INFLUENCE OF PROBIOTICS ON PHYSIOLOGICAL AND IMMUNE RESPONSES IN TILAPIA *Oreochromis mossambicus* (PETERS)

THESIS
SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN ZOOLOGY

By

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Under the guidance of

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DEPARTMENT OF ZOOLOGY
UNIVERSITY OF CALICUT
KERALA-673635
2021

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CERTIFICATE

This is to certify that the thesis entitled **"INFLUENCE OF PROBIOTICS ON PHYSIOLOGICAL AND IMMUNE RESPONSES IN TILAPIA *Oreochromis mossambicus* (PETERS)"** submitted to University of Calicut, in partial fulfillment of the requirements for the award for the degree of Doctor of Philosophy in Zoology is a record of original and independent research work carried out by **Ms. Mariyam K. H.**, Department of Zoology, University of Calicut, under my guidance and supervision. The thesis has not formed the basis for the award of any other Degree/Diploma of this or any other university.

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DECLARATION

I do hereby declare that this thesis entitled "**INFLUENCE OF PROBIOTICS ON PHYSIOLOGICAL AND IMMUNE RESPONSES IN TILAPIA *Oreochromis mossambicus* (PETERS)**", submitted to the University of Calicut in partial fulfillment for the Doctoral degree in Zoology is a bonafide research work done by me under the supervision and guidance of Dr. E. Pushpalatha, Head of the Department & Associate Professor, Department of Zoology, University of Calicut and no part of the thesis has been presented by me for the award of any other degree, diploma or similar title.

Place: CU campus
22 February 2021

Mariyam, K. H

ACKNOWLEDGEMENT

With the blessings from **God Almighty** *and the support from many human beings on the earth my dream has come to a successful reality.*

I use this opportunity to express my profound sense of indebtedness to my supervisor **Dr. E. Pushpalatha***, Associate Professor and Head of Department of Zoology, University of Calicut for her instructions, keen interest to the topic, sustained encouragement and affectionate advice throughout the entire period of the study. It was indeed a great pleasure to work under her guidance. The scholastic inspiration and pertinent criticisms rendered by her always kept my research work in progress.*

It's a matter of great satisfaction and privilege to express my highly respected regards to the Head of the Department and previous heads of Department of Zoology, University of Calicut for providing the facilities and logistic support throughout the study. Additionally, I am grateful to all faculties of the Department of Zoology for their encouragement and support. I would like to express my deep sense of gratitude to **Dr. M. Nasser, Dr. V.M. Kannan, Dr.Y. Shibuvardhanan, Dr. E.M. Manogem, Dr. K.C. Chitra, Dr. C.D. Sebastain** *and* **Dr. M. Zubair***, Faculties, Department of Zoology, University of Calicut for their suggestions and support.*

Words are inadequate to thank **Dr. Mashhoor***, Assistant Professor, Department of Biotechnology, EMEA College, Kondotty,*

Malappuram, who helped me to do the microbiology works in his guidance. Also I am grateful to the Council for Scientific and Industrial Research, New Delhi for the financial support through Junior Research Fellowship during the tenure of the study.

I would like to express my sincere gratitude to **Mr. Ramesh***, for his insightful suggestions and support for the completion of my thesis. I also express my thanks to* **Mr. Santhosh Kumar, K.V.***, Librarian, Department of Zoology for his help and services and other non-teaching staff.*

A special thanks to all lab members of Biopesticides and Toxicology Laboratory, **Dr. Anju Viswan, K., Dr. Rahana, V.K., Dr. Aiswarya, A., Shabna Asmi** *and* **Shibu Anand** *for their support.*

Words cannot express how grateful I am towards my friends **Greeshma, K.P., Athira, A., Reshma, V., Manjusha, K., Bobika, V.K., Jalaala Najath., Sreejina sreedharan, Ranjith, A.P., Nidhish, G., Safeer Mohammad, P.P., Arun, A***., for their wholehearted support and encouragement and bearing many of my rants and flaws during the period. Without them I wouldn't have been completed within time. I wold like to express my thanks to* **Mr.Rajesh***, Bina Photostat, for the timely help to submit my thesis.*

I express my sincere thanks to all my family members. I am greatly indebted to my parents for their prayers for me. I always failed to express the profoundness towards my family members for their never-wavering moral support.

Mariyam, K.H.

Dedicated to

My Family

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1. INTRODUCTION

Man's effort to meet the ever-increasing demand for food and food resources has led to the domestication of plants and animals and the development and dissemination of techniques to raise them both in land and waterbodies with high yield and productivity, thus agriculture and aquafarming together became the major activity of human race around the globe. Aquaculture and its practices increased globally at a fast pace and it remain the second largest food producing industry next to agriculture. Global aquaculture practice contributes, nearly 47% of the total fish production (FAO, 2018). With an increase in aquaculture production come more environmentally suitable production systems and particularly land-based aquaculture facilities strive for a more efficient utilization of physical space and water resources (FAO, 2008). However, intensification of aquaculture with high stocking densities, high feed inputs and high organic load is said to be paralleled with a corresponding increase in the occurrence and spread of pathogenic and opportunistic bacteria causing infectious diseases.

Conventional approaches such as disinfectants and antimicrobial drugs are used for prevention and cure of diseases, but all some side effects as fish constitutes major animal protein source for world's population. The wide application of broad-spectrum chemotherapeutics has not only led to the emergence of antibiotic

resistant bacterial strains but also cause environmental degradations and food security problems. Besides, the antibiotics ingested by aquatic animals may be excreted as metabolites which may also be harmful to the animal and environment (Panigrahi and Azad, 2007). Moreover, chemotherapy may kill or inhibit the normal and beneficial microflora of the animal digestive tract and in the culture environment (Aly et al., 2008a). In addition to chemotherapeutics, vaccines are being developed and marketed to address this problem but it cannot be treated as a worldwide disease control measure in aquaculture (Lara-Flores, 2011). Intensive culture system, antibiotic therapy, stress, or inappropriate diet cause problems in the balance of the intestinal microflora which leads to improper digestion, the reduced assimilation of nutrients, growth rates, and development of fish (Kazuń et al., 2018). Therefore, the application of ecofriendly agents such as microbial and herbal supplements, to improve the physiology, growth performance and immune responses of aquaculture related species have gained much more attention during recent years. There has been intense research in developing and evaluating such dietary supplementation strategies in which various health and growth enhancing compounds such as probiotics, phytobiotics, synbiotics, prebiotics and other functional dietary supplements have been tested. Among these, probiotics emerges as promising alternative to antibiotics and improves overall health of organism.

The term probiotic means “for life” and it has been originated from two Greek words “pro” and “bios” (Gismondo et al., 1999). The idea of probiotic was originally used by Lilley and Stillwell (1965) to mean a substance, which has microbiological origin that stimulates growth of other organisms. The word “probiotics” was first used by Parker (1974) and proposed that probiotics are ‘microorganisms and substances which contribute to host intestinal microbial balance’. According to Fuller’s (1989) definition of probiotics, it is, the live microbial feed element beneficial for the host by improving microbial balance in the intestine. Moriarty named probiotics shortly as ‘‘water additives’’ in 1998. Therefore, several terms are generally used to illustrate probiotics such as “beneficial”, “eco-friendly”, or “healthy” bacteria (Moriarty, 1998). Gatesoupe (1999) defined probiotics for aquaculture “as microbial cells that are administered in such a way as to enter the gastrointestinal tract and to remain alive, with the target of improving health”. The definition provided by Verschuere et al. (2000) provides that “it is 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 improving the host response towards disease, or by positively changing the quality of its ambient environment”. As per Food and Agricultural Organization and World Health Organization, “probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits

on the host" (FAO/WHO, 2002). Merrifield et al. (2010a) defined probiotics "as any microbial cell administered through the feed or rearing water that benefits the host fish, fish farmer or fish consumer, which is achieved, in part at least, by improving the microbial balance of the fish". Probiotics can be emphasized as a nutritious food source and as an eco-friendly biological control mediator (Ahmad, et al., 2017). Thus, the application of probiotics in aquaculture is expected to be an excellent strategy for the prevention of infectious microbial diseases and to replace chemotherapeutics and antibiotics.

Naturally occurring microorganisms play a key role in aquatic environments, as they can fulfil a wide range of roles, including recycling nutrients, degrading organic matter, and protecting fish against infections. All these roles conduced to use these microorganisms in aquaculture as probiotics. Overall, probiotics are considered eco-friendly agents that can be administered in aquatic culture environments to control pathogens and enhance feed utilization, survival and growth rate of farmed species. Probiotics positively influences the host by their ability to increase intestinal permeability, exogenous production of digestive enzymes, promote the immunological and non- immunological defense barriers in the gut, and alter gut microflora. Probiotic bacteria adheres to the intestine of the host and produce a wide range of chemical compounds including lysozymes, proteases, siderophores, bacteriocins and hydrogen peroxides. Adhesion of

probiotics to the intestine and production of chemicals creates a barrier against the multiplication of opportunistic pathogens as well as regulate the changes in the intestinal pH through the secretion of organic acids (Zai et al., 2009; Strom-Bestor and Wiklund, 2011). The use of probiotics has increased in the aquaculture sector due to the positive influences in livestock production (Fulton et al., 2002) and in human health effects (Gill, 2003). However, Probiotics for aquatic practice are different from those of terrestrial environment as aquatic animals have a close interaction with the extrinsic environment. Probiotics can also be either used as biocontrol agents when the treatment is antagonistic to pathogen or as an aid to bioremediation when water quality is to be improved.

The gastrointestinal microbiota of the fish is changed according to the surroundings since the digestive tract experienced uninterrupted flow of water. The gut microbiota is also transient because of the continuous intrusion of microbes from food and water. The probiotics is successful when the administered microbes, either through water or feed, survive in the gastrointestinal tract. According to Belicova et al. (2013) an organism should be defined as a probiotic when it has no pathogenic reveal, antibacterial activities toward potential pathogens, tolerate lower range of pH and high concentrations of conjugated and deconjugated bile salts, be accepted by the immune system, and does not result in formation of antibodies. In addition, the probiotics must not transfer antibiotic resistance genes to pathogens through horizontal gene transfer. The

purpose of the probiotic application is to replace or compensate for the functions of the indigenous microbiota that inhabit in the gut or the surface of the body. The selection criteria for a probiotic demand that, the strain that it adheres to the intestinal mucosa and produce antimicrobial components (Bandyopadhyay and Mohapatra, 2009). The requirements that a probiotic organism must meet are 1. Resistance to the acidic stomach environment, bile and pancreatic enzymes; 2. Accession to the cells of the intestinal mucosa; 3. Capacity of colonization; 4. Staying alive for a long period of time, during the transport, storage, so that they can colonize the host efficiently; 5. Production of antimicrobial substances against the pathogenic bacteria; and 6. Absence of translocation.

The mode of action of probiotics has been classified and presented by Oelschlaeger (2010) as follows: (1) Probiotics might be able to improve the host's gut defense including the innate as well as the acquired immune system and this mode of action is most likely essential for the prevention and cure of infectious diseases, but also for the treatment of inflammation of the digestive tract or parts thereof. (2) Probiotics can have a direct influence on other microorganisms, commensal or pathogenic ones and this principle is in many cases important for the prevention and therapy of infections and restoration of the microbial equilibrium in the gut. (3) Finally, probiotic effects may be due to actions affecting microbial and host products and food ingredients; such actions may result in inactivation of toxins and detoxification of host and food

components in the gut. In aquaculture, probiotics also help to improve the quality of water due to the ability of the probiotic bacteria to participate in the turnover of organic nutrients in the ponds (Moriarty, 1997). In general, benefits of probiotics to the host help improve metabolism by escalating enzyme activity, improve feed uptake and digestion, maintain healthy intestinal microflora through antagonism against pathogens, strengthen the immune system and neutralize the entero-toxins and stimulate the immune system (Oelschlaeger, 2010).

Fish health status and growth rate are the reflection of ingested feed ingredients which is the modifiers of the enzyme physiology and digestive tract functions. Feed is a major requirement for all living organisms including fish for growth, reproduction and maintenance. In order to enhance the utilization of feed ingredients, dietary substances has a major role in regulating the metabolic functions in fish through modification of enzyme production. Optimal intestinal functionality is essential for better animal health. The digestive tract of fish is a complex ecosystem that contains a large number of microorganisms. Intestinal anaerobic bacteria can speed up the digestive process, providing a variety of extracellular enzymes such as protease, lipase, carbohydrase, phosphatase, esterase and peptidase, which facilitate the efficient absorption of nutrients and provide growth factors such as amino acids, vitamins and fatty acids (Ramirez and Dixon, 2003). Microbiota also play major role in reducing or eliminating the incidence of opportunistic

pathogens in the gastrointestinal tract of aquatic animals through competitive exclusion which is a common phenomenon in nature (Balcázar et al., 2006a). Competitive exclusion is the process by which an established microflora eliminates or diminishes the microbial colonization, which competes for the alike resources in the intestine. The probiotics blocks the intestinal infection route common to many pathogens by its capacity of adhesion to the intestinal mucus (Gatesoupe, 1999; Ringø et al., 2010). They can enhance nutrition by the breakdown of indigestible components, production of vitamins, detoxification of compounds in the diet and also increase the appetite (Abdelhamid et al., 2009). Therefore, the effective metabolism and nutrients absorption occurs when the host fed with probiotics supplemented feed (El-Haroun et al., 2006).

The gut microbiota with the epithelium and mucosal immune system orchestrate a network of immunological and non-immunological defenses, providing both protection against pathogens and tolerance to commensal bacteria and harmless antigens (Sanz and Palma, 2009). Enhancement of the immune system is the most promising method for prevention of diseases. Like other higher animals, fish immune system also has two fundamental components the natural or innate, nonspecific defense system and the acquired, adaptive or specific immune system. Innate immunity mediated by several cellular and humoral components, in which innate humoral components include antimicrobial lysozyme, peptides, lectins, antiproteases, complement components and natural

antibodies, whereas phagocytes and nonspecific cytotoxic cells constitute innate cellular immune effectors. Innate immune system is influenced by the indigenous micro biota in the gastrointestinal system, which plays a major role in the host's resistance against pathogens. Probiotics act on the non-specific immune responses thereby boost the immunity of the host. Probiotics interact with the immune cells such as polymorphonuclear leucocytes (neutrophils), mononuclear phagocytic cells (macrophages, monocytes) and natural killer (NK) cells to induce innate immune responses. Further, probiotics can enhance the number of erythrocytes, granulocytes, macrophages and lymphocytes in different fish (Kumar et al., 2008) and increases the immunoglobulin level (Nayak et al., 2007). Application of probiotics improves the non-specific immune system by means of cellular systems, e.g. increase phagocytosis, lysozyme activities (Irianto and Austin, 2002) and antibody production. Further, probiotics have been reported to improve the respiratory burst of phagocytic cells, which play a central role in the protection of non-specific cells (Panigrahi et al., 2004; Balcázar et al., 2007a, b).

The architectural dynamics of a tissue is very essential for maintaining the structural integrity and for effective physiological, biochemical and metabolic functions. The cellular and sub-cellular constituents of tissue in terms of size, shape, number and position play an important role in the physiological and metabolic functions. Therefore, the histological structure of tissue in an animal has a

profound influence on its function. Hence, it is useful to have an insight into the histological analysis as they act as biological markers to assess the status of the fish. Digestion and absorption of dietary nutrients in fishes mainly occur in the intestine and the anatomical and histological characteristics of fish intestine are expected to be helpful for understanding the digestive physiology and feeding habits, which can further be helpful for diagnosing some intestinal diseases and formulating suitable fish feeds that ensure better growth benefits (Logothetis et al., 2001; Chatchavalvanich et al., 2006; Rsfstie et al., 2006).

Antibacterial activity of probiotics can be used to eliminate pathogen, which is achieved by the productions of antibiotics, bacteriocins, siderophores, lysozyme, protease, hydrogen peroxide, alteration of pH values, and the production of organic acids and ammonia (Verschuere et al., 2000). Probiotics act as a sustainable tool to reduce or eliminate the prevalence of opportunist pathogens through its ability to alter the microbiota (Balcázar, 2002). Bacterial infections are considered to be the major cause of mortality in aquaculture (Govind et al., 2012). They can pass on a disease to a single fish and that can spread rapidly to cause a substantial fish kill in a few days or weeks. Bacterial infections of both farmed and wild fish are mostly due to the growth of several obligate or facultative pathogenic bacterial strains like *Aeromonas, Pseudomonas, Citrobacter, Proteus, Streptococcus, Edwardsiella, Staphylococcus* and different species of *Vibrio*, which cause huge mortality in both

freshwater and brackish water fish (Sihag and Sharma, 2012). *Streptococcus* bacterium is a Gram negative, pleomorphic, coccobacillus, which is a facultative intracellular pathogen able to infect many fish species, including tilapia. *Streptococcus agalactiae* and *Streptococcus iniae* are most common strains found in infected tilapia. The site of infection of *S. agalactiae* is the liver, spleen, heart, kidney and brain, but mortality is usually associated with the infection of the brain (Amal and Saad, 2011).

A wide range of microorganisms are evaluated as probiotics, it includes microalgae (*Tetraselmis*), yeast (*Phaffia, Saccharomyces* and *Debaromyces*), gram negative bacteria (*Vibrio, photorhodobacterium, Alteromonas, Pseudomonas* and *Aeromonas*) and gram positive (*Lactobacillus, Micrococcus, Bacillus, Carnobacterium, Weissella, Lactococcus, Streptococcus, Enterococcus*) (He et al., 2011; Nwanna, 2015). Usually, non-pathogenic species from normal microflora is used as probiotics in animal nutrition. The way of administration of probiotics to the aquaculture includes either as dietary supplements (live feed such as artemia or rotifers or pellet feed) or directly added to the water (Ramos et al., 2005; Mahdhi et al., 2011). Furthermore, probiotic delivery via injection has also been reported (Lapatra et al., 2014). Commercially available probiotics are in dry and liquid forms; when compared to liquid forms, dry forms have higher shelf life. However, in most of the studies, probiotics in liquid forms show better results than spore and dry probiotics, it may be because of their lower

density (Decamp and Moriarty, 2007). Probiotics can also be classified into two based on the mode of administration. The first one is mixing of probiotics with the feed to intensifying the beneficial bacteria inside the intestinal tract and the second one is the direct addition of probiotics to the water to inhibit the proliferation of pathogen in the medium by consuming nutrients available in the water. The probiotics is called as putative probiotics when it is isolated from different sources such as gastrointestinal tract, stomach, gonads, kidney, gill and other internal organs.

Of the bacteria colonizing the gastrointestinal tract, lactic acid bacterium is considered as the most promising bacterial genera as probiotic due to its abilities to stimulate the development of gastrointestinal tract, digestive function including digestive enzymes, mucosal tolerance, stimulating immune response and improved disease resistance. The most commonly used probiotic candidates belong to Gram-positive of LAB group or *Bacillus* species family (Banerjee and Ray, 2017). Lactic acid bacteria are classified as phylum Firmicute, class Bacilli, and order Lactobacillales. They are gram positive non-endosporin, morphologically rod shaped or coccid, catalase and oxidase negative, acid tolerant, facultative anaerobes and most of them are non-motile. Among the lactic acid bacteria, *Lactobacillus plantarum* are widespread on fermenting food, plants, and in the digestive tract of humans and animals, including fish. These bacteria can survive very well in the digestive system, produce compounds

which exert antimicrobial effects, adhere to the mucous membrane of the digestive tract, which facilitates their colonization and persistent presence in the intestines and act antagonistically towards pathogenic bacteria. (Kazuń et al., 2018).

Numerous strains of *Bacillus* species have been documented as safe for food or industrial applications and importantly as they have been documented as probiotics (Khochamit et al., 2015). *Bacillus* bacteria have been used as putative probiotics as they secrete many exoenzymes. Commonly used strains are *Bacillus subtilis, B. cereus, B. amyloliquefaciens, B. clausii, B. coagulans, B. megaterium, B. licheniformis, B. circulans*, and *B. polymyxa* (Sutyak, et al., 2008). *Bacillus* is a genus of gram-positive, nonpathogenic, rod shaped bacteria of phylum Firmicutes and these are saprophytic, spore forming organisms normally found in air, water, dust, soil and sediments (Gatesoupe, 1999; Moriarty, 1999). These bacteria are considered allochthonous and enter the gut through association with food. *Bacillus* species are able to produce antibiotics, amino acids and enzymes (Sanders et al., 2003). Some *Bacillus* sp., such as *Bacillus subtilis* and *Bacillus licheniformis* are generally recognized as 'safe' bacteria in aquaculture (Teo and Tan, 2005). Spores are being heat-stable and have a number of advantages over other non-spore-formers such as *Lactobacillus* sp., as the product can be stored at room temperature in a desiccated form without any deleterious effect pertaining to viability and the spore is

capable of surviving in low pH of the gastric barrier (Spinosa et al., 2000; Barbosa et al., 2005).

In aquaculture, a number of commercially formulated probiotics are being tried but success rates differ. It is possible that for a given probiotic to be effective, they need to be isolated from the same environment where they will work (Kato et al., 2016). The endospore forming *Bacillus* sp. have been found to enable them to endure extreme stresses, and they are suitable for formulation of stable probiotics; they could tolerate the acidic and alkaline conditions in gastro-intestinal tracts (Doan et al., 2016). Most of these species are both aerobic and facultative anaerobic, which means they can grow in various places to compete against potential pathogens. Moreover, *Bacillus* sp. has the potential to produce antimicrobial metabolites with an amazing variety of structures. Recent advances in genome sequencing have highlighted the genus *Bacillus* as an unexpected source of antimicrobial compounds (Grubbs et al., 2017).

The present study uses two probiotics, *Lactobacillus plantarum* and *Bacillus coagulans*, isolated from *Oreochromis mossambicus* (Tilapia) itself. Lactic acid bacteria and *Bacillus* sp. produce several chemical substances that may inhibit the growth of competing bacteria. Tilapia is a good experimental model for physiological and genetic studies in relation to stress, pollution, and growth promoters (Ebanasar and Kavitha, 2003). However, tilapia

holds vast promise to become an important species for aquaculture in India, considering the demand for more fish (NFDB, 2015). It also has worldwide economic importance. Tilapia culture is increasing because it is an affordable source of animal protein. Tilapia is an omnivore that feeds on phytoplankton, aquatic plants, small invertebrates, benthic fauna, detritus and bacterial films associated with detritus. It tolerates low water quality and wide range of environmental conditions. It is a prolific breeder and all sizes of fish is available throughout the year.

Disease control in aquaculture is largely depended on probiotics nowadays and hence it has been widely accepted in recent years. Thus, research on probiotics for fish and other aquatic animals is increasing to develop sustainable environment friendly aquaculture. It is anticipated that it can persuade the nutritional security in the next millennium (Patra and Bandyopadhyay, 2002). Several studies are conducted using different species and genus of bacteria to determine the probiotic potentials of different strains on different host. Most of the studies in finfish reported probiotic benefits on growth, reproductive performance, immune responses and disease resistance except limited contradictory reports. However, the fate of probiotics in the rearing medium and in gastrointestinal tract still remains unanswered. The present study has been conducted with the following objectives:

1. To study influence on the growth performance in tilapia fed with *Lactobacillus plantarum* and *Bacillus coagulans* as probiotics at different doses.

2. To determine the effect on digestive enzyme activities in tilapia fed with *Lactobacillus plantarum* and *Bacillus coagulans* as probiotics at different doses.

3. To estimate the changes in haematological and immunological parameters in tilapia fed with *Lactobacillus plantarum* and *Bacillus coagulans* as probiotics at different doses.

4. To analyze the influence of probiotics on histology of gill, intestine and liver of Tilapia.

5. Resistance against *Streptococcus agalactiae* in tilapia fed with probiotics.

6. Histopathological aspects of fish fed with probiotic supplemented diet.

2. REVIEW OF LITERATURE

Aquaculture is a rapidly growing sector as it plays an important role to achieve global protein food demand. The role of aquaculture to improve the socio-economic status of any region is highly appreciable because it is not only limited to the source of essential nutrients but it also generates various employment opportunities (Araujo et al., 2015; Handbook on Fisheries Statistics, 2014). India ranks second in the world after China in fish production through aquaculture with a contribution of 6.3% of the global aqua production, which is very less as compared to that of China (60.5%) (Chavan, 2018; Mo et al., 2018). Due to the increase in demand for aquaculture, intensification of culture system results in the spread of infectious diseases and cause great economic loss. Reverter et al. (2014) stated that the intensive culture systems in aquaculture cause stress through overcrowding, very low water quality, periodic handling, poor nutritional status, and sudden changes in temperature. The intensive rearing of fish species in aquaculture generates a potentially stressful environment to the fish, with the possible suppression of the immune system, rendering the fish more susceptible to different diseases (Austin and Austin, 1999). Frequent incidence of diseases has thus become a major hurdle for the highly intensified fish farming industry (Hai, 2015).

Chemotherapeutic agents like antibiotics and chemicals, are being employed as an option to cure common diseases prevailing in fish farming industry (Hambali and Akhmad, 2000). However, the extensive usage of these chemotherapeutic drugs leads to their accumulation in aquatic habitat and results in harmful consequences such as emergence of antibiotic resistant bacteria, accumulation of antibiotic residues in the flesh, kill the beneficial microbes of the gastrointestinal tract and alterations of gut microbiota by affecting non-target microorganisms of the aquatic environment (Munoz-Atienza et al., 2013; Azevedo et al., 2015). Therefore, the use of antibiotics as chemotherapeutic drugs in aquaculture has become a risk factor and it has not been promoted by authorities (Balcázar et al., 2006a &b, Balcázar et al., 2008; Mancuso et al., 2015). A promising emerging alternative approach to prevent fish diseases is the use of probiotics, which helps fishes to fight against pathogens through various mechanisms.

Probiotics are live microorganisms or a component of bacteria which when administered in adequate amounts confers a health benefit on the host or to its environment (Merrifeld et al., 2010b). Chai et al. (2016) have reported that the probiotics have been widely used in aquaculture as an effective way to control diseases, improves the immunity, provides nutrition, promotes proper digestion and helps in controlling the water quality.

Probiotic is a relatively new term which is used to name microorganisms that are associated with the beneficial effects for the host. Kozasa (1986) made the first empirical application of probiotics in aquaculture by considering the benefits exerted through the application of probiotics on humans and poultry. He used spores of *Bacillus toyoi* as probiotic feed supplement and observed increase in the growth rate of yellow tail, *Seriola quinqueradiata*. Later, Porubcan (1991a, b) documented the use of *Bacillus* sp., to test its ability to increase productivity of *Penaeus monodon* farming and to improve water quality by decreasing the concentrations of ammonia and nitrite. According to Irianto and Austin (2002), the more general and common concept of probiotic is "one or more microorganisms with beneficial effects for the host, able to persist in the digestive tract because of its tolerance to acid and bile salts". The application of probiotics is to maintain a healthy relationship between beneficial and unhealthy bacteria present in gastrointestinal tract of the fish (Olsson et al., 1992; Thirumurugan and Vignesh, 2015). Probiotics can meet the needs to develop successful aquaculture because it enhances the key factors that assure yield in growth and disease resistance in cultured organisms (Dawood and Koshio, 2016).

Microorganisms intended to be used as probiotics in aquaculture should perform functions that should be considered safe not only for aquatic hosts but also for their environments, other organisms and humans (Munoz-Ateinza et al., 2013). According to FAO (2016), the probiotic effect on food can have the desired impact

only if it contains at least 10^6–10^7 live probiotic bacteria per gram or milliliter. Probiotics also have a direct influence on other microbes, either commensal or pathogenic, which is very important for the prevention, treatment and restoration of the bacterial equilibrium inside the gut of the host (Oelschlaeger, 2010). The beneficial effects of probiotics used in aquaculture is not only limited to gastrointestinal tract, but also plays a major role in the enhancement of overall health of an organism (Mehrabi et al., 2018) such as: it acts as growth promoter (Gobi et al., 2018), prevents diseases (Meidong et al., 2018), enhances the immune response (Ramesh and Souissi, 2018) and improves the water quality by modifying microbiota of water and sediments (Verschuere et al., 2000; Deng et al., 2018).

It is suggested that the farmers can regulate the sedimentation of organic carbon in growing season by using high concentration of probiotics in the ponds (Balcázar et al., 2006a; Mohapatra et al., 2013). Some probiotic bacteria possess significant algicidal activity and affects many microalgae species (Fukami et al., 1997). The probiotic bacteria are valuable as it increases the number of good bacteria in water and improve the water quality by eliminating ammonia and nitrate toxicity (Mohapatra et al., 2013; Zorriehzahra et al., 2016). The other water quality parameters like pH, temperature, dissolved oxygen, ammonia and hydrogen sulfide contents improve by the utilization of probiotics. Thus, the use of probiotics in aquaculture system can achieve a positive and healthy

culture environment (Banerjee et al., 2010; Aguirre-Guzman et al., 2012).

According to some authors, probiotic bacteria can be used as ecological biocontrol or bioremediation agent for the sustainable development of aquaculture (Dimitroglou et al., 2011; Iribarren et al., 2012; Ibrahem, 2015). Several authors reported that the application of *Lactobacillus* species as probiotics removes the nitrogenous waste from the ponds and use of *Bacillus* species improves the water quality by converting organic carbon to slime (Verschuere et al., 2000; Ma et al., 2009; Kolndadacha et al., 2011). Reduced algae growth and organic load, increase in nutrient concentration and dissolved oxygen, enhancement of beneficial microbiota and inhibition of potential pathogens are benefits attributed to water additive probiotics (Ibrahem, 2015). Probiotics can also produce inhibitory substances against pathogens, competition for essential nutrients and adhesion sites (Ringø and Gatesoupe, 1998). Silva et al. (2013) have reviewed that the probiotics do not produce residues or drug resistance in animals, and thus probiotics act as a substitute for antibiotics. In addition, they supply essential nutrients and enzymes resulting in enhanced nutrition in the host. Furthermore, the modulation of interactions with the environment and the development of beneficial immune responses are exerted by probiotics (Ringø and Gatesoupe, 1998; Balcázar, et al., 2008). Thus, the use of probiotics, in the culture of aquatic organisms, is increasing with the demand for more

environment-friendly aquaculture practices (Gatesoupe, 1999). The use of probiotics in humans, pigs, steers and poultry has already been studied, but the studies on the use of probiotics in aquaculture has been started later (Harimurti and Hadisaputro, 2015; Uyeno et al., 2015; Daniel, 2017; Chua et al., 2017; Jiang et al., 2017). Most of the reports are focused on beneficial effects of probiotics based on their ability to produce antimicrobial substances, competition for attachment sites and adhesion properties but the dose, duration and way of administration of bacteria have been rarely performed (Araujo et al., 2015; Mancuso et al., 2015; Alonso et al., 2019).

The main sources for the isolation of appropriate probiotics in aquaculture are intestines, gills, skin, mucus of aquatic animals, habitats or even culture collections and commercial products and are mainly identified as Gram-positive, Gram-negative bacteria, bacteriophages, microalgae and yeast which have been widely used in aquaculture via water additive or feed supplement (Llewellyn et al., 2014). The most frequently used probiotic microorganisms in aquaculture belong to *Bacillus, Lactobacillus, Saccharomyces, Enterococcus* and *Bifidobacterium* species (Tinh et al., 2008; Rahiman et al., 2010; Nwanna, 2015). Various species of *Lactobacillus* and *Bifidobacterium* reported for use in aquaculture as probiotics, include *Lactobacillus acidophilus, L. casei, L. fermentum, L. gasseri, L. plantarum, L. salivarius, L. rhamnosus, L. johnsonii, L. paracasei, L. reuteri, L. helveticus, L. bugaricus, Bifidobacterium bifidum, B. breve, B. lactis, B. longum,* and

Saccharromyces boulardii (Nwanna, 2015). Lazado et al. (2015) have reviewed and found that several bacterial species have been identified, characterized and applied in aquaculture. Currently, there are lots of commercially available probiotics in use which are of mono or multi-strains (Doan et al., 2017).

The ability of probiotics to enhance the animal's health is through many positive ways, viz; (i) competitive exclusion of pathogenic microorganism, (ii) production of nutrients and enzymatic contribution to digestion, (iii) Production of inhibitory substance, (iv) improvement of water quality, (v) Growth performance and (vi) enhancement of immune response (Defoirdt et al., 2007; Muñoz-Atienza et al., 2013; Zokaeifar et al., 2014).

The most common method for administration of probiotics in aquaculture is through water or oral routine (Huang et al., 2006). But most of the probiotics are designed in such a way that they can be mixed with the feed additives to show high efficiency against pathogens (Austin et al., 1992; Gildberg and Mikkelsen, 1998; Gomes et al., 2009; Hai et al., 2009). Aquaculture probiotics must be able to colonize the gastrointestinal tract of aquatic species which is constantly been affected by the flow of water passing through the digestive tract (Gatesoupe, 1999). Fuller (1989) emphasized on the dietary application of probiotics to the host in order to improve the gastrointestinal microbiota. The dietary probiotics usually consist of spore-forming microorganisms. They can be directly incorporated

into the basal feed with the help of a binder. The basal feed often possesses vitamins and other nutritional additives (Bandyopadhyay and Mohapatra, 2009). Probiotic strains can be used individually or in a combination of different strains (Havenaar et al., 1992; Salinas et al., 2005; Kesarcodi-Watson et al., 2008, 2012; Lin et al., 2012). Several probiotics either as monospecies or multispecies supplements are commercially available for aquaculture practices (Decamp and Moriarty 2007; Ghosh et al., 2007). In aquaculture, the addition to feed is considered the most effective way to administer probiotics, and the development of fish feed containing natural immunostimulants and probiotics has grown exponentially in recent years (Bahi et al., 2017). The frequency of administration and period of administration of probiotics also play a very important role in maintaining the effectiveness and function of probiotics. The duration for administration of the potential probiotic can be as short as 6 days or as long as 5 to 8 months (Joborn et al., 1997; Aubin et al., 2005; Aly et al., 2008b). Prolonged administration of probiotics can sometimes cause immunosuppression of continuous response of nonspecific immune system (Sakai, 1999). According to Guo et al. (2009), daily application of probiotics is better than using thrice a week during the culture period. Aubin et al. (2005) checked the recovered amounts of probiotics over a time period and observed that recovery levels were found to be higher after 20 days than 5 months.

Several literatures are available that report probiotics can improve the overall growth performance of the fish. Yassir et al. (2002)

reported *Micrococcus luteus* as probiotics in tilapia *Oreochromis niloticus*, in which highest growth rate and feed conversion ratio were observed in fish fed with probiotic diet. Another research work also indicates the lactic acid bacteria as the growth enhancer as a result of its effectiveness on the growth performance in immature carp (Shishehchian et al., 2001). Probiotics have been used in aquaculture to increase the growth of cultivated species as it increases the appetite and improve digestibility. Some people inclined to think that it would be important to determine whether probiotics actually taste good for aquaculture species (Irianto and Austin, 2002). Boyd (2015) have stated that the probiotics treatment potentially reduce eutrophication, induce weight gain, and hence it could be a viable option to promote sustainable aquaculture management practices. Diet of Nile tilapia (*Oreochromis niloticus*) was amended with a probiotic *Streptococcus* strain, increasing significantly the content of crude protein and crude lipid in the fish, also weight has increased from 0.154 g to 6.164 g in 9 weeks of culture (Lara-Flores et al., 2003). Due to the commercial importance of this species of fish, the effect of supplementing diet with probiotics produced an increase of 115.3% when commercial dietary formulations were used at a concentration of 2% (El-Haroun et al., 2006).

Abdel-Tawwab et al. (2008) reported the assessment of commercially available *Saccharomyces cerevisiae* as an immunity stimulator and growth promoter for Nile tilapia fry, *Oreochromis*

niloticus (L.) challenged with fish pathogen *Aeromonas hydrophila*. Wang et al. (2007) analyzed the effect of a probiotic bacterium, *Enterococcus faecium* ZJ4 on growth performances and immune responses of tilapia, *Oreochromis niloticus*. The tilapias were treated with *E. faecium* ZJ4 at a final concentration of $1x10^7$ cfu ml^{-1} in aquaria water every 4 days. After 40 days, the tilapias supplemented with the probiotic showed significantly better final weight and daily weight gain (DWG) than those fed the basal diet in the control set ($P<0.05$). Rahman et al. (2019) studied the influence of probiotics (*Lactobacillus plantarum* and *Bacillus coagulans*) on the Growth Performance of sex reversed Nile tilapia (*Oreochromis niloticus*) fry. After 100 days of investigation, it was observed that the fish groups fed with probiotics supplemented diets revealed significant improvement in aspect of growth. The addition of probiotics could improve feed utilization even under stress conditions (Lara-Flores et al., 2003). The best FCR values were observed when probiotics containing diets were fed to Nile tilapia (*Oreochromis niloticus* L.). The use of Spirulina as a probiotic in Nile tilapia diet improved feed conversion ratio compared to the control fish fed with diet not containing probiotics (Abdel-Tawwab and Ahmad, 2009). In another study, Nile tilapia treated with commercially available probiotic containing feed showed improved feed conversion efficiency (El-Haroun et al., 2006). The positive effects on nutrient digestibility in Rohu fish (*Labeo rohita*) were observed when the diets supplemented with different microbial probiotics (Mohapatra et al.,

2012). Tovar-Ramirez et al. (2002) recorded an increase in the digestive enzyme activities of amylase, trypsin and lipase in sea bass (*Dicentrarchus labrax*) using live yeast. Wang and Xu (2006) investigated the effect of *Bacillus* sp. probiotics on protease, amylase and lipase specific activities in the common carp and a significant increase in digestive enzyme activities in the all probiotics treatment groups were observed.

Suzer et al. (2008) demonstrated that probiotics affect the digestive process by enhancing the population of beneficial microorganisms and then microbial enzyme activity, consequently improving the digestibility and absorption of feed and feed utilization. They also illustrated that the high growth performance can enhance specific activities of digestive enzymes as well. Campa-Cordova et al. (2009) recorded growth performance and survival rate in juvenile *Crassostrea corteziensis* incorporated with probiotic microorganisms. Ziaei-Nejad et al. (2006) studied the effect of *Bacillus* species used as probiotics in the activity of the digestive enzyme in the Indian white shrimp, *Fenneropenaeus indicus*. In various functions, they improve digestibility of feed through the advancement of the diverse excavators such as proteases, amylases and alginate lyases (Zokaeifar et al., 2012). Khattab et al. (2005) have studied the effect of *Micrococcus luteus* as probiotics in Tilapia (*Oreochromis niloticus*) and showed improvement in growth performance and feed conversion ratio (FCR) in fishes fed with probiotic supplemented diets. Reda and Selim (2015) have

concluded that the dietary supplementation of *Bacillus amyloliquefaciens* improved the growth performance in *Oreochromis niloticus* fingerlings and found to influence the gut morphology.

Aly et al. (2008a) compared the activity of mixed strains of *Lactobacillus acidophilus* and *B. subtilis* in Nile tilapia in which serum bactericidal activity and hematocrit values were higher in comparison to single strain. Beck et al. (2015) also conducted a study to enhance the immunity against *Streptococcus iniae* by a combination of *Lactobacillus plantarum* and *Lactococcus lactis* in Japanese flounder. Multi strain probiotics have been efficiently used which enhance the growth and survival of rohu at hatchling and fry stages (Jha et al., 2015). The probiotics such as *Lactobacillus rhamnosus* were reported to improve the fecundity of *Danio rerio* (Gioacchini et al., 2010). Feeding of probiotic strains such as *Shewanella xiamenensis* and *Aeromonas veronii* to grass carp for about 28 days reduced the cumulative mortality when challenged with *Aeromonas hydrophila* (Wu et al., 2015). The feeding of synbiotic *Enterococcus faecalis* and mannan-oligosaccharide (MOS) showed better FCR (feed conversion ratio) in fish as compared to feeding of probiotic and prebiotic individually (Rodriguez-Estrada et al., 2009). The application of probiotics, prebiotics and synbiotics have improved the survival of aquatic organisms against pathogenic bacteria. The survivability was found to be maximum in the group treated with probiotics followed by

prebiotic and synbiotics (Decamp and Moriarty, 2007; Daniels et al., 2013). Yakubu et al. (2016) used commercial probiotic strain as a feed supplement for *Clarias gariepinus* and reported increased growth and survivability.

Al-Dohail et al. (2009) observed enhanced growth, survivability and feed utilization efficiency in *Clarias gariepinus* by supplementing the feed with *Lactobacillus acidophilus*. Falaye et al. (2017) reported enhanced growth, weight gain and FCR in *Clarias gariepinus* fingerlings through applying fortified diet infused with *L. plantarum*. Dey et al. (2016) isolated autochthonous putative probiotic strain *Bacillus aryabhattai* KP784311 from the foregut of adult *C. batrachus* and obtained better growth performance in juvenile fish by encapsulating the probiotic with chironomid larvae. El-Haroun (2007) used a commercial strain of *Bacillus* to obtain better growth performance, protein efficiency ratio, protein productive value and comparatively better feed conversion ratio in *C. gariepinus.*

According to many reports, lactic acid bacteria are normal flora in gastrointestinal (GI) tract of healthy animals like mammals and aquaculture animals (Nikoskelainen et al., 2001) with no harmful effects (Ringø and Gatesoupe, 1998). Lactic acid bacteria also had an effect as growth promoters on the growth rate in juvenile carp though not in Sea bass (Dhanaraj et al., 2010). The *Bacillus* sp. and *Lactobacillus* sp. are great candidates as probiotics used in

aquatic animals because they are capable to survive in high temperatures (El-Haroun et al., 2006) such as after the pelleting process of feed. This feed can be stored at room temperature without any deleterious effect and resisted to the low pH that can reach intact to the small intestine (Cutting, 2011). Lactic acid bacteria as a main group of probiotics which are used in animal nutrition to improve growth, survivability, feed efficiency, and also to prevent intestinal disorders and neutralize antinutritional factors present in the feedstuffs (Ringø and Gatesoupe, 1998; Rastall and Maitin, 2002; Suzer et al., 2008). They are also applied to increase microbial monitoring, growth and feed efficiency (Panigrahi et al., 2005; Suzer et al., 2008). Moreover, some reports have noted that the gut microorganisms are important for fish health by inhibiting the establishment of pathogenic bacteria in the alimentary tract (Ringø et al., 2006). *Bacillus* sp. is one of the most studied probiotics in fish and has been reported to have various beneficial properties, including immunostimulation and increased disease resistance, when added as a supplement in fish diets (Aly et al., 2008c, Kumar et al., 2008). Similarly, many authors mention an improvement in the immune response of fish treated with *Lactobacillus* sp. owing to their ability to colonize the digestive tract, altering the natural balance of the intestinal microbiota which could enhance the immune system and confer protection against several major fish pathogens (Reyes-Becerril et al., 2008). Recently, the effects of two probiotic strains *Bacillus subtilis* and *Rhodococcus* sp. have been

evaluated on gut microbiota of *Oreochromis niloticus* (Kathia et al., 2018) and the results of their study clearly indicated a significant shifting of gut microbial community (increasing percentage of proteobacteria and bacteroidetes) in fish fed with probiotics when compared to the control.

The enhancement of the immune response is proposed as one of the main modes of action of probiotics for increasing fish resistance to infection (Salinas et al., 2005). Several reviews have documented the benefits of probiotics in fish and particularly their effects on immunity (Cordero et al., 2015). Probiotics often exert signaling molecules to stimulate humoral or cellular immune response against pathogenic invasion (De et al., 2014). Dahiya et al. (2012) applied probiotic *Lactobacillus sporogenes* and *Saccharomyces boulardii* to *Clarias batrachus* fingerlings against pathogenic *Aeromonas hydrophila* and *Micrococcus* sp. and observed increased level of immunity and haematological profile in cat fish. Probiotics enhances the number of leucocytes (Korkea-Aho et al., 2012), lymphocytes (Gobi et al., 2016), erythrocytes (Zhou et al., 2010), neutrophil adherence, migration of neutrophils, plasma bactericidal activity (Taoka et al., 2006a), complement activity (Sun et al., 2010), cytotoxicity (Salinas et al., 2005), phagocytic and superoxide dismutase activities (Cha et al., 2013). An increase in total globulin, serum bacterial agglutination titres, an enhancement of phagocytic, lysozyme activities (Ridha and Azad, 2012), albumin levels (Sharifuzzaman and Austin 2010a), serum peroxidase and

blood respiratory burst activities (Heo et al., 2013) are also been reported. Also, increase in respiratory burst (Chen et al., 2019), antiprotease and peroxidase activities (Newaj-Fyzul et al., 2007; Sharifuzzaman and Austin, 2010a) have been reported.

The pathogens attack the immune system in the fish and causes infectious diseases in fish. The major components of the innate immune system are macrophages, monocytes, granulocytes and humoral elements, such as lysozymes or the complement system (Secombes and Fletcher, 1992; Galina et al., 2009). The stimulation of the immune system of the fish can protect the fish from infectious diseases. Thy et al. (2017) have demonstrated that the dietary supplementation of a mixture of *Bacillus amyloliquefaciens* and *Bacillus pumilus*, which colonized in striped catfish intestine, enhanced the growth performance, innate immunity and protection of fish (*Pangasianodon hypophthalmus*) against *Edwardsiella ictaluri* at the dose of 5×10^8 CFU g^{-1} which also increased the stress tolerance of fish at three different doses of 1, 3, and 5×10^8 CFU g^{-1}. Cao et al. (2011) have concluded *B. amyloliquefaciens* as a promising probiotic for the biocontrol of *Aeromonas hydrophila* infections in *Anguilla anguilla* (L.). Based on the above literature, it is clear that the uses of probiotic cellular components to enhance fish innate and adaptive immune response, thus impart beneficial properties to eliminate the disease in aquaculture. The effects of these supplemented diets on growth performance parameters and the humoral immune response (natural haemolytic complement,

peroxidase, total IgM levels, protease and antiprotease activities) were evaluated after 2 and 3 weeks of feeding. The results showed a significant increase in the immune parameters, principally in fish fed only fenugreek or fenugreek combined with *B. subtilis*. An experimental report supported that probiotics supplemented at 10^2 CFU/g diet for 2 weeks act as an immunomodulator by binding its MAMPs (microbial associated molecular patterns) to pathogen pattern recognition receptors (PRRs) on immunogenic cells like dendritic cells, macrophages, which trigger intracellular signaling cascade, resulting in the release of specific cytokines and interleukins by the activated T cells to exert anti-viral, pro- or anti-inflammatory exercise effects (Balcázar et al., 2006c; Akhter et al., 2015). A number of research works shed light on the useful impact of probiotics on the organism's gut resistances, which are quite significant for not only diseases deterrence but also digestive tract tenderness management (Azimirad et al., 2016; Modanloo et al., 2017).

Probiotic microorganisms may inhibit fish pathogens by producing wide-spectrum of bactericidal or bacteriostatic chemical substances (e.g., siderophores, bacteriocins, enzymes etc.). Ogunshe and Olabode (2009) isolated *Lactobacillus plantarum* LbOGI and *Lactobacillus fermentum* LbFF4 strains from *Clarias gariepinus*, and observed antibacterial activity against pathogenic *Salmonella, Klebsiella, E. coli, Citrobacter, Proteus* and *Pseudomonas.* Strains of *Lactococcus* and *Lactobacillus*, isolated from the surface of *C.*

gariepinus executed significant antimicrobial activity against aquaculture pathogens (Kato et al., 2016). *Bacillus subtilis, Bacillus licheniformis, Bacillus* sp., *Pediococcus* sp. administration in *Oreochromis* sp. as feed supplement enhanced resistance to *Streptococcus agalactiae* infection and survivability in fish (Ng et al., 2014). Fish fed a diet containing *Lactobacillus plantarum* CR1T5 (10^8 CFU g^{-1} feed) displayed not only no mortality but also growth improvement. At the end of feed-trial, fish were challenged by intramuscular injection of *Aeromonas hydrophila* ($3.1x10^5$ CFU/ml). The *Lactobacillus plantarum* CR1T5-fed fish survived (87.5%) better than the fish fed a control diet (12.5%) after a two week-challenge. Munisaru et al. (2017) studied the effects of *Bacillus subtilis* and *Lactobacillus rhamnosus* application in *Labeo rohita* as multi-strain probiotics increased the value of biochemical components, growth, immune parameters and protection against *Aeromonas hydrophila*.

CHAPTER 1

EFFECT OF PROBIOTICS ON GROWTH PERFORMANCE AND DIGESTIVE ENZYME ACTIVITY

3.1 INTRODUCTION

Probiotics are beneficial microorganisms that when administered in adequate amounts provide overall enhancement of growth performance and digestive enzyme activities in host. Dall and Moriarty (1983) proposed that, microbiota may serve as complementary source of food and microbial activity in the digestive tract and an excellent source of vitamins or essential amino acids. Prieur et al. (1990) said that, some beneficial bacteria may participate in the digestion processes of bivalves via producing extracellular enzymes, such as lipases, proteases, as well as providing essential growth factors. They enhance growth by stimulating fish appetite and production of vitamins, fatty acids and additional digestive enzymes thereby breaking down indigestible feed components and improving digestion (Wee, 1991; Lara-Flores et al., 2013; Abdelhamid et al., 2014). The production of vitamins, fatty acids and additional digestive enzymes by probiotics cause breakdown of indigestible feed components and thereby overall improvement in growth performance of the fish. Research on nutritional parameters of the fish has been expanding for the proper administration of the new functional feed ingredients including

probiotics to improve growth, feed utilization and overall health. The nutrition of the aquatic organisms is essential for their profitable aquaculture, and the formulation of effective feed depends on our knowledge of the nutritional biochemistry and physiology of the cultured species (Lemos et al., 2000).

Digestive enzymes reflect the development of the digestive tract and digestive capability of the organism under study and can thus be used as an indicator of digestion and nutritional status at an early life stage (Ueberschar, 1993; Alvarez-Gonzalez et al., 2006; Comabella et al., 2006; Chen et al., 2006; Yufera and Darias, 2007) and provide information for determining the appropriate time for adjusting feeding strategies or weaning in fish culture (Chen et al., 2006; Hamza et al., 2007). Digestive enzyme studies are essential to explain nutrient digestibility (Kolkovski, 2001) in aquatic organisms. In fish, data pertaining to digestive enzyme activity and profiles have helped to overcome nutritional problems associated with formulation of artificial diets that meet an animal's nutritive capability (Furne et al., 2005).

Bacillus have been widely used as potential probiotics (Ziaei-Nejad et al., 2004), since they secrete a variety of antimicrobial compounds and exoenzymes (Moriarty, 1996, 1998). Wang (2007) showed that, *Bacillus* species as probiotic on shrimp *Penaeus vannamei* stimulated the activities of some digestive enzymes such as protease and amylase. Bagheri et al. (2008) proved that, *Bacillus*

sp., significantly stimulate the enzymatic activity and the digestive development in rainbow trout fry. Feng et al. (2008) observed that the probiotics significantly increased the activity of lipase enzyme in juvenile Japanese flounder *Paralichthys olivaceus*. Ghosh et al. (2008) viewed that, *Bacillus subtilis* increase the specific activity of protease and amylase enzymes in the digestive tract of *Anabas testudineus*. Suzer et al. (2008) observed that, *Lactobacillus* sp. enhance the specific activities of both the intestinal and the pancreatic enzymes in gilthead sea bream.

Gatesoupe (1991) reported the benefit of using *Lactobacillus plantarum* and *Lactobacillus helveticus* in turbot, *Scophthalmus maximus* (L.) leading to enhanced growth. Bandyopadhyay and Mohapatra (2009) incorporated *Bacillus circulans* in the diet of Indian major carps and reported significantly high growth performance, low FCR, high carcass protein and lipid content and high protease activity in the fishes fed with diet containing probiotics. Alterations in endogenous microbiota may offer an alternative method to increase feed utilization, control disease and promote health management (Suzer et al., 2008; Rodrigáñez et al., 2009).

However, only a few studies have been published on the nutritional effects of the *Bacillus coagulans* and *Lactobacillus plantarum*, particularly their effects at different doses in *Oreochromis mossambicus.*

3.2. MATERIALS AND METHODS

Oreochromis mossambicus, the tilapia fish was purchased from Aqua fish farm, Kottakkal, Malappuram District, Kerala. Total length and weight of the fishes used for the study was 3.45±0.61 cm and 1.75±0.54 g respectively. The fish were acclimatized for one week before conducting the experiment. During the adaptation period, the fish were fed twice a day with pellet without the addition of probiotics at a dose of 3% body weight.

3.2.1. Probiotic strains and culture conditions

Bacillus coagulans and *Lactobacillus plantarum* used in this study were isolated from gut of *O. mossambicus* and identified based on the phenotypic characteristics and 16S rRNA gene sequencing. They were grown at 37^0C for 24 h in nutrient agar broth. The pellets were washed once, and then resuspended in sterile 0.85% NaCl solution. The number of the bacterial cells in the suspension was determined by MRS agar plate count method (Zheng et al., 2018).

3.2.2. Diet preparation

Diet preparation procedure of Jana et al. (2012) and Rani and Rani (2014) was used with slight modification to prepare the experimental diets. Groundnut oilcake 30%, rice bran 25%, soya bean 23%, fish meal 20%, tapioca flour 1% and Vitamin mineral mix 1% used for the preparation of basal diet. Firstly, full fat soybean was autoclaved at 121^oC at 15 lbs to reduce the level of heat-labile

antinutritional factors (Garg et al., 2002). Mixer grinder was used to grind all the feed ingredients and made into small pellets. Probiotic supplemented feeds are prepared by the addition of *Bacillus coagulans* and *Lactobacillus plantarum* separately at different levels such as 10^2 (Diet 1), 10^4 (Diet 2), 10^6 (Diet 3), 10^8 (Diet 4) cfu/g feed.

3.2.3. Experimental design

After acclimatization process, the fishes were distributed into aquaria with a capacity of 20 liters. Twenty fishes were kept per aquaria and each experiment with three replications. Each aquarium was equipped with an aerator and a submersible water pump for recirculation system. Fishes were fed probiotic-supplemented pellets that had been previously prepared for one week at 3% per body weight, two times a day. Uneaten food were collected and dried and weighed. Water replacement was also carried out every 2–3 days as much as 30% of the water volume. The growth parameters measured were % weight gain, specific growth rate, feed conversion ratio and feed efficiency and digestive enzyme assays such as amylase, protease and lipase. All the parameters were measured for a duration of 15 days (DG15), 30 days (DG30), 45 days (DG45) and 60 days (DG60) separately.

3.2.4. Estimation of growth parameters

At the end of each feeding trial fish in each aquarium were individually weighed and growth performances were calculated

according to Sevier et al. (2000) and Dawood et al. (2016) by using the following equations:

$$\%\ weight\ gain = \frac{final\ weight - initial\ weight}{final\ weight} \times 100$$

Specific growth rate

$$= \frac{Ln\ (\text{Final weight}) - Ln\ (\text{Initial weight})}{\text{Experimental period in days}} \text{X } 100$$

$$\text{Feed Conversion Rate (FCR)} = \frac{\text{Feed given (g)}}{\text{Weight gain (g)}}$$

$$\text{Feed Efficiency (FE\%)} = \frac{\text{Weight gain}}{\text{Feed intake (g)}} \text{X } 100$$

3.2.5. Digestive enzyme assays

Isolation and homogenization of digestive tract

The whole digestive tract was isolated and homogenized at 4ºC using 0.85% NaCl solution (1:5 ration w/v). The contents were centrifuged at 4ºC at 13000 g for 20 min and the supernatant was used for the estimation of digestive enzymes.

3.2.5.1. Amylase assay

Amylase enzyme activity was assayed by following the procedure of Bernfeld (1955) and where one unit of enzyme activity represents the amount of enzyme required to release one µg of maltose per minute during assay conditions.

Reagents used

- Starch solution (1%): 1 g of starch dissolved in 100 ml distilled water
- NaCl solution (1%): 1 g of NaCl dissolved in 100 ml distilled water
- 3, 5 Dinitrosalicylic reagent: 100 ml Distilled water with 1g of 3, 5 Dinitrosalicylic acid, 30g sodium-potassium tartarate and 1.6g NaOH in it
- 0.1 M phosphate buffer (pH 7.0)
- Standard maltose solution: 100 mg of maltose in 100ml distilled water

Procedure for Amylase assay

1ml of 1% of starch solution as substrate, 1 ml of 0.1 M phosphate buffer (pH 7.6), 1 ml of 1% NaCl and 1 ml of crude enzyme extract as homogenate were taken in a test tube and this solution was incubated in an incubator at 37° C for 1 hour. After 1 hour, the reaction was arrested by adding 0.5 ml 3, 5 Dinitrosalicylic reagent into it. Absorbance was taken at 540 nm using spectrophotometer and the obtained value was deducted from the standard curve prepared from maltose monohydrate. Soluble protein (mg/g) was calculated from the crude enzyme extract using Lowry's method (Lowry et al., 1951).

3.2.5.2. Protease assay

Protease activity was measured following the procedure of Walter et al. (1984).

Reagents used

- 0.1 M Sodium phosphate buffer pH 7
- 0.6% Casein dissolved in tris buffer
- 12% Trichloroacetic acid
- Tyrosine

Procedure for Protease assay

The reaction mixture containing 4 ml of 0.6% casein in 0.1 M sodium phosphate buffer (pH 7) and 200 µL of enzyme extract was incubated for 1 h at 37^0C. 4 ml of chilled 12% TCA was then added to the reaction mixture to stop the enzyme action. Blanks were obtained by adding TCA to the substrate prior to the addition of enzymes. The reaction mixture was then filtered and the optical density was read at 273 nm in a spectrophotometer. The tyrosine content was measured in the test samples using a calibration curve of tyrosine. The enzyme activity was expressed as µg of tyrosine liberated per ml of enzyme extract min^{-1}

3.2.5.3. Lipase assay

Lipase assay was measured following the method described by Bier (1955).

Reagents used

- Phosphate buffer (0.1 M, pH 7.4)
- Pure olive oil as substrate
- NaOH (0.02 N)
- Polyvinyl alcohol (2%)
- Phenolphthalein indicator

Procedure for Lipase assay

Lipase enzyme activity was measured using olive oil emulsion in 2% polyvinyl alcohol as substrate. 1 ml of enzyme extract was added to 2 ml of substrate followed by 0.5 ml of 0.1 M sodium phosphate buffer and 3 ml distilled water. The reaction mixture was taken in a conical flask and incubated at 37^0C for 1 h in a shaker incubator with continuous shaking. Then 3 ml of 95% ethanol was added to stop the reaction. Few drops of phenolphthalein indicator were added and fatty acid liberated as a result of enzymatic action titrated with 0.02 N NaOH solutions till the appearance of faint pink color. 1 ml of 0.02 N NaOH is equivalent to 100 µM of free fatty acid. Blanks were obtained by boiled enzyme. Lipase activity was expressed as µmole of fatty acid liberated ml^{-1} of enzyme extract min^{-1}.

3.3. RESULTS

Data observed on Growth performance parameters of *Oreochromis mossambicus* fed on diets supplemented with *Bacillus coagulans* at different probiotic levels are provided in table 1. In the treatment with different probiotics concentrations, % weight gain for DG15 was observed as 7.42%, 11.94%, 11.96% and 13.76% for the probiotic's concentrations of 10^2, 10^4, 10^6 and 10^8 respectively. Percent weight gain of the samples DG30, DG45 and DG60 ranged between 8.48 and 13.86 %, 9.29 and 16.18% and 11.21 and 17.54% respectively for probiotic concentration between 10^2 and 10^8 (Fig. 1). Likewise, Specific Growth Rate increased in the treatment with 10^2 to 10^8 from 0.193 to 0.355, 0.215 to 0.376, 0.230 to 0.397 and 0.242 to 0.450 for DG15, DG30, DG45 and DG60 respectively (Fig. 2). Feed conversion ratio observed at various probiotics concentrations from 10^2 to 10^8 was ranged from 7.486 to 3.729, 6.470 to 4.419, 5.855 to 3.195 and 4.751 to 3.108 for DG15, DG30, DG45 and DG60 respectively (Fig. 3). In control, feed conversion ratio observed was 8.478, 8.598, 7.359 and 6.417 for DG15, DG30, DG45 and DG60 respectively (Fig. 3). Feed efficiency (%) observed at various probiotics concentrations from 10^2 to 10^8 was ranged from 8.185±0.16 to 12.389±0.15, 8.716±0.37 to 11.932±0.64, 8.774±0.67 to 10.870±0.69 and 9.7012±0.46 to 14.565±0.54 for DG15, DG30, DG45 and DG60 respectively (Fig. 4). For the groups of 15 days (DG15), 30 days (DG30), 45 days (DG45) and 60 (DG60) days administration of *Bacillus coagulans* as feed supplemented

probiotics at different concentration, growth parameters such as weight gain % (% WG), Specific growth rate (SGR), Feed conversion ratio (FCR) and Feed efficiency % (FE %) were found to be increasing significantly in each experimental group ($P<0.05$) (Table 1).

Table 1: Growth performance parameters of *Oreochromis mossambicus* fed on diets supplemented with different concentrations of *Bacillus coagulans*.

Duration of Expt.	Conc.of probiotics (cfu/g feed)	% weight gain	Specific growth rate (SGR)	Feed conversion ratio (FCR)	Feed efficiency (FE%)
DG15	Control	6.609±0.17	0.103±0.15	8.478±0.15	7.503±0.26
	10^2	7.420±0.19	0.193±0.16	7.486±0.13	8.185±0.16
	10^4	11.938±0.16	0.227±0.14	4.426±0.16	11.192±0.14
	10^6	11.955±0.64	0.321±0.17	3.759±0.17	12.231±0.18
	10^8	13.763±0.16	0.355±0.15	3.729±0.15	12.389±0.15
DG30	Control	6.523±0.45	0.111±0.24	8.598±0.12	6.912±0.24
	10^2	8.487±0.63	0.215±0.16	6.470±0.15	8.716±0.37
	10^4	9.728±0.39	0.241±0.41	5.568±0.14	9.996±0.37
	10^6	11.362±0.37	0.359±0.57	4.681±0.13	11.512±0.51
	10^8	13.860±0.19	0.376±0.37	4.419±0.15	11.932±0.64
DG45	Control	7.538±0.69	0.105±0.54	7.359±0.12	7.463±0.75
	10^2	9.295±0.61	0.230±0.44	5.855±0.15	8.774±0.67
	10^4	13.838±0.63	0.364±0.54	3.736±0.14	10.854±0.45
	10^6	15.812±0.43	0.377±0.53	3.527±0.15	10.718±0.67
	10^8	16.183±0.36	0.397±0.60	3.195±0.14	10.870±0.69
DG60	Control	8.551±0.50	0.064±0.62	6.417±0.15	7.870±0.14
	10^2	11.213±0.41	0.242±0.53	4.751±0.12	9.7012±0.46
	10^4	13.315±0.62	0.400±0.67	3.906±0.14	13.131±0.46
	10^6	16.971±0.71	0.417±0.49	3.695±0.15	14.562±0.52
	10^8	17.539±0.96	0.450±0.63	3.108±0.12	14.565±0.54

DG15-15 days group, DG30-30 days group, DG45-45days group and DG60-60 days group.

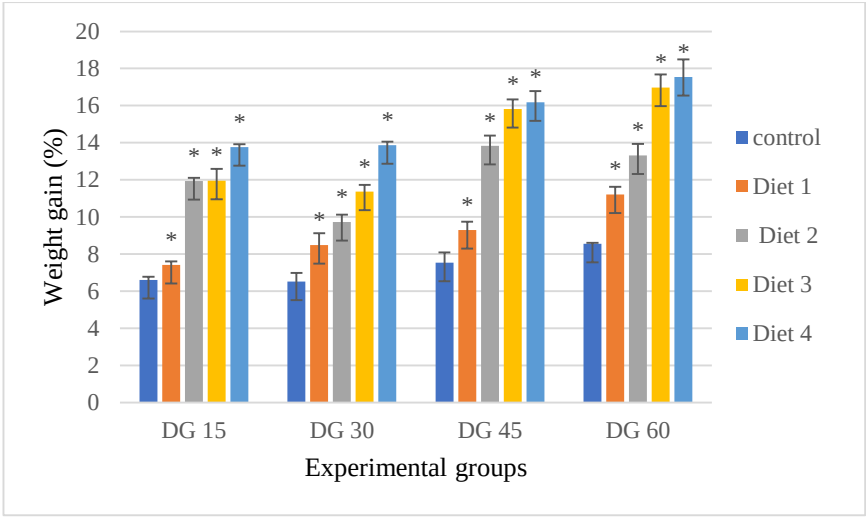

Figure 1: percent weight gain in *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

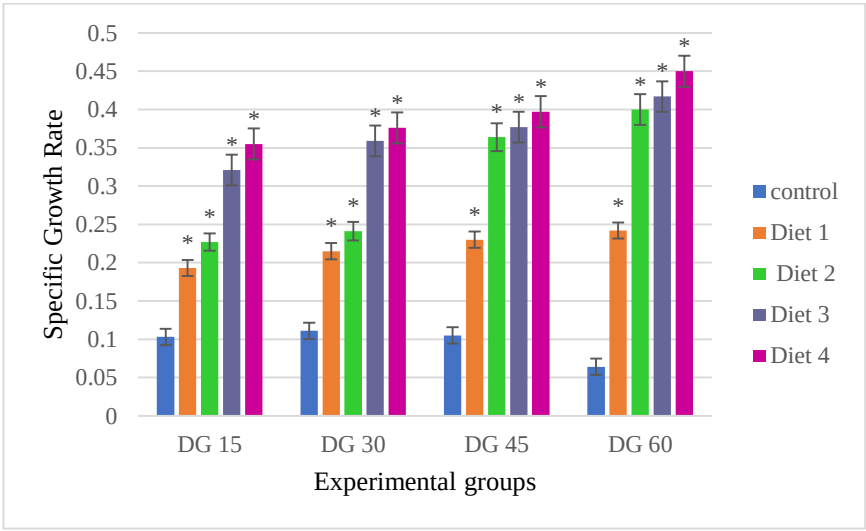

Figure 2: Specific growth rate in *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

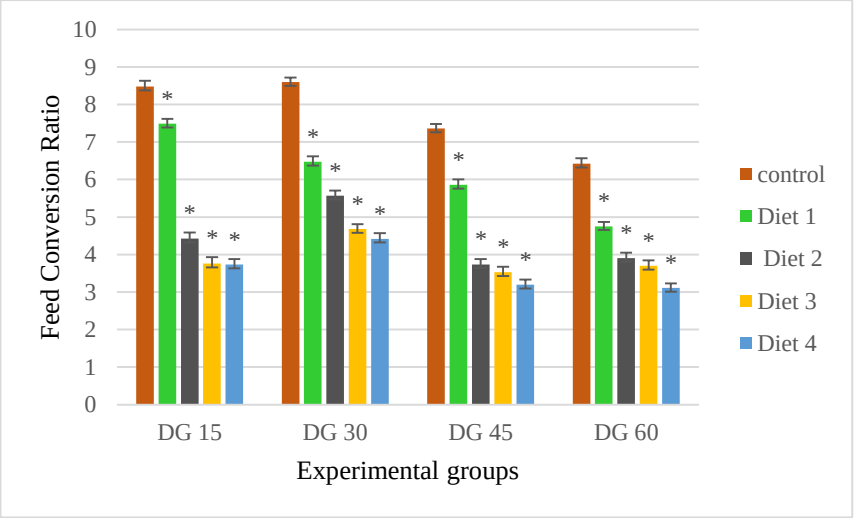

Figure 3: Feed conversion ratio in *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

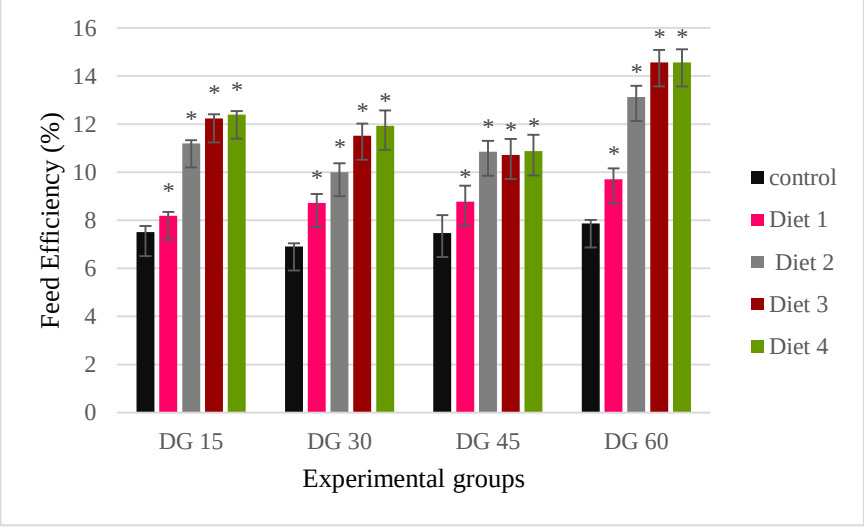

Figure 4: Feed efficiency (%) in *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

Growth parameters such as percent weight gain, Specific growth rate, Feed conversion ratio and Feed efficiency also increased significantly ($P<0.05$) when compared to the control after administration of *Lactobacillus plantarum* as feed supplemented probiotics at different concentrations and the data were presented in table 2. All the growth parameters increased proportionate to the concentration of probiotics per diet and duration of administration of probiotics. In the treatment with different probiotics concentrations, % weight gain for DG15 was observed as 2.85%, 3.35%, 4.69% and 5.17% for the probiotic's concentrations of 10^2, 10^4, 10^6 and 10^8 respectively. Percent weight gain of the samples DG30, DG45 and DG60 ranged between 6.25 and 10.67%, 9.82 and 16.37% and 13.52 and 23.68% respectively for probiotic concentration between 10^2 and 10^8 (Fig. 5). Likewise, Specific Growth Rate increased in the treatment with 10^2 to 10^8 from 0.126 to 0.246, 0.591 to 0.849, 0.650 to 1.047 and 0.793 to 1.177 for DG15, DG30, DG45 and DG60 respectively (Fig. 6). Feed conversion ratio observed at various probiotics concentrations from 10^2 to 10^8 was ranged from 7.071 to 5.068, 6.989 to 5.022, 5.510 to 3.064 and 3.838 to 2.111 for DG15, DG30, DG45 and DG60 respectively (Fig. 7). In control, feed conversion ratio observed was 8.107, 8.379, 8.965 and 8.211 for DG15, DG30, DG45 and DG60 respectively. Feed efficiency (%) observed at various probiotics concentrations from 10^2 to 10^8 was ranged from 4.906±0.16 to 9.104±0.15, 11.111±0.26to 19.912±0.62, 18.148±0.35 to 32.640±0.59 and 26.056±0.64 to 51.724±0.69 for DG15, DG30, DG45 and DG60 respectively (Fig. 8).

After the fish were fed with *L. plantarum* for DG60, the high % weight gain %WG, SGR, FCR and FE % observed in fish at the concentration 10^8 cfu/g with values 23.684±0.52, 1.177±0.12, 2.111±0.05, 51.724±0.69, whereas fish were fed with *B. coagulans* at the concentration 10^8 cfu/g for DG60 had highest values of %WG, SGR, FCR and FE as 17.539±0.96, 0.450±0.63, 3.108±0.12, 14.565±0.54 at concentration 10^8 cfu/g.

Table 2: Growth performance parameters of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

Duration of Expt.	Conc. of probiotics (cfu/g feed)	% weight gain (%WG)	Specific growth rate (SGR)	Feed conversion ratio (FCR)	Feed efficiency (FE %)
DG15	Control	1.539±0.15	0.107±0.18	8.107±0.05	2.606±0.15
	10^2	2.859±0.16	0.126±0.15	7.071±0.06	4.906±0.16
	10^4	3.352±0.14	0.202±0.17	7.047±0.05	5.780±0.14
	10^6	4.695±0.17	0.240±0.19	5.802±0.04	8.211±0.17
	10^8	5.179±0.15	0.246±0.12	5.068±0.09	9.104±0.15
DG30	Control	3.269±0.16	0.450±0.36	8.379±0.08	5.633±0.19
	10^2	6.250±0.23	0.591±0.43	6.989±0.05	11.111±0.26
	10^4	6.986±0.42	0.682±0.47	6.526±0.04	12.518±0.36
	10^6	10.207±0.67	0.804±0.34	5.278±0.06	18.946±0.61
	10^8	10.672±0.39	0.849±0.51	5.022±0.05	19.912±0.62
DG45	Control	4.617±0.15	0.523±0.67	8.965±0.08	8.068±0.62
	10^2	9.820±0.43	0.650±0.46	5.510±0.08	18.148±0.35
	10^4	15.123±0.53	0.993±0.65	4.433±0.06	29.696±0.47
	10^6	15.613±0.49	1.003±0.69	3.243±0.05	30.837±0.55
	10^8	16.377±0.61	1.047±0.15	3.064±0.06	32.640±0.59
DG60	Control	3.771±0.50	0.596±0.12	8.211±0.05	6.532±0.42
	10^2	13.520±0.51	0.793±0.62	3.838±0.04	26.056±0.64
	10^4	21.358±0.62	0.953±0.51	3.367±0.08	45.265±0.67
	10^6	22.131±0.45	1.147±0.56	2.209±0.07	47.367±0.42
	10^8	23.684±0.52	1.177±0.12	2.111±0.05	51.724±0.69

DG15-15 days group, DG30-30 days group, DG45-45days group and DG60-60 days group.

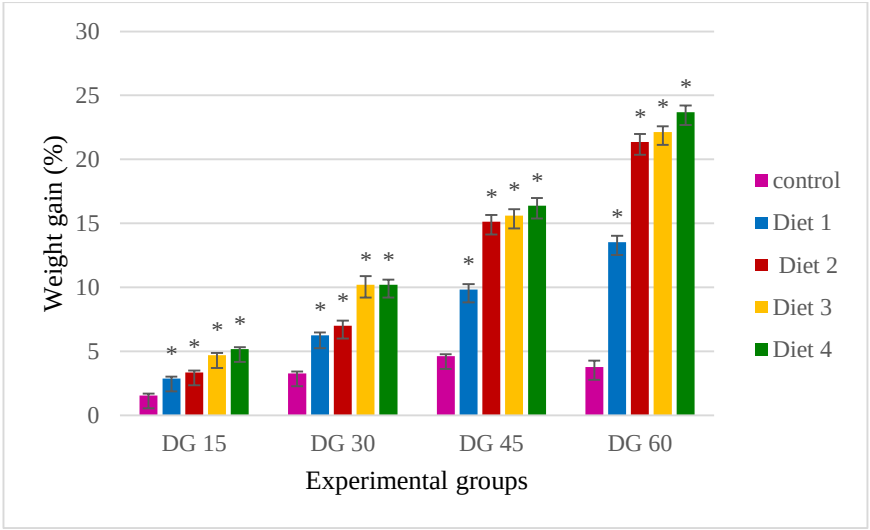

Figure 5: Percent weight gain in *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

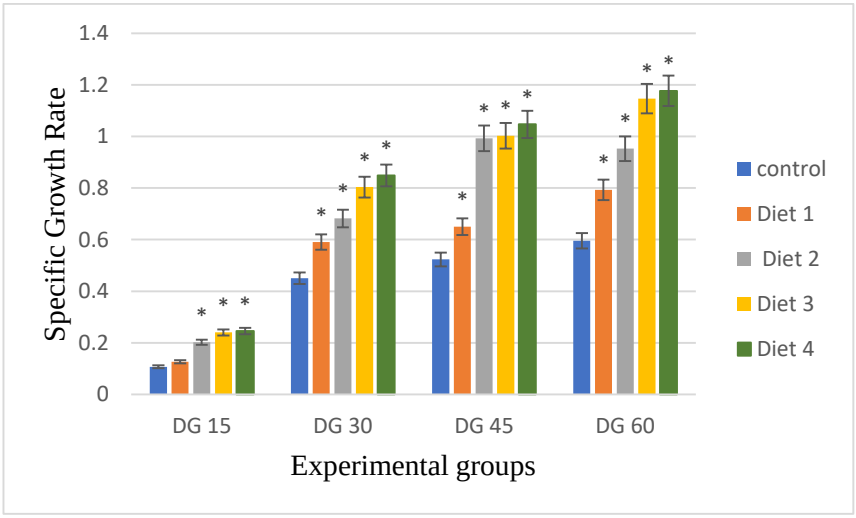

Figure 6: Specific growth rate in *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

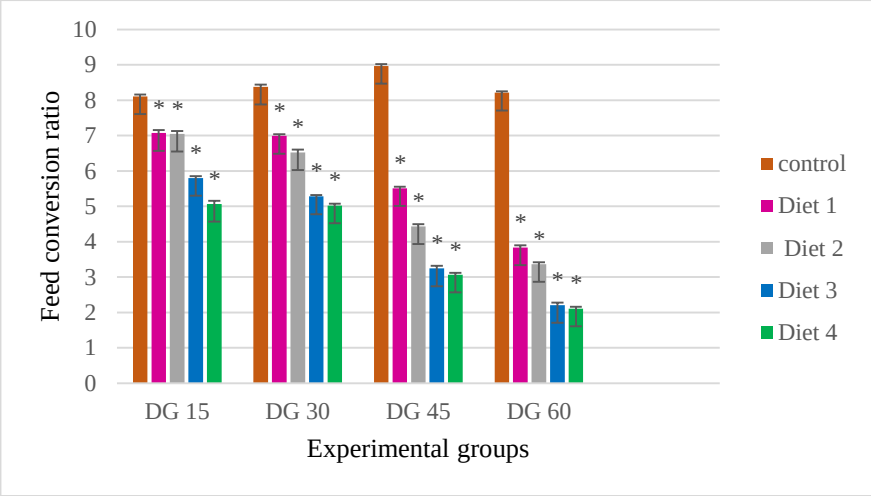

Figure 7: Feed conversion ratio in *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

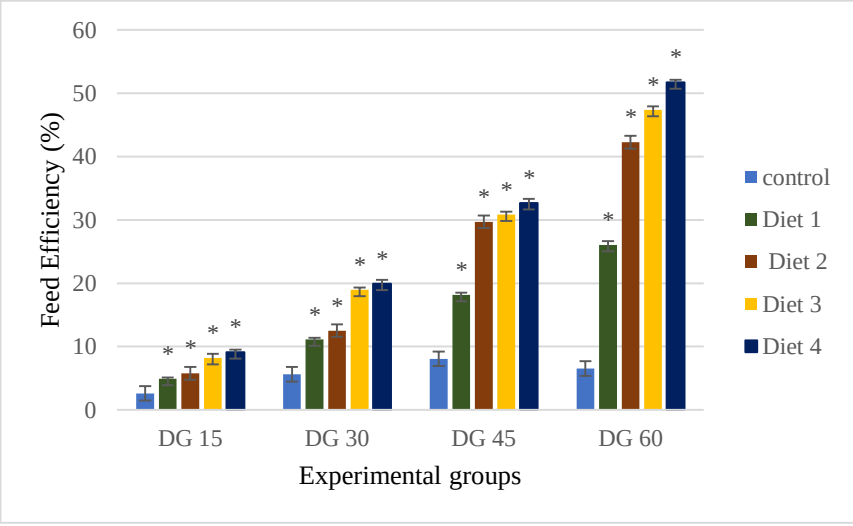

Figure 8: Feed efficiency (%) in *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

Concerning the growth performance of *O. mossambicus* treated with two different probiotics supplemented diet containing *Bacillus coagulans* and *Lactobacillus plantarum*, the results revealed that both groups received probiotics supplemented diets showed higher growth rate than those kept on a basal diet, suggesting that the addition of probiotics enhanced the growth performance and feed utilization and mitigated the effects of growth-inhibiting factors in intensive aquaculture systems.

The probiotic supplemented diet significantly increased enzyme activities of amylase, protease and lipase when compared to the control group for all the 15 days interval groups with a gradient. In each experimental group, fish received higher probiotic supplemented diets revealed increase in enzymatic activity of amylase, protease and lipase. Amylase activity in fishes fed with *Bacillus coagulans* as probiotic supplements at concentrations 10^2, 10^4, 10^6 and 10^8 were 12.87±0.31, 13.55±0.38, 15.45±0.30, 16.58±0.42 respectively for DG15 experiments and in the control amylase activity was 11.82±0.23 (Fig. 9). For DG30 enzyme activity were 11.95±0.40, 13.87±0.20, 15.56±0.29, 16.91±0.53, and 19.08±0.65 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG45 enzyme activity were 12.09±0.37, 15.19±0.49, 16.84±0.60, 18.86±0.17, and 19.08±0.65 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG60 enzyme activity were 12.27±0.36, 16.56±0.18, 18.95±0.13,

22.14±1.13 and 23.56±0.19 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively (Fig. 9).

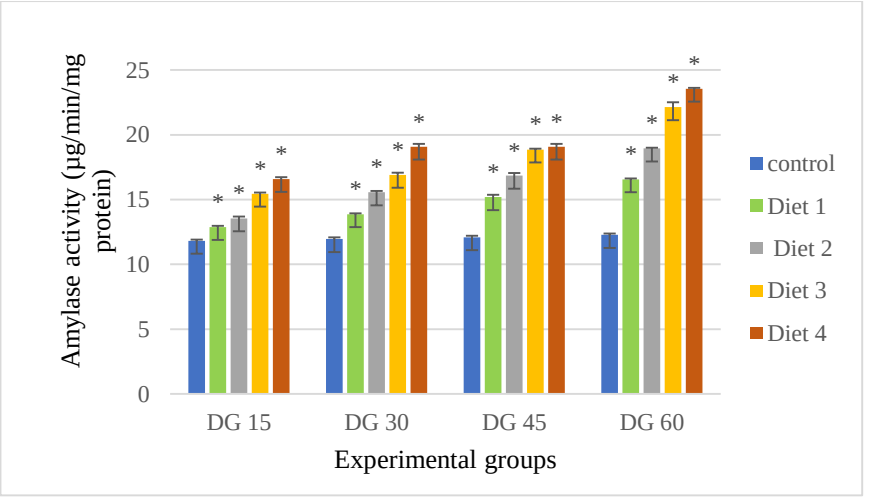

Figure 9: Amylase activities of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

Protease activity in fishes fed with *Bacillus coagulans* as probiotic supplements at concentrations 10^2, 10^4, 10^6 and 10^8 were 16.33±1.50, 18.18±1.49, 19.81±1.38 and 23.61±1.33 respectively for DG15 experiments and in the control protease activity was 15.40±0.67 (Fig 10). For DG30 enzyme activity were 15.53±0.77, 18.28±0.37, 19.68±0.29, 20.94±0.69 and 27.91±1.12 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG45, activity was 15.52±0.75, 20.10±0.62, 22.71±0.72, 29.43±0.71 and 30.05±0.94 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG60 enzyme activity was 15.52±0.71,

20.51±0.65, 23.03±0.73, 30.52±1.32 and 33.65±1.25 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively (Fig. 10).

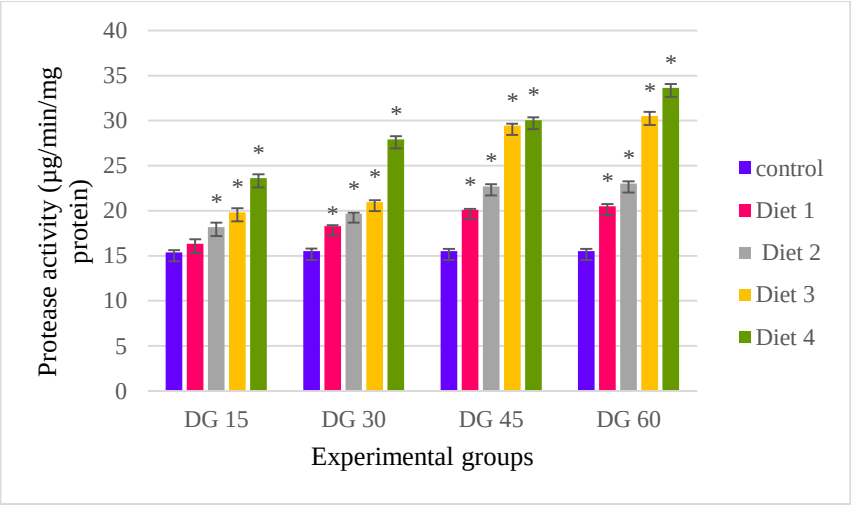

Figure 10: Protease activities of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

Lipase activity in fishes fed with *Bacillus coagulans* as probiotic supplements at concentrations 10^2, 10^4, 10^6 and 10^8 were 0.58±0.07, 0.63±0.09, 0.95±0.05 and 1.27±0.07 respectively for DG15 experiments and in the control lipase activity was 0.47±0.07 (Fig. 11). For DG30 enzyme activity were 0.48±0.07, 0.71±0.05, 1.03±0.07, 1.33±0.10 and 1.76±0.07 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG45 enzyme activity were 0.49±0.08, 0.82±0.10, 1.54±0.05, 2.02±0.07 and 2.37±0.08 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG60 enzyme activity were 0.49±0.04, 0.90±0.08, 1.84±0.09,

2.21±0.20 and 2.83±0.16 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively (Fig. 11).

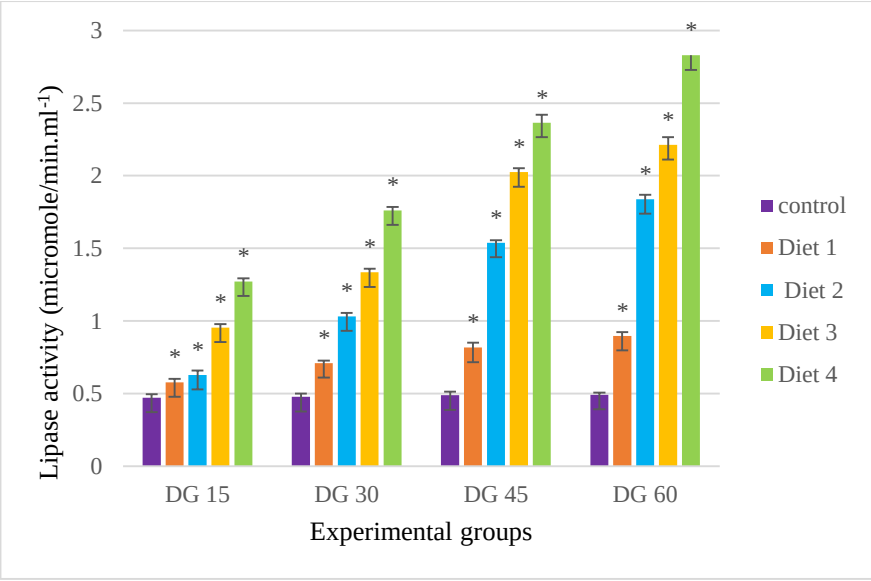

Figure 11: Lipase activities of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

The amylase activity in fishes fed with *Lactobacillus plantarum* as probiotic supplements at concentrations 10^2, 10^4, 10^6 and 10^8 were 12.67±0.54, 13.37±0.41, 14.97±0.45 and 15.21±0.34 respectively for DG15 experiments and in the control amylase activity was 12.10±0.54 (fig 12). For DG30 enzyme activity were 12.13±0.23, 14.51±0.76, 16.01±0.43, 18.01±0.88 and 18.94±0.55 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG45 enzyme activity were 12.39±0.53, 17.23±1.47, 18.47±0.48, 20.72±0.60 and 22.67±0.64 for control, 10^2, 10^4, 10^6

and 10^8 experimental groups respectively. For DG60 enzyme activity were 12.72±0.27, 17.35±1.34, 19.46±0.43, 21.70±0.25 and 23.79±0.38 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively (Fig. 12).

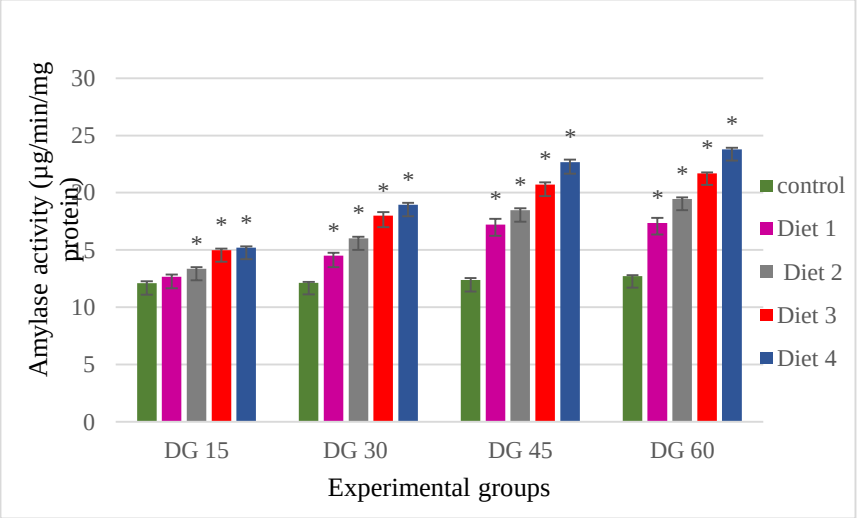

Figure 12: Amylase activities of *Oreochromis mossambicus* fed on diets supplemented with different concentrations of *Lactobacillus plantarum*.

Protease activity in fishes fed with *Lactobacillus plantarum* as probiotic supplements at concentrations 10^2, 10^4, 10^6 and 10^8 were 17.26±0.68, 18.40±1.09, 21.00±0.61, 22.71±0.66 respectively for DG15 experiments and in the control protease activity was 15.85±0.71 (Fig. 13). For DG30, enzyme activity was 16.10±0.73, 17.02±0.43, 18.89±0.52, 21.40±0.60 and 25.65±1.76 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG45 enzyme activity were 17.23±0.36, 19.43±0.52, 21.40±0.60,

25.35±1.59 and 28.50±1.05 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG60 enzyme activity were 17.74±0.59, 19.50±0.52, 23.33±0.27, 25.65±0.45 and 29.18±0.47 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively (Fig. 13).

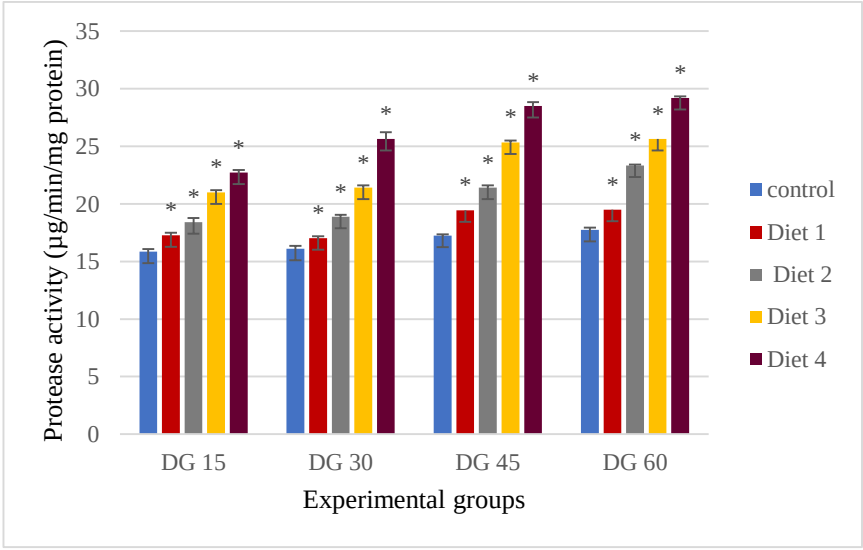

Figure 13: Protease activities of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

Lipase activity in fishes fed with *Lactobacillus plantarum* as probiotic supplements at various concentrations are provided in figure 14. The activity with *Lactobacillus plantarum* as probiotic supplements at concentrations 10^2, 10^4, 10^6 and 10^8 were 1.01±0.11, 1.31±0.06, 1.58±0.08 and 1.81±0.10 respectively for DG15experiments and in the control lipase activity was 0.83±0.13 (Fig. 14). For DG30 enzyme activity were 0.78±0.11, 1.15±0.09,

1.32±0.06, 1.56±0.05 and 1.89±0.06 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG45 enzyme activity were 0.73±0.16, 1.11±0.06, 1.47±0.13, 1.80±0.05 and 2.13±0.04 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively. For DG60 enzyme activity were 0.84±0.12, 1.24±0.14, 1.74±0.06, 1.95±0.08 and 2.16±0.08 for control, 10^2, 10^4, 10^6 and 10^8 experimental groups respectively (Fig. 14).

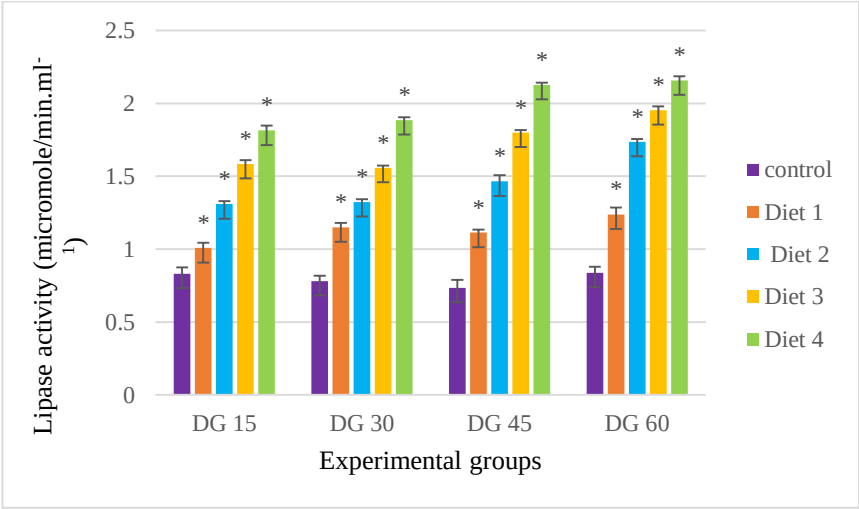

Figure 14: Lipase activities of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

Amylase enzyme activity was found to be maximum in the fishes for DG60 of *Bacillus coagulans* administration at 10^8 cfu/g concentration, with the value 23.56±0.19 which is significantly increased from the control enzyme activity 12.27±0.36 at $p<0.05$ (Fig. 9) Whereas, amylase activity was found to be maximum for

DG60 of *Lactobacillus plantarum* administration at 10^8 cfu/g concentration, with the value 23.79±0.38 which is significantly increased from the control enzyme activity 12.72±0.27 at $p<0.05$ (Fig. 12). Protease enzyme activity found to be maximum in the fishes for DG60 of *Bacillus coagulans* administration at 10^8 cfu/g concentration, with the value 33.65±1.25 which is significantly increased from the control enzyme activity 15.52±0.71 at $p<0.05$ (fig.10) Whereas, protease activity was found to be maximum for DG60 of *Lactobacillus plantarum* administration at 10^8 cfu/g concentration, with the value 29.18±0.47 which is significantly increased from the control enzyme activity of 17.74±0.59 at $p<0.05$ (Fig.13). Lipase enzyme activity found to be maximum in the fishes for DG60 of *Bacillus coagulans* administration at 10^8 cfu/g concentration, with the value of 2.83±0.16 which is significantly increased from the control enzyme activity 0.49±0.04 at $p<0.05$ (Fig.11) whereas, lipase activity was found to be maximum for DG60 of *Lactobacillus plantarum* administration at 10^8 cfu/g concentration, with the value 2.16±0.08 which is significantly increased from the control enzyme activity 0.84±0.12 at $p<0.05$ (Fig.14). At every 15 days intervals, amylase, protease, lipase activity in the intestine extracts was found significantly higher in the all the experimental groups than in the control group. Increase in the digestive enzyme activities is observed to be proportionate to the concentration of probiotics incorporated in the diet and duration of administration of probiotics.

3.4 DISCUSSION

Probiotics are known as live microorganisms that promote the health of their host by improving the balance of the gastrointestinal tract microbial flora (Nayak, 2010b). Lactic acid bacteria are widely used as feed supplemented probiotics for promoting growth performance and disease resistance of aquatic animals including tilapia (Doan et al., 2016). The present study shows that supplementation of probiotic strains *Bacillus coagulans* and *Lactobacillus plantarum* with tilapia diets enhances the growth performance of fish which is dependent to the dose of probiotics used and duration of administration (Table 1 & 2). The same diets also improved digestive enzyme activities such as amylase, protease and lipase (Figs 9–14). The present study reports are in agreement with several related reports. This study observed highest activity of digestive enzymes, amylase, protease and lipase were in fish fed with highest concentration of probiotics 10^8 cfu/g feed in DG60 groups.

Digestive enzyme activity can help fish degrade nutrients in feed and subsequently increase digestibility and feed efficiency (Cerezuela et al., 2011; Widanarni et al., 2015). These enzymes are responsible for the hydrolysis of the major components of the diet such as proteins, lipids, and carbohydrates, so that the use of a probiotic as a feed supplement which stimulates the action thereof, resulting in a higher quantity and quality of the produced fish, effectively acts as a growth promoter, characteristic of great interest

to the aquaculture production. Thus, it can be stated that the positive effect of probiotic on overall growth performance of fish is a reflection of its stimulating action on the activity of enzymes protease, lipase, and amylase.

Probiotic bacteria are capable of producing digestive enzymes that help fish for proper utilization of feed nutrients through digestion (Bairagi et al., 2002). Even though, exogenous enzymes produced by probiotic bacteria give only a small contribution to total enzyme activity in digestive tract (Ziaei-Nejad et al., 2006; Zhang et al., 2010), such high enzyme activity in gastrointestinal tract was alleged because the probiotic bacteria stimulate the synthesis of endogenous digestive enzyme production in fish. Mohapatra et al. (2012) also stated probiotics improve digestive enzyme activity by stimulating the synthesis of endogenous enzyme in the digestive tract. In brief, fish can produce endogenous digestive enzymes, but the presence of probiotics can improve the production of digestive enzymes or stimulate the activity of enzymes. The high digestive enzyme activity in fish fed with probiotic supplemented feed increases nutrient digestibility which indicated that the fish are capable of digesting nutrients in feed properly.

The results on feed efficacy indicated that supplementing diets with probiotics significantly improved feed utilization in tilapia. This helps to optimize protein use for growth which is the most expensive feed nutrient. The improvement in the biological

value of the supplemented diets in these treatments with high population and low dietary protein demonstrated that the probiotics supplements performed more efficiently in stress situations. This agreed with the results obtained by Ringø and Gatesoupe (1998). The best FCR values observed with probiotic-supplemented diets suggested that, the addition of probiotics improved feed utilization, ie., the reduction of production cost by supplementation of probiotics, reduce the quantity of feed essential for animal growth. Similar results have been reported by Lara-Flores et al. (2003). Probiotics are known to degrade the anti-nutritional factors in foods by improving the quality of the fish flesh. The higher growth efficiency was reported by Chandra and Rajan (2009) in Koi carp. The food conversion ratio parameters indicate the capability of any fish species to convert diet into body weight gain that keeps healthy (Hepher, 1988) relationship between food intake and weight gain. Growth parameters influenced by many factors such as the dose, the origin of probiotic strain and duration of administration plays an important role in the growth parameters (Irianto and Austin 2002).

It is reported by Lamari et al. (2013) that *L. casei* X2 could promote the growth of sea bass larvae. Andani et al. (2012) found that commercial feed containing 5×10^7 cfu/g of *Lactobacillus casei* could improve growth parameters of rainbow trout. This growth enhancement by Lactic acid bacteria is probably due to its effect on intestinal microbiota which has been demonstrated to play important roles in digestion, the production of essential vitamins, and

protection of the gastrointestinal tract from pathogen colonization (O'Hara and Shanahan, 2006). In accordance with this assumption, we found that Lactic acid bacteria containing feed changed the relative abundance of many genes enriched in metabolism pathways including lipid metabolism, metabolism of terpenoids and polyketides, metabolism of other amino acids, nucleotide metabolism and carbohydrate metabolism, xenobiotics biodegradation and metabolism, which could potentially improve growth rate and feed utilization.

Although the favorable effects of *Lactobacillus plantarum* in aquaculture animals have been investigated and it includes enhancement of the growth performance (Son et al., 2009; Doan et al., 2016; Yu et al., 2017). A similar kind of result was also found in *Dicentrarchus labrax* (Carnevali et al., 2004), *Labeo rohita* (Saini et al., 2014) and *Clarias gariepinus* (El-Feky et al., 2017). The presence of Enterobacter species in the intestinal flora of the fish improves the probiotic nature and helps the nutritional benefits for the fish (Sivakumar et al., 2014). Lara-Flores et al. (2003) stated that absorption of *Lactobacillus* by tilapia larvae for 9 weeks enhanced growth and feed conversion rate. Several studies have shown enhancement in growth rate and feed utilization parameters in different fish including Nile tilapia (Lara-Flores et al., 2003), Indian major carp, rohu, *Labeo rohita* (Sinha and Pandey, 2013) and rainbow trout, *Oncorhynchus mykiss* (Bagheri et al., 2008)

The obtained results could be attributed to the ability of *B. coagulans* and *L. plantarum* to adhere to the intestinal mucosa of *O. mossambicus* producing a wide range of relevant digestive enzymes (amylase, lipase and protease) which have the ability to breakdown the indigestible components in the diets, the ability to detoxify the potentially harmful components of feed and the ability to produce a lot of essential vitamin B. complex members particularly Biotin and vitamin B12, the matter of which resulted in increased feed utilization and digestibility of various diet components. These results supported those of Kennedy et al. (1998) who used *B. subtilis* in the food of common snook, *Centropomus undecimalis* and found that these probiotic bacteria increased the food absorption by enhancing the protease level and consequently gave a better growth. El-Haroun et al. (2006) in his study with Biogen® as food additive containing *B. subtilis* came to the conclusion that, this organism germinates in the intestine of fish, using a large number of sugar (carbohydrates) and produces a wide range of digestive enzymes (amylase, lipase and protease) which have a beneficial effect including higher growth rate and higher feed efficiency. Also, the incorporation of *S. cerevisiae* as a probiotic in fish diet was investigated and similar results were obtained. There are several reports available regarding the influence of probiotics on digestive enzyme activity in fish (Tovar-Ramirez et al., 2002, 2004; El-Haroun et al., 2006; Wache et al., 2006; Ghosh et al., 2008; Suzer et al., 2008; Rodrigáñez et al., 2009).

It has been reported that dietary supplementation of probiotic yeasts could increase digestive enzyme activity in sea bass, *Dicentrarchus labrax* (Tovar-Ramirez et al., 2002), rainbow trout, *Oncorhynchus mykiss* (Adel et al., 2017), abalone, *Haliotis midae* (Macey and Coyne, 2006) and sea cucumber (Yang et al., 2014). Study on the growth performance of saline Tilapia, *Oreochromis mossambicus* treated with probiotics showed a higher significant effect ($P<0.05$) on final weight, %weight gain, SGR and FCR compared to control (El-Feky et al., 2017). In tilapia, that showed improvement in feed digestibility and growth by adding *Bacillus* NP5 as feed probiotics (Putra and Widanarni, 2015). Application of probiotic bacteria *Bacillus megaterium* increases the activity of digestive enzyme and growth rate of catfish, *Clarias* sp. (Afrilasari et al., 2016). Lara-Flores et al. (2003) reported that probiotics strains can produce vitamins, detoxify compounds in the diet and the breakdown of the indigestible components thereby stimulate appetite and improve nutrition. Aly et al. (2008a) reported that diets supplemented with probiotics increased the weight gain in *O. niloticus*. The present study also observed significant increase in growth rate as observed in the groups fed with *Bacillus coagulans* or *L. acidophilus* than the untreated control group. Increased body weight gain in fish could be attributed to the improved digestive activity by improving the synthesis of vitamins, cofactors and enzymatic activity reported by Gatesoupe (1999), Jory (1998) and Ziemer and Gibson (1998). In addition, it has been reported that the

protein digestibility of fish was improved by oral administration of a diet containing probiotics. Some of bacteria can promote digestive enzyme synthesis of fish and digestion and high digestibility should be related to high growth rate in fish (Taoka et al., 2006a).

Supplement of probiotic bacteria resulted in conversion of high molecular weight protein to low molecular weight compounds. In this study, results showed enhancement of digestive enzyme activities in the gastrointestinal tract of tilapia after probiotics-application, especially the two groups given with highest concentration of probiotic cells. Zieai-Nejad et al. (2006) observed that the administration of probiotics containing *Bacillus* sp. enhanced the enzyme activity in the digestive tract of Indian white shrimp *Fenneropenaeus indicus*. They described that endogenous enzymes synthesized in the digestive tract of shrimp were stimulated rather than exogenous enzymes synthesized by probiotic bacteria because high enzyme activity was observed under the low population of *Bacillus* sp. This study demonstrated that the probiotic treatment stimulated the digestive enzyme synthesis in the gastrointestinal tract of the tilapia, especially with administration of high concentration of bacterial cells, suggesting that the concentration and duration of administration are key-factors. However, it is still unclear about the mechanisms of probiotic effects on the digestive enzyme synthesis of fish and whether the enhancement of digestive enzyme activities induces the high growth rate of host fish or not in this manner (Taoka et al., 2006a) Therefore,

studies on the mechanisms of the stimulation of digestive enzyme synthesis by probiotic treatment should be done further in detail.

It is reported that the digestive organs are very sensitive to food constituents and followed changes in the activities of the digestive enzymes (Bolasina et al., 2006; Shan et al., 2008), which is finally reflected in fish health and growth. In most cases, the mechanism for improved growth performance is unknown or not reported. An enhanced activity of lipolytic enzymes has been found in *Bacillus* fed groups. Many of the probiotics are efficient of secreting lipase, which stimulate production and assimilation of essential fatty acids resulting in higher growth and immunity in fish.

CHAPTER 2

EFFECT OF PROBIOTICS ON HAEMATOLOGICAL AND IMMUNOLOGICAL PARAMETERS

4.1 INTRODUCTION

Probiotics is one of the most promising methods for aquaculture, to strengthen the defense mechanisms of fish through prophylactic administration of natural immunostimulants and these agents are well known to increase resistance to infectious diseases by enhancing innate or nonspecific immunity (Raa et al., 1992). In recent years, increasingly more attention has been paid to the development of immunostimulants for both fish and other animals. A number of biological and synthetic compounds have been found to enhance the non-specific system in fish, which in turn protect the fish against infection caused by the pathogens (Raa et al., 1992; Jeney and Anderson, 1993; Sakai, 1999; Raa, 2000; Yin et al., 2006). Probiotic bacteria are used for prevention and cure of various diseases in aquaculture by using its ability to enhance non-specific immune responses in fishes. Probiotics protect fish by various mechanisms, it includes the activation of innate and adaptive immune responses to amplify killing of pathogenic agents (Bloch et al., 2013). Probiotic microorganisms will of course, have to be non-pathogenic and non-toxic when applied to fish in order to keep away detrimental side-effects. The indigenous micro biota in the

gastrointestinal system has influence on the innate immune system, which is crucial for the fish resistance against pathogenic microorganism. It is reported that the non-specific immune system can be strengthened by probiotics (Lara-flores, 2011).

In fish, the primary lines of non-specific defenses are the skin and mucus, when pathogens enter into the body, cellular and humoral non-specific defenses are mobilized (Dugenci et al., 2003). The major components of the innate immune system are macrophages, monocytes, granulocytes and humoral elements, such as lysozymes or the complement system (Secombes et al., 2009). The pathogen attacks the immune system in the fish and causes infectious diseases.

The benefits of probiotics on growth, the intestinal microbial population and immunity of fish in aquaculture, and in particular in tilapia farming, have been extensively studied and reviewed in recent years (Najeeb et al., 2015; Goutam and Kumar, 2017; Truong-Giang et al., 2017; Emmanuel et al., 2018; Narayanan et al., 2018). Rengpipat et al. (2000) reported that use of *Bacillus* sp. provides protection by stimulating responses of both cellular and immune defenses. Furthermore, induction of a particular immune response with respect to different tissue or organ also varies with dose. Therefore, the concentration of the individual probiotics needs to be estimated for a particular host. The concentration of probiotics consumed is an important factor to obtain high concentrations in the

various regions of the gastrointestinal tract. It is often said that probiotic concentrations must be greater than or equal to 10^8 cfu g^{-1} in the colon and 10^6 cfu mL^{-1} in the small intestine (Sanders, 2003). In aquaculture, the concentration of probiotics applied usually in the range 10^6- 10^{10} cfu g $feed^{-1}$, but the optimum concentration of a probiotics can vary with respect to host and also type of immune parameters (Panigrahi et al., 2004). Estimation of the optimum concentration of live probiotic bacteria to be consumed to fish is not an easy task (Aureli et al., 2011). The insufficient concentration of probiotics administered could limit the achievement of the optimum effects. A lower dose can be inadequate to induce the piscine immune system, whereas too high a dose can impose deleterious effects such as immunosuppression. Nikoskelainen et al. (2001), reported higher percentage of mortality in *Oncorhynchus mykiss* fed at high dose of *Lactobacillus rhamnosus* (10^{12} CFU g $feed^{-1}$) compared to lower dose (10^9 CFU g $feed^{-1}$). The optimum concentration of probiotics is not only required for bacteria colonization and multiplication in the intestine but it is also essential to effectively exert the beneficial effects, including immunostimulatory activity, enhancing growth and host protection. Commercially available probiotics are sometimes ineffective due to some or the other reasons. They are unable to survive or remain viable at optimum concentration in gut, possibly due to their non-fish origin (Abraham et al., 2008). Different strains of the same species may impose different effects on the host, as well as strains

of the same species can exert different, and sometimes, opposite effects (Aureli et al., 2011).

Al-Dohail et al. (2011) observed that serum total immunoglobulins concentration was significantly higher in African catfish fed with probiotic supplemented diet, compared to the control. Marzouk et al. (2008) showed that *Bacillus subtilis* and *Saccharomyces cerevisiae* improved the non-specific immune response of *Oreochromis niloticus*, through the stimulation of macrophage cells and increased phagocytic activity. Probiotic fed fishes exhibited an increase in the number of lymphocytes, monocytes and total white blood cell count and also a high resistance to the challenge with *Pseudomonas fluorescens*. Balcázar et al. (2007b) estimated the influence of probiotic strains in Rainbow trout (*Oncorhynchus mykiss*) on the humoral and cellular immune responses. The alternative complement activity in serum and phagocytic activity of leukocytes were significantly enhanced in the probiotic fed group when compared to untreated control fish. Kamgar et al. (2013) observed significant difference in the serum total protein, serum albumin, IgM and lysozyme of probiotic fed Rainbow trout, compared to control. Kim et al. (2012) studied the influence of a probiotic, *Enterococcus faecium*, on the immune responses against infection with *Lactococcus garvieae* in Olive flounder (*Paralichthys olivaceus*) and found elevated levels of lysozyme activity, complement activity and antiprotease activity on

probiotic treatment. El-Ezabi et al. (2011) investigated the effect of yeast, *Saccharomyces cerevisiae* and mixture of bacterial isolates such as *Bacillus subtilis* and *Lactobacillus plantarum*, on the immune response of the *Oreochromis niloticus* (Nile tilapia) and the results showed significantly higher phagocytic activity, acid phosphatase activity, lysozyme activity and total immunoglobulin in probiotic fed fish as compared with the control.

The administration of *Lactobacillus plantarum* induced immune modulation, enhances the growth performance, and increases disease resistance in fishes (Son et al., 2009; Giri et al., 2013, 2014). The lactic acid bacteria (LAB) are known to produce plantaricin that is active against certain pathogens (Cebeci and Gurakan, 2003). Among natural immunostimulants, lactic acid bacteria, especially from fish gastrointestinal tracts, have become potential candidates to replace antibiotics for controlling diseases in fish due to their generally recognized safe status and participation as key components in fish immune responses. Several research works have successfully utilized LAB to enhance fish immunity (Panigrahi et al., 2004; Salinas et al., 2005; Wang et al., 2008) and disease resistance ability (Gatesoupe, 1994; Gildberg et al., 1995, 1997; Gildberg and Mikkelsen, 1998; Verschuere et al., 2000).

The haematological evaluations are also inevitable to assess the health status of fish and monitoring stress responses. Nwanna and Tope-Jegede (2016) reported that probiotic *Lactobacillus*

plantarum administered to *Clarias gariepinus* had positive impact on blood profile, carcass protein and mineral composition of the catfish. Feed-probiotic *Lactobacillus acidophilus* administered to juvenile *C. gariepinus* increased the relative growth rate, specific growth rate, feed conversion ratio, protein efficiency ratio, haematological parameter and survival rate (Al-Dohail et al., 2009).

Pirarat et al. (2006) reported that supplementing the probiotic feed (*L. rhamnosus*) to tilapia (*Oreochromis niloticus*) stimulates the phagocytic activity. Further, probiotics have been reported to improve the respiratory burst of phagocytic cells, which play a central role in the protection of non-specific cell (Panigrahi et al., 2004; Balcázar et al., 2007b). *Bacillus subtilis* and *Lactobacillus* groups can stimulate the respiratory burst activity in the cultured fish (Nikoskelainen et al., 2003; Zhou et al., 2009). The present study determine the effects of *Bacillus coagulans* and *Lactobacillus plantarum* on haematological parameters such as haemoglobin concentration, haematocrit, total leucocyte and total erythrocyte count and immunological parameters such as total serum immunoglobulin, lysozyme activity and respiratory burst activity in *Oreochromis mossambicus*.

4.2 MATERIALS AND METHODS

4.2.1 Haematological parameters

Blood samples of treated fish were taken at 15 days (DG15), 30 days (DG30), 45 days (DG45) and 60 days (DG60) duration. Blood samples were also taken from the control group. Blood was drawn from the caudal peduncle region using a sterile syringe of 2 ml. Rinsed with 2.7% Ethylene Dimethyl Tetra Amine (EDTA) solution. Blood was collected in Eppendorf tubes.

4.2.1.1 Estimation of haemoglobin concentration

Haemoglobin concentration was estimated by following the procedure of Drabkin, 1946.

Reagents used

- 2.7% EDTA
- Drabkin's reagent

Procedure

The haemoglobin content of blood was analyzed following the Cyanmethemoglobin methods using Drabkins Fluid. Twenty microliters of collected blood was mixed with 5 ml working solution of Drabkin's fluid. The absorbance was read at a wavelength of 540 nm with a spectrophotometer. Haemoglobin contents were expressed as g/d1.

4.2.1.2 Haematocrit percentage

There is a linear relationship between haemoglobin and haematocrit and the percentage of haematocrit was calculated from the haemoglobin concentration using the formula (Sorell-Rashi and Tomasic, 1998)

$$Hct\ (\%) = 2.941\ X\ ctHb\ (g/dl)$$

4.2.1.3 RBC and WBC count

The blood samples were used for the estimation of total erythrocyte and leukocyte count with the help of hemocytometer using a Neubauer's counting chamber following the methods given by Dacie and Lewis (1963).

4.2.1.3.1 Total Erythrocyte Count (TEC)

Reagents used

- Dacies fluid (Formaldehyde-10 ml, Trisodium citrate-31.3 g, Brilliant cresyl Blue-1.0 g, Distilled water-1 litre).

Procedure

The blood was drawn up to 0.5 mark in RBC pipette of hemocytometer. The pipette was immediately filled to 101 mark with the diluting fluid. The pipette was shaken for 30 seconds and then few drops of diluting blood were expelled from it. The tip of the

pipette was touched to Neubauer's slide and cover slip junction. The Neubauer's slide is divided into ruled areas of 1 sq. mm with the center sq. mm divided into 25 groups of 16 small squares each. The cells within the boundaries of these small squares (80 smallest squares) were counted. TEC = total number of cells in five small squares x 10,000 cu. mm-1 of blood.

4.2.1.3.2 Total Leukocyte Count (TLC)

Reagent required

- Shaw's solution (Solution A- Neutral red-25 mg, Sodium Chloride-0.9 g, Distilled water 100 ml; Solution B-Crystal violet 12.0 mg, Sodium Citrate-3.8 g, Formaldehyde-0.4 ml, Distilled water-100 ml)

Procedure

Shaw's solution was used after filtration. Blood was diluted 1:20 with WBC diluting fluids using WBC counting pipette. To suspend the cells uniformly in the solution, mixture was shaken well. Then the cells in four large squares of hemocytometer were counted.

TLC = total number of cells in four small squares x 500 cu. mm^{-1} of blood.

4.2.2 Immunological parameters

4.2.2.1 Total serum immunoglobulin concentration

Total serum immunoglobulin was measured according to the Siwicki and Anderson, (1993) and Milla et al. (2010) and serum total protein was measured using Bradford (1976).

Reagents used

- Polyethylene glycol

Procedure

Serum immunoglobulin precipitated out with polyethylene glycol. For this, 100 µl of the serum were mixed with an equal amount of 12% polyethylene glycol and incubated for 2 hours under constant agitation at room temperature. After centrifugation at 3000 g for 15 min, the supernatant was removed and the remaining protein was determined and it was subtracted from the total serum protein concentration.

4.2.2.2 Lysozyme activity

Reagents used

- Lyophilized *Micrococcus lysodeikticus*
- 0.05M PBS buffer, pH 6.2

Procedure

The turbidometric assay used to determine serum lysozyme activity was that of Ellis (1990), with slight variations. Briefly, 190-µl of a 0.2 mg/ml lyophilized *Micrococcus lysodeikticus* suspension in PBS (pH 6.2, 0.05 M) were added to wells of a 96-well plate and 10-µl of serum was then added. Absorbance was read at 530 nm after incubation at room temperature for 0.5 and 4.5 min by a microplate reader. One unit of lysozyme activity corresponded to a reduction in absorbance of 0.001/min.

4.2.2.3 Nitroblue tetrazolium assay

Reagents used

- 0.2% NBT
- N, N-dimethyl formamide

Procedure

The oxygen radical production by phagocytes in blood during respiratory burst activity was measured through nitroblue tetrazolium (NBT) assay as described by Anderson and Siwicki (1995). Briefly, 0.1 ml of EDTA mixed blood from each treatment group was taken in Eppendorf to which 0.1 ml of 0.2% NBT solution was added. The mixture was incubated for 30 minutes at 25°C. From the suspension, 50 µl was taken, added to 1.0 ml N, N-dimethyl formamide in Eppendorf tube and centrifuged at 3000 g for 5 minutes. The optical density (OD) of the supernatant was measured at 540 nm in spectrophotometer.

4.3 RESULTS

HAEMATOLOGICAL PARAMETERS

Haemoglobin concentration in fishes fed with *B. coagulans* as probiotic feed supplements is presented in Figure 15. Haemoglobin (Hb) value observed for DG15 was 4.772±0.09, 4.948±0.02, 5.088±0.06, 5.243±0.01 for concentration of probiotics 10^2, 10^4, 10^6 and 10^8 respectively. The values observed for DG60 were 5.203±0.10, 5.372±0.02, 6.253±0.03, 6.350±0.14 for concentration of probiotics 10^2, 10^4, 10^6 and 10^8 respectively and for the control group in DG 60 was 4.713±0.22.

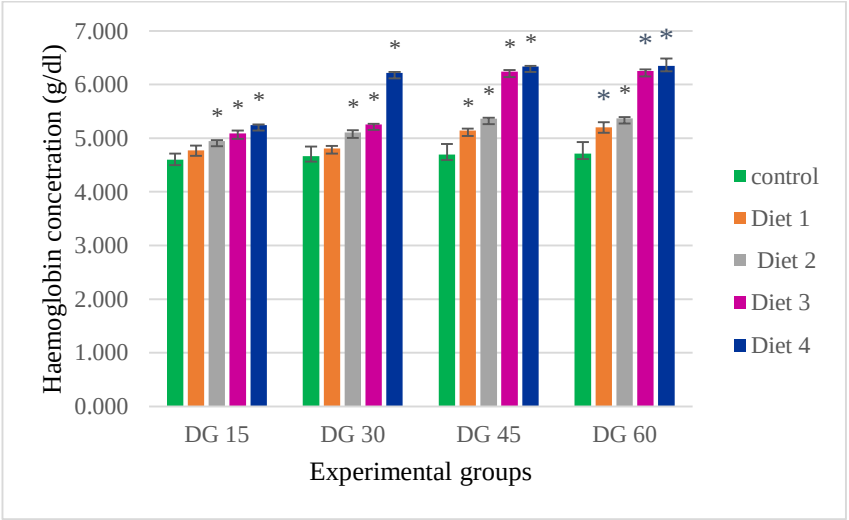

Figure 15: Haemoglobin concentration of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

Fish fed with *L. plantarum* as probiotic supplements, the Hb concentration in blood is represented in Figure 16. Hb values increased with the treatment 10^2 to 10^8 from 4.745±0.07 to 5.275±0.05, 4.815±0.04 to 6.228±0.02, 5.142±0.07 to 6.403±0.06, 5.205±0.10 to 6.460±0.11 for DG15, DG30, DG45 and DG60 experimental groups respectively.

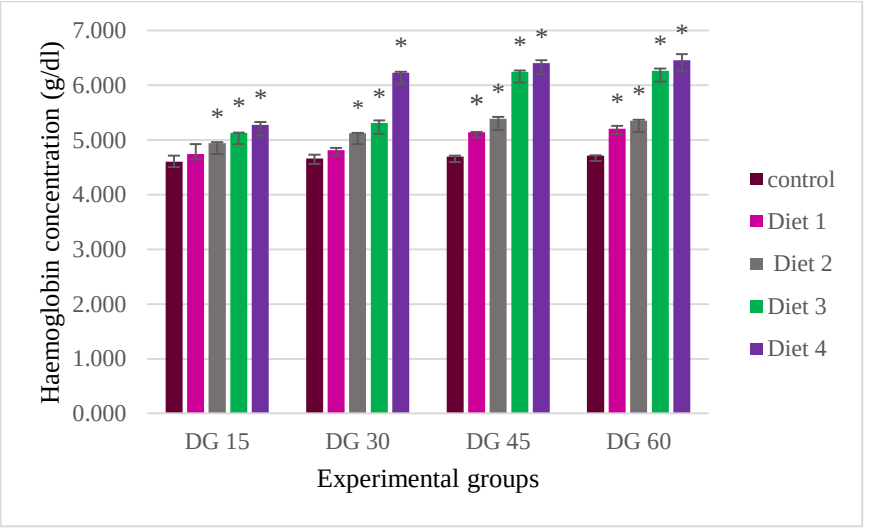

Figure 16: Haemoglobin concentration of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

Haematocrit (Hct) percentage in fishes fed with *B. coagulans* as probiotic feed supplements is presented in Figure 17. Hct percentage observed in DG15 were 21.898±0.47, 22.684±0.39, 23.493±0.07, 24.135±0.24, 24.844±0.04 for control, 10^2, 10^4, 10^6 and 10^8 respectively, whereas values observed in DG60 were

22.417±0.91, 24.661±0.40, 25.432±0.08, 29.470±0.13, 29.913±0.58 for control, 10^2, 10^4, 10^6 and 10^8 respectively. Haematocrit percentage in fishes fed with *L. plantarum* as probiotic feed supplements is presented in figure 18. Hct percentage observed in DG 15 were 21.898±0.47, 22.562±0.29, 23.455±0.07, 24.310±0.02, 24.990±0.21 for control, 10^2, 10^4, 10^6 and 10^8 respectively, whereas values observed in DG60 were 22.417±1.00, 24.669±0.44, 25.325±0.11, 29.524±0.17, 30.417±0.48 for control, 10^2, 10^4, 10^6 and 10^8 respectively.

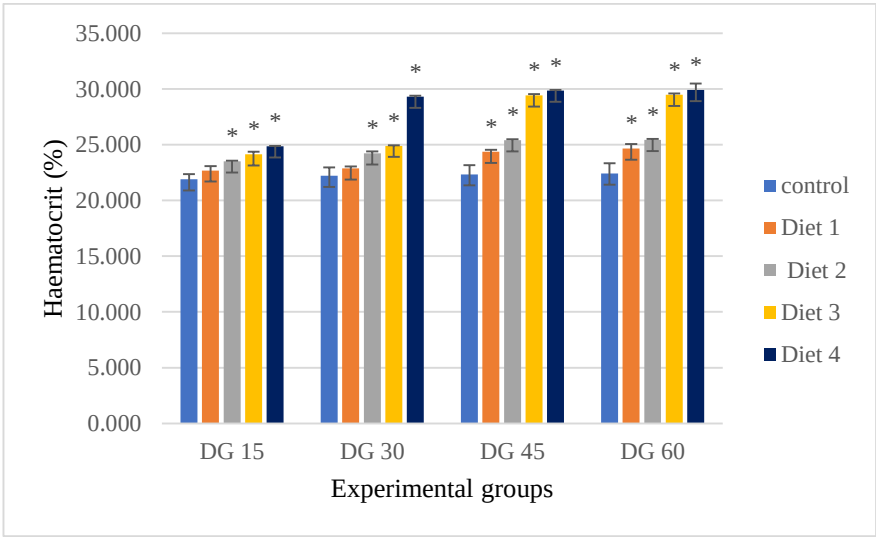

Figure 17: Haematocrit of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

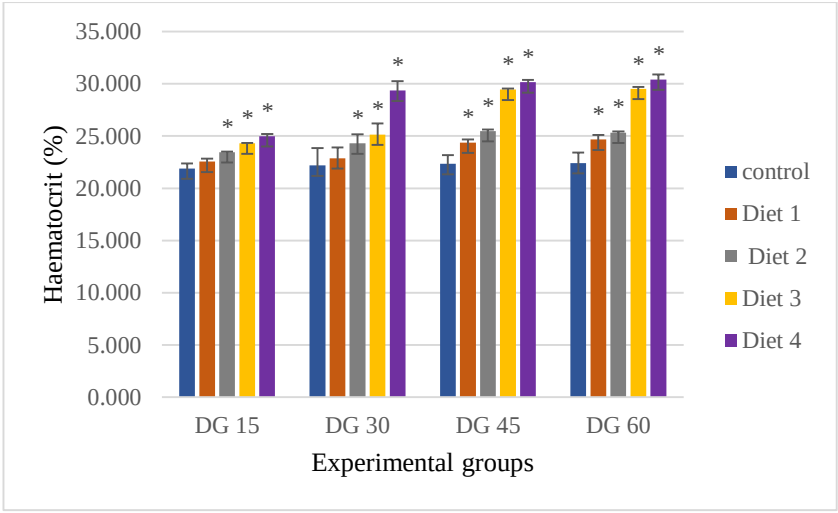

Figure 18: Haematocrit of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

Total erythrocyte count (TEC) observed during 60 days experiment was presented in Figure 19 and 20 for fish fed with *B. coagulans* and *L. plantarum* as probiotic feed supplement respectively. Total erythrocyte count in fish fed with *B. coagulans* as probiotic supplement increased from DG15 to DG60 as 1.993±0.02 to 2.090±0.48, 2.515±0.04 to 2.677±0.30, 3.187±0.08 to 3.603±0.28 and 3.580±0.06 to 3.924±0.05 for 10^2, 10^4, 10^6 and 10^8 respectively. TEC in fish fed with *L. plantarum* as probiotic feed supplement increased from DG15 to DG60 as 1.830±0.02 to 1.940±0.48, 2.797±0.04 to 2.940±0.30, 3.393±0.08 to 3.885±0.28 and 3.693±0.06 to 4.013±0.05 for 10^2, 10^4, 10^6 and 10^8 respectively.

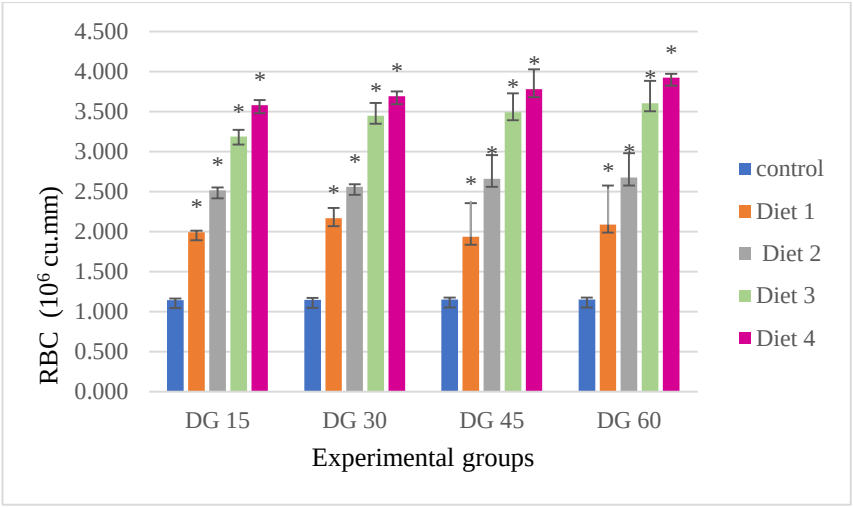

Figure 19: Total erythrocyte count of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

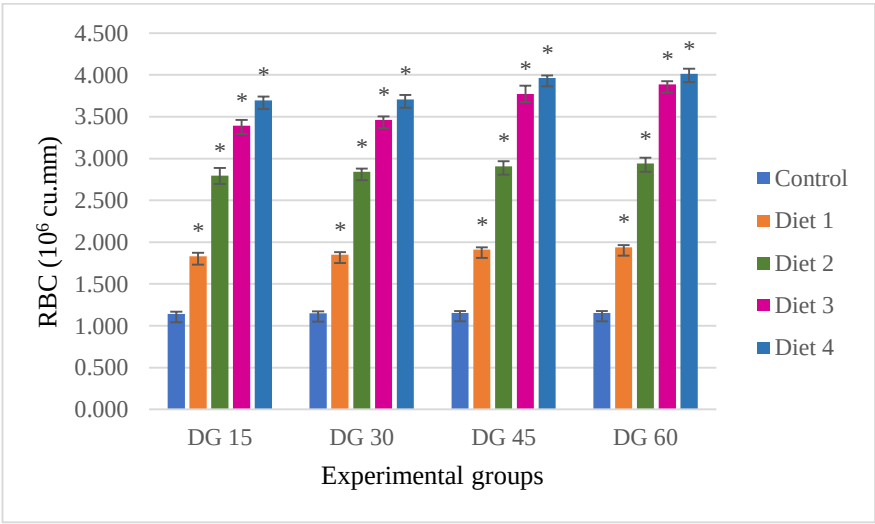

Figure 20: Total erythrocyte count of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum.*

Total Leucocyte Count (TLC) estimated from the blood samples of the fishes fed with *B. coagulans* and *L. plantarum* as probiotic feed supplement is provided in Figure 21 and 22 respectively. Total leucocyte count in fish fed with *B. coagulans* as probiotic supplement increased from DG15 to DG60 as 21.417±0.40 to 22.462±0.40, 22.047±0.19 to 23.047±0.19, 22.715±0.27 to 23.358±0.27 and 23.218±0.41 to 23.947±0.41 for 10^2, 10^4, 10^6 and 10^8 respectively (Fig. 21). TLC in fish fed with *L. plantarum* as probiotic feed supplement found to increase from DG 15 to DG 60 as 21.035±0.11 to 21.747±0.12, 21.523±0.06 to 22.695±0.22, 21.941±0.31 to 22.95±0.10 and 22.97±0.06 to 23.797±0.06 for 10^2, 10^4, 10^6 and 10^8 respectively (Fig. 22).

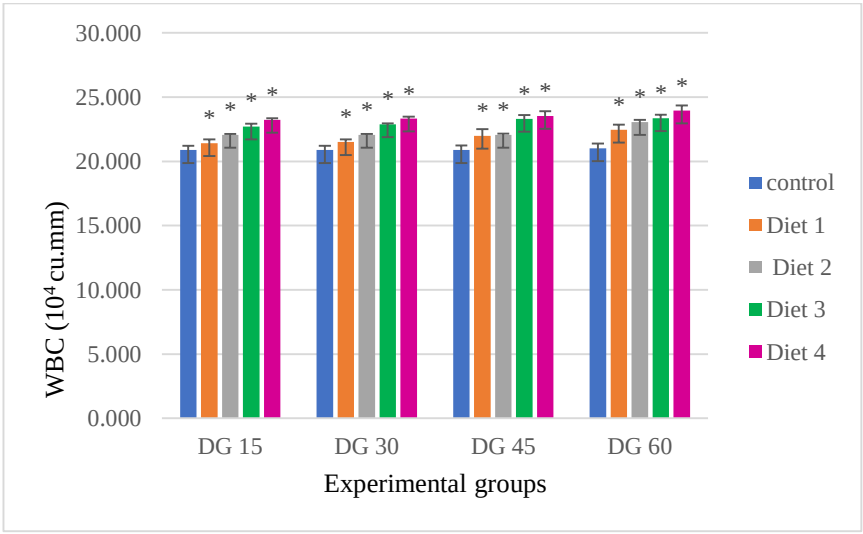

Figure 21: Total leucocyte count of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

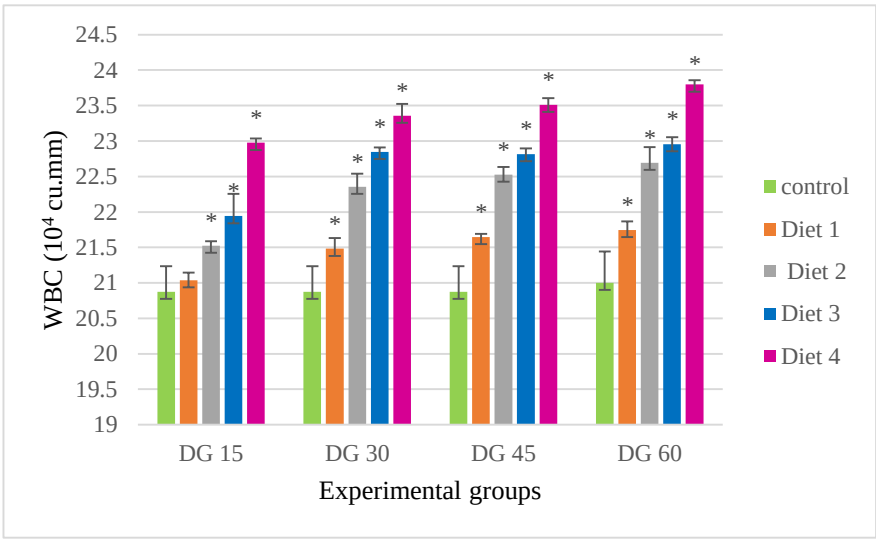

Figure 22: Total leucocyte count of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum.*

IMMUNOLOGICAL PARAMETERS

Total serum immunoglobulin was measured in fishes fed with *B. coagulans* and *L. plantarum* and presented in Figure 23 and 24 respectively. Total serum immunoglobulin observed in DG60 in treatment with *B. coagulans* were 21.903±0.33, 23.892±0.47, 26.843±0.42 and 27.687±0.40 for 10^2, 10^4, 10^6 and 10^8 respectively and the value for the control group in DG60 was 19.998±0.14. Whereas total serum immunoglobulin observed in DG60 with *L. plantarum* was 21.127±0.56, 23.055±0.51, 25.937±0.33, 27.228±0.46 for 10^2, 10^4, 10^6 and 10^8 respectively and the value for the control group in DG60 was 19.998±0.12.

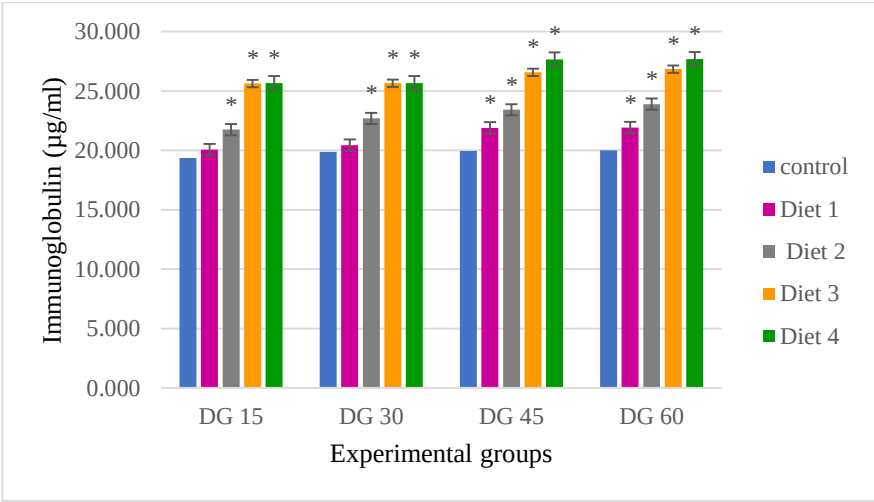

Figure 23: Total serum immunoglobulin of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

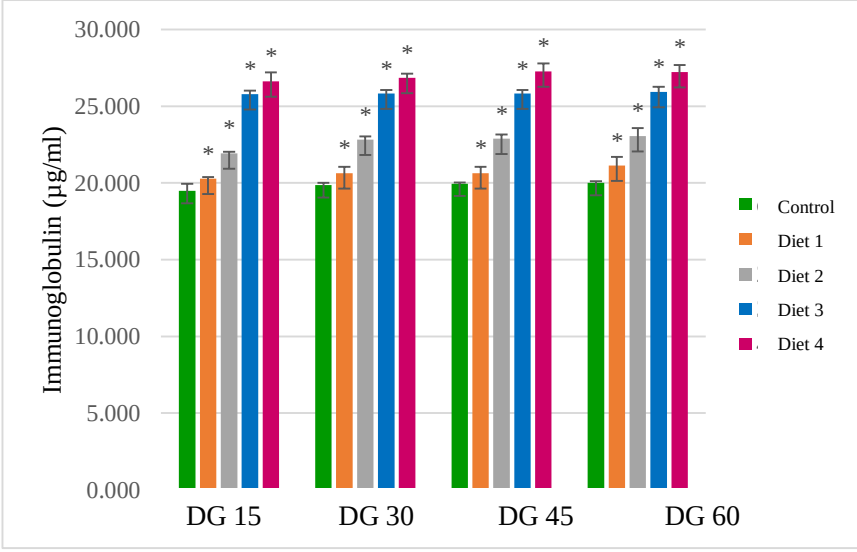

Figure 24: Total serum immunoglobulin of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

Lysozyme activity in fishes fed with *B. coagulans* and *L. plantarum* probiotics estimated and were presented in Figure 25 and 26 respectively. The lysozyme activity in fish fed with *B. coagulans* at different doses in DG15 were 4.788±0.09, 5.255±0.08, 5.515±0.11, 6.303±0.09 and 6.653±0.09 for control, 10^2, 10^4, 10^6 and 10^8 respectively, whereas values observed in DG60 were 4.871±0.13, 5.553±0.04, 5.902±0.03,6.577±0.03 and 7.255±0.09 for control, 10^2, 10^4, 10^6 and 10^8 respectively (Fig. 25). The lysozyme activity in fish fed with *L. plantarum* at different doses in DG15 were 4.788±0.09, 5.157±0.06, 5.505±0.06, 5.975±0.01, 6.408±0.04 for control, 10^2, 10^4, 10^6 and 10^8 respectively, whereas values observed in DG60 were 4.872±0.15, 5.553±0.07, 5.623±0.01, 6.088±0.10,6.713±0.12 for control,10^2, 10^4, 10^6 and 10^8 respectively (Fig. 26).

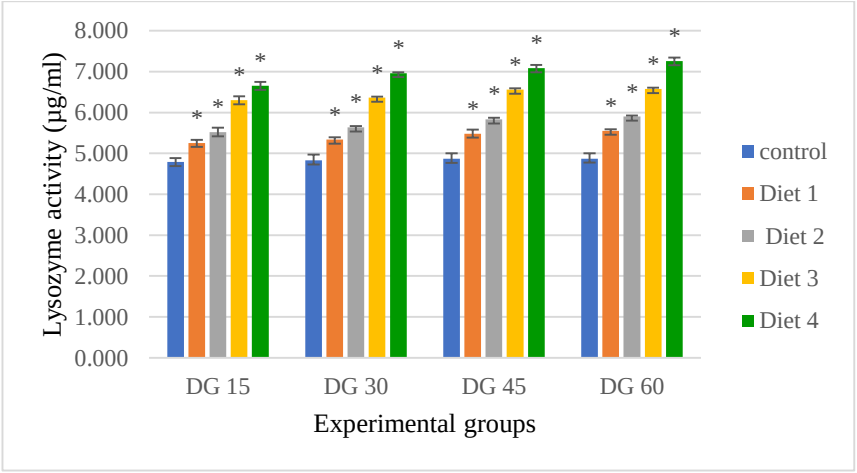

Figure 25: Lysozyme activities of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

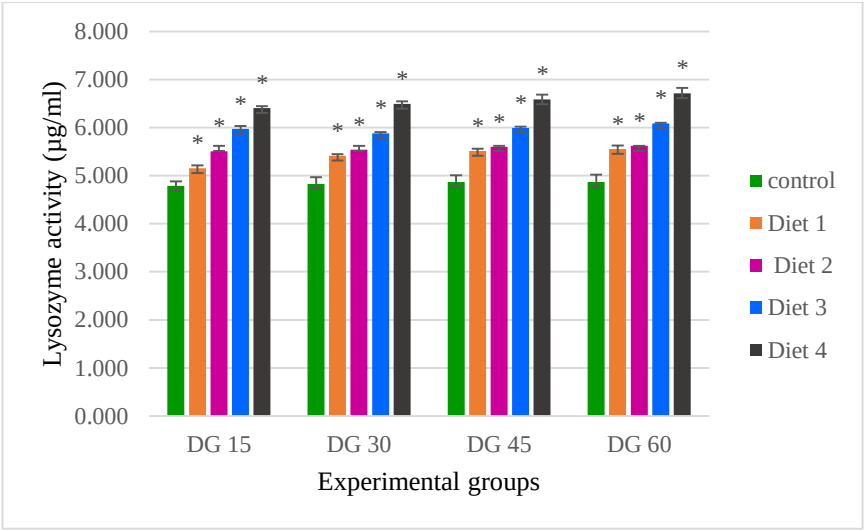

Figure 26: Lysozyme activities of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

Respiratory burst activity measured by NBT assay were presented in Figure 27 and 28 respectively. Respiratory burst activity in fish fed with *B. coagulans* at different doses in DG15 were 0.880±0.05, 1.148±0.03, 1.787±0.18, 2.338±0.04 and 2.482±0.04 for control, 10^2, 10^4, 10^6 and 10^8 respectively, whereas values observed in DG60 were 0.935±0.08, 1.393±0.04, 2.082±0.13, 2.613±0.01, 2.722±0.05 for control, 10^2, 10^4, 10^6 and 10^8 respectively (Fig 27). Respiratory burst activity in fish fed with *L. plantarum* at different doses in DG15 were 0.880±0.05, 1.155±0.03, 1.790±0.06, 2.203±0.10, 2.870±0.05 for control, 10^2, 10^4, 10^6 and 10^8 respectively, whereas, values observed in DG60 were 0.935±0.08 1.393±0.06, 2.342±0.03, 2.613±0.03 and 2.927±0.07 for control, 10^2, 10^4, 10^6 and 10^8 respectively (Fig. 28).

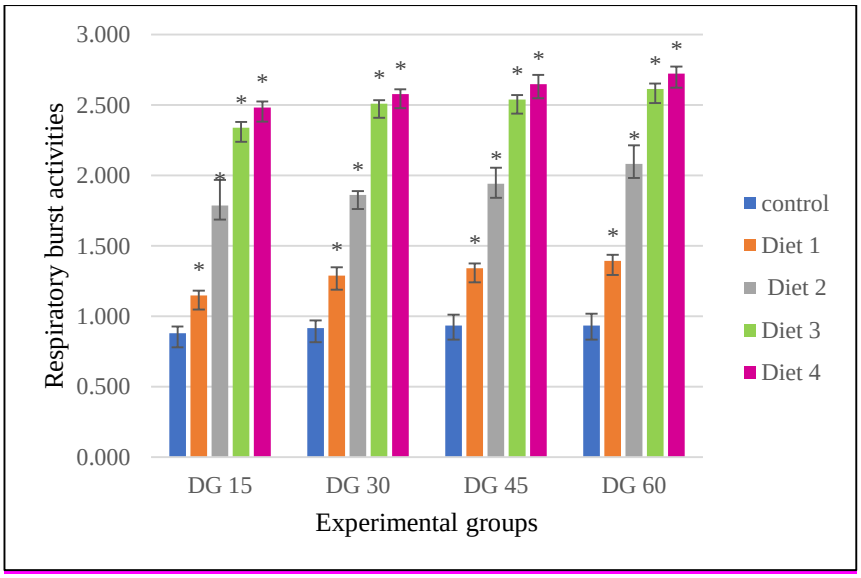

Figure 27: Respiratory burst activities of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Bacillus coagulans*.

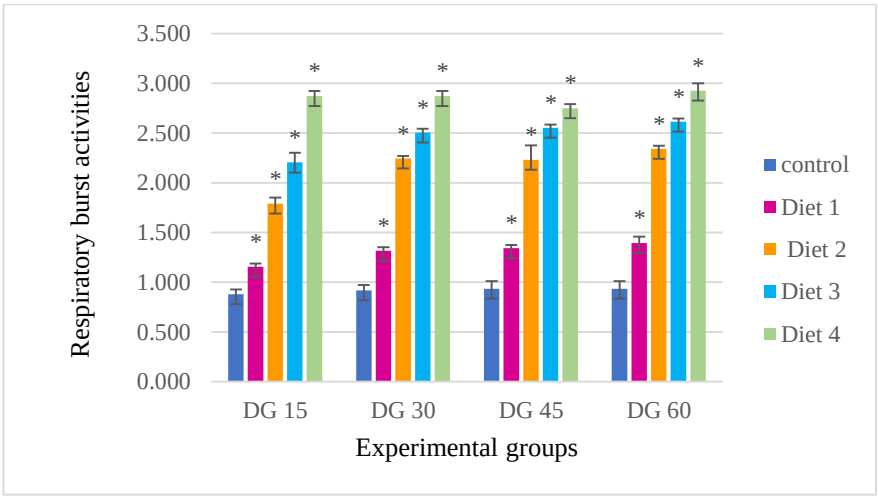

Figure 28: Respiratory burst activities of *Oreochromis mossambicus* fed on diet supplemented with different concentrations of *Lactobacillus plantarum*.

4.4 DISCUSSION

The probiotics have been recognized to function as immune-modulators in finfish which is, often through stimulation of innate and cellular immunity, including enhanced phagocytic, lysozyme, respiratory burst, cytotoxicity, complement activity, superoxide dismutase, increased numbers of leukocytes, erythrocytes, monocytes and lymphocytes, migration of neutrophils, neutrophil adherence, antiprotease and peroxidase activities, and plasma bactericidal activity (Newaj-Fyzul and Austin, 2015). Nonetheless, various probiotics may show different type of immune responses (Kane et al., 2016).

In the present study, haemoglobin concentration in fish fed with probiotics, *Bacillus coagulans* and *Lactobacillus plantarum* significantly increased ($p<0.05$) when compared to the control groups in each experimental duration of DG15, DG30, DG45 and DG60 (Figs 15 & 16). In DG15 and DG30 no significant increase ($P>0.05$) was observed for fishes fed with probiotics at the level of 10^2. It may be due to the fact that this level of probiotics are too low to initiate response in fish. Even though highest Hb value was observed in DG60, there is no significant changes ($P>0.05$) in Hb values between DG45 and DG60. The results are in agreement with several authors. The application of a probiotic species of *Lactococcus sporogenes* in Indian magur *Clarias batrachus* (Dahiya et al., 2012) and combined dosage of these probiotics *L.*

sporogenes, *L. acidophilus*, *B. licheniformis*, *B. subtilis* *Saccharomyces cervisiae* in *Cirrihinus mrigala* (Sharma et al., 2013), *Bacillus* np5 in *Oreochromis niloticus* (Tanbiyaskur et al., 2015), *Lactobacillus* and *Bifidobacterium* in *Clarias gariepinus* (Kiron and Watanabe, 2010) *B*acillus *subtilis*, *Saccharomyces cerevisiae* in mori, *L. acidophilus* and β-glucan, in snakehead (Talpur and Ikhwanuddin, 2013), *Bacillus cereus* in juvenile Nile tilapia (Garcia-marengoni et al., 2015), *B. pamillus* in *Labeo rohita* (Rajikkannu et al., 2015), *B. subtilis* and *B. licheniformis* in *Rutilus frisii* (Azarin et al., 2015) have been reported to enhance the haemoglobin levels. Previous studies reported an increase in the immunoglobulin in fish fed probiotic using *Lactobacillus rhamnosus*, *Lactobacillus acidophilus* and *Lactobacillus fermentum* respectively (Panigrahi et al., 2004; Al Dohail et al., 2009; Can et al., 2012; Akanmu et al., 2016). These results are in line with the present study.

Haematocrit (Hct) is the measure of capacity of blood to carry oxygen (Gallaugher, 1994). In this study, haematocrit percentage in fish fed with probiotics, *B. coagulans* and *L. plantarum* significantly increased ($p<0.05$) when compared to the control groups in each experimental duration of DG15, DG30, DG45 and DG60 (Figs 17 & 18). There is significant increase ($p<0.05$) in Hct percentage in fish fed with different doses of probiotics at each of the experimental group. Hct values found to increase depending to

the concentration of fed probiotics. The present observations are in agreement with the previous reports in this regard. Supplementation with commercial probiotics containing *Lactobacillus acidophilus, Lactobacillus casei, Enterococcus faecium and Bifidobacterium bifidum have been* have been reported to increase the Hct levels in Caspian roach fry (Imanpoor and Roohi, 2015). In other research reports, the application of a mixed probiotic species of *Lactococcus rhamnosus* and *Lactococcus lactis* in red seabream (Dawood et al., 2017), *Bacillus* sp. in Nile tilapia (Feliatra et al., 2018), a combination of *B. cereus* and *B. subtilis* in Nile tilapia (Garcia-marengoni et al., 2015) and *L. rhamnosus* on rainbow trout (Kiron and Watanabe, 2010) have been reported to increase the Hct levels. Increase in Hct value indicates that the fish can respond to stress factors in better ways.

The haematological parameters such as red blood cells, white blood cells, lymphocytes, heterocytes and respiratory burst have significantly increased in common carp fed diets fortified with *Lactobacillus acidophilus* (Adeshina, 2018). The present study also observed significant increase ($p<0.05$) in total erythrocyte count and total leucocyte count in fish fed with probiotics as feed supplement (Figs 19–22). TEC and TLC gradually increased depending upon on the concentration of probiotics and duration of administration. Highest counts were observed in fish fed with high concentration of probiotics in DG60 group, but there is no or less significant increase

in TEC and TLC between DG45 and DG60 at different concentration of probiotics. However, there is high significant difference ($p<0.05$) in TEC and TLC in between DG15 and DG60. The results obtained are in accordance with the findings of Faramarzi et al. (2011) in rainbow trout and Al-Dohail et al. (2009) in African catfish fed diets contain *Lactobacillus acidophilus*. *Oreochromis niloticus* fed a diet with *B. subtilis* improved the growth and positive effects on haematological parameters (Soltan et al., 2008; El-Rhman et al., 2009). The same results reported that the haematological parameters consistently increased in the probiotic diet (Al-Dohail et al., 2009). The haematological indices like haematocrit (Hct) and haemoglobin (Hb) levels, MCV, MCH and MCHC values increased in the fish, *L. rohita* fed with a formulated pelleted diet with probiotic isolated from gut *B. subtilis* for 15^{th}, 30^{th}, 45^{th} and 60^{th} days of the exposure period (Jayapraksh and Parvathi, 2019). Duncan and Klesius (1996) had reported that fish fed with diets containing probiotic *S. cervisiae* shows significant increases in the TEC (total erythrocyte count). Kumar et al. (2006), also stated that significant increase in TEC (total erythrocyte count) and TLC (total leukocyte count) was found in fishes fed with *B. subtilis*, which is an indication of improved health and immune response of fishes. Maqsood et al. (2009) and Misra et al. (2009) also observed significantly ($P<0.05$) high WBC count in fish administered with low and medium dosages of levamisole when compared to control group.

The innate immune system is an important defensive tool in invertebrates and a fundamental defense mechanism in fish (Magnadóttir, 2006). The *Bacillus coagulans* and *Lactobacillus plantarum* treated as probiotics in the present study showed improvement in immunological parameters such as total serum immunoglobulin, lysozyme activity and respiratory burst activity.

Total immunoglobulin is considered to be a key constituent of the humoral immune response in teleost (Ingram, 1980). It plays important roles in both nonspecific and specific immunity and its activity can be promoted by several immunostimulants (Magnadóttir, 2006). Enhanced Ig levels following probiotic administration have been documented in many animals, including fishes (Lee et al., 2017a, 2017b; Ramesh and Souissi, 2018). The present study also observed increase in total Ig level in blood of fish fed with probiotic feed supplements (Figs 23 & 24). Highest values, which is significantly higher when compared to the control ($P<0.05$) were observed in fish fed with probiotics at the concentration of 10^8 cfu/g feed in DG60 experimental group (Figs 23 & 24). In each experimental group, DG15, DG30, DG45 and DG60, highest values were observed in fish fed with higher concentration (10^8 cfu/g) of probiotics and there is significant difference ($p<0.05$) in the values of Ig in fish fed with different levels of probiotics. Improvement of Ig levels by *Bacillus subtilis* administration has been reported

previously in triangular bream (*Megalobrama terminalis*), rohu, and grouper (Sun et al., 2010; Zhang et al., 2013, Nandi et al., 2017).

Lysozyme constitutes an essential defense mechanism against bacterial infection and it plays an important role in the nonspecific humoral defense system of fish (Saurabh and Sahoo, 2008). The production of lysozyme has been reported to be one of the mechanisms through which probiotics provide protection against pathogens (Galagarza et al., 2018; Adorian et al., 2019; Mohammadian et al., 2019; Zhou et al., 2019). Probiotics trigger lysozyme activity in teleost when they are applied as single probiotics or in combination probiotics (Nayak, 2010a). In this study, lysozyme activity in fish fed with probiotics, *B. coagulans* and *L. plantarum*, significantly increased ($p<0.05$) in each experimental group when compared to the control (Figs 25 & 26). Fish fed with B. *coagulans* as probiotic feed supplement, lysozyme activity in DG60 significantly increased from DG45 at the concentration of 10^8 cfu/g feed, but there is no significant change ($p>0.05$) in lysozyme activity between DG45 and DG60 at lower concentrations (Fig. 25). This study observed significant increase ($p<0.05$) in lysozyme activities in fish fed with *B. coagulans* from DG15 to DG60 at every different concentration, whereas there is no significant increase ($p>0.05$) from DG15 to DG60 at every different concentration in *L. plantarum* fed fishes (Fig. 26). But there is significant difference in lysozyme activity in fish fed with different concentration of

probiotics in each experimental group. This is in agreement with results obtained with rohu *Labeo rohita* (Giri et al., 2014; Ramesh and Souissi, 2018), Japanese eel *Anguilla japonica* (Lee et al., 2018), hybrid Hulong grouper (Zhou et al., 2019), and Nile tilapia *Oreochromis niloticus* (Galagarza et al., 2018).

The lysozyme activity was reported to be dependent on the adherence of probiotic strains to host intestinal mucus (Balcázar et al., 2007a). Lysozyme has bactericidal activity and can act as an opsonin to activate the complement system and phagocytosis (Jollés and Jollés 1984). In agreement with our study, increased lysozyme activity was observed in some fish species that had given *Bacillus* sp. such as Nile tilapia, *O. niloticus* (Aly, et al., 2008a), common carp, *Cyprinus carpio* (Wang et al., 2014) and rainbow trout, *Onchorhynchus mykiss* (Merrifield et al., 2010a, c). Robertsen et al. (1994) showed an increased protection against the fish bacterial infection correlated to an increment in the serum lysozyme levels. The lysozyme activity levels have been previously found to be increased in fish fed diets supplemented with probiotics (Panigrahi et al., 2004; Kim and Austin, 2006; Aly et al., 2008c). While some studies indicate that probiotics do not have significant impact on this non-specific defense mechanism of fish (Nayak et al., 2007; Diaz-Rosales et al., 2009; Sharifuzzaman and Austin, 2010b). Lysozyme is a lytic protein that is important in the non-specific defense system. In particular, this protein causes the lysis of the cell walls of Gram-

positive bacteria observed in fish serum, mucus and tissues where in leucocytes are present (Ellis, 1990; Lie et al., 1989; Paulsen, 2003). The enhanced serum lysozyme level would have been mainly due to phagocytic cells, neutrophils and monocytes (Murray and Fletcher, 1976).

Phagocytes produce huge quantities of superoxide anion during phagocytosis or upon stimulation which can be reduced by NBT. The NBT reduction product obtained after reaction with superoxides is hence, a good indicator of the health status or the immunization effectiveness in fish (Anderson, et al., 1992). The NBT assay is a rapid inexpensive test focusing on the efficiency of phagocytes to reduce the dye by the production of oxygen radicals. Intracellular superoxide radicals produced by leucocytes reduce NBT and it can be estimated as respiratory burst activity of phagocytes, studies shows increased activity in host fed with diet supplemented with probiotics (Sumathi et al., 2014). In animals, the oxygen radicals are focused at the destruction of bacterial invaders. The ability of macrophages to kill pathogenic microbes is probably one of the most important mechanisms of protection against disease among fishes (Maqsood et al., 2009). The present study observed significant increase ($p<0.05$) in respiratory burst activity in fish fed with probiotics as dietary supplement (Figs 27 & 28). In fish fed with *B. coagulans* as feed supplement, respiratory burst activity was significantly increased ($p<0.05$) at different concentrations. But

there is no significant difference ($p>0.05$) between respiratory burst activity induced by 10^6 cfu g^{-1} and 10^8 cfu g^{-1} when compared to each other. In fishes fed with *L. plantarum* as feed supplement induce respiratory burst activity depending on the concentration of the probiotics. The values were significant when compared with each other in each experimental group. Similar results were obtained by Gopalakannan and Arul (2006) for *Cyprinus carpio*. Kumari and Sahoo (2006) also reported significantly high respiratory burst (NBT) activity in commercial probiotic fed group as compared to the control group. Sharp and Secombes (1993) suggested that enhancement in respiratory burst activity can be correlated with enhanced phagocytosis of bacterial pathogen by phagocytes.

It has been known that the innate immunity of fish can be enhanced by interactions between probiotics and intestinal epithelial cells (Lee et al., 1999) and according to Dawood et al. (2017) the immunological or physical barrier properties of the intestine could be controlled by those interactions. Probiotic bacteria can adhere and proliferate in host intestinal mucus, where they can then interact with epithelial cells. In animals including fish, the mucus secreted from intestinal epithelial cells contains many immune-related factors such as lectins, mucins and antimicrobial peptides (Lazado and Caipang, 2014) and plays an important role as a protective barrier to pathogenic infections (Zaineldin et al., 2018).

These study results are in agreement with, the report showed improvement in certain immunological and haematological parameters of rainbow trout Gullian et al. (2004). Probiotic bacteria exerted their beneficial effects through many ways, among those, there are strong evidences that they could also effectively strengthen host adaptive and innate immunity (Heyman and Ménard, 2002; Isolauri et al., 2002; Taoka et al., 2006a, b). Probiotics acts as immunostimulants through its capability to increase the nonspecific immunity of host either by enhancing the number of phagocytes or accelerating phagocytosis and respiratory burst activity to fight against microbes (Shoemaker et al., 2010). The significant changes in lysozyme activity, the respiratory burst, and the SOD levels constitute clear evidence that the synbiotic exercised immunomodulatory effects through positive alteration of different immune cells and enzyme activities in olive flounder (Hasan et al., 2018). These are in agreement with Taoka et al. (2006a, b), who studied the influence of commercially available probiotics on the nonspecific immune parameters of tilapia (*O. niloticus*) and reported an increase in lysozyme activity after the direct addition of probiotics to the rearing water. The fish given *B. coagulans* B16 as probiotic showed higher values than the fish fed without probiotic supplemented feed groups, suggesting that the probiotics can arouse nonspecific immune responses (Zhou et al., 2010). Salinas et al. (2006) reported that an increased respiratory burst activity of teleost fish *Sparus aurata* L. in vitro after supplementation of the heat-

inactivated *Lactobacillus delbrueckii* sp. *Lactis*. these are also in agreement with reports by Nikoskelainen et al. (2003), that showed a significant enhancement in respiratory burst activity when compared with the control group in rainbow trout fed with *L. rhamnosus* (8 X 10^4 cfu g^{-1}) for 2 weeks.

Faramazi et al. (2011) studied *O. mykiss* fed diets with *Lactobacillus acidophilus* and showed higher RBC and haemoglobin values when compared with fish that received the control diet. The numbers of leukocytes and thrombocytes are considered important indicators of fish health. The fish reared at the low stocking density and fed with the probiotic-supplemented diet showed higher values of leukocytes compared with the fish fed with control diet and raised at the same stocking density (Telli et al., 2014). The consumption of the probiotic diet induces the production of thrombocytes when these fish are raised at low stocking densities, which may be interesting because these cells play an important role in the immune system.

Newaj-Fyzul et al. (2007) observed higher lysozyme levels, phagocytic activity, and respiratory burst activity than the control group in rainbow trout fed a diet supplemented with 1X 10^7 CFUs of *B. subtilis* AB1 per gram of feed during a period of 14 days. Telli et al. (2014) demonstrated that the dietary administration of *B. subtilis* at the level of 5 X 10^9 CFU kg^{-1} of feed has a positive effect on the hematology parameters and the non-specific immunity parameters of tilapia (*O. niloticus*) such as the mean hemoglobin corpuscular

content, lysozyme level, and phagocyte activity of tilapias exposed to a high stocking density. Oral delivery of live yeast *Debaryomyces hansenii* modulates the main innate immune parameters and the expression of immune-relevant genes in the gilthead seabream (*Sparus aurata* L.) after the administration of 10^6 CFU g^{-1} for weeks (Reyes-Becerril et al., 2008). Giri et al. (2013) observed that the oral administration of *Bacillus subtilis* and *Lactobacillus delbrueckii* sp., single or combined, on gilthead seabream enhanced cellular innate immune responses like phagocytic activity and cytotoxicity. Respiratory burst activity did not showed any significant changes throughout the experimental period in any of the study groups.

In aquaculture, the haematological indices could be used as essential diagnostic tools to evaluate the health status of fish and the level of these indices in fact depends on species, age, nutritional parameters and environmental conditions to which the cultured fishes are subjected to. Though many potential probiotic bacteria have been identified for use in aquaculture, Lactic acid bacteria and *Bacillus* species are the common probiotics that have been reported to modulate most of these haematological indices. Besides, research data available indicate probiotic investigation on haematology is mostly based on optimal culture conditions as well as responses following pathogen infections. However, many environmental perturbations such as changes in temperature, the concentration of pollutants, nutrition among others are known to

cause physiological stress in fish. Even though many interventions have been made by various researchers on the use of probiotics in improving blood profiles of fish (Kiron and Watanabe, 2010; Dahiya et al., 2012; Da Paixão et al., 2017), the present work has thrown some light on the comparative dose dependent effects of two different strains of probiotic bacteria isolated from the gut of the same fish species on the haematological parameters of the fish, *Oreochromis mossambicus*. Even though both strains of probiotics are isolated from the gut of tilapia itself, the present study observed that there is difference in haematological and immunological responses induced by *B. coagulans* and *L. plantarum* in *Oreochromis mossambicus*. Therefore, it was necessary to consider the possibility of species and host differences (Lara-Flores et al., 2003).

CHAPTER 3

EVALUATION OF EFFICIENCY OF PROBIOTICS CHALLENGED BY *Streptococcus agalactiae*

5.1 INTRODUCTION

Fish are always susceptible to a wide range of bacterial, fungal, parasitic and viral diseases mainly owing to the characteristics of the immediate environment they live in as they are constantly in contact with facultative and obligatory pathogens most of which are opportunistic. Aquaculture to be commercially feasible, the basic requirement that maximum output with minimum space and time is to be satisfied. This often leads to poor water quality, high stocking densities and over feeding which in turn enhance the disease susceptibility of aquatic animals (Banerjee et al., 2017) in aquaculture systems. In aquaculture, infectious diseases are major problem that limits output worldwide and there are different methods available to diminish the effect of pathogenic microorganism in farmed aquatic animals (Newaj-Fyzul et al., 2014). Antimicrobials, immunostimulants and feed supplements are available to augment the health status of farmed animals, to eradicate or cure diseases and check disease outbreaks.

Application of probiotics is being practiced as an effective and attractive way to modifying the intestinal microbial composition

of aquaculture fauna and, to nourish and promote host health (FAO/WHO, 2001). The major mechanism of action of probiotics includes creation of epithelial barrier and pathogen elimination through adhesion to the intestinal cells, production of antibacterial substances and modulation of the immune function (Rijkers et al., 2010). Through the above mechanisms, the probiotics can achieve the modification of microbial balance and limiting the growth of pathogens (Almada et al., 2015). Recently, the administration of beneficial bacteria as a probiotics, either through direct addition to water or feed supplement has been demonstrated to be useful in aquaculture (Pérez-Sánchez et al., 2014). The species belonging to lactic acid bacteria (LAB) and *Bacillus* sp. are leading probiotic microorganisms used in aquaculture (Sun et al., 2011; He et al., 2013; Beck et al., 2015; Chai et al., 2016; Giatsis et al., 2016; Liu et al., 2012, 2016).

In recent years, the great challenge for the culture of tilapia is the disease streptococcosis caused by pathogenic *Streptococcus* sp. and it result in huge losses for tilapia farmers all over the world (Amal and Saad, 2011). Streptococcal diseases in fish initially affect the skin, fins, gills, and external organs. Various bacterial agents cause streptococcosis; *Streptococcus parauberis*, *Streptococcus iniae*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae* are the prominent species regardless of geographical region (Toranzo et al., 2005; Vendrell et al., 2006, Agnew and Barnes, 2007; Nho et al.,

2009, 2013). *Streptococcus agalagtiae* and *Streptococcus iniae* are important among the pathogens cause streptococcosis in farmed tilapia. *Streptococcus* sp. are Gram positive, non-acid fast, non-motile, oxidase-positive, catalase-negative cocci. Control of streptococcus infection mainly relies on the use of antimicrobial compounds, vaccinations, and environmental strategies (Darwish and Hobbs, 2005; Hastein et al., 2005; Sommerset et al., 2005; Cheng et al., 2010; Woo and Park, 2014), of which vaccines and antimicrobial compounds have been ineffective for various reasons (Shoemaker et al., 2001; Toranzo et al., 2005, Agnew and Barnes, 2007; Park et al., 2009). Environmental strategies have been used to control fish infections in their natural and artificial habitats by several methods (Holmer, 2010). Probiotics can eliminate pathogenic bacteria through competitive exclusion especially by antagonistic probiotics, provide nutrients and enzymes to enhance host growth, strengthen the immune response by immune stimulation, and do not cause secondary pollution problems. In sight of this, finding *S. agalactiae* antagonizing probiotics appropriate for tilapia culture is of great practical significance for increasing the resistance of tilapia and also help bringing down the use of antibiotics.

There are several literature available on probiotics that disclosed favorable effects on host's defense system which has vital importance in disease prevention as well as digestive tract

inflammation treatment (Azimirad et al., 2016; Modanloo et al., 2017). Apart from immunomodulation, probiotic microorganisms, such as lactic acid bacteria, *Vagococcus fluvialis, Brevibacillus brevis* and *Vibrio harveyi* (Arijo et al., 2008; Lazado et al., 2011; Sugimura et al., 2011; Korkea-aho et al., 2012; Mahdhi et al., 2012; Sorroza et al., 2012), adhere to the mucosal epithelium of gastrointestinal tract and provide resistance against pathogens (Luis-Villaseñor et al., 2011). Beck et al. (2015) investigated the influence of single or mixture administration of two host associated probiotics including *Lactobacillus plantarum* FGL0001 isolated from olive flounder (*Paralichthys olivaceus*) hindgut and *Lactobacillus lactis* BFE920 isolated from bean sprout, in olive flounder. After challenge with *Streptococcus iniae* ($\log_{10}$ 6.0 CFU/fish), the survival rate in the groups fed mixed probiotics and *L. plantarum* FGL0001, were found improved than the control. *Lactobacillus lactis* in rainbow trout diet and observed increased immune parameters as well as protection against furunculosis (Balcázar et al., 2007b). Kim et al. (2013) reported that *Lactobacillus lactis* BFE920 inhibits the growth of different pathogenic bacteria including *Streptococcus parauberis, S. iniae, Enterococcus viikkiensis* and *Lactococcus garviae* under *in vitro* condition.

Dietary administration of *Lactobacillus lactis* (10^8 CFU g^{-1}) increased serum immune responses as well as resistance against *S. iniae* in olive flounder (Heo et al., 2013). Raida et al. (2003) reported

that the dietary administration of *Bacillus licheniformis* and *Bacillus subtilis* (BioPlus2B) enhanced resistance against infection with *Yersinia ruckeri* in trout. Also, Kumar et al. (2006) reported increased resistance against *Aeromonas hydrophila* infection in Indian major carp *Labeo rohita* fed with *Bacillus subtilis* at 1.5×10^7 CFU g^{-1}. Newaj-Fyzul et al. (2007) studied influence of probiotics in rainbow trout by administrating in different forms such as viable, cell-free supernatant, formalized or sonicated cells and reported higher resistance against *Aeromonas* sp. in fish fed with probiotics when compared to the control. Liu et al. (2012) reported enhanced relative survival rate percentages of grouper fish, *Epinephelus coioides* challenged with pathogenic *Streptococcus* sp. after fed with diet supplemented with *B. subtilis* (10^4, 10^6, and 10^8 CFU g^{-1}) for 14 and 28 days. Red hybrid tilapia fed with diet supplemented with 0.1 or 0.3% *B. subtilis* showed improved prophylactic property and survival rate after challenged with pathogenic *Streptococcus agalactiae* (Ng et al., 2014).

Histological techniques can be used as potential biomarkers to detect and explain the effects of immunostimulants on the internal organs of organisms. Histopathology may be used to establish the patterns of both acute and chronic effects on tissues and organs and provide the prognostic evidence of the potential pathophysiological effects on organisms. The literature available on the effects of probiotics on histology and histopathology of fishes are really scarce. Several literatures explained the histopathological changes in fishes infected with different pathogens. *Streptococcus agalactiae*

infection in fish causes necrotic foci between foci and mucosa in intestine, vacuolization of hepatocytes in liver and lesions similar to epitheliocystis on the gill lamella (Chen et al., 2007).

It has been reported that diet supplemented with multispecies probiotics (*Pediococcus acidilactici*, *Enterococcus faecium*, *Bacillus subtilis* and *Lactobacillus reuteri*) significantly alter the intestinal morphology. It increased mid-intestinal microvilli density after 8 weeks of probiotic administration (Pirarat et al., 2011). They also suggested improvement in ratio of internal perimeter of the intestine lumen to external perimeter of the intestine. Saad (2006) suggesting that the probiotic possibly had positive effects on the immune system without triggering harmful inflammatory response by histological analysis. Nile tilapia, *Oreochromis niloticus* supplemented with probiotics (*Lactobacillus plantarum*) showed lower congestion degree in liver tissue when compared to the fish fed without probiotic diet (Ruiz et al., 2020). Mello et al. (2013) concluded that the use of *Bacillus subtilis* and *Bacillus cereus* at a concentration of 4×10^8 CFU g^{-1} caused an increase in the number of goblet cells. The increase in goblet cell number is a beneficial feature induced by supplementation of probiotic bacteria, since they increase the production of mucus. The intestinal mucus forms a gel that adheres to the epithelium and constitutes the first line of defense against chemical, mechanical, damages and affictions caused by bacterial toxins and enzymes that can damage the integrity of the intestinal epithelium (Finnie et al., 1995; Gaudier et al., 2009; Carnevali et al., 2017).

The present study investigated the effects on Relative Percent Survival (%) in fish fed with *Bacillus coagulans* and *Lactobacillus plantarum* as feed supplement after challenge with *Streptococcus agalactiae* and analyzed the histology of the internal organs. Histopathological observations also made after the fish challenged with *S. agalactiae* infection.

5.2 MATERIALS AND METHODS

5.2.1 Challenge Test with *S. agalactiae*

Streptococcus agalactiae strain were purchased from National Collection of Industrial Microorganisms (NCIM Accession no: 5659). *S. agalactiae* were freshly prepared by inoculating a single colony of the bacterial strain into nutrient broth and culturing at 32 °C for 24 h. The cultures were centrifuged at 5000 g for 5 min. The pellets were washed twice with sterile 0.85% NaCl solution. The number of the bacterial cells in the suspensions was measured by nutrient agar plate count. At the end of the feeding trail, 20 fish from each group (*Bacillus coagulans, Lactobacillus planatrum*) were challenged intraperitoneally with 100 µl (10^8 cfu/mL) of *S. agalactiae*. After the challenge test, fish were observed for two weeks. Fish mortality for each tank was recorded daily, and the Relative Percent Survival (%) were calculated using the formula:

$$\text{Relative Percent Survival (\%)} = \left(1 - \frac{\text{\% Mortality in treated group}}{\text{\% Mortality control}}\right) \times 100$$

5.2.2 Histology and histopathology

After the 60 days of probiotic feed administration, intestine, liver and gill tissue were analyzed by histological techniques. Fish were dissected and intestine, liver and gill removed and fixed in 10% formaldehyde solution. Histological slides were prepared after processing, sectioning and staining. Slides were observed under compound microscope and photographed using digital camera.

Histological slides were also prepared after two weeks of challenge test. Intestine, liver and gill tissue were analyzed by histological techniques. Slides were prepared by the following procedure.

Tissue processing

After fixation tissues were dehydrated by passing through different grades of alcohol, 70%, 80%, 90%, 95% alcohol for 1hour each orderly. After that two changes of absolute alcohol were also given for 1 hour. Then the tissues were placed in absolute alcohol and xylene (1:1 ratio) for 30 minutes. Then the tissues were cleaned by washing in xylene. Then the tissues were put in liquid paraffin wax and kept in hot air oven (58ºC–60ºC) for 24 hours. Finally, the tissues were embedded in paraffin wax and blocks were prepared by using L mold.

Sectioning

Sections were taken by using microtome, the thickness of the tissues were 6 µ (Thermo scientific Microm HM 325). Then the sections were mounted on individual microscopic slides smeared with Mayer's albumin. Slides were air dried.

Staining

The slides containing sections were stained serially as follows;

- The slides were transferred to xylene for 5 minutes and repeated 2 times.
- Slides were passed through different grades of alcohol (absolute alcohol, 95%, 90%, 80%, 70%, 50%, and 30%) for 5 minutes.
- Slides were washed under tap water
- Staining of slides was done with hematoxylin for 10 minutes counter stained with eosin for 5minutes.
- Dehydration of slides was done by passing them through 30%, 50%, 70%, 80%, 90%, and 95% grades of alcohol.
- Two changes of absolute alcohol were given for 5 minute.
- Finally slides were dipped in xylene and mounted using DPX.
- Then slides were examined under microscope and photographed.

5.3 RESULTS

The present study observed the tissues of intestine, liver and gill of the fishes after fed with probiotics for 60 days and image were presented in Plate 1.A–1.F, Plate 2.A–2.F and Plate 3.A–3.F respectively. Compared with the control group, tilapia fed with probiotics supplements has longer and denser microvilli. Morphological changes in intestine was observed in fish fed with *B. coagulans* as probiotic feed supplement than the fish fed with *L. plantarum* (Plate 1.C & 1.E). Histological images of the liver tissue of the fishes fed with control diet (without probiotics), diet supplemented with *B. coagulans* and diet supplemented with *L. plantarum* were presented in Plate 2.A, 2.C & 2.E respectively. There is no prominent changes were observed among the control and experimental fish tissue. Histological images of the gill tissue of the fishes fed with control diet (without probiotics), diet supplemented with *B. coagulans* and diet supplemented with *L. plantarum* were presented in Plate 3.A, 3.C & 3.E respectively. There are no changes observed in gill tissue of fish fed with control diet and probiotic supplemented diet. Both *B. coagulans* and *L. plantarum* has no significant effect on morphology of liver and gill tissue.

After the feeding trail of 60 days and fishes were infected with *Streptococcus agalactiae* and Relative Percent Survival (%) after 1 weeks and 2 weeks of challenge test were presented in Table 3. In the control fish (fed without probiotics and infected with *S.*

agalactiae) Relative Percent Survival (%) decreased from 41.67% to 24% after 2 weeks. In experimental group, fish fed with *B. coagulans* observed 91.30% Relative Percent Survival (%) and fish fed with *L. plantarum* observed 86.36% Relative Percent Survival (%) after 2 weeks.

Table 3: Relative Percent Survival (%) in fishes challenged with *Streptococcus agalactiae* after fed with probiotics, *B. coagulans* and *L. plantarum* at the dose of 10^8 cfu/g feed.

Duration after challenge test	**Relative Percent Survival (%)**		
	Control	***B. coagulans***	***L. plantarum***
1 week	41.67 ± 0.86	76.67 ± 0.94	73.33 ± 0.84
2 week	24.00 ± 0.92	91.30 ± 0.82	86.36 ± 0.78

Histopathology of fish intestine, liver and gill tissue were observed and images are presented in Plate 1.B–1.F, Plate 2.B–2.F and Plate 3.B–3.F respectively. In control fish tissues were damaged after the infection. Villi shrinks and reduced in intestine, degeneration of hepatocytes and hyperplasia and necrosis were observed in fish fed with diet devoid of probiotics. When compared to the control pathogenic effect of *S. agalactiae* were comparatively less in fish fed with *B. coagulans* and *L. plantarum* as dietary probiotic supplement.

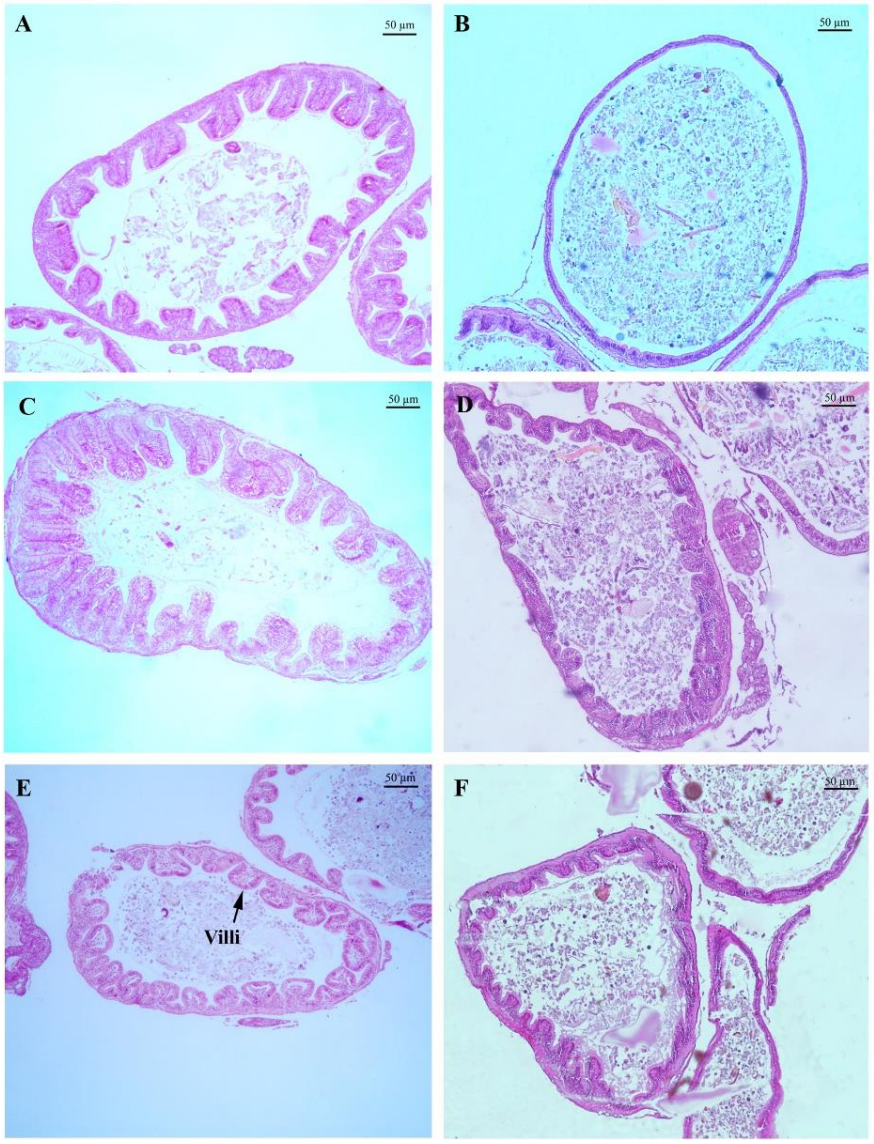

Plate 1: A. Cross section of intestine of the control fish showing villi; **B**. Cross section of intestine of the control fish after challenged by *Streptococcus agalactiae*; **C**. Cross section of intestine of the fish fed with *B. coagulans* as probiotic feed supplement; **D.** Cross section of intestine of the fish challenged with *S. agalactiae* after fed with *B. coagulans* as probiotic feed supplement; **E**. Cross section of intestine of the fish fed with *L. plantarum* as probiotic feed supplement; **F**. Cross section of intestine of the fish challenged with *S. agalactiae* after fed with *L. plantarum* as probiotic feed supplement.

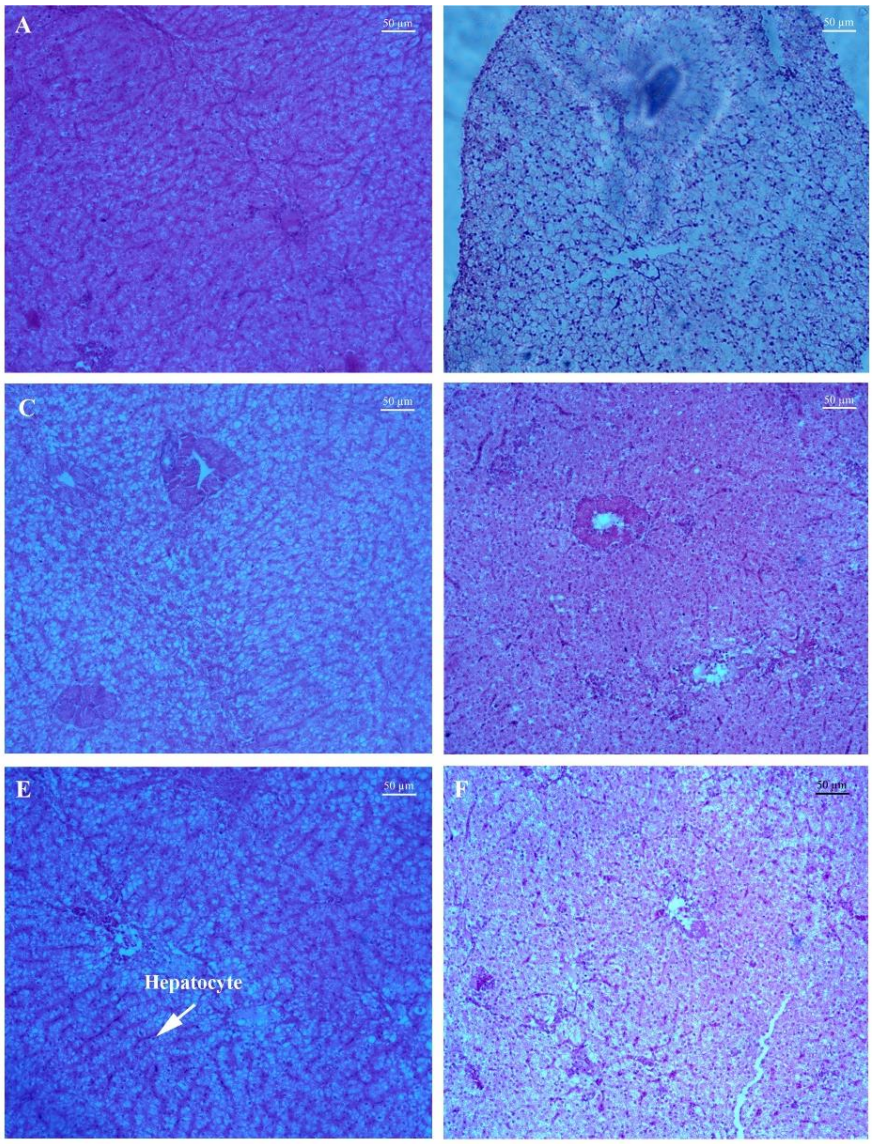

Plate 2: A. Cross section of liver of the control fish showing hepatocytes; **B**. Cross section of liver of the control fish after challenged by *Streptococcus agalactiae* showing degeneration of hepatocytes; **C**. Cross section of liver of the fish fed with *B. coagulans* as probiotic feed supplement; **D**. Cross section of liver of the fish challenged with *S. agalactiae* after fed with *B. coagulans* as probiotic feed supplement; **E**. Cross section of liver of the fish fed with *L. plantarum* as probiotic feed supplement; **F**. Cross section of liver of the fish challenged with *S. agalactiae* after fed with *L. plantarum* as probiotic feed supplement.

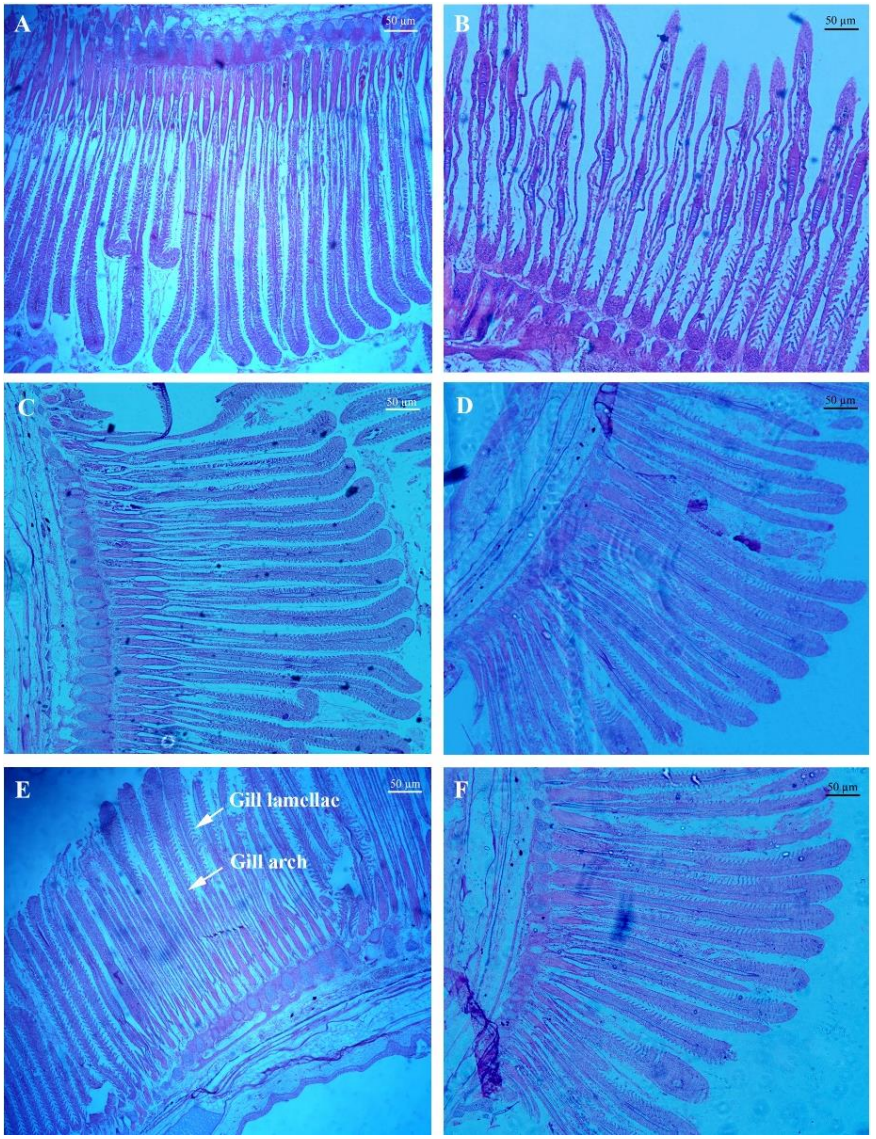

Plate 3: A. Cross section of gill of the control (without probiotics) fish; **B**. Histological section of gill of control fish challenged by *Streptococcus agalactiae* showing hyperplasia and necrosis; **C**. Histological section of gill of the fish fed with *B. coagulans* as probiotic feed supplement; **D**. Histological section of gill of the fish challenged with *S. agalactiae* after fed with *B. coagulans* as probiotic feed supplement; **E**. Histological section of gill of the fish fed with *L. plantarum* as probiotic feed supplement. **F**. Cross section of gill of the fish challenged with *S. agalactiae* after fed with *L. plantarum* as probiotic feed supplement.

5.4 DISCUSSION

This study shows difference among morphological structures in the intestine of the fish fed diets without probiotics, with *B. coagulans* and *L. plantarum* (Plate 1.A, 1.C & 1.E). This study observed denser microvilli in intestine of fish fed *L. plantarum* as probiotic feed supplement whereas denser and longer microvilli in intestine of fish fed *B. coagulans* as probiotics feed supplement when compared to the intestine of fish fed without probiotics. These results are in agreement with others reported morphological changes in intestine according to changes in diet components. It has been reported that after the 8 weeks of administration of multistrain probiotics such as *Pediococcus acidilactici, Lactobacillus reuteri*, *Enterococcus faecium* and *Bacillus subtilis* supplemented diet significantly enhanced tilapia mid-intestinal microvilli density and also showed the numerical increases of microvilli length and perimeter ratio which indicates that intestinal morphology is influenced by probiotic administration (Pirarat et al., 2011). Jesus et al. (2017) using *Weissella cibaria* at concentration of 10^9 cfu mL^{-1} for hybrid surubim (*Pseudoplatystoma reticulatum* female × *P. corruscans* male) observed that supplemented fish has significant increase in the length and width of their intestinal villi, in the number of villi and bigger perimeter of the villi. Application of whole yeast and its by-products as feed supplement influence the intestinal tract morphology and its microbiota and also observed that it may be due

to the presence of nucleotide in the diet (Hisano et al., 2006). These results are in agreement with the reports by Silva et al. (2005) with *Steindachnerina notonecta.* In fish fed with probiotics, there is statistically significant increase in mean values of proximal, middle and distal length of the intestine and also in total surface area of the intestinal tract (Burrells, 2001). This study results are in agreement with others reported increase in villi length and surface area of the intestine which helps in absorption. The overall changes in intestine morphology induced by probiotics lead to enhancing the absorption of feed ingredients which results in overall growth performance of the fish. There is an increase in the length and thickness of the intestinal villi in rainbow trout after fed with soybean protein, which is a vegetal nutrient abundant with structural polysaccharides (Escaffre et al., 2007). In addition to that, the increase in microvilli density is favorable factor to the enhancement of host resistance against entry of pathogens by reduction in the extent to which the inter enterocyte junctions are exposed.

The liver is the key organ of metabolism and excretion, being responsible for detoxification, removing toxic substances from the blood, and excreting them (Surai, 2015). In this study, application of *B. coagulans* and *L. plantarum* as probiotic feed supplement in fish presented no effect on the morphology of the liver. There is a previous report that *L. plantarum* has the ability to regulate trace element imbalance, alleviating oxidative stress and pathological

alterations in hepatic and renal tissues (Yu et al., 2017). Probiotic *Acinetobacter* KU011TH administration in the Bighead Catfish (*Clarias macrocephalus*) showed no significant changes in control and experimental fish intestine, liver and gill tissue (Bunnoy et al., 2019). The present study also observed no changes between histological structures of gill tissue in control and experimental fish. It can be inferred that the probiotics, *B. coagulans* and *L. plantarum*, can improve the growth parameters and immunity in fishes without causing damage to the internal organs.

Probiotics application in aquaculture enhance the immunity and control disease caused by pathogenic bacteria. The present study observed resistance against *S. agalactiae* infection in fish fed with probiotic supplemented diet. Both *B. coagulans* and *L. plantarum* significantly enhanced the Relative Percent Survival (%) rate in fishes fed with probiotics when infected with *S. agalactiae* (Table 3). Relative Percent Survival (%) rate in control fishes were decreased after two weeks whereas it was observed to be increasing in experimental fishes fed with probiotics. Relative Percent Survival (%) rate percentage were higher in fish fed with *B. coagulans* as probiotic feed supplement than *L. plantarum* fed fish. In tilapia, administration of *B. pumilus* supplemented diet increased host resistance against various bacterial diseases including those caused by *S. agalactiae* (Srisapoome and Areechon, 2017). These results are in agreement with others, who reported that administration of

Edwardsiella ictaluri in striped catfish (*Pangasianodon hypophthalmus*) increased resistance against streptococcosis (Ho et al., 2017). *Streptococcus iniae, Aeromonas hydrophila,* and *E. tarda* are the primary bacterial pathogens that have been evaluated in tilapia probiotic studies. Aly et al. (2008b) found that supplementing *Bacillus pumilus* at 10^{12}/g diet increased protection of Nile tilapia against A. *hydrophila* after 1 and 2 months but not 8 months of feeding. In another study, Aly et al. (2008c) also found that dietary supplementation of *L. acidophilus, B. subtilis,* or a mixture of the two, generally provided greater protection against *A. hydrophila, P. fluorescens,* and *S. iniae* after 2 months of feeding compared to 1 month. Resistance of grouper (*Epinephelus coioides*) to iridovirus is enhanced with supplementation of *Lactobacillus plantarum* in diet (Son et al., 2009), and *Pseudomonas* sp., *Vibrio* sp., *Aeromonas* sp., and groups of coryneform show antiviral activity to infectious hematopoietic necrosis virus (IHNV) (Kamei et al., 1988). Administration of *Bacillus* sp. is effective for integrated prevention, cure and treatment of streptococcus infections (Widanarni and Tanbiyaskur, 2015). Histopathological images show tissue damages caused by *S. agalactiae* infection in tilapia. Necrosis and reduction in villi of intestine, hepatocyte degeneration in liver and severe hyperplasia and necrosis of gill tissues were observed in fish fed without probiotic diet infected with *S. agalactiae.* Similar tissue damages were observed by other fish infected with *Streptococcus* sp. when compared to the control, tissue damages were less in fish fed

with probiotics and it may be due to the competitive exclusion of pathogenic bacteria by probiotics. The present study also observed higher Relative Percent Survival (%) rate in fish fed with probiotics. *Streptococcus* infection cause changes in the gill secondary lamellae inflicting hyperplasia with mononuclear infiltration and severe necrosis (Velappan and Munusamy, 2018). This is in accordance with the previous study performed in experimentally infected Nile Tilapia where histopathological changes noticed, multifocal fusion of the secondary lamellae of the basal epithelium of the primary gill lamellae with mononuclear infiltration (Perera et al., 1998). Moreover, retrogression such as hydropic degeneration of hepatic cells was evident. This is similar to the previous investigation performed in *Labeo rohita* fingerlings and in Common carp (*Cyprinus carpio*) by Yanong and Floyd (2002). Ulceration of intestinal villi and necrosis was also observed in the intestinal epithelium (Velappan and Munusamy, 2018). Histopathological changes observed and it can be concluded that application of probiotics *B. coagulans* and *L. plantarum* can helps fish to maintain the morphology of the internal organs such intestine, liver and gill during the infection of *S. agalactiae.*

SUMMARY

Aquaculture and its practices increased globally at a fast pace and it remain the second largest food producing industry next to agriculture. In order to meet the increasing demand, aquaculture industry is being intensified with high stocking densities, feed inputs and organic load. Intensification may lead to stress in fish thereby reducing the immunity and paralleled with a corresponding increase in the occurrence and spread of pathogenic and opportunistic bacteria causing infectious diseases. Conventional approach for cure and prevention of disease in aquaculture is the use of wide spectrum chemotherapeutics. It has not only led to the development of antibiotic resistant bacterial strains but also cause environmental degradations and food security problems as fish constitutes major animal protein source for world's population. It may also cause damage to the beneficial microflora of the animal digestive tract and in the culture environment. Therefore, the application of ecofriendly agents such as microbial and herbal supplements, to improve the physiology, growth performance and immune responses of aquaculture related species have gained much more attention during recent years. Probiotics are the live microorganisms that improve the health of the organism including disease resistance when administered in adequate amounts.

The present study investigated the effect of probiotic strains, *Bacillus coagulans* and *Lactobacillus plantarum* on the

physiological parameters, immune parameters and disease resistance against *Streptococcus agalactiae* in tilapia, *Oreochromis mossambicus*. The *B. coagulans* and *L. plantarum* were isolated from the gut of tilapia itself and identified by biochemical characteristics and 16S rRNA sequencing. The fish, *O. mossambicus* were purchased from Aqua fish farm, Kottakkal, Malappuram District, Kerala. They were acclimatized in lab condition before the experiment. The experiment was conducted on tilapia feeding with *B. coagulans* and *L. plantarum* separately at different concentrations such as 10^2, 10^4, 10^6, 10^8 cfu/g feed for 60 days. The parameters were measured for 15 days, 30 days, 45 days and 60 days and grouped into DG15, DG30, DG45 and DG60 respectively.

The results obtained indicated improvement in % weight gain, Specific Growth Rate, Feed Conversion Ratio (FCR) and feed efficiency (FE) % in fish fed with probiotic diet than the control fish fed without probiotic supplements. Highest % weight gain and SGR were observed in fish fed with highest concentration of probiotics in DG60 group and it were 17.54±0.96, 0.45±0.63 in fish fed with *B. coagulans* and 23.684±0.52, 1.177±0.12 in fish fed with *L. plantarum*. The FCR in fish fed with probiotics is improved and best FCR were observed as 3.108±0.12, 3.838±0.05, in fish fed with *B. coagulans* and *L. plantarum* respectively in DG60 group. The feed efficiency % also improved by the supplementation of probiotics and FE % observed were 14.56±0.54, 51.72±0.69 in fish fed with *B.*

coagulans and *L. plantarum* respectively in DG60 group. There is significant difference ($p<0.05$) in % weight gain, SGR, FCR and feed efficiency % improved by different concentration of probiotics.

Incorporation of probiotics in feed influenced the activity of digestive enzymes, amylase, protease and lipase. Highest enzyme activity was observed in fish fed with *B. coagulans* for amylase, protease and lipase were 23.56±0.19, 33.65±1.25 and 2.83±0.16 in DG60 group whereas highest enzyme activity was observed in fish fed with *L. plantarum* for amylase, protease and lipase were 23.79±0.38, 29.18±0.47 and 2.16±0.08 in DG60 group. In each experimental group, DG15, DG30, DG45 and DG60, digestive enzyme activity was significantly increased ($p<0.05$) when compared to the control. Amylase, protease and lipase activity were found to be changed in fish fed with different concentration of probiotics supplemented feed. Both, *B. coagulans* and *L. plantarum* influenced the activity of digestive enzymes. In fish fed with *B. coagul*ans as feed supplement, there is significant difference ($p<0.05$) among DG15, DG30, DG45 and DG60 groups whereas in fish fed with *L. plantarum*, there is no significant difference between DG45 and DG60 at higher concentration of probiotics. However, maximum activity was observed in DG60 group and it can be inferred that concentration and duration of administration of probiotics influence the growth parameters and digestive enzyme activity in tilapia.

Haemoglobin concentration and hematocrit percentage values in fishes fed with *B. coagulans* and *L. plantarum* as probiotic feed supplements significantly increased ($p<0.05$) from 10^2 to 10^8 cfu/g probiotic feed in each experimental duration of DG15, DG30, DG45 and DG60. Highest haemoglobin concentration and haematocrit percentage observed were, 6.35±0.14, 29.91±0.58 respectively when fed with *B. coagulans* whereas haemoglobin concentration and haematocrit percentage observed were, 6.46±0.11, 30.42 ±0.48 respectively in fed with *L. Plantarum*. In DG15 and DG30 no significant increase ($P>0.05$) was observed for fishes fed with probiotics at the level of 10^2. It may be due to the reason that this level of probiotics is too low to initiate response in fish. Even though highest haemoglobin concentration and haematocrit percentages were observed in DG60, there is no significant changes ($P>0.05$) in haemoglobin and haematocrit values between DG45 and DG60. Improvements in haemoglobin concentration and haematocrit percentage indicates that the fish can respond to stress factors in better ways.

The total erythrocyte count (TEC) and Total leucocyte count (TLC) were also influenced by supplementation of probiotics with the diet. TEC and TLC were gradually increased depending on the concentration of probiotics and duration of administration. Highest count was observed in fish fed with high concentration of probiotics in DG60 group. The TEC observed were 2.09±0.48, 2.67±0.30,

3.60±0.28 and 3.92±0.05 for 10^2, 10^4, 10^6 and 10^8 respectively in fish fed with *B. coagulans* as dietary supplement whereas TEC observed were as 1.94±0.48, 2.94±0.30, 3.88±0.28 and 4.01±0.05 for 10^2, 10^4, 10^6 and 10^8 respectively in fish fed with *L. plantarum* as dietary supplement. TLC observed were 22.46±0.40, 23.05±0.19, 23.36±0.27 and 23.95±0.41 for 10^2, 10^4, 10^6 and 10^8 respectively in fish fed with *B. coagulans* as dietary supplement whereas TLC observed were as 21.75±0.12, 22.69±0.22, 22.95±0.1 and 23.79±0.06 for 10^2, 10^4, 10^6 and 10^8 respectively in fish fed with *L. plantarum* as dietary supplement. There is significant increase ($p<0.05$) in both TEC and TLC values from in DG15 to DG60 groups.

The innate immune system is an important defensive tool in invertebrates and a fundamental defense mechanism in fish. The *B. coagulans* and *L. plantarum* treated as probiotics in the present study showed improvement in immunological parameters such as total immunoglobulin, lysozyme activity and respiratory burst activity. The present study observed increase in total Ig level in blood of fish fed with probiotic feed supplements. Highest values of Ig observed were 27.68±0.4, 27.23±0.46 in fish fed with probiotics, *B. coagulans* and *L. plantarum* respectively at the concentration of 10^8 cfu/g feed in DG60 experimental group. The values are significantly higher ($P<0.05$) when compared to the control group. In each experimental group, DG15, DG30, DG45 and DG60 higher values were observed

in fish fed with higher concentration (10^8 cfu/g) of probiotics and there is significant difference ($p<0.05$) in the values of Ig in fish fed with different doses of probiotics in each experimental group.

Lysozyme activity in fish fed with probiotics, *B. coagulans* and *L. plantarum*, significantly increased ($p<0.05$) in each experimental group when compared to the control. Highest activity of lysozyme was 7.25±0.09 and 6.71±0.12 in fish fed with *B. coagulans* and *L. plantarum* respectively at concentration 10^8 cfu/g feed in DG60 group. In fish fed with *B. coagulans* as probiotic feed supplement, lysozyme activity in DG60 significantly increased from DG45 at the concentration of 10^8 cfu/g feed, but there are no significant changes ($p>0.05$) in lysozyme activity between DG45 and DG60 at lower concentrations. This study observed significant increase ($p<0.05$) in lysozyme activities in fish fed with *B. coagulans* from DG15 to DG60 at every different concentration, whereas there is no significant increase ($p>0.05$) from DG15 to DG60 at every different concentration in *L. plantarum* fed fishes. However, there is significant difference ($p<0.05$) in lysozyme activity in fish fed with different concentration of probiotics in each experimental group.

Respiratory burst activity of phagocytes is measured as reduction of NBT by intracellular superoxide radicals produced by leucocytes. Respiratory burst activity was also influenced by *B. coagul*ans and *L. plantarum* as probiotic feed supplement.

Respiratory burst activity observed in fish fed with *B.* coagulans were 1.39±0.04, 2.08±0.13, 2.61±0.01, 2.72±0.05 for 10^2, 10^4, 10^6 and 10^8 respectively. In fish fed with *B. coagulans* as feed supplement, respiratory burst activity was significantly increased ($p<0.05$) at different concentrations when compared to the control. But there is no significant difference ($p>0.05$) between respiratory burst activity induced by 10^6 cfu/g and 10^8 cfu/g feed when compared to each other. In fishes fed with *L. plantarum* as feed supplement induce respiratory burst activity depending on the concentration of the probiotics. The values were significant when compared each other in each experimental group.

Resistance against *S. agalactiae* infection in fish fed with probiotics was estimated by Relative Percent Survival (%) in fish after challenge test. In the control fish (fed without probiotics and infected with *S. agalactiae*) Relative Percent Survival (%) was decreased from 41.67% to 24% after 2 weeks. In experimental group, fish fed with *B. coagulans* observed 91.3% Relative Percent Survival (%) and fish fed with *L. plantarum* observed 86.36% Relative Percent Survival (%) after 2 weeks.

The application of probiotics as dietary supplement influenced the morphology of the intestine whereas it has no influence on the morphology of gill and liver tissue of the fishes. Morphological changes in intestine were observed in fish fed with *B. coagulans* as probiotic feed supplement than the fish fed with *L.*

plantarum. Histopathology of fish intestine, liver and gill tissue were observed and in control, fish tissues were found to be damaged after the infection. Villi shrunk and reduced in intestine; degeneration of hepatocytes and hyperplasia and necrosis were observed in fish fed with diet without probiotics. When compared to the control, pathogenic effect of *S. agalactiae* were comparatively less in fish fed with *B. coagulans* and *L. plantarum* as dietary probiotic supplement.

In a nutshell, *B. coagulans* and *L. plantarum* isolated from the gut of *O. mossambicus* itself have positive effects on the growth parameters, amylase, protease, lipase enzyme activity and also improve the hematological parameters like hemoglobin concentration, hematocrit, total erythrocyte and leucocyte count and immunological parameters like total immunoglobulin, lysozyme and respiratory burst activity. In addition to this, these probiotics improves the Relative Percent Survival in fish challenged with *S. agalactiae*. The administration of supplements as probiotics with the proper percentage of *Bacillus coagulans* and *Lactobacillus plantarum* for two months could enhance the growth rate, digestive enzyme activity and immunity in fish thereby improve overall health performance of tilapia. The concentration and duration of administration of probiotics are the factors which contribute the beneficial effects of probiotics on the health of the host. The probiotics *B. coagulans* and *L. plantarum* provide resistance against *S. agalactiae* in tilapia. Probiotics also provides immunity against

pathogenic bacterial strain, may be through competitive exclusion. Histopathological changes were observed and it can be concluded that application of probiotics *B. coagulans* and *L. plantarum* can help fish to maintain the morphology of the internal organs such intestine, liver and gill during the infection of *S. agalactiae*. Probiotic bacteria showed its potential to be used as supplement in feed for aquaculture, practical viability must be confirmed by studies performed under field condition.

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