EFFECTS OF PROBIOTIC SUPPLEMEN TATION ON GROWTH AND HEALTH PROMOTION OF THE NILE TILAPIA, *Oreochromis niloticus* (Linnaeus, 1758)

M.Sc. THESIS

By

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HAWASSA UNIVERSITY, HAWASSA, ETHIOPIA

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ADVISORS' APPROVAL SHEET

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We, the undersigned, Members of the Boards of Examiners of the final Open Defense have read and evaluated his thesis entitled "**Effects of probiotic supplementation on growth and health promotion of the Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758)**" and examined the candidate. This is therefore, to certify that the thesis has been accepted in partial fulfillment of the requirements for the Degree of Master of Science in Aquaculture and Fishery Management.

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I hereby declare that this MSc thesis entitled "**Effects of Probiotic Supplementation on Growth and Health Promotion of the Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758)**" is my original work and it has not been presented for a degree in any other university, and all sources of material used for this thesis have been properly acknowledged.

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DEDICATION

I dedicate this thesis to my father Mr. Koysha Oyato, who was largely responsible for my current state of being. Additionally, I dedicate it to my beloved brother Mr. Sintayehu Koysha and my sister Ms. Lemlem Koysha, who have been a guiding force throughout my studies and research.

BIOGRAPHICAL SKETCH

Kassahun Koysha Oyato was born on September 1998 GC, at Damot Gale Wereda, Wolaita Zone, and South Ethiopia Regional State, Ethiopia. He finished his Junior and Secondary School education at Edget Primary School and Boditi Secondary and Preparatory schools respectively. He got his first degree in Applied Biology from the Wolaita Sodo University in February 2021 GC. After his successful completion of his first degree education, he joined Wolaita Sodo University Biology Department and worked as Graduate Assistant for 7 months (February 2021 to August 2021). He then joined as M.Sc. student at the College of Natural and Computational Sciences, Hawassa University in September 2021 with specialization in Aquaculture and Fisheries Management. He has now completed his work and ready to defend it.

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LIST OF ABBREVIATIONS

ANU	Apparent nitrogen utilization
BHI	Brain heart infusion broth
BWG	Body weight gain
CARE	Center for aquaculture research and education
CFU	Colony forming unit
CP	Crude protein
CRD	Complete randomized design
DO	Dissolved oxygen (of the water)
DWG	Daily weight gain
EDTA	Ethylene-di-amine-tetra-acetic acid
EMB	Eosin methylene blue agar
FBW	Final body weight
FCR	Feed conversion ratio
Hb	Hemoglobin
IBL	Initial body length
IBW	Initial body weight
LABs	Lactic acid bacteria
Ln	natural logarithm

lnW_f	Lan (natural logarithm) of final weight
lnW_i	Lan (natural logarithm) of initial weight
MFU	McFarland unit
MLG	Mean length gain
PCV	Packed cell volume
PER	Protein efficiency ratio
ROS	Reactive oxygen species
SAS	Statistical analysis software
SEM	Standard error of the mean
SGR	Specific growth rate
SR	Survival rate
TSI	Triple sugar iron agar
XLD	Xylose lysine deoxycholate agar

ABSTRACT

Probiotics have emerged as promising feed additives in aquaculture, offering potential benefits for fish growth and health promotion. This study was intended to evaluate the efficacy of three probiotic strains, *Bacillus subtilis subtilis* (p1), *Bacillus subtilis inaquosorum* (p2), and *Saccharomyces cerevisiae* (p3), on the growth and health of *Oreochromis niloticus* fingerlings. A total of 300 fingerlings with an average weight of 7.7 ± 0.103g and length of 7.46 ± 0.073cm were randomly divided in to five experimental groups: control (p0), p1, p2, p3, and a combination of all three probiotics (p4). The fish were fed a diet containing 30% crude protein (CP) at 5% of their body weight either with a basal feed (control group, p0) or with probiotic-supplemented feed (p1, p2, p3, and p4 groups at a dosage of $1.5*10^8$ CFU/mL) for 90 days. Water quality parameters, including dissolved oxygen (DO), pH, and temperature, were measured every 15 days, three times a day. Growth performance parameters, including body weight gain (BWG), specific growth rate (SGR), daily weight gain (DWG), feed conversion ratio (FCR), protein efficiency ratio (PER), and survival rate (SR), were assessed at the end of the experimental period. Additionally, gut microbiota analysis, hematological parameters, and proximate composition of fish were evaluated. The results indicated that water quality parameters remained stable throughout the experiment, except for DO, which showed significant differences ($P < 0.05$) among the groups. The probiotic-supplemented groups (p1, p2, p3, and p4) exhibited significantly higher growth performance parameters compared to the control group (p0). The group fed with *Bacillus subtilis inaquosorum* (p2) demonstrated the most remarkable growth performance, with the highest BWG (128.4 ± 0.146 g), SGR (2.66 ± 0.015% day^{-1}), DWG (1.43 ± 0.001 g day^{-1}), and PER (4.28 ± 0.004), and the lowest FCR (1.04 ± 0.003) and SR (100 ± 1.291%). Additionally, the probiotic diet effectively eliminated pathogenic microflora, including *Salmonella* spp., *Escherichia coli*, and *Candida albicans*, from the gut of *O. niloticus* compared to the control group. Hematological examination revealed significantly improved packed cell volume, RBC, hemoglobin, WBC, and differential leukocyte counts in the probiotic-fed groups. Proximate composition revealed better Crude protein, Dry matter and Carbohydrate content in probiotic diet and decreased Moisture, Crude fat, Ash and Crude fiber. In conclusion application of probiotics in aquaculture is crucial to improve growth and health of *O. niloticus* as well as to improve water quality.

Key words: Aquaculture, *Bacillus subtilis*, Gut Micro-flora, Hematology, *O. niloticus*, Probiotics

1. INTRODUCTION

1.1. Background of the Study

Aquaculture, or fish farming, is the fastest growing food production sector in the world. It provides high quality animal protein that supports the nutritional and food security needs of human populations (Subedi and Shrestha, 2020). However the demand for fish exceeds the supply from natural habitats, which are limited and degrading due to human activities (Hines *et al*., 2022). Therefore, there is a need to enhance fish farming practices to meet the demand. The Nile tilapia (*O. niloticus* L.) is the most commonly cultured fish after carps, and its culture is being practiced throughout the world. It is the most prominent species used in fish production in Ethiopia (Kassahun Mereke, 2015).

The ultimate goal of aquaculture is to increase production rates to maximize profitability. The cost of production is likely to be reduced if growth performance and feed efficiency are increased in commercial aquaculture. However, in the large-scale production system, fish may be exposed to various diseases especially if they are stressed by lack of quality feeds, deteriorated water quality and exposed to stressful conditions resulting in severe economic losses (Abdel-Latif and Khafaga, 2020). One of the key factors that affect the success of aquaculture is the quality of the feed. Feed is the major input and cost in fish farming, and it influences the growth, health, and profitability of the fish (Munguti *et al.*, 2012). To improve the feed efficiency and quality, various feed additives can be used, such as vitamins, minerals, enzymes, Phytobiotic or phytogenic compounds, prebiotics, and probiotics (Gupta and Banerjee, 2016).

Probiotics are live microorganisms that confer beneficial effects on the host when administered in adequate amounts (Hoseinifar *et al*., 2016). They can improve the water quality, growth performance, feed utilization, health status, and body composition of fish by modifying the gut microflora, enhancing the immune response, and stimulating the digestive enzymes (Hoseinifar *et al*., 2016). Probiotics can also reduce the environmental impact of aquaculture by reducing the organic waste and nutrient loading in the water (Hui *et al*. 2019). They are beneficial for the hosts and are able to persist in the digestive tract because of their tolerance to acid and bile salts (Martinez-Cruz et al., 2012).

Probiotic feeds are gaining widespread adoption in aquaculture due to their safety, pharmaceutical-free nature, environmental friendliness, and effectiveness in controlling aquaculture diseases. They are gaining traction in aquaculture due to their safety, pharmaceutical-free nature, eco-friendliness, and ability to control aquaculture diseases (Balcazar *et al*., 2006). When probiotics are incorporated into the diet, can induce beneficial alterations in the gut morphology, composition, and activity of the intestinal microbiota, leading to enhanced nutrient absorption and digestion, as well as improved immune function (Dawood and Koshio, 2016). They also benefit farmed aquatic organisms that cannot utilize some of these essential nutrients from their diets, through converting them into usable end products like short-chain fatty acids. Therefore, modulating the intestinal microbiota of aquatic organisms positively is crucial, and probiotics offer a promising approach to achieving this goal (Dawood and Koshio, 2016).

Ethiopia is one of the developing countries where food security is not ensured yet and there is a need to expand aquaculture, particularly in rural areas where there is a high shortage of

proteinaceous foods. The country has a huge potential for aquaculture in the form of vast water resources, agro-ecology, enriched land along with labor and presence of suitable fish species (Ayana Chimdo, 2022). It is in this respect, the factors that limit aquaculture production have to be identified and suitable measures have to be taken to solve such challenges. Probiotics have been widely used in controlling infectious diseases thereby increasing the production of fish under culture practice (Hoseinifar *et al*., 2016). Despite the potential benefits of probiotics, their use in aquaculture is still limited and not well understood. Especially in Ethiopia, where aquaculture is an emerging sector, there is a lack of research and knowledge on the effects of probiotics on fish farming. Therefore, the aim of this thesis is to explore the effects of probiotic supplementation on the growth and health of Nile tilapia, *O. niloticus*, which is the most widely cultured fish species in Ethiopia.

1.2. Statement of the Problem

Aquaculture, or fish farming, is the fastest growing food production sector in the world, contributing to more than half of the global fish supply (FAO, 2018). Aquaculture provides high quality animal protein that supports the nutritional and food security needs of human populations, especially in developing countries. Aquaculture also generates income and employment for millions of people, especially smallholder farmers and women (Odende *et al*., 2022). However, aquaculture faces many challenges, such as environmental degradation, disease outbreaks, low productivity, and market constraints. These challenges limit the potential of aquaculture to meet the increasing demand for fish and to contribute to sustainable development (Hines *et al*., 2022).

One of the major problems affecting aquaculture production and fish health is the occurrence of diseases caused by various pathogens, such as bacteria, viruses, fungi, and parasites. Disease can cause high mortality, reduced growth, and poor quality of fish, resulting in economic losses and food insecurity for the farmers and consumers. To prevent and control diseases, antibiotics are widely used in aquaculture. However, the use of antibiotics has negative impacts on the environment, human health, and fish welfare. Antibiotics can pollute the water, create antibiotic resistance, and affect the gut microflora of fish. Moreover, antibiotics are often expensive, inaccessible, or ineffective for the farmers (Subedi and Shrestha, 2020). Therefore, there is a need for alternative and sustainable methods to improve fish health and aquaculture production. One of the promising alternatives to antibiotics is the use of probiotics.

Probiotics are live microorganisms that confer beneficial effects on the host when administered in adequate amounts. Probiotics can improve the water quality, growth performance, feed utilization, health status, and body composition of fish by modifying the gut microflora, enhancing the immune response, and stimulating the digestive enzymes. Probiotics can also reduce the environmental impact of aquaculture by reducing the organic waste and nutrient loading in the water (Kassahun Mereke, 2015). The use of probiotics in aquaculture can potentially increase the productivity, profitability, and sustainability of fish farming, as well as the food security, nutrition, and income of the farmers and consumers. The aim of this study is to evaluate the effects of probiotics on the growth and health of Nile tilapia, *Oreochromis niloticus*, which is the most widely cultured fish species in Ethiopia.

1.3. Objectives of the study

General Objective: To evaluate the effects of probiotic supplementation on the growth, feed utilization and health of *O. niloticus.*

Specific Objectives are to:

- To compare the water quality parameters of *O. niloticus* hapas supplemented with different probiotics and a control group.
- To measure the growth performance indicators of *O. niloticus* fed with different probiotic diets and a control diet.
- To analyze the feed utilization efficiency of *O. niloticus* fed with different probiotic diets and a control diet.
- To assess the gut microflora composition of *O. niloticus* fed with different probiotic diets and a control diet.
- To examine the hematological parameters of *O. niloticus* fed with different probiotic diets and a control diet.
- To determine the biochemical composition of *O. niloticus* fed with different probiotic diets and a control diet.

1.4. Research questions

- How do the supplementation of different probiotics and a control group on *O. niloticus* hapas affects the water quality parameters?
- What is the effect of growth performance indicators of *O. niloticus* fed with the supplementation of different probiotics and a control group?

- How do supplementation of different probiotic diets and a control diet improve the feed utilization efficiency of *O. niloticus?*
- What is the effect of supplementing different probiotic diets and a control diet on gut microflora composition of *O. niloticus?*
- What is the influence of supplementing different probiotic diets and a control diet on the hematological parameters of *O. niloticus?*
- What are the effects of supplementation of different probiotic diets and a control diet on the biochemical composition of *O. niloticus?*

1.5. Significance of the Study

Aquaculture production is generally affected due to improper water quality, lack of appropriate fish feeds and disease outbreaks in culture systems. Integrated systems to overcome these problems are imperative, and it is in this direction the use of probiotics in fish feeds assume exceptional significance. The study on fish feeds incorporated with probiotics especially bacteria and yeast with basal diets will not only enhance fish production potential and health status, it also helps increase beneficial gut micro-flora there by increasing absorption of nutrients which promotes fish growth (Ayisi *et al*., 2017). It also helps reducing pathogenic micro-flora which normally occurs in culture systems due to the release of metabolites of fish during metabolism. Use of probiotics enhances production potential by reducing microbial pathogens and by controlling disease incidences in fish culture thereby increasing economic profitability

1.6. Scope of the Study

The experiment has been carried out in the Center for Aquaculture Research and Education (CARE), Hawassa University. Fish fingerlings of Nile tilapia are the experimental objects. Probiotic test feeds were prepared by incorporating probiotic strains with locally available feed ingredients. Growth, feed utilization and health of *O. niloticus* were assessed in terms of various growth parameters. Fish gut pathogenic microorganisms fed with control and probiotic diets were investigated. Investigations of hematology and body composition of *O. niloticus* were studied to understand the influence of probiotics. Influence of probiotics on water quality of the experimental pond was assessed using standard methods.

1.7. Limitation of the Study

The sample size of 300 fingerlings of Nile tilapia may not be representative of the whole population of the species in Ethiopia, and thus the results may not be applicable to other regions or conditions. The experimental design of using hapas in a pond may not be able to control for all the confounding factors that may affect the growth and health of the fish, such as the water temperature, the dissolved oxygen, the pH, the ammonia, the nitrite, and the nitrate levels, and the presence of other microorganisms or predators in the pond. The data analysis of using descriptive statistics and LSMeans Tukey HSD may not be able to capture the complex interactions and relationships among the different variables, such as the probiotic strains the dosage, the duration, the water quality, the growth performance, the feed utilization, the gut microflora, the hematological parameters, and the biochemical composition of the fish.

The generalizability of the findings may be limited by the use of only one species of fish, Nile tilapia, and only three strains of probiotics, *B. subtilis subtilis*, *B. subtilis inaquosorum* and *S. cerevisiae*. The effects of probiotics may vary depending on the fish species, the probiotic strains, and their interactions. The validity and reliability of the findings may be affected by the accuracy and precision of the methods and instruments used to measure and analyze the different parameters, such as the feed formulation, the probiotic enumeration and preparation, the fish sampling, the water quality monitoring and analysis, the estimation of growth performance and feed utilization, the isolation and identification of gut pathogenic microflora, the hematological examination, and the biochemical composition of the fish.

2. LITERATURE REVIEW

2.1. Definition of Probiotics

Probiotics are organisms which contribute to intestinal micro-flora (Parker, 1974), and when consumed in adequate amounts, it confer health benefits to the host (Lazado and Caipang 2014). According to Cingelova-Maruscakova *et al*. (2021), probiotics are microorganisms when applied in aquaculture, it stabilize and control microbial populations, maintain stable water quality parameters, prevent bacterial and viral infections, improve the quality of feed, support the growth of aquatic organisms by producing vitamins, minerals, and nucleic acids, and increase the survival of aquatic organisms.

2.2. Methods of Administration of Probiotics

Probiotics in aquaculture can be administered through different routes as feeding, injection or direct immersion in water, which can be applied singly or in combination (Hai, 2015).The most commonly used methods for administrating probiotic mixtures is incorporation into the feed (92.8%), followed by direct incorporation into the water (4.8%) and in live food (1.6%) (Melo-Bolívar *et al*., 2020). Some bacterial and fungal strains can be mixed with feeding pellets or by encapsulating into live feed stock or administered orally to feed for rearing animals. Furthermore, probiotics can also be administrated as water additives in addition to the above methods. In addition to this, probiotics can be applied singly or in combination in different forms such as multi-strain probiotics, probiotic with plant extract and probiotics with yeast extract (Shefat, 2018). Most of the study on probiotics in aquaculture focused on the use of single probiotics but the combination of probiotics is more beneficial.

2.3. Dosage

Probiotic dosage is important for the establishment of bacterial population in fish gut and to maintain fish health. For the optimum effectiveness of the probiotics on the particular species, the probiotics and gut micro-biota at appropriate dosage should be administrated. Appropriate probiotic levels depend on the probiont species, fish species and their physiological status, rearing conditions and the specific goal of the applications (Hai, 2015). Most report suggested the use of probiotic concentrations of approximately $1*10^6$CFU/g for significant improvement in growth performance, resistance to infection, and immune modulation. Some studies also reported that higher concentration may not be able to maintain overall body physiology of fish and it may create disturbances in carbohydrate and fat metabolism (Gourab *et al*., 2020).

2.4. Effects of Probiotics on Selected Parameters

2.4.1. Growth Promoter

One of the most expected consequences of using bacterial probiotics is the growth increment in fish (Roque *et al*., 2020), and this may be due to the increased release of digestive enzymes, production of vitamins, and breakdown of indigestible components (Van-Doan *et al*., 2018). The common probiotics include *Lactobacillus, Lactococcus, Leuconostoc, Carnobacterium, Shewanella, Bacillus, Aeromonas, Vibrio, Enterobacter, Pseudomonas, Bifidobacteria, Clostridium* and *Saccharomyces* (Nayak, 2010). Dietary application of *Pediococcus acidilactici* promoted growth performance in zebra fish *(Danio rerio)* without impairing its appetite (Ahmadifar *et al*., 2020). Supplementation of *Bacillus pumilus* on juvenile golden pompano, *Trachinotus ovatus* improved the weight gain and specific growth

rate (Liu *et al*., 2020). Supplementation of *Bacillus circulans* PB7 in the formulated diets promoted the growth in *C. catla* (Bandyopadhay and Das-Mohapatra, 2009). Single or combined supplementation of *B. megaterium* and *Pediococcus pentosaceus* improved the growth performance of catfish *Clarias* sp (Hamka and Meryandina, 2020). Dietary supplementation of *L. plantarum* at the dose of 10^8 CFU/g for 4 weeks enhanced the growth performance in *O. niloticus* (Zhai *et al*., 2017). The growth and feed utilization performance of *O. pabda* were significantly higher when dietary commercial probiotics was supplied since probiotics increased the digestive enzyme activity, elevated health status and stimulated the gastric development (Gourab *et al*., 2020).

2.4.2. Feed Utilization

Many researches have concluded that the use of probiotics results in the alteration of enzymes and hence improved feed utilization. Incorporation of heat killed *L. plantarum* at 50, 100 or 1000 mg/kg for 12 weeks significantly enhanced amylase, lipase and protease activity of Nile tilapia (Dawood *et al*., 2019). Wang and Xu (2006), investigated the effect of probiotic *Bacillus* sp. and reported increased protease, amylase and lipase activities in common carp. A similar study report was reported by Valipour *et al*. (2019) who observed elevation in protease, amylase, alkaline phosphatase and lipase activity in crayfish, *Astacus leptodactylus* fed with *L. plantarum* at a concentration of 10^7, 10^8 and 10^9 CFU/g.

2.4.2.1. Feed Conversion Ratio (FCR)

The addition of probiotics could improve feed utilization even under stressed conditions (Lara-Flores *et al*., 2003). Nile tilapia treated with commercial probiotic showed significantly higher

feed conversion efficiency compared to the control (Abdel-Tawwab and Ahmad, 2009). While Mohapatra *et al*. (2012), reported a lower FCR for Rohu fingerling when they were fed with diets containing probiotics, Hidalgo *et al*. (2006), did not observe any significant influence of FCR when the probiotics were used for juvenile dentex *(Dentex dentex)*.

2.4.2.2. Protein Efficiency Ratio (PER) and Digestibility

Some reports indicated that probiotics fortified diets could significantly improve the protein efficiency ratio (PER) or apparent nitrogen utilization (ANU). Abdel-Tawwab and Ahmad, (2009), showed a significant effect of probiotics on the protein efficiency ratios compared to the control in Nile tilapia. In general, the application of probiotics in diets results in more nutrient digestibility for feedstuffs. This suggests that the addition of probiotics will improve the diet and protein digestibility, which may in turn explain the better performance. Probiotics enhances feed nutrient digestion which is attributable to the digestive enzyme activity of bacteria (Mohapatra *et al*., 2012). The positive effects on nutrient digestibility in Rohu were observed when the diets supplemented with different microbial probiotics (Mohapatra *et al*., 2012). Dawood *et al*. (2020), recorded an increase in the digestive enzyme activities of amylase, trypsin and lipase in sea bass using live yeast. Roque *et al*. (2020), demonstrated that probiotics affect the digestive process by enhancing the population of beneficial microorganisms and then microbial enzyme activity, consequently improving the digestibility and absorption of feed and feed utilization. They also illustrated that the high growth performance can enhance specific activities of digestive enzymes as well.

2.5. Effects of Probiotics on Health of Fish

2.5.1. Increase Disease Resistance

Probiotic microorganisms have the ability to release chemical substances with bactericidal or bacteriostatic effect on pathogenic bacteria that are in the intestine of host (Martinez-Cruz *et al*., 2012). Supplementation of probiotic *Enterobacter* sp enhances the disease protection against *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Laptra *et al*., 2014). Supplementation of *Lactococcus garvieae* isolated from the raw cow milk at the dose of 10^7cells/g for 10 days increased resistance against *Staphylococcus aureus* in Nile tilapia (Abdelfatah and Mahbouh, 2018). Supplementation of *Lactococcus lactis* isolated from *Cromileptes ativelis* gut at the dose of 10^6, 10^8, 10^{10} CFU/g for 4 weeks enhanced resistance against *Vibrio harveyi* in *C. altivelis* (Sun *et al*., 2018). Banos *et al*. (2019), reported that administration of *Enterococcus faecalis* isolated from commercial probiotic at 10^8 CFU/g for 30 days increased the disease resistance against *L. garvieae* in rainbow trout.

2.5.2. Enhancement of the Immune Response

Probiotics can enhance the various immunological parameters in aquaculture species. Probiotics can inhibit the pathogen infection by enhancing the host immune through the stimulation of body non-specific and cellular immunity (Hamka and Meryandina, 2020). Administration of viable lactic acid bacteria, *L. lactis, Leuconostoc mesenteroides* and *Lactobacillus sakei* enhanced both cellular and hormonal immune functions in rainbow trout by increasing the proportion of phagocitically active cells from head kidney and activating the complement receptor expression. Host associated probiotics *L. plantarum* and *Bacillus*

velezensis enhanced the innate immune parameters as skin mucus lysozyme and peroxidase activity, serum lysozyme, serum peroxidase, alternative complement, phagocytosis and respiratory burst activities in Nile tilapia (Van-Doan *et al*., 2018).

Supplementation of host gut derived probiotic, *B. pumilus* on juvenile golden pompano, *Trachinotus ovatus* increased lysozyme activity and total protein of fish (Liu *et al*., 2020). Dietary supplementation of *L. plantarum* at 10^8 and 10^9 CFU/g significantly improved complement activity after 15 days and significantly enhanced respiratory activity and lysozyme activity in black eared catfish, *Pangasius larnaudii* after 30 days and 45 days of feeding respectively (Silarudee *et al*., 2019). Ahmadifar *et al.* (2020), reported that dietary supplementation of *P. pentosaceus* in common carp result in increased in red blood cells, white blood cells and hematocrit as well as total serum antibody level, alternative complement, protease and lysozyme activities and antibacterial activity. *Saccharomyces cerevisiae* supplemented in date palm seed meal enhanced the lysozyme and phagocytic activity in Nile tilapia (Dawood *et al*., 2020).

2.5.3. Stress Tolerance

Supplementation of probiotics significantly improves the stress tolerance of aquaculture species. Stress tolerance in fish to ammonia indicated that fish fed with probiotics *L. plantarum* performed a lower increment of cortisol concentration to those of control diet as cortisol is a stress hormone (Dawood *et al*., 2019). Supplementation of dietary commercial probiotics at 0.2% has significantly higher saline water stress tolerance than higher and lower doses in pabda catfish (Gourab *et al*., 2020). This can be concluded from the result that

probiotics supplemented groups have lower level of plasma glucose which is the stress indicator in fish.

2.6. Effects of Probiotics on Gut Pathogenic Microbial Load

The microbial colonization of the fish gut is mainly influenced by the rearing water and nutritional status of fish (Giatsis *et al*., 2016). Imbalanced fish gut micro-biota leads to poor metabolism, reduced growth, stress, and disease onset. *Aeromonas* is a specific primary pathogen in freshwater, and it also acts as a secondary opportunistic pathogen attacking immune-compromised or stressed freshwater fish where it normally inhabits in the gut of *O. niloticus* (Sherif *et al*., 2020). The gut micro-biota markedly influences homeostasis and digestion, modulating not only the host's physiology and immune responses but also the morphology and function of the intestinal epithelium (Butt and Volkoff, 2019). So modulating the indigenous micro-biota by probiotic administration might thus regulate the dynamic basis of host-micro-biome symbiosis, playing a pivotal role against pathogens. Nile tilapia fed with *Rummeliibacillus stabekisii* probiotic showed higher counts of beneficial bacteria, *Bacillus* and *Lactobacillus* and lower counts of pathogenic *Streptococcus* and *Staphylococcus*, suggesting that *R. stabekisii* can modulate the intestinal micro-biota and contribute to immunomodulation and disease resistance (Tan *et al*., 2019).

2.7. Effects of Probiotics on Hematology of Fish

Hematological indices are used as vital diagnostic tools to assess the health status of fish (Pradhan *et al*., 2012). Probiotics play beneficial role to enhance hematological parameters of fish species. Supplementation of Primalac probiotics containing *Lactobacillus acidophilus, Lactobacillus casei, Enterococcus faecium* and *Bifidobacterium bifidium* increased the

hematocrit levels in Caspian roach fry (Imanpoor and Roohi, 2015). The application of a mixed probiotic species of *Lactococcus rhamnosus* and *L. lactis* in red seabream; *Bacillus* sp in Nile tilapia; and combination of *B. cereus* and *B. subtilis* in Nile tilapia have been reported to increase the hematocrit levels (Feliatra *et al*., 2018). Fish fed with probiotic-supplemented diets with probiotic *L. sporogenes* in *Clarias batrachus* and a combined dosage of *L. sporogenes, L. acidophilus, B. subtilis, B. licheniformis* and *S. cervirial* in *Cirrhinus mrigala* and *L. plantarum* in Nile tilapia were reported to have significantly higher RBC compared to fish fed with supplemented diets (Sharma *et al*., 2013). Fish fed with probiotic supplemented diets also showed better immune response compared to those fed with control diets (Munir *et al*., 2018).

2.8. Effects of Probiotics on Histopathology of Fish

Recently, it was found that the inclusion of *L. plantarum* in the diet alleviates aluminum toxicity in tilapia, reducing hepatic lesions such as necrosis, vacuolar degeneration, nuclear pyknose, and sinusoid narrowing (Yu *et al*., 2017), a strong indication that probiotics can act in the maintenance of the organ's structure that are directly involved in fish health. However, many effects on animal health still need further research. According to Gaffar *et al*. (2023), administration of commercial probiotic in fish diet positively enhanced various histo-morphometric dimensions of intestine such as villi length, width, area, crypt depth and thickness of intestine muscle.

2.9. Water Quality Improvement

In aquaculture ponds, the quality of water normally deteriorates mainly due to the accumulation of metabolic wastes, decomposition of unutilized feed and decay of biotic materials. Probiotic *Bacillus* can decompose organic matters, diminishes nitrogen and phosphorus availability, and controls ammonia, nitrate, and hydrogen sulfide thus improving the water quality (Abarike *et al.,* 2018). Ammonia is the most dangerous to cultured organisms and it exists in water as ionized and un-ionized forms and their levels are balanced depending on the pH and water temperature (El-Sayed, 2006). *Bacillus* spp. are known to utilize different nitrogen sources, including both NH3 and NH4+ for catabolism of proteins and subsequently use H+ particle (Yin *et al*., 2018). According to Hui *et al*. (2019), gram positive *Bacillus* species can remove the different forms of nitrogen from aquaculture wastewater through ammonification, nitrification, de-nitrification as well as nitrogen fixation.

Nimrat *et al*. (2012), reported that administration of mixed *Bacillus* probiotics significantly enhanced the water quality for the levels of pH, ammonia and nitrite of culture water. The water addition of probiotic *B. subtilis* at 10^3-10^5 CFU/mL effectively reduced the total ammonia and enhanced water quality. Administration of probiotics *B. cereus* and *Pediococcus acidilactici* at 10^6CFU/mL to the pond water positively decreased nitrate, ammonia and biological oxygen demand (Khademzade *et al*., 2020). Many researchers have reported that *Bacillus* species play significant roles in removing nitrogenous and phosphorus compounds and maintaining the balance of the microbial community structure (Soltani *et al*., 2019).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted in Center for Aquaculture Research and Education (CARE) Experimental Fish Ponds in Hawassa University, Sidama Region, Ethiopia (Fig. 1). Hawassa is found in the southern part of Ethiopia at 275km south of Addis Ababa, the capital city of Ethiopia. It is located at $6^0$54'-$7^0$3'N latitude and $38^0$27'-$38^0$33'E longitude and situated at 1,686m above sea level. The city is characterized by mean annual precipitation of 933.4 mm. Temperatures vary between 5^0C in winter and 34^0C in summer. The city experiences sub humid-called 'WoyinaDega' type of climate. It has the highest and lowest temperature of 34^0C and 3^0C respectively. The average annual temperature is 20.3^0C. There are three rainfall seasons in the study area: Kiremt (summer), also called the main rain season (June, July, August, and September); Belg (Spring) season, also called the short rainy season (February, March, April, and May); and the Bega (dry) season (October, November, December and January) (Alemayehu & Bewket, 2017). The amount of rainfall received in the three seasons varies significantly while Kiremt is the main rainy season. Due to the city's location in the Great Rift Valley on the shore of rift valley Lake Hawassa, its weather condition changes dramatically from day to night.

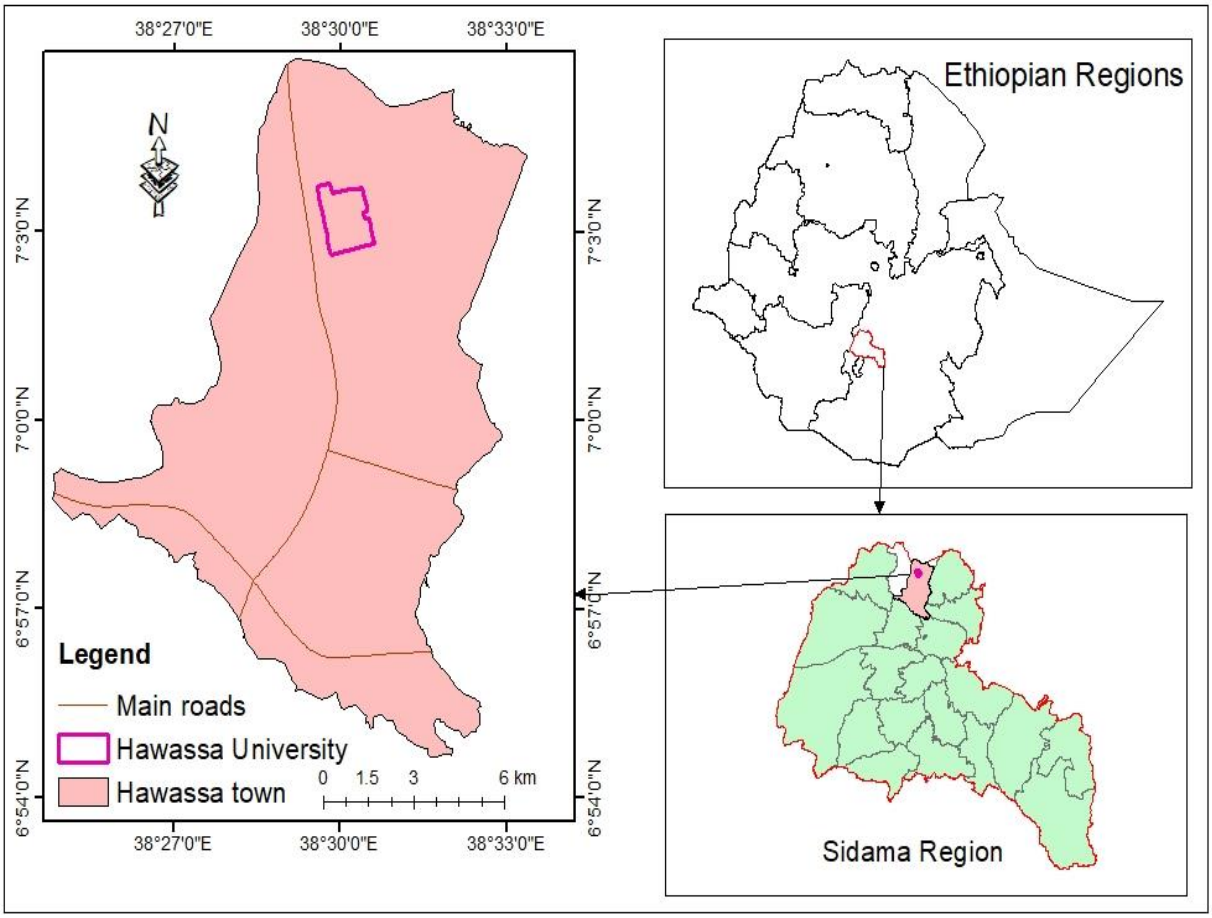

Figure 1*: Location map of the study area (Source: ArcGIS)*

3.2. Sampling Design

This study includes both laboratory and on-station experiments using concrete ponds. Complete randomized design (CRD) having five treatments with triplicates were used to improve the accuracy of the comparison among control and experimental groups. The experimental study was conducted to determine the potential role of probiotics on water quality, growth performance, feed utilization, pathogenic gut micro-flora, hematological parameters and proximate composition of *O. niloticus*. Mixed sex *O. niloticus* fingerlings used in this experiment were collected using seine net (50 m*2.5 m) with a mesh size of 20mm

from the experimental fish farm. Three hundred (300) *O. niloticus* fingerlings were collected and twenty (20) fingerlings each were stocked in 15 individual hapas (mosquito net enclosue). Fingerlings were acclimatized in the hapas 4.5m^3 (2m * 1.5m * 1.5m size) for 5 days before the experiment. They were fed with control diet containing 30% crude protein (CP) at 5% of their body weight. During this period, fish were adapted on feeding of control diet (without any additives).

Water was changed once a week to maintain good water quality by removing wastes. The experiment was carried out for three months with control diet (P0) and treatment with probiotics (P_1-P_4). Probiotics both in the form of single-use and combination were supplemented in fish diet at appropriate level. They were designated as P0-treatment as Control, whereas, experimental groups were designated as P_1-treatment (feed supplemented with probiotic *Bacillus subtilis subtilis*), P_2 treatment (feed supplemented with probiotic *Bacillus subtilis inaquosorum*), P_3 treatment (feed supplemented with probiotic *Saccharomyces cerevisiae*) and P_4-treatment (feed supplemented with probiotic *S. cerevisiae*, *Bacillus subtilis subtilis* and *Bacillus subtilis inaquosorum*) were supplied to fingerlings with mean initial body weight of 7.7±0.103g and mean initial body length of 7.46±0.073cm for three months.

3.3. Feed Formulation and Methods Used to Supplement Probiotics in Pelleted Diet

The basal diet was formulated to meet the nutritional requirements of *O. niloticus* fingerlings. A basal diet contains bone and meat meal 12.12 Kg (30.3%), full fat soybean meal 10.4 Kg (26%), maize flour 8.8 Kg (22%), and wheat flour 8.68 Kg (21.7%), and 30% crude protein ratio was analyzed by using feed excel. The basal diet has no probiotic additives. Feed

formulation was done in Fish Nutrition Laboratory at CARE, Hawassa University. Pre-ground dry ingredients were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 minutes. The feed ingredients were mixed to produce slight dough in boiled water and then the mixture was pelleted using mechanical pelletizer. The pellets were put in trays and dried under shaded environmental condition until four days in order to prevent excess removal of the moisture from the formulated feed. After drying, 40kg pelleted diet was prepared and stored in water impermeable container. Prepared probiotics were fortified with pelleted feed at appropriate level (Hogendoorn, 1983).

The determination of probiotic doses were adjusted based on the study with optimal probiotic doses of 10 mL/kg of feed adjusted with McFarland turbidity standard level at 0.5MFU (McFarland, 1907), as reported by first international conference on fisheries and marine science in 2019 (Setiawati *et al*., 2013). The pelleted feed was given to the fingerlings and the feed was offered daily at a rate of 5% of their body weight. The frequency of feeding was three times per day at morning 8:00, afternoon 12:00 and late afternoon 6:00 for the consecutive weeks of the experimental period. The feeding allotment was adjusted as recommended by Hogendoorn (1983).

3.4. Enumeration and Preparation of Probiotic Feed

Probiotics of live bacterial and yeast species were prepared according to the standard dose of McFarland at cell density of $1.5*10^{8}$CFU/mL cells turbidity standard at 0.5MFU on the McFarland scale to incorporate with pelleted fish diet (McFarland, 1907). Bacterial species such as *B. subtilis subtilis* and *B. subtilis inaquosorum* extracted from shrimp gut were brought from the Centre for Marine Sciences and Technology (CMST), Manonmaniam Sundaranar

University, Tirunelveli, India and *S. cerevisiae* from the Veterinary Medicine Microbiological Laboratory of Hawassa University. Probiotics were kept in brain heart infusion broth (Oxoid) with adding 20% glycerol buffer at -20^{0}C deep freezer until used for the experiment. Probiotics were re-enumerated before incorporated with pelleted feed to refresh and propagate its number. Materials and media such as nutrient agar and potato dextrose agar for enumeration of *B. subtilis* and *S. cerevisiae* respectively were prepared and autoclaved at 121^{0}C for 15min and cooled at room temperature. The inoculating loop was frequently sterilized to prevent cross contamination. Saline solution was prepared from 8.5g of NaCl with 500ml of distilled water (Das *et al*., 2013).

B. subtilis subtilis and *B. subtilis inaquosorum* were incubated at37^{0}C and *S. cerevisiae* at 25^{0}C for 24 hours. Both *B. subtilis* and *S. cerevisiae* were grown after 24 hours in Petridish at room temperature. Then 20mL of nutrient broth were prepared in the Falcon, and the microbial sample was added in to nutrient broth by inoculating loop and shaken by vortex shaker to mix bacteria and broth media equally. After 24 hours, the microbial sample was inoculated into sterilized test tube at concentration of 0.1mL by using micropipette. Aseptically prepared saline solution was added into test tubes containing microbial sample until the solution was equivalent to McFarland turbidity standard at 0.5MFU on the McFarland scale. After mixing probiotics with pelleted feed, saline solution was sprayed to feed since they do not harm the bacterial cells or affect the palatability of the feed when added in low percentage. This decreases the loss of probiotic bacteria in water before fish ingests the pellet. Then the four probiotic strains were fortified with feed and it was then fed to fingerlings of *O. niloticus* every day by hand feeding (Das *et al*., 2013).

3.5. Fish Sampling

Fish were sampled twice a month using a clean bucket from the hapas in the pond. All the fish were collected for individual weight and length measurements. Each fish was weighed with a digital balance (0.01g sensetivity) (model SF-400A, Germany) and measured total length using a measuring board (1mm) as described by Caspers (1969). Fish were returned to their respective hapas after measurements. At the end of the experimental period, fish were deprived of feed for 24 hours and were then harvested, counted and weighed individually. The fish growth performances under different treatments were evaluated in terms of final total length (cm), final weight (g), body weight gain (g), specific growth rate (SGR, % day^{-1}), daily weight gain (DWG, g day^{-1}), feed conversion ratio (FCR), protein efficiency ratio and survival rate (%).

3.6. Water Quality Monitoring and Analysis

Water quality parameters such as temperature, dissolved oxygen (DO), and pH were determined for the duration of the experiment with the same stocking density at twice a month and three times a day (morning 8:00-8:30, afternoon 12:00-12:30 and late afternoon 5:30-6:00). Water samples from each treatment were measured *insitu* by following the methods adopted Boyd & Tucker (1998**).**

3.7. Estimation of Growth Performance Parameters

3.7.1. Body Weight Gain

Final body weight was measured at the end of the experimental period. The average weight in gram was used to calculate the mean weight gain. Therefore, the following formula was used. (BWG)=Final weight (g) − initial weight (g) computed by (Ricker, 1975).

3.7.2. Specific Growth Rate

The average specific growth rate for each stocking density was calculated by following the equation $\mathbf{SGR\%=\frac{(LnWf-LnWi)}{T} * 100}$ as proposed by (Ricker, 1975) where: SGR%=Percentage increase in body weight per fish per day. LnWf =natural log of final weight, LnWi. = natural log of initial weight, T= number of culture days.

3.7.3. Daily Weight Gain (DWG) $= \frac{\mathbf{Mean\ Final\ Weight(gm) - Mean\ Intial\ Weight(gm)}}{\mathbf{number\ of\ culture\ days}}$ as computed by Eyo *et al.* (2013).

3.7.4. Mean Length Gain

It was calculated as: **MLG = Mean Final Length - Mean Initial Length** as computed by Eyo *et al.* (2013).

3.7.5. Survival Rate (SR)

Survival rate of the fingerlings were determined after final harvesting of the fingerlings. The total numbers of fingerlings harvested were counted and then it was computed as:

Survival Rate (%) = $\frac{\textbf{Number of survivals at the end of the experiment}}{\textbf{Number of fingerlings stocked}} * \mathbf{100}$ as computed by Eyo *et al.* (2013).

3.8. Feed Utilization

3.8.1. Feed Conversion Ratio

The feed conversion ratio is a measure of feed efficiency that is used for all livestock production. It can provide a good indication of how efficient a feed or a feeding strategy can be. It was calculated by the formula; **FCR** = $\frac{\textbf{Total feed consumed by fish(g)}}{\textbf{Total weight gain by fish(g)}}$ as computed by Mohanty (2004)

3.8.2. Protein Efficiency Ratio

PER = $\frac{\textbf{Weight gain per fish(g)}}{\textbf{Protein intake (g)}}$ as computed by Mohanty (2004)

3.9. Isolation and Identification of Gut Pathogenic Micro-flora from *O. niloticus* Fed with Control and Probiotic Diet.

Isolation of bacteria from the gut of fish fed with probiotics diet and control (diet without probiotics) was done according to Venkat *et al.* (2004). At the end of the feeding period, fish were starved for 24 hours to allow gut evacuation and one hapa from each treatment groups was purposively selected. From selected hapa three fish were collected using simple random sampling and brought to Veterinary Medicine Microbiological Laboratory for gut pathogenic microbial content analysis. Before the dissection of the gut, their body weight (g) and length (cm) were measured. Selected fish sample for gut dissection were placed on a tray and they were externally disinfected with 96-99% alcohol and anesthetized by clove oil. The entire fish

intestine was carefully removed and longitudinally dissected via sterilized scissors and divided into three parts (anterior, middle and posterior). The contents of the gut were squeezed out and washed with distilled water to remove feed residues. Each part of inoculum samples were transferred to liquid media of brain heart infusion broth (BHI)**.** Broth containing inoculum samples was incubated at 37^0C for 24 hours.

To investigate the presence of *Salmonella* spp. from *O. niloticus* gut, 9mL of buffered peptone water and 1mL of microbial sample from the brain heart infusion broth media were added in each sterilized test tubes. Next 10 mL of Rappaport vacillidius media were prepared in the sterilized test tubes and 0.1mL sample from buffered peptone water were transferred to the media and incubated at 42^0C for 24hr. Then after, the color of the media changed due to the presence of microorganisms. The homogenate from Rappaport Vacillidius media were transferred to XLD-media which were prepared in appropriate amount for isolation of S*almonella* spp. The samples from test tubes were transferred by inoculation needle and streaked in XLD- media and incubated at 37^0C for 24 hours. Thereafter, black centered and slightly red colored colonies were observed in the media. Finally pure culture isolates were identified to confirm the bacteria based on colony morphology, gram stain and biochemical tests (Methyl Red test and TSI test**)** according to Holt *et al.* (1994).

To investigate the presence of *Escherichia coli* from *O. niloticus* gut, the microbial samples were inoculated on Eosin Methylene Blue (EMB) agar and MacConkey agar and incubated at 37^0C for 24 hours. Typical *E. coli* colonies showed characteristic metallic sheen with dark center colonies on EMB agar and colonies that are medium sized, bright pink to red with flat or elevated surface and complete white edges on MacConkey agar confirmed the existence of

E. coli in the gut of *O. niloticus.* The purified cultures of *E. coli* were further identified based on colony morphology, gram stain and biochemical tests (Methyl Red test, Citrate Utilization test and Indole test) according to Holt *et al. (*1994).

To investigate the presence of *C. albicans* from *O. niloticus* gut, samples were transferred to liquid media of brain heart infusion broth (BHI). Microbial samples were spread plating 0.1 mL of the homogenate on Sabouraud's dextrose agar and incubated at 37^0C for 24hours. Then gram staining was used for primary identification of the isolates as well as colony morphology (white to cream colored colonies) according to Holt *et al. (*1994).

3.10. Pure Pathogenic Microbial Culture

3.10.1. Morphology of the Microbial Colony

On solid medium the following characters were observed: Different types of bacterial shapes such as: circular, irregular, radiate or rhizoid and colors such as colorless, pink, black, red, bluish and green were observed. For *Candida albicans* white to cream colored colonies were observed.

3.10.2. Gram Staining: A thin smear of the bacterial/fungal isolates were made on a clean glass slide and heat fixed. Then the smear was stained with crystal violet for 1 minute and washed with tap water. Next gram's iodine was added for 1 minute and decolorized with alcohol. After de-colorization the smear was counter stained with saffranin for 1 minute and washed with water. Finally the smear was air dried and observed for the color. The color was examined under 100x light microscope and produced violet color for gram positive bacteria/fungus and pink color for gram negative.

3.10.3. Biochemical Test

Methyl Red Test: The test could be used in differentiating *Escherichia coli* and *Enterobacter aerogenes* (both coliform bacteria) that are used as indicator of the sanitary quality of water, foods etc. The suspected bacteria were inoculated into appropriately labeled tubes containing MR broth by means of loop inoculation and an un-inoculated tube was kept as control. Both tubes were incubated at 37^0C for 24-48 hours. After proper incubation 5 drops of MR indicator was added to both tubes including control. It was mixed well and the tubes were observed for changes in the color of Methyl Red. The color of MR reagents at a pH of 4 remaining red was a positive test, it indicates the presence of *E. coli, Salmonella* sp. and the color at pH 6, still indicating the presence of acid but with a lower hydrogen ion concentration, the indicators turn yellow, which was indicating the negative test, it shows the presence of *Klebsiella, Enterobacter* sp (Holt *et al*., 1994)

Triple Sugar Iron Test: This test was designed to differentiate microorganisms that belong to the *Enterobacteriaceae* family which was all gram negative intestinal bacteria. The test medium was made up of three sugars: 0.1 percent glucose, and 1% lactose and sucrose. The glucose was used by the inoculated bacteria, and glucose intake is kept low in comparison to other sugars. The test bacteria were inoculated into the TSI media by means of streak inoculation and an un-inoculated tube was kept as control. Both tubes were incubated at 37^0C for 24 hours and the tubes were observed for color changes of both the butt and slant and also gas production by means of cracks or bubble or blackness of butt. The production of hydrogen sulphide in the medium was indicated by the formation of a black precipitate that was blackening the medium in the butt of the tube. The result was observed for

alkaline slant/acid butt (K/A) with glucose fermentation only and H_2S production which showed the existence of *Salmonella* spp. (Hemraj *et al*., 2013).

Citrate utilization test: The Simmons citrate agar slant was inoculated with the test bacteria by means of a stab and streak inoculation. An un-inoculated tube was kept as control and both tubes were incubated at 37^0C for 24 – 48 hours. The tubes were observed for growth and coloration of the medium. Growth in the medium was shown by turbidity and change in color. Color of the medium were turned blue indicated positive result. Citrate positive bacteria: *Klebsiella* spp. Color of the medium remained as green indicated a negative result. Citrate negative bacteria: *E. coli* (Holt *et al.,* 1994).

Indole Test: This enzyme converts the amino acid tryptophan to indole gas by using tryptone broth. The peptone water tubes were inoculated with bacterial broth culture using sterile needle technique. An un-inoculated tube was kept as control and both tubes were incubated at 37^0C for 24-48 hours. After proper incubation, 5 drops of Kovac's reagent was added to both tubes including the control. The tubes were shaken gently after an interval for 10 – 15 minutes. The tubes were observed for the color in the top reagent layer. A positive indole test was observed by the formation of a pink to red color ("cherry red ring") in the reagent layer on top of the medium within seconds of adding the reagent while in negative reaction a yellow ring was formed. Indole positive bacteria: *E. coli*, Indole negative bacteria: *Klebsiella* sp. and *Proteus mirabilis* (Holt *et al.,* 1994).

3.11. Hematological Examination

Blood Sample Collection: Three fish from each hapa were randomly selected and anesthetized with clove oil at a concentration of 1mg/L. Fish were well wiped and cleaned in order to avoid mucus mixing into the blood. Blood samples were then collected by puncturing the caudal peduncle vessel with the use of 5mL syringe and needle that has been treated with anti-coagulant such as heparin to prevent clotting into small sampling bottles containing Ethylene diamine tetra-acetic acid (EDTA). After the collection, the blood samples were taken to the Veterinary Laboratory of Hawassa University for hematological analysis. Blood aliquots was used to determine the total number of erythrocytes (RBC) according to Dianti *et al.* (2013), packed cell volume (or hematocrit percentage) (Zuhrawati, 2014), hemoglobin (Simanjuntak, *et al*., 2018), leukocyte counts (Dianti *et al*., 2013), as well as differential leukocyte counts (Yılmaz *et al*., 2016).

Packed cell volume: PCV (hematocrit) was determined by collecting the fish blood into micro-hematocrit heparinized tube which was sealed with critaseal at one end to ensure that the column of blood is not charred by the application of heat. The sampled tubes were then placed in the micro-hematocrit centrifuge with the sealed end outermost. The tubes were loaded symmetrically to ensure good balance and it was placed correctly on to the rubber gasket. The tubes were screwed on rotary cover. The centrifuge lid was closed and centrifuged within the speed of 5 minutes at 12000rpm using micro-hematocrit centrifuge. The hematocrit (PCV) values were read on a micro-hematocrit reader (hematocrit table scale) and were expressed as of packed red blood cells to total volume of whole blood. A mean of three readings was recorded as percentage for the fish (PCV) hematocrit (Zuhrawati, 2014).

Erythrocyte count (RBC): Red blood cell counts were determined by aspirating blood in to a special pipette for measuring the number of erythrocytes, with red stirring grains in it until the scale of 1 mark was reached. The blood was then mixed with 1:100 Hayem's solution (RBC diluting fluid) up to 101 marks on the pipette and then the pipette was quickly holds in a horizontal position. After that, the pipette containing the fish blood and Hayem Solution were shaken for ±30 seconds to ensure the contents were thoroughly homogenized. Before the sample was being dripped on hemocytometer, the amount of the sample was reduced for 2-3 drops and covering it with a glass cover to avoid the appearance of air bubbles. Counts were performed under a binocular microscope with a magnification of 40x. The number of erythrocytes were counted in 10 small compartments in the hemocytometer and converted to obtain the number of blood cells per $mm^{3.}$ Erythrocytes were counted by using the formula of Dianti *et al*. (2013).

Number of Erythrocyte = $\sum N * 10^4$ cells/mm^3

Note: N: number of the calculated Erythrocyte

Hemoglobin Determination:

The measurement of hemoglobin was performed by using Sahli's Haemoglobino Meter method. This method is also known as the acid hematin method, where the blood sample was obtained by using Sahli's pipette up to 20 mm (the line limit of Sahli's pipette) and added in 0.1 N HCl Solution up to number 2 (the yellow line boundary). Blood sample was inserted in Sahli's tube and waited until turning into blackish brown. For this distilled water was added drop by drop and was stirred and mixed thoroughly using the stirrer after the addition of each

drop so that the color of the solution corresponded with the two tubes of the color standard of the hemometer. Furthermore, the hemoglobin value was directly read in gram per deciliter from the graduated hemoglobin tube (Simanjuntak, *et al*., 2018).

Leukocyte count: The numbers of leukocyte count were determined by aspirating the blood sample in to special pipette up to 0.5, and then it was diluted by using Turk Solution until reaching the 11 limits. After that, the pipette containing fish blood and Turk Solution were shaken for ±30 seconds to ensure the contents were thoroughly homogenized. Before the sample was being dripped on hemocytometer, the first 2-3 drops of the solution were discarded. Next, the hemocytometer was slowly covered with a glass cover to avoid the appearance of bubbles. The numbers of leukocytes were counted in 5 out of the 16 small compartments in the hemocytometer and converted to obtain the number of cells per mm^3. Leukocyte was counted by using the formula of Dianti *et al*. (2013).

Number of Leukocyte = $\sum$ N * 50 cells/mm^3

Note: N: number of the calculated leukocyte

Differential leukocyte count: The observation of leucocyte differential count was done by taking one drop of blood from tube by using syringe, and then it was placed on glass object. Next, it was flattened by using the smear method. The smear method was carried out by pulling forward the blood that had been dripped on the glass object using another glass object at an angle of 45^0. After that, the samples on the slide was then air dried before being placed in methanol for five minutes, and then placed in Wright dye for three minutes. The slides were washed by running water for 5 minutes and dried. Each slide was observed under oil-

immersion microscope at 100x magnification. For each slide, 100 leukocytes were identified as (lymphocyte, monocyte, eosinophil, basophil, and neutrophil) (Yılmaz *et al*., 2016).

3.12. Biochemical Composition of *O. niloticus*

Two fish from each hapa were randomly selected for whole body analysis. The fish were dried in oven at 60^0C for 24hrs, grounded and chemically analyzed. Standard methods were used for analyzing the proximate composition of whole-body samples (Association of Official Analytical Chemists, 1995). The crude protein (N*6.25) was analyzed by Micro Kjeldahl apparatus. The moisture content of fish body was measured by oven drying the sample at 105 ^{0}C for 12 hours. Petroleum ether extraction method through Soxtec system was used to analyze the percentage of crude fat. Crude fiber was obtained as a loss on combustion of lipid-free dry residues after digestion with 1.25% H_2SO4 and 1.25% NaOH. Ash was determined at 650^0C for 12 hours at constant heat in the electric furnace (Eyela-TMF 3100). Total amount of carbohydrates was calculated by the following formula: Total carbohydrates (%) = 100 − (crude protein% + ether extract (crude fat %) + crude fiber% + ash %)

3.13. Data Analysis

Descriptive statistics including means and standard errors of the growth variables, hematological parameters, body composition of the fish and water quality parameters were used. The effects of probiotics on the growth performance and feed utilization of Nile tilapia (based on mean weight gain, specific growth rate; daily weigh gain, feed conversion ratio, protein efficiency ratio, survival rate), hematology, body composition of fish and average water quality parameters were computed. Estimation of various growth performance parameters and feed utilization potentials over time was presented in table. Microsoft Excel

sheet were used to compute some of the descriptive statistics and to plot graphs. SAS (Statistical Analysis Software) at P= 0.05 was further used to test the effects of probiotics on the growth performance, feed utilization, hematology, body composition of the fingerlings and water quality parameters during the experimental period. Analyses were carried out with SAS software. LSMeans Tukey HSD was used to compare the means of the different treatment groups for each Water quality parameters, growth performance parameter, Hematological parameters and Biochemical composition. All data were expressed as means ± standard error of the mean (SEM).

4. RESULTS AND DISCUSSION

4.1. RESULTS

4.1.1. Water Quality Parameters

The mean values of water temperature were ranged between 27.67 and 28^0C among the treatments. The highest mean temperature during the experimental period was 28^0C in p0 treatment and followed by 27.81^0C in p1, 27.72^0C in p4, 27.7^0C in p3 and 27.67^0C in p2 treatments. But the mean temperature among all treatments did not show significant difference ($p>0.05$). Dissolved oxygen ranged between a minimum of 4.7 in control and a maximum of 4.85mg L^{-1} in p3 followed by p4 (4.84 mg L^{-1}), p2 (4.83 mg L^{-1}) and p1 (4.8 mg L^{-1}). However, the results of dissolved oxygen in control diet (p0) indicated that the difference in DO level among the treatment were statistically significant ($p<0.05$). The results of pH during the experimental period were 8.2, 7.92, 7.86, 7.94 and 8 in p0, p1, p2, p3 and p4 respectively. All the pH values were same during the experimental period and thus they did not show any significant difference ($P>0.05$).

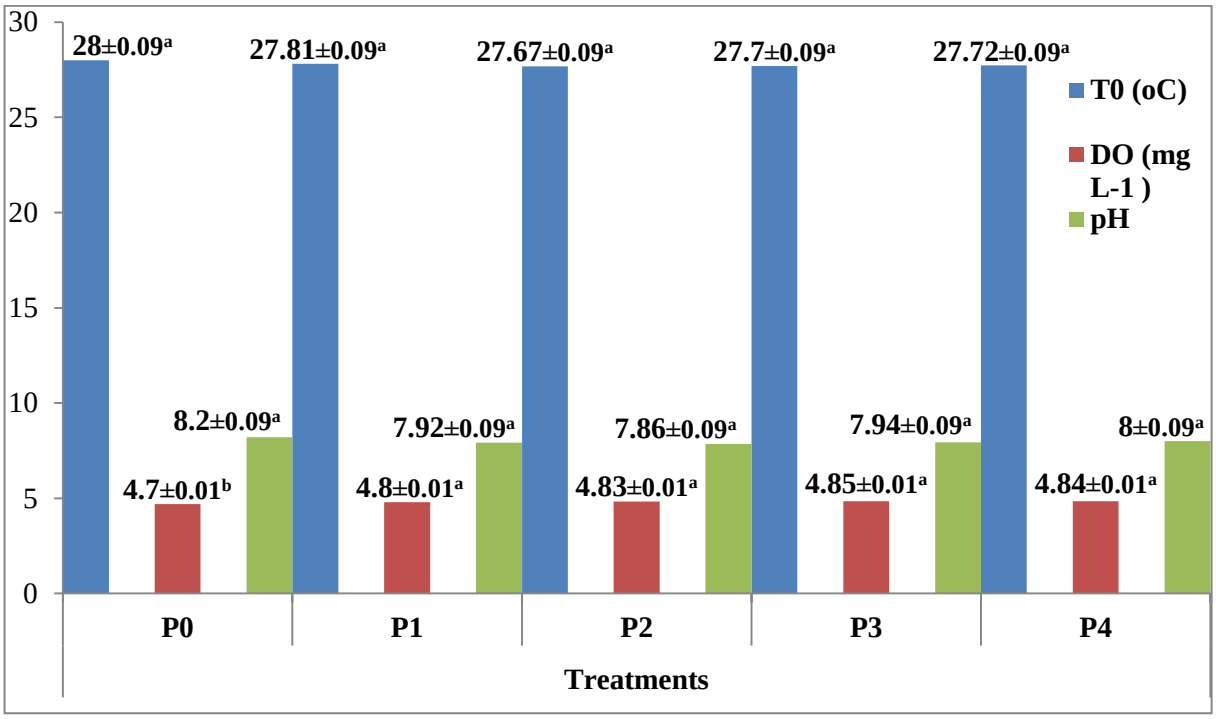

Figure 2: *Mean Values of Water Quality Parameters*

Mean values ± standard error (±SE) means levels that are not connected by the same letter (subscripts) are significantly different (p≤0.05).

4.1.2. Estimation of Growth Performance of Nile Tilapia

The results of the growth performance of *O. niloticus* fingerlings fed with diets supplemented with, *B. subtilis subtilis, B. subtilis inaquosorum S. cerevisiae*, and combination of the three are presented in Table 1. The response of fish fed with probiotic supplemented diet during the experimental period showed better improvement on growth performance parameters and nutrient utilization. The fish fed with p2 and p4 diet showed highest improvement in FBW, FBL, BWG, SGR, DWG, FCR, PER, MLG and SR followed by p3 and p1 diet. It was observed that application of probiotics such as *B. subtilis subtilis, B. subtilis inaquosorum* and *S. cerevisiae* alone and in combination to fish diet enhanced growth performance and

improved nutrient utilization in each treatment group than the control diet. The inclusion of effective bacterial and yeast species in fish diet at concentration of $1.5*10^8$ CFU/mL ensured better fish weight and length increment.

Table 1: *Mean Values of Growth performance parameters of O. niloticus*

Growth Performance Parameters	Treatments				
	P0	P1	P2	P3	P4
IBW(g)	8 ± 0.10^{a}	7.4 ± 0.10^{b}	7.6 ± 0.10^{ab}	7.8 ± 0.10^{ab}	7.7 ± 0.10^{ab}
FBW(g)	90.1 ± 0.10^{e}	108.2 ± 0.10^{d}	136 ± 0.10^{a}	122.3 ± 0.10^{c}	134.7 ± 0.10^{b}
IBL(cm)	7.5 ± 0.07^{a}	7.5 ± 0.07^{a}	7.3 ± 0.07^{a}	7.6 ± 0.07^{a}	7.4 ± 0.07^{a}
FBL(cm)	13.3 ± 0.09^{c}	14.8 ± 0.09^{b}	15.7 ± 0.09^{a}	15 ± 0.09^{b}	15.6 ± 0.09^{a}
BWG(g)	82.1 ± 0.15^{e}	100.8 ± 0.15^{d}	128.4 ± 0.15^{a}	114.5 ± 0.15^{c}	127 ± 0.15^{b}
SGR(%/day^{-1})	2.19 ± 0.02^{c}	2.46 ± 0.02^{b}	2.66 ± 0.02^{a}	2.52 ± 0.02^{b}	2.64 ± 0.02^{a}
DWG(g/day^{-1})	0.91 ± 0.00^{e}	1.12 ± 0.00^{d}	1.43 ± 0.00^{a}	1.27 ± 0.00^{c}	1.41 ± 0.00^{b}
FCR	1.63 ± 0.00^{a}	1.32 ± 0.00^{b}	1.04 ± 0.00^{d}	1.16 ± 0.00^{c}	1.05 ± 0.00^{d}
PER	2.74 ± 0.00^{e}	3.36 ± 0.00^{d}	4.28 ± 0.00^{a}	3.82 ± 0.00^{c}	4.23 ± 0.00^{b}
MLG	5.8 ± 0.09^{c}	7.3 ± 0.09^{b}	8.4 ± 0.09^{a}	7.4 ± 0.09^{b}	8.2 ± 0.09^{a}
SR (%)	95 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}

Mean values ± standard error (±SE) means levels that are not connected by the same letter (subscripts) are significantly different ($p\leq0.05$).

Mean final body weight of *O. niloticus* fed with (p2) at a dosage of $1.5*10^8$CFU/mL showed highest final mean body weight (136g) followed by p4 (134.7g), p3 (122.3g) and p1 (108.2g), respectively. On the other hand control diet showed lowest mean final body weight (90.1g) compared with probiotic supplemented treatments. Mean final body weight gain indicated that there was a significant difference among all the treatments ($p<0.05$). Mean final body length of *O. niloticus* was highest in fish received p2 (15.7cm) and p4 (15.6cm) incorporated diet followed by p3 (15cm) and p1 (14.8cm), and the lowest mean final body length was observed in control diet p0 (13.3cm) compared with probiotic supplemented treatments. However, the mean final body length between p2 and p4 are statistically uniform ($p>0.05$), and also p1 and p3 did not show any significant difference ($p>0.05$) between each other. However, mean final body length among the treatments showed statistically significant difference ($p<0.05$).

Body weight gain of *O. niloticus* obtained during the experimental period showed that there was variation among all treatments and better body weight gain was observed in fish fed with probiotic supplemented diet at a dosage of $1.5*10^8$CFU/mL. Among five experimental groups of fish fed with control diet and probiotic supplemented diet such as p1, p2, p3 and p4, the maximum body weight gain was observed in p2 (128.4±0.15g) followed by p4 (127±0.15g), p3 (114.5±0.15g) and p1 (100.8±0.15g) respectively (Table 1). The lowest body weight gain was found in p0 (82.1±0.15g) and the means of the weight gains among all the treatment groups were significantly different ($P<0.05$). The highest mean specific growth rate of *O. niloticus* recorded during the study period was p2 (2.66) and p4 (2.64) which were statistically uniform between each other. The mean specific growth rate of *O. niloticus* fed with p3 (2.52) and p1 (2.46) also did not show variation between each other ($p>0.05$) and the lowest SGR

percentage was recorded in treatment that received control diet (2.19) (Table 1). The result of SGR between p2 and p4 did not show any significant difference and also p3 and p1 did not show significant difference between each other. However, the SGR showed that there was statistically significant difference among the treatments ($p<0.05$).

Daily weight gain of *O. niloticus* was recorded as 1.43±0.00, 1.41±0.00, 1.27±0.00, 1.12±0.00 and 0.91±0.00 in p2, p4, p3, p1 and p0 respectively (Table 1). It was observed that p2 treatment was highest mean daily weight gain followed by p4, p3, p1 respectively, whereas the lowest values of DGR was recorded in control diet (P0) treatment. The mean daily weight gain in all of the treatments obtained during the study period showed that there was a significant difference among all treatments ($p<0.05$). Mean length gain of *O. niloticus* that consumed feed fortified with p2 (8.4±0.09) and p4 (8.2±0.09) was highest among the treatments which did not show variation between each other ($p>0.05$). Fish fed with p3 (7.4±0.09) and p1 (7.3±0.09) also did not show statistically significant difference between each other ($p>0.05$). On the other hand fish fed with control diet in the study period resulted in lowest mean length gain (5.8±0.09). The results of mean length gain in the treatment during the study period showed that there was a significant difference among the groups ($P<0.05$). Survival rate of *O. niloticus* ranged between 95% and 100% which received control diet treatment and treatments with P1, p2, p3 and p4. The survival rate of fish received control diet with the same stocking densities showed 95% survival and in all other probiotic supplemented treatments the survival rate was 100% during the study period. However, survival rate did not show any significant difference among all the treatments ($p>0.05$).

4.1.3. Estimation of Feed Utilization Potential

The feed conversion ratio obtained during the study period showed that there was variation among treatments ($p<0.05$) (Table 1). The highest improvement in FCR was observed in fish fed with p2 (1.04±0.00) and p4 (1.05±0.00) which was statistically uniform ($p>0.05$) followed by p3 (1.16±0.00) and p1 (1.32±0.00), whereas the lowest mean feed conversion ratio was recorded in control diet P0 (1.63±0.00). The mean values of protein efficiency ratio of all treatments observed during the study period indicated that there was a variation among all the treatments ($p<0.05$) (Table 1). It ranged between 2.74±0.00 and 4.28±0.00 and the highest mean value of protein efficiency ratio was recorded in treatment p2 (4.28±0.00) followed by p4 (4.23±0.00), p3 (3.82±0.00) and p1 (3.36±0.00), whereas the lowest mean value of protein efficiency ratio was recorded in control diet (2.74±0.00).

4.1.4. Isolation and Identification of Gut Pathogenic Microbial Flora of *O. niloticus* Fed with Control and Probiotic Diet.

Isolation, identification and characterization of pathogenic microbial species from gastro-intestinal tract (GIT) of *O. niloticus* fed with control diet showed the growth of different opportunistic pathogenic micro flora such as *Salmonella* spp. *E.coli* and *Candida albicans.*

While pathogenic microbial flora in the intestine of *O. niloticus* that received probiotic diet namely p1, p2, p3 and p4 showed the disappearance of *Salmonella* spp., *E.coli,* and *C. albicans.* It was confirmed by colony morphology, gram stain and biochemical tests.

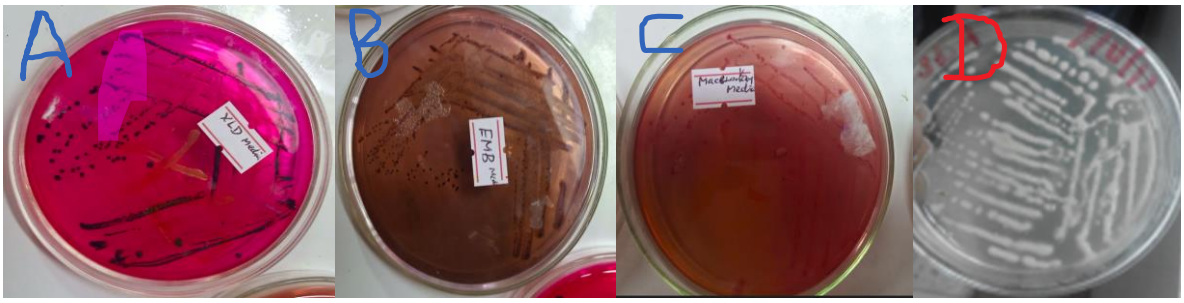

Figure 3: *Photographs showing the growth of micro-flora isolates on solid media.*

A: *is Salmonella spp. grown on XLD agar with black center and slightly red colored colonies,* ***B*** *and* ***C:*** *are E. coli grown on EMB agar with metallic sheen with a dark center colonies and MacConkey agar with bright pinky-red colonies respectively and* ***D:*** *is C. albicans grown on Sabouraud dextrose agar with white to cream colored colonies.*

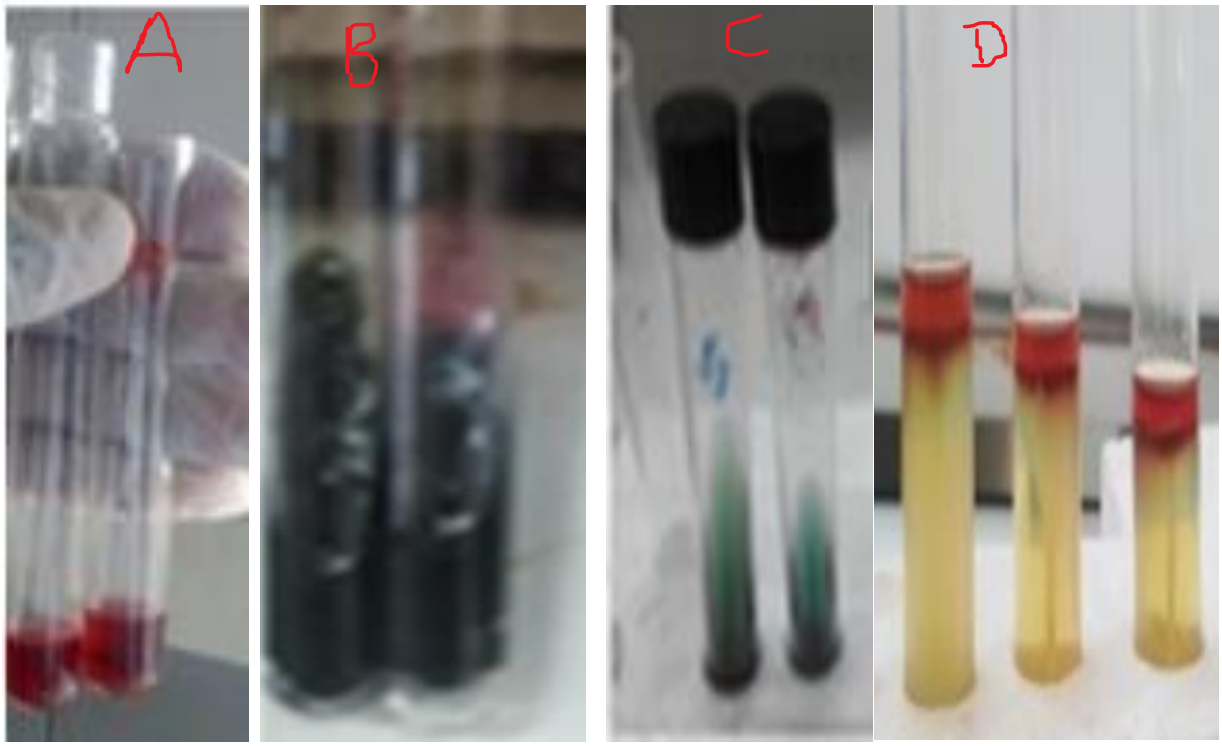

Figure 4: *Photographs showing the biochemical tests of microbial isolates*

A: *is MR* ***positive,*** ***B:*** *is TSI which is Alkaline slant / Acid Butt / H2S Positive,* ***C:*** *is Citrate negative and* ***D:*** *is Indole positive*

4.1.5. Hematological Parameters

The results of hematological examination of fish fed with probiotic and control diet are presented in Table 2.

Table 2: *Mean Values of Hematological Examination*

Parameters	Treatments				
	P0	P1	P2	P3	P4
PCV%	15 ± 0.10^{e}	22.5 ± 0.10^{c}	24.17 ± 0.10^{a}	20 ± 0.10^{d}	23.33 ± 0.10^{b}
Erythrocyte ($*10^{6}$ cells/mm^{3})	0.89 ± 0.01^{c}	1.43 ± 0.01^{b}	1.91 ± 0.01^{a}	1.44 ± 0.01^{b}	1.88 ± 0.01^{a}
Hemoglobin (g/dL)	12 ± 0.09^{c}	14 ± 0.09^{a}	14.07 ± 0.09^{a}	13.4 ± 0.09^{b}	14.4 ± 0.09^{a}
Leukocyte ($*10^{3}$ cells/mm^{3})	8.13 ± 0.08^{a}	5.15 ± 0.08^{b}	4.2 ± 0.08^{c}	5.3 ± 0.08^{b}	4.4 ± 0.08^{c}
Differential leukocyte count					
Lymphocyte%	47 ± 0.12^{e}	50 ± 0.12^{c}	54 ± 0.12^{a}	48 ± 0.12^{d}	53 ± 0.12^{b}
Neutrophil%	30 ± 0.16^{a}	29 ± 0.16^{b}	28 ± 0.16^{c}	30 ± 0.16^{a}	26 ± 0.16^{d}
Monocyte%	19 ± 0.12^{a}	16 ± 0.12^{c}	15 ± 0.12^{d}	17 ± 0.12^{b}	17 ± 0.12^{b}
Eosinophil%	2 ± 0.07^{b}	3 ± 0.07^{a}	2 ± 0.07^{b}	3 ± 0.07^{a}	3 ± 0.07^{a}
Basophil%	2 ± 6.66^{a}	2 ± 6.66^{a}	1 ± 6.66^{b}	2 ± 6.66^{a}	1 ± 6.66^{b}
Total%	100	100	100	100	100

Mean values ((±SE) (standard error)) means levels not connected by the same letter (subscripts) are significantly different ($p\leq0.05$).

The results showed that fish fed with probiotic diet had the highest PCV value in p2 treatment (24.17%) followed by p4 (23.33%), p1 (22.5%) and p3 (20%) whereas, the lowest result of 15% was recorded in fish fed with control diet (p0). These results indicated that, all the values observed were significantly different among the treatment ($p<0.05$). The result of erythrocyte counts in fish fed with probiotic at a dosage of $1.5*10^8$ CFU/mL showed 1.91, 1.88, 1.44, 1.43, and 0.89 $*10^6$ cells/mm^3 in p2, p4, p3, p1 and p0, respectively. The results indicated that, the erythrocyte cell count was highest in p2 treatment, whereas the lowest value was in p0 treatment. The fish consumed the diet fortified with p2 and p4 did not show any significant difference ($p>0.05$) between the treatments. In the same way, fish consumed p3 and p1 diet did not show significant difference between the treatments. However, the results observed showed that there was significant variation among the treatments ($p<0.05$).

The value of hemoglobin content in *O. niloticus* fed the diet that contained probiotics resulted 14.4, 14.07, 14, 13.4 and 12 g/dL in p4, p2, p1, p3, and p0 respectively. It showed that, the highest values were observed in p4, p2 and p1 probiotic supplemented diets in the treatments and there were no statistically significant difference among p4, p2 and p1 treated group followed by p3 treated group. However, fish treated with control diet showed lowest hemoglobin concentration and showed significant variation among the group ($p<0.05$). The highest leukocyte counts were observed in control diet (8.13) followed by p3 (5.3), p1 (5.15), p4 (4.4) and p2 (4.2) consecutively. Statistical analysis showed that, p3 and p1 did not exhibit any significant difference, so also p4 and p2, but among the treatments the difference was statistically significant. Differential leukocyte cell counts showed that, lymphocytes alone

constituted about half of the leukocyte cells in all treatments. The highest percentages were in the order of lymphocyte, neutrophils, monocyte, eosinophil and basophils (Table 2).

4.1.6. Biochemical composition of *O. niloticus*

Table 3 presents the mean percentage value of Moisture, Dry matter, Crude protein, Crude fat, Ash, Crude fiber and total Carbohydrate of *O. niloticus* fed with probiotic feed at an inclusion level of 1.5*108 CFU/mL. The results on body composition of *O. niloticus* fed with probiotic diet showed better improvement in Crude protein, Dry matter and total Carbohydrate percentage than the control diet. On the other hand *O. niloticus* fed with probiotic diet showed significant decrease in mean Moisture content, Crude fat, Ash and Crude fiber percentage compared with control. However, the analysis of biochemical composition of *O. niloticus* showed statistically significant difference among the experimental and control treatments ($P<0.05$).

Table 3: *Mean Values of Biochemical composition of O. niloticus*

Parameters	Treatments				
	P0	P1	P2	P3	P4
Moisture%	63 ± 0.07^{a}	59.87 ± 0.07^{b}	58.77 ± 0.07^{c}	59.9 ± 0.07^{b}	58.57 ± 0.07^{c}
Dry matter (%)	92.6 ± 0.07^{c}	92.2 ± 0.07^{d}	93.2 ± 0.07^{b}	92.3 ± 0.07^{cd}	93.6 ± 0.07^{a}
Crude protein%	53.9 ± 0.06^{e}	55.3 ± 0.06^{c}	56.6 ± 0.06^{b}	54.9 ± 0.06^{d}	57.5 ± 0.06^{a}
Crude fat (%)	22.53 ± 0.08^{a}	19.8 ± 0.07^{c}	19.2 ± 0.07^{d}	20.2 ± 0.07^{b}	19.2 ± 0.07^{d}
Ash (%)	16.03 ± 0.08^{a}	15.67 ± 0.08^{ab}	15.5 ± 0.08^{bc}	15.2 ± 0.08^{c}	15.7 ± 0.08^{ab}
Crude fiber%	0.75 ± 0.01^{a}	0.15 ± 0.016^{d}	0.42 ± 0.01^{b}	0.28 ± 0.01^{c}	0.17 ± 0.01^{d}
Total Carbohydrates (%)	6.78 ± 0.17^{c}	9.08 ± 0.17^{ab}	8.3 ± 0.17^{b}	9.42 ± 0.17^{a}	7.43 ± 0.17^{c}

Mean values ± standard error (±SE) means levels that are not connected by the same letter (subscripts) are significantly different ($p\leq0.05$).

Percentage Moisture content was highest in fish fed with the control diet (63±0.07) followed by p3 (59.9 ±0.07), p1 (59.87±0.07), p2 (58.77±0.07) and p4 (58.57±0.07) respectively in fish fed with probiotics diet (Table 3). The mean percentage value of Moisture among the treatments obtained showed that there was statistically significant variation ($P > 0.05$). Percentage Dry matter (93.6±0.07) was highest in fish fed with p4 probiotics and followed by p2 (93.2±0.07), p0 (92.6±0.07), p3 (92.3±0.07), and p1 (92.2±0.07) respectively. Mean percentage of Dry matter indicated that there was significant difference among all the treatments ($p<0.05$). Percentage of mean Crude protein was highest in *O. niloticus* with p4

(57.5±0.06) diet followed by p2 (56.6±0.06), p1 (55.3±0.06) and p3 (54.9±0.06) respectively, whereas control diet (53.9±0.06) showed lowest percentage of Crude protein. Mean percentage of Crude protein difference among treatments was statistically significant ($p<0.05$).

Percentage of mean Crude fat was highest in fish with control diet (22.53±0.08), followed by p3 (20.2±0.07), p1 (19.8±0.07), p2 (19.2±0.07) and p4 (19.2±0.07) respectively. Mean percentage Crude fat indicated that there was statistically significant variation among the treatments ($p<0.05$). Percentage of mean Ash was highest in fish fed with control diet (16.03±0.08) whereas, fish that received probiotic diet showed significant decrease in the amount of Ash which were 15.7±0.08, 15.67±0.08, 15.5±0.08 and 15.2±0.08 in p4, p1, p2 and p3 respectively. The results of Ash showed that, the difference in values of ash among the treatments were statistically significant ($p<0.05$). The results of Crude fiber ranged between a maximum of 0.75±0.01 to a minimum of 0.15±0.01 in fish with control diet and diet with p1 respectively (Table 3). The results showed significant variation among the treatments ($p<0.05$). The total mean Carbohydrate was highest in fish fed with p3 (9.42±0.17) followed by p1 (9.08±0.17), p2 (8.3±0.17), p4 (7.43±0.17) and control diet (6.78±0.17) respectively and the difference found were statistically significant among the treatments ($p<0.05$).

4.2. DISCUSSION

4.2.1. Water quality parameters

The values of temperature and pH in the current results in Hapas did not show any significant difference ($p \geq 0.05$) and this is because Hapas were installed in the same pond. The amount of dissolved oxygen measured among the treatments showed that, the control diet had less DO than the probiotic incorporated diet. However, the values of water quality parameters are within the acceptable range recommended for the culture of *O.niloticus.* The current result of mean temperature ranges from 27.67-28 ^{0}C, dissolved oxygen from 4.7-4.85mg/L and pH from 7.86-8.2 whch is in agreement with the studies of Hasan *et al.* (2021), who reported that the optimum temperature required for *O.niloticus* is 25-27^{0}C, dissolved oxygen 5mg/L, and pH 5.5-9.0, in the rearing water. In aquaculture, probiotics could improve water quality by influencing the water born microbial population, by decomposing organic matter and by reducing the number of pathogens in the vicinity of farming species (Soltani *et al*., 2019).

Temperature: The findings of the current study is in accordance with the report of Samuel Mengistu *et al.* (2020), who reported 27-32^{0}C as the optimal thermal range for the proper growth of *O. niloticus*. Application of probiotics has a significant effect on water temperature, where temperature is a major factor that affects the metabolism of cultured organism. According to Johnson *et al*. (2017), administration of probiotics *Bacillus* sp. significantly affects the temperature in the aquaculture system. They found that, the population of *Bacillus* sp. helps in regulating water temperature, reducing excessive temperature fluctuations, and maintaining optimal temperature condition for good growth. Therefore, probiotics were

suggested to maintain high levels in production ponds to reduce the organic carbon load and to enhance the water quality and fish health (Hasan and Banerjee, 2020).Temperature plays a crucial role in fish feed consumption. Probiotics has the potency in maintaining water quality and controlling diseases by reducing the number of harmful microbes as reported by Dalmin *et al*. (2001).

Dissolved Oxygen: Dissolved oxygen in the current study showed the highest value in P3 treatment (4.85mg/L) whereas, the lowest DO was in P0 treatments (4.7mg/L). However, all the treatments including control treatment showed that the dissolved oxygen content in hapas was at optimum level to culture *O. niloticus*. This might be due to the better management strategies followed during the experimental period. According to Makori *et al*. (2017), the amount of dissolved oxygen, which is a crucial factor for fish growth, health, and physiology, should be over 5 mg/L for sustainable growth of *O. niloticus*. Dissolved oxygen (DO) is one of the important indicators of water quality because it plays a role in the metabolism and health of aquatic organisms. According to research conducted by Nguyen *et al*. (2021), the use of probiotics *Nitrosomonas* sp. significantly influenced the concentration of dissolved oxygen in the aquarium. They found that the presence of *Nitrosomonas* sp. increases DO concentration, because these microorganisms play a role in the nitrogen cycle and assist in the removal of harmful compounds such as ammonia.

pH: The present results of pH (7.86-8.2) is strongly agreed with the results of Makori *et al*. (2017), who reported that pH is an imperative factor which specifies the health and production output of a water body and the optimum range normally ranges between 6.1-8.3 for *O. niloticus*. According to Smith *et al*. (2018), the use of certain probiotics, such as *Lactobacillus*

acidophilus, has been shown to have a significant effect on water pH. They reported that administering *L. acidophilus* in an aquaculture system resulted in a steady increase in the pH of the water, keeping the pH value within the optimal range for fish growth.

4.2.2. Estimation of Growth Performance Parameters

It is a known fact that inclusion of live Bacteria and Yeast in fish diet at appropriate dosage has crucial role on growth and feed utilization of fish. According to Abdel-Tawwab *et al*. (2020) microbial species has been tested as potential probiotics for aquaculture such as *Bacillus* spp., *Lactobacillus* spp. and *Saccharomyces* spp. and they were incorporated as feed or water additive. The probiotic diets such as p1, p2, p3 & p4 when incorporated alone or in combination showed improved fish body weight gain, specific growth rate, daily weight gain, feed conversion ratio, protein efficiency ratio and survival rate of *O. niloticus* than the control diet. This study agrees with the report of Lara-Flores *et al*. (2003), who reported that although both bacteria and yeast gave better results, the growth performance was better in bacteria than the yeast in *O. niloticus.* According to Allameh *et al*. (2020), Probiotics are able to balance the digestive tract microbes so that they can increase the digestibility of fish by converting carbohydrates into lactic acid which can lower pH, thereby stimulating the production of endogenous enzymes to increase nutrient absorption, feed consumption, growth, and inhibition of pathogenic organisms.

In the current study, fish fed with p2 and combination of p4 probiotics showed maximum improvement on BWG, SGR, DWG, FCR, PER, MLG and SR than control diet and this study aligned with the studies of Mohapatra *et al.* (2012), who reported that, Rohu fingerling *(Labeo*

rohita) fed with a combination of three probiotic diets *(B. subtilis, L. lactis and S. cerevisiae)* showed a higher growth, protein efficiency ratio, nutrient digestibility and lower feed conversion ratio compared to the other groups.

Merrifield *et al*. (2010), also reported that, the mixture of *B. subtilis* and *B. licheniformis* significantly improved FCR, SGR and PER in rainbow trout. In contrast to the current study, Gomez-Gil *et al*. (2000), reported no significant growth performance in fish fed with a bacterial mixture when compared to the control and yeast treatments. These results may be due to the greater adaptive capacity of yeasts in aquatic environments than bacteria. They further described that, the addition of probiotics mitigated the effects of the stress factors and resulted in better fish performance, with better growth in the diets containing the yeast.

Body Weight Gain: The best mean body weight gain was observed in fish fed with p2 treatment (128.4g), and the lowest mean body weight gain was in p0 (82.1g). The best mean body weight gain observed in p2 treatment might be due to the fact that the selected bacterial strain has the highest potential to make available the nutrients to fish than others. This observation coincides with the observation of Merrifield *et al*. (2010), who observed better growth performance in fish when native bacterial strains were isolated and given as probiotic feed. The present results are also very well in support of the study results of Zhai *et al*. (2017), who reported better body weight gain in *O.niloticus* when fed with diet containing *L. plantarum* at a dose of 10^8 CFU/g for 4 weeks. Liu *et al*. (2020), reported that supplementation of *B. pumilus* as growth promoter, improved the weight gain and specific growth rate of juvenile golden pompano, *Trachinotus ovatus*. Bandyopadhay and Das-Mohapatra (2009), observed growth promotion in *Catla catla* fed with *B. circulans* PB7. Hamka and Meryandina

(2020), reported improved body weight gain of catfish *Clarias* sp when the fish was fed with single or combined supplementation of *B. megaterium* and *Pediococcus pentosaceus*

In contrast to the present study, Silva *et al*. (2015) and Garcia-Marengoni *et al*. (2015), who were investigated the effects of probiotic bacteria *(B. amyloliquefaciens)* with a concentration of $1*10^6$ CFU/g, $1.5*10^6$ CFU/g, and $1*10^7$ CFU/g; *(B. subtilis* C-3102, $5*10^8$ CFU/g) respectively on growth performance of Nile tilapia reported that, the supplementation of probiotics did not affect the growth performance of Nile tilapia. This might probably be due to the inappropriate concentration of probiotic supplementation in fish feed. The results of the current study is also inconsistent with the report of Akanmu *et al*. (2022), on African catfish *(Heterobranchus bidorsalis*) juveniles; the reason for this variation could be attributed to the difference of fish species and level of S. *cerevisiae* at 10^2 CFU/mL and *L. fermentum* 10^1 CFU/mL probiotic inclusion.

Specific Growth Rate: The specific growth rate of *O.niloticus* in the treatment with p2 was the highest (2.66) among the groups whereas; the lowest SGR was recorded in control treatment (2.19) and this could be recognized by the action of p2 cells in the gut and secretion of enzymes which promote the digestibility of the diet. The increase in specific growth rate ($p<0.05$) among different treatments observed in the present study is similar with the reports of Al-Dohail *et al*. (2009) who also reported significant difference in the specific growth rate (SGR) of *C. gariepinus* fed with diet containing probiotics.

Daily Weight Gain: The mean daily weight gain of fish plays a crucial role to monitor the growth performance of fish on a daily basis. In the present result, the maximum mean daily

weight gain (1.43 g/day$^{-1)}$ observed in fish fed with p2 might be owing to improved water quality, detoxification of formulated diet by p2 bacteria, and dosage level of probiotics inclusion in prepared diet. The current result is in agreement with the results of Li *et al*. (2016), who found significant increase in daily weight gain in freshwater pomfret with the administration of probiotics containing *S. cerevisiae.* As reported by Irianto and Austin (2002), the positive impacts of probiotics can also be traced to their role in boosting appetite which is achieved through production of vitamins and detoxification of feeds or because of decomposition of undigested compounds. This might be attributed to the generation of certain enzymes such as peptide by probiotic bacteria that hydrolyze micro molecular compounds converting them to peptides and amino acids (Ghosh *et al*., 2002).

The current study results on daily weight gain are not comparable with the results of Hasan and Banerjee (2020). While the present study reported a higher daily growth rate (1.43 g/day) with probiotic feed, the report of Hasan and Banerjee (2020) is much lower (0.26g/day) when Nile tilapia was fed with a combination of commercial feed and commercial probiotics. This variation might be occurred due to the difference in probiotics used.

Survival Rate: Probiotic utilization in fish improves nutrient absorption, as well as survival rate of the rearing fish. According to Merrifield *et al.* (2010), probiotic consumption has been developed to enhance fish appetite, digestibility, stimulating the secretion of digestive enzymes and maintaining the balance of intestinal beneficial micro-flora. This might be exhibited to proper digestion and better nutrient absorption in fish body and enhances survival rate. While Smith *et al*. (2018) reported significant survival of freshwater pomfret with the addition of probiotic bacteria *L. acidophilus*, the survival rate of Nile tilapia fed with

probiotics in the current study did not shows any statistically significant difference among the treatments. The difference in survival rates reported by authors may be due to the duration of the culture period, probiotic species administered, difference in fish species stocked, stocking density, water quality and the quality of basal diet (Sultana and Hossain, 2018)

4.2.3. Feed utilization

Feed Conversion Ratio: Feed conversion ratio (FCR) is used to assess the amount of feed utilized and convert in to flesh. The best feed conversion ratio in the study (FCR) ((1.04) was observed in p2 and this indicate that p2 has the ability to build more flesh at low amount of feed. The current result is in alignment with the report of EL-Haroun *et al*. (2006), who reported significantly higher feed conversion efficiency with commercial probiotics compared to the control.

Johnson and Wagner (2017), reported that, the administration of probiotics containing *B. subtilis* to freshwater pomfret resulted in an increase in the feed conversion ratio. According to Lara-Flores *et al.* (2010), probiotics has the ability to improve the intestinal microbial flora, absorption ability, enzyme activity and degradation of higher molecular weight protein to lower molecular weight peptides and amino acids. On the contrary, Hidalgo *et al*. (2006), did not observe any significant influence for FCR when the probiotics were added in fish diet for juvenile dentex *(Dentex dentex).* The contradiction between the current and previous study may be due to the selection of probiotics and the difference in fish species selected.

Protein Efficiency Ratio: Protein efficiency ratio is based on the weight gain of fish with respect to its intake of a particular feed during the experimental period. This indicates high

PER will be obtained if the weight gain is high compared to the dietary protein intake. In the present study, fish fed with diet blended with p2 had higher mean PER value (4.28) and this indicated bulk of the diets containing probiotics were fully consumed and converted in to fish flesh. The better PER contributes to optimizing protein use for growth which is the most expensive feed nutrient. The current finding is similar with the results of Abdel-Tawwab and Ahmad (2009), who reported that probiotics supplemented diet, had a significant effect on protein efficiency ratios compared to the control in *O. niloticus.* The lowest PER was found in control diet (2.74). The current study shows that high protein diets supported the increased body weight gain which is an indication for adequate amount of protein content, higher protein intake and optimal usage of feed consumed.

4.2.4. Gut Pathogenic Micro-flora from *O. niloticus* Fed with Control Diet.

Bacterial and fungal contamination of fish is considered the main cause of signs of spoilage as off flavor and unpalatable taste and it may constitute a public health hazard as well as many of economic losses (Hassan *et al*., 2012). The current result is in agreement with the studies of Nebyu Kassa and Marshet Adugna Mitiku (2021), who isolated pathogenic bacteria such as *A. hydrophila*, *Flavobacter* spp., *Vibrio* spp., *E. coli*, and *Aeromonas salmonicida* from the intestine, skin, gill and kidney of *O. niloticus* and observed comparably higher bacterial load in the intestine than other organs like gills, skin and kidney. In similar manner to the current study, Begonesh Bekele *et al*. (2019), isolated pathogenic bacteria belonged to the genera *Vibrio, Escherichia, Aeromonas, Pseudomonas, Salmonella* and *Streptococcus* from the intestine, gill, skin and liver and observed highest isolates in the intestine of *O. niloticus*

Similar to this, Cardozo *et al*. (2018), recovered pathogenic *E. coli* from the intestine of *O. niloticus* in the northeast region of the state of Sao Paulo. Daoust *et al*. (1992), also isolated pathogenic *Salmonella* spp. from the intestine of *O. niloticus.* The current result is similar with the studies of Zaky and Ibrahim (2017), who isolated pathogenic Bacterial spp. such as *E. coli, Proteus mirabilis* and *Klebsiella* and fungal species, particularly toxigenic species, such as, *Aspergillus* spp, *Penicillium* spp and *Fusarium* spp. from the intestine, gills and skin of *O. niloticus*. According to Hashem (2011), pathogenic fungal species such as *Paecilomyces lilacinus, P.variotii* and *Phoma herbarum* as well as mycotoxin producing fungi including *A. flavus, A. clavatus, A. ochraceous* and *A. parasiticus,* were isolated from the intestine and gills of *O. niloticus, C. gariepinus* and *Tilapia zilli* and the highest number of species was isolated from *C. garipinus* followed by *O. niloticus, and Tilapia zilli*.

As reported by Tartor *et al*. (2018), pathogenic *Candida* spp. such as *C. albicans, C. lipolytica* and *Trichosporon mucoides* were isolated from the intestine, gills, and skin of *O. niloticus, C. garipinus* and *Mugil cephalus*. Similar observations were made by Chi *et al*. (2010), who reported that most fungal attacks in fish are associated with unsuitable environmental conditions, bacterial or viral infections, or when fish, are lost their mucus protection. Gatesoupe *et al*. (2005), reported the presence of *Candida* sp. in the gut of healthy rainbow trout. However, he added that, the affected fish exhibited normal swimming behavior, feed intake and no disease symptoms. This result is similar with the present study. However, in contrast to the current study, Park *et al.* (2012) observed some abnormality such as distended stomach and swim bladder of the fingerlings of Sturgeon, *Acipenser ruthenus* due to *Candida* infection. This difference may be due to the severity of infection and the stages of the

fish. In the present study, the growth parameters such as body weight gain, daily weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio, length gain, survival rates; hematological parameters such as PCV, Hb, RBC, WBC and Crude protein are affected in control treatment where as in probiotic treatment, all the growth parameters were not affected.

4.2.5. Gut Pathogenic Micro-flora from *O. niloticus* Fed with Probiotic Diet

It is an accepted fact that preparing quality fish diet incorporated with probiotics has profound beneficial effects on fish gut micro flora. Supplementation of non-pathogenic live microorganism in fish nutrition has a beneficial effects on the host intestinal health as well as the general wellness of the host. The disappearance of *Salmonella* spp *E.coli* and *C. albicans* in four probiotic supplemented (p1, p2, p3, p4) treatments might be due to the capacity of these probiotics to bind the intestinal mucosal cell receptors for immune-stimulation, eliminates some members of pathogenic *Enteriobacteriaceae* and fungal strains, reduced stress response, and improved gastro-intestinal morphology (Van-Nguyen *et al*., 2019).

The current study aligned with the studies of Irianto and Austin (2002), who reported that probiotics has the ability to inhibit the colonization of potential pathogens in the digestive tract by their anti-microbial compound. The current finding also support the report of Marzouk *et al*. (2008), who stated that co-culture of yeast and bacteria in fish would significantly reduce coliform bacteria. As reported by Faramarzi *et al*. (2011), probiotic will improve digestibility and growth of fish. The current study reported 100% survival in fish fed with probiotic feed. Abd El-Rhman *et al*. (2009), reported 75% survival of tilapia fed with diets containing probiotic feed, *Micrococcus luteus.* This difference in survival rate of fish may be due to the

type of probiotics used. Similarly, the dietary inclusion of *B. subtilis* and *B. cereus* at 10^8 CFUg−l in the current study increased intestinal cell count and microvilli density, thereby enhancing disease resistance by altering the intestinal micro-biota composition of tilapia. The increase in microvilli density suggests that an increase in the absorptive surface area of the intestine reduces the colonization rate by pathogenic bacteria, consequently increasing the resistance of fish to pathogenic microorganisms (Xia *et al*., 2020).

As reported by Merrifield *et al.* (2010), Probiotics may minimize the incidence of diseases or lessen the severity of outbreaks in aquaculture or fish culture. Zapata and Lara-Flores (2013), also reported that *Leuconostoc mesenteroides* inhibited the growth of pathogenic bacteria in *O. niloticus* particularly *Vibrio* spp. and *Mycobacterium* spp. Nile tilapia that fed with diets containing *Rummeliibacillus stabekisii* probiotic showed higher counts of beneficial bacteria such as *Bacillus* and *Lactobacillus* and lower counts of pathogenic bacteria such as *Streptococcus* and *Staphylococcus*, suggesting that *R. stabekisii* can alter the intestinal micro-biota and contribute to immunomodulation and disease resistance (Tan *et al*., 2019). Probiotics are primarily used as feed additives to prevent infectious intestinal diseases through the secretion of micro-toxins that inhibit the growth of other virulent micro-organism (such as *E. coli* and *Salmonella* spp) in the intestinal lumen of the host (Barth *et al.,* 2009). The current findings contradict with the study of Shelby *et al*. (2006), who found that feeding commercial probiotics for 94 days did not prevent *Streptococcal* disease. The variation between the current findings and the previous might be potentials of probiotics used and dosage supplemented in the diet.

As reported by Balcázar *et al*. (2009), probiotics may positively affect gut defenses of the host by creating a hostile environment for pathogens, competing for essential nutrients, blocking adhesion sites on the epithelium, and modulating immune responses at physiological and molecular levels. Feeding tilapia with 10^{10} CFUg−1 of *B. licheniformis* improved the disease resistance against *Streptococcus iniae* (Han *et al*., 2015). Similarly, dietary *Bacillus* spp. increased resistance to *S. iniae* infection in rohu, *Labeo rohita* (Ramesh *et al*., 2017), and Atlantic salmon *Salmo salar* (Wang *et al*., 2019). *Bacillus* enables the occurrence of many overlapping protection mechanisms such as synergistic, antagonistic, and competitive exclusion and immune-stimulating effects, being the most widely used probiotics in aquaculture (Nayak, 2021). In addition to the protection against bacterial pathogens, probiotics can also protect against pathogenic viruses, fungi, and protozoa (Chauhan and Singh, 2019). According to Abdel-Tawwab *et al*. (2020), probiotic yeast *S. cerevisiae* supplemented to *O. niloticus* diets increased resistance against the pathogenic fungus *Aspergillus flavus* at an optimum inclusion level of 6 g kg−1 diet. Lactic acid bacteria compete with pathogens for adhesion sites on the mucosal epithelium of the gastrointestinal tract in several fish, including tilapia (Van-Nguyen *et al*., 2019).

4.2.6. Hematological Examination

Examinations of hematological parameters help in monitoring disease conditions and physiology of fish in natural and culture environment (Fazio *et al*., 2015).

Packed cell volume Determination: PCV (or Hematocrit) is the measure of erythrocyte content in blood as a percentage of erythrocytes in blood volume. Anemia is most simply and

reliably estimated by measuring the packed cell volume. The current result of PCV cells showed that the highest percentage was observed in fish fed with p2 (24.17%) treatment and it might be because of good potential of supplemented probiotics in the diet. The lowest results of PCV were observed in fish treated with control diet (15%) and this might be due to the infection in fish with bacteria and fungus which caused reduced PCV value in the fish. According to Tamamdusturi and Yuhana (2016), the decreased number of hematocrits, hemoglobin and erythrocytes could be caused by an infection that may be resulted in the changing of red blood cells to become lysis. As reported by McBeath *et al*. (2015), PCV value depends on erythrocyte size and number which can be affected by various factors, such as water quality parameters, drugs and some infectious diseases. The current result of PCV value was within the acceptable range according to Grant (2015), who stated that PCV values for different fish species range from 9.4 to 33.53%.

The present findings are also in agreement with the results of Fazio *et al*. (2019), who stated that PCV measured in different fish species ranged from 17.80 to 53.33%. However the current result of PCV of fish fed with control diet showed below the range compared with the results of Osman *et al*. (2018), who found that the PCV value of Nile Tilapia ranged from 20.49 - 38.76%. The reduced value of PCV might be because of the existence of pathogenic bacterial and fungal micro-organism in fish. In the case of *Cyprinus carpio,* the value of PCV obtained by different authors ranged from 14.0–44.0% (Witeska *et al*., 2016). The current findings of PCV value in all treatment among the groups are in acceptable range reported by Grant (2015).

Erythrocyte (RBC) Count: Erythrocytes are the most abundant blood cells in fish and usually constitute 98–99% of all blood cells in animals. It reflects the total number of red blood cells in the circulating blood. Erythrocyte count was an important parameter to evaluate fish health status and it depends on various environmental factors like water temperature (Paul *et al*., 2019).

The current findings of erythrocytes in fish fed with probiotic fortified diet are coincided with that of Osman *et al*. (2018), who stated that, the average number of erythrocyte cells in *O. niloticus* was 1.49 - 2.39*10^6/mm^3 whereas the reduced value of erythrocyte counts in fish fed with control diet observed in this study might be owing to the low quality feed. The range of current findings (0.89*10^6 cells/mm^3 - 1.91*10^6 cells/mm^3) are also in contradiction with the findings of Ismail and Mahboub (2016), who stated that, the average number of erythrocyte cells in Nile Tilapia was ranged from 1.13-1.31*10^6/mm^3 the variation might be because of types of feed supplementation, frequency of feeding per day and variation in the environmental factors. This was supported by Osman *et al*. (2018), who stated that there was a significant reduction in the value of red blood cells, hemoglobin, and hematocrit of tilapia samples taken from polluted environments compared to those which were taken from less polluted environments. The variation might also be due to biological factors such as age, sex and reproductive status among various populations of the same species.

The current findings are in agreement with the results of Witeska *et al*. (2016), who observed the RBC of *C. carpio* was found within 0.33 to 2.95*10^6/mm^3. As stated by Fazio *et al*. (2019), RBC determined in different fish species ranged from 0.81 to 3.73*106/mm^3 which were somewhat consistent with the current findings. Basically, the number of erythrocytes and

hemoglobin were interconnected. If there is a decrease in the number of erythrocyte cells and hemoglobin levels, it would affect oxygen transportation and metabolism. In addition, changes in the number of erythrocyte cells could be a stress indicator in fish due to the presence of toxins or pollutants in aquatic environments (Kori-Siakpere *et al*., 2009).

Hemoglobin Determination: Measurement of hemoglobin concentration in fish blood directly reflects the ability of erythrocyte to carry oxygen. The current findings are parallel with the results of Yaghobi *et al*. (2014), who observed hemoglobin values from 12.58 to 13.98 g/dL. According to Fazio *et al*. (2019), who determined Hb values of different species of fish reported that the values ranged between 4.70 and 16.6 g/dL and the current result agrees with their findings.

The present results conflict with those of Osman *et al*. (2018), who stated that, hemoglobin content ranged from 7-11.55 g/dL in tilapia. The better result of hemoglobin value observed in the current study of (14.4) might be due to fish fed with quality diet and the management of water quality parameters during the experimental period. According to Summarwar (2012), there was a remarkable decrease in the number of red blood cells, hemoglobin, and hematocrit in contaminated environments compared to uncontaminated environments. The present result opposes with the study results of Hasan *et al*. (2021), who found that the highest mean hemoglobin (5.70 g/dL) was recorded in *O. niloticus* fry fed with a locally available commercial probiotics. This variation in the finding might be because of different factors such as age, species variation and reproductive status. In addition, Daneshvar *et al*. (2012), stated that the main function of hemoglobin is to transport oxygen and it is one of the parameters of fish health. The decrease in hemoglobin concentration could be caused due to infections

within stressful conditions. Stress that occurred could be caused by the uncontrolled environmental conditions (Valenzuela *et al*., 2007).

Leukocyte (WBC) Count: Leukocyte count is an important parameter in the assessment of the immune status in fish. These present findings of WBC count support the results of Abdel-Tawwab *et al*. (2020), who reported that probiotics such as *Psychrobacter namhaensis, B. subtilis, L. plantarum, Lactobacillus rhamnosus*, and *S. cerevisiae* were found to reduce white blood cells in *O. niloticus*. The reduction of WBC count in fish fed with probiotic supplemented diet is an indication of a good health status and they were not subjected to stress from disease and infection compared to the control. In the current results, fish fed with probiotic supplemented diet showed better health than fish with control diet.

The growing leukocyte value resulted in fish fed with control diet might be due to stressful condition developed through infection by bacteria and fungi. This was supported by Mazrouh *et al*. (2015), who explained that, leucocyte number in fish blood increases when fish are exposed to pollutants. The current result opposes the findings of Osman *et al*. (2018), who stated that the total leucocyte number of Nile Tilapia was about 35.7 – 50.7*10^3 cells/mm^3. The highest value observed in previous findings might due to fish exposed to infections by toxins, bacteria and fungus. According to Svobodova *et al*. (2012), WBC in *C. carpio* was 10–80*10^3/mm^3. Fazio *et al*. (2019), reported that WBC in different fish species ranged from 9.41 to 829.33*10^3/mm^3. Moreover, many intrinsic and environmental factors may affect leukocyte count in fish such as sex, season, feeding habits, stress, aquatic pollution and diseases (Ahmed *et al*., 2020).

Differential Leukocyte Count: Differential leukocyte counts such as lymphocyte, neutrophil, monocyte, eosinophil and basophil are important to assess fish health. In the current study, lymphocytes alone constituted about half (50%) of the leukocyte cells in all experimental treatments and served as the body's defense system against infection. The present study is comparable with the findings of Osman *et al*. (2018), who reported more similar value of lymphocyte cell as 59.5%. According to Witeska *et al*. (2016), lymphocytes are the most abundant leukocytes in healthy teleost and the average percentage are usually varied from about 50 to 99%. Changes in the number of leukocyte cells could be caused due to the stress resulted in the declining of fish's immune system which made it susceptible to disease attack (Ariweriokuma *et al*., 2016). Neutrophils are the second most common fish blood leukocytes and are capable of eliminating pathogens through multiple complementary mechanisms. It is identified as round cells larger than lymphocytes, condensed nucleus that is either round, oval, kidney-shaped or lobed. Activated neutrophils become powerful killers, utilizing toxic intracellular granules; secrete neutrophil extracellular traps which consist of antimicrobial granular proteins that prevent the dissemination of invading pathogens (Pijanowski *et al*., 2013). The current results of neutrophil ranged from 26-30% in the treatment are in agreement with the report of Witeska *et al*. (2016), who reported that, the percentages of neutrophils reported by various authors in *C. carpio* were 0.4–34%. The current finding opposed the result of Kumar and Ramulu, (2013), who reported that the average value of neutrophils in Striped Catfish was 8-23%. This variation might be because of difference in fish species and feeding strategy.

Monocytes are the largest leukocytes, usually round to oval or sometimes irregular-shaped cells with abundant light gray-blue cytoplasm, often containing vacuoles. The nucleus can be round, oval, kidney-shaped with loose chromatin (Bianchi *et al*., 2014). Blood monocytes contribute to tissue-resident macrophage populations during inflammatory conditions and the depletion of resident macrophages in their environment (Varol *et al*., 2015). In the present findings monocyte ranged from 15-19% and the results are in contradiction with the study conducted by Osman *et al*. (2018), who observed monocyte cell values ranged from 12-14.47% in Striped Catfish. This variation might be due to age of fish, species characteristics or nutritional differences. According to Witeska *et al*. (2016), the percentage range of monocytes in *C. carpio* by various authors are 0.1–13.6%.

The eosinophils of fish are effective in controlling parasitic infections. According to Old and Huveneers (2006), eosinophils are identified as large round cells with pale blue-gray cytoplasm filled with large round to oval and the color of the granules varies with species, from bright red or orange to pale pink with round or lobed nucleus. In the current study, the percentage of eosinophil ranged from 2-3% in laboratory investigation and the result is in conflict with the study conducted by Osman *et al*. (2018), who stated that, the result of Striped Catfish differential leucocyte obtained from eosinophil cell values was 1.8%. This variation might be because of age of fish to species characteristics or nutritional differences. Vazquez and Guerrero, (2007), also reported that the occurrence of blood eosinophils in teleost blood ranges from 0–1.87%. Basophils are the least abundant leukocyte in most fish species and it triggers the production of antimicrobial factors (Edholm *et al*., 2011). The present result however differed with the result of Osman *et al*. (2018), who observed greater differential

leucocyte value as 3.72% in Striped Catfish. The variation might be due to differences in fish species and the method of processing of the feed.

4.2.7. Biochemical composition of *O. niloticus*

In the current findings Crude protein, Dry matter and total Carbohydrate contents are improved in fish fed with probiotic diet than control and the results were similar with the study of Jan *et al*. (2023), who reported that Grass Carp Fingerlings fed with probiotics *(Lactobacillus rhamnosus)* diet showed significant increase in Crude protein and decrease in the other parameters such as Moisture content, Crude fat and Ash. Similar results were observed with the study of Wu *et al*. (2022), who reported that dietary inclusion of vitamin A significantly decreased Crude fat, Ash and total Moisture content of grass carp *(Ctenopharyngodon idella*) compare to that of control group.

In similar manner to the current study, Opiyo *et al*. (2019), reported that Nile tilapia fed with *B. subtilis* containing diet showed increase in Crude protein and reduction in Crude fat when compared to control diet. According to Bagheri *et al*. (2008), who reported an increase in the level of Crude protein and reduction in Crude fat content were observed in *O. niloticus* and *O. mykiss* fed with probiotic supplemented diets. This manifested the big role of effective microorganism (bacteria) to stimulate protease enzymes and inducing higher availability of feed protein which was more beneficial to fish (Gao *et al*., 2021). As reported by Abdel-Tawwab *et al.* (2008), *S. cerevisiae* plays a key role in enhancing food intake resulting to improvement of fish body composition. Therefore, the higher carcass protein content in this study could be attributed to more proteins secreted by the probiotics in the gut of *O. niloticus*

and effective conversion of ingested food in to structural protein building more muscle (Zapata and Lara-Flores, 2013).

This represents fish with more protein and less fat is desirable for aquaculture (Hassaan *et al*., 2018). The current result was in agreement with the report of Bahnasawy *et al*. (2020), who found that *O. niloticus* fed with control diet showed highest moisture content compared with probiotic (EM.1) bacteria in the diet. Ayoola *et al*. (2013), who found that C. *gariepinus* fed with control diet has the highest moisture content than that of probiotics (*Lactobacillus* and *Bifidobacterium*) diet. In the current finding the percentage of Dry matter was significantly increased in fish fed with probiotic feed compared with control. According to Eissa *et al*. (2022), *O. niloticus* fed with *Pediococcus acidilactici* probiotics in the diet showed the highest percentage of Dry matter compared with control diet.

In contradiction to the current results EL-Haroun *et al*. (2006), suggested that *O. niloticus* fed with different commercial probiotics in the diet were observed as no statistical differences in protein, carcass moisture and ash content. The same contradicting results were reported by Lara-Flores *et al*. (2010), who reported that diets containing probiotics could not significantly affect the body composition of Nile tilapia. In addition, the effects of administration of *B. subtilis* in the diet on body composition showed that this probiotic improved the fat content of the carcass, but no significant differences were observed for moisture, ash and protein content. Therefore, it seems that probiotics have no significant influence on the body composition of fish and do not affect strongly tissue synthesis (Ghosh *et al*., 2008). The difference might be due to variation of probiotics, dosage and environment in which fish where cultured.

5. CONCLUTIONS AND RECOMMENDATIONS

5.1. CONCLUSIONS

From the findings of the current study, the following conclusions are drawn

- Probiotic bacteria tested in the current study ***improve water quality*** by decomposing unutilized feed, fish excreta and directly up-taking toxic material in the water and maintained good water quality environment for the culture of *O. niloticus*.
- Supplementation of probiotic bacteria such as *Bacillus subtilis subtilis and Bacillus subtilis inaquosorum* and yeast (*Saccharomyces cerevisiae*) alone and in combination to *O. niloticus* diet at an inclusion level of $1.5*10^{8}$CFU/mL ***improve FBW, FBL, BWG, SGR, DGR, FCR, PER and SR*** because the probiotics has the potential to produce digestive enzymes to reduce the amount of anti-nutritional factors of the feed ingredients, enhance the appetite, improving the digestion and nutrient absorption of the host.
- The feed of *O. niloticus* blended with probiotics at an inclusion level of $1.5*10^{8}$CFU/mL ***eliminate pathogenic gut micro-flora and enhance the beneficial gut micro-flora,*** because it has the ability to compete for binding sites to preventing the connection by pathogenic bacteria, produce of antibacterial substances, compete for nutrients and stimulation of immune system by increasing the production of antibodies.
- Probiotics enhance ***hematological parameters*** such as percentage of PCV, hemoglobin concentration, number of RBC, WBC as well as percentage of differential leukocyte counts in *O. niloticus* fed with probiotic blended diet than those fed with control diet, and the hematological parameters are in acceptable range in fish fed with probiotic

incorporated diet whereas in control diet some of the values are below the acceptance level.

- Supplementation of probiotics in feed improve ***biochemical constituents*** such as Crude protein, the most expensive feed nutrient for the growth of fishes, Total carbohydrate and Dry matter content in *O. niloticus*.

5.2. RECOMMENDATIONS

Based on the findings of this study, the following recommendations are forwarded:

- The study suggests that fish farmers should use proper and effective probiotics, create awareness and training about the benefits and methods of probiotics, and develop diverse and locally available probiotics and ensure their supply.
- The study also suggests that future research should explore the effects of probiotics on other fish species, other probiotic strains, and their interactions.
- Future research should also use more advanced methods and instruments to measure and analyze the different parameters, and test the validity and reliability of the findings.
- Future research should also address the challenges and constraints of using probiotics in aquaculture, such as the cost, accessibility, stability, and regulation of probiotics.

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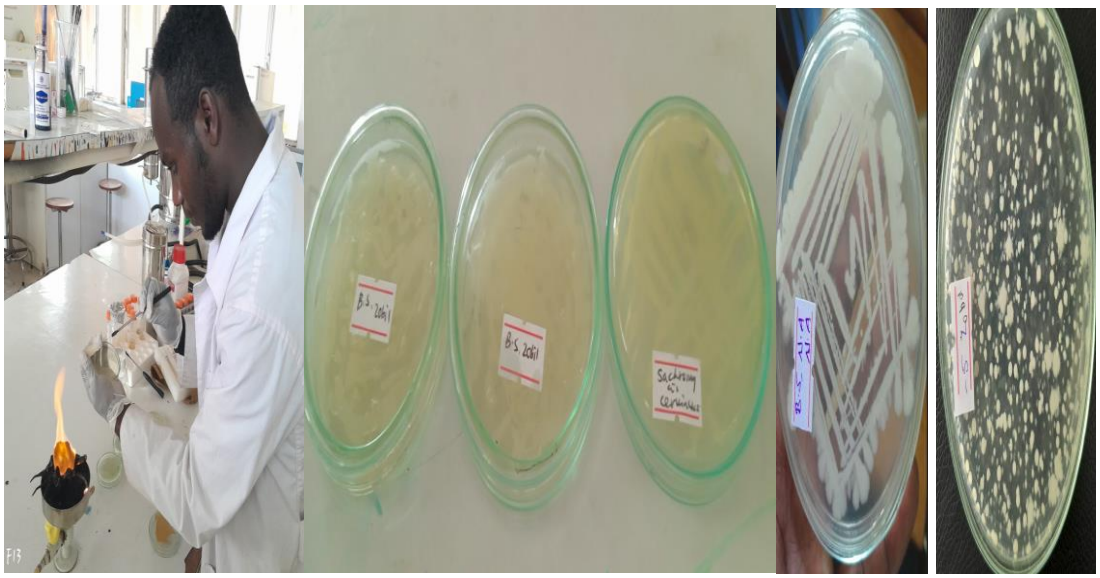
APPENDICES

Appendix 1: *Preparation of pelleted fish feed.*

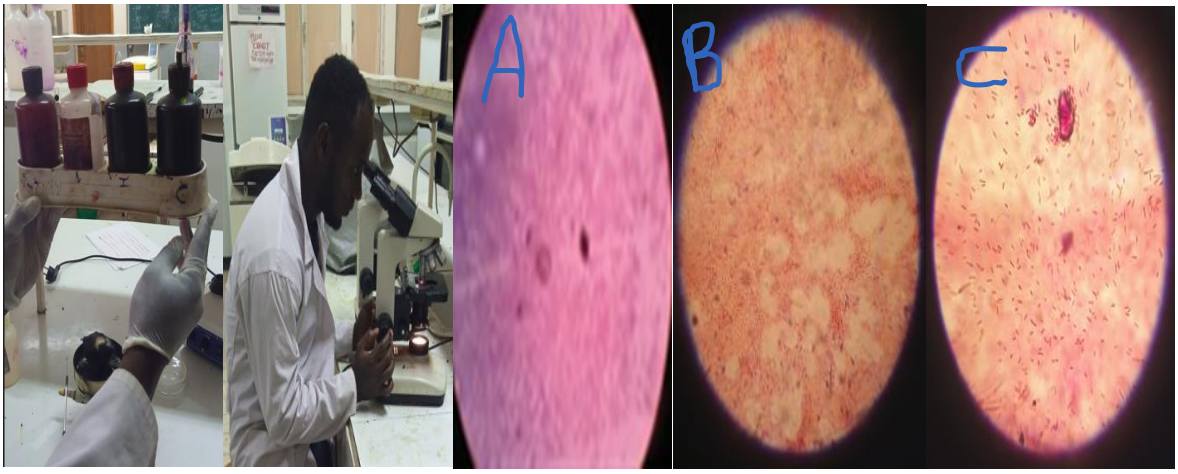
Appendix 2: *Feeding and recording growth and water quality data.*



Appendix 3: *Enumeration of probiotics.*

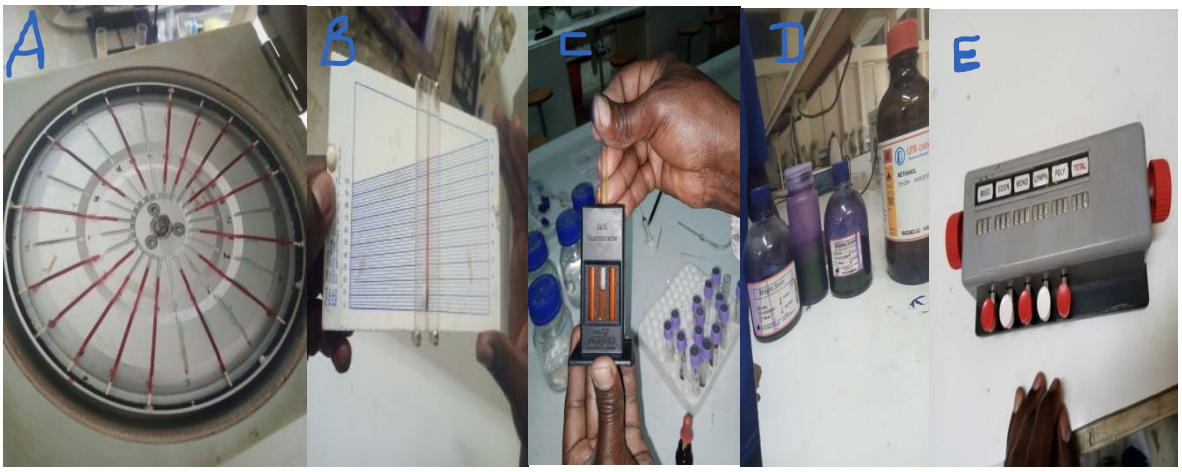
Appendix 4: *Standardizing probiotics according to the standard dose of McFarland.*

Appendix 5: *A: Fish gut dissection and media preparation for microbial isolates.*



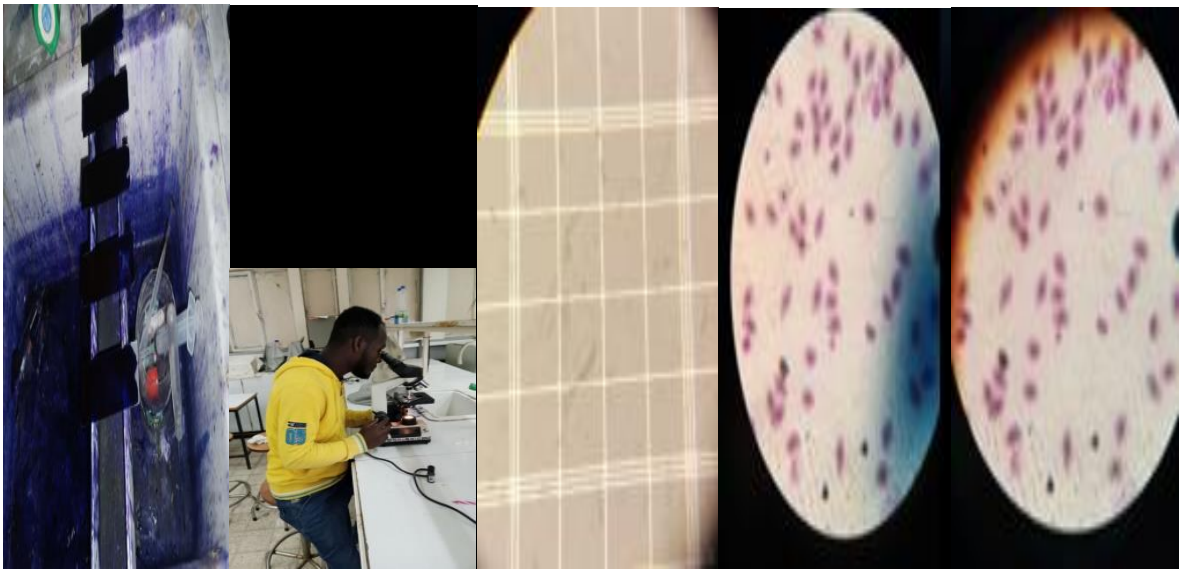
Appendix 6: *Microscopic observation of microbes.*

A: *C. albicans*, ***B:*** *Salmonella spp. and C: E. coli respectively*



Appendix 6: *Determination of hematological parameters.*

A *and* ***B****: PCV determination* ***C****: hemoglobin determination* ***D****: Giemsa stain, wright stain and methanol* ***E****: Differential leukocyte cell counting machine*



Appendix 7: *Counting of RBC and WBC within the square.*

Appendix 8: *Trashing and oven drying whole fish body.*