

The effect of grain and soya bean-based diets on chicken production, some egg quality traits, and the potential for allergen carryover to eggs and meat

By

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Declaration

I, Adia-En-Michelle Dokora, hereby declare that this research is an outcome of my own investigation under the supervision of Prof. V. Muchenje, Prof. L.C. Hoffman, Dr. D.M. Cawthorn and Dr. E. Pieterse and has not been previously submitted to any other University. Where reference to other researchers' work has been made and where assistance was rendered; it has been duly acknowledged in the text.

Signature..... Date

Abstract

This study determined the effect of maize-, whole wheat-, soya bean-based-diets on the growth performance, egg quality and dressing percentage of chickens, as well as the potential for soy and gluten allergen carryover to eggs and meat from chickens. Twenty 36-week-old Lorham White (LW) hens were divided into two groups and kept in individual cages until they reached 39-weeks of age, with water and feed supplied *ad libitum*. Ten LW hens were fed a maize/soya bean-based diet (T₁) and the other ten birds were fed a maize/soya bean-based diet with a 15% whole wheat inclusion (T₂) for a period of four weeks, with weekly individual hen weights and group feed consumption figures being recorded. After a two-week diet adaptation period, six eggs per treatment group were collected every second day from the LW hens' to measure egg quality traits. Every third day, over a 29-day period, six eggs per treatment were collected and analysed for the presence of soy, gluten and gluten-derived peptides using allergen specific enzyme linked immunosorbent assays (ELISA) kits. For the broiler trial, a total of 160 Ross 308 mixed sex day old chicks were used in a completely randomized design. Two dietary treatments were assigned to eight cages (replicate) per treatment, with ten birds per cage. The Ross 308 broiler feeding programme consisted of three phases, starter (day 1 to day 10), grower (day 11 to day 20) and finisher (day 21 to day 28). At the beginning of the trial all chicks were fed T₁ which contained a maize/soya bean-based diet for a period of 10 days and then eight cages with 10 birds per cage were randomly selected and fed the T₂ diet, which contained a maize/soya bean-based diet with a 15% whole wheat inclusion, until they reached 28 days of age. Weekly live weights, feed intake, average daily gain and the average daily feed intake were recorded and calculated. At the end of the grower (day 21) and finisher phase (day 28), one broiler bird was selected per cage to have eight (8) birds per treatment and 16 birds per phase that were slaughtered and breast meat samples were analysed in duplicate for the presence of soy and gluten allergens using ELISA

kits. The dressing percentage of hot carcasses was also determined at the end of the finisher phase on eight birds per treatment. For LW hens, live weight (LW) at 39 weeks of age, the average daily feed intake (ADFI) and weekly feed intakes (FI) were significantly different ($P < 0.05$) between treatments with birds on T₂ recording higher gains. As birds age in weeks increased, significant differences ($P < 0.05$) in live weight values were recorded. Egg quality traits measured (shell weight, yolk weight, albumen weight, albumen height and Haugh units) were significantly different ($P < 0.05$) between treatments, with eggs from hens receiving T₁ recording higher mean values. As hens got older, the colour of egg yolk improved, producing eggs with higher b^* (yellowness) values and lower L^* (lightness) and a^* (redness) mean values. The Haugh unit, significantly improved ($P < 0.05$) as the hens got older, signifying better quality of eggs. For the broiler birds, growth traits measured indicated that there were no significant differences ($P > 0.05$) in live weight (LW), average daily feed intake (ADFI), weekly feed intake (FI), feed conversion ratio (FCR), cumulative feed intake (CFI) and cumulative gain (CG) between treatments. No significant differences ($P > 0.05$) between dressing percentage of broilers from the two treatments were observed. As birds grew, significant differences ($P < 0.05$) in LW, FI, AFI, FCR, and CFI, over a four week period were recorded. Results of the allergen analyses indicated that soy and gluten allergens were not carried over from feed into eggs and meat of chickens fed a maize/soya bean-based diet and a maize/soya bean-based with a 15% whole wheat inclusion, respectively. In conclusion, a maize/soya bean-based diet and maize/soya bean-based diet with a 15% whole wheat inclusion that contained soy and gluten allergens, are feed sources that promote and stimulate 36-week-old LW hen and Ross 308 chicken growth. Egg production was also maintained without the possibility of soy and gluten allergens being carried over from the feed into the eggs and meat.

Keywords: Allergens, chicken growth, carryover, carcass yield, and egg quality

Dedication

Para Lazarus Dagwa Kambarami Dokora y Grace Dadirai Gapare Dokora, gracias por todo mis padres.

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List of Abbreviations

α	Alpha
a^*	Colour- redness coordinate
AA	Amino Acids
ABA	Allergen Bureau of Australia and New Zealand
ADG	Average Daily Gain
AFI	Average Feed Intake
AFMA	Animal Feed Manufacturers Association
AOAC	Association of Official Analytical Chemists
β	Beta
b^*	Colour – yellow coordinate
BRICS	Brazil Russia India China and South Africa
BSE	Bovine Spongiform Encephalopathy
°C	Degree Celsius
CD	Celiac Disease
CF	Crude Fiber
CHD	Coronary Heart Disease
e	Error term
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay

EU	European Union
FA	Fatty Acids
FAO	Food and Agricultural Organization of the United Nations
FCR	Food Conversion Ratio
FDA	Food and Drug Administration
FI	Feed Intake
GIT	Gastrointestinal system
GLM	General Linear Model
GMO	Genetically Modified Organisms
HLA	Human Leukocyte Antigen
IgE	Immunoglobulin E
INFOSAN	International Food Safety Authorities Network
<i>L*</i>	Colour – lightness coordinate
LOAELs	Lowest-Observed Adverse Effect Level
LSD	Least Significant Difference
LWG	Live Weight Gain
LW	Lorham White
NOAELs	No-Observed Adverse Effect Level
NRC	National Research Council
NSP	Non-Starch Polysaccharide

OECD	Organization for Economic Cooperation and Development
PSFA	Polysaturated Fatty Acids
PUFA	Polyunsaturated Fatty Acids
SAPA	South African Poultry Association
SAS	Statistical Analysis System
μ	Mu, population mean (average)
UK	United Kingdom
vCJD	Creutzfeldt Jakob Disease
VITAL	Voluntary Incidental Trace Allergen Labelling
WCRF	World Cancer Research Fund

Chapter 1: General Introduction

1.0 Background

Chickens have been recognised as the livestock of the poor and have been incorporated into small-holder farming systems as a quick and cheap source of animal protein. Approximately 85% of rural families in Sub-Saharan Africa keep chickens for egg and meat production (Food and Agriculture Organization (FAO), 2010). Eggs and meat are important animal products that help in maintaining the health and nutritional status of communities, especially in developing countries, where sources of protein are usually limited (Capper, 2013). Layer and broiler production in most countries aims at closing the gap of animal protein deficiency (Ebenebe *et al.*, 2012). With an increase in poultry meat and egg production, raw materials used in the poultry feed industry have become more diverse.

Crops such as barley, sorghum and wheat are now used as sources of animal energy and protein whilst soybean has further become a primary source of protein in most, if not in all livestock feed production systems. These crops have significantly helped reduce the cost of poultry production, while simultaneously producing good quality products such as eggs and meat from chickens (Kiarie *et al.*, 2014). With reported increase in growth performance and egg quality from chickens fed maize, wheat and soya bean containing diets (Safaa *et al.*, 2009). Sources of protein such as fish meal are expensive versus soya bean and the fish aroma can be carried over onto animal products hence the shift to the use of soya bean by most poultry producers. In Canada, Europe and Australia, soya bean is the most commonly used energy and protein source (Mahammadi Ghasem Abadi *et al.*, 2014; Amerah, 2015).

In the quest to produce food that is cheap, potential harmful health effects of cheap feed sources were neglected. In recent years the emergence of health related problems, such as food allergies that have been associated with ingesting poultry products such as eggs has been

documented (Sampson, 2004). According to Sampson (2004), a food allergy is an adverse immunological reaction that might arise due to an immunoglobulin E (IgE) - or non- IgE - mediated immune mechanisms following the ingestion of certain proteins present in a foodstuff. Symptoms of allergies range from mild skin reactions to life-threatening anaphylaxis (Waugh and Grant, 2006). Food allergies or food hypersensitivity have now been recognised as a worldwide problem taking precedence in Western nations, but varying within Western Europe (Sampson, 2004; Burney *et al.*, 2014). According to Umetsu *et al.* (2015), approximately 10% of children in industrialized countries suffer from food allergies. In America alone, 1.3% of children and 0.2% of the total adult population suffer from food allergies as a result of milk ingestion alone (Sampson, 2004). Various foodstuffs have been declared to cause allergies despite any food being capable of eliciting an allergic reaction.

The common protein allergens include; cow's milk, crustaceans, eggs, molluscs, peanuts, soya bean, tree nuts and fish (Ogawa *et al.*, 2000; Herman *et al.*, 2003; Alsaeed *et al.*, 2013; Burney *et al.*, 2014; Fernández-Rivas and Asero, 2014). Soya bean, fish and wheat are common allergens that are currently used in the poultry feed manufacturing industry but laws in South Africa, Europe, Canada and Australia require these to be declared on food product labels.

Some studies could be found in literature, which have evaluated the potential of allergen carryover from feed to animal-derived products such as pork and fish (Buur *et al.*, 2008; Faeste *et al.*, 2015). Investigations with other monogastric species such as layer and broiler chickens are yet to be determined. Therefore, the following study aimed at investigating growth performance of broiler and layer chickens, dressing percentage, egg quality and the possibility of allergen carryover from formulated poultry diets having quantified sources of allergens.

1.1 Problem statement

In recent years, the use of cereals as a source of energy in most monogastric livestock feeds has decreased because of its relatively high content of non-starch polysaccharide (NSP) that make digestion a problem despite recommended inclusion levels in various literature sources (Mahammadi Ghasem Abadi *et al.*, 2014; Amerah, 2015). Although various wheat and soya bean inclusion levels have been investigated, a more suitable one is still yet to be determined. Soya bean and wheat are currently being used in the livestock industry and there is a need to determine their effect on growth performance and egg quality of broilers and layers respectively.

Numerous questions have been raised by food producers and the public relating to the possibility of allergens consumed by animals from feeds being carried over into the eggs, milk and meat, and be potentially consumed by allergic humans (Colchester and Cholchester, 2005; Buur *et al.*, 2008). Although there is limited evidence of such reactions occurring in the general consuming public, little or no experimental work has been conducted to date to determine and inform the public the associated safety concerns of such a possibility. In general, proteins present in animal-derived products are synthesized from amino acids made available during the breakdown and reassembly of proteins and their metabolites during digestion in the gastrointestinal (GIT) system of livestock. However, intact and fragments of melamine, a non-protein nitrogen chemical source has been detected in fish, fresh eggs and pork meat with peanut allergens being detected in human breast milk following maternal ingestion (Frank *et al.*, 1999; Ding *et al.*, 2012; Faeste *et al.*, 2015) which suggests that there is a hypothetical risk that allergenic carryover could occur in monogastric animals that have similar gastro-intestinal characteristics. Undoubtedly this deserves further research.

1.2 Justification

Although various studies have recommended certain inclusion levels of wheat and soya bean in diets, it is still yet to be determined if these levels normally used in industry, not only produce the expected qualities in growth performance, eggs and meat but could actually be potential sources of allergens to would be egg and broiler meat consumers. Although some studies could be found in literature that have assessed parasitic and transgenic protein carryover from feed to animal-derived products such as fish and fresh eggs, none have been found in literature that have investigated plant allergen carryover in chicken products such as eggs and meat (International Food Safety Authorities Network (INFOSAN), 2008; Faeste *et al.*, 2015). Currently no indication exists to propose that plant proteins can be expressed in tissues of animals that have consumed plant protein containing feeds (European Food Safety Authority (EFSA), 2007).

The natural GIT system of healthy livestock is a protective barrier against the absorption of macromolecules such as proteins, which could have missed digestion (Sampson, 2004; Netting *et al.*, 2013). However, previous investigations have demonstrated the presence of the chemical melamine and fragment proteins of the fish parasite *Anisakis simplex (s.l.)* in pig and fish flesh respectively following intentional inclusion in diets (Buur *et al.*, 2008; Faeste *et al.*, 2015) showing the possibility of carryover. Therefore the current study was aimed at analysing the possibility of soy and gluten allergen carryover from feed consumed by chickens to eggs and meat.

1.3 Objectives

The broad objective of this study was to determine growth performance, egg quality, dressing percentage and the potential for allergen carryover in Lorham White hens and Ross 308 broilers fed poultry feed containing soya bean and whole wheat. The specific objectives were:

i)-To determine the effect of maize/soya bean and maize/soya bean-based diet with a 15% whole wheat inclusion on growth performance, egg quality and the potential for allergen carryover into eggs from layer chickens.

ii)-To determine the effect of maize/soya bean and a maize/soya bean-based diet with a 15% whole wheat inclusion on growth performance, dressing percentage and the potential for allergen carryover into meat from broiler chickens.

1.4 Hypothesis

The null hypothesis being tested included

i)-Maize/soya bean and maize/soya bean-based diet with a 15% whole wheat inclusion do not have an effect on growth performance, egg quality and no allergen carryover onto eggs from layer chickens.

ii)-Maize/soya bean and wheat/maize/soya bean-based diet with a 15% whole wheat inclusion do not have an effect on growth performance, dressing percentage and allergen carryover onto meat from broiler chickens.

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Chapter 2: Literature Review

2.0 Introduction

The current global population stands at approximately 7.3 billion with an anticipated 9 billion expected to be surpassed by the year 2050 (Food and Agricultural Organization (FAO), 2009a). This growth has placed a tremendous strain on the agricultural sector to meet current and anticipated global food demand (FAO, 2010). Global food production must rise by 70% in developed and in developing nations. This figure must double if food demand is to be met in the year 2050 (FAO, 2009a). Although this production level is feasible, global food production faces many challenges. Such challenges include and are not limited to: unforeseen rapid increase in the global population in the past 10 years, land and space restrictions, high feedstock prices, limited forage production, climate change, water shortage, carbon emission and the recent welfare legislations that govern animal and plant use (Liverman and Kapadia, 2010; Cawthorn and Hoffman, 2014). Despite these challenges food consumption patterns have shown an increase in the demand for some agricultural products (FAO, 2010).

Current food consumption patterns have revealed that meat production has risen in the past 50 years over crops. This observed trend indicates a change in demand and price signals in some nations (Liverman and Kapadia, 2010). Generally, in developing countries, income growth, urbanization and a rapid population growth are attributable as having increased the demand for animal products such as eggs, meat and milk (FAO, 2009b). This is unlike in developed countries where consumers are becoming more health conscious and making more informed choices on the quality of animal product they would like on their meal plate (FAO, 2009b).

Animal product health consciousness arose as a result of the various food scandals involving animal products such as meat. The recent reported cases of the presence of the allergen

chemical melamine in pet food, meat species substitution and mislabelling in the European Union (EU), Finland and South Africa (SA), as well as the presence of Bovine Spongiform Encephalopathy (BSE) in meat, that led to the emergence of Creutzfeldt Jakob Disease, vCJD ('mad cow disease') in humans, has raised concerns on how safe animal products are (Burton and Young, 1996; Burns, 2007; Higgs, 2000; Cawthorn *et al.*, 2013; Tähkäpää *et al.*, 2015). Producers attempt to cut costs through the use of cheap harvests that then replace genuine animal products such as meat in order to meet production demands (Burns, 2007; Cawthorn *et al.*, 2013). Red meat and its products are highly susceptible to food fraud because of the wide range of animal species that have a general high redness a^* colour that ranges from 11.1 to 23.6 and does not turn white during cooking (Muchenje *et al.*, 2009). Despite all these concerns, red meat is still extensively consumed in some global regions such as North and South America, the EU, and China (Cawthorn and Hoffman, 2014).

Red meat consumption remains high in certain regions because meat is an important source of proteins, essential nutrients such as folic acid, selenium, zinc, iron, vitamin B12, and polyunsaturated fatty acids (PUFAs) (McAfee *et al.*, 2010). These nutrients are all essential for wholesome human development and daily functions of vital organs and processes. Despite this, currently in some consumer spheres, red meat is termed unhealthy and harmful to human health. According to Rutsaert *et al.* (2015), the perception of red meat being detrimental to human health, is greatly fuelled by free non-peer reviewed information in today's online environment. The fear of developing coronary heart diseases (CHDs) and cancer has seen consumers cutting down on red meat consumption as highly publicised in media recently. In a recent peer reviewed article, the authors concluded that not enough evidence is available to link meat consumption to CHDs such as myocardial ischaemia (Lippi *et al.*, 2015). Although the cognitive behaviour of individuals that usually avoid information that is not consistent with their cognitions can also play a role in the ultimate perception to

red meat, therefore most consumers are misinformed on the actual benefits or otherwise of red meat consumption (Gaspar *et al.*, 2015).

However, according to several studies strong evidence exists that indicates the link between red meat consumption and several health related ailments such as, an increased rate in aging, CHDs such as arteriosclerosis, diabetes mellitus (type 2), and colon cancer (McAfee *et al.*, 2010; Kim, *et al.*, 2015; Lippi *et al.*, 2015). Coronary Heart Diseases have been linked with red meat consumption because of the elevated cholesterol and polysaturated fatty acids (PSFA)-content found in most red meat species (Higgs, 2000). This was revealed in studies done in Brazil and America (Pan *et al.*, 2012; Cocate *et al.*, 2015). The PSFAs are deposited in arteries when in excess and this reduces or blocks the diameter of arteries. The tissues distal to the narrow point became ischaemic which leads to infarction therefore when a coronary artery is affected, myocardial infarction occurs (Waugh and Grant, 2006). Experiments by Bingham *et al.* (2002), revealed that heterocyclic amines that are carcinogenic in nature develop after cooking red meat at high temperatures, thus individuals that consume red meat cooked at high temperatures are susceptible to developing cancer. As a result the World Cancer Research Fund (WCRF) has reported that red meat has a considerable role to play in colon cancer and the organization advocates for a low red meat consumption in preference to white meat (McAfee *et al.*, 2010).

Animal species known to provide white meat include fish, chicken, and rabbit. According to recent statistics, white meat production has increased, in particular poultry as compared to bovine red meat production (FAO, 2009b; Cawthorn and Hoffman, 2014; FAO, 2015). In 2012, 112.4 million tonnes of meat from pigs, 105.4 million tonnes of meat from poultry and 67 million tonnes of bovine meat was produced (FAO, 2015). In the year 2014, poultry production numbers were projected to increase to 108.7 million tonnes, pigs to 115.5 and bovine to 68 million tonnes (FAO, 2015). White meat is documented to contain high quality

protein, relatively less fats, less PSFA and more PUFA as compared to red meat (Maragoni *et al.*, 2015). It is considered to be healthier as opposed to red meat (Cocate *et al.*, 2015).

In this regard, broilers, with a short production life span and layer birds that produce eggs, have become increasingly popular. This popularity is a result of broilers having a short generation interval, (broiler chicks can be hatched and harvested within 35 days), as well as efficient feed conversion ratios, and minimal space requirements when compared to other livestock species (FAO, 2009a). In Malaysia, a fast developing country, broiler meat has become the staple meat (Majid and Hassan, 2014).

2.1 Poultry production and projections: a look at *Gallus gallus domesticus*

2.1.1 Current global trends: chicken eggs and meat

Chickens form an integral part of the livestock sector as they make a significant contribution to poverty alleviation and poverty reduction because of their edible products (FAO, 2009b). *Gallus gallus domesticus* (chickens) were domesticated in the early Neolithic period for egg and meat production. Currently chickens can now be grown under a wide range of husbandry conditions, from free range to intensive production (Siegel, 2014). The wide production range, from egg to meat and efficiency of feed utilization has enabled the species to be established in various environments globally as seen by current production numbers.

Global egg production in the past 15 years has increased. In 1980, total egg production in developing nations was 9.5 million tonnes and in developed nations it was 17.9 million tonnes and these figures increased to 39.4 million tonnes of eggs in both developed and developing countries respectively in the year 2007 (FAO, 2009b). The exponential growth in egg production recorded was as a result of an increase in layer birds and the need to close the protein deficiency gap with eggs (FAO, 2009a). Eggs not only provide protein but essential

nutrients such as vitamins D, K, and 12, folic acid, riboflavin, as well as energy despite having relatively high cholesterol content (Applegate, 2000).

According to FAO (2013), there was an anticipated global increase in broiler meat production of 106 million tonnes in the year 2013 (FAO, 2013). Current figures reported for the year 2012 surpassed the 2013 projections by 600 000 tonnes of chicken meat produced and the global projection exceed 108 million tonnes (FAO, 2015). This increase is as a result of the increase in the demand for white meat from chickens and consumer preference.

2.1.2 Local trends

In South Africa, the per-capita broiler meat consumption projected for 2013 was 39 kg per person per annum an increase from 35.5kg per person per annum in 2008 (South African Poultry Association (SAPA), 2013a). The recorded increase in consumption is as a result of urbanization, health awareness on the benefits of white meat, and a change in the consumer basket (FAO, 2009b). This is as a result of the long term profits that are obtained in a short space of time per unit area of land as compared to other livestock species (SAPA, 2013a; Majid and Hassan, 2014). As seen in global trends, more commercial and emerging farmers are venturing into broiler production despite current and projected increase in feed prices that account for 70% of production costs (Majid and Hassan, 2014). Current broiler animal feed sales statistics in South Africa stand at 2 852 107 tonnes for broiler and layer feed respectively as from April 2014 to March 2015 (Animal Feed Manufacturers Association (AFMA), 2015). Ten years ago, figures of sales stood at 2 114 156 tonnes as from April 2005 to March 2006 (AFMA, 2007). As the figures indicate, poultry feed manufacturing is increasing which is in accordance with previously reported figures on broiler numbers. As broiler birds increase so too is the amount of feed.

In 2008, egg consumption figures were set at 153 eggs per person per annum and in 2013, with a projected decrease to 147 eggs per person per annum in the following year (SAPA, 2013a). A decrease in layer numbers from 25.037 million in 2012 to 24.528 million could have also contributed to the low egg numbers reported in South Africa (SAPA, 2013b). Another reason could be the increasing cost of raw materials required for feed production that constitute approximately 70% -75% of total production costs (Majid and Hassan, 2014; Rufino *et al.*, 2015). It takes approximately 17-20 weeks for layers to start laying eggs and at approximately 30-32 weeks to reach peak period at the same time consuming approximately 120g of feed per day. Current layer feed sales statistics in South Africa stand at 952 607 tonnes as from April 2014 to March 2015 (AFMA), 2015). Ten years ago, figures of sales stood at 667 417 for layer feed as from April 2005 to March 2006 (AFMA, 2007). As the figures indicate, poultry feed manufacturing is increasing in the layer sector although bird numbers are low. This is because, layer birds consume more feed and produce more eggs compared to broiler birds where the number of birds is approximately proportional to the amount of feed consumed. Farmers need substantial amounts of capital just to cover feed costs of pullets till they commence to lay eggs, hence the reported increase in feed production in this group of chickens.

2.2 Plant poultry feed sources, Soya bean and Wheat

2.2.1 Fish meal to soya bean

In order to reduce feed costs and increase production in the poultry industry, conventional plant feed sources such as wheat and soya bean are being used. This is because, protein is the most limiting and expensive dietary nutrient constituent (Ncobela and Chimonyo, 2015) and soya bean has been documented to be relatively cheap at the same time supplying the required protein. A summary of the nutritional composition of soya bean is tabulated in Table 2.1 and Table 2.2.

Table 2. 1 A summary of proximate chemical composition of soya bean meal (as % dry weight)

Nutrient	Range	Source
Dry matter	86.98 - 93.86	Grieshop and Fahey (2001)
Organic matter	94.56 - 95.1	Grieshop and Fahey (2001)
Ash	5.1 - 5.5	Grieshop and Fahey (2001)
Crude protein	42.14 - 50	García <i>et al.</i> (1997); Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004); Thakur and Hurburgh, (2007); Medic <i>et al.</i> (2014)
Lipid	8.1-24	García <i>et al.</i> (1997); Grieshop and Fahey (2001); Medic <i>et al.</i> (2014)
Carbohydrate	35 – 40	García <i>et al.</i> (1997); Grieshop and Fahey (2001); Thakur and Hurburgh (2007)

Table 2. 2 : A summary of amino acid composition of soya bean meal (% dry weight)

Essential and Non-essential amino acids		
Nutrient	Range	Source
Arginine	2.87 - 3.08	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Histidine	0.9 - 1.21	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Isoleucine	1.51 – 2.06	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Leucine	2.47 - 3.32	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Lysine	2.07 – 2.69	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Methionine	0.28 – 0.64	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Phenylalanine	1.63 – 2.33	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Threonine	1.25 – 1.75	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Tryptophan	0.36 – 0.58	Karr-Lilienthal <i>et al.</i> (2004)
Valine	1.69 – 2.20	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Alanine	1.46 – 1.79	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Aspartate	3.44 – 5.00	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Cystine	0.50 – 0.88	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Glutamate	5.35 – 8.00	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Glycine	1.48 – 1.83	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Proline	1.31 – 2.44	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Serine	1.31 – 2.24	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Tryosine	1.15 – 1.56	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)
Total AA	30.9- 42.80	Grieshop and Fahey (2001); Karr-Lilienthal <i>et al.</i> (2004)

Therefore, soya bean of the family *Leguminosae* is a rich source of protein and other nutrients and it is currently being used as a substitute for fish meal in livestock diets (Irish and Balnave, 1993; Denbow *et al.*, 1995; Pieterse *et al.*, 2013). One of the reasons is that, fish meal is becoming expensive. This might cause adulteration due to different aquatic animals and different fish species and portions used and thereby compromising fish meal quality (Verrez-Bagnis, 2010; Sun *et al.*, 2014). Other reasons include but are not limited to the following: the remnant fish odour that may be carried over onto eggs and milk that were produced from livestock that were fed fish meal containing diets (Wu *et al.*, 2014). Chemical contamination of fish meal is likely to occur, as preservatives used to conserve may be toxic to animals (Karimi, 2006). Saw dust and sand presence in fish meal, and the risk of the occurrence of BSE in livestock feed animal protein can contribute to the gradual decline in fish meal use in livestock diets (Karimi, 2006; Verrez-Bagnis, 2010; Wu *et al.*, 2014). Currently the FAO has recommended the ban on the use of animal derived protein sources in the advent of vCJD that has been widely documented to be detrimental to both human and animal health (FAO, 2004).

2.2.2 Soya bean use in poultry feeds

Soya bean is an important agro-economic crop that is produced and consumed globally by both humans and livestock because of its high nutritional value and low cost (García *et al.*, 1997). In the EU, soya bean has gained considerable popularity with 59% of total produce being used in the livestock feed industry, poultry included (deVisser *et al.*, 2014). Soya bean has a wide range of use when compared to other legume plants, primarily as a protein source for broilers and layers (deVisser *et al.*, 2014). Apart from being a good source of protein, most soya bean varieties have been reported to contain high levels of polyunsaturated fatty acids (PUFAs), antioxidants, fibre, vitamins, minerals, and low PSFA that can be of benefit to layer and broiler growth, development and health maintenance (García *et al.*, 1997; He and

Chen, 2013; Natarajan, 2014). However, antinutritional factors such as trypsin inhibitors, lectin and saponins are present and these can have harmful effects on chicken growth performance (Senkoylu *et al.*, 2005). Despite this, soya bean use is increasing worldwide (FAO, 2004). Currently the EU is working to increase its soya bean production as a measure to increase the protein pool and meet protein demand for the animal feed industry. This strategy will go a long way in alleviating concerns by health conscious consumers (deVisser *et al.*, 2014).

Soya bean has been reported in several studies as a good alternate protein source in poultry feed production and its benefits have been, and continue to be exploited. Layer birds fed graded levels of full fat soya bean improved their feed conversion ratio (FCR) improved despite birds being reported to have consumed less feed as the inclusion rate of FF SB was increased to 22% (Senkoylu *et al.*, 2005). Egg quality traits such as egg weight, shell thickness, yolk colour, and Haugh units were reported to have either improved or maintained in studies where soya bean was the main CP source. The differences that arose were as a result of different additives, enzymes and micro-nutrient substitution in layer diets and alternate energy sources such as wheat (Ketelaars *et al.*, 1982; Um and Paik, 1999; Silversides *et al.*, 2006; Aziza *et al.*, 2013).

Similarly in earlier studies where fermented soya bean was used in broiler diets, there was a significant improvement in weight gain and carcass traits of broiler chickens (Chah *et al.*, 1975). In studies where enzymes were incorporated in soya bean based diets, as the only crude protein (CP) source, it was revealed that CP digestibility was improved, therefore the authors recommended that CP levels can be reduced and the same results of a good growth performance can be obtained (Zanella *et al.*, 1999). According to Zhaleh *et al.* (2014), using full fat soya bean (FFSB) in diets up to an inclusion level of 15% of the total protein resulted

in decreased duodenal surface area but this did not have a negative effect on broiler performance.

2.2.3 Wheat as an alternate energy source in chicken production

The increased competition between livestock and humans for maize, the universally acknowledged energy source has led the research in the use of alternate grain energy sources (Davis *et al.*, 2003; Yunusa *et al.*, 2014). Alternate energy sources currently being used in livestock diets include, barley, rye, oats, rice and wheat (Davis *et al.*, 2003). Wheat use in livestock diets has gained popularity because of its high yield, low production costs and high amount of available energy similar to that of maize (Yunusa *et al.*, 2014; Pirgozliev *et al.*, 2015). The crop has an annual global production that ranges from 600 to 700 million tonnes per year with 20% being used as animal feed (Shewry and Lovegrove, 2014; Pirgozliev *et al.*, 2015). The chemical and physical composition is highly variable as a result of differences in growing location, fertilizer use, moisture conditions, and other agronomic factors (Amerah, 2015). Table 2.3 summarizes the chemical composition of wheat as reviewed in literature.

Table 2. 3: A summary of the chemical composition of wheat in g/kg

Nutrient	Average	Range
Protein	120	80-201
Starch	585	402-712
Amylose: Amylopectin	0.46	-
Fat	20	9-34
Ash	16	15-18
Water-insoluble cell walls	102	94-118
Non-starch polysaccharide		
Total	115	66-146
Soluble	28	8-41
Insoluble	87	-
Pentosans	53	45-61
Arabinoxylans		
Soluble	18	-
Insoluble	63	-
Total P	3.6	2.3-8.3
Phytate P	2.8	0.9-3.2
Endogenous enzymes		
Xylanase XU	0.48	0.27-0.68
Phytase (FTU/kg)	508	206-775
α -Amylase activity (AU)	0.12	-
Lipase	7.93	2.0-27.3
Xylanase inhibitor		
TAXI	94	17-137
XIP	299	234-355
Xylanase inhibition activity (InhU)	183	-

Source: Amerah (2015)

2.2.4 Wheat use in the poultry industry

The relatively high energy and protein content makes wheat a good feed component with the latter being preferred in the livestock feed industry (Hell *et al.*, 2015). Wheat is commonly used in the manufacture of ruminant feed because ruminants contain enzymes and bacteria that can easily break down the non-starch polysaccharides (NSPs) through digestion, this is unlike in monogastric animals. However, in recent years wheat use in monogastric feed for pigs and chickens has increased despite digestion difficulties being reported in literature (Amerah, 2015).

Digestion problems in monogastric animals arise as a result of arabinoxylan, cellulose and other NSPs which are present in wheat. Non-starch polysaccharides form alkali-labile ester-like cross linkages with the cell wall of wheat grains (Amerah, 2015). This makes digestion difficult, as monogastrics do not contain digestive enzymes and bacteria that are able to break down the cross linkages making GIT contents highly viscous and difficult to digest (Hell *et al.*, 2015). The anti-nutritive properties of NSP are such that viscosity and nutrient unavailability make wheat use in monogastric diets a challenge. In this regard poultry feed manufacturers have employed the use of enzymes to help curb this problem (Pirgozliev *et al.*, 2015).

Although NSP has detrimental effects in general digestion, it is still beneficial to stimulate gizzard activity. In a study where layer birds were fed diets containing coarse wheat and housed in cages with paper or wood shaving, results indicated that layer birds with access to coarse wood shavings had a higher gizzard and content weight in floor pens with wood shavings unlike layer birds fed the same wheat diet but kept in cages (Hetland *et al.*, 2005). This ultimately improved feed utilization, egg production and egg quality traits of the layer birds.

In a recent study where the effect of wheat on growth performance of broilers was compared against maize containing diets, results indicated that wheat based diets had better and superior growth performance compared to maize diets (Kiarie *et al.*, 2014). This had an impact on chicken cuts and portion size leading to improved meat quality traits. Various studies where soya bean was used as the source of protein, broiler carcass traits and meat quality traits improved significantly (Ravindran *et al.*, 2014). Differences that arose were as a result of the different additives included in the diets. However according to Pirgozliev *et al.* (2015), the growth performance of broiler chickens fed different wheat levels was reduced as wheat content increased. This can be attributed to the presence of high levels of NSP as wheat content increased, which lead to high viscosity, leading to a low available apparent metabolisable energy (AME) as starch, lipid and protein digestion was inhibited in the small intestine (Steenfeldt, 2001). Higher dietary content in broiler diets has been noted to promote bacterial populations in the GIT, synthesising and deconjugating bile salts, making them less reabsorbable (Casparly, 1992). This leads to nutrient malabsorption leading to low broiler productivity.

With this in view, the importance of plant sources such as wheat and soya bean as feed for chickens cannot be disputed. These two plant feed sources are favoured by most animal feed manufacturers for their high nutrient content even though they have been proven to contain allergens (Cawthorn *et al.*, 2009; Inomata, 2009; Galan *et al.*, 2011). In South Africa and other global regions, wheat and soya bean are considered common sources of allergen and thus carry the potential risk of allergen carryover into animal products meant for human consumption. Humans that are allergic to these two plant protein sources might suffer from food allergies.

2.3 Food allergens

2.3.1 Prevalence and global distribution

According to the World Allergy Organization (WAO) (2011), approximately 1 - 2% of adults and 5 - 8% of children suffer from food allergies worldwide. In America alone, 1.3% of children and 0.2% of the total adult population suffer from food allergies through milk ingestion (Sampson, 2004). Globally, children are the ones that are mostly affected. Food allergies or food hypersensitivities have now been recognised as worldwide problems because of recorded increase, taking precedence in westernised nations but varying within Western Europe (Sampson, 2004). The major risk factors for food allergy are a family history of allergy and sensitization to aero allergens.

A food allergy or hypersensitivity is an immune response by the body to the presence of a specific protein antigen or allergen in food (Sicherer, 2002). The allergen itself is usually harmless but it is the immune response that causes the damage to the body (Waugh and Grant, 2006). All food allergic reactions are a result of the body's immune response to certain proteins in food once ingested. Symptoms of food allergy vary depending on the organs affected. A reaction can be mild, like a slight skin urticaria, to annoying such as when an individual develops a running nose (Waugh and Grant, 2006). Rhinitis, asthma, nausea, vomiting, diarrhoea, conjunctivitis, and angioedema are some of the signs and symptoms that can be experienced by individuals' affected by food allergens (vanRee *et al.*, 2015). Occasionally a food allergic reaction can be life-threatening, overwhelming body systems and can cause death as in the case of anaphylactic shock (Sicherer, 2002; Cawthorn *et al.*, 2009). The only known preventive treatment is avoiding the offending food source (vanRee *et al.*, 2015).

2.3.2 Food Allergy (hypersensitivity) pathophysiology

Food hypersensitivity is caused by a malfunction of the body's normal immune system response to the presence of ingested proteins. A healthy gastrointestinal system has mechanisms that properly identifies and ignores harmless dietary protein antigens (Sicherer, 2011). Some physiological barriers at the intestinal wall, include, epithelial cells, glycocalyx, and enzymes, of which a small portion of ingested antigen penetrates the gut barrier and then stimulates the systemic immune response leading to an allergic (hypersensitivity) reaction.

There are generally four mechanisms of hypersensitivity in which the body reacts in the presence of allergens (Waugh and Grant, 2006). Type I is usually termed anaphylactic hypersensitivity and it occurs in individuals who would have inherited very high levels of immunoglobulin E (IgE-). Once an antigen containing food is ingested, soluble antigens are differentiated from particulate antigens accomplished by differential processing of luminal contents (Sicherer, 2002). The follicle-associated epithelium (M cells) overlying Peyer's patch is believed to be responsible for sampling particular antigens where macrophages and T cells induce the requisite IgA- responses (Sicherer, 2002; Sicherer and Sampson, 2010). In the presence of a food allergen, these IgE- activate mast and basophils which then release histamine. Histamine acts by causing vasoconstriction of smooth muscles of the respiratory way as well as induce vasodilation. Anaphylactic hypersensitivity is usually fatal and can lead to death (Sicherer, 2002; Waugh and Grant, 2006). Figure 2.1 summarizes an anaphylactic hypersensitivity reaction.

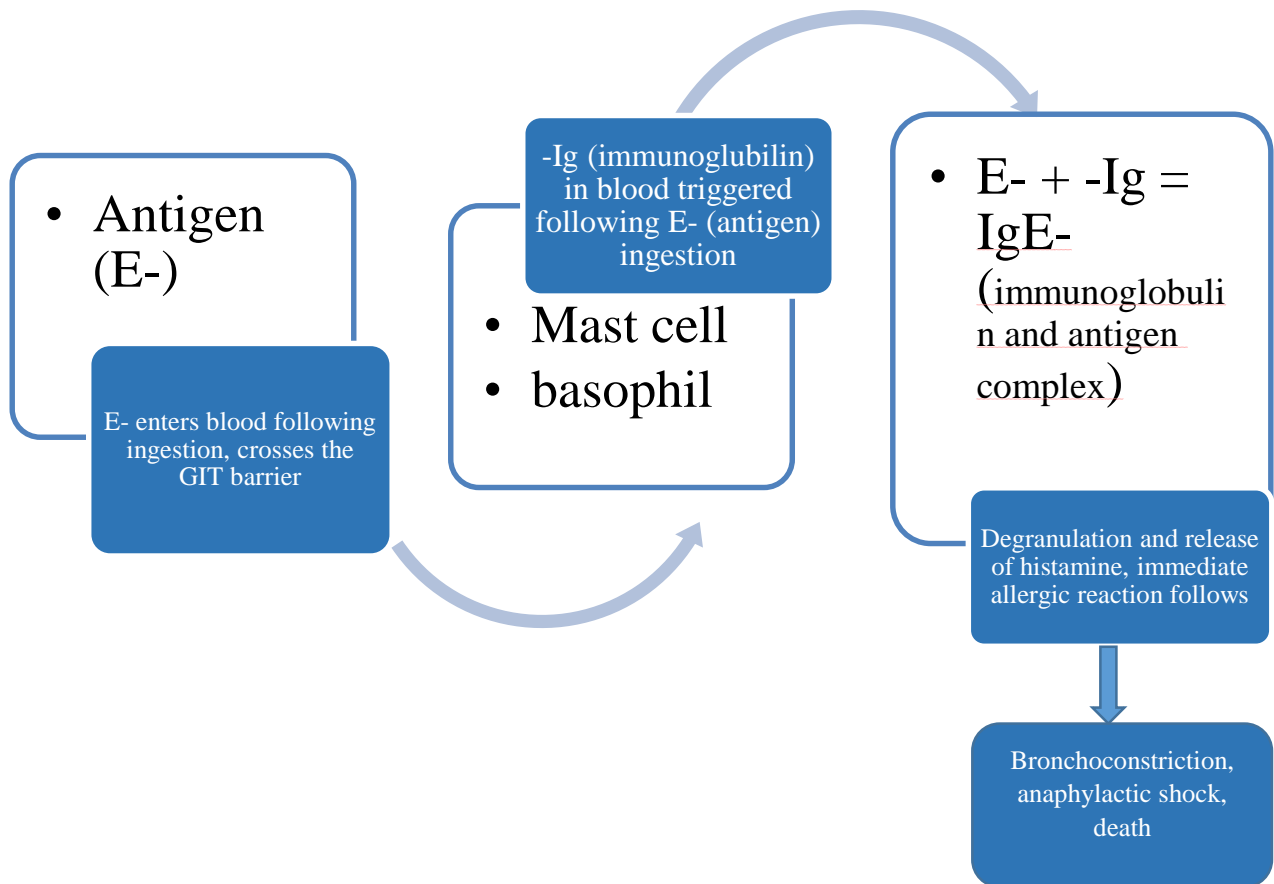


Figure 2. 1: Anaphylactic hypersensitivity (Adapted from: Waugh and Grant, 2006)

The other type of an allergic response that can occur following ingestion of a food allergen is cytotoxic hypersensitivity. This occurs when an ingested protein allergen attaches itself to a cell in the body and can lead to body releasing antigens against itself (Waugh and Grant, 2006; Breiteneder and Clare Mills, 2013). Similarly, an immune-complex-mediated hypersensitivity occurs when following an antigen-antibody complex, if not effectively removed from the body, they are deposited in tissues resulting in mild rashes, joint pains and sometimes haematuria when the kidney glomeruli became blocked by immune complexes (Waugh and Grant, 2006). It should be noted that upon an individuals' initial exposure to an allergen, the individual becomes sensitized and on subsequent exposures, the immune system mounts a response entirely out of proportion to the perceived threat therefore in some cases, following the ingestion of an allergen an individual can collapse and die immediately (Sicherer, 2002; Waugh and Grant, 2006). This can occur in any individual that is hypersensitive to allergens, such as soy and gluten that are found in soya bean and wheat, two of the most commonly used animal feed sources.

2.4 Wheat and soya bean, known allergens in poultry feed

While any food can theoretically induce an adverse allergic response in a susceptible individual, there are eight food groups or 'common allergens' that are responsible for instigating most known food-allergic reactions. The common allergens include the following: cow's milk, eggs, peanuts, tree nuts, fresh fruit, fish, soya bean, crustaceans and molluscs (Herman, *et al.*, 2003; Sicherer and Sampson, 2010; Herman and Ladics, 2011; Alsaeed *et al.*, 2013; Fernández-Rivas and Asero, 2014). In South Africa and Canada, wheat is also amongst the list of 'common allergens' (Cawthorn *et al.*, 2009). These allergens are incorporated daily in animal feed and are consumed daily through various animal products thus posing a potential risk to consumers. As previously discussed, soya bean and wheat are

commonly used as raw materials in the poultry feed industry as sources of protein and energy, respectively, and hence dietary avoidance is difficult (Amerah, 2015).

Limited data is available on the epidemiology of specific food allergens as a result of the wide variations in the methodologies used in food allergy studies across the world (vanRee *et al.*, 2015). Some challenges in determining the prevalence of specific food allergy include and not limited to; misclassification, biased participation, the lack of simple diagnostic tests, rapid evolution of disease, large numbers of potential triggers and the varied sign and symptoms per individual (Sicherer, 2011). This makes evaluation of available literature difficult.

According to Kattan *et al.* (2011), soy allergy is not as common as cow's milk with soy allergy affecting 0.7% of children in the EU and 1.4% of 1 year old infants being affected in the USA. In overall, soy allergy prevalence affects approximately 0 - 0.7% of the total global population (Luccioli *et al.*, 2008). Other researchers have indicated that, 10% of the total population of children affected with cow's milk allergy also suffer from soy allergy (vanRee *et al.*, 2015). According to Zuidmeer *et al.* (2008), approximately 0 – 0.5% of the global population is affected by wheat (gluten) allergy. In the US, 0.5% of the infant population and approximately two million of the adult population suffer from wheat allergy known to cause celiac disease (Westerberg, 2006). A 0.4% incidence in three year old children was recorded in the United Kingdom (UK) (Luccioli *et al.*, 2008). Currently no literature is available on the prevalence of the specific allergen proteins in both wheat and soya bean.

2.4.1 Specific protein allergens in soya bean and wheat

Proteins in soya bean are subdivided into, storage proteins, anti-nutritional and allergic proteins (Hossain and Komatsu, 2014). The allergic proteins are of particular interest and with the aid of genome sequencing, this has helped in proper allergic protein identification

(Hossain and Komatsu, 2014). Major storage proteins in soya bean include; β -conglycinin and Glycinin which constitute approximately 70% of seed protein and are known sources of soy allergen (Medic *et al.*, 2014; Natarajan, 2014).

Major allergen proteins include Gly m Bd 60K, Gly m Bd 30k, Gly m Bd 28k, Glycin, and Kunitz trypsin (KTI) (Natarajan, 2014). Glycinin is mainly composed of 5 subunits, mainly G1, G2, G3, G4, and G5 (Hossain and Komatsu, 2014; Natarajan, 2014). Gly m Bd 60K the major soya bean allergen protein, is composed of β -conglycinin and glycinin which all have α and β amino acid subunits and α -conglycinin, G1 and G2 containing both α and β subunits which have been reported to induce allergic reactions in humans (Natarajan, 2014). Kunitz trypsin inhibitor apart from being an allergen, has been characterised as an antinutritional protein that inhibits trypsin, a digestive enzyme (Breiteneder and Clare Mills, 2013; Medic *et al.*, 2014; Natarajan, 2014). Soy allergen proteins have been reported to contain approximately 20 to 240 amino acid (AA) per polypeptide chain (Natarajan, 2014). This range enables identification using conventional ELISA methods.

Further, wheat seeds are mainly composed of four protein classes that can all potentially induce an allergic reactions in hypersensitive individuals (Inomata, 2009). These classes are water/salt-soluble albumin and globulins, ethanol-soluble gliadins, urea detergent and potassium hydroxide-soluble glutenins (Sander *et al.*, 2001; Inomata, 2009). However according to Larré *et al.* (2011), only two main groups of wheat allergy inducing reactions can be separated, that is, salt-soluble fraction composed mainly of albumin/globulins and the gluten fraction composed of gliadins and glutenins. According to Inomata (2009), another class exists, that is the water/salt-insoluble gliadins particularly *w*-5 gliadin that are known to trigger Celiac disease in susceptible individuals.

2.4.2 Celiac disease

Gluten containing wheat proteins induce Celiac disease (CD) (Lamacchia *et al.*, 2014). Celiac disease has been described as a syndrome characterised by damage of the small intestine mucosa by the gliadin fraction of wheat gluten and similar prolamines of rye and barley (secalin and hordein) in genetically susceptible individuals (Catassi and Fasano, 2004; Food and Drug Administration (FDA), 2011). Genetically susceptible individuals have genes that code for the human leukocyte antigen (HLA) DQ2 and DQ8 which are strongly associated with celiac disease (Alaedini, 2015).

Once gluten (gliadin) peptides containing products are consumed, they are modified by TG2, an enzyme found in the small intestine. This modification results in the high affinity of gliadin to bind to HLA-DQ2 or HLA-DQ8 molecules found in genetically predisposed individuals to CD (FDA, 2011; vanBergen *et al.*, 2015). The gliadin peptide-loaded HLA-DQ2 or HLA-DQ8 molecules become recognised by CD4⁺ T cells, and B cells in the lamina propria of the small intestines and these activate intraepithelial lymphocytes that promote destruction of mucosal epithelial cells as summarized in Figure 2.2 (Westerberg *et al.*, 2006; vanBergen *et al.*, 2015). The chronic allergic inflammatory process ultimately results in lesions in the small intestine and nutrient malabsorption occurs (Lamacchia *et al.*, 2014). Nutrient malabsorption and small intestine lesions lead to reported symptoms by affected individuals, these include diarrhoea, nausea, vomiting and in severe cases ulceration of the small intestine leading to tarry stools when intra-abdominal bleeding becomes severe as a result of continued gluten exposure (Lamacchia *et al.*, 2014; Alaedini, 2015).

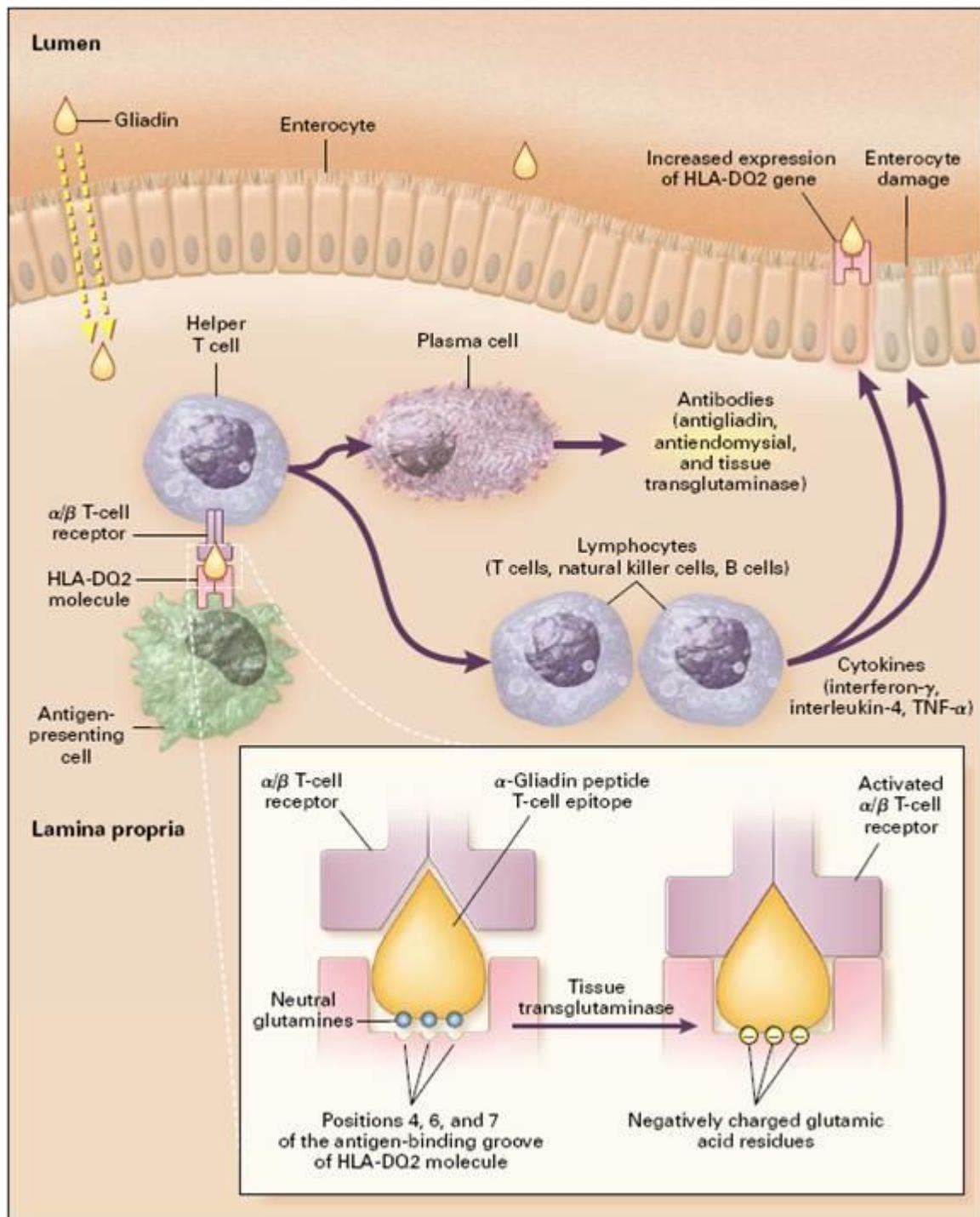


Figure 2. 2: Pathogenesis of celiac disease in the small intestine (Source: Westerberg *et al.*, 2006)

Extra intestinal complications have been reported as a result of continued exposure to gluten containing peptides. Dermatitis Herpetiformis (DH) is one such case. This condition is associated with prolonged exposure to wheat gluten and protein allergens in rye and barley (Fasano and Catassi, 2001). It is an autoimmune skin disease characterised by intensely pruritic skin rash characterised by papules and vesicles that disappear once the gliadin source is removed (Fasano and Catassi, 2001; Green and Jabri, 2003). Skeletal health problems comprising of reduced mineral density and increased risk of fractures as a consequence of malabsorption of essential nutrients such as calcium (Alaedini, 2015). Diagnosis of celiac disease is based on serologic testing and intestinal biopsy once individuals have reported some of the signs and symptoms (Catassi and Fasano, 2004; Alaedini, 2015). A gluten free diet is the recommended treatment of Celiac disease, the reversal of symptoms occurring as quickly as they commence upon removal of the allergen source as low as a few milligrams (Lamacchia *et al.*, 2014).

2.4.3 Threshold values of allergens

Soy and gluten allergic reactions differ from individual to individual depending on the threshold of the allergen in the food (Moneret-Vautrin and Kanny, 2004; FDA, 2011). Threshold values are continuously being revised due to a change in available information and scientific research. Currently using population data new approaches to detect the minimum amount of allergen required to cause a hypersensitivity reaction in susceptible individuals has been discussed (Taylor *et al.*, 2014). Currently the no-observed adverse effect levels (NOAELs) and lowest observed adverse effect level (LOAELs) of a specific food allergen that would elicit mild, and objective symptoms in highly sensitive individuals has been and is being used to determine at what level a food allergen will elicit a hypersensitive reaction (Mills *et al.*, 2004; FDA, 2011). For eggs, cow's milk and peanuts, the LOAELs is in the low milligram range (Mills *et al.*, 2004). According to Moneret-Vautrin and Kanny (2004), the

LOAELs for natural foods is between 1-2 mg of natural foods, representing a few hundred micrograms of protein with a few tens of micrograms for NOAELs.

In 2011, as part of the Voluntary Incidental Trace Allergen Labelling (VITAL) programme of The Allergen Bureau of Australia and New Zealand (ABA), an expert panel was established in order to come up with appropriate reference doses for allergic food residues (Taylor *et al.*, 2014). The recommendations were done in order to protect allergic consumers and help in implementation of safe and avoidance diets (Taylor *et al.*, 2014). According to the Allergen Bureau (2011), a level of 1mg wheat protein and soya bean protein is the reference dose that will not cause any allergic reactions in consumers allergic to soya bean although for soya bean milk this level might be lower. The FDA of the United States also came up with similar values. For soya bean containing soy, the NOAELs ranges from 1 – 2 mg/day and LOAELs from 88 to 552mg/day and for gluten NOAELs values of 2.4 – 200 mg/day and LOAELs ranging from 2.4 to 10 000 mg/day (FDA, 2006; FDA, 2011). The values differ as a result of the difference within individuals leading to a wide range in NOAELs and LOAELs (FDA, 2011). The FDA of the United States, Allergy Bureau, the Foodstuff, Cosmetics and Disinfectants Act of South Africa and other various global legislation instruments have made progress in trying to come up with acts to help protect allergic consumers and ensuring adherence by food manufacturers to recommended threshold values.

2.4.4 Legislation and consumer awareness

Management strategies for allergic consumers have focused on providing information about allergen presence through label declaration. According to Gendel (2012), risk management options for allergic reactions are limited because the hazard is food. Therefore allergic consumers rely on food labels which must declare all ingredients therein. Legislation plays a major role in consumer awareness and protection. Food safety issues and labelling matters are clearly discussed and what producers can and cannot do is clearly outlined in various laws in

most nations. The FDA of the United States of America and The Allergen Bureau of Australia and New Zealand (ABA), have been reliable by actually having NOAELs and LOAELs values to which food manufacturers should adhere to (Allergen Bureau, 2011; FDA, 2011).). Their reports have been reviewed by external and independent expert panels and recommendations have been useful to both consumers and food manufacturers. Food manufacturers are mandated by the FDA to declare any allergen in their foods or face heavy penalties and potential bans if not adhered to (FDA, 2011).

According to the Foodstuff, Cosmetics and Disinfectants Act (Act 54 of 1972) of 1972 of South Africa, all ingredients including allergens in a product must be properly labelled on a food product (Department of Health, 2010). The act also states claims such as gluten-free and naturally gluten free can only be used when the tested level of gluten does not exceed 20mg/kg of the tested feed. Other levels for other cereals, legume and other potential sources of allergens are clearly stated. This is to ensure the food industry has all the required information and all food products manufactured adhere to the set standards.

Recently, 19 laws, directives, regulations, rules and ministerial statements on food labelling concerning allergens in the European Union, America, Asia and South America were reviewed. In the review it was noted that all legislation concerning allergens are quite different as most of them do not state how they arrived at their priorities and what food sources they claimed to be allergens as seen with the case of Japan, where a literature review was used to list allergens of concern within their population (Gendel, 2012). In Canada, the list of food allergens is also, not clearly stated how it was compiled. However, recently a guide was developed by health authorities for the addition of food allergens and it is stated categorically in Health Canada (Health Canada, 2013). This clearly shows that there is still room for further research and opportunities to ensure all allergens are properly identified to ensure food safety.

The various laws discussed also take into consideration the feed manufacturers that produce animal feed as they are required by legislation to declare ingredients. This arose as a result of the uncertainty regarding the prevalence of allergies from plant food which most if not all feed manufacturers use daily (Zuidmeer *et al.*, 2008). The well documented cases of BSE in humans, that cause ‘mad cow disease’ and the recent scandal that rocked the pet industry where cats and dogs developed kidney failure and died as a result of ingesting feed containing wheat that was tainted with melamine (Colchester and Colchester, 2005; Burns, 2007). This indicates the possibility of allergen carryover from animal feed to animal products.

2.5 Potential for allergen carryover from feed to animal products

The possibility of allergen carryover from animal feed to animal products is of great concern in order to maintain food safety. This is because of the catastrophic health repercussions and tremendous commitment that is required for individuals that are allergic to certain foods to change their diets and lifestyle (Silvester *et al.*, 2015). This has an enormous impact on the quality of life of affected individuals. Some individuals can die suddenly as a result of ingesting allergen protein and developing anaphylactic shock (Sicherer, 2002). With this view and in order to avoid misleading allergic consumers and protect them, immunochemical tests were developed in order to test for intact or fragments of protein allergens in food.

Reports of infants becoming sensitized to cow’s milk and eggs are as a result of ingesting allergens through breast milk (Warner, 1980; Restani *et al.*, 2000). The presence of melamine in pig and fish meat after it was intentionally added to animal diets (Buur *et al.*, 2008; Faeste *et al.*, 2015). This demonstrates clearly that intact or fragment of allergen proteins can be carried over from feed to breast milk and animal flesh that is meant for human consumption.

2.5.1 Allergen protein digestion

Allergens are proteinous in nature as previously discussed thus their digestion is similar to that of general proteins. Proteins are composed of amino acids, the final product of protein digestion are absorbed through the intestinal wall in most livestock species. Absorption and assimilation occurs in the small intestine with help from several enzymes and precursors found in this section of the gastrointestinal (GI) tract (Waugh and Grant, 2006). Villi and microvilli in the small intestine increase the surface area to enable greater assimilation through the small intestine walls (Picariello *et al.*, 2013). Enzymes and precursors in the GI tract play a vital role in protein digestion.

The inactive enzyme precursor's trypsinogen and chymotrypsinogen are activated by enterokinase into active proteolytic enzymes trypsin and chymotrypsin in the small intestine (Waugh and Grant, 2006). These enzymes convert polypeptides to tripeptides, then dipeptides and eventually amino acids (Waugh and Grant, 2006). Amino acids are then absorbed through the intestinal walls via the brush borders of the villi, end up in the circulatory system and are transported to areas where required. In the eventuality that some proteins may escape digestion in the gastrointestinal tract, these are absorbed in the intestinal lymphatic system (Picariello *et al.*, 2013) and do not end up in the blood stream. Therefore it is unlikely that intact allergen proteins, peptides or amino acids may find their way into the blood and eventually become deposited in animal products such as meat and eggs as the various stages that an allergen protein passes through in the small intestine ensure the protein is efficiently digested. The gastrointestinal mucosal barrier is a complex barrier that does not allow intact proteins to be absorbed in the small intestines (Sampson, 2004) unless the integrity of the epithelial layer has been compromised. Allergen proteins can be carried over in the event of malabsorption, reduced integrity of the epithelial lining in the small intestine as a result of the presence of disease. This will lead to peptide carry over into animal flesh and eventually

products such as meat which could potentially lead to allergic reactions in susceptible individuals (Flachowsky *et al.*, 2005).

Egg and cow's milk are two known sources of allergens (FDA, 2011). There have been reports of infants becoming sensitized to cow's milk and eggs as a result of ingesting allergens through breast milk (Warner, 1980). Other non-protein allergen inducing chemicals such as melamine have been absorbed by the GIT of pigs and fish and melamine molecules have been deposited in meat from the two species in experiments where melamine was intentionally added to their diets (Buur *et al.*, 2008; Faeste *et al.*, 2015). The accidental contamination of pet food with melamine that led to the death of cats and dogs is also evidence that non-protein nitrogen chemicals can be carried over into animal products (Burns, 2007). This clearly demonstrates that intact or fragment of allergen proteins have the potential to be carried over from feed to breast milk and animal flesh, although proteins from transgenic crops such as wheat and soya bean have not been reported to be carried over onto animal products, as they are termed safe (Dumont *et al.*, 2010). This raises the question; if humans and pigs, that have a monogastric GIT system can experience this phenomenon what about monogastric bird species?

2.6 Summary

Wheat and soya bean are good sources of energy and protein respectively and relatively cheap thus their projected and continued use in the animal feed industry. Their continued and favoured use in broiler, layer and egg production have been extensively documented in various literature sources as a result of their significant effect on growth performance and egg quality. Currently no studies have been carried out to investigate the possibility of allergen carryover from poultry feed to animal derived food products such as eggs and chicken meat. In view of reported protein peptide carryover from feed to the meat of pigs and fish, it is

important to investigate if a similar phenomenon might occur in broiler and layer birds fed known allergen plant sources incooperated in daily poultry feed.

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Chapter 3: Growth Performance, some egg quality traits of 36-week-old Lorham White hens fed diets containing wheat, maize and soya bean and the potential for soy and gluten allergen carryover from feed to eggs

Abstract

The current study examined the effect of maize/soya bean and a maize/soya bean-based diet with a 15% whole wheat inclusion on growth performance, some egg quality traits and the potential for soy and gluten allergen carryover into eggs. Twenty 36-week-old Lorham White (LW) hens were divided into two groups and kept in individual cages till they reached 39 weeks of age with water and feed supplied *ad libitum*. Ten hens were fed the maize/soya bean diet (T₁) and the other ten birds were fed a maize/soya bean with a 15 % whole wheat inclusion (T₂) diet for a period of four weeks with weekly individual hen weights and group consumption figures being recorded. Six eggs per treatment group were collected every second day and after the two week diet adaptation period and some egg quality traits were measured the following morning. Every third day six eggs per treatment for 29 days were also collected and analysed for the presence of whole and peptide fragments of soy and gluten (gliadin) allergen proteins using specific enzyme linked immunosorbent assays (ELISA) kits. Growth traits measured indicated that live weight (LW) and average daily feed intake (ADFI) and weekly feed intakes (FI) were significantly different ($P < 0.05$) between treatments with birds on T₂ recording higher gains. Birds recorded significant differences ($P < 0.05$) in live weight values as weeks increased. Egg quality traits measured (shell weight, yolk weight, albumen weight, albumen height and HU units) were significantly different ($P < 0.05$) between treatments, eggs from hens receiving T₁ recording higher values. As hens got older the colour of egg yolk improved producing eggs with higher b^* (yellowness) values and lower L^* (lightness) and a^* (redness) values. The Haugh unit which measures egg quality in relation to consumer acceptance significantly improved ($P < 0.05$) as hens got older

signifying better quality eggs. Results indicated that feed containing gluten and soy allergens were not carried over into eggs. In conclusion, a maize/soya bean-based diets and maize/soya bean-based diet with a 15% whole wheat inclusion that contain soy and gluten allergens are potential sources of nutrients that can promote and stimulate 36-week-old LW hen growth and produce eggs of quality without the possibility of soy and gluten allergen being carried over into eggs from the feed.

Keywords: Allergens, egg colour, haugh units, Lorham hens, soya bean, and wheat.

3.1 Introduction

Eggs contribute significantly to human's daily nutritional requirement due to their protein, energy and nutritional composition. Eggs also play a vital role in health as seen by the presence of docosahexaenoic acid, an important fatty acid reported to help in the reduction of the incidence of coronary heart disease (CHD) in humans (Beynen, 2004). Although eggs are important to humans, production is hindered by feed costs which account for approximately 70% to 75% of total production costs, with fish meal being the most expensive component (Zarei *et al.*, 2011; Manju *et al.*, 2015).

Reports have proven that remnant fish odour from fish meal can be carried over onto milk and eggs produced from livestock, hence the shift away from using fish meal in layer diets (Wu *et al.*, 2014). Consumers do not appreciate a fishy aroma in their eggs. Possible chemical contamination of fish meal with potentially toxic material can occur, as preservatives used to conserve fish meal may be toxic to animals (Karimi, 2006). With this in view livestock feed producers have looked at alternative plant sources such as wheat and soya bean that are highly nutritious and do not negatively affect, the quality of animal products such as eggs (Zarei *et al.*, 2011).

Soya bean is a popular legume protein source used in the livestock sector. It has been reported to contain a crude protein content similar to that of fish meal (Geleta and Leta, 2015). Various studies have documented the positive effects of soya bean as a protein source in growth performance of layers and egg quality measurements (Geleta and Leta, 2015). Soya bean has been reported to contain up to 60 % linoleic acid of total amino acid which has been reported to improve egg quality traits such as egg weight even though being a known source of soy allergen (Mirzaie *et al.*, 2012). However linoleic acid has also been reported to inhibit the conversion of α -linoleic acid into eicosapentaenoic acid and docosahexaenoic acid which are both fatty acids (FA) of great importance to human health, however more research needs

to be done to ascertain these claims (Beynen, 2004). Therefore for this reason some producers still prefer to use fish meal despite having a negative odour and taste effect on animal products. Continued research of soya bean benefits in egg and layer production is still ongoing despite the fact that it's widely used in the poultry industry (deVisser *et al.*, 2014).

A balanced diet that contains optimum energy and protein must be provided to layers during the laying period to ensure optimum egg production (Geleta and Leta, 2015). Apart from being a good source of energy, wheat has various health beneficial bioactive compounds such as minerals, polyphenols, amino acids, and vitamin B and E (Rosa-Sibakov *et al.*, 2015). Continued use of wheat in poultry and human diets has been extensively documented in literature because of its nutritional composition (Manju *et al.*, 2015; Rosa-Sibakov *et al.*, 2015). However, the high content of non-starch polysaccharide (NSP) mainly composed of arabinoxylans has been reported to increase viscosity of digesta as seen by sticky droppings (Zarei *et al.*, 2011). Non-starch polysaccharides reduce feed intake and bird performance as nutrients are trapped in the complex beneficial food matrix microstructure. This makes bioactive nutrient release extremely difficult without the aid of enzymes (Rosa-Sibakov *et al.*, 2015). Little to no information is available on the effect of a 15% wheat inclusion in a maize/soya bean-based diet on growth performance and egg quality of 36 to 39-week-old Lorham White (LW) hens without the addition of enzymes and antibiotics.

Although soya bean and wheat are popularly used in the poultry industry, it is interesting to note that, they are both sources of food allergens, soy and gluten, respectively. A food protein allergenic epitope tends to be a small, water-soluble glycoprotein that is generally resistant to denaturation by heat or acid. With this perspective in view, some allergic proteins remain intact after processing, storage, cooking and digestion. As a result, intact or fragments of allergenic inducing proteins may be found in most food matrices and form the basis of most allergic reactions (Waserman and Watson, 2011). Allergic individuals, when exposed to food

allergens can develop mild dermatitis to life threatening anaphylaxis that can lead to death (Cawthorn *et al.*, 2009).

Cross-contamination of food containing allergens has been reported to occur (Health Canada, 2013). As part of long-standing agricultural practices cereal grains are grown, harvested, stored and transported using the same equipment in close proximity to other grains therefore it is extremely difficult to keep all traces of these different crops from getting mixed with each other at low levels, this usually referred to as co-mingling or agricultural cross-contamination (Health Canada, 2013).

There has been reports of infants becoming sensitized to cow's milk and eggs as a result of ingesting these allergens through breast milk (Warner, 1980). This clearly demonstrates that intact or fragment of allergen proteins can be carried over from feed to breast milk and animal flesh. This raises the question; if humans, that have a monogastric gastrointestinal system can experience this phenomenon what of monogastric bird species? Currently, no studies have been done to warrant food safety concerns in terms of food grains i.e. soya bean and wheat containing allergens being used in the poultry industry to manufacture relatively low cost feeds. Therefore the current study aims at determining the effect of a 15% wheat/soya bean-based diet in layer growth performance, its effect on egg quality and the potential for allergen carryover from feed to eggs.

3.2 Materials and Methods

3.2.1 Site description

The study was conducted at the University of Stellenbosch Mariendahl Experimental Farm. The farm is situated 15 km outside Stellenbosch in the Western Cape Province of South Africa. The farm lies 177m above sea level, longitude of 18⁰50'E and latitude of 33⁰51'S in the Renosterveld region. The soils are mostly dark alluvium to clay (Acocks, 1988). The

seasons are divided into spring (hot-dry), summer (hot-wet), winter (cool dry) and autumn (post-rainy). The climate is typical Mediterranean and receives an average of 622.7mm of rainfall and around 84% occurs between April and October. The temperature averages 19.2 °C for most of the year.

3.2.2 Dietary Treatments

Two dietary treatments were assigned to two groups of Lorham White layers, one composed of maize/soya bean and the other with a 15% whole wheat inclusion in a maize/soya bean-based diet. A 15% whole wheat inclusion was chosen as the lowest inclusion rate to assess if allergen carryover will occur. Each group was assigned 10 birds in a completely randomized design. Iso-nitrogenous, iso-caloric diets were formulated using Win-Feed Formulation Software version 3.0 so as to meet all dietary requirements of the birds (National Research Council (NRC), 1994). The feeding programme consisted of one phase for a period of 29 days. Diets were mixed on site at Mariendahl Research Farm. Formulated diets were analyzed for nutrient composition of experimental diets on dry matter basis for each phase and are clearly described in Table 3.1. Proximate analysis for moisture, crude fiber, ash and ether extract was analyzed according to methods described by the Association of Official Analytical Chemists and the DUMAS LECO FO method was used to determine crude protein content (AOAC, 2002), results are recorded in Table 3.1. The metabolisable energy (ME) in kcalkg⁻¹ of layer diets was calculated using the method of Ponzenga (1985), were;

$$ME = 35 \times CP \% + 81.8 \times EE\% + 35.5 \times NFE\%.$$

Table 3. 1: Dietary composition of layer diets (kg/t)

Diet components	T₁ (maize/soya bean)	T₂ (15% whole wheat and maize/soya bean)
Maize	588.8	447.7
Wheat	-	150.000
Soya bean full fat	226.4	240.5
Soybean 46	41.6	18.4
L-lysine	-	0.137
DL-methionine	1.3	1.4
Vitamin mineral premix	2.5	2.5
Limestone	85.8	86.0
Salt	2.8	2.8
Monocalcium phosphate	14.9	14.3
Sodium bicarbonate	0.9	0.7
Oil (sunflower)	35.5	35.6
Analysed composition % DM basis		
CP	12.5	15.4
CF	3.5	3.7
Ash	5.7	6.2
EE	4.7	5.2
Moisture	8.6	9.3
NFE%	26.4	30.5
Calculated composition		
ME (kcal/kg)	1759.2	2047.1

3.2.3 Experimental design and general management

The diets were mixed onsite. Ten birds were randomly allocated to ten battery cages and each bird served as an experimental unit within the specified treatment. Lorham White layer birds were housed in a layer house during the experimental period. Layers at peak of lay, 36 weeks old, which were already on site and were housed for a period of four weeks. Birds were housed in individual cages measuring approximately 800cm² to allow enough room for movement, with water given via nipple drinkers and 120 g/day/hen of feed being provided *ad libitum* twice daily according to ethical clearance guidelines, Stellenbosch University, Animal Ethics Unit:SU-ACUM14-00032(UFH counter EC certificate No: MUC131SDOK01). Temperatures inside the house were maintained by opening or closing door and opening a sprinkler controlled cooling system on top of the layer house.

3.2.4 Growth performance

The amount of feed at the start of the trial was recorded and on weekly basis before feeding using an electronic scale (Micro A12E, Germany). Body weight in grams was measured at start of the trial and thereafter measurements were done on weekly basis for a group of ten birds per cage. Feed consumed by each group of 10 birds per treatment was recorded so as to determine the weekly feed consumption patterns. The live weight gain (LWG), average daily gain (ADG), the feed intake (FI) and the average feed intake (AFI) were calculated. For all calculations mortalities were taken into consideration so as to be more representative. The formulas used are depicted:

Live weight gain (g) = recorded weekly weights of LW hens over a four week period

Average daily gain (g/day) = average body weight (g) / total number of days (days)

Feed intake (g) = feed given (g) /remaining feed (g)

Average daily feed intake (g) = given feed per week (g/week/ remaining feed (g/week)

3.2.5 Egg collection and egg quality

A total of six eggs were randomly selected from the ten Lorham white hens per treatment every second day as not all hens laid eggs every day. Egg collection commenced after a two week period to ensure all residual diets was out of the birds system. Collected eggs were as a result of the experimental diets, since it takes approximately two weeks for an egg to be formed. Each collected egg was clearly labelled according to day and treatment. Egg quality was measured immediately the following day in the morning. Eggs that were cracked, deformed or were small were not considered and were discarded.

Each randomly selected egg was cleaned carefully with a soft cloth to remove any dirt that might influence results. Thereafter the egg was weighed. After weighing, each egg was carefully broken and its contents were placed on a glass. The shell thickness was recorded by obtaining an average of the narrow, broad and middle thickness of the egg shell using a digital calliper manufactured by MAJOR TECH (South Africa), KTV 150 with a resolution of 0.001mm and size 180 x 86 x 25 mm. Albumen height was measured at two spots using a meniscus to obtain an average (AMES 5-6428. IMM.BC. AMES CO. Waltham Mass, U.S.A.). Albumen height obtained was used to calculate Haugh units using the following formula;

$$HU = 100 \log \frac{[H - \sqrt{G(30(W)^{0.37} - 100)} + 10.57]}{100}$$

Where H is the average height of albumen and G is a constant standing for gravitational force (981cm/s) and W is the weight of the egg (Haugh, 1937).

Since the colour of foodstuffs can be determined objectively using portable instruments with predefined traits, a spectrophotometer was used to minimize bias based on ones perception of the colour yellow (Dvořák *et al.*, 2010). Colour of the egg yolk was thus measured using a

BYK Spectro-Guide 45⁰/0⁰ (BYK-Gardner GmbH, serial number 874210, Germany) colour guide with a 20 mm diameter measurement area and an illuminant D65-day light at a 10⁰ standard observer. The LAB system, (L^* =Lightness, a^* =Redness and b^* =Yellowness) was used to determine the colour of the yolk. The colour guide measurement area was placed flat on a portion of the yolk and a reading was obtained and recorded in terms of the colour coordinates L^* , a^* , b^* . Colour coordinates obtained were used to calculate Chroma ($C^* = \sqrt{a^{*2} + b^{*2}}$) and Hue angle ($H^\circ = \tan^{-1} (b^*/a^*)$) of the yolk colour. The colour-guide was standardized before each day's reading to minimize bias and errors. The green standard was used to check if calibration was needed before each days measurement using the black and white standards ($L^*=95.13$, $a^*=-0.89$, $b^*=0.66$). Colour was determined every second day for a period of 29 days for the six randomly selected eggs per treatment.

3.2.6 Egg collection and preparation for allergen analysis

Egg collection procedure is clearly described under Section 3.2.5 in the current chapter. A total of three eggs per treatment every 3rd day were randomly selected for the first two weeks of the trial, this was the adaptation phase. On day 15, 6 eggs were randomly selected and collected, there after 5 eggs every 2 days for 18 days except for day 22 and day 33 where 6 eggs were collected per treatment. Day 15, marked the end of the adaptation period, day 22, seven days after and day 29 marked the end of the trial. This was done for both bird groups receiving T₁ and T₂. A total of 50 eggs per treatment were sampled. Egg yolk and egg white of each egg was effectively mixed and poured in polyethylene bags that were clearly labelled with the date of collection, hen number and treatment. After labelling and sealing, eggs were stored in crates and placed in a freezer at -20 °C till required for soy and gluten analysis.

3.2.7 Soy Allergen Analysis

Enzyme-linked immunosorbent assays (ELISA) were used for the detection of possible carryover allergens in formulated feeds, and collected egg samples from layers. Table 3.2 summarises the specificity of each test not included in specific tests subheadings. All assays were carried out at the Food and Allergy Consulting and Testing Services (FACTS), Stellenbosch, Western Cape, South Africa. A Veratox[®] for Soy Allergen quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kit (Neogen Corporation, Cat. No. 8410, supplied by Analytical & Diagnostic Products (ADP), Gauteng, and South Africa). The kit detects both native and processed soya protein. Results obtained were expressed as mg.kg⁻¹(ppm) soy flour, from which the concentration of soy protein isolates (SPI) was calculated by multiplying results by a factor of 4.

All surfaces were wiped clean with ethanol before the test was carried out. Feed and egg samples were prepared in conformity with the following rubric. A total of 5g per each duplicate sample of feed and 5ml of egg was used in the assays. Sample preparation and extraction, and test procedure was done according to set guidelines in the Veratox[®] for Soy Allergen quantitative test (Neogen Corporation, Cat. No. 8410, supplied by ADP). Five standards supplied by the test kit, i.e., 0 ppm, 2.5 ppm, 5.0 ppm, 10 ppm, 25 ppm were utilized during the assay so as to compare the obtained results. Immediately after assay performance, the test results were read in an Anthos microtiter plate spectrophotometer, with optical densities being determined at 650nm. Quantification of the soya levels in feed, egg and control samples was performed using Veratox for Windows Software (Neogen, Cat. No. 9305W, supplied by ADP).The optical densities read from the standards were used to construct a curve and the optical densities of the samples were plotted against the curve to calculate the concentrations of soya present in each, with results obtained being expressed as mg.kg⁻¹ (ppm) soy flour from which soy protein isolates (SPI) were obtained.

Table 3. 2: Summary of ELISA kits used

Protein allergen (targeted)	Lower limit of quantification	Quantification range	Results expressed ppm of	Time (Preparation and Test implementation)	Supplier and Kit name
Soy	2.5 mg.kg ⁻¹ (ppm) soya	2.5 - 25 mg.kg ⁻¹	Soy flour	70 minutes (for 12 samples)	Neogen, Veratox [®]
Gluten	2.5 mg.kg ⁻¹ gliadin (5.0mg.kg ⁻¹ gluten)	2.5 - 40.0 mg.kg ⁻¹ gliadin (5.0 – 80.0 mg.kg ⁻¹ gluten)	Gliadin/gluten	210 minutes (for 10 samples)	r-Bioparm, RIDASCREEN [®]
Gluten (peptide fragment from wheat)	5.0 mg.kg ⁻¹ gliadin	5.0 – 135 mg.kg ⁻¹ gliadin	Gliadin	70 minutes (for ten samples)	r-Bioparm, RIDASCREEN [®]

3.2.8 Gliadin (Gluten) Allergen Analyses

3.2.8.1 Gliadin (Gluten) Allergen Analysis

A RIDASCREEN® Gliadin quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kit (r-Biopharm AG, Darmstadt, Germany, Art. No. R7001, supplied by AEC Amersham, Cape Town, South Africa) was used to analyse for the presence of gliadin in feed, and eggs. This method is recognized as the official method for gluten analysis by the Association of Official Analytical Chemists (AOAC), and has been performance tested by the AOAC Research Institute (licence No. 120601) and it's the Codex Alimentarius Method (Type I). The kit contains R5 monoclonal antibodies to detect the prolamines from wheat (gliadin), rye and barley in raw and processed foods. No cross reaction with soy, oats, maize, rice, teff, buckwheat, quinoa and amaranth have been confirmed. The kit used R5-Mendez ELISA, is specified for gluten testing as written in the South African labelling regulations (R.146/2010).

Before the ELISA was commenced, all surfaces were wiped clean with disposable tissues using 70% ethanol to ensure no contamination on samples to be analysed. Gloves were worn at all times to eliminate any external source of contamination. All tests were done in duplicate per individual sample. Eggs samples were allowed to thaw in a fridge set at 4 ± 1 °C before being analysed. Feed and egg samples were analysed for the presence of gliadin (gluten). For the extraction purposes, 0.25g of fine crushed feed samples from T₁ and T₂, 2.5 ml of homogenized eggs were mixed individually with 2.5ml of Mendez Cocktail (patented) solution. Gliadin was extracted following the exact procedures recommended in the RIDASCREEN® Gliadin ELISA of gliadins and corresponding prolamines (Art. No. R7001, R-Biopharm AG, Damstadt, Germany, supplied by AEC Amersham, South Africa) according to AOAC standards of 2012.

Six standards supplied in the test kit (0 ppb, 5 ppb, 10 ppb, 20 ppb, 40 ppb, and 80 ppb) were also prepared concurrently and utilised during the assay performance as controls and for verification and accuracy of the method (R-Biopharm AG, Art No.70001, Darmstadt, Germany, supplied by AEC Amersham, South Africa). Immediately after finishing the extraction assay, for the controls, eggs, were read in an Anthos 2010 microtiter plate spectrophotometer (Biochrom, supplied by SepSci, Gauteng, South Africa), with optical densities being read at 450nm.

The quantification of gliadin levels in the analysed samples was performed using RIDA[®]SOFT Win software (Z9999, r-Biopharm, supplied by AEC-Amersham). The optical densities of the standards were used to construct a standard curve and the optical densities of the samples were plotted against standard curve to calculate the concentration of gliadin in each sample, following which the values were multiplied by the dilution factor to express results as mg.kg⁻¹(ppm) gliadin. As per the kit instruction, the gliadin results were multiplied by a factor of 2 in order to obtain the gluten concentrations in each sample.

3.2.8.2 Gliadin (Gluten) Fragment Allergen Analysis

A RIDASCREEN[®] Gliadin quantitative competitive enzyme-linked immunosorbent assay (ELISA) kit (r-Biopharm AG, Art. No. R7021, Darmstadt, Germany supplied by AEC Amersham, South Africa) was used for the detection of peptide fragments of gliadins and corresponding prolamins. The kit used has been approved by the American Association of Cereal Chemists (AACC) (38.55.01) and it's recognised by the Association of European Coeliac Societies (AOECS) as the Standard R5 ELISA for hydrolysed food. The kit contains specific R5 monoclonal antibodies that detect potentially toxic peptide fragments of prolamins from wheat (gliadins), rye (secalins) and barley (hordeins) that might not have been detected in the gliadin kit. The antibodies do not cross react with soy, oats, maize, rice, teff, buckwheat, quinoa and amaranth.

Before the assay was done all surfaces were cleaned and made sterile with ethanol. Clean latex gloves were worn at all times to eliminate sources of contamination. All assays were done in duplicate. Egg samples were first thawed in a fridge set at 4 ± 1 °C and feed samples from T₁ and T₂ were crushed using a pestle and mortar. For feed samples 1g of samples were weighed and 1ml of eggs were measured out and mixed with 10 ml of 60% (v/v) ethanol solution (Sigma-Aldrich, Gauteng, South Africa) for extraction purposes.

All samples were extracted, implemented and tested according to the specific steps described out in the RIDASCREEN® Gliadin competitive ELISA (Art. No. R7021, R-Biopharm AG, Germany, supplied by AEC Amersham, South Africa). Five standards supplied in the test kit, 0 ng/ml, 10ng/ml, 30ng/ml, 90ng/ml and 270ng/ml gliadin were utilized during the assay performance and a set of gliadin assay controls of known concentrations were also included to verify the recovery and accuracy of the method.

Immediately following the extraction assays, the test results were read in an Anthos 2010 microtiter plate spectrophotometer (Biochrom, supplied by SepSci, Gaunteng, South Africa) with optical densities determined at 450nm. Results were interpreted using a RIDA®SOFT Win software (Z9999, R-Biopharm, supplied by AEC-Amersham, South Africa). The optical densities of the standards were used to construct a standard curve and the corresponding optical densities of the standards were used to construct a standard curve and the optical densities of the samples were plotted against the curve so as to calculate the concentration of gliadin in each sample, following which the values were multiplied by a dilution factor to express as mg.kg^{-1} (ppm) gliadin. The obtained results were multiplied by a factor of 2 in order to obtain the gluten concentration in each sample as indicated in the kit manual.

3.2.9 Statistical Analysis

3.2.9.1 Layer growth traits

The data was presented as a Completely Randomized Design. The response variables measured for layer growth was analysed using the General Linear Model (GLM) procedure of SAS (2010). Means for layer growth traits were separated using the least significant difference method (LSD). Differences were considered to be significant at $P < 0.05$.

The model used was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$$

Y_{ijk} = is the dependent variable live weight gain (LWG), average daily gain (ADG), feed intake (FI) and average feed intake (AFI)

μ = overall mean of the observations

α_i = effect of treatment (maize/soya bean diet and maize/soya bean diet with a 15% whole wheat inclusion)

β_j = effect of duration (Weeks 36, 37, 38, 39)

$\alpha\beta_{ij}$ = interaction between treatment and duration

e_{ijk} = random error

3.2.9.2 Egg quality traits and allergen analysis

The data was presented as a Completely Randomized Design. The response variables for egg quality traits were analysed using the General Linear Model (GLM) procedure of SAS (2010). Means for egg quality traits were separated using the least significant difference method (LSD). Differences were considered to be significant at $P < 0.05$. Since soy and gluten (gliadin) allergens were not detected in the analysed egg samples thus no further data analysis was performed. Results were simply discussed.

The model used was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$$

Y_{ijk} = is the dependent variable egg quality traits measured, which are; egg weight, shell weight, albumen weight, shell thickness, yolk height, albumen height, Haugh units (HU), yolk colour in terms of L^* , a^* , b^* , C^* and Hue angle

μ = overall mean of the observations

α_i = effect of treatment (maize/soya bean diet and maize/soya bean diet with a 15% whole wheat inclusion)

β_j = effect of duration (day 15, 17, 19, 21, 23, 25, 27, 29)

$\alpha\beta_{ij}$ = interaction between treatment and duration

e_{ijk} = random error

3.3 Results and Discussion

3.3.1 Effect of treatments on layer growth performance traits

There was no interaction ($P > 0.05$) between treatments and duration on layer growth traits studied in this experiment, therefore main effects were only discussed. The effect of maize/soya bean and a 15% wheat inclusion in a maize/soya bean-based diets on some growth performance traits was determined and recorded in Table 3.3. There was a significant ($P < 0.05$) difference in growth performance traits measured in the fourth week period where layer birds receiving a maize/soya bean-based diets with a 15% wheat inclusion (T_2) recording the highest values as compared to birds receiving a maize/soya bean diet (T_1). Live weight gain and the average daily gain for birds receiving T_2 were significantly different ($P < 0.05$). Although different, it is important to note that a difference of 0.1 for live weight gain and 0.001 for average daily gain although statistically significant, in practical terms no difference was actually seen between treatments.

Birds consuming a 15% wheat-maize substituted diet grew equally as their counterparts receiving a maize/soya bean-based diet. Similarly in experiments were Lorham Brown hens were fed a maize/soya bean diet substituted with 50% wheat, no significant differences ($P > 0.05$) in growth performance traits were reported (Lázaro *et al.*, 2003; Safaa *et al.* 2009). This is despite the fact that wheat with a known high NSP have been reported to affect growth performance of layers as a result of the presence of NSP (Pirgozliev *et al.*, 2015).

Table 3.3: Effect of treatments on growth traits of Lorhams White layer birds from 36 to 39-weeks of age.

Traits	Treatments	
	T ₁ (maize/soya bean) n=10	T ₂ (maize/soya bean with 15% whole wheat inclusion) n=10
Live weight (kg) at 39- weeks of age	1.8 ^b ± 0.002	1.9 ^a ± 0.002
Average daily gain (g/day)	0.03 ^b ± 0.001	0.04 ^a ± 0.001
Feed intake (g/week)	810.0 ^b ± 0.00	823.0 ^a ± 0.00
Average daily feed intake (g/day/hen)	115.7 ^b ± 0.00	117.0 ^a ± 0.00

^{a-b} Means within the same row that do not share a common superscript are significantly different ($P < 0.05$).

As seen in Table 3.3, feed intake and the average feed intake was significantly different ($P < 0.05$) between the two treatments. Layer birds receiving T₂ had a higher feed intake and average feed intake. This resulted in the slightly higher live weight gains recorded. This is similar to results observed by Mirzaie *et al.* (2012), where layer birds fed diets containing wheat inclusions of up to 69 % performed the same as birds fed a normal diet. However in experiments by Jahanian and Goldshadi (2015), the average daily feed intake for layer hens receiving maize/soya bean-based diets was 113.7 g/day/hen and 114.4 g/day/hen for hens on a wheat/soya bean-based diet respectively. These values are slightly lower than what was recorded for LW layer birds used in this trial being fed similar maize and wheat containing diets. Lorham White layers receiving maize/soya bean based diets in the current trial had an average ADFI of 115.7g/day/hen and those on the maize/soya bean-based diet with a 15% whole wheat inclusion had an ADFI of 117 g/day/hen. Recorded values are within the recommended 119g/day/hen theoretical feed required per day per hen based on a 105% rate (ISA-Hendrix, 2011). The overall significant difference ($P < 0.05$) in growth traits in Lorham White layer birds receiving diets with a 15% wheat inclusion indicate that wheat had an overall positive effect on some growth traits of Lorham White layers. This can be explained by an increase in size of the gizzard that resulted in more feed being used up resulting in the observed results as the gizzard activity was stimulated and more feed was effectively utilised (Hetland *et al.*, 2005).

3.3.2 Changes in some growth traits of Lorham White hens over a period of four weeks.

Changes in some growth performance traits of twenty 36-week-old Lorham White was recorded over a four week period till birds were aged 39-weeks. Results are tabulated in Table. 3.4. There was a significant difference ($P < 0.05$) in some growth traits except for the average daily gain.

Table 3.4: Summarised changes in growth traits of 20 Lorham White layer birds from 36 to 39-weeks of age

Traits	Age in weeks			
	n= 20			
	36	37	38	39
Live weight (kg)	1.84 ^d ± 0.003	1.85 ^c ± 0.003	1.89 ^b ± 0.003	1.92 ^a ± 0.003
Average daily gain (g/d)	0.01 ^a ± 0.001	0.01 ^a ± 0.001	0.01 ^a ± 0.001	0.01 ^a ± 0.001
Feed intake (g/kg)	815.00 ^c ± 0.00	820.00 ^a ± 0.00	815.00 ^c ± 0.00	817.00 ^b ± 0.00
Average feed intake (g/day/hen)	116.40 ^c ± 0.00	117.10 ^a ± 0.00	116.40 ^c ± 0.00	116.80 ^b ± 0.00

^{a-d} Means within the same row that do not share a common superscript are significantly different (P < 0.05).

It is interesting to note that at the age of 38 weeks of age, birds ate approximately the same amount of feed as when they were 36-week-old, upon being commenced on the experimental diets. The slight difference when the birds were 36-weeks-old and 38 weeks old, in feed intake could be attributed to two week diet adaptation period. Feed adaptation was noted to take approximately seven days in most commercial hen strains (Jahanian and Golshadi, 2015), but this can differ within breeds. During their first week of feeding when birds were 36-week-old, Lorham White layer birds were adapting to the diet and then a slight increase was recorded and then feed consumption leveled off and birds consumed an approximate 116g of feed on daily basis. Similarly, in a study by Traineu *et al.* (2013) where sequential layer diets containing whole wheat were fed to layer birds, these birds had a significant ($P < 0.05$) improvement in growth traits as compared to birds fed maize-based diets. Most growth performance trials are done over a longer period of time but in our current trial, growth performance was measured over a 4 week period.

3.3.3 Effects of treatments on egg quality traits

The effect of maize/soya bean (T_1) and maize/soya bean (T_2) on egg quality traits of LW hens reared for a period of 36 weeks to 39 weeks is summarised in Table 3.5. There was no significant difference ($P > 0.05$) in egg weight, yolk height and average shell thickness between treatments. Eggs generally weighed approximately the same from both treatments. However in similar studies, hens receiving maize diets had heavier eggs, weighing above 63g compared to hens fed diets containing durum wheat (Safaa *et al.*, 2009). Similar results were also observed by Mathlouthi *et al.* (2015) where hens reared from 28 to 40 weeks of age fed maize/soya bean-based laid eggs weighing approximately 62g as compared to hens on wheat/soya bean-based diets that laid eggs weighing an average of 59g.

Table 3. 5: Effect of maize/soya bean and a maize/soya bean-based diet with a 15% whole wheat inclusion on egg quality traits

Traits	Treatments	
	T ₁ (maize/soya bean) n=50	T ₂ (maize/soya bean with a 15% whole wheat inclusion) n=50
Egg weight (g)	59.3 ^a ± 0.50	58.6 ^a ± 0.50
Shell weight (g)	7.6 ^a ± 0.11	7.2 ^b ± 0.11
Yolk weight (g)	15.8 ^b ± 0.21	16.5 ^a ± 0.21
Albumen weight (g)	35.8 ^a ± 0.39	34.9 ^b ± 0.34
Yolk height (mm)	17.9 ^a ± 1.86	20.9 ^a ± 1.62
Albumen height (mm)	6.9 ^b ± 0.20	8.1 ^a ± 0.17
Average shell thickness(mm)	0.4 ^a ± 0.01	0.4 ^a ± 0.01
HU units	66.3 ^b ± 3.53	79.0 ^a ± 3.09
<i>L</i> *	57.2 ^b ± 0.60	59.4 ^a ± 0.52
<i>a</i> *	9.9 ^a ± 0.26	7.2 ^b ± 0.23
<i>b</i> *	43. 4 ^a ± 0.88	42.1 ^a ± 0.78
<i>C</i> *	44.6 ^a ± 0.88	42.7 ^b ± 0.78
<i>H</i> •	76. 8 ^b ± 0.36	80.2 ^a ± 0.32

^{a-b} Means within the same row that do not share a common superscript are significantly different (P < 0.05).

Differences observed could be as a result of the short trial period. In most hen trials in literature, significant differences in egg weight, yolk height and average shell thickness are observed when birds are on a specific diet for a minimum of 6 weeks for up to 50 weeks (Van Der Klis *et al.*, 1997; Jahanian and Golshadi, 2015; Mathlouthi *et al.*, 2015).

Wheat has been reported to reduce linoleic acid content, from 1.5% to 0.7% in diets where maize was substituted with 69% wheat (Mirzaie *et al.*, 2012). Linoleic acid has been reported to have a significant effect on egg weight (Jahanian and Golshadi, 2015). Maybe in our case since only 15% wheat was replaced, there could have been a relatively lower linoleic acid content resulting in relative similar egg weight, yolk height and shell thickness, Although linoleic acid content was not determined in both diets, to ascertain these results.

The colour of an egg yolk is an important egg quality feature that egg producers make efforts to produce eggs with a rich colour using suitable feeds (Dvořák *et al.*, 2010). For colour coordinates measured, there was a significant difference in L^* , a^* , C^* and H° colour variables between treatments as seen in Table 3.5. Eggs from hens receiving T₂ had higher L^* and H° values. Lightness (L^*) values for egg yolks from hens receiving T₁ were lower compared to eggs from hens receiving T₂. Lower L^* values are indicative of higher concentration of colour pigments. This was observed in a^* values. The high a^* values observed can be as a result of higher incidence of red blood/ meat spots in eggs from hens receiving T₁. Blood spots occur on egg yolks as a result of a ruptured blood vessel during egg formation with meat spots being formed from degenerated blood spots (Burmester and Card, 1938; Nalbandov and Card, 1944). These abnormalities are thought to be as a result of lack of vitamins in a hen's diets, disease presence and inheritance (Chen *et al.*, 2015). All diets that were given to hens were balanced and animals were disease free so we can assume some individual hens that consumed T₁ had some inherent abnormalities that made them have some meat and blood spots on the yolk of eggs they laid.

Blood /meat spots are usually pink, red to brown and even black in colour and this could have affected the recorded L^* values, since a mixture of red and yellow of yolk colour will give an orange colour and this eventually reduced the L^* values recorded (Nalbandov and Card, 1944). It is interesting to note that there was no significant ($P > 0.05$) difference in b^* colour coordinates between treatments, the degree of yellowness was the same in eggs from hens fed the two different diets despite the presence of blood and meat spots in eggs from hens. Chroma which measures colour saturation was higher in eggs from hens fed T_1 . This was expected since a^* values were higher in eggs recorded from T_1 and a^* are used to calculate C^* values.

3.3.4 Changes in egg quality parameter over a 29 day period

Changes in egg quality traits over a period of fourteen days was recorded in Table 3.6. No significant differences ($P > 0.05$) in egg weights were recorded as the days increased, from day 15 to day 29. Egg weights ranged from 58 g to 59.3 g over the two week period. Similar results were obtained by Safaa *et al.* (2009), where dietary treatment containing either maize/soya bean or wheat/soya bean had no effect of egg weight in birds fed those two different diets over a period of 28 weeks. Changes in the egg shell thickness were noted with thicker egg shells being recorded after day 21, they averaged approximately 0.4 mm. However results obtained by Safaa *et al.* (2009) indicated that diets containing maize and wheat had no significant effect on egg shell, albumen weight and other egg quality related traits. In our experiment it can be concluded that thicker shells were obtained because as hens got older they produce larger eggs because of the possible high linoleic acid content in the diet as well as a result of better and more phosphorus being deposited in the egg as it was formed (Jahanian and Golshadi, 2015).

Table 3.6: Changes in egg quality traits of 100 sampled eggs over a period of 29 days

Egg quality Traits	Days							
	15	17	19	21	23	25	27	29
Egg weight (g)	58.2 ^a ± 0.87	58.8 ^a ± 0.87	59.4 ^a ± 1.09	57.8 ^a ± 0.80	58.8 ^a ± 0.97	59.7 ^a ± 0.87	59.7 ^a ± 0.89	59.3 ^a ± 1.07
Shell weight (g)	7.2 ^b ± 0.19	7.9 ^a ± 0.19	7.9 ^a ± 0.24	7.2 ^b ± 0.17	7.0 ^b ± 0.21	7.1 ^b ± 0.19	7.2 ^b ± 0.19	7.7 ^a ± 0.23
Yolk weight (g)	15.5 ^b ± 0.38	15.8 ^b ± 0.38	15.6 ^b ± 0.47	16.1 ^{ab} ± 0.35	16.2 ^{ab} ± 0.42	16.7 ^a ± 0.38	16.7 ^a ± 0.39	16.6 ^a ± 0.45
Albumen weight (g)	35.4 ^a ± 0.68	35.1 ^a ± 0.68	35.9 ^a ± 0.85	34.6 ^a ± 0.62	35.6 ^a ± 0.75	35.9 ^a ± 0.68	35.8 ^a ± 0.69	35.0 ^a ± 0.83
Yolk height (mm)	17.3 ^b ± 3.25	18.2 ^b ± 3.25	27.5 ^a ± 4.09	18.3 ^b ± 2.99	18.3 ^b ± 3.61	18.3 ^b ± 3.25	18.62 ^b ± 3.34	18.83 ^b ± 4.01
Albumen height (mm)	6.6 ^c ± 0.35	8.1 ^{ab} ± 0.35	8.0 ^{ab} ± 0.44	7.2 ^{bc} ± 0.32	6.9 ^c ± 0.39	7.3 ^b ± 0.35	7.5 ^b ± 0.36	8.9 ^a ± 0.43
Average shell thickness (mm)	0.4 ^b ± 0.01	0.4 ^b ± 0.01	0.35 ^c ± 0.02	0.45 ^a ± 0.01	0.4 ^b ± 0.01	0.4 ^b ± 0.01	0.4 ^b ± 0.01	0.4 ^b ± 0.02
Hu units	66.8 ^{bc} ± 6.17	73.3 ^b ± 6.17	78.1 ^{ab} ± 7.78	70.4 ^b ± 5.68	74.6 ^b ± 6.86	70.3 ^b ± 6.17	63.4 ^c ± 6.35	83 ^a .9 ± 7.63
<i>L</i> *	60.8 ^a ± 1.05	63 ^a ± 1.32	62.1 ^a ± 1.32	55.5 ^b ± 0.97	55.1 ^b ± 1.17	56.5 ^b ± 1.05	57.2 ^b ± 1.08	56 ^b ± 1.34
<i>a</i> *	8.8 ^a ± 0.46	8.8 ^a ± 0.46	9.8 ^a ± 0.58	8.9 ^a ± 0.42	8.9 ^a ± 0.51	8.4 ^{ab} ± 0.46	7.7 ^b ± 0.48	7.3 ^b ± 0.57
<i>b</i> *	43.7 ^{ab} ± 1.55	39.2 ^b ± 1.55	39.3 ^b ± 1.96	45.8 ^a ± 1.43	41 ^b ± 1.73	46 ^a ± 1.55	43 ^{ab} ± 1.6	43.6 ^{ab} ± 1.92
<i>C</i> *	44.7 ^{ab} ± 1.55	40.3 ^b ± 1.55	40.6 ^b ± 1.96	46.7 ^a ± 1.43	42 ^b ± 1.72	46 ^a ± 1.55	43.7 ^{ab} ± 1.6	44.3 ^{ab} ± 1.92
<i>H</i> ^o	78.3 ^a ± 0.64	77.1 ^b ± 0.64	75.7 ^c ± 0.81	79.1 ^a ± 0.59	77.7 ^b ± 0.71	79.7 ^a ± 0.64	79.9 ^a ± 0.66	80.6 ^a ± 0.79

^{a-c} Means within the same row that do not share a common superscript are significantly different (P < 0.05).

The Haugh unit (HU) is the worldwide accepted measure of choice for egg quality despite several authors disputing the fact that this measure bears little relation to any consumer standard, or nutritional quality as it only takes into account the egg weight and albumen height (Hunton, 1985; Sauveur, 1988; Williams, 1992; Silversides *et al.*, 1993). As seen in Table 3.6, as the days increased, the HU units increased. The HU score of freshly laid eggs has been extensively documented to decrease with increasing age of the layer bird (Silversides *et al.*, 1993). A possible explanation for the observed results could be since HU is a unit that looks at the relationship of albumen height and egg weight, and since no egg weight changes were seen but albumen height was increasing, it resulted in the increasing calculated HU units as days increased. The obtained values indicate larger albumen heights and eggs of better quality as Lorham White hens got older. It has been widely documented that as hens get older, the egg quality improves (Mathlouthi *et al.*, 2010).

Both T₁ and T₂ diets had no significant effect on egg colour as hens got older therefore they were not reported in this study. There was a significant difference ($P < 0.05$) in all the measured and calculated colour variables on colour coordinates measured. Lightness (L^*) values ranged from 55.1 to 62.1, b^* from 39.2 to 46 and a^* from 7.3 to 9.8. C^* and Hue angle values also changed depending on a^* and b^* values measured. As the days increased L^* and a^* values, initially increased and then significantly decreased ($P < 0.05$) starting from day 21. However L^* values have been documented to increase to give lighter eggs as the hen gets older (Odabasi *et al.*, 2007). In the current study as the birds got older egg colour was increasing with intensity as observed by the high Hue angle values of above 77° . Eggs from hens receiving both diets, generally increased in weight and had a relatively higher colour intensity that is normally associated with greater consumer acceptance.

3.3.5 Analysed feed samples for soy and gluten allergen presence

The ELISA kits used are highly, sensitive and precise and all protocols in the manuals were followed precisely. All feed samples were tested in duplicate for soy and gluten (gliadin) to confirm the presence of allergens in feed. All layer diets and broiler finisher diets were tested for the presence of soy and gluten (gliadin) allergens and their fragments. All sample layer diets had soy allergens above 25 ppm of soy flour, the highest concentration signifying the presence of soy allergens in all tested feed samples as indicated in Table 3.6. Results indicate that, all mixed feeds had intentional soya bean containing soy allergens. The protein allergen did not disintegrate and it was active even after feed processing thus the feed cannot be labelled as soybean free.

All feed diets were analysed for the presence of gliadin (gluten) and gliadin peptide fragments. Diets that had 15% wheat added to a soya bean-maize diet, all tested positive for the presence of gluten (gliadin) and gliadin peptide fragments. All values for gliadin (gluten) were above 135ppm gliadin and gliadin peptides had values above 40ppm gliadin. Table 3.7 summarises the results of analysed feed samples. These feeds, since they contained above 40 and 135 ppm of gliadin, cannot be labelled as gluten free or very low gluten according to the general guidelines in the kits used. Results obtained show that a considerable amount of allergenic peptides had been retained during feed ingredient processing and mixing indicating that allergens are bioactive, heat stable and resistant to some form of degradation (Faeste *et al.*, 2015). These results were similar to other studies where peptide allergens were intentionally mixed with feeds (Pariza and Cook, 2010; Faeste *et al.*, 2015).

Table 3.7: Levels of soy, gliadin (gluten) and gluten peptides in layer feed

Feed sample	Results (/ppm)				
	Soy (ELISA)	Gluten-peptides (competitive ELISA)		Gluten (sandwich ELISA)	
	Soy	Gliadin	Gluten	Gliadin	Gluten
T ₁ (maize/soya bean)	>25	32.96	65.9	36.3	72.7
T ₂ (maize/soya bean with a 15% whole wheat inclusion)	>25	>135	>270	>40	>80

However feed samples from T₁ of layer birds (maize/soya bean-based diets) were analysed for the presence of gluten in order to rule out cross contamination from wheat. The feed source tested positive for gluten. Soy was expected although gliadin (gluten) that was never incorporated in the diet was also detected as indicated in Table 3.6. Contamination levels ranged from 28.46 to 42.73 ppm of gliadin. The observed doses are below the recommended 100 ppm gliadin levels to term a product gluten free. These feed can be labelled as containing very low gluten as indicated in the RIDASCREEN[®] kits used for gluten analysis. This a classic case of agricultural grain co-mingling. Soya bean and wheat grains at one point could have been harvested, stored or transported using the same equipment and on the same facilities. Therefore extremely difficult to keep these grain from being mixed together at low amounts (Health Canada, 2013).

These results also indicate that there was cross contamination with wheat, as a result of remnant traces of wheat bean found in the mixing equipment. Cross contamination could have come as a result of some wheat grains remaining in the mixing equipment after cleaning. This is similar to the recent scandal involving wheat grain. In the recent reports, wheat imported from China, is assumed to have been accidentally mixed with melamine and this was detected in the final pet food product that resulted in the death of cats and dogs in the United States (Burns, 2007). Results indicate that protein allergens are heat stable, have degradation resistant immunoglobulin- binding sites that are retained during feed processing (Faeste *et al.*, 2015).

3.3.6 Potential for allergen carryover from feed to eggs of Lorham White layer hens

Results revealed that feed sources containing soya bean and a 15% whole wheat inclusion in maize/soya bean diets, had no significant effect on the presence of soy, gliadin (gluten) and gliadin peptide fragments in egg samples as indicated in Table 3.8. No allergens were detected.

Table 3.8: Level of soy, gliadin (gluten) and gliadin peptide fragments in analysed egg samples

Sample	Dietary Treatment	Veratox Soy Allergen Test	RIDASCR EEN [®] Gliadin Kit R7001 Gluten test		RIDASCREEN [®] Gliadin Kit R7021 Competitive Gluten Test	
		Soy (ppm)	Gliadin (ppm)	Gluten (ppm)	Gliadin (ppm)	Gluten (ppm)
Eggs n=50	T ₁ (maize/soya bean)	<2.5	<2.5	<5.0	<5.0	<10.0
Eggs n=50	T ₂ (maize/soya bean with a 15% whole wheat inclusion)	-	<2.5	<5.0	<5.0	<10.0

Eggs were collected as from Day 1, of commencement of feeding trial, to detect any allergens that might present themselves in the eggs as a result of a hen's problem, thus act as a control. At the end of the feed adaptation period, eggs were also sampled and this is because every 15th day, hens lay a new egg since it takes 14 days for an egg to be formed and be laid (Bellairs and Osmond, 2014). Similar studies have detected the presence of allergen such as Bovine Spongiform Encephalopathy (BSE) peptides, *Anisakis simplex* peptides, peanuts, and melamine in meat of fish, chickens and pigs and breast milk of breast feeding mothers (Frank *et al.*, 1999; Colchester and Colchester, 2005; Buur *et al.*, 2008). However no human allergic reactions to common animal feed proteins have been reported to occur as a result of consuming animal products (Pariza and Cook, 2010). This study is the first to investigate the potential for gluten and soy allergens being present in eggs since most studies looked at the egg itself as it is a confirmed allergen source to some consumers (Dumont *et al.*, 2010). The results in this experiment support these assumptions, indicating that current feed formulation for layer birds are balanced enough to meet bird nutritional needs and safe enough not to cause any harm to allergic individuals as no soy or gluten allergens were detected.

3.4 Conclusions

In conclusion, the current study demonstrated that a 15% whole wheat inclusion in a maize/soya bean diet can be given to layer diets and encourage growth performance that is similar to hens fed a maize/soya bean diet. Eggs produced in this study had improved egg quality especially yolk colour indicating that a maize/soya bean and a maize/soya bean-based diet with a 15% whole wheat, can be used to improve layer growth performance and egg quality. This study was able to demonstrate that no soy and gluten allergen carryover occurs from feed to eggs of healthy broilers and hens receiving a maize/soya bean and a 15% whole wheat inclusion in a maize/soya bean-based diet.

3.5 References

A **RIDASCREEN**[®] Gliadin quantitative competitive enzyme-linked immunosorbent assay (ELISA) kit (r-Bioparm AG, Art. No. R7021, Darmstadt, Germany supplied by AEC Amersham, South Africa).

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Chapter 4: Growth performance, dressing percentage and the potential for soy and gluten allergen carryover onto meat of broiler chickens fed maize/soya bean and a maize/soya bean-based diet with a 15% whole wheat inclusion

Abstract

The current study examined the effect of maize/soya bean and a maize/soya bean-based diet with a 15% whole wheat inclusion on growth performance, dressing percentage and the potential for allergen carryover from feed to meat of broiler chickens. A total of 160 Ross 308 day old chicks of mixed sex were used in a completely randomized design. Two dietary treatments were assigned to eight replicate birds per treatment group, with ten birds per cage. At the beginning of the trial all chicks were fed T₁ which contained a maize/soya bean diet for a period of ten days and then eight cages with ten birds per cage were randomly selected and had T₂ which contained a 15% wheat inclusion to the maize/soya bean-based diet fed to them. The feeding programme consisted of three phases, starter (day 1 to day 10), grower (day 11 to day 20) and finisher (day 21 to day 28). Weekly live weights and feed intake were recorded and used to calculate growth performance traits. One broiler bird was selected per cage, to have a total of eight birds per treatment that were slaughtered at the end of the grower (day 21) and finisher phase (day 28) and breast meat samples in duplicate were analysed for the presence of soy and gluten allergens using ELISA kits. The dressing percentage of hot carcasses was also determined at the end of the finisher phase on 8 birds per treatment. Growth traits measured indicated that there was no significant difference ($P > 0.05$) between live weight (LW) and average daily feed intake (ADFI) and weekly feed intakes (FI) and FCR and CFI and CG between treatments. As birds grew over a period of four weeks, significant differences ($P < 0.05$) between weeks was noted. No significant differences ($P > 0.05$) between dressing percentage of broilers from the two treatments were also noted. The results obtained indicated that diets formulated with maize and a 15% wheat inclusion can be

given to broiler birds without affecting their growth and development. Therefore a maize/soya bean-based diet and a 15% whole wheat inclusion in a maize/soya bean-based diets are equally good sources of nutrients to promote growth in Ross 308 broiler birds. Results for soy and gluten allergen analysis indicate that feed containing gluten and soy allergens were not carried over into the meat of broiler chickens.

Keywords: Broilers, carcass, non-starch polysaccharide, soya bean, wheat.

4.1 Introduction

A projected increase in poultry production and consumption in Brazil, Russia, India, China and in South Africa (BRICS) by the year 2022 is seen as a result from an anticipated growth in an aging population, changing lifestyles, diets and consumer preferences (OECD/FAO, 2013). Consumers prefer a healthier diet for a longer life span. With this in view plant derived feed raw materials such as wheat and soya bean use is increasing in order to appease health conscious consumers, reduce production costs and increase nutritional content of livestock products (FAO, 2009). This is despite the fact that wheat and soya bean are both known sources of protein allergens (Cawthorn *et al.*, 2009).

Wheat production is driven by food consumption patterns with human accounting for 68% use of total produce and livestock feed manufacturing using the remainder (OECD/FAO, 2013). The relatively high energy and protein content makes wheat a preferred livestock energy and protein source in most livestock feed (Hell *et al.*, 2015). Wheat is used in ruminant feed manufacture because in the rumen, enzymes and bacteria that can break down the various non-starch polysaccharide (NSP) are present. However in recent years use in monogastric animals such as pigs and chickens has increased despite digestion problems being reported in literature (Amerah, 2015).

Digestion problems in monogastric animals arise as a result of arabinoxylan, cellulose and other various NSP which are present in wheat bran. Non-starch polysaccharides form alkali-labile ester-like cross-linkages with the cell wall of wheat grains (Amerah, 2015). This makes digestion difficult, as monogastric animals do not contain digestive enzymes that are able to break down the cross-linkages making GIT contents more viscous and difficult to digest (Hell *et al.*, 2015). Phytate an enzyme, is also present in wheat grains and it has been known to reduce nutrient availability (Logan, 2014; Amerah, 2015).

Despite these anti-nutrient properties of wheat, in recent studies, antioxidant properties of arabinoxylan have been reported and these seem to have an effect on protecting food matrices and the GIT against free radicals (Malunga and Beta, 2015). According to Hell *et al.* (2015), arabinoxylan is the most abundant valuable component of wheat bran as it has both nutritional and rheological benefits. In monogastric animals digestion, NSP is also required to help in digestion, for example in poultry the gizzard requires some fibre for efficient functioning. These benefits should be taken into consideration and continued research into the benefits of wheat inclusions in broiler diets should be continued.

Soya bean is a legume with confirmed high protein content as extensively discussed in literature (deVisser *et al.*, 2014; Logan, 2014; Amerah, 2015). Soya bean use in the livestock feed industry has been seen as a production reducing cost measure. Soya bean production is lower compared to fishmeal as it provides cheap good quality protein. Since fishmeal has become expensive due to an increased demand and low availability of fish raw material. Soya bean use has enabled feed costs to be reduced at the same time maintaining targeted broiler production quotas. According to Logan (2014), the high quality protein content of soya bean, can provide an individual's daily requirement thus favoured by health conscious consumers. Soya bean has been seen as an alternative to good protein production and its benefits are being explored already. Research on soya bean use in conjunction with wheat is still an area of research that still needs establishment of an optimal wheat inclusion level in broiler diets. Therefore the current study aimed at investigating the effect of a low level of 15 % whole wheat inclusion and soybean formulated diets on growth performance and dressing yield of broiler chickens.

As previously discussed, both soya bean and wheat are known food allergens and their fatal effects on human health are well known and discussed in various literature (Buur *et al.*, 2008; Cawthorn *et al.*, 2009). Currently in order to avoid misleading consumers and in the interest

of protecting allergic individuals, legislation and various research around the world has helped in the development of immunochemical analytical methods to help test and monitor adherence to “allergen free” claiming labels in the food and animal feed industry (Health Canada, 2013). Currently, no studies have been done to warrant food safety concerns in terms of food grains, that is, soya bean and wheat containing allergens being used in the poultry industry to manufacture relatively low cost feeds. Thus the current study also aims at investigating the possibility of allergen carryover from feed containing two known sources of allergen to meat from broiler birds and eggs from broiler chickens.

4.2 Materials and Methods

4.2.1 Site description

Broilers were kept at Mariendal Experimental Farm. The site has already been described under Section 3.2.1 in Chapter 3.

4.2.2 Experimental design and Broiler management

Broiler birds were housed and cared for in an environmentally controlled broiler house for 28 days within ethical guidelines (Stellenbosch University, Animal Ethics Unit: SU-ACUM14-00032, UFH counter EC certificate No: MUC131SDOK01). All chicks were vaccinated against Marek's disease and Fowl pox at the hatchery. Feed and water was provided *ad libitum* throughout the 28 days. Internal house humidity was kept constant between 60-70% depending on external environmental conditions by constant rotating fans. The broiler house temperature was set at 34 °C on the day of chick placement and for seven days afterwards and there after reduced by 3 °C every week till 22 °C reached and maintained till the end of the 28 days.

Lighting was provided for 23 hours to maximize feed intake and 1 hour of darkness was allowed to boost the birds' immunity as per requirements in the Ross 308 broiler manual.

Biosecurity measures were set in place. Chicks were checked two hourly for the first week of life and thereafter twice daily to ensure their safety and removal of dead birds by recording their body weight, date and cage number.

A total of 160 Ross 308 day old chicks of mixed sex were used in a completely randomized design. Two dietary treatments were assigned to eight replicate birds per treatment group, with ten birds per cage. At the beginning of the trial all chicks were fed T₁ which contained a maize/soya bean diet for a period of ten days and then 8 cages with 10 birds per cage were randomly selected and had T₂ which contained a 15% wheat inclusion to the maize/soya bean-based diets applied to them as depicted in Figure 4.1.

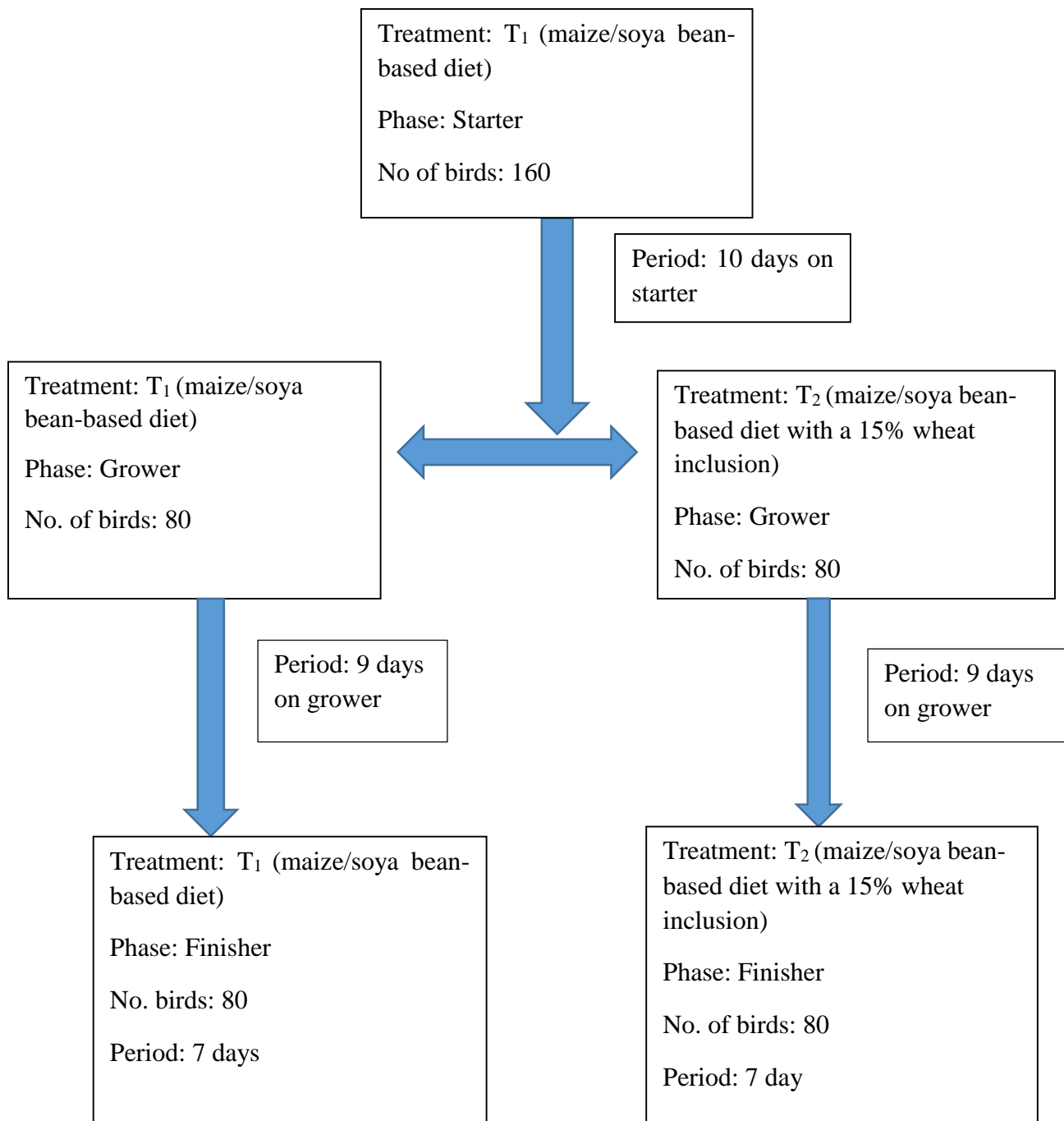


Figure 4. 1: Schematic diagram of feeding programme of Ross 308 broilers

4.2.3 Dietary Treatments

Iso-nitrogenous, iso-caloric diets were formulated using Win-Feed Formulation Software version 3.0 so as to meet all dietary requirements of the birds (National Research Council (NRC), 1994). The feeding programme consisted of three phases, starter (day 1 to day 10), grower (day 11 to day 20) and finisher (day 21 to day 28), all diets were mixed on site at Mariendahl Research Farm. Two dietary treatments were assigned to 160 broilers in a completely randomized design. Treatment 1 (T₁) consisted of a soya bean/maize-based diet and served as a control for Treatment 2. Treatment 2 (T₂) had a soya bean/maize-based with a 15% whole wheat inclusion, and it commenced on day 11 till day 28 to the second group of birds. Figure 4.1 clearly describes this process. Formulated diets and analyzed nutrient composition of experimental diets on dry matter basis for each phase of growth are clearly described in Table 4.1. Proximate analysis for moisture, crude fiber, crude protein ash and ether extract was performed according to methods of the Association of Official Analytical Chemists (AOAC, 2002) and described in Table 4.2. The metabolisable energy (ME) in kcalkg⁻¹, was calculated using the method of Ponzenga (1985).

Table 4. 1: Dietary composition of starter, grower and finisher diets of Ross 308 broiler chickens

Ingredient	Feed phases				
	Starter (maize/soya bean) T ₁ (day 1- day 10)	Grower (maize/soya bean) T ₁ (day 11- day 20)	Finisher (maize/soya bean) T ₁ (day 21- day 28)	Grower (15% whole wheat and maize/soya bean)) T ₂ (day 11- day 20)	Finisher (15% whole wheat and maize/soya bean) T ₂ (day 21- day 28)
White maize	477.552	485.485	496.356	351.485	362.344
Wheat	-	-	-	150.000	150.000
Maize gluten 60	-	47.974	75.215	51.917	79.239
Soya full fat	321.009	413.695	373.230	389.842	349.370
Soya bean 46	77.177	-	-	-	-
Fish meal 65	92.453	-	-	-	-
L-lysine HCL	0.625	2.750	1.550	3.232	2.032
DL-methionine	3.583	3.173	1.910	3.185	1.920
L-threonine	0.792	0.915	-	1.098	0.123
Vitamin and mineral premix	4.500	4.500	4.500	4.500	4.500
Limestone	11.740	15.042	14.479	15.339	14.776
Salt	0.772	2.509	2.732	2.407	2.630
Monocalcium phosphate	8.732	14.377	13.178	13.810	12.611
Sodium bicarbonate	1.066	1.585	1.2979	1.528	1.221
Oil (Sunflower)		7.995	15.571	11.658	19.235

Table 4. 2: Analysed nutrient composition of experimental diets on dry matter basis

Dietary composition							
Feed phase/Treatments	Crude protein (CP) %	Crude fiber (CF) %	Moisture %	Ash %	Ether extract (EE) %	Nitrogen-free extract (NFE) %	Metabolizable energy (ME) kcalkg⁻¹
Starter (0-10 day)							
T₁	27.1	4.7	8.8	1.2	5.6	38.6	2776.9
Grower (11-20 day)							
T₁	22.4	4.2	8.6	2.7	5.3	34.6	2445.8
T₂	22.3	5.0	8.3	3.5	5.4	36.2	2507.3
Finisher (21-28 day)							
T₁	21.8	5.1	8.0	3.2	5.6	35.7	2488.4
T₂	23.0	3.8	8.2	2.9	5.5	35.2	2504.5

4.2.4 Growth performance

The amount of feed at the start of the trial was recorded and on weekly basis before feeding using an electronic scale (Micro A12E, Germany). Body weight in grams was measured at placement and thereafter measurements were done on weekly basis and recorded. Feed consumed per week was measured, at the end and beginning of each phase so as to determine feed consumption per phase and thus calculate feed conversion ratio, the average feed intake. The body weight gain (BWG), average daily gain (ADG), feed conversion ratio (FCR) and the average feed intake (AFI) were calculated. For all calculations mortalities were taken into consideration so as to be more representative. The formulas used are depicted:

Body weight gain (g) = final body weight (g) day 28 - initial body weight (g) at start of trial placement

Average daily gain (g/day) = average body weight (g) / total number of days (7)

Average feed intake (g/day) = given feed per day (g/day) / remaining feed (g/day)

Feed conversion efficiency = Average feed intake (g) / Average body weights (g)

4.2.5 Slaughter management and dressing yield

At the end of the finisher phase (day 28), one bird per cage was slaughtered, 8 birds per treatment in total. All birds in each cage were initially weighed and an average was calculated. The bird with the weight closer to the average was chosen as the representative bird. A total of 16 birds were chosen at the end of each phase. Eight hours prior to slaughter, feed was removed but water was offered *ad libitum* to allow emptying of the gut. At harvest, lights were dimmed to reduce stress and birds were transported to the abattoir onsite. At the abattoir, birds were stunned with a voltage of 70V to render them unconscious and immediately slaughtered by cervical dislocation using a sharp knife and allowed to bleed as indicated in the Meat Act of 2000. After bleeding, scalding, plucking, and washing, the head,

neck and internal organs were removed and discarded. The hot carcass weight was then weighed and used to calculate dressing percentage using the following formula;

$$\text{Dressing \%} = \text{Hot Carcass weight} / \text{Body Weight} \times 100\%$$

4.2.6 Breast meat sample collection

At 21 and 28 days of age, eight birds per treatment (one bird per cage) were chosen for allergen analysis. Birds were sacrificed according to the procedure described under section 4.2.5. Following the plucking feathers, head, feet and internal organs were removed and vacuum packed and placed in a fridge set at 4 ± 1 °C for 24 hours. After 24 hours both breasts from each bird were removed and homogenized separately. The homogenizer was thoroughly washed in-between samples to minimize any risk of cross contamination or comingling of breast samples. Homogenized breast samples were vacuum packed and placed in freezer at -20°C and stored there until required for gluten and soy allergen analysis.

4.2.7 Soy allergen analysis

Analysis for the presence of soy allergens in meat samples was done using a Veratox® for Soy Allergen quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kit (Neogen Corporation, Cat. No. 8410, supplied by Analytical & Diagnostic Products (ADP), Gauteng, South Africa). The procedure used is clearly described in Chapter 3 under Section 3.2.7 with the following exceptions to meat and broiler feed samples. A total of 5g per each duplicate sample of meat and feed and control samples was performed using Veratox for Windows Software (Neogen, Cat. No. 9305W, supplied by ADP). The optical densities read from the standards were used to construct a curve and the optical densities of the samples were plotted against the curve to calculate the concentrations of soya present in each, with results obtained being expressed as mg.kg^{-1} (ppm) soy flour from which soy protein isolates (SPI) were obtained by multiplying results by a factor of 4.

4.2.8 Allergen analysis: Gliadin (Gluten) allergen analysis

The analysis for the presence of gliadin (gluten) in breast meat and broiler feed samples was conducted exactly the same as for eggs in Chapter 3 under Section 3.2.8 with the following changes as per meat sample requirements. Meat samples were allowed to thaw in a fridge set at 4 ± 1 °C before being analysed. Meat and feed samples were analysed for the presence of gliadin (gluten). For the extraction purposes, 0.25g of breast samples and 0.25g of feed were mixed individually with 2.5ml of Mendez Cocktail (patented) solution. Gliadin was extracted following the exact procedures recommended in the RIDASCREEN® Gliadin ELISA of gliadins and corresponding prolamines (Art. No. R7001, R-Biopharm AG, Darmstadt, Germany, supplied by AEC Amersham, South Africa) according to AOAC standards of 2012. All assays were done in duplicate.

4.2.8.1 Gliadin (Gluten) Fragment Allergen Analysis

Analysis for the presence of peptide fragments and corresponding prolamins of gliadin (gluten) in meat and feed of broiler chickens were done using a RIDASCREEN® Gliadin quantitative competitive enzyme-linked immunosorbent assay (ELISA) kit (r-Biopharm AG, Art. No. R7021, Darmstadt, Germany supplied by AEC Amersham, South Africa). The analysis was the same as used for eggs. This is described in Chapter 3 under Section 3.2.8 with the following exceptions to meat and feed samples in particular. Meat samples were first thawed in a fridge set at 4 ± 1 °C and feed samples from T₁ and T₂ from the finisher phases were crushed using a pestle and mortar. For meat samples and feed, 1g of samples were weighed mixed with 10 ml of 60% (v/v) ethanol solution (Sigma-Aldrich, Gauteng, South Africa) for extraction purposes. All samples were analysed in duplicate.

4.2.9 Statistical Analysis

The data for growth performance and dressing yield was analysed using the General Linear Model (GLM) procedure of SAS (2010). Means were compared using the least significant method (LSD). Differences were considered to be significant at $P < 0.05$. Since soy and gluten (gliadin) allergens were not detected in the analysed meat samples, no further data analysis was performed. Results were simply discussed.

The model used was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta_{ij}) + e_{ijk}$$

Y_{ijk} = response variables such as, BWG, ADG, AFI, CFI, FCR and Dressing percentage

μ = overall mean of the observations

α_i = effect of treatment (maize/soy diet and maize/soy/wheat diet)

β_j = effect of duration (weeks 1, 2, 3, 4) on BWG, ADG, AFI, FCR

$\alpha\beta_{ij}$ = interaction between treatment and duration

e_{ijk} = random error

4.3 Results and Discussion

4.3.1 Effect of a maize/soya bean and a maize/soya bean-based diet with a 15% wheat inclusion on the growth performance and dressing percentage of broiler chickens

There was no interaction ($P > 0.05$) between treatments and duration on broiler growth traits studied in this experiment, therefore main effects only were discussed. The effect of a maize/soya bean-based diet (T_1) and a maize/soya bean-based diet with a 15% wheat inclusion (T_2) on growth performance traits of broiler chickens is reported in Table 4.3. There was no significant difference ($P > 0.05$) in all measured live weight, feed intake, average daily gain, average feed intake, feed conversion ratio, cumulative gain measured and hot dressing percentage of birds receiving both treatments. However, there was a significant difference in the cumulative feed intake, with birds receiving diets containing a 15% whole wheat inclusion in a maize/soya bean-based diet being higher.

Results obtained are similar to those reported by Kiarie *et al.* (2014), where there was no significant difference ($P > 0.05$) between similar growth traits measured when broiler birds were fed maize/soya bean and wheat/soya bean diets. However, Pirgozliev *et al.* (2015) recorded a significant decrease ($P < 0.05$) in growth traits of broiler chickens fed maize/soya bean and wheat/soya bean-based diets. Contrary to our expectations, results in the current study could be as a result of the level of NSP present in the diets (Wiseman, 2000; 2006; Tancharoenrat *et al.*, 2015). Although NSP levels were not measured it can be concluded that a 15% whole wheat inclusion in broiler diets has relatively low NSP levels to cause any detrimental effect on growth performance of broiler chickens without the addition of enzymes such as xylanase (Wu and Ravindran, 2004; Liu *et al.*, 2015). Birds fed a 15% wheat diet performed equally as their counterparts.

Table 4.3: Effect of a maize/soya bean and a maize/soya bean-based diet with a 15% wheat inclusion on the growth performance of broiler chickens

Growth traits	Treatments	
	maize/soya bean (T ₁) n=80	maize/soya bean and a 15% whole wheat inclusion (T ₂) n=80
Live weight (g/28 days)	812.2 ^a ± 11.45	807.8 ^a ± 11.45
Weekly Feed Intake (g/day)	520.0 ^a ± 6.93	532.8 ^a ± 6.93
Average daily gain (g/day)	116.0 ^a ± 6.93	115.4 ^a ± 6.93
Average feed intake (g/day)	74.3 ^b ± 0.99	76.1 ^a ± 0.99
Feed conversion ratio	0.9 ^a ± 0.01	0.9 ^a ± 0.01
Cumulative gain (g)	212.5 ^a ± 2.90	210.0 ^a ± 2.90
Cumulative feed intake (g)	177.7 ^b ± 1.87	182.0 ^a ± 1.87
Dressing percentage (%)	70.0 ^a ± 0.63	70.5 ^a ± 0.63

^{a-b} Means within the same row that do not share a common superscript are significantly different ($P < 0.05$).

4.3.2 Changes in growth traits over a time

In the current study all growth traits measured increased over time with highest reading being recorded in the last week of the trial. These readings are recorded in Table 4.4. Live weight was 1500g at four weeks (28 days) of age which is within the expected range of 4 week Ross broilers as indicated in the Ross 308 manual. As birds grew older they ate more, as seen by a feed intake of 770g when birds reached four weeks of age. All recorded results are in accordance with similar studies where broiler birds were fed maize/soya bean and a maize/soya bean diet with a 15% whole wheat inclusion containing diets (Wiseman, 2000; Attaj, 2002; Mohamed *et al.*, 2015).

Table 4. 4: Changes in growth traits of broiler birds over a period of four weeks

Growth traits	Weeks			
	1	2	3	4
Live weight (g)	208.7 ^d ± 16.20	517.0 ^c ± 16.20	1014 ^b ± 16.20	1500.2 ^a ± 16.20
Total Feed intake (g)	235.2 ^d ± 9.80	416.6 ^c ± 9.80	682.8 ^b ± 9.80	770.9 ^a ± 9.80
Average daily gain (g/day)	29.8 ^d ± 2.31	73.9 ^c ± 2.31	144.9 ^b ± 2.31	214.3 ^a ± 2.31
Average feed intake (g/day)	33.60 ^d ± 1.40	59.51 ^c ± 1.40	97.6 ^b ± 1.40	110.1 ^a ± 1.40
Feed conversion ratio	0.3 ^d ± 0.02	0.7 ^c ± 0.02	1.1 ^b ± 0.02	1.5 ^a ± 0.02
Cumulative gain (g)	29.8 ^d ± 4.10	103.7 ^c ± 4.10	248.6 ^b ± 4.10	462.0 ^a ± 4.10
Cumulative feed intake (g)	33.6 ^d ± 2.64	143.7 ^c ± 2.64	241.3 ^b ± 2.64	300.8 ^a ± 2.64

^{a-d} Means within the same row that do not share a common superscript are significantly different (P < 0.05).

4.3.3 Analysed feed samples for the presence of allergens

All feed samples were tested in duplicate for soy and gluten (gliadin) to confirm the presence of allergens in feed. All broiler finisher diets were tested for the presence of soy and gluten (gliadin) allergens and their fragments. Only the finisher diet for the two treatments was tested for the presence of soy and gluten (gliadin) as birds were being fed the same diet throughout the experimental diet. It is assumed that no changes in nutrient composition occurred, just the quantities of the broiler finisher diets (T₁ and T₂) as seen by the analysed nutrient composition in section 4.2.3. All broiler feed finisher diets had soy allergens above 25 ppm of soy flour, the highest concentration signifying the presence of soy allergens in all tested feed samples as indicated in Table 4.5. Results indicate that, all mixed feeds had intentional soya bean containing soy allergens. The protein allergen did not disintegrate and it was active even after feed processing thus the feed cannot be labelled as soy free.

The finisher diets were analysed for the presence of gliadin (gluten) and gliadin peptide fragments. Diets that had 15% whole wheat added to a soya bean-maize diet (T₂), all tested positive for the presence of gluten (gliadin) and gliadin peptide fragments. All values for gliadin (gluten) were above 135ppm gliadin and gliadin peptides had values above 80ppm gliadin. Results indicate that the wheat inclusion contained the allergen gluten (gliadin) was present in T₂ of broiler and layer diets as indicated in Table 4.4 These feeds, since they contained above 80 and 135 ppm of gliadin, cannot be labelled as gluten free or very low gluten according to the general guidelines in the kits used. Results obtained show that a considerable amount of allergenic peptides had been retained during feed ingredient processing and mixing indicating that allergens are bioactive, heat stable and resistant to some form of degradation (Faeste *et al.*, 2015). These results were similar to other studies where peptide allergens were intentionally mixed with feeds (Pariza and Cook, 2010; Faeste *et al.*, 2015).

Table 4. 5: Levels of soy, gliadin (gluten) and gluten peptides in broiler finisher feeds

Feed sample	Results (/ppm)				
	Soy (ELISA)	Gluten-peptides (competitive ELISA)		Gluten (sandwich ELISA)	
		Soy	Gliadin	Gluten	Gliadin
T₁ (maize/soya bean)	>25	34.5	69.0	42.7	85.5
T₂ (maize/soya bean plus a 15% wheat inclusion)	>25	>135	>270	>40	>80

However feed samples from T₁ fed to broilers and layers (soya bean and maize based diets) were analysed for the presence of gluten in order to rule out cross contamination from wheat. The feed source tested positive for gluten. Soy was expected although gliadin (gluten) was never incorporated in the diet was also detected as indicated in Table 4.4. Contamination levels ranged from 28.46 to 42.73 ppm of gliadin. The observed doses are above the recommended 20 ppm gliadin levels to term a product gluten free but they are below 100 ppm gliadin. These feed can be labelled as containing very low gluten as indicated in the RIDASCREEN[®] kits used for gluten analysis. This a classic case of agricultural grain co-mingling. Soya bean and wheat grains at one point could have been harvested, stored or transported using the same equipment and on the same facilities. Therefore it is extremely difficult to keep these grains from being mixed together at low amounts (Health Canada, 2013).

These results also indicate that there was cross contamination with wheat, as a result of remnant traces of wheat being found in the mixing equipment. Cross contamination could have come as a result of some wheat grains remaining in the mixing equipment after cleaning as seen in various literature (Burns, 2007). Soy and gluten presence in feed indicate that protein allergens are heat stable, have degradation resistant immunoglobulin-binding sites that are retained during feed processing (Faeste *et al.*, 2015).

4.3.4 Potential for allergen carryover from feed to meat of broiler chickens

Homogenized breast meat samples were analysed for the presence of soy, gliadin (gluten) and gliadin peptide fragments at the end of the grower and finisher stage. These two stages of broiler growth were chosen as most commercial broiler producers slaughter birds ready for the meat market at approximately five (35 days) weeks of age and other producers preferring younger birds, four week old. Birds were slaughtered at these stage as according to previous studies, which concluded that if broiler birds are kept above the age of 35 days, thus will

result in a decrease in carcass traits, development of ascites thus production will be at a loss (Chivizhe and Rushizha, 2011; Baèza *et al.*, 2015).

Breast muscle meat from birds from T₁ and T₂ were analysed for the presence of soy, gliadin (gluten) and gliadin peptide allergen fragments. All analysed meat samples had no detectable soy allergen (<2.5 soy/ppm), which is the lowest detectable standard as indicated in Table 4.5. Breast samples were also used to determine the presence of gluten and to act as a control for meat samples from T₂. It was done to rule out any presence of gluten in the meat samples.

Feed contaminated with gluten from wheat had no significant effect on the presence of gliadin (gluten) and gliadin peptide fragments on all meat samples at days 21 and 28 respectively as indicated in Table 4.5. All results were below the lowest range of quantification of gliadin (gluten) and gliadin peptide fragments <2.5 gliadin/ppm and <5.0 gliadin/ppm respectively thus can be labelled 'gluten free'. However in similar experiments, where *Anisakis simplex*, a naturally occurring parasite of marine fish that contains proteins to which some individuals are allergic to, carryover from feed to flesh was established (Faeste *et al.*, 2015). *Anisakis simplex* peptide allergens were also detected in the serum and meat samples of chickens fed diets formulated with fish-meal containing *A. simplex* (Armentia *et al.*, 2006).

To note also is peanut allergens that were detected in breast milk (Frank *et al.*, 1999). Infants being breast fed from mothers who had ingested peanuts suffered an allergic reaction indicating allergens can cross the mammalian placental barrier and induce illness in an unborn foetus (Frank *et al.*, 1999). Similarly, intact parasitic proteins of *Anisakis simplex* (*s.l.*) have been detected in meat from fish that had their feed intentionally infected with the parasite (Faeste *et al.*, 2015). These results indicate that allergic peptides and their fragments can be transferred from animal feed into final animal products such as meat. In the present

study, the recommended levels of soya bean and wheat that are presently used in the feed industry were used. Thus this is the first study to determine the possibility of soy and gluten carryover from feed to meat from broiler chickens. It can be concluded that common animal feed proteins fed allergic plant sources within recommended levels will not be carried over to meat of chickens as similarly concluded by Pariza and Cook (2010).

4.4 Conclusions

In conclusion, the current study demonstrated that a 15% whole wheat inclusion in a maize/soya bean diets can be given to broiler diets and produce growth performance and dressing percentage that is similar to broilers fed a maize/soya bean diet. This study was able to also demonstrate that no soy or gluten allergen carryover occurs from feed to meat of broiler birds.

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Chapter 5: General Discussion

The current study aimed at investigating the effect of wheat-, maize-, soya bean-based diets on the growth performance, egg quality, dressing percentage and the potential for allergen carryover from feed to eggs and meat from Lorham White layer hens and Ross 308 broiler chickens. It was hypothesised that healthy Lorham White hens and Ross 308 chicks used in this study that were kept under standard commercial set-ups within bio-secure facilities would grow and achieve their potential within the trial period using the available nutrients in the formulated diets. It was further hypothesised that allergens present in poultry diets would not be carried over into eggs and meat from chickens.

Egg and meat production from chickens is still facing some challenges of high feed costs that make production generally expensive (Kwari *et al.*, 2011; Ncobela and Chimonyo, 2015). According to Manju *et al.* (2015), poultry production is hindered by feed costs that account for approximately 70 to 75% of production costs. Fishmeal, the commonly used protein source is the most expensive poultry feed ingredient thus the shift to use other protein sources like insects and plant protein sources such as soya bean (Ncobela and Chimonyo, 2015).

The commonly available and most suitable energy source used in animal feed production is maize as stated in various literature. The available maize produced is shared between humans and livestock thus an increased pressure for the limited resources. This in turn increases the price of raw materials such as maize as well. Producers in trying to cut costs, have implemented the use of soya bean and wheat as alternate protein and energy sources respectively, in most livestock feeds (Kwari *et al.*, 2011; Logan, 2014). Although, highly documented that wheat has high levels of non-starch polysaccharide (NSP), this makes its use in monogastric animals limited (Amerah, 2015).

However current literature has documented the fact that maize/soya bean-based diets have been able to produce eggs of higher weight and quality traits compared to eggs from wheat based diets (Safaa *et al.*, 2009; Mathlouthi *et al.*, 2015). Although wheat has high levels of NSP, in order to reduce feed costs, maize is partially being replaced with wheat up to 69% level of substitution with the aid of enzymes and without having detrimental effects on egg production, egg quality and general chicken production (Mirzaie *et al.*, 2012).

In Chapter 3, of this study it became evident that healthy Lorham White hens of superior genetic worth were fed a maize/soya bean and a 15% whole wheat in a maize/soya bean diet and performed well. The 15% wheat inclusion in the diet of layer birds had a significant ($P < 0.05$) effect on live weight of hens at 39 weeks of age, average daily gain, feed intake and the average feed intake. A 15% wheat inclusion in T₂ had no detrimental effects on hen growth parameter. The recorded gains could be as a result of an increased gizzard activity that resulted in increased feed retention and better nutrient assimilation (Hetland *et al.*, 2005). As the number of weeks increased, there was a significant indicated increase ($P < 0.05$) in growth traits.

Some egg quality traits measured, which include, egg weight, shell weight, albumen height, yolk height and Haugh units were significantly different ($P < 0.05$) for eggs from hens that received T₂ with no significant difference noted in yolk weight and average shell thickness. For colour coordinates measured, lower L^* and higher a^* values were documented for eggs from hens that received T₁. This could be attributed to meat and blood spots that were observed in some of the eggs (Burmester and Card, 1938; Nalbandov and Card, 1944). The higher values noted were seen in eggs from hens fed T₂ diets that contained a 15% wheat inclusion. Results obtained indicate that a 15% wheat inclusion improved general hen performance and egg quality.

Currently, broiler chicken producers are using soya bean and wheat in feed in order to reduce feed costs as extensively discussed (Kwari *et al.*, 2011; Amerah, 2015). In Chapter 4, there was no significant ($P > 0.05$) difference observed between treatments in some growth performance traits measured in Ross 308 broiler chicks reared for 28 days. Broiler birds fed maize/soya bean and a maize/soya bean diet with a 15% whole wheat inclusion grew equally and produced carcass weights of no significant difference between treatments ($P > 0.05$).

No soy and gluten allergens were detected in eggs and meat from chickens as indicated at the end of Chapters 3 and 4. This is despite various literature indicating the possibility of protein and non-protein allergens such as melamine, peanuts and vCJD being carried over from feed to milk and fish and pig meat (Burton and Young, 1996; Buur *et al.*, 2008; Faeste *et al.*, 2015). The results obtained for both bird species, indicate that a 15% wheat inclusion in poultry diets will encourage growth, egg production and broiler production without the risk of allergen carryover into eggs and meat of chickens.

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Chapter 6: Conclusions and Recommendations

In conclusion, the current study demonstrated that a 15% whole wheat inclusion in a maize/soya bean diet can be given to layer diets and encourage growth performance that is similar to LW hens and Ross 308 broilers fed a maize/soya bean diet. Eggs from Lorham hens, used in this study had improved egg quality especially yolk colour indicating that a maize/soya bean and a maize/soya bean based diet with a 15% whole wheat inclusion can be used to improve layer and broiler growth performance and egg quality. This study was able to demonstrate that no soy and gluten allergen carryover occurs from feed to eggs and meat of healthy broilers and hens receiving a maize/soya bean and a maize/soya bean-based diet with a 15% whole wheat inclusion.

As a recommendation:

- Carcass traits in broiler chickens should be determined to see if the diets had any effect on commercial cuts.
- A fatty acid profile of the diets, eggs and meat should be conducted.
- A prolonged trial in both bird species above 29 days with graded and higher levels of whole wheat and soya bean should be conducted to rule out allergen carryover into eggs and meat at different stages of growth.
- Other analytical methods such as the use of spectrometry and DNA based methods need to be used in order to improve in accuracy as well as obtain a reference point and see variability within methods although the ELISA has been recommended due to its sensitivity, rapidity and user friendliness.