and antibiotics to prevent disease in prawn aquaculture system.

The present investigation also demonstrated that, *Streptococcus faecalis*-supplemented diet was found to be enhanced the growth performances, digestive enzyme (protease and amylase) activity, immunity and disease resistance against pathogenic bacteria, *Vibrio* spp. in freshwater prawn aquaculture, it seems likely that the use of probiotics will gradually increase in the days to come that will open new vistas in the arena of aquaculture. It can be concluded that instead of using antibiotic, *Streptococcus faecalis* might be used as an effective antibacterial agent to combat the diseases in freshwater prawn culture, which may be screened and exploited for an environment-friendly aquaculture practice.

Chapter Seven

References

Chapter 7. References

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Appendix-1

Table: Load of *Streptococcus faecalis* in the intestinal tracts of collected prawn.

Sample No.	Replication No.	Load of *Streptococcus faecalis* in intestinal tracts (CFU/g)	Average load in intestinal tracts (CFU/g)
01	01	5.26×10^4	5.35×10^4
	02	5.36×10^4	
	03	5.42×10^4	
02	01	4.57×10^4	4.30×10^4
	02	4.18×10^4	
	03	4.16×10^4	
03	01	3.45×10^4	3.03×10^4
	02	2.70×10^4	
	03	2.96×10^4	

Appendix-2

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

Sample No.	Replication No.	Load of *Vibrio* spp. in intestinal tracts (CFU/g)	Average load in intestinal tract (CFU/g)
01	01	1.64×10^4	1.29×10^4
	02	1.31×10^4	
	03	9.20×10^3	
02	01	1.25×10^4	1.90×10^4
	02	1.77×10^4	
	03	2.69×10^4	
03	01	3.28×10^3	1.40×10^4
	02	2.04×10^4	
	03	1.84×10^4	

Appendix-3

Table: *In-vitro* challenge test and enumeration of *Vibrio harveyi* load with and without probiotic (*Streptococcus faecalis*).

Time Interval	Sample No.	Without Probiotic		With Probiotic	
		Vibrio harveyi load (CFU/g)	Average load (CFU/g)	*Vibrio harveyi* load (CFU/g)	Average load (CFU/g)
0 hour	01	2.01×10^3	**$(4.69\pm0.29)\times 10^3$**	2.01×10^3	**$(4.69\pm0.29)\times10^3$**
	02	1.18×10^3		1.18×10^3	
	03	1.09×10^4		1.09×10^4	
4th hour	01	1.89×10^4	**$(2.30\pm0.08)\times10^4$**	9.56×10^3	**$(1.11\pm0.08)\times10^4$**
	02	1.76×10^4		8.20×10^3	
	03	3.25×10^4		1.56×10^4	
8th hour	01	3.50×10^4	**$(2.36\pm0.24)\times10^5$**	6.32×10^3	**$(7.34\pm0.17)\times10^3$**
	02	1.92×10^5		8.25×10^2	
	03	1.66×10^5		1.43×10^4	
12th hour	01	4.32×10^5	**$(4.24\pm0.12)\times10^5$**	1.48×10^3	**$(1.05\pm0.24)\times10^3$**
	02	3.71×10^5		2.32×10^2	
	03	4.70×10^5		1.46×10^3	

Appendix-4

Table: *In-vivo* challenge test and enumeration of *V. harveyi* load with and without probiotic.

Time Interval	Sample No.	Without Probiotic *Vibrio harveyi* load (CFU/g)	Average load (CFU/g)	With Probiotic *Vibrio harveyi* load (CFU/g)	Average load (CFU/g)
10 days	01	2.30×10^5		2.30×10^5	
	02	2.27×10^5	**$(2.37\pm0.06)\times10^5$**	2.19×10^5	**$(2.31\pm0.08)\times10^5$**
	03	2.54×10^5		2.46×10^5	
20 days	01	2.81×10^5		2.16×10^5	
	02	2.65×10^5	**$(2.65\pm0.10)\times10^5$**	2.26×10^5	**$(2.21\pm0.09)\times10^5$**
	03	2.48×10^5		2.20×10^5	
30 days	01	1.37×10^6		1.32×10^5	
	02	1.48×10^6	**$(1.49\pm0.09)\times10^6$**	1.42×10^5	**$(1.42\pm0.08)\times10^5$**
	03	1.62×10^6		1.52×10^5	
40 days	01	3.52×10^6		2.58×10^4	
	02	3.45×10^6	**$(3.45\pm0.08)\times10^6$**	2.67×10^4	**$(2.70\pm0.08)\times10^4$**
	03	3.38×10^6		2.84×10^4	
	01	5.72×10^6		1.41×10^4	
50 days	02	5.82×10^6	**$(5.74\pm0.07)\times10^6$**	1.37×10^4	**$(1.44\pm0.09)\times10^4$**
	03	5.68×10^6		1.53×10^4	
	01	6.87×10^6		1.01×10^4	
60 days	02	6.74×10^6	**$(6.85\pm0.08)\times10^6$**	1.03×10^4	**$(1.03\pm0.10)\times10^4$**
	03	6.95×10^6		1.06×10^4	

Appendix-5

The growth performance of *M. rosenbergii* fed the control diet and probiotic, *Streptococcus faecalis*-supplemented diet after 60 days in the experiment. Each value is given as mean ± standard deviation of three individual observations. The mean differences are significant at ($P < 0.05$).

Variable	Control	Treatment
Average Initial Weight (g)	2.19±0.05	2.90±0.16
Average Final Weight (g)	10.34±1.03	16.84±0.24
Weight Gain (%)	371.31±43.95	481.22±36.61
Specific Growth rate (%)	2.60±0.24	2.93±0.14

Appendix-6

Digestive enzyme activity of *Macrobrachium rosenbergii* fed the control diet and probiotic, *Streptococcus faecalis*-supplemented diet after 60 days in the experiment. Each value is given as mean ± standard deviation of three individual observations. The mean differences are significant at ($P < 0.05$).

Experimental group	Enzyme Activity	
	Amylase (Unit/mg intestine)	Protease (Unit/mg intestine)
Control	0.70±0.03	1.79±0.02
Treatment	1.26±0.08	2.78±0.02

Appendix-7

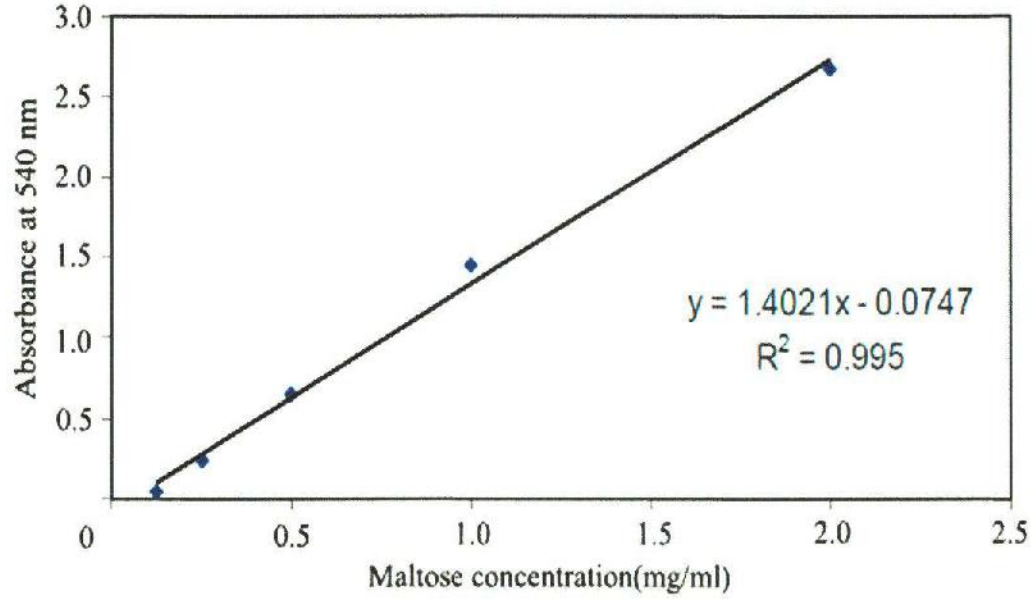


Figure: Standard calibration curve for the determination of maltose released in the amylase assay. (Mehrabadi and Bandani, 2009).

Appendix-8

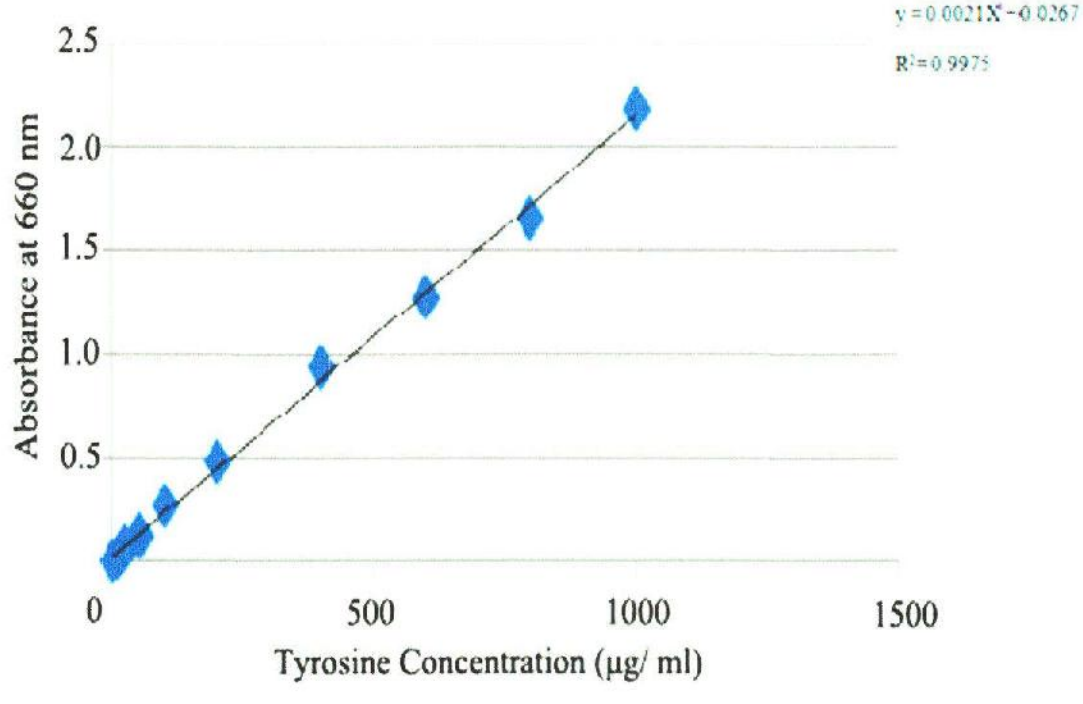


Figure: Standard calibration curve for the determination of tyrosine released in the protease assay (Prasad *et al.*, 2013).

Appendix-9

Table: Statistical analysis of *In-vitro* challenge test

Independent Sample T Test		
	df	**Sig. (2-tailed)**
0 Hour	4	1.000
4 Hour	4	0.053
8 Hour	4	0.034
12 Hour	4	0.010
Confidence level=95%		

Appendix-10

Table: Statistical analysis of *In-vivo* challenge test

Independent Sample T Test		
	df	**Sig. (2-tailed)**
Day_10	4	.066
Day_20	4	.083
Day_30	4	.000
Day_40	4	.000
Day_50	4	.000
Day_60	4	.000
Confidence level=95%		

Appendix-11

Table: Statistical analysis of weight gain

Independent Sample T Test		
Amylase activity	**df**	**Sig. (2-tailed)**
	4	.029
Confidence level=95%		

Appendix-12

Table: Statistical analysis of specific growth rate

Independent Sample T Test		
Amylase activity	**df**	**Sig. (2-tailed)**
	4	.042
Confidence level=95%		

Appendix-13

Table: Statistical analysis of amylase activity

Independent Sample T Test		
Amylase activity	**df**	**Sig. (2-tailed)**
	4	.001
Confidence level=95%		

Appendix-14

Table: Statistical analysis of protease activity

Independent Sample T Test		
Amylase activity	**df**	**Sig. (2-tailed)**
	4	.000
Confidence level=95%		

Figure: Prawn farm

Figure: Application of probiotic treated feed

Figure: Capture of fish for sampling

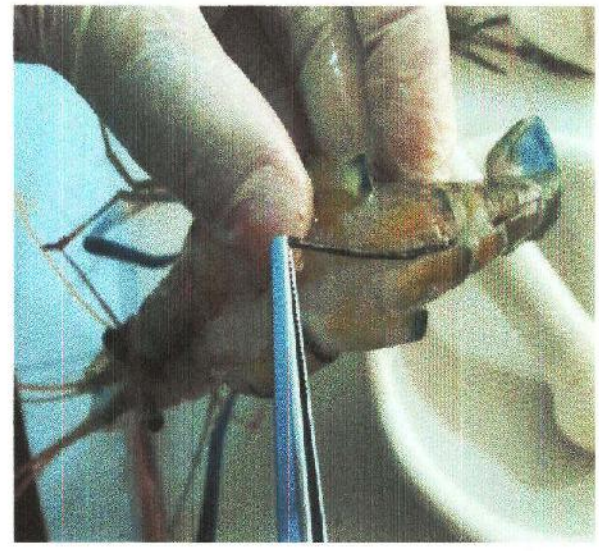


Figure: Dissection of the prawn sample

Figure: Inoculation of culture media

Figure: Incubation of culture media

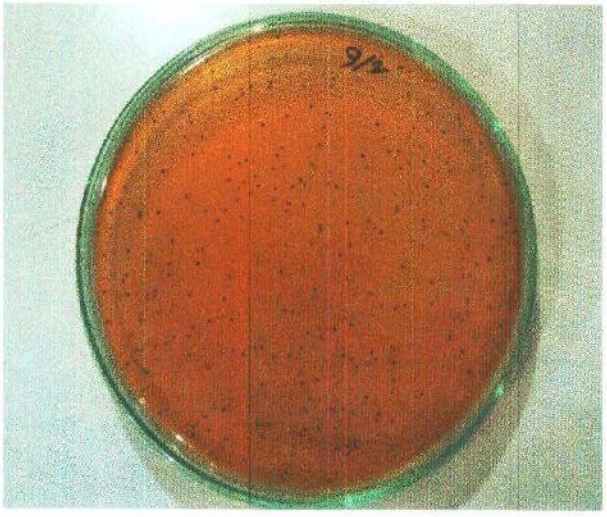


Figure: Colony of *Streptococcus faecalis*

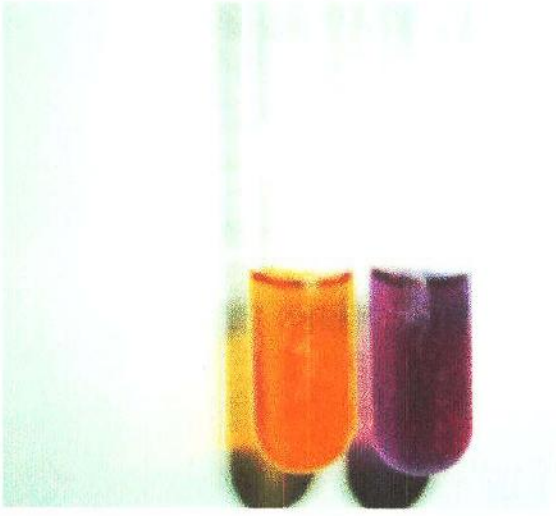


Figure: Yellow color indicate the presence of *Streptococcus faecalis*

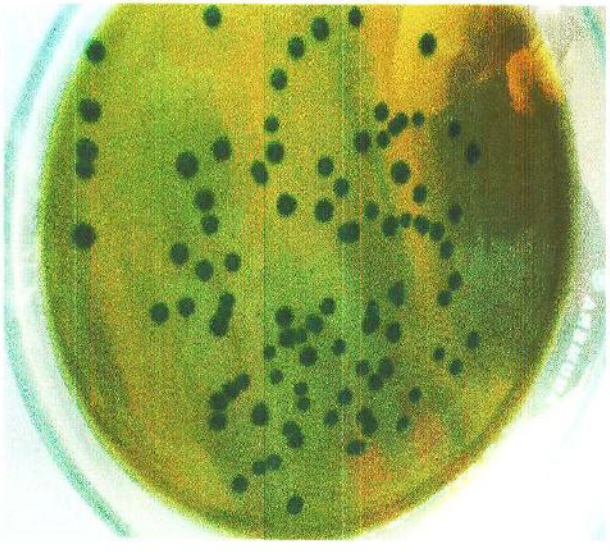


Figure: Colony of *Vibrio* spp.

Figure: Result of VP test

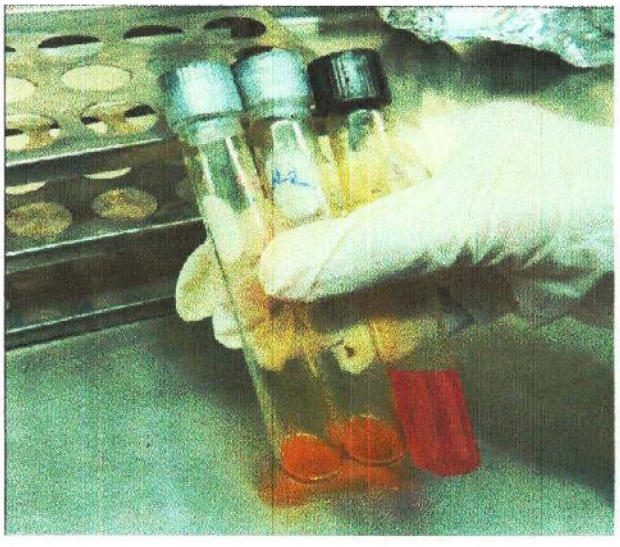


Figure: Result of MR test

Figure: Result of indole test

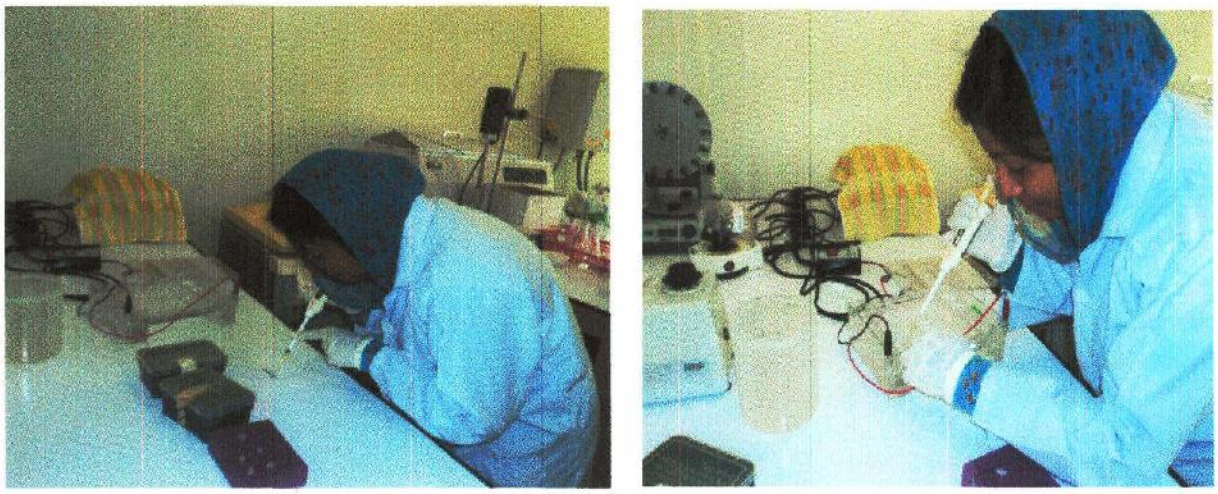


Fig: Gel Electrophoresis to evaluate PCR product.

Figure: Identification of haemocyte

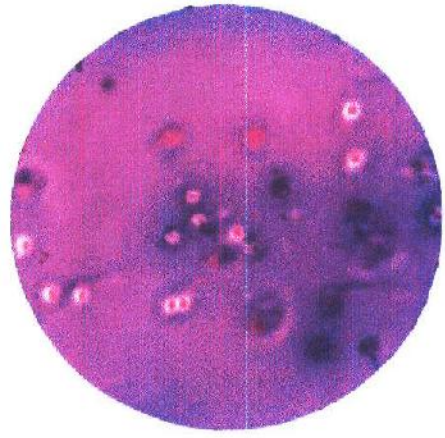
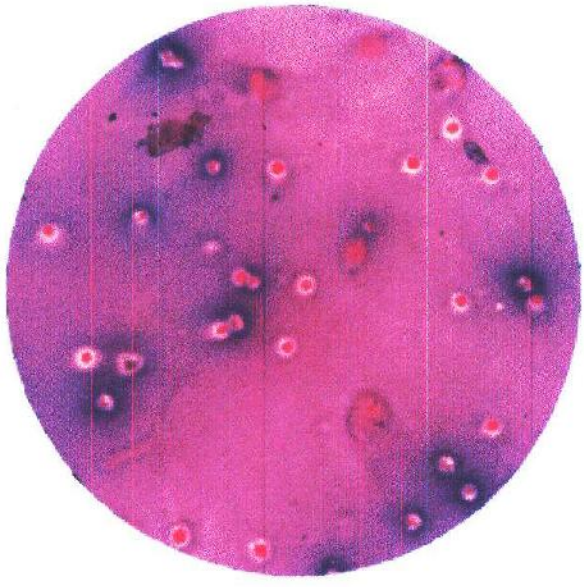


Figure: Haemocyte in prawn haemolymph



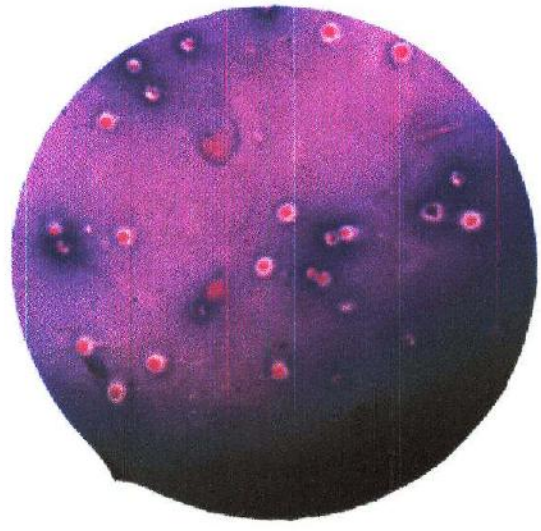


Figure: Haemocyte in prawn haemolymph