EFFECTS OF PROBIOTICS ON GROWTH, FLESH QUALITY AND HEMATO-IMMUNOLOGICAL STATUS OF CULTURED NILE TILAPIA (*Oreochromis niloticus*) IN KIRINYAGA COUNTY, KENYA

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AUGUST, 2020

DECLARATION

This thesis is my original work and has not been presented for degree or other awards in any other University.

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DEDICATION

I dedicate this work to my late dad, Mr. Boniface Opiyo, you were my greatest mentor and you taught me never to give up. I salute you for the efforts you made to bring me up. This is my tribute to you!

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
AU-IBAR	African Union -InterAfrican Bureau for Animal Resources
BCA	Bicinchoninic Acid
CF	Crude Fiber
CFU	Colony Forming Units
CP	Crude Protein
df	Degrees of freedom
DMRT	Duncan Multiple Range Test
DWG	Daily Weight Gain
EC	European Commission
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization of the United Nations
FCR	Food Conversion Ratio
FL	Final Length
FW	Final Weight
GALT	Gut Associate Lymphoid Tissue
GIT	Gastro Intestinal Tract
GOK	Government of Kenya
ICMSF	International Commission on Microbiological Specification for Foods

IL	Initial Length
IW	Initial Weight
KMFRI	Kenya Marine and Fisheries Research Institute
KNBS	Kenya National Bureau of Statistics
LC-PUFAs	Long-Chain Poly-Unsaturated Fatty Acids
LWR	Length Weight Relationship
MRL	Maximum Residue Limits
NACA	Network of Aquaculture Centers in Asia and the Pacific
NRF	National Research Fund
OD	Optical Densities
OIE	Office International des Epizooties
RBC	Red Blood Cell
SEM	Standard Error of the Mean
SGR	Specific Growth Rate
SPSS	Statistical Package and Service Solutions
TAN	Total Ammonia Nitrogen
TPC	Total Plate Count
TSA	Tryptone Soy Agar
TSI	Triple Sugar Iron
USA	United States of America
WBC	White Blood Cell
WHO	World Health Organization

ABSTRACT

Nile tilapia (*Oreochromis niloticus*) culture in Kenya is mainly conducted in low input ponds where supplementary feed is given alongside pond fertilization. Excess nutrients in the culture water in form of nitrogen (N) and phosphorus (P) may make the culture environment susceptible to invasion by disease-causing microorganisms. In the recent past, probiotics have received much attention as a new strategy in fish health management and have been documented to improve fish growth performance and immunity in fish cultured in recirculating systems. However, their effect in tilapia cultured in low input ponds is still relatively unknown. In this study, monosex *O. niloticus* fingerlings with a mean weight of 39.75 ± 0.05 g were randomly stocked at 50 fish m^{-3} in 1.25 m^3 cages in low input earthen ponds. The fish were fed twice daily at 3% body weight on seven isonitrogenous (28% crude protein) diets supplemented with either *Saccharomyces cerevisiae* (1×10^{10} CFU g^{-1}) or *Bacillus subtilis* (1×10^{9} CFU g^{-1}) at different levels as follows: No probiotic (Diet 0); *S. cerevisiae* at 2 g kg^{-1} (Diet 1), 4 g kg^{-1} (Diet 2) and 6 g kg^{-1} (Diet 3); and *B. subtilis* at 5 g kg^{-1} (Diet 4), 10 g kg^{-1} (Diet 5) and 15 g kg^{-1} (Diet 6) for a period of 7 months. The fish were sampled monthly for weight and length measurements. Hemato-immunological parameters were determined by blood sampling and hematological analysis for red blood cells, white blood cell and haemoglobin counts. Blood serum assay was conducted using commercial enzyme-linked immunosorbent assay (ELISA) kits to determine the serum protein, albumin, globulin levels and lysozyme activity. Microbiological samples were analyzed through sub-culturing to obtain pure cultures on nutrient media and enumerated through standard methods. Results of the trials indicated that the highest performance was achieved with Diet 2. The highest final weight (255.31 ± 3.19 g), Specific growth rates (SGR) (0.77±0.01% day^{-1}) and feed conversion ratio (FCR) (1.61 ± 0.02) were recorded in fish fed Diet 2. This was followed by fish fed Diet 5. Results of growth performance analysis indicated that fish fed on probiotic-supplemented diets had significantly better growth, nutrient utilization and FCR than fish fed on the control diet ($P < 0.05$). Probiotic supplementation significantly affected the body composition of the fish ($P < 0.05$). *Saccharomyces cerevisiae* at 4 g kg^{-1} (Diet 2) led to significantly high protein (86.06%) ($P < 0.05$) while *B. subtilis* at 5 g kg^{-1} (Diet 4) led to significantly higher protein (89.40%) ($P < 0.05$). Crude lipid and ash content were significantly lower in the fish fed probiotic-supplemented diets ($P < 0.05$) compared to the control. Results of hemato-immunological analysis indicated that haemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), serum protein, albumin, globulin and lysozyme activity were higher in fish fed on probiotic-supplemented diets and lower in the control group (Diet 0). Probiotic significantly affected hemato-immunological parameters ($P < 0.05$). Fish fed on probiotic-supplemented diets retained the probiotics in their guts and had lower microbial load in their muscle ($P < 0.05$). This study shows that incorporation of probiotics in diets of Nile tilapia in low input ponds promotes growth performance, enhances body composition, improves immunity and manipulates gut microbiota of fish. The two probiotics differ in effect at different levels of application. Probiotic *S. cerevisiae* exhibited the best performance at 4 g kg^{-1} while *B. subtilis* had the best performance at 10 g kg^{-1}. Probiotics are therefore recommended for use in low input fish culture systems for better nutrient utilization, higher yields and improved fish health for increased aquaculture production.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Global fish production has grown steadily in the last five decades, with food fish supply rising at an average annual rate of 3.2%, against a population growth of 1.6% (FAO, 2018). On the other hand, global per capita fish consumption has increased from an average of 9.0 kg in the 1960s to 20.5 kg in 2017 (FAO, 2016c, 2018). Despite the increase in global fish consumption, per capita consumption of food fish in Africa is still a factor of seasonality and availability of the fish. The growth in per capita consumption is still low (3.4 to 7.7 kg) between 1961 and 2015, compared to developed countries (24.9 kg) in 2015 (Subasinghe, 2017; FAO, 2018). In global markets, more food fish products are traded than beef, pork and poultry (FAO, 2018). Moreover, the quantity of fish produced has been reported to be two times that of poultry and three times that of beef (Béné *et al*., 2016).

Fish accounts for 17% of animal protein consumed globally and have provided more than 3.2 billion people with almost 20% of their average per capita intake of animal protein (FAO, 2016c, 2018). It has also become an important alternative provider of much needed high quality animal protein and other essential nutrients particularly important for the rural poor and food insecure population in the world (Béné *et al*., 2016). In addition to proteins, fish contains the unique long-chain poly-unsaturated fatty acids (LC-PUFAs) and highly bio-available essential micronutrients (vitamins D and B), minerals (calcium, phosphorus, iodine, zinc, iron and selenium) (Kobayashi *et al*., 2015; FAO, 2018).

Globally, fish production from aquaculture is low and a production of 80 million tonnes was recorded in 2017 compared to 90 million tons from capture fisheries (FAO, 2018). In Kenya, aquaculture production increased by more than 300% since 2007 (Rothuis *et al*., 2011) with a total production of 24,098 tons being recorded in 2014 contributing 15% of the total national fish production (Opiyo *et al*., 2018; KNBS, 2019).

Aquaculture is the fastest growing food production sector with an annual global growth of 11% since 1984 in comparison to poultry and beef (Béné *et al*., 2016). Aquaculture has outpaced wild capture fisheries in production of food fish and it represents one sixth of the total global animal protein consumption (Kobayashi *et al*., 2015; FAO, 2017). It contributed 45% of the world total fish production in 2015 which was an increase from 42.1% in 2012 and 31.1% in 2004 (FAO, 2016c, 2018). The current production from aquaculture is at 76.6 million tons which is an increase from the 70.2 million tons produced in 2013 (Figure 1.1) (FAO, 2018). Increase in aquaculture production is a general trend worldwide with 35 countries producing more fish from aquaculture compared to the static capture fisheries (Subasinghe, 2017). The growth in aquaculture has been attributed to increased per capita fish consumption, increased wealth in western countries, increased urbanization in developing countries and increased international trade (Little *et al*., 2016). Small holder farmers have also responded to the increasing fish demand and seasonal shortfall of food fish by commercialization of farming ventures leading to better production (FAO, 2018). Per capita fish consumption in 2015 was estimated at 20.3 kg up from 19.7 kg in 2013 (FAO, 2018). To maintain the current fish consumption level, and to reduce

pressure on the declining stocks of capture fisheries, FAO estimates that global aquaculture production should reach 80 million tons by 2050 (FAO 2016c).

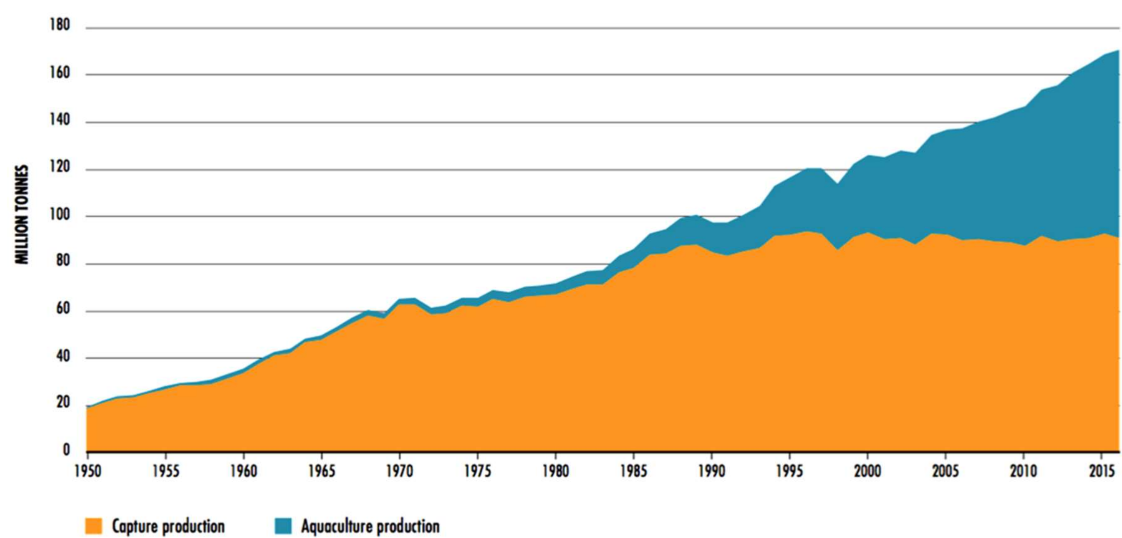

Figure 1.1: World capture and aquaculture production from 1950 to 2015 (FAO, 2018).

In Sub-Saharan Africa, aquaculture contributed 556, 950 tons in 2014; a 7% increase and a 21% average annual increase from 2004 with the production majorly dominated by tilapias and African catfishes. The seven top aquaculture producers in Sub-Saharan Africa include Nigeria (313,231 tons), Uganda (111,023), Ghana (38,535), Kenya (24,098), Zambia (19,281), Madagascar (8,470) and South Africa (4,160 tons) (FAO, 2017). The lead countries account for 93% of the total fish production in Sub-Saharan Africa with Kenya ranking 4th among major aquaculture producers in the region (FAO, 2017).

In Kenya, aquaculture started in 1920's and became popular in 1960s with the introduction of pond culture of Nile tilapia and African catfish (Kagai, 1975; FAO, 2016a). Fish production from aquaculture stagnated for a long time despite numerous efforts to boost fish production through the "Eat More Fish Campaigns" championed by the government of Kenya. This was until 2007 when the production rose from 1,012 tons to 4,240 tons (Ngugi and Manyala, 2009). Between 2009 and 2014, production increased from 4,895 tons to 24, 098 tons (Figure 1.2) as a result of the government funded project Economic Stimulus Project - Fish Farming Enterprise Productivity Program (ESP-FFEPP) which triggered a rapid growth in the sector (FAO, 2016a; FAO, 2017). Despite the increase in aquaculture production, the contribution of aquaculture to the national fish production still stands at 14% with Nile tilapia contributing 78% of the total farmed fish (Obwanga and Lewo, 2017). The growth of aquaculture in Kenya over the past three years has been attributed to intensification of pond systems and introduction of intensive systems like cages in Lake Victoria (Aura *et al*., 2018; Opiyo *et al*., 2018).

Nile tilapia, *Oreochromis niloticus* is one of the major cultured finfishes in the world after carps (Subasinghe, 2017; FAO, 2018). The vast culture is attributed to its ability to feed on various feeds, fast growth, tolerance to a wide range of culture conditions, popularity with consumers, ease of breeding and wide availability to farmers (Pullin, 1991; Pullin *et al*., 1991; Charo-Karisa, 2006). In Africa, Nile tilapia is either cultured in monoculture or polyculture with African catfish, *Clarias gariepinus* in semi-intensive earthen ponds which are fertilized with livestock manure alongside supplementary feeding to reduce the cost of feeds (El-Sayed, 2008; Elnady *et al*., 2010).

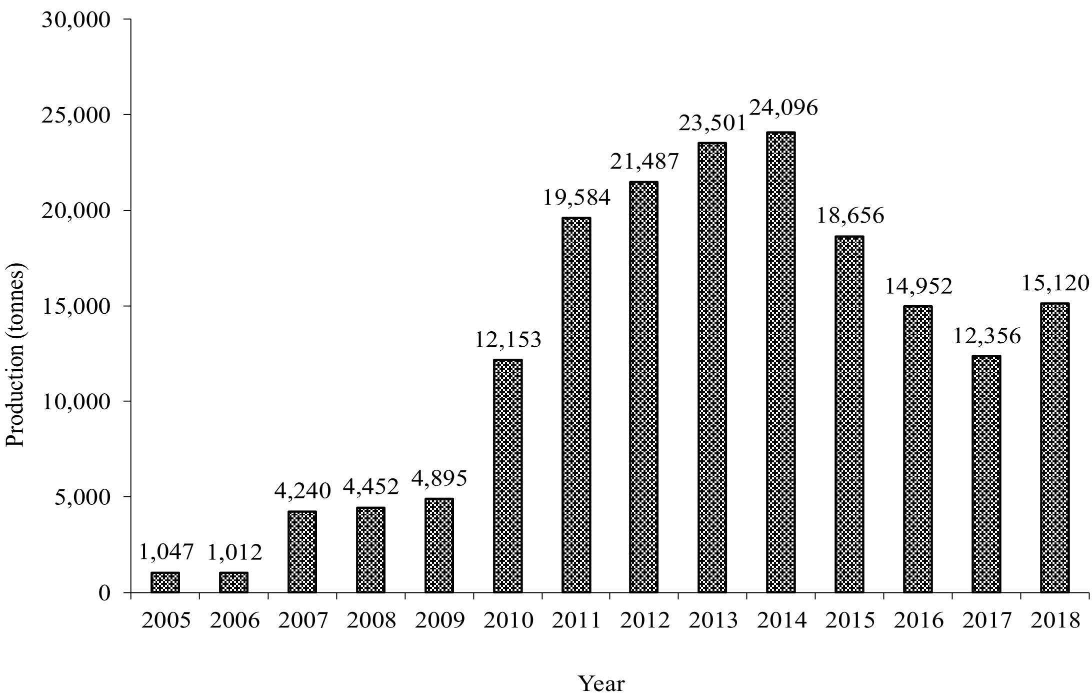

Figure 1.2: Trends in aquaculture production in Kenya between 2005 and 2018. Source: (FAO, 2016a; KNBS, 2019).

Natural foods produced in fertilized ponds increase efficiency of supplemental feeds significantly and is usually indicated by low feed conversion ratio (FCR) in such systems (Diana *et al*., 1994; Charo-Karisa, 2006). Low input systems are an old tradition in Asia and are well established in many parts of the world (Knud-Hansen *et al*., 1991; WHO, 2006b; Terziyski *et al*., 2007). Livestock wastes have commonly been used to augment primary productivity in ponds in China, Vietnam, India, Indonesia, Bangladesh and Thailand (Little and Edwards, 2003; Elnady *et al*., 2010; Rapatsa and Moyo, 2013). In most cases, animal houses are constructed on top of the ponds and the droppings are eaten directly by fish (Tabaro *et al*., 2012). Bangladesh has dominated the production of Nile

tilapia in low input ponds characterized by direct use of livestock production wastes and manures in the ponds (Little and Edwards, 2003). In Vietnam, latrines are built hanging on ponds to provide fertilization of the ponds while 'night soil' have been reported to be used to fertilize ponds in China, Indonesia and Thailand (NACA, 1989; Little and Edwards, 2003; WHO, 2006b).

Manures used in low input systems originate from different animals including chicken, ducks, rabbits, cows, sheep, goats, pigs, buffaloes and other livestock. The manures are a source of nitrogen (N) and phosphorus (P) which stimulate natural food webs in the ponds by increasing primary productivity, dissolved oxygen, pH and total phosphorus in ponds (Little and Edwards, 2003; Rapatsa and Moyo, 2013). Edwards (1993) reported that only 5-15% of the nutrient inputs in the fertilized ponds are converted to harvestable products. Other workers subsequently reported that unutilized nutrients make the environment susceptible to invasion by disease causing microorganisms to both fish and humans (Mente *et al*., 2011; Abu-Elala *et al*., 2016).

Microbial levels of especially faecal coliform and *Salmonella* have been reported to increase with loading levels for buffalo manure added to ponds (Little and Edwards, 2003). Generally, common pathogens of warm-blooded animals do not cause disease in fish but the role of cultured fish in the possible transfer of pathogens between livestock and humans is now gaining importance (WHO, 2006b). Fish reared in low input systems may passively transmit pathogens from their body surfaces to the fish handlers and consumers as a result

of concentration of bacteria and other microbes (viruses and protozoa) in their body and intestines (WHO, 2006a).

Rapid growth and intensification of aquaculture systems has brought in issues related to food safety, human health and environmental sustainability. The immense use or misuse of antibiotics to enhance growth, increase yields and overcome possible fish diseases in aquaculture has raised public concerns on human health, safety and environmental impacts (Singh and Yadava, 2005; Caruso, 2016). To improve the overall nutrient utilization by fish and immunity of fish, use of dietary supplements such as probiotics are essential in the low input systems (Verschuere *et al*., 2000).

Probiotics are live microbes that when administered in sufficient amounts improve digestion, growth and enhances fish welfare (Ringø and Gatesoupe, 1998; Bomba *et al*., 2002; Abdel-Tawwab *et al*., 2008; De *et al*., 2014). Probiotics have received much attention as a new strategy in feeding and health management in intensive aquaculture systems (Balcázar *et al*., 2006; Hai, 2015a). The benefits derived from use of probiotics in fish culture include; enhancement in digestibility of the proteins and nutritional value of food; enzymatic contribution to digestion and absorption of nutrients; inhibition of pathogens; growth promotion and enhancement in immune response of cultured fish (Verschuere *et al*., 2000; Merrifield *et al*., 2010a; de Azevedo and Braga, 2012; Lara-Flores and Olvera-Novoa, 2013; Standen *et al*., 2013).

Studies indicate that probiotics in feeds improve growth of different fish species including African catfish (Al-Dohail *et al*., 2009; Essa *et al*., 2011), Senegalese sole (*Solea senegalensis*) (Sáenz de Rodrigáñez *et al*., 2009), Nile tilapia (Lara-Flores *et al*., 2003; El-Haroun *et al*., 2006; Lara-Flores *et al*., 2010; Lara-Flores and Olvera-Novoa, 2013; Hai, 2015b), gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) (Carnevali *et al*., 2006), Indian major carp (rohu, *Labeo rohita*) (Sinha and Pandey, 2013) and Rainbow trout (*Oncorhynchus mykiss*) (Kim and Austin, 2006; Bagheri *et al*., 2008).

Saccharomyces cerevisiae and *Bacillus subtilis* are two of the commonly used probiotics to enhance growth and improve feed utilization of cultured fish due to their low cost, viability, ability to withstand the digestion process of fish and the ability to colonize the gut of the fish (Lara-Flores *et al*., 2003; Bagheri *et al*., 2008; He *et al*., 2013; Iwashita *et al*., 2015; Allameh *et al*., 2016; Hassaan *et al*., 2018). *Bacillus subtilis* and *S. cerevisiae* have been used to improve growth performance, feed efficiency and disease resistance in fish (Lara-Flores *et al*., 2003; He *et al*., 2013; Hai, 2015a; Iwashita *et al*., 2015).

1.2 Statement of the problem

Use of animal manure for Nile tilapia culture has been in existence for years as a source of soluble phosphorus, nitrogen and carbon for algal growth in ponds and is usually preferred by farmers since manures are cheaper compared to chemical fertilizers. However, use of these manures has raised concerns on fish quality, fish contamination and human health due to the occurrence of pathogenic bacteria in fish cultured in low input systems. For example, *Escherichia coli* has been reported in carps cultured in ponds fertilized with

animal manure in India and *Edwardsiella*, *Pasteurella*, *Pseudomonas* and *Salmonella* spp. in muscle of tilapia from ponds fertilized with chicken manure due to the poor sanitary condition in fertilized ponds. The presence of these bacteria in fish intended for human consumption may lead to a potential risk not only in causing disease, but also because of the possible transfer of antibiotic resistance from aquatic bacteria to humans due to antibiotic residue from animal husbandry. The wellbeing and safety of fish from low input ponds is a concern to the end consumers and there is a need to reduce incidences of disease outbreaks and to ensure safety in Nile tilapia produced from low input ponds.

Due to the differences in the control and management of the intensive recirculating systems and the low input ponds, the results from application of probiotics in the intensive recirculating systems may not be similar or applicable to low input ponds where application of manure make the environment more susceptible to invasion by disease causing microorganisms. Though probiotics are widely used in the livestock industry in Kenya, their use in aquaculture appears to occur inadvertently. This may be related to lack of knowledge on their importance, efficacy and application levels. Furthermore, no studies have documented the use of probiotics on fish cultured in low input ponds. Therefore this study investigated the effects of commonly used probiotics on growth performance, feed utilization, survival, flesh quality and immunity of Nile tilapia cultured in low input ponds.

1.3 Study justification

Increase in pathogenic bacteria populations such as *Vibrio* spp., *Salmonella* spp. and *Aeromonas* spp. which poses a health risk to the cultured fish and render them immune-

compromised have been reported in fertilized ponds (Moriarty, 1999; Molinari *et al*., 2003). Moreover, fish has an intimate interaction with the culture environment which has both pathogenic and saprophytic microorganisms. Therefore, enhancement of the immune system of cultured fish and establishment of a normal gut microbiota is vital, as it affects a wide range of biological processes including; nutrients utilization, development and assembly of gut associate lymphoid tissue (GALT) and ability to fight infections (Merrifield *et al*., 2011; Ringø *et al*., 2016). Use of probiotics is gaining acceptance in fish farming as a means of improving growth, immunity response and control of potential pathogens in fish (Nayak, 2010a). All probiotic applications in tilapia aquaculture have typically been done in favorable controlled recirculating systems where fish receive high protein complete feeds and the environment is efficiently controlled (Lara-Flores and Olvera-Novoa, 2013; Standen *et al*., 2013; Abdelhamid *et al*., 2014a). However, in most of Asia and Africa, tilapia is produced in low input ponds (Charo-Karisa, 2006; Elnady *et al*., 2010; Rapatsa and Moyo, 2013).

1.4 Research questions

This study was undertaken to answer the following research questions:

i. What is the effect of different levels of probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* on weight gain, growth rate, feed conversion ratio and survival of Nile tilapia reared in low input ponds?

ii. What is the effect of probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* on body composition of Nile tilapia reared in low input ponds?

iii. What is the effect of probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* on hemato-immunological parameters of Nile tilapia cultured in low input ponds?

iv. What is the effect of probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* on the fish muscle microbial content and gut microbiota of Nile tilapia cultured in low input ponds?

1.5 Hypotheses

i. Probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* have no significant effects on weight gain, growth rate, feed conversion ratio and survival of Nile tilapia reared in low input ponds.

ii. Probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* have no significant effects on body composition of Nile tilapia reared in low input ponds.

iii. Probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* have no significant effects on hemato-immunological parameters of Nile tilapia cultured in low input ponds.

iv. Probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* have no significant effects on the fish muscle microbial content and gut microbiota of Nile tilapia cultured in low input ponds.

1.6 Objectives of the study

1.6.1 General objective

To evaluate the effects of probiotics on the growth performance, feed utilization, flesh quality and immunity status of Nile tilapia reared in low input ponds.

1.6.2 Specific objectives

i. To determine the effect of probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* on weight gain, growth rate, feed conversion ratio and survival of Nile tilapia reared in low input ponds.

ii. To determine the effect of probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* on body composition of Nile tilapia reared in low input ponds.

iii. To determine the effects of probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* on hemato-immunological parameters of Nile tilapia cultured in low input ponds.

iv. To determine the effects of probiotic *Saccharomyces cerevisiae* and *Bacillus subtilis* on the fish muscle microbial content and gut microbiota of Nile tilapia cultured in low input ponds.

1.7 Significance of the study

Application of probiotics in aquaculture reduces the virulence of diseases and multiplication of pathogens since they enhance the immune response of the fish against pathogenic bacteria (Ali *et al*., 2010). Efficacy of probiotics application depends on various factors, such as; species composition, application level, frequency of application, environmental conditions and application period (Gomez-Gil *et al*., 2000; Hai, 2015b). In the present study, dietary probiotics were incorporated in supplementary diet to enhance the utilization of nutrients from the feed thus improving growth, body composition and immunity of the fish. The results of the this study contribute to promotion of best

management practices for Nile tilapia production in low input ponds and improvement of aquaculture production in Kenya and other countries facing similar conditions. The study also avails information that is beneficial to Nile tilapia farmers in adoption of use of probiotics in low input systems to lower cost of production of healthy fish, free from contamination. Such fish are preferred by consumers both nationally and in the international export market.

CHAPTER TWO: LITERATURE REVIEW

2.1 Fish production in low input ponds

Fish culture in low input ponds is the major contributor to aquaculture for food security and nutrition worldwide (FAO, 2016c). Additionally, more than half of the food fish produced from aquaculture come from non-fed species cultured in low input ponds in Asian countries (Subasinghe, 2017). Production of fish in low input ponds is popular in developing countries because low input systems requires minimal input (start-up capital, fingerlings, feed, technology and general management practices) which are affordable to small holder farmers (Charo-Karisa, 2006; Terziyski *et al*., 2007). Fish culture in Kenya is mostly under small holder low input systems whereby earthen ponds are fertilized with organic manure and fish are fed on locally available, low-cost agricultural by-products (FAO, 2017; Obwaga and Lewo, 2017). Low input systems have been defined as aquaculture systems where animal manure provides natural food for fish and supplementary feeds formulated from locally available agricultural by products are used to increase yields (Charo-Karisa, 2006; El-Sayed, 2008; Elnady *et al*., 2010). Tilapia and carp farming in Asia and Africa are largely conducted in low input systems (Knud-Hansen *et al*., 1991; WHO, 2006b; Terziyski *et al*., 2007).

Nile tilapia is considered as a hardy fish with the ability to perform better in low input systems (Charo-Karisa, 2006). The fish has the ability to reproduce easily in captivity, relative resistance to handling stress and disease-causing agents compared to other farmed finfish species, good flesh quality, ability to feed at a low trophic levels and high growth

rate when fed on a wide variety of natural and artificial diets (Ngugi *et al*., 2007; Welker and Lim, 2011). These characteristics make the fish suitable for culture in most parts of Kenya and explain its widespread culture in the country. According to Charo-Karisa *et al*. (2008) use of low inputs ponds is considered to lower the cost of fish production in places where feeds are expensive as a result of use of fish meal and oil from the overexploited capture fishery for feeds. The high cost of fish meal has called for the use of organic or inorganic fertilizers or their combinations as the sole nutrient inputs in low input ponds or supplementation of fertilization with farm made feed to optimize production (Diana *et al*., 1994; El-Sayed, 2008; Elnady *et al*., 2010; Mente *et al*., 2011).

A wide variety of agricultural by products used as pond inputs in low input systems include; grasses, weeds, poultry and livestock manure, rice bran, and leftover food (Wee, 1991; Little and Edwards, 2003; Elnady *et al*., 2010). Livestock manure has been used for ages as a source of soluble phosphorus, nitrogen and carbon for algal growth in aquaculture and are preferred since they are cheaper compared to inorganic fertilizers (Terziyski *et al*., 2007). Among the livestock manure, chicken manure has been recommended for use in low input systems as it produces the highest phytoplankton and zooplankton abundance in ponds (Charo-Karisa *et al*., 2008; Rapatsa and Moyo, 2013; Abu-Elala *et al*., 2016). Although supplementary feeds usually contain low protein, natural food which contains high protein partially compensates the protein deficit in low input systems (Little and Edwards, 2006).

2.2 Productivity and nutrients in low input ponds

Productivity of low input ponds has been reported to be 500 kg ha^{-1} yr^{-1} in manure fed only and up to 6,000 kg ha^{-1} yr^{-1} in manure and supplementary fed ponds. These are much lower compared to the productivity of commercial farms (10,000 - 15,000 kg $ha^{-1}yr^{-1}$) which offers complete feeds (Yi *et al*., 2008; Charo-Karisa, 2006; Gindaba *et al*., 2017). Yi *et al.* (2008) reported annual production of 14 tons ha^{-1} for Nile tilapia reared in fertilized ponds alongside supplementary feeding while Green (1992) reported yield of 4,351 kg ha^{-1} of Nile tilapia in low input ponds where chicken manure was applied simultaneously alongside feeding with pelleted feed. A gross yield of 3,600 to 3,900 kg ha^{-1} yr^{-1} was reported by Yi *et al*. (2004) for Nile tilapia cultured in ponds fertilized by chicken manure and 7,100 kg ha^{-1} yr^{-1} for ponds fertilized with both inorganic fertilizer (urea) and organic fertilizer (chicken manure).

In cases where supplemental feed was used, Nile tilapia production was reported to increase to 19,600 kg ha^{-1} yr^{-1}. Fertilization with inorganic fertilizers led to better yields compared to use of chicken manure (Yi *et al*., 2008; Gindaba *et al*., 2017). In polyculture systems with Indian major carps (rohu *Labeo rohita* and mrigal *Cirrhina mrigala*), Nile tilapia yields from low input system was at 2.96 g m^{-1} day^{-1} which was better than carps from similar system (Hossain *et al*., 2003). Additionally, integration with different livestock to provide manure for primary productivity has been reported to result to different yield of Nile tilapia. Integration with chicken resulted to 6,500 kg ha^{-1}, rabbits resulted 6,000 kg ha^{-1}, pigs led to 5,000 kg ha^{-1}, while ducks led to 4,500 kg ha^{-1} (Tabaro *et al*.,

2012). In most cases, supplementary feeding alongside pond fertilization has been reported to result to better yield in low input systems (Yi *et al*., 2008; Gindaba *et al*., 2017).

Nutrients in low input ponds mainly consist of Nitrogen (N) and Phosphorus (P). Studies have indicated that whereas about 15-30 % of the nutrient input in pellet-fed pond systems is converted into harvestable products (Acosta-Nassar *et al*., 1994; Gross *et al*., 2000), only 5-15% of the nutrients input in the fertilized pond systems are converted to harvestable products (Edwards, 1993). Excess nutrients in the form of nitrogen (N), phosphorus (P) and organic matter have been reported in ponds as a result of feed remnants, feaces and excreta (Boyd and Tucker, 1998). These nutrients are associated with algal blooms which lead to water quality problems, physiological stress, high susceptibility to pathogens and even mortality of cultured fish (Li and Yakupitiyage, 2003; Mohamed *et al*., 2013; Abu-Elala *et al*., 2016).

Shevgoor *et al*. (1994), reported deterioration in water quality when buffalo manure was applied in ponds at 100 kg dry matter ha^{-1} while Tabaro *et al*. (2012) reported an increase in ammonia and nitrites with increase in rabbit manure application in earthen ponds. Changes in the pond environment as a result of excess nutrients have been reported to increase bacterial population of pathogenic bacteria such as *Salmonella* spp., *Vibrio* spp. and *Aeromonas* spp. (Wanja *et al*., 2019). The presence of these pathogenic bacteria in fertilized ponds poses a health risk to the cultured fish and render them immune-compromised (Moriarty, 1998; Moriarty, 1999; Lucas *et al*., 2010; Molinari *et al*., 2003).

2.3 Safety of fish produced in low input systems

Production of fish in low input ponds requires close monitoring of fish health especially when inputs such as animal manures are used. Microorganisms are always present in the aquatic environment, and are in direct contact with the cultured fish and with the food given to the fish (Al-Harbi and Uddin, 2004). This makes it easier for microorganisms to accumulate in the skin of the fish and even access the digestive tract of the fish (WHO, 2006b; de Azevedo and Braga, 2012). Bacteria accumulating in the skin of fish are rarely found in the flesh of fish and can only penetrate into the fish muscle under stressful conditions as a result of overcrowding and poor water quality (Edwards, 1993).

According to World Health Organization (WHO), fish can accumulate microbial contaminants including bacteria, viruses and protozoa in their guts (WHO, 2006b). Among the microorganisms present in the aquatic environment are potentially pathogenic microorganisms, which are opportunist. They take advantage of the fish environment to cause infections, reduce growth and even cause death (Ampofo and Clerk, 2010). In Bangladesh, fish health problems have been experienced in Nile tilapia and shrimps cultured in fertilized earthen ponds (Ahmed *et al*., 2012; Hossain *et al*., 2013). Furthermore, the use of organic manure in fish culture makes the fish more susceptible to infections by the opportunistic pathogens present in the culture environment (Mente *et al*., 2011; de Azevedo and Braga, 2012).

The interaction between fish and the environment is such that the microorganisms present in the water manipulates the microbiota of the fish's intestine and vice versa (WHO,

2006b). Pathogenic bacteria for example, *Salmonella* spp. and *Escherichia coli* have been found to survive and multiply in the guts of Nile tilapia and carps cultured in fertilized ponds (WHO, 2006b). Moreover, livestock faecal waste used as inputs in fish ponds contains varying quantities of viruses and bacteria which can pose health risks to human beings (Edwards, 1993). Little and Edwards (2003) have reported faecal coliform, *Salmonella* and bacteriophages in ponds fertilized with organic manure. In Kenya, *Serratia plymuthica, S. ficaria, S. marcescens*, *Pseudomonas luteola, Klebsiella oxytoca* and *Raoultella terrigena* have been reported in fish and culture water from fertilized ponds (Wanja *et al*., 2019). The presence of these bacterial species in cultured fish poses a great risk to aquaculture as they may lead to high mortalities and economic losses.

2.4 Fish disease occurrence in aquaculture

Disease challenges have been experienced in various countries in Africa including Egypt, Algeria, Nigeria and Uganda (AU-IBAR, 2016). Kenya is highly vulnerable to spread of the diseases due to cross border movement of live fish and trade of fish in the region which may lead to introduction of fish with unknown health history (AU-IBAR, 2016). Additionally, the global climatic changes are also likely to cause changes in aquatic ecosystems that may lead to increased susceptibility of farmed fish to infections and diseases (Kobayashi *et al*., 2015; FAO, 2016c). Disease incidences in the aquaculture and fisheries industry have the capacity of causing huge economic losses as seen in Asian countries where massive expansion in the aquaculture industry preceded fish health capabilities, costing the industry heavily and could lead to significant negative impacts to the industry (FARM AFRICA, 2016a; Opiyo *et al*., 2018).

Limited information exists on disease outbreaks and fish health management practices in fish farms in Kenya (Wanja *et al*., 2019). Most fish health studies have paid attention to parasites in wild fish populations of the two most culture species, *O. niloticus* and *C. gariepinus* dwelling on their descriptions, biology and pathology (Akoll and Mwanja, 2012; Ochieng *et al*., 2012). Lack of information on fish diseases could be linked to poor diagnostic infrastructure, lack of human resource with expertise in fish health management, high cost of diagnosis and lack of well-equipped veterinary laboratories and expertise to undertake identification of fish pathogens. Farmers have also contributed to the deficiency of information by poor record keeping and misreporting of the causes of mortalities in their farms (Akoll and Mwanja, 2012).

A study conducted in 2014 investigating bacterial and fungal infections in farmed fish established that fish hatcheries lost most of their stocks to fungal diseases mainly saprolegniasis and bacterial diseases mainly hemorrhagic disease and pop eye diseases (Njagi, 2016). Major bacterial infections among fish in Kenya are caused by species of *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *P. aeruginosa*, *Edwardsiella tarda*, *Flavobacterium columnare*, *Mycobacterium fortuitum* and *Streptococcus iniae* (Defoirdt *et al*., 2011; Akoll and Mwanja, 2012). Most hatcheries have been affected by *S. iniae* which makes fish to have a C- shape in newly stocked production systems (Akoll and Mwanja, 2012). These infections are believed to be caused by use of untreated water sourced directly from rivers and streams which have a high bacterial load (Opiyo *et al*., 2018).

Increased intensity in fish farming has increased the vulnerability of the fish to infections in crowded conditions (Obwanga and Lewo, 2017). Grow-out fish have also been affected by fish louse (*Argulus* spp.) and white spot disease (*Ichthyophthirius multilifis*) (Yongo *et al*., 2012). Disease occurrences in grow-out farms have been attributed to poor husbandry practices including use of on-farm formulated feed with high bacterial load and use of water directly from the river without prior treatment (Yongo *et al*., 2012). Some cage fish farmers have experienced mortality of fish, losing 40-100% of the stock due to diseases (Aura *et al*., 2018). In intensive cage farming, serious suspected disease problems have been encountered in fish reported to have symptoms like skin lesions and fin rot which are suspected to be fungal infections (Aura *et al*., 2018; Njiru *et al*., 2019). Although massive mortalities in ponds and cages have been associated with poor water quality, disease incidences in the country are often attributed to inadequate biosecurity measures and poor management practices (Opiyo *et al*., 2018).

2.5 Fish health management practices in Kenya

Fish farms in Kenya use preventive measures to reduce chances of disease occurrence (AU-IBAR, 2016). Contrary to biosecurity in grow-out systems, hatcheries routinely include disinfection of farm equipment and culture facilities in their fish health management schemes. The preference of management practice and application of prophylactics are based on farmers' knowledge and experience (Yongo *et al*., 2012). Commonly used prophylactics to get rid of fungal and bacterial infections include; potassium permanganate, copper sulphate, formalin and sodium chloride (Akoll and Mwanja, 2012). Oxytetracycline is the only antibiotic used by private hatcheries for apparent bacterial infections in African

catfish broodstock. The use/misuse of chemicals and drugs in aquaculture should be discouraged due to possible residue problems in fish and the development of drug resistance among pathogens (Defoirdt *et al*., 2011).

Limited biosecurity measures have been put in place to monitor new introductions and occurrence of fish diseases in Kenya (Obwaga and Lewo, 2017). This is due to non-reported fish disease challenges making the establishment of such facilities unappealing, inadequate human resource specialized in fish diseases and poor infrastructure in disease management (Opiyo *et al*., 2018). The substantive growth of aquaculture in Kenya has led to the introduction of non-indigenous species which are possible sources of diseases and parasites, posing a danger to the wild stocks (FAO, 1996; Hickley *et al*., 2008). The inadequate biosecurity measures in these activities may result into rapid spread of disease pathogens within the country. The recent sampling and detection of Tilapia lake virus (TiLV) in Lake Victoria in both fish in cages and open waters put the farmed fish in high density cages in lake Victoria Kenya at high risk of infections since the lake is a common property shared among the three East African countries (Uganda, Tanzania and Kenya) (Aura *et al*., 2018; Mugimba *et al*., 2018).

Kenya has no specialized fish diagnostic laboratories recognized by the World Animal Health Organization (known by its French name Office International des Epizooties (OIE) (Opiyo *et al*., 2018). In the event of a fish health challenge, diagnoses are always performed at public research institutes and universities that conduct research on fisheries and aquaculture (Akoll and Mwanja, 2012). FARM AFRICA, (2016b) recommended that

farmers should use preventative measures in intensive farming systems like cages due to inadequacy of specialists in fish disease in Kenya. Farmers are always advised to maintain suitable environmental conditions, stock healthy fish, provide a nutritious diet and limit stress to prevent fish diseases (Akoll and Mwanja, 2012).

2.6 The need for probiotics application in aquaculture

Microorganisms are always present in fish culture water and are in direct contact with the fish, gills and the food supplied. These are potentially pathogenic microorganisms, which are opportunists. They take advantage of the fish stress situation (poor nutrition and high density) to cause infections, worsening its performance and eventually leading to mortalities (de Azevedo and Braga, 2012).

A wide range of veterinary drugs including hormones, antibiotics and nutrient mixtures have been used on farmed fish for disease prevention, treatment and as growth promoters (Lara-Flores *et al*., 2003; Caruso, 2016). These growth promoters and antibiotics have been linked to adverse effects such as accumulation in the flesh, negative impacts on microbial populations in the aquaculture environment, immune suppression and emergence of multiple drug resistant microorganisms (Kesarcodi-Watson *et al*., 2008; Nayak, 2010a, Welker and Lim, 2011; Nayak, 2013; Caruso, 2016). Most antibiotic resistance in farmed fish has been reported for oxytetracycline (Miranda and Zemelman, 2002) and tetracycline (Miranda *et al*., 2003). Antibiotic resistance to chloramphenical, amoxylin, sulphonamide and streptomycin have been reported for *Aeromonas salmonica* in Japan and Ireland (Alderman and Hastings, 1998). These antibiotic-resistant bacteria remain in sediments

and farm environment and act as a source of antibiotic resistance genes for fish pathogens in neighboring farms.

Global concerns over antibiotic resistance from farmed fish have been recognized by the European Union and a set of regulations (EC No. 470/2009) was put in place to regulate the antibiotic maximum residue limits (MRL) in foodstuff of animal origin including fish. As a result of antibiotic resistance in cultured animals, concerns have been raised regarding the impact of antibacterial use in aquatic environment and the risk associated with the possible transfer of pathogens in fish to humans (WHO, 1999; Caruso, 2016; FAO, 2018).

In some countries especially the developing countries, there are no effective regulations and control of antibiotics usage in food fish posing a great risk of antibiotic resistant pathogens in farmed fish getting transmitted to humans (Alderman and Hastings, 1998). Although it is rare for bacterial pathogens in aquatic environment to be transmitted to humans, in warm climatic condition experienced in tropical countries, fish pathogens especially *Aeromonas hydrophila* and *Edwardsiella tarda* are likely to be transferred to humans (Alderman and Hastings, 1998; WHO, 1999). Therefore, the use of natural additives and biological control measures to substitute antibiotics has become an area of great interest in aquaculture to reduce infections and emergence of antibiotic resistant bacteria in farmed fish (Martínez Cruz *et al*., 2012; Abdelhamid *et al*., 2014b; Caruso, 2016; Ridha and Azad, 2016).

2.7 Probiotic use in aquaculture

The use of probiotics in fish farming is on the rise with the increasing demand for more environment-friendly practices and sustainability in aquaculture (Gatesoupe, 1999; He *et al*., 2013; De *et al*., 2014; Lazado and Caipang, 2014). According to Soccol *et al*. (2013), the global market for probiotics in 2008 was valued at US $15, 900 million, and is expected to increase at a rate of 4.3% annually. However, the benefits to the host depends largely on the type of the probiotic used, dosage, mode of the application, duration, age and size of the host and water quality parameters (Ridha and Azad, 2016).

In recent years, emphasis have been put on dietary manipulation of gut microbiota of cultured fish to improve overall fish health status by promoting growth and immunity of cultured fish using probiotics (Merrifield *et al*., 2010a; Nayak, 2010a, 2010b; Standen *et al*., 2013). Probiotics are gaining importance in controlling potential pathogens in aquaculture by decreasing colonization and adherence of pathogenic bacteria and improving fish health (Merrifield *et al*., 2010a; Abdelhamid *et al*., 2014b; Ringø *et al*., 2016).

The use of probiotics is regarded as a promising strategy and their wide acceptance for use in aquaculture has been shown by the number of research studies published over the last ten years (Irianto and Austin, 2002a, 2002b; Balcázar *et al*., 2006; Merrifield *et al*., 2010b; Martínez *et al*., 2012; De *et al*., 2014). The advantages of probiotics use in aquaculture include; improvement of water quality in the culture environment, increased food absorption and utilization, reduction of suspected pathogenic bacteria in the gut of fish,

enhancement of nutrition of the fish through production of supplemental digestive enzymes, reduction in disease incidences, greater survival and improvement of fish immune response (Boyd and Massaut, 1999; Gatesoupe, 1999; Verschuere *et al*., 2000; Irianto and Austin, 2002a; Yanbo and Zirong, 2006; Zhou *et al*., 2009; Merrifield *et al*., 2010b; de Azevedo and Braga, 2012; Martínez *et al*., 2012; De *et al*., 2014; Ridha and Azad, 2016).

2.8 Types of probiotics and application levels in aquaculture

Application of probiotics is done either in the culture water or by inclusion in feeds, the latter being the common practice (Gatesoupe, 1999). The different probiotics used in aquaculture are either monospecies or multispecies combinations from different microorganisms (Verschuere *et al*., 2000; Nayak, 2010a; Allameh *et al*., 2016). The commonly used probiotics in aquaculture belongs to microalgae (*Tetraselmis*), yeast (*Debaryomyces*, *Phaffia and Saccharomyces*); gram-negative bacteria (*Aeromonas*, *Alteromonas*, *Photorhodobacterium*, *Pseudomonas and Vibrio*) and gram-positive bacteria (*Bacillus*, *Lactococcus*, *Micrococcus, Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Weissella*) (Gatesoupe, 1999; Verschuere *et al*., 2000; Irianto and Austin, 2002a; Nayak, 2010a; Pandiyan *et al*., 2013; De *et al*., 2014).

Monospecies probiotic supplementation in fish feed have been reported to result in better growth performance of fish compared to multispecies (Gomez-Gil *et al*., 2000; Irianto and Austin 2002a; Allameh *et al*., 2016). On the other hand, improvement in growth and non-specific immunological parameters have been reported when a mixture of probiotics were

administered to olive flounder (*Paralichthys olivaceus*) and rohu (*Labeo rohita*) (Harikrishnan *et al*., 2010; Giri *et al*., 2014). In-feed probiotics are usually associated with mucosal surfaces, particularly the gastrointestinal tract and their occurrence have been established in the normal intestinal flora of different fish species such as salmon, Arctic char, Atlantic cod, rainbow trout, Indian major carps and Nile tilapia (Ringø and Gatesoupe, 1998; Kim and Austin, 2006; Lara-Flores and Olvera-Novoa, 2013).

Among the probiotics used in aquaculture, the most studied are lactic acid bacteria, *Bacillus* spp. and yeasts (*Saccharomyces cerevisiae)* (Gatesoupe, 1999, 2007; Nayak, 2010a, Martínez *et al*., 2012; Nayak, 2013; Pandiyan *et al*., 2013; Silva *et al*., 2015) due their low cost, viability and ability to participate in the digestion process, survive in the digestive tract of fish and colonize the gut of the fish (de Azevedo and Braga, 2012; Hai, 2015b; Iwashita *et al*., 2015; Allameh *et al*., 2016; Hassaan *et al*., 2018). Despite great attention as a viable alternative to antibiotics, use of probiotics have been faced by numerous challenges including; possible negative impact of untested probiotics on the environment, regulatory constraints in most developing countries, food safety concerns, difficulty in maintaining viable microorganisms through feed manufacturing process and potency during storage (Welker and Lim, 2011; De *et al*., 2014).

Use of microorganism that are known to be safe for the environment and humans, and maintaining viable populations during storage could be the most appropriate way for testing prospective probiotics for use in aquaculture (Welker and Lim, 2011). The study of the effects of probiotics supplementation in the diets of tilapias have not advanced as far

as it has in other fish species, like salmonids and shrimps (Balcázar *et al*., 2007; Bagheri *et al*., 2008; Ninawe and Selvin, 2009; Zhou *et al*., 2009; Merrifield *et al*., 2010a; Merrifield *et al*., 2011; Hossain, *et al*., 2013; Ghosh *et al*., 2016). Therefore, research in application of the probiotics in tilapia still warrants studies to establish the suitable probiotic treatment in different environments where tilapias are cultured.

The effect of dietary probiotics depends on the level of application. Different levels of probiotic applications have been studied and recommended for use in fish culture (Barman *et al*., 2013). Red tilapia (*Oreochromis mossambicus*) fed on a higher dosage of commercial probiotic (BZT® BIO-AQUA) exhibited a higher final body weight compared to the control (Mohamed *et al*., 2013). On the other hand, Nile tilapia fed on commercial probiotic (Biogen®) supplemented feed, had no significant differences in fish fed at different levels of probiotic supplementation (El-Haroun e*t al*., 2006).

According to Mohamed *et al*. (2013), supplementation of commercial probiotic (BZT® BIO-AQUA) did not affect survival of *O. mossambicus* reared in tanks. However, the growth of African catfish was reported to improve when 2% baker's yeast was included in the diet (Essa *et al*., 2011). In a separate study in Nile tilapia, baker's yeast (*S. cerevisiae*) supplementation at 1 g kg^{-1} diet resulted to improved growth performance and nutrient utilization compared to control and higher levels of 2 g kg^{-1} diet (Asadi *et al*., 2012). De *et al*. (2014) recommended that the best dose of the probiotic *B. subtilis* is 0.1 %, for improvement in the growth performance, innate cellular responses and microbial profile of fish intestines.

2.9 Probiotics use in enhancement of fish nutrition and growth performance

The role of probiotics in enhancement of nutrition of fish has been reported by earlier studies in tilapia and other fish species (Lara-Flores *et al*., 2010; Newsome *et al*., 2011; Welker and Lim, 2011; Lara-Flores and Olvera-Novoa, 2013). Probiotics in fish feed stimulates fish appetite and improves nutrition by production of additional vitamins and detoxification of compounds in the diet hence breaking down indigestible feed components (Abdelhamid *et al*., 2014a).

Probiotics have been found to participate in fish digestion processes by supplying fatty acids and production of vitamins some of which are lacking in fish diets especially in plant based proteins (Nayak, 2010a; Newsome *et al*., 2011). Moreover, fish generally do not possess cellulase enzyme or relevant symbiotic gut flora capable of breaking down the cellulose usually present in plant materials (Wee, 1991). After probiotics transit through the stomach, they attach in the intestine and use a large number of carbohydrates for their growth and produce a range of relevant digestive enzymes (amylase, protease and lipase) that increases the digestibility of organic matter and protein especially from plant sources (Holzapfel *et al*., 1998; Lara-Flores *et al*., 2010; Lara-Flores and Olvera-Novoa, 2013).

Lactic acid bacteria probiotics are known to stimulate feed conversion efficiency, growth performance and survival in fish and shrimp culture, preventing intestinal disorders, neutralizing anti-nutritional factors and myco-toxinogenic moulds present in the feeds (Ringø and Gatesoupe, 1998; De *et al*., 2014). *Bacillus coagulans* SC8168 applied in shrimp culture water was beneficial on the survival rate of shrimp (*P. vannamei*) larvae

and commercial probiotic *Bacillus* spp. increased the survival rate of India white shrimp (*Fenneropenaeus indicus*) (Ziaei-Nejad *et al*., 2006) while *Thalassobacter utilis* resulted to increased survival of blue crab (*Portunus trituberculatus*) cultured in sea water (Nogami and Maeda, 1992).

Low FCR is an indicator of better feed utilization and has been reported in several fish species fed on probiotic-supplemented diets. Better feed utilization indicates a reduction in the amount of feed necessary for the growth of fish hence cost reduction (Ringø and Gatesoupe, 1998). Low FCR has been reported in Nile tilapia fed on commercial probiotic Biogen® (*Bacillus licheniformis* and *Bacillus subtilis*) compared to the control (El-Haroun *et al*., 2006). Additionally, lower FCR and growth performance have been experienced in common carp fed photosynthetic bacteria and *Bacillus* spp. (Yanbo and Zirong, 2006). *B. subtilis* significantly improved the growth performance of *O. niloticus* through enhanced absorption of nutrients (Hassaan *et al*., 2018). Growth performance was enhanced in Nile tilapia juveniles fed with heterologous autochthonous *Bacillus amyloliquefaciens* isolated from the local yellowfin bream, (*Acanthopagrus latus*) (Ridha and Azad, 2012).

Brewer's yeast, (*S. cerevisiae*) is rich in protein and can be used as a supplement to compensate for amino acid and vitamin deficiencies in animal feed exemplified by Koi carp (*Cyprinus carpio*) which achieved higher growth and better FCR when fed on feed supplemented with *S. cerevisiae* for 45 days (Dhanaraj *et al*., 2010). Additionally, Ghazalah *et al*. (2010) reported that commercial probiotic mixtures (Premalac® or Biogen®) used in diets containing different levels of protein, produced better growth

performance in tilapia fed 25% CP diet, suggesting improved protein utilization compared to the control groups.

A probiotic mixture containing a combination of *Lactobacillus acidophilus, Streptococcus faecium* and *S. cerevisiae* improved nutrient utilization in Nile tilapia (Lara-Flores *et al*., 2003) while *B. subtilis* led to better nutrient intake by Nile tilapia cultured in polyculture with fresh water prawn (*Macrobrachium rosenbergii*) (Günther and Jiménez-Montealegre, 2004). Enhancement of nutrition is associated with an increase in specific activities of digestive enzymes in probiotic treatments and have been documented to lead to enhanced digestion and increased absorption of food (Ziaei-Nejad *et al*., 2006; Ninawe and Selvin, 2009; Zhou *et al*., 2009; Welker and Lim, 2011; Allameh *et al*., 2016).

2.10 Role of probiotics in enhancement of body composition of fish

Body composition of fish depends on the quality of the diet, nutrient concentration, feeding levels, feeding regime and other factors. Fish farmers and consumers prefers fish with more protein and less fat (Hassaan *et al*., 2018). Dietary probiotics have had significant effects on body composition of fish. It has been reported that, a combination of two dietary probiotic *B. licheniformis* and *B. subtilis* led to significant increase in crude protein levels, reduction of crude lipid and moisture content in Kutum (*Rutilus frisii kutum*) (Azarin *et al*., 2015). Similar results were obtained when *S. cerevisiae* was used as a probiotic in Koi carp (Dhanaraj *et al*., 2010). Increase in protein level has also been reported in rainbow trout fed on a combination of *B. licheniformis* and *B. subtilis* (Bagheri *et al*., 2008). On the other

hand, combination of *L. acidophilus* and *S. cerevisiae* led to higher protein but lower lipid content in Koi carp (Dhanaraj *et al*., 2010).

Nile tilapia fed on malic acid and/or *B. subtilis* exhibited a significant decrease in lipid content compared with the control diet (Hassaan *et al*., 2018). The greater protein value of carcass was attributed to proteins produced by members of genus *Bacillus.* Nevertheless, commercial probiotic Biogen® did not have any effect on the carcass moisture, ash and protein content of *O. niloticus* but resulted to low lipid content (El-Haroun *et al*., 2006). In a separate experiment with probiotic *S. faecium* on Nile tilapia, Lara-Flores *et al*. (2003) realized a significant increase in the crude protein and crude lipid contents in fish flesh.

2.11 Effect of probiotics on water quality

In aquaculture, fish and microorganisms share an ecosystem. Therefore, the well-being of fish is dependent on the quality of the rearing water. Boyd and Massaut (1999) described the beneficial effect of probiotics in pond water as an aid in organic matter decomposition, reduction of the harmful organic waste that accumulates in the pond bottom and reduction of phosphate levels and nitrogen compounds when added to fish culture water. The nitrogen wastes are reduced through the re-mineralization by probiotic populations (De *et al*. 2014; FAO, 2016b).

Probiotics have been used to improve culture environment of shrimps in ponds by enhancing the microbial and phytoplankton populations which results to a healthy ecosystem (FAO, 2016b). Particularly, bacterial treatments to improve water quality and

production yield of *Penaeus monodon* have led to reduction in ammonia and nitrite in the rearing water (Gatesoupe, 1999). Higher levels of gram-positive bacteria have been used to reduce the accumulation of particulate organic carbon and minimize the buildup of dissolved and particulate organic carbon during the culture cycle of shrimp while promoting more stable phytoplankton blooms through the increased production of CO_2 (Verschuere *et al*., 2000). Furthermore, introduction of *Bacillus* spp. in proximity to pond aerators have been reported to lead to degradation of organic matter, reduced chemical oxygen demand, thus improving the water quality and increased shrimp harvest (FAO, 2016b).

Effects of probiotics on water quality in Northern white shrimp (*Penaeus vannamei*) ponds have been investigated and the results indicated that commercial probiotics could reduce the concentrations of nitrogen and phosphorus in pond water (Wang *et al*., 2005). However, Zhou *et al*. (2009) reported no effect on water quality when *B. coagulans* SC8168 was used in shrimp (*P. vannamei*) ponds. It was explained that *B. coagulans* resulted to stable water quality parameters throughout the study period. The use of probiotics have been reported to directly regulate water quality especially in terms of a stable pH within 7.5 to 8.0 or healthy growth of shrimps (*P. monodon* and *Litopenaeus vannamei)* in Thailand (FAO, 2016b). Wang *et al*. (2005) indicated that a commercial probiotic made from *S. cerevisiae*, *Bacillus* spp., *Nitrosomonas* spp. and *Nitrobacter* spp. had the ability to reduce the concentrations of phosphates from 0.1105 to 0.0364 mg L^{-1} and inorganic nitrogen from 3.74 to 1.79 mg L^{-1}. In Nile tilapia, commercial probiotics made from *Bacillus*

licheniformis and *B. subtilis* led to optimized dissolved oxygen and ammonia levels (El-Haroun *et al*., 2006).

2.12 Use of probiotics in enhancing fish immunity

Defense system of fish against pathogens and microorganisms is important; therefore, organic, inorganic, or synthetic matter have been evaluated as immunostimulant which are better alternative to antibiotics to control fish diseases (Findlay and Munday, 2000; Cuesta *et al*., 2004). Natural immunostimulants are considered promising alternatives to chemotherapy and vaccines because of their cost effectiveness, broad spectrum activity and eco-friendly measures (Anderson, 1992; He *et al*., 2009; Elkamel and Mosaad, 2012).

Since the year 2009, there has been increasing attention in the modulation of the non-specific immune system of fish as prophylactic measures against diseases and treatment using probiotics (Nayak, 2010a; Elkamel and Mosaad, 2012; Navarrete and Tovar-Ramrez, 2014; Hai, 2015a; Newaj-Fyzul and Austin, 2015). Immunomodulation or stimulation of immune system is considered important mechanisms supporting probiosis. Probiotics have been used to enhance immune responses in gut-associated lymphoid tissue and systemic immunity of fish; for example, phagocytic, lysozyme activities, alternative complement activities, superoxide anion production and expression of certain cytokines and antibodies (Balcázar *et al*., 2007; Nayak, 2010a; Newaj-Fyzul and Austin, 2015).

Lactobacillus plantarum have been reported to increase the immune system of Pangasius catfish (*Pangasius bocourti*) by increasing the level of serum lysozyme activity and

respiratory burst activity (Van Doan *et al*., 2016). Baker's yeast (*S. cerevisae*) has been used to serve as dietary additive to improve fish growth and immune responses in different species of fish (Irianto and Austin, 2002a). Additionally, it has been reported to contain various immunostimulating compounds such as β-glucans, nucleic acids as well as mannonoligosaccharides which have the capability to enhance immune response in fish (Osman *et al*., 2010; Abu-Elala *et al*., 2013; Navarrete and Tovar-Ramrez, 2014). Baker's yeast has the advantage of fast growth, low cost, high stability and is a common constituent of fish feed (Abdel-Tawwab *et al*., 2008; Irianto and Austin, 2002b; Osman *et al*., 2010).

Diet supplementation with baker's yeast leads to reduced bacterial counts in intestines of tilapia indicating that it can be an alternative to antibiotics and can be used in disease prevention in tilapia culture (Abdel-Tawwab *et al*., 2008). *Bacillus* spp. has been reported to compete for nutrients thus inhibiting other bacteria from multiplying in the gut of fish (Verschuere *et al*., 2000; Hassaan *et al*., 2018). It also produces a heat liable siderophore with the ability to control bacterial population in the gut of fish (Singh and Yadava, 2005). Additionally, *S. cerevisiae* has been reported to reduce gram-negative bacteria like *Vibrio* spp., *Pseudomonas* spp. and *Edwardsiella tarda* in the gut of shrimp (Rengpipat *et al*., 1998).

2.13 Effect of probiotics on fish gut microbiota

Fish gastrointestinal tract (GIT) consists of the oesophagus, stomach, pyloric caeca, small and large intestine (Ringø *et al*., 2016). Interaction between the fish and the environment is such that, microorganisms present in the culture water influences the microbiota of the

fish's intestine making the gastrointestinal tract one of the entry points of some pathogens into the fish body (Al-Harbi *et al*., 2004; Silva *et al*., 2015). The function of gut microbiota includes; influencing nutrient partitioning, degrading dietary compounds, lipid metabolism, providing essential nutrients generated as a result of microbial metabolism, protecting against invading pathogens by blocking their attachment to gut binding sites and stimulating gut morphology (Strom and Ringø, 1993; Holzapfel *et al*., 1998; Mulder *et al*., 2009; De *et al*., 2014). A balanced gut microbial community has been recommended to be essential for fish health and well-being (Garrett *et al*., 2010; Ringø *et al*., 2016). According to Welker and Lim, (2011) the gut microbial population is important in improvement of fish nutrition by increasing nutrient uptake and utilization, enhancing the production of digestive enzymes, amino acids, short chain fatty acids and vitamins hence improving digestion of food.

Fish gut is usually colonized by indigenous microorganisms (Standen *et al*., 2013; Ringø *et al*., 2016). Under normal conditions, the dominant microbial species in the intestine of fish are anaerobic, *Lactobacillus*, *Bacillus, Bifidobacterium* and *Saccharomyces* spp. accounting for 99% of the microbial community. The aerobic and facultative bacteria account for only 1% of the population microorganisms (Ringø *et al*., 2016). Gram-positive bacteria including lactic acid bacteria have been reported to be numerically dominant members of the normal microbiota in the gastrointestinal tract of larval fish (Ringø and Gatesoupe, 1998).

Indigenous gut microbiota is usually affected by the culture environment and the diets eaten by the fish (Al-Harbi and Uddin, 2004). For example, the gut of tilapia cultured in freshwater earthen ponds has a greater diversity of gut bacteria, predominately comprising of gram-negative bacteria (*Shewanella putrefaciens*, *A. hydrophila*, *Corynebacterium urealyticum, Vibrio cholera* and *Escherichia coli*) (Al-Harbi and Uddin, 2003; Ringø *et al*., 2016). These bacteria are always ubiquitous in the aquatic environment and can influence the gut microbial communities and population (Al-Harbi and Uddin, 2004). Different dietary compositions including probiotics, plant proteins, vitamins and organic salts have been reported to influence the gut microbiota of both freshwater and brackish water fish (Al-Harbi and Uddin, 2003; Merrifield *et al*., 2010a; Standen *et al*., 2013; Hoseinifar *et al*., 2017).

Probiotics have been reported to establish favorable microbial communities, such as lactic acid bacteria and *Bacillu*s spp. in the gastrointestinal tract of fish (Ringø et al., 2016). The microbial communities alters gut morphology and produce certain enzymes and inhibitory compounds which improves digestion and absorption of nutrients, as well as enhanced immune response (Standen *et al*., 2013). This is as a result of their ability to colonize and adhere to the fish gastrointestinal tract hence maintaining a favorable relationship between the beneficial and pathogenic microorganisms in the gastrointestinal tract of the host (Ferguson *et al*., 2010; Ringø *et al*., 2010; De *et al*., 2014; Ringø *et al*., 2016; Adel *et al*., 2017).

The adherence of probiotic bacteria to the gut of fish either increases or reduces depending on the species of fish and the type of feed fed to the fish (Ringø and Gatesoupe, 1998; Al-Harbi and Uddin, 2003; Hartviksen *et al*., 2014; Ringø *et al*., 2016). Several studies have reported modulations of gut microbiota due to adding probiotics to the diet of fish (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Irianto and Austin, 2002a; Balcázar *et al*., 2006; Merrifield *et al*., 2010b; Nayak, 2010b; Ringø *et al*., 2016; Standen *et al*., 2013).

Continuous application of probiotics cells containing 10^5 to 10^9 colony forming units (cfu) have been reported to colonize gastrointestinal tracts of different fish species (Bagheri *et al*., 2008; Kim and Austin, 2006; Merrifield *et al*., 2010b). For example, ingestion of *Lactobacillus* products and supplements containing viable lactic bacteria resulted to their establishment in the gastrointestinal tract of Nile tilapia (Abdelhamid *et al*., 2014b) whereas *Bacillus* spp. have been reported to colonize the digestive tract of black tiger shrimp (*P. monodon*) (Rengpipat *et al*., 1998) and Nile tilapia (Hassaan *et al*., 2018). Provision of dietary probiotics elevated the ability of *Carnobacterium divergens* to adhere to the intestine of Atlantic salmon (*Salmo salar*) (Abdelhamid *et al*., 2014b).

Different probiotic bacteria adhere differently in fish intestines and some have been reported to persist in the digestive tract several weeks after treatment and after reverting to non-supplemented diets (Balcázar *et al*., 2006; Kim and Austin, 2006). Probiotic *Carnobacterium divergens* and *C. maltaromaticum* were reported to persist in the intestine of rainbow trout for more than 3 weeks (Kim and Austin, 2006). Lactic acid bacteria have the ability to survive for several days in the intestine of larval and juvenile fish (Strom and

Ringø, 1993) while different species of yeast were reported to be persistent in the digestive tract of rainbow trout (Andlid *et al*., 1995) and Koi carp (Dhanaraj *et al*., 2010). Additionally, yeasts has been reported to have a great potential to adhere and to colonize the intestine of most fish species (Gatesoupe, 1999).

Several studies have demonstrated that probiotic colonization of the gastrointestinal tract alters the indigenous microbiota composition and population of the host (Strom and Ringø, 1993; Bagheri *et al*., 2008). Aubin *et al*. (2005) reported that feeding rainbow trout on *Pediococcus acidilactici* or *Saccharomyces cerevisiae* var. *boulardii* for 5 months led to changes in the relative and absolute abundance of the indigenous bacteria in the gut of the fish. Presence of bacteria of the genera *Buttiauxella* and *Citrobacter* was confirmed in the fish fed control diet while *Serratia* spp. was detected in the fish fed diets containing *P. acidilactici* (Aubin *et al*. 2005).

Data from most studies have indicated the sensitivity of gut microbiota to probiotic modulation across larval stages to juvenile stages (Strom and Ringø, 1993; Carnevali *et al*., 2006; Kim and Austin, 2006; Balcázar *et al*., 2007; Newaj-Fyzul *et al*., 2007; Bagheri *et al*., 2008; Merrifield and Carnevali, 2014) This necessitates long-term trials monitoring the gut microbiota throughout maturation and development of the gastro intestinal tract (GIT) until the adult stage.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Area

3.1.1 Geographical location

The study was carried out at Kenya Marine and Fisheries Research Institute (KMFRI) Sagana fish farm (altitude 1230 m above sea level, latitude 0°39´S and longitude 37°12´E) (Munguti *et al*., 2012). The fish farm is located in Kenya's Kirinyaga County, 2 km outside of Sagana town and 105 km Northeast of Nairobi city (Figure 3.1 and 3.2).

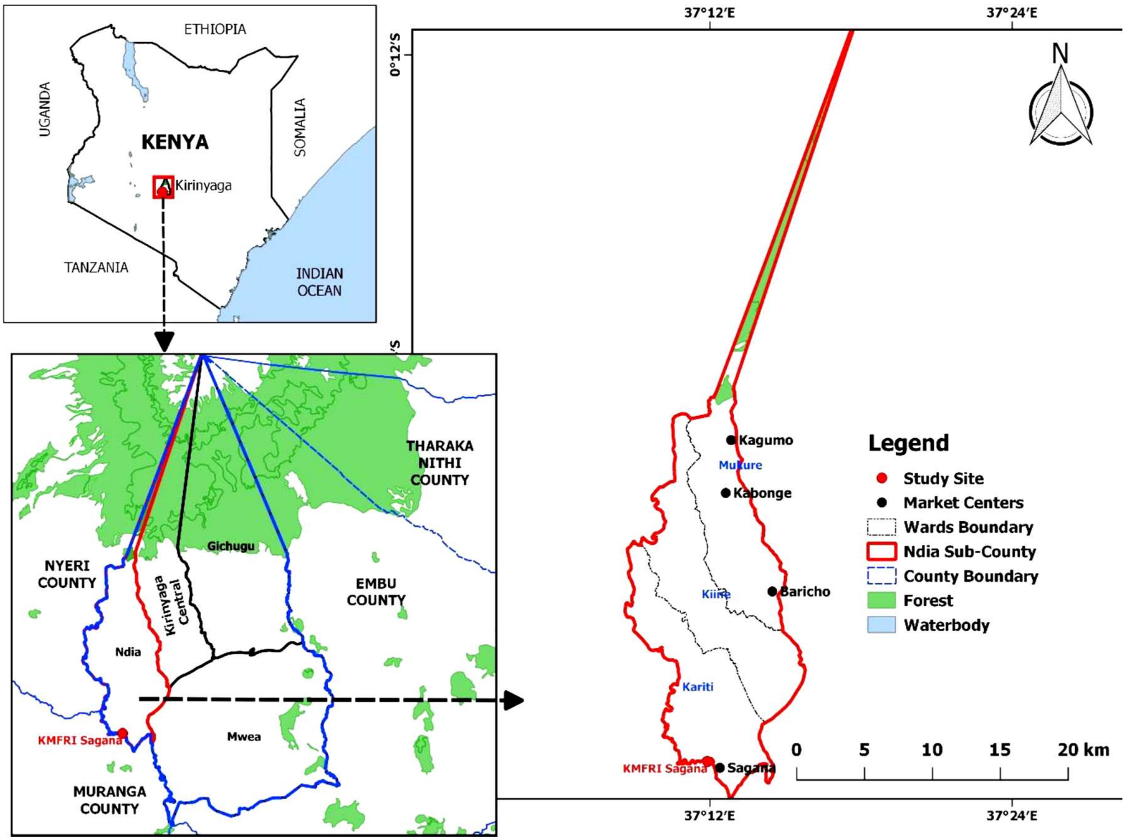

Figure 3.1: Map of Kirinyaga County showing the study site (https://www.google.co.ke/maps/place/Kirinyaga County/) (Google 2019).

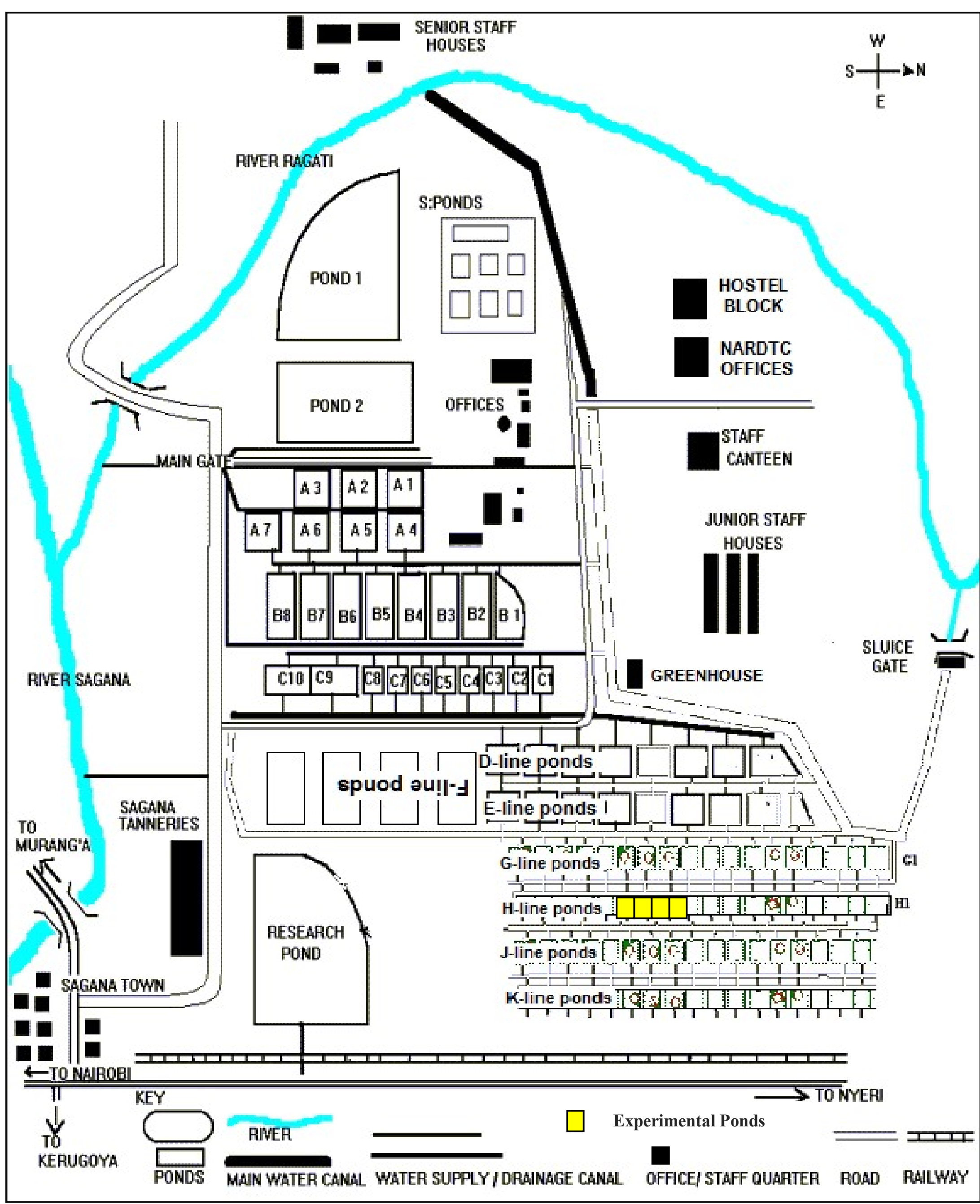

Figure 4.2: A sketch plan of KMFRI Sagana showing ponds where the study was carried out (*Drawing not to scale*).

3.2 Climate

3.2.1 Topography and soils

The Sagana fish farm is located at the edge of a large plain at the southern leeward side of Mt. Kenya and is characterized by a warm climate. The fish farm has a gently sloping topography with several hills in the immediate surroundings. Ponds lie on a relatively flat area with a gentle slope from north to south. The pond soils are generally black cotton soils, high in 2:1 type mineral clays with cation exchange capacity (CEC) typical for the type of soils (30-50 meq/100 g) with pH values of 5.4 to 7.5 (Munguti, 2007).

3.2.2 Temperature and rainfall

Kirinyaga County has two distinct dry and rainy seasons. Annual precipitation is high with a 30-year average of 1,166 mm and a distinct cold season between June and August when rainfall is at minimum and temperature at low levels of an average of 15°C. Short and long rains occurs from March to May and October to December respectively with one month peak of 500 mm or more around April (Munguti, 2007). Daily mean temperatures ranged between 15 to 23°C with daily minimum range of between 14 to 19°C and daily maximum range of 20 to 30°C. The dry period is experienced between from February to April and September to November annually.

3.3 Study design

Nile tilapia fingerlings used in the experiment were produced at Kenya Marine and Fisheries Research Institute, Sagana fish farm hatchery. Sex reversed male *O. niloticus* fingerlings were produced through hormonal sex reversal as described by Phelps and

Popma (2000). The fish fingerlings with a mean weight of 39.75 ± 0.05 g were acclimatized for 10 days while being fed on a control diet 28% crude protein (CP) at 3% body weight. During acclimatization, dead fish were replaced with fish of similar size reared in similar conditions. Thereafter, the fish fingerlings were randomly distributed in net cages and assigned seven treatments in four replicates.

Fish were stocked in 1.25 m^3 net cages (1.0 × 1.0 × 1.25 m); with a mesh size of 1.80 cm. The cages were placed in 4 earthen ponds measuring 150 m^2 each (Plate 1). Each pond had 7 cages representing replicates of each treatment. Cages were floated by wooden bars 30 cm above the pond bottom and 25 cm above the water surface and placement was done at 2 m from each other with a distance of 2 m allowed from the sides of the pond to the cages on all pond sides. Fish were stocked at 50 fish m^{-3} according to Yi *et al*. (1996) and Chakraborty *et al*. (2010). Each cage was fitted with a polyvinyl chloride (pvc) feeding ring of 30cm diameter suspended at the midpoint of each cage to prevent direct spillage of the experimental feed.

3.4 Pond fertilization, diet preparation and feeding

Pond preparation was done by drying and conditioning with agricultural lime ($CaCO_3$). Lime application was at 100 g m^{-2} as described by Pillai and Boyd (1985). Pond fertilization was done 2 weeks before stocking using dry chicken manure at 50 g of dry matter m^{-2} and thereafter on a weekly basis to stimulate natural productivity of the pond following Charo-Karisa (2006). Dry feed ingredients were used to formulate seven isonitrogenous (28% crude protein) basal diet (Table 3.1). Different experimental diets were prepared by

supplementing the basal diet with dietary commercial probiotic *Saccharomyces cerevisiae* (1×10^{10} CFU g^{-1}) FURAHA ® (Agro Chemical and Food Company Limited, Kenya) at 3 concentrations of 2 g kg^{-1} (Diet 1); 4 g kg^{-1} (Diet 2) and 6 g kg^{-1} (Diet 3); and *Bacillus subtilis* (1×10^{9} CFU g^{-1}) ULTRALACT® (Gee Dee Enterprises, India) at 3 concentrations of 5 g kg^{-1} (Diet 4); 10 g kg^{-1} (Diet 5) and 15 g kg^{-1} (Diet 6) according to Abdel-Tawwab *et al*. (2008) and Hai, (2015a). The control diet (Diet 0) was not supplemented with any probiotic.

Plate 1: Arrangement of experimental cages in ponds where *O. niloticus* were cultured using probiotic-supplemented diets

Table 3.1: Ingredient composition and chemical proximate composition of the experimental diets

Ingredients (g kg^{-1})	Experimental Diets						
	Diet 0 (Control)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Fish meal	190	190	190	190	190	190	190
Wheat bran	390	390	390	390	390	390	390
Wheat pollard	160	160	160	160	160	160	160
Maize germ	120	120	120	120	120	120	120
Cotton seed cake	120	120	120	120	120	120	120
Soybean oil	20	20	20	20	20	20	20
**S. cerevisiae*	0	2	4	6	0	0	0
** *B. subtilis*	0	0	0	0	5	10	15
Chemical analysis (% of dry matter)							
Dry matter	88.9	89.2	88.3	87.9	88.4	87.6	88.7
Crude protein	29.4	29.9	30.2	29.7	29.3	29.9	29.4
Crude lipids	3.4	4.1	3.4	3.1	3.8	3.5	4.1
Crude fiber	5.7	6	6.2	4.7	5.1	5.8	6.1
Moisture	8.2	9.2	9.2	9.1	8.4	8.6	8.7
Ash	8.9	8.9	7.5	9.2	10.4	10.9	11.3

* *Saccharomyces cerevisiae*: - Diet 1 (2 g kg^{-1}); Diet 2 (4 g kg^{-1}) and Diet 3 (6 g kg^{-1}),
***Bacillus subtilis:* - Diet 4 (5 g kg^{-1}); Diet 5 (10 g kg^{-1}) and Diet 6 (15 g kg^{-1}).

The basal diets were blended with the respective proportion of probiotics, soybean oil at 20 g kg^{-1} of the feed and 100 ml of water per 1 kg diet. The diets were pelleted using an electric meat mincer (2 to 3mm die) (Plate 2), dried at room temperature, packed in plastic bags and refrigerated at 4°C to maintain microbial viability as described by Allameh *et al*. (2016) Diets were repeatedly prepared every 2 weeks during the experiment (Rengpipat *et al*., 2008). Fish were hand-fed twice daily at 3% of the total biomass at 1000 h and 1500 h for a period of 7 months. Feed adjustments were done for each cage every month after sampling. The growth experiment was conducted for a period of 7 months (from November 2016 to May 2017). Laboratory samples analysis for body composition, hemato–immunological parameters, microbiological content of fish muscle and gut microbiota was done from June 2017 to March 2018.

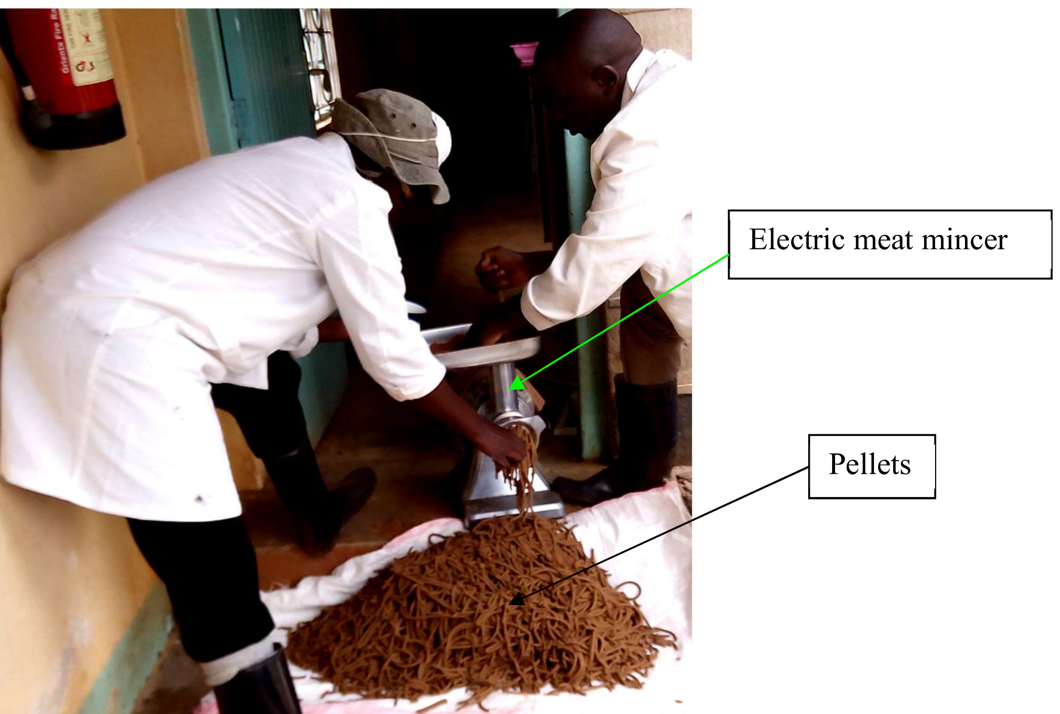

Plate 2: Feed preparation and pellet production of probiotic supplemented diets used for *O. niloticus* culture at KMFRI, Sagana

3.5 Feed analysis

All the feed ingredients were sampled for proximate analyses before feed formulation and the formulated experimental diets were analyzed to determine their nutritional composition (Table 3.1). All biochemical analyses were done on dry matter basis using standard methods of the Association of Official Analytical Chemists (AOAC, 2003). Analysis of dry matter was done by drying pre-weighed samples in an oven at 105°C for 16 hours to reach a constant weight. Nitrogen was analyzed using the Kjeldahl method. Crude protein, lipids and fiber were determined using procedures outlined by (AOAC, 2003). Ash content was determined by burning the samples in a muffle furnace at 550°C for 4 hours.

3.6 Water quality monitoring and analysis

Water quality parameters i.e. temperature, dissolved oxygen, pH, total ammonium nitrogen (NH_3-N), nitrites-nitrogen (NO_2-N) and total phosphorus (P) were determined for the duration of the experiment. Temperature (°C), dissolved oxygen (mg L^{-1}) and pH, were measured *in situ* using a multi-parameter water quality meter model number H19828 (Hanna Instruments Ltd., Chicago, USA). Readings were recorded weekly at (1000 h). Water samples from each pond were analyzed for total ammonium nitrogen (NH_3-N) (mg L^{-1}), nitrites-nitrogen (NO_2-N) (mg L^{-1}) and total phosphorus (P) (mg L^{-1}) weekly using standard methods by Boyd and Tucker, (1998). Water samples were filtered through microfiber glass filter paper (Whatman GF/C) using a vacuum pressure air pump before nutrient analysis.

3.7 Fish sampling

Fish were sampled monthly using a scoop net. Random samples of 30 fish were collected from each cage for individual weight and length measurements (Plate 3). Fish were weighed with a digital balance (0.01g) (model KERN 572-33, Germany) and total length was measured using a measuring board (0.10 cm) as described by Caspers, (1969). Fish were returned to their respective cages after measurements. At the end of the experimental period, fish were deprived of feed for 24 hours; all the fish were harvested, counted and weighed individually. Fish performances under different treatments were evaluated in terms of final total length (cm), final weight (g), daily weight gain (DWG, g day^{-1}), weight gain, specific growth rate (SGR, % day^{-1}), survival (%) and feed conversion ratio (FCR). The following standard formulae were used for the calculation;

SGR (%) = 100 ($\ln W_t$ - $\ln W_0$ / t) where: - (ln = Natural logarithm, W_0 = initial weight (g), W_t = final weight (g) and t = time in days from stocking to harvesting)……………….. (1)

FCR = feed given (g)/body weight gain (g)...(2)

Weight gain (g) = W_t - W_0..(3)

Survival (%) = (number of fish harvested/number of fish stocked) ×100………………...(4)

Logarithmic regression formula, $W = aL^b$ was used to calculate the length-weight relationship (LWR) whereas condition factor (K) was calculated by the formula, $K = 100(W/L^3)$; where W = weight (g) and L = total length (cm), a and b are the regression slope and intercept (regression coefficient), respectively, according to Froese, (2006).

Plate 3: Sampling of experimental fish from the cages (a) Fish removal from the experimental cages (b) Measuring of fish length using a measuring board and recording of data

3.8 Fish body composition analysis

At the beginning of the experiment, a pooled sample of ten fish was taken randomly from the experimental batch of fish to serve as an initial carcass composition sample. At the end of the experiment, a random sample of three fish was collected from each replicate for the final body composition analyses. The analysis of dry matter was done by drying pre-weighed samples in an oven at 105°C for 16 hours to reach a constant weight. The proximate analysis for crude protein (CP), crude lipids (CL), moisture and ash were carried out according to the standard methods by AOAC (2003).

3.9 Immunological parameters analysis

3.9.1 Blood sample collection

At the end of the seven-month trial, three fish were sampled randomly from each replicate (12 samples per treatment) for immunological analysis. The fish were anaesthetized using clove oil (20 mg L^{-1}) of water. Blood samples (1 ml from each fish) were drawn from the

caudal vein of each fish using a sterile syringe, previously rinsed with 2.7% Ethylenediaminetetraacetic acid (EDTA) solution as an anticoagulant (Plate 4). Blood samples were collected and processed according to standard methods described by Svobodová *et al*. (1991). The blood samples were used immediately for analysis of haemoglobin, red blood cells (RBC) and white blood cells (WBC). Extra blood sample (2 ml from each fish) were collected without anticoagulant and allowed to clot for 2 hours in Eppendorf tubes and centrifuged at 3000 rpm using an Eppendorf centrifuge (Centrifuge 5415 R®) for 10 minutes (Plate 5). Blood serum was collected from each centrifuged sample with a micropipette and stored at -20°C in Eppendorf tubes for analysis of serum total protein, albumin and lysozyme activity.

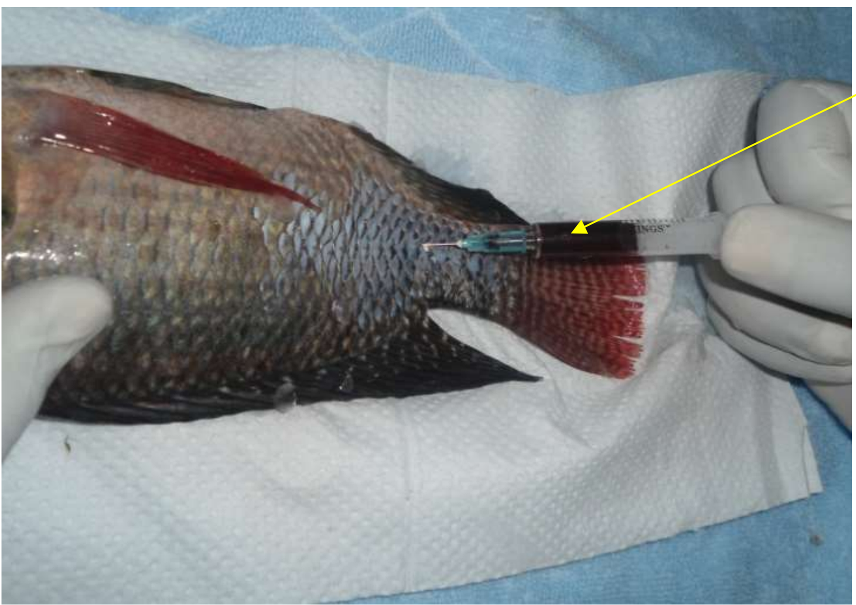

Plate 4: Blood collection from the caudal vein using a syringe

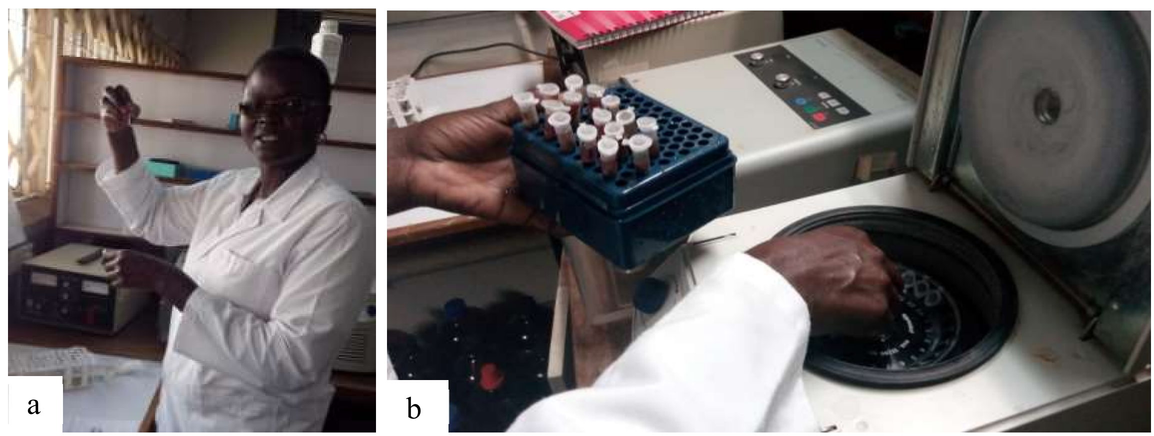

Plate 5: Preparation of samples for immunological analysis at the University of Nairobi (a) Blood sample held in an Eppendorf tube (b) Clotted blood samples being put in a centrifuge for serum separation

3.9.2 Hemato-immunological analysis

Haemoglobin (Hb) was determined by a commercial haemoglobin kit (Marienfeld®, Germany) using Sahli's/acid hematin method described by Wintrobe and Greer, (2009). Red blood cells (RBC) and white blood cells (WBC) were counted after dilution with respective diluting fluids prepared according to Svobodová *et al*. (1991). Twenty microlitres of blood were mixed with 3980 µL of diluting fluid in a clean glass vial. The mixture was shaken by hand to suspend the cells uniformly in the solution. The red blood cell (RBC: 10^6 mm^{-3}) and white blood cells (WBC: 10^4 mm^{-3}) were counted using haemocytometer.

Serum lysozyme activity was determined using commercial fish lysozyme enzyme-linked immunosorbent assay (ELISA) kit (Mybiosource®, USA) as per manufacturer's instructions. The optical densities (O.D) of the well plates were read in an ELISA plate reader (BioTekPowerwave ELx808 Microplate Reader/KC Junior software) at 450 nm (Plate 6). Serum total protein was determined by Bicinchoninic Acid (BCA) method using

commercial total protein assay kit (Mybiosource®, USA). The samples were diluted 9 times with saline water before assay. The absorbance of the standard and sample were measured against a blank in an ELISA plate reader (BioTek Powerwave ELx808® Microplate Reader/KC Junior software) at O.D of 630 nm. The absorbance readings were fitted in a normal curve and the actual O.D value of sample was derived from the logistic curve fit. Serum total protein was calculated using the following formulae provided by the assay kit manufacturer:-

Serum total protein (μg dl^{-1}) = (O.D sample) - (O.D blank)/O.D standard-O.D blank ×562 μg L^{-1}× Dilution factor of sample before testing.

Serum albumin was measured using commercial fish serum albumin ELISA kits (Mybiosource®, USA). The O.D values of the well plates were read in an ELISA plate reader at 450 nm. Serum globulin was determined by subtracting the albumin values from the total serum protein. The albumin: globulin (A/G) ratio was calculated by dividing albumin by globulin values.

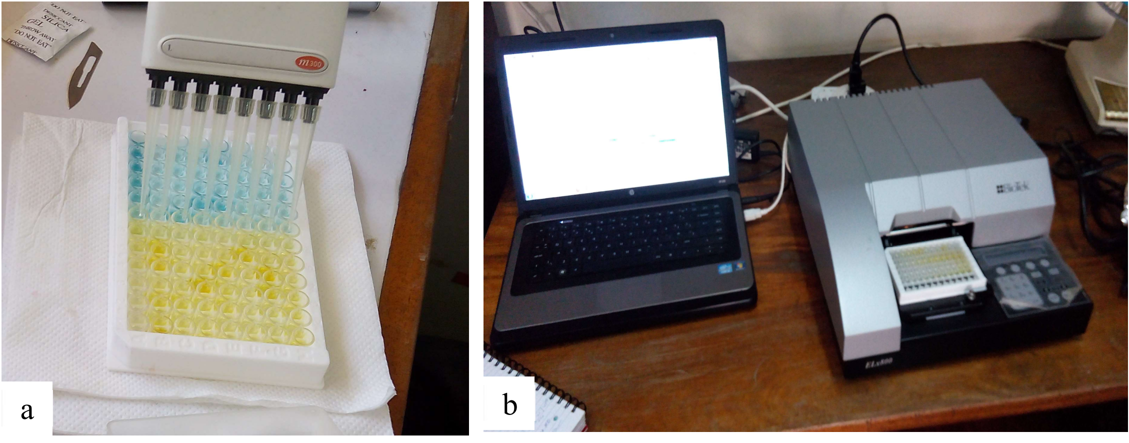

Plate 6: Serum samples analysis at the University of Nairobi (a) Preparation of serum samples in a well plate (b) Reading of processed samples in an ELISA plate reader

3.10 Fish muscle microbiological analysis and identification

Microbiological analysis of fish muscle was done according to the standard procedures for enumeration of respective group of microorganisms (MacFaddin, 1980; Murray *et al.*, 1995). All equipment, chemicals and media were sterilized in an autoclave at 121ºC (15 lb pressure) for 15 minutes before use. Three fish from each treatment were rinsed with de-ionized water and the surface of the fish sterilized using 70% ethyl alcohol. Ten grams of muscle along with skin were taken randomly from 4 parts different parts of the fish and were homogenized for 1 min with 90 ml of sterile saline (0.85% sodium chloride) solution in a stomacher-400 lab blender making 12 sub samples per treatment. The homogenate was serially diluted to 10^{-2} and 10^{-4} for bacterial and yeast analysis respectively.

Total plate count of aerobic bacteria was done by spread-plating 0.1 ml of the diluents in tryptone soy agar and incubated at 37°C for 16 hours. The colony forming units (CFUs) were counted from each plate using a colony counter. Total coliforms were estimated by membrane filtration method where 1 ml of the homogenate was aseptically filtered through a membrane filter (Whatman filter pore diameter 0.45 µm) placed on Eosin Methylene Blue (EMB) agar and incubated at 37°C for 24 hours. Typical *Escherichia coli* colonies (pink with a metallic sheen) were counted. Aliquots of the homogenate were inoculated into lactose broth in Durham tubes and incubated at 44.5°C for 24 hours to selectively isolate faecal coliforms. Sub-culturing was done from tubes with gas in the Durham tubes on MacConkey sorbitol agar and incubated at 37°C for 48 hours. Translucent to white colonies (no sorbitol fermentation) were purified for biochemical characterization.

Salmonella spp. were selectively isolated by inoculating aliquots of the homogenate in selenite enrichment broth and thereafter incubated at 37 °C for 16 hours. A loopful from each tube was sub-cultured on *Salmonella shigella* agar (SS) and deoxycholate citrate agar (DCA). Colonies that did not ferment lactose (i.e. did not have a pink colour) and/or produced hydrogen sulphide (i.e. black or black spotted) were purified for biochemical characterization. Pure culture isolates were identified based on colony morphology, gram stain and biochemical characterization according to Holt *et al*. (1994). These tests included triple sugar iron (TSI) test, sulphide production, motility, citrate utilization, urease test, methyl red and Voges Proskauer test. Total yeast cell counts were done by spread plating 0.1 ml of the homogenate on Sabouraud's agar and incubation done at 25°C for 5 days. Microorganisms were identified up to the genus level according to MacFaddin, (1980). Readings obtained with 30 to 300 colonies on plate were used to calculate bacterial and yeast populations. Colony counts were log transformed and recorded as log CFU g^{-1} of muscle (Waché *et al*., 2006).

3.11 Analysis and identification of gut microbiota

At the end of the feeding period, fish were starved for 24 hours to allow gut evacuation and a random sample of 3 fish were taken from each treatment. Fish were sacrificed by icing, dissected and longitudinally opened. The entire fish intestine was aseptically removed and homogenized in 90 ml, 0.85% sodium chloride solution in a stomacher-400 lab blender and divided into 12 sub samples per treatment. The final suspension was coarse-sieved using sterile nylon mesh (100 µm). The homogenates were serially diluted to 10^{-4} in 9 ml volumes

of sterile 0.85% saline solution. Total plate counts were done by spread plating 0.1 ml of each homogenate on tryptone soy agar (TSA) and incubation done at 37 °C for 16 hours.

Yeasts cells enumeration was done by spread-plating 0.1ml of the homogenate on Sabouraud's agar. The plates were incubated at 25°C for 5 days and yeast cells counted using a colony counter. Dominant bacterial and yeast colonies from the cultures were purified and identified based on morphological characteristics and growth parameters using biochemical tests and standard techniques for isolating *Bacillus* spp. and yeast (MacFaddin, 1980; Holt *et al*., 1994; Murray *et al*., 1995). The bacterial and yeast cell counts were expressed as log CFU g^{-1} intestine.

3.12 Data analysis

Percent survival data were arcsine-transformed before statistical analysis. Data on growth performance parameters, survival and body composition were analyzed using one-way ANOVA at $P \leq 0.05$ for significance differences among groups. Differences between means were further analyzed using Duncan Multiple Range Test (DMRT) at $P \leq 0.05$ since the parameters had equal datasets. The quantities of bacteria and yeast cells in the gut and muscle were log-transformed before analysis. One-way ANOVA test was used to test significant differences among groups at $P \leq 0.05$. Differences between means for quantities of bacteria in the gut and muscle identified were subjected to pairwise comparisons using Tukey HSD Test at $P \leq 0.05$ as a result of unequal data sets from microbiological sample analysis. All analyses were carried out with the Statistical Package and Service Solutions (SPSS version 20). All data were expressed as means ± standard error of the mean (SEM).

CHAPTER FOUR: RESULTS

4.1 Fish growth performance

Fish growth performance parameters are presented in Table 4.1. Feeding probiotic-supplemented diets led to higher final weight and SGR and weight gain. The mean final weight of the fish was between 180.96 (Diet 0) to 255.31 g (Diet 2). Fish fed the probiotic-supplemented diets had higher final weight compared to the control (Figure 4.1). Final weight, SGR and weight gain were highest in fish fed Diet 2 followed by fish fed Diet 5. FCR was more than 1.00 in all the treatments. The lowest FCR (1.61±0.02) was recorded in fish fed Diet 2 while the highest FCR (2.03±0.03) was in fish fed the control diet. Fish growth in terms of final weight was significantly affected by the levels of probiotics in the diets (F = 230.07, df = 6, $P < 0.001$). Fish fed the control diet had significantly lower growth, SGR and weight gain ($P < 0.05$) compared to other dietary groups. The mean daily weight gain ranged from 0.59 to 0.90 g day $^{-1}$ and was significantly affected with the different levels of probiotics in the diets (F = 226.47, df = 6, $P < 0.001$).

Highest weight gain was recorded in fish fed on Diet 2 followed by Diet 4. Condition factor (K) was affected by probiotic supplementation (F = 49.30, df = 6, P < 0.001). Fish fed Diet 2 and 5 having significantly higher condition factor compared to the fish fed on the control diet and Diet 6 (P < 0.05). However, there were no significant differences (P > 0.05) in condition factor between fish fed on Diet 2, 3, 4, and 5 (Table 4.1). Results of length-weight relationships (LWRs) indicated that the values of b during the culture period were not significantly different and ranged from 2.84 to 3.05. The mean R^2 values recorded

per dietary groups were: 0.95, 0.97, 0.96, 0.96, 0.97, 0.97 and 0.98 for fish fed on Diets 0,1,2,3,4,5,6 respectively; while regression slopes (b) were 2.86, 2.84, 3.02, 2.93, 2.98, 3.05 and 2.99 for fish fed on control, diet,1,2,3,4,5,6 respectively. Survival of the fish was between 77.00 and 89.50%. The different diets significantly affected the survival of fish (F = 2.14, df = 6, P = 0.045). The highest survival (89.50±0.56%) was recorded in fish fed on Diet 2 while the lowest (77.00±1.00%) was in fish fed on the control diet. Fish fed on *S. cerevisiae* supplemented diets had better growth performance and survival compared to fish fed on *B. subtilis* supplemented diets.

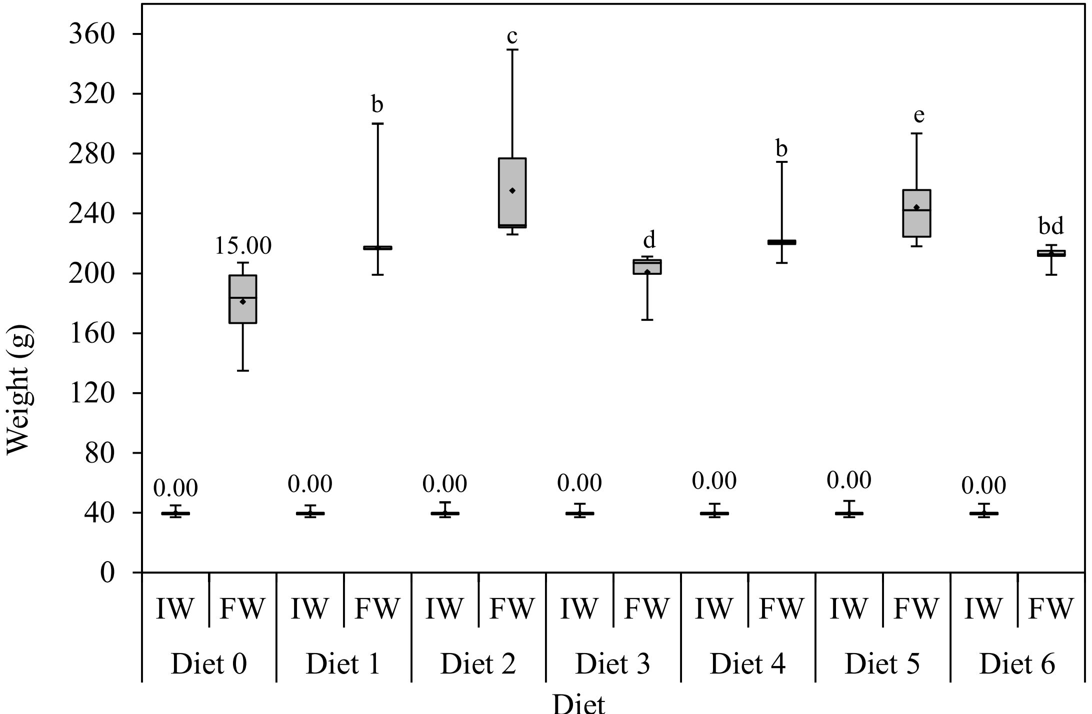

Figure 4.1: A box plot showing the initial (IW) and final weight (FW) of *O. niloticus* fed on diets supplemented with different levels of probiotics in low input ponds. The box plot indicates the lower and upper quartiles (bottom and top box lines), the median (horizontal line in the box), the minimum and maximum values (top and bottom whiskers) and the means (black circles inside the box). Different letters on top of the box denote significant differences at $P < 0.05$ (one-way ANOVA, Duncan Multiple Range Test (DMRT). For every diet, n=30.

Table 4.1: Growth performance of *O. niloticus* fed on diets supplemented with different levels of probiotic in low input ponds for 7 months

Parameter	Diet 0 (Control)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Initial length (cm)	13.24±0.08[a]	13.20±0.04[a]	13.26±0.04[a]	13.35±0.04[a]	13.26±0.04[a]	13.28±0.04[a]	13.22±0.04[a]
Initial weight (g)	39.90±0.10[a]	39.75±0.14[a]	39.99±0.15[a]	39.65±0.14[a]	39.63±0.15[a]	39.57±0.14[a]	39.90±0.15[a]
Final length (cm)	22.18±0.08[a]	23.46±0.06[a]	23.88±0.05[a]	23.10±0.05[a]	23.31±0.07[a]	23.93 ±0.06[a]	23.29±0.07[a]
Final weight (g)	180.96±1.74[a]	216.93±1.74[b]	255.31±3.19[c]	200.84±1.08[d]	220.62 ±1.59[b]	243.99±1.89[e]	212.93±1.33[bd]
SGR (% day $^{-1}$)	0.63±0.01[a]	0.71±0.01[b]	0.77±0.01[c]	0.67±0.01[d]	0.72±0.01[b]	0.76±0.01[e]	0.70 ±0.01[b]
Weight gain (g)	140.92±1.76[a]	177.18 ±0.75[b]	215.32±3.22[c]	161.29±1.08[d]	204.17±1.00[b]	173.03±0.40[e]	179.07±1.02[b]
FCR	2.03±0.03[a]	1.87±0.01a[b]	1.61±0.02[c]	1.95±0.02[d]	1.85±0.01[b]	1.67±0.02[e]	1.73±0.01[f]
Condition factor (K)	1.74±0.01[a]	1.78±0.01 [b]	1.83±0.01 [c]	1.82±0.01[c]	1.80±0.02[bc]	1.83±0.01[c]	1.75±0.02[a]
Survival (%)	77.00±1.00[a]	83.50±0.50[a]	89.50±0.56[b]	81.50±5.25[ab]	87.50±2.06[b]	88.50±1.50[b]	80.00±6.00[a]

* *Saccharomyces cerevisiae* treatments: 2 g kg^{-1} (Diet 1); 4 g kg^{-1}(Diet 2) and 6 g kg^{-1} (Diet 3)
** *Bacillus subtilis* treatments: 5 g kg^{-1} (Diet 4); 10 g kg^{-1} (Diet 5); and 15 g kg^{-1} (Diet 6)
*** Means within the same row with different superscript letters are significantly different at $P < 0.05$.

4.2 Water quality parameters

The mean values for water quality parameters during the experiment were as follows: water temperature was between 25.35 - 25.50°C; dissolved oxygen, 4.71 - 4.81 mg L^{-1}; pH , 8.00-8.09), total ammonium-nitrogen, 0.01 mg L^{-1}; nitrates-nitrogen ,0.03 mg L^{-1} and total phosphorus , 0.02 mg L^{-1} (Table 4.2). All the water quality parameters were constant during the experimental period and were not significantly affected by the different probiotic supplementation ($P > 0.05$).

Table 4.2: Water quality parameters of *O. niloticus* fed on diets supplemented with different levels of probiotics in low input ponds for 7 months

Parameter	Diet 0 (Control)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Temperature (°C)	25.35±0.17[a]	25.38±0.21[a]	25.40±0.18[a]	25.39±0.19[a]	25.42±0.09[a]	25.40±0.09[a]	25.50±0.09[a]
Dissolved oxygen (mg L^{-1})	4.78±0.08 [a]	4.77±0.09 [a]	4.76±0.08[a]	4.75±0.09 [a]	4.71±0.10[a]	4.76±0.04[a]	4.81±0.10[a]
pH	8.00±0.05[a]	8.09±0.06[a]	8.05±0.06[a]	8.08±0.05[a]	8.09±0.05[a]	8.06±0.03[a]	8.05±0.05[a]
Total ammonium-nitrogen (NH_3-N) (mg L^{-1})	0.01±0.0[a]	0.01±0.00[a]	0.01±0.01[a]	0.01±0.01[a]	0.01±0.00[a]	0.01±0.01[a]	0.01±0.01[a]
Nitrites-nitrogen (NO_2-N) (mg L^{-1})	0.03±0.02[a]	0.03±0.01[a]	0.03±0.01[a]	0.03±0.00[a]	0.03±0.00[a]	0.03±0.01[a]	0.03±0.00[a]
Total phosphorus (P) (mg L^{-1})	0.02±0.01[a]	0.02±0.00 [a]	0.02±0.00 [a]	0.02±0.10 [a]	0.02±0.00[a]	0.02±0.01[a]	0.02±0.00[a]

* Means within the same row with the same superscript letters are not significantly different at $P < 0.05$.

4.3 Fish body composition

Results for body composition of *O. niloticus* fed on diets supplemented with probiotic *S. cerevisiae* and *B. subtilis* showed that fish fed on Diet 4 had the highest muscle protein level (89.40±0.16%) while lipid level was highest in the control diet (Diet 0) (22.16±0.00%) (Table 4.3). Moisture levels were lowest in fish fed on Diet 5 (70.80±0.01%), while ash content was lowest in fish fed on Diet 4 (10.53±0.12%). The initial values for crude protein and crude lipid were lower than the levels after feeding experimental feeds, while ash content was higher before the experiment (24.33±0.33%). Different probiotic levels significantly affected the protein content of the fish ($F = 95.17$, $df = 6$, $P < 0.001$). Crude protein content significantly increased while lipid content reduced in *S. cerevisiae* treated groups compared to the control ($P < 0.05$). *B. subtilis* supplemented-diets led to significantly higher protein content than the control ($P < 0.05$).

Lipid content was significantly affected by the different probiotic levels in the diets ($F = 1005.03$, $df = 6$, $P < 0.001$) and was significantly higher in fish fed on the control diet compared to fish fed on Diet 4 ($P < 0.05$). Moisture content was significantly affected by the probiotic treatment ($F = 36.06$, $df = 6$, $P < 0.001$) and was significantly higher in fish fed on Diet 6 and lower in fish fed on the other diets. Ash content was significantly affected by the different probiotic levels ($F = 184.15$, $df = 6$, $P < 0.001$) and was significantly lower ($P < 0.05$) in fish fed on Diets 2 and 6 compared to the control group (Diet 0). Additionally, ash content was significantly higher in fish fed on Diet 0 ($P < 0.05$) and no significant difference was recorded between fish fed on Diet 2, Diet 4 and Diet 6 ($P > 0.05$).

Table 4.3: Body composition of *O. niloticus* fed on diets supplemented with different levels of probiotics in low input ponds for 7 months

Parameter (% dry matter)	Initial	Diet 0 (Control)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Protein	69.95±0.00	83.33±0.28[a]	83.73±0.36[a]	86.06±0.18[b]	85.81±0.05[b]	89.40±0.16[c]	86.63±0.09[b]	78.94±1.68[d]
Lipids	18.90±0.00	22.16±0.00[a]	12.85±0.06[b]	13.13±0.47[b]	12.54±0.21[b]	11.81±0.01[c]	12.56±0.03[b]	12.06±0.05[bc]
Moisture	74.00±0.00	74.18±0.01[a]	73.28±0.20[a]	71.20±0.14[b]	72.53±0.18[a]	71.29±0.05[b]	70.80±0.01[b]	76.55±0.86[c]
Ash	24.33±0.33	16.78±0.33[a]	14.67±0.34[b]	11.31±0.93[c]	13.22±0.30[b]	10.53 ±0.12[c]	12.47±1.65[bc]	11.52±1.53[c]

* *Saccharomyces cerevisiae* treatments: 2 g kg^{-1} (Diet 1); 4 g kg^{-1}(Diet 2) and 6 g kg^{-1} (Diet 3)
** *Bacillus subtilis* treatments: 5 g kg^{-1} (Diet 4); 10 g kg^{-1} (Diet 5); and 15 g kg^{-1} (Diet 6)
*** Means within the same row with different superscript letters are significantly different at $P < 0.05$.
**** Initial values were excluded in the comparisons between treatments. For every diet, n=12

4.4 Hemato-immunological parameters

Fish fed on diets containing probiotics had higher haemoglobin than the control group (Diet 0) (Table 4.4). However, the increase in haemoglobin was not proportional to the probiotic inclusion level, with fish fed on Diet 5 having the highest haemoglobin (7.71±0.27 g dl^{-1}). White blood cell (WBC) counts increased with increase in probiotic level but reduced at the highest level of inclusion. The highest value of WBC (60.83×10^4 mm^{-3}) was recorded in fish fed on Diet 2 with the lowest (41.40×10^4 mm^{-3}) in the control group. The haemoglobin levels were significantly different among the groups (F = 11.98, df = 6, P = 0.004) with the fish fed on Diet 2 and Diet 5 having significantly higher haemoglobin levels ($P < 0.05$). Red blood cell (RBC) counts were significantly affected by the different probiotic levels (F = 6.84, df = 6, P = 0.0021) and were significantly higher in fish fed on Diet 5 compared to the other treatments ($P < 0.05$). White blood cell counts were significantly affected by the probiotic treatments (F = 4.86, df = 6, P= 0.002) and were significantly higher in fish fed on Diet 2 and Diet 5 respectively ($P < 0.05$). However; no significant differences were recorded for haemoglobin and RBC between fish fed on Diet 0 and Diet 6 ($P > 0.05$).

Serum protein and albumin increased with increase in *S. cerevisiae and B. subtilis* levels in the diets. Serum protein was higher in fish fed on Diet 3 (5.30±0.41 μg dl^{-1}) and Diet 6 (5.22±0.27 μg dl^{-1}) for S. *cerevisiae* and B. *subtilis* based diets respectively. The serum protein levels were significantly different among the groups (F =1.85, df = 6, P = 0.048) with fish fed on Diet 6 exhibiting significantly higher serum protein ($P < 0.05$).

Table 4.4: Hemato-immunological parameters of *O. niloticus* fed on *S. cerevisiae and B. subtilis* supplemented diets in low input ponds for 7 months

Parameter	Diet 0 (Control)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Haemoglobin (g dl^{-1})	4.46 ± 0.39^{a}	6.66 ± 0.41^{b}	7.28 ± 0.39^{b}	6.76 ± 0.47^{b}	5.14 ± 0.33^{a}	7.71 ± 0.27^{b}	5.10 ± 0.19^{a}
RBC (10^6 mm^{-3})	1.67 ± 0.17^{a}	2.03 ± 0.18^{a}	2.97 ± 0.19^{ab}	2.46 ± 0.26^{a}	2.25 ± 0.20^{ac}	3.11 ± 0.17^{d}	2.69 ± 0.15^{a}
WBC (10^4 mm^{-3})	41.40 ± 2.99^{a}	51.04 ± 2.30^{b}	60.83 ± 4.23^{c}	51.58 ± 3.27^{b}	51.40 ± 1.65^{b}	56.43 ± 1.61^{bc}	50.38 ± 1.88^{b}
Total protein (µg dl^{-1})	4.11 ± 0.30^{a}	5.07 ± 0.23^{b}	5.12 ± 0.44^{b}	5.30 ± 0.41^{b}	4.85 ± 0.22^{ab}	4.95 ± 0.19^{ab}	5.22 ± 0.21^{b}
Total albumin (µg dl^{-1})	1.55 ± 0.22^{a}	1.79 ± 0.22^{b}	1.86 ± 0.45^{ab}	2.20 ± 0.28^{b}	2.15 ± 0.19^{b}	2.49 ± 0.21^{b}	2.56 ± 0.15^{b}
Globulin (µg dl^{-1})	2.34 ± 0.47^{a}	3.21 ± 0.55^{ab}	3.31 ± 0.34^{b}	3.10 ± 0.55^{b}	2.70 ± 0.31^{c}	2.74 ± 0.30^{c}	2.38 ± 0.31^{a}
Albumin globulin (A/G) ratio	2.11 ± 1.00^{a}	1.01 ± 0.37^{ab}	0.57 ± 0.17^{b}	1.26 ± 0.49^{b}	0.97 ± 0.21^{b}	1.09 ± 0.25^{b}	1.68 ± 0.54^{ab}
Lysozyme activity (U ml^{-1})	9.42 ± 1.51^{a}	11.86 ± 1.46^{b}	18.00 ± 2.11^{c}	14.51 ± 1.88^{b}	11.96 ± 0.79^{b}	17.56 ± 2.25^{c}	15.02 ± 1.51^{b}

*****Data represented as means ± SEM (n =12). Mean values in the same row having different superscript letters (a, b and c) are significantly different at $P \leq 0.05$

Albumin levels were significantly affected by the different probiotic levels (F = 1.77, df = 6, $P = 0.045$) and fish fed on Diet 6 exhibited significantly higher albumin levels compared to the control (Diet 0) ($P < 0.05$). However, no significant differences were found in serum albumin ($P > 0.05$) in fish fed on Diet 4, 5 and 6. The lowest globulin level was recorded in fish fed Diet 0 (control diet) (2.34±0.47 µg dl^{-1}). The different probiotic levels significantly affected the serum globulin levels (F =1.80, df = 6, $P = 0.035$) with highest level being recorded in fish fed Diet 2. The lowest globulin level was recorded in fish fed on the control diet (Diet 0) but was not significantly different from fish fed on Diet 6 ($P > 0.05$). Fish fed on Diet 1, 2 and 3 had significantly higher globulin levels ($P < 0.05$) compared to those fed on Diet 4, 5 and 6.

The albumin/globulin ratio (A/G) was lowest in fish fed on Diet 2 (0.57±0.07) and was highest (2.11±1.00) in fish fed the control diet (Diet 0). The different probiotic levels significantly affected the A/G and there was a significant decrease in the A/G levels in all the fish fed probiotic-supplemented diets compared to the control (F=1.86, df = 6, $P = 0.052$). Serum lysozyme activity ranged from 6.17 to 20.50 U ml^{-1} and was highest in fish fed Diet 2 (18.00±3.11 U ml^{-1}) and Diet 5 (17.56±5.46 U ml^{-1}) for the S. *cerevisiae and B. subtilis* based diets respectively. Different probiotic levels in the diets significantly affected the lysozyme activity in the fish and increased with increase in probiotic dosage (F =1.66, df = 6, $P = 0.0044$). Serum lysozyme activity was significantly lower at the highest level of each probiotic ($P < 0.05$) but no significant differences was realized in fish fed on Diet 1, 3, 4 and 6 ($P > 0.05$).

4.5 Fish muscle microbiological content

Probiotic treatments resulted in low microbial counts in the muscle of Nile tilapia (Table 4.5). High total plate counts were found in fish fed on the control diet (Diet 0) (2.27×10^{-2} log CFU g^{-1}) while the lowest counts were recorded in fish fed on Diet 2 (1.44×10^{-2} log CFU g^{-1}) (Plate 7). *Escherichia coli* counts were present in all the treatment and were higher in the control group (Diet 0) (1.99×10^{-2} log CFU g^{-1}) (Table 4.5). Probiotic treatments significantly affected the levels of total plate counts (F = 5.77, df = 6, $P < 0.0001$) and *Escherichia coli* counts (F = 16.23, df = 6, $P < 0.0001$) in the fish muscle and were significantly higher in the control group (Diet 0) compared to fish fed on probiotic treated diets ($P < 0.05$). Faecal coliform and *Salmonella* spp. were only detected in the control group (Diet 0) with mean levels of 1.14×10^{-2} and 1.01×10^{-2} log CFU g^{-1} respectively (Table 4.5). Probiotic treatment significantly affected the total yeast cells counts (F = 6.01. df = 6, $P < 0.0001$) with higher values being recorded in the control group (Diet 0) compared to other treatments. Fish fed on Diet 5 had significantly lower yeast cell counts than the control ($P < 0.05$). Plate 7 and 8 shows images of microbial cells isolates from the fish muscle during microbiological analysis.

Table 4.5: Microbial content of muscle of *O. niloticus* fed on *S. cerevisiae and B. subtilis* supplemented diets in low input ponds for 7 months

Parameter	Diet 0 (Control)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Total plate count (log CFU g^{-1}) (10^{-2})	2.27±0.16^{ac}	1.83±0.22^{bc}	1.44±0.09^{b}	1.61±0.31^{bc}	2.08 ±0.01^{bc}	1.49±0.07^{b}	2.00±0.03^{bc}
Escherichia coli (log CFU g^{-1}) (10^{-2})	1.99±0.05^{a}	1.75±0.04^{bc}	1.48±0.04^{c}	1.70±0.02^{cd}	1.82±0.04^{ac}	1.59±0.05^{c}	1.80±0.03^{ac}
Feacal coliform (log CFU g^{-1}) (10^{-2})	1.14±0.03	n.d	n.d	n.d	n.d	n.d	n.d
Salmonella spp. (log CFU g^{-1}) (10^{-2})	1.01±0.01	n.d	n.d	n.d	n.d	n.d	n.d
(Total yeast cell counts) (log CFU g^{-1}) (10^{-4})	2.10±0.04^{a}	1.73±0.02^{ab}	1.23±0.22^{b}	1.97±0.08^{ab}	1.18±0.18^{b}	1.59±0.06^{ab}	1.81±0.10^{ab}

* n.d - Not detected.
** Data represented as means ± SEM, (n =12). Mean values in the same row having different superscript letters (a, b, c and d) are significantly different at $P \leq 0.05$.

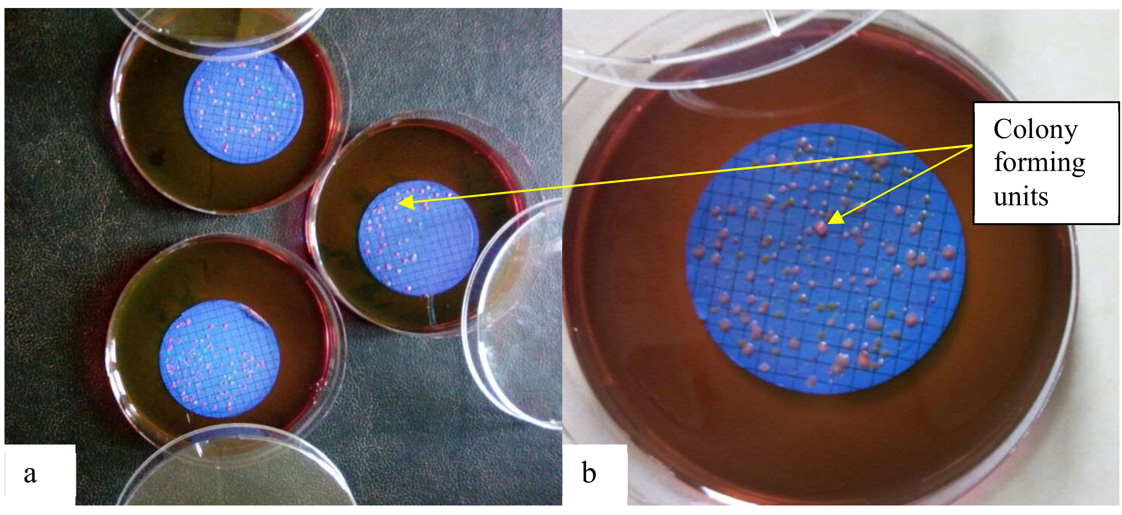

Plate 7: Microbiological content of the muscle of *O. niloticus* fed on probiotic-supplemented diets. (a) and (b) Colony forming units in membrane filters of bacterial isolates of *O. niloticus* fed on control diet

Plate 8: *Escherichia coli* colonies isolated from the muscle of *O. niloticus* fed on control diet.

4.6 Gut microbiota

The highest levels of bacterial total plate count (TPC) were recorded in the gut of fish fed on Diet 4 (1.94×10^{-4} log CFU g^{-1}) and lowest levels were in fish fed on Diet 6 (1.63×10^{-4} log CFU g^{-1}) (Table 4.6). Total plate count was significantly affected by the probiotic-supplemented diets ($F = 3.53$, df = 6, P=0.008). Levels of TPC were significantly higher in fish fed on Diet 4 followed by the fish fed on the control diet (Diet 0) and Diet 1 ($P < 0.05$). Highest yeast cell levels were recorded in fish fed Diet 3 (1.64×10^{-4} log CFU g^{-1}) while the lowest were in fish fed on the control (Diet 0) and Diet 6 (1.35×10^{-4} log CFU g^{-1}). Yeast cells counts were significantly affected by the probiotic diets ($F = 14.58$, df = 6, $P = 0.001$) and were higher in fish fed on the *S. cerevisiae* based diets (Diet 1, 2 and 3). Yeast levels were significantly lower in the gut of the control group (Diet 0) ($P < 0.05$). However, fish fed on yeast-based diets (Diet 1, 2 and 3) had a significant higher number of yeast cells counts ($P < 0.05$) compared to the control. Plate 9 and 10 shows images of yeast cells isolated from the intestine of *O. niloticus* fed on the control and probiotic-supplemented diets respectively.

Bacillus spp. counts in the gut were highest in fish fed Diet 2 (2.44×10^{-4} log CFU g^{-1}) and lowest in the fish fed the control group (Diet 0) (1.48×10^{-4} log CFU g^{-1}). Different levels of probiotics affected *Bacillus* spp. counts in the gut of fish ($F = 6.37$, df = 6, $P < 0.001$) and higher *Bacillus* spp. counts were recorded in all fish fed on probiotic-supplemented diets compared to the control ($P < 0.05$). Fish fed on Diet 2 had the highest levels of *Bacillus* spp. in the gut ($P < 0.05$) followed by fish fed on Diet 5.

Plate 9: Yeast cells isolated from the intestine of *O. niloticus* fed on the control diet.

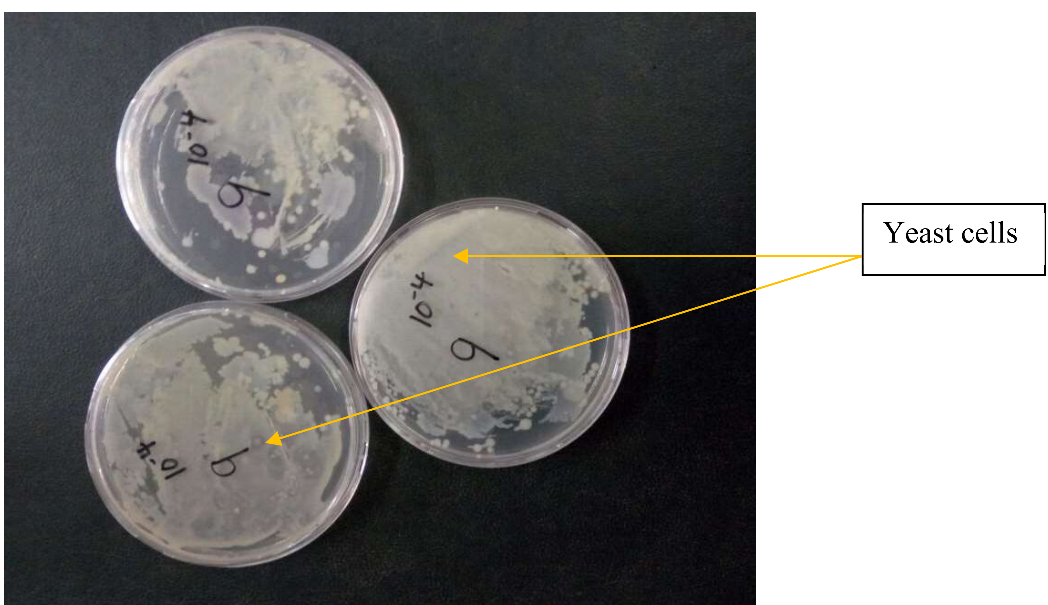

Plate 10: Yeast cells isolated from the intestine of *O. niloticus* fed on probiotic-supplemented diets.

Table 4.6: Gut microbiota of *O. niloticus* fed on *S. cerevisiae and B. subtilis* treated diets in low input ponds for 7 months

Parameter	Diet 0 (Control)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Total plate count (log CFU g^{-1}) (10^{-4})	1.85 ± 0.14^{ab}	1.87 ± 0.02^{ab}	1.70 ± 0.02^{ab}	1.73 ± 0.02^{ab}	1.94 ± 0.02^{a}	1.75 ± 0.03^{ab}	1.63 ± 0.03^{b}
Yeast cell count (log CFU g^{-1}) (10^{-4})	1.35 ± 0.02^{a}	1.63 ± 0.05^{b}	1.62 ± 0.04^{b}	1.64 ± 0.06^{b}	1.34 ± 0.02^{a}	1.36 ± 0.02^{a}	1.35 ± 0.03^{a}
Bacillus spp. (log CFU g^{-1}(10^{-4})	1.48 ± 0.03^{a}	2.01 ± 0.06^{b}	2.44 ± 0.17^{c}	2.01 ± 0.07^{b}	2.05 ± 0.12^{b}	2.30 ± 0.05^{b}	2.03 ± 0.10^{b}

**Data represented as means ± SEM (n =12). Mean values in the same row having different superscript letters (a, b and c) are significantly different at $P \leq 0.05$.

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Growth performance and feed utilization

Diet supplementation with probiotics (*S. cerevisiae* and *B. subtilis*) in the current study resulted to better growth and feed utilization but indicated a non-linear relationship between the level of probiotic supplementation and the growth performance of fish. The highest growth performance exhibited in fish fed on probiotic-supplemented diets in the present study could be attributed to improved nutrient digestibility and availability to the fish. According to Merrifield, *et al*. (2010a) and Welker and Lim (2011), probiotics have been reported to improve digestion of feed by producing digestive enzymes or alterations of the gut environment, translating to better growth. Several studies have demonstrated that *S. cerevisiae* and *B. subtilis* affect growth of several fish species including Nile tilapia (Abdel-Tawwab *et al*., 2008; Hassaan *et al*., 2018), rainbow trout (*Oncorhynchus mykiss*) (Adel *et al*., 2017) and common carp (*Cyprinus carpio*) (Yanbo and Zirong, 2006).

Enhanced growth observed in fish fed on *S. cerevisiae* supplemented diets at 4 g kg^{-1} compares well with the results of Diab *et al*. (2006) who recorded high average body weight of Nile tilapia fed on diets containing dried yeast at 1% to 5%. Hassaan *et al*. (2014) also reported increased final weight and improved FCR of *O. niloticus* with increasing yeast inclusion level from 0% to 0.5% or 1.0%. Better FCR exhibited in fish fed on probiotic-supplemented diets could be attributed to enhanced levels of gastrointestinal bacteria involved in the decomposition of nutrients thereby providing additional enzymes, vitamins

and amino acids to the fish (Ringø *et al*., 2016). Lara-Flores *et al*. (2003) reported that live yeast supplementation in diets of Nile tilapia improved feed and protein digestibility which could explain the improved feed utilization and efficiency recorded in the present study. The higher growth reported in fish fed on diets supplemented with *B. subtilis* is in agreement with El-Haroun *et al*. (2006) who realized an increase in the daily growth rate by 33% and lower FCR by 43% in *O. niloticus* fed on Biogen® (a commercial product that contains *B. subtilis*).

The non-linear relationship between the level of *S. cerevisiae* and *B. subtilis* supplementation and growth performance of the fish in this study is in agreement with the results of Goda *et al*. (2012) who recorded reduced growth in fish fed on high levels of baker's yeast. Rainbow trout (*O. mykiss*) had significantly lower growth when fed on commercial probiotic containing *S. cerevisiae* and *S. elipsoedas* at 1.5% and above (Adel *et al*., 2017). Additionally, Bagheri *et al*. (2008) established that higher levels of a combination of *B. subtilis* and *B. licheniformis* led to lower growth in rainbow trout fry. Reduced growth at higher levels of probiotic supplementation could be an indicator of depressed nutrient utilization attributed to variations in experimental conditions and duration of probiotic administration. This suggests that higher probiotic levels do not necessarily result in improved growth and fish fed intermediary levels of probiotics may have acquired better health conditions compared to the other treatments hence an increase in growth.

The observed higher growth rates could be as a result of the enhanced presence of beneficial gut microbes (from the probiotics) which leads to improvement of feed digestibility and nutrient utilization since gut microbes are known to produce additional amino acids that are beneficial to the fish especially when there is nutrient deficiency in the fish diet (Nayak, 2010b; Newsome *et al*., 2011; Welker and Lim, 2011; Zhang *et al*., 2015).

The length weight relationship (LWR) during the culture period indicates that fish fed on Diet 2 and Diet 5 had isometric growth which is the ideal growth recommended by Froese, (2006). The improved survival of the fish fed on probiotic-supplemented diets could be an indication of better health conditions. This concurs with the findings of Welker and Lim (2011) and Hai, (2015a) who established that *S. cerevisiae* and *B. subtilis* contains peptide antibiotics, including subtilin and bacitracin which improves immunity hence higher survival. The water quality parameters were constant during the experimental period and were within the recommended levels for tilapia culture (Boyd and Tucker, 1998).

5.1.2 Fish body composition

Fish fed on probiotic-supplemented diets had significantly higher protein content and lower lipid content compared to the control. The increase in protein content could have resulted from increased nutrient deposition. This is in agreement with El-Haroun *et al*. (2006) and Bagheri *et al*. (2008) who reported an increase in the level of protein and reduction in crude lipid content in Nile tilapia and *O. mykiss* fed probiotic-supplemented diets. According to

Abdel-Tawwab *et al*. (2008), *S. cerevisiae* plays a key role in enhancing food intake resulting in improvement of fish body composition.

The higher carcass protein content in this study could be attributed to secretion of more proteins by the probiotics in the gut of Nile tilapia and effective conversion of ingested food into structural protein building more muscle (Rosovitz *et al*., 1998; Lara-Flores and Olvera-Novoa, 2013). On the contrary, other studies have documented that probiotic treatments have no significant effect on protein, lipid or ash content (Merrifield *et al*., 2010b; Hassaan *et al*., 2018). Crude lipids were lower in fish fed on probiotic-supplemented diets compared to the control. This represents fish with more protein and less fat which is desirable in aquaculture (Azarin *et al*., 2015; Hassaan *et al*., 2018). The changes in protein and lipid levels in fish body could be attributed to the changes in their synthesis, deposition rate in muscle and different growth rates (Abdel-Tawwab *et al*., 2006).

5.1.3 Hemato-immunological parameters

Hematological parameters are indicators for fish well-being, health, physiological responses, nutritional status and environmental conditions (Ranzani-Paiva *et al*., 2005; Abdel-Tawwab *et al*., 2008; Iwashita *et al*., 2015). Blood samples from fish fed on probiotic-supplemented diets contained a significantly higher number of haemoglobin, red blood cells (RBC) and white blood cells (WBC) compared to the control group. Particularly, fish fed on Diet 2 and Diet 5 exhibited higher levels of haemoglobin compared to other treatments. Similarly, increase in levels of haemoglobin have been reported in Nile

tilapia fed on *S. cerevisiae* and *Bacillus* spp. treated diets (Abu-Elala *et al*., 2013; Selim and Reda, 2015; Addo *et al*., 2017a; Liu *et al*., 2017; Elsabagh *et al*., 2018).

Red and white blood cells are essential components in both innate and adaptive immune response and a higher abundance indicates a stronger immune system (Standen *et al*., 2013). Levels of RBC and WBC were significantly higher in fish fed on Diet 2 and Diet 5. Likewise, higher levels of RBC and WBC were observed in Nile tilapia fed on *S. cerevisiae* treated diets at a dosage between 1 to 6 g kg^{-1} indicating that, increase in probiotic supplementation may reflect improved immunity of fish (Osman *et al*., 2010). On the contrary, Ali *et al*. (2018) found no remarkable differences in hematological parameters of Nile tilapia fed on commercial probiotic, Biogen (containing *B. subtilis*) and attributed it to the differences in the composition of Biogen and the dosage levels. Therefore, improvement in hematological parameters in the fish fed probiotic treated diets in the current study indicates the role of single species probiotics in stimulating immune responses of fish under stressful conditions, thereby reducing the deleterious effects caused by biological, chemical and physiological stress in the culture system (Nayak, 2010a; Mohapatra *et al*., 2012a; Mohapatra *et al*., 2014).

In the current study, an increase in serum protein and serum albumin with increased level of probiotic inclusion was observed. A similar trend was observed in *O. niloticus* fingerlings fed on baker's yeast up to 1 g kg^{-1} (Abdel-Tawwab *et al*., 2008) and in adult *O. niloticus* fed on baker's yeast up to 6 g kg^{-1} (Osman *et al*., 2010). According to Wiegertjes *et al*. (1996), high level of serum protein and serum albumin are associated with strong

innate immune response in fish. Moreover, a study carried out on *Labeo rohita* fed on a mixture of probiotics (*B. subtilis, Lactococcus lactis* and *S. cerevisiae*), resulted in an increase in the level of serum protein, albumin and globulin with a reduction in A/G ratio (Mohapatra *et al*., 2012b).

This study realized a significant increase in globulin accompanied by a significant decrease in Albumin/Globulin (A/G) ratio in fish fed on Diet 2 with the control having the highest A/G ratio. This is an indication that probiotic administration promoted the immune system of Nile tilapia. Similarly, increase in globulin levels have been reported in Nile tilapia fed on *Bacillus* spp. based probiotics (Zhou *et al*., 2010; Elsabagh *et al*., 2018) and a reduction of A/G ratio have been demonstrated in rainbow trout (*Oncorhynchus mykiss*) fed on multi-strain probiotic bacteria (*Bacillus* spp., *Pediococcus* spp., *Enterococcus* spp., *Lactobacillus* spp.) (Ozório *et al*., 2016). Consequently, increase in the total serum globulin and the decrease in the A/G ratio realized in this study could be attributed to a high level of specific immunoglobulin (antibody) in the blood of the fish hence enhanced protective mechanisms for fish (Kumar *et al*., 2006).

Serum lysozyme was higher in fish fed on probiotic-supplemented diets than the control. This is an indicator of the ability of the probiotic to kill pathogenic bacteria by breaking down the cell wall of both gram-positive and gram-negative bacteria as suggested by earlier studies (Paulsen *et al*., 2001; Uribe *et al*., 2011; Ridha and Azad, 2016). *Saccharomyces* spp. have been found to trigger increase in serum lysozyme activity in *O. niloticus* and other teleosts (Abdel-Tawwab *et al*., 2008; Abu-Elala *et al*., 2013). Similarly,

higher serum lysozyme activity has been reported in Nile tilapia (Shelby *et al*., 2006; Ferguson *et al*., 2010; Addo *et al*., 2017b), rainbow trout (*O. mykiss*) (Merrifield *et al*., 2010b; Ozório *et al*., 2016), brown trout (*Salmo trutta*) (Balcázar *et al*., 2007) and common carp (*Cyprinus carpio*) (Wang *et al*., 2010), fed on various probiotics.

High doses of probiotics, *Saccharomyces cerevisiae* (6 g kg^{-1}) and *Bacillus subtilis* (15 g kg^{-1}) resulted into low lysozyme activity in the current study. Similar results were reported for rainbow trout fed on diets supplemented with a commercial probiotic (*B. subtilis* + *B. cereus toyoi*) at 0.03 to 0.06% of the diet (Ramos *et al*., 2017). Low lysozyme activity has been associated with immunosupression after long-term exposure to immunostimulants (He *et al*., 2009). However, the contradictory effect of probiotics on the immune response of fish could also be related to differences in microbial concentration, viability, type of probiotic used and duration of treatment (Merrifield *et al*., 2010b; Ringø *et al*., 2016).

5.1.4 Fish muscle microbiological content

Microorganisms in fish culture system are either saprophytic or pathogenic and enter the fish through the body and intestine surfaces before causing infections and are often associated with post - harvest quality of the fish (Al-Harbi and Uddin, 2003; Ringø *et al*., 2016; Li *et al*., 2019). Fish fed on probiotic-supplemented diets had lower microbial load than the control in the current study. *E. coli* were significantly higher in the control compared to other treatments while faecal coliform and *Salmonella* spp. were only detected in the control. Total yeast cells counts were detected in all treatments but were significantly lower in fish fed on Diet 2 and 5 respectively. This is associated with more mucus

secretions which have been reported to acts as a barrier to pathogenic bacteria entering the body of fish fed probiotic-supplemented diets (Al-Harbi and Uddin, 2003).

Feeding fish on probiotic-supplemented diets has been reported to lead to sufficient mucus secretion by the fish inhibiting transfer of microorganisms from the environment to the flesh (Uribe *et al*., 2011). The mucus layer in fish skin contains lysozymes, pentraxins, lectins, complement proteins, antibacterial peptides and immunoglobulin (IgM, IgT/IgZ), which have an important role in inhibiting the entry of pathogens to the fish (Uribe *et al*., 2011; Lazado and Caipang, 2014; Rodiles *et al*., 2018). It's worth noting that the microbiological content in the muscle of fish realized in this study are lower than the limits of safety acceptance recommended for microbiological limits for fish (ICMSF, 1986).

5.1.5 Gut microbiota

In the this study, an increase in yeast cells in the gut of fish fed on Diet 1, 2 and 3 and an increase in *Bacillus* spp. counts in the gut of the fish fed on Diet 4, 5 and 6 was realized. This indicates that the respective probiotic in the diet led to an increase of the respective bacteria in the gut of the host. Additionally, fish fed on probiotic-supplemented diets had less pathogenic bacteria load in their gut suggesting enhanced immunity. According to Ringø *et al*. (2016), the increase in beneficial microbes in the gut of fish is an indication of the positive role probiotics play in improving the intestinal microbial balance of the fish by replacing harmful bacteria with beneficial bacteria.

Gut microbiota often plays an important role in preventing pathogens from colonizing the gut, and maintaining health, but this depends on the type of probiotic used (Gómez and Balcázar, 2008; Ringø *et al*., 2016). Results of the present study confirm earlier studies that demonstrated the antagonistic effect of *Bacillus* spp. against pathogenic bacteria by competing for the same nutrients and adhesion sites resulting in stimulation of the immune system and improvement of the intestinal microbial balance (Balcázar *et al*., 2006; Merrifield *et al*., 2010b; Ringø *et al*., 2016).

Presence of yeast cells in the gut of experimental fish in the current study concurs with the results of He *et al*. (2009), who observed growth stimulation of a variety of beneficial bacteria and yeasts in the gut of hybrid tilapia (*O. niloticus* ♀ × *O. aureus* ♂) fed on commercial *S. cerevisiae* product (DVAQUA®). Probiotic adhesion in the gut of fish is very important in improving intestinal microbial balance and modulation of non-specific immunity (Gómez and Balcázar, 2008; Nayak, 2010b). The presence of the administered probiotics in the gut of the fish in the current study is an indicator that the ingested probiotics remained viable in the gut of fish during the growth period and were able to survive the digestion process. This shows that the two probiotics were beneficial to the host (Li *et al*., 2019; Sayes *et al*., 2018). The adhesion of the probiotic in the gut can be related to improved internal environmental conditions for beneficial microbial growth and a suitable environment that inhibits the growth of harmful microbial cells in the intestine of the host (Bagheri *et al.*, 2008; Gómez and Balcázar, 2008). Furthermore, past studies have established that probiotics have strong adhesion to fish intestinal mucus and outcompetes

pathogenic microorganisms for available receptor sites for attachment in fish gut (Navarrete and Tovar-Ramrez, 2014; Ringø *et al*., 2016; Li *et al*., 2019).

5.2 Conclusions

i. This study has demonstrated that feed supplementation with baker's yeast (*Saccharomyces cerevisiae*) at 4 g kg^{-1} and *Bacillus subtilis* at 10 g kg^{-1} led to improved growth performance of *O. niloticus* in low input ponds indicated by higher final weight, weight gain, SGR and FCR.

ii. Continuous administration of dietary *S. cerevisiae* at 4 g kg^{-1} and *Bacillus subtilis* at 10 g kg^{-1} increased the hemato-immunological parameters hence the improved innate immune condition of the fish.

iii. *S. cerevisiae* led to higher immunity compared to *B. subtilis* demonstrating that it's a more effective probiotic compared to bacteria.

iv. Administration of dietary *S. cerevisiae* and *Bacillus subtilis* lowered fish muscle contamination with pathogenic bacteria and modulated the gut microbiota of *O. niloticus*.

5.3 Recommendations from study

i. Feed manufacturers/farmers should include baker's yeast (*S. cerevisiae*) and *B. subtilis* to supplementary feeds used by majority of farmers in low input ponds to promote growth and enhance immunity of Nile tilapia.

ii. Animal manure should include nutrient and microbial load analysis of the manure and treatment before application to low input ponds.

iii. Regulations and standards on probiotics usage in aquaculture should be developed to enhance probiotic usage and reduce possibility of antimicrobial resistance in culture fish.

5.4 Areas for future research

i. Challenge trials using the recommended probiotic levels should be conducted for fish cultured in stressful conditions to establish their resistance to environmental stress.

ii. Further studies should focus on morphometric assessment of the intestinal villi of fish fed on probiotics to evaluate the effect of probiotics on gut morphology of fish cultured in low input ponds.

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APPENDICES

Appendix I: Additional pictures

Dietary probiotics used in the experiments (a) Baker's yeast (*Saccharomyces cerevisiae*); (b) *Bacillus subtilis*

Weighing of experimental fish during sampling at KMFRI Sagana

Length measurements of experimental fish during sampling at KMFRI Sagana

Appendix II: Proposal approval letter

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke

Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 810901 Ext. 57530

Internal Memo

FROM: Dean, Graduate School

DATE: 19th October, 2016

TO: Ms. Opiyo Mary
C/o Department of Zoological Sciences
Kenyatta University

REF: I84/27364/14

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board at its meeting of 7th October, 2016 approved your Research Proposal for the Ph.D. Degree, entitled **"Effects of Probiotics on Growth Performance, Immunity and Flesh Quality of Pond Cultured Nile Tilapia (*Oreochromis niloticus L.*) in Kenya".**

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology & Innovation

As you embark on your data collection, please note that you will be required to submit to Graduate School completed supervision Tracking Forms per semester. The form has been developed to replace the progress Report Forms. The Supervision Tracking Forms are available at the University's Website under Graduate School webpage downloads.

By copy of this letter, the registrar (Academic) is hereby requested to grant you Substantive registration for your Ph.D. studies.

Thank you.

REUBEN MURIUKI
FOR: DEAN, GRADUATE SCHOOL

c.c. Chairman, Department of Zoological Sciences

Registrar Academic – Att. J. Likam

RM/cao

Appendix III: Research permit

THIS IS TO CERTIFY THAT:
MISS. MARY ADHIAMBO OPIYO
of KENYATTA UNIVERSITY, 0-10230
SAGANA,has been permitted to conduct
research in *Kirinyaga County*

Permit No : NACOSTI/P/17/24101/16412
Date Of Issue : 13th April,2017
Fee Recieved :Ksh 2000

on the topic: *EFFECTS OF PROBIOTICS ON GROWTH PERFORMANCE, IMMUNITY AND FLESH QUALITY OF POND CULTURED NILE TILAPIA (OREOCHROMIS NILOTICUS L.) IN KENYA*

for the period ending:
13th April,2018

Applicant's Signature

Director General
National Commission for Science, Technology & Innovation

CONDITIONS

1. You must report to the County Commissioner and the County Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit.
2. Government Officer will not be interviewed without prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two(2) hard copies and one (1) soft copy of your final report.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice

REPUBLIC OF KENYA

National Commission for Science, Technology and Innovation

RESEACH CLEARANCE PERMIT

Serial No.A 13736

CONDITIONS: see back page

Appendix IV: Research authorization

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone: +254-20-2213471, 2241349,3310571,2219420
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when replying please quote

9th Floor, Utalii House
Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref. No. **NACOSTI/P/17/24101/16412**

Date: **13th April, 2017**

Mary Adhiambo Opiyo
Kenyatta University
P.O. Box 43844-00100
NAIROBI.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Effects of probiotics on growth performance, immunity and flesh quality of pond cultured Nile Tilapia (Oreochromis Niloticus L.) in Kenya,"*** I am pleased to inform you that you have been authorized to undertake research in **Kirinyaga County** for the period ending **13th April, 2018.**

You are advised to report to **the County Commissioner and the County Director of Education, Kirinyaga County** before embarking on the research project.

On completion of the research, you are expected to submit **two hard copies and one soft copy in pdf** of the research report/thesis to our office.

BONIFACE WANYAMA
FOR: DIRECTOR-GENERAL/CEO

Copy to:

The County Commissioner
Kirinyaga County.

The County Director of Education
Kirinyaga County.

National Commission for Science, Technology and Innovation is ISO 9001: 2008 Certified