Effect of dietary synbiotic on phenotypic traits and metabolic activities in monosex tilapia (*Oreochromis niloticus*)

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Declaration

Effect of dietary synbiotic on phenotypic traits and metabolic activities in monosex tilapia (*Oreochromis niloticus*)

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

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DEDICATED TO

My Beloved Parents

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ABSTRACT

The present study evaluated the effects of dietary synbiotic on growth performance, some selected phenotypic traits and digestive enzymes activities of Nile tilapia (*Oreochromis niloticus)*. There had four types of treatment groups in the 45 days long study. Only commercial feed was used as a control (C) and other three different concentrations of synbiotics with commercial feed were used as treatments (T_1, T_2 and T_3). The synbiotic composition contained the mixture of probiotic bacteria *Lactococcus lactis* strain D1813 and prebiotic $CaCO_3$, starch QS2. The probiotics doses of 1gm/kg, 2gm/kg and 3gm/kg was given to these three treatments T_1, T_2, and T_3, respectively. The present results revealed that the diets containing different doses of synbiotic significantly ($p<0.05$) increased growth performance of Nile tilapia. The experiment documented significant changes ($P<0.05$) in phenotypic traits of total length, standard length, trunk length, eye diameter, body area and dressing percentage except in tail length and color patterns. The mixture of feed with synbiotic showed a significantly higher amylase and protease activity in intestine of fish than the fish fed with controlled diet; however the enzyme activity in muscle were also increased but it was not significant ($P>0.05$). This result suggested that the incorporation of dietary synbiotic (mixture of *Lactococcus lactis* D18113, CaCO3 and starch QS2) had beneficial effect on growth performance, phenotypic traits and digestive enzyme activity of Nile tilapia.

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research species. It can exist in a very wide range of conditions. Not only that, but also their high protein content, survival capacity at low dissolved oxygen, efficient food conversion and palatability bring it on the focus of the researchers (Asad et al., 2010). Besides, tilapia gets consumer preferences due to its low price, easy preparation and mild taste. Moreover, the production of all male populations is an important tool to avoid energy consumption in reproduction and to produce the sex with the larger growth potential (Green et al., 1995). As it has many other potentialities like high growth rate, high reproductive capability within a very short time etc. and is also available in local market, it gives priority for this experiment.

High quality formulated feeds are used to achieve high yields and large sized fish (600-900 g) within a short period of time. Tilapia feeds accounted for about 8.1 percent of global aquafeed production in 2003. Commercial tilapia feeds are mainly dry sinking pellets and extruded floating pellets (Tacon et al., 2006). The main issue in formulating feed is to meet the protein and essential amino acids (EAAs) requirements of the species. the optimum dietary protein level for growth of Nile tilapia was 30% crude protein (Wang et al., 1985). Fishmeal is generally the preferred protein source because of the high quality of the protein and its EAA profile. However, fishmeal is generally expensive and is not always available. Nile tilapia (*O. niloticus*) can be fed with a high percentage of plant proteins. It is economically judicious to replace fishmeal with alternative protein sources including animal by-products, oilseed meal and cakes, legumes and cereal by-products and aquatic plants. Most of these ingredients are deficient in some EAA and hence require supplementation or be compensated with other feedstuffs (Tacon et al., 2006).

Though tilapia are considered to be relatively resistant to a number of diseases encountered in other farmed fishes, a number of bacterial, fungal, protozoan and parasitic diseases have been reported in this fish (FAO, 2005). In addition, some viral diseases, though not very common, have been reported in tilapia. These disease hampered the production a lot, causes a loss in aquaculture (Jobling and Sumpter, 1993). To get a better production performance, it should be emphasized on the disease resistance along with optimum feeding mechanism. There have several remedies for diseases like antibiotic, probiotic, prebiotic, synbiotic, etc.

Antibiotics are used as traditional strategy for fish diseases management during the last decades. Excess use of antibiotics to control the incidence of diseases not only resulted in the emergence

of multiple antibiotic resistant bacterial strains (Karunasagar et al., 1994) but also cause critical environmental and human health concerns. If antibiotics are used to kill bacteria, some bacteria will survive, because they develop antibiotic resistance genes. These will then grow rapidly because their competitors are removed (Moriarty, 1999). On the other hand, beneficial micro-biota in the gastrointestinal (GI) ecosystem are also reduced by inhibitory action of antibiotics and it is alarming that antibiotic residues accumulated in fish products are harmful for human consumption (Lange et al., 2016). Consequently, eco-friendly and sustainable aquaculture treatments came into existence to prevent the diseases such as probiotic, prebiotic, synbiotic, biofloc etc. The quality and production of fish can be enriched with proper application of those technologies in a lower operating cost of feed and disease prevention.

Probiotics are live microorganisms intended to provide health benefits when consumed, generally by improving or restoring the gut flora (Rijkers et al., 2011). Probiotics are considered to be able to make cultured animals healthier by increasing digestibility and immune performance in fish body. On the other hand, Prebiotics are non-digestible oligosaccharides that can stimulate selectively the growth of probiotic-like bacteria. Probiotics and prebiotics in the diet can modify the composition and some metabolic activities of the microflora (Macfarlane and Cummings, 1999). The application of probiotic and prebiotic enriches the activity of the digestive enzyme like protease, amylase, cellulose, and lipase. The microbe of intestine is either modified or the probiotic bacteria itself secrets the enzyme to enhance the activeness (Adel et al., 2017). Good aquaculture practices at all levels and application of measures for quality control and food safety would ensure sustainable development of fish farming in Bangladesh (Gatesoupe, 1999). The use of prebiotics and probiotics separately is not very recent issue but use of synbiotics in aquaculture is increasing with demand recently for more environment friendly aquaculture practices. These technologies are also being used to improve production performances by using lower feeding level and reducing disease accumulation in the production area (Cerezuela et al., 2011).

The term synbiotic is used when a product contains both probiotics and prebiotics. Synbiotics refer to food ingredients or dietary supplements combining probiotics and prebiotics in a form of synergism (Pandey et al., 2015).The synbiotic concept was first introduced as "mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and

2.2 Problems in aquaculture

Aquaculture creates several problems as well there have several problems to adopt an aquaculture. Generally, it can be noticed that, water color turns to deep green because of the presence of excessive algae in water, which is the result of overabundance of nutrients. Even heavy fish loads contribute to green pond water because fish waste is broken down by beneficial bacteria through a series of steps into nitrate, which is food for plants and algae. Excessive algae growth can lead to a lack of dissolved oxygen in the water, particularly, dissolved oxygen content drops during night. The appearance of muddy water in farm ponds can have negative effects other than detracting from the aesthetics of the pond (Hussan et al., 2016).

2.2.1 Disease problem in aquaculture

Outbreak of disease is more common in farming operations than the wild as a result of higher levels of stress in fish, high stocking densities and establishment of conditions conducive to incubation of disease organisms. Aquaculture provides opportunity for amplification of disease, though notably it also facilitates early detection of outbreaks due to frequency of testing to protect valuable fish stocks. Additionally, increased food resources near farm cages attract large concentrations of escaped and wild fishes, which may act as vectors for the transfer of disease and parasites to other native fish (Carss, 1990).

Disease problems constitute the largest single cause of economic losses in aquaculture. The major disease problems that occur in aquaculture ponds are parasitic, fungal, and bacterial in origin (Reno, 1998). Bacterial infections constitute the most important source of disease problems in all the various types of production. Gram-negative bacteria cause epizootics in nearly all cultured species (Austin et al., 2012). Aquaculture operations can spread parasites and disease into the wild. Also, farmed fish have an increased chance of getting parasites such as sea lice, as opposed to fish that live and breed in their natural environment (Woo, 1992). Fungal diseases constitute the second most important source of losses, especially in the culture of crustaceans and salmon. External protozoan parasites are responsible for the loss of large numbers of fry and fingerling fin fishes and are a cause of epizootics among young shellfish (Meyer, 1991). Farmed fish are also exposed to disease through the use of unprocessed fish used as a food source, as opposed to safer processed fish pellets (Bryan, 1977).

2.2.2 Environmental problem

Fish farming operations result in the release of a number of wastes into the aquatic environment. The release of faeces, nutrients and chemicals can result in algae blooms which eventually remove dissolved oxygen in the receiving waterway, or eutrophication. Zero oxygen content results in deadly fish kills (Camargo, 2006). Additionally, chemicals such as antibiotics and water treatment agents are commonly used in the aquaculture industry and aquaculture systems need to be closed, or wastewater treated prior to discharge (Cabello, 2006).

The effects on the food chain from this additional organic input are many and varied, the input leading to water column nutrient enrichment and accumulation of organic matter in the sediments. In the water column, soluble nutrients can alter the species composition and density of phytoplankton, increasing the risk of toxic algal blooms (DPIF, 1997). Chemicals are used in finfish aquaculture for a wide range of applications. Chemicals are not only used in fish health, but also to control nuisance organisms on equipment such as nets, and to disinfect and improve water quality. The use of such chemicals raises a number of environmental concerns, and they must be registered with the National Registration Authority before use (NPI, 2001).

2.2.3 Feed problem

Aquaculture can also negatively impact the environment through overfeeding fish. In addition, feed can also account for up to 60% of the total production costs in commercial aquaculture. Aquaculture feed management is absolutely vital because the schedule that fish farmers follow to feed their fish is directly correlated to economic and environmental sustainability. The universal and primary concern among aquaculturists is finding a way to distribute feeds that meet the nutritional standards of the fish at ration sizes that promote both growth and proper feed conversion ratio (FCR) (White, 2013).

2.3 Synbiotics in aquaculture

There have an ecofriendly and cost effective way to solve the problem of aquaculture discussed above. It is the application of Synbiotic technology. Synbiotics refer to food ingredients or dietary supplements combining probiotics and prebiotics in a form of synergism (Pandey et al., 2015). The synbiotic concept was first introduced as "mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and

implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, thus improving host welfare" (Gibson and Roberfroid,1995). Synbiotics may be complementary synbiotics, where each component is independently chosen for its potential effect on host health, or synergistic synbiotics, where the prebiotic component is chosen to support the activity of the chosen probiotic (Rastall, 2017). Probiotics are live bacteria which are intended to colonize the large intestine, although as of 2018, there is no evidence that adding dietary bacteria to healthy people has any added effect (Rijkers et al., 2011). A prebiotic is a food or dietary supplement product that may induce the growth or activity of beneficial microorganisms. A prebiotic may be a fiber, but a fiber is not necessarily a prebiotic (Rastall, 2017). Using prebiotics and probiotics in combination may be described as synbiotic, but the United Nations Food & Agriculture Organization recommends that the term "synbiotic" be used only if the net health benefit is synergistic (Pineiro et al., 2008).

2.4 Tilapia fish

Nile tilapia is one of the most important and useful species in freshwater aquaculture and an important research model species in several fields of biology (Tilápia and Nilo, 2014). Nile tilapia (*O. niloticus*) is considered as a standout amongst the most critical freshwater species for commercial aquaculture because of its high nutritional qualities, quick development rate and resistance to illnesses. However, *O. niloticus* is cultured widely because of high strength; sometime it causes misfortune for the farmer. A few methodologies has done like inoculation and chemotherapy have been done to build fish immune competence and prevent aquatic diseases (Nakkina, 2016).The uncontrolled breeding of tilapia in ponds, which led to excessive recruitment, stunting and a low percentage of marketable-sized fish, dampened the initial enthusiasm for tilapia as a food fish. Commercially, tilapia are the second most important group of wild-captured fish, after carps, with a global capture (harvest reaching) 769,936 MT in 2007 (FAO, 2009). It is used as a model organism for research in physiology, endocrinology, genomic biology and molecular genetics because it shows a wide range of biological responses to different environmental conditions both in culture and in nature (Anuario, 2016).

2.5 Economical benefit of tilapia

It was mentioned that tilapia has become the third most important fish species in aquaculture. They are the easiest and most profitable fish to farm due to their omnivorous diet, mode of reproduction, tolerance of high stocking density and rapid growth rate. Commercially grown tilapias are almost male. Those are called monosex tilapia. Generally, they have high ability of taking natural feed from pond, good interest in supplementary feed, surviving capacity in adverse weather and they have high diseases resistance power. Not only that, but also larger in size, high protein content and palatability are the focus of major aquaculture efforts. Along with this, the demand of this fish in international market is increasing day by day (Tilápia and Nilo, 2014).

2.6 Effects of probiotics

The name probiotic comes from the Greek "pro bios" which means "for life" (Fečkaninová et al., 2017). According to FAO "probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host". In the field of aquaculture, probiotic can be defined 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" (FAO, 2002).

Probiotics are a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring an improved use of the feed or enhancing its nutritional value, by increasing the host response towards disease, or by improving the quality of its environment (Verschuere et al., 2000).

It has been studied about the use of probiotics, a live microbial feed supplement which benefits the host by modifying the host-associated or ambient microbial community, by enhancing the host response towards disease, by ensuring improved use of feed or enhancing its nutritional value, or by improving the quality of its ambient environment, in aquaculture is being encouraged probiotics are commonly used to substitute and restrict the use of antibiotics or chemical drugs in aquaculture, with the aim of improving growth and disease resistance (Fečkaninová et al., 2017).

There are increasing reports of research works on the application of probiotics in aquaculture though very few studies related to the usage of probiotics in prawn culture available in Bangladesh (Ghosh et al., 2016). Most research has focused on the use of single cultures, and it is largely speculative whether two or even multiple combinations of probiotics are beneficial (Fečkaninová et al., 2017). Ghosh has concluded that probiotics plays an important role in growth and production but it is very necessary to identify the suitable and profitable stocking density and to increase our prawn production in comparing with other developing countries, application of probiotics is essential (Ghosh et al., 2013).

Multi-strain and multi-species probiotics have proven that it is possible to provide synergistic bacteria with complementary modes of action to enhance protection (Kesarcodi-Watson et al., 2008). Interaminense observed that the probiotic candidates produced an inhibition effect against vibrio pathogens under different growth periods, pH and NaCl concentrations. Regarding pH and NaCl conditions, the maximum antagonistic level were in same range of optimum shrimp culture and able to grow inside the *Litopenaeus vannamei* digestive system (Interaminense et al., 2018). Defoirdt mentioned that probiotics should preferentially be selected for more than one characteristic or probionts with different modes of action should be combined to maximize the chance of success (Defoirdt et al., 2011).

The beneficial effect of probiotics on the commercial culture of shrimp, *Penaeus monodon* was studied in grow out pond by Kalimuthu. An effort was made to evaluate the differences between regular and irregular application of soil probiotics in the *P. monodon* shrimp farming. Subsequently the performance of using probiotics frequently and the total average yield attained was 1900 Kg /0.5 ha. Contrarily, in irregular treated ponds the total yield achieved only 1150 Kg /0.5 ha (Kalimuthu, 2015).

The effect of probiotics (*Lactobacillus sp.*) on growth performance and biochemical activity of the shrimp *Litopenaeus vannamei* was investigated by Mg and Ammani. After three weeks, shrimp receiving the diets supplemented with probiotics showed significantly better growth performance than those fed the basal diet (Control). Lactic acid levels were significantly ($P<0.01$) increased in 10% and15% treated groups. The 10% probiotic treated group showed highest protein levels and all the treated groups showed significant difference when compared to control (Mg and Ammani, 2015).

The study investigated the effects of dietary mono- and multiprobiotic strains of *Enterococcus faecalis, Lactobacillus fermentum* and *Leuconostoc mesenteroides* on growth performance, intestinal bacteria and body composition of Javanese carp *(Puntius gonionotus).* They observed that compared to the control group, significant ($P < 0.05$) effect on weight gain, feed conversion ratio, specific growth rate and protein efficiency ratio of probiotic fed Javanese carp was observed. No significant variations were observed in the chemical composition of the carcass by probiotics (Allameh et al., 2016).

Probiotics use in aquaculture show great impact on aquatic organisms. Probiotics decrease accumulation of organic load and maintain water quality in an efficient way. A modern probiotic organism can easily fulfill the desires of sustainable aquaculture development because it can heighten two major key factors of growth performance and disease resistance (Dawood and Koshio, 2016).

Prasad et al. (2003) reported that Lactic acid bacteria, a popular probiotic strain, can be applied to control bacterial pathogen. In addition, another well-known probiotic organism, *Bacillus* sp. is used to diminish metabolic waste in aquatic system. Many strains of *Aeromomas* sp., *Pseudomonas* sp., *Vibrio* sp. act against infectious hematopoietic necrosis virus to show antiviral activity (Kamei et al., 1988). These probiotic organisms may be used singly or in combination such as incorporation of individual or combined supplementation of *Lactobacillus rhamnosus* and *Lactobacillus sporogenes* enhance health and disease resistance of common carp (Allameh et al., 2016).

In 2009, Ma et al. described that *Lactobacillus* sp. simultaneously eliminates nitrogen and pathogens from polluted shrimp farms. According to Stanier et al., gram-positive bacteria are usually more efficient in transforming organic matter back to CO_2 than gram-negative bacteria, which would convert organic carbon to bacterial biomass or slime. The aerobic gram-positive bacteria, such as *Bacillus* sp. were associated with development of water quality, reduction of pathogenic population in culture environment, enhancement of survival and growth rate, and the better health condition of juvenile *Penaeus monodon* (Dalmin et al., 2001).

Administration of probiotic can reduce the use of antibiotics and synthetic chemicals in the fish feed (Fuller, 1989). The effect of probiotic organisms is achieved through improving the immune system of culture organism, enhancing their disease resistance potential or generating inhibitory-substance that prevent the pathogenic organisms from disease

formation in the host (Dawood and Koshio, 2016). As per the use of *Bacillus* sp. showed disease protection by initiating both cellular and humoral immune resistances in tiger shrimp (*P. monodon*) (Rengpipat et al., 2000).

Probiotics have been used in aquaculture to enhance the growth of cultivated species and yet no side effect on the host. Yassir et al. (2002) used probiotic bacteria as growth promoter on tilapia (*Oreochromis niloticus*) in his work and the highest growth performance with *Micrococcus luteus* was noted and the best feed conversion ratio was seen with the same probiotic organism. So they mentioned *M. luteus* as a growth promoter in fish culture. Lactic acid bacteria also referred as growth promoters due to effect on the growth rate in juvenile carp (Noh et al., 1994).

According to Michael et al., 2014 various microorganisms have a valuable effect in the digestive system of aquatic organisms. It has also been stated that *Bacteroides* and *Clostridium* sp. have supplied nutrients like fatty acids and vitamins to the host in fish aquaculture. Some microorganisms such as *Agrobacterium* sp., *Pseudomonas* sp., *Microbacterium* sp. and *Staphylococcus* sp. may contribute to nutritional processes in Arctic charr (*Salvelinus alpines*) (Ringø et al., 1995).

In 1995, Sakai et al. has been demonstrated that oral administration of *Clostridium butyricum* bacteria to rainbow trout, improved their resistance to vibriosis by developing the phagocytosis of leucocytes. Wang, Y. B. (2007) investigated the effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. The mean digestive enzyme activity of each treatment groups was significantly different ($P<0.05$) from that of the Control. Both treatment groups had significantly higher lipase and cellulase activity compared to the Control.

Zokaeifar et al. (2012) studied the effect of two probiotic (*Bacillus subtillis*) strains on the growth performance, digestive enzyme activity, immune gene expression and disease resistance of juvenile white shrimp *(Litopenaeus vannamei*). The findings demonstrated that administration of *B. subtillis* strains, L10 and G1, can improve growth performance and disease resistance through an enhanced immune response in shrimp.

Sumon et al. (2018) studied to determine antagonistic effect of *Clostridium butyricum* against *Vibrio harveyi* and its probiotic effect on growth performance, digestibility and immune response of fresh water prawn, *Macrobrachium rosenbergii.* This study revealed that

probiotic, *C. butyricum* incorporated diets were found to be beneficial for *M. rosenbergii* culture in terms of hindering the growth of pathogenic bacteria and increasing the growth, protease and amylase activities of prawn.

In 2018, Enferadi et al. studied the effects of *Lactobacillus plantarum* on growth performance, proteolytic enzymes activity and intestine morphology of *Oncorhynchus mykiss*. Results showed a positive effect on growth performance in both treatment groups compared to the control group. Acidic protease activity in the lower dose of administration was higher in comparison with the higher dose and control groups. Alkaline protease activity and trypsin activities showed the same pattern with the higher level in treatment groups compared to the control group. The current study showed that *Lactobacillus plantarum* can improve growth performance and protease activity in juvenile rainbow trout.

Hai et al. (2009) examined the effects of two selected probiotics (*Pseudomonas synxantha* and *Pseudomonas aeruginosa*) on the specific growth rate (SGR), survival and immune parameters of juvenile western king prawns (*Penaeus latisulcatus*). The results showed that applications of the probiotics resulted in no signi¢cant di¡erence ($P>0.05$) in the SGR and survival of the prawns, but significantly decreased the food conversion ratios ($P<0.05$) compared with the control where no probiotics were applied.

Sahandi et al. (2013) evaluated the dietary effects of *Saccharomyces cerevisiae* and *Bacillus* spore (*B. licheniformis* and *B. latrospore*) probiotic blend in guppy (*Poecilia reticulata*). The result indicates that the blend of *Saccharomyces cerevisiae* and *Bacillus* spore can act as immune stimulator to treat fish ectoparasites, *Ichthyophtirius multifilus* instead of chemical drugs. Wang et al. reported that use of *Bacillus sp.* improve water quality, survival and growth rates and the health status of juvenile *Penaeus monodon* and reduce the pathogenic vibrios (Wang et al., 2008).

2.7 Effects of prebiotics

The term prebiotic was introduced by Gibson and Roberford who exchanged "pro" for "pre," which means "before" or "for." They defined prebiotics as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon" (Gibson and Roberford, 2012). When the probiotic bacteria do not get enough nutrients, the bacteria in the digestive tract will wash out and then there needs to be another approach to overcome these limitations, such as the application of

prebiotics (Arisa et al., 2015). Prebiotics beneficially affect the host by selectively stimulating the growth and activity of a limited number of bacteria, usually bifidobacteria and *lactobacilli*, and thus improves host health (Mona et al., 2015).

Prebiotics are food ingredients that cannot be digested and that provide beneficial effects to the host by stimulating the growth and activity of one or more of the digestive bacterias in the colon (Arisa et al., 2015). A huge number of prebiotics are used as feed supplement to achieve better growth performance. Growth parameters vary on the basis of aquatic organisms as well as prebiotic supplementation. A diet containing 20 g kg^{-1} oligofructose, a fructo-oligosaccharide produced by partial enzymatic hydrolysis of inulin, resulted in increased growth of turbot larvae, but 20 g kg^{-1} inulin itself had no effect on growth (Mahious et al., 2006).

In 2018, Guerreiro et al. evaluated the effects of short-chain fructo-oligosaccharides (scFOS), xylo-oligosaccharides (XOS) and galacto-oligosaccharides (GOS) on growth performance, hepatic metabolism, gut microbiota and digestive enzymes activities of White Sea bream juveniles. Prebiotics had no effect on growth, feed efficiency or gut microbiota. Plasmatic triglycerides were lower in fish fed XOS than FOS and GOS diets. Malic enzyme activity was lower in fish fed XOS than FOS diet. Fish fed XOS diet had lower fatty acid synthetase (FAS), a key lipogenic enzyme and higher alanine aminotransferase activities.

As per Yousefian and Amiri (2009), last few decades, antibiotics were used to control bacterial diseases but this type of chemical substance is advisable to avoid in aquaculture. In recent years in the aquaculture industry, alternative strategies have been developed for disease control as well as reduction in the widespread use of antibiotics. Prebiotic is a well-known group of these strategies which enhances non-specific immune response. According to Bailey et al. (1991) prebiotic can modify microbes of GI tract by increasing immune responses.

The gastrointestinal track of all invertebrates and vertebrates plays a vital role for providing habitat to different kinds of microorganisms (Flickinger et al., 2003). Various prebiotic oligosaccharides such as inulin and oligofructose are fermented in the colon where they stimulate the growth of bacterial populations related with a well-functioning colon and this stimulation occurs because oligosaccharides are readily fermented by beneficial colonic bacteria and are not used effectively by pathogenic bacteria species (Yousefianl and Amiri, 2009).

Meng et al. (2017) conducted a research to evaluate the effects of five different levels of low molecular weight chito-oligosaccharides (LMW-COS) (0, 0.1, 0.2, 0.4, and 0.8 g kg^{-1}) on growth performance, serum parameters, body composition, and non-specific immunity in Nile tilapia (*Oreochromis niloticus*). The results showed that dietary supplementation with 0.4 or 0.8 g kg^{-1} COS significantly improved the final body weight, specific growth rate, feed efficiency rate, and protein efficiency ratio of fish ($P < 0.05$).

In 2016, El-Gawad et al. fed experimental diets containing different levels (0, 1, 2, and 3%) of fructo-oligosaccharide (FOS) for 6 weeks to investigate its effect on the antioxidant activity, non-specific immunity and growth performance of Nile tilapia. The results indicated that dietary FOS supplementation could significantly enhance the antioxidant activities, non-specific immune response and growth performance of *Oreochromis niloticus*.

Widanarni et al. (2018) aimed to determine the effect of dietary mannan-oligosaccharides (MOS) delivered through *Artemia* sp. at different concentrations (i.e. 0, 3, 6 and 12 mg L^{-1}) on the survival, growth, immunity, digestive enzymes and resistance against *Vibrio harveyi* of Pacific white shrimp. the present study demonstrated that dietary MOS supplementation through *Artemia* enrichment at the concentration up to 12 mg MOS L^{-1} could significantly improve Pacific white shrimp post larval digestive enzymes activities, growth, survival and resistance against *V. harveyi* infection.

2.8 Influence of prebiotic on probiotic

A large number of studies have combined probiotics with prebiotics, a selectively fermented ingredient that allows specific changes both in the composition and activity of the gastrointestinal microflora that confers benefits upon host well-being and health (Zhou and Wang, 2012).

Akhter mentioned that a variety of useful feed additives, including probiotics and prebiotics having beneficial effects to the host was used in aquaculture to combat diseases such as supplements, to improve growth include increasing the size and weight gain, and in some cases, act as an alternative antimicrobial compounds, as well as to stimulate immunity response of the host. Probiotics live in microbial feed additives that modulate gastrointestinal microbial communities whereas Prebiotics, non-digestible forage additives stimulate the activity or abundance of beneficial gastrointestinal bacteria. Both of these have received

widespread attention, showing the improved production, health and disease resistance of aquatic animals (Akhter et al., 2015).

2.9 Effect of synbiotic

As per Cerezeula et al. 2011, synbiotics significantly effect on fish immune system considering a number of activities such as lysozyme activity, phagocytic activity, alternative complement pathway, respiratory burst, mucus production and superoxide dismutase activity. Lysozyme is the vital bactericidal enzymes of innate immunity, and shows a crucial defense mechanism against various pathogens in fish (Lindsay, 1986). The efficiency of synbiotic therapy in terms of protection against infectious agents could be assessed by a challenge test because of its controlling power on pathogens and disease resistance capability (Cerezuela et al., 2011).

Dehaghani conducted experiment on *Enterococcus faecium* bacteria with fructo-oligosaccharide, which are available in synbiotic Biomin IMBO and concluded that they have increased the digestibility and absorption of feed and consequently the SGR by enhancing the activities of digestive enzymes and their secretion. *E. faecium* bacteria are capable of creating dominant bacterial flora in guts of experimental carp and consequently LAB are also increased and experimental treatments which are influenced by this bacteria have obviously demonstrated the increasing activity of trypsin and chymotrypsin enzymes (Dehaghani et al., 2015).

There have beneficial effects of the combined supplementation of *Bacillus subtilis* and MOS as synbiotic on growth performance, body composition, digestive enzyme activity and intestinal microbiota of *Cirrhinus mrigala* fingerlings by kumar (Kumar et al., 2018).

Hassaan et al. clearly indicated that the supplementation of *B. licheniformis* not only enhanced the growth performance and feed utilization of Nile tilapia, but also hematological and biochemical blood parameters (Hassaan et al., 2014).

Dehaghani experimented on the body analysis, showed the percentages of body protein and lipid in fish fed with synbiotics was significantly higher than that from the control fish whereas the percentage of ash content was not. Synbiotic diet displayed improved growth performance, including weight gain, SGR, FCR and CF. Furthermore, roach fed 1.0g and 2.0g synbiotics diet had significantly higher survival compared to the control (Dehaghani et al., 2015).

Kumar et al. (2018) evaluated effects of dietary symbiotic in terms of changes in innate immunity, antioxidant activity and disease resistance against *Aeromonas hydrophilla* infection. *Bacillus subtilis* used as a probiotic source and MOS used as a prebiotic source in the experiment. The results of this study showed that under the experimental conditions, dietary supplementation of synbiotic had a synergestic effect on enhancing innate immunity and disease resistance of *Cirrhinus mrigala* ($P < 0.05$).

In 2016, Azevedo et al. investigated the supplementation of prebiotic (Mannan oligosaccharide – MOS, from yeast *Saccharomyces cerevisiae*), probiotic (*Bacillus subtilis* – BS, C-3102 strain) and their combination in diets for Nile tilapia. Fishes fed diets pre-, pro- and synbiotic supplemented performed better in average daily gain, feed conversion rate, specific growth rate, protein efficiency ratio, carcass yield, total and standard length and body height than those maintained on control diets. The results of this study indicates that the mannan oligosaccharide and *Bacillus subtilis* supplementation, isolated or combined (synbiotic), can improve growth, body index, intestine morphometry and carcass composition in Nile tilapia.

In 2014, Eleraky et al. evaluated the effect of prebiotics and probiotics on the growth performance, non-specific immunity and chemical composition of *Cyprinus carpio*. The study revealed that a dietary supplementation 2.5 g/kg prebiotics improves growth performance, and nonspecific immunity of *Cyprinus carpio* fry.

Mehrabi et al. (2018) examined the effects of Immunowall® prebiotic and Primalac® probitic on common carp (*Cyprinus carpio*) fry from the Caspian Sea. BWG, SGR, TL, and survival rate were significantly influenced in the fish fed 1.5 g of mixed Immunowall® and Primalac® ($P<0.05$). The experimental carp fry displayed marked improvements in some of carcass qualities ($P<0.05$). Also, the fry fed Primalac® showed greater white blood cell (WBC) counts than the control group ($P<0.05$). The positive effects of Primalac®, and to a lesser extent, Immunowall®, or their mixture on the parameters examined in the carp fry signify improved growth performance, enhanced body composition, and stimulated fish immune system.

Salih and Mustafa (2017) studied to assess the efficiency of synbiotic on hematological, histopathological changes and resistance against *Saprolegnia spp.* in *Cyprinus carpio*. The results indicated that supplementation of dietary synbiotic at 2.0 % in diet are more resistant against infection by *Saprolegnia spp.* Therefore, dietary treatment using probiotic and

prebiotic combination have a positive influence on the survival rate and disease resistance in *C. carpio.*

Toutou et al. (2016) investigated the effect of probiotic (yeast *Saccharomyces cerevisiae, Bacillus subtilis* and *Bacillus cereus*) and synbiotic (Microban aqua®) on growth performance, nutrient utilization, biochemical composition, blood parameters, gut pathogens and stress response of the fingerlings of grass carp *Ctenopharyngodon idella.* Results indicated an enhancement in growth and feed utilization for all fish groups fed by probiotic followed by synbiotic compared with the control group. The results indicate the effectiveness of food supplementation with Probiotic and Synbiotic in fish diet with the preference of probiotic to improve the growth Performance and Healthy Status of fishes particularly Grass Carp, *Ctenopharyngodon idella.*

In 2017, Safari et al. evaluated the effects of prebiotics (mannanoligosaccharide and xylooligosaccharide), probiotics (*Enterococcus faecalis* and *Pediococcus acidilactici*) and synbiotics on the immune responses, hemolymph indices, antioxidant enzymes, and biological responses after a 48- hour *Aeromonas hydrophila* exposure of sub-adult crayfish. Feeding crayfish a (xylooligosaccharide + *E. faecalis*) diet increased the growth rate and the resistance to the *A. hydrophila*-injection challenge ($p < 0.05$). These results revealed that feeding crayfish with synbiotic diets are more effective than a single administration with prebiotics and probiotics.

In 2016, the effects of dietary synbiotics was evaluated by Chitsaz et al. on growth performance, survival, stress resistance, body composition and immune response in the Caspian roach (*Rutilus rutilus*). Immune responses (Ig levels, lysozyme activity and ACH50) were significantly higher in 2 g kg-1 synbiotics fed fish ($p<0.05$). The intestinal tract of the fish with synbiotic diet supplementation had higher concentrations of lactic acid bacteria (7.13 ± 0.32 log CFU g^{-1}). The protein and lipid contents in the whole body increased in the 2 g kg^{-1} synbiotics fed group. At the end of experiment the fish fed synbiotics had the highest survival index after 40 hours exposure to salinity stress (13.8 ppt).

Wang et al. (2017) investigated the effects of different dietary additives [w/w: 2% small peptides, 0.01% probiotics (*Bacillus licheniformis*) and 0.2% prebiotics (inulin)] on growth performance, digestive enzyme activities, and oxidative stress in juvenile *Epinephelus coioides* reared in artificial seawater. Overall, the three types of additives improve growth

rate of juvenile grouper and digestive enzymes activities to varying degrees but do not effectively improve antioxidant capacity under low-salinity stress conditions.

In 2019, Sewaka et al. evaluated the effect of supplementing the diet of red tilapia *(Oreochromis* spp.) with Jerusalem artichoke (*Helianthus tuberosus*) and *Lactobacillus rhamnosus* GG (LGG). Growth performance, serum biochemical parameters, intestinal morphology, goblet cell counts, immune parameters and protection against *Aeromonas veronii* challenge were determined. The results showed that fish fed with synbiotic-supplemented diets has a significantly higher ($P< 0.05$) feed conversion ratio (FCR), specific growth rate (SGR), and average daily gain (ADG) than fish fed with a control diet. The synbiotic-supplemented diet increase glucose, total protein and the total cholesterol levels. Fish fed the synbiotic-supplemented diets also exhibit significantly higher ($P < 0.05$) lysozyme activity. The cumulative mortalities of fish fed with a synbiotic-supplemented diet are significantly lower than those of fish fed other diets.

Alak and Hisar (2012) studied the effects of probiotic and/or prebiotic on intestinal flora used in raising rainbow trout. Feed mixtures including the probiotic caused a 1 logarithmic unit increase in the number of lactic acid bacteria. The number of enterobacteriaceae was found to be at a lower level in the fish fed with the probiotic, including feed compared to control and prebiotic groups.

González-Félix et al. (2018) evaluated the growth, innate immune responses, and transient intestinal microbiota and histology of *Totoaba macdonaldi* after being fed commercial prebiotic and probiotic supplements singularly and in combination. Histometric analysis of the gastrointestinal tract revealed that fold length in the proximal section was significantly larger ($P = 0.0045$) in fish fed the diet containing the probiotic (435.6 μm), compared to those of fish fed the basal diet (382.3 μm). Results showed different bacterial clustering patterns between the basal diet and the diets with additives (prebiotic, probiotic, pre + pro). Initial weight of totoaba, or the length of the trial, could partly explain the absence of growth differences among treatments. From these results, it was evident that studies with probiotics and prebiotics fed to sturdy fish like totoaba will benefit from including a challenge experiment in order to assess their potential role in preventing infections.

In an another study in 2015, Azevedo et al. evaluated economically the inclusion of prebiotics, probiotics and symbiotics in diets of Nile tilapia, at two stocking densities. Total feed consumption (FC), final biomass (FB), relative gain in biomass (RGB), apparent feed

conversion (AFC), survival rate (SUR), average cost of feed per kg of live weight gain (ACF), total cost of feed (TCF), total cost of production (TCP), gross income (GI), operating profit (OP) and economic efficiency index (EEI) were determined. There was no significant influence from the inclusion of prebiotics, probiotics and symbiotics on FC, SUR and TCF, however there was an influence ($p<0.05$) on the parameters FB, RGB, AFC, ACF, TCP, GI and OP. The control diet at the higher density displayed the worst EEI. The best EEI was obtained by fish at the lower density which received feed with added probiotics and synbiotics.

In 2013, Addo studied the Effects of pre- and probiotics on pond production, growth and disease susceptibility of channel catfish *Ictalurus punctatus* and Nile tilapia *Oreochromis niloticus*. He found that the prebiotic and probiotic strains used as water or feed additives in combination or individually effectively reduce disease mortality in channel catfish and Nile tilapia under laboratory conditions.

2.10 Effect on Growth performances, Survival rate, and Feed utilization

In rainbow trout, administration of *Enterococcus faecalis* and mannanoligosaccharides / polyhydroxybutyrate acid for 12 weeks not affects the survival rate of fish as well as the experimental fish was in good condition and there was no mortality during the feeding trial (Cerezuela et al., 2011). According to Ai et al. (2011) and Geng et al. (2011) administration of *Bacillus subtillis*/ fructooligosaccharides in yellow croaker or *Bacillus subtillis*/chitosan in cobia, do not affect the survival rate, showing no changes among different dietary treatments. In case of Japanese flounder nourishing with *Bacillus clausii* and mannanoligosaccharides/ fructooligosaccharides, in which fish retain active ingestion, show proper growth and survival for all time (Ye et al., 2011).

Administration of *E. faecalis* and MOS/PHB in rainbow trout for 12 weeks not affects the survival rate of fish (Rodriguez-Estrada et al., 2009). In case of growth parameters, almost all references stated a positive effect of synbiotics application. In case of rainbow trout, dietary mannan-oligosaccharides combined with *Enterococcus faecalis* significantly enhanced growth performance and nutrient utilization of fish, on the other hand, combination of *Enterococcus faecalis* and polyhydroxybutyrate acid developed growth performance of rainbow trout to some extent (Cerezuela et al., 2011).

However, in case of rainbow trout fingerlings, all synbiotic treatments test fishes were survived significantly in higher rate toward the end of experimental period compared to those from the control. The highest average of survival rate was observed in the T2 that was statistically different from T1, T3 and the control groups (Mehrabi et al., 2012). In yellow croaker and cobia, administration of *B. subtillis*/FOS or *B. subtillis*/chitosan respectively, not affect the survival rate, with no differences among different dietary treatments (Ai et al., 2011).

In terms of growth parameters, almost all references reported a positive effect of synbiotics on any parameter. Rainbow trout fed with diets M, EM and EMP recorded significantly higher weigh gain rate (WGR) and specific growth rate (SGR) than those of the rest experimental groups. Differences among experimental groups E and P were not significant with respect to the control group, whereas fish fed the EP diet showed significant better growth and SGR. In general, major effects were observed in EM and EMP, with no differences between the two groups (Rodriguez-Estrada et al., 2009).

The study of Mehrabi et al. (2012) showed that after 60 days groups fed diets containing different levels of synbiotics (0.5, 1.0 and 1.5) increased body weight gain about 50, 59 and 53%, respectively, in comparison with the control group. The addition of synbiotic to the feed also produced the better SGR, FCR and conversion factor (CF) with values significantly higher than the control, more specifically in groups treated with 0.1% synbiotic. In the study, significant better growth performance was observed in *O. mykiss* fingerlings maintained on the diet supplemented with synbiotic.

2.11 Effect on phenotypic trait

Beak studied that lactic acid bacteria (LAB) probiotics showed a significant elevation of skin mucus lysozyme activity of flounder fed *Lactobacillus plantarum* FGL0001 alone or a mixture of *Lactobacillus plantarum* FGL0001 and *Lactococcus lactis* BFE920 has much higher than that of the flounder fed only *Lactococcus lactis* BFE920 (Beck et al., 2015).

Another study showed that total protein content significantly increased when the probiotic was applied firstly, and then further increasing the amount of probiotic would decrease the protein level (Hassaan et al., 2014).

The addition of synbiotic to the feed has shown better specific growth rate (SGR), food conversion ratio (FCR) and conversion factor (CF) with values significantly higher than those of the control (Kumar et al., 2018).

Hughes et al. (2005) investigated Genetic and environmental effects on secondary sex traits in guppies (*Poecilia reticulata*). Male guppies (*Poecilia reticulata*) exhibit extreme phenotypic and genetic variability for several traits that are important to male fitness and several lines of evidence suggest that resource level affects phenotypic expression of these traits in nature. They also found that male colour pattern elements had nearly an order of magnitude more genetic variation than did male size.

In 2012, Devigili et al. studied expression of pre- and postcopulatory traits under different dietary conditions in guppies. The results revealed that diet manipulations influence the expression of both pre-copulatory (sexual behavior and ornamentation) and post-copulatory sexually selected traits (sperm viability), reinforcing the importance of resource acquisition in sexual selection.

Olsson and Eklöv (2005) studied on the contribution of habitat structure and feeding mode to morphological divergence within Eurasian perch (*Perca fluviatilis* L.). The result indicated that both habitat structure and feeding mode contribute to morphological divergence (40.7% and 4.9% of the total variation respectively), which suggests that both habitat complexity and prey type diversity influence morphological adaptations in this fish species.

In 2013, Kapusta et al. studied to determine the impact of diet and culture conditions on the morphometric features of crucian carp (*Carassius carassius*), which is a species characterized by a highly variable body shape. Feed type and culture conditions significantly impacted the shape and the morphology of crucian carp. Fish-fed larval chironomids exhibited the least variation in biometric characters, while those fed high-energy-formulated feed exhibited the greatest variation. The body depth index of fish fed different feeds or cultured under controlled conditions did not differ significantly in comparison to wild crucian carp. The phenotypes of the cultured fish from the two experiments were similar to those of individuals inhabiting the natural environment.

Adro'i et al. observed the phenotypic variation of guppy fish population in 2018 living in several aquatic environments with different quality levels in Surabaya, Indonesia. The results of this study consist of data on phenotype structure and phenogram tree. The phenotypic

character of guppy fish that have different values between sites is body length, body area, the relative area of structural color, and relative area of carotenoid color.

Choubert et al. (2009) studied to determine the effects of the use of astaxanthin alternate feeding on rainbow trout pigmentation in term of astaxanthin serum concentration, muscle colour and astaxanthin muscle retention. There were no significant differences among fish fed the different feeding schedules in term of final mean weight, specific growth rate and feed efficiency ratio. Muscle chroma showed the most pronounced effect. It increased significantly for all fish groups during the experiment. Muscle astaxanthin concentrations increased significantly during the experiment whatever the astaxanthin feeding schedule.

Yanar et al. (2007) investigated the effects of carotenoid sources on pigmentation, sensory properties and fatty acid composition of rainbow trout (*Onchorhynchus mykiss*). Dietary carotenoid sources do not significantly affect fatty acid composition of the fish fillets. Trout muscle colored with commercial astaxanthin are more preferred than the others by the sensory panellists.

In 2010, Yasir and Qin evaluated the role of supplemented dietary carotenoids in regulating the skin color and pigments of the false clownfish, *Amphiprion ocellaris*. This study suggests that astaxanthin has the potential to enhance the red hue on clownfish skin and its withdrawal from the diet do not fade the red hue of the skin.

Cejas et al. (2003) conducted an experiment on red porgy alevins to investigate the influence of dietary supplementation with shrimp on pigmentation and lipid composition of carcass (muscle and skin) and eyes. They conclude that the natural carotenoids supplied by the shrimp are effectively assimilated by the red porgy and allow the cultured fish to acquire skin coloration similar to that of wild fish.

Again, Kalinowski et al. (2005) conducted the study to evaluate the effect of diet supplementation with two carotenoid sources, at two different concentrations, on growth and skin coloration. The results of this study suggests that the inclusion of astaxanthin, from shrimp shell meal, in red porgy commercial diets significantly improves skin coloration and markedly enhances the commercial value of this cultured species.

In 2011, Zaťková et al. experimented the effect of carotenoid-rich microalgal biomass as feed supplement to increase the coloration of a freshwater ornamental fish species – an albinic

form of wels catfish (*Silurus glanis*). In the treatment groups fed with Algadiets, the specific growth rate and physical condition index (weight/length) increased by 11– 58% and 6–26%, respectively, compared to the control. An intensive yellow or yellow–orange colouration appeared in all Alga-diet fed fish groups because colour saturation increased 1.4–2.4 times within 20–40 days of the trial. A change from an albinic to an intensive golden colour was visible as the shorter-wavelength shift from 595– 607 to 584–586 nm.

2.12 Effect on enzyme activity

Administration of synbiotics may effect on enzymes of fish digestive system. The increase in digestive enzyme activities would permit the host degrades more nutrients, enhancing digestion and promoting a probable increase in the weight gain rate and/or feed efficiency (Cerezuela et al., 2011).

Ghosh stated that there has a benefit of such supplements (probiotics) including improved feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, anti-mutagenic and anti-carcinogenic activity, growth-promoting factors, and increased immune response (Ghosh et al., 2013). Large amounts of Alanine aminotransferase (ALT) and aminotransferase (AST) are released into animal blood, mostly during liver cell damage by the influence of probiotic (Hassaan et al., 2014).

Kumar mentioned that digestive enzyme activity are high in the synbiotic fed groups, a significantly higher amylase and protease activity had been found in (high probiotic + high prebiotic) group followed by the medium and low groups as compared to control. But there have no significant difference for lipase activity among the synbiotic fed groups (Kumar et al., 2018).

The study of Ye et al. in 2011 evaluated the activities of amylase and protease which found that protease activity was significant higher in fish fed diets BM and BFM than in fish fed the control diet, obtaining similar value in both diets. No any other supplementation could positively affect the protease activity. These results indicated that administration of FOS in combination with MOS and *B. clausii* not determine a major increase in protease activity than the observed with the BM diet. The increase in digestive enzyme activities would allow the host degrades more nutrients, improving digestion and promoting a possible increase in the WGR and/or feed efficiency.

Afrilasari and Meryandini (2016) studied to analyze the effect of *Bacillus megaterium* on the population of intestinal microflora, digestive enzyme activity, and the growth of catfish. The activity of protease and amylase enzymes, and specific growth rate in normal plus treatment were significantly higher ($p < 0.05$) than other treatments. *Bacillus megaterium* increased the activity of digestive enzymes and the growth of catfish.

2.13 About the present study

From the above review of previous literature, it is clear that a few studies have been conducted on the effect of synbiotic on growth performance, several phenotypic traits as well as on the activity of enzyme activities of monosex tilapia. But no specific study has been conducted to explore whether they show pronounced compensatory growth and expression of some important phenotypic traits and changes in enzyme activities when the synbiotic is added to tilapia culture. So, there has a knowledge gap issue on that topic. So, the present study was conducted to mitigate the knowledge gap according to the specific methodology.

Chapter- 3. Material and methods

3. Materials and method

3.1 Sample collection

A total of 360 monosex tilapia were collected from BRAC hatchery, Dumuria, Khulna, Bangladesh. The age of randomly collected fingerlings was 30 days. The mean total lengths and average body weights of fingerlings were 4.129 ± 0.323 cm and 1.15 ± 0.19 mg, respectively. The collected fishes were transported carefully in oxygenated plastic bags to avoid any injury to the Fish Physiology laboratory, Fisheries and Marine Resource Technology (FMRT) Discipline, Khulna University.

3.2 Fish acclimatization

The fingerlings were acclimatized for seven days at a room temperature in glass tanks (50cm×29cm×30cm). Commercial fish feed was used during the acclimatization period of fish. Prior to acclimatization tanks were washed with freshwater and fishes were dipped into $KMnO_4$ (5 ppm) to prevent introduction of any diseases into the experimental area. Then the apparently healthy and disease free fishes were sorted. Water exchange (60% every day) and removal of fecal matter were done every day from the continuously aerated acclimatization tanks.

3.3 Experimental set up

The pictorial presentation of the experimental set up has been given to the Table 1:

Groups	Description of group	Replication	Stocking density
Control	Only feed	3	30
T_1	1 gm of synbiotic/kg of feed	3	30
T_2	2 gm of synbiotic/kg of feed	3	30
T_3	3 gm of synbiotic/kg of feed	3	30

The experiment was carried out over a period of 45 days in glass tanks. Twelve tanks each of 1500 cm^2 size were used for this trial. Water level in each tank was maintained 20 cm throughout the experimental period by exchanging freshwater from a tube-well. For the convenience of the study, the tank were numbered as 1,2,3,4,5,6,7,8,9,10,11,12. The tanks were divided into four treatments namely C, T_1, T_2, T_3; each having three replicates. Four different feeds namely feed C, feed D_1, feed D_2, feed D_3 were applied to the treatment C, T_1, T_2, T_3, respectively. Acclimatized fish fry were randomly distributed to the rate of 30

individuals /tank. Fish during the experimental period were fed twice daily. Feeds were the commercial pelleted floating feed named 'Ruposhibangla feed' collected from 'Chowa agro products ltd'. Feeds D_1, D_2, D_3 were prepared with the combination of 1gm, 2gm and 3gm synbiotics with per kg of feed, respectively. Randomized block design is applied to conduct the experiment and make the experiment easier.

3.4 Synbiotics

The trade name of the used synbiotics was Power Lac. The mixture of probiotic, *Lactococcus lactis* D18113 ($>1\times10^{11}$ CFU/ Kg) and prebiotic, $CaCO_3$ + starch QS2 (1 Kg) were used as the synbiotic composition in the study which was manufactured by 'Kyushu Medical Co., Ltd., Japan' and marketed in Bangladesh by 'ACI Animal Health'.

3.5 Doses of probiotic

The probiotic was given as a mixture with feed and there was a juice which act a binder for probiotic and feed. The feed was dried after the introduction of probiotic and binder gel 'ACP Vita Fish Gel' manufactured by 'ACP Agro Science Ltd' at least 30 min in room temperature. One caution was that the prepared feed or mixture of the feed and synbiotic could be used only one day after mixing. The mixture of the diet should not be dried on sunlight.

3.6 Determination of growth performance

Growth performance was determined by measuring final weight of experimental fishes. Weight of each fishes was measured two times, before starting the experiment and after experiment. Firstly, each male was anaesthetized with MS222 and its body surface was gently dried with blotting paper. The male was then weighted on an electric balance and recorded the data. Then the data was analyzed with MiniTab.

3.8 Measurements of phenotypic traits

After forty five days of the experiment, some selected phenotypic traits were studied to explore whether they were affected by different doses of synbiotics or not. The selected phenotypes were total length (TL), standard length (SL), trunk length, head length, tail length, body area (BA), body depth, eye diameter, dressing percentage (DP) and color patterns. At first, fish was placed on a graph paper together with its ID and photographed under standard lighting (two 13W fluorescent bench lamps) against a measurement scale on a white background using a digital camera (Canon 700d with Nikon 18-135mm macro lens) from a distance of 30 cm. Each image included a unique code so that subsequent analyses of

male traits were performed blind of treatment. The raw uncompressed images (NEF) were converted to TIFF files and imported into *ImageJ* software (http://rsbweb.nih.gov/ij/) for the measurement of total length (the distance in cm from the tip of the snout to the tip of the longer lobe of the caudal fin, Figure 1), standard length (the distance in cm from the male's snout to the tip of his caudal peduncle, Figure 2), head length (Figure 3), trunk length (the distance in cm from the head to caudal peduncle of fish, Figure 4), eye diameter [in cm], see Figure 5), tail length (the distance in cm from the fish caudal peduncle to the end of the tail, Figure 6), body depth (Figure 7) and the body area (the area [in cm^2] of the body excluding all fins and tail, Figure 8). Moreover, the dressing percentage was calculated by the equation of-

Dressing percentage = (trunk length/total length) * 100.

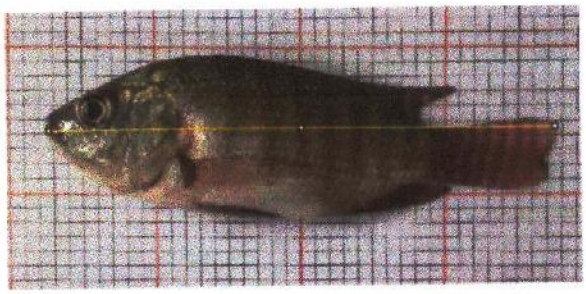

Figure 1: Measurement of total length (cm).

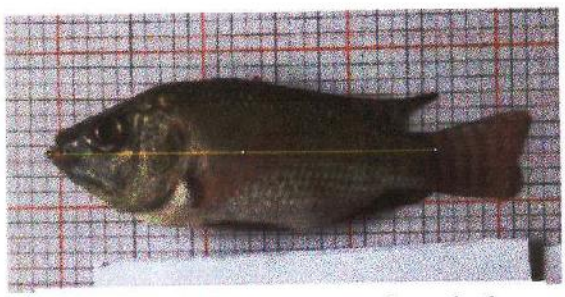

Figure 2: Measurement of standard length (cm).

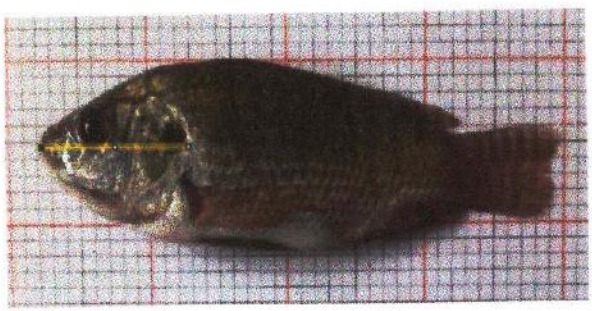

Figure 3: Measurement of head length (cm).

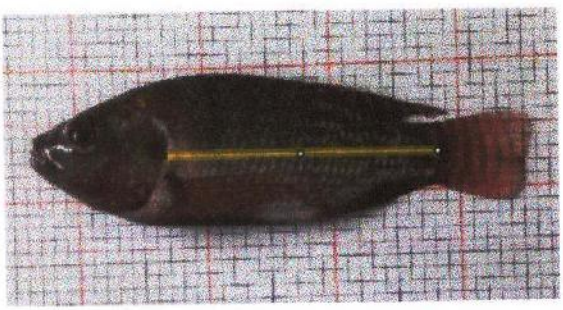

Figure 4: Measurement of trunk length (cm).

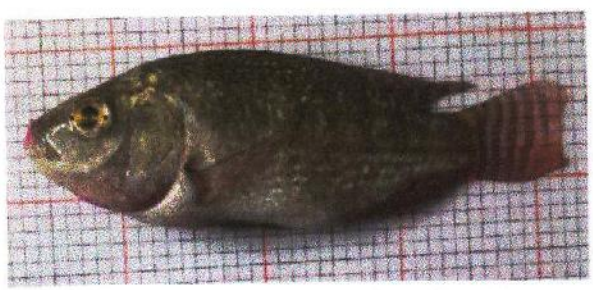

Figure 5: Measurement of eye diameter (cm).

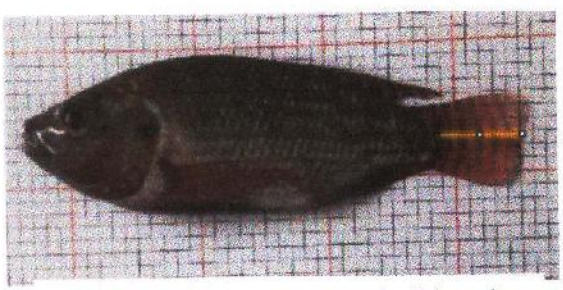

Figure 6: Measurement of tail length (cm).

3.10 Reagent preparation

By measuring with a cylindrical flask, 100 ml of distilled water was taken in a 500 ml conical flask. 0.285 g of Tris-HCl and 0.111 g of EDTA-Na_2 were weighted on an electric balance and poured in the flask to make a solution. Then, the solution was stirred for few minutes with the magnetic power until the chemicals were dissolved. It is used as mother solution in this experiment.

After preparing Tris- EDTA buffer, 50ml was taken from there into an another conical flask and 0.5 g starch (Merk, Darmstadt, Germany) was measured through an electric balance and put into that flask. Until the chemicals were dissolved, it was stirred with magnetic power. It was used as a substrate to analyze amylase activity.

In 100ml distilled water, 2.189 g DNS (Loba Chemie Pvt. Ltd) was given. Then, it was placed on hot plate for giving heat with rotation by the magnetic power. After few minutes, it was turned into yellow color with 0.96 mM. It is used as a stop solution in amylase activity assay.

Casein (10 mg/ml) was dissolved in 0.1 M Tris- EDTA buffer at pH 8. That was used to measure protease activity according to Casein method.

In 100ml of distilled water, 1.79729 g TCA was given to make it 110mM. It was performed a role as stop solution in protease activity assay.

3.11 Preparation of crude enzyme

After measuring weight and taking images of fishes, samples were rinsed with distilled water and kept into transparent square shaped polybags (15 cm x 15 cm) with labeling was stored at -80°C until enzyme assayed. From each replication tank, three samples were taken individually for preparation of crude enzyme. By using tissue paper, excess water of fishes was removed. Then, the total digestive tract and muscle were taken from each fish and weighted in electronic balance. Then, the whole muscle and intestine were kept in the falcon tube separately and tissue of 0.5 g was homogenized with 50 ml of 0.1 M tris EDTA-buffer, pH 8.0, at 2-4° C with high speed laboratory homogenizer. Then, the samples were homogenized by a Wise Tis Homogenizer with 800-1000 rpm for five minutes with ice. After completing homogenization, the crude solution was kept in eppendorf tube and placed in 4 °C immediately. Then the sample was centrifuged in 12000 rpm for 10 min at 4 °C according to the method of Lovette and Felder (1990). The supernatant was separated from the pellet portion by micro pipette and stored at -80 °C until further analysis.

3.12 Metabolic activity assay

3.12.1 Determination of amylase activity

The amylase activity was assayed according to dinitrosalicylic acid (DNS) method (Bernfeld, 1955). In this procedure, 1% soluble starch was used as substrate. 200µl of crude enzyme from each sample was taken in two eppendorf tubes and added 200µl soluble starch into one of them and another one was filled with 200µl of Tris- EDTA buffer. Then, it was incubated for 30min at 35°C. After that, 100 µl of DNS solution was added into both of them and heated for 10 min at 70°C to stop the reaction. 3, 5-dinitrosalicylic acid is a color reagent that the reducing groups released from starch by amylase action were measured by the reaction. Then it was centrifuged in 12000 rpm for 10 min at 4°C. Then the sample was taken into the cuvette of a double beam spectrophotometer (HITACHI, U-2910, Japan) and absorbance was read at 540 nm after centrifuging and enzyme activities were measured as the change in absorbance. One unit of amylase activity was defined as the amount of enzyme required to produce 1µg maltose in 1 min at 35°C. In the same way, the absorbance was read at 540 nm against a blank consisting of the same assay mixture without substrate. The absorbance of blank was subtracted from the absorbance of substrate. A calibration was carried out using maltose under the same condition. The calibration mixture consists of 200 µl distilled water, 200 µl Tris-HCL buffer and 20-100 (µg/ml) of maltose.

3.12.2 Determination of protease activity

Protease activity was measured according to the Casein method (Walter, 1984). The amount of 200µl of crude sample from each replication was taken into an individual eppendorf tube. Then, it was incubated for 30min at 35°C with 200µl soluble casein. Casein was used as a substrate in this experiment. After that, the reaction was stopped by addition of 200µl 110mM TCA. Next this mixture was centrifuged in 12000 rpm at 4°C for 10min. Then it was taken into a cuvette of the double beam spectrophotometer (HITACHI, U-2910, Japan) and the absorbance was read at 280 nm after centrifuging. Enzymes activities were measured as the change in absorbance. One unit of protease activity was defined as 1 µg tyrosine produced within 1min from 1mg crude enzyme. After a further incubation for 30 min at 35°C, the absorbance was read at 280 nm against a blank consisting of the same assay mixture without substrate. Then the absorbance of blank was subtracted from the absorbance of

mixture with substrate. A calibration was carried out using tyrosine under the same condition. The calibration mixture consists of 200 µl distilled water, 200 µl Tris- HCl buffer and 20-100 (µg/ml) of tyrosine.

3.13 Statistical analyses

All types of statistical analysis were carried out with statistical software MINITAB, Version 17 (Minitab, 2014). One-way ANOVA test for growth and enzyme activities were done after confirming the normality and homogeneity of the data. All the data sets were normally distributed. Tests for normality of the data were done according to Anderson and Darling (1954). Test for Homogeneity of Variances was done based on Levene (1960). Post-hoc analysis of the means were accomplished with Tukey's HSD (Honest Significant Difference) test (Tukey, 1949). Count data of the colour patterns were analyzed with Pearson Chi-square test of association/independence.

that the standard length of control fishes was significantly different from T_1, T_2 and T_3 ($P<0.05$). Consequently, there was no significant variation among T_1, T_2 and T_3 (figure 14).

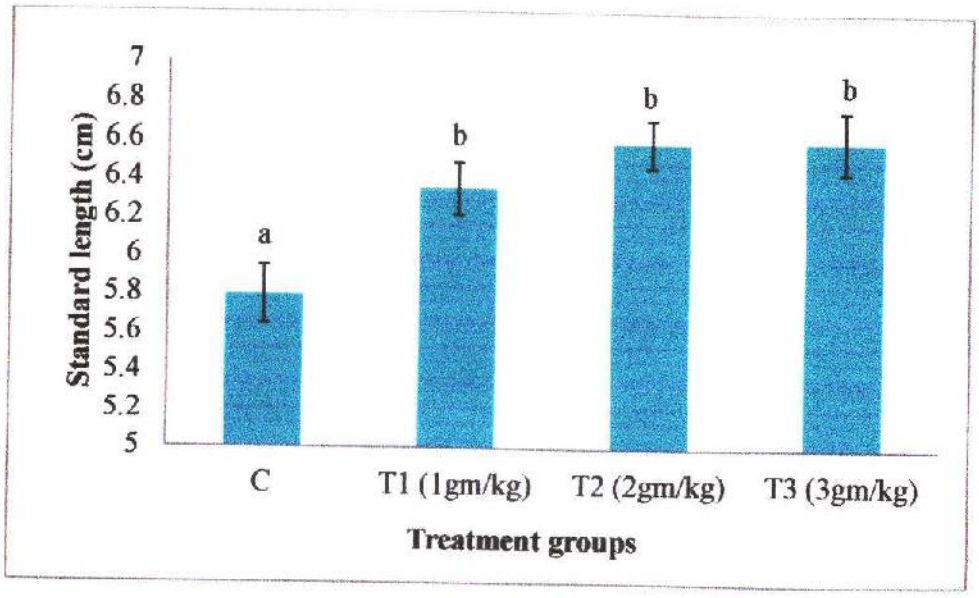

Figure 14: Changes in standard length (mean ± SE) of monosex tilapia under synbiotic treatments.

4.2.3 Head Length

Firstly, the head length had no significant variation among the treatment groups which data has not shown in the graph. After 45 days, the model was revealed highly significant difference in head length among the treatment groups ($F_{3, 179} = 5.48$, $P<0.05$).The subsequent post-hoc test showed that control group had significant lower head length than other treatments. The Tukey test resulted that C was significantly differed from T_2 and T_3, however, no significant difference was found between $T_1$and C, and also, between T_1 and other two synbiotic treatment groups (figure 15).

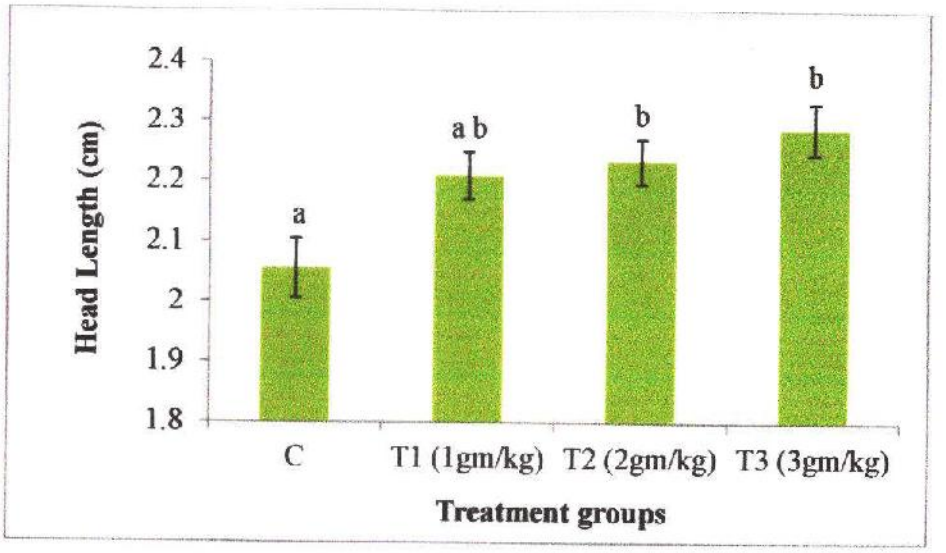

Figure 15: Effects on head length (mean ± SE) of monosex tilapia under synbiotic treatments.

4.2.4 Trunk length

The initial data of trunk length differed not significantly of which has not mentioned in the graph. Finally, the experiment had revealed highly significant variation among the treatments ($F_{3, 179} = 7.02$, $P<0.05$). The subsequent post-hoc test was shown that the trunk length of control fishes was significantly different from T_1, T_2 and T_3 ($P<0.05$). Consequently, there was no significant variation among T_1, T_2 and T_3 (figure 16).

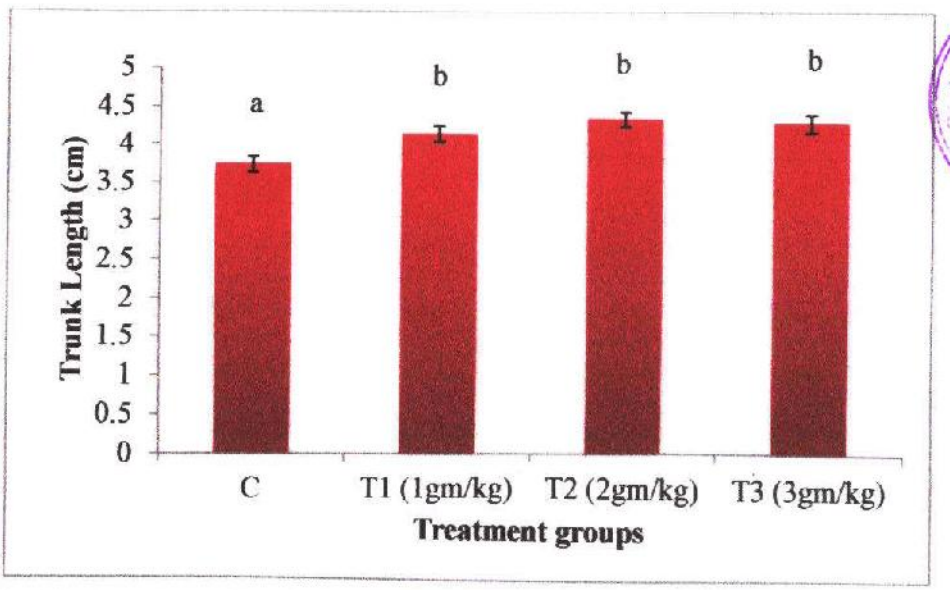

Figure 16: Effects on trunk length (mean ± SE) of monosex tilapia under synbiotic treatments.

4.2.5 Tail length

The tail length revealed no significant difference at the beginning of experiment (data not shown). At the end of experiment, the post hoc test also showed no significant difference ($F_{3, 179} = 2.77$, $P>0.05$) between control and treatments as well as among treatment groups (figure 17).

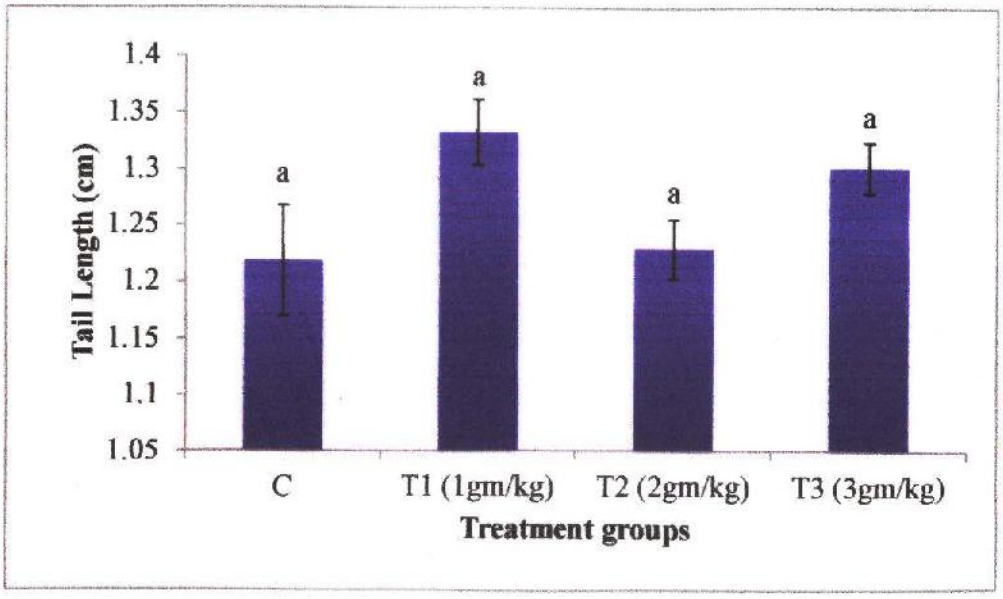

Figure 17: Effects of synbiotic on tail length (mean ± SE) of monosex tilapia.

4.2.6 Eye diameter

At the starting of the experiment, the eye diameter did not vary significantly of which data has not shown. But the final data of the experiment had revealed highly significant variation in eye diameter among the treatments ($F_{3, 179} = 3.33$, $P<0.05$). The subsequent post-hoc test was shown that the eye diameter of control fishes was significantly different from T_2 and T_3 ($P<0.05$). But, there had no significant variation among the T_1, T_2 and T_3 groups (figure 18).

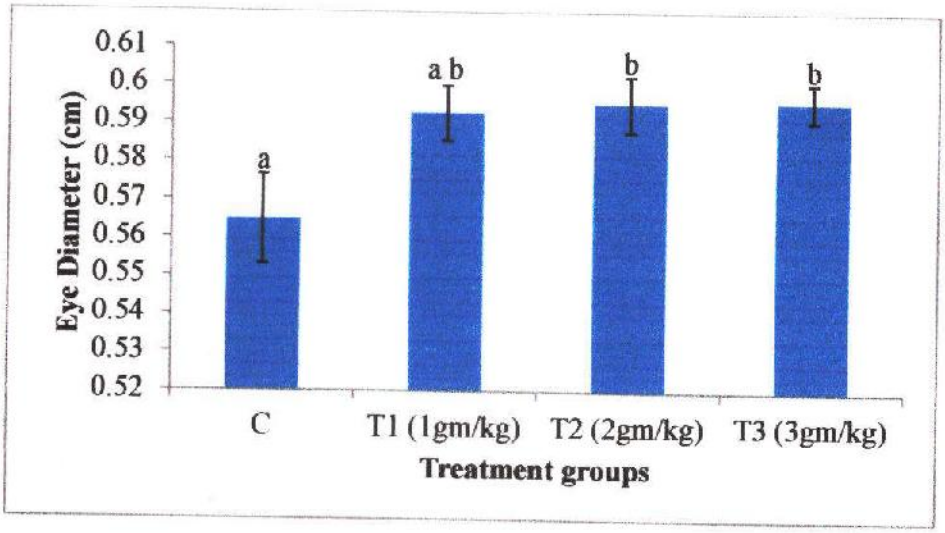

Figure 18: Effects of synbiotic on eye diameter (mean ± SE) of monosex tilapia.

4.2.7 Body depth

The body depth of 1st day had no significant difference among the treatment groups (data not shown). At 45 days, the experiment had revealed highly significant variation among the treatments ($F_{3, 179} = 6.78$, $P<0.05$). The subsequent post-hoc test was shown that the body depth of control fishes was significantly different from T_1, T_2 and T_3 ($P<0.05$). Consequently, there was no significant variation among T_1, T_2 and T_3 (figure 19).

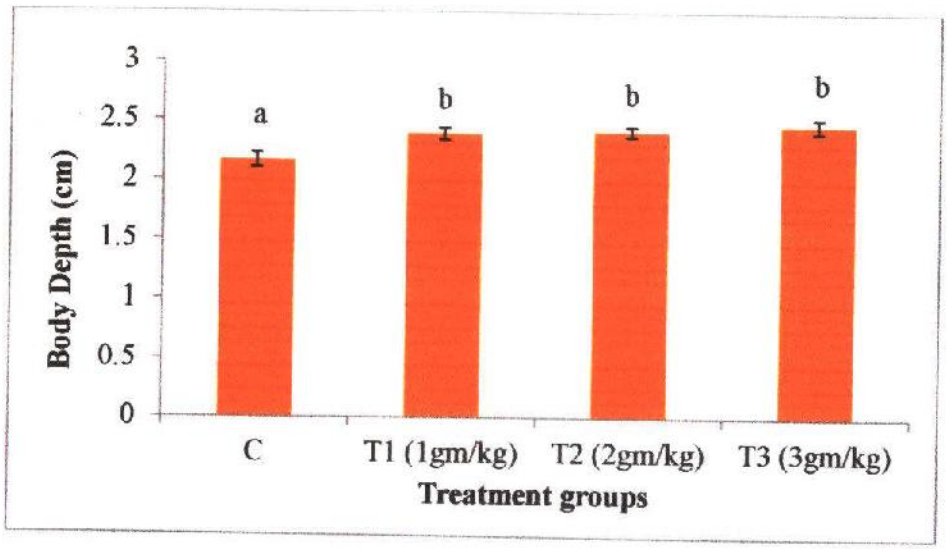

Figure 19: Effects of synbiotic on body depth (mean ± SE) of monosex tilapia.

4.2.8 Body area

Initially body area had no significant variation among the treatments which data has not shown in the graph. But finally, significant difference ($F_{3, 179} = 5.5$, $P<0.05$) was found in body area among the treatment groups after 45 days. The subsequent post-hoc test was shown that the body area of control fishes was significantly different from T_1, T_2 and T_3 ($P<0.05$). Consequently, there was no significant variation among T_1, T_2 and T_3 (figure 20).

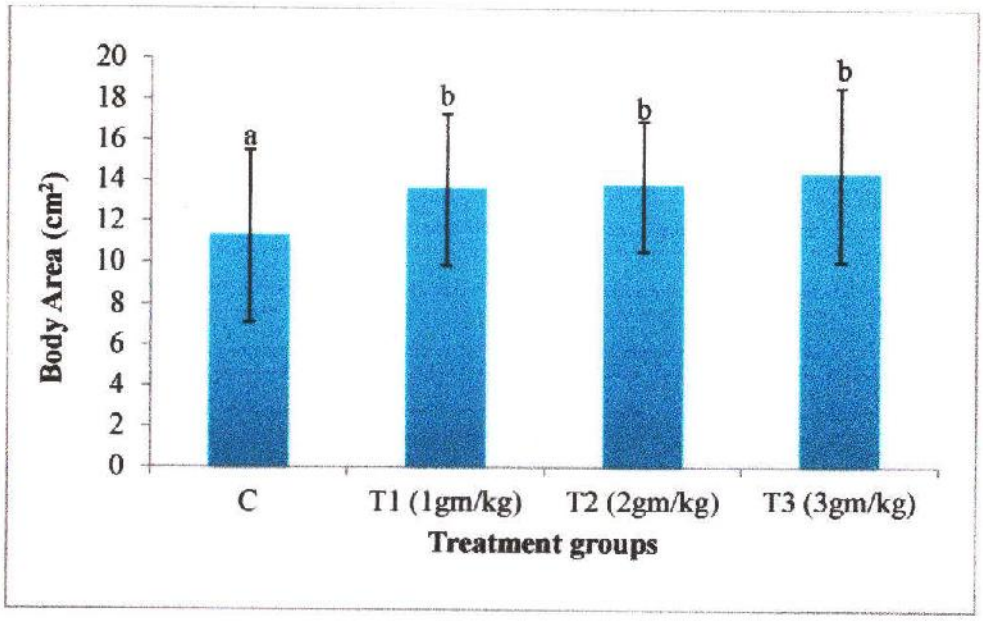

Figure 20: Effects of synbiotic on body area (cm^2) (mean ± SE) of monosex tilapia.

4.2.9 Dressing percentage

The data of dressing percentage revealed not significantly at the beginning of the experiment (data not shown). At the end of the experiment, There was significant difference in dressing percentage among treatments groups ($F_{3, 179} = 6.42$, $P<0.05$). The post hoc test revealed that T_1 and T_3 groups did not perform significant difference with control, but T_2 had significant difference with control and T_1.

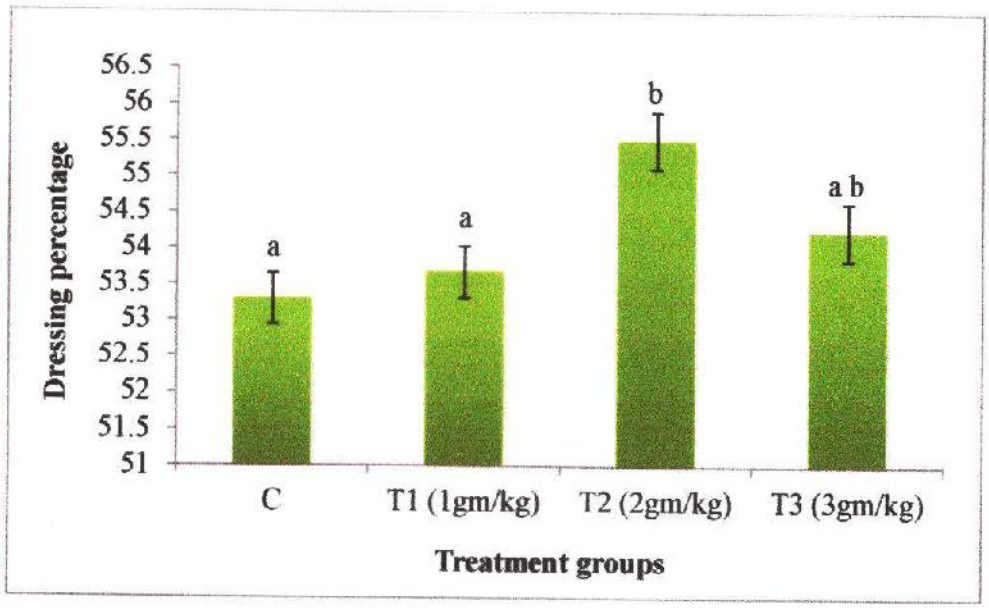

Figure 21: Effects of synbiotic on dressing percentage (mean ± SE) of monosex tilapia.

4.2.10 Color patterns

At the end of experiment, the Pearson's Chi-square test revealed no significant variation in color patterns among different treatment groups (χ^2 (1) = 9.519, P-Value = 0.146). The results shown that the number of dark stripped fish were 28 in control group followed by T_3 (29), T_2 (30) and T_1 (34). On the other hand, the number of light stripped fish was 15 in control followed by T_3 (12), T_2 (9) and T_1 (5). In case of no stripped fish, control had 2 fishes compared with T_1 (6) and T_2 (6) T_3 (4) (Figure 22).

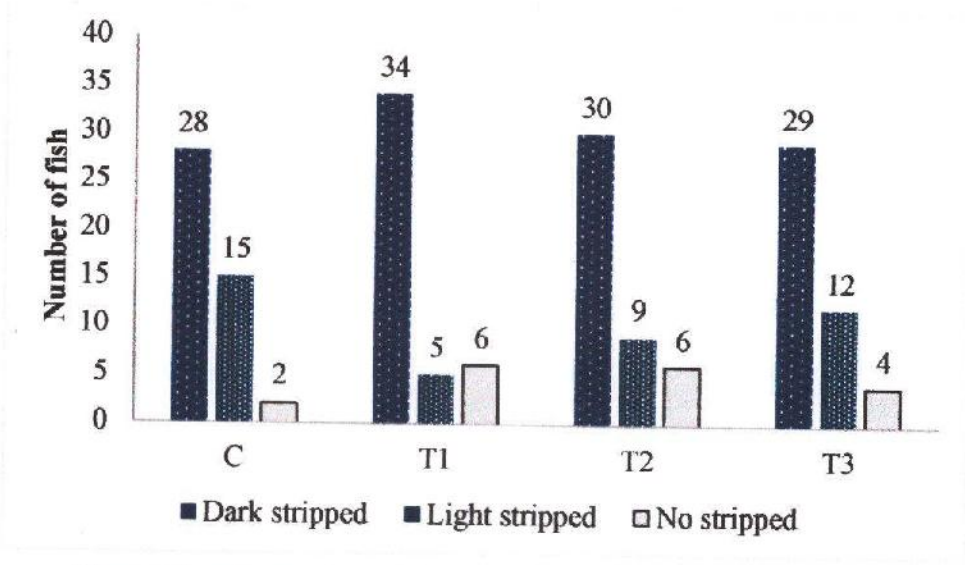

Figure 22: Effects of synbiotic on color patterns of monosex tilapia.

4.3 Metabolic activity assessment

4.3.1 Amylase enzyme activity

Standard calibration curve is a general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration. Standard maltose curve was generated using maltose of different concentrations ranged from 20-100 mg/ml. This curve was used to know the concentration of maltose produced by amylase against absorbance 540 nm (Figure 23).

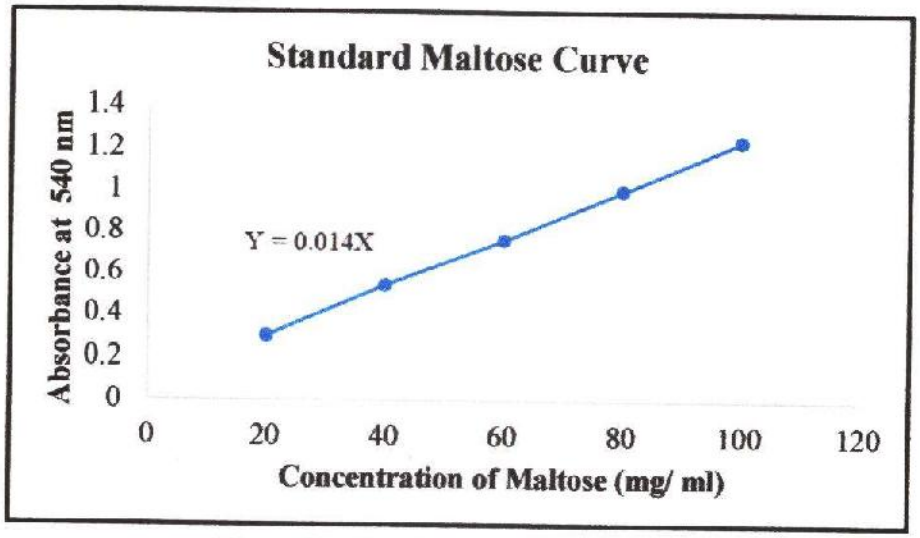

Figure 23: Standard Maltose curve

In intestine, the amount of average maltose were found 0.1658 ± 0.0238 µg/min/mg, 0.2753 ± 0.0449 µg/min/mg, 0.3428 ± 0.0303µg/min/mg and 0.3833 ± 0.0256 µg/min/mg in the Control, T_1, T_2 and T_3, respectively. These result showed that amylase expression and activity among control and treatments increased constantly from Control to T_3. There was significant differences ($F_{(3, 35)} = 8.67$, $p<0.05$) in amylase enzyme activity of intestine among control, T_1, T_2 and T_3 treatments. The subsequent post-hoc test was shown that the amylase activity of control fishes was significantly different from T_2 and T_3 ($P<0.05$). Consequently, there had significant variation between control and T_2 as well as between control and T_3 but there had no significant variation between control and T_1 (Figure 24).

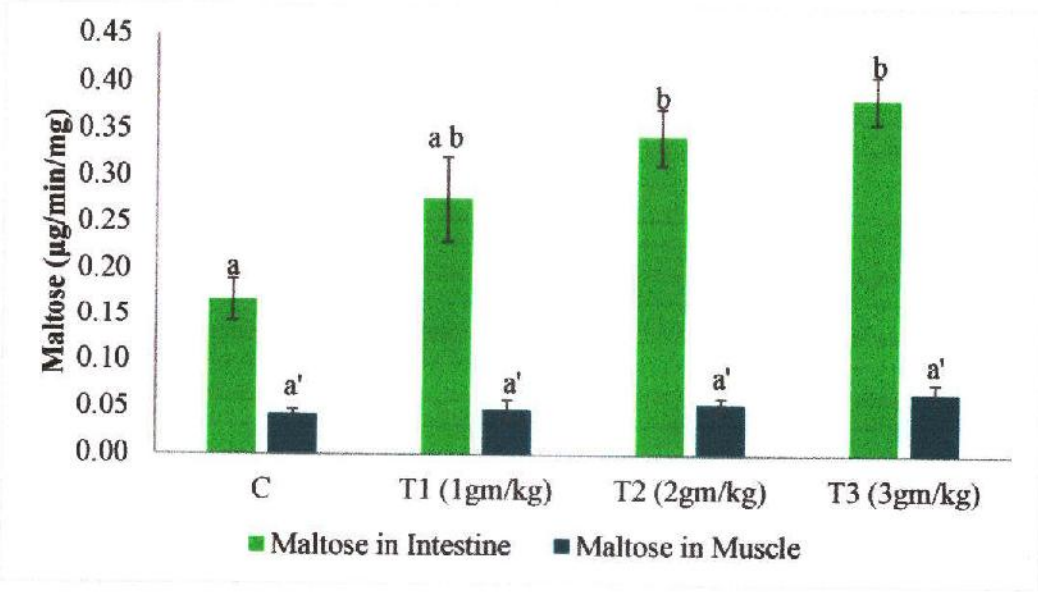

Figure 24: Amylase activity in intestine and muscle of monosex tilapia with different treatment groups.

On the other hand, in Muscle, the amount of average maltose were found 0.0428 ± 0.0044 µg/min/mg, 0.0476 ± 0.0105 µg/min/mg, 0.0547 ± 0.0065 µg/min/mg and 0.0674 ± 0.0096 µg/min/mg in the control, T_1, T_2 and T_3, respectively. These result showed that amylase expression and activity in muscle among control and treatments increased constantly from

control to T_3. But, there was no significant difference ($F_{(3, 35)} = 1.72$, $P > 0.05$) in amylase enzyme activity among control, T_1, T_2 and T_3 treatments (Figure 24).

4.3.2 Protease enzyme activity

Standard tyrosine curve was generated using tyrosine of different concentrations ranged from 50-250 mg/ml. This curve was used to know the concentration of tyrosine produced by protease against absorbance 280nm (Figure 25).

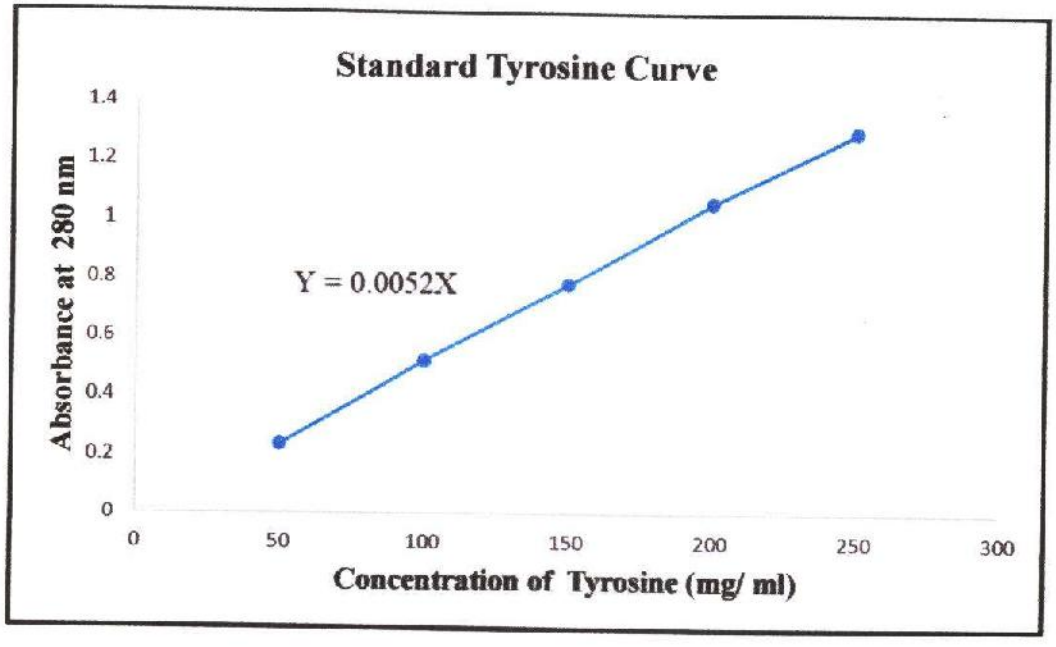

Figure 25: Standard Tyrosine Curve

In Intestine, the amount of average tyrosine were found 4.2478 ± 0.2114 µg/min/mg, 5.4145 ± 0.2759 µg/min/mg, 7.6474 ± 0.2573 µg/min/mg and 7.7115 ± 0.1405 µg/min/mg in the Control, T_1, T_2 and T_3, respectively. There was significant differences ($F_{(3, 35)} = 56.69$, $P<0.05$) in protease enzyme activity between control and T_1, T_2 and T_3 treatments. The subsequent post-hoc test was shown that the protease activity of control fishes was significantly different from T_1, T_2 and T_3 ($P<0.05$). Besides, there was also significant variation between T_1 and T_2 as well as between T_1 and T_3 (Figure 26).

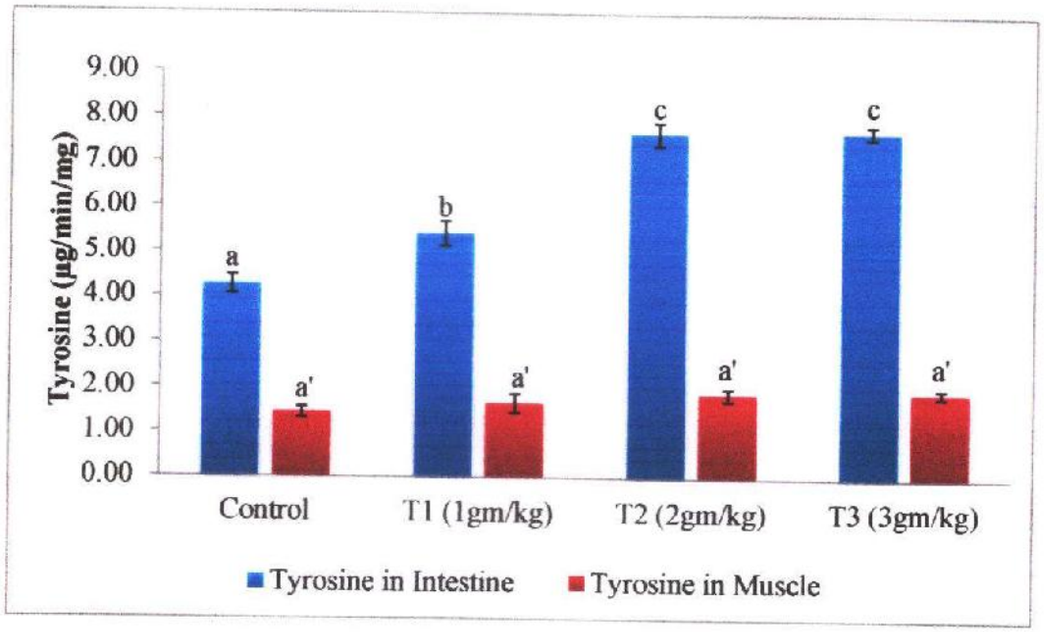

Figure 26: Protease activity in intestine and muscle of monosex tilapia with different treatment groups.

Whereas in Muscle, the amount of average tyrosine were found 1.4294 ± 0.1215 µg/min/mg, 1.6435 ± 0.2077 µg/min/mg, 1.8632 ± 0.1318 µg/min/mg and 1.9102 ± 0.0859 µg/min/mg in the Control, T_1, T_2 and T_3, respectively. These result showed that protease activity between treatments increased constantly from T_1 to T_3. But, there was no significant difference ($F_{(3, 35)} = 2.37$, $P > 0.05$) in protease enzyme activity among T_1, T_2 and T_3 treatments (Figure 26).

Chapter- 5. Discussion

5.1 Growth performance of monosex tilapia

In this experiment, significant enhancement of weight was found in treatment groups in which fish were fed diets supplemented with *Lactococcus lactis* probiotic and starch QS2 prebiotic than control group. These results suggest that dietary prebiotic and probiotic significantly improved growth performance and nutrient utilization of fish. This idea is supported by the fact that faster growth was observed in rainbow trout fed probiotic (*Enterococcus faecalis*) and prebiotic (PHB) supplemented diets (Rodriguez-Estrada et al., 2009). The study of Mehrabi et al. (2012) also support the result where significant better growth performance was observed in *Oncorhynchus mykiss* fingerlings maintained on the diet supplemented with synbiotic. Furthermore, Ye et al. (2011) reported that the diets M (MOS), FM (FOS + MOS), BM (*Bacillus clausii* + MOS) and BFM (*Bacillus clausii* + FOS + MOS) led to an in-crease in final body weight gain (FBW), whereas diets MB and BFM increased WGR ($P < 0.05$).

Results in cobia (Geng et al., 2011) showed that SGR in the fish fed with different diets was significantly higher than in the control fish group except the diet 6 groups, which was fed diet with 2.0 g/kg *Bacillus subtilis* and 3.0 g/kg chitosan. At 1.0 and 2.0 g/kg supplemental *B. subtilis*, the cobia fed diets supplemented with 6.0 g/kg chitosan had a significantly higher SGR than those fed diets supplemented with 3.0 g/kg chitosan. At 6.0 g/kg supplemental chitosan, cobia in the group fed diets supplemented with 1.0 g/kg supplemental *B. subtilis* had a significantly higher SGR. The significant and maximum SGR was observed in the fish fed with 1.0 g/kg *B. subtilis* and 6.0 g/kg chitosan, suggesting the supplementation level was optimal for the growth of cobia.

Although there is higher growth of fish fed the extra doses of synbiotics but there had no significant difference between the treatments. These findings suggest that increased doses of combination of *Lactococcus lactis* and starch QS2 slightly improved growth performance of Nile tilapia. Until now, most of the research using prebiotics and probiotics have been focused in the synbiotic effects in the gut (Hai and Fotedar, 2009) where the prebiotic is fermented by the ingested probiotic conferring health benefits to the host (Gibson, 2004).

Chapter- 6. Conclusion

The present study clearly demonstrated that synbiotic treatment caused considerable alterations in growth performance, some phenotypic traits (e.g. total length, standard length, head length, tail length, trunk length, body depth, body area, eye diameter, dressing percentage and color patterns) and metabolic (amylase and protease) enzymes activities of monosex tilapia. The amylase and protease activities of muscle and intestine were lower in control fish while those were found higher with the increasing of synbiotic doses. Due to increase in digestive enzyme activity, the growth was also increased in the different treatments of synbiotic. Though significant differences of phenotypic traits cannot be found in between synbiotic treatment groups, they were significantly higher than the control group. Again in case of the synbiotic (mixture of *Lactococcus lactis* strain D1813 and $CaCO_3$, starch QS2) significantly increased the activity of amylase a protease in intestine but no significant change was found on muscle. The present results recommend the incorporation of dietary synbiotic to Nile tilapia feeds as supplements to stimulate fish growth and digestion as well as development of phenotypic traits. Development of synbiotic mixtures may prove to be the most effective means of probiotic use, allowing fish culturists to increase fish production and provide favorable conditions to the animals. Future work must focus on applications of synbiotics in order to achieve the maximum efficiency, and synbiotic treatments must be used in concern with effective farm management and husbandry. The findings from the study must be helpful for the farmers to increase the overall fish production in a shortest period of time.

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Chapter- 8. Appendixes

PHOTO GALLERY

Figure 1. Fish stocking

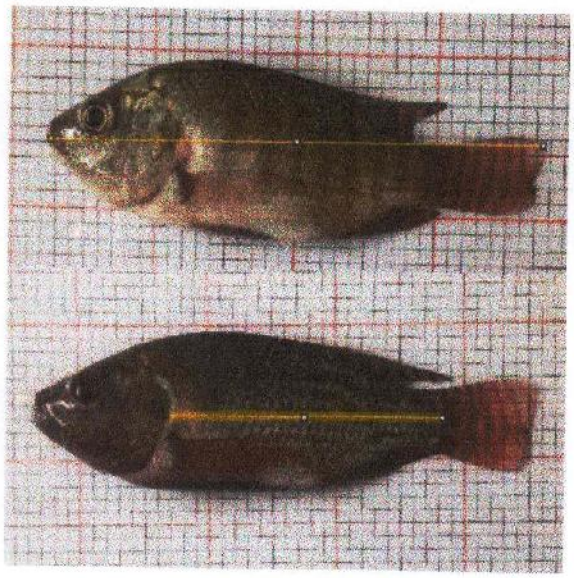

Figure 2. Length measurement under Image *J* sofware

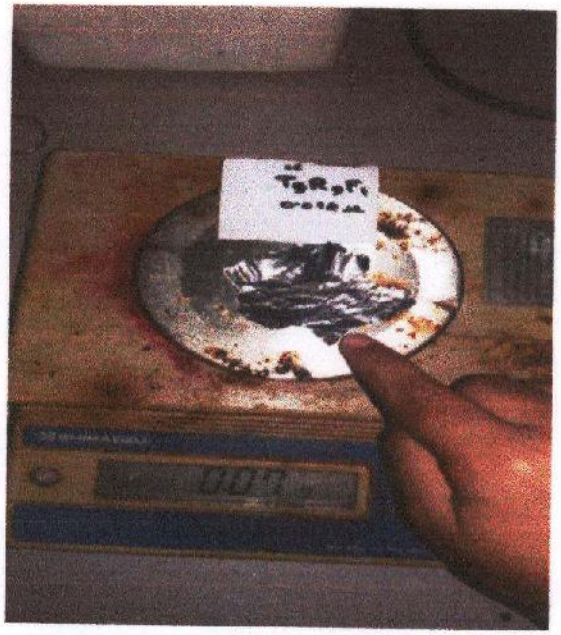

Figure 3. Weight measurement of fingerlings

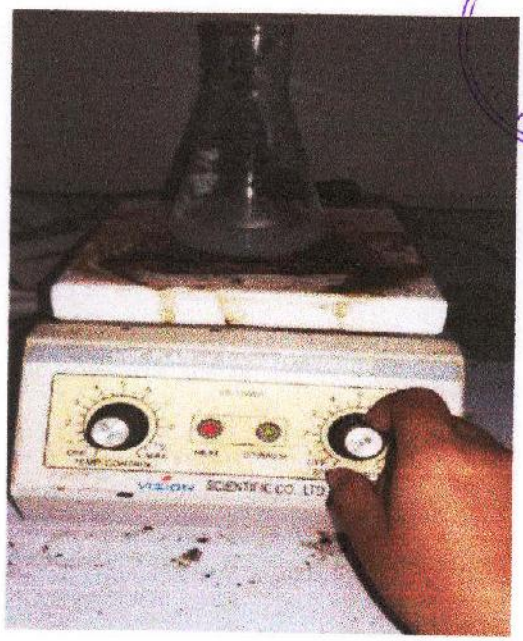

Figure 4. Preparation of casein reagents

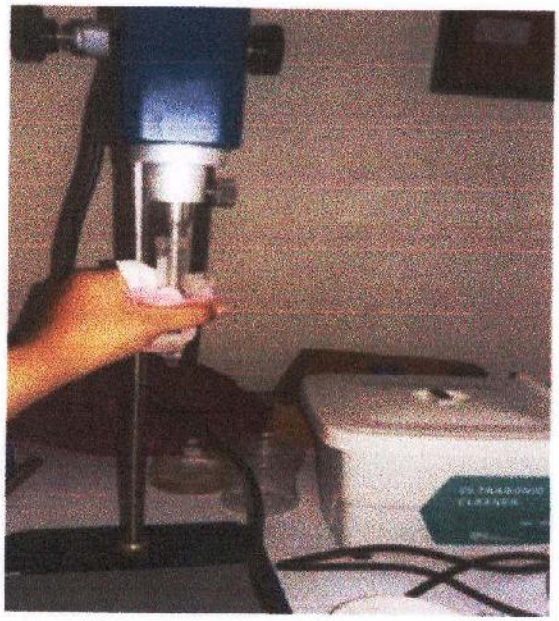

Figure 5. Preparation of crude enzyme

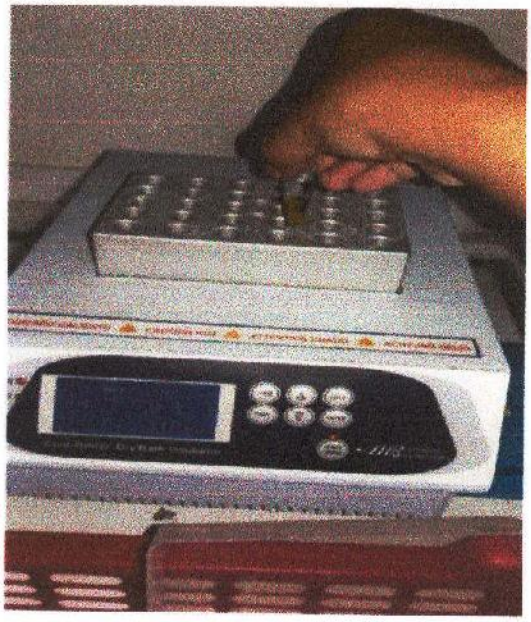

Figure 6. Placing of sample into incubator

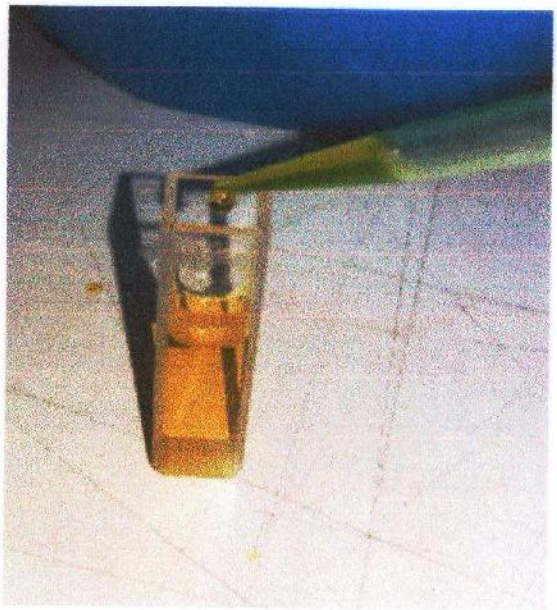

Figure 7. Taking of sample into cuvette for absorbance test

Figure 8. Recording of absorbance after spectrophotometric test