Effect of probiotics and biofloc on total cultivable bacteria and *Vibrio* spp. in shrimp culture

A Thesis

by

Sumaiya Sarmin
Roll No: MS-180613
Session: 2018-2019

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

of

Master of Science (MSc)

in

Aquaculture

September, 2019

Effect of probiotics and biofloc on total cultivable bacteria and *Vibrio* spp. in shrimp culture

Approved as to style and content

by

14.10.19

Dr. Muhammad Abdur Rouf

Professor and Head

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

September, 2019

DECLARATION

Effect of probiotics and biofloc on total cultivable bacteria and *Vibrio* spp. in shrimp culture

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

it is being concurrently submitted for any degree.

Sumaiya Sarmin
15-09-19
....
(Signature of the candidate)

Sumaiya Sarmin
Roll No: MS-180613
Session: 2018-19

....
(Signature of co-supervisor)

Shikder Saiful Islam

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

G.R. Banu
....
(Signature of the supervisor)

Dr. Ghausiatur Reza Banu

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

September, 2019

Dedicated To

My Beloved Parents

Contents

ACKNOWLEDGEMENT

First of all, my admiration goes to the Almighty for His mercy to enable me to pursue my education in Fisheries and Marine Resource Technology Discipline. It is my great pleasure for being able to complete this thesis work for the degree of Masters of Science in Aquaculture for which I am completely indebted to the Creator of this Universe, The Almighty.

I would like to express my profound appreciation and sincere gratitude to my honorable supervisor, **Dr. Ghausiatur Reza Banu,** Professor, Fisheries and Marine Resource Technology (FMRT) Discipline for her scholastic guidance, constructive criticism, constant encouragement and sustained support that rationalized me to complete this thesis paper successfully.

I would also like to express my profound gratitude 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 and facilities that he offered me during my experiment.

I would like to acknowledge here the ministry of Science and Technology for providing me NST fellowship to conduct this thesis.

I wish to give special thanks to Hazrat Ali, Divisional Administrator, Value Chain and Nutrition Program for his informative suggestion, guidance and provided facilities.

My heartiest thanks go to all my respected teachers in Fisheries and Marine Resource Technology Discipline, Khulna University and friends whose inspiration was all along with me in the hard time during the course of the present study and for their endless support and encouragement.

Finally, I would like to express my deepest gratitude to my beloved parents for their immeasurable blessing and continuous inspiration.

Sumaiya Sarmin
Roll No: MS-180613
July, 2019

List of Tables

List of figures

List of Abbreviations and non-English Words

Abbreviation	Elaboration
BFT	Biofloc Technology
CFU	Colony Form Unit
DoF	Department of Fisheries
et al.	And Others
FAO	Food and Agricultural Organization
FCR	Food Conversion Ratio
GDP	Gross Domestic Product
GIT	Gastrointestinal Tract
GWT	Green Water Technology
IMNV	*Infectious Myonecrosis Virus*
UK	United Kingdom
L	Litre
MBV	*Monodon baculovirus*
Ml	Milliliter
MS	Microsoft
MT	Metric Tonne
PL	Post Larvae
SPF	Specific Pathogen Free
Spp	Species
TBC	Total Bacteria Count
TCBS	Thiosulphate Citrate Bile Salt Sucrose
TVC	Total *Vibrio* Count
WSSV	*White spot syndrome virus*

Abstract

The successful entrepreneurship of aqua farming relies on the production of aquatic animals in the cost effective, social and environmental friendly approach. Nevertheless, presently fish farming is suffering from various problems related to environmental damages and low productivity of various crops. Biofloc technology and/or application of probiotics provide promising results to aquaculture in terms of improvement in the growth and survival of aquatic animals, along with other benefits such as maintaining water quality without causing pollution to the environment and microbial management. Therefore, this study was conducted to assess the effect of probiotics and biofloc on total bacteria and *Vibrio* spp. load in grow-out culture of shrimp which was treated in nursery phase. Two commercial probiotic (PRO-2 and PRO-W) and biofloc (C:N=1:12) were applied for 27 days in nursery tank at Redient Shrimp Hatchery in Shaymnagar, Satkhira. The populations of total bacteria and pathogenic bacteria of *Vibrio* spp. were monitored in shrimp after 61 days of grow-out culture where no probiotic and biofloc were applied. The results of this study reported that probiotic and biofloc treatment in the nursery phase of *P. monodon* culture did not have any impact of the total microbial load throughout the grow-out culture period. In contrast, in feed probiotics (T1, T3), and biofloc (T4) as well as combination of biofloc and in feed probiotics (T5) was found to have good contribution to develop a microbial community in the gut of *P. monodon*, which may capable to protect exploring *Vibrio* spp. community during the grow-out culture. Thus, application of probiotics and bioflocs could lead to disease-free and profitable shrimp culture operations which will be helpful for shrimp farmers as most of them are now-a-days severely affected by vibriosis disease.

Chapter...........1

Introduction

1 Introduction

Aquaculture is recognized as the fastest growing food producing sector in the world. With the global population still increasing and capture fisheries leveling off, aquaculture is seen in the future as the hope to fill the gap between fish supply and demand. Aquaculture production in Southeast Asia has contributed significantly to worldwide supply and in the process, to the countries' economic development (Inui, 2002). Bangladesh is an agro-based developing country and is exclusively gifted with natural fisheries resources. It is one of the world's leading inland fisheries producers with a wide range of marine aquatic bio-diversities, estuaries and huge water resource all over the country (Ghose, 2014). As the second most valuable agricultural crop, fish contributes 3.61% to our national GDP and around one-fourth (24.41%) to the agricultural GDP (DoF, 2017) and to the livelihoods and employment of millions (more than 11% of total population) of people in Bangladesh. FAO reported, Bangladesh's position is now after China and India in inland fish production, which ranked first and second respectively this year, while the country's position was the fifth in 2017. In last 10 years the average growth performance of this sector is almost 5.43% which eventually ensures to achieve the governments projected production target of 4.55 million MT by 2020-21. The increasing production rate of shrimp (*Penaeus monodon*), the major export items in Bangladesh from 1.60 lakh MT in 2002-03 to 2.46 lakh MT in 2016-17 contributes the main part of the growth performance of fisheries sector (DoF, 2017). The demand of seafood throughout the world has been leading to a worldwide expansion of aquaculture which will meet half of the world's seafood demand by 2020, as wild capture fisheries are overexploited and are in decline.

However, world aquaculture production has grown more rapidly compared to Bangladesh. In Bangladesh, most of the culture techniques are done through extensive method where subsequently yield is very low because of inefficient control of predators and competitors and disease and their surroundings "space", "water", "biomass" and "landscapes" are limited due to strong anthropic pressure. In large scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses (Hernandez-Llamas *et al.*, 2016). Disease is the main restraining factor for the development of shrimp sector in Bangladesh. The diseases of cultured penaeid shrimp include syndromes with infectious (viral, bacterial, fungal), as well as a number of noninfectious diseases, which are also of importance for the industry, caused by environmental extremes, nutritional imbalances, toxicants and genetic factors (Kautsky, 2000)). In recent years, the viral and bacterial diseases have affected the shrimp farms causing pronounced economic losses. Black tiger shrimp (*Penaeus monodon*), the most widely cultured species; is now endangered by vibriosis disease mostly due to *Vibrio* spp like *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio alginolyticus* (Hasan *et al.*, 2012).

Prevention and control of diseases have led during recent decades to a substantial increase in the use of veterinary medicines. Most of the fish diseases in cultured pond were treated with drugs such as antibiotics, sulfonamides, nitrofurans, methylene blue and others (Sorum *et al.*, 2006). Consequently, wide ranges of chemicals particularly antimicrobial agents were used in shrimp farming to prevent and to treat diseases. The usage of these antimicrobial agents has increased enormously and tones of antibiotics are distributed in the biosphere during an antibiotic era of only about 60-year duration. The indiscriminate use of antibiotics had led to the appearance of antibiotic-resistant strains and led to the contamination of shrimp meat and also the environment (Holmstrom *et al.*, 2003). Thus it forces the control measure switch to new drugs or higher doses and also the aquatic organisms which are useful for the maintenance of favorable water quality could be affected. Antibiotic is also harmful for aquatic animals because it kills beneficial microbiota in gastrointestinal system of animals and it also accumulates in fish products to be unsafe for human consumption (Das *et al.*, 2017). However, the utility of antimicrobial agents as a preventive measure has been questioned, given the extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria (Balcazar, 2003).

Nevertheless, shrimp aquaculture constantly requires modern technologies and other sciences such as biotechnology and microbiology in order to increase higher quality and greater quantity of products (Farzanfar, 2006) and to avoid antibiotics. However, the best mean to control the bacterial diseases of aquatic animals is not the control but the prevention, in other words the avoidance of exposure to pathogen for infection. However, a new and better technology (Green Water Technology) to culture shrimp is being used by many enterprising shrimp farmers nowadays. It is a technique that cultures shrimps in water that is abundant in phytoplankton, turning the water green hence. The reason for the wide application of phytoplankton is due to the fact that they compose the base food chain in nature, stabilize the water quality through removal of metabolic waste and produce oxygen. Besides, it can be grazed on by fishes; thereby recycling waste nutrients and lowering feed conversion ratios and also stimulate non-specific immune system of fish larvae. But GWT required both enriched water and a suitable substrate for the growth of these nutrient-rich natural foods. Moreover, if nitrates rise to high levels, the enrichment causes algae blooms that reduce oxygen levels to near-critical levels at night (James, 2018).

For a sustainable further development of the aquaculture industry, novel strategies to control bacterial infections are needed. In aquaculture, the cultured organisms have a direct interaction with the environment. The natural and external micro biota are in permanent contact with the animals; considering it, Verschuere *et al.* (2000) suggested a new term of probiotic for aquaculture, which includes their effect over the microbial community of the water, and over the quality of the farming environment. In addition, probiotics are nonpathogenic and nontoxic microorganisms without undesirable side-effects when administered to aquatic organisms (Farzanfar, 2006). The common probiotics that are used for aquaculture practices include *Lactobacillus, Lactococcus, Enterococcus, Bacillus, Enterobacter, Pseudomonas,* and *Saccharomyces* species. 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. The involvement of probiotics in nutrition, disease resistance and other beneficial activities in fish has proven beyond any doubt (Nayak, 2010). Probiotics shows a new dimension in disease resistance and improving water quality in aquaculture industry (Mukherjee *et al.*, 2016). They perform several mechanisms to prevent the harmful effect of intestinal pathogens such as competitive exclusion of pathogen creating competition for nutrients, adhesion sites, and inhibition of interaction between pathogens and intestinal mucosa, and production of antimicrobial substances (Steer *et al.*, 2000). The application of probiotics lesser pathogenic Vibrios and enhance beneficial *Bacilli* in the culture leading to improve water quality, promoted growth, and survival rates and increased health status of the shrimp without stress and disease outbreak (Dalmin *et al.*, 2001).

Also, in recent years the searching of new sustainable aquaculture technologies allows the development of culture method called Biofloc (Zhao *et al.*, 2012) which uses food waste, organic matter and compounds produced during the production, through proliferation of heterotrophic microorganisms which develop in water using an external carbon source and high oxygen level (Ferreira *et al.*, 2015). Biofloc comprises various microorganisms (phytoplankton, bacteria, rotifers, nematodes, and protozoa), uneaten feed, dead cells, detritus, and feces (Emerenciano *et al.,* 2017) which facilitates degradation of organic wastes and assimilation and nitrification of nitrogen (Avnimelech, 2007). Application of Biofloc Technology in aquaculture offers a solution to avoid the environmental impact of high nutrient discharges and to reduce the use of artificial feed (Crab *et al.*, 2012). This technology, to a certain extent, has also the capacity to control pathogens in aquaculture. If carbon and nitrogen are well balanced in the solution, ammonium in addition to organic nitrogenous waste will be converted into bacterial biomass. By adding carbohydrates to the pond, heterotrophic bacterial growth is stimulated and nitrogen uptake through the production of microbial proteins takes place.

As the human population continues to grow, food production industries such as aquaculture is need to expand as well. In order to preserve the environment and the natural resources, this expansion is needed to take place in a sustainable way. Recently, in this study, both technologies were implemented, through endogenous addition of probiotic bacteria in Biofloc system, considering that they improve the obtained results with respect to their application separately.

Objectives

- To determine the effect of probiotics and biofloc on the total cultivable bacteria in shrimp body from a grow-out culture system.
- Investigating the controlling effect of probiotics and biofloc against the *Vibrio* spp.

Chapter...........2

Review of Literature

2 Literature Review

The fisheries sector provides immense opportunities to accomplish food security, create economic growth to lessen poverty, as well as to confirm better utilization of all types of fisheries assets (Bondad-Reantaso *et al.*, 2012). It plays a very important role in the socio-economic development, satisfying the demand of animal protein, opening for employment, poverty improvement of large number of population and earning foreign exchange for Bangladesh. Over the last decade, dramatic increases in the production of a variety of species from commercial aquaculture systems and sharp increases in per capita fish consumption have occurred in Bangladesh. For enhancing shrimp production and promote business friendly supply chain, good aquaculture practices, cluster farming approach are being adopted. Culture systems using high density and minimal or zero water exchange have been used to increase aquaculture sustainability (Crab *et al.,* 2012). With the increased intensification and commercialization of aquaculture practice, fish health has turned into a most important issue to aqua culturists (Bondad-Reantaso *et al.*, 2005).

However, the rapid and generally uncontrolled development of aquaculture led to frequent occurrence of infectious diseases which have been threatening the sustainability of aquaculture in the region. Also, environmental problems faced by the shrimp culture industry today are the major constraints to its future growth and in some cases even raise doubts over its continued existence (Faruque, 2008). So are bacterial diseases, primarily luminous vibriosis in shrimp is more harmful for aquaculture (Inui, 2002). Even though antibiotics were used in shrimp farming for controlling disease, the passage and permanent existence of large amounts of antibiotics in the environment of water and sediments also have the potential to affect the presence of the normal flora and plankton in those niches, resulting in shifts in the diversity of the microbiota (Davies *et al.*, 1999; Miranda and Zemelman, 2001).

In this view, the development of non-antibiotic agents for health management in shrimp farming is necessary. The control measure by immunization of fish with vaccines gains the effort and rapid development. Quorum quenching is another disease controlling factor which inhibits the behavior of bacterial quorum sensing which regulate certain phenotypic expressions such as biofilm formation, virulence factor expression, motility and also to regulate bioluminescence, nitrogen fixation and sporulation (Pan and Ren, 2009). Inhibition of quorum sensing can be accomplished in several ways, including: enzymatic degradation of signaling molecules; blocking signal generation; and blocking signal reception (Majerczyk et al., 2016).

The shrimp farmers need to give emphasis on providing a healthy environment for the shrimp and provide more effort to control adverse environmental impacts and disease outside the shrimp farm. The environmental management both within the shrimp pond and outside the pond is concerned to farming success (Faruque, 2008). Recent studies suggest that proper handling of microbial communities can help to get better water quality, on one side reducing nutrient levels (nitrogen compounds and bacteria biomass) and for other side, reducing pathogen bacteria abundance, consequently increasing survival of cultured species, without using toxic chemical substances or antimicrobials (Bentzon-Tilia *et al.*, 2016). Considering this point of view, one of the most used technology in aquaculture to modified microbiota is the application of probiotics (Pandiyan *et al.*, 2013) which have shown health benefits and water quality of aquatic cultured species, through different action mechanisms like: stimulation of immune response, substances segregation which inhibited the microorganisms pathogen growth, enzyme production which induce absorption and improve fish and crustacean nutrition and nitrogen compound reduction which were transformed by microorganism in culture systems (Yamashita *et al.*, 2016; Das *et al.*, 2017). Some other studies have tried to clarify the different mechanisms by which probiotics modulate the immune system of fish, including the stimulatory effect of pro-inflammatory cytokines on the activity of immune cells, antibodies, acid phosphatase, lysozymes, complement, and antimicrobial peptides, in response to invasive pathogens (Pirarat *et al.*, 2006).

Probiotics are live non-pathogenic microorganisms providing colonization resistance to the pathogenic microbes and thus are effective in prevention and treatment of some diseases (Hossain *et al.*, 2013). Verschuere *et al.* (2000) extended the concept of probiotics as a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease or by improving the quality of its ambient environment.

Probiotics, prebiotics, synbiotics and medicinal plants were among the most promising feed supplements for control or treatments of bacterial, viral and parasitic diseases of fish and shellfish. The possible mode of action of probiotics (Strengthening immune response, competition for binding sites, production of antibacterial substances, and competition for nutrients) is providing protection against diseases (Hoseinifar, *et al.*, 2018). Hasan *et al.* (2012) studied disease resistance activity of probiotics and found no disease incidence (swollen gut and vibriosis) appeared in ponds treated with probiotics and *Vibrio* spp. (green colonies) was dominant only in the control ponds similar to the findings of Lakshmanan and Soundarapandian, (2008). They conclude that application of probiotics in the treatment ponds hindered the growth of green *Vibrio* and luminous due to the colonization of the beneficial microbes like *Bacillus* spp., *Pseudomonas* spp., and *Lactobacillus* spp. in the shrimp gut and growth, immunity and survival rate of shrimp were improved.

Rengpipat *et al.* (2003) performed an experiment to see the growth and resistance to *Vibrio* in black tiger shrimp (*P. monodon*) by using feed with a *Bacillus* probiotics (BS11). The result showed that the growth and survival rates of shrimps fed with probiotic supplement were significantly greater than those of the controls. Similar results were observed by Karthik *et al.* (2014) while checking other probiotics *Lactobacillus* spp (AMET1506) for the same purpose. The effect of gut probiotics mixed in a feed was studied by Jha, (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.

Fish and other marine animals have a unique and intimate interaction with their surrounding environment and, in turn, with the microorganisms that co-exist there. Soundarapandian and Babu, (2010) examined the effect of probiotics on microbial load in 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. Similarly, Souza *et al.* (2012) conducted a study about the use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* 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.

Probiotic bacteria can be used as ecological biocontrol or bioremediation agent for the sustainable development of aquaculture. Probiotics can decrease algal growth and organic load, increase nutrient concentration and beneficial bacterial population, inhibit potential pathogens, and increase concentration of dissolved oxygen. It has been proven that the addition of probiotic bacteria reduces the load of pollutants such as heavy metals (Pb, Cd, Hg, Ni, etc.) (Sayes *et al.*, 2018). According to Prasad *et al.* (2003) water probiotics are applied to reduce organic pollutants and various contaminants in water by directly applying to rearing pond. These improve water quality by converting organic matter to smaller units. Probiotic bacteria such as *Bacillus* spp. can convert organic matter to CO_2 so that organic effluent can be minimized in aquatic system. They also reported that Lactic Acid bacteria, a popular probiotic strain can be applied to control bacterial pathogen. 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* (1.62kg/ha) and *Nitrobacter* (0.82kg/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.

Venkatrayulu *et al.* (2009) investigated the effect of both water and feed probiotic combinely on the population density of *Vibrio* (yellow and green colonies) bacteria and total yield in semi intensive shrimp culture ponds. The results showed that the use of probiotics has reduced *Vibrio* bacterial count and improved total yield of shrimp. The average *Vibrio* bacterial (yellow and green colonies) counts were found to be significantly ($p<.01$) lower in probiotic treated (PB) culture ponds compared to control pond.

The use of beneficial bacteria (probiotics) to displace pathogens by competitive processes is being used in the animal industry as a better remedy than administering antibiotics and is now gaining acceptance for the control of pathogens in aquaculture. Health management of shrimp requires consideration of delicate balance between the host, pathogen and environment (Wang *et al.*, 2016). Moriarty, (1999) reported his successful experiences of using probiotic bacteria instead of antibiotics to control Luminus vibrios in shrimp farms in Negros, Philipine. He found luminous *Vibrio* numbers in the hepatopancreas of shrimps fell from around 1×10^4 per gut when antibiotics were used in the feed, to zero when probiotics were applied to the pond.

Another recent technology used in aquaculture is the Biofloc Technology. The biofloc is a protein rich macro aggregate of organic material and micro-organisms including diatoms, bacteria, protozoa, algae, faecal pellets, remains of deals organisms and other invertebrates (Avnimelech, 1999). Biofloc technology is a technique of enhancing water quality through the addition of extra carbon to the aquaculture system, through an external carbon source or elevated carbon content of the feed. This promoted nitrogen uptake by bacterial growth decreases the ammonium concentration more rapidly than nitrification. The technology has recently gained attention as a sustainable method to control water quality, with the added value of producing protein rich feed in situ. Biofloc technology is also used for feed utilization. The excessive nitrogen that is leached out in water can be utilized by biofloc. It can convert the excessive nitrogen into protein that is ultimately used as a good protein source of shrimp.

Biofloc technology provide promising results to aquaculture in terms of improvement in the growth and survival of aquatic animals, along with other benefits such as maintaining water quality without causing pollution to the environment (Daniel and Nageswari, 2017). This technology has demonstrated positive results in fish and crustacean production with significantly saving of commercial food, also in medicines and chemical supply for disease control and reduction of water exchange which reduce significantly the environmental impact on water and limit pollution reducing aquaculture effluent discharges (Avnimelech, 2007).

Eskahari *et al.* (2014) reported higher phenoloxidase activity (an immune indicator) in response to carbon loading in a biofloc system. Chen and Avnimelech, (2012) showed significantly lower infection of tilapia by *Streptococcus* in biofloc compared to clear water system. It is widely recognized that many of the deadly shrimp diseases such as *White spot syndrome virus* (WSSV), *Infectious myonecrosis virus* (IMNV) and *Early mortality syndrome virus* (EMS), find it harder to infect shrimp grown in a biofloc system.

Kasan *et al.* (2018) conducted an experiment aiming to assess the impact of BFT to water quality and shrimp health. To achieve maximum growth of BFT in *P. vannamei* cultures, an isolated biofloc boost-up bacteria inoculum was added during each new stocking program of shrimp post-larvae (PL). Interestingly, biofloc was observed started to be formed as early as 10 days after shrimp cultivation periods, where the biofloc formation was speed up by the addition of the inoculum and thus effectively enhanced good water quality which resulted in the increasing shrimp biomass.

Manan *et al.* (2016) applied Biofloc Technology (BFT) in a closed water exchange shrimp hatchery of University Malaysia Terengganu (UMT) and found no mass mortality in the biofloc tank except for the first tank, T1 that infected with the *Vibrio* sp. Water parameter such as DO, salinity, pH, temperature and nutrients level include ammonia, nitrite and nitrate were in good condition in biofloc treatment compared to the control tank, respectively. Biofloc microorganisms successfully work in removing the nitrogenous wasted in the treatment tanks and maintain the nutrients and water quality in the safety level for the shrimp.

Kathia *et al.* (2017) demonstrated that 80% of scientific studies reported some benefit with probiotic use, either in growth, immune response or also water quality from cultured species. Likewise, culture with Biofloc have shown better productive yields in fish and crustaceans like shrimp at intensive system, in addition to reduce commercial food up to 30%, decrease the exchange of water of ponds almost in 100% and enhance water quality by stabilization of nitrogen compounds for bacterial action. Unfortunately, the effects of adding of probiotics in Biofloc are not clear yet according to their concentration and used type.

Hafsari, (2016) conducted a study with juveniles of *Clarias gariepinus* in Biofloc system added with *Bacillus* sp. molasses and fish pellets. The result showed better growth with Biofloc and probiotics (30%) with respect experimental treatment without Biofloc.

During the study of Krummenauer *et al.* (2014), commercial probiotics was added in Biofloc system with *Litopenaeus vannamei* infected with *Vibrio parahaemolyticus* and vibriosis was found to be controlled but with respect to growth rates, it did not show significant differences.

However, significant differences were not observed when commercial probiotic was added, composed by *Bacillus* spp. and *Lactobacillus* spp. in Biofloc system, in shrimp culture. These results could be assigned to anaerobiosis and deplections that can exist at pond bottom, could be influenced in action reduction of probiotic. Another factor that could affect was commercial probiotic concentration with respect to recommended probiotic concentration.

Chapter...........3

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 World Fish Bangladesh, Sterling University, UK and Khulna University. The study was continued from June 2018 to June 2019. Subsequent laboratory analysis of the shrimp samples was done in Fish Pathology and Quality Control 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 application of probiotics and biofloc in nursing of *P. monodon*, and its effect on controlling total bacteria and *Vibrio* spp. load in grow-out culture. Therefore, the treatments were applied at the nursery stage of *P. monodon* and the juveniles were allowed to grow in grow-out hapas for 61 days according to section 3.4. The nursing of shrimps was conducted under five treatments, such as the application of in feed probiotics (PRO-2) (T_1), water probiotics (PRO-W) (T_2), combination of both in feed and water probiotics (T_3), application of biofloc (C:N = 1:12) (T_4) and in feed probiotics and biofloc (T_5). In addition, a control group (C) was maintained without any application of probiotic and/or biofloc in shrimp nursing. Each of the treatment was replicated into five times and the nursing was continued for 27 days. After nursing, the juveniles from different treatments were stocked in hapas for grow-out culture for 61 days without applying any treatment. At the end of grow-out culture shrimp samples were collected from each hapas and the total bacterial load and *Vibrio* spp. load was determined through microbiological assay.

Table 3.1 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
T_4	Biofloc (C:N = 1:12)	5
T_5	In-feed probiotic and biofloc	5
C	Control/ Non-treated tank	5

3.3 Nursery rearing

3.3.1 Tank preparation

A total of 30 tanks of 500L volume were set randomly at Redient Shrimp Hatchery. The tanks were disinfected with chlorine water and filled with 450L disinfected water (water was disinfected by 35% active chlorine at 60 ppm for 72 hours).

3.3.2 Stocking and feeding

After that, shrimp larvae, SPF PL15 were stocked in the tanks for nursery rearing at the rate of 700PL/tank. The shrimps were fed 5 times a day with commercial diet (CP shrimp feed) having the protein level of 46%. The experimental system was a zero water exchange system and up to 20% of water was exchanged in case of emergency.

3.3.3 Composition and application of probiotics

The treatments were applied during the nursery phage of the experimental culture period. The feed probiotics Sanolife^{R} PRO-2, containing *Bacillus subtilis, Bacllus licheniformis, Bacillus pumilus* strains of 2×10^{10}cfu/g was applied through mixing with the feed at the rate of 5g/Kg (as per label instructions) of feed. The function of PRO-2 was recorded to have suppressing capability on the growth of pathogenic bacteria (*Vibrio* spp.) in the gut. PRO-2 has another function in breaking down uneaten feed and faces rapidly. Sanolife PRO-W, containing *Bacillus subtilis, Bacllus licheniformis* strains of 5×10^{10}cfu/g was applied at the rate of .005g/L (as per label instructions) of water to keep the water quality optimal, stabilizing the phytoplankton bloom. Biofloc was added at the C:N ratio of 1:12. The nursing was continued up to 27 days.

3.4 Grow-out culture

3.4.1 Pond preparation

Grow out ponds were drained, left to dry under sun light for 15 days, embankments were repaired and reconstructed. The pond sides were fenced by blue net for biosecurity at a height of 1.25m. The ponds were disinfected with bleaching powder (35% active chlorine). Pond was filled with water of 9ppt salinity to a depth of 1.5m. Pond water was chlorinated with bleaching powder at 60ppm. The pond water was then conditioned with minerals before setting up hapas and stocking. And then 24 hapas (16m^2 water area each: 4m x 4m, with a height of 1.5m and 0.5mm mesh), 4 replicates per treatment, were fixed.

3.4.2 Stocking

The juvenile tiger shrimp was transferred from nursery pond to separate synthetic nylon made hapas in grow out earthen pond (102m×22m=2244m^2) for culture up to harvest for 61 days. The juveniles were pooled by treatment and each hapa was stocked with 240 juveniles at a density of 15 juveniles/m^2.

3.4.3 Feed and feeding management

For the grow-out phase in hapas, commercial CP shrimp feed (crumble and pelleted feed) were used according to the CP instructions. Shrimp was fed 4 times per day at 6:00, 11:00, 16:00 and 22:00 h and feeding rates ranged from 7.0 to 3.5% of the biomass. Throughout grow-out culture period no treatment was applied and after completing the culture period, shrimps were collected for bacteriological analysis.

3.5 Microbiological analysis

Shrimp samples were analyzed for the total heterotrophic bacterial count and total *Vibrio* count (TVC) at the end of the nursery phase and grow-out phase. Total bacterial count (TBC) was determined by using nutrient agar and thiosulfate citrate bile salt-sucrose (TCBS) agar media was used for the TVC.

3.5.1 Laboratory equipments

Autoclave machine (ALP), centrifuge machine (Eppendrof AG 22331, Hamburg), electric balance (OHAUS CORPORATION), spirit lamp and burning spirit, Petri dish, biological dissection box, vortex mixer (Thermolyne), bacteria isolating loops, glass rods, oven (memmert), incubator (SANYO), refrigerator (Rangs), bio-safety cabinet (LABCONCO), micro-pipette (Thermo Scientific), tips and tips box were needed for conducting the study.

3.5.2 Reagents and chemicals

Peptone water (HiMedia), absolute alcohol (Merck), sodium chloride (NaCl), yeast extract (HiMedia), ethyl alcohol (70% and 100%), distilled water, TCBS agar media (HiMedia), nutrient agar media (HiMedia) powder were required for the study.

3.6 Lab work

3.6.1 Sterilization of lab accessories

Test tubes, petri dishes, conical flask, plastic tips and other glassware were sterilized by autoclaving 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.

3.6.2 Preparation of stock solution from shrimp samples

At the nursery phase, the whole animal was processed while at the grow-out phase, mixture of hepatopancreas and intestine were used for preparation of stock solution and weighed by an electric balance aseptically (HR-200). The weighed samples were then taken in a sterilize vortex mixer with 5ml 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 serial dilution and further works.

3.7 Preparation of media

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

TCBS agar was prepared by adding 89.0g TCBS powder in 1L demineralized water. The mixture was subsequently heated to boiling point using a magnetic stirrer. Then the media was kept in the bio-safety cabinet until it cools at 45-50°C and then transferred to the required number of petri-dishes by 20-25ml volume for preparing media plates and finally the plates were left at least 30 min to solidify.

3.7.2 Preparation of nutrient agar media

Nutrient agar media was prepared by adding 28g agar powder in 1L distilled water. Mixing it properly, for dissolving the powder and autoclaved at 121°C for 15 minutes. After autoclaving the agar media was kept under bio-safety cabinet and transferred to the required number of petri-dishes by 20-25ml volume in each petri-dishes. After pouring into the plates it was kept under the laminar flow to solidify and used for bacterial inoculation.

3.7.3 Inoculation and incubation

After preparing the agar plates it was poured with 1ml of diluted solution aseptically by sterile micropipette, followed by spreading with a L-shaped glass rod. The Petri-dishes were covered with aluminum foil and incubated at 30°C for 24 hours. After incubation the bacterial colony grown in the plates in between 10-300 were counted and recorded. The bacterial colonies were expressed as cfu/g.

3.8 Data collection and analysis

The obtained data during the experiment was recorded, accumulated, grouped and interpreted according to the objectives. Collected data were stored, explored and analyzed using Microsoft Excel 10.0 and SPSS 25.0. The means of the treatments were compared by One-way Anova ($P<0.05$) and pair-wise comparison was done by Tukey-HSD post-hoc analysis.

Chapter...........4

Results

4 Results

This experiment was conducted to evaluate the effect of probiotics and biofloc in grow-out phage of shrimp (*Penaeus monodon)* when the treatments were applied in nursery phage. Following the nursery treatment, the shrimps were cultivated for two months for the marketable size. This experiment was an approach of microbial community management from the beginning of the culture period. Through-out the culture period in hapa, growth parameters and water quality data was collected; however, incorporating those parameters was not the aim of this thesis. For understanding the contribution of probiotics and biofloc in microbial community management total bacterial loads and *Vibrio* spp. loads were investigated at the end of the grow-out culture of *P. monodon.*

4.1 Total bacterial count in *P. monodon*

Although not significant, the samples from the tested shrimp cultured in hapas showed lower total bacterial loads in feed probiotic (PRO-2) (T1) $((3.48\pm1.51)\times10^{14}$cfu/g), compared to other control group $((6.60\pm3.45)\times10^{14}$cfu/g) as well as other treatments, such as combination of in feed probiotic and water probiotic (PRO-2+PRO-W) (T3) $((4.12\pm2.03)\times10^{14}$cfu/g) and water probiotic (PRO-W) (T2) $((5.09\pm2.21)\times10^{14}$cfu/g) and also treatment with development of biofloc (C:N=1:12) (T4) $((6.55\pm3.71)\times10^{14}$cfu/g). In-feed probiotic and biofloc (T5) $((7.32\pm5.31)\times10^{14}$cfu/g) (Figure 4.1). Highest number of bacteria in the treatment group T5 indicated that combination of probiotic and biofloc treatment helped to develop a comparatively stable community compared to other groups.

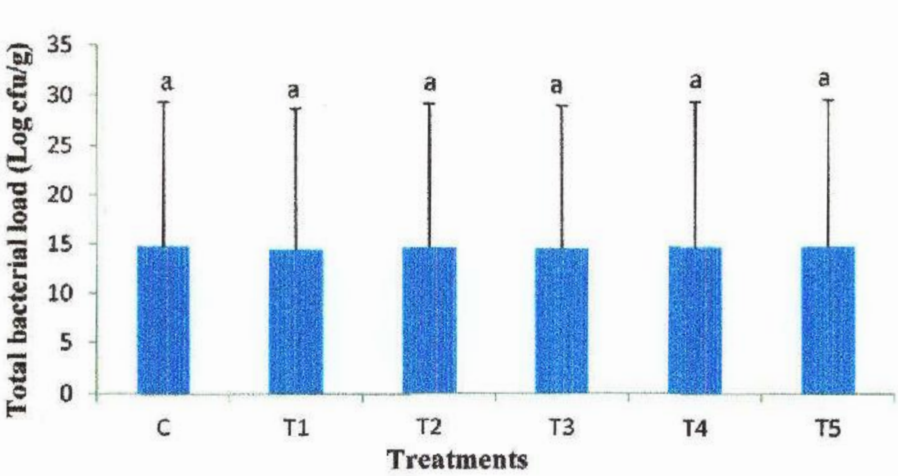

Figure 4.1 Total bacterial loads in shrimp after 61 days of grow-out culture using only feed, previously treated with feed probiotic (T1), water probiotic (T2) and combination of feed and water probiotics (T3), biofloc (T4) and combination of biofloc and feed probiotics (T5) in nursery phage. The error bars represent the standard error of five replicates. Same letters indicate no significant difference among the treatments (one-way anova, P<0.05).

4.2 *Vibrio* count in *P. monodon*.

The abundance of total *Vibrio* spp. was observed comparatively lower in the treatment with feed probiotic (PRO-2) (T1) ((2.37±2.23)×10^4cfu/g) compared to control group C ((5.69±3.52)×10^5cfu/g). Similarly, *Vibrio* spp. load was found to be lower in T1 than that of T3 ((11.65±8.31)×10^4cfu/g), T5 ((1.87±1.82)×10^5cfu/g), T4 (2.14±1.24)×10^5cfu/g), and T2 ((4.13±2.22)×10^5cfu/g) respectively (Figure 4.2). The results of this experiment indicated that the developed microbial community after application of in feed probiotics as well as biofloc was capable to control pathogenic *Vibrio* spp. load throughout the culture period of *P. monodon*.

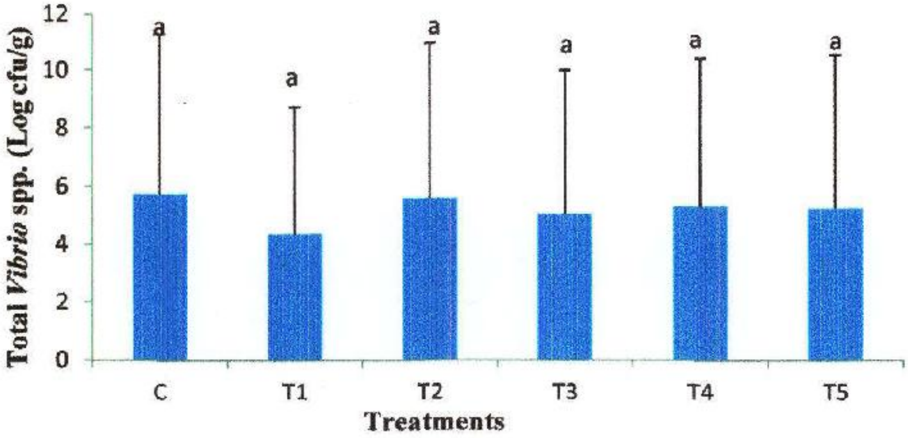

Figure 4.2 Total *Vibrio* loads in shrimp after 61 days of grow-out culture using only feed; previously treated with feed probiotic (T1), water probiotic (T2) and combination of feed and water probiotics (T3), biofloc (T4) and combination of biofloc and feed probiotics (T5) in nursery phage. The error bars represent the standard error of five replicates. Same letters indicate no significant difference among the treatments (one-way anova, P<0.05).

Chapter...........5

Discussion

5 Discussion

In shrimp aquaculture, farming systems are carefully managed to avoid rearing failure due to stress, disease, or mass mortality, and to achieve optimum shrimp production (Alfiansah *et al.*, 2018). So water quality control and microbial community balance are necessary for successful shrimp cultivations to prevent disease mostly caused by *Vibrio* spp. which are responsible for mass mortalities (Raman *et al.,* 2013). For microbial community management, use of beneficial bacteria (probiotics) and biofloc (aggregation of microorganisms) instead of antibiotics in aquaculture is an increasing demand for environment friendly aquaculture practices throughout the globe (Kumar *et al.*, 2013). The present experiment was undertaken to know the effect of probiotics and biofloc treatments in the early life stage of *P. monodon* on the bacterial loads of cultivable shrimp in grow-out ponds.

The results of this study reported that after 61 days of grow-out culture, we did not find any significance difference among the treatments, although total cultivable bacterial load was found to be bit lower in T1 where shrimp were treated with in feed probiotics compared to water probiotics (T2) and the combination of feed and water probiotics (T3). However, shrimp treated with biofloc, feed probiotics and biofloc and also the control group showed same type of bacterial community but that was higher than the T1, T2 and T3 (Figure 4.1). The total bacterial number in the biofloc group probably was higher than the probiotic group might be due to free-living flocs with a dense microbial biomass and they tend to congregate and create flocs, conglomerates of microbes having a diameter in the range of 0.1 to several mm (Browdy *et al.*, 2012). The total micro-organism counts in the carbon added groups were higher than the control throughout the duration of the experiment. These might be as a result of the carbon sources added in the system as compared in the control (Rono *et al.*, 2018).

Similar enumeration of total bacterial counts in all the treatments might be due to absence of probiotic application throughout the grow-out phase. That means that the probiotics and bacterial community enriched bioflocs used in the nursery phase might not stable enough to support the animals for further 61 days for influencing the microbial community development in the gut of the animals. Therefore, the treatments should be continued in the grow-out phase also, at least in a time interval. In previous study, Sivakumar *et al.,* (2012) stated that higher total bacterial count was recorded in shrimp fed with feed probiotics ($5.2\pm0.53\times10^8$cfu/g) compared to control tank. In an another observation which was conducted by Aftabuddin *et al.* (2013) declared that total heterotrophic bacteria was $5.4\pm1.70\times10^2$ to $2.38\pm0.78\times10^4$cfu/g was higher than control tank ($2.3\pm0.3\times10^3$cfu/g). The number of total bacteria in biofloc group was also significantly higher than that of control as proposed by Burford *et al.* (2003), but in present study, bacterial abundance in biofloc group is similar to the control group. The reason of variation between the present study and the previous study might be the application of different treatments during various time of culture period.

In another case, the abundance of total *Vibrio* spp. was observed comparatively higher in control/ non-treated hapas than any of the treated groups and the lowest *Vibrio* spp. density was found in shrimp treated with feed probiotics in this study (Figure 4.2). It means that feed probiotics might have controlling capability of pathogenic bacteria or these bacteria may improve some immune activity against pathogenic *Vibrio* spp. Rengpipat *et al.* (2003) stated that the growth and resistance to *Vibrio* in black tiger shrimp (*P. monodon*) were significantly greater by using feed with a *Bacillus* probiotics (BS11) than those of the controls. Generally, probiotics actively inhibit the colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients and space, as well as alteration of the microbial metabolism and stimulating host immunity (El-Haroun *et al.*, 2006).

Likewise, Sakai *et al.* (1995) demonstrated that oral administration of *Clostridium butyricum* bacteria to rainbow trout enhanced their resistance to vibriosis by increasing the phagocytic activity of leucocytes. Similarly, Balcazar, 2006 reported that the administration of a mixture of bacterial strains (*Bacillus* spp. and *Vibrio* spp.) positively influenced the growth and survival of white shrimp juveniles and gave a protective effect against pathogens *V. harveyi* and *White spot syndrome virus*. This protection was attributed to the stimulation of the immune system by increasing phagocytosis and antibacterial activity. Sivakumar *et al.* (2012) reported that total *Vibrio* spp. count was recorded in GI tract of control group ($50\pm0.21\times10^{10}$cfu/g) and due to introduction of probiotic feed total *Vibrio* count was decreased in GI tract of shrimp which is same as the result of present study. Results of this study showed that *Vibrio* spp. counts were reduced successively in the treated shrimp because of using feed probiotic (T1), combined application of feed probiotic and water probiotic (T3), combination of feed probiotic and biofloc (T5), Biofloc (T4) and water probiotic treatment (T2), compared to control (C) as suggested by other studies by Moriarty (1998), Prabhu *et al.* (1999), Dalmin *et al.* (2001), Aguilera-Rivera *et al.* (2014) and Das *et al.* (2006) where treated shrimp showed lower *Vibrio* colony compared to control group. This experiment demonstrates that *Vibrio* spp. are more sensitive to probiotics and biofloc and they affect *Vibrio* spp. community development even after a long period of application. The lower *Vibrio* growth also indicates the enhancement of shrimp immunity against *Vibrio* spp. duo to the application of probiotics and biofloc.

Chapter……….6

Conclusion

6 Conclusions and recommendations

From the results of this study the following conclusions can be drawn:

1. Application of in feed and water probiotics, and bioflocs in shrimp nursing could not be able to control the total bacterial counts during the grow-out culture of *P. monodon.*
2. Although not significant, but the probiotics and bioflocs application in shrimp nursing might have controlling capability of pathogenic microbial community or enhance the immunity of shrimp.

Recommendation

1. Microbial community structure should be determined through molecular approach using 16s rRNA gene analysis during the experimental time.

Chapter...........7

References

7 References

Aftabuddin, S., Kashem, M. A., Kader, M. A., Sikder, M. N. A., 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*, *6*(3), 253-267.

Aguilera-Rivera, D., Prieto-Davo, A., Escalante, K., Chavez, C., Cuzon, G., and Gaxiola, G. (2014). Probiotic effect of FLOC on Vibrios in the pacific white shrimp *Litopenaeus vannamei*. *Aquaculture*, *424*, 215-219.

Alfiansah, Y. R., Hassenruck, C., Kunzmann, A., Taslihan, A., Harder, J., and Gardes, A. (2018). Bacterial abundance and community composition in pond water from shrimp aquaculture systems with different stocking densities. *Frontiers in Microbiology*, *9*, 2457.

Avnimelech, Y. (1999). Carbon/nitrogen ratio as a control element in aquaculture systems. *Aquaculture*, *176*, 227-235.

Avnimelech, Y. (2007). Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. *Aquaculture*, *264*, 140-147.

Balcazar, J. L. (2003). Evaluation of probiotic bacterial strains in *Litopenaeus vannamei*. Final Report. *National Center for Marine and Aquaculture Research*, *6*(5), 337–341.

Balcazar, J. L., De Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., and Muzquiz, J. L. (2006). The role of probiotics in aquaculture. *Veterinary Microbiology*, *114*(4), 173-186.

Bentzon-Tilia, M., Sonnenschein, E. C., and Gram, L. (2016). Monitoring and managing microbes in aquaculture–Towards a sustainable industry. *Microbial Biotechnology*, *9*(5), 576-584.

Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R., and Shariff, M. (2005). Disease and health management in asian aquaculture. *Veterinary Parasitology*, *132*, 249-272.

Bondad-Reantaso, M. G., Subasinghe, R. P., Josupeit, H., Cai, J., and Zhou, X. (2012). The role of crustacean fisheries and aquaculture in global food security: past, present and future. *Journal of Invertebrate Pathology, 110*(2), 158-165.

Browdy, C. L., Ray, A. J., Leffler, J. W., and Avnimelech, Y. (2012). Biofloc-based aquaculture systems. *Aquaculture Production Systems, 5*(3), 153-154.

Burford, M. A., Thompson, P. J., Bauman, H., and Pearson, D. C. (2003). Microbial communities affect water quality, shrimp performance at Belize Aquaculture. *Global Aquaculture Advocate, 6*, 64-65.

Chen, Y., and Avnimelech, Y. (2012). The role of organic matter in modern agriculture. Retrived from https://www.springer.com/gp/book/9789024733606.

Crab, R., Defoirdt, T., Bossier, P., and Verstraete, W. (2012). Biofloc technology in aquaculture: beneficial effects and future challenges. *Aquaculture, 356*, 351-356.

Dalmin, G., Kathiresan, K., and Purushothaman, A. (2001). Effect of probiotics on bacterial population and health status of shrimp in culture pond ecosystem. *Indian Journal of Experimental Biology,* 39, 939-942.

Daniel, N., and Nageswari, P. (2017). Exogenous Probiotics on Biofloc based Aquaculture: A Review. *Current Agriculture Research Journal, 5*(1), 88-107.

Das, S., Lyla, P. S., and Ajmal Khan, S. (2006). Application of Streptomyces as a probiotic in the laboratory culture of *Penaeus monodon* (Fabricius). *The Israeli Journal of Aquaculture – Bamidgeh 58*(3), 198-204.

Das, S., Mondal, K., and Haque, S. (2017). A review on application of probiotic, prebiotic and synbiotic for sustainable development of aquaculture. *Journal of Entomology and Zoology Studies,* 5(2), 422-429.

Davies, J. E., Roberts, M. C., Levy, S. B., Miller, G. H., O'Brien, T. F., and Tenover, T. C. (1999). Antimicrobial resistance: an ecological perspective. A Report from the American Academy of Microbiology, Washington, DC, USA.

DoF (2017). Yearbook of Fisheries Statistics of Bangladesh 2016-17. Fisheries Resources Survey System (FRSS), Department of Fisheries. Bangladesh: Director General. *34*,129.

Ekasari, J., Azhar, M. H., Surawidjaja, E. H., Nuryati, S., De Schryver, P., and Bossier, P. (2014). Immune response and disease resistance of shrimp fed biofloc grown on different carbon sources. *Fish and Shellfish Immunology*, *41*(2), 332-339.

El-Haroun, E. R., Goda, A. S., and Chowdhury, K. M. A. (2006). Effect of dietary probiotic Biogen® supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *Oreochromis niloticus*. *Aquaculture Research*, *37*(14), 1473-1480.

Emerenciano, M. G. C., Martinez-Cordova, L. R., Martinez-Porchas, M., and Miranda-Baeza, A. (2017). Biofloc technology (BFT): a tool for water quality management in aquaculture. Retrieved from http://dx.doi.org/10.5772/66416.

Faruque, A. M. O., Ahmed, G. U., Hasan, M. R., and Hossain, M. A. (2008). Disease problems in shrimp sector in Khulna district of Bangladesh: a question of sustainability. *Journal of the Indian Fisheries Association*, *35*, 121-129

Farzanfar, A. (2006). The use of probiotics in shrimp aquaculture. *Federation of European Microbiological Societies Immunology & Medical Microbiology*, *48*(2), 149-158.

Ferreira, G. S., Bolivar, N. C., Pereira, S. A., Guertler, C., do Nascimento Vieira, F., Mourino, J. L. P., and Seiffert, W. Q. (2015). Microbial biofloc as source of probiotic bacteria for the culture of *Litopenaeus vannamei*. *Aquaculture*, *448*, 273-279.

Ghose, B. (2014). Fisheries and aquaculture in Bangladesh:challenges and opportunities. *Annals of Aquaculture and Research*, *1*(1), 1-5.

Hafsari, F. (2016). The effect of fermented and non fermented biofloc inoculated with bacterium *Bacillus* cereus for catfish (*Clarias gariepinus*) juveniles. *Aquaculture, Aquarium, Conservation and Legislation*, *9*(2), 334-339.

Hasan, B.M.A.; Guha, B. and Datta, S. (2012). Efficacy of probiotics on growth and sustainable production of black tiger shrimp, *Penaeus monodon. Asian Fisheries Science*, 25, 303-316.

Hernandez-Llamas, A., Cabanillas-Ramos, J., and Magallon-Barajas, F. J. (2016). Estimating impact of white spot disease on economic risk in semi-intensive shrimp farms in Mexico: the case of the State of Sinaloa. *Reviews in Aquaculture*, *8*(2), 111-120.

Holmstrom, K., Graslund, S., Wahlstrom, A., Poungshompoo, S., Bengtsson, B. E., and Kautsky, N. (2003). Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International Journal of Food Science and Technology*, *38*(3), 255-266.

Hoseinifar, S. H., Sun, Y., Wang, A., and Zhou, Z. (2018). Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives. *Frontiers in Microbiology*, *9*, 2429.

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), 363-370.

Inui, Y., and Cruz-Lacierda, E. R. (2002). Disease control in fish and shrimp aquaculture in Southeast Asia: diagnosis and husbandry techniques. Retrieved from https://repository.seafdec.org.ph/bitstream/handle/10862/1998/disease-control-in-fish-and-shrimp-aquaculture-in-SE-Asia.pdf?sequence=1&isAllowed=y.

James, N. (2018, June 28). Green water fish culture: the perfect alternative. Retrieved from https://www.farmersweekly.co.za/animals/aquaculture/green-water-fish-culture-perfect-alternative.

Jha, A. K. (2014). Probiotics: an effective feed supplement in Shrimp culture. *Journal of Bangladesh Academy of Sciences*, *38*(2), 127-130.

Karthik, R., Hussain, A. J., and Muthezhilan, R. (2014). Effectiveness of *Lactobacillus* sp (AMET1506) as probiotic against vibriosis in *Penaeus monodon* and *Litopenaeus* *vannamei* shrimp aquaculture. *Biosciences Biotechnology Research Asia, 11*, 297-305.

Kasan, N. A., Dagang, A. N., and Abdullah, M. I. (2018). Application of biofloc technology (BFT) in shrimp aquaculture industry. *IOP Conference Series: Earth and Environmental Science*, 196(1), 012043.

Kathia, C. M., Carmen, M. D. M., Aida, H. P., Jorge, C. M., and Daniel, B. C. (2017). Probiotics used in Biofloc system for fish and crustacean culture: A review. *Aquaculture, 23*, 28.

Kautsky, N., Ronnback, P., Tedengren, M., and Troell, M., (2000). Ecosystem perspectives on management of disease in shrimp pond farming. *Aquaculture, 191*(3), 145-161.

Krummenauer, D., Samocha, T., Poersch, L., Lara, G., and Wasielesky, W. (2014). The reuse of water on the culture of Pacific white shrimp, *Litopenaeus vannamei*, in BFT System. *Journal of the World Aquaculture Society, 45*(1), 3-14.

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), 6-11.

Lakshmanan, R., and Soundarapandian, P. (2008). Effect of commercial probiotics on large scale culture of black tiger shrimp *Penaeus monodon* (Fabricius). *Research Journal of Microbiology, 3*(3), 198-203.

Majerczyk, C., Schneider, E., and Greenberg, E. P. (2016). Quorum sensing control of Type VI secretion factors restricts the proliferation of quorum-sensing mutants. *eLife, 5*, 14712.

Manan, H., Moh, J. H. Z., Kasan, N. A., and Mhd, I. (2016). Biofloc application in closed hatchery culture system of Pacific white shrimp, *Penaeus vannamei* in sustaining the good water quality management. *Journal of Fisheries and Aquatic Science, 11*(4), 278-286.

Miranda, C. D., and Zemelman, R. (2001). Antibiotic resistant bacteria in fish from the Concepcion Bay, Chile. *Marine Pollution Bulletin, 42*(11), 1096-1102.

Moriarty, D. J. (1998). Control of *luminous Vibrio* species in penaeid aquaculture ponds. *Aquaculture, 164*, 351-358.

Moriarty, D. J. (1999). Disease control in shrimp aquaculture with probiotic bacteria. *Microbial Interactions in Aquaculture,* 237-243.

Mukherjee, A., Dutta, D., Banerjee, S., Ringo, E., Breines, E. M., Hareide, E., and Ghosh, K. (2016). Potential probiotics from Indian major carp, *Cirrhinus mrigala.* characterization, pathogen inhibitory activity, partial characterization of bacteriocin and production of exoenzymes. *Research in Veterinary Science, 108,* 76-84.

Nayak, S. K. (2010). Probiotics and immunity: a fish perspective. *Fish and Shellfish Immunology, 29*(1), 2-14.

Padmavathi, P., Sunitha, K., and Veeraiah, K. (2012). Efficacy of probiotics in improving water quality and bacterial flora in fish ponds. *African Journal of Microbiology Research, 6*(49), 7471-7478.

Pan, J., and Ren, D. (2009). Quorum sensing inhibitors: a patent overview. *Expert Opinion on Therapeutic Patents, 19*(11), 1581-1601.

Pandiyan, P., Balaraman, D., Thirunavukkarasu, R., George, E. G. J., Subaramaniyan, K., Manikkam, S., and Sadayappan, B. (2013). Probiotics in aquaculture. *Drug Invention Today, 5*(1), 55-59.

Pirarat, N., Kobayashi, T., Katagiri, T., Maita, M., and Endo, M. (2006). Protective effects and mechanisms of a probiotic bacterium Lactobacillus rhamnosus against experimental Edwardsiella tarda infection in tilapia (Oreochromis niloticus). *Veterinary Immunology and Immunopathology, 113*(3), 339-347.

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*, 227-236.

Prasad, L., Baghel, D. S., and Kumar, V. (2003). Role and prospects of probiotics use in aquaculture. *Aquaculture*, *4*(2), 247-251.

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), 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), 169-173.

Rono, K., Tarus, A., Manyala, J. O., Obado, E., Ngugi, C., Egna, H., and Fitzsimmons, K. (2018). Effects of biofloc technology on growth performance of nile tilapia (*Oreochromis niloticus*) fingerlings and microbial colonization in the system. *International Journal of Agriculture, Environment and Bioresearch,* 3(5), 2456-8643.

Sakai, M., Yoshida, T., Atsuta, S., and Kobayashi, M. (1995). Enhancement of resistance to vibriosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum), by oral administration of *Clostridium butyricum* bacterin. *Journal of Fish Diseases*, *18*(2), 187-190.

Sayes, C., Leyton, Y., and Riquelme, C. (2018). Probiotic bacteria as a healthy alternative for fish aquaculture. *Antibiotic Use in Animals. Intech Open, London*, 115-132.

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), 15811-15818.

Sorum, H., Aarestrup, F.M., and Washington, D.C. (2006). Antimicrobial drug resistance in fish pathogen. *Antimicrobial Resistance in Bacteria of Animal Origin*, 213–223.

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), 9-15.

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), 815-819.

Steer, T., Carpenter, H., Tuohy, K., and Gibson, G. R. (2000). Perspectives on the role of the human gut microbiota and its modulation by pro-and prebiotics. *Nutrition Research Reviews*, *13*(2), 229-254.

Venkatrayulu, C., Rani, V. K., and Reddy, D. C. (2009). Influence of commercial probiotics on bacterial (*Vibrio*) load and total yield in semi intensive *Penaeus monodon* culture ponds. *Asian Journal of Animal Science*, *4*(1), 117-122.

Verschuere, L., Rombaut, G., Sorgeloos, P., and Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews*, *64*(4), 655-671.

Wang, Y., He, J., Chen, H., Chen, J., Zhu, R., Ma, P., and Dong, Y. (2016). Ultrastable, highly luminescent organic–inorganic perovskite–polymer composite films. *Advanced Materials*, *28*(48), 10710-10717.

Yamashita, T., Emoto, T., Sasaki, N., and Hirata, K. I. (2016). Gut microbiota and coronary artery disease. *International Heart Journal*, 57(6), 663-671.

Zhao, P., Huang, J., Wang, X. H., Song, X. L., Yang, C. H., Zhang, X. G., and Wang, G. C. (2012). The application of bioflocs technology in high-intensive, zero exchange farming systems of *Marsupenaeus japonicus*. *Aquaculture*, *354*, 97-106.