Effect of biofloc technology on gut microbial proliferation of *Macrobrachium rosenbergii*

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Declaration

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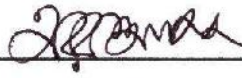
The results submitted in this project thesis are entirely of the candidate's own investigations and no part of the results has been accepted for any degree, nor is it being concurrently submitted for any degree.

Candidate



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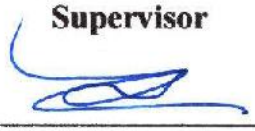

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DEDICATED TO

My Beloved Parents

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Abstract

A new approach of microbial community management like biofloc technology in the culture of aquatic organisms is very needed to increase the production along with less feed application and environmental friendly culture practice. The present study was conducted from August to December, 2017 at water quality laboratory, Fisheries and Marine Resource Technology discipline, Khulna University. The study was furnished to investigate the effect of biofloc technology and C/N ratios on the potential gut microflora of *Macrobrachium rosenbergii*. In this experiment different C/N ratios e.g. 10:1 (T_1), 15:1 (T_2) and 20:1 (T_3) along with a control group (C) without any additional carbon source were planned to apply in prawn culture. The results of this study demonstrated that although it was not significant ($P>0.05$) but we observed highest heterotrophic bacterial count in T_2 followed by T_1 and T_3 while lowest in control. However, *Enterococcus* spp. density was found in T_2 (9 ± 1.1) $\times 10^4$ MPN/g) and T_3 (6.7 ± 1.5) $\times 10^4$ MPN/g) compared to T_1 (8.8 ± 1.08) $\times 10^3$ MPN/g) and C (1.07 ± 0.4) $\times 10^3$ MPN/g) ($P<0.05$). Similarly, although it was not significant ($P>0.05$), but *Lactobacillus* spp. was found higher in the T_1 (9.9 ± 5.2) $\times 10^5$ CFU/g), T_2 (1.3 ± 0.5) $\times 10^6$ CFU/g) and T_3 (1.5 ± 0.7) $\times 10^6$ CFU/g) compared to C (6.3 ± 5.4) $\times 10^5$ CFU/g). In conclusion, different C/N ratios have different effects on potential bacterial composition in prawn gut. Therefore, further study should be conducted for total bacterial composition determination through 16s rRNA gene analysis.

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Chapter One

INTRODUCTION

Chapter- 1

INTRODUCTION

Bangladesh is covered with a large number of aquatic resources like as various rivers, ponds, beels, lakes and extensive flood plain areas etc. As a riverine country, Bangladesh possesses an immense potential for fisheries which provide 60% of total animal protein supply needed in the nationwide. One of the main cultured species is the freshwater prawn (*Macrobrachium rosenbergii*). Bangladesh is considered as one of the most suitable country in the world for freshwater prawn, *Macrobrachium rosenbergii* farming due to its climatic conditions. South-western region of Bangladesh, especially Khulna, Satkhira, Bagerhat are most suitable for the aquaculture practice of the freshwater prawn (Ahmed, 2008b).

There are twenty-four species of freshwater prawn are found in Bangladesh of which ten of them are *Macrobrachium*. Among all these freshwater prawn only *M. rosenbergii* has aquaculture potential and is commercially cultured (Akand & Hasan, 1992; Ahmed, 2001; Muir, 2003a). A sub-tropical climate and a vast area of water bodies provide a unique opportunity for the production of *Macrobrachium spp.*

Freshwater prawn (*M. rosenbergii*) farming is currently one of the most important sectors of the national economy and during the last two decades. Its development has attracted considerable attention because of its export potential. The prawn and shrimp sector as a whole is the second largest export industry after readymade garments, which generating US$ 526 million annually and 4.6% of the total value of export earnings (DoF, 2017). There are 1.0 million people employed in prawn and shrimp production and a further 4.5 million household members are associated with the sector (USAID, 2016). Unfortunately the export value and the number of people involved in prawn farming is not known because statistics often do not distinguish between prawn and shrimp. Despite the growth of this sector, a number of issues are important for freshwater prawn farming in Bangladesh including production technology, socioeconomic and environmental aspects all of these are important parameters of sustainability.

Biosecurity is a priority in aquaculture industry. For example, in shrimp farming, considerable impact of disease outbreaks during the past two decades greatly affected the operational management of shrimp farms worldwide (Wasielesky *et al.*, 2006). Infected PLs

and incoming water seem to be the main pathway for pathogen introduction. This scenario forced farmers to look for more biosecure culture practices to minimize the risk associated with exposure to pathogens (Browdy *et al.*, 2001).

Biofloc technology is mainly based on the principle of waste nutrients recycling, in particular nitrogen, into microbial biomass that can be used *in situ* by the cultured animals or be harvested and processed into feed ingredients (Avnimelech, 2009; Kuhn *et al.*, 2010). Heterotrophic microbiota is stimulated to grow by steering the C/N ratio in the water through the modification of the carbohydrate content in the feed or by the addition of an external carbon source in the water (Avnimelech, 1999), so that the bacteria can assimilate the waste ammonium for new biomass production. Hence, ammonium/ammonia can be maintained at a low and non-toxic concentration so that water replacement is no longer required. Biofloc technology brings an obvious advantage of minimizing consumption and release of water, recycling in situ nutrients and organic matter. Furthermore, pathogens introduction is reduced, improving the farm biosecurity.

Lactobacillus is a genus of Gram-positive, facultative anaerobic or microaerophilic, rod-shaped, non-spore-forming bacteria (Makarova *et al.*, 2006). They are a major part of the lactic acid bacteria group (i.e. they convert sugars to lactic acid). In humans, they constitute a significant component of the microbiota at a number of body sites, such as the digestive system, urinary system, and genital system. *Lactobacillus* forms biofilms in the vaginal and gut microbiota, allowing them to persist during harsh environmental conditions and maintain ample populations (Salas-Jara *et al.*, 2016). *Lactobacillus* exhibits a mutualistic relationship with the human body as it protects the host against potential invasions by pathogens, and in turn, the host provides a source of nutrients (Martin *et al.*, 2013). *Lactobacillus* is the most common probiotic found in food such as yogurt, and it is diverse in its application to maintain human well-being as it can help treat diarrhea, vaginal infections and skin disorders such as eczema (Inglin R).

Lactobacilli are members of the lactic acid bacteria whose primary fermentation end product is lactic acid. They are commercially important bacteria with a wide variety of application both in food and nonfood industries due to their "Generally Recognized as Safe" (GRAS) status. Lactobacilli have been extensively studied for their molecular biology in order to improve their specific beneficial characteristics (Pouwels and Leer, 1993).

Enterococcus faecalis, a gram positive bacterium was formerly known as *Streptococcus faecalis*. In addition to being gram positive it is also a commensal bacterium that lives in a mammal's gastrointestinal tract. *E. faecalis* is typically considered to be a beneficial gut bacterium that has been shown to be capable of modulating the activities of specific animal pathogens and the host immune response; consequently, several strains have been applied as probiotics for the maintenance of a healthy gut and the treatment of gut diseases, including diarrhea, irritable bowel syndrome, and ulcerative colitis (Hadji-Sfaxi *et al.*, 2011).

Enterococcus is a large genus of lactic acid bacteria (LAB) of the phylum Firmicutes. Enterococci are Gram-positive cocci that often occur in pairs (diplococci) or short chains, and are difficult to distinguish from streptococci on physical characteristics alone (Gilmore *et al.*, 2002). Enterococci are facultative anaerobic organisms, i.e., they are capable of cellular respiration in both oxygen-rich and oxygen-poor environments (Fischetti *et al.*, 2000). Members of the genus *Enterococcus* were classified as group D *Streptococcus* until 1984, when genomic DNA analysis indicated a separate genus classification would be appropriate (Schleifer and Kilpper-Balz, 1984).

Enterococci are part of the normal intestinal flora of fish and shrimp (Gomes *et al.*, 2010). *Enterococcus faecalis* and *Enterococcus faecium* are the most prevalent species cultured from humans, accounting for more than 90% of clinical isolates. Enterococci have both an intrinsic and acquired resistance to antibiotics, making them important nosocomial pathogens (Liaskovs'kyĭ, 2008). Some strains present probiotic properties due to health promoting effects. Some of them may inhibit growth of pathogenic and spoilage microorganisms, presenting a potential application in food preservation. In the past, enterococci were considered commensals but lately there has been increasing concern about the prevalence of virulence factors and antibiotic-resistance genes, which could compromise their application in foods.

Salmonella is a genus of rod-shaped (bacillus) gram-negative bacteria of the family Enterobacteriaceae. The two species of *Salmonella* are *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is the type species and is further divided into six subspecies (Su LH and Chiu CH, 2007) that include over 2,500 serotypes.

Salmonella species are non-spore-forming, predominantly motile enterobacteria with cell diameters between about 0.7 and 1.5 μm, lengths from 2 to 5 μm, and peritrichous flagella (all around the cell body) (Fabrega *et al.*, 2013). They are chemotrophs, obtaining their

energy from oxidation and reduction reactions using organic sources. They are also facultative anaerobes, capable of generating ATP with oxygen ("aerobically") when it is available; or when oxygen is not available, using other electron acceptors or fermentation ("anaerobically") (Fabrega *et al.*, 2013). *S. enterica* subspecies are found worldwide in all warm-blooded animals and in the environment. *S. bongori* is restricted to cold-blooded animals, particularly reptiles (Tortora, 2008).

Salmonella species are intracellular pathogens (Jantsch *et al.*, 2011) certain serotypes cause illness. *Nontyphoidal serotypes* can be transferred from animal-to-human and from human-to-human. They usually invade only the gastrointestinal tract and cause *Salmonella* food poisoning; symptoms resolve without antibiotics. However, in sub-Saharan Africa they can be invasive and cause paratyphoid fever, which requires immediate treatment with antibiotics. *Typhoidal serotypes* can only be transferred from human-to-human, and can cause *Salmonella* food poisoning, typhoid fever and paratyphoid fever (Ryan *et al.*, 2004). Typhoid fever occurs when *Salmonella* invades the bloodstream—the *typhoidal form*; or in addition spreads throughout the body, invades organs, and secretes endotoxins—the *septic form*. This can lead to life-threatening hypovolemic shock and septic shock and requires intensive care including antibiotics.

Microorganisms play a key role in BFT systems. The maintenance of water quality, mainly by the control of bacterial community over autotrophic microorganisms, is achieved using a high carbon-to-nitrogen (C:N) ratio, since nitrogenous by-products can be easily taken up by heterotrophic bacteria. High carbon-to-nitrogen ratio is required to guarantee optimum heterotrophic bacteria growth, using this energy for maintenance (respiration, feeding, movement, digestion, etc.) but also for growth and to produce new bacterial cells.

The environmental friendly aquaculture system called "Biofloc Technology (BFT)" is considered as an efficient alternative system since nutrients could be continuously recycled and reused. The sustainable approach of such system is based on growth of microorganism in the culture medium, benefited by the minimum or zero water exchanges (Ray A 2012). These microorganisms (biofloc) has two major roles: (i) maintenance of water quality, by the uptake of nitrogen compounds generating "*in situ*" microbial protein; and (ii) nutrition, increasing culture feasibility by reducing feed conversion ratio and a decrease of feed costs. Increasing concerns in recent years about the environmental impact of marine shrimp farms,

associated with the incidence of diseases, has led to the development of production systems with little or no water exchange (Hopkins *et al.*, 1995).

Objectives

Considering the above facts, the present study will be undertaken with following objectives:

1. To investigate the effect of C/N ratios on the potential gut microflora of *Macrobrachium rosenbergii* under biofloc developing cuture system.
2. To determine the microbial content on the gut of *M. rosenbergii*.

Chapter Two

REVIEW OF LITERATURE

Chapter- 2

REVIEW OF LITERATURE

Approximately 16.7% of animal protein consumed by the world's population is originated from fish, and over one billion people worldwide depend on fish as their main source of animal protein (FAO, 2006). Aquaculture offers one way to supplement the production of wild capture fisheries and it will continue to increase in importance as demand increase in future (White *et al.*, 2004). The ever growing demand for seafood leads to the intensification of aquaculture through high stocking density and intensification of the artificial feeds, leading to the aquaculture sector as most cost-effective as well as waste promoting industry. Like other form of intensive food production, industrial-scale fish farming generates significant environmental costs (White *et al.*, 2004). Aquaculture development should be in a sustainable manner. Sustainable development is defined as the management and conservation of the natural resource-base and the orientation of technological and institutional change in such a manner as to ensure the attainment and continued satisfaction of human needs for present and future generations. Such sustainable development conserve land, water, plant and animal genetic resources, is environmentally non-degrading, technically appropriate, economically viable and socially acceptable (FAO, 1991).

Aquaculture production has increased at an average annual rate of 8.9% since 1970, as compared with an annual growth rate of 1.2% and 2.8% for capture fisheries and terrestrial farmed meat production over the same frame. Yet, to supply demands, aquaculture production must grow by five fold in the next five decades. This development has to overcome three major constraints: a) Produce more fish without significantly increasing the use of basic natural resource of water and land, b) Develop sustainable systems that will not damage the environment, c) Develop systems providing a reasonable cost/benefit ratio, to support the economic and social sustainability of aquaculture (Avnimelech, 2009).

The nutrient rich environment of aquaculture tanks and ponds favors the proliferation of microbes. Exposed body surfaces of fish, including the gut, are quickly colonized right after hatching mostly due to the influence of water communities (Reitan *et al.*, 1998; Verschuere *et al.*, 2000). Feed microbiota becomes important later mostly from the time that active feeding occurs (Hansen and Olafsen, 1999; Ringo and Birkbeck, 1999). This

dynamic community is subjected to changes related to the developmental stage of the host and constantly adapts to shifts in the nutritional and environmental situation (Nayak, 2010b; Romero and Navarrete, 2006; Navarrete *et al.*, 2012; Li *et al.*, 2012b). Fish gut microbiota vary within species due to factors such as season, salinity, pH, temperature, trophic level and feed (Nayak, 2010b; Sullam *et al.*, 2012).

Although water and feed are the main sources of microbes, fish and their gut bacteria seem to have more complex relationships than one would expect from a simple reflection of the free-living microbial community in the environment (Sullam *et al.*, 2012). For the ingested microbes, the gut provides different (micro) habitat niches leading to the establishment of a very complex microbial community upon which the host has a great influence (Sommer and Bäckhed, 2013; Hansen and Olafsen, 1999).

The majority of the ingested microbes are only transient in the gut (Sugita *et al.*, 1996). For ingested bacteria to proliferate and persist as "resident" microbiota, they must be capable to adapt to the environmental conditions inside the gut, such as nutrient availability, pH and digestive enzymes (Hansen and Olafsen, 1999). Host dependent selective pressures impacting gut microbiota in fish, are mostly attributed to gut habitat (ecology) and host genotype (Yan *et al.*, 2012; Rawls *et al.*, 2006; Navarrete *et al.*, 2012).

Fish microbial communities are strongly influenced by, but also influence, their hosts. (Sullam *et al.*, 2012). The beneficial role of the intestinal microbiota in fish is recently being explored, suggesting that microbiota stimulates nutrient metabolism, innate immune response and epithelial proliferation (Roeselers *et al.*, 2011). Additionally, gut microbiota constrains the colonization of infection agents and, by interacting with the host, mediate the development, maintenance and effective functionality of the intestinal mucosa (Dimitroglou *et al.*, 2011).

Alagarswami (1995) identified adverse impacts of aquaculture on social and physical environments and emphasized the need to adopt eco-friendly technologies. Intensive aquaculture systems are used to efficiently produce dense biomasses of fish or shrimp, intensive aquaculture industry faces two major problems. The first is the water quality deterioration caused by the high concentrations of metabolites and the second is the low feed utilization in cases when high water exchange, within or outside the pond system, is practiced

(Avnimelech, 2007). Artificial formulated feed is the main investment in aquaculture i.e.; feed is the largest single cost item, as it constitutes 40-60% of operational cost in prawn production (Mitra *et al.*, 2005).

The major portion (>80%) of artificial feed is lost in the aquaculture system as uneaten feed and faeces (Daniels and Boyd, 1989; Siddiqui and Al-Harbi, 1999; Rahman, 2006). Artificial feed, which is lost in the system, has a great effect on water quality through decomposition (Horner *et al.*, 1987; Poxton and Allouse, 1987; Cowey and Walton, 1989; Poxton and Lloyd, 1989; Wilson, 1994; Moreira *et al.*, 2008). Higher dietary protein deteriorates the water and soil qualities in shrimp grow-out ponds (Boyd, 1989). To great extent water quality determines the success or failure of aquaculture operation. Physical and chemical characteristics such as suspended solids, temperature, dissolved gases, pH, mineral content and the potential danger of toxic metals must be considered in the selection of a suitable water source (Boyd and Tucker, 2009).

The salmon and shrimp aquaculture have proven to be destructive to the natural environment and population of aquatic animals (Gowen and Bradbury, 1987; Folke *et al.*, 1994; Kautsky *et al.*, 1997; Naylor *et al.*, 2000; Milewski, 2001). Crustacean aquaculture is fraught with environmental problems that arise from: (i) the consumption of resources such as land, water, seed and feed; (ii) their transformation into products valued by society; and (iii) the subsequent release into the environment of wastes (Kautsky *et al.*, 2000; Ronnback, 2001). The direct impacts include release of eutrophicating substances and toxic chemicals, the transfer of diseases and parasites to wild stock, and the introduction of exotic and genetic material into the environment. The environmental impact can also be indirect through the loss of habitat and niche space, and changes in food webs. Whereas traditional and extensive shrimp aquaculture uses natural production in the ponds or in the incoming waters, semi-intensive and intensive production systems are heavily dependent on formulated feeds based on fishmeal and fish oils. These latter systems use more than two times more protein, in the form of fishmeal, to feed the farmed shrimps than is ultimately harvested (Tacon, 1996). Most aquaculture systems are so-called throughput systems (Daly and Cobb, 1989). This means that resources, collected over large areas, are introduced and used in the aquaculture production site, and released back into the environment in concentrated forms as nutrients and pollutants, causing various environmental problems (Folke and Kautsky, 1992). Uneaten food, faecal and urinary wastes may lead to eutrophication and oxygen depletion, the

magnitude of which is dependent on the type and size of operation as well as the nature of the site, especially size, topography, and water retention time (Kautsky *et al.*, 2000). In semi-intensive and intensive farms, artificial feeds provide most of the nitrogen (N), phosphorus (P) and organic matter inputs to the pond system. Only 17% (by dry weight) of the total amount of feeds applied to the pond is converted into shrimp biomass (Primavera, 1993). The rest is leached or otherwise not consumed, egested as faeces, eliminated as metabolites, etc. Effluent water during regular flushing and at harvest can account for 45% of nitrogen and 22% of organic matter output in intensive ponds (Briggs and Funge-Smith, 1994). Consequently, pond sediment is the major sink of N, P and organic matter, and accumulates in intensive shrimp ponds at the rate of almost 200 t (dry weight) per ha and production cycle (Briggs and Funge-Smith, 1994). During pond preparation between cropping the top sediment is removed and usually placed on pond dikes, from where it continuously leaks nutrients to the environment. Several methods have been proposed to ameliorate the impact of shrimp pond effluents on the water quality of the recipient: improved pond design (Dierberg and Kiattisimkul, 1996); construction of waste-water oxidation-sedimentation ponds, reduction of water exchange rates (Hopkins *et al.*, 1995); reduction of nitrogen and phosphorus input from feed (Jory, 1995); removal of pond sludge; a combination of semi-closed farming systems with settling ponds and biological treatment ponds using polycultures (Dierberg and Kiattisimkul, 1996; Troell *et al.*, 1999) and the use of mangroves as biofilters for pond discharge prior to the release of effluent to estuarine waters (Robertson and Phillips, 1995). Furthermore, the use of fertilisers should be restricted to organic products..

Knoesche and Tscheu (1974) already adopted the idea of intensive heterotrophic bacteria growth in aquaculture systems and could retain 7% feed N and 6% feed P (estimated from 1% P feed, *KarpiCo Supreme-7Ex, Coppens International*, The Netherlands). They used an activated sludge process to treat water in a recirculation system, and proposed to mix produced sludge with grains for later re-use as fish feed for carps. The principles of growing fish or shrimp in limited water exchange intensive ponds were developed simultaneously for shrimp in the Waddel Mariculture Centre in the USA and for fish, mostly tilapia, in Israel (Avnimelech *et al.*, 1989, 1994; Hopkins *et al.*, 1993; Chemberlain and Hopkins, 1994) and practiced in the USA (Serfling, 2000), in the beginning of the 1990's. The idea of addition of carbohydrate for the immobilization of ammonia excreted by the fishes was suggested by Avnimelech and Lacher (1979), Boyd (1985), and Muthuwani and Lin (1996). But idea about

the general water quality of the pond is essential before any modification or manipulation in aquaculture systems (Boyd and Tucker, 2009).

Good water quality is the key factor for the success of aquaculture and that ensures the survival, production and growth rate of the cultured animals (Boyd, 1990; Burford, 1997). Biofloc technology, BFT, (called also active suspension ponds, heterotrophic ponds, green soup and other terms) was first developed to solve water quality problems. Water quality management is based upon developing and controlling dense heterotrophic bacteria within the culture component (Avnimelech, 2007). The addition of fertilizers or carbon sources directly to the pond water is a way to augment the natural productivity (Crab *et al.*, 2007; Uddin *et al.*, 2007). The removal of toxic nitrogenous compounds, especially ammonium, from water through its assimilation into microbial protein by the proper addition of carbonaceous materials to the culture system is the basic principle of biofloc technology. The success of this technology mainly depend on the selection of species, because the cultured animal should have the ability to harvest the bacterial floccules developed in the system, and should have the ability to digest and utilize the microbial protein. The bacterial floc produced as the result of biofloculation serve as an important source of feed protein. Experimental trials showed that microbial flocs of different sizes can be taken up by fish or shrimp and serve as a feed source (Avnimelech *et al.*, 1989; Beveridge *et al.*, 1989; Rahmathulla and Beveridge, 1993; Tacon *et al.*, 2002; Burford *et al.*, 2004) which will help to reduce the cost of production by reducing the protein content of the artificial feed and improving the overall economics (Mc Intosh, 1999; Moss, 2002).

Avnimelech (2007) schematically represented and explained the process behind biofloc production and harvest by the fishes as

$$D[BF] / dt = BF_{production} - (BF_{harvesting} + BF_{degradation})$$

Where $D[BF] / dt$ is the bio-floc concentration change with time, as affected by production, harvesting by fish and biodegradation. The process shown in this equation depends on a verity of factors:

1) Production of biofloc depends on the supply of organic substrates to the microbial community, both external sources (feed supply, algal activity) or by the excretion of un-utilized feed components by fish. In addition bioflocs production most probably

depends on the quality of the added substrates, its C/N ratio, bio-availability and other factors.

2) Uptake of the bio-flocs by fish depends most probably on the fish species and feeding traits, fish size, floc size and floc density. It is possible that bioflocs harvesting depends also on the presence and rate of formulated feed added to the pond. In addition, feed eaten by the fish may be utilized and accumulate in the fish, or excreted and serve as a substrate for the production of more bioflocs.
3) Biodegradation of the floc depends on the microbial community associated with the bioflocs, be it bacteria, protozoans or others. 4) Finally, all of these processes may be affected by environmental and operational conditions such as temperature, water salinity, water exchange rate (affecting floc mean residence time), mixing intensity and many others.

Biofloc aquaculture is a sustainable solution for the development of aquaculture and this technology is fully based on the concept of carbon nitrogen (C/N) ratio. The control of inorganic nitrogen accumulation in the pond is based upon carbon metabolism and nitrogen immobilization into microbial cells (Avnimelech *et al.*, 1989; Avnimelech, 1999; Crab *et al.*, 2007). Bacterial cells are composed of proteins. carbon nitrogen ratio of most microbial cell is about 4-5 (Rittmann and Mc Carty, 2001). When bacteria fed with organic substrates that contains mostly carbon and little or no nitrogen (sugar, starch, molasses, cassava meal, etc.), they have to take up nitrogen from the water in order to produce the protein needed for the cell growth and multiplication. C/N ratio in an aquaculture system can be increased by the addition of cheap carbohydrate source or reduction of protein content in the feed (Avnimelech, 1999; Hargreaves, 2006).

Avnimelech and Mokady (1988) and Avnimelech *et al.*, (1992) suggested managing the heterotrophic food web by the development of active suspension ponds, intensive ponds with zero or limited water exchange, accumulating high amounts of organic substrates.

In summary, inorganic nitrogen accumulation is controlled through the addition of carbonaceous substrates, raising the C/N ratio and leading to the immobilization of nitrogen through the production of microbial proteins (Avnimelech *et al.*, 1989, 1992, 1994; Crab *et al.*, 2007).

Fish harvest the bacteria, as bacterial flocs and utilize this protein source (Avnimelech *et al.*, 1989; Milstein *et al.*, 2001; Mc Intosh, 2000a).The amount of carbohydrate needed for reducing the ammonium was worked by Avnimelech (1999). The equation proved its success both in indoor and farm level experiments by various researchers (Hari *et al.*, 2004, 2006; Varghese, 2007; Saritha, 2009).

Kurup (2009) summarised the advantages of the application of BFT to shrimp culture systems in India as:

- It is the best means for the control of toxic inorganic nitrogen in water and for accumulating production of microbial protein by adjusting C/N ratio.
- It can convert uneaten nitrogen for being utilized to produce microbial protein rather than generating toxic component.
- Microbial protein, the end product which is suspended in the system as microbial flocs can be utilized as feed by shrimps.
- The level of protein utilization is doubled in microbial reuse system.
- The dense heterotrophic microbial biomass decreases the outbreak of microbial diseases and finally.
- The technology enables high yield in environmentally and economically sustainable system.

Minimal-exchange, intensive aquaculture systems offer an environmentally attractive means of shrimp and fish production, allowing for high density culture and little or no water exchange (Ray *et al.*, 2010b). The basic principle of the activated suspension technique and the C/N ratio controlled systems were recently referred as biofloc technology (BFT) is the retention of waste and its conversion to biofloc as a natural food within the culture system (Azim and Little, 2008). One of the features of natural aquatic environment is the ability to recycle the nutrients. Microbial floc generally consists of floc formers, filamentous bacterial particles, protozoans, zooplankton, colloids, organic polymers, cations and dead cells surrounded by a gelatinous matrix, which contains extra-cellular polymeric substances that encapsulate the microbial cells and play a major role for binding the floc components together (Jorand *et al.*, 1995; Avnimelech, 2007). The grouping of bacteria within the floc mainly depends upon the zeta potential and Van der Waals forces (Sobeck and Higgins, 2002). Typical bacterial flocs are irregular by shape have a broad distribution of particle size, are fine compressible, highly porous and permeable to fluids (Chu and Lee, 2004).

Accumulation of toxic inorganic nitrogen species (NH4, NO2) is prevented in bio-flocs system by maintaining a high C/N ratio and inducing the uptake of ammonium by the microbial community (Avnimelech *et al.*, 1994; Mc Intosh, 2000b). Nitrogen removal from aquaculture pond water by heterotrophic nitrogen assimilation in labscale sequencing batch reactor was studied by Schryver and Verstraete (2009).

Sesuk *et al.* (2009) demonstrated Inorganic nitrogen control in a novel zero-water exchanged aquaculture system integrated with airlift-submerged fibrous nitrifying biofilters. The microorganisms not only remove excess nutrients, but have been implicated in nutritional provision for animals, including shrimp and tilapia, that can result in improved growth rate, feed conversion ratio (FCR) and weight gain (Moss and Pruder, 1995; Burford *et al.*, 2004; Wasielsky *et al.*, 2006 Azim and Little, 2008). This process was quantitatively formulated (Avnimelech, 1999), verified and practiced by farmers world-wide (Browdy *et al.*, 2001; Panjaitan, 2004).

There are several factors that influencing floc formation and floc structure (Schryver *et al.*, 2008). Ritvo *et al.* (2003) studied the salinity and pH effect on the colloidal properties of suspended particles in super intensive aquaculture systems. The mixing intensity imposed by a chosen aeration device at a certain power input will determine the steady-state floc size, this is in equilibrium between the rate of aggregation and the rate of breakage, and floc size distribution (Spicer and Pratsinis, 1996; Chaignon *et al.*, 2002). Dissolved oxygen is another factor that influences the bioflocs. Previous studies revealed that biofloc with a higher floc volume index (FVI) are produced at lower dissolved oxygen levels. The organic carbon source of choice will to a large degree determine the composition of floc produced (Mikkelsen *et al.*,1996; Hollender *et al.*, 2002; Oehmen *et al.*, 2004). Organic loading rate, temperature and pH are the other factors that affect the biofloc formation (Schryver *et al.*, 2008). pH level has a significant role in the floc formation both qualitatively and quantitatively in the culture of *P. monodon*, pH of 7.5 is found to be optimum for better biofloculation in shrimp aquaculture (Devi, 2009; Kurup and Devi, 2010).

Schneider *et al.* (2006) used molasses as carbon source for heterotrophic bacteria production on solid fish waste. Crab *et al.* (2010a) monitored effect of different carbon sources on the nutritional value of biofloc, in the culture system of *M. rosenbergii* post-larvae. In that experiment flocs were grown on acetate, glycerol and glucose as sources. Varghese (2007) evaluated performance of various locally available carbohydrate sources for biofloculation

the culture system of *P. Monodon*; tapioca powder was the biofloculating agent selected by Hari *et al.* (2004, 2006), Varghese (2007), and Kurup and Varghese (2007a, 2007b).

Initial studies were conducted to optimize the addition of carbohydrate source in aquaculture systems for the process of biofloculation and standardize the quantity of carbohydrate source required for biofloculation. Mechanism and principle behind this technology was also interesting field of research (Avnimelech, 1999, 2009). Eminent Israeli scientist Yoram Avnimelech is the pioneer in this field. Species selection is one of the major factors that decide the success of the BFT aquaculture (Avnimelech, 2007; Azim and Little, 2008). BFT is proved to be beneficial for both shrimp and finfish culture (Milstein *et al.*, 2001; Burford *et al.*, 2003; 2004; Serfling, 2006; Wasielsky *et al.*, 2006). Tilapia was the first studied species for the biofloc ponds, the nibbling habit and feeding behaviour were the reasons for the selection of this fish. Later, marine shrimps were also identified as the suitable species for BFT aquaculture (Burford *et al.*, 2003, 2004; Wasielsky *et al.*, 2006; Hari *et al.*, 2004, 2006; Varghese, 2007; Kurup and Varghese, 2007a, 2007b; Arnold *et al.*, 2009; Ballester *et al.*, 2009; Kuhn *et al.*, 2009).

Studies by Avnimelech (2007) revealed that microbial flocs developing in BFT ponds are effective potential food source for tilapia; according to his observation, fish growing in the BFT pond did not rush on to the added feed pellets, since the pond contained flocs as a potential feed, feed that is available 24 hours per day. Avnimelech *et al.*, (1994) estimated that feed utilization is higher in BFT systems, while tilapia in such ponds is fed a ration 20% less than conventional one. Varghese (2007) studied various aspects of BFT in the culture of *P. monodon.* Optimization of protein in the feed by the application of C/N ratio, effect of various modes of carbohydrate application, on farm application of BFT, optimization of stocking density in BFT tanks, performance of carbohydrate sources, and combined application of BFT with periphyton based aquaculture were the focal theme of the study. The author summarises the study as, C/N ratio optimization by the addition of suitable carbohydrate sources is a potential method for controlling the inorganic nitrogen species in aquaculture systems.

Kurup and Varghese (2007a), and Varghese (2007) explained the suitability of various carbohydrate sources for controlling C/N ratio in shrimp culture system. They also studied the combined effect of BFT and periphyton based aquaculture. Biofloc can act as a feed for different cultured species such as Nile tilapia (*Oreochromis niloticus*) and whiteleg shrimp, *L.*

vannamei (Azim and Little, 2008; Crab *et al.*, 2009; Kuhn *et al.*, 2009). Burford *et al.* (2004) suggested that "flocculated particles" rich in bacteria and phytoplankton could contribute substantially to the nutrition of *L. vannamei* in intensive shrimp ponds. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for *L. vannamei* was studied by Wasielsky *et al.* (2006).

Several researchers followed the uptake of microbial protein through the use of 15N enriched microbial biomass (Preston *et al.*, 1996; Epp *et al.*, 2002; Burford *et al.*, 2004; Avnimelech, 2007). Avnimelech and Kochba (2009) conducted a detailed study on the uptake and utilization of microbial protein by tilapia, using N15 tagging to get data on the dynamics of the biofloc system and to critically evaluate the available experimental procedures. Authors also estimated the residence time of bioflocs. It was estimated that the residence time of bioflocs was about 8 hours, i.e. bioflocs were regenerated three times a day.

Ray *et al.* (2010b) characterized microbial communities in minimalexchange, intensive aquaculture systems and studied the effects of suspended solids management. Characterisation of microbial community structure in shrimp biofloc cultures using biomarkers and analysis of floc amino acid profile was done by Ju *et al.* (2008). Ray *et al.* (2010 a) also studied the mechanism behind the suspended solids removal to improve production of *L. vannamei* and part of the same study the team has evaluated a plant based feed in minimal-exchange, super intensive culture systems. According to their study, shrimp biomass production (kg/m−3) was increased 41% when biofloc concentration was managed through the use of external settling chambers. They also showed a 60% reduction in nitrate-nitrogen concentration and a 61% reduction in phosphate concentration when biofloc concentration was managed.

Michaud *et al.* (2006) studied the effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. Addison *et al.* (2010) studied the effect of biofloc replaced fish meal and soybean meal in semi-purified shrimp diets. Ju *et al.* (2008) studied the enhanced growth effect on shrimp (*L. vannamei*) from inclusion of whole shrimp floc or floc fractions to a formulated diet. The results suggest that inclusion of the floc material in shrimp diets could enhance the shrimp growth. They observed enhanced growth when compared with that of the control ($P<0.05$); the diet preference and pellet stability study were also positive. Shrimp preferred the experimental diets over commercial diet and the feed stability was same as that of commercial diet.

Addison *et al.* (2010) reported increased growth of *L. Vennamei* by replacing fishmeal and soybean with biofloc. Microbial flocs produced in suspended growth bioreactors could offer the shrimp industry a novel alternative feed. Study by Kuhn *et al.* (2009) showed that bioflocs harvested from the sequencing batch reactors (SBRs) and membrane biological reactors proved to be a suitable and often superior ingredient, to soybean protein and fishmeal in lab-scale feeding trials with *L. vannamei*. High quality control diets were compared against experimental diets in 35- day feeding trials. Result was that experimental diets were varied greatly and notable independent variables included complete replacement of soybean protein, two-thirds replacement of fishmeal, and no fish oil. Biofloc inclusion always increased growth rates and ranged from a low average increase of 4% to a high average of 67% over the control diets; the latter percent increase was significant at $P < 0.01$. It seems that biofloc technology represents a promising option for sustainability of the aquaculture industry. Ballester *et al.* (2010) compared the performance of *Farfantepenaeus paulensis* reared with partial diet with different protein levels under zero exchange microbial floc intensive system. Forty five days experimental trial concluded that shrimps fed with a protein percentage of 35 which are maintained in biofloc ponds showed maximum growth parameters. It was significantly greater when compared to the feed with 25, 30, 40 and 45% of crude protein. Kuhn *et al.* (2010) compared the possibilities of incorporating two types of microbial flocs in the feed of *L. vannamei* derived from biological treatment of fish effluent. Logan (2010) presented the possibilities of commercializing the biofloc feeds in recent World Aquaculture Society conference held at California, the product is expected to be available in the market by 2012 as the trade name *Obron SCP ingredient*.

In addition to being a tool for water quality and feed management, biofloc technology also has potential to protect the cultured organisms from infections with pathogenic bacteria, which are responsible for major economic losses in aquaculture (Crab *et al.*, 2010b). Biofloc can be a novel strategy for disease management on a long-term basis, in contrast to conventional approaches such as antibiotic, probiotic and prebiotic application (Sinha *et al.*, 2008). Recent research questions focus on the possible extra added value of bioflocs, more specifically regarding pathogen control, because infectious diseases burden the aquaculture industry. It was observed that the regular addition of carbon to the culture is known to select for polyhydroxyalkanoate (PHA) accumulating bacteria (Salehizadeh and Von Loosdrecht, 2004) such as *Alcaligenes eutrophus, Azotobacter vinelandii, Pseudomonas oleovorans* and others that synthesise PHA granules. Such granules are synthesised under conditions of

nutrient stress, that is, when an essential nutrient like nitrogen is limited in the presence of an excess carbon source (Avnimelech, 1999). These PHAs are polymers of β-hydroxy short chain fatty acids and if degraded in the gut, they could have antibacterial activity similar to short chain fatty acids (SCFAs) or organic acids. The breakdown of PHA inside the gastrointestinal tract can be carried out via enzymatic and chemical hydrolysis (Yu *et al.*, 2005). Apart from inhibiting the growth of pathogenic bacteria by lowering the pH of surrounding milieu, SCFA have also been shown to specifically down-regulate virulence factor expression and positively influence the gut health of animals (Teitelbaum and Walker, 2002). Moreover, these compounds are capable of exhibiting bacteriostatic and/or bacteriocidal properties, depending on the physiological status of the host and the physiochemical characteristics of the external environment (Ricke, 2003). Defoirdt *et al.* (2006) reported increased survival of artemia nauplii when fed formic, acetic, propionic, butyric and valeric acid and challenged with a luminescence pathogenic *Vibrio campbellii* strain. In another study, the same authors (Defoirdt *et al.*, 2007) reported that commercial polyhydroxy butyrate (PHB) particles or PHB accumulating bacteria offered a preventive and curative protection to artemia against luminescent vibriosis. Crab *et al.* (2010b) studied the use of bioflocs as new bio-control agents for aquaculture using a model system with gnotobiotic brine shrimp (*Artemia franciscana*). They found that the bioflocs can also be used by brine shrimp (*A. franciscana*) nauplii as a feed. The technique has also proved to be beneficial in the nursery rearing of *P. monodon*, wherein the use of carbon source and artificial substrates was found to positively influence growth and production of shrimp juveniles in addition to providing more favourable water quality conditions irrespective of elevated stocking densities (Arnold *et al.*, 2009; Anjalee and Kurup, 2011).

Eventhough more than 25 species of freshwater prawns have been reported from Kerala, *M. rosenbergii* is the only species with aquaculture importance. There is a vast potential for the development of prawn culture and nearly 65,000 ha comprising ponds, tanks and check dams are found suitable for freshwater prawn farming in monoculture or polyculture with carps (Kumar and Velayudhan, 2006). Prawn farming has been considered as an important aquaculture activity in the state due to its domestic as well as export market and the possibility of integration or rotation with paddy cultivation which in turn provides an additional income. The increase in the aquaculture production from the state of Kerala mentioned earlier may be due to the utilisation of many of these potential areas for culture purposes as denoted by the increase in the area brought under the culture. Raising of

freshwater prawn production through expansion of pond area would demand large additional quality of water and land area, both are very scarce resources. The most practical way to raise freshwater prawn production is by increasing pond productivity per unit land area and water (Asaduzzaman *et al.*, 2008). *M. rosenbergii* is omnivore in the feeding behaviour and is more or less similar to the feeding of the tiger shrimp, *Peanaeus monodon*. The benefits of BFT has been described extensively for shrimp culture (Buford *et al.*, 2003, 2004; Hari *et al.*, 2004, 2006; Wasielsky *et al.*, 2006) and for finfish culture (Avnimelech, 1999; Avnimelech, 2007; Milstein *et al.*, 2001; Serfling 2006). There are only few works on its applicability and advantages of the technology to *M. rosenbergii* grow outs (Asaduzzaman *et al.*, 2008, 2009a, 2009b 2010a, 2010b) and in larviculture (Saritha, 2009; Saritha and Kurup, 2011).

Asaduzzaman *et al.* (2008, 2009a, 2009b, 2010a, 2010b) conducted a series of experiments on diversified application of C/N ratio control in giant freshwater prawn culture. In 2009, the research team studied the effects of addition of tilapia, *Oreochromis niloticus,* and substrates for periphyton developments on pond ecology and production in C/N-controlled *M. rosenbergii* farming systems. In the same year the team investigated effects of stocking density of *M. rosenbergii* and addition of different levels of *Oreochromis niloticus* on production in C/N controlled periphyton based system. Asaduzzaman *et al.* (2010a) explained the effects of C/N ratio and substrate addition on natural food communities in freshwater prawn monoculture ponds. Asaduzzaman *et al.* (2010b) investigated the possibilities of combining C/N ratio control, fish-driven re-suspension and periphyton based aquaculture in freshwater prawn culture with varying stocking densities of tilapia and *Labeo rohita*. They also studied the effect of two carbohydrate sources for controlling the C/N ratio in the culture pond. In the same year, the team scanned various carbohydrate sources for maintaining a high C/N ratio and fish driven re-suspension on pond ecology and production in periphyton-based freshwater prawn culture systems.

The biofloc technology has been widely used in shrimp culture for reducing the accumulation of toxic metabolites like ammonia by its conversion to heterotrophic bacterial biomass, especially in static systems. The modified static green water system of seed production of *M. rosenbergii* faces the problem of elevated levels of metabolites like ammonia and nitrite. Saritha (2009), Kurup and Saritha (2010) and Saritha and Kurup, 2011) made an attempt to evaluate the effectiveness of biofloc technology in larval rearing of the giant freshwater

prawn wherein the quantity of carbohydrate addition has been optimised to assimilate the toxic metabolites generated and consequently converting them into bacterial floccules.

Application of BFT making culture more sustainable by 54% increase of total revenue from the harvested shrimp, reduce water based nitrogen species discharge to the environment and also reducing the protein content of the feed substantially (Kurup, 2009). Enhanced pond productivity through stimulation of suspended and attached algal development and by using them to improved water quality, provide additional food, higher carrying capacity in combination with reduced nutrient discharge, improving environmental sustainability. This technology is simple and cheap, making it also socially and economically sustainable, even for small scale or poor farmers (Hari *et al.*, 2004, 2006; Varghese, 2007; Kurup, 2009).

Chapter Three

MATERIALS AND METHODS

Chapter- 3

MATERIALS AND METHODS

3.1 Study area

The experiment was conducted in the laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline of Khulna University. For the determination of the *Lactobacillus* spp., *Enterococcus* spp. and *Salmonella* spp. the freshwater prawn *Macrobrachium rosenbergii* were cultured with calculated feed application along with molasses application and then the samples were taken in the Biochemistry and Molecular Genetics Laboratory of FMRT Discipline, Khulna University for culture and enumeration of *Lactobacillus* spp., *Enterococcus* spp. and *Salmonella* spp. bacteria.

Figure 1: Map of Bangladesh indicating the study area.

Figure 2: Tank in laboratory

In the study area, twelve fiber glass tanks were selected to carry out the experiment. Each of them was rectangular in shape and had water holding capacity of 10 litres. The sample was collected from the experimental tanks and bacterial load were counted in the Biochemistry and Molecular Genetics Laboratory of FMRT Discipline, Khulna University.

pen, foil paper, pipette, micro-pipette, tips and tips box (Swanson *et al.*, 2001) were needed for conducting study.

3.3.2 Reagents and chemicals

Peptone water, absolute alcohol, Ethyl alcohol (75%), distilled water and detergent powder were required during the study periods.

3.3.3 Types of media needed during culture period

Lactobacillus MRS agar medium was required for the culture and enumeration of *Lactobacillus* spp. SF (*Streptococcus faecalis*) broth medium was required for culture and estimation of *Enterococcus* spp. This media was specific or selective for *Enterococcus* spp. bacteria. Broth Medium I (Tetrathionate Bile Brilliant Green Broth) will be required. This media will be specific or selective for harmful *Salmonella* spp.

3.3.4 Sample collection

About three samples of gut were collected from each experimental tank for each and every enumeration at every three days' interval. So, twelve samples were collected under each treatment. As a result, there were total twenty-four sampling days from twelve experimental tanks under three treatments. Then collected sample was carried immediately to the Molecular Biology Laboratory of FMRT Discipline, Khulna University using plastic vial maintaining the cool chain system. Gut and intestine of the collected prawn were separated by dissecting the body. To avoid cross contamination, separate sterile surgical blade, dissection tray was used for each individual.

Two gram of pooled gut sample of fresh prawn (*Macrobrachium rosenbergii*) was collected from each experimental tank. As the prawn individual was smaller in size, it was needed to catch 4-5 individual prawn for the collection of two-gram gut sample. The sample was kept in 1.5 ml tube with 1 ml distill water. All the samples were instantly used for microbial analysis and also properly labeled and stored in -20°C temperature for further analysis.

3.4 Laboratory work

3.4.1 Sterilization of equipment

Test tubes, 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 (Swanson *et*

al., 2001). For completing the autoclaving procedure about 2 hours and 45 minutes was needed.

3.4.2 Sample preparation and dilution

The prawn individual was dissected and gut sample was separated. Accurate amount (2 g) of pooled sample was weighed. Then the sample was taken into a sterile mortar for blending. The sample was taken into a flask containing 18 ml 0.1 % sterile peptone water. The mixture was homogenised for 2 min. This provided a dilution of 10^{-1}. This was the stock solution for the bacterial culture.

Samples from each of the experimental tank was taken and prepared in the same way. The prepared samples of the twelve tanks were kept in separate conical flask.

3.5 Enumeration of *Lactobacillus* spp.

3.5.1 Culture media and bacteria culture

Lactobacillus spp. bacteria were cultured in the Lactobacillus MRS agar medium. The composition of the media is the following:

Ingredients	**Gms/Litre**
HiVegPeptone	6.0
HiVeghydrolysate	4.0
Yeast extract	3.0
HiVeg extract	1.5
Dextrose	1.0
Agar	15.0
Total	**30.5**

30.5 g of the media was dissolved in 1 L of distilled water, followed by heat sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes. By streaking the bacteria was inoculated into the media and incubated for 18-48 hours at 35-37 °C.

3.5.2 Cultural Response

Cultural characteristics observed during incubation at 35 - 37°C after eight hours of incubation.

3.5.3 Confirmation tests of *Lactobacillus* spp.

For the confirmation of *Lactobacillus* spp., the following biochemical tests will be conducted:

1. Catalase test
2. Oxidase test

3. Urease test
4. Methyl red test
5. Voges-proskauer test

3.5.4 Counting of colonies

After the specified period of incubation, the bacterial colonies of each Petri dish were counted by using the colony counting equipment. To calculate the number of bacteria per gram of diluted sample following the equation (Herigstad *et al.*, 2001):

$$\frac{\text{Number of CFU}}{\text{Volume plated (ml) x total dilution used}} \longrightarrow \frac{\text{Number of CFU}}{\text{ml}}$$

3.6 Enumeration of *Enterococcus* spp.

3.6.1 Composition of culture media

Enterococcus spp. bacteria were cultured in the SF Broth media. SF Broth was produced by Hi Media Laboratories according to the formulation developed by Hajna and Perry (1943). The media composition has been given in the section of appendix-1.

3.6.2 Preparation of culture media

36.032 g media (SF Broth) was mixed in 1000 ml of distilled / deionized water. Heat was provided to dissolve the medium completely. Then it was dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The Final pH of the media was adjusted to 6.9 ± 0.3 at 25°C before autoclaving. The media was stored below 30°C in tightly closed container and the prepared medium was at 2 - 8°C.

3.6.3 Principle and Interpretation of culture media

SF Broth is prepared according to the formula of Hajna and Perry (1943) for the detection of faecal Streptococci in the swimming pools, water and milk samples. SF Broth is also recommended for detection of faecal streptococci in water and other samples (Eaton et.al, 2005). Streptococci grow luxuriantly at 45.5°C with an acidic reaction, seen as color change from purple to yellow.

In this medium, the indicator turning yellow in presence of Enterococci is evident after 18-20 hours but to proceed for the isolation, a supplementary incubation in Petri plates is recommended. A positive reaction for the presence of group D Enterococci is indicated by

turbidity and a color change in the medium from purple to yellow. A negative reaction is indicated by absence of growth and a purple color (no color change).

3.6.4 Culture of *Enterococcus* spp.

Then the bacteria were cultured in the prepared culture media (SF broth). The bacteria were cultured in five dilutions and each dilution had three tubes. So, fifteen test tubes were needed for one stock solution. Twelve stock solutions were prepared from the twelve experimental tanks and used for bacterial analysis. (12×12) = 144 test tubes were needed for the culture of bacteria of tanks.

9 ml of prepared media was taken in each of the test tubes. Then 1 ml of stock solution was taken in a test tube with a micro pipette. It was the culture of 10^{-1} dilution. This tube was centrifuged in a vortex machine for 2 minute. Then 1 ml was taken from the tube with micro pipette after changing tip and poured into another test tube. It was the culture of 10^{-2} dilution. The culture of 10^{-3}, 10^{-4} and 10^{-5} dilutions were done in the same way. There were three replication tubes for the culture of each dilution. Thus the samples taken from the twelve replication tanks were cultured in the same manner. The test tubes were then incubated at specific temperature (45.5^0 C) for bacterial growth.

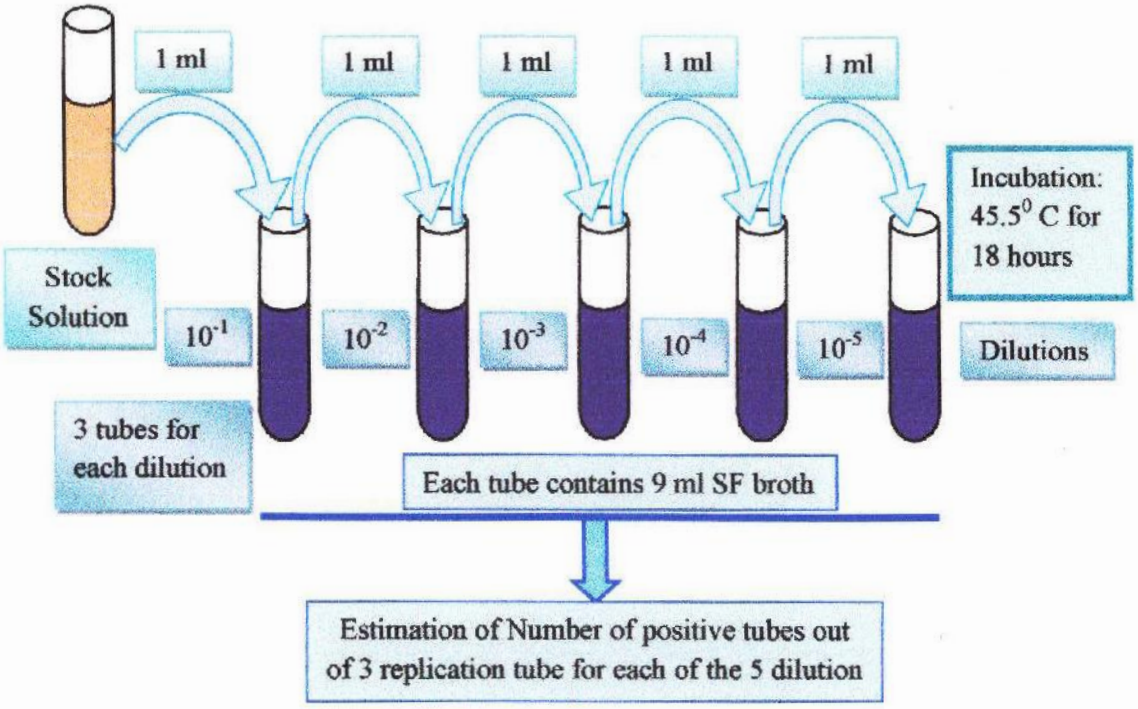

Figure 3: Flow chart of culture techniques of *Enterococcus* spp. in SF broth.

3.6.5 Cultural Response

Cultural characteristics were observed after incubation at 45-46°C for 18-48 hours. The color of the tubes was changed from purple to yellow giving the positive result in which the growth *Enterococcus* spp. was luxuriant. Some tubes remained unchanged (purple color) which gave the negative result due to lack of growth. As the SF broth media is selective for Enterococci and the presence of *Enterococcus* spp. was identified by the color change (from purple to yellow) at the time of culture, so any further confirmation test was not necessary.

3.6.6 Data collection

In the tube culture method, the number of bacteria cannot be found directly. Only the number of positive and negative tube for each dilution were found and recorded. Then an established Most Probable Number (MPN) chart was used to estimate the number of bacterial cell by using the number of positive tube for each dilution. The MPN chart was prepared by J.L. Oblinger and J.A. Koburger in 1975 on the basis of poisson counting statistics regarding the number of positive tubes out of all the cultured tubes. The bacterial culture was done in a five-fold dilution each of with three replication tubes. So, three tubes MPN chart was used for the estimation of the load of *Enterococcus* spp. Then the Most Probable Number (MPN) bacterial data was recorded, accumulated, grouped and interpreted according to the objectives as well as parameters.

3.7 Enumeration of *Salmonella* spp.

3.7.1 Culture media and bacteria culture

Salmonella spp. bacteria were cultured in the broth medium I (Tetrathionate Bile Brilliant Green Broth media). The composition of the media is the following:

Ingredients	**Gms/Litre**
Peptone	8.600
Ox-bile dried	8.000
Sodium chloride	6.400
Calcium carbonate	20.000
Potassium Tetrathionate	20.000
Brilliant green	0.070
Total	**63.07**

63.07 gm of the media were dissolved in 1 L of distilled water, followed by heat sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes. By streaking the bacteria were inoculated into the media and incubated at anaerobically at 41-43°C for 18-24 hours.

3.7.2 Data collection

In the tube culture method, the number of bacteria cannot be found directly. Only the number of positive and negative tube for each dilution were found and recorded. Then an established Most Probable Number (MPN) chart was used to estimate the number of bacterial cell by using the number of positive tube for each dilution. The MPN chart was prepared by J.L. Oblinger and J.A. Koburger in 1975 on the basis of Poisson counting statistics regarding the number of positive tubes out of all the cultured tubes. The bacterial culture was done in a five-fold dilution each of with three replication tubes. So, three tubes MPN chart was used for the estimation of the load of *Salmonella* spp. Then the Most Probable Number (MPN) bacterial data was recorded, accumulated, grouped and interpreted according to the objectives as well as parameters.

3.7.3 Biochemical tests for *Salmonella* spp.

Test	**Results**
Indole test	Negative (-)
Methyl Red/Voges Proskauer	Negative (-)
Motility Test	Positive (+)
Gas Production	Positive (+)
Catalase test	Positive (+)
Oxidase Test	Negative (-)
Glucose fermentation	Positive (+)
Sucrose fermentation	Negative (-)
H_2S	Positive (+)

3.8 Data analysis

Collected data were stored, explored and analyzed using Microsoft Excel, SPSS and Microsoft Word program to present the results and discussion. The normality test of the data was done in SPSS at first. But they were not normally distributed and as they were the bacterial count data, the Chi- square test was conducted in SPSS to compare the data of control and treated ponds.

Chapter Four

RESULTS

Chapter- 4

RESULTS

To analyze the proliferation of microbial content in the gut of fresh water prawn in biofloc contained water, four different treatments were used. One control and three treatments with three replications were used in the laboratory of FMRT discipline in Khulna University.

4.1 Total bacterial count

The mean number of total heterotrophic bacteria (THB) in T_2 was (11.3 ± 5.7) $\times 10^9$ CFU/g higher in the treated group and lowest mean number found in control group (2.0 ± 0.3) $\times 10^9$ CFU/g. The mean volume of THB in T_3 and T_1 was (9.8 ± 3.3) $\times 10^9$ CFU/g and (4.0 ± 2.4) $\times 10^9$ CFU/g respectively.

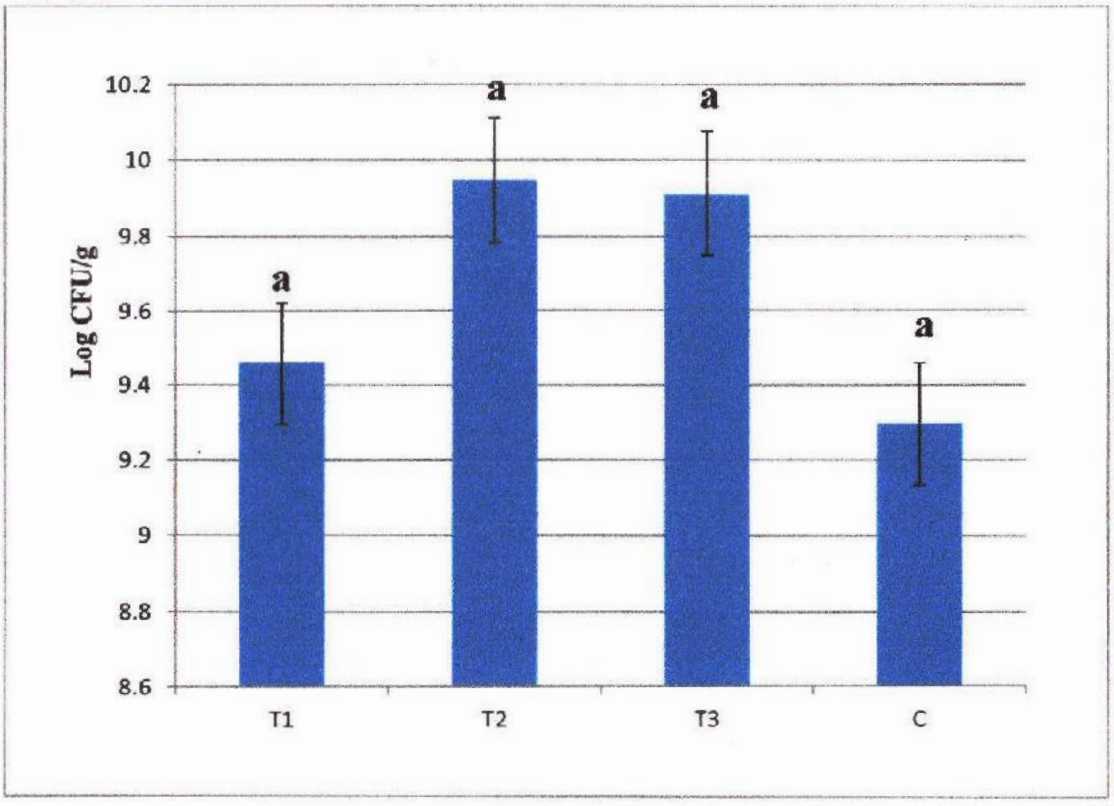

Figure-4.1: Count of total heterotrophic bacteria (Log CFU/g) in the gut of *M. rosenbergii* cultured at different C/N ratio based biofloc culture system. The error bars indicate the standard

deviation of three replications. Same letters indicate there had no significant difference among the treatments (One-way anova, $P<0.05$).

4.2 *Enterococcus* spp. count

The mean number of *Enterococcus* spp. was the higher in T_2 (9 ± 1.1) $\times 10^4$ MPN/g and T_3 (6.7 ± 1.5) $\times 10^4$ MPN/g compared to T_1 and C ($P<0.05$). In control group, lowest number (1.07 ± 0.4) $\times 10^3$ MPN/g of *Enterococcus* spp. was counted in the gut of *M. rosenbergii*.

There had significant differences ($P<0.05$) between control and T_2 as well as T_3.

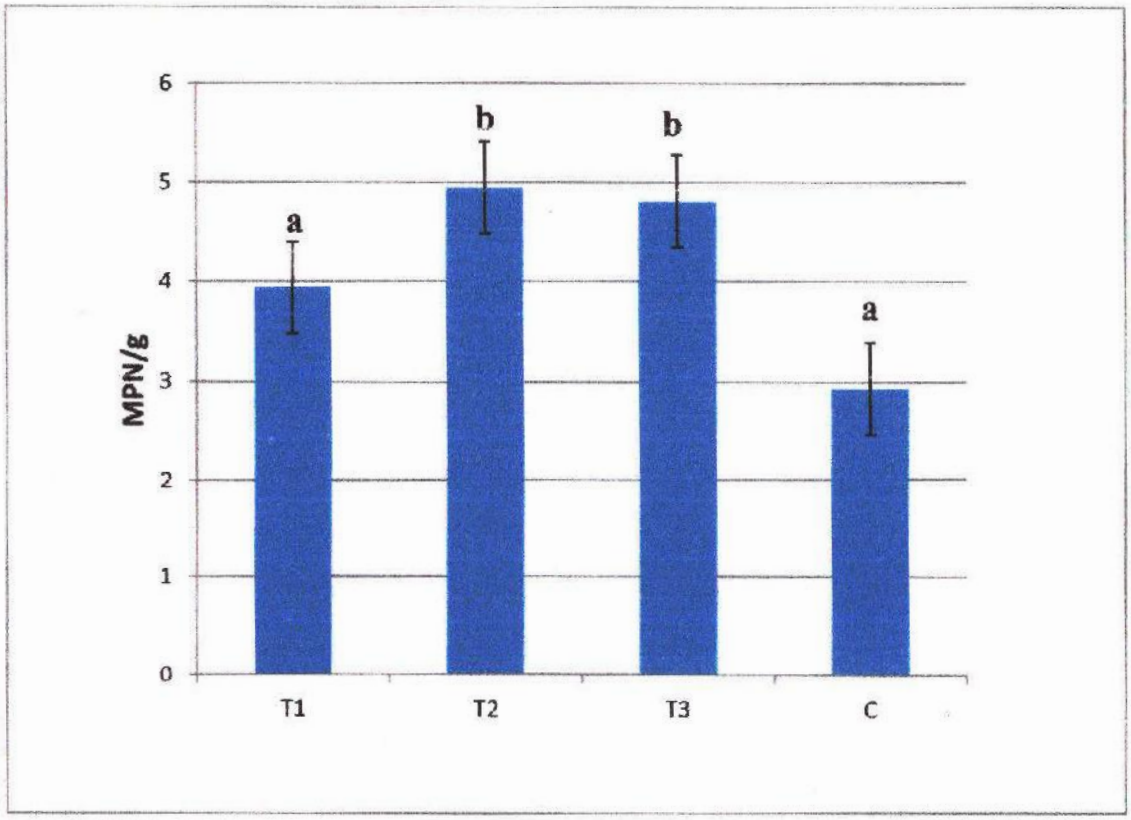

Figure-4.2: Count of *Enterococcus* spp. (MPN/g) in the gut of *M. rosenbergii* cultured at different C/N ratio based biofloc culture system. The error bars indicate the standard deviation of three replicates. Different letters indicate significant difference among the treatments (One-way anova, $P<0.05$).

4.3 *Lactobacillus* spp. count

In case of *Lactobacillus* spp. although not significant, but higher load was recorded in T_3 (1.5 ± 0.7) × 10^6 CFU/g and T_2 (1.3 ± 0.5) × 10^6 CFU/g compared to T_1 and C ($P<0.05$). Similar to *Enterococcus* spp., *Lactobacillus* spp. was found lower (6.3 ± 5.4) ×10^5CFU/g in control group.

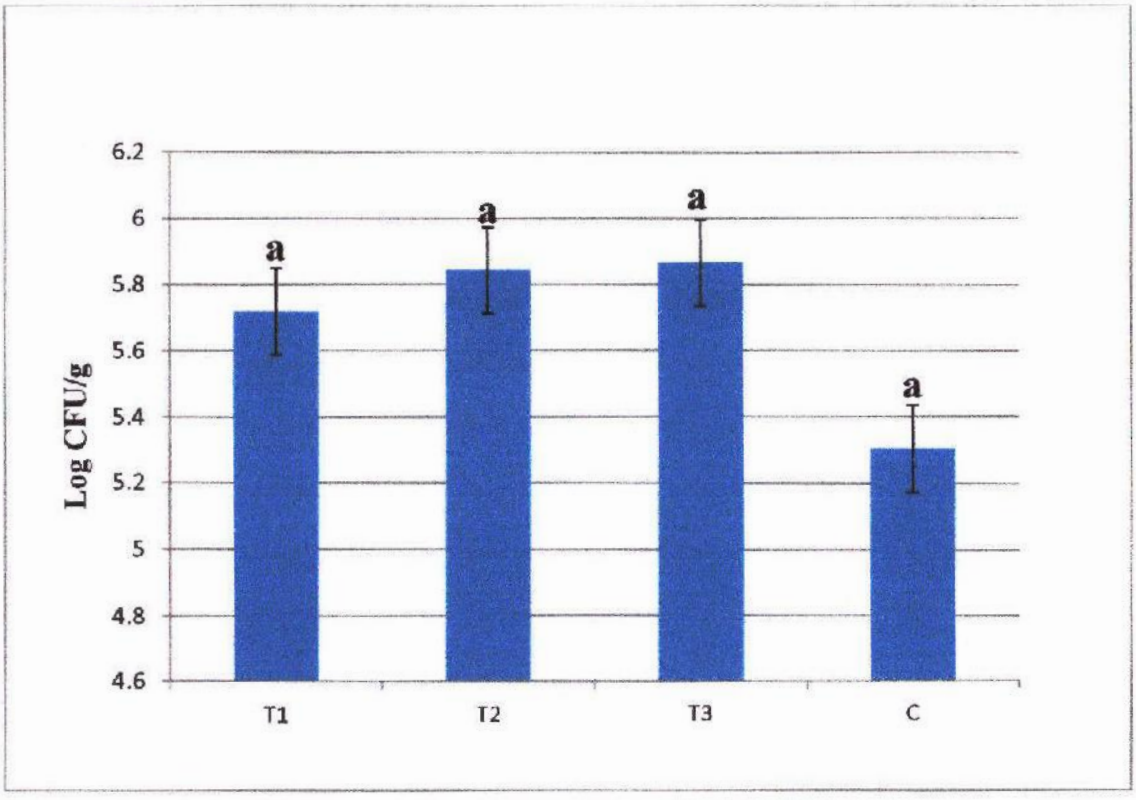

Figure-4.3: Count of *Lactobacillus* spp. (Log CFU/g) in the gut of *M. rosenbergii* cultured at different C/N ratio based biofloc culture system. The error bars indicate the standard deviation of three replications. Same letters indicate there had no significant difference among the treatments (One-way anova, $P<0.05$).

4.4 *Salmonella* spp. count

The presence of *Salmonella* spp. was not found in any of the experimental tanks.

Chapter Five

DISCUSSIONS

Chapter- 5

DISCUSSIONS

In this study, proliferation of bacterial colonies and microorganisms in the gut of *Macrobrachium rosenbergii* was directly influenced by the C/N ratio. There were three C/N ratio (10:1, 15:1: 20:1) in the study and abundance of bacterial colonies are comparatively higher in C/N ratio in 15:1 than other C/N ratios except *Lactobacillus* spp. Bakar *et al.* (2015) showed that among various C/N ratios, and when using molasses, the optimal ratio was 15 in terms of removing ammonia in a biofloc based system culturing *C. gariepinus*.

Smith *et al.* (2004) showed that bacteria use nitrogen in the nitrate formation. In Biofloc culture system, total bacteria take nitrogen as food and increase their community. Avnimelech *et al.* (1992); Kochva *et al.* (1994); Avnimelech (1999) who found that numbers of heterotrophic bacteria increased in response to increasing levels of C:N ratio.

Addition of carbon source in a limited amount leads to the development of beneficial bacteria in fish culture medium. Thus cost of production can be reduced as fish take these bacteria as food. Ebeling *et al.* (2006); Hari *et al.* (2006); Avnimelech and Kochva (2009); Crab *et al.* (2010) showed that adding a carbon source (direct or indirect Carbon sources) to the culture medium in limited-discharge systems (changing C/N ratio), cause significant enhancement of useful microbial growth and the fixation of toxic nitrogen metabolites.

Proliferations of *Enterococcus* spp in C/N ratio 15:1 shows higher abundance than C/N ratio 10:1 and 20:1. The mean number of *Enterococcus* spp. was $(9 \pm 1.1) \times 10^4$ MPN/g. Where lowest amount of *Enterococcus* spp. found in control and was $(1.07 \pm 0.4) \times 10^3$ MPN/g using no additional carbon source. Proliferations of *Lactobacillus* spp. in C/N ratio 20:1 shows higher abundance than C/N ratio 10:1 and 15:1. The mean number of *Lactobacillus* spp. was $(1.5 \pm 0.7) \times 10^6$ CFU/g. Where lowest amount of *Lactobacillus* spp. found in control and was $(6.3 \pm 5.4) \times 10^5$ CFU/g using no additional carbon source. Chamberlain and Hopkins (1995) showed that if a carbon source such as wheat bran, rice bran, molasses and cellulose is sprayed on the surface of

pond water with continuous aeration, at the optimum C/N ratio 15:1; bacterial growth will increase. The bacteria consume the carbon source as energy and reduce the ammonia concentration through nitrification, while the fish feed on the bacteria.

Finally, the study shows the abundance of *Salmonella* spp. was nil. It might be that the cultural system was good and quite hygienic, so the beneficial microbes might exclude the growth of the pathogenic agent. Like as, the association of flocs acts as a bio regulator of water quality and as an inhibitory agent to prevent the growth of *Vibrio* spp. during the cultivation of shrimp in the biofloc system (Pacheco-Vega *et al.*, 2018).

Moreover, the addition of lactic acid bacteria in shrimp diets has been suggested to decrease the adherence and colonization of pathogenic bacteria and to improve fish health (Ringo *et al.*, 2010). So, the *Lactobacillus* spp. may be the cause of inhibiting the *Salmonella* spp.

In the study, the mean number of total heterotrophic bacteria (THB) in T_2 was the highest and it was $(11.3 \pm 3.7) \times 10^9$ CFU/g. According to Zhu and Chen (2001), the increase in the C/N ratio from 0 to 15 reduced the efficiency in 70% of the nitrification rate. In this way, the authors verified a reduction in the nitrification rate due to the increase in organic carbon promoting the dominance of heterotrophic bacterial community.

In this study, the mean number of the total heterotrophic bacteria (THB) in control unit is higher than the treatment unit. There, in control unit no external carbon source was added. Without balanced C/N ratio, there is a possibility to grow pathogenic bacteria along with the beneficial or probiotic bacteria. It is similar to the Sthephaniee *et al.* (2016), noted that a better removal of contaminant nitrogen compounds, the growth increase of heterotrophic bacteria, which together probiotic bacteria, inhibit the development of potential pathogenic bacteria in aquaculture and recognized the beneficial effect of external carbon source used to obtain the type and quantity of bacteria that development in culture production systems.

That is similar to the Hari *et al.* (2004) showed that the addition of a carbohydrate source to the pond water column benefited the extensive shrimp culture practices in three ways (1) increased heterotrophic bacterial growth supplying bacterial protein to augment the shrimp production, (2)

reduced demand for supplemental feed protein and subsequent reduction in feed cost and (3) reduced toxic inorganic nitrogen levels in the pond as well as effluents.

Chapter Six

CONCLUSION

Chapter- 6

CONCLUSION

From this present study the following conclusions can be drawn

1. Higher C/N ratio, 15 and 20 have significant effect on the *Enterococcus* spp. growth in the gut of *M. rosenbergii*. However, with the increasing the ratio the bacterial load can be decreased.
2. There were no significant differences, but carbon sources can increase the *Lactobacillus* spp. load in prawn gut.
3. Higher carbon source can increase total heterotrophic bacteria, C/N 15 and 20 compared to 10. However, no carbon source might cause risk of higher load of pathogenic bacteria.

Recommendations:

1. Further research should be conducted to investigate other potential beneficial and harmful bacteria in prawn gut in biofloc based culture system at different C/N ratios using different available carbon sources.
2. Micro-environments of gut bacteria should be studied through other microbial community management approach.

Chapter Seven

REFERENCES

Chapter- 7

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APPENDIXES

Appendix- 1

SF (*Streptococcus faecalis*) broth medium composition:

Ingredients	Amounts per liter*	Amounts per 100ml	Amounts per 200ml
Tryptone	20.0 g	2.0 g	4.0 g
Dextrose	5.0 g	0.5 g	1.0 g
Dipotassium Phosphate	4.0 g	0.4 g	0.8 g
Monopotassium Phosphate	1.5 g	0.15 g	0.30 g
Sodium Chloride	5.0 g	0.5 g	1.0 g
Sodium Azide	0.5 g	0.05 g	0.10 g
Bromcresol Purple	0.032g	0.0032g	0.0064g
Sterile Distilled Water	1000.00ml	100.00ml	200.00ml
Final pH 6.9 ± 0.2 at 25°C			

(Hajna and Perry, 1943)

*Adjusted and/or supplemented as required to meet performance criteria

Appendix-2

PHOTO GALLERY

Figure 1. Dissection of the prawn sample

Figure 2. Sample preparation in mortar

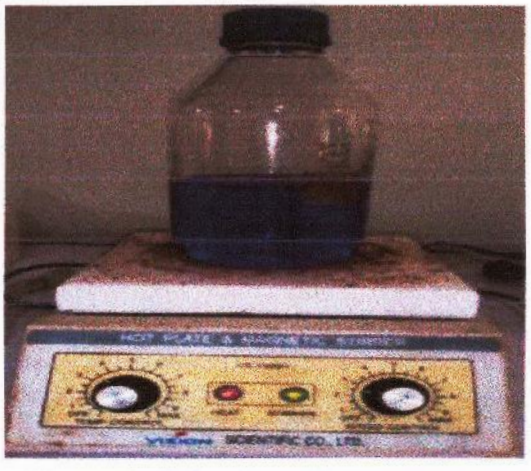

Figure 3. SF broth media preparation

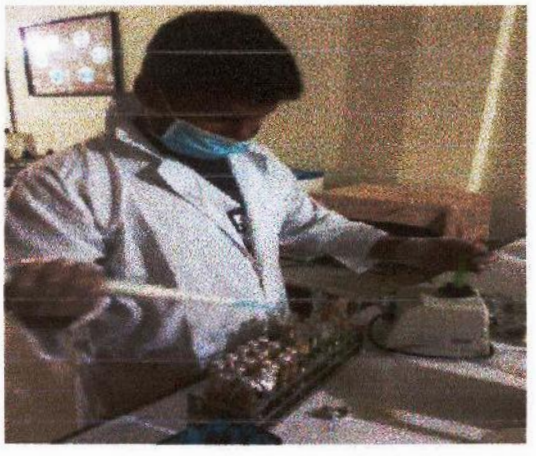

Figure 4. Using Vortex mixer

Figure 5. Sample in the media for bacteria culture

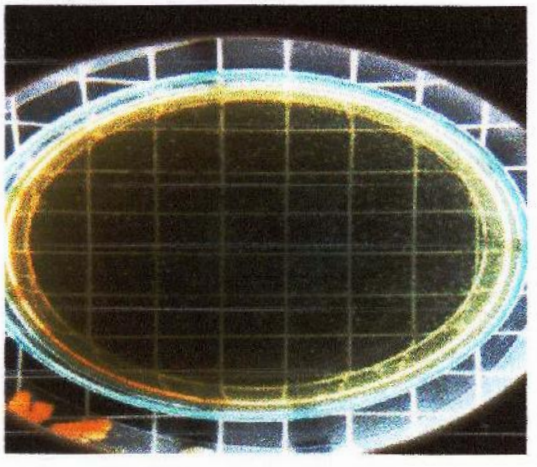

Figure 6. Bacteria colony counting

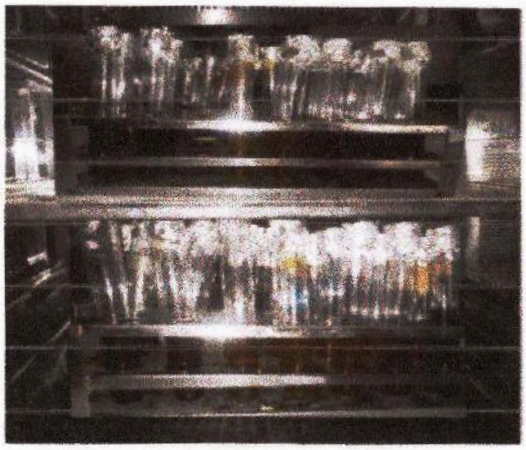

Figure 7. Incubation of culture media

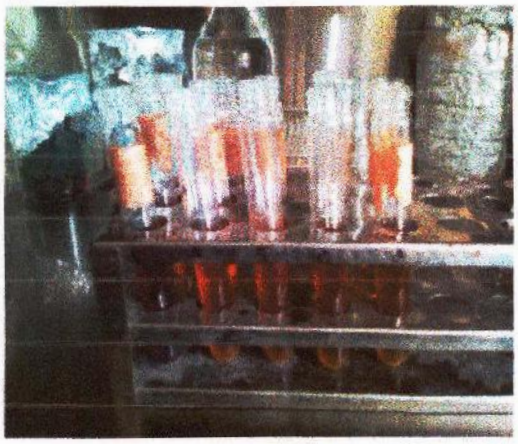

Figure 8. Color identification after incubaion

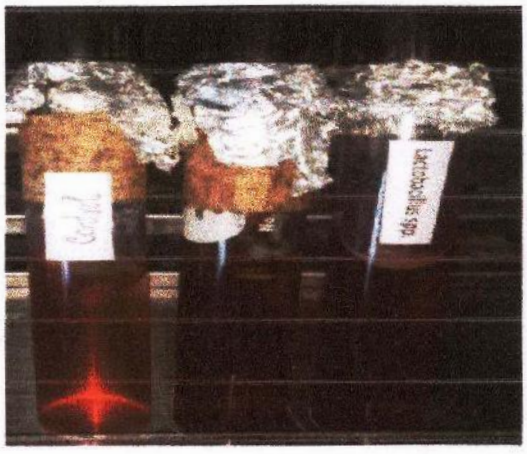

Figure 9. Biochemical test for *Lactobacillus* spp.

Figure 10. Biochemical test for Enterococcus spp.