

**Effect of Commercial Probiotics on Total Bacteria and *Vibrio* spp.
in Shrimp (*Penaeus monodon*) Nursing**



TANAY BISWAS

Examination Roll: MS - 170603

Session: 2016-17



Fisheries and Marine Resource Technology Discipline

Khulna University

Khulna-9208, Bangladesh

DECEMBER 2018

**Effect of Commercial Probiotics on Total Bacteria and *Vibrio* spp.
in Shrimp (*Penaeus monodon*) Nursing**

A Thesis By

TANAY BISWAS

Examination Roll: MS -170603

Session: 2016-17



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

M. Sc. in Fish Processing and Quality Control

Fisheries and Marine Resource Technology Discipline

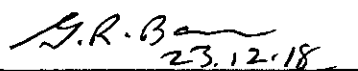
Khulna University

Khulna-9208, Bangladesh

DECEMBER 2018

**Effect of Commercial Probiotics on Total Bacteria and *Vibrio* spp.
in Shrimp (*Penaeus monodon*) Nursing**

Approved as to style and content by


23.12.18

(Signature of the Head)

DR. GHASIATUR REZA BANU

Professor and Head

Fisheries and Marine Resource Technology Discipline

Life Science School, Khulna University

Khulna-9208, Bangladesh

DECEMBER 2018

DECLARATION

Effect of Commercial Probiotics on Total Bacteria and *Vibrio* spp. in Shrimp (*Penaeus monodon*) Nursing

The results presented in the 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.

Candidate

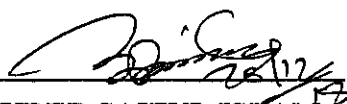
Tanay Biswas
20.12.18

TANAY BISWAS

Examination Roll: MS - 170603

Session: 2016-2017

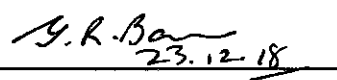
Co-Supervisor


SHIKDER SAIFUL ISLAM

Assistant Professor

Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208, Bangladesh

Supervisor


DR. GHASIATUR REZA BANU

Professor

Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208, Bangladesh

DECEMBER 2018

Dedicated to

My Beloved Parents



Acknowledgement

At the outset, I wish to express my deepest sense of gratitude to Almighty Allah, whose boundless blessings enabled me to successfully complete the research work and prepare this dissertation.

I wish to express my profound appreciation and heartiest gratitude to my supervisor **Dr. Ghausiatur Reza Banu**, Professor, Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna. Her kind and continuous inspiration, scholastic guidance, constructive criticism, constant encouragement and providence of all necessary facilities help me to complete thesis paper successfully.

My sincere gratitude goes to my Co-supervisor **Shikder Saiful Islam**, Assistant Professor, Fisheries and Marine Resource Technology Discipline, Khulna University, for his very careful and sincere advice and guidance that he offered me during my experiment and thesis write up. I am grateful to World Fish Bangladesh for financial support to conduct the research.

I am also grateful to my friends at Khulna University, who always appreciated me during my thesis work, my bad time and sadness.

Finally, I would like to express my earnest appreciation to my parents, for their immeasurable blessing, continuous inspiration.

TANAY BISWAS

KHULNA UNIVERSITY

DECEMBER 2018

Abstract

Shrimp hatcheries often be set with diseases, mainly the bacterial infection and antibiotics are widely used for prevention of disease. Presently, beneficial bacteria (probiotics) are used to prevent diseases instead of antibiotics and increasing the production. In the present study a twenty seven (27) days experiment was conducted to determine the effect of probiotics on total bacterial load and specific pathogenic *Vibrio* spp. in shrimp (*Penaeus monodon*). Two types of probiotics were applied viz; feed probiotic (PRO-2) and water probiotic (PRO-W) for the juvenile rearing of *P. monodon* nursing system which was compared with control culture tanks (without probiotics). Total bacterial count was found higher in shrimp treated with feed probiotics (T1) $((2.75 \pm 1.3) \times 10^7$ CFU/g) compared to control group $((5.9 \pm 0.8) \times 10^6$ CFU/g) as well as in case of water body it was higher in T2 $((4.2 \pm 0.5) \times 10^6$ CFU/ml) where only water probiotics (PRO-W) was used. In contrast total *Vibrio* spp. in shrimp juvenile was found to have lowest in T3 $((1.37 \pm 0.8) \times 10^3$ CFU/g) and T1 $((4.8 \pm 2.4) \times 10^3$ CFU/g) compared to control $((1.02 \pm 0.6) \times 10^4$ CFU/g) and T2 $((1.79 \pm 0.8) \times 10^4$ CFU/g). *Vibrio* spp. was also lowest in T1 $((7.2 \pm 3.8) \times 10^3$ CFU/ml) on water body where only feed probiotic was used but *Vibrio* spp. was observed higher in T2 and T3 because of using water probiotic and combine application of feed and water probiotics compared to control treatments (C). So from the results it might conclude that probiotics had no effect on water body it was only effective in shrimp's body. The reason was might be the use of feed probiotic and combination of both probiotics used individually in the treatment T1 and T2.

Table of Contents

Title	Page No.
Acknowledgement	I
Abstract	II
Table of contents	III- IV
List of Tables	IV
List of Figures	V
Chapter-1: 1. Introduction	01-05
1.1 Background of the study	01-05
1.2 Objective of the study	05
Chapter-2: Literature Review	06-10
Chapter-3: Materials and Methods	11- 15
3.1 Study area and duration	11
3.2 Experimental Design	11
3.3 Tank preparation	12
3.4 Stocking and feeding	12
3.5 Composition and application of probiotics	12-13
3.6 Laboratory Requirements	13
3.6.1 Laboratory Equipment's	13
3.6.2. Reagents and chemicals	13
3.7 Laboratory work	13
3.7.1 Sterilization of equipment's	13
3.7.2 Preparation of Stock Solution from Shrimp samples	13-14
3.8 Preparation of Media	14
3.8.1 Preparation of Thiosulfate Citrate Bile Salt-sucrose	14
(TCBS) Agar Media	
3.8.2 Preparation of Nutrient Agar (NA) Media	14

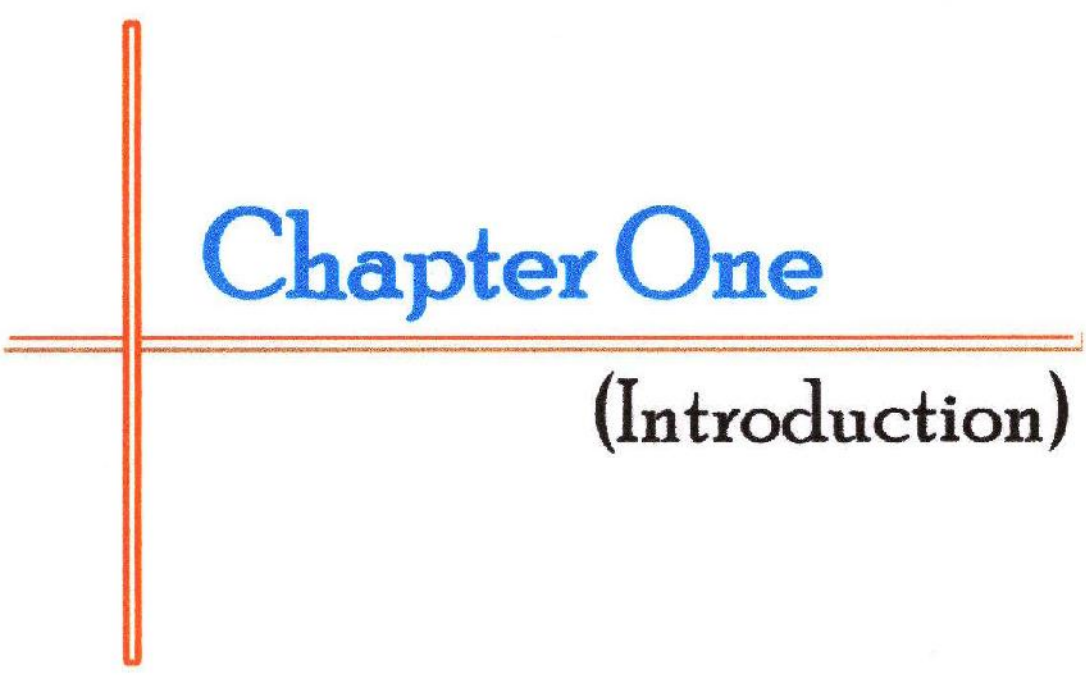
3.9 Serial dilution	14-15
3.10 Inoculation	15
3.11 Incubation	15
3.12 Data collection and analysis	15
Chapter-4: Result	16-19
4.1 Total bacteria count in <i>Penaeus monodon</i> juvenile	16
4.2 Total <i>Vibrio</i> spp. count in <i>Penaeus monodon</i> juvenile	17
4.3 Total bacteria count in water body	18
4.4 Total <i>Vibrio</i> spp. count in water body	19
Chapter-5: Discussion	20-23
Chapter-6: Conclusion	24
Chapter-7: References	25-36

List of tables

Table No	Title	Page
Table 1	Layout of the experiment	11

List of Figures

Figure No	Title	Page
Figure 1	Figure 1. Total heterotrophic bacterial load in shrimp juvenile after a 27 days nursing using in feed, water and combination if in feed and water probiotics. The error bars represent the standard deviations of five replicates. Different letters indicate significant difference among the treatments (one-way anova, $P<0.05$).	16
Figure 2	Figure 2. Total <i>Vibrio</i> spp. load in shrimp juvenile after a 27 days nursing using in feed, water and combination if in feed and water probiotics. The error bars represent the standard deviations of five replicates. Different letters indicate significant difference among the treatments (one-way anova, $P<0.05$).	17
Figure 3	Figure 3. Total heterotrophic bacterial load in the water of <i>P. monodon</i> nursing tanks after a 27 days nursing using in feed, water and combination if in feed and water probiotics. The error bars represent the standard deviations of five replicates. Different letters indicate significant difference among the treatments (one-way anova, $P<0.05$).	18
Figure 4	Figure 4. Total <i>Vibrio</i> spp. load in the water of <i>P. monodon</i> nursing tanks after a 27 days nursing using in feed, water and combination if in feed and water probiotics. The error bars represent the standard deviations of five replicates. Different letters indicate significant difference among the treatments (one-way anova, $P<0.05$).	19

A decorative graphic consisting of a vertical orange line and a horizontal orange line intersecting at the center. The vertical line is slightly thicker than the horizontal line.

Chapter One

(Introduction)

1. Introduction

1.1 Background of the study

Bangladesh is one of the world's leading fish producing countries with a total production of 4,134 million MT, where aquaculture contributes 56.44 % to total production. The average growth performance of this sector is almost 5.43 % in last 10 years. Considering this high growth performance Government is trying to develop this sector income generating industry, which eventually ensures to achieve the projected production target of 4.55 million MT by 2020-21 (DoF, 2017). According to FAO (2017), Bangladesh is not only ranked 5th in world aquaculture production but also recorded surplus fish production with an annual output of 0.84 million in 2016-17. It is a big achievement for the country and thus fisheries and aquaculture sectors are making crucial contributions to the world's wellbeing and prosperity (FAO, 2012). Only fisheries sectors contributes 3.61 % to our national GDP and around one-fourth (24.41 %) to the agricultural GDP and among the exportable fish and fisheries items of Bangladesh the contribution of shrimp is 57% (DoF, 2012).

Aquaculture, directly or indirectly play a vital role in the livelihoods and income of millions of people, particularly in developing countries like Bangladesh (Akter *et al.*, 2017). More than 17 million people including about 1.4 million women depend on fisheries sector for their livelihoods through fishing, farming, fish handling, and processing (Shamsuzzaman *et al.*, 2017). A different survey revealed that more than 80% of labors engaged in the fish processing industries are women (DoF, 2015) and almost 11 % of total populations of Bangladesh are engaged with this sector directly or indirectly on full time and/or part time basis for their livelihoods (DoF, 2017).

This sector also has high potential for the perspective of economic development of the country. Bangladesh earns a considerable amount of foreign currencies by exporting fish, shrimps and other fisheries products. Bangladesh exports frozen shrimp and other fish and

fisheries products to more than 50 countries, including Belgium, UK, Netherlands, Germany, USA, China, France, Russian Federation, Japan and Saudi Arabia (DoF, 2017).

Bangladesh is very much blessed with brackish water shrimp (*Penaeus monodon*) culture because of some suitable conditions like vast amount of freshwater, geographic position, and brackish water, agro climatic environment, suitable resources, water, soil, available culture technique and availability of hatchery PL (Wahab *et al.*, 2012; Ahmed *et al.*, 2008). Brackishwater shrimp (*P. monodon*) is widely cultured due to its advantages over other in terms of size, growth, salinity tolerance, taste and high price in international markets (DoF, 2010). But the growth of aquaculture industry is hampered day by day due to unpredictable mortalities, many of which are caused by pathogenic microorganisms. One of the major problems at present in the shrimp aquaculture is the microbial disease (Hossain *et al.*, 2013). Bacterial diseases caused by *Vibrio* spp. are generally the major causes of mortality in shrimp hatcheries, particularly at very early larval stage. It results in significant low production of farmed shrimp and financial loss to commercial growers, which accounts around 25% of the total world aquaculture product (Kumar *et al.*, 2016b).

Although the shrimp hatchery technology has advanced over the decades, the hatchery production is more often hampered by severe mortalities caused mostly by bacteria. Increasing *Vibrio* spp. population in larvae and rearing tank water has been reported to reduce the survival rate of larvae and post larvae (Singh, 1986; Hameed, 1993). Luminous species of *V. harveyi* have been associated with causing mass mortalities in shrimp hatcheries (Sunaryanto and Mariam, 1986; Baticados *et al.*, 1990; Karunasagar *et al.*, 1994). Added to this, significant stock mortality has been reported due to poor environmental conditions on farms, unbalanced nutrition, generation of toxins, and genetic factors and for this causes country faces a vast economic losses (Kautsky *et al.*, 2000). In recent decades, prevention and control of animal diseases has focused on the use of chemical additives and veterinary

medicines, especially antibiotics, are a common practice in shrimp culture for many decades which generate significant risks to public health by promoting the selection, propagation (OIE/WHO FAO, 2006 and Nomoto, 2005) at the same time instead of preventing and arresting the disease, the unwanted and unwarranted usage of the chemicals damages the environments and develops antibiotics resistant bacteria (Karunasagar *et al.*, 1994). Although antibiotics application in hatcheries may be cost-effective due to the limited water volume, antibiotic treatment in grow-out systems with large-volume water exchange is costly and avoided by most producers (Harikrishnan *et al.*, 2010). On the other hand antibiotics kill beneficial micro-biota in the gastrointestinal (GI) ecosystem and it is alarming that antibiotic residues accumulated in fish products that are harmful for human consumption (WHO, 2006). These alarming disadvantages prompted the aquaculture industry to explore and develop strategies that are as equally effective as antibiotics, eco- and consumer-friendly and most importantly sustainable (Standen *et al.*, 2013; Lazado *et al.*, 2015). For accounting this sense, several alternative strategies due to the prevention and control of diseases have been proposed, such as the use of vaccines, immunostimulants and probiotics (Gatesoupe, 1999; Balcazar *et al.*, 2006). The concept of probiotics is one of the promising areas for research and development (Kumar *et al.*, 2016a) and it is environmentally safe and cost effective (Gatesoupe, 1999). The research into the use of probiotics for aquatic animals is increasing with the demand for environment-friendly sustainable aquaculture (Lester *et al.*, 1999). Probiotics are usually members of the healthy micro-biota associated with the host; therefore, they may provide an alternative way to reduce the use of antibiotics in aquaculture (Perez *et al.*, 2010).

Probiotics, known as beneficial microbes, are being proposed as an effective and eco-friendly alternative to antibiotics. They were first applied in aquaculture species more than three decades ago, but considerable attention had been given only in the early 2000s but recently

the use of probiotics have been increased (Zorriehzahra *et al.*, 2016). Probiotics are live nonpathogenic microorganisms that provide colonization resistance to the pathogenic microbes and thus are effective in prevention and treatment of some diseases (Hossain *et al.*, 2013). The application of probiotics in aquaculture has been widely used as a means of controlling disease, enhancing immune response, providing nutritional and enzymatic contributions to the digestion of the host, and improving water quality (Qi *et al.*, 2009).

Different species of probiotics used in aquaculture for their beneficial effects and the most commonly used probiotic species include genera *Lactobacillus*, *Bifidobacterium*, *Aeromonas*, *Plesiomonas*, *Bacteroides*, *Fusobacterium*, *Carnobacterium* and *Eubacterium* and strains of *Bacillus*, *Enterococcus*, *Bacteroides*, *Clostridium*, *Agrobacterium*, *Pseudomonas*, *Brevibacterium*, *Microbacterium*, *Staphylococcus*, and *Aspergillus* (Balcazar *et al.*, 2006; Nayak 2010; Lakshmi *et al.*, 2013; Tuan *et al.*, 2013 and Lazado *et al.*, 2015).

Though bacteria live in every corner of the aquatic environment, there are no serious problems for water quality during the initial stages of farming aquatic organisms, when the stocked organisms are small and their metabolism rate and amounts of supplementary feed are low but with the progress of culture the organisms grow, leading to a rapid increase in biomass, and water quality deteriorates, mainly due to the accumulation of metabolic waste of cultured organisms, decomposition of unutilized feed, and decay of biotic materials (Prabhu *et al.*, 1999). At this time, the application of a group of beneficial microorganisms (such as *Lactobacillus*, *Bacillus*, *Nitrosomonas*, *Cellulomonas*, *Nitrobacter*, *Pseudomonas*, *Rhodoseudomonas*, *Nitrosomonas* and *Acinetobacter*) would be very useful for controlling the pathogenic microorganisms and water quality (Prabhu *et al.*, 1999; Shariff *et al.*, 2001; Irianto and Austin, 2002).

Based on the previous research results on probiotics suggest that the use of probiotic both of feed and water probiotics in aquaculture has tremendous scope and the study of the



application of probiotics in aquaculture have a glorious future. The role of probiotics bacteria in small culture is studied but commercial level is not that much reported especially in giant tiger shrimp, *P. monodon*. Hence in Bangladesh the beneficial effect of probiotics on the commercial culture of shrimp, *P. monodon* is very much need of the hour to improve the fisheries sectors (Ramanan *et al.*, 2010).

In Bangladesh, a few studies have so far been made to evaluate the effects of probiotics on controlling pathogenic bacteria in *P. monodon* under the hatchery management framing with formulated feed in high stocking density with a view to producing good quality seed of shrimp.

1.2 The objective of the study was to -

Evaluate the effect of commercial probiotics on total bacteria and *Vibrio* spp. in *Penaeus monodon* nursing.



Chapter Two

(Literature Review)

2. Literature Review

Shrimp aquaculture, as well as other fisheries industries, constantly requires new techniques in order to increase production yield. Feeding and new practices in farming usually play an important role in hatchery to produce disease free and bacteria resistance seed, and the addition of various additives to a balanced feed formula to achieve better growth is a common practice of many fish and shrimp feed manufacturers and farmers. Probiotics, as 'bio-friendly agents' can be introduced into the culture environment to control and compete with pathogenic bacteria as well as to promote the growth of the cultured organisms. In addition, probiotics are nonpathogenic and nontoxic microorganisms without undesirable side effects when administered to aquatic organisms (Farzanfar, 2006).

In 1986 the application of probiotics were occurred first time, to test their ability to prompt growth of hydrobionts (organisms that live in water) then it was applied to improve water quality and control of bacterial infections. Nowadays, there is documented evidence that probiotics can improve the digestibility of nutrients, increase tolerance to stress, and encourage reproduction. Currently, there are commercial probiotic products prepared from various bacterial species such as *Bacillus* spp., *Lactobacillus* spp., *Enterococcus* spp., *Cyanobacterium* spp., and the yeast *Saccharomyces cerevisiae* (Martínez et al., 2012).

The Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO, 2002) defined probiotics as living microorganisms, which, once administered in appropriate amounts, confer a health profit on the host. Stimulation or improvement of the defense system may be a mode of action by that probiotic exerts a helpful impact to the host. The definition was re-improved by Fuller in 1989 whose explanation was as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance."

Probiotics are used for quite long time in aquaculture. The effects of probiotics in fish and shrimp have been reported (Wang *et al.*, 2005; Son *et al.*, 2009). But from few years they become an integral part of the culture practice for growth improvement disease resistance bacteria and many microbial compounds (Wang, 2007)). Probiotic bacteria also have their effects against viruses like WSSV and *Vibrio* spp. (Gullian *et al.*, 2004).

De Souza *et al.* (2012) conducted a study about the use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817) in a zero exchange system. According to their results, they reported that bacteriological concentration of *Vibrio* spp. measured in probiotic treatment tanks was lower than that recorded in the control tanks.

Kennedy (1998) used probiotic bacteria in the culture of marine fish larvae. They observed that the periodic addition of bacteria (probiotics) to the tanks altered the microbial communities of both tanks and fish. In addition, they noticed that the fish eggs incubated with probiotic bacteria were less likely to develop bacterial overgrowth and die than those incubated without probiotic bacteria.

Soundarapandian and Babu in (2010) conducted a study on the effect of probiotics on the hatchery seed production of black tiger shrimp, *P. monodon* (Fabricius). In their study, the probiotics was applied (experimental) for the larval rearing of *P. monodon*, which is compared with control tanks (without probiotics). They conclude that the probiotics plays a vital role in maintaining the microbial load that *vibrio* sp. is dominant in the control tanks than in experimental tanks.

The effect of a probiotic supplemented diet on shrimp growth, digestive tract bacterial microbiota, survival, and some hemato-immunological parameters after an experimental challenge with *Vibrio harveyi* was studied by Vieira *et al.* (2010). Total bacteria count and *Vibrio* spp. count in the digestive tract were not different ($P>0.05$) until day 40, but they were lower

($P < 0.05$) in the probiotic-supplemented group on day 60. Total lactic bacteria in the shrimp digestive tract was higher after day 20 in the probiotic-supplemented group. Bacterial counts in hemolymph and hepatopancreas was lower ($P < 0.05$) in the probiotic-supplemented group than in the control group after *V. harveyi* challenge.

Gildberg (1997) had analyzed the effect of a probiotic of lactic acid bacteria in the feed of Atlantic cod fry (*Gadus morhua*) on growth and survival rates. A certain improvement in disease resistance was obtained, and at the end of the experiment lactic acid bacteria dominated the intestinal flora in surviving fish given feed supplemented with lactic acid bacteria.

Moriarty (1999) reported on his successful experiences of using probiotic bacteria instead of antibiotics to control *Luminus vibrios* in shrimp farms in Negros, Philippine. Meunpol (2003) recorded the effects of ozone and probiotics on the survival of black tiger shrimp (*P. monodon*). According to the results of their study the antagonistic effect of *Bacillus* against the pathogenic *Vibrios* was evaluated in black tiger shrimp (*P. monodon*), and it was suggested as an alternative treatment factor instead of antibiotics in shrimp aquaculture (Vaseeharan and Ramasamy, 2003ab).

Soundarapandian *et al.* (2010) studied the effect of probiotics on the growth and survival of shrimp *Penaeus monodon* (Fabricius). In the study the probiotics, viz., Super biotic, Super Ps, Zymetin and Mutagen was applied. According to their observed value they conclude that the pathogenic bacterial population decreased at the end of culture in probiotic ponds, but in control ponds the bacterial population was not showing a significant decrease.

The effect of gut probiotics mixed in a feed was studied by Jha in (2014) for its effect on black tiger shrimp (*P. monodon*) in the Aquaculture Department Laboratory of College of Marine Science and Technology, Massawa, Eritrea- (Northeast Africa) and was found to be effective in providing higher growth, better FCR and resistant to pathogenic infections.

According to Bhavani *et al.* (2015) the probiotics either individually or in combined action, induced the best growth potentials in fresh water prawn *M. rosenbergii* compared to control pond where in which only commercial feed C. P. Brand was broad casted. The use of water probiotics and feed probiotics in the culture activity indicated that, probiotics play a vital role in inducing growth and survival rates by maintaining good water quality parameters throughout the culture operation.

Kumar *et al.* (2016a) to evaluate the physico-chemical parameters of the *Litopenaeus vannamei* culture ponds by applying water probiotic (PRO-W) conducted a study. The major water quality parameters like salinity, dissolved oxygen, pH and total ammonia nitrogen were recorded by standard measurements. The study results showed that the probiotic (PRO-W) plays a prominent role in maintain good water quality parameters throughout the culture period.

Padmavathi *et al.* (2012) studied on efficacy of probiotics in improving water quality and bacterial flora in fish ponds cultured with *Pangasius sutchi*, *Catla catla* and *Labeo rohita*. Two ponds were treated with probiotics having *Nitrosomonas* (1.62 kg/ha) and *Nitrobacter* (0.82 kg/ha) species and one pond was kept as control. It was observed that in treated ponds, total heterotrophic bacterial and beneficial bacterial load increased and pathogenic *Pseudomonas* load decreased. The bacterial population changed during every fortnight sampling of the culture period.

A study was carried out by Sivakumar *et al.* (2012) to evaluate the probiotic potential of *Lactobacillus acidophilus* 04 (homemade curd isolate) on pathogenic *Vibrio* spp. in shrimp *P. monodon* juveniles. *L. acidophilus* showed antibacterial activity against *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Vibrio harveyi* and *Vibrio alginolyticus*. Results of the study validated *L. acidophilus* 04 has potential probiotic principles to control pathogenic *V. alginolyticus* in shrimp aquaculture.

Pattukumar *et al.* (2010) studied on the population of *Vibrio Parahaemolyticus* (Pathogen) and *Bacillus* (Beneficial Bacteria) in *P. monodon* (Fabricius, 1798) Culture. The present work was attempted to evaluate the probiotic activity of *Bacillus* spp. in shrimp health management. Based on this, it is concluded that application of suitable and effective probiotics (*Bacillus*) will certainly reduce population of pathogens and provide a congenial pond environment for shrimp culture. However, this could be achieved by the effective usage of commercially available probiotics for maintaining good pond environment.

Souza *et al.* (2012) conducted a study on probiotic actions of *Bacillus cereus* var. *toyoi* and *Saccharomyces boulardii* in silver catfish (*Rhamdia quelen*) larvae culture. According their study they declared that both probiotics have an inhibitory effect in vitro against *Vibrio carchariae*.

De Paiva-Maia *et al.* (2013) conducted a study to know the Effect of a commercial probiotic on bacterial and phytoplankton concentration in intensive shrimp farming (*Litopenaeus vannamei*) recirculation systems. These studies showed significant differences for total heterotrophic bacteria in the sediment and total heterotrophic bacteria in surface water respectively, for the control and probiotic treatment. These results conclude that the probiotic causes changes in the total heterotrophic bacteria in the sediment and percentage values of Pyrrophyta concentration, improving the environmental quality of the sediment and water in ponds with closed recirculation systems.

According to the study of Aftabuddin *et al.* (2013), the average total heterotrophic bacteria (THB) and total presumptive *Vibrio* bacteria both in culture water and post larvae were lower during experimental culture in compared to control culture with the introduction of probiotics.

K.V. V. A-4/NO 3686, D-19.2.19

Chapter Three

(Materials and Methods)

3 Materials and Methods

3.1 Study area and duration

This experiment was conducted at Redient Shrimp Hatchery in Shaymnagar, Satkhira by the joint effort of WorldFish Bangladesh, University of Stirling, UK and Khulna University. The study continued from 15 May to 11 June, 2018. The sample of juvenile shrimp for total cultivable bacteria were prepared and *Vibrio* spp. analysis was done in Fish Pathology and Molecular Laboratory of Fisheries and Marine Resource Technology Discipline, Khulna University.

3.2 Experimental design

The idea for commencing this experiment was to understand the consequence of using in feed and water probiotics in shrimp nursing. As an approach of microbial community management, the scope in the master thesis was to work with the effect of those probiotics and its combination on the load of total cultivable bacteria and total *Vibrio* spp. Therefore, the experiment was conducted under three treatments, such as shrimp nursing under the application of in feed probiotics (PRO-2) (T1), water probiotics (PRO-W) (T2), and combination of both in feed and water probiotics (T3). In addition, a control group (C) was maintained without any application of probiotic in shrimp nursing. Each of the treatment was replicated into five times and the nursing was continued for 27 days.

Table 1: Layout of the experiment.

Group	Description of Group	Replications
T ₁	Treated with in feed probiotic (PRO-2)	5
T ₂	Treated with water probiotic (PRO-W)	5
T ₃	Combination of in feed probiotic and water probiotic (PRO-2+PRO-W)	5
C	Control/ Non-treated tank	5

3.3 Tank preparation

A total of 20 black fiber glass tanks with the volume of 500 liter water holding capacity was set up randomly at Redient Shrimp Hatchery. The tanks were washed with chlorine water to disinfect it and tanks were filled with 450 liter water before starting the experiment. and each tank volume was 500 L/tank and then it was filled with water. During the study period 20% water exchange was done from each tank and it was done total 5 times.

3.4 Stocking and feeding

After tank preparation and set up shrimp larvae, SPF PL15 were stocked in the tanks for nursery rearing at the rate of 700 PL/ tank. The animals were fed with a diet having the protein level of 46%, and the feeding frequency was 6 times a day.

3.5 Composition and application of probiotics

Sanolife^R PRO-2 shrimp probiotic is a well-defined mixture of selected *bacillus* strains. The selected *bacillus* strains are able to grow and perform in the gut of shrimp. Minimum concentration of *bacillus* strains bacteria is 2×10^{10} CFU/g. Sanolife PRO-2 increase immunity, growth rate and survival while reducing feed conversion ratio (FCR). The use of the Sanolife PRO-W feed probiotic also suppresses the growth of pathogenic bacteria (*Vibrio* spp.) in the gut and breakdown uneaten feed and faces rapidly. PRO-2 commercial probiotics was applied through mixing with the feed at the rate of 5 g per kilogram of feed.

PRO-W is a water probiotics made with beneficial bacteria. Sanolife PRO-W has been specifically formulated for use in both fresh and sea water. It is composed mainly with *Bacillus* strains is 5×10^{10} CFU/ml. The activity of the *Bacillus* in Sanolife PRO-W keeps the water quality optimal, stabilizing the phytoplankton bloom. It promotes rapid decomposition of waste material and helps to control pathogenic bacteria (*Vibrio* spp.). The product reduces

the development of blue-green algae, controls the concentration of ammonia and eliminates nitrate. PRO-W probiotics was applied at 5 g per tonnes of water.

3.6 Laboratory requirements

3.6.1 Laboratory equipment's

Autoclave machine, centrifuge machine, electric balance, spirit lamp and burning spirit, Petri dish, biological dissection box, vortex mixer, bacteria isolating loops, glass rods, oven, incubator, refrigerator, bio-safety cabinet, micro-pipette, tips and tips box (Swanson *et al.*, 2001) was needed for conducting the study.

3.6.2 Reagents and chemicals

Peptone water, absolute alcohol, sodium chloride (NACL), yeast extract, ethyl alcohol (70% and 100%), distilled water, TCBS agar media, nutrient agar media powder was required in this study.

3.7. Laboratory work

3.7.1 Sterilization of equipment's

Test tubes, petri dishes, conical flask, plastic tips and other glassware was sterilized by autoclaving (VS-1221, Vision Scientific., LTD) at a temperature of 121°C and a pressure of 15 pound per square inch for 15 minutes. Petri dishes, isolating loop, etc. were subjected to dry heat sterilization at 170 °C for 1 hour in an electric oven (Swanson *et al.*, 2001).

3.7.2 Preparation of stock solution from shrimp samples

The live shrimp samples and water samples were collect from the culture tanks. To prepare stock solution from collected shrimp larvae, it was weighed by an electric balance aseptically (HR-200). Samples weight ranged from 0.94g to 2.947g. The weighed samples were then taken in a sterilize vortex mixer with 5 ml alkaline saline peptone water for proper mixing. The mixed samples were taken in autoclaved eppendorf tubes for centrifugation at 3000 rpm

for 4 minutes (Centrifuge (5415 D)). After centrifugation the supernatant was collected in an autoclaved falcon tube for further work.

3.8 Preparation of media

Media were prepared by compounding the required individual ingredients or more conveniently, by adding distilled water to a dehydrated product which contain all the ingredients. All Media were available commercially in powdered form.

3.8.1 Preparation of thiosulfate citrate bile salt-sucrose (TCBS) agar media

TCBS agar was prepared by adding 89.0 g TCBS powder in 1 L demineralized water. The mixture was subsequently heated to boiling point using a magnetic stirrer. Plates were immediately poured (15 ml per plate) under laminar flow and left to solidify for 30 minutes. The plates were stored in plastic bags at room temperature.

3.8.2 Preparation of nutrient agar (NA) media

Nutrient agar media was prepared by adding 20 g agar powder in 1 liter distilled water, mixing it properly by desolving it, followed by autoclaving at 121 °C for 15 minutes. After autoclaving the agar media was kept under bio-safety cabinet and 20 ml was transferred in each petri-dishes. After pouring into the plates it was kept under the laminar flow to solidify and used for bacterial inoculation.

3.9 Serial dilution

- ❖ Serial dilutions were done in sterile falcon tubes each containing 4.5 ml of sterile normal saline peptone water.
- ❖ 0.5 ml of stock solution was taken by micropipette and was mixed with 4.5 ml of sterile normal saline water which gave 10^{-1} dilution.
- ❖ From 10^{-1} dilution 0.5 ml of solution was taken in another falcon tube containing 4.5 ml saline which gave 10^{-2} dilution.

- ❖ In this way 10 fold dilutions of the samples were made up to 10^{-5} .

3.10 Inoculation

- ❖ 0.5ml of diluted solution was poured on solid media aseptically by sterile micropipette.
- ❖ After this spreading was done by glass L-shaped rod until proper mixing and then properly covered the Petri dish with aluminum foil and turn opposite and was level each Petri dish.

3.11 Incubation

After spreading, all the plates were incubated at 30 °C for 24 hours. After incubation the bacterial colony grown in the plates in between 10-300 were counted and recorded.

3.12 Data collection and analysis

- The obtain data during the experiment was recorded and collected data were accumulated, grouped and interpreted according to the objectives. Collected data were stored, explored and analyzed using Microsoft Excel, SPSS and Microsoft Word program to present results and discussion. One way anova ($P < 0.05$) was done to see the significance difference among the treatments and pair-wise comparison was done by Tukey post-hoc analysis.



Chapter Four

(Results)

4. Results

This experiment was conducted as an approach of microbial community management in aquaculture, especially focusing on tiger shrimp *Penaeus monodon* nursing using different probiotic application. The major goal in the scope of this master thesis was to investigate the effect of different commercial probiotics on the total heterotrophic bacteria and total *Vibrio* spp. load in water as well as in the gut of experimental shrimps.

4.1 Total bacteria count in *P. monodon* juvenile

The result of this study demonstrated that although not significant, but total heterotrophic bacterial count was found higher in shrimps treated with in feed probiotics (PRO-2) (T1) $((2.75 \pm 1.3) \times 10^7$ CFU/g) compared to control group $((5.9 \pm 0.8) \times 10^6$ CFU/g). Similar observation on total load was observed in *P. monodon* treated with water probiotics (PRO-W) $((5.60 \pm 3.2) \times 10^6$ CFU/g) and combination if in feed and water probiotics $((6.03 \pm 1.6) \times 10^6$ CFU/g).

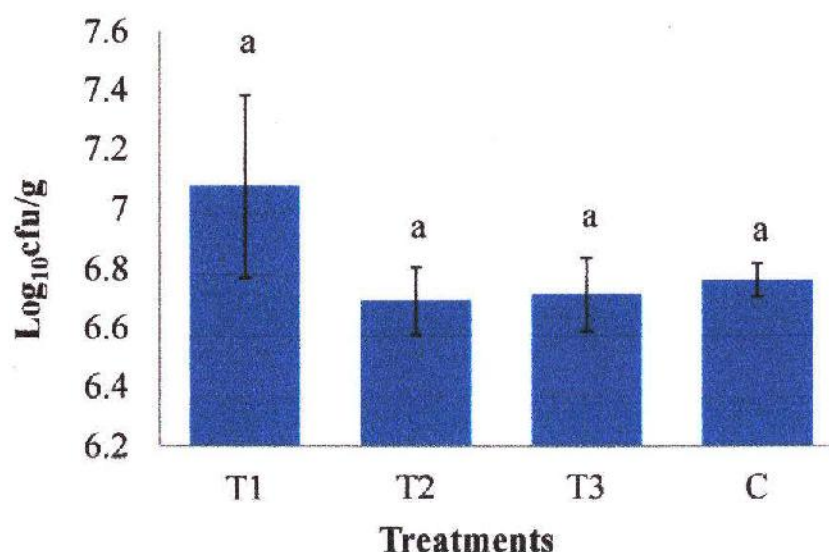


Figure 1. Total heterotrophic bacterial load in shrimp juvenile after a 27 days nursing using in feed, water and combination if in feed and water probiotics. The error bars represent the standard deviations of five replicates. Different letters indicate significant difference among the treatments (one-way anova, $P < 0.05$).

4.2 Total *Vibrio* spp. count in *P. monodon* juvenile

In contrast to total heterotrophic bacteria, although not significant, but total *Vibrio* spp. count in juvenile after 27 days nursing was found to have lowest in T3 ($(1.37 \pm 0.8) \times 10^3$ CFU/g) and T1 ($(4.8 \pm 2.4) \times 10^3$ CFU/g) compared to control ($(1.02 \pm 0.6) \times 10^4$ CFU/g) and T2 ($(1.79 \pm 0.8) \times 10^4$ CFU/g). That means *P. monodon* juveniles under the treatment of in feed probiotics (T1) and its combination with water probiotics (T3) had good effect on the lowering of total *Vibrio* spp. load in the animals body (Figure 2).

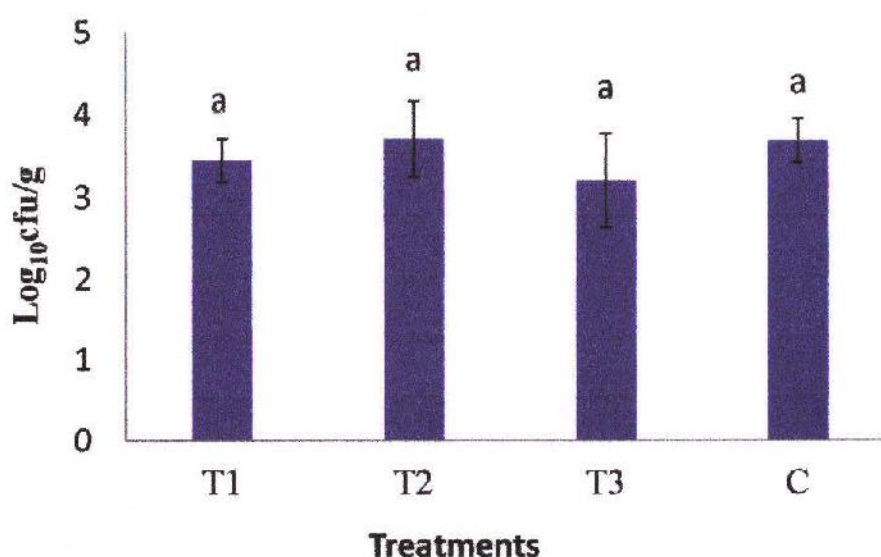


Figure 2. Total *Vibrio* spp. load in shrimp juvenile after a 27 days nursing using in feed, water and combination if in feed and water probiotics. The error bars represent the standard deviations of five replicates. Different letters indicate significant difference among the treatments (one-way anova, $P < 0.05$).

4.3 Total bacteria count in water body

The results of this experiment revealed that T2 ($(4.2 \pm 0.5) \times 10^6$ CFU/ml), where water probiotics(PRO-W) was used showed to have higher total heterotrophic bacterial count compared to control group ($(1.36 \pm 1.1) \times 10^6$ CFU/ml) as well as T3 ($(7.57 \pm 3.5) \times 10^5$ CFU/ml) ($P < 0.05$) (Figure 3).

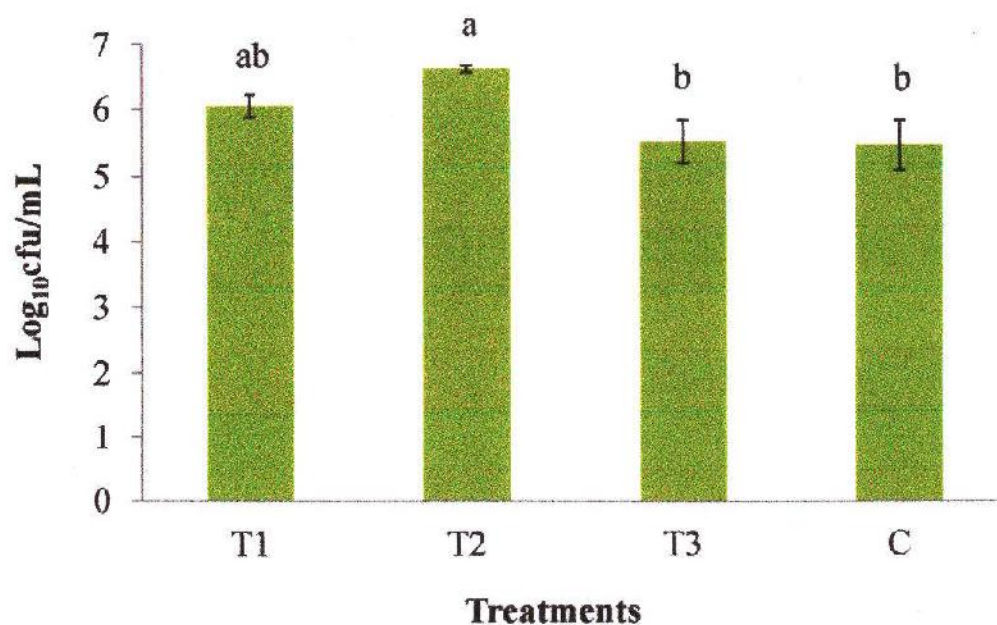


Figure 3. Total heterotrophic bacterial load in the water of *P. monodon* nursing tanks after a 27 days nursing using in feed, water and combination if in feed and water probiotics. The error bars represent the standard deviations of five replicates. Different letters indicate significant difference among the treatments (one-way anova, $P < 0.05$).

4.4 Total *Vibrio* spp. count in water body

From this experiment, although not significant higher number of *Vibrio* spp. was found in T2 $((2.9 \pm 1.7) \times 10^5$ CFU/ml) compared to other treatments. *Vibrio* spp. was lowest in T1 $((7.2 \pm 3.8) \times 10^3$ CFU/ml), followed by C $((1.11 \pm 0.6) \times 10^4$ CFU/ml) and T3 $((1.18 \pm 0.4) \times 10^4$ CFU/ml) (Figure 4).

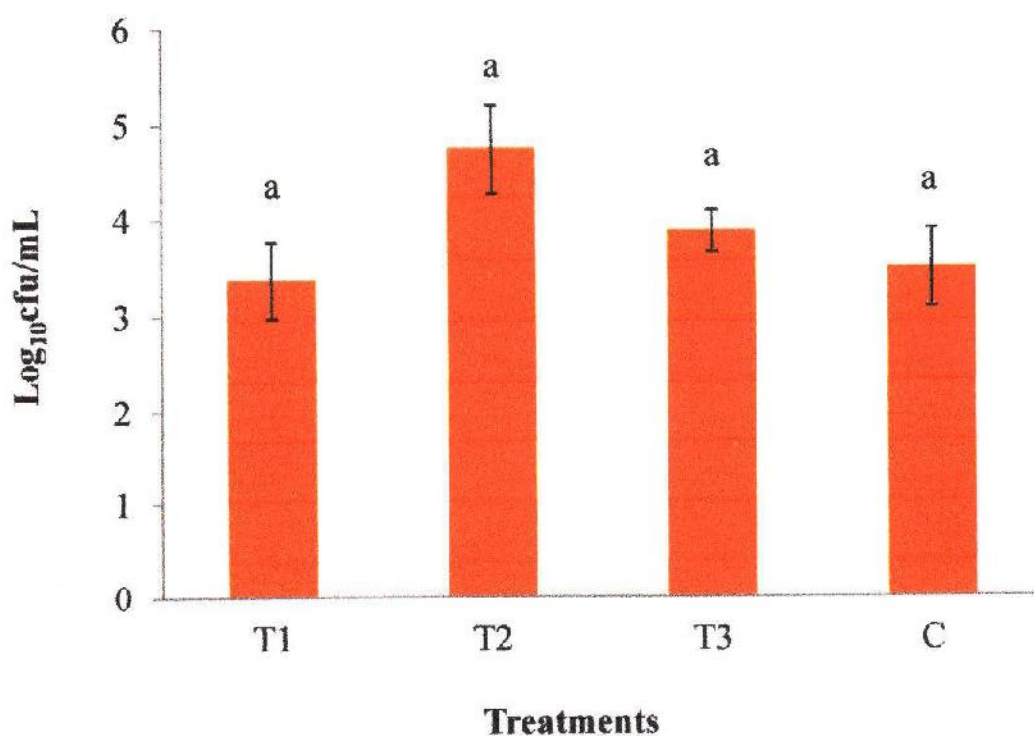


Figure 4. Total *Vibrio* spp. load in the water of *P. monodon* nursing tanks after a 27 days nursing using in feed, water and combination if in feed and water probiotics. The error bars represent the standard deviations of five replicates. Different letters indicate significant difference among the treatments (one-way anova, $P < 0.05$).

Chapter Five

(Discussion)



5. Discussion

In general, diseases in aquaculture practices are mostly caused by *Vibrio* spp. and it has been referred as the largest economic loss in the shrimp hatchery due to mass mortalities (Shivakumar *et al.*, 2012). So water quality control and microbial community balance are necessary for successful shrimp cultivations (Raman *et al.*, 2013). To control the microbial community and the pathogens, the use of beneficial bacteria (probiotics) instead of antibiotics in aquaculture is increasing demand for its more environment friendly aquaculture practices throughout the globe (Kumar *et al.*, 2013). Now a day, the use of probiotics in aquaculture might represent a valuable mechanism to increase shrimp growth and survival rate. In general, the Gastro Intestinal Tract (GIT) of the aquatic animal is mainly composed of gram negative bacteria being the genera *Vibrio*, *Pseudomonas*, and *Aeromonas* predominant (Vine *et al.*, 2006). So, the incorporation of beneficial gram positive (probiotic) bacteria in feed can modify its gastro intestinal tract (Vieira *et al.*, 2007). Several studies have demonstrated that such bacterial micro-biota can be modified by the addition of Gram-positive bacteria (probiotics) in the feed (Ziaei-Nejad *et al.*, 2006; Vieira *et al.*, 2008) or in the culture water (Rengpipat *et al.*, 1998).

At present the culture of brackish water shrimp, *P. monodon* is increasing due to its taste, price, candidate of human nutrition and market demand for both national and international markets. To increase the production of shrimp, disease free and healthy shrimps seed are important criteria. There are several traditional and non-scientific farms that's mostly depend on shrimp seeds which are caught from the river as a result demand of shrimp seeds did not fulfill. So considering the need of healthy and quality shrimp seeds throughout the year hatchery management is practice but hatchery faces many problems like unpredictable mortalities due to bacterial attack and controlling *Vibrio* spp. is a major challenge for hatchery operations (Soundarapandian and Babu, 2010). In order to overcome from this

situation microbial management in the hatchery is mandatory. The present investigation was undertaken to know the effect of probiotics on the bacterial growth of cultivable shrimp larvae and water in hatchery. In the present conducted study the abundance of bacterial growth of marine water shrimp *P. monodon* was monitored after subjecting to the treatments with water probiotics and feed probiotics for a culture period of twenty seven (27) days. *P. monodon* larvae were cultured in four different tanks, subjected to different treatments. The first tank was treated with commercial feed probiotic (PRO-2); the second tank was treated with commercial water probiotic (PRO-W). In the case of third tank both in feed probiotic and water probiotic (PEO-2+PRO-W) were used and the fourth tank was control. In the present study *Vibrio* spp. were enumerated using widely recognized culture based techniques. In the experiment, total heterotrophic bacterial count was found higher in feed probiotic treatment (T1) in shrimp than control and other tanks. The results of this study is also similar to the previous observation of Sivakumar *et al.* (2012) where they stated that higher total bacterial count was recorded in shrimp fed with feed probiotics ($5.2 \pm 0.53 \times 10^8$ CFU/g) compared to control tank. In an another observation which was conducted by Aftabuddin *et al.* (2013) declared that in post larvae the recorded total heterotrophic bacteria was $5.4 \pm 1.70 \times 10^2$ to $2.38 \pm 0.78 \times 10^4$ CFU/g was higher than control tank ($2.3 \pm 0.3 \times 10^3$ CFU/g) that is nearly same of this present observation value.

Sivakumar *et al.* (2012) reported that total *Vibrio* spp. count was recorded in GI tract of control group ($50 \pm 0.21 \times 10^{10}$ CFU/g) and due to introduction of probiotic feed total *Vibrio* count was decreased in GI tract of shrimp. Results of this study showed that *Vibrio* spp. counts were reduced in the probiotic treated shrimp larvae because of using feed probiotic in treatment tank (T1) and combined application of feed probiotic and water probiotic tank (T3) compared to control tank (C) and water probiotic treatment tank (T2). Same results like this study in order to Vibriosis was estimated by Aftabuddin *et al.* (2013) and they said that the

Vibrio spp. count decreased with the introduction of probiotics and increased in control tank and also said that this finding coincide with the works of Moriarty (1998), Prabhu *et al.* (1999), Dalmin *et al.* (2001) and Das *et al.* (2006).

In contrast, total heterotrophic bacteria in water were counted more in T2 than control tank and T3. Same results were investigated by Otta *et al.* (2001) in total plate count of shrimp rearing water in a number of *P. monodon* hatcheries in India was reported in ranges of 10^2 to 10^4 CFU/ml and 10^4 to 10^7 CFU/ml. In an another experiment conducted by Sung *et al.* (2001) put out their finding on total bacterial count of three *P. monodon* grow out ponds which were in a range of 2×10^3 to 3×10^6 CFU/ml that's revealed agree with the present experiment. Vaseeharan and Ramasamy (2003ab) were broadcasted the mean value of total plate count of shrimp rearing tanks of nine different *P. monodon* in India, which was $3.08 \pm 0.28 \times 10^5$ CFU/ml. The finding of total bacterial count from the current study in shrimp rearing water is almost similar of the previous reports.

In the present experiment the *Vibrio* spp. counts were generally lower in water for feed probiotic treatment (T1) compared to other treatment. The investigated finding of this study is related with the observed value of Srinivas *et al.* (2014). In their study they observed that total *Vibrio* count in the water of the control pond at ninety days of culture was $30.0 \times 10^2 \pm 0.47$ CFU/ml and the total *Vibrio* counts in the experimental pond A and pond B were recorded as $29.0 \times 10^2 \pm 0.23$ CFU/ml and $2.63 \times 10^2 \pm 0.32$ CFU/ml in the pond water.

It is demonstrated that the combined application of feed probiotic and water probiotic presented a protective effect against the pathogens *Vibrio harvei* and white spot syndrome virus (Balca'zar, 2003). Srinivas *et al.* (2014) also conclude that all the culture ponds or tanks where the application of probiotics is followed there was reduction of the proliferation of the bacteria. Shakibazadeh *et al.* (2012) reported that total aerobic heterotrophic bacteria count or total plate count of shrimp rearing water on TSA was 2.9×10^4 CFU/ml and bacterial counts of

different rearing tanks did not exhibited any significant difference ($P < 0.05$). The observed findings of the current study are quite similar of the previous study. According to the study of Aftabuddin *et al.* (2013) following their study conclude that the average total heterotrophic bacteria (THB) and total presumptive *Vibrio* spp. bacteria both in culture water and post larvae were lower during experimental culture in compared to control culture with the introduction of probiotics. Supamattaya *et al.* (2005) reported according to their results value and finally revealed that the use of AquaStar® Hatchery in feed is effective in reducing the total number of *Vibrio* spp. bacteria found in the hepatopancreas and intestine of white shrimp (*Penaeus vannamei*), which can reduce the risk of infection and in the present study total *vibrio* count was lower in number both in shrimp body than control tank. It has been reported that use of probiotics improved water quality and reduced the pathogenic *Vibrio* spp. (Dalmin *et al.*, 2001).



Chapter Six

(Conclusion)

6. Conclusion

In the present work two types of probiotics like feed probiotic (PRO-2) and water probiotic (PRO-W) and combination of both probiotics were applied in culture of shrimp nursing system due to know the effect of probiotic on shrimps larvae and water body. Probiotics played a vital role in bacterial disease resistance and proliferation of gram positive bacteria. From the results of the present study it might say that *Vibrio* spp. were counted lower on shrimp's body when treated with feed probiotics (PRO-2) and combination of feed probiotic and water probiotic (PRO-2+PRO-W) but probiotic might have no effect on water body.



Chapter Seven

(References)

7. References

- Aftabuddin, S., Kashem, M. A., Kader, M. A., Sikder, M. and Hakim, M. A. (2013). Use of *Streptomyces fradiae* and *Bacillus megaterium* as probiotics in the experimental culture of tiger shrimp *Penaeus monodon* (Crustacea, Penaeidae). *Aquaculture, Aquarium, Conservation and Legislation-International Journal of the Bioflux Society (AACL Bioflux)*, 6(3), P(253-267).
- Ahmed, N., Demaine, H., Muir, J.F. (2008). Freshwater prawn farming in Bangladesh: history, present status and future prospects. *Aquaculture Research*, 39 (8), P(806–819).
- Akter, R., Hossain, M. R., Rahman, M. M., Rahman, B. S. and Ahmed, K. K. U. (2017). The effect of commercial probiotics on health and production of shrimp (*Penaeus monodon*). *Asian Journal of Medical and Biological Research*, 3(1), P(88-93).
- Balcázar, J. L., De Blas, I., Ruiz-Zarzuola, I., Cunningham, D., Vendrell, D. and Muzquiz, J. L. (2006). The role of probiotics in aquaculture. *Veterinary Microbiology*, 114(3-4), P(173-186).
- Balcazar, J. L. (2003). Evaluation of probiotic bacterial strains in *Litopenaeus vannamei*. Final Report. *National Center for Marine and Aquaculture Research, Guayaquil, Ecuador*, 8(3), P(123-130).
- Baticados, M.C.L., C.R., Lavilla-Pitogo, E.R., Cruz- Lacierda, L.D., de la Pena and N.A. Sunaz, (1990). Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. *Diseases of Aquatic Organisms*, 9(2), P(133-139).

- Bhavani, M., Suneetha, Y., Kumaraswamy Naidu C. and M. Srinivasulu Reddy (2015). Monitoring the effect of water and feed probiotics on the growth potentials in fresh water prawn *Macrobrachium rosenbergii*. *International Journal of Fisheries and Aquaculture Sciences*. ISSN 2248-9975, 5(1), P(59-70).
- Bright Singh, I. S., Lakshmana, P. and Chandramohan, D. (1986). Studies on the bacteria associated with *Penaeus indicus* in a culture system (Doctoral dissertation, Cochin University of Science and Technology), 11(1), P(79-95).
- Dalmin G., Kathiresan K. and Purushothaman A. (2001). Effect of probiotic on bacterial population and health status of shrimp in culture pond ecosystem. *Indian Journal of Experimental Biology*, 39(9), P(939-942).
- Das, S., Lyla, P.S. and Ajmal K.S. (2006). Application of streptomyces as a probiotic in the laboratory culture of *Penaeus monodon* (Fabricius). *The Israeli Journal of Aquaculture*, 58(3), P(198-204).
- De Paiva-Maia, E., Alves-Modesto, G., Otavio-Brito, L., Olivera, A. and Vasconcelos-Gesteira, T. C. (2013). Effect of a commercial probiotic on bacterial and phytoplankton concentration in intensive shrimp farming (*Litopenaeus vannamei*) recirculation systems. *Latin American Journal of Aquatic Research*, 41(1), P(126-137).
- De Souza, D. M., Suita, S. M., Leite, F. P. L., Romano, L. A., Wasielesky, W. and Ballester, E. L. C. (2012). The use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817) in a zero exchange system. *Aquaculture Research*, 43(12), P(1828-1837).

- DoF (2017). Yearbook of Fisheries Statistics of Bangladesh 2016-17. Fisheries Resources Survey System (FRSS), Department of Fisheries. Bangladesh: Director General. 34(5), P(129).
- DoF (2015). National fish week, compendium (In Bengali). Dhaka: Department of Fisheries, Ministry of Fisheries and Livestock, Government of Bangladesh.
- DoF (2012). Unnata projuktite bagda chingri chas sohayeka (Matshabhabon, Ramna, Dhaka, Bangladesh).
- DoF (2010). Exported fish and fisheries by product of Bangladesh. Department of Fisheries, Ministry of Fisheries and Livestock, Dhaka, Bangladesh. P(107).
- FAO (2017). The State of World Fisheries and Aquaculture. Contributing to Food Security and Nutrition for All.
- FAO (2012). The State of World Fisheries and Aquaculture. FAO Fisheries and Aquaculture Department, Food and Agriculture Organization of the United Nations, Rome.
- Farzanfar, A. (2006). The use of probiotics in shrimp aquaculture. *Federation of European Microbiological Societies, Immunology and Medical Microbiology*, 48(2), P(149-158).
- Fuller, R. (1989). Probiotic in man and animals. *Journal of Applied Bacteriology*, 66(5), P(131-139).
- Gatesoupe, F. J. (1999). The use of probiotics in aquaculture. *Aquaculture*, 180(1-2), P(147-165).

- Gildberg, A., Mikkelsen, H., Sandaker, E, and Ringo, E. (1997). Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (*Gadus morhua*). *Hydrobiologia*, 352(1-3), P(279-285).
- Gullian, M., Thompson, F. and Rodriguez, J. (2004). Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. *Aquaculture*, 233(1-4), P(1-14)
- Hameed, A. S. (1993). A study of the aerobic heterotrophic bacterial flora of hatchery-reared eggs, larvae and post-larvae of *Penaeus indicus*. *Aquaculture*, 117(3-4), P(195-204).
- Harikrishnan, R., Balasundaram, C. and Heo, M. S. (2010). Effect of probiotics enriched diet on *Paralichthys olivaceus* infected with lymphocystis disease virus (LCDV). *Fish and Shellfish Immunology*, 29(5), P(868-874).
- Hossain, M. I., Kamal, M. M., Mannan, M. A. and Bhuyain, M. A. B. (2013). Effects of probiotics on growth and survival of shrimp (*Penaeus monodon*) in coastal pond at Khulna, Bangladesh. *Journal of Scientific Research*, 5(2), P(363-370).
- Irianto, A. and Austin, B. (2002). Probiotics in aquaculture. *Journal of Fish Diseases*, 25(11), P(633-642).
- Jha, A. K. (2014). Probiotics: an effective feed supplement in Shrimp culture. *Journal of Bangladesh Academy of Sciences*, 38(2), P(127-130).
- Karunasagar, I., Pai, R., Malathi, G. R. and Karunasagar, I. (1994). Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. *Aquaculture*, 128(3-4), P(203-209).

- Kautsky, N., Rönnbäck, P., Tedengren, M. and Troell, M. (2000). Ecosystem perspectives on management of disease in shrimp pond farming. *Aquaculture*, 191(1-3), P(145-161).
- Kennedy, S. B., Tucker, J. W., Thoresen, M. and Sennett, D. G. (1998). Current methodology for the use of probiotic bacteria in the culture of marine fish larvae. *Aquaculture*, 98, P(286).
- Kumar, N. J. P., Srideepu, K., Reddy, H. M. and Reddy, S. K. (2016a). Effect of water probiotic (Pro-W) on *Litopenaeus vannamei* culture ponds of Nellore, Andhra Pradesh, India. *International Journal of Environmental Sciences*, 6(5), P(846-850).
- Kumar, V., Roy, S., Meena, D. K. and Sarkar, U. K. (2016b). Application of probiotics in shrimp aquaculture: importance, mechanisms of action, and methods of administration. *Reviews in Fisheries Science and Aquaculture*, 24(4), P(342-368).
- Kumar, P. N. J., Jyothsna, R. S., Reddy, M. H. and Sreevani, S. (2013). Effect of *Bacillus subtilis* and *Lactobacillus rhamnosus* incorporated probiotic diet on growth pattern and enzymes in *Penaeus vannamei*. *International Journal of Life Science and Pharma Research*, 3(4), P(6-11).
- Lakshmi, B., Viswanath, B. and Sai Gopal, D. V. R. (2013). Probiotics as antiviral agents in shrimp aquaculture. *Journal of Pathogens*. Article Id-424123, 8(2), P(13).
- Lazado, C. C., Caipang, C. M. A. and Estante, E. G. (2015). Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions. *Fish and Shellfish Immunology*, 45(1), P(2-12).
- Lester, L.J. and Pante, M.J.R. (1999). Penaeid temperature and salinity responses. In: Fast A W, Lester L. J. (ed) *Shrimp Culture: Principles and Practices*, 9(3), P(37-46).

- Martinez Cruz Patricia, Ana L. Iba'nez, Oscar A. Monroy Hermosillo, and Hugo C. Ramirez Saad (2012). Use of Probiotics in Aquaculture: Review Article. *International Scholarly Research Network Microbiology*, Article Id- 916845, 11(4), P(13).
- Meunpol, O., Lopinyosiri, K., and Menasveta, P. (2003). The effects of ozone and probiotics on the survival of black tiger shrimp (*Penaeus monodon*). *Aquaculture*, 220(1-4), P(437-448).
- Moriarty, D. J. (1999). Disease control in shrimp aquaculture with probiotic bacteria. *In Proceedings of the 8th international symposium on microbial ecology*. Halifax, Canada: Atlantic Canada Society for Microbial Ecology. 21(6), P(237-243)
- Moriarty, D. J. W. (1998). Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture*, 164(1-4), P(351-358).
- Nayak, S. K. (2010). Probiotics and immunity: a fish perspective. *Fish and Shellfish Immunology*, 29(1), P(2-14).
- Nomoto, K. (2005). Prevention of infections by probiotics. *Journal of Bioscience and Bioengineering*, 100(6), P(583-592).
- Otta, S. K., Karunasagar, I. and Karunasagar, I. (2001). Bacteriological study of shrimp, *Penaeus monodon* (Fabricius), hatcheries in India. 17(5), P(59-63).
- Padmavathi, P., Sunitha, K. and Veeraiah, K. (2012). Efficacy of probiotics in improving water quality and bacterial flora in fishponds. *African Journal of Microbiology Research*, 6(49), P(7471-7478).



- Pattukumar, V., Sahu, M. K., Murugan, M., Sethubathi, G. V., Sivakumar, K. and Arul, V. (2010). Population of *Vibrio parahaemolyticus* (Pathogen) and *Bacillus* (Beneficial bacteria) in *Penaeus monodon* (Fabricius, 1798) Culture. *International Information System for the Agricultural Science and Technology*, 4(8), P(81-92).
- Pérez, T., J. L. Balcázar, I. Ruiz-Zarzuela, N. Halaihel, D. Vendrell, I. De Blas, and J. L. Múzquiz (2010). Host-microbiota interactions within the fish intestinal ecosystem. *Mucosal Immunology*, 3(4), P(355).
- Prabhu, N.M., Nazar, A.R., Rajagopal, S. and Khan, S.A. (1999). Use of probiotics in water quality management during shrimp culture. *Journal of Aquaculture in the Tropics* 14(10), P(227-236).
- Qi, Z., Zhang, X. H., Boon, N. and Bossier, P. (2009). Probiotics in aquaculture of China—current state, problems and prospect. *Aquaculture*, 290(1-2), P(15-21).
- Raman, M., Ambalam, P., Kondepudi, K. K., Pithva, S., Kothari, C., Patel, A. T. and Vyas, B. R. M. (2013). Potential of probiotics, prebiotics and synbiotics for management of colorectal cancer. *Gut microbes*, 4(3), P(181-192).
- Rengpipat, S., Tunyanun, A., Fast, A. W., Piyatiratitivorakul, S. and Menasveta, P. (2003). Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic. *Diseases of Aquatic Organisms*, 55(2), P(169-173).
- Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S. and Menasveta, P. (1998). Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture*, 167(3-4), P(301-313).

- Shakibazadeh, S., Che Roos, S., Hafezieh, M., Christianus, A., Saleh Kamarudin, M. and Kamaruzaman, S. (2012). A putative probiotic isolated from hatchery reared juvenile *Penaeus monodon*. *Iranian Journal of Fisheries Sciences*, 11(4), P(849-866).
- Shamsuzzaman, M. M., Islam, M. M., Tania, N. J., Al-Mamun, M. A., Barman, P. P. and Xu, X. (2017). Fisheries resources of Bangladesh: Present status and future direction. *Aquaculture and Fisheries*, 2(4), P(145-156).
- Shariff, M., Yusoff, F. M., Devaraja, T. N. and Rao, P. S. (2001). The effectiveness of a commercial microbial product in poorly prepared tiger shrimp, *Penaeus monodon* (Fabricius), ponds. *Aquaculture Research*, 32(3), P(181-187).
- Singh, B.I. (1986). Studies on the bacteria associated with *Penaeus indicus* in a culture system. Ph.D. Thesis. Cochin University of Science and Technology, Cochin, India, 12(9), P(230).
- Sivakumar, N., Sundararaman, M. and Selvakumar, G. (2012). Probiotic effect of *Lactobacillus acidophilus* against vibriosis in juvenile shrimp (*Penaeus monodon*). *African Journal of Biotechnology*, 11(91), P(15811-15818).
- Son, V. M., Chang, C. C., Wu, M. C., Guu, Y. K., Chiu, C. H. and Cheng, W. (2009). Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses, and disease resistance of the grouper *Epinephelus coioides*. *Fish and Shellfish Immunology*, 26(5), P(691-698).
- Soundarapandian, P. and Babu, R. (2010). Effect of probiotics on the hatchery seed production of black tiger shrimp, *Penaeus monodon* (Fabricius). *International Journal of Animal and Veterinary Advances*, 2(1), P(9-15).

- Soundarapandian, P., Ramanan, V. and Dinakaran, G. K. (2010). Effect of probiotics on the growth and survival of *Penaeus monodon* (Fabricius). *Current Research Journal of Social Sciences*, 2(2), P(51-57).
- Souza, D. M. D., Martins, G. B., Piedras, S. R. N., Pouey, J. L. O. F., Robaldo, R. B., and Leite, F. P. L. (2012). Probiotic actions of *Bacillus cereus* var. *toyoi* and *Saccharomyces boulardii* in silver catfish (*Rhamdia quelen*) larvae culture. *Revista Brasileira de Zootecnia*, 41(3), P(815-819).
- Srinivas, R., Manjulatha, C., Raju, M. R. and Reddy, K. R. B. K. S. (2014). Effect of probiotics on *Vibrio* bacterial populations in culture ponds of *Litopenaeus vannamei* at Vadacheepurupalli, Andhra Pradesh, India. *Asian Journal of Experimental Science*, 28(2), P(1-7).
- Standen, B. T., Rawling, M. D., Davies, S. J., Castex, M., Foey, A., Gioacchini, G. and Merrifield, D. L. (2013). Probiotic *Pediococcus acidilactici* modulates both localised intestinal-and peripheral-immunity in tilapia (*Oreochromis niloticus*). *Fish and Shellfish Immunology*, 35(4), P(1097-1104).
- Sunaryanto, A. and A. Mariam, (1986). Occurrence of a pathogenic bacteria-causing luminescence in penaeid larvae in Indonesian hatcheries. *Brackish water Aquaculture Development Centre*, 8(2), P(64-70).
- Sung, H. H., Hsu, S. F., Chen, C. K., Ting, Y. Y. and Chao, W. L. (2001). Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation. *Aquaculture*, 192(2-4), P(101-110).

- Supamattaya, K., Viriyapongsutee, B., Ruangsri, J., Encarnacao, P. and Schatzmayr, G. (2005). Effect of probiotic *Enterococcus faecium* and phycophytic substances on growth performance and health condition of white shrimp (*Penaeus vannamei*). *Aquaculture*, 7(11), P(35-42)
- Tuan, T. N., Duc, P. M. and Hatai, K. (2013). Overview of the use of probiotics in aquaculture. *International Journal of Research in Fisheries and Aquaculture*, 3(3), P(89-97).
- Vaseeharan, B. A. R. P., and Ramasamy, P. (2003a). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Letters in Applied Microbiology*, 36(2), P(83-87).
- Vaseeharan, B. and Ramasamy, P. (2003b). Abundance of potentially pathogenic microorganisms in *Penaeus monodon* larvae rearing systems in India. *Microbiological Research*, 158(4), P(299-308).
- Vieira, F. N., Buglione, C. C., Mourino, J. P. L., Jatobá, A., Martins, M. L., Schleder, D. D. and Vinatea, L. A. (2010). Effect of probiotic supplemented diet on marine shrimp survival after challenge with *Vibrio harveyi*. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 62(3), P(631-638).
- Vieira, F. D. N., Buglione Neto, C. C., Mouriño, J. L. P., Jatobá, A., Ramirez, C., Martins, M. L. and Vinatea, L. A. (2008). Time-related action of *Lactobacillus plantarum* in the bacterial microbiota of shrimp digestive tract and its action as immunostimulant. *Pesquisa Agropecuária Brasileira*, 43(6), P(763-769).

- Vieira, F. D. N., Pedrotti, F. S., Buglione Neto, C. C., Mourão, J. L. P., Beltrame, E., Martins, M. L. and Arana, L. A. V. (2007). Lactic-acid bacteria increase the survival of marine shrimp, *Litopenaeus vannamei*, after infection with *Vibrio harveyi*. *Brazilian Journal of Oceanography*, 55(4), P(251-255).
- Vine, N. G., Leukes, W. D. and Kaiser, H. (2006). Probiotics in marine larviculture. *Federation of European Microbiological Societies, Microbiology Reviews*, 30(3), P(404-427).
- Wahab, M.A., Ahmad-Al-Nahid, S., Ahmed, N., Haque, M.M. and Karim, M. (2012). Current status and prospects of farming the giant river prawn *Macrobrachium rosenbergii* (De Man) in Bangladesh. *Aquaculture Research*, 43(7), P(970–983).
- Wang, Y. B. (2007). Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. *Aquaculture*, 269(1-4), P(259-264).
- Wang, Y. B., Xu, Z. R. and Xia, M. S. (2005). The effectiveness of commercial probiotics in northern white shrimp *Penaeus vannamei* ponds. *Fisheries Science*, 71(5), P(1036-1041).
- World health organization (WHO), (2006). Report of a joint FAO/OIE/WHO Expert Consultation on antimicrobial use in aquaculture and antimicrobial resistance, Seoul, Republic of Korea, 13–16 June 2006.
- World health organization (WHO), (2002). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, a joint FAO/WHO expert consultation. Cordoba, Argentina, 1-4 October 2001.

- Ziaei-Nejad, S., Rezaei, M. H., Takami, G. A., Lovett, D. L., Mirvaghefi, A. R. and Shakouri, M. (2006). The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture*, 252(2-4), P(516-524).
- Zorriehzahra, M. J., Delshad, S. T., Adel, M., Tiwari, R., Karthik, K., Dhama, K. and Lazado, C. C. (2016). Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: a review. *Veterinary Quarterly*, 36(4), P(228-241).

