Isolation of potential probiotic (*Clostridium butyricum*) for controlling pathogenic bacterial growth using PCR technique in Prawn Farm

A Thesis

By

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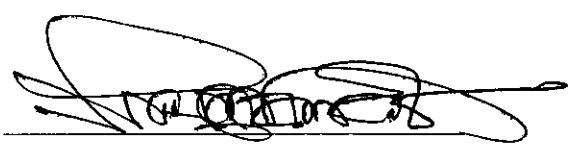
Approved as to the style and content

By



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Declaration

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The results presented in this thesis with the above mentioned title 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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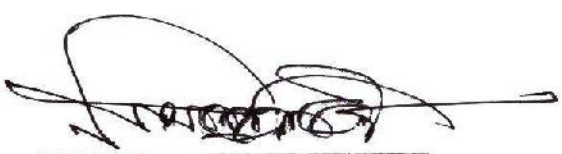

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DEDICATED

TO

MY BELOVED PARENTS

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Abstract

The present study was carried out from January to November, 2014 to evaluate the antagonistic effect of probiotic species, *Clostridium butyricum*, on *vibrio* spp. by means of *in-vitro* and *in-vivo* test as well as to determine the impact of this probiotic species on the growth and immunity of fresh water prawn (*Macrobrachium rosenbergii*) based on enzyme activity and haemocite count respectively. Both the pathogenic and probiotic bacterial species were isolated from fresh water prawn collected from local market of Khulna. The probiotic incorporated basal diet and diet without any probiotic served as treatment group and control group respectively. These two types of diets were fed to *M. rosenbergii* separately under treatment group and control group for a period of about 60 days. After the feeding trail, the antagonistic effect as well as the growth parameters such as weight gain, specific growth rate and enzyme activity (amylase and protease) were found to be significantly ($P < 0.05$) higher in treatment group compared with control group. Although immunity determining parameter (haemocyte count) was exhibited to be non-significantly ($P > 0.05$) higher in treatment from control. Ultimately this study revealed that probiotic, *C. butyricum* incorporated diets were beneficial for *M. rosenbergii* culture in terms of decreasing pathogenic bacterial load, increasing growth, enzyme activity and immunity. Thus the present study will be helpful to improve fresh water prawn farming.

Keywords: Probiotic, *Clostridium butyricum*, pathogenic, antagonistic effect, growth, immunity.

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Introduction

Introduction

Aquaculture remains a growing, vibrant and important production sector of high-protein animal food that is easily digestible and having a high biological value. It's contribution to world food production, raw materials for industrial and pharmaceutical use, and aquatic organisms for stocking or ornamental trade has increased dramatically in recent decades. The report World Aquaculture 2012 revealed that global production of fish from aquaculture enhanced more than 30 percent between 2006 and 2011, from 47.3 million tons to 63.6 million tons. It also forecasts that by 2012 more than 50 percent of the world's food fish consumption will come from aquaculture, so it is expected to overtaking capture fisheries as a source of edible fish (FAO, 2012). In addition, prawn is being considered as very popular aquatic food item in the international fish market. It is in an industry set for a period of strongly growing demand, and is currently worth around US$10 billion. It (both fresh and frozen) contributes about 6.03% of the world's total aquatic food export (FAO, 2009). In Bangladesh prawn represents the country's second largest export after garments from which Bangladesh earned about 4312.61 crore BDT in the year 2013 (DOF, 2014). It is nothing but to say that Bangladesh secured a rank of fourth with respect to area of prawn farming and sixth in volume of production (DOF, 2007).

One of the major constraints for the development of prawn aquaculture is the mortality due to the outbreaks of different viral, bacterial, and fungal diseases especially vibriosis caused by different *Vibrio* spp. including *V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. splendidusthat* as well as luminous bacterial disease caused by *Vibrio harveyi* that cause devastating economic losses worldwide (Shruti and Soumya, 2012). According to a recent World Bank Report, global losses due to diseases are around US $ 3000 million (Lundin, 1996). *Vibrio harveyi* is a gram-negative, curved-rod shape (comma shape), luminous bacterium, facultative anaerobes found both in freshwater and marine ecosystems. This species become become opportunistic pathogens when natural defence mechanisms of prawn are suppressed (Brock and Lightner, 1990). It is one of the important etiologic agents of mass mortalities in prawn farm (Thompson *et al.*, 2005). Luminescent *V. harveyi* appears to release exotoxins (Liu *et al.*, 1996) and may cause 80-100% mortality in *P. monodon* hatcheries (Harris, 1995).

In order to control these diseases, a number of chemotherapeutic agents including antibiotics are used as effective agents in farms. However, the continuous use of antimicrobials increases

the antibiotic resistance (Karunasagar *et al.*, 1994). According to WHO fact sheet 194, the massive use of antimicrobials for disease control and growth promotion in animals increases selective pressure on the microbial world and encourages the emergence of resistant bacteria which can transfer their resistance genes to other bacteria (WHO, 194). Another major concern associated with the use of antibiotics is the problem of residues which has already led to 'red alert' on imported prawn in the European Union. Therefore, it is essential to find an effective and non-resistance alternate strategies for pathogen control in aquaculture systems. In this situation, there is no alternative of probiotics. Probiotics are viable cell preparations that have beneficial effects on health of the host by improving its intestinal balance (Fuller, 1989) via producing nutrients, enhancing immune responses and improving the water quality in aquaculture (Gatesoupe, 1999; Verschuere *et al.*, 2000). In addition it produces an antibiotic that inhibited a *Vibrio*, and then the *Vibrio*'s mortality rate would increase. Ultimately enhancing the survivality and production of prawn in the farms (David ,1999).

Clostridium butyricum is a mesophilic, butyricum-acid as well as lactic acid producing, strictly anaerobic, endospore-forming gram-positive and rod shape bacterium. They are distributed in soils, sediments, the decaying heartwood of living trees, the stool of healthy children and adults, soured milk and cheeses and intestines of animals (fish) and humans (Cato *et al.*, 1986; Beckers *et al.*, 2010,Meng *et al.*, 1997; Wiegel *et al.*, 2006). It is able to survive in media of low pH and relatively high bile concentrations, and it produces endospores. These properties of *C. butyricum* make it suitable as a probiotic supplement in animal feed (Sato and Tanaka, 1996; He *et al.*, 2004; Kong *et al.*, 2011). Today it is widely being used as a probiotic for humans and animals in Eastern Asia countries, such as Japan, Korea and China (Ito *et al.*, 1997; Kamiya *et al.*, 1997). This species produces short chain fatty acids (SCFAs), such as butyric acid, propionic acid, and acetic acid. Several reports have suggested that SCFAs have potential beneficial effects; in particular, butyric acid has a proliferative effect on intestinal mucosal cells (Sakata *et al.*, 1995) and anti-inflammatory effects (Mishiro, 2013). It could inhibit the growth of various enteropathogens (Kuroiwa *et al.*, 1990). *C. butyricum*, itself and its spent culture supernatants exhibite significant inhibitory activity on *S. enteritidis* and *V. parahaemolyticus* growth. Besides this it is capable to enhance resistance power to Vibrosis in fish by leucocyte activation, including phagocytosis and increased superoxide anion production (Sakai *et al.*,1995). It can enhance resistance to *V. harveyi* infection by increasing total haemocyte as well as serum

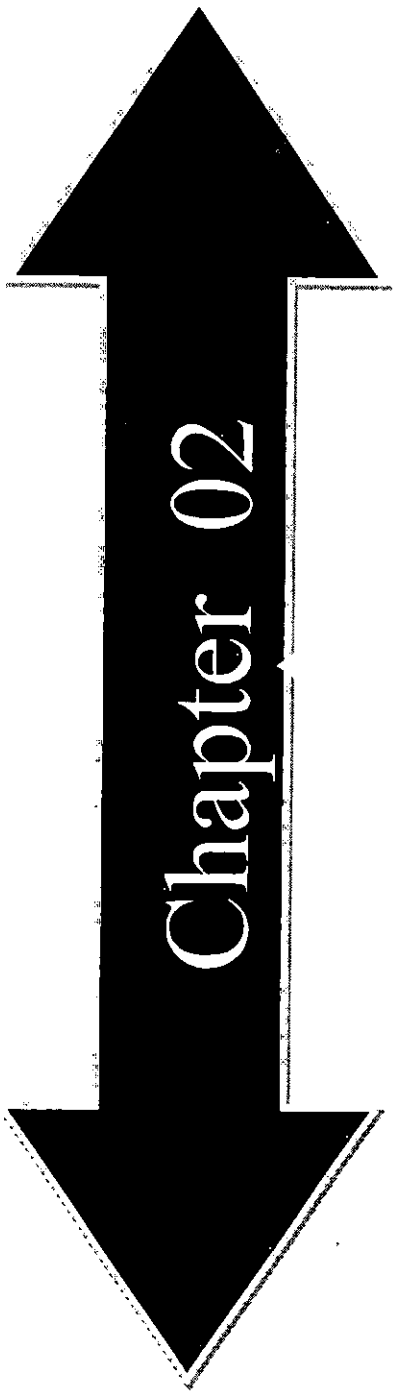
agglutination activity in prawn body (Vieira *et al.* 2010*)*. This bacterial species also exhibit antagonistic interactions with *Candida albicans*, *C. difficile*, *Klebsiella spp.*, *Salmonella spp.*, *Vibrio spp.* (Chen *et al.*, 1987; Fujita and Takashi, 1987; Kuroiwa *et al.*, 1990). The highest level of bactericidal and bacteriostatic properties against *Pseudomonas aeruginosa, Salmonella enteritidis* and *Escherichia coli* was found with the *C. butyricum* (Szymanowska-Powalowska *et al.*, 2014). Thus this probiotic species exhibits a preventative and therapeutic effects on bacterial infections in fish as well as in crustacean. Moreover, dietary supplementation of *C. butyricum* has been demonstrated by several researchers to promote growth performance (Han *et al.*, 1984; Song *et al.*, 2006; Mountzouris *et al.*, 2010), improve immune function (Chen *et al.*, 1993; Murayama *et al.*, 1995; Wang *et al.*, 1996; Song *et al.*, 2006; Zhang *et al.*, 2009; Cao *et al.*, 2012; Yang *et al.*, 2012). In addition, this bacterial species plays a vital role in increasing enzyme activity including amylase activity, pretease activity and lipase activity in animal body (Yang *et al.*,2005; Nimrat and Vuthiphandchai, 2011; Ziaei-Nejad *et al.*,2006; Wang and Xu,2006). For these reasons, *C. butyricum* is receiving renewed attention, and it is very important to isolate the stain.

Unfortunately, commercially available different probiotics in the market are very much expensive that is almost at out of the purchasing capacity of a larger portion of farmer. Moreover, almost all of these probiotics are composed of *Bacillus*, *Lactobacillus* and *Enterococus* species because a couple of experiments have already been performed on these species. In spite of being sorrow, it is true that experiments based on *C. butyricum* are hardly exist. Therefore, investigation of the characteristics of *C. butyricum* is desired. That's why the prime objective of the present experiment is to isolate *C. butyricum* from prawn intestine and apply in the farm incorporated with feed as well as to observe their antagonistic effect on pathogenic bacteria in terms of *in-vitro, in-vivo* test and their role in the growth performance of prawn in terms of enzyme activity and immunity. Thus this study will be capable to reduce the dependency on commercially produced probiotics as well as to evaluate the effect of this bacterial species as a probiotic in aquaculture.

Objective of the study

- Isolation of the pathogenic (*Vibrio* spp.) and probiotic (*C. butyricum*) bacterial species.
- Determination of the antagonistic effect of the probiotic species on the pathogenic species.

- Evaluation of the role of probiotic species on the growth of farming prawn by the means of enzyme activity as well as immunity determination.



Literature Review

Literature review

The following information was reviewed in favor of the present study that was performed elsewhere in the world and relevant to the study.

In 1995, Sakai *et al.* conducted an experiment based on *Enhancement of resistance to vibriosis in rainbow trout, Oncorhynchus mykiss Walbaum by oral administration Clostridium butyricum species* and from the result of that experiment they concluded that *C. butyricum* and its spent culture supernatants exhibite significant inhibitory activity on *S. enteritidis* and *V.parahaemolyticus* growth. It also shows significant inhibitory effects on *S. enteritidis*and *V. parahaemolyticus* induced apoptosis, which may due to its growth and adhesion inhibitory effects. Thus the probiotic bacterium *C. butyricum* has preventive and therapeutic effects on *S. enteritidis*and *V. parahaemolyticus* infections in fish.

Zhang *et al.* (2011) studied on the effects of dietary lipids and *Clostridium butyricum* on the performance and the digestive tract of broiler chickens and found that this bacterial species plays a vital role in their growth, besides this the load of pathogenic bacterial species are also decreased.

Seki *et al.* (2003) conducted an experiment on Prevention of antibiotic-associated diarrhea in children by *Clostridium butyricum* MIYAIRI and observed that this strain control the diarrhea of children more effectively even better than of antibiotic. So, this study proved that this strain is capable to prevent the pathogenic bacterial species.

In 2008, Imase *et al.* performed a research work named the efficacy of *Clostridium butyricum* preparation concomitantly with Helicobacter pylori eradication therapy in relation to changes in the intestinal microbiota and got a better result from this experiment so he concluded that this bacterium has an antagonistic effect with different harmful bacteria.

Kamiya *et al.* (1997) studied on Bacterioprophylaxis using *Clostridium butyricum* for lethal caecitis by *Clostridium difficile* and he found a positive result from his research.

Kong *et al.* (2011) carried out a study on the Oral administration of *Clostridium butyricum* for modulating gastrointestinal microflora in mice and provided valuable information about *C. butyricum* including they promotes the growth of *Lactobacillus* and *Bifidobacterium* and inhibits diarrhea.

In 1995, Murayama *et al.* revealed, this strain may enhance immunity in the respiratory organs of mice by preventing different types of pathogenic bacteria,based on his research about the effects of orally administered *Clostridium butyricum* MIYAIRI 588 on mucosal immunity in mice.

A research, conducted by Yang *et al.* in 2012 named 'effects of probiotic, *Clostridium butyricum*, on growth performance, immune function, and cecalmicroflora in broiler chickens', provides valuable information about *C. butyricum* as a probiotic. The result section of this research elucidated that this bacterial species not only increase the growth rate of broiler chickens but also plays an important role in enhancing the immunity of broiler chickens through increasing serum Immunoglobulins as well as Complement Components.

Research carried out by Nimrat and Vuthiphandchai in 2011 based on the topic, "In vitro evaluation of commercial probiotic products used for marine shrimp cultivation in Thailand" stated that *C. butyricum* as a probiotic is capable to enhance the growth of shrimp by considerably increasing enzyme activity in the shrimp body.

The output of an research experiment named Inhibition of enteropathogens by *Clostridium butyricum* MIYAIRI 588 conducted by Kuroiwa et al. in 1990 revealed that C. butyricum could inhibit the growth of various enteropathogens.

Gildberg *et al.* (1997) had analyzed the effect of a probiotics of lactic acid bacteria in the feed of Atlantic cod fry (*Gadus morha*) on growth and survival rates. At the end of the experiment they concluded that certain improvement in disease resistance could be obtained by using lactic acid bacteria as a probiotic in fish because they usually dominate the intestinal flora in fish.

Sivakumar *et al.* (2012) carried an experiment based on "Probiotic effect of *Lactobacillus acidophilus* against vibriosis in juvenile shrimp (*Penaeus monodon*)" and from this experiment they study concluded that the lactic acid bacteria including *Lactobacillus acidophilus* as well as *C. butyricun* will be helpful in the management of *V. alginolyticus* related bacterial disease in tiger shrimp *P. monodon.*

Seenivasan *et al.* (2012) conducted a research named the effects of Probiotics on Survival, Growth and Biochemical Constituents of Freshwater Prawn *Macrobrachium rosenbergii* Post

Larvae and based on their findings from the research they recommended that lactic acid bacteria *C. butyricum* can be used as a feed additive either as enrichment material of live feeds or as supplementary material in formulated feeds for enhancing the growth and production of *M. rosenbergeii* PL.

In 2014 Seenivasan *et al.* concluded that lactic acid bacteria incorporation has significantly improved survival, growth, specific growth rate (SGR), feed conversion rate (FCR), feed conversion efficiency (FCE) and protein efficiency rate (PER) biochemical constituents and energy utilization in post larvae of *M. rosenbergii* based on a research work named effect of *Lactobacillus sporogenes* on survival, growth, biochemical constituents and energy utilization of freshwater prawn *Macrobrachium rosenbergii* post larvae.

Yang *et al.* (2010) observed that *C. butyricum* prevent disturbances of microflora in animal intestine, enhance animal immune response, promote digestion, improve meat quality and increase growth performance from findings of a research named effects of dietary lipids and *Clostridium butyricum* on fat deposition and meat quality of broiler chickens.

A scientific research named "Effect of probiotic supplemented diet on marine shrimp survival after challenge with *Vibrio harveyi*" conducted by Vieira *et al.* in 2010. According to the findings of this research it can be concluded that Probiotic-supplemented diet modifies shrimp digestive tract bacterial microbiota, enhance resistance to *V. harveyi* infection by increasing total haemocyte as well as serum agglutination activity.

Song *et al.* (2006) conducted an experiment 'Effects of dietary supplementation with *Clostridium butyricum* on the growth performance and humoral immune response in Miichthys miiuy". He concluded from this experiment *C. butyricum* incorporated diet has a great impact on the growth and immunity of animals.

Takahashi *et al.* (2004) found that *C. butyricum* exhibited a significant antagonistic effect on Escherichia coli from the experiment named the effect of probiotic treatment with *Clostridium butyricum* on enterohemorrhagic *Escherichia coli* O157:H7 infection in mice.

In 2008, Pan *et al.* showed that *Clostridium butyricum* is very much effective against *Aeromonas hydrophila* and *Vibrio anguillarum* based on the findings of a study "In vitro

evaluation on adherence and antimicrobial properties of a candidate probiotic *Clostridium butyricum* CB2 for farmed fish".

Nayak (2010) carried out a study named and based on the output of that study he concluded that *C. butyricum* has the capability to prevent certain pathogenic bacteria.

Most clostridial species, especially gut-associated clostridial species such as *Clostridium butyricum*, are non-pathogenic gut commensals, which dominate and form an important part of the lower gut flora of both man and animals (Finegold *et al.*, 1983).

Rengpipat *et al.* (2000) conducted a study named Use *Carnobacterium sp.* as probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*Walbaum) and found that when this bacterial strain was administered as probiotics in the *P. monodon* culture, its growth and survival were improved and immunity was enhanced. Administration as probionts has been shown to increase shrimp survival by enhancing resistance to pathogens by activating both cellular and humoral immune defenses in shrimp. The higher survival in probiotics fed shrimps might be attributed to the presence of this species which is able to out-compete other bacteria for nutrients and space and can exclude other bacteria through the production of antibiotics.

Based on the experiment conducted by Sakata *et al.* 1995, it could be said that *C. butyricum* produces short chain fatty acids (SCFAs), such as butyric acid, propionic acid, and acetic acid. Moreover they have potential beneficial effects; in particular, butyric acid has a proliferative effect on intestinal mucosal cells.

Mishiro *et al.* 2013 carried out a study named "Butyric acid attenuates intestinal inflammation in murine DSS-induced colitis model via milk fat globule-EGF factor 8" and concluded that butyric acid provides an anti-inflammatory effect. It should be mentioned that *C. butyricum* usually produces butyric acid that's why they are called butyric acid bacteria.

A research experiment, enzyme producing bacterial flora isolated from fish digestive tracts, conducted by Bairagi *et al.* in 2002, found that *C. butyricum* produces digestive enzymes, which facilitate feed utilization and digestion, antibacterial activity against pathogenic microorganisms.

In 2013, Elumalai *et al.*, performed a study to evaluate the performance of commercially available probiotics (the major active ingredients include *Clostridium butyricum, Streptococcus faecalis, Streptococcus faecium, Bacillus mesentericus, Bacillus subtilis, Bacillus natto, Saccharomyces cerevivisiae*, Alkaline Protease and Lipase) in the growth and survival of *Peneaus monodon* in grow-out conditions. At the end of the experiment,it was found that the ponds with probiotic provided much more srvibility as well as growth than the ponds without probiotics. From this research, it is evident that application of probiotics has improved the growth and survival of *P. monodon* and which in turn paved way to reap better profit for the farmers.

Chapter 03

Materials and Methods

Methods and Material:

1. Study Site and period

The laboratory work was performed in the Biochemistry and Molecular Genetics Lab, Khulna University, Khulna. The field work was conducted in the research pond complex 2 of Fisheries and Marine Resource Technology Discipline at Khulna University, Khulna, Bangladesh. The total experiment took a period of time from January to November, 2014.

2. Sample collection

Prawns samples were collected from local market of Khulna. These samples were used for detecting the contamination of pathogenic bacteria. Only prawn intestine was used for screening probiotic bacteria and *Vibrio* spp. The standard lengths of collected prawn were between 14cm-17cm and the weights were between 20.12g-28.6g.

3. Pathogenic and Probiotics bacterial species isolation

3.1. Preparation of stock solution: The target organs of the sample named intestine was separated and weighted in electric balance. The target organs of the samples were collected aseptically and weighed in electric balance. Weights of intestinal tracts were between 0.13g-0.25g. Organs were taken into eppendorf tubes with peptone water (James and Hirsch, 1960) for isolating *Clostridium butyricum* and alkaline saline peptone water was used for isolating *Vibrio harveyi*. 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 freeze.

3.2. Media preparation and sterilization of equipment

3.2.1. 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.
- 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 coolingand then transferred in required number of petridishes by 25ml volume for preparing plates.

3.2.2. Preparation of Rainforced clostridia bacterial Media (RCM)

Rainforced clostridia bacterial media was used for *Clostridium* species culture (About 37.54 gm media/ 1000ml distilled water + 20gm Bacteriological agar/ 1000ml distilled water).

The following steps were followed in the preparation of Rainforced clostridia bacterial Media:

- Definite amount of Rainforced clostridia bacterial media (18.77gm) and Bacteriological agar (10gm) were 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.
- The volumetric flasks were kept in autoclave for sterilization. Autoclaving was done at a temperature of 121°C and at a pressure of 15 lbs/sq inch for 15 minutes.
- After autoclaving the agar media was kept under the bio-safety cabinet for 20-30min for cooling and then transferred in required number of petridishes by 25ml volume for preparing the plates.

3.2.3. Sterilization of Ager Media and all required equipment

All required equipment such as test tubes, eppendorf tubes, plastic tips, other glassware and agar media were sterilized by autoclaving at a temperature of 121°C and a pressure of 15 lbs/sq inch. for 15 minutes. But petridishes, isolating loops etc. were subjected to dry heat sterilization at 170°C for 1 hour in electric oven.

3.3. Isolation of *Vibrio harveyi* and confirmation

3.3.1. Isolation procedure

Vibrio harveyi was isolated from the intestinal tracts of the collected shrimp samples. ISO method was followed for isolating *Vibrio harveyi*. 0.1ml stock solution of each gill and intestinal tract was taken and mixed with 0.9ml alkaline saline peptone water (ASPW) in sterilized test tubes. Then the mixture was incubated at 37 °C for 6 h ± 1. This was the first selective enrichment. After that, whole culture of each test tube obtained in first selective enrichment was taken and transferred into other test tubes each containing 10 ml ASPW. Then the solution was incubated at 41.5 °C for 18 h ± 1. This was the second selective enrichment. Then serial dilution (tenfold) was done and 0.1ml suitable dilution of each

culture was inoculated in thiosulfate citrate bile and sucrose (TCBS) agar plates. Then the inoculated TCBS agar plates were incubated at 37 °C. After 24 h ± 3 h of incubation, the plates were examined for the presence of typical colonies of presumptive Vibrio spp. (ISO/TS 21872-1, 2007). The total process was done according to the following flowchart.

3.3.2. Confirmation of the isolated species

Confirmation by biochemical test

Different types of conformation tests such as Gram Staining, Indole test , MR test, Voges-Proskauer(VP) test etc were performed for the conformation of the *Vibrio* species(*Vibrio harveyi*).

1. Gram Staining

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

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 & Steel's, 1993).

3. 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 & Steel's, 1993).

4. 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 & 1 h. A positive reaction is indicated by a strong red color (Cowan and Steel's, 1993).

5. MR test

The MR test inoculated the isolated bacteria with buffered glucose broth & 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).

6. 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.

7. Growth at different temperature range

The isolated *Vibrio* spp. was kept in incubator after inoculation at subsequent interval at temperature (4^0C, 28^0C, 37^0C, 55^0C).

Confirmation by PCR test

1. Preparation of media and culture of organism

Nutrient (NB) broth was prepared by mixing 2g NB powder in 100 ml water and then it was autoclaved (1210 C, 20 min), then 5 ml NB broth was taken into test tube and one colony was taken into it shaking incubator at 37°C overnight. Then stock solution was prepared by adding 150 ml of cultured sample into 850 ml glycerin in a vial. From stock solution10 µl samples were was used for DNA extraction (Ausubel *et al.*, 1990).

2. DNA Extraction

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

3. Qualitative and quantitave determination of extracted DNA

Integrity of extracted DNA sample was studied by agarose gel electrophoresis as described by Sambrook *et al.* (1989). Briefly, electrophoresis was performed using a horizontal electrophoresis apparatus (Bioneer, Korea) at a constant volt of 100 with a 1% agarose gel containing 1.2 µl

ethidiumbromide in 120 ml 1x TAE buffer. Supply of power for electrophoresis was continued until DNA migrated near about the two-third of the gel towards the positive electrode. DNA size was determined by comparison with a DNA size marker (100 bp DNA ladder, Bioneer, Korea). DNA bands were visualized by UV illumination and photographed using the High Performance UV Transilluminators, (Ultra-Violet Products Ltd., UK).

4. Description of Target genes and primers

Target genes: virA gene for *Vibrio* (Table 1)

Table 1: Specific gene for pathogenic bacteria detection.

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

5. Oligonucleotide primers

The DNA fragment to be amplified is determined by selecting primers. Primers are short, artificial DNA strands that are complementary to the beginning and the end of the DNA fragment to be amplified. They anneal to the DNA template at these starting and ending points, where the DNA-polymerase binds and begins the synthesis of the new DNA strand. In this study, seven pairs of primers were used. Sequences of the seven PCR primer pairs and size of expected amplification products are shown in table 2.

Table 2: Primers and expected size of PCR amplified gene targets of pathogenic bacteria.

Specie	Primers	Sequence (5' →3')	Product size (bp)
Vibrio	Vibr-F	CGGTGAAATGCGTAGAGAT	663
	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 3: The component of reaction mixture for the PCR.

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

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 mMTris-HCL, 0.1 mM EDTA, 1mM DTT, Stabilizers, 50% Glycerol, pH 8.2) at 4: 1 ratio (Buffer : Enzyme).

7. Deoxynucleotide Triphosphates (dNTPs) mixture

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

8. Buffers used for PCR

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

9. DNA amplification by a thermal cycler

Desired DNA amplification and target gene amplification were conducted by PCR analysis. In this method, following DNA extraction from samples with forward and reverse primers and *Taq* polymerase. PCR amplification was performed in a Gene Cycler under the following conditions: In brief, heat denaturation at 94°C for 2 min followed by 35 cycles of heat denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and DNA extension at 72°C for 1 min. This is followed by incubation at 72°C for 10 min followed by cooling at 4°C (Table 3) and the PCR product was analyzed by agarose gel electrophoresis.

Before PCR, made PCR sample by the following way, in Brief, 3 µl DNA extract were directly used as template in each PCR-tube for the reaction. The reaction mixture consisted of 1 µl primer (Forward 0.5 µl + Reverse 0.5 µl), 2 µl 10x Reaction buffer, 2 µl dNTPs, 1 µl 1 unit Top DNA Polymerase enzyme as well. These all components were mixed in 13 µl distill

water and thus a total of 20 µl PCR sample was prepared in a 0.2 ml PCR-tube (Eppendrof, Humburg, Germany) to operate in a thermal cycler.

10. Evaluation of PCR products

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

3.4. Isolation of probiotic species (*Clostridium butyricum*) and confirmation

3.4.1. Isolation procedure

Clostridium butyricum was isolated from intestinal tracts of the collected shrimp samples. The stock solution was diluted (tenfold dilution) with peptone water (James and Hirsch, 1960). 0.1 ml suitable dilution of each stock solution was inoculated in Rainforced Clostridia bacterial media with bacteriological agar and incubated at 37^0C temperature for 48 hours under anaerobic condition (By warping each petridish with parafilm paper). After incubation at 37^0C temperature for 48 h, white, glistening, with rhizoidrhizoid (has thin, branching projections) edges colonies were collected. Then colony screening was done and for this the selected colonies from the RCBM based on morphology were inoculated in *Clostridium butyricum* isolation medium (BIM) culture media separately and incubated as like as the above mentioned. After that, the pure culture was done.

3.4.2. Confirmation Test

Phenotypic characteristics such as cell morphology were observed under a light microscope. In addition, among very few of lactic acid bacteria under clostridia species only the *Clostridium butyricum* is capable to fermented lactose as well as to produce hydrogen gas (Beckers *et al.*, 2010). Lactose fermentation and hydrogen gas production were observed as described by Tanasupawat *et al.* (1998). In a conical flask 0.18gm beef extract, 0.075gm phenol red, 0.3gm peptone were taken and added 60ml distilled water then sterilized. And about 6gm lactose was sterilized with petroleum ether and 60ml distilled water was added with it. After sterilized 9ml of sterilized media and 1ml of lactose solution were taken in each test tube containing durham tube. After that bacteria was inoculated in each tube and incubated over night at 37^0C. Some tubes show yellow colour and others showed red colour. The tubes showed yellow colour with hydrogen gas in durham tube also represented a difference in p^H before and after incubation and other didn't show. Tubes with yellow colour with hydrogen gas in durham tube indicated positive result and conformed that it contains

lactic acid producing bacteria, *Clostridium butyricum* because phenol red shows red colour at higher p^H and yellow colour at very low p^H.

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

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 subsequent intervals of 4 hour up to 12hour. This procedure was done for 2 times. Test solution of *V. harveyi*; without probiotic was also inoculated in TCBS agar media at 0 hour, 4^{th} hour, 8^{th} hour and 12^{th} hour subsequently. All the inoculated TCBS agar plates were incubated at 37^0 C for 48hours. Standard plate count was done after incubation.

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

5.1. Experimental design

Six earthern ponds were selected for the experiment. There were one control group and one treatment group and each containing three replicates. Prawns of control group were given commercial feed without any probiotic and prawns of treatment group were given feed mixed with probiotics for about 60 days (Figure 1).

5.2. 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 or bicar and then examined holding against the sun. So the ponds were ready for stocking.

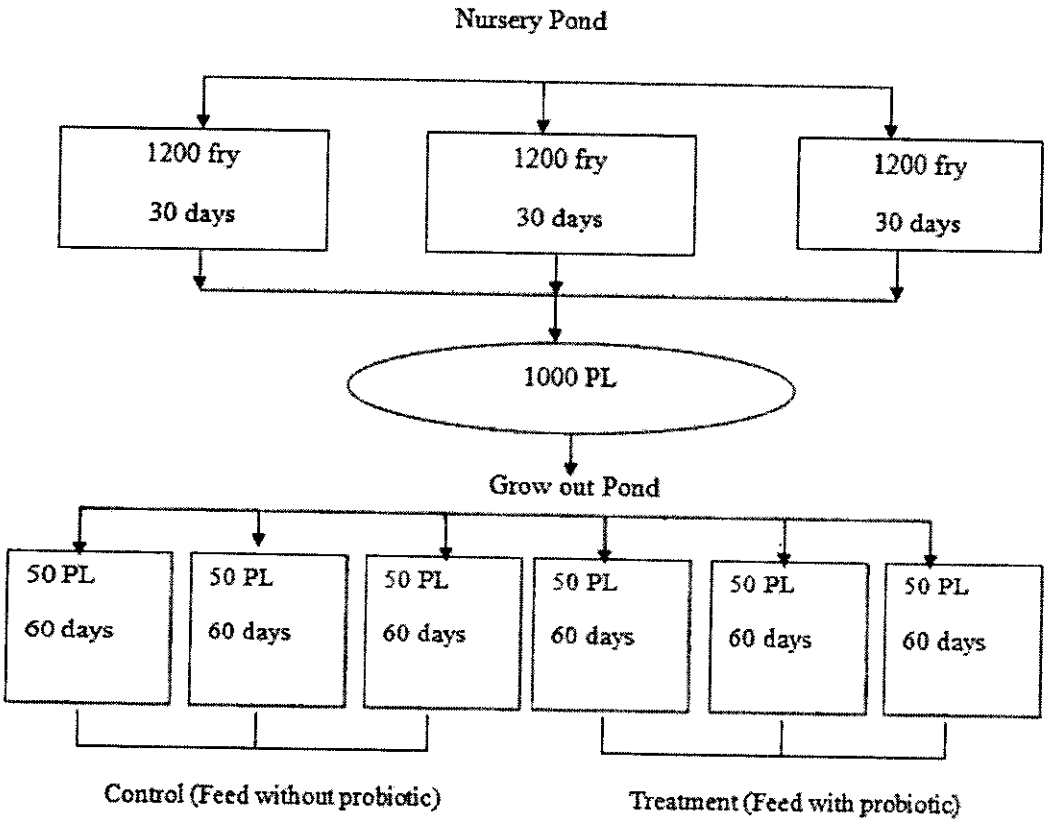


Figure 1: Experimental design

5.3. Fry Nursing and stocking

After pond preparation, prawn fry nursing was done in the pond to reach the fry into juvenile to reduce the mortality rate of prawn into grow out pond. The fry was nursed for 30 days from May 15 to june 15. After nursing the fry for 30 days, they were stocked in grow out pond on 16th June when the average individual weight was about 1g. Before releasing in the pond, acclimatization of fry in the new environment was done by keeping the pots in pond water for a while and then gradually mixing pond's water with fry containing water, which was very important to reduce mortality. The on-station experiment was carried out in 4 ponds of 70 m^2 area and 1 m mean depth of each whereas each pond contains 50 fingerlings. Ponds

under treatment group were given feed mixed with probiotic whereas ponds under control group were provided feed without any probiotic 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.

5.4. 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 probiotic bacteria g-1 infeed and the mixture was spread out and aseptically dried in an oven for 1-2h at 60^0C(Rahiman et al.,2010). Then the feed was applied in the research ponds at a rate of 7-11% of prawn body weight for the first month and from the next month 5-6% of body weight per day at a frequent like twice per day until harvested. Thus the prawn was cultured in the research ponds about 60 days (Ziaei-Nejad *et al.*, 2006).

The stocked probiotic solution was cultured in nutrient broth media. The quantity of bacteria means probiotic was measured by using expectophotometer . The espectophotometere read that one ml of cultured solution contains about 1.44×10^9 probiotic bacteria that mean *Clostridium butyricum.*

5.5. Antagonistic effect analysis

After a culture period of 60 days with probiotic incorporated feed and pure feed, the *viorio* load in the prawn body was further determined by following the before mentioned procedure to determine the antagonistic effect of this probiotic spp. on the *Vibrio* spp.

6. Growth and Digestive enzyme analysis

6.1. Determination of Growth performance

At the end of the experiment, the growth parameters such as weight gain (WG) and specific growth rate (SGR) of different treatments were determined by following equations used by Olvera-Novoa *et al.*1990; Ziaei-Nejad *et al.* 2005.

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

$$\text{SGR} = \frac{(\ln Wt - \ln Wo) \times 100}{t}$$

[Where, t is the culture period, ln Wo is the natural logarithm of the weight of the prawn at beginning of the experiment, and ln Wt is the natural logarithm of the weight of the prawn at day t.]

6.2. 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.

6.2.1. Amylase Activity Assay

The amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1995), using 1% soluble starch (Merck, product number 1257, Darmstadt, Germany) as substrate. 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 that was used by Mehrabadi and Bandani (2009) to enable

calculation of the amount of maltose released during amylase assays. The standard curve has been given in the appendix.

6.2.2. Protease Activity Assay

Sigma's non-specific protease activity assay was used as a standardized procedure (Anson, 1938) to determine the proteases activity, using 0.65% weight/volume casein solution (Merck, product number 1257, Darmstadt, Germany) as substrate. To assay the protease activity two vials were used. One vial was used as a blank, and other was used to assay activity of the protease. In each vials added 5mls of our 0.65% casein solution, and submerged in a water bath at 37°C for 5 minutes. Then added 1ml of enzyme solution to the test sample, 1ml buffer in the blank. The solution was mixed by swirling and incubated for 37°C for ten minutes. After that in each tube added 5 mls of the TCA (Trichloroacetic acid) reagent to each tube to stop the reaction. Then the final solution was incubated at 37°C for 30 minutes. Then 5mls of sodium carbonate and 1 ml of Folin's reagent are added immediately. Sodium carbonate is added to regulate any pH drop created by the addition of the Folin's reagent. These solutions became cloudy after the addition of sodium carbonate and the Folin's reagent was reacted with free tyrosine. Then the solution was mixed by swirling and incubated at 37°C for 30 minutes. After this incubation, appreciable colour change was noticed. 2mls of these solutions were filtered using a 0.45 um 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 that was used by Prasad *et al.* (2014) to enable calculation of the amount of maltose released during protease assays. The standard curve has been given in the appendix.

7. Immunity assay

7.1. Prawn Haemocyte Analysis

Use of an electric Microscope and clinical haemocytometer for differential counts of prawn *Macrobrachium rosenbergii* haemocytes following the method of Owens & O'Neill's (1997) with some modifications.

7.1.1. Haemolymph collection

Prawns were collected from the experimental ponds and carried out alive to the laboratory using bucket. The prawns were washed thoroughly with clean freshwater and kept in freshwater for around 15 minutes. Then it was pulled from water, gently wiped to minimize water content. After that, fresh haemolymph were extracted in an anti-coagulant 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 10 ml-syringe along with 25 gauge needle 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; NaCI 0.45 M; glucose 0.1 M, adjusted to pH 4.6 with 1 M HC 1(Soderhall & Smith, 1983)], and 0.5 m 1 of haemolymph was extracted.

7.1.2. 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 I: 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).

7.1.3. Haemolymph slide preparation for Haemocyte count

After mixing with anticoagulants, a drop of the liquid was quickly poured on the corner of a slide and a spreader is utilized to first draw back the sample to spread it both left and right side and then push the sample to the entire front side of the slide at a single pressure in around 45^0 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.

7.1.4. Cell counting from slide

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

7.1.5. Cell counting using Haemocytometer

Total haemocyte counts (THC) were performed with an improved Neubauer haemocytometer using a modification of the method described by Bain *et. al.*,2001 for analysis of human blood samples. First, a special cover slip was taken on the improved Neubauer chamber. The WBC pipette containing anti-coagulated 0.05 ml blood sample were mixed well with 0.95m1 WBC fluid and a drops were discarded outside first, then I 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 4X16=64 squares (volume of each large corner square =1mmX1mmX0.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).

7.1.6. Calculating Formula

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

7.1.6.1. Determining Blood dilution

Since, 0.05mI (50 µL, 0.5 marked) blood mixed with preloaded 0.95m1 (950 µL, llmarked) anticoagulants and made lml of sample. Thus the dilution of blood sample was I in 20. At that point, 10.5µL anti-coagulant (instead of WBC fluid) and 0.5 µL haemolymph was mixed in 1: 20 ration.

7.1.6.2. Determining chamber volume

Determining volume of each large corner

Volume = lmm X lmm X 0.1mm=1mm X lmm X 1/10mm =1/10 (10 X 10 X 1) mm^3 = 1/10 (100) mm^3 = 1/10 X 100 mm^3 =10 mm^3

THC/ mm^3 = {(Cell counted) X (20) X (10)} / 4

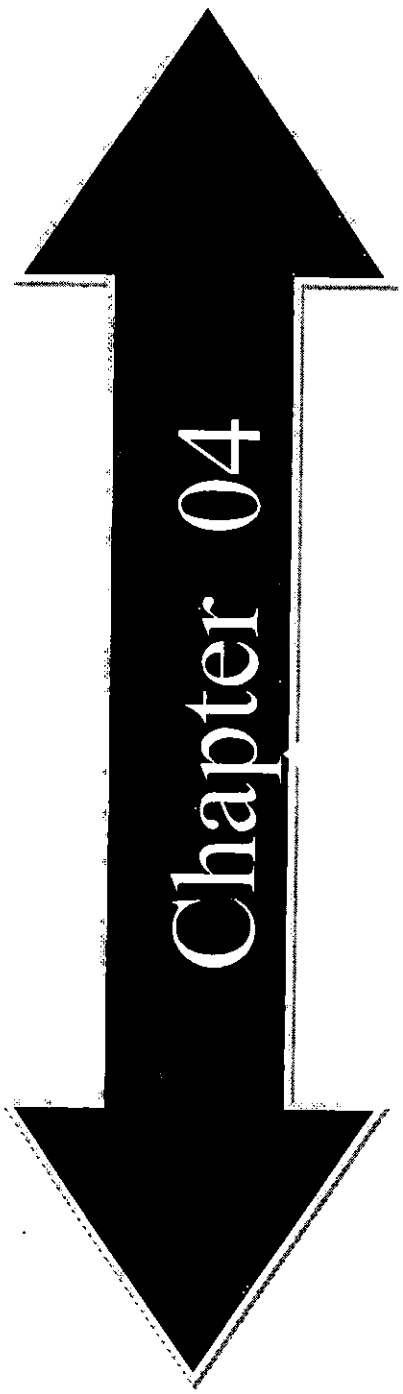
THC/ mm^3 = (Cell counted) X 50

7.1.7. Sequential Procedure of Total Haemocyte Count

- A special cover-slip and haemocytometer were ensured as grease-free and clean (alcohol used to clean).
- Moistened (with water or exhaled breath) and affixed cover-slip to the haemocytometer. 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.
- The WBC pipette was used to take well-mixed blood cell suspension (where 0.5 ml blood sample stained with equal volumes of Giemsa were mixed gently with 0.95ml anticoagulants e.g. mix 0.5mlGiemsa stained cell sample with 0.95ml anticoagulants).
- Then first 1/2 drops of sample were discarded outside and one drops were pipetted (approximately 10µl) at the edge of the cover-slip and allowed to run under the cover slip to disperse throughout the Neubauer chamber.
- 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.
- The cells were counted in one or more large corner squares and cell counts were recorded. To obtain an accurate cell count result, more than one large corner square of the haemocytometer were counted.
- Calculate the cells using formula.

8. Data collection and analysis

Collected data were stored, explored and analyzed using Microsoft Excel, SPSS and Microsoft Word program to present the results and discussion. Data are reported as means ± standard deviations. Independent samples t test using SPSS (version16.0) was applied to determine whether significant variations between the treatments existed.



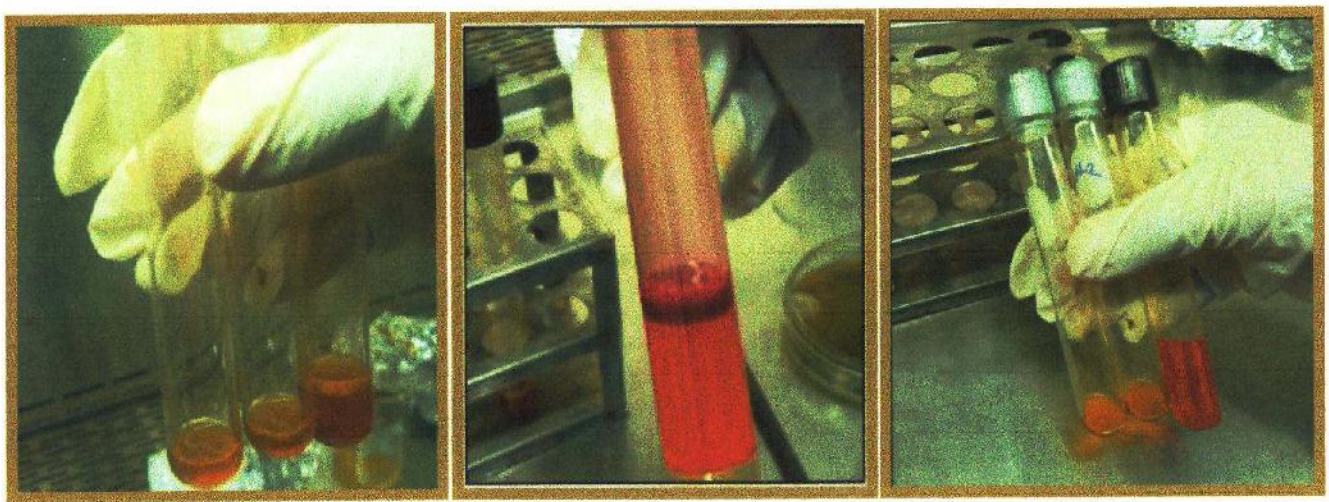
Result

Result

1. Biochemical test

1.1. Result of different biochemical tests for pathogenic spp.

Eight biochemical tests including 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 were performed (Table-4) to identify *vibrio harveyi* from the isolated colonies of *Vibrio* spp. 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 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 60% 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 *Clostridium butyricum* on *vibrio harveyi.* Snap shots of VP test; indole test and MR test are given in Figure 2.



VP test Indole test MR test

Figure 2: Different confirmation tests for *Vibrio harveyi*

Table 4: Biochemical tests 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%	+	+	+	+	+	+	+	+		+
5%	+	+	+	+	+	+	+	+	N/D	+
7%	-	-	-	-	+	-	-	-		-
10%	-	-	-	-	N/D	-	-	-		-
Growth at										
4°C	-	-	-	-		-	-	-		-
28°C	+	+	+	+	N/D	+	+	+	N/D	+
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.*

1.2. Result of biochemical test for probiotic spp.

In addition, among very few of lactic acid bacteria under clostridia species only the *Clostridium butyricum* is capable to fermented lactose as well as to produce hydrogen gas. The isolated species showed a positive result in lactose fermentation (yellow color indicates positive result where red color indicates negative result) (Figure 3).

Figure 3: Confirmation test of *C. butyricum*

2. PCR result

PCR test was performed to confirm the presence of *Vibrio sp.* in the sample. Three bands of target genes were found on electrophoresis gel at 663 bp position for *Vibrio* sp. which indicated the presence of pathogenic *Vibrio* in prawn sample. Figure 4 show the presence of pathogenic bacteria in prawn.

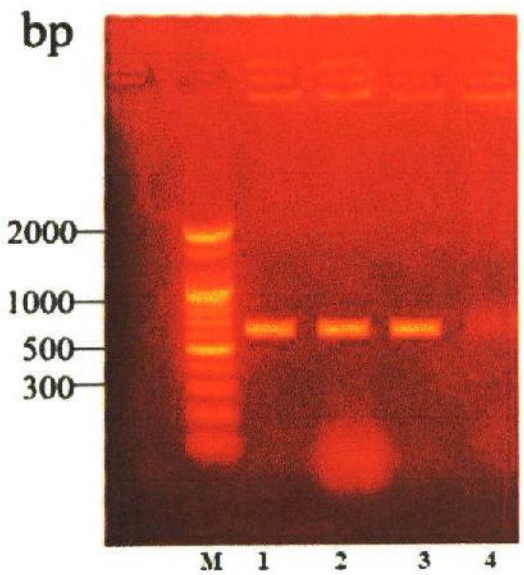


Figure 4: Electrophoretic analysis of PCR-amplified target genes from *Vibrio* sp. obtained under optimal conditions of PCR of isolates from biochemical test. Mobilities of the different target genes are indicated on the right. Lane M, 100-bp DNA ladder (size marker); lane 1-2, gene for stock sample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

3. Output of *In-vitro* challenge test

When *in-vitro* challenge was performed, the average load of *Vibrio* spp. in the intestinal tract at zero (0) hour was was $4.69\times 10^3\pm5.39\times 10^2$ CFU/g and it was $2.30\times 10^4\pm8.25\times 10^2$, $2.36\times 10^5\pm2.93\times 10^2$ and $4.24\times 10^5\pm4.92\times 10^4$ at 4th, 8th and 12th hour respectively. However, after treatment of isolated probiotics, *Clostridium butyricum* the average load of *Vibrio* spp. in the intestinal tract at 4th, 8th and 12th hour was reduced to $1.34\times 10^4\pm7.89\times 10^2$, $2.69\times 10^3\pm2.72\times 10^2$and $5.75\times 10^3\pm3.26\times 10^2$respectively. The present study showed a non-significant reduction of *Vibrio* spp. load in the experimental intestinal tracts until 8th hour of probiotic application whereas a drastic and significant reduction ($P < 0.05$) in the load of *Vibrio* spp. had been obtained at the 12th hour of probiotic application. The result of the *in-vitro* challenge test of the experimental intestinal tracts with and without probiotics had been presented in Table 05.

Table 5: *In-vitro* challenge test and enumeration of *Vibrio harveyi* load with and without probiotics (*Clostridium butyricum*).

Time (hours)	Control (*V. harveyi*, CFU/g)	Probiotic (*V. harveyi*, CFU/g)
0	$4.69\times 10^3\pm5.39\times 10^2$	$4.70\times 10^3\pm5.02\times 10^2$
4	$2.30\times 10^4\pm8.25\times 10^2$	$1.34\times 10^4\pm7.89\times 10^2$
8	$2.36\times 10^5\pm2.93\times 10^2$	$2.69\times 10^3\pm2.72\times 10^2$
12	$4.24\times 10^5\pm4.92\times 10^4$	$5.75\times 10^3\pm3.26\times 10^2$

4. Output of In-*vivo* challenge test

In *in-vivo* challenge test the average load of *Vibrio* spp. in the intestinal tract of freshwater prawn in the control group at 0 day, 10 days, 20 days, 30 days, 40 days, 50 days and 60 days were $3.25\times 10^4 \pm 7.02\times 10^2$, $3.73\times 10^4 \pm 1.73\times 10^2$, $2.23\times 10^5 \pm 1.32 \times 10^3$, $5.32\times 10^5\pm 1.19 \times 10^3$, $7.61\times 10^6\pm 4.9 \times 104$, $8.26 \times 10^6\pm 2.53 \times 10^4$ and $9.79 \times 10^6\pm 7.12 \times 10^4$ respectively. But after the application of probiotic, *Clostridium butyricum* the average load of *Vibrio* spp. in the intestinal tract of freshwater prawn at the before mentioned days became $3.61\times 10^4\pm 2.11\times 10^2$, $2.43\times 10^4\pm 1.21\times 10^2$, $7.53\times 10^3\pm 3.11\times 10^2$, $2.21 \times 10^3 \pm 2.37 \times 10^2$, $1.03\times 10^3\pm 3.17 \times 10^2$, $6.32 \times 10^2 \pm 1.32 \times 10^1$ and $2.91 \times 10^2 \pm 7.02 \times 10^1$respectively. The present study also showed that no significant reduction of *Vibrio* spp. load in the experimental intestinal tract was found until 10 days of probiotic application whereas at 20, 30, 40, 50 and 60 days a significant reduction of *Vibrio* spp. was observed ($P < 0.05$). The result of the *in-*

vivo challenge test of the experimental intestinal tract with and without probiotics was presented in Table-06.

Table 6: *In-vivo* challenge test and enumeration of *V. harveyi* load with and without probiotics.

Time (days)	Control (*V. harveyi*, CFU/g)	Probiotic (*V. harveyi*, CFU/g)
0	$3.25\times 10^4 \pm 7.02\times 10^2$	$3.61\times 10^4 \pm 2.11\times 10^2$
10	$3.73\times 10^4 \pm 1.73\times 10^2$	$2.43\times 10^4 \pm 1.21\times 10^2$
20	$2.23\times 10^5 \pm 1.32\times 10^3$	$7.53\times 10^3 \pm 3.11\times 10^2$
30	$5.32\times 10^5 \pm 1.19\times 10^3$	$2.21\times 10^3 \pm 2.37\times 10^2$
40	$7.61\times 10^6 \pm 4.9\times 10^4$	$1.03\times 10^3 \pm 3.17\times 10^2$
50	$8.26\times 10^6 \pm 2.53\times 10^4$	$6.32\times 10^2 \pm 1.32\times 10^1$
60	$9.79\times 10^6 \pm 7.12\times 10^4$	$2.91\times 10^2 \pm 7.02\times 10^1$

5. Growth performance

In this study the effects of diets containing probiotic (*Clostridium butyricum)*, on growth performance were evaluated. Data analyzed, on the growth performance of prawn in treatments and control, including initial weight, final weight, weight gain as well as SGR is showed in appendix. There were no significant differences for initial weight between treatment and control at the start of the experiment. At the end of the experiment, statistical analysis showed that prawn fed probiotic supplemented diets grew significantly faster than the control group. Average final weight was recorded significantly different ($P < 0.05$) for treatment (16.82±0.49 g) compared to the control (10.34±1.03 g). In addition significant differences ($P < 0.05$) were recorded in weight gain for treatment (476.25±35.72)% in comparison with control (371.31±43.95) % (Figure 5). Moreover, SGR in the prawn fed *Clostridium butyricum* diet were significantly different ($P < 0.05$) from those of the control group (Figure 6)

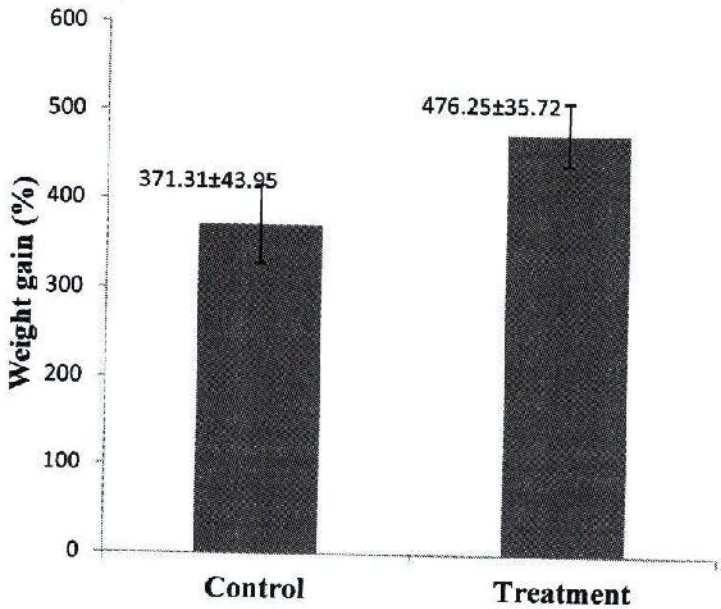


Figure 5: The weight gain of *M. rosenbergii* reared with and without *Clostridium butyricum* probiotic added to fed. Each value is mean ± standard deviation of three individual observations. The mean differences are significant at (P<0.05).

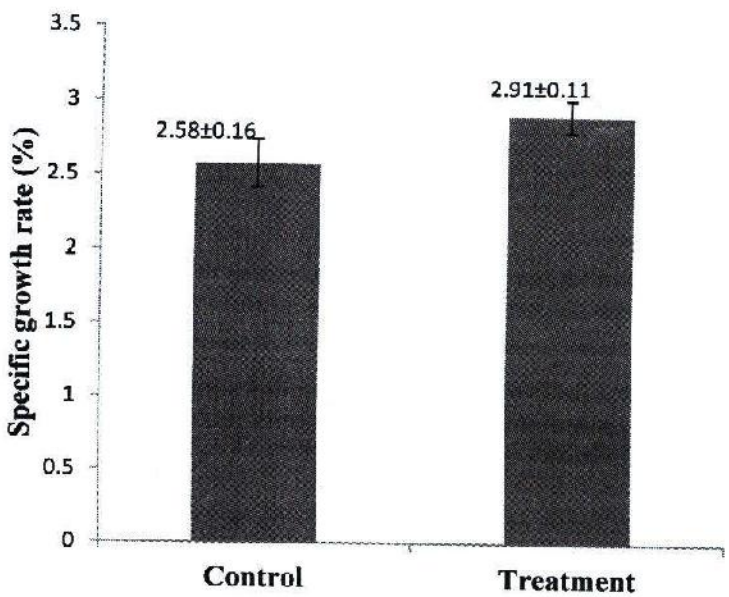


Figure 6: The specific growth rate of of *M. rosenbergii* reared with and without *Clostridium butyricum* probiotic added to feed. Each value is mean ± standard deviation of three individual observations. The mean differences are significant at (P<0.05).

6. Digestive Enzyme Activity

The digestive enzyme activity in crustacean plays a central role in nutritional physiology and may directly or indirectly regulate growth, moult cycle and dietary formulation. In the present study, the activities of digestive enzymes such as protease and amylase were elevated. The specific enzyme activity including amylase as well as protease of treatment group was significantly higher ($P < 0.05$) with that of the control examined after 60 days of feeding trial (Figure 7-8). It clarifies the fact that probiotic, *Clostridium butyricum* showed their ability to increase digestive enzyme activities in prawn body.

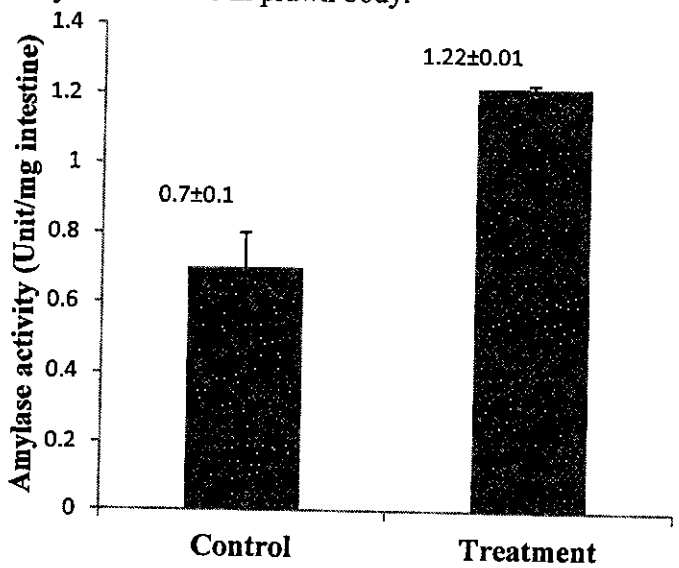


Figure 7: Amylase activity in digestive tract of *Macrobrachium rosenbergii* reared with and without *Clostridium butyricum* probiotic added to fed. Each value is mean ± standard deviation of three individual observations. The mean differences are significant at ($P<0.05$).

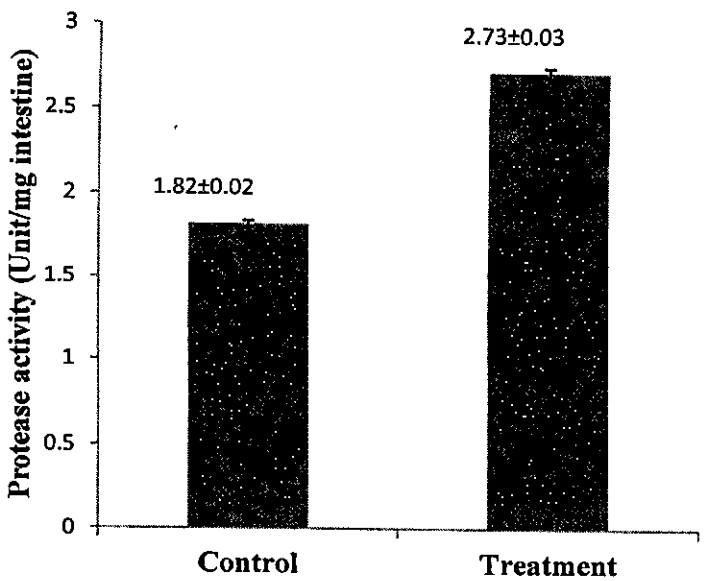


Figure 8: Protease activity in digestive tract of *Macrobrachium rosenbergii* reared with and without *Clostridium butyricum* probiotic added to fed. Each value is mean ± standard deviation of three individual observations. The mean differences are significant at ($P < 0.05$).

7. Status of haemocite

Haemocytes play important roles in their immune response including recognition, phagocytosis, melanization, cytotoxicity and cell-cell communication in Crustacean. In this present study, the heterogeneous prawn haemocyte population of the prawn haemolymph was presented mainly based on based on the cell size and presence of the granules, three major groups of hemocytes, including nongranular haemocyte (NGH), small granular haemocyte (SGH) and large granular (LGH) haemocyte were identified in the present study. The total haemocytes counts (THC) were found about $(5.2 \pm 0.73) \times 10^6$ and $(5.6 \pm 0.67) \times 10^6$ in control group without any probiotic and probiotic treated group with probiotic *C. butyricum* respectively (Table-7). So to say, the ponds with probiotic revealed higher THC than the ponds without probiotic. It clarifies the fact that probiotic are capable to increase THC in the prawn haemolymph. In control group nongranular haemocyte represented the highest percentage of haemocyte (75.33 ± 15.93%) followed by small granular haemocyte (22.24 ± 9.13%) and large granular haemocyte (2.43 ± 2.2%). In case of probiotic treated group about 76.56 ± 17.33% of haemocyte were NGH whereas only about 21.13 ±10.32% and 2.31 ± 1.9% were SGH and LGH respectively (Table-7).Thus both in the control and treatment most of the haernocytes were belonged to nongranular haemocytes whereas a very few were belonged to large granular haemocyte. Although there were no significant differences of THC, DHC (NGH, SGH, LGH) between treatment and control group ($P > 0.05$) (Figure 9-10). A snap shot from the light microscope represents three major types of haemocyte (Figure 11)

Table 7: Average total haemocyte count (THC) and differential haemocyte count (DHC) of *Macrobrachium rosenbergii* fed with pure diet and probiotic, *Clostridium butyricum* - supplemented diet after 60 days in the experiment.

Treatment	THC (cmm)	DHC (%)		
		NGH	SGH	LGH
Control	$(5.2 \pm 0.73) \times 10^6$	75.33± 15.93	22.24± 9.13	2.43± 2.2
Treatment with probiotic	$(5.6 \pm 0.67) \times 10^6$	76.56± 17.33	21.13±10.32	2.31± 1.9

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

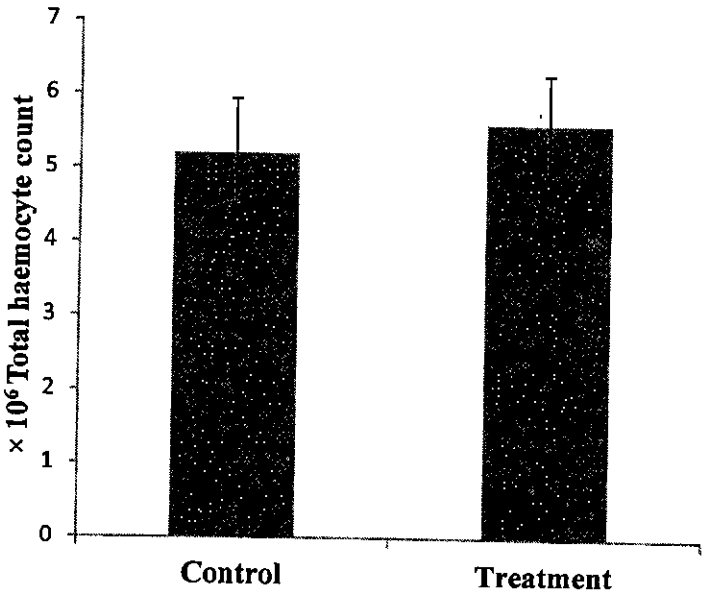


Figure 9: Average total haemocyte count of *Macrobrachium rosenbergii* fed with pure diet and probiotic, *Clostridium butyricum* -supplemented diet after 60 days in the experiment.

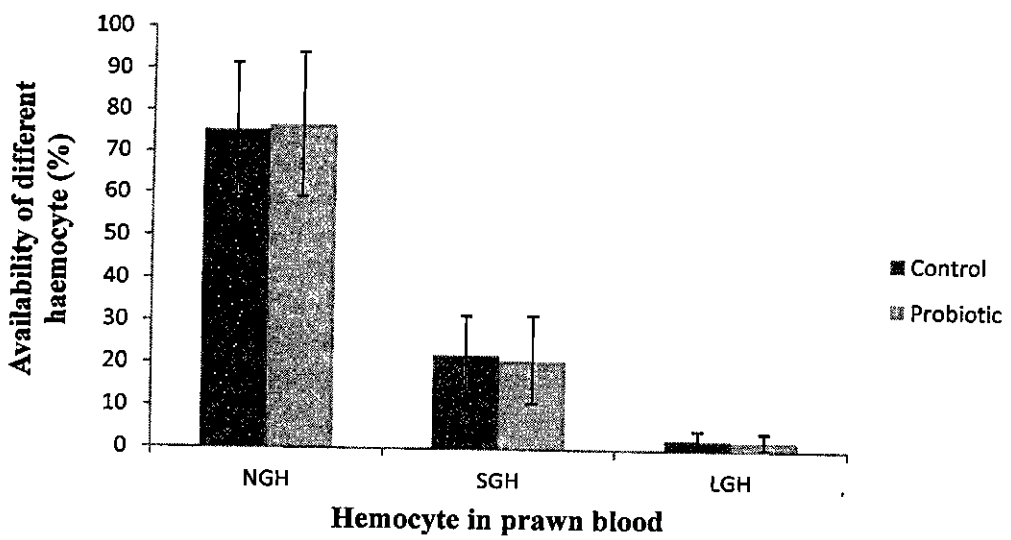


Figure 10: Average differential haemocyte count of *Macrobrachium rosenbergii* fed with pure diet and probiotic, *Clostridium butyricum* -supplemented diet after 60 days in the experiment.

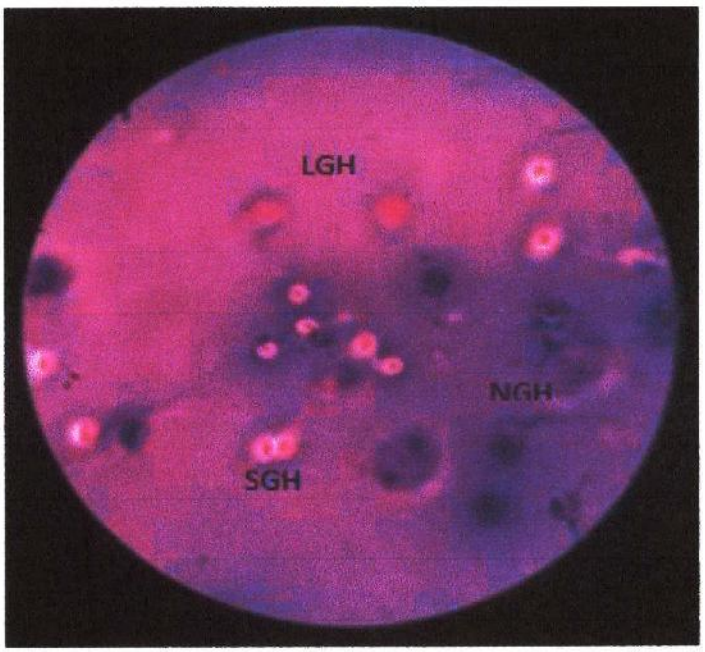
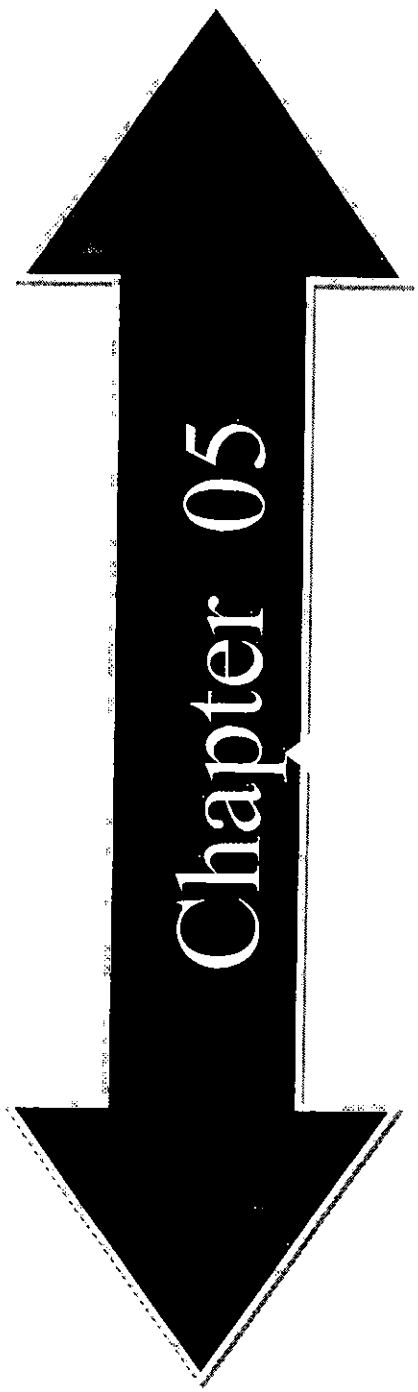


Figure 11: Three major types of haemocyte found in prawn blood [Large Granular Haemocyte (LGH); Small Granular Haemocyte (SGH); Non Granular Haemocyte (NGH)]



Discussion

Discussion

Today probiotics, well-known for their antagonistic effect, disease preventing and health promoting properties, are quite commonplace in health-promoting functional foods as well as growth supplements in fish production (Senok *et al.*, 2005). Typically, the *C. butyricum* that is a natural resident of human and animal gut, has been widely used to prevent disturbances of microflora in the host intestine and to treat pathogenic diseases, enhance immune response, promote digestion and increase growth performance in fish (Song et al., 2006; Yang *et al.*, 2010). In the present study, this bacterial species was chosen in order to evaluate its potential capacity for controlling pathogenic bacterial load in prawn body, which may help to reach a better understanding *of C. butyricum.*

Data, obtained from the *in-vitro* test of the present study, revealed that *C. butyricum* non significantly reduced the load of *Vibrio* spp. just after 4 hours passed of the combined culture of *Vibrio* spp. and *C. butyricum* but a significant reduction of the growth of *Vibrio* spp. was observed after passing 12 hours of the culture period ($P < 0.05$). *C. butyricum* is able to produce different types of organic acids including butyric acid as well as lactic acid at the time of fermentation. Thus, these organic acids produced by *C. butyricum* during the process of fermentation, which lower the pH of the culture medium, play an important role in inhibiting the growth of pathogens. A couple of research findings including Gao *et al.* (2013), Balcázar *et al.*(2006), Senok *et al.* (2005) also exhibited the similar findings.

The results of *in-vivo* test of the present experiment demonstrates that probiotic (*Clostridium butyricum)* significantly reduced the load of *Viorio spp.* in the intestine of prawn cultured with *C. butyricum* incorporated diet for a period of about 60 days ($P < 0.05$). This is because the fact that probiotics 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 *et al.* 2000). A couple of previous research findings also support the findings of the present study. Sakai *et al.* (1995) concluded that *C. butyricum* and its spent culture supernatants exhibite significant inhibitory activity on *S. enteritidis* and *V.parahaemolyticus* growth and this probiotic bacterium *C. butyricum* has preventive and therapeutic effects on *S. enteritidis* and *V. parahaemolyticus* infections in fish. In 2008, Imase *et al.* explained that this bacterium has an antagonistic effect with different harmful bacteria. Zhang *et al.* (2011) showed that this probiotic species significantly decreased the load of pathogenic bacterial species. It has been proved that Clostridium butyricum MIYAIRI strain is capable to prevent the growth of

pathogenic bacterial species. The highest level of bactericidal and bacteriostatic properties against *Pseudomonas aeruginosa, Salmonella enteritidis* and *Escherichia coli* was found with the *C. butyricum* from the research output of Szymanowska-Powalowska *et al.*, 2014. Kuroiwa *et al.* in 1990 revealed that C. butyricum MIYAIRI 588 could inhibit the growth of various enteropathogens. *Clostridium butyricum* has previously shown effectiveness against *Aeromonas hydrophila* and *Vibrio anguillarum.* Another research findings showed that *C. butyricun* will be helpful in the management of *V. alginolyticus* related bacterial disease in tiger shrimp *P. monodon.* Vieira *et al.* in 2010 revealed that *C. butyricum* can enhance resistance to *V. harveyi* infection by increasing total haemocyte as well as serum agglutination activity in prawn body. It needs to be stressed that *C. butyricum* bacteria also exhibit antagonistic interactions with *Candida albicans, C. difficile, Klebsiella spp., Salmonella spp., Vibrio spp.* (Chen *et al.*, 1987; Fujita and Takashi, 1987; Kuroiwa *et al.*, 1990). Numerous reports based on the inhibitory effect of *C. butyricum* on the pathogenic bacteria (Tanaka *et al.*, 2000; Takahashi *et al.*, 2004; Shimbo *et al.*, 2005, Elumalai *et al.*, 2013, Bairagi *et al.* 2002 and Sakata *et al.* 1995) also supports the findings of the present study.

In this study dietary administration of *Clostridium butyricum* significantly improved final weight, weight gain and SGR ($P < 0.05$). This is due to the fact that probiotics have a beneficial effect on the digestive processes of aquatic animals because probiotic strains synthesize extracellular enzymes such as proteases, amylases, and lipases as well as provide growth factors such as vitamins, fatty acids, and aminoacids (Balcázar *et al.* 2006). Therefore, nutrients are absorbed more efficiently when the feed is supplemented with probiotics (Haroun *et al.* 2006). Although Gibson and Roberfroid (1995) concluded that probiotics improve the fish health and growth through modifing the fish associated microbial community. Research findings of Zhang *et al.* (2011) revealed that *Clostridium butyricum* treated feed significantly increased the growth of broiler chickens. A research, conducted by Yang *et al.* in 2012 also supports the finding of the present study and elucidated that this bacterial species increase the growth rate of broiler chickens. Similar results have been reported in marine shrimp fed with *Clostridium butyricum* incorporated diets had significantly higher growth performance. It has been reported that lactic acid bacteria (*Clostridium butyricum* is also a lactic acid bacteria) incorporated diets had significantly increased growth performance in Atlantic cod fry (*Gadus morha*) (Gildberg *et al.* 1997).

Seenivasan *et al.* (2012) reported that lactic acid bacteria *C. butyricun* can be used as a feed additive either as enrichment material of live feeds or as supplementary material in formulated feeds for enhancing the growth and production of *M. rosenbergeii* PL. In 2014 Seenivasan *et al.* concluded that lactic acid bacteria incorporation has significantly improved survival, growth, specific growth rate (SGR), feed conversion rate (FCR), feed conversion efficiency (FCE) and protein efficiency rate (PER) biochemical constituents and energy utilization in post larvae of *M. rosenbergii* based on a research work named effect of *Lactobacillus sporogenes* on survival, growth, biochemical constituents and energy utilization of freshwater prawn *Macrobrachium rosenbergii* post larvae. Liao *et al.* (2015) found that *C. butyricum* incorporated feed reveals a significantly higher growth performance in the growth of broilers in terms of average weight gain and specific growth rate. Rengpipat *et al.* (2000) reported in Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) fed with *Clostridium bacterium* incorporated diets had significantly improved the growth performance than the control. Based on the experiment conducted by Sakata *et al.* 1995, it could be said that *C. butyricum* produces short chain fatty acids (SCFAs), such as butyric acid, propionic acid, and acetic acid. Moreover they have potential beneficial effects; in particular, butyric acid has a proliferative effect on intestinal mucosal cells. In 2013, Elumalai *et al.*, revealed that *Clostridium butyricum, Streptococcus faecalis, Streptococcus faecium, Bacillus mesentericus, Bacillus subtilis, Bacillus natto, Saccharomyces cerevivisiae* incorporated diets had significantly higher growth performance in case of *Peneaus monodon.* In contrast, Shariff *et al.* (2001) and McIntosh *et al.* (2000) found that treatment of *P. monodon* and *Litopenaeus vannamei* with a commercial *Bacillus* probiotic did not significantly increase growth. This is due to the probiotic species variation that means the probiotic species used in the that expeiment is different from the probiotic species used in the present study.

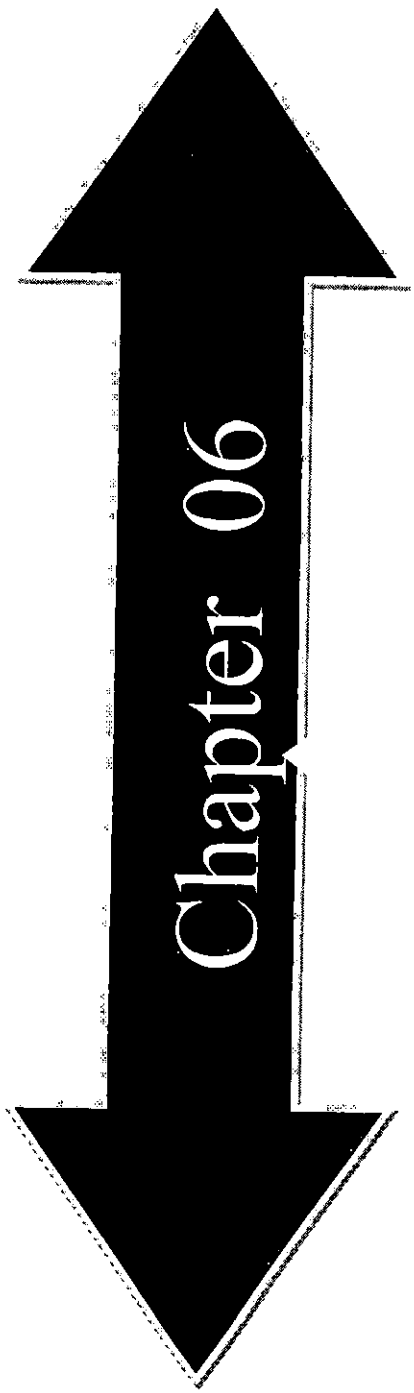
The digestive enzyme activity in crustacean plays a central role in nutritional physiology and may directly or indirectly regulate growth (Lovett and Felder, 1990), moult cycle (Van Wormhoudt and Favrel, 1988) , and dietary formulation (Moullac *et al.*, 1996).The dietary administration of this species also significantly ($P<0.05$) enhanced the amylase and protease activity in prawn body. Yang et al. (2005) explained that the increase in digestive enzymes activity may be caused by the synbiotic that stimulates a healthier digestive tract microbial ecology and modifies the selection of bacterial enzymes. Research carried out by Nimrat and Vuthiphandchai in 2011 showd that *C. butyricum* as a probiotic is capable to enhance the growth of shrimp by considerably increasing enzyme activity in the shrimp body which

supports the findings of the present study. Kennedy *et al.* (1998) showed that this type of probiotics are able to increse protease enzyme activity in the fish body that ultimately increase diet absorbtion by fish (BB). It has been reported that *C. butyricum* incorporated diets had significantly increased digestive enzymes activity in prawn body (Bairagi *et al.*, 2002). It also a couple of research findings including. In Seenivasan et al.(2014); Ziaei-Nejad *et al.* (2006); Wang and Xu (2006);Verschuere *et al.* (2000), Mohapatra *et al.* (2012) supports the findings of the present study.

In this study, the total haemocytes counts (THC) were found at values of $(5.2 \pm 0.73) \times 10^6$ and $(5.6 \pm 0.67) \times 10^6$ per cubic milimeter in control and treatment respectively. That means prawn treated with probiotics incorporated feed represented a little bit higher THC than prawn treated with pure feed, although this difference was not significant ($P > 0.05$). Owens and O'Neill (1997) showed 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) found that the total haemocyte number didn't vary significantly in the prawn given probiotic mixed feed from the prawn feed without any probiotic mixed feed. Saraswati *et al.* (2013) also showed that *C. butyricum* increase total haemocyte count in prawn body but it is not significant enhancement. Althogh Partida-Arangure *et al.* (2013) observed that prawn feed with probiotic incorporated diet revealed significantly higher total haemocyte count than those feed with pure feed ($P = 0.04$). Owens and O'Neill (1997) found that the total haemocyte count of *Penaeus monodon* was 23.3×10^6 cells mL^{-1} using a haemocytometer whis is almost simiar with the value of the present experiment. Similarly Tsing et al. (1989) reported about $5\text{-}14 \times 10^6$ cells/mm^3 in the hemolymph of the *Penaeus japonicas* as our record in *F. indicus*. In shrimp, *Penaeus setiferus* the THC was approximately recorded 8.9×10^6 (Yeager and Tauber, 1935). Vieira *et al.* in 2010 showed that C. *butyricum* can enhance resistance to *V. harveyi* infection by increasing total haemocyte as well as serum agglutination activity in prawn body. In contrastr, Martin and Graves (2005) reported a relatively low THC (about 11×103 cells/mm3) in the *Penaeus californiensis*. Balasundaram *et al.*, (2013) calculated the haemocyte of normal *Penaeus monodon* after 30 days of probiotic supplementation and found that THC in control prawn was 1442 cells/cu.mm, THC in *B. coagulans* supplemented prawn was 2757 cells/cu.mm. This lower THC might be due to the differences of methodology and species variation.

Different fisheries researchers have adopted different criteria for hemocytes classification although the classification of crustacean hemocytes remains controversial (Hose et al. 1987,

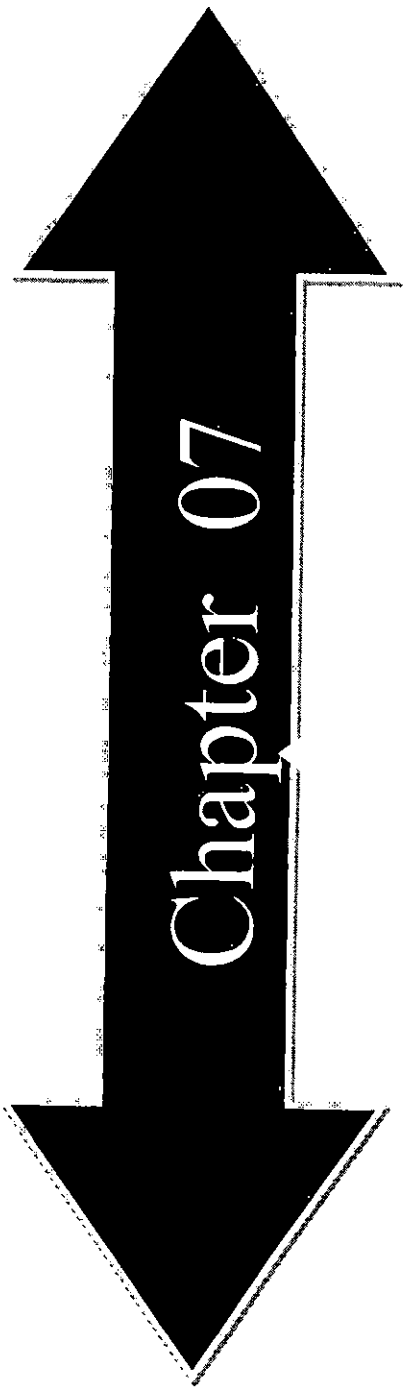
1990a). The results of the present study *Macrobrachium rosenbergii* comprise three major groups, including non granular hemocyte (NGH), small granule hemocyte (SGH) and large granule hemocyte (LGH) depend on granules size. Differential hemocyte count values were recorded in the relative percentage of the hemocytes. No granular haemocyte (NGH) represented about 75.33 ± 15.93% and 76.56 ± 17.33% of the circulating hemocytes in control and treatment respectively. Although SGH represented about 22.24 ± 9.13% and 21.13 ±10.32% whereas LGH represented 2.43 ± 2.2% and 2.31 ± 1.9% in control and treatment respectively. There was no significant differences between control and treatment in terms of NGH, SGH and LGH (P> 0.05). In *Sicyonia ingentis*, Hose *et al.* (1992) recorded that the NGH comprises 50–60% of the circulating hemocytes, whereas the SGH and LGH represented 30% and 10%, respectively. NGH in *F. indicus* is varied and approximately was 10-15%, lower value compared to former species and LGC and SGC were 20-25% and 60-65%, respectively (Kakoolaki *et al.*, 2010). The NGH of *Macrobrachium rosenbergii*, posses 70% of hemocytes (Vazquez *et al.*, 1997) that also supports the findings of the present research.



Conclusion

Conclusion

The present experiment revealed that antagonistic effect to pathogens, growth, enzyme activity as well as immunity were obtained significantly higher in *M. rosenbergii* fed with *C. butyricum* incorporated diet than those fed with diet without probiotic .Take together, this work strongly suggest that the probiotic species, C. *butyricum* could provide protection against *Vibrio spp.* infections in prawn as well as ehhance the growth of prawn. From this study it is evident that application of this probiotic species will be capable to improve the growth and immunity of *M. rosenbergii* and which in turn will pave the way to reap better profit for the fish farmers.



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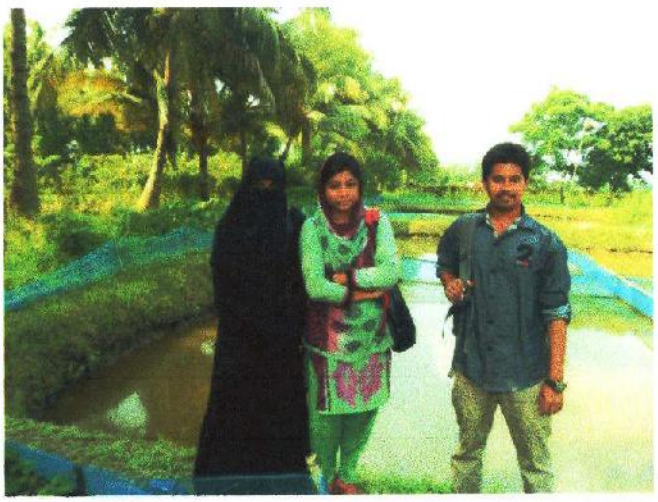
Appendix

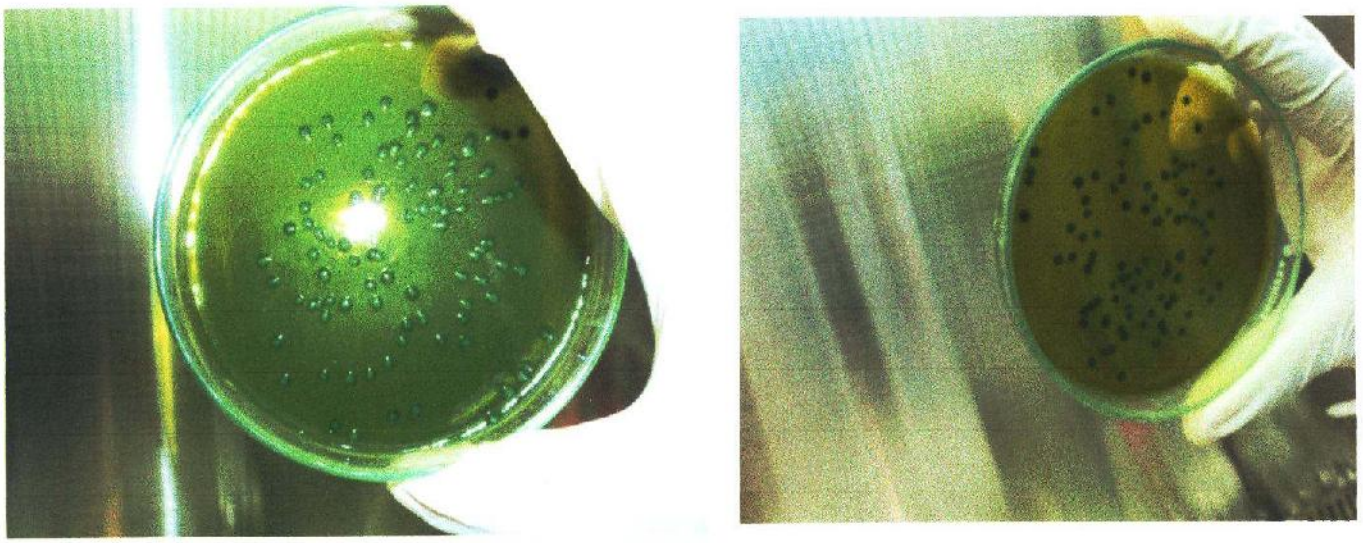
Photo Gallery

Site of the present experiment

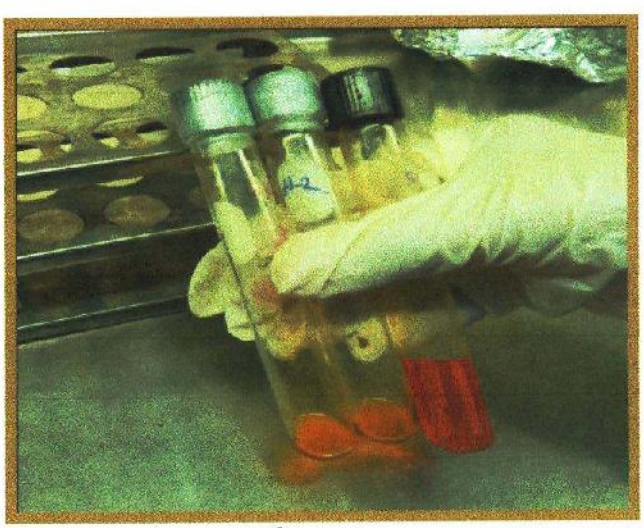

Giving probiotic incorporated feed in the treatment


Harvesting of prawn after 60 days culture



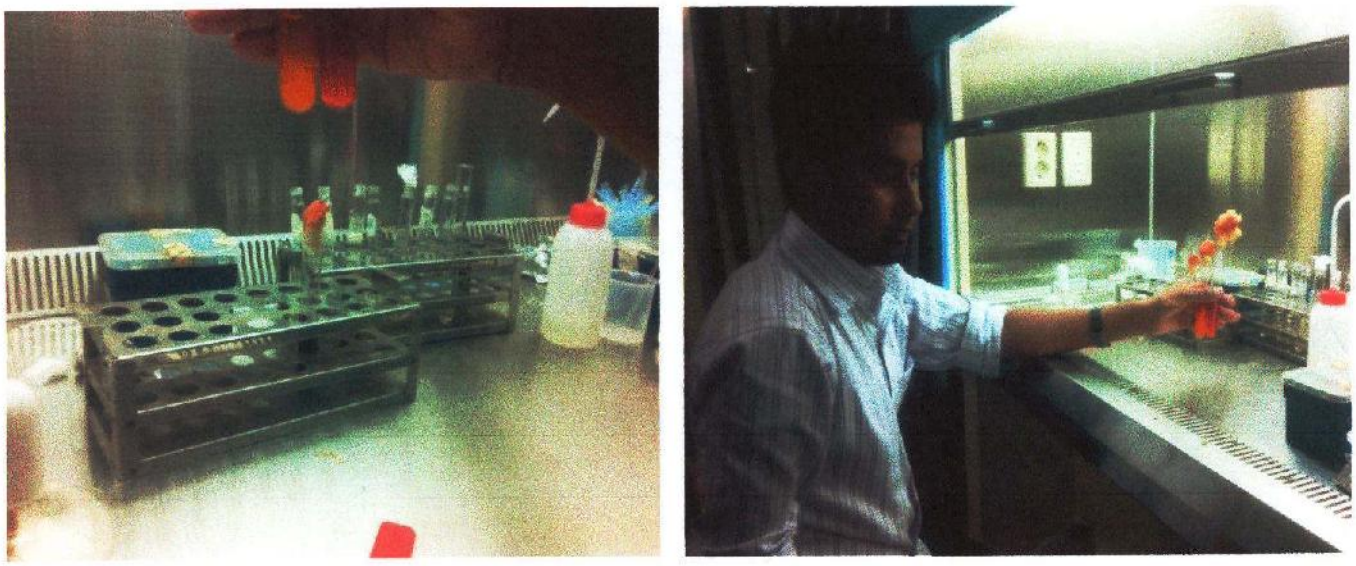
Vibrio spp. in patridish

1 2

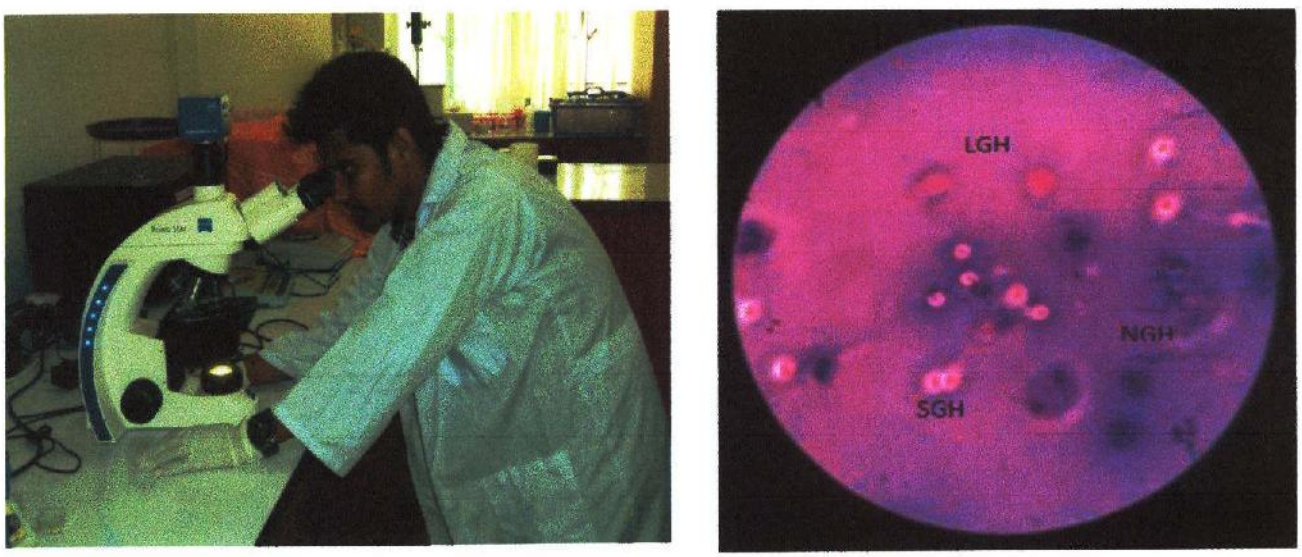


3

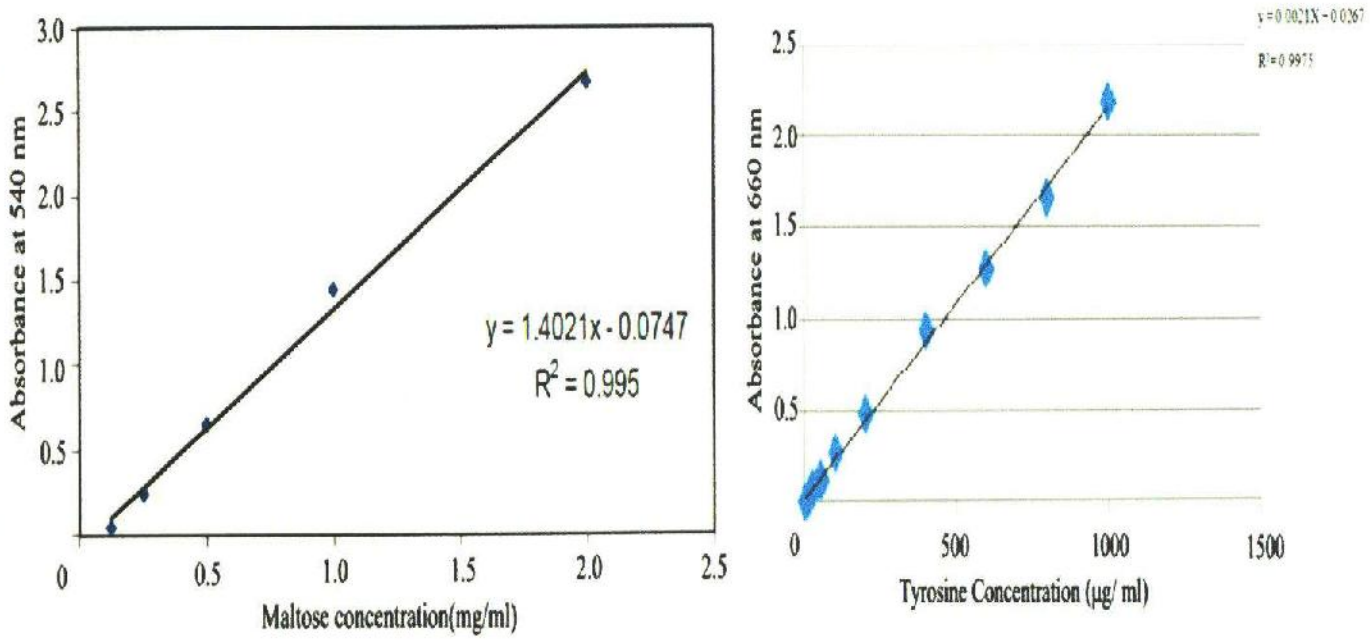
Snap shots of *vibrio harveyi* conformation test



Snap shots of *Clostridium butyricum* conformation test (Lactose fermentation test)



Snap shots of detectable haemocytes in microscope



Standard curves for determining enzyme activity

Raw data that were not showed in the main body

Amylase Activity

	R1	R2	R3	Average	SD
Control	0.6	0.7	0.8	0.7	0.1
Treatment	1.22	1.21	1.23	1.22	0.01

Protease Activity

	R1	R2	R3	Average	SD
Control	1.83	1.82	1.8	1.82	0.02
Treatment	2.75	2.72	2.73	2.73	0.03

Initial weight

	R1	R2	R3	Average	SD
Control	2.25	2.18	2.15	2.19	0.05
Treatment	2.79	2.96	3.02	2.92	0.12

Final weight

	R1	R2	R3	Average	SD
Control	11.12	9.17	10.73	10.34	1.03
Treatment	17.21	16.27	16.98	16.82	0.49

Weight Gain

	Avearge	R1	R2	R3	SD
Control	371.31	394.22	320.64	399.07	43.95
Treatment	476.25	516.85	449.66	462.25	35.72

Specific Growth

	R1	R2	R3	Average	SD
Control	2.67	2.4	2.67	2.58	0.16
Treatment	3.03	2.83	2.87	2.91	0.11

t - test result

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
h0	Equal variances assumed	.002	.964	-.002	4	.999	-6.66667	4364.09084	-12123.32533	12109.99199
	Equal variances not assumed			-.002	3.999	.999	-6.66667	4364.09084	-12124.59310	12111.25977

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
h4	Equal variances assumed	.003	.959	1.403	4	.233	9596.66667	6840.03493	-9394.31482	28587.64815
	Equal variances not assumed			1.403	3.997	.233	9596.66667	6840.03493	-9400.82713	28594.16046

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
h8	Equal variances assumed	12.259	.025	2.640	4	.058	1.28310E5	48608.65081	-6649.25060	2.63269E5
	Equal variances not assumed			2.640	2.004	.118	1.28310E5	48608.65081	-80418.51239	3.37039E5

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
h12	Equal variances assumed	5.456	.080	14.495	4	.000	4.18913E5	28900.62533	3.38672E5	4.99154E5
	Equal variances not assumed			14.495	2.018	.005	4.18913E5	28900.62533	2.95638E5	5.42189E5

Independent Samples Test

	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Days (0) Equal variances assumed	4.103	.113	-2.576	4	.062	-3266.66667	1267.98177	-6787.14846	253.81512
Equal variances not assumed			-2.576	2.447	.101	-3266.66667	1267.98177	-7870.83758	1337.50425

Independent Samples Test

	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Days (10) Equal variances assumed	8.532	.043	25.511	4	.000	33000.00000	1293.57386	29408.46319	36591.53681
Equal variances not assumed			25.511	2.024	.001	33000.00000	1293.57386	27497.09105	38502.90895

Independent Samples Test

	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
D (20) Equal variances assumed	15.675	.017	215.456	4	.000	2.15470E5	1000.06666	2.12693E5	2.18247E5
Equal variances not assumed			215.456	2.001	.000	2.15470E5	1000.06666	2.11168E5	2.19772E5

Independent Samples Test

	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Day(30) Equal variances assumed	3.989	.116	43.696	4	.000	5.29790E5	12124.36802	4.96127E5	5.63453E5
Equal variances not assumed			43.696	2.000	.001	5.29790E5	12124.36802	4.77623E5	5.81957E5

Independent Samples Test

	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Day (40) Equal variances assumed	3.999	.116	65.896	4	.000	7.60897E6	1.15470E5	7.28837E6	7.92957E6
Equal variances not assumed			65.896	2.000	.000	7.60897E6	1.15470E5	7.11214E6	8.10580E6

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Day(50)	Equal variances assumed	3.999	.116	286.113	4	.000	8.25937E6	28867.51358	8.17922E6	8.33952E6
	Equal variances not assumed			286.113	2.000	.000	8.25937E6	28867.51358	8.13516E6	8.38357E6

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Day(60)	Equal variances assumed	13.247	.022	101.880	4	.000	9.78971E6	96090.23537	9.52292E6	1.00565E7
	Equal variances not assumed			101.880	2.000	.000	9.78971E6	96090.23537	9.37627E6	1.02032E7

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Amylase	Equal variances assumed	3.208	.148	-8.962	4	.001	-.52000	.05802	-.68110	-.35890
	Equal variances not assumed			-8.962	2.040	.011	-.52000	.05802	-.76502	-.27498

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Protease	Equal variances assumed	.000	1.000	-73.497	4	.000	-.91667	.01247	-.95130	-.88204
	Equal variances not assumed			-73.497	4.000	.000	-.91667	.01247	-.95130	-.88204

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
WG	Equal variances assumed	.341	.591	-3.210	4	.033	-104.94333	32.69639	-195.72306	-14.16361
	Equal variances not assumed			-3.210	3.839	.035	-104.94333	32.69639	-197.23965	-12.64702

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
SGR	Equal variances assumed	1.116	.350	-3.034	4	.039	-.33000	.10878	-.63202	-.02798
	Equal variances not assumed			-3.034	3.521	.046	-.33000	.10878	-.64896	-.01104

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
THC	Equal variances assumed	.800	.422	-3.098	4	.06	-.40000	.12910	-.75844	-.04156
	Equal variances not assumed			-3.098	2.941	.055	-.40000	.12910	-.81554	.01554

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
NGH	Equal variances assumed	.800	.422	-.953	4	.395	-1.23000	1.29099	-4.81438	2.35438
	Equal variances not assumed			-.953	2.941	.412	-1.23000	1.29099	-5.38540	2.92540

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
SGH	Equal variances assumed	.004	.952	1.459	4	.218	1.15333	.79070	-1.04201	3.34868
	Equal variances not assumed			1.459	3.982	.219	1.15333	.79070	-1.04582	3.35249

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LGH	Equal variances assumed	3.208	.148	2.068	4	.107	.12000	.05802	-.04110	.28110
	Equal variances not assumed			2.068	2.040	.172	.12000	.05802	-.12502	.36502