

**PERFORMANCE OF IMPROVED INDIGENOUS LAYER CHICKEN FED ON  
ENZYME-TREATED MULBERRY (*Morus alba*) LEAF MEAL-BASED DIETS**

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for the Master of Science Degree in Animal Nutrition of Egerton University**

**EGERTON UNIVERSITY**

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## DECLARATION AND RECOMMENDATION

### Declaration

This thesis is my original work and has not been presented in this university or any other for the award of a degree.

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## **DEDICATION**

This work is dedicated to the Almighty God for giving me knowledge, understanding and a sound mind to tackle this project. To my husband Titus Kibet and our daughter Angela Jepchumba for the encouragement and push to succeed and for all the sacrifices you have made for me.

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## ABSTRACT

Poultry production, particularly indigenous chicken (IC) plays a significant role as an affordable source of protein and improves the livelihoods of smallholder farmers through income generation. However, its production is hampered by an inadequate supply of feed, which makes up the major cost of production which results in low chicken productivity. Mulberry leaves are locally available, and have high protein content,  $\beta$ -carotene and ascorbic acid. Using mulberry leaf meal (MLM) as an alternative protein source to soybean and fish meal in poultry diets reduces feed costs. However, the incorporation of MLM in layer diets is limited by high crude fibre content resulting in poor nutrient utilization. This study evaluated the *in vitro* digestibility of enzyme-treated and fermented MLM. The experiment was laid out in a completely randomized design (CRD) with four treatments replicated three times. The treatments were: T1: MLM treated with an enzyme, T2: untreated MLM, T3: MLM fermented with *Bacillus coagulans* and T4: MLM treated using natural fermentation. Data on *in-vitro* digestibility was collected and a feeding trial was conducted for 10 weeks based on the results of the *in-vitro* digestibility experiment. In the feeding trial, experimental diets were formulated to meet the nutritional requirement of indigenous layer chicken as at 4 inclusion levels of MLM (0, 10, 20 and 30%) and 2 levels of an enzyme (0 & 0.035 g). The experiment was set up in  $4 \times 2$  factorial arrangements in a CRD. Data on external (shell thickness and weight) and internal (yolk colour and yolk-to-albumen ratio) egg quality parameters was collected during the feeding trial. Data were subjected to analysis of variance (ANOVA) using the general linear model (GLM) procedure in the statistical analysis system (SAS, 2009). Significant differences among means for chicken performance and egg qualities were separated using Duncan's New Multiple Range test ( $p < 0.05$ ) and Tukey's test ( $p < 0.05$ ) for the IVDMD. The results showed that pre-treatment of MLM with enzyme (Natuzyme®) enhanced *in-vitro* digestibility as compared to untreated, natural, and *Bacillus coagulan* induced fermentation. In the feeding trial, results showed that feed intake, increased with an increase in treated MLM inclusion levels in the diet, while egg production decreased with an increase in MLM in diet after exceeding up to 10%. In conclusion, enzyme-treated MLM should be incorporated up to 10% in the indigenous chicken layer diet to improve egg production and quality.

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## LIST OF ABBREVIATIONS AND ACRONYMS

|              |   |
|--------------|---|
| <b>AA</b>    | Amino acids   |
| <b>ANF</b>   | Anti-nutritive factor                                   |
| <b>ANOVA</b> | Analysis of variance                                    |
| <b>CF</b>    | Crude fibre   |
| <b>CP</b>    | Crude protein   |
| <b>CRD</b>   | Completely randomized design                            |
| <b>DNJ</b>   | Deoxynojirimycin  |
| <b>FAO</b>   | Food and Agriculture Organisation of the United Nations |
| <b>FCR</b>   | Feed conversion ratio                                   |
| <b>FI</b>    | Feed intake   |
| <b>FRS</b>   | Free range system                                       |
| <b>GLM</b>   | General linear model                                    |
| <b>KALRO</b> | Kenya agricultural livestock and research organization  |
| <b>ME</b>    | Metabolizable energy                                    |
| <b>MLM</b>   | Mulberry leaf meal                                      |
| <b>NDF</b>   | Neutral detergent fibre                                 |
| <b>NSRC</b>  | National Sericulture Research Centre                    |
| <b>NRC</b>   | National Research Council                               |
| <b>SBM</b>   | Soybean meal  |

## **CHAPTER ONE**

### **INTRODUCTION**

#### **1.1 Background Information**

According to the 2019 household and livestock census, the Kenyan poultry population was estimated to be over 43,796,477 birds. About 36,578,441 were free-ranging indigenous chicken, 7,218,035 commercial layers and broilers, while the rest were other species (ducks, turkeys, pigeons, ostriches, guinea fowls and quails) (MoALFC, 2019). Indigenous chickens are kept by 90% of the rural communities in small flocks of up to 30 birds, mainly under the free-range system (Magothe *et al.*, 2012). Poultry production is flexible and does not require much space compared to other livestock enterprises. Indigenous chickens are widely distributed in the rural areas of tropical countries, where they are kept by most rural poor and contribute more than 50% of the total eggs and meat consumed (King'ori *et al.*, 2014). Although indigenous chicken (IC) contribute a significant proportion of egg requirements, their productivity is low. To boost the productivity of indigenous chicken in Kenya, a research was done by Kenya Agriculture and Livestock Research Organization (KALRO) Naivasha over years of intensive research under the Nation Poultry Development Programme for the improvement of pure IC. Improved IC are easy to establish for low-income families, grows faster, are highly resistant to diseases and have high productivity compared to the pure indigenous chicken (KALRO, 2013) and high commercial hybrid (Hassan *et al.*, 2004). They are easy to maintain, are suitable for free range and can be utilized as a dual-purpose breed by among small scale farmers. They are less fatty, and tastier compared to the hybrid chicken, lay more eggs than the local chicken and have softer meat associated with improved growth (KALRO, 2013). However, their productivity is faced with many challenges due to inadequate supply of feeds all year round. The feeds are limited during the dry season, consequently decreasing the productivity of IC. One way of improving productivity of IC is by providing adequate (quality and quantity) feed throughout the year. This can be accomplished by using feed resources that are locally available and affordable such as mulberry leaf meal (MLM).

The poultry production is highly dependent on feed prices because feeds account for about 60-70% of poultry production costs (Khairu *et al.*, 2014). Soybean meal (SBM) remains poultry's most essential and preferred protein feed source. However, the supply and

quality of SBM fluctuate and the meal is expensive due to processing and transportation costs (Erdaw *et al.*, 2016). In addition, it does not have sufficient  $\beta$ -carotene, which imparts the yellow yolk colour preferred by most consumers. The increasing costs of conventional feedstuffs like corn, soybean meal and fish meal for poultry diets are making poultry feed producers to find less expensive alternatives (Olteanu *et al.*, 2012). One possible feed alternative is mulberry leaf meal (*Morus alba*) (Al-Kirshi *et al.*, 2013). Mulberry leaf meal (MLM) has been identified as a locally available and affordable alternative protein source in poultry feeds and contains  $\beta$ -carotene that may improve egg production and yolk colour. Its leaves are considered a high-quality forage plant resource because of its high crude protein content (22-29.8%), balanced amino acid composition, rich in vitamins, trace elements, phytosterols, flavonoids, alkaloids, polysaccharides, and other bioactive substances (Ding *et al.*, 2021). Due to their high protein content, mulberry leaves are thought to be suitable for feed supplementation and have been tried on pigs and chickens. However, due to the high content of crude fibre in mulberry leaves and branches and the presence of anti-nutritional factors such as tannins, high inclusion of mulberry leaves and branches in diet would affect the performance and health of poultry. This, to a certain extent, limits its use in poultry feed production. Mulberry leaf meal has been used in laying hens up to 10% without adversely affecting egg production and quality (Al-kirshi *et al.*, 2010). This rate of inclusion in layer diets is low and can be improved through the use of suitable biotechnology.

Incorporation of enzymes in poultry diets has been one of the significant nutritional advances in the last fifty years (Slominski, 2011). Feed industry experts have long known that applying “best-generation” feed enzymes can be a potential solution to the ever-increasing feed costs by optimizing the ingredients’ usage and unlocking unavailable nutrients. Enzymes are used in feed supplements in increasing nutrient, the number of enzymes availability to the animal, mitigating the negative influences of anti-nutritional factors, such as  $\beta$ -glucan and arabinoxylans and increase the availability of nutrients and energy and enhance their absorption from feed components. Moreover, they alter the microbial environment of the gut by raising beneficial organisms at the expense of harmful organisms. Enzymes that have been established as efficient in animal feed include the Natuzyme which is a highly effective multi-enzyme feed additive developed by Bioproton that has been shown to significantly enhance the nutrition and

health benefits for livestock and aquaculture. It is composed of Phytase, hydrolyses phytate substrates to release phosphorus, which is essential for bone health and growth. Non-starch polysaccharide (NSP) enzymes, including xylanase, mannanase,  $\beta$ -glucanase, and cellulase, break down plant cell walls, leading to the degradation of cellulose, hemicellulose and  $\beta$ -glucan into simple sugars such as glucose, mannose, and xylose.  $\alpha$ -amylase catalyses the breakdown of starch into maltose, while protease catalyses the breakdown of proteins into amino acids (Al-Harthi *et al.*, 2018). Natuzyme helps animals get more out of their feed, leading to better growth rates, improved overall health and lower feeding costs.

## **1.2 Statement of the Problem**

The feed cost in poultry production usually constitutes the major proportion which ranges between 60-75% of the total cost. Soybean meal is the main protein ingredient in poultry feeds. It is expensive, inadequate in supply and of variable quality. It also does not have sufficient  $\beta$ -carotene which gives the egg yolk the deep yellow colour. Due to high cost of protein (conventional) feed sources such as soybean and fish meal, and which is further exacerbated by high competition between humans and animals, there is need for exploration of locally available and cheaper alternative protein feed sources. One possible alternative protein source that is locally available and affordable is Mulberry leaves. Despite Mulberry leaves being a locally available source of protein, it contains high fibre content determined as neutral detergent fibre (NDF) and also anti-nutritive compound known as 1-deoxynojirimycin (DNJ). These anti-nutritive factors lower digestibility and absorption in mulberry leaves by preventing polysaccharide hydrolysis. This study therefore evaluated the effect of incorporating enzyme in a mulberry leaf meal-based indigenous chicken layer diet on performance.

## **1.3 Objectives**

### **1.3.1 General Objective**

To contribute to food and nutrition security in Kenya through sustainable chicken production by use of mulberry leaf meal as a source of protein.

### **1.3.2 Specific Objectives**

- i. To determine the effect of fermentation and enzyme treatment of Mulberry leaf meal on *in vitro* digestibility

- ii. To determine the effect of inclusion of enzyme-treated MLM on indigenous layer chicken performance
- iii. To determine the effect of inclusion of enzyme-treated MLM on external and internal egg quality of indigenous layer chicken

#### **1.4 Hypotheses**

- i. Fermentation and enzyme treatment of Mulberry leaf meal has no significant effect on *in vitro* digestibility
- ii. Incorporation of enzyme-treated MLM in IC layer diet has no significant effect on performance.
- iii. Incorporation of enzyme-treated MLM in IC layer diet has no significant effect on external and internal egg quality.

#### **1.5 Justification of the study**

The cost of poultry production in Kenya is high due to the high cost of feeds, mainly protein sources which include soybean and fish meal (Chia *et al.*, 2019). The supply of these protein sources in Kenya is limited, mostly, because of importation tariffs and competition for use as human food, without a guarantee of quality. They are, therefore, expensive to procure, and this makes the prices of the feed expensive, leading to low profitability and unsustainable poultry production. To make poultry production economically viable in Kenya, there is a need to identify cheap and locally available feed resources for use as a protein supplements. Mulberry leaf meal can be one such alternative with a huge potential for use in poultry feeds. The government is therefore implementing a policy of promoting utilization of locally available feed resources. Mulberry is locally available, it is utilized by people as fruit, and leaves are used to feed silkworms, consumed as vegetables, medicine, as source of wood fuel. It is rich in  $\beta$ -carotene, which can be converted to vitamin A and contains xanthophyll that imparts yellow pigmentation in the egg yolk (Tuigong *et al.*, 2015). Dried mulberry leaves contain 5.11-7.24% moisture, 15.31-30.91% crude protein, 14.59-17.24% total ash, 27.60-36.66% NDF, 2.09-4.93% crude fat, 9.70-29.64% carbohydrate, 113-224 kcal/100 g energy, 100-200 mg/100 g ascorbic acid and 13.12- 84.38g/100 g  $\beta$ -carotene, 19.00-35.72 mg/100 g iron, 0.72-3.65 mg/100 g zinc and 786.66-2226.66 mg/100 g calcium, the ranges vary depending on the stage of maturity of the leaves (Ustundag *et al.*,2015). However, the high crude fibre in mulberry is

an anti-nutrition factor which prevent nutrient digestibility; therefore, limiting its use as poultry feed (Mwai *et al.*, 2022). This therefore, calls for biotechnological intervention such as the use of prebiotics and probiotics, addition of vaccines or antibodies in feeds, metabolic modifiers, genetic manipulation of microbes and use of enzymes. Enzymes can enhance the feed utilization by improving the availability of nutrients from feed and to reduce the wastage of the feed and fodder. Enzymes can be used for removal of ant nutritional factors (e.g.,  $\beta$ -glucan and arabinoxylans in barley grain cell-wall), increasing the digestibility of nutrients (e.g. Phytate phosphorus in grains) and non-starch polysaccharides (e.g., xylose and arabinose in plants). Improvement in weight gain and feed utilisation efficiency with supplementation of enzymes such as cellulase and hemicellulase has been reported. Therefore, the application of enzyme in this study is expected to enhanced poultry productivity by improving the digestibility of crude fibre. The results of this study will therefore provide a guide in utilization of mulberry leaves treated with enzyme as a locally available alternative protein source in poultry feed. This will improve the growth of the industry and livelihoods of poultry farmers in Kenya.

## **CHAPTER TWO**

### **LITERATURE REVIEW**

#### **2.1 Poultry Industry in Kenya**

Poultry (avian species) have been recognised as affordable source of high-quality protein worldwide in the forms of meat and eggs. The poultry sector has been shown to become the world's largest meat sector by 2020. Besides, the sector continues to record high global output of eggs as additional high-quality food protein. The rapid growth of the poultry sector is driven by several factors such as an increasing human population, greater purchasing power in developing economies, increased urbanisation and industrialisation in developing countries, development and transfer of feed, relatively short production cycle and advances in poultry breeding, and improved processing technologies. Of these factors, feed has been recognised as the most important factor controlling profitability and product quality (Dei, 2017).

According to the 2019 household and livestock census, the Kenyan poultry population was estimated to be over 38 million birds with 78% being free-ranging indigenous chicken, 21% commercial layers and broilers, while the rest being other poultry species (ducks, turkeys, pigeons, ostriches, guinea fowls and quails) (KNBS, 2019). While indigenous chickens are mainly found in rural areas, broilers and layers are kept in urban areas (Magothe *et al.*, 2012). Currently the commercial poultry sector is producing over one million chicks per week, 14% being layers and the rest being broilers. Based on the number of animals, poultry represents the largest domestic animal stock in the world and poultry meat was the fastest growing component of global meat production in the early 21st century. Poultry meat and eggs provide affordable high-quality protein. Poultry farming, especially on a small scale, is renewable and efficient and can provide a ready source of income and nutrition. The key drivers of the commercial market are a growing urban population and a growing retail sector such as fast-food branches, supermarket branches and restaurants. The demand for commercial chicken (whole, half, parts, grilled and fried chicken) and eggs is high and growing.

#### **2.2 Chicken Production Systems in Kenya**

Poultry farming in the world involves different types of production systems depending on the conditions in each region. Production systems has been classified according to production objectives, commercial or subsistence (Magothe *et al.*, 2012). Based on husbandry practices

and levels of inputs and outputs, Menge *et al.* (2005) identified and categorised IC production systems in Kenya into free range systems (FRS), semi-intensive systems (SIS) and intensive systems (IS). Whereas all production systems are practised in both rural and urban residential areas, the choice of a particular system depends on a households' land availability and the objective attached to the enterprise. The main aim of keeping chicken include subsistence or family use only, subsistence and cultural use, home consumption and income, and income only.

### **2.2.1 Free Range Systems (FRS)**

This chicken production system is practiced in most rural areas of the country and objectives of production under this system are for household consumption and as a source of additional income. Management of birds in this system is mainly based on available indigenous technical knowledge. Small flocks of less than 30 chickens per household are kept most being indigenous breeds and dependant on locally available feed material as supplement, with low health services and other management practices. The birds are mostly left to scavenge for feeds during the day and confined at night (Larsen *et al.*, 2017). During cropping seasons, birds are sometimes confined and supplemented with maize, kitchen leftovers and any other available feed resource. Night shelters include rudimentary coops, kitchens, stores and human habitats. Due to low inputs, production is also low but the cost per unit of egg or meat is nearly negligible. There is also long broody periods and risk of exposure to diseases and predators (Magothe *et al.*, 2012).

Semi-intensive system requires low to medium inputs depending on the value attached to the birds. Small flocks of chicken (5 to 50 birds) are kept mainly for family use and sale. Most chickens reared under this system are mainly crosses of indigenous and exotic. In this system, birds are left to free range (to source for any available feed resources) during the day around the homestead or in fenced runs and confined to shelters during the night (Magothe *et al.*, 2012). They are supplemented with grains, kitchen leftovers and commercial feeds. Water and veterinary care are also provided (King'ori *et al.*, 2010). In this system, there is complete control over operation, record keeping throughout the year, economic use of land and there is better protection during the cold season. Healthcare depends on the commercial value attached to the enterprise. However, water and supplementary feeds are provided. Because input levels are low, production is lower than in intensive system. The system is common in high human population density rural and peri-urban areas.

### **2.2.3 Intensive Production System**

In this system, more inputs are used than in free range and semi-intensive systems. It is market oriented and focuses on profit maximization. The flock is confined full time and supplied with a balanced diet. Vaccination against endemic diseases is common under this system. However, the system is not common in most field situations because of the high level input required. The type of housing used includes; use of cages and deep litter system of housing. The intensive system is common in urban and peri-urban areas and it is mostly practiced by financially stable individuals who want to invest in poultry farming (Magothe *et al.*, 2012). The number of chickens involved are relatively high (more than 200 chicken). Breeds used are specialized improved breeds (layer or broiler). In addition, the relative economic importance of each trait is needed to ensure that genetic improvement is proportional to the overall objective of the production system. Improvement in genetic potential of the indigenous chicken should be accompanied by a concomitant improvement in the standard of management (Menge *et al.*, 2005).

### **2.2.4 Deep Litter Housing System**

Deep litter system is the most popular intensive system of rearing poultry in East Africa (Okedere *et al.*, 2020). It involves rearing of chickens on a floor littered by 5-10 cm thickness litter. The litter can be made from locally available material such as dry hay, straw, coffee pulp and wood shavings. In this system of poultry production, birds are kept on the litter throughout their lifecycle. In addition to provision of comfort for the chicken, the litter absorbs any waste material excreted from the chicken and make the house dry (Ovwigho *et al.*, 2009). The structures of the deep litter system must include a large, well-ventilated house without draught, feeders and drinkers should be provided for the birds to eat and drink from comfortably, and perches and laying nests should also be provided for laying birds (Okedere *et al.*, 2020).

### **2.2.5 Battery Cage Housing System**

The system is best for layers whereby the hens live in cages where feeds and water are provided. The main housing systems found in current egg production are conventional cages, enriched cages and cage-free systems, which include multi-tier, barn, free-range and organic

productions (Hardin *et al.*, 2019). For controlled-environment housing of layers, multi-tier cage systems are common.

Egg production is a dependent variable and is influenced by the breed of chicken, age at point of-lay, and the environment (Kassa *et al.*, 2021) In this system, there is accommodation, prompt culling of unproductive birds, proper control of diseases and predators, good record keeping and high egg production (Ovwigho *et al.*, 2009). Land requirement is minimum, easy and economic management, scientific feeding and management, high degree of supervision, minimum labour and automation is possible and manure value is increased.

### **2.3 Overview of Livestock Feed Industry in Kenya**

Kenya's livestock sub-sector plays an essential role in the country's economy through directly providing food, income and employment to millions of Kenyans and indirectly providing raw materials to the agro-processing industry (Njagi *et al.*, 2013). However, the livestock's contribution to agricultural GDP has been declining. Poor animal husbandry, mainly because of poor feeding practices, has led to low livestock productivity. In part, the high costs of commercial livestock feed, the unfriendly business environment for trade in livestock feed, and the weak regulatory environment have affected the availability, affordability, and utilisation of quality commercial feeds to grow the livestock value chains. This has resulted in increased competition from imports for livestock products and animal feed ingredients from neighbouring countries. The costs of animal feeds have been increasing since 2021, with the price increases attributed to a shortage of raw materials such as soya and oil cakes seeing the most significant price increases. According to the Association of Kenya Feed Manufacturers (AKEFEMA), the price of soya and oil cakes increased by more than 60% in 2021. The Ministry estimates that the country utilises about 12.5% of the total maize demand on animal feeds. Kenya only has a stable production of maize. However, due to unstable production of and a proportion being used as a staple food commodity, and also competition from other uses, Kenya must rely on imports to meet her total maize demand. Maize is usually imported from neighbouring countries, especially Uganda and Tanzania. Other raw materials used for animal feed production, such as wheat, rice, oil cakes and vitamins, are primarily imported. Maize germ, wheat grain, wheat pollard, rice bran, rice polish and millet are mainly sourced from Uganda. Soya bean, Soy meal, and groundnut cake are primarily sourced from Tanzania (Oloo, 2011). Sunflower meal, cotton

seed meal, and omena are sourced from Uganda and Tanzania. Vitamins, mineral premixes, and amino acids are mainly imported from Asia and South Africa, with China being a key source country in Asia.

## **2.4 Poultry Nutrition and Feeding**

Indigenous chickens (*Gallus domesticus*) are chickens that are adapted to harsh environmental conditions that include extensive small-scale village, free-range and organic production systems. Numerous studies have shown that local chickens play a key role in improving the socio-economic status of many rural communities. However, poor housing, lack of coordinated disease control mechanisms, poor feeding and the absence of conservation strategies are some of the challenges facing local chicken production systems in Africa (Mtileni *et al.*, 2009). Providing the right nutrition is important for the growth, production and health of poultry. Furthermore, depending on factors such as bird age and production status, different nutrients are required. This also applies to local chickens to ensure that they achieve their productive potential and remain healthy. Poor-quality feeds and incorrect mixing of dietary nutrient levels such as energy and protein can potentially cause nutritional stress and health concerns among local chickens (Hatori *et al.*, 2012). Chickens vary greatly according to the purpose they have been developed for (Khobondo *et al.*, 2015). Those raised for egg production have a small body size and are known as layers, whereas those raised for meat are known as broilers.

Several studies have been conducted to improve the productivity of local chickens through efficient feeding. According to Alabi *et al.* (2014) protein, lysine (a base for all other amino acids) and energy requirements of local chickens must be met optimally in order to improve and maximise productivity (Tables 2.1 & 2.2). According to King'ori *et al.* (2010), a protein level of 16% in local chickens aged between 14 and 21 weeks has been observed to optimise feed intake and growth. However, protein levels of 17% to 23% have been reported as not to have any effect on the growth and feed intake of local chickens (Mujyambere *et al.*, 2022).

**Table 2. 1 Recommended Protein and Energy Levels for Indigenous Chicken**

| <b>Nutrient level<br/>protein</b> | <b>Age (weeks)</b> | <b>Researchers</b>            |
|-----------------------------------|--------------------|-------------------------------|
| 17-23%                            | 1-6                | Ndegwa <i>et al.</i> (2001)   |
| 18-19%                            | 1-6                | Mbajorgu <i>et al.</i> (2010) |
| 18%                               | 13                 | Manyelo <i>et al.</i> (2020)  |
| 15.53%                            | 14                 | King'ori <i>et al.</i> (2014) |
| 16%                               | 14-21              | King'ori <i>et al.</i> (2014) |
| <b>Energy</b>                     |                    |                               |
| 14 MJME/kg                        | 1-6                | Mbajorgu <i>et al.</i> (2010) |
| 1234 MJME/kg                      | 7                  | Alabi <i>et al.</i> (2014)    |
| 2842–3200 kcal/kg ME              | 6-9                | Ndegwa <i>et al.</i> (2001)   |
| 12.91 MJ ME/kg                    | 8-13               | Alabi <i>et al.</i> (2014)    |
| 2750 kcal/kg ME                   | 9-20               | Mohammad <i>et al.</i> (2012) |

**Table 2. 2 Nutrient Requirement of Indigenous Chicken**

| <b>Nutrient</b>     | <b>Chicks</b> | <b>Growers</b> | <b>Layers</b> |
|---------------------|---------------|----------------|---------------|
| Energy ME MJ/kg     | 11.9          | 11.2           | 11.2          |
| Crude protein %     | 19-20         | 13-14          | 15-17         |
| Crude fibre (min) % | 3.0           | 4.0            | 4.5           |
| Calcium %           | 1.0-1.1       | 1.3-3.0        | 3.3-3.7       |
| Phosphorus %        | 0.6           | 0.5            | 0.5           |
| Lysine %            | 1.0           | 0.6            | 0.7           |
| Methionine %        | 0.4           | 0.3            | 0.4           |

**Source:** King'ori (2004)

## **2.5 Feed Resources**

In Africa and other developing countries feeds and feeding comprise 60-70% of total production costs (Lukuyu *et al.*, 2011). With the present trend of rising feed stuff prices and global inflation, livestock production is increasingly constrained by feed scarcity and the high cost of feeds. Not

every country has access to soybeans and corn, generally accepted as the basis for pig and poultry diets. Some countries may not have the financial resources to import these feedstuffs; others may not have the land or favourable climate to grow them (Farrell, 2005). Those that do import them may be in a precarious position when difficult economic circumstances arise such that their industry can easily collapse (Meremikwu, 2009). Food agricultural organization (FAO) predicts that in the next 30 years developing countries will treble their cereal imports and their meat imports will rise five-fold. The rapid increase in the price of oil has helped to push up the cost of conventional feedstuffs and affect other activities, ranging from transport to chicken processing.

Protein and carbohydrate are by far the two most important nutrients in poultry diets due not only to their market effect on voluntary feed intake of the bird, but also the fact that they represent approximately 90% of the total cost of the ingredients in a ration (Dei, 2017). Birds eat primarily to satisfy their energy needs, provided that the diet is adequate in all other essential nutrients. The energy level in the diet is therefore a major determinant of poultry's feed intake. When the dietary energy level changes, the feed intake will change, and the specifications for other nutrients must be modified to maintain the required intake. For this reason, the dietary energy level is often used as the starting point in the formulation of practical diets for poultry (FAO,2013). Cereal grains constitute a large proportion (>50%) of poultry diets and contribute largely carbohydrates and to some extent proteins. They are mainly dietary source of energy, but can vary widely between grain types and animal species (Gopi, 2020). The common feed grains used for manufacturing poultry feeds include poultry are corn or maize (*Zea mays*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and sorghum or milo (*Sorghum bicolor*).

## **2.6 Protein Feed Ingredients**

Plant and animal origin feed ingredients are used as protein sources when formulating poultry feeds. The function of dietary protein is to supply amino acids for maintenance, muscle growth and synthesis of egg protein. Plant protein ingredients includes: sunflower meal, soybean meal, canola meal, beans and peas, while meat meal and fish meal are the common animal protein sources (Suthama & Wibawa, 2018). At the moment, soybean has been the main protein source for poultry feeds. It has an excellent amino acid profile that complements cereal grains in diet formulation, as methionine is typically the only limiting amino acid for poultry (Dozier & Hess, 2011). The amino acid requirements of poultry are influenced by several factors, including

production level, genotype, sex, physiological status, environment and health status. For example, high levels of lean meat deposition require relatively high levels of lysine. High levels of egg output or feather growth require relatively high levels of methionine. Animal origin protein sources have traditionally been used for animal feeding. However, they pose a high risk of introducing diseases to animals through this practice. Consequently, feed ingredients containing or contaminated by animal matter from any source must not be fed to livestock in Africa (Akhter *et al.*, 2008). According to Al-Qazzaz *et al.* (2016), many of the traditional ingredients used in poultry diets are foreseen to be in short supply within ten years. Thus, there is a need for identifying alternative protein sources that meet dietary requirements and reduce feed costs.

## **2.7 Soybean (*Glycine max*) Meal in Poultry Nutrition**

Soybean meal (SBM) is the dominant protein supplement used in poultry diets and is the standard to which alternative protein sources are compared. Soybean meal has relatively high crude protein (CP) level, good amino acid profile and high digestibility making it standard as compared to other plant protein sources. It represents two-thirds of the total world output of protein feedstuffs, including all other major oil meals and fish meal (Jannathulla *et al.*, 2019). Soybeans can be fed to poultry whole or as soybean meal, a by-product of oil extraction (Waldroup & Smith, 2018). Pressure is applied to the soybeans in order to extract the oil using methods such as mechanical extraction, or by solvent extraction, even though mechanically extracted soybean meal is used in organic poultry diets. The expansion of aquaculture and prohibitions on the feed use of slaughterhouse by-products have also increased the demand for this high-quality source of protein. Soybean meal (SBM) also has an excellent amino acid profile that complements that of corn, the primary energy source in poultry diets. Antinutritive factors in SBM include, lectins, protease trypsin inhibitors (heat sensitive), isoflavones and oligosaccharides. Protease trypsin inhibitors are mostly considered during evaluation of SBM nutritive value (Facey, 2022).

Fifty-five to 60% of the total phosphorous found in soybeans is bound to phytate. Poultry possess insufficient endogenous phytase to liberate substantial quantities of phosphorous from the phytate compound. The high phytate content of soybean meal requires supplementation with inorganic sources of phosphorus in monogastric animals.

## 2.8 Mulberry Plant

Mulberry belongs to the *Morus* genus of Moraceae. Mulberry is a fast-growing deciduous plant found in wide variety of climatic, topographical and soil conditions, and is widely distributed from temperate to subtropical regions. Due to presence of valuable phytochemical constituents, mulberry as a whole plant has been utilized as a functional food for long time. *Morus alba* (white mulberry), *Morus nigra* (black mulberry) and *Morus rubra* (red mulberry) are all commonly accepted worldwide species of genus *Morus* as they exhibit maximum medicinal properties (Ercisli & Orhan, 2007). Roots, leaves, bark, stem twigs, and fruits of mulberry possess valuable bioactive constituents that can be explored in food, health care, and cosmetic industries. Its fruits, leaves, and barks in traditional Turkish folk medicine have been utilized as an anti-fever, an expectorant, assists in the discharge of urine, to lower blood pressure, as a folk remedy to treat dental diseases, in dysentery, as a de-worming agent, laxative, anthelmintic, odontalgic, treat diabetes, hypertension, arthritis, and anaemia. Leaves of mulberry have been used as a feed for silk worm larvae and therefore making a major contribution in the sericulture industry since ancient times. The extract of mulberry leaves may play role in catalysing the metabolism of glucose. Devi *et al.* (2013) and Lown *et al.* (2017), reported that mulberry leaf extract lowered high blood glucose levels in animal studies. Mulberry leaves contain an important flavonoid called resveratrol. This flavonoid may help to remove constriction in blood vessels and thus may have an effect on reducing the chances of heart failure.

Mulberry leaf meal is a protein source which is available in rural areas in Kenya. The fruits/berries are consumed by people, the leaves are used for feeding silkworms (Tuigong *et al.*, 2015), while the tree branches are used for wood fuel and timber. Mulberry leaves contain  $\beta$ -carotene, which can be converted by poultry to vitamin A and xanthophylls, which can be a source of pigmentation of egg yolk (Srivastava *et al.* 2006). Mulberry is high quality forage which is palatable to both monogastric and ruminant livestock (Sergon, 2020). The nutrient composition of Mulberry leaves shows that it is a formidable forage for monogastric with 70 -90% palatability and 15 to 28% essential amino acid profile depending on the variety (Vijayan, 2009) and has a high mineral content with no known anti-nutritional factors.

### 2.8.1 Varieties of Mulberry

There are about 68 species of the genus *Morus* in the world, the majority of which occur in Asia. In Kenya, the dominant species is *Morus alba*. Although these species may have originated from foreign countries during colonial times, the naming of the varieties depended on where the mulberry tree was first sited in Kenya. These include

*Morus alba* - *Ex-Embu* variety is characterized by short internodes; purplish coloured bark prominent at the shoot tips. The variety has many small leaves and is drought resistant. This variety is more susceptible to leaf spot than other varieties but can be controlled by timely harvesting of leaves.

*Morus alba* - *Ex-Thika* is characterized by large light green slightly drooping leaves, has long internodes and whitish bark. It is fairly drought tolerant. The young shoot is weak and may need support to avoid falling or bending.

*Morus alba* - *Ex-Limuru* is characterized by small finger shaped deeply serrated leaves, very thin shoots with short internodes. It is a high berry producer and is thus not recommended for silkworm rearing due to low leaf harvest but recommended for berry production

*Morus alba Ex-Ithanga* is characterized by medium heart shaped and smooth light green leaves. It may sometimes produce a few lobed leaves. Roots easily and is fairly drought tolerant. It is suitable for both silkworms rearing and berry production (Mwai *et al.*, 2021).

### 2.8.2 Nutritive Value of Mulberry Leaves

Mulberry leaves are very rich in protein (15-35%), minerals (2.42-4.71% calcium (Ca); 0.23-0.97% phosphorus (P)) and metabolizable energy (1130-2240 kcal/kg) with absence of or negligible anti-nutritional factors (Sanchez, 2002). The amino acids composition of mulberry leaf meal indicates it is a good source of essential amino acids, especially lysine 1.80% and leucine 2.58%. Excellent results have been obtained with mulberry leaves as poultry feed (Al-kirshi *et al.*, 2010). The ratio of Ca:P in mulberry leaves was found to be 10:1, which suggests the Ca and P in MLM is unbalanced (Al-Kirshi *et al.*, 2013). The consequence would be that P supplementation would be required when high levels of MLM are included in diets. The recommended level of Ca:P for other animals including chickens is 2:1, according to NRC (1994), dried mulberry leaves contain 5.11-7.24% moisture, 15.31-30.91% crude protein, 14.59-17.24% total ash, 27.60-36.66% NDF, 2.09-4.93% crude fat, 9.70-29.64% carbohydrate, 113-224 kcal/100 g energy, 100-200

mg/100 g ascorbic acid and 8438-13.12 g/100 g  $\beta$ -carotene, 19.00-35.72 mg/100 g iron, 0.72-3.65 mg/100 g zinc and 786.66-2226.66 mg/100 g calcium (Ustundag *et al.*, 2015). Srivastava *et al.* (2006) reported that fresh mulberry leaves contain 71.13-76.68% moisture, 4.72-9.96% crude protein, 4.26-5.32% total ash, 8.15- 11.32% neutral detergent fibre (NDF), 0.64- 1.51% crude fat, 8.01-13.42% carbohydrate, 69-86 kcal/100 g energy, 160-280 mg/100 g ascorbic acid, 10.000-14.688  $\mu$ g/100g  $\beta$ -carotene, 4.70-10.36 mg/100 g iron, 0.22-1.12 mg/100g zinc and 380-786 mg/100 g calcium.

### **2.8.3 Mulberry Leaves in Poultry Nutrition**

Although mulberry leaves are generally used to feed the silkworms, many researchers have studied it as an alternative feed source for animals due to the high fibre content (Sujathamma *et al.*, 2013). Mulberry leaves powder has also been used to feed poultry (Simol *et al.*, 2012). Digestibility of mulberry leaves is very high by ruminants (Huyen *et al.*, 2012). However, digestibility of mulberry leaves dry matter is poor (35-37%) by poultry due to the high neutral detergent fibre (NDF). Despite poor utilization of mulberry leaves dry matter, crude protein and ether extract are highly digested at 73% and 88%, respectively (Al-Kirshi *et al.*, 2013).

Besides the nutritive value, mulberry leaves are nontoxic natural therapeutic agents known to possess antidiabetic, antimicrobial, antimutagenic, antioxidant, anticancer, anxiolytic, anthelmintic, anti-stress, immunomodulatory, hypo-cholesterolemic, nephroprotective, hepatoprotective activities (Devi *et al.*, 2013; Yang *et al.*, 2012). They have been reported to significantly increase yolk weight, shell weight, shell strength, shell thickness, Haugh unit, and yolk colour (Lokaewmanee *et al.*, 2009). Mulberry has an anti-nutritive compound called 1-deoxy nojirimycin (DNJ). Energy source absorption can be affected by this anti-nutritive compound, therefore resulting to prevention of polysaccharide hydrolysis and decreasing metabolic energy. DNJ from mulberry can block  $\alpha$ -glycosidase activity which hydrolyzes polysaccharide into plain molecule (Has *et al.*, 2013). In poultry, mulberry leaves dry matter digestibility is poor as a result of high neutral detergent fibre (NDF) content (Ustundag & Ozdoga, 2015). The leaves contain high crude fibre and deoxynojirimycin. In human, deoxynojirimycin becomes active compound for diabetes, but in poultry feed serve as anti-nutrition preventing carbohydrate digestibility; therefore, its limited use as feed.

## **2.9 Approaches to Enhance Utilization of Fibrous Diets by Layer Chicken**

Dietary fibre (DF) has been considered as an ant nutritional factor and a diluent in poultry diets. To enhance the utilization of fibrous diets by layer chicken such as Cellulose and hemicellulose that are not well digested, there is need for the use of chemical, mechanical, and biological methods of treatment. These have been proven to be effective in rendering dietary fibre more degradable (Kanengoni *et al.*, 2015). These dietary fibre cannot be hydrolyzed by the digestive enzymes in the small intestine but can be fermented to a certain degree by the microflora (Jha *et al.*, 2015).

### **2.9.1 Exogenous Enzyme Treatment**

Exogenous enzymes are potentially important alternatives to antibiotics for improving growth performance, particularly in poultry and swine, although research in other animals such as ruminants, fish, fur-bearers, and pets has been done in recent years (Mael *et al.*, 2019). Enzymes break down the non-starch polysaccharides (NSPs), decreases intestinal viscosity and eventually improve the digestibility of nutrients by improving gut performance (Jha *et al.*, 2015). The value of added feed enzymes in promoting growth and efficiency of nutrient utilization in animal production is clearly recognized. Enzymes as feed additives for food production animals are biologically active proteins that facilitate chemical breakdown of nutrients to smaller compounds for further digestion and absorption (Saeed *et al.*, 2019).

In poultry, feed enzymes generally increase its digestibility, which could be particularly beneficial to young animals; thus, young birds may benefit from a wide spectrum of enzymes, such as lipase, proteases, and amylases. Many other enzyme classes can significantly improve the utilization of feed such as xylanases,  $\beta$ -glucanases, pectinases, amylases, and proteases.

The use of exogenous feed enzymes in poultry diets is becoming a norm to overcome the adverse effects of ant nutritional factors (ANFs) that are present in plant-based feedstuffs such as phytic acid, non-starch polysaccharides (NSP), and cell wall complex carbohydrates, and to improve digestion of dietary components and bird performance. The cell walls of cereal grains, legumes, and oilseed meals are made up of complex carbohydrates commonly referred to as NSP (Choct, 2009). NSP are a complex group of components differing widely in chemical composition, physical properties, and physiological activity. NSP consist of a wide range of polymers including hemicellulose, pectins,  $\beta$ -glucans (consisting of either a more soluble or a non-soluble fraction),  $\alpha$ -galactosides (raffinose, stachnyose, verbascose) and xylans.

The enzymes currently used in monogastric diets are predominantly glycanases, which cleave NSPs into smaller polymers, thereby removing their ability to form viscous digesta and enhancing nutrient digestibility. The effects of glycanases are generally nonspecific, except for their effect on fat (greater effect on saturated fat than on unsaturated fat). Another enzyme used in feed is phytase, which increases the utilization of phytate phosphorus.

### **2.9.2 Fermentation Treatment**

Fermentation has been used for food and feed processing and preservation, respectively, for a long time in history. Fermentation increased crude protein content but decreased crude fibre content (Sugiharto & Ranjitkar, 2019) several ANF and toxic compounds in feed ingredients. Apart from improved nutritional properties, fermentation is associated with a high number of lactic acid bacteria (LAB), a low pH and a high concentration of organic acids (Jakobsen *et al.*, 2015). In terms of cost efficiencies, the replacement of expensive conventional feedstuffs such as yellow maize in broiler diets may further encourage the use of cheaper unconventional fermented feedstuffs in broiler nutrition.

Depending on the type of microorganisms involved, fermentation will result in the formation of different final products such as lactic acid, ethanol or acetic acid, as different microorganisms may react differently to each substrate e.g. *Lactobacillus* produce lactic acid, mould yield citric acid, whereas yeasts generate ethanol and CO<sub>2</sub> (Couto & Sanroman, 2006).

## CHAPTER THREE

### EFFECT OF FERMENTATION AND ENZYME TREATMENT OF MULBERRY LEAF MEAL ON *IN VITRO* DIGESTIBILITY

#### Abstract

*In-vitro* digestibility is a technique used to improve the level of precision while mimicking the physiological and chemical characteristics of the digestive system of the animal to which the ingredient will be feed. This method is simple, reproducible, less expensive and less time-consuming when compared to the *in vivo* models. This study examined the effect of (Natuzyne®) enzyme and fermentation treatments on *in-vitro* dry matter digestibility (IVDMD) of mulberry leaf meal (MLM). Pepsin-pancreatin hydrolysis was used in this experiment to mimic the chicken stomach. The MLM was subjected to four treatments replicated 3 times in a completely randomized design (CRD). The treatments were T1: untreated MLM, T2: enzyme-treated MLM, T3: MLM fermented with *Bacillus coagulans* and T4: Naturally fermented MLM. Data (moisture, ash, crude protein, crude fibre, NDF and ADF) on proximate analysis and digestibility were analysed using Statistical Analysis Software SAS version 9.4, general linear model (GLM). Tukey's test at ( $p < 0.05$ ) was used to do means separation. The results showed that the treatment method had a significant effect ( $p < 0.05$ ) on the *in-vitro* dry matter digestibility (IVDMD). MLM treated with enzyme (Natuzyne®) and *Bacillus coagulans* had improved IVDMD by 7.31% and 3.99% respectively compared to untreated ( $p < 0.05$ ). Enzyme treatment significantly differed from natural fermentation ( $p < 0.05$ ). Based on the results of this study, pre-treatment of MLM with enzyme (Natuzyne®) enhanced *in vitro* digestibility as compared to untreated, natural, and *Bacillus coagulans* induced fermentation.

#### 3.1 Introduction

Chicken have been a widespread poultry species worldwide due to the cultural, social, and economic role they play in the daily livelihoods of the population. Indigenous chickens have played a substantial role by contributing to the food security of rural households across the developing world (Padhi, 2016). Their products, meat and eggs often constituted the main source of protein and income, and they served as a source of investment and security for rural households. However, utilisation of intensive system (IS) should be considered because land availability for

practicing free range system (FRS) is reducing due to the ever-increasing human population, and therefore the production systems may shift to intensive system (Magothe *et al.*, 2012). Feed prices affect the poultry sector significantly since feed costs account for 60–70% of poultry production costs (Khairu *et al.*, 2014). Owing to the rising costs of conventional poultry feeds such as maize, soybean meal and fish meal, consumers are looking for less expensive locally available alternatives (Olteanu *et al.*, 2012). Among the possible feed substitutes is mulberry leaf meal (*Morus alba*) (Al-Kirshi *et al.*, 2013).

Due to its high crude protein content (22-29.8%), balanced amino acid composition, and high content in vitamins, trace elements, phytosterols, flavonoids, alkaloids, polysaccharides, and other bioactive compounds, its leaves are regarded as a high-quality forage plant resource (Ding *et al.*, 2021; Rizk *et al.*, 2005) despite the high protein content in mulberry leaves, it has high content of crude fibre which limits high levels of inclusion in poultry feeds. Furthermore, the presence of anti-nutritional factors such as tannin in mulberry leaves and branches makes its addition in high proportion affect the production performance and health of livestock and especially, poultry thus to a certain extent this limits its large-scale application in animal production. The gastrointestinal tract (GIT) of chicken lacks the necessary microbes (fungi and bacteria) to digest fibre into products that are utilizable, in contrast to ruminants (cattle, sheep, and goats). Chicken also lack cellulase, an enzyme that breaks down cellulose, non-starch polysaccharides and oligosaccharides (Abu, 2019). This makes feed and forages that are high fibre content, in general to be unsuitable to poultry. Plant cell walls are composed of cellulose, non-starch polysaccharides (NSP), pectin and lignin (Cowieson *et al.*, 2013). The plant's NSP (e.g. pectins and lupins) component is associated with antinutritive factors that can make it difficult for chickens to digest nutrients (Cowieson *et al.*, 2013). Thus, this study evaluated the impact of mulberry leaf meal treatment with enzyme and fermentation on *in vitro* dry matter digestibility.

## **3.2 Materials and Methods**

### **3.2.1 Area of Study**

The experiment was conducted at the Animal Nutrition laboratory, Department of Animal Sciences, Egerton University, which is located in the Njoro Sub-County of Nakuru County at 0° 23 S and 35° 55 N. The region is 1,800 M above sea level with an average annual rainfall of 900-

1,200 mm and temperatures ranging from 17°C to 22°C (Egerton University Weather Station, 2019).

### 3.2.2 Experimental Layout and Samples Preparation

Mulberry (*Morus alba*) leaves were collected from trees at National Sericulture Research Centre (NSRC). The centre is located in Gatanga sub-county, Muranga County, S 1°0'9.894" Latitudes, E 37°4'42.661" Longitude (Mwai *et al.*, 2022). They were harvested by plucking the whole branches from the plant. After collection the leaves were oven dried at a temperature of 60°C for 48 hours. The dried mulberry leaves were finely ground to powder using a universal hammer mill (Number of Hammers :24 Rotor Dia:220 mm) and stored in air-tight polythene bags in a cool and dry place. Proximate analysis of the samples was done at Egerton University, Animal Science Nutrition Laboratory following the association of official analytical chemists (AOAC) protocols (AOAC, 2006 Version 3).

The *in-vitro* digestibility trial consisted of four treatments replicated 3 times, in a completely randomized design (CRD). The treatments were; T1: Untreated MLM, T2: Enzyme-treated MLM T3: *Bacillus coagulans* fermented MLM and T4: MLM treated using natural fermentation. In preparation of enzyme- treated MLM (T2), the Natuzyme® enzyme powder containing (12,000 parts/g of xylanase, 6,000 parts of cellulase, 1,500parts/g of phytase 700 parts/g of beta-glucanase, 700 parts/g protease and 400 parts/g of alpha-amylases) was utilized. It was incorporated at 350 mg/kg and thoroughly mixed with the dry MLM samples based on the guidelines and instructions from the manufacturer. For T3 preparation, an inoculant containing a single strain of *Bacillus coagulans* powder sourced from the feed biotechnology laboratory, China Agricultural University with a concentration of  $2.0 \times 10^4$  CFU/g was utilized as the starter culture. To ensure the samples were uncontaminated, they were sterilised in an autoclave at 121°C for 10 minutes. The culture was added to three separate samples of 1 kg of MLM and then mixed with distilled water at a ratio of 1:2.50 (wt/vol) (Chuah *et al.*, 2021). The inoculated MLM was cultured in the laboratory at 22°C in tightly wrapped 2 kg plastic bottles for 7 days. The natural fermentation (T1) was prepared by incubating a mixture of 1 kg MLM and 1:2.75 (wt/vol) distilled water in triplicate for seven days at room temperature (22°C) in two 2 kg sealed plastic bottles (Jørgensen *et al.*, 2010). The pH of each sample was determined using a portable pH meter (pH/ORP/Temperature Combo Tester -

HI98121 HANNA instruments) after 7 days, whereupon a sample was collected for proximate analysis.

### 3.2.3 Proximate Analysis

Proximate composition was determined by the AOAC (2006) procedures, Dry matter method.930.15, ash content method.942.05, ether extract method 920.39 and total nitrogen for crude protein (N x 6.25) by Kjeldahl method 984.13. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined using the Van Soest method (Van Soest *et al.*, 2010). Hemicellulose was calculated as the difference between neutral detergent fibre (NDF) and acid detergent fibre (ADF).

### 3.2.4 Enzymatic Pre-Digestion of Mulberry Leaf Meal

The experiment was conducted to mimic the digestion in the chicken digestive system as described by Zhao *et al.* (2010). A ground MLM sample (0.4 g) was weighed and put in a 100 ml digestion tube before the addition of simulated gastric fluid. To mimic the *in vivo* activity of pepsin in chicken stomach fluid, a fluid containing 1,550 U/mL of pepsin (Sigma 10070; Sigma-Aldrich Co., St. Louis, MO) was used (Sturkie, 2012). The gastric buffer solution contained 16.9 mmol/L of NaCl, 9.6 mmol/L of KCl, and 10 mmol/L of HCl to match the *in vivo* ionic concentration of gastric fluid from roosters (Sturkie, 2012). The pH was raised to 2.0 at 41°C by adding 200 mmol/L of HCl. Each digestibility test tube was put 2 ml Chloramphenicol C-0378; Sigma-Aldrich Corp., St. Louis, MO, USA (0.5 g/100 ml 19 ethanol) to prevent bacterial growth. The test tubes were then sealed and incubated in a water bath at 39°C with continuous stirring for 2 hours.

The first step's mixture was combined with 20 ml of 0.6 M NaOH and 80 ml of phosphate buffer (0.2 M, pH 6.8). The pH was brought down to 6.8 using 1 M HCl or 1 M NaOH to establish a stable environment for intestinal enzymes to perform well. To the mixture, 10.6 ml of artificial pancreatin P-1750 Sigma-Aldrich Corp., St. Louis, MO, USA (porcine grade enzyme with 3 x USP activities) containing 100 mg/1 litre buffer was added and incubated at 39°C with constant stirring for 4 hours. Remains were put in 1.5 ml centrifuge tubes and centrifuged (12700×g) for 2 min. The mixture was carefully withdrawn, washed twice with 20 ml of 95% ethanol and 99.5% acetone, and then rinsed with distilled water. Those that remained were then dried for 12 hours at 70°C in the oven prior to weighing.

### 3.2.5 Computation of Dry Matter Digestibility

The following formulae were used to determine the *in-vitro* digestibility (IVDMD) of dry matter (DM) as per the formulae by Boisen & Fernández (1997)

$$DM \text{ digestibility} = \left( \frac{DM_{In} - DM_{RS}}{DM_{In}} \right) \times 100$$

where:

Where the initial (DM) and residual (DM) are denoted by DM<sub>In</sub> and DM<sub>RS</sub>, respectively

### 3.2.6 Statistical Analysis

Data on proximate analysis and digestibility were analysed using Statistical Analysis Software SAS version 9.4, general linear model (GLM). Tukey's test ( $p < 0.05$ ) was used to do means separation.

### 3.2.7 Statistical Model

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk}$$

where;

$Y_{ijk}$  = overall effect of treatment

$\mu$  = overall mean

$\alpha_i$  = effect due to fermentation

$\beta_j$  = effect due to enzyme

$\varepsilon_{ijk}$  = error term component

### 3.3 Results

The nutrient composition of the treated mulberry leaf meal is presented on Table 3.1

**Table 3. 1 Nutrient Composition (%) of Treated Mulberry Leaf Meal**

| Parameters    | T1                       | T2                       | T3                        | T4                        | P value |
|---------------|--------------------------|--------------------------|---------------------------|---------------------------|---------|
| DM            | 93.07 <sup>a</sup> ±0.14 | 92.84 <sup>b</sup> ±0.07 | 93.07 <sup>a</sup> ±0.03  | 84.52 <sup>c</sup> ±0.13  | <.0001  |
| Ash           | 11.09 <sup>a</sup> ±0.33 | 11.34 <sup>a</sup> ±0.05 | 10.75 <sup>ab</sup> ±0.03 | 10.11 <sup>b</sup> ±0.03  | 0.0309  |
| CF            | 12.05 <sup>a</sup> ±0.04 | 10.58 <sup>b</sup> ±0.09 | 11.24 <sup>ab</sup> ±0.03 | 12.02 <sup>a</sup> ±0.33  | 0.0123  |
| CP            | 28.42 <sup>a</sup> ±0.2  | 28.42 <sup>a</sup> ±0.2  | 26.94 <sup>b</sup> ±0.08  | 24.43 <sup>c</sup> ±0.16  | <.0001  |
| EE            | 14.18 <sup>a</sup> ±0.05 | 11.91 <sup>b</sup> ±0.12 | 2.53 <sup>d</sup> ±0.13   | 9.75 <sup>c</sup> ±0.06   | <.0001  |
| NDF           | 25.00 <sup>b</sup> ±1.00 | 39.00 <sup>a</sup> ±1.00 | 33.00 <sup>ab</sup> ±3.00 | 30.00 <sup>ab</sup> ±2.00 | 0.0484  |
| ADF           | 20.48 <sup>b</sup> ±0.32 | 25.58 <sup>a</sup> ±0.32 | 23.34 <sup>a</sup> ±0.28  | 20.00 <sup>b</sup> ±0.32  | 0.0063  |
| ADL           | 5.39 <sup>a</sup> ±0.21  | 2.10 <sup>b</sup> ±0.10  | 2.09 <sup>b</sup> ±0.30   | 3.69 <sup>ab</sup> ±0.51  | 0.0281  |
| Hemicellulose | 4.52                     | 13.75                    | 9.66                      | 10                        |         |

<sup>a,b,c,d</sup> Means within a row with different superscripts differ significantly ( $P<0.05$ ). T1: Untreated MLM, T2: Enzyme-treated MLM, T3: *Bacillus coagulans* fermented MLM T4: MLM treated using natural fermentation. DM= Dry matter, EE= Ether extract, NDF= Neutral Detergent Fibre, ADF= Acid Detergent fibre, ADL= Acid Detergent lignin.

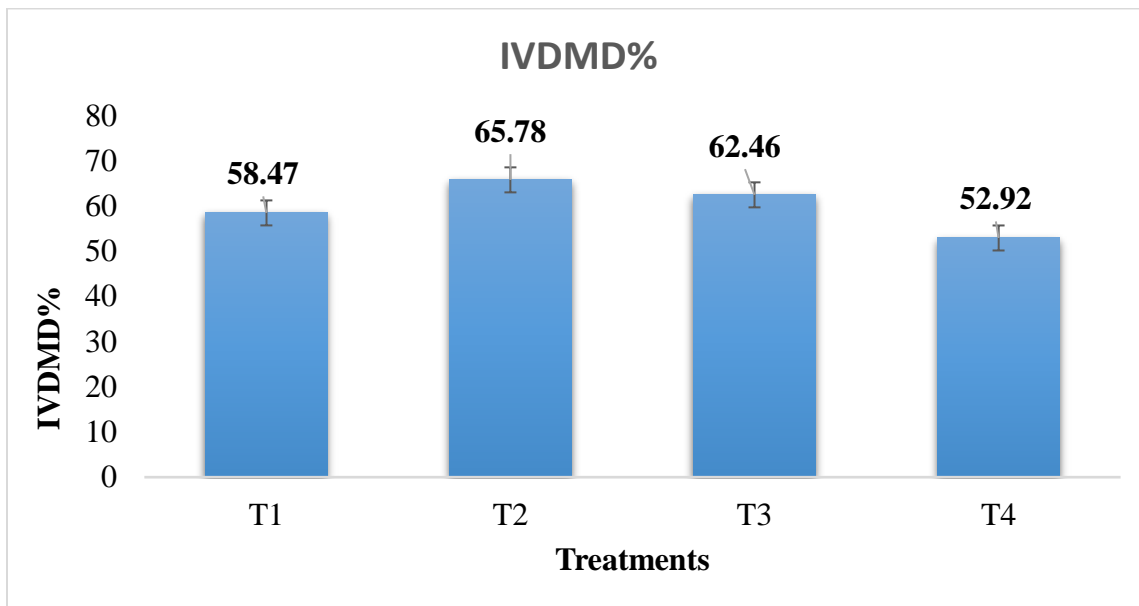
#### 3.3.1 Nutritional Quality of Treated MLM

The moisture content of MLM in the four treatments were significantly different ( $P<0.05$ ). The moisture content ranged between 6.92- 15.48%. DM content differed significantly in the four treatments at ( $P<0.05$ ). Crude protein content differed significantly. Enzyme-treated MLM had the highest crude protein content of 28.42% while naturally fermented MLM had the least protein

content of 24.43%. The crude fat content of the four treatments varied significantly from one another. The *Bacillus coagulan* treated MLM contained crude fat of 2.53% while the Untreated MLM had 14.98%. The ash content varied from 10.11 to 11.34%. The highest ash content (11.34%) was recorded in enzyme-treated MLM. This could be because of the impact of ash on enzymatic hydrolysis because the ash buffer pH levels potentially hindering the enzymatic conversion. The NDF, ADF and ADL of MLM differed significantly among various treatments.

### 3.3.2 The Enzymatic Pre-Digestion of Untreated, Enzyme-Treated and Fermented Mulberry Leaf Meal

The highest digestibility was achieved with enzyme treatment ( $65.78 \pm 0.22$ ), while natural fermentation had the lowest digestibility ( $52.98 \pm 0.75$ ). Natural fermentation and enzyme treatment were significantly different ( $p < 0.05$ ). In contrast to natural fermentation, IVDMD was enhanced by *Bacillus coagulans* inoculation. The IVDMD increased by 7.31% (Fig.3.1) following the MLM's treatment with the enzyme ( $65.78 \pm 0.22$ ), relative to the untreated ( $58.47 \pm 0.29$ ). The digestibility results are presented in Fig 3.1.



**Figure 3.1** In vitro dry matter digestibility results. Error bars represents the Mean ( $\pm$ SE) IVDMD of MLM

### 3.4 Discussion

Since chicken have single-stomach, they lack the enzymes needed to break down complex carbohydrates like cellulose, hemicellulose, and lignin (Swain, 2016). Complex carbohydrates make up a sizable portion of fibrous by-products, therefore, it is essential to develop methods to make better use of these materials so that they can be added to poultry feed without negatively affecting the health and productivity of the birds. Enzyme treatments and natural fermentations can be used to improve the nutritive value of alternative feed resources such as MLM. Furthermore, the effectiveness of such intervention can rapidly be assessed using *in vitro* digestibility as opposed to the expensive and time-consuming *in vivo* digestibility method.

Several variables, including the feed-to-water ratio, the inoculant used in the fermentation process and temperature changes between the environment and the fermentation process, can affect how feed material ferments (Marii *et al.*, 2022). After the 7<sup>th</sup> day of fermentation both pH of naturally and *Bacillus coagulans* fermented MLM decreased. *Bacillus coagulans* recorded lower pH compared to natural fermentations. This could be because *Bacillus coagulans* is a sporogenic lactic acid bacterium that grows optimally and produces lactic acid as the primary fermentation product from both hexoses and pentoses thus leading to decrease in pH. The pH value is a critical component influencing and improving the fermentation quality of ensiled forage (Denek *et al.*, 2011). In both the control and treated silages, the final pH (4.5 or less) promotes optimal fermentation (Chen *et al.*, 2016). The pH for *Bacillus coagulans* and natural fermentation in this study varied between 4.6 and 5.67, which is slightly higher than the pH of silage. The possible explanation for the difference in pH could be differences in mulberry leaves' DM contents, which was 93.07% for *Bacillus coagulans* and 84.52% for naturally fermented MLM. High dry matter content usually means more substrates for microorganisms to consume, potentially increasing fermentation rates. Therefore, the reason for the high pH value of mulberry leaf meal in this study could be due to the high dry matter content and less fermentable carbohydrates which led to high acid production. According to studies, the population of LABs reduces because bacterial development slows down in low water activity due to the high dry matter content of ensiled forages (Rizk *et al.*, 2005; Whiter *et al.*, 2001). The MLM treated with *Bacillus coagulans* however, had a lower pH (4.6) compared to natural fermentations, this could be due to higher water-soluble carbohydrates available for utilization by epiphytic lactic acid bacteria, which then transforms

them into lactic acid with a lesser proportion of acetic acid. This lowers the pH of the silage and inhibits unfavourable microbial growth, allowing it to be preserved for a long period (Soundharrajan *et al.*, 2021).

Mulberry leaf meal's nutritional content increased following fermentation using *Bacillus coagulans*, as fermentation increases the nutritional quality of MLM by increasing protein levels, reducing antinutritional factors, and improving the bioavailability and bioaccessibility of nutrients in this study this was indicated by the increase in CP and the decrease in CF, NDF, ADF and ADL compared to the natural fermentations. In this study, the CF of untreated MLM was 12.05, naturally fermented MLM 12.02 %, and *Bacillus coagulans* inoculated 11.24% while for enzyme treatment the CF was 10.58% (Table 3.1) respectively. The findings of this study are consistent with those of Yu *et al.* (2018) and Ding *et al.* (2021) who reported that unfermented and fermented mulberry leaf meal had a crude fibre range between 11-12.30%. Furthermore, Cubas-Cano *et al.* (2013) found reduced NDF and ADF contents in treated silages after *Lactobacillus plantarium* and *Bacillus coagulans* were added because of the acid broke down hemicellulose during ensiling. Additionally, *Bacillus coagulans* produced the enzymes cellulase and feruloyl esterase, which accounted for the pH drop caused by fermentation of the released fermentable carbohydrates. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) levels decreased indicating that there was destruction of plant structural carbohydrates, which may be connected to the production of fibrinolytic enzymes by *Bacillus coagulans* during the fermentation of silage (He *et al.*, 2020). This might be the explanation why NDF and ADF levels decreased after fermentations.

Fermenting feeds lowers the quantity of anti-nutritional factors in the feed, increases the protein content (lysine, histidine, and methionine), improves the bioavailability of minerals (such as P, Ca, Mg, and Cu), and breaks down indigestible carbohydrates (Njenga *et al.*, 2011). Lactic acid bacteria (LAB) fermentations have been proven to increase the amount of key amino acids like lysine, methionine, and tryptophan in cereals. This is because Lactic acid fermentation modifies the content and the composition of proteins due to the presence of enzymes and due to the presence of other components such as acids. Microbial proteases are able to break peptide bonds and produce new polypeptides or even free amino acids, hence enhancing their nutritional value (Mohapatra *et al.*, 2019).

Mulberry leaves can be conserved effectively by ensiling and the addition of additives could enhance the silage's quality forages (Dong *et al.*, 2016; Trabi *et al.*, 2017). In this study, fermentation of mulberry leaves with *Bacillus coagulans* and natural fermentation led to the loss of organic matter, which was in agreement with other studies that also reported decreased DM losses due to LAB inoculation in the combined silage of sorghum and soybean (Al-Kirshi *et al.* 2013). During fermentation the soluble carbohydrates are converted into lactic acid, acetic acid, ethanol, CO<sub>2</sub>, and water by LAB fermentation and which represent a modest loss of DM and energy value (Pahlow *et al.*, 2003). According to Zhao *et al.* (2018) additives (hemicellulose and *Lactobacillus plantarum*) induced a rapid pH drop at the early stage of ensiling and significantly decreased DM loss. In the present study, *Bacillus coagulans* inoculation, increased acid production and lowered the pH, inhibiting protein hydrolysis. It could be that inoculation with *Bacillus coagulans* increased the initial LAB load, accelerating fermentation, promoting acid production and pH drop, and therefore suppressing the activities of spoilage organisms and protein hydrolysis (Heinritz *et al.*, 2012). The crude protein of inoculated MLM increased in comparison to naturally fermented MLM. This could be explained by the efficient bioconversion of highly polymerized carbohydrates into microbial protein, production of several enzymes, many of which are protein in nature that could be responsible for the fermentation's notable increase in protein content (Imelda *et al.*, 2008). The increase in protein content in this study is consistent with the study by Gao *et al.* (2017) that also reported increase in crude protein mulberry leaves following fermentation. In this study, both enzyme and *Bacillus coagulans* treatment increased *in-vitro* dry matter digestibility of MLM by 7.31 and 3.99 % respectively, while natural fermentation decreased it by 5.49% compared to untreated. The increased IVDMD in enzyme treatment could be because of the enzyme (Natuzyme®) that contained cellulase, xylanase and phytase that decreased the anti-nutrient factors and crude fibre (Ali *et al.*, 2020; Hong *et al.*, 2019). The enzyme (Natuzyme®) also contained xylanase and cellulase, which have both been shown to hydrolyze plant cell wall polysaccharides and degrade their structure, permitting the release of oligosaccharides and other desirable compounds (Ning *et al.*, 2021). This could explain the improved digestibility of enzyme-treated MLM. While the digestibility of *Bacillus coagulans* treated MLM might be explained by reducing the main fermentation phase and achieving the expected fermentation quality. *Bacillus coagulans*, can consume oxygen sources to generate an anaerobic environment, which fastens the

growth of lactic acid bacteria (Pattnaik *et al.*, 2001) and the acidity of silages, reducing nutrient loss by limiting the evolution of spoilage microbes. Additionally, increased IVDMD could be explained by lower fibre content in *Bacillus coagulans* fermentation compared to natural fermentation.

### **3.5 Conclusion**

Based on the findings of this study, mulberry leaf meal treated with an exogenous enzyme had higher *in vitro* digestibility compared to untreated, natural, and *Bacillus coagulans* induced fermentation.

### **3.6 Recommendation**

Exogenous enzyme (Natuzyme®) may should be used to treat MLM to be incorporated in IC layer diets to hydrolyse the lignin -cellulose bonds and improve digestibility.

## CHAPTER FOUR

### EFFECT OF INCLUSION OF ENZYME-TREATED MLM ON THE PERFORMANCE OF IMPROVED INDIGENOUS LAYER CHICKEN

#### **Abstract**

Poultry production, particularly indigenous chicken (IC) plays a significant role as an affordable source of protein and improves the livelihoods of smallholder farmers through income generation. Compared to other livestock species, indigenous chicken (IC) rearing remains attractive to poor households because the IC are hardy; adapt well to the rural environment; require less start-up capital. However, its production is hampered by the high cost of feeds, quality, variability and inadequate supply resulting in poor chicken productivity. Using mulberry leaves as a locally available alternative protein source instead of the conventional protein sources like soybean meal and fish meal in poultry diets is expected to play a vital role in the poultry industry because it can reduce feed costs. However, the incorporation of MLM in layer diets is limited by its high crude fibre content resulting in poor nutrient utilization. Therefore, this study evaluated the effect of varied inclusion levels of treated MLM in indigenous layer chicken diet on performance (feed intake, hen day production, feed conversion ratio, egg production and weight). The experiment was conducted for ten weeks after a one-week adaptation period. Eight experimental diets were formulated to meet the requirements of indigenous layer chicken as per King'ori (2004). There were 4 inclusion levels of MLM (0, 10, 20, and 30%) and 2 levels of an enzyme (0 & 0.035 g). The experimental design was a completely randomized laid out in a 4×2 factorial arrangements. Data was collected on feed intake, hen day production, egg weight and feed conversion ratio and subjected to analysis of variance (ANOVA) using the general linear model (GLM) procedure in the statistical analysis system (SAS, 2009). Significant differences among means were separated using Duncan's New Multiple Range test ( $p < 0.05$ ). Results showed that feed intake was highest in birds fed the diet with enzyme and 30% MLM, the lowest egg production was recorded in birds fed on diet with enzyme and 30% MLM, the highest HDP was observed in control diet, lowest FCR was recorded in hens fed diet with enzyme and 30% MLM. Based on the results of this study, it was concluded that the inclusion of enzyme-treated MLM in improved indigenous layer diets up to 20% adversely affect egg production, egg weights and feed conversion ratio. Therefore, enzyme-treated MLM should not be incorporated in the diets above 10 %.

## 4.1 Introduction

Poultry production is one of the most significant livestock sectors that provides the cheapest animal protein (egg and meat) for human consumption within the shortest time (Mottet and Tempio, 2017). However, the scarcity, fluctuating feed supply and the price are the major constraints to poultry production in underdeveloped countries. The feed cost usually constitutes the major proportion of the cost of poultry production which ranges between 60-75% (Khairu *et al.*, 2014). The prices of conventional protein sources for animal feed like groundnut cake; fish meal and soybean meal usually are always high and cannot permit profit maximization in poultry production. In view of this, current research interest in the poultry industry is aimed at finding alternatives to those expensive feed ingredients. Mulberry leaf (*Morus alba*) is one such alternative source of dietary protein for livestock and poultry production. Mulberry is a tree which grows well in the tropics and subtropics where it has multiple uses. For example, it is reported to have excellent nutritional value as forage. It is also grown extensively for its leaves which are used for raising silkworms in the sericulture industry, mulberry is highly appreciated for its delicious fruit, its wood is used for handicrafts, cabinetwork and for sporting woods, mulberry trees are utilized for landscaping, variety of medicinal properties have been attributed to the different parts of the mulberry plant and as a forage for feeding farm animals. Mulberry leaf meal contain protein (15-35%) (Vu *et al.*, 2011) balanced amino acid composition, rich vitamins, trace elements, phytosterols, flavonoids, alkaloids, polysaccharides, and metabolizable energy (1130-2240 kcal/kg) (Ding *et al.*, 2021). Mulberry leaf contains  $\beta$ -carotene which can be converted with varying efficiency by animals to vitamin A as well as xanthophylls which can potentially impart yellow pigmentation in egg yolk (Kamruzzaman *et al.*, 2014). However, the high content of crude fibre in mulberry leaves and the presence of anti-nutritional factors such as tannin tend to a certain extent limits its use in poultry production. Mulberry leaf meal (MLM) has been incorporated in laying hens' diet up to 10% (Al-kirshi *et al.*, 2010). The low inclusion level of MLM in poultry diets has led to the need to use biotechnologies to decrease the effects anti-nutritional factors and increase the level of incorporation in poultry diets. Therefore, this study was carried out to investigate the response of improved indigenous layer chicken to varied levels of MLM inclusion (10, 20 and 30%) with or without supplementation with an enzyme (Natuzyne®) as a feed additive.

## **4.2 Materials and Methods**

### **4.2.1 Study Site**

The feeding experiment was conducted at Centre of Excellence in Livestock Innovation and Business (CoELIB), Egerton University. The university is located at Latitude S 0°22'11.0" and Longitude E 35°55'58.0" within Rift valley in Njoro Sub-County, Nakuru County Kenya. It is at an altitude of 1,800 metres above sea level with average temperatures ranging between 17 to 22 °C. The average annual rainfall in the area is upto 1,200 mm per year (Egerton University Weather Station, 2019).

### **4.2.2 Source and Preparation of Mulberry Leaf Meal**

This is as described in chapter three, section 3.3.2

### **4.2.3 Experimental Diets**

The experimental diets (**Table 1**) were formulated to contain all nutrients to meet the nutritional requirement for indigenous layer chicken as per the (King'ori *et al.*, 2004) at the inclusion of 4 levels of MLM (0, 10, 20 and 30%) and 2 levels of an enzyme (0 & 0.035 g) the experiment was laid out in a 4×2 factorial in a completely randomized design. Diets were iso-nitrogenous (16% crude protein) and iso-caloric (2800-2850 kcal/kg). Dietary ingredients for the study comprised ground white maize, wheat pollard, soybean meal, omena (*Rastrineobola argentea*), and mulberry leaf meal. A layer premix was added to supply minerals and vitamins (**Table 4.1**).

**Table 4. 1 Nutrient Composition (kg) of Enzyme-Treated Mulberry Leaf Meal**

| <b>Ingredients</b>           | <b>T1</b> | <b>T2</b> | <b>T3</b> | <b>T4</b> | <b>T5</b> | <b>T6</b> | <b>T7</b> | <b>T8</b> |
|------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Maize grain                  | 67.7      | 67.7      | 65.8      | 65.8      | 63.5      | 63.5      | 58.6      | 58.6      |
| Wheat pollard                | 5.5       | 5.5       | 3.2       | 3.2       | 1         | 1.0       | 0         | 0         |
| Mulberry leaf meal           | 0         | 0         | 10        | 10        | 20        | 20        | 30        | 30        |
| Soya bean meal               | 15        | 15        | 8.4       | 8.4       | 2.9       | 2.8       | 0         | 0         |
| Omena ( <i>R. argentea</i> ) | 3.5       | 3.5       | 3.8       | 3.8       | 3.3       | 3.4       | 1         | 1         |
| DCP (granular 24%)           | 2.5       | 2.5       | 2.5       | 2.5       | 2.5       | 2.5       | 2.5       | 2.5       |
| Limestone                    | 5         | 5         | 5         | 5         | 5         | 5         | 5         | 5         |
| Salt                         | 0.3       | 0.3       | 0.3       | 0.3       | 0.3       | 0.3       | 0.3       | 0.3       |
| Premix                       | 0.5       | 0.5       | 0.5       | 0.5       | 0.5       | 0.5       | 0.5       | 0.5       |
| vegetable oil                | 0         | 0         | 0.5       | 0.5       | 1         | 1         | 2.1       | 2.1       |
| Enzyme                       | 0         | 0.035     | 0         | 0.035     | 0         | 0.035     | 0         | 0.035     |
| Total                        | 100       | 100       | 100       | 100       | 100       | 100       | 100       | 100       |
| <b>Calculated analysis</b>   |           |           |           |           |           |           |           |           |
| ME (kcal/kg)                 | 2834.3    | 2833.7    | 2835.9    | 2835.2    | 2832.3    | 2831.9    | 2834.8    | 2833.6    |
| CP                           | 16.1      | 16.1      | 16.0      | 16.0      | 16.0      | 16.0      | 16.1      | 16.1      |
| CF                           | 2.6       | 2.5       | 3.5       | 3.5       | 4.4       | 4.4       | 5.4       | 5.4       |

**4.2.4 Feed Analysis**

Chemical analysis of experimental diets was done at Egerton University, Animal Nutrition laboratory following the procedures AOAC (2006). Crude fibre (CF) was analysed according to the AOAC, Method 978.10, Crude protein (CP) by Method 984.13, Ether extract (Soxhlet using ether) (EE) by Method AOAC.920.39 and Moisture by Method 934.01, 2006.

**4.2.5 Birds and Management**

The house and the cages were fumigated properly with kupacide® before placing of birds. A total of seventy-two (72), 18-week-old pullets fully vaccinated improved indigenous layer chickens sourced from KALRO Naivasha were used in this study. They were randomly allocated to the eight dietary treatments replicated three times. The experimental birds were weighed and housed in individual deep litter cages measuring 1 m ×1 m ×1 m, each with three hens. Wood

shavings were placed inside the cages as beddings to absorb moisture from the droppings and were changed regularly upon caking. The chicken was dewormed before the start of the experiment and physical indicators of a good layer were factored during the selection. The birds were allowed one week of adaptation before the feeding trial commenced and continued for ten weeks. The cages were equipped with drinkers and feeders which were cleaned on daily.

#### 4.2.6 Data Collection

##### i. Feed intake

Feed intake was calculated and recorded daily as the difference in the amount of feed offered and the amount left over before the next feeding as shown in the formula below;

$$\text{Feed Intake (FI)per hen (g)} = \frac{\text{Feed offered (g)} - \text{Feed remain (g)}}{\text{Number of hens/treatment}}$$

##### ii. Feed conversion ratio (FCR)

Feed conversion ratio (FCR) was calculated as average feed intake (g) divided by average egg weight (g) during each week.

##### iii. Egg production (%)

Eggs laid were recorded per cage and hen-day production (HDP) calculated as:

$$\text{HDP} = \frac{\text{Total number of eggs produced in aday}}{\text{Total number of hens present on that day}} \times 100$$

#### 4.2.7 Experimental Design

A Completely randomized design (CRD) with a 4×2 factorial layouts was used in this study with Seventy-two (72), 18-week-old, layer improved indigenous chicken. There were 24 experimental units (cage with 3 hens) per treatment, each replicated 3 times.

#### 4.2.8 Statistical Model

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$$

where;

$Y_{ijk}$  = overall effect due to treatments

$\mu$  = overall mean

$\alpha_i$  = effect due to the  $i = \{1, 2, 3, 4\}$  treatment MLM

$\beta_j$  = effect due to  $j = \{1, 2\}$  treatment enzymes

$\alpha\beta_{ij}$  = interaction effect due to MLM and enzyme

$\varepsilon_{ijk}$  = random error effect

#### **4.2.9 Statistical Analysis**

Data was analysed using Statistical Analysis Software (SAS) Version 9.4, general linear model (GLM). Duncan's New Multiple Range test ( $p < 0.05$ ) was used to do mean separation where the means were significantly different.

#### **4.3 Results**

The results of the diets' chemical composition and the hens' performance are presented in Tables 4.2 and 4.3, respectively.

**Table 4. 2 Chemical Composition of Experimental Diets**

| Nutrients | T1         | T2         | T3         | T4         | T5         | T6         | T7         | T8         | P value |
|-----------|------------|------------|------------|------------|------------|------------|------------|------------|---------|
| DM        | 91.49±0.17 | 91.09±0.03 | 91.05±0.01 | 90.5±0.05  | 91.1±0.13  | 90.86±0.01 | 91.14±0.28 | 91.26±0.01 | <.0001  |
| EE        | 5.87±0.13  | 5.17±0.03  | 5.37±0.17  | 5.62±0.42  | 4.50±0.05  | 4.02±0.13  | 4.17 ±0.08 | 4.15 ±0.20 | <.0001  |
| CF        | 6.07±0.18  | 5.22±0.03  | 4.35±0.11  | 3.17±0.18  | 5.72±0.08  | 4.62±0.08  | 5.97±0.08  | 5.62±0.07  | <.0001  |
| CP        | 16.95±0.13 | 16.21±0.07 | 16.99±0.13 | 16.28±0.26 | 16.41±0.14 | 16.02±0.07 | 16.41±0.07 | 16.27±0.08 | <.0001  |
| Ash       | 12.44±0.36 | 13.08±0.31 | 13.25±0.04 | 12.19±0.07 | 11.74±0.25 | 13.62±0.95 | 13.48±0.42 | 13.77±0.47 | 0.0072  |

± represents the standard deviations, T1 0% MLM 0 Enzyme, T2 0% MLM 0.035 g Enzyme, T3 10% MLM 0 Enzyme, T4 10% MLM 0.035 g Enzyme, T5 20% MLM 0 Enzyme, T6 20% MLM 0.035 g Enzyme, T7 30% MLM 0 Enzyme, T8 30% MLM 0.035 g Enzyme, MC: moisture content, EE: ether extract, CF: crude fibre, CP: crude protein. The proximate analysis showed a significant increase of crude fibre as the level of inclusion of MLM in the diet increased. Diets with 30% Mulberry leaf meal (T7 and T8) had the highest 5.97% and 5.62% respectively. The ash content also increased as the level of inclusion of MLM increase

#### 4. 4.1 Performance of Indigenous Layer Chicken

**Table 4. 3 Effects of Dietary Diets on IC Performance**

| Parameters        | T1                  | T2                  | T3                  | T4                  | T5                  | T6                  | T7                  | T8                  | <i>P</i><br><i>value</i> |
|-------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|--------------------------|
| ADFI (g)          | 116.37 <sup>e</sup> | 118.31 <sup>e</sup> | 130.33 <sup>c</sup> | 131.48 <sup>c</sup> | 134.99 <sup>b</sup> | 127.05 <sup>d</sup> | 137.84 <sup>a</sup> | 139.29 <sup>a</sup> | <.0001                   |
| HDP (%)           | 47.62 <sup>a</sup>  | 50.79 <sup>a</sup>  | 32.54 <sup>b</sup>  | 38.36 <sup>b</sup>  | 13.76 <sup>c</sup>  | 17.72 <sup>c</sup>  | 9.79 <sup>cd</sup>  | 2.65 <sup>d</sup>   | <.0001                   |
| FCR (g)           | 0.26 <sup>d</sup>   | 0.25 <sup>d</sup>   | 0.44 <sup>d</sup>   | 0.35 <sup>d</sup>   | 1.31 <sup>b</sup>   | 1.96 <sup>b</sup>   | 2.03 <sup>b</sup>   | 2.62 <sup>a</sup>   | <.0001                   |
| Egg<br>weight (g) | 55.44 <sup>ab</sup> | 56.11 <sup>a</sup>  | 52.77 <sup>ac</sup> | 54.89 <sup>a</sup>  | 53.94 <sup>ac</sup> | 51.72 <sup>cd</sup> | 50.50 <sup>cd</sup> | 49.38 <sup>d</sup>  | <.0001                   |

<sup>a,b,c,d</sup> Means within a row with different superscripts differ significantly ( $p < 0.05$ ). T1 0% MLM 0 Enzyme, T2 0% MLM 0.035 g Enzyme, T3 10% MLM 0 Enzyme, T4 10% MLM 0.035 g Enzyme, T5 20% MLM 0 Enzyme, T6 20% MLM 0.035 g Enzyme, T7 30% MLM 0 Enzyme, T8 30% MLM 0.035 g Enzyme, ADFI: Average daily feed intake, HDP: Hen day production, FCR: Feed conversion ratio.

#### 4.5 Discussion

##### 4.5.1 Average Daily Feed Intake

The average daily feed intake increased as the levels of MLM in diet increased. There was higher feed intake at 30% MLM inclusion level compared to the control diets (**Table 4.3**). This could probably be due to the hens trying to meet their nutritional requirements due to depressed feed digestibility due to the high fibre content (Chong *et al.*, 2008). High fibre content in the diet increase the viscosity of the intestinal content. Thus, makes it difficult to mix well with digestive enzymes which reduces the digestion and absorption of nutrients in the diet (Molist *et al.*, 2014) This results in a decrease in the bioavailability and utilization of feed nutrients like crude protein, carbohydrates, vitamin A and of dietary fats, and ultimately decreasing the efficiency of feed and nutrient utilization (De Vries, 2015). This low efficiency of nutrient utilization results in high feed intake in order for the hens to meet nutritional requirements. However, high feed intake in this study could also be due to the use of improved indigenous hens which are or may be better adapted

to high non-starch polysaccharides (fibre) intake as scavenging chicken (Kondra *et al.*, 1974). The results of this study disagree with the findings of Al-kirshi *et al.* (2010) who reported a reduction in feed intake with increased dietary leaf meals in the diets for broilers and laying hens. This difference could be because indigenous chicken possibly being better adapted to high non-starch polysaccharides (fibre) intake as scavenging chicken (Mattioli *et al.*, 2022).

#### **4.5.2 Feed Conversion Ratio (FCR)**

Feed conversion ratio (FCR) is an indicator of how efficiently a feed is converted into egg. This study showed that FCR was not significantly different up to 10% MLM (Table 4.3) inclusion level. This could be because enzyme-treated MLM improved the digestive tract by increasing the activity of digestive enzymes of gastric mucosa, potentially improving laying performance (Lin *et al.*, 2017). These results agree with the findings of Lin *et al.* (2017) which also reported that feeding mulberry leaves improved FCR and egg mass due to the antioxidant activities of MLM, which provided healthy GIT, uterine and ovarian environments. However, higher FCR was observed in chickens fed with diets containing 30% MLM because the diet was high in fibre content and thus chicken forced to consume more feed for their survival rather than production. The significant increase in the FCR and decrease in laying percentage indicated a lower feed utilization efficiency with increased MLM content in the diets due to high fibre content.

#### **4.5.3 Hen Day Production (HDP)**

The egg production differed significantly among the layers fed the diets with different levels of MLM. The results indicated that feeding of MLM up to 10% with an enzyme in the diet of the layer chicken had no negative effect on egg production. However, there was a decrease in egg production in hens that were fed diets with 20 and 30% MLM with or without enzymes because high fibre in diets overwhelmed enzymes, therefore passing through the digestive system without being broken down by enzymes. This could be due to increasing of fibre level with the increased inclusion of mulberry leaf meal in the diets. This might have lowered the digestibility of the feed and therefore decreasing availability of nutrients, particularly protein and energy resulting in low egg production. The results of this study agree with the observations of Kamruzzaman *et al.* (2014), who also reported decreased egg production with the increased level of the mulberry leaf meal. Egg weight did not differ significantly for birds fed control diets and enzyme at 10% MLM. The results indicate that the inclusion of mulberry leaf meal up to 10% in the diet of laying hens had

no inimical effect on egg size. However, feeding chicken with diets containing 20 and 30% MLM resulted in decreased egg weights. This could be due to lack of adequate sources of supplemental oil in diets which are higher in linoleic acid, like soybean oil. The results are consistent with Kamruzzaman *et al.* (2018) who found no significant difference in egg size when birds are fed diets with 15% MLM.

#### **4.6. Conclusion**

Based on the results of this study, it was concluded that the inclusion of enzyme-treated MLM in improved indigenous layer diets up to 20% adversely affect egg production, egg weights and feed conversion ratio. Therefore, enzyme-treated MLM should not be incorporated in the diets above 10 %.

#### **4.7 Recommendation**

This study recommends incorporation of 10% enzyme-treated MLM and enzymes in diets of improved IC diets.

## CHAPTER FIVE

### EFFECT OF INCLUSION OF ENZYME-TREATED MLM ON EXTERNAL AND INTERNAL EGG QUALITY OF INDIGENOUS LAYER CHICKEN

#### Abstract

The study was performed to determine the effects of inclusion of various dietary levels of enzyme treated mulberry (*Morus alba*) leaf meal in improved IC layer diet on external and internal egg qualities. Seventy-two (72), 18-week-old laying hens were grouped into 8 treatments each with 3 replications (3 birds/replication). They were offered the experimental diets with 0, 10, 20 and 30% enzyme treated mulberry leaf meal together with an enzyme for 10 weeks. Eggs were collected and weighed daily, egg quality analysis (external and internal egg parameters) was performed weekly on 3 eggs sampled from each treatment repeatedly for 10 weeks. Results showed that there was no significant effect of dietary treatments on egg shape index, egg: shell ratio, egg: yolk ratio and yolk: albumin ratio. However, significant differences were observed in Shell thickness, Shell weight and yolk colour with increase in the inclusion levels of MLM. Based on the findings of this research, it was concluded that inclusion of enzyme treated MLM up to 10% in IC layer diets did not adversely affect the external and internal egg qualities.

#### 5.1 Introduction

Mulberry leaf meal (MLM) is a plant protein source, which is readily available in rural areas of Kenya. Mulberry tree is hardy, deep-rooted and does well in almost all types of soils, and it is locally available in Kenya because of favourable climatic conditions. It is drought tolerant and can survive in arid and semi-arid lands (ASALs), which is 88% of Kenya land mass. Mulberry trees are commonly found in most rural compounds because they produce fruits which are consumed by people, leaves are used for feeding silkworms and livestock and also used as a source of fuel (Mwai *et al.*, 2021). It is easy to propagate mulberry, hence it is easily available as an alternative source of protein. The leaves are highly palatable and digestible for herbivores and monogastric animals (Petkov, 2016). According to Huang *et al.* (2022), mulberry leaves can also be used as a vitamin and mineral supplement in the diets of poultry and can improve egg production. Mulberry leaf contains carotene, which can be converted with varying efficiency by animals to vitamin A and xanthophylls, which may have potential in egg yolk pigmentation (Islam *et al.*, 2014). One of

the most important egg qualities for consumers buying eggs is egg yolk colour. Most consumers desire a darker yolk colour (Alkan & Berber, 2022). According to the findings of Mwai *et al.* (2021) and Al-Kirshi *et al.* (2010) the use of MLM in laying hens diet up to 10% did not adversely affect egg production and egg quality. However, little is known about the effect of treated MLM in poultry diets on egg production and egg quality. Therefore, this study was conducted to evaluate the effect of enzyme-treated MLM at varied inclusion levels of 0, 10, 20 and 30% as a replacement of soybean meal in IC diets on external and internal egg qualities.

## **5.2 Material and Methods**

### **5.2.1 Study Site**

The study site has been described in Chapter three, section 3.3.1.

### **5.2.2 Experimental Birds and Management**

The experiment was conducted for 10 weeks using 72 improved indigenous layer chicken, 19 weeks of age. The layers were randomly assigned to 8 treatments, each with 3 layers replicated three times. There were 24 experimental units. The chicken was housed in in deep litter cages equipped with drinkers and feeders. The chicken were fed with experimental diets formulated meet the nutritional requirement of indigenous layer chicken (King'ori *et al.*, 2004). The inclusion levels of MLM were 0,10,20 and 30% with enzyme at 2 levels (0 and 0.035g). The diets were isonitrogenous (16%) and iso-caloric (2800-2850 kcal/kg).

### **5.2.3 Experimental Diets**

This is as described in chapter four, Table 4.1.

### **5.2.4 Data Collection**

Egg quality analysis (external and internal egg parameters) was performed weekly using 3 eggs sampled from each treatment. Eggs were collected twice daily at 09.00 and 18.00hr, then labelled per treatment (T) and replicate (R) and placed in egg trays.

### **Measurement of External Egg Parameters**

A digital balance was used to weigh each egg to the nearest 0.01 g accuracy (Şekeroğlu & Altuntaş, 2009). The length and width of the egg was measured using a digital Vernier calliper and the shape index was calculated by multiplying the width to length ratio by 100 (Duman *et al.*, 2016). Egg shells were weighed 24 hrs after removing the shell membrane and drying in the open air. All of the dried shells were weighed from four pieces of the eggshell, two pieces from the two

ends (pointed and rounded ends) and two pieces from the midpoint were weighed using a digital balance with 0.01 g accuracy. Shell ratio was calculated by dividing shell weight by egg weight. The thickness of 4 (bottom, 2 pieces centre, top) portions of shells were measured to the nearest 0.01 mm using a digital Vernier calliper.

where W is the width of the egg at the widest point and L, is the length of the egg.

i.  $shape\ index(\%) = \frac{width(mm)}{length(mm)} \times 100$

ii.  $shell\ thickness\ (mm) = \frac{bottom\ thickness + centre\ piece\ thickness + top\ piece\ thickness}{4}$

iii.  $Eggshell\ ratio\ \% = \frac{shell\ weight(g)}{egg\ weight(g)} \times 100$

### Measurement of Internal Egg Parameters

An electronic balance (0.1 g sensitivity) was used to determine yolk and albumin weight. Yolk colour was visually examined using a Roche colour fan (1 to 15) (Kumar *et al.*, 2022). As shown in **Figure 5.1 and 5.2** respectively



**Figure 5.1** Weight of the Yolk



**Figure 5.2** Roche colour fan

- i.  $Yolk\ ratio\ (\%) = \frac{yolk\ weight\ (g)}{egg\ weight\ (g)} \times 100$
- ii.  $Albumin\ ratio\ (\%) = \frac{albumin\ weight\ (g)}{egg\ weight\ (g)} \times 100$

### 5.2.5 Experimental Design

The experiment was laid out in completely randomized design (CRD) in a  $4 \times 2$  factorial layout. There were 8 treatments (Treatment 1 up to 8) which were replicated thrice. Each replicate had 3 hens, hence a total of 9 hens per treatment.

### 5.2.6 Statistical Model

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$$

where;

$Y_{ijk}$  = overall effect due to treatments

$\mu$  = overall mean

$\alpha_i$  = effect due to the  $i^{\text{th}}$  treatment MLM

$\beta_j$  = effect due to  $j^{\text{th}}$  treatment enzymes

$\alpha\beta_{ij}$  = interaction effect due to MLM and enzyme

$\varepsilon_{ijk}$  = random error effect

### **5.2.7 Data Analysis**

Analysis of variance of data on the external and internal egg parameters were done using SAS Version 9.4. The significant differences between the treatment means were separated by Duncan's Multiple Range Test at ( $p < 0.05$ )

### 5.3 Results

**Table 5. 1 External and Internal Egg Qualities of an Egg**

| Parameters              | T1                  | T2                  | T3                  | T4                  | T5                  | T6                  | T7                  | T8                 | P value |
|-------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|--------------------|---------|
| Shape index (%)         | 74.7 <sup>bc</sup>  | 73.37 <sup>c</sup>  | 75.82 <sup>bc</sup> | 75.66 <sup>bc</sup> | 76.40 <sup>b</sup>  | 75.50 <sup>bc</sup> | 75.65 <sup>bc</sup> | 79.25 <sup>a</sup> | 0.0091  |
| Shell thickness(mm)     | 0.41 <sup>a</sup>   | 0.31 <sup>ab</sup>  | 0.33 <sup>ab</sup>  | 0.29 <sup>cb</sup>  | 0.18 <sup>ed</sup>  | 0.21 <sup>cd</sup>  | 0.11 <sup>ef</sup>  | 0.02 <sup>f</sup>  | <.0001  |
| Shell weight (g)        | 4.44 <sup>ab</sup>  | 4.78 <sup>a</sup>   | 4.48 <sup>ab</sup>  | 4.30 <sup>ab</sup>  | 2.74 <sup>cd</sup>  | 3.56 <sup>cb</sup>  | 2.56 <sup>d</sup>   | 0.70 <sup>e</sup>  | <.0001  |
| Egg: shell ratio        | 12.24 <sup>ab</sup> | 11.53 <sup>a</sup>  | 11.91 <sup>ab</sup> | 11.40 <sup>b</sup>  | 10.28 <sup>b</sup>  | 11.76 <sup>ab</sup> | 11.03 <sup>b</sup>  | 13.60 <sup>a</sup> | 0.1729  |
| Egg: yolk ratio         | 26.74 <sup>ab</sup> | 26.48 <sup>ab</sup> | 26.60 <sup>ab</sup> | 25.05 <sup>ab</sup> | 24.80 <sup>ab</sup> | 26.03 <sup>ab</sup> | 22.62 <sup>a</sup>  | 19.27 <sup>b</sup> | 0.5582  |
| Yolk: albumin ratio (%) | 42.57 <sup>ab</sup> | 44.19 <sup>ab</sup> | 45.53 <sup>ab</sup> | 44.71 <sup>ab</sup> | 50.44 <sup>a</sup>  | 43.57 <sup>ab</sup> | 37.61 <sup>b</sup>  | 50.44 <sup>a</sup> | 0.2681  |
| Yolk colour             | 1.83 <sup>c</sup>   | 1.83 <sup>c</sup>   | 10.33 <sup>b</sup>  | 10.33 <sup>b</sup>  | 10.67 <sup>b</sup>  | 10.67 <sup>b</sup>  | 12.0 <sup>a</sup>   | 12.0 <sup>a</sup>  | <.0001  |

<sup>abcd</sup> Means within a row with different superscripts differs significantly ( $p < 0.05$ ) and means separation by Duncan's New Multiple Range test with or without enzyme (0,0.035 g) at inclusion levels of 0, 10,20 and 30% MLM.

## 5.4 Discussion

Both egg shape index and egg: shell ratio were not significantly different among treatments. Shell thickness and shell weight decreased with increased inclusion levels of MLM. This could be because of the high fibre content in the diets (5.97%) that depressed nutrient digestibility. Hens require dietary crude fibre content of 4%. High crude fibre increases the viscosity of the intestinal content and thus decreases the digestibility of feeds therefore impairing nutrient utilization (De Vries, 2015). The reduction in shell weight might have been due to insufficient Ca intake from the diets due to depressed diet digestibility. The results of this study agree with the findings of studies done by Al-Kirshi *et al.* (2010) who reported that shell weight decreased with 15 and 20% MLM inclusion in diet due to insufficient Ca intake by the hens.

Since thickness does not imply stiffer or stronger eggs, shell thickness is regarded as one of the primary indirect parameters for evaluating shell quality (Gibson, 2022; Yan *et al.*, 2014). More feed mineral consumption is correlated with increased shell strength. The thicker shell is an implication that there was more mineral consumption from feed (Sun *et al.*, 2012). Egg shape index is defined as the ratio of width to length of the egg, and it is an important criterion in determining egg quality. Domestic hen eggs that are unusual in shape, such as those that are long and narrow, round, or flat-sided, cannot be placed in grade AA (nearly perfect) or A (slightly worse than AA) since an egg is generally oval (72–76) (Duman *et al.*, 2016). Round eggs and unusually long eggs have poor appearances and do not fit well in egg trays and cartons; therefore, they are much more likely to be broken during transportation than the eggs of normal shape. Egg shape index and shell thickness affect the risk of cracked eggs. Larger and rounder eggshells have a higher resistance to breaking forces (Ketta & Tůmová, 2018). In this study however, there was no significant difference in shape index.

Yolk colour intensity increased with increased inclusion levels of MLM, Egg: yolk ratio and yolk: albumin ratio while did not differ significantly among the treatments, this could be because the layers were of the same breed. Yolk colour was significantly higher in diets with 30% MLM compared to diets with 10% MLM. The increase in egg yolk colouration could be due to high bio-availability of the xanthophylls in the mulberry leaf meal (Huikhiaw *et al.*, 2021). According to the studies conducted by Lokaewmanee *et al.* (2009) and Al-Kirshi *et al.* (2010) mulberry leaves are rich in  $\beta$ -carotene which can be converted by poultry to vitamin A and xanthophylls which are

good sources of egg yolk pigmentations. Thus, the addition of mulberry leaf meals in the diets up to 30% resulted in yolk with deeper yellow colour. The significant difference observed in the results of egg yolk colour indicated that the MLM inclusion improved the yolk quality due to higher supply of xanthophyll. Therefore, the MLM-based diets resulted in a higher yolk quality than those in control group.

According to Wang *et al.* (2022), colour is an important quality trait of foods since it affects the consumers' perception of quality, intensity of aroma, flavour and their decision on purchase. Most consumers associate yolk colour to age and health status of the animal and to the quality of eggs and egg products, though objectively this is not the case (Cayan *et al.*, 2015). Feed additives and system of production (e.g. free range) are primary factors in pigmentation of egg yolk. For consumers, one of the most important characteristics of eggs is the colour of the egg yolk (Berkoff *et al.*, 2020). Both consumers and individuals/businesses that process egg products (e.g. shops) have a greater preference for eggs with deep yellow-coloured yolks. The changes observed in yolk colour are largely associated with the ingredients used in diets. Carotenoids play an important role in the development of different colour scores and tones in egg yolk (Bidura *et al.*, 2020), lutein which is also a xanthophyll is an active yolk colourant. The deepest egg yolk colour was observed in the groups fed mulberry leaf meal diets at 20 and 30% levels. This increase in egg yolk colour intensity can be attributed to the carotene contents of MLM. Addition of MLM to layer diet can boost the colouring compound in diets and the colouring ingredients from yellow corn (51 µg/100 g of β-carotene and of 780 µg/100 g of lutein) which is mostly used in poultry diets (Yilmaz, 2019). The MLM has 13.12- 84.38 g/100 g β-carotene (Ustundag and Ozdogan, 2015). Similarly, Lokaewmanee *et al.* (2009) and Feng *et al.* (2017) reported that MLM increased the egg yolk colour intensity. This implies that treated MLM can be used as a source of β-carotene and protein. These results agree with the findings of Al-Kirshi *et al.* (2010) who reported that there was both negative and positive impact in internal and external egg qualities of the egg with MLM inclusion above 10%.

## **5.5 Conclusion**

The results indicated that inclusion of more than 10% treated or untreated mulberry leaf meal in indigenous layer chicken diet negatively affected the internal and external egg qualities except the yolk colour that deepens in colour.

## **5.6 Recommendation**

Mulberry leaf meal should not be incorporated as a protein source in IC layer diet beyond 10% level to replace soya bean meal.

## CHAPTER SIX

### GENERAL DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

#### 6.1 General Discussions

Poultry farming in Kenya is a widespread occupation. It is mostly practiced on a small-scale, and predominantly for domestic consumption. The majority of the Kenyan population resides in rural areas characterized by low income and food insecurity which leads to high levels of poverty. Poultry production plays a key role in contributing to a cheap source of animal protein and cash income. The production is driven by increased demand for white meat and emerging niche in urban markets for quality organic products. The indigenous chicken population continues to increase with time thereby leading to the need for feed ingredients that are locally available and facing less competition from human. Thus, this calls for search of locally available feeds ingredients that are also affordable. Among them is mulberry leaf meal (MLM) which is a good source of protein to poultry as it is rich in protein and carotene which improves the yolk colour of an egg, an attribute that is widely preferred by consumers. Despite MLM being a good source of protein, increasing the percentage of mulberry leaf meal in the diet as replacement for soybean meal led to an increase in the proportion of crude fiber in the diet (De Vries, 2015). This therefore, becomes a major constraint in the inclusion of MLM in poultry diets at higher levels, unless intervention measures are put in place to ameliorate this hurdle with enzyme treatment and fermentation being possible option. However, there is limited information that treatment with exogenous enzyme or fermentation could improve the digestibility of MLM and therefore improve performance of improved indigenous layer chicken. This study therefore, was conducted to:

- i. To determine the effect of fermentation and enzyme treatment of Mulberry leaf meal on *in vitro* digestibility.
- ii. To determine the effect of inclusion of enzyme-treated MLM on indigenous layer chicken performance.
- iii. To determine the effect of inclusion of enzyme-treated MLM on external and internal egg quality of indigenous layer chicken.

These objectives were achieved using *in-vitro* and *in-vivo* experiments and the hypotheses tested by analysis of variance using SAS version 2009. From the findings discussed in Chapters 3, 4 and 5 processing techniques was found to improve the nutritive value of MLM, utilization and performance of improved indigenous layer chicken. As described in the following paragraphs.

### **6.1.1. To determine the effect of fermentation and enzyme treatment of Mulberry leaf meal on *in vitro* digestibility.**

Many fibrous ingredients incorporated in poultry feed to reduce production costs have low digestibility and cause poor growth in poultry. However, all plant-based fibres are not equal, and thus exert variable physiological effects on the birds, including but not limited to, digestibility, growth performance, and microbial fermentation (Jha *et al.*, 2021). Therefore, overcoming the limitation of poor digestibility associated with high fibre through biotechnological interventions can be a viable option. This objective determined the effect of fermentation and enzyme treatment of Mulberry leaf meal on *in vitro* digestibility. The findings of this study showed that treatment of MLM with an exogenous enzyme (Natuzyne®) resulted in better IVDM compared to untreated, natural, and *Bacillus coagulans* induced fermentation.

### **6.1.2 To determine the effect of inclusion of enzyme-treated MLM on the performance of indigenous layer chicken.**

The inclusion of MLM with Natuzyne® multi-enzyme complex up to 30% resulted in an increase in the feed intake. This could probably be due to the birds trying to meet their nutritional requirements due to depressed feed digestibility as a result of the high fibre content (Chong *et al.*, 2008). High fibre content in the diet increase viscosity of the intestinal content. The viscosity of ingesta, makes it inaccessible to digestive enzymes, reduces the digestion and absorption of nutrients in the diet (Molist *et al.*, 2014). However, higher FCR was observed in chickens fed with diets containing 30% MLM because the diet was high in fibre content and thus chicken forced to consume more feed for their survival rather than production. The significant increase in the FCR and decrease in laying percentage indicated a lower feed utilization efficiency with increased MLM content in the diets due to high fibre content.

### **6.1.3 To determine the effect of inclusion of enzyme-treated MLM on external and internal egg quality of indigenous layer chicken.**

Shell thickness and shell weight decreased with increased inclusion levels of MLM. This could be because of high fibre content in the diets (5.97%) that depressed nutrient digestibility. Hens require dietary crude fibre content of up to 4%. Higher levels of crude fibre, especially the insoluble fibre tend to decrease digestibility, while the soluble fibre increases the viscosity of the intestinal content which similarly decreases the digestibility of feed and absorption of nutrients and therefore impairing nutrient utilization (De Vries, 2015). The reduction in shell weight might

also have been due to insufficient Ca intake from the diets due to depressed diet digestibility. Yolk colour intensity increased with increase in inclusion levels of MLM, Egg: yolk ratio and yolk: albumin ratio while did not differ significantly among the treatments this could be because the layers were of the same breed. Yolk colour was significantly higher in diets with 30% MLM compared to diets with 10%. The increase in egg yolk colouration could be due to high bio-availability of the xanthophylls in the mulberry leaf meal (Huikhiaw *et al.*, 2021).

## **6.2 Conclusions**

- i. Treatment of MLM with an exogenous enzyme (Natuzyne®) resulted in better IVDM digestibility compared to untreated, natural, and *Bacillus coagulans* induced fermentation.
- ii. Inclusion of enzyme-treated MLM in IC layer diets up to 10% had no negative effect on IC performance.
- iii. Inclusion of 10% enzyme-treated mulberry leaf meal in indigenous layer chicken diet had no negative effects on internal and external egg qualities.

## **6.3 Recommendations**

- i. Enzyme should be used in the treatment of MLM for incorporation in chicken diets to enhance digestibility
- ii. Enzyme-treated MLM should not be included in indigenous layer chicken diets at a level above 10%
- iii. Feed manufacturers can include MLM in IC layer diets instead of using synthetic  $\beta$ -carotene to enhance egg yolk colour.

## **6.4 Areas for Further Research**

- i. A study should be conducted to determine the cost benefit analysis of MLM inclusion in IC layer diet
- ii. A study should be conducted to evaluate the effects of feeding treated MLM-based diet to IC layers on egg organoleptic attributes.
- iii. Conduct a study to determine actual inclusion level between 10-20% and the safety aspect on animal feed as well as human health.

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APPENDICES

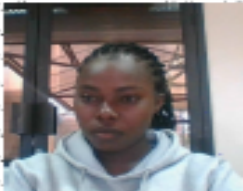
Appendix A: National Commission for Science, Technology and Innovation Research

Permit

Republic of Kenya  
Ministry of Science, Technology and Innovation  
National Commission for Science, Technology and Innovation  
Date of Issue: 25/May/2023

**RESEARCH LICENSE**

**Ref No: 186984**




**License No: NACOSTIP/23/26002**

**Applicant Identification Number: 186984**

**Director General**  
*Walter Wambui*  
**NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION**

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**See overleaf for conditions**

## Appendix B: Ethical clearance

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**EGERTON**

TEL: (051) 2217808  
FAX: 051-2217942



**UNIVERSITY**

P. O. BOX 536  
EGERTON

**EGERTON UNIVERSITY INSTITUTIONAL SCIENTIFIC AND ETHICS  
REVIEW COMMITTEE**

**EU/RE/DIR/009**

Approval No. **EUISERC/APP/223/2023**

10<sup>th</sup> March, 2023

Naomi Chebet  
Department of Animal Sciences,  
Egerton University  
Telephone + 2540742178619  
E-mail: naomichebet@gmail.com

Dear Naomi,

**RE: ETHICAL APPROVAL: PERFORMANCE AND EGG QUALITY OF IMPROVED  
INDIGENOUS LAYERS FED ENZYME-BASED MULBERRY (Morus alba) LEAF  
MEAL DIETS**

This is to inform you that *Egerton University Institutional Scientific and Ethics Review Committee* has reviewed and approved your above research proposal. Your application approval number is **EUISERC/APP/223/2023**. The approval period is **10<sup>th</sup> March, 2023 –11<sup>th</sup> March, 2024**

This approval is subject to compliance with the following requirements;

- i. Only approved documents including (informed consents, study instruments, MTA) will be used.
- ii. You are required to adhere Institutional Experimental Animals use and Care policy.
- iii. All changes including (amendments, deviations, and violations) are submitted for review and approval by *Egerton University Institutional Scientific and Ethics Review Committee*.
- iv. Death and life-threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to *Egerton University Institutional Scientific and Ethics Review Committee* within 72 hours of notification
- v. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to *Egerton University Institutional Scientific and Ethics Review Committee* within 72 hours.

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*"Transforming Lives through Quality Education"*

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- vi. Clearance for Material Transfer of biological specimens must be obtained from relevant institutions.
- vii. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
- viii. Submission of an executive summary report within 90 days upon completion of the study to *Egerton University Institutional Scientific and Ethics Review Committee*.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) <https://oris.nacosti.go.ke> and also obtain other clearances needed.

Yours sincerely,

  
Prof. Raphael M. Ngure  
**CHAIRMAN, EGERTON UNIVERSITY INSTITUTIONAL SCIENTIFIC AND ETHICS  
REVIEW CTTEE**  
*RAC/BE*



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*"Transforming Lives through Quality Education"*

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## Appendix C. Publication Abstract

International Journal of Veterinary Sciences and Animal Husbandry 2023; 8(4): 227-231



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### Effect of fermentation and enzyme treatment of mulberry leaf meal on enzymatic pre-digestion in the chicken simulated digestive system

Naomi Chebet, Anthony Macharia Kingori and Mary Kivali Ambula

#### Abstract

This study examined the effect of (Natuzyme®) enzyme and fermentation treatments on *in-vitro* dry matter digestibility (IVDMD) of mulberry leaf meal (MLM). Pepsin-pancreatin hydrolysis was used in this experiment to mimic the chicken stomach. The MLM was subjected to four treatments replicated 3 times in a completely randomized design (CRD), Treatment 1: enzyme-treated MLM, Treatment 2: untreated MLM, Treatment 3: MLM fermented with *Bacillus coagulans* and Treatment 4: Naturally fermented MLM. Data on proximate analysis and digestibility were analysed using Statistical Analysis Software SAS version 9.4, general linear model (GLM). Tukey's test at ( $p < 0.05$ ) was used to do means separation where means were significantly different. This study's findings showed that the treatment method had a significant effect ( $p < 0.05$ ) on the *in-vitro* dry matter digestibility (IVDMD). MLM treated with enzyme (Natuzyme®) and *Bacillus coagulans* had improved IVDMD by 7.31% and 3.99% respectively compared to untreated ( $p < 0.05$ ). Natural fermentation significantly differed from enzyme treatment ( $p < 0.05$ ). Based on the outcome of this research pre-treatment of MLM with enzyme (Natuzyme®) enhanced *in-vitro* digestibility as compared to untreated, natural, and *Bacillus coagulans* induced fermentation.

**Keywords:** Chicken, enzyme, *in-vitro* digestibility, Mulberry leaf meal

**Appendix D. Statistical Outputs**

The GLM Procedure

Dependent Variable: IVDMD

|  |          |
|--|----------|
| <b>Alpha</b>                               | 0.05     |
| <b>Error Degrees of Freedom</b>            | 6        |
| <b>Error Mean Square</b>                   | 0.702189 |
| <b>Critical Value of Studentized Range</b> | 4.89559  |
| <b>Minimum Significant Difference</b>      | 2.3685   |

**Means with the same letter are not significantly different.**

| <b>Tukey Grouping</b> | <b>Mean</b> | <b>N</b> | <b>TRT</b> |
|-----------------------|-------------|----------|------------|
| A                     | 65.7833     | 3        | T1         |
| B                     | 62.4567     | 3        | T3         |
| C                     | 58.4733     | 3        | T2         |
| D                     | 52.9800     | 3        | T4         |

| <b>Source</b>          | <b>DF</b> | <b>Sum of Squares</b> | <b>Mean Square</b> | <b>F Value</b> | <b>Pr &gt; F</b> |
|------------------------|-----------|-----------------------|--------------------|----------------|------------------|
| <b>Model</b>           | 5         | 274.4341333           | 54.8868267         | 78.17          | <.0001           |
| <b>Error</b>           | 6         | 4.2131333             | 0.7021889          |                |                  |
| <b>Corrected Total</b> | 11        | 278.6472667           |                    |                |                  |

|                 |                  |                 |                   |
|-----------------|------------------|-----------------|-------------------|
| <b>R-Square</b> | <b>Coeff Var</b> | <b>Root MSE</b> | <b>IVDMD Mean</b> |
| 0.984880        | 1.398399         | 0.837967        | 59.92333          |

| Source | DF | Type I SS   | Mean Square | F Value | Pr > F |
|--------|----|-------------|-------------|---------|--------|
| TRT    | 3  | 273.2092667 | 91.0697556  | 129.69  | <.0001 |
| REPS   | 2  | 1.2248667   | 0.6124333   | 0.87    | 0.4650 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|--------|----|-------------|-------------|---------|--------|
| TRT    | 3  | 273.2092667 | 91.0697556  | 129.69  | <.0001 |
| REPS   | 2  | 1.2248667   | 0.6124333   | 0.87    | 0.4650 |

### Appendix E.

#### Data analysis on feed intake, FCR and egg production Feed intake

The GLM Procedure

| Class Level Information |        |  |
|-------------------------|--------|--|
| Class                   | Levels | Values   |
| TRT                     | 8      | T1 T2 T3 T4 T5 T6 T7 T8  |
| REPS                    | 3      | 1 2 3  |
| DAYS                    | 70     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35<br>36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66<br>67 68 69 70 |

|                             |      |
|-----------------------------|------|
| Number of Observations Read | 1680 |
| Number of Observations Used | 1680 |

The SAS System

The GLM Procedure

Dependent Variable: FI

| Source | DF   | Sum of Squares | Mean Square | F Value | Pr > F |
|--------|------|----------------|-------------|---------|--------|
| Model  | 78   | 1707724.785    | 21893.907   | 15.53   | <.0001 |
| Error  | 1601 | 2256723.710    | 1409.571    |         |        |

| Source          | DF   | Sum of Squares | Mean Square | F Value | Pr > F |
|-----------------|------|----------------|-------------|---------|--------|
| Corrected Total | 1679 | 3964448.495    |             |         |        |

| R-Square | Coeff Var | Root MSE | FI Mean  |
|----------|-----------|----------|----------|
| 0.430760 | 9.666968  | 37.54426 | 388.3768 |

| Source | DF | Type I SS   | Mean Square | F Value | Pr > F |
|--------|----|-------------|-------------|---------|--------|
| TRT    | 7  | 951714.5565 | 135959.2224 | 96.45   | <.0001 |
| REPS   | 2  | 1494.5250   | 747.2625    | 0.53    | 0.5886 |
| DAYS   | 69 | 754515.7030 | 10935.0102  | 7.76    | <.0001 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|--------|----|-------------|-------------|---------|--------|
| TRT    | 7  | 951714.5565 | 135959.2224 | 96.45   | <.0001 |
| REPS   | 2  | 1494.5250   | 747.2625    | 0.53    | 0.5886 |
| DAYS   | 69 | 754515.7030 | 10935.0102  | 7.76    | <.0001 |

The GLM Procedure

Dependent Variable: ADFI

| Source          | DF   | Sum of Squares | Mean Square | F Value | Pr > F |
|-----------------|------|----------------|-------------|---------|--------|
| Model           | 78   | 189747.3905    | 2432.6589   | 15.53   | <.0001 |
| Error           | 1601 | 250750.9716    | 156.6215    |         |        |
| Corrected Total | 1679 | 440498.3621    |             |         |        |

| R-Square | Coeff Var | Root MSE | ADFI Mean |
|----------|-----------|----------|-----------|
| 0.430756 | 9.667038  | 12.51485 | 129.4590  |

| Source | DF | Type I SS   | Mean Square | F Value | Pr > F |
|--------|----|-------------|-------------|---------|--------|
| TRT    | 7  | 105745.8937 | 15106.5562  | 96.45   | <.0001 |
| REPS   | 2  | 165.9709    | 82.9855     | 0.53    | 0.5888 |
| DAYS   | 69 | 83835.5259  | 1215.0076   | 7.76    | <.0001 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|--------|----|-------------|-------------|---------|--------|
| TRT    | 7  | 105745.8937 | 15106.5562  | 96.45   | <.0001 |
| REPS   | 2  | 165.9709    | 82.9855     | 0.53    | 0.5888 |
| DAYS   | 69 | 83835.5259  | 1215.0076   | 7.76    | <.0001 |

The SAS System

The GLM Procedure

Class Level Information

| Class | Levels | Values   |
|-------|--------|--|
| TRT   | 8      | T1 T2 T3 T4 T5 T6 T7 T8  |
| REPS  | 3      | 1 2 3  |
| DAYS  | 70     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35<br>36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66<br>67 68 69 70 |

|                             |      |
|-----------------------------|------|
| Number of Observations Read | 1680 |
| Number of Observations Used | 1680 |

The SAS System

The GLM Procedure

Dependent Variable: FI

| Source          | DF   | Sum of Squares | Mean Square | F Value | Pr > F |
|-----------------|------|----------------|-------------|---------|--------|
| Model           | 78   | 1707724.785    | 21893.907   | 15.53   | <.0001 |
| Error           | 1601 | 2256723.710    | 1409.571    |         |        |
| Corrected Total | 1679 | 3964448.495    |             |         |        |

| R-Square | Coeff Var | Root MSE | FI Mean  |
|----------|-----------|----------|----------|
| 0.430760 | 9.666968  | 37.54426 | 388.3768 |

| Source | DF | Type I SS   | Mean Square | F Value | Pr > F |
|--------|----|-------------|-------------|---------|--------|
| TRT    | 7  | 951714.5565 | 135959.2224 | 96.45   | <.0001 |
| REPS   | 2  | 1494.5250   | 747.2625    | 0.53    | 0.5886 |
| DAYS   | 69 | 754515.7030 | 10935.0102  | 7.76    | <.0001 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|--------|----|-------------|-------------|---------|--------|
| TRT    | 7  | 951714.5565 | 135959.2224 | 96.45   | <.0001 |
| REPS   | 2  | 1494.5250   | 747.2625    | 0.53    | 0.5886 |

| Source      | DF | Type III SS | Mean Square | F Value | Pr > F |
|-------------|----|-------------|-------------|---------|--------|
| <b>DAYS</b> | 69 | 754515.7030 | 10935.0102  | 7.76    | <.0001 |

The SAS System

The GLM Procedure

Dependent Variable: ADFI

| Source                 | DF   | Sum of Squares | Mean Square | F Value | Pr > F |
|------------------------|------|----------------|-------------|---------|--------|
| <b>Model</b>           | 78   | 189747.3905    | 2432.6589   | 15.53   | <.0001 |
| <b>Error</b>           | 1601 | 250750.9716    | 156.6215    |         |        |
| <b>Corrected Total</b> | 1679 | 440498.3621    |             |         |        |

| R-Square | Coeff Var | Root MSE | ADFI Mean |
|----------|-----------|----------|-----------|
| 0.430756 | 9.667038  | 12.51485 | 129.4590  |

| Source      | DF | Type I SS   | Mean Square | F Value | Pr > F |
|-------------|----|-------------|-------------|---------|--------|
| <b>TRT</b>  | 7  | 105745.8937 | 15106.5562  | 96.45   | <.0001 |
| <b>REPS</b> | 2  | 165.9709    | 82.9855     | 0.53    | 0.5888 |
| <b>DAYS</b> | 69 | 83835.5259  | 1215.0076   | 7.76    | <.0001 |

| Source      | DF | Type III SS | Mean Square | F Value | Pr > F |
|-------------|----|-------------|-------------|---------|--------|
| <b>TRT</b>  | 7  | 105745.8937 | 15106.5562  | 96.45   | <.0001 |
| <b>REPS</b> | 2  | 165.9709    | 82.9855     | 0.53    | 0.5888 |
| <b>DAYS</b> | 69 | 83835.5259  | 1215.0076   | 7.76    | <.0001 |

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for FI

Note: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

|                                 |          |
|---------------------------------|----------|
| <b>Alpha</b>                    | 0.05     |
| <b>Error Degrees of Freedom</b> | 1601     |
| <b>Error Mean Square</b>        | 1409.571 |

|                        |          |          |          |          |          |          |          |
|------------------------|----------|----------|----------|----------|----------|----------|----------|
| <b>Number of Means</b> | <b>2</b> | <b>3</b> | <b>4</b> | <b>5</b> | <b>6</b> | <b>7</b> | <b>8</b> |
| <b>Critical Range</b>  | 7.187    | 7.567    | 7.821    | 8.009    | 8.156    | 8.277    | 8.377    |

| <b>Means with the same letter are not significantly different.</b> |             |          |            |
|--|-------------|----------|------------|
| <b>Duncan Grouping</b>   | <b>Mean</b> | <b>N</b> | <b>TRT</b> |
| A  | 417.862     | 210      | T8         |
| A  |             |          |            |
| A  | 413.524     | 210      | T7         |
|  |             |          |            |
| B  | 404.976     | 210      | T5         |
|  |             |          |            |
| C  | 394.448     | 210      | T4         |
| C  |             |          |            |
| C  | 390.981     | 210      | T3         |
|  |             |          |            |
| D  | 381.162     | 210      | T6         |
|  |             |          |            |
| E  | 354.943     | 210      | T2         |
| E  |             |          |            |
| E  | 349.119     | 210      | T1         |

---



---

The SAS System

The GLM Procedure

Duncan's Multiple Range Test for ADFI

Note: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

|                                 |          |
|---------------------------------|----------|
| <b>Alpha</b>                    | 0.05     |
| <b>Error Degrees of Freedom</b> | 1601     |
| <b>Error Mean Square</b>        | 156.6215 |

|                        |          |          |          |          |          |          |          |
|------------------------|----------|----------|----------|----------|----------|----------|----------|
| <b>Number of Means</b> | <b>2</b> | <b>3</b> | <b>4</b> | <b>5</b> | <b>6</b> | <b>7</b> | <b>8</b> |
| <b>Critical Range</b>  | 2.396    | 2.522    | 2.607    | 2.670    | 2.719    | 2.759    | 2.792    |

| Means with the same letter<br>are not significantly different. |         |     |     |
|--|---------|-----|-----|
| Duncan Grouping  | Mean    | N   | TRT |
| A  | 139.287 | 210 | T8  |
| A  |         |     |     |
| A  | 137.841 | 210 | T7  |
|  |         |     |     |
| B  | 134.992 | 210 | T5  |
|  |         |     |     |
| C  | 131.482 | 210 | T4  |
| C  |         |     |     |
| C  | 130.327 | 210 | T3  |
|  |         |     |     |
| D  | 127.054 | 210 | T6  |
|  |         |     |     |
| E  | 118.314 | 210 | T2  |
| E  |         |     |     |
| E  | 116.373 | 210 | T1  |

---

The SAS System

The MEANS Procedure

| TRT | N Obs | Variable | Mean   | Std Error |
|-----|-------|----------|--------|-----------|
| T1  | 210   | FI       | 349.12 | 2.92      |
|     |       | ADFI     | 116.37 | 0.97      |
| T2  | 210   | FI       | 354.94 | 3.86      |
|     |       | ADFI     | 118.31 | 1.29      |
| T3  | 210   | FI       | 390.98 | 2.64      |
|     |       | ADFI     | 130.33 | 0.88      |
| T4  | 210   | FI       | 394.45 | 2.64      |
|     |       | ADFI     | 131.48 | 0.88      |

| TRT | N Obs | Variable | Mean   | Std Error |
|-----|-------|----------|--------|-----------|
| T5  | 210   | FI       | 404.98 | 2.27      |
|     |       | ADFI     | 134.99 | 0.76      |
| T6  | 210   | FI       | 381.16 | 3.14      |
|     |       | ADFI     | 127.05 | 1.05      |
| T7  | 210   | FI       | 413.52 | 2.43      |
|     |       | ADFI     | 137.84 | 0.81      |
| T8  | 210   | FI       | 417.86 | 3.21      |
|     |       | ADFI     | 139.29 | 1.07      |

### Feed Conversion Ratio (FCR)

The SAS System 01:40 Thursday, June 24, 2023

The GLM Procedure

Class Level Information

| Class | Levels | Values                  |
|-------|--------|-------------------------|
| TRT   | 8      | T1 T2 T3 T4 T5 T6 T7 T8 |
| REPS  | 3      | 1 2 3                   |

Number of observations 145

The SAS System 01:40 Thursday, June 24, 2023 40

The GLM Procedure

Dependent Variable: FCR

| Source | DF  | Sum of Squares | Mean Square | F Value | Pr > F |
|--------|-----|----------------|-------------|---------|--------|
| Model  | 9   | 38.27092769    | 4.25232530  | 10.80   | <.0001 |
| Error  | 111 | 43.72083760    | 0.39388142  |         |        |

|                 |          |             |             |          |        |  |
|-----------------|----------|-------------|-------------|----------|--------|--|
| Corrected Total | 120      | 81.99176529 |             |          |        |  |
|                 | R-Square | Coeff Var   | Root MSE    | FCR Mean |        |  |
|                 | 0.466766 | 85.46941    | 0.627600    | 0.734298 |        |  |
| Source          | DF       | Type I SS   | Mean Square | F Value  | Pr > F |  |
| TRT             | 7        | 35.00192418 | 5.00027488  | 12.69    | <.0001 |  |
| REPS            | 2        | 3.26900352  | 1.63450176  | 4.15     | 0.0183 |  |
| Source          | DF       | Type III SS | Mean Square | F Value  | Pr > F |  |
| TRT             | 7        | 34.23884896 | 4.89126414  | 12.42    | <.0001 |  |
| REPS            | 2        | 3.26900352  | 1.63450176  | 4.15     | 0.0183 |  |

The SAS System 01:40 Thursday, June 24, 2023 41  
The GLM Procedure

Duncan's Multiple Range Test for FCR

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha 0.05  
Error Degrees of Freedom 111  
Error Mean Square 0.393881  
Harmonic Mean of Cell Sizes 13.09091

NOTE: Cell sizes are not equal.

|                 |       |       |       |       |       |       |       |
|-----------------|-------|-------|-------|-------|-------|-------|-------|
| Number of Means | 2     | 3     | 4     | 5     | 6     | 7     | 8     |
| Critical Range  | .4861 | .5116 | .5285 | .5409 | .5506 | .5584 | .5648 |

Means with the same letter are not significantly different.

| Duncan Grouping | Mean   | N  | TRT |
|-----------------|--------|----|-----|
| A               | 2.1300 | 6  | T8  |
| B               | 1.4060 | 10 | T7  |
| B               | 1.1967 | 18 | T6  |
| B               | 1.1387 | 15 | T5  |
| C               | 0.4411 | 18 | T3  |
| C               | 0.3489 | 18 | T4  |
| C               | 0.2589 | 18 | T1  |
| C               | 0.2506 | 18 | T2  |

**Hen Day Production (HDP)**

The SAS System 01:40 Thursday, June 24, 2023

The GLM Procedure  
Class Level Information

| Class | Levels | Values                  |
|-------|--------|-------------------------|
| TRT   | 8      | T1 T2 T3 T4 T5 T6 T7 T8 |

REPS 3 1 2 3  
 Number of observations 144

The SAS System 01:40 Thursday, June 24, 2023  
 The GLM Procedure

Dependent Variable: HDP

|                 |          | Sum of      |             |          |        |
|-----------------|----------|-------------|-------------|----------|--------|
| Source          | DF       | Squares     | Mean Square | F Value  | Pr > F |
| Model           | 9        | 42002.79993 | 4666.97777  | 27.35    | <.0001 |
| Error           | 134      | 22863.26213 | 170.62136   |          |        |
| Corrected Total | 143      | 64866.06206 |             |          |        |
|                 | R-Square | Coeff Var   | Root MSE    | HDP Mean |        |
|                 | 0.647531 | 49.00754    | 13.06221    | 26.65347 |        |
| Source          | DF       | Type I SS   | Mean Square | F Value  | Pr > F |
| TRT             | 7        | 41414.69584 | 5916.38512  | 34.68    | <.0001 |
| REPS            | 2        | 588.10409   | 294.05205   | 1.72     | 0.1824 |
| Source          | DF       | Type III SS | Mean Square | F Value  | Pr > F |
| TRT             | 7        | 41414.69584 | 5916.38512  | 34.68    | <.0001 |
| REPS            | 2        | 588.10409   | 294.05205   | 1.72     | 0.1824 |

Duncan's Multiple Range Test for HDP

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

|                 |                          |          |      |      |      |      |       |  |
|-----------------|--------------------------|----------|------|------|------|------|-------|--|
|                 | Alpha                    | 0.05     |      |      |      |      |       |  |
|                 | Error Degrees of Freedom | 134      |      |      |      |      |       |  |
|                 | Error Mean Square        | 170.6214 |      |      |      |      |       |  |
| Number of Means | 2                        | 3        | 4    | 5    | 6    | 7    | 8     |  |
| Critical Range  | 8.61                     | 9.06     | 9.36 | 9.59 | 9.76 | 9.90 | 10.01 |  |

Means with the same letter are not significantly different.

| Duncan Grouping | Mean   | N  | TRT |
|-----------------|--------|----|-----|
| A               | 50.792 | 18 | T2  |
| A               |        |    |     |
| A               | 47.619 | 18 | T1  |
| B               | 38.361 | 18 | T4  |
| B               |        |    |     |
| B               | 32.541 | 18 | T3  |
| C               | 17.726 | 18 | T6  |
| C               |        |    |     |
| C               | 13.757 | 18 | T5  |
| C               |        |    |     |
| D C             | 9.788  | 18 | T7  |
| D               |        |    |     |
| D               | 2.645  | 18 | T8  |

| Class | Levels | Values                  |
|-------|--------|-------------------------|
| TRT   | 8      | T1 T2 T3 T4 T5 T6 T7 T8 |
| REPS  | 3      | 1 2 3                   |

Dependent Variable: Egg weight

| Source          | DF       | Sum of Squares | Mean Square | F Value         | Pr > F |
|-----------------|----------|----------------|-------------|-----------------|--------|
| Model           | 9        | 55855.7130     | 6206.1903   | 19.00           | <.0001 |
| Error           | 206      | 67284.8796     | 326.6256    |                 |        |
| Corrected Total | 215      | 123140.5926    |             |                 |        |
|                 | R-Square | Coeff Var      | Root MSE    | Egg weight Mean |        |
|                 | 0.453593 | 48.60212       | 18.07279    | 37.18519        |        |
| Source          | DF       | Type I SS      | Mean Square | F Value         | Pr > F |
| TRT             | 7        | 54611.03704    | 7801.57672  | 23.89           | <.0001 |
| REPS            | 2        | 1244.67593     | 622.33796   | 1.91            | 0.1514 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|--------|----|-------------|-------------|---------|--------|
| TRT    | 7  | 54611.03704 | 7801.57672  | 23.89   | <.0001 |
| REPS   | 2  | 1244.67593  | 622.33796   | 1.91    | 0.1514 |

The SAS System 01:40 Thursday, June 24, 2023  
The GLM Procedure

Duncan's Multiple Range Test for Egg weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

| Alpha                    | 0.05     |       |       |       |       |       |       |
|--------------------------|----------|-------|-------|-------|-------|-------|-------|
| Error Degrees of Freedom | 206      |       |       |       |       |       |       |
| Error Mean Square        | 326.6256 |       |       |       |       |       |       |
| Number of Means          | 2        | 3     | 4     | 5     | 6     | 7     | 8     |
| Critical Range           | 9.70     | 10.21 | 10.55 | 10.80 | 11.00 | 11.15 | 11.29 |

Means with the same letter are not significantly different.

| Duncan Grouping | Mean   | N  | TRT |
|-----------------|--------|----|-----|
| A               | 52.111 | 27 | T1  |
| A               |        |    |     |
| A               | 51.778 | 27 | T2  |
| A               |        |    |     |
| A               | 51.333 | 27 | T4  |
| A               |        |    |     |
| A               | 48.593 | 27 | T3  |
| B               | 36.519 | 27 | T6  |
| C               | 26.370 | 27 | T5  |
| C               |        |    |     |
| C               | 25.111 | 27 | T7  |
| D               | 5.667  | 27 | T8  |

**Appendix F.  
Data analysis on external egg qualities**

The SAS System 22:49 Saturday, July 10, 2023 1  
The GLM Procedure  
Class Level Information

| Class | Levels | Values                  |
|-------|--------|-------------------------|
| TRT   | 8      | T1 T2 T3 T4 T5 T6 T7 T8 |
| REPS  | 3      | 1 2 3                   |

Number of observations 216

NOTE: Due to missing values, only 153 observations can be used in this analysis.

The SAS System 22:49 Saturday, July 10, 2023 2  
The GLM Procedure

Dependent Variable: Shape index

| Source          | DF  | Sum of Squares | Mean Square | F Value | Pr > F |
|-----------------|-----|----------------|-------------|---------|--------|
| Model           | 9   | 207.278962     | 23.030996   | 2.57    | 0.0091 |
| Error           | 143 | 1283.239761    | 8.973705    |         |        |
| Corrected Total | 152 | 1490.518723    |             |         |        |

|        | R-Square | Coeff Var   | Root MSE    | SHAPE Mean |        |  |
|--------|----------|-------------|-------------|------------|--------|--|
|        | 0.139065 | 3.981104    | 2.995614    | 75.24582   |        |  |
| Source | DF       | Type I SS   | Mean Square | F Value    | Pr > F |  |
| TRT    | 7        | 181.1589185 | 25.8798455  | 2.88       | 0.0076 |  |
| REPS   | 2        | 26.1200432  | 13.0600216  | 1.46       | 0.2367 |  |
| Source | DF       | Type III SS | Mean Square | F Value    | Pr > F |  |
| TRT    | 7        | 170.5456857 | 24.3636694  | 2.72       | 0.0113 |  |
| REPS   | 2        | 26.1200432  | 13.0600216  | 1.46       | 0.2367 |  |

The SAS System 22:49 Saturday, July 10, 2023 3  
The GLM Procedure

Duncan's Multiple Range Test for SHAPE

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha 0.05  
Error Degrees of Freedom 143  
Error Mean Square 8.973705  
Harmonic Mean of Cell Sizes 11.57699

NOTE: Cell sizes are not equal.

| Number of Means | 2     | 3     | 4     | 5     | 6     | 7     | 8     |
|-----------------|-------|-------|-------|-------|-------|-------|-------|
| Critical Range  | 2.461 | 2.590 | 2.677 | 2.740 | 2.789 | 2.829 | 2.862 |

Means with the same letter are not significantly different.

| Duncan Grouping | Mean   | N  | TRT |
|-----------------|--------|----|-----|
| A               | 79.253 | 3  | T8  |
| B               | 76.398 | 13 | T5  |
| B               |        |    |     |
| C B             | 75.819 | 26 | T3  |
| C B             |        |    |     |
| C B             | 75.659 | 26 | T4  |
| C B             |        |    |     |
| C B             | 75.647 | 13 | T7  |
| C B             |        |    |     |
| C B             | 75.504 | 20 | T6  |
| C B             |        |    |     |
| C B             | 74.698 | 26 | T1  |
| C               |        |    |     |
| C               | 73.370 | 26 | T2  |

**Shell thickness**

The SAS System 22:49 Saturday, July 10, 2023 4  
The GLM Procedure

Class Level Information

| Class | Levels | Values                  |
|-------|--------|-------------------------|
| TRT   | 8      | T1 T2 T3 T4 T5 T6 T7 T8 |
| REPS  | 3      | 1 2 3                   |

Number of observations 192

The SAS System 22:49 Saturday, July 10, 2023 5

The GLM Procedure  
 Dependent Variable: shell thickness

| Source          | DF  | Sum of Squares | Mean Square | F Value | Pr > F |
|-----------------|-----|----------------|-------------|---------|--------|
| Model           | 9   | 2.76623021     | 0.30735891  | 12.13   | <.0001 |
| Error           | 182 | 4.60996979     | 0.02532950  |         |        |
| Corrected Total | 191 | 7.37620000     |             |         |        |

R-Square 0.375021  
 Coeff Var 67.72445  
 Root MSE 0.159152  
 THICKNESS Mean 0.235000

| Source | DF | Type I SS  | Mean Square | F Value | Pr > F |
|--------|----|------------|-------------|---------|--------|
| TRT    | 7  | 2.72615833 | 0.38945119  | 15.38   | <.0001 |
| REPS   | 2  | 0.04007187 | 0.02003594  | 0.79    | 0.4549 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|--------|----|-------------|-------------|---------|--------|
| TRT    | 7  | 2.72615833  | 0.38945119  | 15.38   | <.0001 |
| REPS   | 2  | 0.04007187  | 0.02003594  | 0.79    | 0.4549 |

The SAS System 22:49 Saturday, July 10, 2023 6  
 The GLM Procedure

Duncan's Multiple Range Test for Shell thickness

NOTE: This test controls the Type I comparison wise error rate, not the experiment-wise error rate.

| Alpha                    | 0.05    |       |       |       |       |       |       |
|--------------------------|---------|-------|-------|-------|-------|-------|-------|
| Error Degrees of Freedom | 182     |       |       |       |       |       |       |
| Error Mean Square        | 0.02533 |       |       |       |       |       |       |
| Number of Means          | 2       | 3     | 4     | 5     | 6     | 7     | 8     |
| Critical Range           | .0907   | .0954 | .0986 | .1009 | .1028 | .1042 | .1055 |

Means with the same letter are not significantly different.

| Duncan Grouping | Mean    | N  | TRT |
|-----------------|---------|----|-----|
| A               | 0.40542 | 24 | T1  |
| A               |         |    |     |
| B A             | 0.33042 | 24 | T3  |
| B A             |         |    |     |
| B A             | 0.31542 | 24 | T2  |
| B               |         |    |     |
| B C             | 0.29917 | 24 | T4  |
| C               |         |    |     |
| D C             | 0.21542 | 24 | T6  |
| D               |         |    |     |
| D E             | 0.18542 | 24 | T5  |
| E               |         |    |     |
| F E             | 0.10792 | 24 | T7  |
| F               |         |    |     |
| F               | 0.02083 | 24 | T8  |

Shell weight

The SAS System 10:39 Sunday, July 11, 2023 4  
 The GLM Procedure

Class Level Information

| Class                  | Levels | Values                  |
|------------------------|--------|-------------------------|
| TRT                    | 8      | T1 T2 T3 T4 T5 T6 T7 T8 |
| REPS                   | 3      | 1 2 3                   |
| Number of observations |        | 216                     |

The SAS System 10:39 Sunday, July 11, 2023 5  
 The GLM Procedure

Dependent Variable: Shell weight

| Sum of          |          |             |             |             |        |
|-----------------|----------|-------------|-------------|-------------|--------|
| Source          | DF       | Squares     | Mean Square | F Value     | Pr > F |
| Model           | 9        | 377.8425926 | 41.9825103  | 16.09       | <.0001 |
| Error           | 206      | 537.4907407 | 2.6091784   |             |        |
| Corrected Total | 215      | 915.3333333 |             |             |        |
|                 | R-Square | Coeff Var   | Root MSE    | WEIGHT Mean |        |
|                 | 0.412792 | 46.89567    | 1.615295    | 3.444444    |        |
| Source          | DF       | Type I SS   | Mean Square | F Value     | Pr > F |
| TRT             | 7        | 361.4814815 | 51.6402116  | 19.79       | <.0001 |
| REPS            | 2        | 16.3611111  | 8.1805556   | 3.14        | 0.0456 |
| Source          | DF       | Type III SS | Mean Square | F Value     | Pr > F |
| TRT             | 7        | 361.4814815 | 51.6402116  | 19.79       | <.0001 |
| REPS            | 2        | 16.3611111  | 8.1805556   | 3.14        | 0.0456 |

The SAS System 10:39 Sunday, July 11, 2023 6  
 The GLM Procedure

Duncan's Multiple Range Test for Shell weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

| Alpha                    |       | 0.05     |       |       |       |       |       |
|--------------------------|-------|----------|-------|-------|-------|-------|-------|
| Error Degrees of Freedom |       | 206      |       |       |       |       |       |
| Error Mean Square        |       | 2.609178 |       |       |       |       |       |
| Number of Means          | 2     | 3        | 4     | 5     | 6     | 7     | 8     |
| Critical Range           | 0.867 | 0.912    | 0.943 | 0.965 | 0.983 | 0.997 | 1.009 |

Means with the same letter are not significantly different.

| Duncan Grouping | Mean   | N  | TRT |
|-----------------|--------|----|-----|
| A               | 4.7778 | 27 | T2  |
| A               |        |    |     |
| B A             | 4.4815 | 27 | T3  |
| B A             |        |    |     |
| B A             | 4.4444 | 27 | T1  |
| B A             |        |    |     |
| B A             | 4.2963 | 27 | T4  |
| B               |        |    |     |
| B C             | 3.5556 | 27 | T6  |
| C               |        |    |     |
| D C             | 2.7407 | 27 | T5  |
| D               |        |    |     |

D 2.5556 27 T7  
 E 0.7037 27 T8

**Egg: shell ratio**

The SAS System 10:39 Sunday, July 11, 2023 1  
 The GLM Procedure  
 Class Level Information

| Class | Levels | Values                  |
|-------|--------|-------------------------|
| TRT   | 8      | T1 T2 T3 T4 T5 T6 T7 T8 |
| REPS  | 3      | 1 2 3                   |

Number of observations 216

NOTE: Due to missing values, only 164 observations can be used in this analysis.

The SAS System 10:39 Sunday, July 11, 2023 2  
 The GLM Procedure

Dependent Variable: Egg: shell ratio

| Sum of          |          |             |             |            |        |
|-----------------|----------|-------------|-------------|------------|--------|
| Source          | DF       | Squares     | Mean Square | F Value    | Pr > F |
| Model           | 9        | 79.547017   | 8.838557    | 1.45       | 0.1729 |
| Error           | 154      | 940.891770  | 6.109687    |            |        |
| Corrected Total | 163      | 1020.438787 |             |            |        |
| R-Square        |          | Coeff Var   | Root MSE    | RATIO Mean |        |
|                 | 0.077954 | 21.27499    | 2.471778    | 11.61823   |        |
| Source          | DF       | Type I SS   | Mean Square | F Value    | Pr > F |
| TRT             | 7        | 68.53406750 | 9.79058107  | 1.60       | 0.1386 |
| REPS            | 2        | 11.01294981 | 5.50647490  | 0.90       | 0.4082 |
| Source          | DF       | Type III SS | Mean Square | F Value    | Pr > F |
| TRT             | 7        | 66.70322975 | 9.52903282  | 1.56       | 0.1514 |
| REPS            | 2        | 11.01294981 | 5.50647490  | 0.90       | 0.4082 |

The SAS System 10:39 Sunday, July 11, 2023 3  
 The GLM Procedure

Duncan's Multiple Range Test for Egg: shell ratio

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha 0.05  
 Error Degrees of Freedom 154  
 Error Mean Square 6.109687  
 Harmonic Mean of Cell Sizes 15.28958

NOTE: Cell sizes are not equal.

| Number of Means | 2     | 3     | 4     | 5     | 6     | 7     | 8     |
|-----------------|-------|-------|-------|-------|-------|-------|-------|
| Critical Range  | 1.766 | 1.859 | 1.921 | 1.966 | 2.002 | 2.030 | 2.054 |

Means with the same letter are not significantly different.

| Duncan Grouping | Mean    | N  | TRT |
|-----------------|---------|----|-----|
| A               | 13.6000 | 5  | T8  |
| A               |         |    |     |
| B A             | 12.2493 | 27 | T1  |

|   |   |         |    |    |
|---|---|---------|----|----|
| B | A |         |    |    |
| B | A | 11.9148 | 27 | T3 |
| B | A |         |    |    |
| B | A | 11.7627 | 22 | T6 |
| B |   |         |    |    |
| B |   | 11.5352 | 27 | T2 |
| B |   |         |    |    |
| B |   | 11.4038 | 24 | T4 |
| B |   |         |    |    |
| B |   | 11.0319 | 16 | T7 |
| B |   |         |    |    |
| B |   | 10.2831 | 16 | T5 |

**Yolk: albumin ratio (%)**

The SAS System 10:39 Sunday, July 11,

2023 7

The GLM Procedure  
Class Level Information  
Class Levels Values  
TRT 8 T1 T2 T3 T4 T5 T6 T7 T8  
REPS 3 1 2 3

Number of observations 121

NOTE: Due to missing values, only 102 observations can be used in this analysis.

The SAS System 10:39 Sunday, July 11, 2023 8

The GLM Procedure

Dependent Variable: yolk: albumin ratio

| Sum of                                 |          |             |             |          |        |
|--|----------|-------------|-------------|----------|--------|
| Source                                 | DF       | Squares     | Mean Square | F Value  | Pr > F |
| Model                                  | 9        | 804.375558  | 89.375062   | 1.26     | 0.2681 |
| Error                                  | 92       | 6513.940607 | 70.803702   |          |        |
| Corrected Total                        | 101      | 7318.316165 |             |          |        |
| R-Square Coeff Var Root MSE RATIO Mean |          |             |             |          |        |
|  | 0.109913 | 19.13229    | 8.414494    | 43.98059 |        |
| Source                                 | DF       | Type I SS   | Mean Square | F Value  | Pr > F |
| TRT                                    | 7        | 799.4703693 | 114.2100528 | 1.61     | 0.1415 |
| REPS                                   | 2        | 4.9051889   | 2.4525944   | 0.03     | 0.9660 |
| Source                                 | DF       | Type III SS | Mean Square | F Value  | Pr > F |
| TRT                                    | 7        | 797.6488448 | 113.9498350 | 1.61     | 0.1425 |
| REPS                                   | 2        | 4.9051889   | 2.4525944   | 0.03     | 0.9660 |

The SAS System 10:39 Sunday, July 11, 2023 9

The GLM Procedure

Duncan's Multiple Range Test for yolk: albumin ratio

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha 0.05

Error Degrees of Freedom 92  
 Error Mean Square 70.8037  
 Harmonic Mean of Cell Sizes 11.23063

NOTE: Cell sizes are not equal.

|                 |       |       |       |       |       |       |       |
|-----------------|-------|-------|-------|-------|-------|-------|-------|
| Number of Means | 2     | 3     | 4     | 5     | 6     | 7     | 8     |
| Critical Range  | 7.052 | 7.421 | 7.666 | 7.845 | 7.984 | 8.096 | 8.189 |

Means with the same letter are not significantly different.

| Duncan Grouping |   | Mean   | N  | TRT |
|-----------------|---|--------|----|-----|
| A               |   | 50.444 | 5  | T8  |
| A               |   |        |    |     |
| A               |   | 46.346 | 12 | T5  |
| A               |   |        |    |     |
| B               | A | 45.533 | 15 | T3  |
| B               | A |        |    |     |
| B               | A | 44.719 | 14 | T4  |
| B               | A |        |    |     |
| B               | A | 44.189 | 15 | T2  |
| B               | A |        |    |     |
| B               | A | 43.569 | 15 | T6  |
| B               | A |        |    |     |
| B               | A | 42.568 | 15 | T1  |
| B               |   |        |    |     |
| B               |   | 37.609 | 11 | T7  |

**Egg yolk ratio**

The SAS System 10:39 Sunday, July 11, 2023 ,10

The GLM Procedure

Class Level Information

| Class | Levels | Values                  |
|-------|--------|-------------------------|
| TRT   | 8      | T1 T2 T3 T4 T5 T6 T7 T8 |
| REPS  | 3      | 1 2 3                   |

Number of observations 120

NOTE: Due to missing values, only 104 observations can be used in this analysis.

The SAS System 10:39 Sunday, July 11, 2023 , 11

The GLM Procedure

Dependent Variable: egg: shell ratio

| Sum of          |          |             |             |          |        |
|-----------------|----------|-------------|-------------|----------|--------|
| Source          | DF       | Squares     | Mean Square | F Value  | Pr > F |
| Model           | 9        | 2985.43195  | 331.71466   | 0.87     | 0.5582 |
| Error           | 94       | 36002.66794 | 383.00711   |          |        |
| Corrected Total | 103      | 38988.09989 |             |          |        |
|                 | R-Square | Coeff Var   | Root MSE    | Mean     |        |
|                 | 0.076573 | 72.08389    | 19.57057    | 27.14971 |        |
| Source          | DF       | Type I SS   | Mean Square | F Value  | Pr > F |
| TRT             | 7        | 2533.624999 | 361.946428  | 0.95     | 0.4760 |
| REPS            | 2        | 451.806949  | 225.903475  | 0.59     | 0.5565 |
| Source          | DF       | Type III SS | Mean Square | F Value  | Pr > F |

|      |   |             |            |      |        |
|------|---|-------------|------------|------|--------|
| TRT  | 7 | 2322.890078 | 331.841440 | 0.87 | 0.5361 |
| REPS | 2 | 451.806949  | 225.903475 | 0.59 | 0.5565 |

The SAS System 10:39 Sunday, July 11, 2023 12  
The GLM Procedure

Duncan's Multiple Range Test for egg: shell ratio

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha 0.05  
Error Degrees of Freedom 94  
Error Mean Square 383.0071  
Harmonic Mean of Cell Sizes 11.86517

NOTE: Cell sizes are not equal.

|                 |       |       |       |       |       |       |       |
|-----------------|-------|-------|-------|-------|-------|-------|-------|
| Number of Means | 2     | 3     | 4     | 5     | 6     | 7     | 8     |
| Critical Range  | 15.95 | 16.79 | 17.34 | 17.75 | 18.06 | 18.32 | 18.53 |

Means with the same letter are not significantly different.

| Duncan Grouping | Mean   | N  | TRT |
|-----------------|--------|----|-----|
| A               | 40.619 | 11 | T7  |
| A               |        |    |     |
| B A             | 26.741 | 15 | T1  |
| B A             |        |    |     |
| B A             | 26.603 | 15 | T3  |
| B A             |        |    |     |
| B A             | 26.481 | 15 | T2  |
| B A             |        |    |     |
| B A             | 26.032 | 15 | T6  |
| B A             |        |    |     |
| B A             | 25.052 | 15 | T4  |
| B A             |        |    |     |
| B A             | 24.796 | 12 | T5  |
| B               |        |    |     |
| B               | 19.265 | 6  | T8  |

The SAS System 22:48 Sunday, July 18, 2023 1  
The GLM Procedure

Class Level Information

| Class | Levels | Values                  |
|-------|--------|-------------------------|
| TRT   | 8      | T1 T2 T3 T4 T5 T6 T7 T8 |
| REPS  | 3      | 1 2 3                   |

Number of observations 144

The SAS System 22:48 Sunday, July 18, 2023 2  
The GLM Procedure

Dependent Variable: **yolk colour**

| Source | DF | Sum of Squares | Mean Square | F Value | Pr > F |
|--------|----|----------------|-------------|---------|--------|
|--------|----|----------------|-------------|---------|--------|

|                 |          |             |             |             |        |
|-----------------|----------|-------------|-------------|-------------|--------|
| Model           | 9        | 2324.750000 | 258.305556  | 245.48      | <.0001 |
| Error           | 134      | 141.000000  | 1.052239    |             |        |
| Corrected Total | 143      | 2465.750000 |             |             |        |
|                 | R-Square | Coeff Var   | Root MSE    | COLOUR Mean |        |
|                 | 0.942817 | 11.77937    | 1.025787    | 8.708333    |        |
| Source          | DF       | Type I SS   | Mean Square | F Value     | Pr > F |
| TRT             | 7        | 2324.750000 | 332.107143  | 315.62      | <.0001 |
| REPS            | 2        | 0.000000    | 0.000000    | 0.00        | 1.0000 |
| Source          | DF       | Type III SS | Mean Square | F Value     | Pr > F |
| TRT             | 7        | 2324.750000 | 332.107143  | 315.62      | <.0001 |
| REPS            | 2        | 0.000000    | 0.000000    | 0.00        | 1.0000 |

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The GLM Procedure

Duncan's Multiple Range Test for yolk colour

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

|                          |          |       |       |       |       |       |       |
|--------------------------|----------|-------|-------|-------|-------|-------|-------|
| Alpha                    | 0.05     |       |       |       |       |       |       |
| Error Degrees of Freedom | 134      |       |       |       |       |       |       |
| Error Mean Square        | 1.052239 |       |       |       |       |       |       |
| Number of Means          | 2        | 3     | 4     | 5     | 6     | 7     | 8     |
| Critical Range           | .6763    | .7118 | .7354 | .7528 | .7663 | .7772 | .7863 |

Means with the same letter are not significantly different.

| Duncan Grouping | Mean    | N  | TRT |
|-----------------|---------|----|-----|
| A               | 12.0000 | 18 | T7  |
| A               |         |    |     |
| A               | 12.0000 | 18 | T8  |
| B               | 10.6667 | 18 | T5  |
| B               |         |    |     |
| B               | 10.6667 | 18 | T6  |
| B               |         |    |     |
| B               | 10.3333 | 18 | T3  |
| B               |         |    |     |
| B               | 10.3333 | 18 | T4  |
| C               | 1.8333  | 18 | T1  |
| C               |         |    |     |
| C               | 1.8333  | 18 | T2  |

**Appendix E. Mulberry plant**



**Appendix F. Roche yolk colour fan**

