EFFECTS OF SUPPLEMENTATION WITH DIFFERENT LEVELS OF A MULTI STRAIN PROBIOTIC ON THE PERFORMANCE OF LAYING CHICKENS

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DECLARATION AND APPROVAL

I Nkiambuo Brian Ndambuo hereby declare that this thesis is my original and independent research work. The thesis work was carried out under the supervision of Dr. Judith Atela and Prof. Charles Gachuiri. This thesis or any part of it has not been previously presented or submitted in any University or Institution of higher learning for the award of any Degree, Diploma or Certificate.

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DEDICATION

This work is dedicated to my very supportive family headed by Mr. Nkiambuo Charles Mbiluefeh and Mrs. Nkiambuo Caroline Feneh who have stood by me throughout my study and contributed towards its success financially, socially and morally. Not forgetting my siblings Nkwentie Joseph Anombie, Nkiambuo Gillian Peleke, Nkiambuo Barry Ntumbiwoh, Nkiambuo Jerry Mbipowoh, and Nkiambuo Nadesh for their support. I thank each of them for encouraging and motivating me especially in my education. May The Almighty God bless and protect you.

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LIST OF ABBREVIATIONS

°C- Degree Celsius

AAS- Atomic absorption spectrophotometer

AGP- Antibiotic Growth Promoters

AME- Apparent Metabolizable Energy

ANOVA- One-way analysis of Variance

AOAC- Association of official analytical chemists

CP- Crude protein

CRD- Complete Randomize Design

ECW- Egg content weight

EW- Egg weight

FCR- Feed Conversion Ratio

FI- Feed intake

GIT- Gastrointestinal track

HCl- Hydrochloric acid

HDEP- Hen Day egg production

KeBS- Kenya Bureau of Standards

NaCl- Sodium Chloride

NRC- National Research Council

PC- Protein consumed

SG- Specific gravity

ST- Shell thickness

SW- Shell weight

WI- Water intake

YC- Yolk colour

ABSTRACT

Use of antibiotic growth promoters (AGP) in poultry production to enhance performance is not recommended due to accumulation of antibiotic residues end products which can confer antimicrobial resistance (AMR) to consumers. Inclusion of probiotics in layers diets has been reported to enhance laying performance and egg quality without the risks of AMR. A study to determine the effect of supplementation of a multi-strain probiotic on performance and egg quality (internal and external) was carried. The specific objectives were 1.) To investigate the effects of supplementation of different levels of a multi-strain probiotic on the performance of ISA Brown and 2.) To investigate the effects of supplementation of different levels of a multi-strai probiotic on egg quality of ISA Brown layers. A multi-strain probiotic (MolaPlus®) was purchased from a reputable supplier and administered to laying birds via drinking water at different levels; Prob0 (control), Prob2.5(2.5ml/L), Prob5(5ml/L), Prob10(10ml/L) and Prob15(15ml/L). A layer's mash feed (Unga feeds) was purchased from a reputable feed manufacturer. One hundred and fifty (150) 65 weeks old ISA Brown were recruited from a laying flock at the Poultry Unit, University of Nairobi and assigned in CRD of five (5) treatments each replicated five times with six birds per replicate. The feed intake, body weight, egg weight, egg specific gravity, yolk colour, eggshell weight, and thickness was recorded weekly for 5 weeks. Hen day egg production and water intake were recorded daily while the mineral content of the eggshells (Ca & P) was determined during the 1st, 3rd and 5th week. The mean daily feed intake was not significantly affected ($p>0.05$) by treatment Prob0 (151.6g), Prob2.5 (145.6g), Prob5 (143.5g), Prob10 (139.4g) and Prob15 (145.4g) respectively. Supplementation tended to increase egg weight in treatment groups though non-significantly ($P>0.05$) compared to Prob0. The feed conversion ratio (FCR), body weight, hen day egg production, water intake, protein consumed and yolk colour were not ($P>0.05$) affected by inclusion levels of probiotics. The specific gravity, shell weight, shell thickness and eggshell % were not influenced significantly ($p > 0.05$) by probiotic inclusion levels. There was a significant ($p < 0.05$) increase in calcium content of the shells with inclusion of probiotics with Prob5 (52.8%) being the highest. The phosphorus content of the eggshells was significantly higher ($p < 0.05$) for Prob5 (0.5311) and Prob15 (0.5093) compared to control (Prob0), Prob2.5 and Prob10. From the findings, it can be concluded that a multi-strain probiotic (MolaPlus®) can be included in layers diet via drinking water to improve egg quality.

Keywords: Probiotics, Performance, egg quality, layers, drinking water

CHAPTER ONE

1.0 Introduction

1.1 Background Information

A significant portion of players in the poultry industry within different states have eliminated antibiotics usage to promote growth due to the rising concerns regarding the development of resistant microorganisms on prolonged use of antibiotics (Van *et al.*, 2020). Alternatives such as probiotics, prebiotics, synbiotics, essential oils and organic acids are instead being used to achieve similar benefits as antibiotics (Abd el-hack *et al.*, 2022). Probiotics supplements are live microbes made from various unique strains of bacteria that have beneficial effects on poultry (Krysiak *et al*., 2021). The microorganisms have the ability to contribute greatly to improving the host's health benefits (Krysiak *et al*., 2021). The probiotic properties, benefits, and purpose depend on the specific strains of bacteria used in the manufacture (Abd el-hack *et al*., 2020). Microorganism's strain, inclusion levels and the age of the birds further affects the effectiveness of probiotics. (Park *et al*., 2016).

Youssef *et al*., (2013) conducted research supplementing different feed additives in layers diet on the performance and egg quality. The results showed a significant difference in the rate of egg production by birds under probiotic and symbiotic treatments compared to those under the control group. The egg weight and egg mass also showed significant improvement on inclusive of the additives. Upadhaya *et al*., (2019) supplemented different levels of bacillus-based probiotics in laying hens' diet and reported positive effects when administered during the laying period on feed intake, egg production and egg quality. According to a study conducted by Park *et al*., (2016), E.faecium probiotic had a positive effect as a general feed additive in the poultry feeds. They concluded that probiotics with the E.faecium bacterial strain had a significant impact in increasing the egg production, nutrient digestibility, the eggshell thickness and the reduction of ammonia emission from poultry houses.

1.2 Statement of the Problem

The use of Antibiotic Growth Promoters (AGP) in the livestock industry has led to many concerns as a result of development of resistant microorganisms and the residual antibiotics in the animal products. The ban on antibiotics usage by the European Union, Japan, Australia and New Zealand has forced many poultry farmers to seek alternative methods of enhancing layers performance and immunity. Some of the viable alternatives that exist currently in the market to replace the use of antibiotics in improving performance include probiotics, prebiotics, synbiotics, and organic acids. Multi-strain probiotics have showed a lot of promising effects in improving layers feed intake, reducing pathogenic load, improving egg quality, and increasing the nutrient digestibility in layers. This study therefore focused on the supplementation of ISA Brown laying hens' diets with a commercially produced probiotic, MolaPlus® poultry microbes that has four strains of bacteria. The bacterial strains include Bacillus safensis, Cupriavidus mellidurans, Bacillus subtilis and Bacillus megaterium. The study investigated the effects of different levels of a multi-strain probiotic supplementation in layer performance and egg quality of ISA Brown birds.

1.3 Justification

The rapid and tremendous growth experienced in the poultry industry has led to the rise in demand for quality feed and feed additives to improve productivity (Hafez & Attia, 2020; Vernooij *et al.*, 2018). There is an increase in the number of small and large-scale poultry farmers especially those keeping layers and broilers (Hafez & Attia, 2020). There is also the need to improve performance and egg quality of layers since consumers purchasing preference is based on quality (yolk colour, shell thickness and strength) than quantity and eggs remain an important source of protein for most people globally (Krysiak *et al*., 2021). The use of antibiotics initially had a positive effect in improving the growth rate and performance (Krysiak *et al*., 2021). However, this effect has been re-evaluated and since their discovery, some pathogens have established resistant mechanism with the antibiotics making it transferable from animals to humans. Most countries have placed a ban on the use of antibiotics and there is urgent need for alternatives (Krysiak *et al*., 2021). Some of the suggested alternatives have been proven to have beneficial effects on the host when used as feed additives (Selaledi *et al*., 2020). Several studies are currently being done globally and more so in Africa owing to the fact that Africa has been a victim of deaths resulting from food borne diseases (Sasson, 2012). It is therefore essential to seek new ways of improving poultry production while maintaining quality and eradicating the use of antibiotics (Agyare *et al*., 2019).

1.4 Objective

1.4.1 Overall objective

To evaluate the effect of supplementation of multi strain probiotics via drinking water on performance and egg quality of layers.

1.4.2 Specific objectives

1. To determine the effect of supplementation of different levels of multi-strain probiotics on performance of ISA Brown layers.
2. To determine the effect of supplementation of different levels of multi-strain probiotics on internal and external egg quality of ISA Brown layers.

1.5 Hypothesis to be tested

H_0: Supplementation of different levels of multi-strain probiotics via drinking water has no effect on laying performance and egg quality of ISA Brown layers.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Importance of Chicken Production

Poultry production has been a driver raising the living standards of many people globally thus improving their welfare (Mottet and Tempio,2017). Studies are now focusing on how to improve chicken production through the application of various feeding strategies. Some of the benefits that accrue from rearing chickens include cash through sales, socio-cultural roles, and the production of high-quality food in the form of meat and eggs (Dumas *et al*., 2018). The production systems in the developing world however face challenges such as feed shortage, housing management and health issues that require improvement (Mapiye *et al*., 2008).

The global poultry production industry is growing tremendously due to various innovations and technological advancements (Gerber *et al*., 2007). The world agribusiness on poultry products has become competitive among different nations practicing poultry production with global egg production at 83 million tonnes in 2019, a 63% increase from 2000 (FAO, 2021). The 21st century has experienced a great increase in chicken meat and egg production (Gerber *et al*., 2007).

2.2 The Kenyan Poultry Industry

The Kenyan poultry population has been estimated at approximately 31 million birds (KeBS, 2019). The layers and broiler population make up 22% of these birds (KeBS, 2019). The commercial poultry market produces a high number of chicks to meet the rising demand for quality chicken meat and eggs (Nguyen *et al*., 2020). Some of the factors that affect the poultry industry include expensive feeds, inadequate housing and equipment, lack of farmers education and technology application (Mottet and Tempio., 2017). Poultry farming is a major source of income among many communities in Kenya. Most rear broilers and layers with the egg consumption rate higher than the meat consumption in the country (Onono *et al*., 2018). The demand for poultry eggs from schools, hotels, and homes in Kenya has increased making the commercial layers keeping business profitable (Omondi, 2019). Poultry products are a major source of protein among Kenyan communities. Poultry farming in Kenya is practiced on both large and a small scale (Okello *et al*., 2010).

2.3 Probiotics, Antibiotics, Organic acids, and Prebiotics in layer chickens' production

Probiotics in poultry are defined as living feed additives that have a positive impact on poultry through modification of gut microflora, improved digestive process, enhancing the chicken's health status (Youssef *et al*., 2013). Its efficiency is however affected by the inclusion levels, age and strain of animal (Park *et al*., 2016). The use of probiotics in poultry production has increased due to the discovery of their benefits in birds such as lessening of nitrogenous gas emissions, growth promotion, and immunity boost (Hatab *et al*., 2016). Probiotics reduce the number of gut pathogens in the host's body, have a metabolism that kills pathogenic bacteria, and controls the gastrointestinal immune response (Deng *et al*., 2020). They also trigger the poultry's appetite, promote antioxidative enzymes and compete with other microbes for the active sites (Lei *et al*., 2013).

Inclusion of probiotics as feed additives for both animal and poultry diets has increased in commercial feed production and confer-the following benefits in layer diets: improving egg quality traits, increasing the growth and performance of layers, and a significant increase in the mass of egg produced (Krysiak *et al*., 2021). Other studies have shown that probiotics dietary supplementation affects the eggshell strength, increase egg production by 2.8%, feed intake, conversion ratios, and utilization (Mikulski *et al*., 2020; Xiang *et al*., 2019). Probiotics increase the birds' immunocompetence, reduced the mortality rate, and improves their growth performance (Fathi *et al*., 2018). The impact of probiotics on layers' performance depends on the method of administration, frequency of administration, dosage of the supplements, bird's age, the microbial composition of the probiotics, diet composition, environmental stress factors, and diet composition (Karimi *et al*., 2010).

Probiotics supplementation in layers helps to reduce the effect of heat stress on the quality of the eggs produced (Krysiak *et al*., 2021). They also reduce the number of eggs that get damaged and improves the eggshell quality (Krysiak *et al*., 2021). Additionally, some studies have shown that probiotics inclusion in laying hens diet alters their lipid metabolism through symbiotic relationship existing between the microbes and intestinal flora reducing lipid peroxidation and improving metabolism in vivo. It is also speculated that probiotics containing microbes settle in the small intestine, decrease bile acid absorption and has an inhibitory effect on cholesterol absorption (Al-Khalaifa *et al*., 2019; Dev *et al*., 2021; Rafiq *et al*., 2022). Other researchers have indicated that probiotics also reduce the cholesterol amounts in the egg yolk by 11.7% (Sheoran *et al*., 2017; Fathi *et al*., 2018).

The livestock industry has experienced a challenge since the ban on antibiotics by the European Union which were initially used for poultry production to improve their general performance (Apata, 2009). Prior to the ban of antibiotic growth promoters, their use resulted in a positive impact in the poultry industry through enhancing growth performance by increasing feed efficiency, reduce mortality, and increase weight gain (Glasgow *et al*., 2019). They however resulted in the development of drug-resistant microorganisms that were likely to affect humans consuming animals/animal products whose production and health were improved using antibiotics (Lillehoj and Lee, 2012).

Research, however showed that growth promoters such as antibiotics has led to the development of resistance hence the need to find other supplements that can be used to serve the same purpose (Selaledi *et al*., 2020). Various alternative solutions have been proposed such as the use of prebiotics, probiotics, organic acids, and synbiotics in chicken diets to help improve their productive performance (Youssef *et al*., 2013). Probiotics have been shown to affect eggshell quality parameters, mineral utilization rate and increase production of volatile fatty acids. Increased volatile fatty acid is achieved through microbes' colonization and growth leading to higher proximal and distal colonic concentration which in effect, stimulate the growth of lactate-consuming bacteria resulting in increased short chain fatty acid production, especially butyrate (Park *et al*., 2016). Commercial probiotics in the market are manufactured using various bacterial strains such as Lactobacillus, Pediococcus, Bacillus, and Enterococcus (Park *et al*., 2016). A combination of probiotics and prebiotics produces synbiotics (Vyas & Ranganathan, 2012). The advantage of the mixture is the adaptation of the probiotics in the prebiotic's substrate, therefore, stimulating and improving the impact of both (Pandey *et al*., 2015).

Organic acids have been used in the poultry industry to help control the microorganisms found in the gastrointestinal tract and respiratory organs that are harmful to the host (Dittoe *et al*., 2018). Additionally, when used in poultry diets, they help in maintaining the pH levels in the gut and stomach of poultry, thereby enhancing the birds' immune response (Hajati, 2018). The use of organic acids in layers feeding has indicated that acids such as citric, lactic, and fumaric acid have an impact on the eggshell quality and increase the performance of the birds (Youssef *et al*., 2013).

2.3.1 Bacterial Composition in Probiotics

The type of bacterial species commonly used in probiotics is the lactic acid bacteria (Vieco-Saiz *et al*., 2019; Chen *et al*., 2020). Different types of bacteria have been included in different brands of probiotics some of which include; Escherichia, Prevotella, Streptococcus, Clostridium, Enterococcus, Bacillus and Lactobacillus species (Anee *et al*., 2021). The type of bacteria used has an impact on the effect that particular probiotic has on the chicken (Park *et al*., 2016). A study conducted by Park *et al*., (2016) used a probiotic containing a bacterial strain Enterococcus faecium as supplement to layer chicken. The researcher concluded that Enterococcus faecium had an effect on the nutrient utilization of Isa Brown layers which in turn increases egg production, eggshell thickness, decline in ammonia emissions, improved nutrient digestibility, decreases faecal coliform counts (Park *et al*., 2016). Furthermore, it was shown that feeding layers with probiotics containing Enterococcus faecium bacteria helped to ensure maximum retention of the nutrients fed to the chicken instead of them being excreted and an increase in chicks' growth rate and health status (Park *et al*., 2016).

Bacillus-based probiotics also have an impact on the laying performance of chickens (Mazanko *et al*., 2018). In a study, layers chicken diet was supplemented with probiotics containing Bacillus subtilis and Bacillus lecheniformis to determine its effects on egg production, excreta microflora, and egg quality (Mazanko *et al*., 2018; Upadhaya *et al*., 2019). The result showed that Bacillus based probiotic impact on performance, was resistant to heat, stable and tolerant to high pH thus enabling it to survive adverse conditions (Mazanko *et al*., 2018). In other studies, Bacillus licheniformis had a better impact on the reduction of the population of E. coli compared to Bacillus subtilis (Kan *et al*., 2021). Of the two species, Bacillus subtilis proved to have a greater impact on the egg production rate, egg yolk and the eggshell strength (Upadhaya *et al*., 2019; Souza *et al*., 2021). Upadhaya *et al*., (2019) concluded that the inclusion of probiotics containing bacillus strain of bacteria in layers' chicken feed had a positive impact on the performance and to some point the egg quality. Radiati *et al*., (2013) conducted a study on the impact of a heterogeneous probiotic composed of lactobacillus bacterial strain and the bacillus species and the results obtained from the study indicated that supplementation of layers diets with the mixed cultured probiotic has an impact on layers feed conversion ratio (FCR), hen day production, egg weight, lowers yolk cholesterol.

Fathi *et al*., (2018) analysed the effects of probiotics supplemented diet fed to layers and its effect on their egg production traits and the ratio of damaged eggs. The bacterial strain used in this study was Bacillus subtilis. The study results showed that probiotics affected the eggshell weight, shell thickness, eggshell strength, a darker coloured yolk, and improved cellular immune response. Epithelial cells in the intestinal layer protects the host from pathogenic invasion (Shalaei *et al*., 2014). A reduction in these cells leads to pathogenic invasion of the host intestinal lumen (Shalaei *et al*., 2014). Probiotic act by competing with the pathogen for adhesion receptor sites in the host intestine, stimulation of immune response, increase enzymatic action hence greater absorption of nutrients and better egg quality (Shalaei *et al*., 2014).

The Bacillus species have several strains of bacteria with different capabilities and survival conditions (Menconi *et al*., 2013). The Bacillus strains are known to survive under acidic conditions and, can metabolize various carbohydrates (Luise *et al*., 2022). The MolaPlus® Probiotic has four strains of bacteria i.e., the Bacillus megaterium, Bacillus subtilis, Cupriavidus metallidurans, and Bacillus safensis (Atela *et al*., 2019).

2.4 Effects of Probiotics on Layers Performance

This sections reviews literature on effect of different levels of probiotics on the overall performance of laying chickens. It covers aspects such as feed intake, egg weight, body weight gain, hen day egg production and feed conversion ratio.

2.4.1 Effects of Probiotics on laying performance in layers

The performance of laying hens can be assessed by hen-day egg production, egg weights, feed conversion ratio and mortality rates. In a study where layers were supplemented with a probiotic containing B. mesentericus, C. butyricum, and Faecalis bacterial strains there was no effects on the number of eggs laid by each hen per day or the egg weight but had a significant increase in the birds feed conversion rate (Selvin *et al*., 2020).

Another experiment conducted by Hayirli *et al*., (2005) tested the impact of probiotic supplementation and cage density on the laying performance, egg quality, and the metabolic profile. Analysis of the collected data indicated that probiotics improved the laying performance. Youssef *et al*., (2013) investigated the effects of probiotics and organic acids on the layer performance and egg quality and reported that organic acid significantly increased egg production by 9.94% while egg mass was increased by 14.18% and 6.61% by organic acid and probiotics respectively.

Layer chicken farmers are always focused on various ways of increasing egg yield and egg weight (Peralta-Sánchez *et al*., 2019). The mechanism applied in the egg production process and the health of the birds affects the quality of the eggs produced (Zhang *et al*., 2012). An experiment by Pan *et al*., (2011) tested the effect of probiotics on egg quality, laying performance, and egg composition. The study concluded that dietary probiotics supplementation has an impact on the egg-laying rate, egg weight, and egg cholesterol content.

2.4.2 Feed intake and Feed conversion ratio

Inclusion of probiotics in poultry diets leads to an increase in the body weight gain in chicken, feed consumption and FCR (Aziz Mousavi *et al*., 2018; Agustono *et al*., 2022). The level of inclusion of probiotics in poultry diets also have been reported to impact on the average body weight gain, average daily feed intake and FCR (Mikulski *et al*., 2020).

Other studies conducted on the effect of probiotics supplementation on layer chicken performance show significant improvement in parameters such as the feed intake, body weight, and the FCR (Zhao *et al*., 2019; Jha *et al*., 2020; Sheoran *et al*., 2020). The productive performance improvement is attributed to the ability of probiotics to compensate low apparent metabolizable energy AME (Jha *et al*., 2020). Mikulski *et al*., (2020) reported a 3.8% decrease in AME/kg from 2,650 to 2,550 kcal with no effect on compensatory feed intake. This could be as a result of layers ability to fine-tune feed intake relative to dietary energy, and a decline in dietary energy content leads to an increase in feed intake (Mikulski *et al*., 2020). Probiotics improves layers productivity which is mainly associated with the fact that its administration to layers increases the nutrient use efficiency (Piqué *et al*., 2019).

The increase in feed consumption by layers whose diets are supplemented with probiotics has not been clearly established (Bhogoju, S., & Nahashon, 2022). Different studies have been inconsistent effect of probiotics on feed consumption. A decrease in the feed intake of layers chicken whose diet has been supplemented with probiotics has been reported (Siadati *et al*., 2018). Additionally, studies have also reported an improvement on the feed conversion ratio of layers (Macit *et al*., 2021). Probiotics improves gram of feed consumed per gram of egg weight produced in layers when included in their diets (Mikulsk *et al*., 2020).

2.4.3 Body weight and body weight gain

Probiotics improved the body weight by 3.4% of commercial layers at week -32 (Neijat *et al*., 2019). The mode of action of probiotics is mainly through maintaining the gut environment and supporting the function of beneficial intestinal microbes (Markowiak and Śliżewska, 2018). Probiotics also stimulate poultry immune system and exclude the competitive pathogens that reduce growth rate (Abd El-Hack *et al*., 2020). An improved body weight gain in poultry is mostly because of the improved feed conversion ratio and increased average daily feed intake (Aziz Mousavi *et al*., 2018).

Dietary inclusion of probiotics in layer chicken positively affected the body weight gain (Macit *et al*., 2021). A study conducted by Jha *et al*., (2020) indicated an increase in the final weight and also weight gain on feeding layer chicken diets supplemented with probiotics. Sobczak & Kozłowski, (2015) also observed a significant body weight gain (12.8%) in layer chicken after 24 weeks of probiotics supplementation.

2.5 Effects of Probiotics on egg and shell quality

External egg quality are those physical characteristics of an egg that affects consumers acceptability and preference such as cleanliness, egg weight, eggshell weight, eggshell thickness, egg specific gravity and eggshell shape (Onunkwo & Okoro, 2015). Several factors affect the external quality of an egg including hen age, temperature, relative humidity, handling, storage and nutrition (Chambers *et al*., 2017).

The overall egg quality may not be improved by including probiotics in the laying hens' diet but the eggshell thickness and the shell strength are however increased (Inatomi, 2016). A study by Lei *et al*., (2013) investigated the effect of dietary inclusion of the B. licheniformis bacterial strain on the egg quality and laying performance in laying birds and reported that probiotics significantly increased the egg mass, eggshell thickness, and shell strength.

An important aspect to consumers in egg quality is a strong shell which is resistant to breakage to protect the egg against pathogenic organism (Sreekumar & Mohan, 2018). The metabolism of macro minerals (Ca and P) is of vital importance in the formation of quality shell structure (Kristl *et al*., 2019). The economies of egg production are highly depended on eggshell quality with its main factor been the deposition of calcium carbonate in the shells (Kristl *et al*., 2019). Probiotics supplementation to layer chicken has an effect of increasing eggshell thickness significantly as well as eggshell weight (Yan *et al*., 2019; Dey *et al*., 2021). This is because metabolic activity of beneficial bacteria colonies positively influences bone mineralization by increasing absorption of Ca and P and eventual deposition in shells (Yan *et al*., 2019; Sjofjan *et al*., 2021). Additionally, increase in eggshell thickness and egg shell weight is attributed by probiotics ability to improve volatile fatty acid production and promotion of an acidic pH in the intestinal tract of layer chicken which favours gut environment thus increase mineral absorption (Ca and P) and deposition in shells (Sjofjan *et al*., 2021;Yaqoob *et al*., 2021). The increase in egg shell thickness and weight was also observed when probiotics administered to layers between the ages of 28-32 and 32-36 weeks (Beshara and Ayman, 2019). The improvement in egg shell parameters is majorly due to the improved metabolic activities of beneficial bacteria and also the improved magnesium and calcium mineral assimilation (Shehata *et al*., 2022).

2.6 Egg weight and Egg mass

The primary criteria that influence the market value of eggs is the egg weight and egg mass (Travel *et al*., 2011). Alaqil *et al*., (2020) reported a significant increase in average egg weight by 1.7 and 1.9g and total egg mass by 1.3 and 4.8% respectively compared to control when layer diet was supplemented with probiotics. Probiotics inclusion in layer chicken diet resulted in an increase in the egg weight by 4.4% as they age (Aalaei *et al*., 2018). Researchers have reported high increase in the egg weight when layer chickens were fed probiotics administered via drinking water (Lokapirnasari *et al*., 2019). The highest egg weights were also recorded between week 20 and 68 of laying (Fathi *et al*., 2018). There was however no significant difference in the egg mass of layer chicken supplemented with probiotics. Additionally, probiotics inclusion in layer chicken diets did not have a significant influence on the egg weight (Neijat *et al*., 2019). These contrasting reports may be due to the difference in probiotic dosages administered and the bacterial concentration used in the diet offered (Neijat *et al*., 2019).

2.7 Effects of probiotics on internal egg quality

2.7.1 Egg Yolk Colour

Yolk colour is the most important internal egg quality factor that informs consumers re-purchase (Rondoni *et al*., 2020). Consumers' preference for egg yolk coloration will decrease with a pale-yellow coloration and increase with a deep yellow coloration as a result of taste, flavour and odour (Kljak *et al*., 2021; Rondoni *et al*., 2020). The main component of the egg yolk is carotenoid (xanthophylls, lutein and zeaxanthin) which plays an important role in human diet in reducing or preventing cataracts and age-related macular degeneration (Kljak *et al*., 2021; Zurak *et al*., 2022). The egg yolk colour usually improves and becomes deep yellow when layers' diets are supplemented with probiotics, synbiotics, or organic acids (Mirza, 2018). The egg yolk colour is a parameter for determining the egg quality (Sjofjan *et al*., 2021). In a study conducted by Aalaei *et al*., (2018) layer chicken eggs yolk scores for both fed probiotic increased. Probiotics induces acidification of the gut thus stimulating gut health and allowing increase intestinal absorption of carotenoid resulting in improved yolk colour (Lokaewmanee *et al*., 2011b).

2.7.2 Albumin Quality

Albumin is affected by various factors including genotype, age, body size, breed of hen, feed ingredients, water consumption and environmental conditions (Nematinia & Abdanan Mehdizadeh, 2018; Şekeroğlu & Altuntaş, 2009). Internal egg quality standard measurement is albumen quality which is often the heigh of the albumen or its derivatives haugh unit (Nematinia & Abdanan Mehdizadeh, 2018). Haugh Unit values ranges from 0 to 130 and a score value below 60 is considered un-fresh eggs (Nematinia & Abdanan Mehdizadeh, 2018).

Probiotics supplementation in the diet of laying hen increases the albumen haugh unit and improved the protein quality (Macit *et al*., 2021). Jha *et al*., (2020) conducted a study with commercial probiotics and reported an increase in the albumen height and a greater haugh unit. Multi-strain probiotics improved the protein quality of laying hen eggs by improving the albumin quality (Siadati *et al*.,2018). Additionally, Mikulski *et al*., (2020) reported that probiotics inclusion in layer chicken diet increases the crude protein content of the egg albumin which in turn improves it quality.

CHAPTER THREE

3.1 MATERIALS AND METHODS

3.1.0 Introduction

This study evaluated the effect of supplementation of different levels of a multi-strain probiotics on the performance of laying chickens and this chapter highlights the methodology used in data collection.

3.1.1 Ethical Approval

The experimental procedure was approved by the faculty of Veterinary Medicine Biosafety, Animal Use and Ethics Committee with Ref: FVM BAUEC/2021/311.

3.1.2 Study Site

The research was conducted at the Poultry unit of the department of Animal Production, Faculty of Veterinary Medicine located at Latitude 1°15'33.84''S and Longitude 36°43'30.828''E.

3.1.3 Experimental Birds Acquisition

A total of 150 Isa Brown 65 weeks old layers birds were recruited from a laying flock at the poultry unit and allocated according to the experimental design.

3.1.4 Probiotics acquisition

The multi strain probiotic preparation was obtained from local 'agrovet' shop. The probiotic, MolaPlus® is composed of four bacterial strains i.e. Bacillus subtilis, Bacillus safensis, Bacillus megaterium and Cupriavidus metallidurans (Atela *et al*., 2019).

3.1.5 Experimental birds, Diet and design

In this study, 150 ISA Brown laying hens were assigned in a completely randomized design to 5 treatments, a control and 4 levels of probiotics provided via drinking water. The control treatment was provided with water containing no probiotics, treatment 2 were provided with water mixed with 2.5 ml of probiotics per litre of water, treatment 3: 5ml/l, treatment 4: 10 ml/l and treatment 5: 15ml/l. The hens were reared in metallic battery cage with fitted feeding and drinking troughs. Each cage held one bird and each replicate 6 birds. The hens were exposed to natural lighting for a relatively consistent period of 12hours daily and house temperature of 26°C in addition to proper ventilation. The layers mash experimental feed was purchased from a reputable local feed manufacturer (Unga feeds) after confirming that it was formulated to meet NRC, 1994 nutritional standards with a minimum of 2850Kcal/kg ME and 16% crude protein from analysis of entire proximate and energy of the diet. All the birds were weighed weekly, and analysis of different egg parameters tested e.g., hen day egg production, feed intake, water intake, egg weight, egg specific gravity, eggshell thickness, the yolk colour, eggshell weight. The mineral content of the eggshell was determined thrice (First week, 3rd week and End week) while the feed conversion ratio was determined at the end of 5 weeks (Bidura *et al*., 2019; Skřivan *et al*., 2016). The experiment lasted 5 weeks (35 days). The layers mash diet was sampled and analysed for entire proximate (dry matter, moisture, crude protein, crude fibre, ether extract, ash, Ca and P) and ME (metabolizable energy) was calculated using the equation below;

$TDN = 54.6 + 3.66 \times e^{-cp} + 0.26 \times CF + e^{EE}$ (AOAC, 2016)

$TDN \times 4.409\% = DE$ (AOAC, 2016)

$ME = 0.45 + (1.01 \times DE)$ (AOAC, 2016)

Where; TDN = Total digestible nutrients, CP = Crude protein, CF = Crude fibre, EE = Ether extract, DE = Digestible energy, ME = metabolizable energy (AOAC, 2016)

3.1.6 Layers Chicken Management

Layers were randomly designated singly in different cages with each replicate consisting of 6 birds. Additionally, to ensure biosafety, a footbath containing 10ml/L disinfectant (Norbrook®) was placed at the entrance. Feeds and clean water were offered *adlibitum*. Water containing the various levels of probiotics, with control receiving no probiotic was provided daily per replicate and intake measured by measuring refusal the following day. At the beginning of every week, known amount of feed was placed in a labelled bucket for each replicate. Feed was provided without restriction and intake measured by weighing the balance at the end of the week.

Figure 1: Layers Chicken Management System

3.2 Assessment of Layer Performance and FCR.

3.2.1 Egg production

The eggs were collected on daily basis. The egg production rate was calculated by dividing the total number of eggs collected by the total number of days (35days) and producing hens (Peralta-Sánchez *et al.*, 2019). The hen-day egg production per replicate for the entire period was calculated using the formula;

$$\% \text{ Hen day Egg Production} = \frac{\text{Number of eggs produced}}{Number\ of\ live\ hens} \times 100$$

3.2.2 Assessment of Laying performance

The hens were weighed individually weekly throughout the experimental period and their body weight changes recorded. The egg numbers were recorded daily while the egg weight was recorded once a week. Feed consumption was determined weekly by subtracting refusal from initial while feed conversion ratio was determined using the formula Grams Feed intake/Grams Egg Weight (Krysiak *et al*., 2021).

3.2.3 Protein Consumed

Protein consumed (PC, g/bird) per replicate was calculated by multiplying the concentration of CP in the diet (g/kg DM consumed) by mean feed intake (g/bird) for the entire period.

$$PC = CP_d \times FI$$

3.3 Assessment of Internal and External Egg quality.

3.3.1 Mineral Content of Eggshells

The eggshells from sampled eggs were grounded into fine powder and ashed (to separate the organic and inorganic matter) (AOAC, 2016). The minerals were extracted via dry ashing where 15ml of 20% dilute HCL was added to the ash in the crucible and allowed to digest for

30minutes (AOAC, 2016). Filtration was done and the filtrate was transferred to 100ml volumetric flask and top up to the mark with distilled water (AOAC, 2016). The standard solution in the volumetric flask contains all the minerals both macro and micro. 2mls of the standard solution was transferred to a 50ml volumetric flask and top up to the 50ml mark with distilled water (AOAC, 2016). For Phosphorus determination, in 2ml of the sample solution was added 15ml colour developer and top up with distilled water to the 50ml mark. Blank standard was used to calibrate the UV-visible spectrophotometer. For Calcium the diluted standard solution was aspirated into the flame and absorbance recorded (AOAC, 2016) (Table 1).

Table 1: Method Used to Analyse Mineral Content of the Eggshell.

Method Used	Mineral Analysed	Equipment
Atomic absorption spectrophotometer (AAS)	Ca	Varian, spectra AA
UV-visible spectrophotometer	P	UV-Visible spectrophotometer-Hitachiu 2900, Model 2JI-0003 Tokyo

Sauce: AOAC, 2016

3.3.2 Egg weight

The eggs were collected twice daily from laying hens under the different treatments, at 8:00h and 17:00h. Seventy-five eggs (3 eggs from each replicate) were randomly sampled and weighed weekly using a 0.0001g precision analytical balance.

3.3.3 Specific gravity

The 75 sampled eggs were used to determine the specific gravity (breaking strength). A saline solution was prepared by dissolving specific amount of common salt (NaCl) in three litres of water (Table 2) with specific gravities ranging from 1.060 to 1.100g/cm^3 with gradient 0.005 (Butcher & Miles, 2017). The eggs were then immersed in each of the saline solution beginning with the lowest (1.060g/cm^3) to the highest (1.100g/cm^3) specific gravity (Butcher & Miles, 2017). The specific gravity at which each egg floats was recorded (Fig 2).

Figure 2: Determination of eggs specific gravity

Table 2: Weight of salt dissolved in three litres of water for a given specific gravity.

NaCl(g)	Specific gravity (g/cm^3)
276	1.060
298	1.065
320	1.070
342	1.075
365	1.080
390	1.085
414	1.090
438	1.095
462	1.100

3.3.4 Yolk Colour

The eggs were broken carefully on a flat white plate and egg yolk pigmentation was measured visually using Roche Yolk Colour Fan (Fig 3) with colour scores ranging from 1 (the light yellow) to 15 (the dark yellow) (Lokaewmanee *et al*., 2011b).

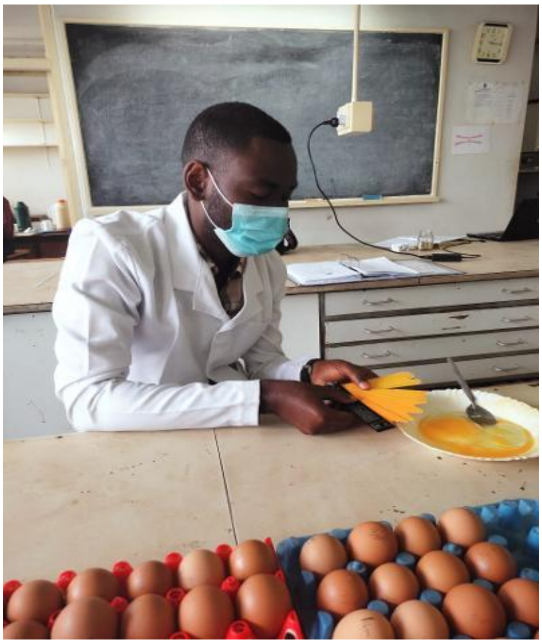


Figure 3: Determination of Egg yolk colour

3.3.5 Eggshell weight

After breaking the egg carefully, and removing the yolk and albumen, the shells were carefully washed under running tap water to remove any remaining traces of albumen (Adegbenro *et al*., 2020). They were then oven dried at 60°C for 12hours and left to cool at room temperature. Finally, they were weighed using a 0.0001g precision analytical balance (Adegbenro *et al*., 2020).

3.3.6 Eggshell thickness

Using a 0.001mm precision micrometer screw gauge, the shell thickness was measured at three locations on the egg (air cell, equator and sharp end) and the mean values represented the shell thickness (Lokaewmanee *et al*., 2011a).

3.3.7 Eggshell percentage

The eggshell percentage was calculated by dividing its weight over the egg weight and expressed as a percentage as shown in the formula below;

Eggshell % = [Weight of eggshell (g)/Egg weight(g)] × 100

3.4 Statistical Analysis

All data obtained on performance and egg quality were subjected to a one-way Analysis of Variance (ANOVA) using GenStat Statistical package version 14. Significant treatment means were separated using Turkey's test and level of significance was set at $P \leq 0.05$.

CHAPTER FOUR

4.0 RESULTS

This chapter highlights results obtained from the various parameters tested in the material and methods chapters.

4.1 Chemical composition of layers mash

The chemical composition of the layers basal diet is shown in Table 3. The layers mash had 91.37% dry matter (DM), 15.05% crude protein (CP), 11.16% crude fibre (CF), 4.79% ether extract (EE), 12.74% Ash, 3.12% calcium (Ca), 0.54% phosphorus (P) and 2752Kcal/kg metabolizable energy (ME).

Table 3: Mean Chemical Composition (%DM) of the Layers Mash fed to experimental birds

Components	Layer Mash (% ± SD)
Dry Matter	91.37 ± 0.61
Crude Protein	15.05 ± 0.43
Crude fibre	11.16 ± 1.44
Ether extract	4.79 ± 0.88
Ash	12.74 ± 1.32
Calcium	3.12 ± 0.48
Phosphorus	0.54 ± 0.04
ME (Kcal/kg) *	2752 ± 73.86

*Calculated metabolizable energy

4.2 Layers Performance

The effect of inclusion of probiotics on the feed intake, initial and final body weight, egg weight, hen/day/egg production, FCR, water intake and protein consumed are shown in Table 4 below.

The average daily feed intake (FI) ranged from 139.4 to 151.6g/d and tended to be lower for the probiotic fed groups but the difference was non-significant ($p = 0.128$) compared to Prob0. The mean egg weight (EW) ranged from 67.99 to 68.63g for treatment groups compared to 67.82 for the control. However, the differences were not statistically significant ($p = 0.948$). The mean final body weight (FBW) ranged between 1992 to 2087g/bird for treatment groups compared to 2088 for control though the differences were not significant ($p = 0.247$). The hen/day/egg production was also not significantly different ($p = 0.246$) between the control and treatment groups. The feed conversion ratio (FCR) and water intake were not significantly affected by treatment. However, the FCR tended to decrease with inclusion of probiotic and was marginally non-significant. The protein consumed (PC) was similar across all treatment, a reflection of similar feed intake of constant protein content.

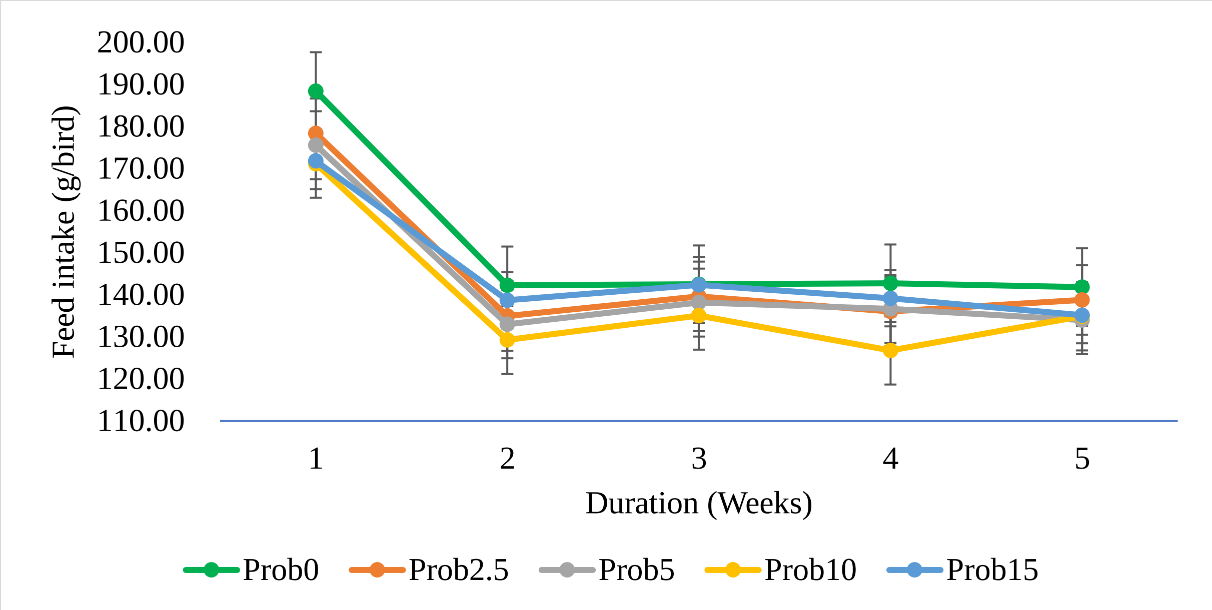
Table 4: Effects of inclusion of different levels of a multi-strain probiotic on layers performance

Parameters	Prob0	Prob2.5	Prob5	Prob10	Prob15	SEM	P-value
FI[1](g/bird/day)	151.6	145.6	143.5	139.4	145.4	3.07	0.128
EW (g/egg)	67.82	68.61	68.63	67.99	68.30	0.866	0.948
IBW (g/bird)	2062.33	1922.33	1963.00	2057.63	2093.00	47.82	0.091
FBW (g/bird)	2088.72	2017.86	2006.61	1992.74	2087.94	37.97	0.247
HDEP (%)	94.48	92.38	94.76	88.38	95.43	2.357	0.246
FCR	2.237	2.123	2.092	2.051	2.131	0.0535	0.193
WI (ml/bird/d)	341.7	341.2	337.9	341.6	347.5	13.59	0.992
PC(g/bird/day)	20.84	20.02	19.73	19.17	20.00	0.423	0.128

Prob0: control, Prob2.5: 2.5ml/L, Prob5: 5ml/L, Prob10: 10ml/L, Prob15: 15ml/L, SEM-Standard Error of the Mean.
mean with no superscripts within a row are not significantly different (P > 0.05)
[1]FI- Feed Intake (as fed basis), EW- Egg Weight, IBW-Initial Body Weight, FBW- Final Body Weight, HDEP- Hen Day egg production, FCR- Feed Conversion Ratio, WI- Water Intake, PC- Protein Consumed

Figure 4: Trend in weekly feed intake of layers fed different levels of a multi-strain probiotics. The bars represent the standard error of the mean.

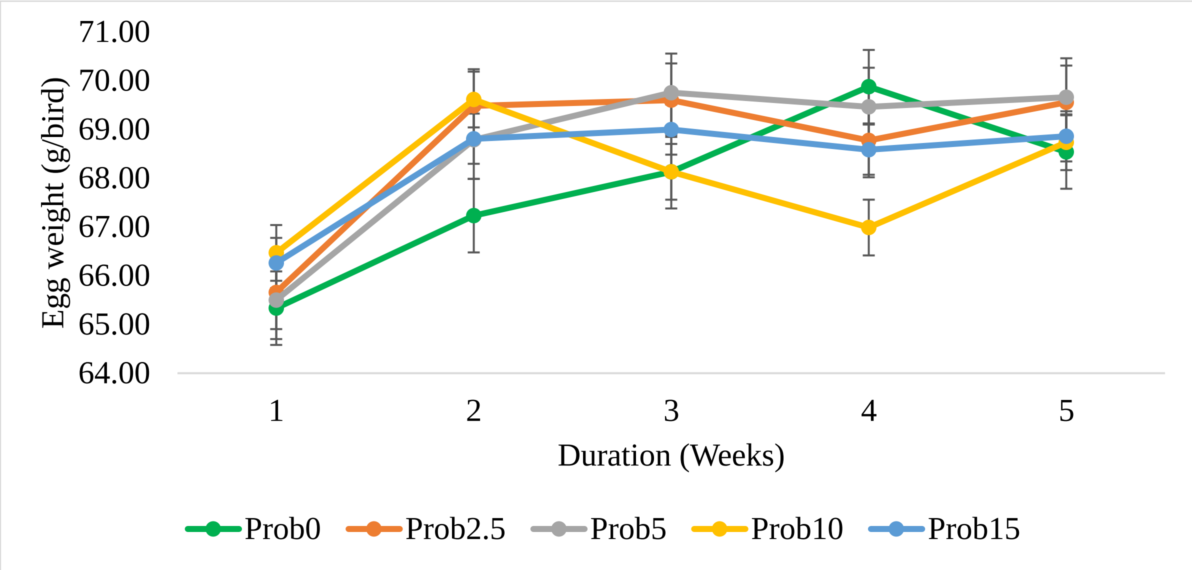
Figure 4 below shows the weekly trend in feed intake across treatment groups fed different levels of a multi-strain probiotic.



It was observed that there was a drop in feed intake across treatments from week 1 to 2 with Prob10 recording the least and Prob0 recording the highest feed intake. Feed intake in layers is affected by temperature, stocking density but were however within the comfort limit of the birds. From week 2 to 5, the feed intake was almost constant but for Prob10 that dropped slightly at week 4. The increase in feed intake at week 1 and drop at 2 could be attributed to the longer acclimatization from the initial week set aside before the start of the experiment.

Figure 5 below shows the weekly trend in egg weight across treatment groups fed different levels of a multi-strain probiotic.

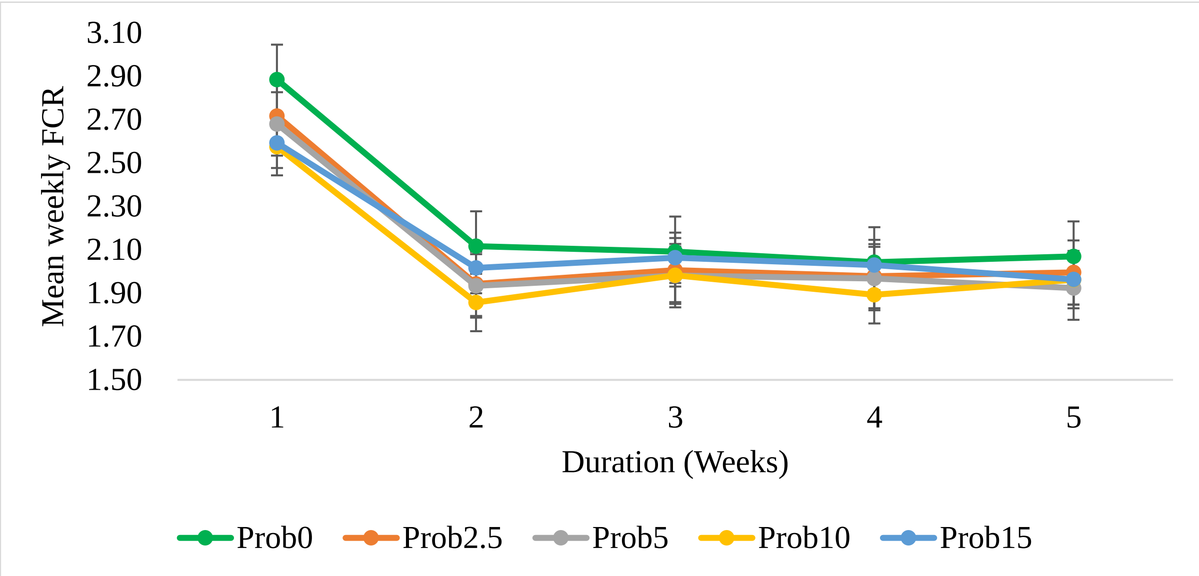
Figure 5: Trend in weekly egg weight fed different levels of a multi-strain probiotic. The bars represent the standard error of the mean.



Egg weight increased across treatment groups from week one to two with Prob0 recording the lowest and Prob10 recording the highest during this period. From week two to four, Prob10 dropped while Prob0 increased and was highest at week four. Prob2.5 & 15 was steady between week 2 and 3 and dropped slightly in week 3. Prob5 increased between week 2 and 3 and maintained an almost even EW from week 3 to 5. Prob0 decreased in EW between week 4 and 5 while Prob10 increase between week 4 and 5. This showed that in treatment groups there was improved efficiency compared to control thus reflecting in the steady EW increase. The increase in egg weight in week 1 could be attributed to an unbalanced diet fed to the birds before the acclimatization (week before the first) period and start of the experiment.

Figure 6 below shows the weekly feed conversion ratio across treatment groups fed different levels of a multi-strain probiotic.

Figure 6: Trend in weekly feed conversion ratio fed different levels of a multi-strain probiotic. The bars represent the standard error of the mean.



FCR improved from week 1 to week 5 throughout the experiment. Prob0 recorded a slightly higher FCR compared to treatment groups at week one. This showed that Prob0 was not efficiently utilized thus leading to low egg weight compared to the treatment groups. By the end of the fifth week, Prob10 was the most utilized diet with a low FCR compared to the other treatment groups. Similar to the current study, Neupane *et al*., (2019) reported no effect of probiotics on FCR on dual purpose chicken. On the contrary, Widya *et al*., (2019) reported a significant increase (11.88%) in average FCR compared to control.

4.3 Egg Quality

The effect of inclusion of probiotic on specific gravity, yolk colour (YC), shell thickness, shell weight, eggshell %, and mineral (Ca & P) content of the eggshells is shown in Table 5 below.

The mean egg specific gravity ranged from 1.08712 to 1.08872 in treatment groups compared to 1.08920 in control, the difference was not significant ($P = 0.513$). The average YC ranged from 13.13 to 13.28 for treatment groups compared to 13.24 for control and were not statistically significant ($p = 0.896$). The shell weight of eggs from layers supplemented with probiotic ranged from 6.3189 to 6.4647g compared to 6.4680g for Prob0. However, the difference was not statistically significant ($P = 0.751$). The shell thickness of eggs from the supplemented groups ranged from 0.44 to 0.45mm compared to 0.45mm in control, the difference not being significant ($p = 0.15$). The % eggshell ranged between 9.207 to 9.424 in treatment groups compared to 9.538 in control, with no significant difference between treatment and control ($P = 0.278$). The calcium content of the eggshell was significantly different ($p < 0.05$) across treatment groups compared to Prob0 with Prob5 recording the highest calcium content. Treatment Prob15 recorded a slightly but significantly lower Ca % compared to control (Prob0). The phosphorus content of the eggshells was highest for Prob5 (0.5311) followed by Prob15 (0.5093), Prob2.5 (0.4907), Prob10 (0.4865) and lowest for Prob0 (0.4731). Prob5 and Prob15 were significantly higher ($p < 0.05$) compared to Prob0, Prob2.5 and Prob10.

Table 5: Effects of inclusion of different levels of a multi-strain probiotic on layers egg quality characteristics

Parameters	Prob0	Prob2.5	Prob5	Prob10	Prob15	SEM	P-value
SG[1] (g/cm^3)	1.08920^{a}	1.08872^{a}	1.08712^{a}	1.08772^{a}	1.08712^{a}	0.001029	0.513
YC	13.24^{a}	13.13^{a}	13.28^{a}	13.27^{a}	13.13^{a}	0.140	0.896
SW (g)	6.4680^{a}	6.4647^{a}	6.3189^{a}	6.3893^{a}	6.3780^{a}	0.09114	0.751
ST (mm)	0.45^{a}	0.45^{a}	0.44^{a}	0.44^{a}	0.44^{a}	0.004	0.150
Eggshell %	9.538^{a}	9.424^{a}	9.207^{a}	9.399^{a}	9.340^{a}	0.1034	0.278
Ca %	49.5229^{b}	50.7926^{d}	52.8265^{e}	49.7886^{c}	49.3890^{a}	0.01780	<.001
P %	0.4731^{a}	0.4907^{ab}	0.5311^{c}	0.4865^{ab}	0.5093^{bc}	0.00617	<.001

Prob0: control, Prob2.5: 2.5ml/L, Prob5: 5ml/L, Prob10: 10ml/L, Prob15: 15ml/L, SEM-Standard Error of the Mean

^{abcde} *means having different superscripts within the same row differ significantly ($P \leq 0.05$)*

[1]*SG- Specific Gravity, YC- Yolk Colour, SW- Shell Weight, ST- Shell Thickness, Ca-Calcium, P- Phosphorus.*

CHAPTER 5

5.0 DISCUSSION

This chapter highlights the comparison of my results in the previous chapter with results obtained from other studies and the reasons for the difference.

5.1 Chemical composition of layers mash diet

The dry matter content was within the >90% range recommended for layers mash diet (Table 3). High moisture feeds could lead to growth of fungus resulting in mycotoxins contamination (Mokubedi *et al*., 2019). The moisture content was 8.63% which was lower than 10-12% reported by Singh *et al*., (2019). The crude protein content was within 14-16% range for layers mash (KeBS., 2019) but lower than 16.1-17.89% reported by Olorunsongo *et al*., (2018). Crude protein content in layers feed affects the egg production, egg weight and size and is necessary for both feather development and carcass growth (Van Emous *et al*., 2015). The crude fibre was higher than the minimum 8% recommended by KeBS., (2019). This higher fibre content in the diet can be attributed to use of cereal milling by-products in feed formulation in the country (Zhang *et al*., 2021). High fibre content in layers mash has been reported to stabilize the chicken guts, reduce the concentration of ammonia in poultry houses and reduces the numbers of dirty eggs collected (Desbruslais *et al*., 2021). The ether extract (representing the crude fat) was also higher than 1.3-2.8% reported by Singh *et al*., (2019) but was within the <6% recommended by KeBS., (2019).

The ash content of the ration was in the range of 11.9-17.6% reported by Ekeocha *et al*., (2021). Layer diets have considerable high ash content due to the high requirement for calcium which is provided through inclusion of limestone in the rations. The calcium and phosphorus content were within the range of 3.0 - 4.20% and 0.40-0.64% respectively, reported by (Rizk *et al*., 2019; Yan *et al*., 2019; Ray *et al*., 2020; Wang *et al*., 2020). Ca and P are the key macro minerals that play a critical role in bone development, mineralization and eggshell formation with the former 94% and latter 1% in eggshell (Aditya *et al*., 2021). The calculated metabolizable energy 2752Kcal/kg was within the range of (2750Kcal/kg) recommended by KeBS., (2019).

5.2 Effects of multi-strain probiotics on laying performance

Feed intake by layer birds is related to several factors including genotype, temperature, light and stocking density (Erensoy *et al*., 2021). The mean daily feed intake in this study ranged from 139.4 to 151.6g/day (as fed) which is equivalent to 127 to 138g/d (on DM basis). (Table 4). The DM intake in laying birds has been reported to range between 125 to 135g/day (Lee *et al*., 2016; Wang *et al*., 2017; Patra *et al*., 2020) which is within the range observed in this study. High stocking density leads to competition for feed and water leading to decrease intake, which however wasn't the case in the current study as birds were placed single in individual cages (Khumput *et al*., 2019). In addition, feed intake is reduced with an increase in temperature as a result of increase in metabolic rate of the birds (Khan *et al*., 2011). Major strategies such as feed restriction, dual feeding regime and wet feeding are been employed to reduce temperature effect. However, the temperature during the study were within the comfort limits (26°C) for laying birds. Neijat *et al*., (2019) observed an increase in feed intake when layers diet was supplemented with low, medium and high single strain Bacillus subtilis by 4.21%, 6.24% and 1.56% respectively at week-20. The researchers attributed the increase to probiotic ability to improve gut health that could mitigate adverse stress effect. Antara *et al*., (2019) and Bidura *et al*., (2019) observed no effect on feed intake with probiotic supplementation in layers diet. Fathi *et al*., (2018) reported a decrease by 5.92% and 1.18% when 200 and 400ppm *Bacillus subtilis* respectively was supplemented in same basal diet compared to control (0ppm supplemented) that recorded an increase. Lack of significant effect on feed intake in this study could be as a result of probiotics not affecting the GI in a way that could increase rate of feed passage.

The mean egg weight in this study was 68.25g (Table 4). Mean average egg weight for hybrid birds has been reported to range between 53.62 to 70.87g (Tang *et al*., 2017; Aalaei *et al*., 2019; Lokapirnasari *et al*., 2019; Alaqil *et al*., 2020; Suswogo *et al*., 2021) which is within the range observed in this study. Egg weight tended to increase with probiotic supplementation though not significantly ($p>0.05$) different from control which could be attributed to the slightly improved feed efficiency. Antara *et al*., (2019) reported an increase ($p<0.05$) in egg weight by 4.25% and 4.35% when layers diet was supplemented with (2% and 4% respectively) fermented extract of Moringa oleifera by probiotic Saccharomyces spp for 8 weeks. Ray *et al*., (2022) also reported an increase ($p<0.05$) with both single strain (5.37%) and multi-strain probiotic (5.54%) supplementation in layers diet. The researchers postulated the increase to proper and efficient nutrient utilization in probiotic supplemented groups. Yan *et al*., (2019) reported a decrease in all multi-strain probiotic supplemented groups. Others reported no effect on egg weight (Aalaei *et al*., 2018; Xiang *et al*., 2019). However, factors such as adhesion and replication of bacteria in the small intestine, the age of the birds, microbe species, single or multi-strain, amount used and method used can influence the positive effect of probiotics on egg weight (Mikulski *et al*., 2012; Forte *et al*., 2016).

The mean final body weight of the birds in this study ranged from 1992.94 to 2088.72g/bird (Table 4). Final body weight of old hybrid layers has been reported to be between 1943 to 2035g/bird (Hossain *et al*., 2016; Ray *et al*., 2022) which is within the range observed in this study. Inclusion of probiotic had no effect on final body. Laying hens are however not expected to gain weight as majority of the feed is used for egg production and maintenance.

The mean daily hen day egg production in this study was in the range 88.38 to 95.43% (Table 4). The observed values fall within the range 83.9 to 98.5% reported from other studies with different production cycles (Bozkurt *et al*., 2011; Inatomi, 2016; Hameed *et al*., 2019). A number of factors affects the hen day egg production including feed intake, water intake, age of birds and light intensity (Philippe *et al*., 2020). However, all the factors affecting hen day egg production were kept constant in this study. Antara *et al*., (2019) reported an increase in egg production by 3.80% and 3.05% in treatment groups compared to control when layers diet was supplemented with 2 and 4% fermented extract of Moringa oleifera by probiotic Saccharomyces spp from week 70 to 78 of age. They attributed this to microbes' ability to survive through the digestion process, growth in the digestive tract and ability to increase digestibility of feed substances. In addition, Ray *et al*., (2022) fed ISA Brown layers with 0.75, 1.00 and 1.25g/kg multi-strain probiotic in feed and recorded a significant increase in egg production by 9.1% compared to single strain fed probiotic and control between 26 and 51 weeks of age. On the contrary, Yan *et al*., (2019) reported that 0.5 and 2.0g/kg inclusion level of probiotic tended to decrease hen day egg production though not significantly compared to control. Several studies have reported no effect on egg production for probiotic supplemented diets (Aalaei *et al*.,2018; Fathi *et al*., 2018; Xiang *et al*., 2019; Mikulski *et al*., 2020; Marwi *et al*., 2021). Lack of effect in the current study on HDEP could be attributed to similar feed intake and protein consumed.

The ratio of grams of feed consumed to grams of egg weight was calculated to obtain feed conversion ratio (Table 4). Since feed intake and egg production were not affected, it was not surprising that the FCR was not significant amongst supplemented groups compared to control. FCR in layers has been reported to be between 1.60 to 2.45 (Inatomi., 2016; Lokapirnasari *et al*., 2019; Yenilmez *et al*., 2021) which is within the range observed in this study. FCR in layers is influence by several factors including feed quality and management practices. Mikulski *et al*., (2020) and Ray *et al*., (2022) reported an improved FCR ($p<0.05$) with probiotic supplemented groups while (Fathi *et al*., 2018; Yan *et al*., 2019) reported no effect. Layers fed on balanced diet that meet their nutritional requirement turn to convert the feed more efficiently than unbalanced diet (Thirumalaisamy *et al*., 2016). In addition, the management welfare of the birds such as protection from diseases influences the FCR (Tsiouris, 2016). However, in the current study, all factors were kept constant thus leading to an improved FCR, though not significant which could be as a result of probiotics competitive exclusion of pathogen through the production of lactic acid and enzymes hence improved intestinal epithelial barrier and nutrient absorption. In the current study, inclusion levels of probiotic had no significant effect on FCR but on a numerical basis, there was marginal improvement which may be attributed to the probiotic enhancing health status thus promoting metabolic processes of digestion and nutrient utilization (Macit *et al*., 2021).

The average water intake in this study ranged from 337.9 to 347.5ml/bird (Table 4) and was not significantly affected by treatment ($p = 0.992$). Several factors have been reported to affect water intake in layers including bird age, feed intake, dry matter content of feed and temperature/heat stress (Orakpoghenor *et al*., 2021). Pambuka *et al*., (2014) monitored water intake for birds fed rations containing 0.15% v/v, 0.30% v/v, and 0.45% v/v liquid probiotic mixed culture (LPMC) via drinking water and reported no effect of probiotic on water intake. Temperature/heat stress leads to increase in water intake as a result of evaporation of water from the respiratory system during panting (Wasti *et al*., 2020). Feed intake and dry matter content of the feed were all within the required limit and thus didn’t affect water intake. Pambuka *et al*., (2014) reported water intake of 253 – 291ml/bird/day for layers birds which is lower than this study. The layers used in their study were younger (52-weeks old) compared to those in this study (65-weeks old) which could explain the difference. The lack of significance in water intake between the birds meant that the probiotic intake via water was at the calculated ratios for different diets.

The mean amount of protein consumed by birds in this study ranges from 20.99 to 22.81g/bird/day (Table 4). Layer birds are expected to consume 16.2 to 18.9g of CP per day for optimum performance (NRC, 1994; Keshavarz, 1998; Novak *et al*., 2006). Excess protein consumption can lead to wastage in terms of energy required to excrete the excess N and the added cost of feed (Nahm, 2007). Consumption of low amount of protein can lead to reduced feed efficiency, growth rate, feather development and egg production (Heuser, 1941) which wasn't the case in the current study. In this study, the amount consumed was higher than the reported (NRC, 1994; Keshavarz, 1998; Novak *et al*., 2006) which could be attributed to high amount of feed consumed.

5.3 Effects of multi-strain probiotic on egg quality

Several factors have been reported to affect layers external egg quality including; bird age, induced moult, nutrition, heat stress, diseases and production system (Roberts., 2004). There was no effect of treatment on the egg specific gravity in this study (Table 5). The specific gravity of an egg gives an indication of eggshell quality with respect to its freshness (Malfatti *et al*., 2021). The specific gravity in the current study ranged between 1.08712 to 1.08920g/cm^3. The observed values fall within the range 1.077 to 1.10g/cm^3 when probiotics were included in layer diets (Kurtoglu *et al*., 2004; Mikulski *et al*., 2012; Youssef *et al*., 2013). Milkuski *et al*., (2012) reported a significant higher egg specific gravity when dietary single strain probiotic (*Pediococcus acidilactici*) was supplemented in layers diet during layer phase 1 (23 – 34wks; 0.37% vs 0.37%), layer phase 2 (35 – 46wks; 0.37%), entire period (23 – 46wks; 0.28% vs 0.37%). The improvement was however attributed to bacteria improving the morphological structure of the small intestine mucosa thus increase absorption of nutrients. On the contrary, others studies where laying birds were fed on different dietary levels of probiotics reported no significant treatment effect on egg specific gravity (Khan *et* *al*., 2011; Yan *et al*., 2019; Mikulski *et al*., 2020). Lack of treatment effect in the current study could attributed to commercial diet sufficient in mineral elements (Ca &P) required for eggshell strength.

Yolk colour in layers chicken eggs is affected primarily by the presence of carotenoids (xanthophylls, lutein and zeaxanthin) in their diets (Marounek & Pebriansyah., 2018; Kavtarashvili *et al*., 2019). Mean egg yolk colour in this study ranges from 13.13 to 13.28 (Table 5). Yolk colour in layers can range between 0 to 15 (Vuilleumier, J.P., 1969), the lighter colour being for diets deficient in carotenoids (will lead to a pale-yellow coloration in the yolk) while carotenoid rich diets will lead to a deep yellow coloration as in the case of our current study. Antara *et al*., (2019) and Macit *et al*., (2021) reported an increase in yolk colour by 17.27% vs 19.24% and 8.44% vs 7.54% respectively in probiotic supplemented groups compared to control while (Aalaei *et al*., 2018; Fathi *et al*., 2018; Marwi *et al*., 2021; Mikulski *et al*., 2020; Ray *et al*., 2022) reported no effect of probiotic supplementation on yolk colour. Neijat *et al*., (2019) reported a decrease in yolk colour by 7.69% when high single strain Bacillus subtilis was supplemented in layers diet which was attributed to the temporal variation in the dose of probiotic used in the study. Lack of effect in the current study could be attributed to sufficient carotenoids present in the diet.

The inclusion of probiotic had no significant effect on eggshell weight (Table 5). The observed range of shell weight (6.3189 to 6.4680g) in this study fell within the range 4.65 to 6.5g reported by several authors when layer chickens were fed incremental levels of probiotics (Gnanadesigan *et al*., 2014; Fathi *et al*., 2018; Sarfo *et al*., 2019; Kinati *et al*., 2021). Fathi *et al*., (2018) reported an increase in eggshell weight by 4% when 200 and 400ppm probiotics were supplemented in layers diet. The increase was attributed to increase intestinal availability of calcium and eventual deposition in shells. Similar to the current study, several authors have reported no effect of probiotic supplementation on eggshell weight (Neijat *et al*., 2019; Macit *et al*., 2021; Marwi *et al*., 2021; Yan *et al*., 2019; Yang *et al*., 2020). Lack of effect on eggshell weight could be attributed to sufficient amount of mineral (Ca & P) in the commercial diet which was efficiently utilized for improved eggshell quality.

Inclusion of probiotic had no significant effect on eggshell thickness (Table 5). The eggshell plays an important role in protecting the egg from physical and pathogenic damage and is affected primary by nutrition (Ca & P content in the diet). The average eggshell thickness in this study ranged from 0.44 to 0.45mm and was within the reported range of 0.35 to 0.51mm (Chung *et al*., 2015; Park *et al*., 2016; Aalaei *et al*., 2018; Fathi *et al*., 2018). Bidura *et al*., (2019) reported a significant increase in eggshell thickness by 18.26% and 23.53% when 0.20% and 0.30% Saccharomyces spp probiotic were incorporated in ducks' diet respectively. Ray *et al*., (2022) reported a significant increase in shell thickness in both single strain and multi-strain probiotic treated groups at week-37 of laying (11.11% vs 16.67% respectively) and week-49 of laying (5% vs 5% respectively) compared to control. The researchers attributed the increase to bacteria proliferation in the gut thus increasing rate of fermentation and fatty acid production that reduce luminal pH which improves calcium solubility and promote absorption. In addition, Mikulski *et al*., (2020) and Fathi *et al*., (2018) reported a significant increase by 1.68% and 2.77% respectively on eggshell thickness in probiotic supplemented groups. They attributed it to probiotic ability to enhance calcium absorption and retention. Other studies have reported no effect of probiotic supplementation on eggshell thickness (Xu *et al*., 2006; Khan *et al*., 2011; Aalaei *et al*., 2018). Lack of effect maybe due to a well-balanced diet sufficient in mineral (calcium and phosphorus) content that have a major effect on eggshell thickness.

The effect of inclusion of probiotic on eggshell % (weight of eggshell relative to whole egg) is shown in Table 5 above. There was no significant treatment effect. The eggshell % as with other eggshell qualities, is affected by age, nutrition, heat stress and diseases. It serves as an indicator of whether sufficient mineral was deposited in the shell relative to its weight. The average eggshell % in this study ranged between 9.207 to 9.538. The observed values fall within the range 9.79 to 11.86 in different studies where laying chicken were fed various diets (Shalaei *et al*., 2014; Sobczak & Kozlowski., 2015). Fathi *et al*., (2018) reported a significant increase in contribution of egg shell to egg weight (7.37%) when 200 and 400ppm probiotics were incorporated in layers diet. Milkuski *et al*., (2012) reported a significant higher shell % when dietary single strain probiotic (*Pediococcus acidilactici*) was supplemented in layers diet during layer phase 1 (23 – 34week; 6.90% vs 5.85%), layer phase 2 (35 to 46week; 6.34%) and entire period (23 to 46week; 4.73% vs 6.20%). The increase in shell percent was attributed to probiotic ability to improve physiological condition of digestion and gut health (intestinal absorption). In addition, Ray *et al*., (2022) reported a significantly higher shell percent (7.36%) at week-37 in multi-strain fed birds while at week-49 both single and multi-strain improved shell percent (2.35 and 2.35% respectively). Several studies have reported no effect of probiotic supplementation on eggshell percent (Shalaei *et al*., 2014; Manafi *et al*., 2016; Yan *et al*., 2019). Lack of effect of probiotic inclusion in the current study on eggshell traits could be attributed to similar feed intake as a result of a well-balanced diet.

The effect of inclusion of probiotic on mineral (Ca & P) content of the eggshells was significant and is shown in Table 5 above. The mean eggshell calcium content in this study was in the range of 49.39 to 52.83% and increase with inclusion of probiotics (except Prob15) while phosphorus was between 0.47 to 0.53% and also increase with inclusion of probiotic. Eggshell Ca and P content can be affected by principally by dietary content of calcium and phosphorus and it is important as they constitute 95% and 3% of the shells respectively. The observed values are however higher than 30.87 to 37.63% for calcium and

0.12 to 0.15% for phosphorus (Abdelqader *et al*., 2013; Bidura *et al*., 2019; Wang *et al*., 2021). This could be attributed to the use of a single strain of bacteria in those studies while in the current study a multi-strain was used. Abdelqader *et al*., (2013) reported a significant increase when probiotics (1g/kg vs 19.65% increase), prebiotics (1g/kg vs 36.99% increase) and synbiotics (1g/kg vs 38.73% increase) were fed to aged layers (64-weeks) on calcium content of the shells compared to control. Wang *et al*., (2021) reported no effect ($p>0.05$) on P content of the eggshell but Ca content of eggshell was significantly increased (8.25%) when *Bacillus subtilis* was supplemented in aged layers (79 weeks). In addition, Bidura *et al*., (2019) reported a significant increase of Ca content of eggshell by 17.28% and 16.85% when 0.20% and 0.30% Saccharomyces spp were incorporated in ducks' diet respectively. Increase mineral content of eggshell could be attributed to probiotic efficacy in increasing intestinal Ca and P availability, absorption and eventual deposition in eggshells (Zou *et al*., 2021). It is however reported that calcium and phosphorus mineral salts require a low pH for solubility which was further enhanced by probiotic supplementation leading to ionization of the minerals and eventual absorption and deposition in eggshells (Soetan *et al*., 2010; Likittrakulwong *et al*., 2021). It has been reported that eggshell quality decrease with age of birds (Robert., 2004). Diet deficient in minerals (Ca & P) will lead to poor eggshell quality which was not the case in the current study. From this study and others, it can be concluded that dietary manipulation through probiotics supplementation is effective in improving mineral content of eggshells.

CHAPTER 6

6.0 CONCLUSIONS AND RECOMMENDATIONS

The aim of these study was to determine the effect of inclusion of probiotics in drinking water on layers performance and egg quality. Five inclusion levels of 0ml/L, 2.5ml/L, 5ml/L, 10ml/L and 15ml/L were provided to 150 ISA Brown layers via drinking water. The five treatments were replicated five times with six layers randomly allocated to each replicate. The layers weekly body weight, weekly feed intake, FCR, weekly egg specific gravity, weekly egg yolk color, weekly eggshell weight, weekly eggshell thickness and weekly egg weight were determined. Layers egg production and water intake were recorded daily and expressed for the entire period. The mineral content of the eggshell was determined thrice (first week, third week and last week).

6.1 CONCLUSIONS

It was concluded that;

1. Supplementation of laying birds with probiotics up to 15ml/L had no significant effect on performance, and egg quality.
2. The supplementation of probiotics up to 15ml/L increased Ca and P deposition in the eggshells.

6.2 RECOMMENDATIONS

From this study it is recommended that, a multi-strain probiotic (MolaPlus®) can be supplemented in layers diet via drinking water up to 10ml/L to improve mineralization of shells which was significant in addition to an improved FCR.

More research should be done to investigate the effect inclusion of probiotic (MolaPlus®) on retention of nutrients. There is also need to look onto for any resistance in the microbes present in probiotics.

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APPENDICES

Appendix 1: Ethical Approval

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

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REF: FVM BAUEC/2021/311

Mr. Nkiambuo Brian Ndambuo.
Dept. of Animal Production, Animal Nutrition & Feed science
University of Nairobi
14/09/2021
Dear Brian,

RE: Approval of proposal by Faculty Biosafety, Animal use and Ethics committee

Performance and egg quality of ISA brown layers supplemented with different levels of multi strain probiotics via drinking water.

Nkiambuo Brian Ndambuo J56/38195/2020

We refer to your MSc. proposal submitted to our committee for review and your application letter dated 16th August 2021.We have reviewed your application for ethical clearance for the study.

The number of ISA Brown hens and protocol that will be used to evaluate the effects of supplementation of multi strain probiotics via drinking water on layin perfomamnce and egg quality meets the minimum standard of the Faculty of Veterinary medicine ethical regulation guidelines.

We also note that registered Veterinary surgeons will supervise the study.

We hereby give approval for you to proceed with the project as outlined in the submitted proposal.

Yours sincerely,

Kaluwa

Dr. Catherine Kaluwa, Ph.D
Chairperson, Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine,
University of Nairobi

Appendix 2: ANOVA table of daily feed intake (g/bird/day)

Source of Variation	Df.	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	384.21	96.05	2.03	0.128
Residuals	20	945.22	47.26		
Total	24	1329.42			

SEM = 3.07

Appendix 3: ANOVA table of daily egg weight (g/egg)

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	2.656	0.664	0.18	0.948
Residuals	20	74.945	3.747		
Total	24	77.601			

SEM = 0.866

Appendix 4: ANOVA table of initial body weight (g/bird)

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	106623.	26656.	2.33	0.091
Residuals	20	228658.	11433.		
Total	24	335280.			

SEM = 47.82

Appendix 5: ANOVA table of final body weight (g/bird)

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	42518.	10630.	1.47	0.247
Residuals	20	144134.	7207.		
Total	24	186652.			

SEM = 37.97

Appendix 6: ANOVA table of daily hen day egg production (%)

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	20.129	5.032	1.48	0.246
Residuals	20	68.044	3.402		
Total	24	88.173			

SEM = 2.357

Appendix 7: ANOVA table of feed conversion ratio

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	0.09629	0.02407	1.68	0.193
Residuals	20	0.28611	0.01431		
Total	24	0.38240			

SEM = 0.0535

Appendix 8: ANOVA table of daily water intake (ml/bird/d)

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	236.4	59.1	0.06	0.992
Residuals	20	18455.3	922.8		
Total	24	18691.6			

SEM = 13.59

Appendix 9: ANOVA table of daily protein consumed (g/bird/day)

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	7.2639	1.8160	2.03	0.128
Residuals	20	17.8705	0.8935		
Total	24	25.1344			

SEM = 0.423

Appendix 10: ANOVA table of egg specific gravity (g/cm^3)

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	1.791E-05	4.478E-06	0.85	0.513
Residuals	20	1.059E-04	5.294E-06		
Total	24	1.238E-04			

SEM = 0.001029

Appendix 11: ANOVA table of yolk colour

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	0.10382	0.02596	0.27	0.896
Residuals	20	1.94667	0.09733		
Total	24	2.05049			

SEM = 0.140

Appendix 12: ANOVA table of egg shell weight (g)

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	0.07958	0.01989	0.48	0.751
Residuals	20	0.83064	0.04153		
Total	24	0.91022			

SEM = 0.09114

Appendix 13: ANOVA table of egg shell thickness (mm)

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	7.5483	1.8871	1.90	0.150
Residuals	20	19.8623	0.9931		
Total	24	27.4105			

SEM = 0.004

Appendix 14: ANOVA table of eggshell %

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	0.29394	0.07348	1.37	0.278
Residuals	20	1.06913	0.05346		
Total	24	1.36307			

SEM = 0.1034

Appendix 15: ANOVA table of % Ca in eggshells

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	24.5603197	6.1400799	6461.11	<.001
Residuals	10	0.0095031	0.0009503		
Total	14	24.5698229			

SEM = 0.01780

Appendix 16: ANOVA table of % P in eggshells

Source of Variation	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Inclusion levels	4	0.0060886	0.0015221	13.32	<.001
Residuals	10	0.0011428	0.0001143		
Total	14	0.0072314			

SEM = 0.00617