Evaluating the efficacy of a modified *E.coli* phytase on growth performance, bone development and strength in comparison to other heat-stable phytases

By

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Declaration

I, the undersigned, declare that this thesis, which I hereby submit for the degree MSc. (Agric) Animal Science: Animal Nutrition at the University of Pretoria, is my own work and has not previously been submitted by me or another individual for a degree at this or any other tertiary institution.

H. S. Solomon

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Abstract

The objective of this study was to determine the optimal dose of the new generation modified *E.coli* 6-phytase (modified *E.coli*) that was developed using gene site saturation mutagenesis, and to compare its efficacy to current heat-stable phytases under typical South African commercial broiler production conditions. A total of 2,340 as-hatched Cobb 500 chicks were randomly allocated to nine treatment groups, each replicated 13 times with 20 chicks per pen. The first three treatments consisted of two negative control diets and a positive control diet as follows: negative control (NC1) – starter diet with 0.23% available phosphorus (avP); NC2 – starter diet with 0.33% avP; positive control (PC) – starter diet with 0.43% avP. For each control, the finisher diets had a 0.11% reduction in avP. The remaining six treatments consisted of NC1 supplemented with three different phytase products (modified *E.coli*, product X and product Y) at two different doses for each product (500 FTU/kg diet and 1,000 FTU/kg diet). Production performance parameters, bone strength, bone ash content and bone mineral content were evaluated.

Broilers fed the NC1 diet showed a significantly lower ($P<0.05$) body weight, feed conversion ratio (FCR), production efficiency factor (PEF), bone strength and ash compared to all other treatment diets. All phytase-supplemented treatments showed results similar to the PC diet ($P>0.05$). No significant differences were detected among phytase-supplemented treatments. Numerically ($P>0.05$), broilers fed the diet supplemented with modified *E.coli* at 500FTU/kg had the greatest body weight, cumulative feed intake and PEF values, and the lowest FCR values on day 35 compared to all other phytase-supplemented diets. Numerically, in terms of bone development broilers fed a diet containing phytase at 1,000FTU/kg had the highest bone breaking strength compared to their 500FTU/kg counterparts, with modified *E.coli* numerically scoring the highest bone breaking strength, ash and phosphorous deposition, followed by product X and then product Y. In conclusion, broiler performance using this new generation modified *E.coli* 6-phytase product is comparable to other phytase products on the South African market at an optimal dose of 500FTU/kg diet.

Keywords: Phosphorus, enzymes, performance efficiency, bone mineralisation, ash content

Table of Contents

List of figures

List of tables

List of acronyms and abbreviations

AIA	Acid insoluble ash
AME	Apparent metabolisable energy
ANOVA	Analysis of variance
ARC	Agricultural Research Council
CAID	Coefficient of apparent ileal digestibility
DDGS	Distillers dried grains with solubles
EGF	Epidermal growth factor
FCR	Feed conversion ratio
FI	Feed intake
GRP	Gastrin-releasing peptides
GSSM	Gene site saturation mutagenesis
HSD	Honestly significant difference
MINPP	Multiple inositol polyphosphate phosphatase
NPP	Non-phytate phosphorus
NSP	Non-starch polysaccharides
PC	Positive control
PCR	Polymerase chain reaction
PEF	Production efficiency factor
PHS	Pulmonary hypertension syndrome
SDM	Site-directed mutagenesis
SGAS	Simple Grow Agricultural Services
SGF	Stimulated gastric fluid

Chapter 1

Introduction

The global human population is predicted to reach just over nine billion by 2050 (Population Reference Bureau, 2011), therefore, meat demand to feed the growing population is expected to double (Rich, 2009; Kruse, 2010). The production of poultry products is expected to increase by 120% as it has the greatest production efficiency, requires the least amount of land, is the cheapest meat source and has the least cultural and religious restrictions (O'Keefe, 2014; Tavárez & de los Santos, 2016). The poultry industry acquired this advantage as a result of decades of successful genetic selection to improve broiler production and efficiency, thereby obtaining the required market broiler weight in a reduced number of days (Havenstein, 2006; Tavárez & de los Santos, 2016).

Nevertheless, intense genetic selection for rapid growth has resulted in an inadequate supply of minerals and metabolic imbalances, leading to a series of unintentional negative effects. These negative effects include reduced reproduction, increased abdominal fat, increased occurrence of pulmonary hypertension syndrome (PHS) and increased skeletal deformities (Julian, 1998; Leeson & Summer, 2009; Shim *et al.*, 2012; Proszkowiec-Weglarz & Angel, 2013; Tavárez & de los Santos, 2016). Distress and skeletal disorders as a consequence of rapid growth rate is a concern, not only for production efficiency and profit for the farmer, but also for the welfare and health of the animal (Julian, 1998; Shim *et al.*, 2012). Modern broilers have high nutritional requirements; thus, optimising nutrition along with improved selection criteria in the breeding stock and optimised farm management are among several strategies used to alleviate the negative effects caused by rapid growth (Julian, 1998; Tavárez & de los Santos, 2016). Furthermore, producers are under pressure to improve animal welfare, reduce greenhouse gas emissions and pollution, and use local raw materials (Charles *et al.*, 2010; South African Poultry Association, 2016). A nutritional strategy is to manipulate the diet of the animal with the use of feed additives, in order to improve bioavailability of the dietary nutrients within the individual raw materials.

Phosphorous is critical to the animal body as it forms part of bone development, ATP production, RNA and DNA, enzymes and phosphoglycerides, and plays an integral role in maintaining acid-base balance and immune function (Cambell & Farrell, 2012; Shim *et al.*, 2012). However, 50-85% of phosphorous from plant sources is in the form of phytate and is not readily digestible; therefore it is poorly absorbed and used by the bird (Proszkowiec-Weglarz & Angel, 2013). The main site for phytate dephosphorylation is in the forestomach, composed mainly of the crop, proventriculus and gizzard (Selle *et al.*, 2011). Gut microflora and endogenous phytase secreted in monogastrics have a limited ability to break down phytate. Additionally, the insoluble complexes formed due to the interaction between phytate and dietary nutrients reduces the efficiency of the endogenous phytase enzymes, resulting in 70% of the total phosphorous being excreted (Selle *et al.*, 2009; Gupta *et al.*, 2015). Leaching, or surface run-off of the excreted phosphorous results in eutrophication of surface water, algae blooms, hypoxia, the death of aquatic species and the production of nitrous oxide (Selle *et al.*, 2011; Gupta *et al.*, 2015).

Phytases (myo-inositol hexakisphosphate phosphohydrolase) are protein enzymes that catalyse the stepwise removal of phosphorous from phytic acid/ phytate releasing the inorganic phosphorous (Dersjant-Li *et al.*, 2015). Numerous published scientific articles have shown that supplementation with exogenous phytase enzymes can improve broiler performance, bone development, apparent metabolisable energy and amino acid digestibility (Selle & Ravindran, 2007).

A naturally occurring wild-type *E.coli* 6-phytase was evolved using a high throughput gene site saturation mutagenesis (GSSM). Put simply, GSSM involves screening and identifying point mutations. Gene reassembly evolution technology is used to substitute the identified amino acid coding sequences in the wild-type *E.coli* 6-phytase with a modified amino acid coding sequence at specified positions that has the best synergistic combinations, resulting in a modified new generation *E.coli* 6-phytase molecule (Pieniazek *et al.*, 2016). Further information on GSSM can be found in an article by Kretz *et al.* (2004). Multiple scientific studies that evaluated the effect of this modified new generation *E.coli* 6-phytase molecule on broiler tibia ash, broiler performance, metabolisable energy, amino acid digestibility and gastric stability have been

published in the USA. However, no studies have been conducted under typical South African conditions, even though the phytase product is currently registered in South Africa.

Therefore, the purpose of this study is to determine the optimal dose of the modified new generation *E.coli* 6-phytase (modified *E.coli*) and to compare its efficacy to other currently-used heat-stable phytase products on a typical commercial South African broiler diet.

Chapter 2

Literature review

2.1. Introduction

The use of exogenous phytase in poultry diets began in the Netherlands in 1987 to reduce phosphorus pollution (Kemme *et al.*, 1997). Increased commercial interest in phytase enzymes with improved efficacy, stability and thermo-stability led to the commercial production of phytase enzymes from various microbial, plant and fungal origins. Countless scientific articles and presentations have recounted the mode of action and beneficial attributes of exogenous phytase on broiler performance, feed efficiency, bone development and environmental pollution at various doses (Selle & Ravindran, 2007; Lei *et al.*, 2013; Dersjant-Li *et al.*, 2015). Over the years, exogenous phytase supplementation in animal diets gained more traction.

Recently, Novus International developed a new generation modified *E.coli* 6-phytase using gene site saturation mutagenesis (GSSM) and gene reassembly evolution technology. This new phytase product is registered under the name Cibenza Phytaverse G10 (to be called modified *E.coli* for the remainder of this paper). The purpose of this study is to determine the optimal dose of modified *E.coli* and to compare its efficacy to other currently-used heat-stable phytase products on a typical commercial South African broiler diet by evaluating broiler performance parameters and bone development parameters.

In order to understand the reasons for the continual technological improvements to commercial phytase enzyme production, we first need to understand how the modern broiler differs from previous generations and the impact that that has on the nutritional requirements of the modern broiler.

2.1.1. Growth of broilers over the years

The world human population had grown considerably between 1900 and 2000, with the growth rate peaking at 2.1% in 1962. Even though the world human population growth rate saw a decline to 1.2% in 2015, an estimated growth of over 9 and 11 billion people by 2050 and 2100 respectively, is still expected (Population Reference Bureau, 2011; Roser & Ortiz-Ospina, 2016).

With this increase in population, the main concern for any country would be providing food security for its population. Food security is defined by du Toit (2011) as the ability of all people to have access to, and be able to, consume safe and nutritional food to meet their daily dietary requirements and live a healthy lifestyle. Africa's population is expected to reach 3.5-4 billion people by 2100 with an expected doubling of meat demand (Rich, 2009; Kruse, 2010). The main reason for this projected increase in meat demand is that protein from animal sources has a high concentration of protein per portion compared to cereal and grain sources. Additionally, animal protein sources have an ideal essential amino acid profile for humans. This was proved in a study conducted by Schönfeldt & Hall (2012) where maternal health, child development and nutritional health of the population is improved when small amounts of protein from animal sources was included into a cereal based diet.

With poultry being the cheapest source of animal protein with the least religious and cultural restrictions, production of poultry products in Africa is expected to increase by approximately 120% (O'Keefe, 2014; Tavárez & de los Santos, 2016). However, the number of farmers has declined as more farms are becoming integrated into larger companies. For instance, in 1980 there were approximately 128 000 commercial farmers in South Africa. This was reduced to 58 000 commercial farmers by 1997 and to just under 40 000 commercial farmers in 2011. It is predicted that the number of commercial farmers in South Africa will drop to 15 000 by 2026 (Gosling & Moolla, 2011).

This has placed, and will continue to place, pressure on meat producers to increase their meat production to address the growing demand. However, in the near future, there will be less land and water available, which will make it difficult to simply increase yields by expanding existing poultry flocks. Furthermore, farmers are experiencing increased pressure from government, and environment and animal activist groups, to reduce greenhouse gas emissions

and pollution, increase animal welfare, and reduce imports by making use of local raw materials (Charles *et al.*, 2010; South African Poultry Association, 2016).

Although one can argue that statistics on meat consumption trends and increases in global human population have little to do with the purpose of this review, it is these circumstances that resulted in the rapid improvement in broiler production and efficiency (Havenstein, 2006). In order to meet the growing demand for meat, mass genetic selection was successfully applied to the breeding stock. Genetic traits that were selected included growth rate, edible meat yield and feed efficiency. The result of this genetic selection is improved broiler production and efficiency. For example, in 1957, a 42-day-old broiler weighed 586g with a feed conversion ratio (FCR) of 2.8. In 2016, a 42-day-old broiler weighed 2.9kg with an FCR of 1.7 (figure 2.1). This is an improvement of 460% in body weight and 50% in FCR (Tavárez & De los Santos, 2016).

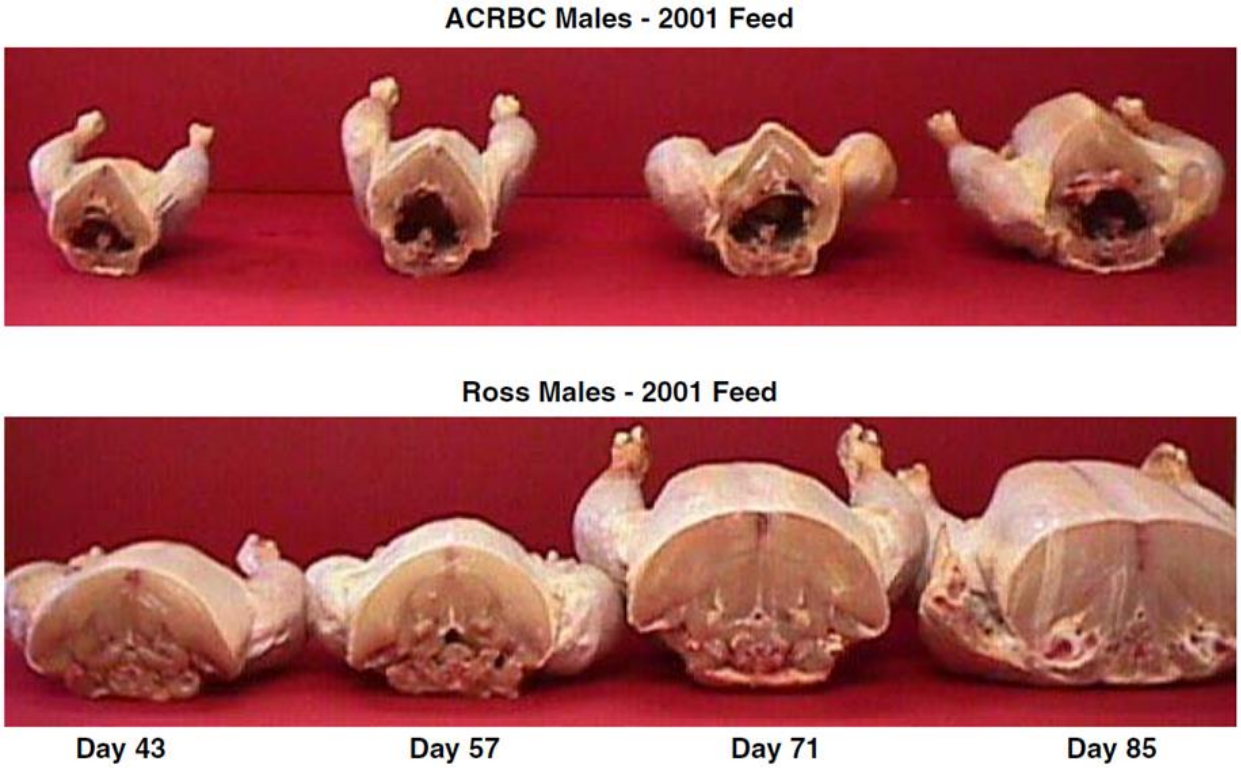

Figure 2.1 Broiler carcasses of a 1957 broiler strain from the University of Alberta (ACRBC) and a modern Ross 308 broiler strain, after being fed typical 2001 feed (Havenstein, 2006).

About 85-90% of all improvements in the efficiency of broiler production have been achieved through genetic selection for economically important traits, while only 10-15% are

attributed to nutrition and management (Havenstein, 2006). Improved growth rates and muscle development through genetic selection have resulted in subsequent changes to the nutrient requirements of the bird. It is the role of nutritionists to ensure that broilers receive an adequate nutrient supply at the cellular level during the different growth phases in order to meet the ever-changing nutrient demand. Some examples of nutritional advances, in diet formulations, to ensure adequate nutrient supply, are formulating to metabolisable energy instead of productive energy, digestible amino acids instead of total amino acids, and calcium: available phosphorus ratio (Rutz, 2012). Further advances include supplementing exogenous enzymes such as proteases, carbohydrases and phytases to broiler diets. The purpose of exogenous enzymes is to increase the bioavailability of inherent nutrients within the raw materials used in diet formulations, reduce anti-nutritional factors in cereal grains, improve nutrient digestion and absorption by the animal, minimise negative nutrient interactions and, quite recently, enhance or alter gene expression (Ferket, 2010; Ribeiro, 2012). Thus, to maximise the genetic potential of an animal, proper nutrition and management must be provided; and the interactions between genetics, nutrition and management must be integrated to obtain optimum broiler efficiency (Havenstein, 2006; Tavárez & de los Santos, 2016).

2.1.2. Negative consequences of rapid growth

Intense genetic selection for rapid growth has resulted in an inadequate supply of minerals and in metabolic imbalances, leading to a series of unintentional negative effects such as reduced reproduction, increased abdominal fat, increased occurrence of pulmonary hypertension syndrome (PHS) and increased skeletal deformities (Leeson & Summer, 2009; Shim *et al.*, 2012; Proszkowiec-Weglarz & Angel, 2013; Tavárez & de los Santos, 2016). The PHS is caused by the combination of a small skeletal frame, large and heavy breast meat, and small lung volume, resulting in right ventricular failure followed by right ventricular dilation and hypertrophy from pulmonary hypertension. PHS leads to illness, death and condemnation of the meat, resulting in reduced meat yield and subsequent reduced profit for the farmer (Julian, 1998).

Genetic selection for improved broiler growth and muscle deposition did not influence the genetic expression for bone developmental pattern. This means that the development of

bones, tendons and ligaments are primarily age dependent, becoming stronger the older the animal gets. Thus, a heavy body weight at a young age places pressure on soft tissue and immature bone causing pain and resulting in lameness. Weak legs also result in bone breakages during processing at the abattoir, which leads to condemned carcasses and/or reduced meat grades. Therefore, leg problems are the biggest cause of economic losses in a poultry house (Julian, 1998; Onyango *et al.*, 2003; Almeida Paz & Bruno, 2006; Shim *et al.*, 2012).

The most common skeletal deformity for broilers fed a diet containing a nutritionally adequate balance is angular bone deformity (figure 2.2). This is a progressive deformity occurring from the age of 6-8 days because not enough time is allowed during growth for the distal tibio-tarsus bones to properly align and remodel. The result is pain and crippling in the legs, which causes reduced activity, feed and water intake. The animal often dies of dehydration and is condemned at processing. Nutritional deficiencies such as vitamin B and trace minerals can also result in angular bone deformity (Julian, 1998).

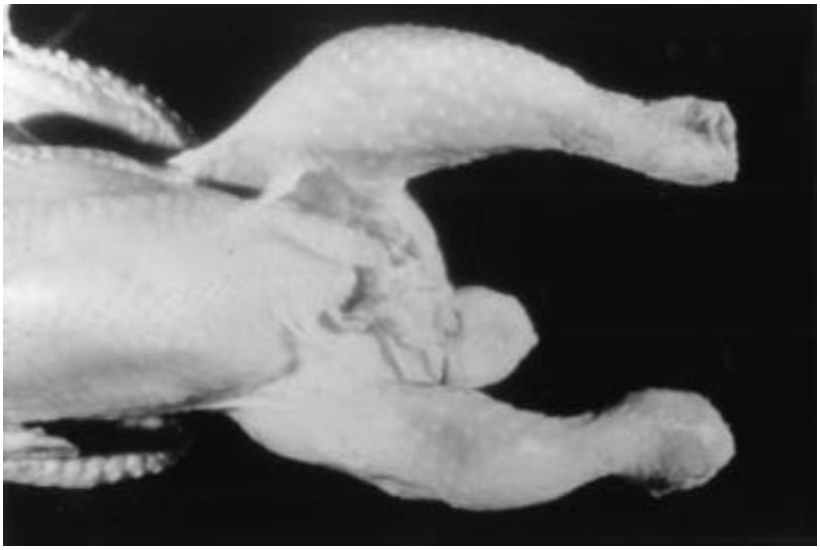

Figure 2.2 A broiler with angular bone deformity and disuse atrophy of the left leg muscle (Julian, 1998)

When chondrocytes in the growth plates do not undergo hypertrophy, vascular penetration of the bone cannot occur and the bone cannot develop. This is known as tibial dyschondroplasia (figures 2.3−2.5). Heavy weight placed on the weakened proximal tibia results in lesions that cause pain when walking or standing, and fractures to the tibia resulting in

crippled birds. Crippled birds have reduced feed and water intake, and do not grow to their genetic potential (Julian, 1998).

Figure 2.3 Severe tibial dyschondroplasia in four broilers (Julian, 1998)

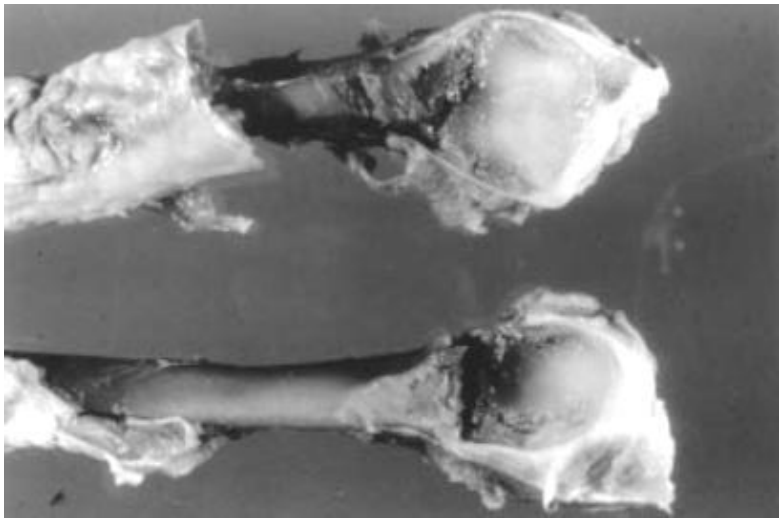

Figure 2.4 Fracture through the proximal tibia of a broiler (Julian, 1998)

Figure 2.5 Crippled broilers unable to rise due to severe tibial dyschondroplasia (Julian, 1998)

When the ligaments in the third and fourth thoracic vertebrae tear, the fourth vertebra dislocates ventrally resulting in the spinal cord pinching and narrowing, causing leg weakness and ataxia. This deformity is known as spondylolisthesis (figure 2.6). Spondylolisthesis is usually caused by heavy breast meat weight placing pressure on immature bones and ligaments (Julian, 1998).

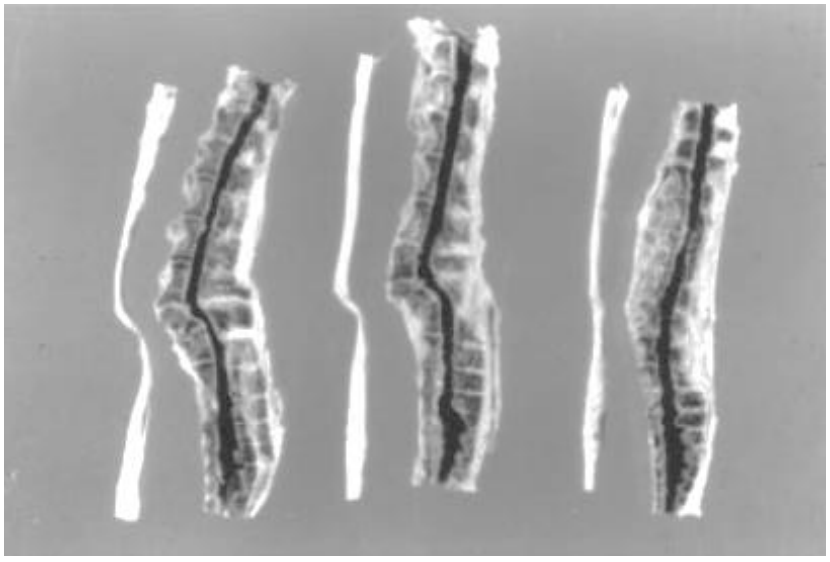

Figure 2.6 Vertebrae of a broiler cut along the midline with the spinal cord removed. The image on the right is a normal spine. The middle image shows mild spondylolisthesis. The image on the left shows severe spondylolisthesis (Julian, 1998)

Distress and pain caused by these adverse consequences of a rapid growth rate are a concern for the production efficiency and profit of the farmer, as well as the welfare and health of the animal (Julian 1998; Shim *et al.*, 2012). In order to reduce the negative consequences of rapid growth on skeletal integrity, broiler breeder companies have included skeletal development traits in their selection criteria. The main problem with using exclusively genetic methods to increase skeletal integrity is that skeletal disorders are caused by a combination of genetics, nutrition and management factors (Shim *et al.*, 2012).

It is a fact that broilers that have been genetically selected for rapid growth have high nutritional requirements (Julian, 1998). As such, optimising nutrition, along with improved selection criteria in the breeding flock and optimised farm management, are processes than can help the bird cope with the negative effects of rapid growth (Tavárez & De los Santos, 2016).

2.2. Importance of phosphorous to the animal body

2.2.1. Biochemical pathways of phosphorous

Elemental phosphorous (P) is a non-metal with an atomic number of 15. Phosphorous can be ionised to have negative charges (anions), which gives it the ability to bind to minerals and dietary nutrients that have a positive charge (cations) (Gagnon, 2017).

Elemental phosphorous is important to the animal body; 80-85% of absorbed dietary phosphorous is directed toward bone formation and is stored in the bone, along with 99% of absorbed dietary calcium (Ca) in the form of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] (R&D Systems, 2007; de Carvalho Mello *et al.*, 2012; Proszkowiec-Weglarz & Angel, 2013).

Bone is a soft tissue that develops and calcifies over time and is dependent on the physiology, nutrition and physical activity of the animal. A typical modern broiler reaches target market weight by 35 days of age. In contrast, bone maturity, as measured by ash content, mineral content, diameter, weight and length, is only reached at 175-245 days (25-35 weeks) of age (Rath *et al.*, 2000). Consequently, the mineralisation of bone is crucial to animal performance because

the extent of mineralisation and the architecture of the bone structure influences bone strength and growth, and therefore the mobility of the animal (Shim *et al.*, 2012). Apart from mobility, bone development is also important for homeostatic regulation by maintaining a consistent ionic environment in the animal, and to support and protect soft tissue and organs (Wasserman, 1984). The three main cells involved in bone development are osteoblasts, osteocytes and osteoclasts (Wasserman, 1984; Liem, 2009):

1) Osteoblasts are single-nucleated cells that contain phosphatase and are involved in bone formation. Osteoblasts are stimulated by the parathyroid hormone (PTH) to secrete RANK-ligand hormone, and inhibit osteoprotegerin (OPG) secretion. When not stimulated by PTH, osteoblasts secrete OPG hormone which inhibits osteoclast activity.
2) Osteocytes are osteoblasts that have become entrapped in the bone lacuna during bone formation.
3) Osteoclasts are multi-nucleated cells involved in bone resorption. Osteoclasts contain RANK receptors on their membrane; when the hormone RANK-ligand binds to the RANK receptors, osteoclast activity is stimulated. Osteoclasts dissolve and remove minerals from bone by creating an acidic environment between the bone and the osteoclast. This promotes the ionisation of minerals, and thus the breakdown of hydroxyapatite and the release of mineral ions.

At hatch, bone is completely composed of an organic matrix and water. Approximately 10-15% of the composition of the organic matrix consists of proteoglygans, lipids and proteins. Collagen makes up the remaining 85-90%. The arrangement of the collagen fibres provides a support for mineral deposition, and thus is a key factor in the tensile strength of the mature bone (Rath *et al.*, 2000). As ossification proceeds the water is displaced by minerals and the cartilage becomes calcified. Different regions of bone have different rates of formation and resorption. There are two main pathways for ossification. Intramembranous ossification occurs in the flat bones of the skull. Endochondral ossification (figure 2.7) occurs in the long bones and is responsible for bone elongation when there is an adequate supply of calcium and phosphorous ions (Wasserman, 1984).

Alkaline phosphatases secreted by osteoblasts hydrolyse phosphate esters to phosphate ions. This increases the formation and precipitation of hydroxyapatite crystals onto the collagen framework. Calcium-acid-phospholipid-phosphate complexes (or calcifiable proteolipids) induce the precipitation of hydroxyapatite crystals. Proteoglycans are involved in bone mineralisation. Matrix vesicles are located in the epiphyseal plate (growth centre for bone elongation) and contain alkaline phosphatases and acid phospholipids that allow for the support of calcification mechanisms. The mitochondria play a role in mineralisation by storing calcium and phosphorous ions. These ions are released during the process of calcification (Wasserman, 1984).

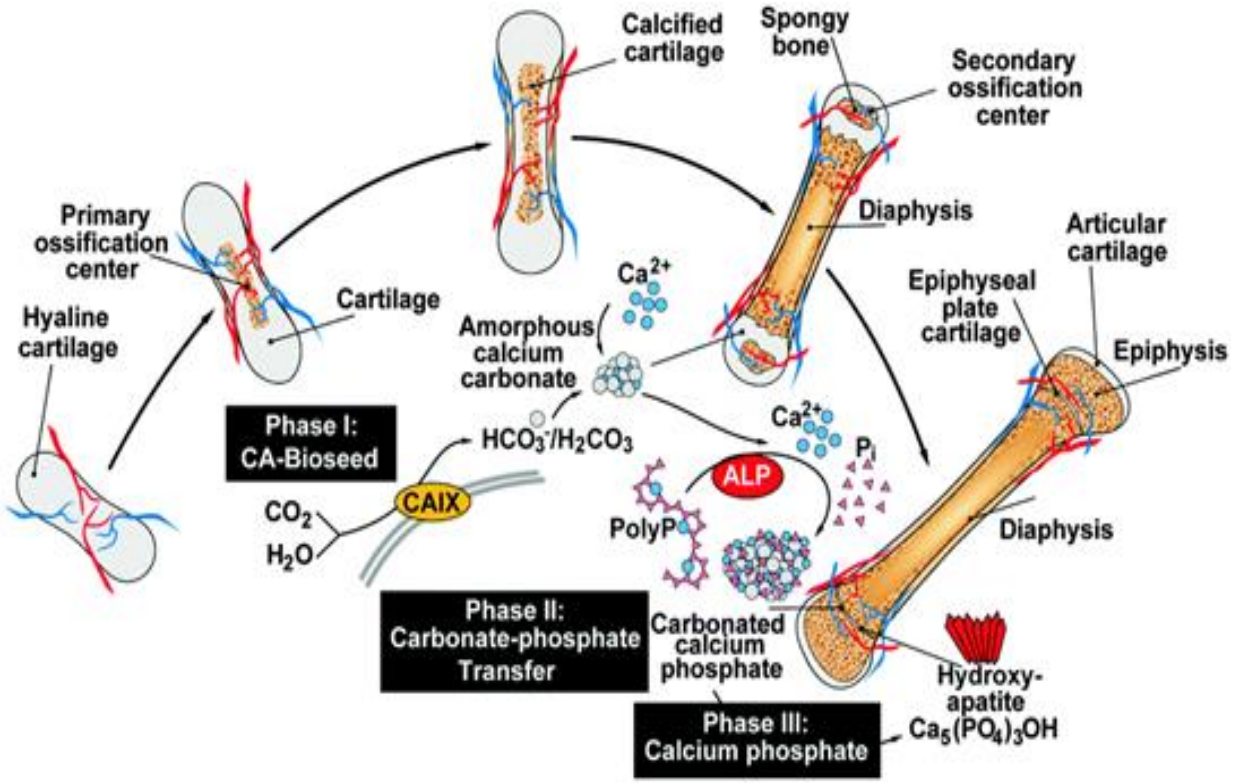

Figure 2.7 The endochondral ossification of the long bone (Talba *et al.*, 2016)

The remaining 15-20% of phosphorous forms part of functional groups, such as phosphoric acid esters and anhydrides, which plays an integral role in energy metabolism, nutrient utilisation, acid-base balance and cellular and membrane function (de Carvalho Mello *et al.*, 2012; Proszkowiec-Weglarz & Angel, 2013). Adenosine triphosphate (ATP), which is composed of phosphoric acid esters and anhydrides linkages, is the main source of energy for cells and metabolic reactions (Cambell & Farrell, 2012). ATP is required for (McDonald *et al.*, 2011(b); Cambell & Farrell, 2012):

1) the synthesis of RNA and DNA
2) increasing the reaction rate of enzymes
3) the phosphorylation of the sodium-potassium pump. ATP donates a phosphate to aspartate 369, changing the conformation of the enzyme and resulting in cyclic changes in the membrane protein
4) the citric acid cycle (convert succinyl-CoA to succinate) and glycolysis (convert glucose to pyruvate)
5) the contraction and relaxation of muscle. ATP is stored in muscle in the form of phosphocreatine.

Phosphorous, in the form of phosphate, forms part of RNA and DNA by forming part of the nucleotides. Phosphoric acid has three different dissociation constants ranging from pka=2.14 to pka=12.40; as a result phosphoric acid functions as a buffer to maintain acid-base balance. Phosphorous forms part of enzymes and co-enzymes; for example glycogen phosphorylase$_a$, which catalyses the first step in stored glycogen breakdown, contains two phosphorous molecules. Pyridoxal phosphate is a co-enzyme in the transamination step of Vitamin B6 synthesis. NAD^+ is an energy source in oxidation-reduction reactions and is composed of two phosphate groups. Phosphorous forms part of phosphoglycerides, which is an important component of phospholipids located in cell membranes. Lastly, phosphorous has an integral role in immune function. For example, N^{α}(5-phosphopyridoxyl)-L-Lysine is a moiety that acts as an antigen when bound to a protein, thereby stimulating antibody production (Cambell & Farrell, 2012).

Phosphorous deficiency results in poor bone quality, muscular weakness, reduced fertility, stiff joints and reduced growth (McDonald *et al.*, 2011(c)). A study by de Carvalho Mello *et al.* (2012) showed that the level of available phosphorous (aP) in the first 10 days of a broilers life greatly influences FCR, bone development, body weight gains and subsequent growth.

2.2.2. Absorption and regulation of calcium and phosphorous in the animal body

The remaining 1% of calcium not found in bone is found in plasma, cells and extracellular fluid. Here calcium has a role in enzyme activation, neuromuscular function, blood clotting, metabolism, cell adhesion, intracellular signalling and muscle contraction (Proszkowiec-Weglarz & Angel, 2013).

Calcium and phosphorous are absorbed via diffusion largely in the small intestine due to the low pH, which promotes ionisation of calcium and phosphorous. In the case of calcium, when the calcium content in the diet is high, paracellular/passive diffusion is favoured over facilitated/active diffusion. When the calcium content in the diet is low, facilitated/active diffusion is favoured over paracellular/passive diffusion. Phosphorous is absorbed by transcellular/saturable diffusion (R&D Systems, 2007).

Passive diffusion mainly occurs in the jejunum/ileum with the aid of the sodium-potassium pump, which creates an electrochemical gradient allowing for the movement of calcium ions from the lumen to the extracellular fluid. Facilitated and transcellular transport mainly occurs in the duodenum/jejunum and is dependent on the concentration of Vitamin D_3. In chickens, the main site for calcium and phosphorous absorption is in the duodenum (R&D Systems, 2007; Proszkowiec-Weglarz & Angel, 2013). The domain channel protein, TRPV, is upregulated in the presence of low Ca^{2+} in the cytosol and by Vitamin D_3. The Ca^{2+} enters the cell by electrochemical gradient. Within the cell calbindins, which are Ca^{2+} binding proteins synthesised only in the presence of Vitamin D_3, mediate the transit of Ca^{2+} through the cell. A similar process occurs in the proximal tubule of the kidney (R&D Systems, 2007).

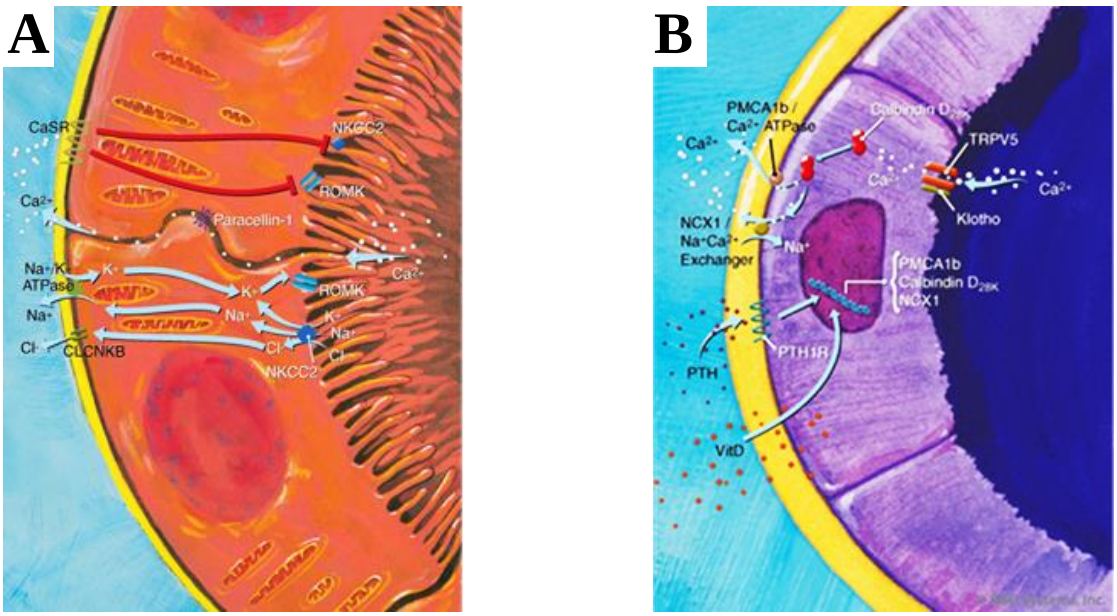

Figure 2.8 Passive diffusion (A**) and facilitated absorption (**B**) of calcium in the renal proximal tubule (R&D Systems, 2007)**

Phosphorous absorption requires the aid of phosphorous co-transporters, namely Na-P, PiT1, PiT2, and Type II (a, b and c). The mRNA for these co-transporters are found in various tissues; however, the intestinal tissue of the duodenum has the greatest mRNA expression, with the ileum containing the lowest mRNA expression. Na-P Type II co-transporters are regulated by PTH, dietary phosphorous concentration and FGF-23 (Proszkowiec-Weglarz & Angel, 2013).

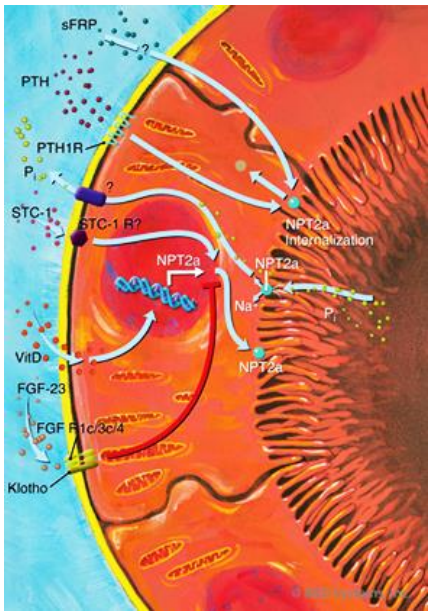

Figure 2.9 Transcellular absorption of phosphorous in the renal proximal tubule (R&D Systems, 2007)

Calcium and phosphorous interact with one another. Ca:aP ratios in the diet greater than 2:1 result in the excess formation of insoluble calcium-phosphorous complexes in the small intestine. This results in reduced phosphorous absorption and utilisation, and increased excretion of phosphorous, leading to a deficiency in phosphorous (Liem, 2009; de Carvalho Mello *et al.*, 2012; Proszkowiec-Weglarz & Angel, 2013).

Calcium and phosphorous levels in the plasma are regulated by the parathyroid gland (the chief cells synthesise and secrete PTH), thyroid gland (parafollicular C cells secrete calcitonin), vitamin D_3 and receptors in the small intestine, bone and kidneys (Liem, 2009; Proszkowiec-Weglarz & Angel, 2013).

Under conditions of low calcium concentration in the plasma, the active form of vitamin D_3 ($1,25(OH)_2D_3$) is synthesised by the activation of renal 1α-hydroxylase (activated by PTH). $1,25(OH)_2D_3$ inhibits renal 1α-hydroxylase and stimulates 24-hydroxylase (converts the active form of vitamin D_3 to the inactive form), thereby allowing self-regulation of vitamin D_3 under different plasma calcium concentrations (Liem, 2009; Proszkowiec-Weglarz & Angel, 2013).

Co-transporter PiT1, located in the parathyroid gland, senses dietary phosphorous and plasma $1,25(OH)_2D_3$ concentration, thereby regulating PTH secretion. Under conditions of high dietary phosphorous levels, PTH inhibits NaP Type II co-transporter, inhibiting renal phosphorous resorption. Under conditions of low dietary phosphorous levels, the NaP transporter recruits Type IIa and Type IIc, increasing phosphorous resorption. PiT2 regulates phosphorous absorption in the intestine when diets low in available phosphorous are consumed. Type II co-transporters are located in the renal and intestinal tissue (Han *et al.*, 2009; Liem, 2009; Proszkowiec-Weglarz & Angel, 2013).

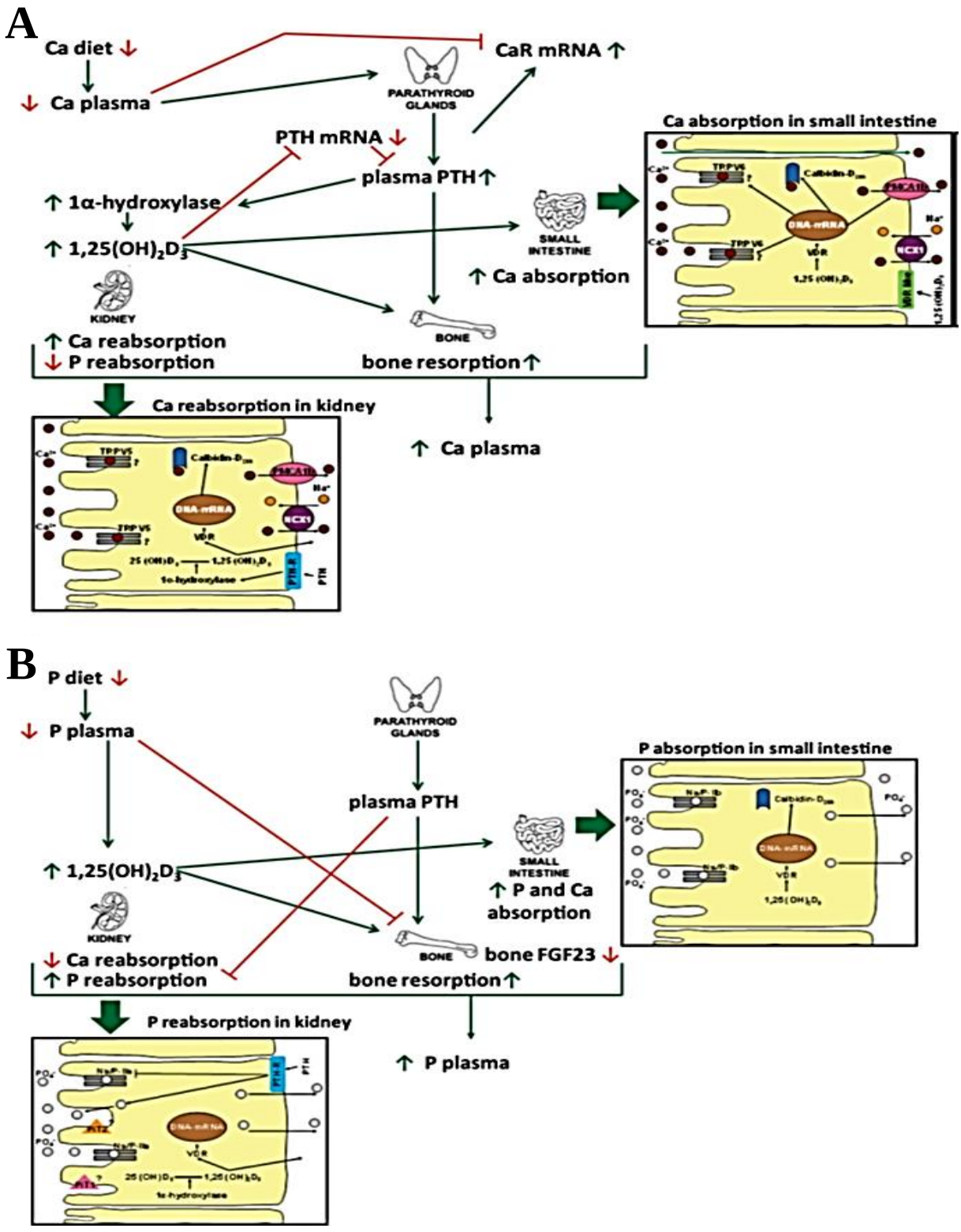

Figure 2.10 Simplified schematic of calcium (A) and phosphorus (B) regulation in the body (Proszkowiec-Weglarz & Angel, 2013)

2.3. Poultry digestive tract

2.3.1. Overview of the digestive tract of poultry

Digestion is defined as the process whereby feed components are chemically and enzymatically broken down in the digestive tract into simple chemical compounds that are absorbed by the body (Merriam-Webster, 2016).

The function of the digestive system is to convert dietary nutrients into chemical complexes that the body can absorb, use and incorporate into biological processes as energy, building blocks or co-factors for maintenance, growth and production (Fernandes *et al.*, 2012; Jacob, 2015).

Feed enters the mouth and travels down the oesophagus to the crop. Amylase in the saliva begins the process of digestion. The oesophagus is a tube that conveys the feed from the mouth to the crop (Jacobs, 2015).

The crop is a sac whose main function is to store feed. The crop controls satiety and hunger. When the crop is nearly empty, receptors send signals to the hypothalamus of the brain. In turn the bird will feel hungry and will eat (Jacobs, 2015). Salivary amaylase and microbial (predominantly *Lactobacilli*) activity occurs in the crop (McDonald *et al.*, 2011(a)). Although the presence of amylase begins the digestive process, very little digestion occurs in the crop (Jacobs, 2015). The initial pH of the crop mimics the pH of the feed. Most common broiler feed has a pH of 5.5−6.5 due to the level of limestone. As retention time in the crop increases, increased microbial fermentation occurs, producing organic acids and resulting in a decline in pH to 4.5−5.9 (Svihus, 2014).

Feed from the crop travels via the oesophagus and enters the proventriculus. The proventriculus is a glandular stomach where digestive enzymes and juices are secreted (Duke, 1984). Feed distends the proventriculus, stimulating the secretion of acetylcholine. Acetylcholine binds to G-cells, which stimulates the secretion of gastrin and gastrin-releasing peptides (GRP). Gastrin and GRP are neurocrine agents that result in the secretion of histamine. All the secreted

substances stimulate the secretion of hydrochloric acid (HCl) by binding to parietal cells. The HCl acts to reduce the pH of the digesta to 2. Pepsinogen is secreted in the proventriculus due to the presence of gastrin. At a pH of 2 the pepsinogen undergoes a chemical reaction resulting in the conversion of pepsinogen to activated pepsin. Pepsin is a proteolytic enzyme that cleaves the N-terminal of amino acids in the protein chain, thereby denaturing the protein in feed (Rynsburger, 2009). Oesophageal contractions move the feed from the proventriculus into the gizzard (McDonald *et al.*, 2011(a); Svihus, 2014; Jacobs, 2015).

The gizzard is a muscular organ whose main function is to grind, mix and mash feed. The muscular wall of the gizzard produces a protein-polysaccharide complex, koilin, which has an amino acid composition similar to that of keratin. When the koilin comes into contact with the hydrochloric acid from the proventriculus, the koilin hardens. This stimulates the rhythmic contractions required to grind feed. The pH of the gizzard ranges from 1.9−4.5 (average is 3.5). This variation in pH is due to the amount of limestone in a diet and the amount of hydrochloric acid secreted from the proventriculus. The gizzard aids in digestion by increasing the time and amount of contact between the dietary nutrients and the digestive enzymes from the proventriculus. The average retention time of feed in the gizzard is 30 minutes to 1 hour. The addition of structural dietary components (such as fibre and hulls) increases the retention time to 2 hours. Increased retention time results in increased hydrochloric acid secretion, thus lowering the pH of the gizzard (Rynsburger, 2009; McDonald *et al.*, 2011(a); Svihus, 2014; Jacobs, 2015).

Fine feed particles travel from the gizzard into the duodenum. The intestinal mucosa of the small intestine − composed of the duodenum, jejunum and ileum − produces mucin, α-amylase, maltase, sucrase, proteolytic enzymes and phosphatases (hydrolyses orthophosphoric acid esters). Digestion continues in the duodenum. The feed from the gizzard is mixed with pancreatic juices and bile resulting in a rapid increase in pH to approximately 6 in the duodenum. The jejunum and ileum are responsible for nutrient absorption. Feed retention time in the jejunum is approximately 60 minutes. The ileum empties into the large intestine (McDonald *et al.*, 2011(a); Svihus, 2014; Jacobs, 2015).

The pancreas, located in the duodenum fold, secretes phospholipase A_2 (lechithins hydrolysis), trypsin and chymotrypsin (protein and peptide hydrolysis) and triacylglycerol lipase

(triacylglycerol hydrolysis) to name a few. At the end of the duodenum two bile ducts and three pancreatic ducts open into the duodenum where their secretions enter the jejunum (McDonald *et al.*, 2011(a); Jacobs, 2015).

The liver plays a role in digestion. The left hepatic duct of the liver secretes enzymes into the duodenum directly. The right hepatic duct enters the gallbladder and stimulates the production of bile, which is then secreted into the duodenum (Duke, 1984).

Two blind sacs, the caeca, are located at the junction where the ileum meets the large intestine. The predominant purpose of the caeca is to reabsorb water and electrolytes that pass through the small intestine (McDonald *et al.*, 2011(a); Svihus, 2014). Nutrients (starch or protein) that bypass the small intestine enter the caeca. The caeca are colonised by microbes that ferment the nutrients to produce volatile fatty acids and B-vitamins (Jacobs, 2015).

The caeca empty into the colon. The colon's main function is water reabsorption. Any solid digestive waste is transported along the colon into the cloaca (Jacobs, 2015). Digestive waste from the colon and urinary waste is mixed in the cloaca. In this way, faeces and urine are excreted together (McDonald *et al.*, 2011(a); Jacobs, 2015).

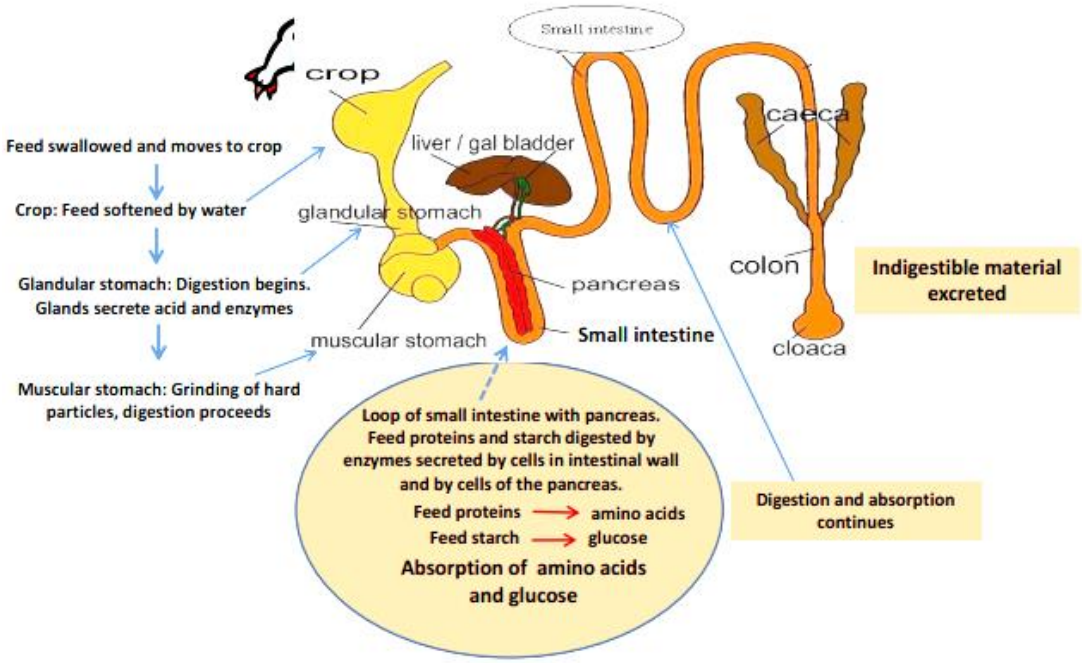

Figure 2.11 Schematic of the functions of the different parts of the digestive tract (Hayes, 2013)

2.3.2. Influence of intensive genetic selection on the digestive tract growth and development

The allometric growth of organs and body tissue over time marks the physiological age of animals (Henn *et al.*, 2014). The first two weeks post-hatch is important for maximum intestinal development as the future performance of the broiler is determined (Hossain *et al.*, 2014). In the first 72 hours post-hatch, nutrients from the residual yolk sac are absorbed (principally fatty acids over glucose and methionine) and directed predominantly toward intestinal development. The growth (as a percentage of bodyweight) of the pancreas and intestine reaches a plateau at approximately 8−14 days old, and then declines gradually with age. Enzyme activity (expressed as per unit of pancreas weight) increases rapidly from 5−8 days of age; however, the magnitude of the activity of the individual enzymes varies according to the dietary nutrients provided. There is a strong dietary nutrient by age interaction in young broilers compared to older broilers (Noy & Sklan, 1997; de Abreu Fernandes *et al.*, 2012; Lilburn & Loeffler, 2015).

Rapid broiler growth has resulted in an increase in the proportion of incubation and neonatal stage of development from 25% to 50% of the broilers production life. This is important as the nutritional intake, ability to resist environmental stress, immune system function and the diversity and stability of gut microorganisms during the neonatal stage determines whether the broilers achieve their genetic potential in terms of performance and production (Ferket, 2010). Another consequence of intense genetic selection for rapid growth is the reallocation of dietary nutrients toward increased breast muscle growth and development at the cost of organ growth and development (Tickle *et al.*, 2014). The first few weeks post-hatch is becoming increasingly important for optimising the efficiency of digestion and performance (Lilburn & Loeffler, 2015).

2.4. Phytate

2.4.1. Definition

Plants have a high phosphorous requirement; however, they can only absorb phosphorous from the soil when the phosphorous is present in its inorganic form. The inorganic form of phosphorous is sensitive to changes in soil pH. At a pH of between 5 and 6, inorganic

phosphorous uptake by plants is at its greatest. At a pH below this range, most of the phosphorous will be present in its organic form (H_3PO_4 and HPO_4^{-2}) therefore limiting phosphorous uptake by plants. Due to the plant's high phosphorous requirement and the relative variability of available phosphorous to the plant, the plant converts absorbed phosphorous to phytic acid (Schachtman *et al.*, 1998).

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphase) is the main storage form of phosphorous in the seeds of plant-based feed ingredients (Gupta *et al.*, 2015). Phytate, also known as $InsP_6$, is the salt form of phytic acid (Dersjant-Li *et al.*, 2015). Phytin is the mixed salt form of phytic acid, ie. $InsP_6$ with potassium, magnesium and calcium deposited as a complex within the plant (Selle & Ravindran, 2007; Dersjant-Li *et al.*, 2015). As the plant enters maturation, the phytic acid content on a dry-matter basis, increases (Liem, 2009). This is because during seed development an increased amount of phosphorus is stored in the seeds for use during seed germination (Selle & Ravindran, 2007). Therefore, approximately 50–85% of the total phosphorous found in the seeds of plant-based feed sources (legumes, cereals, nuts, oil seed) is in the form of phytic acid (Dersjant-Li *et al.*, 2015; Gupta *et al.*, 2015). Typical maize-soybean meal-based broiler diets contain 2.5–4g/kg of phytate phosphorous which, globally, equates to approximately one million tons of phytate phosphorous that is consumed annually by broilers (Selle & Ravindran, 2007).

Table 2.1 Phytic acid content of common feed ingredients used in monogastric diets (Gupta *et al.*, 2015)

Feed ingredient	*Phytic acid (g/100g)*
Maize germ	6.39
Wheat bran	2.1−7.3
Wheat germ	1.14−3.91
Rice bran	2.56−8.7
Barley	0.38−1.16
Sorghum	0.57−3.35
Lentils	0.27−1.51
Peas	0.22−1.22
Soybeans	1.0−2.22
Sunflower meal	3.9−4.3
Oats	0.42−1.16

Phytate is a ployanionic molecule that has 12 negative charges (Selle *et al.*, 2009). Phytate dissociates at a pH of less than 2 and becomes increasingly negatively charged as pH increases. An increase in negative charge is associated with an increase in the solubility of phytate. Thus, as the phytate passes from the gizzard/stomach (low pH) to the lower digestive tract (neutral pH) its negative charge increases, increasing its interaction with cations (Santos, 2012; Dersjant-Li *et al.*, 2015).

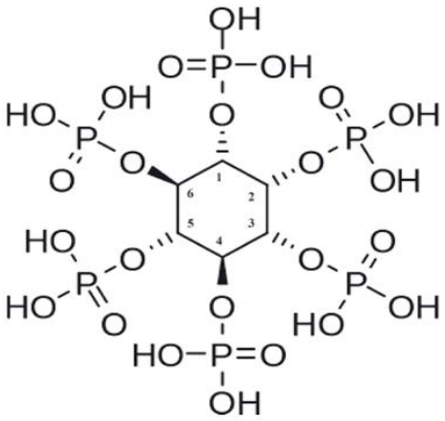

Figure 2.12 Structure of phytic acid (Dersjant-Li *et al.*, 2015)

2.4.2. Anti-nutritional effects of phytate

Cations are minerals, such as calcium, zinc, manganese and copper, which have dissociated at the low pH and become positively charged (Selle *et al.*, 2009). Calcium and phytate have a linear relationship; a diet high in calcium will reduce phytic phosphorous absorption and a diet high in phytate will increase the animals' requirement for calcium (due to decreased calcium absorption) from 0.60% to 0.95%. In the same way, deficiencies in zinc and copper in piglets also occur as zinc and copper have a high inclusion rate in the pre-starter and starter diets for the purpose of improved growth (Santos, 2012). This interaction results in the formation of stable, insoluble salts in the small intestine that are excreted, resulting in mineral deficiency even though the animal is supplemented with minerals at high levels (Santos, 2012; Dersjant-Li *et al.*, 2015). Additionally, the Ca-phytate complex reduces saturated fat digestion as it results in the formation of metallic soaps in the lumen of the gut (Dersjant-Li *et al.*, 2015).

Phytate attracts water molecules. This reduces the amount of water molecules surrounding protein molecules, resulting in reduced protein solubility (Santos, 2012). At an

acidic pH (in the gizzard) phytate non-selectively binds to basic amino acids (such as histidine, arginine and lysine) forming insoluble protein-phytate complexes. Phytate can also bind protein through cations forming protein-mineral-phytate complexes in the small intestine when the pH is above the isoelectric point of the protein (Dersjant-Li *et al.*, 2015). Between pH 0.8 and 2.8 phytate-mineral complexes suppress the activity of pepsin, trypsin, carboxypeptidase-A and α-amylase in the stomach/ gizzard resulting in reduced protein digestion in the upper digestive tract. This leads to an increased amount of protein entering the lower digestive tract to be fermented, which increases the amount of nitrogen available for bacteria. Such an increase leads to optimal conditions for potentially pathogenic bacteria to grow more rapidly than beneficial bacteria, which increases the risk of intestinal disease (Selle *et al.*, 2011; Olukosi, 2012; Santos, 2012).

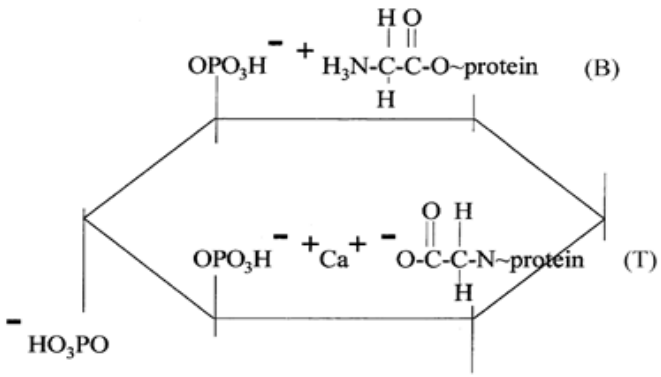

Figure 2.13 Binary (B) and ternary (T) protein-phytate complexes (Selle *et al.*, 2000)

Undigested protein reaching the duodenum results in increased gastrin, HCl and pepsinogen secretions. These secretions result in increased mucin and sodium bicarbonate production because they irritate the gut mucosa. This results in increased endogenous amino acid losses and energy expended by the animal. To compensate for this increase in expended energy the animal will consume more feed, which increases the total feed cost of the operation (Selle *et al.*, 2011; Santos 2012).

The pancreas increases sodium bicarbonate secretion as digesta enters the lower digestive tract in order to increase the pH of the digesta. The sodium deficiency compromises the NaK-ATPase pump, which is responsible for absorbing amino acids and other nutrients; therefore, the

absorption of arginine, glutamine, glycine, leucine and valine is inhibited (Selle *et al.*, 2011; Santos, 2012).

Overall the anti-nutritional (and antiphysiological) effects of phytate result in decreased protein digestibility and nutrient utilisation, increased maintenance energy and decreased energy available for production. These, in turn, result in reduced performance and increased production costs (Liu *et al.*, 2008(b); Dersjant-Li *et al.*, 2015).

2.4.3. Endogenous phytases

The main site for phytate dephosphorylation is in the forestomach, which is composed of the crop, proventriculus and gizzard.

Endogenous plant phytase activity is considered to be negligible in most cereal feed ingredients; however, it has been proven that wheat, barley, rye and triticale contain significant phytase activity. The main disadvantages to endogenous plant phytases are that they are active in a narrow pH spectrum, they are unstable and heat liable, and are often inactivated during feed processing due to the high temperatures. Endogenous plant phytases are more susceptible to endogenous proteolytic enzymes and become inactivated in the acidic environment of the forestomach. Therefore, plant phytases are not efficient or effective in breaking down phytate in the forestomach of broilers (Selle & Ravindran, 2007; Selle *et al.*, 2011).

Gut microflora in monogastrics have a limited ability to break down phytate in the large intestine by secreting endogenous phytase enzymes, resulting in the release of bound phosphorous to be absorbed and used by the animal. However, the insoluble complexes formed due to the interaction between phytate and dietary nutrients reduce the efficiency of the endogenous phytase enzymes. Additionally, the released phosphorous and other dietary nutrients from the microbial breakdown of phytate in the hindgut cannot be absorbed, and thus used, by the animal (Selle *et al.*, 2009; Selle *et al.*, 2011; Gupta *et al.*, 2015).

Endogenous mucosal phytases are located in the small intestine. Cowieson *et al.* (2011) notes the misconception that a lack of endogenous phytase activity is the cause of poorly

digested phytate phosphorous in poultry. The basis of this misconception can be found in the review by Selle & Ravindran (2007) and Dersjant-Li *et al.* (2015), where it is stated that endogenous mucosal phytase has the ability to digest 69.2% of phytate phosphorous when the maize-soy-based diet contained 2g Ca/kg feed, and 89% in maize diets formulated without limestone. It was found that when Ca was increased to 5g Ca/kg, phytate digestion by endogenous mucosal phytases decreased to 25.4%.

Truong *et al.* (2017) stated that both mucosal and microflora phytase activity is affected by the levels of dietary calcium and phosphorous. Calcium is often included in the diet in the form of limestone. Limestone has a high acid-binding capacity (making it an effective pH buffer), resulting in increased crop pH. Increases in crop pH result in increased interaction between phytate and other dietary nutrients. This results in increased precipitation of stable insoluble complexes, which reduces the ability of endogenous and microbial phytase to degrade phytate (Selle & Ravindran, 2007). Additionally, increases in crop pH also reduce the activity of pepsin, which reduces protein digestion (Bedford *et al.*, 2016). Phosphorous and calcium deficient diets result in increased mucosal and microflora phytase activity. A study by Ptak *et al.* (2015) showed that crop pH decreased when calcium and phosphorous-deficient diets were fed.

The above factors result in 70% of the total phosphorous being excreted. Leaching or surface run-off of the excreted phosphorous results in eutrophication of surface water, algae blooms, hypoxia, death of aquatic species and production of nitrous oxide (Selle *et al.*, 2011; Gupta *et al.*, 2015).

2.4.4. Methods to reduce phytate and increase available phosphorous

A typical maize-soybean meal broiler diet contains approximately 0.25% phytate (Liem, 2009). One technique used to reduce the amount of phytate present in a broiler diet, and the impact of phytate on animal performance and the environment, was to genetically modify the cereal plants (Gupta *et al.*, 2015). Depending on the cultivar, maize can contain 0.18% to 0.25% phytate (Liem, 2009). The low-phytic acid (lpa) mutation was located and isolated in maize, barley and rice cultivars. This mutation was used to develop hybrid cultivars that contained

normal levels of phosphorous, but low levels of the phytic acid form and increased levels of the inorganic form of phosphorous. The lpa mutation can reduce the amount of phytic acid in the seeds of cereal grains by 50% to 95% (Raboy, 2002). Low phytate soybean meal contains 0.096% phytate while normal soybean meal contains 0.408% to 0.639% phytate (Liem, 2009). The main drawback to this technique was that low phytate hybrid cultivars had an approximately 5.5% lower yield, caused by reduced seed weight, impaired seed development, poor germination and seedling establishment, and retarded vegetative growth (Raboy, 2002; Shi *et al.*, 2007).

Some studies have shown that phytate phosphorous use is improved when diets are supplemented with organic acids (Liem, 2009). The first known reports of citric acid effects on phytate-containing diets were in 1937 at Harvard, followed by a study conducted by Pileggi *et al.* in 1956 (Boling *et al.*, 2000). The Pileggi *et al.* (1956) study using rats showed that when a 1:1 mixture of citric acid: sodium citrate was added to a diet that contains phytate, femur ash increased by 61% and faecal phosphorous decreased from 94% to 48%. It was reasoned that the effect of citrate on phytate use was because of the ability of citrate to bind to calcium, preventing the formation of insoluble calcium-phytate complexes (Pileggi *et al.*, 1956). Boling *et al.* (2000) evaluated the effect of citric acid on phytate use in young chicks and pigs fed a phosphorous-deficient maize-soybean meal diet. The study found that increasing levels of citrate supplementation resulted in a linear increase in tibia ash and weight gain in broiler chicks. Optimum results were seen when broiler diets were supplemented with citrate at 6% and pig diets at 3%. They suggested that research should be done on supplementing a combination of citrate at lower levels and phytase. Other organic acids that have the potential to improve phosphorous use, but have not been evaluated for it, are gluconic acid (acidifier, coagulant and mineral carrier), fumaric acid, Alimet (organic acid methionine source) and ethylenediaminetetraacetic (chelating agent), also known as EDTA (Rafacz-Livingston *et al.*, 2005). Studies by Rafacz-Livingston *et al.* (2005) and Liem *et al.* (2008) demonstrate that EDTA does not improve phytate phosphorous use. EDTA supplementation reduced feed intake and weight gains. Supplementation with a combination of citric acid, fumaric acid and malic acid increased tibia bone ash numerically. Supplementation with a combination of 1.5–3% gluconic acid and 1% Alimet resulted in significant improvements ($p<0.05$) in tibia ash and weight gain. Methionine source was established as not influencing phytate phosphorous disappearance, tibia ash, feed intake and body weight; however, when phytase was added to the diet, the percentage

of tibia bone ash and phytate phosphorous disappearance was greater in birds fed Alimet compared to birds fed DL-Met. The main drawback in using organic acid supplementation to increase phytate phosphorous use is that it is not economical to supplement these (and lower) levels of organic acids in a diet (Boling *et al.*, 2000).

Research into *in-ovo* feeding gained interest due to its epigenetic effects. Epigenetics is defined as alterations in the physical characteristics of an organism due to changes in gene activity not caused by differences in the DNA sequence. DNA methylation, histone modifications and non-coding RNA cause changes in the expression of genes encoded in the DNA, which changes the gene's activity and the physical characteristic that that gene controls. Epigenetic effects during perinatal development have a life-long influence on the physiology and development of the animal. Thus, nutrition and management during this period is critical (Ferket, 2010; Pierce, 2012). Collaborations between Drs Ferket and Uni at the Hebrew University of Jerusalem led to developments in *in-ovo* feeding. Implementing gene array technology showed that when nutrients are injected into the amnion during late-stage development of the egg, the expression of 50 genes are altered. The physical ramifications are an improved hatchability rate, chick quality and skeletal growth, increased glycogen reserves, advanced intestinal development and muscle development (Ferket, 2010). A study by Sgavioli *et al.* (2016) injected ascorbic acid in-ovo and then subjected the eggs to high incubation temperatures in an attempt to promote the epigenetic adaptation of the broiler chicks to high environmental temperatures, which would reduce heat stress and support their skeletal development. However, the outcome showed that in-ovo injection of ascorbic acid did not reduce heat stress or improve bone development when broilers were subjected to high environmental temperatures. A study by Ebrahimi *et al.* (2015), where 1α-OHD_3 was injected on day 18 of incubation, showed that in-ovo injection resulted in improved body weight gain, FCR and carcass traits compared to the positive control. However, diet supplementation with 1α-OHD_3 post-hatch showed greater improvements in performance and carcass traits, in addition to improved bone strength and mineralisation.

Enviropig was the first transgenic pig developed in 2001 to overexpress the *E.coli* appA gene in the salivary glands. This overexpression led to the constant secretion of phytase directly into the digestive tract, which increased phytate degradation and resulted in a 75% decrease in faecal phosphorous excretion (Cho *et al.*, 2006; Lei *et al.*, 2013). The ability of broilers to digest

phytate was reported to be inheritable. Zhang *et al.* (2003) conducted a study to estimate the heritability and genetic correlation of phytate phosphorous bioavailability in an unselected, random-mating broiler population. The results showed that: phytate phosphorous bioavailability follows a normal distribution curve and, thus, is characterised as a quantitative trait; and the genetic correlation between phytate phosphorus bioavailability and body weight gain and feed intake was moderate and negative. This led to further research into genetically selecting broilers for increased phytate degradation (Selle & Ravindran, 2007). The endoplasmic reticulum in animal cells contains multiple inositol polyphosphate phosphatase (MINPP). MINPP has phytase activity over a pH range of 4.5–7.5, which is similar to that of the poultry digestive tract, indicating the potential to effectively degrade $InsP_6$; however, as it is not secreted in the secretory pathway it cannot digest dietary $InsP_6$. Wild-type MINPP was genetically modified so that the secretion of ADEL-MINPP was 5–6 times that of the wild-type MINPP and was secreted into the secretory pathway. However, the results showed that ADEL-MINPP had no impact on the cellular levels of InsP6 (Cho *et al.*, 2006). The drawbacks of transgenic animals are consumer perception and acceptance, industry hesitation and technological constraints (Lei *et al.*, 2013).

Another method proposed was to chemically degrade the phytate in feed during feed processing; however, this could substantially reduce feed quality. The inoculation of phytase-producing microorganisms directly into the gastro-intestinal tract was considered, but raised a number of concerns such as the over-colonisation of these bacteria resulting is disrupted microbial ecology and balance, the faecal contamination of these microorganisms and the cost of inoculation. Another alternative, which has become the most practical and economical method, is the use of exogenous phytase enzymes to reduce the negative effects of phytate (Lei *et al.*, 2013).

2.5. Exogenous phytase enzyme

2.5.1. Definition

Enzymes are biological catalysts derived from globular proteins that speed up existing chemical reactions in the body, break down large molecules to smaller molecules, and bind two

or more molecules to create a new molecule (Cambell & Farrell, 2012; Castro, 2014). Each enzyme is composed of a unique sequence and number of amino acids arranged in a three-dimensional configuration. This determines the function of the enzyme and the specific substrate that the enzyme can bind with. The active site is where the catalytic reaction occurs (Cambell & Farrell, 2012).

Phytases (myo-inositol hexakisphosphate phosphohydrolase) are protein enzymes that catalyse the stepwise removal of phosphorous from phytic acid/phytate, releasing inorganic phosphorous and myo-inositol ring (Dersjant-Li *et al.*, 2015).

The stepwise dephosphorylation of phytate results in increased concentrations of lower myo-inositol phosphate 1 to 5 ($InsP_{5,4,3,2,1}$) esters (Selle & Ravindran, 2007). The lower InsP esters have increased solubility and, thus, reduced ability to bind to cations and proteins compared to $InsP_6$, greatly reducing the anti-nutritional effects of phytate (Dersjant-Li *et al.*, 2015).

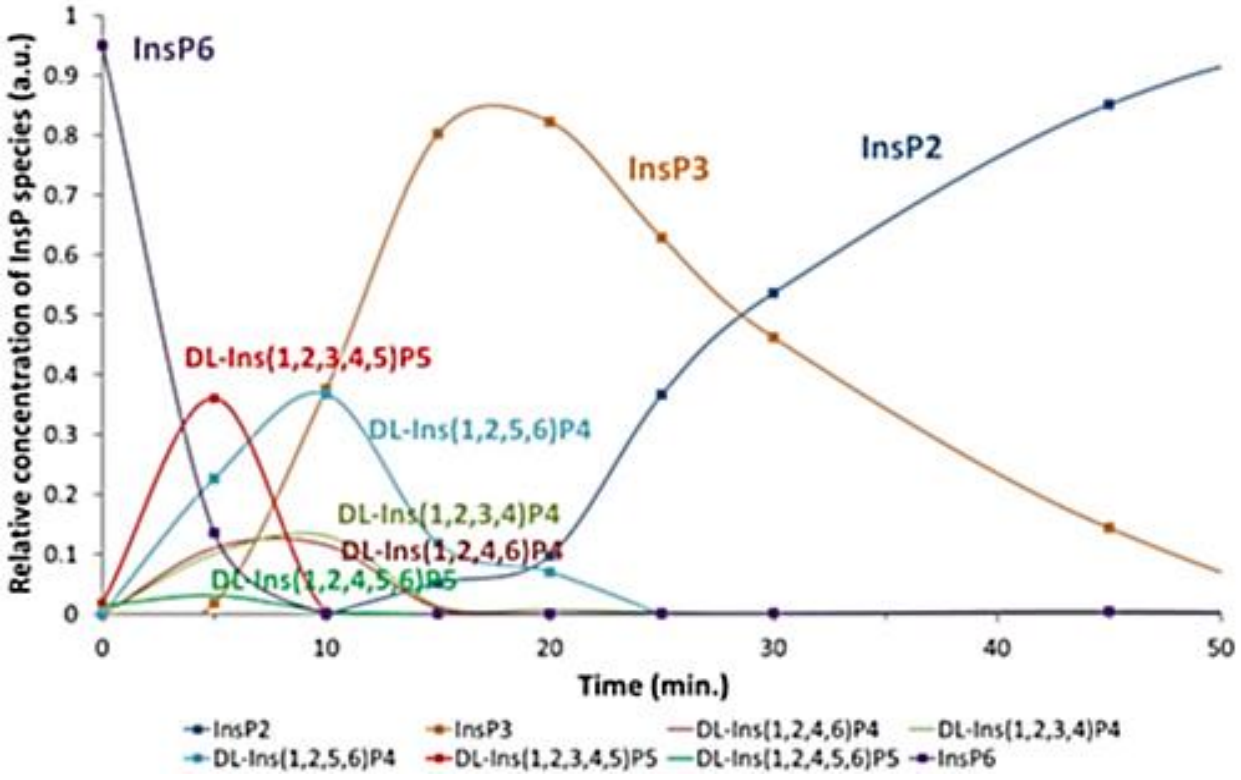

Figure 2.14 Digestion profile of phytate by phytase (Ariza *et al.*, 2013)

Table 2.2 Solubility of the different InsP esters in the digestive tract of a pig (Cowieson *et al.*, 2011)

InsP ester	*Small intestine*	*Large intestine*
InsP$_6$	2%	2%
InsP$_5$	7%	3%
InsP$_4$	8%	0%
InsP$_3$	31%	6%
InsP$_2$	75%	24%

2.5.2. Classification of Phytases

Classification of phytases is based on different properties, as can be seen in table 2.3. Alkaline phytases, such as BPPhy found in *Bacillus spp*, requires Ca^{2+} as a co-factor to reach maximum catalytic activity. When three Ca^{2+} ions bind to the low-affinity Ca-binding sites on the BPPhy molecule, a favourable environment is generated in the cleft, resulting in increased binding of phytate to BPPhy. In contrast, acid phytases such as HAPhy and PAPhy, do not require co-factors for maximum catalytic activity. PAPhy is located in wheat and barley and contains binuclear centres consisting of Fe^{3+} ion bound to Fe^{2+}, Mn^{2+} or Zn^{2+}. HAPhy contains a conserved sequence motif near the N-terminus (RH(G/N)XRXP) and C-terminus (HD-motif). HAPhy activity is inhibited when Zn^{2+}, Fl, Mo and orthophosphate is present (Greiner & Konietzny, 2011).

Table 2.3 Classification of phytase based on different properties (Greiner & Konietzny, 2011; Gupta *et al.*, 2015)

Phytase	*Description*
Based on catalytic mechanism	
HAPhy	Histidine acid phytases
BPPhy	β-propeller phytases
CPhy	Cysteine phytases (also called Protein tyrosine phosphatase [PTPhy])
PAPhy	Purple acid phytases
Based on pH optimum	
Acid Phytase	pH optimum ±5
Alkaline Phytase	pH optimum ±8
Based on carbon in the myo-inositol ring of phytate at which dephosphorylation is initiated	

3-Phytases	Initiates dephosphorylation at the third carbon position (E.C.3.1.3.8) Generally, of bacterial and fungal origin
6-Phytases	Initiates dephosphorylation at the sixth carbon position (E.C.3.1.3.26) Generally, of plant origin
5-Phytases	Initiates dephosphorylation at the fifth carbon position (E.C.3.1.3.72)

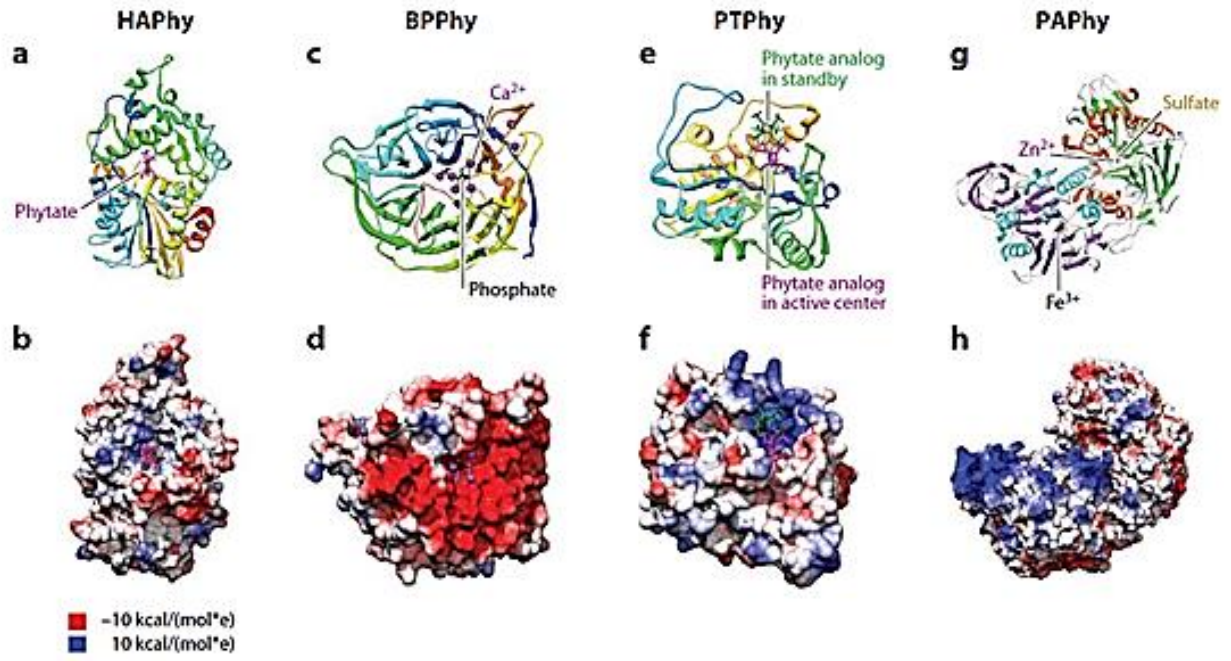

Figure 2.15 Three-dimensional structure of the different phytase classes (Lei *et al.*, 2013)

Natuphos was the first commercially produced 3-phytase derived from *Aspergillus niger* in the Netherlands in 1984. Phytase was first introduced in the Netherlands because of new legislation imposed by the government to reduce nitrogen and phosphorous pollution to protect ground and water quality. Over time, phytase supplementation became accepted globally as inorganic phosphorous is a limited natural resource, which resulted in escalated prices. Consequently, it became more cost-effective to supplement diets with phytases instead of inorganic phosphorous. Another factor contributing to the increased acceptance of exogenous phytase supplementation is the decreased supplementation, and in some countries, prevention, of animal by-product protein meals (such as fish meal, blood meal and meat and bone meal). Protein meals from animal origin are high in bioavailable phosphorous; however, due to the scare of mad cow disease and consumer perception, it is becoming less favourable to supplement diets with these protein meals. Increased global use of supplemental phytase has led to the

development of fungal 6-phytases derived from *Peniophora lycii*, which was characterised by improved thermo-stability and gastric stability (Kemme *et al.*, 1997; Selle & Ravindran, 2007; Selle *et al.*, 2009; Ariza *et al.*, 2013).

Microbial phytases are by far the most used and researched phytase on the market due to their cost-effective and rapid production, high thermo-stability and improved efficiency in phytate degradation, compared to phytases of plant origin (Gupta *et al.*, 2015). Exogenous phytases commercially produced today are HAPhy (can initiate hydrolyses at C3 or C6 position) phytases of bacterial origin, as they have greater resistance to pepsin and pancreatin degradation compared to HAPhy of fungal origin (Griener & Konietzny, 2011; Dersjant-Li *et al.*, 2015; Gupta *et al.*, 2015).

Different techniques have been used to improve the thermo-stability, pH, and gastric stability of wild-type phytases. These techniques are (Lei *et al.*, 2013):

1) Random mutagenesis – involves the use of polymerase chain reaction (PCR) and GSSM, which isolates and exploits mutant genes that manifest the improvements in the desired characteristic
2) Rational design – makes use of site-directed mutagenesis (SDM), which involves substituting amino acids and changing the position and number of disulfide bridges in the proteins' amino acid sequence that will result in the improvement of the desired characteristic
3) Semirational design –makes use of rational design to improve the effectiveness of random mutagenesis
4) Coating the phytase granules to increase the thermo-stability
5) Adding stabilising agents to the phytase product to improve stability.

Table 2.4 Examples of the different classes of phytases with their characteristics (Lei *et al.*, 2013; Derjant-Li *et al.*, 2015)

Classification	*Taxa*		*Protein origin*	*Expression*	*pH optima*	*Temperature optima (oC)*
HAPhy	Bacteria	Gram negative	*E.coli* AppA	S.pombe (ATCC 5233) P.pastoris, T.reesei	4.5	55
			Buttiauxella spp	T.reesei	3.5–4.5	60
	Fungi	Ascomytes	A.niger Phy A	A.niger, T.reesei	2–5.5	65
		Basidiomycetes	P.lycii Phy A	A.oryzae	4–5.5	50–55
	Plants	Monocots	Z. mays Phy S11	-	4.8	55
	Animals		Avian MINPP	-	5	-
BPPhy	Bacteria	Gram positive	B.amyloliquefaciens DS11	-	7-8	70
PAPhy	Plants	Monocots	T.aestivum	-	6	45
		Dicots	G.max	-	4–5.5	58
CPhy	Bacteria	Gram negative	S.ruminantium PhyAsr	-	4.5–5.5	50–55

2.5.3. Factors influencing phytase activity

The aim of nutrition is to formulate a diet that is cost effective while providing the optimal balance of nutrients to the animal. In order to obtain this, over- and underestimation of the nutritional value (matrix) that feed additives contribute to a diet must be avoided (Bedford *et al.*, 2016). Phytases are given calcium, phosphorus, protein and energy matrix values when formulated into feed. Phytase is not given a sodium matrix value even though various studies have observed that phytase supplementation influences the amount of sodium lost. This leads to high dietary sodium levels and results in increased water intake, which, in broilers, results in wet litter. Wet litter increases microbial growth, and ammonia and odourant emissions in the broiler house, resulting in reduced broiler welfare (Sharma *et al.*, 2016). Variation in activity between the different commercial phytases is caused by the difference in enzyme origin, i.e. fungal, bacterial or plant origin. The characteristics of an ideal phytase enzyme are (Gupta *et al.*, 2015; Dersjant-Li *et al.*, 2015):

- Active over a broad pH range

- Catalytically efficient (degrade phytate as quickly as possible to minimise formation of insoluble complexes)
- Thermostable (as feed processing can denature the enzyme)
- Resistant to protease enzymes
- Affordable
- Easily incorporated into feed by the feed manufacturer

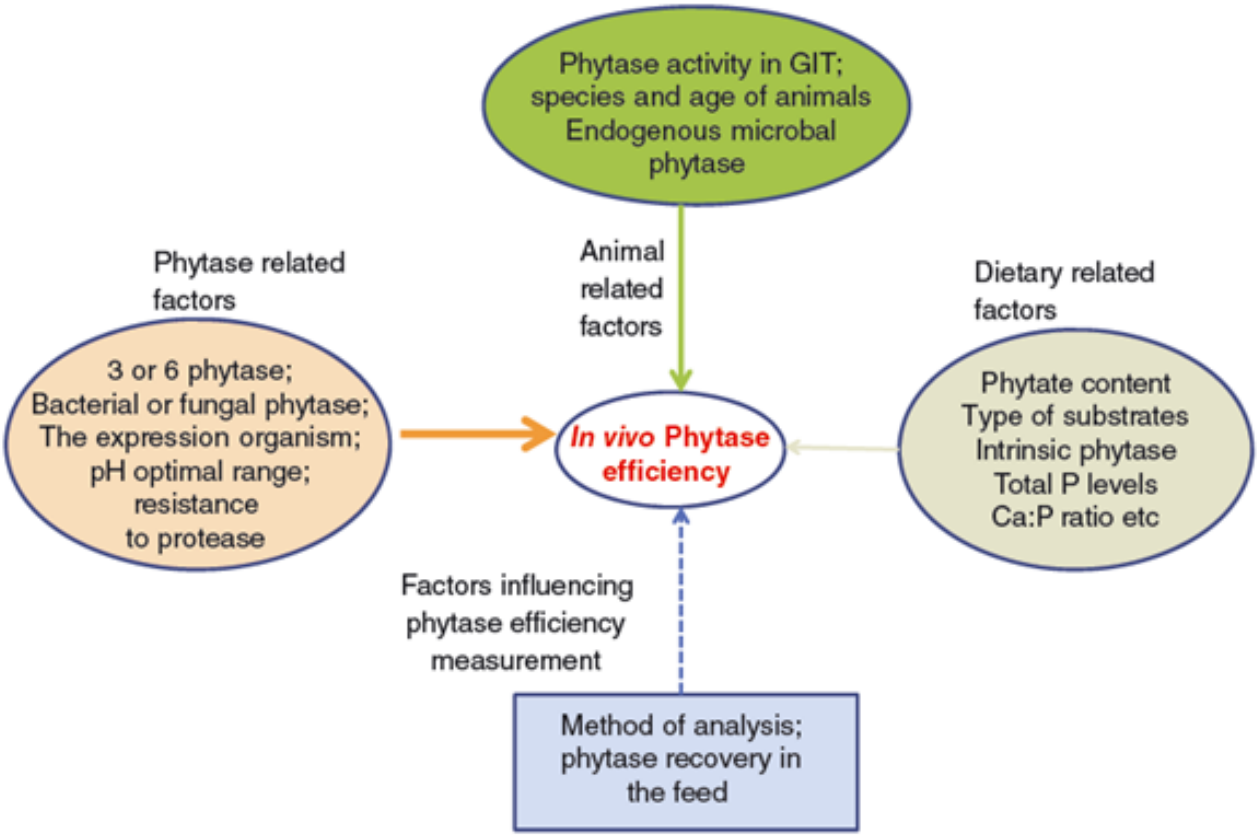

Figure 2.16 Factors influencing phytase activity within the animal (Dersjant-Li *et al.*, 2015)

Phytase is a protein molecule; consequently, it can by digested by endogenous proteases secreted into the proventriculus and gizzard. Phytases from different sources have different molecular structures resulting in differences in activity, optimal pH, catalytic efficiency and thermo-stability. Bacterial phytases have a greater proteolytic resistance and affinity for $InsP_6$ compared to fungal phytases (Gupta *et al.*, 2015). For example, *E.coli*-derived phytase has better activity against soy protein compared to phytase from *A.niger*. The location of the first

hydrolysed phosphate influences the binding capacity of the phytate molecule, and thus its ability to form insoluble complexes. $InsP_3$ has only 11% of the binding capacity of $InsP_6$. As a result, enzymes catalysing phosphate removal stepwise from the carbon 6 position will be the most efficient (Dersjant-Li *et al*., 2015).

Endogenous phytase activity occurs in the colon of pigs and poultry. Supplementing diets with exogenous phytase shifts phytase activity to the stomach (crop in poultry) and upper small intestine. The pH in a pig's stomach is 2–2.5 while the pH in the crop is 5.2–5.8. This difference in pH of the main phytase activity sites between species results in different catalytic efficiencies of the phytase enzyme (Dersjant-Li *et al*., 2015). In poultry, the weight of the gizzard also plays a role in the extent of phytase activity. The larger the gizzard, the greater the retention time of feed and the greater the interaction between phytase and phytate, resulting in a greater extent of phytate dephosphorylation (Selle *et al.*, 2011).

The dietary calcium and phosphorous level, phytase dose, phytate level and their interactions determines the amount of, and rate at which, insoluble mineral-phytate or protein-mineral-phytate complexes are formed (Dersjant-Li *et al.*, 2015). A study conducted by Amerah *et al.* (2012) showed that the degree to which phytase can dephosphorylate phytate is highly dependent on the dietary Ca level, as can be seen in table 2.5. An adequate dietary phosphorus level results in decreased phytase activity. This is because dietary phosphorous is broken down to orthophosphoric acid esters. Microbial phytase activity is inhibited by orthophosphate. Phosphorous released from phytase also reduces phytase activity as it results in imbalances between calcium and phosphorus. Thus, microbial phytase activity is maximised when phosphorous-deficient diets are provided (Kemme *et al.*, 1997; Selle & Ravindran, 2007; Selle *et al.*, 2011). The phytate source influences the magnitude of phytate dephosphorylation. For instance, phytate present in maize is more readily available than phytate in sunflower seed meal; thus, the effect of enzyme supplementation is better observed in maize-based diets compared to sunflower seed meal-based diets (Kemme *et al.*, 1997).

Table 2.5 Influence of calcium to available phosphorous (Ca:AvP) ratio and phytase supplementation on ileal phytate degradation (%) and ileal digestibility of calcium and phosphorous. Values with different superscripts are significantly different (p<0.05) (Amerah *et al.*, 2012)

	Phytate degradation (%)	*Digestible P (%)*	*Digestible Ca (%)*
Ca:AvP			
1.43	69.9[a]	71.0[a]	55.0[ab]
2.14	57.8[b]	62.5[b]	45.6[c]
2.86	59.9[b]	61.5[b]	52.1[bc]
3.57	57.9[b]	59.2[b]	60.2[a]
Phytase (FTU/kg diet)			
0	43.8[b]	55.1[b]	55.2
1000	78.9[a]	72.0[a]	51.2

Apart from the factors affecting phytase activity, there are also factors that affect the magnitude and direction of phytase supplementation response in the animal. Factors that push the response in a negative direction would be the animal's performance in the control group, the year in which the study commenced, dietary phosphorous content, the use of cages or pens, and the percentage of maize in the final diet. Factors that push the response in a positive direction would be the duration of the trial, the phytase dose, mortality in the control group being greater than 5%, inclusion of coccidiostat, and dietary fat content (Bedford *et al.*, 2016).

2.5.4. Super-dosing concept

Commercially, phytase is supplemented at 500FTU phytase/kg diet, as it is the most economical dose for the purpose of improved phosphorus use and broiler performance. The concept of super-dosing arises from the "extra-phosphoric effects" of elevated phytase doses (Lee *et al.*, 2003). Cowieson *et al.* (2011) stated that 500FTU/kg phytase liberated Ca and P to a ratio of, or greater than, 2:1. As inclusion levels of phytase increased, the amount of P liberated increased linearly while the amount of Ca liberated reached a plateau. Increasing the phytase dose from 500FTU/kg to 1000FTU/kg resulted in a further 30% increase in phytate degradation. This translated into further increases in bone mineralisation and more pronounced increases in amino acid digestibility, energy available and improved immune function (Lee *et al.*, 2003). A

study by Manobhavan *et al.* (2015) showed that phytase supplementation between 1000FTU/kg and 5000FTU/kg significantly increased the bone length ($p<0.001$), bone width and mineral content of bone (Ca, P, Mg and Zn) compared to broilers supplemented with the standard 500FTU/kg phytase. Ptak *et al.* (2015) suggested that increased available phosphorus in the lumen of the small intestine increased the number of anaerobic bacteria in the ileum, resulting in increased fermentation of dietary feed. Therefore, high inclusion levels of phytase play a role in modulating microflora in the gastro-intestinal tract.

Myo-inositol has been found to reduce the effects of vitamin E deficiency in chicks, increase leucocyte numbers in turkeys and improve overall performance in swine and poultry (Cowieson *et al.*, 2011). A study by Karadas *et al.* (2010) showed that phytase supplemented at 12500 FTU/kg increased the levels of vitamin E (α-tocopherol), vitamin A (retinol) and co-enzyme Q10 in the liver of growing broilers, in addition to significantly improving body weight gain ($p<0.05$) and FCR compared to 500FTU/kg phytase. Cowieson *et al.* (2011) stated that myo-inositol has a lipotropic effect. Thus, the increased liberation of myo-inositol from phytate could indirectly protect against fatty liver syndrome.

2.5.5. Methods to determine phytase activity

An assay was developed by Harland and Harland in 1980 that directly measures phytase activity by determining the amount of phosphate released by the enzyme. This assay is based on colorimetric measurements of phosphomolybdate (Gupta *et al.*, 2015). Phytase activity is expressed as FTU. FTU is defined as the amount of phytase that liberates 1mmol of inorganic phosphate per minute from 0.0051mol/L sodium phytate at pH 5.5 and temperature 37ºC. The main problem with determining phytase activity directly in the laboratory is that the pH in the stomach/gizzard is less than 5.5; thus, the true activity of the phytase enzyme in the animal is different from the activity measured in the lab (Dersjant-Li *et al.*, 2015).

Another method of measuring phytase activity is by measuring amino acid digestibility using inert markers such as chromium dioxide (CrO_2), acid insoluble ash (AIA) and titanium dioxide (TiO_2). CrO_2, although a cheaper marker, is not uniformly distributed in the digesta

during transit along the digestive tract and does not have complete recovery rates, which overestimates the digestibility of the control diet and results in decreased measured phytase response (Selle & Ravindran, 2007; Cowieson & Bedford, 2009).

Table 2.6 Effect of phytase on the coefficient of apparent ileal digestibility (CAID) of amino acids using different markers (Selle & Ravindran, 2007)

Amino acid	*Acid insoluble ash or titanium oxide*		*Chromic oxide*	
	CAID	**Response (%)**	**CAID**	**Response (%)**
Arginine	0.846	3.48	0.904	1.03
Histidine	0.784	4.64	0.856	1.63
Isoleucine	0.786	4.28	0.836	2.55
Leucine	0.786	4.77	0.867	1.44
Lysine	0.825	3.96	0.878	1.08
Methionine	0.899	1.75	0.907	0.55
Phenylalanine	0.798	4.62	0.865	1.22
Threonine	0.738	6.55	0.784	2.29
Tryptophan	0.783	4.57	0.838	1.59
Valine	0.775	4.97	0.834	1.99

Bone quality can be determined by measuring several parameters. Bone strength is directly correlated to the weight of bone, which is dependent on the growth of the animal. Bone breaking strength is an invasive method developed to indirectly determine bone development and volume (Rath *et al.*, 2000; Almeida Paz & Bruno, 2006). Apart from nutrition, there is a variety of other factors that affect bone strength such as toxins, growth and age, gender, endocrine, disease, genetics and physical weight (Rath *et al.*, 2000). Bone ash can be used as an indirect measure of phytase activity because the amount of ash is directly correlated to bone mineralisation and integrity, and bone mineralisation is directly correlated to phosphorous and calcium deposition (Hall *et al.*, 2003; Shim *et al.*, 2012). There are two methods to determine bone ash; the AOAC method B (1955) and the Bolin-Frankenbach method (2001). The procedures for these methods are summarised in table 2.7. Although the Bolin-Frankenbach method is less time consuming and labour intensive compared to the AOAC method B, it has a greater variance. Therefore, to detect a 2% difference in bone ash with a type I error (false positive result) of 0.05 and a type II error (false negative result) of 0.90 in 21-day-old broilers, the Bolin-Frankenbach method requires a minimum of 114 samples where the AOAC method B

requires a minimum of 40 samples (Hall *et al.*, 2003). Recently, the Bolin-Frankenbach method was successfully used to determine tibia ash in the study by Sharma *et al.* (2016).

Table 2.7 Description of the two methods to determine bone ash (Hall *et al.*, 2003)

AOAC method B (1955)	***Step***	*Bolin-Frankenbach method (2001)*
Bones placed in boiling water for 5 min to loosen the flesh	1.	Tibia is autoclaved under 6.82kg of pressure for 8–12min
Bones chilled and stripped of adhering flesh (using scissors and forceps) and cartilage caps	2.	Bones are cooled and cleaned of all tissue, including cartilage caps
Bones soaked in ethanol overnight	3.	Bones are dried at 105 ^{o}C for 24 hours
Bones extracted for 24 hours with ethanol in a Soxlet extractor	4.	Samples are weighed
Bones extracted for 24 hours with anhydrous ether	5.	Ashed at 600 ^{o}C for 24 hours
Dried at room temperature for 24 hours	6.	Weighed
Weighed	7.	% tibia ash = (ashed weight / dry weight) X 100
Ashed overnight at 600 ^{o}C	8.	
Weighed	9.	

Bone mineral density is a reliable and non-invasive method to determine bone quality. Calcium and phosphorus is deposited in the bone in the form of hydroxyapatite. Hydroxyapatite and aluminium have similar densities. This has resulted in the development of a non-invasive technique to measure bone density by comparing calcium and phosphorus deposition to a pre-determined aluminium scale. This technique is known as optical densitometry in radiographic images (Almeida Paz & Bruno, 2006). A study by Almeida Paz *et al.* (2004) showed that optical densitometry in radiographic images can be successfully applied to determine the incidence of tibial dyschondroplasia in broilers. A study by Onyango *et al.* (2003) found that there is an 86% correlation between bone densitometry and tibia ash percentage; thus, bone densitometry is an effective non-invasive method to determine bone ash percentage in broilers.

2.6. Consequences of improved phosphorous digestibility

2.6.1. Skeletal development

Qian *et al.* (1996) conducted a study to determine the effect of phytase on the histological, mechanical and chemical traits of the tibia. As can be seen in the figures below, phytase supplementation significantly increased tibia length and width ($p<0.01$), shear force ($p<0.05$) and tibia ash percentage ($p<0.01$). Diets high in phytate resulted in reduced mineralisation, osteiods, osteoblasts and osteoclasts, and increased cartilaginous zones that result in weak bones. Phytase supplementation caused an orderly regression of cartilage as bone mineralisation occurred, resulting in the orderly replacement of cartilage with dense bone.

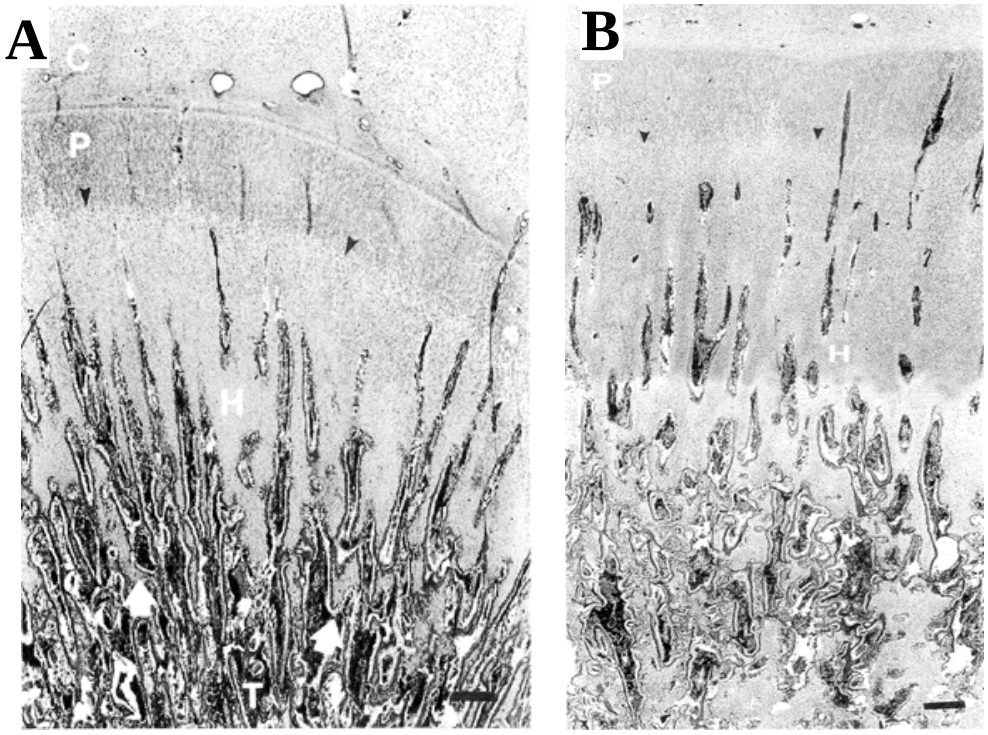

Figure 2.17 A and B **Proximal tibia section of a 21-day-old broiler fed a diet supplemented with phytase (**A**) and without phytase (**B**). The black arrows on** A **between the P and H zone show orderly cell differentiation.** B **shows irregular cell differentiation and increased cartilage (Qian *et al.*, 1996)**

* C= cartilaginous zone. P= proliferative zone. H=hypertrophy zone. T=trabecular bone.

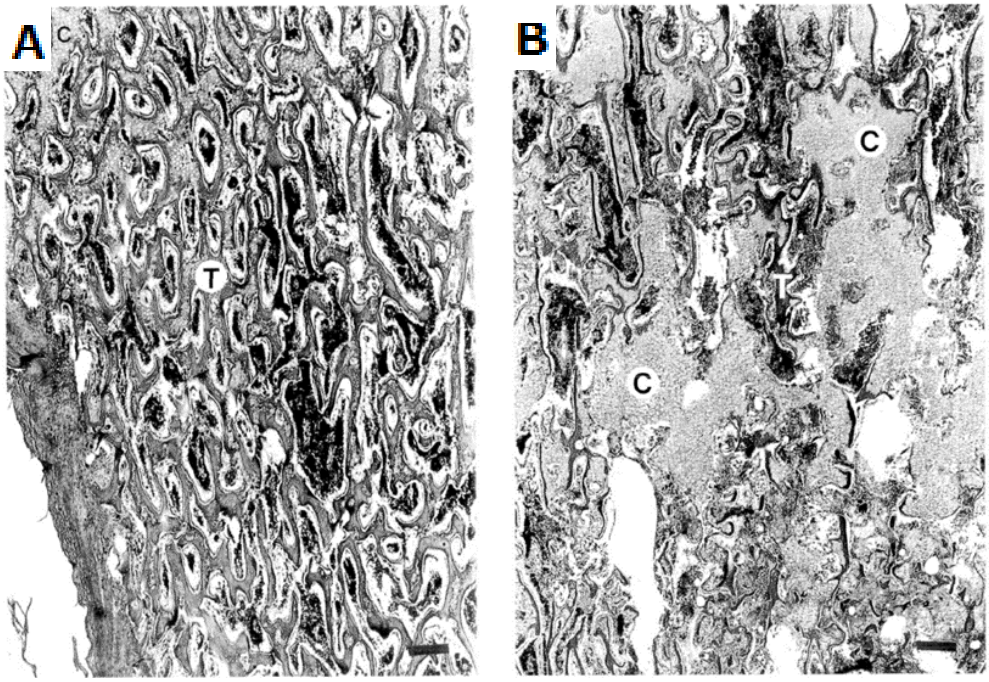

Figure 2.18 A and B **Trabecular bone of a 21-day broiler fed a diet supplemented with phytase (**A**) and without phytase (**B**). Note the normal cartilage regression in the upper left hand corner of** A**, and the abnormal, disorganized enlarged cartilage zones in** B **(Qian *et al.*, 1996)**

* C = cartilage. T= dense trabecular bone formation

More recently, a study by Shaw *et al.* (2011) to determine the impact of a phytase enzyme (HiPhos) on broiler performance, mineralisation and the environment showed that phytase supplementation increased bone breaking strength and tibia ash linearly as the phytase dose increased (figure 2.19 and 2.20).

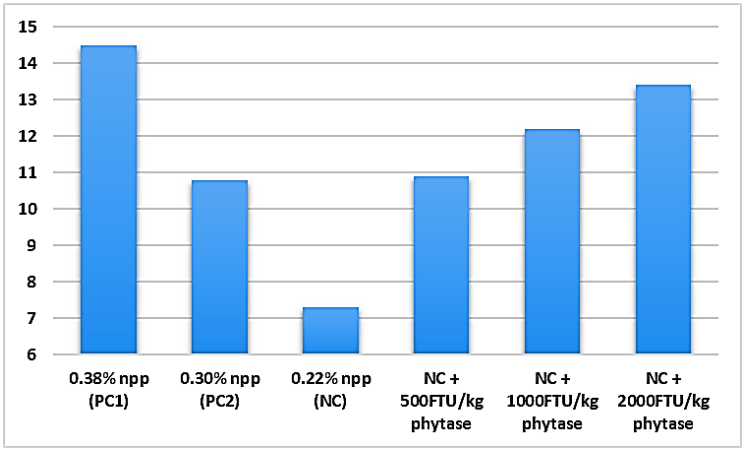

Figure 2.19 Tibia breaking force (kg) of 21-day-old broilers fed diets with different non-phytate phosphorus (npp) levels and different levels of supplemented phytase (Shaw *et al.*, 2011)

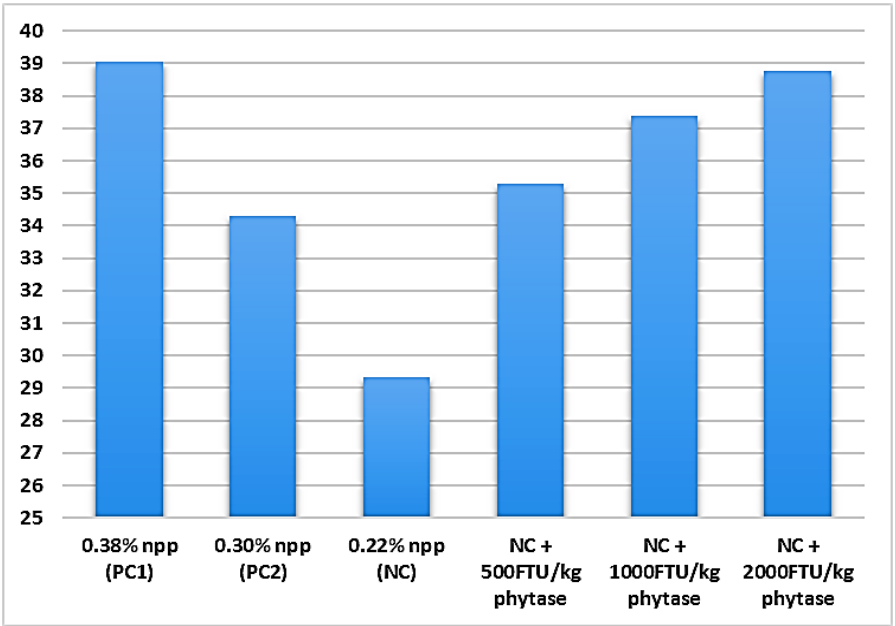

Figure 2.20 Tibias ash (%) of 21-day-old broilers fed diets with different non-phytate phosphorus (npp) levels and different levels of supplemented phytase (Shaw *et al.*, 2011)

2.6.2. Available energy

Energy is a critical component during the first week post-hatch as it will ultimately determine the future performance of the broiler. During this time energy is required to maintain the high metabolic rate of the epithelial cells in the intestine, which are responsible for digestive juice and enzyme secretions and nutrient absorption, and to support the rapid growth of the digestive system (Dibner *et al.*, 1996).

Phytate can bind starch through H-bonds and the proteins associated with starch, inhibit the activity of amylase and reduce glucose absorption. It is thought that the mechanism behind phytate's ability to inhibit amylase is the formation of phytate-Ca complexes. Ca is a co-factor that is required for the stability and optimal performance of amylase (Selle *et al.*, 2000; Liu *et al.*, 2008(b)). Dephosphorylation of phytate decreases the interaction between starch, amylase and glucose, resulting in increased starch digestion. This also prevents the formation of metallic soaps in the gut lumen, resulting in increased fat digestion. Thus, energy derived from fat digestion is available to the animal (Selle *et al.*, 2000; Selle *et al.*, 2011). Phytase supplementation increases the apparent metabolisable energy (AME) of a diet by 0.36MJ/kg DM

(2.8%) when 662FTU/kg phytase is supplemented (Selle *et al.*, 2011). According to Selle & Ravindran (2007) the extent to which phytase supplementation increased AME was negatively correlated ($r = -0.562$ with a significance of $p<0.02$) to the energy density of the control diet. As described previously, phytate resulted in increased energy expenditure due to endogenous losses. Therefore, a third mechanism for phytase to increase available energy is by reducing endogenous losses, which reduces maintenance energy costs (Cowieson *et al.*, 2011).

2.6.3. Trace mineral digestibility

As described in the section on the anti-nutritional effects of phytate, phytate forms insoluble and indigestible complexes with positively charged minerals and causes the increased secretion of some minerals. In the case of sodium (Na), phytate increases Na losses. Phytate from rice bran results in 60% ileal Na loss. Na is important in the animal body to maintain acid-base homeostasis and absorb minerals, glucose and amino acids. The addition of phytase to the diet decreases Na loss by 66% (Selle & Ravindran, 2007). Trace minerals are important in poultry diets as they form a part of many metabolic reactions required for maintenance and performance. The insoluble phytate-mineral complexes formed result in a deficiency of minerals. The metabolic role and the effect of a deficiency of three key trace minerals on poultry are summarised in table 2.8.

Table 2.8 Role of zinc, copper and manganese in poultry (Miles, 2000; Dibner, 2007; Richards *et al.*, 2010)

Trace mineral	*Metabolic role*	*Effect of mineral deficiency*
Zinc	• Synthesis of collagen and keratin (structural proteins) • Development of immune system (differentiates T-lymphocytes) • Regulates cell turnover (important for embryonic and post-hatch development) • Zn containing proteins are receptors for steroid and thyroid hormones • Influences appetite (important for post-hatch development).	• Bone and beak abnormalities • Shortening of bones during embryonic development • Decreased tissue strength • Decreased immune function • Reduced longevity • Reduced shell weight • Poor feathering • Dermatitis • Anorexia and perosis • Increased mortality.

Copper	• Crosslinking of collagen and elastin (performed by Cu-dependent enzyme Lysyl oxidase) • Cartilage formation • Bone structure • Supports skin, bone, tendon and intestinal strength and elasticity • Essential for reproduction and development of embryonic birds • Involved in pigmentation (Cu containing enzyme) • Develop and stabilise tertiary structures of proteins (required in the oxidation reaction).	• Leg problems due to weak bones • Connective tissue weakness • Changes in central nervous system that affects the developing embryo • Retarded chick development • Anaemia • Reduced egg production and hatchability • Aortic rupture.
Manganese	• Antioxidant activity • Keratin formation • Forms proteoglycan matrix, which is important for later stages of bone development • Important for growth and fertility.	• Eggshell abnormalities • Reduced resistance to metabolic stress • Bone weakness and perosis due to the absence of endochondral ossification • Chicks hatched from hens fed Mn-deficient diets have tetanic spasms.

2.6.4. Amino acid digestibility

Because of the increasing meat demand coupled with the increasing cost of raw material, the main objective for meat producers is to maximise the profitability of meat production (Cowieson & Bedford, 2009). Phytase enzymes increase ileal amino acid digestion by hydrolysing phytate, which reduces the formation of insoluble complexes with phytate. Many studies have evaluated the effect of phytase supplementation on amino acid digestibility; however, results are varied.

The causes of variations in these results could include the source of protein used in the studies (soy, SBM, linseed meal), phytate induced changes in the conformation of the protein, inherent amino acids digestibility, the choice of marker (CrO_2 vs TiO_2 and AIA) used in the study, and the age of the birds (Selle & Ravindran, 2007; Selle *et al.*, 2011). Different sources of protein have different amino acid profiles and conformations. The structure of the protein determines how accessible the basic amino acids are to phytate. The more accessible the amino

acids are, the greater the ability of phytate to bind to the protein. It was reported that phytase supplementation increased ileal amino acid digestibility to a greater extent in wheat-based diets (9.2–13.4%) compared to maize-based diets (3.3–3.9%) as phytate tends to bind to wheat proteins more than maize proteins. However, it must be noted that the extent to which phytase supplementation improves amino acid digestibility is dependent on the ability of phytase to rapidly dephosphorylate phytate in the crop and gizzard. If phytase hydrolyses the phytate in the duodenum, breaking the phytate-protein complex, the protein would not have been digested by pepsin would therefore still not be available to the animal.

A study by Cowieson & Ravindran (2007) to determine the effect of phytic acid and phytase on endogenous protein flow and composition shows that increased dietary phytic acid content selectively increased aspartic acid, serine, threonine and tyrosine. This is expected because phytic acid increases the secretion of mucin, which is rich in threonine, serine, proline and cysteine. Another source of endogenous amino acid loss is from brush border enzymes, which are rich in serine, leucine, glycine and aspartic acid. Phytase supplementation reduced the endogenous losses of these amino acids to varying degrees by reducing endogenous amino acid flow, with no influence on the endogenous loss of methionine. Methionine is the least responsive amino acid when phytase is supplemented. In broiler diets methionine is the most limiting amino acid, followed by lysine. Therefore, even though all other amino acids respond positively to phytase supplementation, if methionine is deficient the increase in liberated amino acids will be excreted, negating the positive effects of phytase supplementation on amino acid digestibility (Selle & Ravindran, 2007; Selle *et al.*, 2011). Cowieson *et al.* (2011) states that a dietary phytate: protein ratio of 0.05:1 can minimise phytate-protein formation; however, as far as this author is aware no recent studies have been conducted on this topic.

Table 2.9 Effect of phytase at 1200FTU/kg on apparent ileal digestibility (%) of amino acids for different feed ingredients (Selle *et al.*, 2000)

Amino acid	*Feed ingredient*							
	Maize	**Sorghum**	**Wheat**	**Soybean meal**	**Rapeseed meal**	**Cottonseed meal**	**Sunflower meal**	**Wheat middlings**
Arginine	3.7	6.5	10.3	2.3	1.9	2.9	2.0	1.9
Histidine	2.9	7.0	9.5	4.2	2.1	3.9	5.3	3.1
Isoleucine	2.7	5.8	6.6	3.4	3.1	7.0	4.4	3.3
Leucine	1.0	6.4	6.4	2.5	2.8	5.8	4.4	3.1
Lysine	3.4	3.7	10.9	4.0	0.9	4.7	5.7	2.4
Phenylalanine	2.1	6.5	6.3	4.0	2.6	3.9	4.4	3.0
Threonine	6.7	8.8	15.7	8.0	4.1	6.9	5.1	6.1
Tyrosine	2.7	8.8	11.1	3.5	2.3	3.4	4.3	5.5
Valine	4.1	6.5	8.8	3.4	3.4	5.6	4.8	3.6

2.6.5. Immune function and intestinal microbial population

Liu *et al.* (2008(a)) conducted a study to determine the effect of phytate and phytase on the immune function of broilers. As can be seen from the results summarised in table 10 below, phytase supplementation significantly increased ($p<0.05$) erythrocyte cells, T cells, Newcastle disease antibody titers and SIgA levels. Further, phytase supplementation increased the development and weight of the bursa. B cells develop and proliferate in the bursa, which explains the increase in erythrocyte cells. The increase in T cells indicates an increase in immunocyte activity, specifically CD4+, which enhances the immune response and CD8+, which mediates the cytotoxic death of the target cells. An increase in Newcastle disease antibodies indicates an increase in humoral immunity, decreasing a bird's susceptibility to disease. The increase in SIgA indicates an increase in mucosal humoral immunity in the jejunum. It is thought that the mechanism behind this increase in mucosal immunity is the influence of phytase on mucin secretion. Liu *et al.* (2008(a)) states that with a reduction in mucin secretion, phytase indirectly contributes to maintaining a consistent beneficial microbial ecology in the gastro-intestinal tract. This microbial ecology decreases the concentration of saprogenic

compounds in the mucosa, maintains the integrity of the gastro-intestinal tract and increases SIgA.

Table 2.10 Effect of different levels of phytate and phytase on immunological parameters (Liu *et al.*, 2008(a))

Treatment	*Day 21*					*Day 28*				
	ERFC (%)	**EAC (%)**	**CD4+: CD8+**	**Anti-NDV (log_2)**	**SlgA (µg/ml)**	**ERFC (%)**	**EAC (%)**	**CD4+: CD8+**	**Anti-NDV (log_2)**	**SlgA (µg/ml)**
Phytate P at 0.22%										
0FTU/kg Phytase	25.43^{c}	13.4^{b}	1.5^{a}	3.17^{ab}	3.85^{bc}	25.39^{b}	12.99^{c}	1.54^{b}	3.00^{b}	4.25^{ab}
500FTU/kg Phytase	28.46^{a}	15.23^{a}	1.46^{a}	3.00^{abc}	4.66^{a}	28.15^{a}	15.19^{a}	1.51^{a}	4.00^{a}	4.67^{a}
1000 FTU/kg Phytase	28.35^{a}	14.37^{ab}	1.5^{a}	3.00^{abc}	4.00^{bc}	28.57^{a}	14.57^{ab}	1.57^{a}	2.50^{bc}	4.55^{ab}
Phytate P at 0.44%										
0FTU/kg Phytase	26.07^{bc}	13.91^{ab}	1.49^{a}	2.50^{c}	3.42^{c}	26.98^{ab}	13.32^{bc}	1.49^{b}	2.00^{c}	3.93^{b}
500FTU/kg Phytase	29.06^{a}	14.71^{ab}	1.48^{a}	3.50^{a}	4.24^{ab}	28.43^{a}	15.58^{a}	1.52^{a}	2.83^{b}	4.72^{a}
1000 FTU/kg Phytase	27.81^{ab}	15.29^{a}	1.42^{a}	2.67^{bc}	4.46^{a}	28.61^{a}	15.39^{a}	1.44^{a}	3.00^{b}	4.68^{a}

* Means with different superscripts within the columns are significantly different ($p<0.05$)

* ERFC = erythrocyte rosette-forming cells. EAC= erythrocyte-antibody complement cells. Anti-NDV = antibodies against Newcastle Disease virus. SIgA = jejunal mucosal secretory IgA.

The results from a study by Ptak *et al.* (2015) to determine the effect that exogenous phytase has on intestinal microbial populations and their metabolites can be seen in table 2.11. Overall, Ca- and P-deficient diets reduced the total number of bacteria compared to Ca- and P-adequate diets. In terms of Ca and P-deficient diets, phytase supplementation increased total bacteria, C.perfringens, lactobacillus spp, and C. leptum counts. The numbers of bacteroides, enterobacteria, and bifidobacterium spp decreased. Phytase supplementation did not influence the bacteria counts of streptococcus/lactococcus and C. coccoides–eubacterium rectale. In

addition, Ptak *et al.* (2015) found that phytase supplementation to a Ca- and P-deficient diet resulted in changes in the production of short chain and volatile fatty acids.

Table 2.11 Effect of phytase and dietary phosphorus and calcium levels on the microbial numbers (log cfu/ml digesta) in the ileum of broiler chickens (Ptak *et al.*, 2015)

Treatment	*DAPI*	*Bacto*	*C.perf*	*Entero*	*Lab*	*C.lept*	*Strc*	*Erec*	*Bif*
Ca- and P-adequate	9.139	7.933	8.103	8.225	7.858	7.604	7.656	7.784	7.915
Ca- and P-adequate with Phytase	9.592	7.799	8.048	8.160	8.048	7.571	7.492	7.878	7.628
Ca- and P-deficient	8.572	7.942	7.668	7.985	8.002	7.510	7.567	7.703	7.934
Ca- and P-deficient with Phytase	8.978	7.911	7.961	7.894	8.064	7.675	7.577	7.704	7.748

* DAPI = total number of bacteria. Bacto = bacteroides. C.pref = C. perfringens. Entero = enterobacteria. Lab = lactobacillus spp. C.lept = C. leptum. Strc = streptococcus/lactococcus. Erec = C. coccoides–Eubacterium rectale. Bif = Bifidobacterium spp.

2.6.6. Environmental pollution

The consequence of the projected growth of the livestock and poultry industries is increased environmental pollution. Phytase supplementation has the ability to reduce phosphorous excretion by 30–50% (Gupta *et al.*, 2015). A study by Shaw *et al.* (2011) showed that phytase supplementation reduced phosphorus excretion by 42%, but had no impact on calcium excretion (figure 2.21).

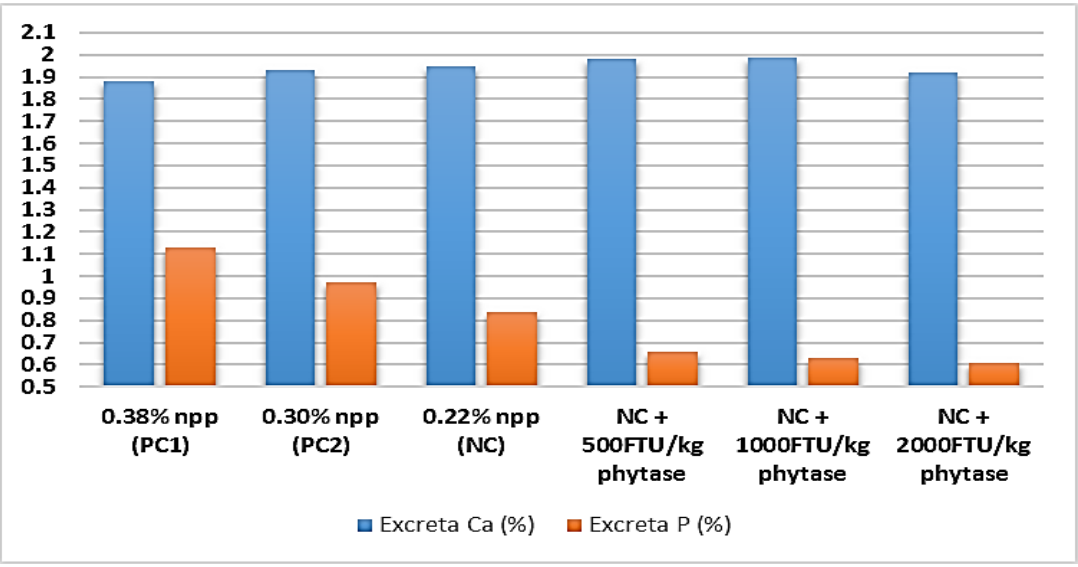

Figure 2.21 Calcium and phosphorus excretion (%) of 21-day-old broilers fed diets with different non-phytate phosphorus (npp) levels and different levels of supplemented phytase (Shaw *et al.*, 2011)

Nagaraju & Nielsen (2011) conducted a study to determine the degree to which phytase (ronozyme) supplementation can reduce the negative impact on the environment compared to inorganic (MCP) phosphorus supplementation. The results have been summarised in the graphs below.

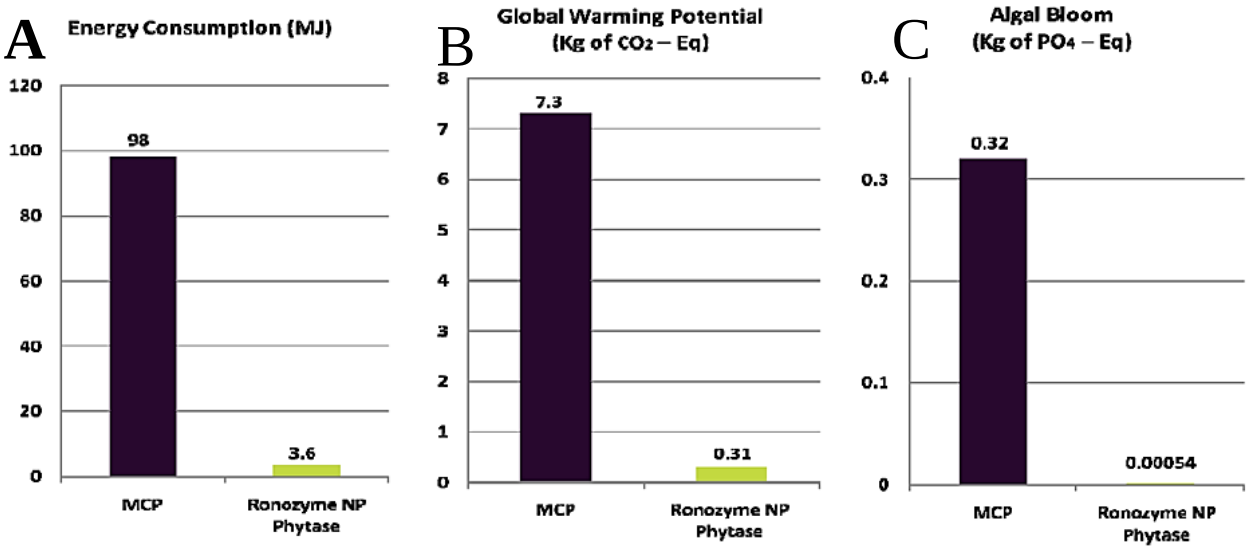

Figure 2.22 Impact of MCP and Ronozyme NP Phytase on (A) energy consumption, (B) global warming potential and (C) eutrophication (algal bloom) per ton of feed (Nagaraju & Nielsen, 2011)

There is, however, concern that although phytase supplementation reduces phosphorous excretion, it can increase the solubility of the remaining phosphorous that is excreted, which would increase eutrophication. There is contradictory literature published that shows that phytase increases (by 31%) and decreases (by 43%) soluble phosphorous pollution. If one looks at the diets provided in these studies, diets that were severely deficient in total phosphorous concentrations resulted in decreased soluble phosphorous pollution, and diets adequately or marginally deficient in phosphorous resulted in increased soluble phosphorous pollution (Selle & Ravindran, 2007).

2.6.7. Enzyme cocktails

The addition of enzyme combinations, such as phytase and carbohydrases, is expected to have additive effects due to the different modes of action of the different enzymes, i.e. dephosphorylation of phytate by phytase and hydrolysis of non-starch polysaccharides (NSP) by carbohydrases. Enzyme cocktails are expected to hydrolyse dietary nutrients more effectively, increasing the nutrients available for absorption. This provides nutritionists the ability to incorporate a larger variety of raw materials in their formulations. For example, the ability to use feed that has not previously been included, or not included at high inclusion levels, such as distillers dried grains with solubles (DDGS) in broiler diets. Consequently, a reduction in feed costs can be realised without sacrificing the nutritional quality of the diet and adversely effecting performance. This is becoming increasingly important due to the biofuels initiative, which produces ethanol from corn (Ferket, 2010). An added benefit is that enzymes can help stabilise the gut microbial ecosystem, resulting in improved animal health, improved welfare and performance, and reduced veterinary costs (Cowieson & Bedford, 2009; Francesch & Geraert, 2009; Ferket, 2010).

The supplementation with xylanase to a diet has been observed to increase amino acid digestibility by approximately 16% (Cowieson & Bedford, 2009). However, Cowieson & Bedford (2009) showed that supplementing xylanase and phytase do not have a complete additive effect on the digestibility of all amino acids, as can be seen in figure 2.23. The additive effect of xylanase and phytase is thought to be due to xylanase increasing mucin (rich in

threonine, serine and aspartic acid) output by reducing luminal viscosity and phytase increasing pepsinogen production. In addition, xylanase effectively increases phytase access to phytate, which increases phytate dephosphorylation and the nutrients released. Full-additivity is expected in wheat-based diets and sub-additivity in maize-based diets. This is because phytate is located in the aleurone layer of wheat and the germ of maize. Due to the carbohydrate composition of the aleurone compared to the germ, xylanase is more effective at degrading the cell wall matrix of the aleurone than the germ (Nyman *et al.*, 1984; Selle & Ravindran, 2007; Cowison & Bedford, 2009).

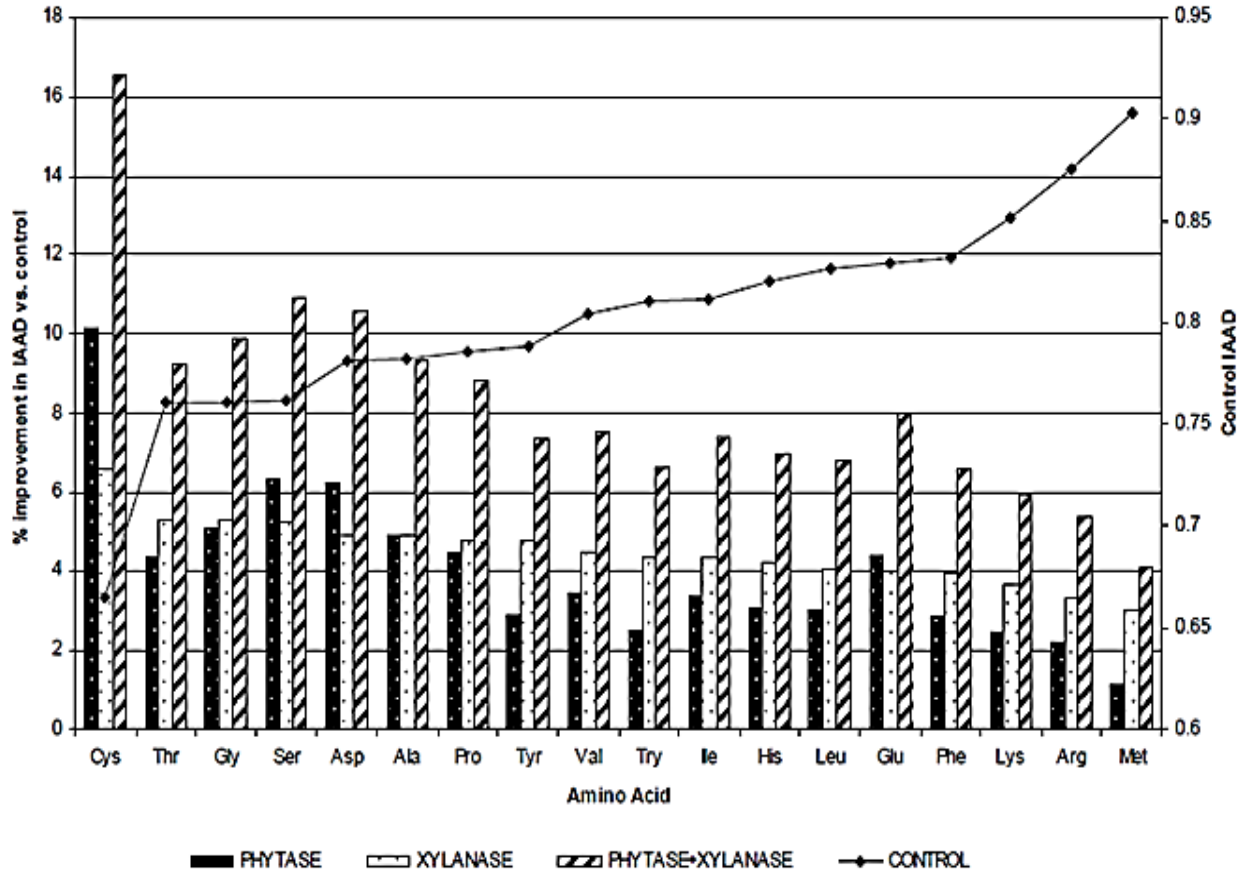

Figure 2.23 Influence of enzyme supplementation on the ileal amino acids digestibility coefficients and the influence of phytase on xylanase efficacy (Cowieson & Bedford, 2009)

2.6.8. Nutrigenomic consequences

Genetic selection had been used as the primary method for improving production traits from one generation to another for many years. Over the past few years, the various disciplines involved in the animal industry have become increasingly integrated. Nutrition, genetics, gut health, management, physiology and the interaction between these disciplines are taken into consideration to achieve the maximum biological and economic performance of the animal. This integration of disciplines gave birth to the fields of nutrigenetics and nutrigenomics. Nutrigenetics is the study of how the genotype of an animal responds to the specific dietary nutrient provided. Nutrigenomics is the study of how a specific dietary nutrient alters the gene expression. Studies in nutrigenomics allow nutritionists to manipulate a specific production trait or metabolic function in order to achieve a desired performance goal (Ferket, 2010; Calsamiglia & Siurana, 2016).

A study by Li *et al.* (2016) aimed to determine the influence of the diet's nutrient density on the expression of NaP Type IIb co-transporter and bone mineralisation. The outcome (table 2.12) showed that high nutrient-dense diets improved overall growth performance parameters compared to low nutrient-dense diets. However, performance was at its greatest when non-phytate phosphorus (NPP) levels were high in low nutrient-dense diets and low in high nutrient-dense diets. It suggested that the ratio of energy to phosphorus in the diet should be considered in feed formulations. Bone mineralisation was greater when low nutrient-dense diets were provided. The NPP level has a significant influence on the mRNA expression of the co-transporters. Extended periods of energy restrictions result in a reduced expression of NaPi Type IIb because the expression is related to glucose and fructose metabolism in the intestine, which is sensitive to energy levels. Glucose and fructose metabolism is regulated by the epidermal growth factor (EGF) protein. EGF results in downregulation of the NaPi Type IIb promoter, resulting in reduced NaPi Type IIb expression. According to Proszkowiec-Weglarz & Angel (2013), phosphorus-deficient diets (0.25%−0.35% npp) increased the intestinal expression of the NaPi Type IIb gene, which is similar to the results from Li *et al.* (2016) when a high nutrient-dense diet is fed, but is contradictory when a low nutrient-dense diet is fed.

Table 2.12 Influence of dietary nutrient density and non-phytate phosphorus (NPP) level on growth performance, bone mineralisation and co-transporter mRNA expression in broilers (Li *et al.*, 2016)

		Growth performance			*Bone mineralisation*			*mRNA expression*		
Diet nutrient density	**NPP (%)**	**BWG (g)**	**FI (g)**	**FCR**	**Tibia ash (%)**	**Tibia P (%)**	**Tibia Ca (%)**	**NaPi Type IIb**	**NaPi Type IIa**	**Calbindin**
Low (ME = 2950kcal/kg)	0.25	1.09	2.33	2.14^{d}	48.36	8.44	16.94	0.84	1.48^{abc}	0.40
	0.30	1.11	2.33	2.11^{d}	50.19	8.52	17.52	0.83	2.21^{bc}	0.53
	0.35	1.13	2.32	2.04^{c}	53.16	9.25	18.61	0.62	1.18^{ab}	0.60
	0.40	1.20	2.39	1.99^{bc}	53.37	9.11	18.54	1.28	2.5^{c}	0.73
High (ME = 3150kcal/kg)	0.25	1.18	2.29	1.94^{ab}	47.77	7.20	16.43	1.28	3.96^{d}	0.82
	0.30	1.21	2.31	1.92^{ab}	51.00	8.35	17.72	1.36	1.11^{ab}	1.19
	0.35	1.18	2.28	1.95^{ab}	51.66	8.92	17.95	0.85	1.36^{ab}	1.02
	0.40	1.23	2.33	1.89^{a}	52.89	9.04	18.25	1.24	0.52^{a}	0.93

*BWG= Body weight gain. FI= Feed intake. FCR= Feed conversion ratio

*NaPi Type IIb expression in the duodenum. NaPi Type IIa expression in the kidney.

*Values not sharing a common superscript are significantly different ($p<0.05$).

As discussed in the section concerning calcium and phosphorus regulation, vitamin D_3 ($1,25(OH)_2D_3$) plays a critical role in the metabolism of Ca and P, and therefore, in skeletal development. Han *et al.* (2009) conducted a study to determine the influence that a vitamin D analogue (1α-OH D_3) would have on NaP Type IIb gene expression and meat quality in broilers. The results showed that 1α-OH D_3 supplementation increased the expression of NaP Type IIb gene expression significantly ($p<0.05$) in the jejunum and ileum, and numerically in the duodenum. Meat quality was also improved as 1α-OH D_3 supplementation increased the lightness and reduced the toughness (shear force) of the meat. Another study by Zhang *et al.* (2002) to determine the influence of dietary phosphorous on the gene expression of renal 1α-hydroxylase, where mice were used as the model animal, concluded that phosphorus-deficient diets result in increased mitochondrial cytochrome P450c1 gene transcription (which is stimulated by an increase in PTH), leading to increased P450c1 mRNA expression. This results in the increased synthesis of renal 1α-hydroxylase, leading to an increase in the production of vitamin D_3.

Liu *et al.* (2008(b)) determined the influence of phytate and phytase on various enzymes and transporters. The results of the study are summarised in table 2.13. In addition to the results obtained in the table, Lui *et al.* (2008(b)) also found that phytate inhibits the activity of endogenous digestive enzymes by binding to the co-factors required for the optimal activity of those digestive enzymes. A contradictory study by Woyengo *et al.* (2011) on piglets found that phytate reduced the expression of SGLT-1 in all segments of the small intestine and reduced crypt depth in the jejunum. The supplementation with phytase increased SGLT-1 expression.

Table 2.13 Summery of the results obtained from the study conducted by Liu *et al.* (2008(b))

	Phytate	*Phytase*
Duodenum	Depressed activity of sucrase (11%), maltase (5%) and Na-K ATPase (6%). Reduced mucosal glucose concentration by 13%. Upregulated SGLT-1 (involved in glucose and Na absorption) mRNA expression.	Increased activity of amylase (23%), sucrase (5%), maltase (6%) and Na-K ATPase (23%). Increased mucosal glucose concentration by 30%. No effect on SGLT-1 expression.
Jejunum	Depressed activity of amylase (6%), sucrase (6%), and Na-K ATPase (4%). Maltase activity is unaffected. Reduced mucosal glucose concentration by 22%.	Increased activity of carbohydrases and Na-K ATPase by 10−16%. Increased mucosal glucose concentration by 33%. No difference was observed between 500FTU/kg phytase and 1000FTU/kg phytase.
Serum	No effect on sucrase, maltase and Na-K ATPase.	Significantly increased ($p<0.05$) carbohydrases and Na-K ATPase activity and glucose concentration. No change in the magnitude of the increase was observed between 500FTU/kg phytase and 1000FTU/kg phytase.

When phytase dephosphorylates phytate, phytate is not completely broken down to myo-inositol in the duodenum. However, it is speculated that $InsP_3$ and $InsP_2$ are absorbed in the jejunum and dephosphorylated in the mucosa and liver to myo-inositol and free phosphorous molecules (Lee *et al.*, 2003). Myo-inositol is thought to mimic insulin and thereby regulate glucose metabolism. When purified myo-inositol is supplemented to a phosphorus-deficient diet, increased body weight of the broilers is observed (Lee *et al.*, 2003; Józefiak *et al.*, 2010). Results from a study by Józefiak *et al.* (2010) showed that when a combination of phytase-carbohydrase was supplemented to a wheat-SBM-based diet, the sensitivity of insulin receptors located in the

liver increased by 12.3% and IGF-1 (related to insulin regulation) gene expression decreased by 32%. Józefiak *et al.* (2010) suggested that one of the reasons for the observed results is increased phytate degradation and thus increased levels of $InsP_{2\ and\ 3}$, and myo-inositol.

2.7. Study considerations and conclusion

Due to the many adverse interactions between phytase and dietary nutrients (described in sections 2.4.2 and 2.5.3), the design of an animal study evaluating the efficacy of a phytase enzyme needs to take into consideration a number of factors. These factors include the characteristics of the enzymes used in the study; dietary factors such as calcium level, Ca:avP ratio, protein and energy level; and animal factors such as sex of the animal and breed.

In this study the three commercial phytases used are modified *E.coli*, product X and product Y. As described by Derjant-Li *et al.* (2015) and Kiearie *et al.* (2015), there are a variety of characteristics that distinguish different phytases produced commercially such as pH range, bacterial origin and expression, specific substrate activity, and resistance to proteolytic degradation. Table 2.14 compares the characteristics of the three phytases used in this study.

Table 2.14 Characteristics of the enzymes used in this study (Manangi *et al.*, 2014; ABVista, 2016; Du Pont, 2016)

Characteristic	***Modified* E.coli***	***Product X***	***Product Y***
Microbial origin	*E.coli*	*E.coli*	Buttiauxella sp.
Microbial expression	Pseudomonas fluorescens	Trichoderma reesei	Trichoderma reesei
Coating	No	No	Yes
Optimal temperature (°C)	90	90	95
Optimal pH	1.5<	4.5	3
Available P (%) / 500FTU/kg	0.15-0.22	0.15	-
Time to reduce IP6 to IP5 (min)	5	6.67	-
Time to complete phytate dephosphorylation (min)	20	-	30
Phytase activity (FTU/g)	10 000	5 000	10 000
Inclusion level (g/MT feed)	25-200	50-400	25-200

* Modified new generation *E.coli* 6-phytase is called modified *E.coli*

Enzymes are biological catalysts and, as such, are susceptible to degradation by high temperatures. Consequently, when pelleting the feed it is critical to monitor and control the conditioning and pelleting temperature to ensure the maximum temperature of the phytase included in the diet has not been exceeded. Phytase activity and dose in the finished feed are dependent on the temperature and time during the pelleting process. The temperature during the pelleting process is a function of the machine model, amount of time it has been running and the amount of steam used, which makes it difficult to control the temperature within a narrow temperature margin. Phytase supplemented at 500FTU is considered to be industry standard in terms of phytate dephosphorylation. However, in South Africa, many feed producers supplement phytase at 1000FTU as a safety margin to ensure that the completed feed contains at least 500FTU of phytase in case the temperature exceeds the maximum temperature that phytase can withstand. In this study, the temperature of the conditioner and pelleting machine was taken every 5 minutes to ensure that the temperature did not exceed the maximum temperature that phytase products can withstand. The temperatures can be found in appendix 3 for each treatment.

Enzymes are composed of amino acids and peptide chains; therefore phytase is susceptible to proteolytic degradation by pepsin in the proventriculus. Consequently, any phytase enzyme produced commercially must be resistant to the action of endogenous proteases (Gupta *et al*., 2015; Dersjant-Li *et al.*, 2015). In addition to the Pieniazek *et al.* (2016) study results on animal performance and bone development, the study also looked at the ability of modified *E.coli* to resist proteolytic degradation. The stimulated gastric fluid (SGF) challenge study implemented was designed to mimic pH and gastric conditions found in the proventriculus and gizzard. The results showed that this new generation modified *E.coli* 6-phytase had 100% activity after 20 minutes of being exposed to the conditions of the SGF challenge, compared to the activity of the wild-type *E.coli* phytase that declined from 68% to 5% after 20 minutes. Interpretation of the results show that this new generation modified *E.coli* 6-phytase product is stable at a pH of 2 and is resistant to proteolytic degradation by endogenous protease enzymes. The study also analysed modified *E.coli* activity at different phytate concentrations. The results show that modified *E.coli* is highly substrate-specific, allowing the enzyme to have high activity at low phytate concentrations, such that at 2mM of phytic acid the enzyme has 120% activity compared to the wild-type *E.coli*-derived enzyme, which has 85% activity. In terms of phytase activity, Pieniazek *et al.* (2016) concluded that this phytase enzyme can dephosphorylate phytate

instantly in the proventriculus as the phytate molecule begins to dissociate regardless of phytate concentration, greatly reducing interactions between phytate and dietary nutrients and resulting in significant improvements in animal performance and bone development. In this study the phytate content in diet and the phytase activity will not be analysed; therefore, we cannot compare the activity of the different phytases used or compare the phytase activity of modified *E.coli* used in this study to that used in the study by Pieniazek *et al.* (2016).

In terms of dietary factors, the diet must always be formulated to meet the minimum requirements set by the breed standard. In this study, the breed used was Cobb 500. Since calcium has a negative impact on the efficacy of the phytase enzyme, the calcium content of the diet must be strictly controlled. The influence of calcium on phosphorus digestibility and absorption has been studied in detail by a multitude of authors; however, there is still uncertainty surrounding the optimum Ca:avP ratio, range of 1.6−2.6 and dietary Ca level, range of 0.8−1.04% (Driver *et al*., 2005; Han *et al*., 2016). In order to accurately determine and compare the efficacy of this modified new generation *E.coli* 6-phytase product to other heat-stable phytases on the South African market, the diets formulated need to be comparable to other studies conducted. Table 2.15 provides the nutrient analysis of the diet used in this study, and compares it to the minimum requirements stated in the Cobb 500 manual, as well as to other studies conducted. The complete formulated diet is provided in appendix 1.

Table 2.15 Comparison of the breed, energy, protein, calcium phosphorus and sodium content of the positive control starter and finisher diet from various studies

Studies	*Broiler breed*	*ME (kcal/kg)*	*CP (%)*	*Ca (%)*	*Total P (%)*	*Ca: Tot P*	*avP (%)*	*Ca:avP*	*Na (%)*
				Starter					
Cobb 500 Min level	Cobb 500	3008	21	0.9	-	-	0.45	2.00	0.16
This study	Cobb 500	2587.78	20.27	0.92	0.82	1.12	0.43	2.14	0.2
***Pieniazek* et al. (2016)**	Cobb 500	3036	22.5	1.1	0.9	1.22	0.45	2.44	-
***Yan* et al. (2003)**	Cobb 500	3200	20	0.7	-	-	0.5	1.40	0.2
***Han* et al. (2015)**	Ross 308	2972	21.68	0.99	0.69	1.43	0.45	2.20	0.3
***Kiarie* et al. (2015)**	Ross 308	3055	22.5	0.95	0.64	1.48	0.4	2.38	-

Liu* et al. *(2014)	Ross 308	2985.57	23.4	1.04	0.69	1.51	0.44	2.36	0.12
Junqueira* et al. *(2011)	Ross 308	3050	21.14	0.9	-	-	0.48	1.88	0.21
Singh* et al. *(2003)	Hubbard	2800	22.91	1.21	1	1.21	0.5	2.42	-
				Finisher					
Cobb 500 Min level	Cobb 500	3167	18	0.76	-	-	0.38	2.00	0.15
This study	Cobb 500	2547.78	19.29	0.79	0.72	1.10	0.32	2.47	0.15
Pieniazek* et al. *(2016)	Cobb 500	3157	18.5	0.77	-	-	0.38	2.03	-
Yan* et al. *(2003)	Cobb 500	3200	16	0.5	-	-	0.35	1.43	0.2
Han* et al. *(2015)	Ross 308	3069	19.69	0.9	0.59	1.53	0.35	2.57	0.3
Junqueira* et al. *(2011)	Ross 308	3200	19	0.78	-	-	0.43	1.81	0.2
Singh* et al. *(2003)	Hubbard	2900	20.1	1.2	0.95	1.26	0.5	2.40	-

The purpose of this study is to determine whether the optimal dose for this modified new generation *E.coli* 6-phytase product is 500FTU/kg or 1000FTU/kg, and to determine the efficacy of this product compared to two other heat-stable phytases.

Chapter 3
Materials and methods

3.1. Animals

This study was approved by the Agricultural Research Council (ARC) ethical committee (Reference number APIEC17/18). A total of 2340 as-hatch day-old Cobb 500 broiler chicks were obtained from Eagles Pride Hatchery and used in the study. Broilers were allocated to pens using randomised complete block design, with the level of pens as the blocking factor. Broilers were also randomly assigned to one of nine treatments with 13 replicates per treatment and 20 birds per pen. The allocation can be found in appendix 2.

Broilers were allowed *ad libtum* access to feed and water during the entire 35-day trial period. Vectormune, Transmune, Avinew and Vitabron were administered to all the broiler chicks at Eagles Pride Hatchery before they were delivered to the broiler house at the ARC Animal Production Institute.

The identification of individual animals was not required for this study. The experimental unit was one pen; thus, a group weight for each pen was recorded. On days 21 and 35 of the trial, two birds from each pen with a weight closest to the average pen weight were euthanised by cervical dislocation.

The left and right drumsticks were removed from the broiler and both the left and right tibias were cleaned of all adhering flesh, the knee cap and the fibula. The right and left tibias were collected in identifying bags (labelled with the treatment number, pen number, left or right tibia and collection day) and kept in a chest freezer at -20^{o}C. All remaining birds that were not sacrificed for tibia collection were then sold by the ARC on day 35.

3.2. Facilities

The broiler house at the ARC Animal Production Institute in Irene is an environmentally controlled house with tunnel ventilation. The house was cleaned and disinfected two weeks prior to the start of the trial. Mixed pine wood shavings were used for bedding, which was distributed evenly on the floor at a depth of 5cm. Pen size was $1m^2$ with a maximum stocking density of $40kg/m^2$, which allowed for a maximum of 21 birds per pen. In this study, 20 birds were allocated per pen. The temperature scale followed the ARC standard operating procedures. At placement, the temperature was set at 32^oC; it was reduced by 3^oC every week until it reached, and was maintained at, 22 ^oC until the end of the trial period. The lighting programme is shown in table 3.1.

Table 3.1 Lighting programme as per ARC standard operating procedures

Age (days)	*Light (hours)*	*Dark (hours)*	*Light intensity (lux)*
0	24	0	20-60
1−5	23	1	20-60
6−9	18	6	5-10
10−15	15	9	5-10
15−21	12	12	5-10

3.3. Dietary treatments

The basal diet composition can be found in appendix 1. The diets were formulated on Format (feed formulation software developed by Format International) using the CVB (Dutch Net energy) system. Treatment diets were mixed at the Simple Grow Agricultural Services (SGAS). Only two diet phases were produced to reduce diet-induced variation. The starter diet was fed from days 0−21 and the finisher diet was fed from days 22−35. Both diets were pelleted. The pelleting temperatures for each treatment can be found in appendix 3.

Table 3.2 Description of the treatments used in the study

Treatment	*Description*
T1	Negative control 1 Starter diet: 0.23% avP with a Ca: avP of 0.33 Finisher diet: 0.12% avP with a Ca: avP of 0.22
T2	Negative control 2 Starter diet: 0.33% avP with a Ca: avP of 0.43 Finisher diet: 0.22% avP with a Ca: avP of 0.35
T3	Positive control Starter diet: 0.43% avP with a Ca:avP of 0.50 Finisher diet: 0.32% avP with a Ca: avP of 0.46
T4	T1 supplemented with modified *E.coli* at 500FTU/kg diet
T5	T1 supplemented with modified *E.coli* at 1000FTU/kg diet
T6	T1 supplemented with product X at 500FTU/kg diet
T7	T1 supplemented with product X at 1000FTU/kg diet
T8	T1 supplemented with product Y at 500FTU/kg diet
T9	T1 supplemented with product Y at 1000FTU/kg diet

* avP = available phosphorus
* Modified new generation *E.coli* 6-phytase product called Modified *E.coli*

3.4. Performance measurements

Feed given and the number of mortalities were recorded daily. Feed refused and pen weight were recorded on days 0, 7, 14, 21, 28, and 35. Feed intake per bird per day (kg), individual bird weight (kg), average daily gain per bird (kg), FCR, and production efficiency factor (PEF) were calculated from this recorded data. The formulae used to calculate each measurement is as follows:

- Daily feed intake (g) = 1000 x [(feed given-feed refused)/ ∑ (number of day-old chicks placed on day 0 – number of mortalities on day Y)/7 days]
- Cumulative feed intake (g/bird) = Cumulative feed intake in previous week + (weekly feed intake/ 7 days)
- Body weight (g/bird) = [pen weight (kg)/ (20 birds-number of mortalities)] x 1000

- Average daily weight gain (g/bird/day) = (body weight (g) on day y – body weight (g) on day x)/ 7 days
- FCR = Daily feed intake / Average daily weight gain/bird
- Cumulative FCR = Cumulative feed intake /(body weight on day Y – body weight on day x)
- Cumulative mortality (%) = (total number of dead birds/ number of birds placed) x 100
- PEF = [(body weight (kg) x 100- cumulative mortality) / 7 days x cumulative FCR] /1000

3.5. Tibia analysis

Both the left and right tibias were removed from the freezer and allowed to thaw at room temperature 24 hours before the start of the analysis.

The left tibias underwent bone breaking strength analysis at the University of Pretoria. The 5 kN Lloyd's press LRX Plus equipment was used to analyse tibia breaking strength. The speed was set to 50mm/minute, with a maximum displacement of 10mm. The support configuration used was a 3-point pending setup with a 40mm support distance between the first and third support.

The right tibias underwent tibia ash and mineral content (Ca and P) analysis at Labworld. The right tibias were split such that 234 bones (13 bones per treatment) underwent tibia ash analysis and 234 bones (13 bones per treatment) underwent mineral analysis. The cartilage caps were removed from all tibias before commencing the analysis.

Ash for each tibia was determined on a fat free dry-matter basis using the AOAC method B of 1995. Each tibia was dried at 50ºC for 72 hours, then wrapped in cheesecloth and placed in the Soxlet extractor for 12 hours. The tibias were removed, allowed to dry at room temperature

for an additional 24 hours and weighed. Once dry, the tibias were ashed at 650^{o}C for 12 hours. Tibia ash percentage was calculated as:

- Tibia ash (%) = (weight ash/weight dry bones) x 100

Calcium and phosphorus content was determined using the method described in AOAC 976.06 (Segmented Flow) official methods of analysis.

3.6. Statistical analysis

Statistical analysis was conducted using the JMP version 13.1.0 software developed by SAS Institute Inc.. Analysis of variance (ANOVA) was used to analyse performance (response) distribution and differences across treatments. The Tukey honestly significant difference (HSD) was used to test for statistical differences across treatment means. $P<0.05$ indicated that there was significant differences across the treatment means, while $P>0.05$ indicated a lack of significant differences across treatment means. Robust fit (which uses the Huber M-estimation) was used to detect outliers and to reduce their influence on the response variables (performance measurements) (SAS Institute Inc. 2017).

Chapter 4

Results

4.1. Performance measurements

To quantify the influence of phytase enzyme supplementation on animal performance, economically important parameters, such as, body weight, feed intake, feed conversion ratio (FCR) and production efficiency factor (PEF) were calculated.

For the duration of the trial, broilers fed the negative control 1 (NC1) diet, which was extremely deficient in available phosphorus (avP), showed significantly lower ($p<0.05$) weekly body weight compared to broilers fed the negative control 2 (NC2) and the positive control (PC) diets, as can be seen in table 4.1. Throughout the trial an incremental increase of avP in the control diets resulted in a subsequent increase in weekly body weight; however, a significant difference ($p<0.05$) between the three control diets was observed only on day 7. By day 35, the NC1 diet resulted in significantly lower ($p<0.05$) weekly body weight compared to the NC2 and PC diets, and the PC diet showed numerically greater ($p>0.05$) body weight compared to the NC2 diet. The supplementation with phytase enzymes to the NC1 diet resulted in a significant increase ($p<0.05$) in body weight compared to the NC1 diet alone, to levels that were comparable ($p>0.05$) to the NC2 and PC diets. There was no significant increase ($p>0.05$) in weekly body weight when modified *E.coli* was supplemented at 500FTU/kg or 1000FTU/kg. Modified *E.coli* that was supplemented at both 500FTU/kg and 1000FTU/kg showed similar ($p>0.05$) body weights to the two other phytase enzymes used at 500FTU/kg and 1000FTU/kg.

Over the full 35-day trial period, the growth of the broilers fed the NC1 diet had significantly lower ($p<0.05$) body weight gain compared to broilers fed the NC2 and PC diets. During the first 21 days of the trial, an incremental increase in avP resulted in a subsequent increase ($p<0.05$) in body weight gain. This difference in body weight gain between the three control diets was maintained until the end of the trial period. However, by day 35 no significant

(p>0.05) difference could be detected in total weight gain between broilers fed the NC2 diet and PC diet. Phytase enzyme added to the NC1 diet increased the growth rate to levels that were comparable (p>0.05) to the NC2 diet during the growth period (days 0−21) and then to both the NC2 and PC diet during the maintenance period (days 21−35). During the growth period, broilers fed the NC1 diet supplemented with modified *E.coli* at 500FTU/kg showed numerically (p>0.05) greater weight gain than broilers fed the NC1 diet supplemented with modified *E.coli* at 1000FTU/kg and broilers fed the NC1 diet supplemented with other phytase enzyme products at 500FTU/kg and 1000FTU/kg.

During the first 14 days, broilers showed a linear average daily weight gain. Weight gain was reduced between days 14 and 21; thereafter, growth rate linearly increased again until day 28. The exception was the NC1 diet, which showed a linear increase until day 21, then linearly increased again from days 28 to 35. Statistically, no significant difference (p>0.05) was observed.

Table 4.1 Weekly individual bird weight (g/bird) and total weight gain (g/bird) over the three critical periods

		Body weight (g/bird)					*Total weight gain (g/bird)*		
Treatment	**0 day**	**7 days**	**14 days**	**21 days**	**28 days**	**35 days**	**0-21 days**	**21-35 days**	**0-35 days**
Negative control 1 (NC1)	45,40	171.33^{d}	420.70^{b}	842.46^{b}	1271.22^{b}	1813.88^{b}	797.06^{b}	971.42^{b}	1768.48^{b}
Negative control 2 (NC2)	45,48	185.73^{ab}	493.33^{a}	873.92^{ab}	1448.20^{ab}	2092.86^{a}	828.45^{ab}	1218.94^{ab}	2047.39^{a}
Positive control (PC)	46,16	186.46^{a}	498.22^{a}	917.46^{a}	1536.56^{ab}	2173.07^{a}	871.30^{a}	1255.61^{a}	2126.91^{a}
NC1 + modified* E.coli *500FTU	45,86	179.01^{abcd}	500.86^{a}	898.32^{a}	1539.85^{a}	2196.00^{a}	852.46^{a}	1297.68^{a}	2150.14^{a}
NC1 + modified* E.coli *1000FTU	45,36	184.32^{abc}	506.30^{a}	888.57^{ab}	1461.33^{ab}	2120.76^{a}	843.21^{ab}	1232.20^{a}	2075.40^{a}
NC1 + product X 500 FTU	45,65	173.98^{cd}	479.96^{a}	857.54^{ab}	1402.63^{ab}	2014.00^{ab}	811.90^{ab}	1156.45^{ab}	1968.35^{ab}
NC1 + product X 1000FTU	45,81	174.51^{bcd}	494.82^{a}	883.79^{ab}	1509.26^{ab}	2154.25^{a}	837.98^{ab}	1270.46^{a}	2108.45^{a}
NC1+ product Y 500FTU	45,42	177.50^{abcd}	495.26^{a}	884.89^{ab}	1469.49^{ab}	2016.16^{ab}	839.46^{ab}	1131.27^{ab}	1970.73^{ab}
NC1 + product Y 1000FTU	45,34	176.08^{abcd}	493.11^{a}	871.54^{ab}	1395.66^{ab}	2045.44^{ab}	826.20^{ab}	1173.90^{ab}	2000.09^{ab}

a-d Means within a column without a common superscript are significantly different (p<0.05)

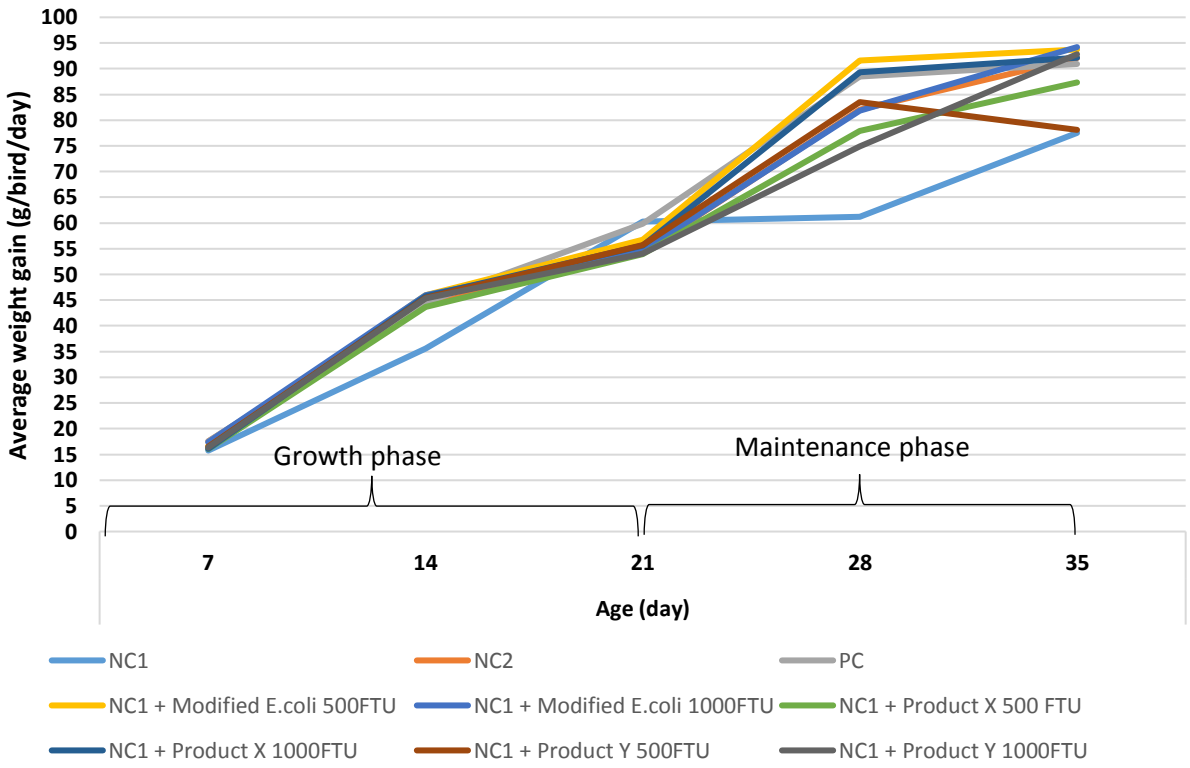

Figure 24.1 Average daily weight gain (g/bird/day) of broilers fed treatments 1−9 from day 7 to day 35 of the trial period.

On day 21, as table 4.2 clearly shows, an incremental increase in avP resulted in a significant increase ($p<0.05$) in feed intake, with broilers fed the NC1 diet having the lowest feed intake, broilers fed the PC diet having the highest feed intake and broilers fed the NC2 diet having a feed intake level between that of broilers fed the NC1 and PC diets. By the end of the trial period, broilers fed the NC1 diet had a significantly lower ($p<0.05$) feed intake compared to broilers fed the NC2 and PC diets. On day 35 there was no significant difference ($p>0.05$) in feed intake between broilers fed the NC2 and PC diets. Phytase supplemented in the NC1 diet resulted in a significant increase ($p<0.05$) in feed intake compared to the NC1 diet alone, to levels that were comparable ($p>0.05$) to broilers fed the PC diet. On both day 21 and day 35 of the trial, modified *E.coli* supplemented at 500FTU/kg resulted in numerically greater ($p>0.05$) feed intake compared to modified *E.coli* supplemented at 1000FTU/kg. No significant difference was detected in feed intake between Modified *E.coli*, product X and product Y supplemented at both 500 FTU/kg and 1000 FTU/kg.

Broilers fed the NC1 diet had significantly higher ($p<0.05$) mortality rates on both day 21 and day 35 of the trial compared to broilers fed all other treatment diets. At the end of the trial period, broilers fed the NC1 diet supplemented with phytase enzymes had numerically greater ($p>0.05$) mortalities than broilers fed the NC2 and PC diets.

Table 4.2 Cumulative feed intake (g/bird) and cumulative mortality (%) during the 35 day trial period for all nine treatment diets

	Cumulative feed intake (g/bird)					*Cumulative mortality (%)*	
Treatment	**7 days**	**14 days**	**21 days**	**28 days**	**35 days**	**21 days**	**35 days**
Negative control 1 (NC1)	170.93^{b}	492.75^{b}	1100.77^{c}	1822.90^{b}	2788.83^{b}	12.31^{a}	23.46^{a}
Negative control 2 (NC2)	178.70^{b}	568.22^{a}	1162.63^{bc}	2075.05^{a}	3206.65^{a}	1.54^{b}	3.85^{b}
Positive control (PC)	181.81^{b}	569.51^{a}	1239.18^{ab}	2146.33^{a}	3263.65^{a}	2.31^{b}	3.85^{b}
NC1 + modified* E.coli *500FTU	191.58^{ab}	577.66^{a}	1230.27^{ab}	2163.24^{a}	3304.80^{a}	4.23^{b}	7.69^{b}
NC1 + modified* E.coli *1000FTU	198.63^{ab}	576.60^{a}	1215.23^{ab}	2095.69^{a}	3254.31^{a}	2.69^{b}	5.77^{b}
NC1 + product X 500 FTU	198.06^{ab}	562.98^{a}	1179.72^{abc}	2047.76^{a}	3120.11^{a}	1.54^{b}	4.62^{b}
NC1 + product X 1000FTU	240.28^{a}	603.46^{a}	1259.72^{a}	2175.62^{a}	3302.19^{a}	3.46^{b}	9.62^{b}
NC1+ product Y 500FTU	200.36^{ab}	578.14^{a}	1231.22^{ab}	2118.77^{a}	3211.26^{a}	2.69^{b}	5.00^{b}
NC1 + product Y 1000FTU	200.40^{ab}	574.52^{a}	1199.34^{ab}	2075.46^{a}	3245.35^{a}	3.08^{b}	6.15^{b}

a-c Means within a column without a common superscript are significantly different ($p<0.05$)

Throughout the trial period no significant ($p>0.05$) difference in FCR could be detected among broilers fed any of the treatment diets, as noted in table 4.3. Numerically ($p>0.05$), on day 35 it can be seen that an incremental increase in avP resulted in a subsequent improvement in FCR. Phytase supplementation did not significantly or numerically improve FCR compared to the NC1 diet.

Table 4.3 Weekly cumulative feed conversion ratio (FCR) from day 7 to day 35 of the trial period for treatment diets 1–9

Treatment	*Cumulative FCR*				
	7 days	**14 days**	**21 days**	**28 days**	**35 days**
Negative control 1 (NC1)	1.36[b]	1.32[a]	1.41[a]	1.50[a]	1.59[a]
Negative control 2 (NC2)	1.27[b]	1.27[a]	1.41[a]	1.50[a]	1.57[a]
Positive control (PC)	1.30[b]	1.26[a]	1.43[a]	1.45[a]	1.54[a]
NC1 + modified* E.coli *500FTU	1.43[b]	1.27[a]	1.45[a]	1.47[a]	1.55[a]
NC1 + modified* E.coli *1000FTU	1.43[b]	1.25[a]	1.45[a]	1.50[a]	1.58[a]
NC1 + product X 500 FTU	1.54[ab]	1.30[a]	1.46[a]	1.52[a]	1.59[a]
NC1 + product X 1000FTU	1.85[a]	1.34[a]	1.51[a]	1.50[a]	1.57[a]
NC1+ product Y 500FTU	1.50[ab]	1.29[a]	1.47[a]	1.51[a]	1.64[a]
NC1 + product Y 1000FTU	1.53[ab]	1.28[a]	1.45[a]	1.56[a]	1.64[a]

a-c Means within a column without a common superscript are significantly different ($p<0.05$)

On both day 21 and day 35, broilers that were fed the NC1 diet had significantly lower ($p<0.05$) PEF compared to broilers fed the NC2 and PC diet. Numerically ($p>0.05$) broilers fed the PC diet had a greater PEF than broilers fed the NC2 diet. Phytase added to the NC1 diet at both 500FTU/kg and 1000FTU/kg showed a significantly greater ($p<0.05$) PEF compared to the NC1 diet alone, to levels that were comparable ($p>0.05$) to the NC2 and PC diets. Modified *E.coli* supplemented at both 500FTU/kg and 1000FTU/kg showed comparable ($p>0.05$) improvements in PEF compared to NC1 diets supplemented with two other phytase enzymes at similar inclusion levels. Modified *E.coli* supplemented at 500FTU/kg showed a numerically ($p>0.05$) greater PEF compared to modified *E.coli* supplemented at 1000FTU/kg, to levels greater ($p>0.05$) than the NC2 diet but lower ($p>0.05$) than the PC diet. All other phytase enzyme supplementation resulted in a PEF lower ($p>0.05$) than the NC2 diet, but greater ($p<0.05$) than the NC1 diet.

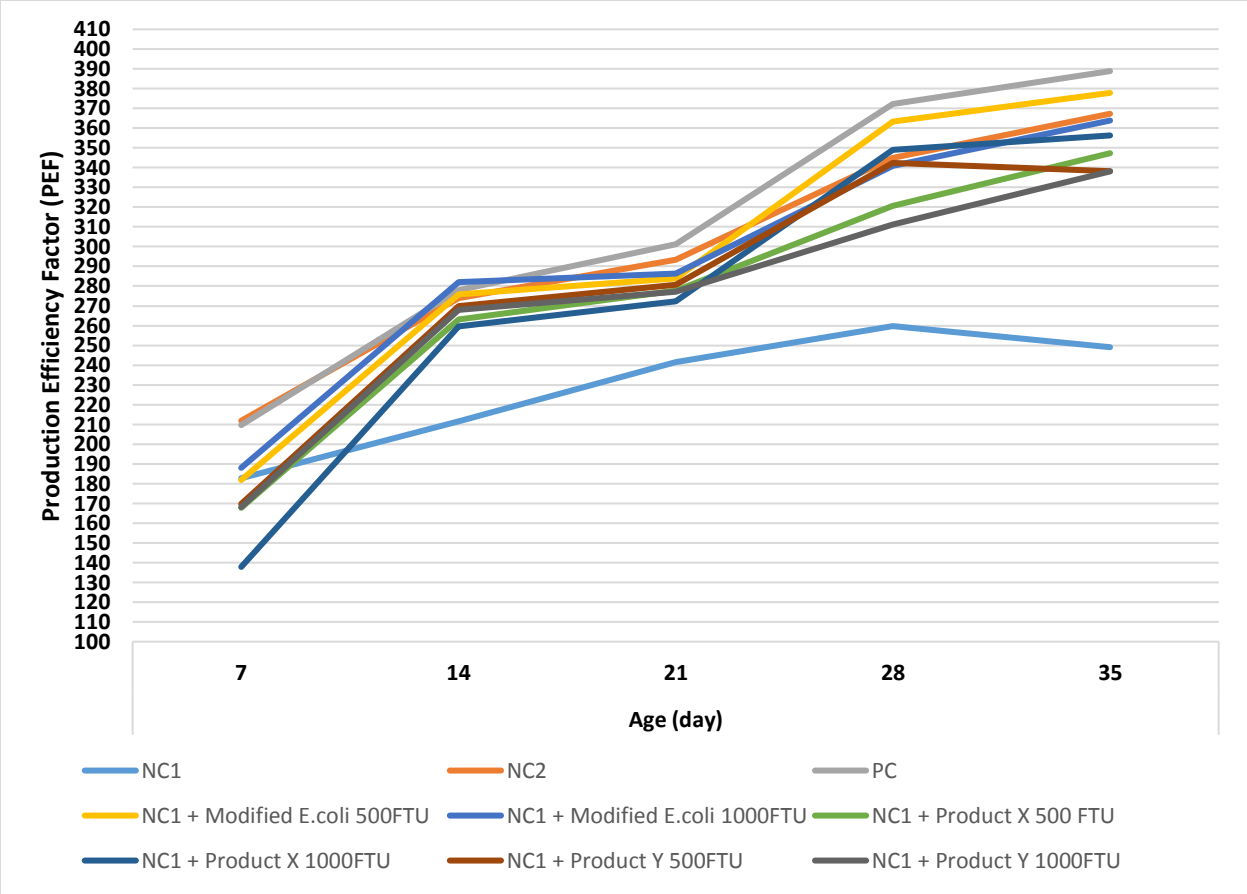

Figure 4.25 Average production efficiency factor (PEF) for broilers fed treatment diets 1–9 over the entire 35 day trial period.

4.2. Tibia analysis

Three measurements, namely; bone breaking strength tibia ash and tibia mineral content (CA and P) were determined to quantify the influence of phytase enzymes on bone development.

On both day 21 and day 35 broilers fed the NC1 diet had significantly ($p<0.05$) weaker bone strength compared to broilers fed the NC2 and PC diet, as seen in table 4.4. No significant ($p>0.05$) difference was seen in bone breaking strength between broilers fed the PC and NC2 diet; however, one can observe numerically that broilers that were fed the NC2 diet had more bone breaking strength than broilers fed the PC diet. Phytase supplemented to the NC1 diet

resulted in a significant ($p<0.05$) increase in bone strength compared to the NC1 diet alone, to levels that were comparable ($p>0.05$) to the NC2 and PC diets. No significant ($p>0.05$) difference could be detected in bone breaking strength between broilers fed a NC1 diet supplemented with modified *E.coli* at 500FTU/kg and a NC1 diet supplemented with modified *E.coli* at 1000FTU/kg. Modified *E.coli* supplemented at 500FTU/kg and 1000FTU/kg showed comparable ($p>0.05$) results to NC1 diets supplemented with other heat-stable phytase enzymes at similar levels.

On days 21 and 35, broilers that were fed the NC1 diet showed significantly lower ($p<0.05$) tibia ash (%) compared to broilers fed all other treatment diets. On day 21, broilers fed the NC2 diet showed significantly higher ($p<0.05$) tibia ash across all treatment groups. By day 35 the PC diet showed significantly higher ($p<0.05$) tibia ash across all the control diet groups. On both day 21 and day 35 broilers fed the NC1 diet supplemented with 500FTU modified *E.coli* was not significantly ($p>0.05$) different from broilers supplemented with 1000FTU modified *E.coli.*

Numerically, on day 21, broilers supplemented with 1000FTU modified *E.coli* showed greater tibia ash content than broilers supplemented with 500FTU modified *E.coli*; however, by day 35 modified *E.coli* supplemented at 500FTU and 1000FTU resulted in similar tibia ash content. Both doses of modified *E.coli* was not significantly ($p>0.05$) different to the PC diet; however, numerically, modified *E.coli* supplementation resulted in greater tibia ash content compared to the PC diet.

On day 21 no significant ($p>0.05$) difference was detected between broilers fed the NC1 diet supplemented with modified *E.coli* and those supplemented with product X at 1000FTU. Product X supplemented at 500FTU resulted in the lowest ($p<0.05$) tibia ash content compared to all other phytase-supplemented treatments. At 500FTU, supplementation with product Y showed lower ash content compared to supplementation with modified *E.coli*. However, product Y supplemented at 1000FTU resulted in greater tibia ash content than modified *E.coli* at the same dose.

On day 35 no significant difference ($p>0.05$) was detected between the supplementation with modified *E.coli*, product X and product Y. A general trend was also observed where tibia

ash content decreased from day 21 to day 35 in the control diet, while it increased in the phytase-supplemented diets.

In terms of mineralisation, product Y at 500FTU resulted in the greatest ($p<0.05$) calcium deposition compared to all other phytase supplemented diets to levels similar ($p>0.05$) to the PC diet. Modified *E.coli* at 1000FTU resulted in the greatest ($p<0.05$) phosphorous deposition compared to all other phytase supplemented diets and the PC diet. Supplementation of product Y at both doses resulted in phosphorus deposition that was similar ($p<0.05$) to the PC diet.

Table 4.4 Average bone breaking strength (N), Ash (%), Ca (%) and P (%) on day 21 and day 35 of broilers fed one of nine treatment diets

	Breaking strength (N)		*Ash (%)*		*Ca (%)*		*P (%)*	
Treatment	**Day 21**	**Day 35**	**Day 21**	**Day 35**	**Day 21**	**Day 35**	**Day 21**	**Day 35**
Negative Control 1 (NC1)	106.42^{b}	149.49^{b}	43.31^{d}	42.52^{b}	15.4^{bc}	13.4^{c}	7.78^{d}	7.35^{c}
Negative Control 2 (NC2)	171.83^{a}	310.88^{a}	49.61^{a}	47.14a^{b}	16.06^{ab}	16.27^{ab}	9.26^{ab}	9.28^{ab}
Positive Control	165.12^{a}	272.08^{a}	47.87^{abc}	47.34^{a}	16.57^{a}	17.43^{a}	9.37^{a}	9.43^{ab}
NC1 + modified E.coli *500FTU*	184.43^{a}	299.1^{a}	47.97^{abc}	49.17^{a}	15.76^{abc}	16.34^{ab}	8.9^{abc}	9.21^{ab}
NC1 + modified E.coli *1000FTU*	182.85^{a}	320.84^{a}	49.01^{abc}	49.82^{a}	16.6^{a}	16.3^{ab}	9.19^{ab}	9.61^{a}
NC1 + product X 500 FTU	170.8^{a}	270.96^{a}	46.68^{c}	47.13^{ab}	15.11^{bc}	15.72^{b}	8.37^{cd}	8.88^{ab}
NC1 + product X 1000FTU	170.93^{a}	317.86^{a}	47.86^{abc}	48.18^{a}	15.07^{bc}	15.45^{b}	8.68^{bc}	8.75^{b}
NC1+ product Y 500FTU	177.72^{a}	278.11^{a}	47.14^{bc}	47.69^{a}	15.28^{bc}	17.15^{a}	9.04^{abc}	9.43^{ab}
NC1 + product Y 1000FTU	168.01^{a}	304.01^{a}	49.26^{ab}	48.64^{a}	14.9^{c}	16.67^{ab}	8.66^{bc}	9.45^{ab}

a-d Means within a column without a common superscript are significantly different ($p<0.05$)

Chapter 5
Discussion

5.1. Animal performance

Animals require phosphorus for growth and development. Phosphorus is involved in bone development, acid-base balance, energy metabolism, and DNA and RNA synthesis (Jiang *et al.*, 2013). Phosphorus stored as phytate in cereal grains adversely influences broiler performance and bone development because of its antinutritional interactions with dietary nutrients. The amount of phytate found in cereal grains is dependent on the cultivar, age and the environmental conditions that the cultivar is exposed to. Consequently, in a typical maize-soybean meal-based diet, maize and soybean meal can have a phytic acid content ranging from 0.80−1.17g/100g and 1.20−1.75g/100g, respectively (Hidvégi & Lásztity, 2002). Consequently, this means that in a complete formulated broiler diet up to two-thirds of the total phosphorus in the diet is in the form of phytate, and thus is not bioavailable to the broiler chick (Pieniazek *et al.*, 2015).

Phytate dissociates at a pH of 2 in the proventriculus and becomes increasingly negatively charged as the pH rises from the proventriculus to the small intestine. Adverse interactions between phytate, cations and proteins occur largely in the gizzard (average pH 3.5) and duodenum (pH 6), impairing nutrient absorption in the jejunum and ileum (Santos, 2012; Dersjant-Li *et al.*, 2015). Phytase is a protein enzyme that dephosphorylates phytate into its lower phosphate esters and increases its solubility. This reduces phytate interactions with dietary nutrients and increases phosphorus bioavailability, consequently promoting animal performance and bone development (Selle & Ravindran, 2007). The objective of this study was to determine whether the optimal dose of this modified new generation *E.coli* 6-phytase (modified *E.coli*) is 500FTU/kg or 1000 FTU/kg, and to compare its efficacy to other currently-used heat-stable phytase products on a typical commercial South African broiler diet.

The growth of a broiler chicken follows a sigmoidal pattern according to the Gompertz function. The curve involves a lag phase (days 0–3), acceleration (growth) phase (days 4–21), inflection point, stationary (maintenance) phase (days 21–35) and deceleration phase (Batt, 1980; Henn *et al.*, 2014). Body weight is an important economic trait as the broiler industry is target-weight driven. This means that at a weight of 1.8–2.0kg slaughter occurs, regardless of age. Typically, under commercial South African conditions this weight is reached at 35 days of age. A body weight below or above the target results in reduced income per broiler for the producer (Tavárez & de los Santos, 2016). In this study, the body weight of broilers, at day 21, fed a severely phosphorus-deficient diet supplemented with phytase was comparable to the body weight of broilers fed a diet containing the nutritionally correct amount of available phosphorus required by a broiler, as stated by the Cobb 500 manuel. The only exception was broilers fed a diet supplemented by product X at 500FTU/kg. Broilers fed a diet containing modified *E.coli* supplemented at 500FTU/kg had numerically greater body weights than all other phytase-supplemented diets and significantly greater ($p<0.05$) body weights compared to broilers given the negative control diets. However, broilers given a diet supplemented with modified *E.coli* had numerically lower body weights than broilers given the positive control (PC) diet, which is consistent with the 21-day observations recorded in the study conducted by Pieniazek *et al.* (2016).

The results from this study contrast with the results from the Henn *et al.* (2014) study. In the Henn *et al.* (2014) study, rapid growth occured from day 3 to day 21, with a deceleration in growth rate from day 21 to day 35, followed by a decline in growth rate from day 35 onwards, as is expected when broilers are provided *ad libitum* access to water and a diet that meets their minimum nutritional requirements for each growth phase. However, in the current study (figure 4.1) for all treatments except negative control 1 (NC1), the growth decelerates at day 14 and accelerates again on day 21 to day 28, with the maintenance phase occurring from days 28 to 35. The NC1 diet follows the general growth curve except at day 28 where accelerated growth is observed. Possible reasons for this could be unintentional restricted feeding during the first 2 weeks, diet and environmental conditions.

Compensatory growth is characterised by a rapid increase in lean muscle deposition, resulting in increased body weight and efficiency of feed use following a period of poor-quality

feed, reduced feed intake and/or environmental stress, such as disease and heat stress (Zubair & Leeson, 1996; Summers, 2008). Multiple scientific review articles and research papers on the topic of compensatory growth have been published. It is observed that broilers that undergo compensatory growth are able to achieve market weight or higher at the same age as broilers which were fed nutritionally adequate diets and experienced no environmental stress. However, the extent to which compensatory growth occurs is largely dependent on the extent and period of time of undernutrition/feed restriction, and the age at which it occurred. For instance, a low energy diet reduces body weight by 4% and feed intake limitations reduce body weight by 33%; however broilers are able to achieve target body weight by 35 days of age if allowed *ad libitum* access to feed from day 14 onwards. Broilers subjected to 20% crude protein diets in the first five weeks can recover from reduced body weight in two weeks (Giachetto *et al.* 2003; Summers, 2008). In this study the energy content of both the starter and finisher PC diets; 2587.78 kcal/kg and 2547.78 kcal/kg, respectively, is below the minimum requirements recommended by the Cobb 500 manual – 3008kcal/kg and 3167 kcal/kg, respectively. The crude protein and sodium content of both diets is adequate. The energy content of the NC1 diet remained consistent from starter to finisher; however, the energy content in both the NC2 and PC diets was reduced. Consequently, on day 21 when the starter diet was changed to the finisher diet, the severity of the energy deficiency remained consistent for broilers fed the NC1 diet and increased for broilers fed the NC2 and PC diets. This allowed for a greater extent of compensatory growth in broilers fed the NC1 diet from day 28.

Production efficiency factor (PEF) is an economic tool designed to allow producers to determine and compare the economic efficiency and performance of their operation to those of other broiler operations in different locations. The PEF values consider the average body weight, livability, age and feed efficiency (FCR) of the broiler flock. FCR is an important parameter as feed costs contribute up to 70% of a broiler production operation. Therefore, the efficiency at which broilers can convert a gram of feed into a gram of muscle is a key economic factor in reducing production costs (Shane, 2017). The two negative control diets resulted in the most efficient feed use numerically compared to all other treatment diets on day 21. On day 35, although both negative control diets showed numerically poorer feed efficiency compared to the PC diet, the phytase-supplemented diets showed even poorer FCR.

A potential explanation for these observed results could be the high mortality rate observed on day 21 and day 35, which was not due to diet. Broilers in pens that experience a high mortality rate often have improved feed and performance efficiency during the following weeks. Diets that contained 500FTU modified *E.coli*, 1000FTU product X and 1000FTU product Y, which had the highest mortality rates, had the greatest increases in growth rate, such that by day 35 these three treatments resulted in a numerically greater growth rate compared to the PC. Unlike FCR values, where lower values indicate greater flock performance efficiency, a higher PEF value is interpreted as greater production efficiency (Marcu *et al.*, 2013; Shane, 2017). Phytase supplementation resulted in numerically greater PEF values compared to the NC1 diet, but just less than the PC diet. A modified *E.coli* supplemented at 500FTU/kg diet resulted in the greatest PEF value compared to all other phytase-supplemented diets.

These results contradict the observations recorded in the study conducted by van Emmenes *et al.* (2014), where broilers fed diets supplemented with 1000FTU phytase showed greater PEF values than those fed diets containing 500FTU phytase. According to Marcu *et al.* (2013) the Cobb 500 strain has an average PEF value of 256.49 on day 14 and 364.24 on day 35. In this study, day 14 PEF values for all treatments were 10 PEF units or more above that stated by Marcu *et al.* (2013). However, by day 35 only NC2, PC, and 500FTU modified *E.coli* diets resulted in PEF values above the stated average value. Modified *E.coli* at 1000FTU was comparable to the average value, and all other phytase-supplemented diets were below the average value. The study by Marcu *et al.* (2013) further determined the influence of PEF on feed cost per kg of weight gained (Euro/kg gain). It was observed that the improved performance efficiency of Cobb 500 compared to Ross 308 resulted in a 0.44%, 1.71% and 14.31% reduction in the costs of the starter, grower and finisher diet respectively, relative to the weight gained. In this study the price of feed per kg weight gained was not evaluated for each treatment; thus we cannot determine the economic benefit of one phytase enzyme over the other compared to the controls. It would be extremely beneficial to calculate this economic parameter in future studies as the result would have a direct tangible impact on the producer.

From the growth performance parameter measures one can observe that a linear increase in avP linearly increases growth performance. Broilers supplemented with modified *E.coli* phytase at 500FTU show greater body weight gain, PEF and improved FCR compared to broilers

supplemented at 1000FTU in both the growth phase (days 0–21) and the maintenance phase (days 21–35). Broilers supplemented with product X phytase showed greater increase in body weight, PEF and improved FCR at 1000FTU compared to 500FTU. During the growth phase broilers supplemented with product Y phytase at 500FTU showed improved performance compared to 1000FTU; however, by day 35 broilers supplemented with product Y phytase at 1000FTU showed improved performance compared to broilers supplemented at 500FTU. Overall, numerically broilers supplemented with modified *E.coli* phytase showed the greatest improvement in performance compared to broilers supplemented with product X or product Y phytases during both the growth and maintenance phase. During the growth phase broilers supplemented with product Y phytase showed numerically higher performance compared to broilers supplemented with product X phytase; however, by the end of the maintenance phase broilers supplemented with product X phytase showed numerically higher performance compared to broilers supplemented with product Y phytase.

5.2. Tibia analysis

The architecture of the bone structure influences bone strength. The consequence of weak bones often entails pain for the animal during rearing, and bone fractures and breakage during catching, transportation and processing. This leads to poor animal welfare, condemned carcasses and/or reduced meat grades. Consequently, leg problems are the biggest cause of economic losses in a poultry house (Almeida Paz & Bruno, 2006; Buijs *et al.*, 2012). Bone breaking strength determines the amount of force the bones can withstand before fractures and breakage occurs, and is a function of its physical, architectural and matrix characteristics. Thus, it is an indirect measure to determine the maximum potential weight of a bird at a specific age and, by inference, incidence of leg problems in a broiler house (Rath *et al.*, 2000). To determine overall phytase activity tibia ash is measured because the proportion of ash to total bone weight is directly associated with the extent of mineralisation of the tibia, while bone mineralisation is sensitive to the bioavailability of minerals within a diet and is directly correlated to phosphorous and calcium deposition (Viveros *et al.*, 2002; Hall *et al.*, 2003; Shim *et al.*, 2012).

Commercially phytase is supplemented at a dose of 500FTU/kg diet. Supplementing phytase from 1000FTU/kg diet upwards has extra-phosphoric benefits due to a 30% additional increase in phytate dephosphorylation at the high phytase doses (Lee *et al.*, 2003). In the current study, it was observed that phytase addition statistically ($p<0.05$) improved bone strength and ash compared to the NC1. This observation is in agreement with a study by Morgan *et al.* (2016) where it was shown that phytase supplemention at 500FTU/kg increased breaking strength ($p<0.05$), regardless of phytate susceptibility to phytase, compared to a diet with no phytase. A study by Viveros *et al*. (2002) agreed, as 5.1% increase in tibia ash was observed in phytase-supplemented NC1 diets compared to the NC1 diet alone.

In this study, no statistical difference in bone breaking strength and tibia ash content was observed between phytase supplemented at 500 and 1000FTU/kg diet. This is in agreement with a study conducted by Singh *et al.* (2003), where phytase supplementation resulted in numerical ($p>0.05$) increase in tibia ash compared to the control diet. Numerically we found that during the growth phase (0–21 days) phytase supplemented at 500FTU showed greater bone breaking strength, but lower tibia ash compared to 1000FTU in the same phase. During the maintenance phase (22–35 days), phytase supplemented at 1000FTU showed greater bone strength and ash content ($p>0/05$). The bone strength values and ash content are in agreement with the findings by Han *et al.* (2015) where bone development increased ($p<0.05$) linearly in the first three weeks of age, thereafter bone development and growth continued to increase but at a slower rate.

Additionally, it can be seen that a linear increase in avP did not result in a subsequent improvement in bone development parameters evaluated as broilers given the NC2 diet had, statistically ($p<0.05$), the greatest bone breaking strength results on day 21 and day 35, and tibia ash results on day 21. By day 35, a linear increase in avP did result in a subsequent increase in tibia ash in the control diets. A potential reason for this is that the total calcium content in the starter and finisher control diets in this study were formulated to increase by 0.07% and 0.08% respectively, such that NC1 contained 0.70% Ca and 0.55% Ca, NC2 contained 0.77% Ca and 0.63% Ca, and PC contained 0.85% Ca and 0.71% Ca in the starter and finisher diets respectively. However, when analysed it was found that the total calcium and phosphorus content in the control diets differed, such that NC1 contained 0.87% Ca and 0.78% Ca, NC2 contains 0.91% Ca and 0.85% Ca, and PC contains 0.93% Ca and 0.79% Ca in the starter and

finisher diets respectively. Consequently, the Ca:avP ratio changed in the starter and finisher diets. The avP content in the diets was not analysed, therefore we cannot say with any certainty what the exact ratio of Ca:avP in the control diets were. One can speculate that if the analysed avP content had increased by the same amount as the analysed total P content compared to the total P formulated content, then the Ca:avP ratio would range between 3.11–3.78 and 5.20–6.50 in the NC1 diet, 2.46–2.76 and 3.15–3.86 in the NC2 diet, and 1.84–2.14 and 1.93–2.47 in the PC diet for the starter and finisher phases respectively. This would suggest that although a lower Ca:avP ratio of 1.8–2.5 is optimal for growth performance, the ratio for optimal bone development appears to be higher at 2.3–2.8. These findings are in agreement with Han *et al.* (2016), who found that the optimal dietary Ca:avP to be 2.2–2.3 for growth performance, 2.2–2.5 for bone development, and 2.3 for optimum growth and bone development and quality.

Bone mineralisation is sensitive to the bioavailability of minerals within a diet and is directly correlated to phosphorous and calcium deposition (Viveros *et al*., 2002; Hall *et al*., 2003). Manobhaven *et al.* (2015) observed that phytase supplemented at doses between 1000 and 5000FTU/kg diet improved bone length, width and mineral content ($p<0.05$) compared to the bones of broilers given a diet containing the standard commercial phytase dose. These results agree with the results found in this study as unlike the performance results where modified *E.coli* supplemented at 500FTU showed greater improvements ($p>0.05$) compared to modified *E.coli* supplemented at 1000FTU, all phytase's supplemented at 1000FTU showed greater improvements in bone development parameters compared to their 500FTU counterparts. Minerals are critical and integral components of many enzyme, metabolic and growth systems and functions in the animal body. For instance, zinc (Zn) is important for immune function, cell differentiation and enzyme function. Magnesium (Mg) interacts with Ca and P during the development of bone and plays a role in enzyme function and ionic balance. Manganese (Mn) forms proteoglycan matrix which is important for later stages of bone development (Kleyn, 2013). Viveros *et al.* (2002) found that the available phosphorus content in the diet and phytase supplemented at 500FTU/kg significantly influenced not only the deposition of Ca and P in the bone, but also of Zn and Mg. The change in Ca:avP ratio between formulated Ca:avP and analysed Ca:avP in the diet would have resulted in differences to the availability of Ca and P for deposition in bone, especially since it has been proven by several authors that calcium content in the diet does impair phosphorus deposition in the bone. Therefore, although phytase

supplementation, specifically that modified *E.coli* supplementation at 500FTU and 1000FTU, resulted in a tibia ash content greater than the PC diet we can see that modified *E.coli* supplementation did not result in greater Ca and P deposition compared to the PC diet. Without analysing for Zn, Mg and Mn, we have no way of knowing how much of the ash content not attributed to Ca and P is made up of Zn, Mg and Mn, and how the extent of deposition of these three minerals influences breaking strength.

Chapter 6

Critical Review

The efficacy of a phytase enzyme and, consequently, the results of an animal trial are influenced by several integrated intrinsic factors. These factors are broadly classified into three characteristics: the enzyme, the diet and the animal (Kiarie *et al.*, 2015).

Variation can be caused by a variety of genetic, environmental, management and nutritional conditions working independently or in combination. In this study, a large variation within treatments resulted in no significant difference being observed between treatments. During the animal trial, a variety of management and environmental issues arose. Management issues included leaking drinkers, incorrect house temperature regulation, broken vents, a tap being left open resulting in feed getting wet and becoming moldy, a broilers foot was stepped on, fly infestation and power outages. These factors contributed to the variation in performance and the high cumulative mortality of 3.76% on day 21 and 7.78% on day 35.

Based on the performance and tibia analysis results, we found that modified *E.coli* supplementation resulted in numerically greater PEF, weight gain, tibia breaking strength and ash content compared to a severely available phosphorus-deficient diet and other phytases supplemented. A possible reason is that modified *E.coli* has greater activity in the proventriculus compared to the other phytases used, if one takes the observations recorded in the study by Pieniazek *et al.* (2016) as a standard. Another possible explanation is the granule size of the phytases used. As can be seen in picture 2 of appendix 4, the individual granule size for each phytase product from biggest to smallest is product X, product Y and modified *E.coli*. Theoretically, a larger granule would result in less phytase intake per gram of feed consumed compared to finer granules. It follows that reduced phytase intake would result in reduced phytase activity per gram of feed entering the proventriculus. However, without the results of phytase activity analysis, this is purely speculation.

In terms of growth performance, one would expect reduced feed intake and dietary nutrients in feed to be directed towards growth parameters more effieciently (measured as FCR) in broilers undergoing compensatory growth as insulin secretion and growth hormone

concentration in the plasma would increase, allowing for a greater proportion of the dietary nutrients absorbed to be directed toward growth instead of maintenance (Zubair and Leeson, 1996; Hornick *et al.*, 2000). However, in this study although feed intake for broilers fed the NC1 diet was lower compared to all other diets, their FCR did not improve from day 28 to 35. This is consistent with a study reviewed in the article by Summers (2008), where feed efficiency was not significantly influenced by compensatory growth; rather, broilers that underwent compensatory growth had significantly increased carcass fat content. In this study carcass fat was not measured so we cannot compare the results.

The use of growth models is an important tool in the broiler industry as it allows producers to estimate the age at which a broiler or flock will reach market weight, the expected feed intake and FCR, thereby allowing for adjustments in diet formulations to provide specific nutrients at specific physiological ages. The result is that poultry meat producers have realised an 8–10% reduction in production costs (Hruby *et al.*, 1996; Henn *et al.*, 2014). However, as far as this author is aware, there appears to be an absence of peer-reviewed published research on the influence of phytase supplementation, either on its own or in combination with other feed enzymes, on the growth curve. This could be a potential area of interest for producers because, if phytase supplementation can shift the growth curve to the left, a greater proportion of the broiler's production life will consist of the growth phase. The consequences are that the broilers' nutritional requirements may alter and broilers could reach market age sooner. To the producer, this means more cycles in a house per year and thus greater income.

A possible explanation for the lack of significant difference observed in bone breaking strength could be slippage of the bones on the support beams as force was applied. This would have resulted in some bones obtaining a hairline fracture before breakage, resulting in lower bone breaking strength values, and some bones were broken closer to the epiphysis rather than in the middle of the diaphysis. This causes a large variation within treatments, resulting in no significant difference being observed. Another factor leading to within-treatment variation is gender. According to Rath *et al.* (2000) and Han *et al.* (2015), male broilers have significantly ($p<0.05$) greater bone development compared to female broilers until 3–5 weeks of age, thereafter no statistical difference is observed between male and female bone development. Gender influences bone development due to differences in the rate of physiological growth of the

different body tissues and muscles, physical activity and hormone concentrations. In the current study, the broilers were not sexed at placement or during tibia collection.

Another potential avenue that requires further research is the use of the femur as a predictive indicator for the incidence of leg problems later in a broilers life. Applegate & Lilburn (2002) suggest that the femur should be the principle bone to measure breaking strength, rather than the tibia as it is a more precise indicator of the maximum load (weight of the broiler) at a specific age and because it is more sensitive to overall skeletal mineralisation, and calcium and phosphorus turnover. This is because the femur responds and adjusts to changes in tibia conformation and changes in body weight, in terms of breast muscle development. The femur responds to align the upper body to the lower skeletal axis, which ultimately influences the load placed on the tibia, walking ability and welfare of the animal.

A dietary factor that could have contributed to the variation within treatment groups is that the matrix value of each phytase enzyme product was not taken into consideration during diet formulations. Sharma *et al.* (2016) observed that phytase supplemented on-top of an already formulated diet increases the amount of dietary sodium excreted, which resulted in increased water consumption. Consequently, the occurrence of wet litter was observed, which is consistent with conditions experienced during the trial run at the ARC. Sodium is an important electrolyte as it is involved in several physiological functions within the animal body such as acid-base and osmotic balance, body fluid regulation, muscle contraction, adrenal gland function, bone development and energy metabolism (Oviedo-Rondón *et al.*, 2001; Živkov Baloš *et al.*, 2016).

According to Živkov Baloš *et al.* (2016) moderate sodium deficiency manifests as reduced growth, weak bones, diarrhea, and renal and digestive disorders. Logically, it follows that the occurrence of diarrhea implies reduced amount of time that the digesta remains in each compartment of the digestive tract and, therefore, reduced nutrient absorption in the small intestine. The treatment diets for this study was formulated to contain adequate sodium content as recommended by the Cobb 500 manual. However, if phytase supplementation did indeed result in increased sodium loss, then the broilers in this study were deficient in sodium, which is a possible explanation for the large variation in performance and the occurrence of wet litter. In addition, wet litter promotes microbial growth, and increases ammonia and odor emissions,

leading to impaired animal welfare. Oviedo-Rondón *et al.* (2001) concluded that a broilers dietary sodium requirement is 0.28% to achieve optimum performance, feed efficiency and reduced incidence of tibial dyschondroplasia. This recommendation contradicts Živkov Baloš *et al.* (2016), who recommends 0.17–0.19% as the dietary level. However, it should be noted that in both studies the aim was not to determine the optimum dietary sodium level in a phytase-supplemented diet. Sharma *et al.* (2016) concluded that when phytase matrix values for protein, energy, Ca, P and Na are considered while formulating the diet, the impact of phytase supplementation on broiler performance and bone development is greater, and sodium loss is minimised.

Another dietary factor that contributes to the observed within-treatment variation is the available phosphorus (avP) and calcium (Ca) content of the diet (Dersjant-Li *et al.*, 2015). The degree to which phytase can dephosphorylate phytate is a function of the dietary Ca level and the ratio of dietary Ca:avP for several reasons. Firstly, Ca is included in a diet in the form of limestone. Limestone increases the alkalinity of the diet, and thus the pH in the crop (and proventriculus to a certain extent), which inhibits the activity of phytase. Secondly, at a pH of approximately 4, phytate binds to Ca^{2+} to form stable insoluble complexes. Thirdly phytate hydrolysis by phytase, results in the release of P, which disrupts the Ca:avP ratio and further results in the increased formation of Ca-P salts that are excreted (Plumstead *et al.*, 2008; Selle *et al.*, 2009; Amerah *et al.*, 2012; Dersjant-Li *et al.*, 2015).

The sensitivity of broilers to the level of Ca and avP in a diet is greater than that of layers due to their differing abilities to efficiently absorb and retain Ca and P from their diet. As a result, the occurance and frequency of bone disorders and impaired performance in broilers persist even though Ca:avP is maintained (Driver *et al.*, 2005). The influence of calcium on phosphorus digestibility and absorption has been studied in detail by a multitude of authors; however, there is still uncertainty surrounding the optimum Ca:avP ratio, which ranges from 1.6 to 2.6, and the Ca level in the diet, which ranges from 0.8% to 1.04% (Driver *et al.*, 2005; Han *et al.*, 2016). The Cobb 500 manual recommends a minimum avP of 0.45% and 0.38%, and Ca level of 0.9% and 0.76%, in the starter and finisher diets respectively.

In this study, the Ca:avP ratio and Ca level was above that recommended by the Cobb 500 manual, while the avP level was just less than the recommended; therefore, one would expect that the dietary Ca and avP would not be a factor contributing to variation in performance and bone development. However, trials by Han *et al.* (2016) and Plumstead *et al.* (2008) on the Ross breed (female Ross 308 and male Ross 344 x 508) suggested a Ca:avP ratio of 2.3–2.5:1 for optimal performance and bone development. This ratio would appear to be ideal when comparing other studies. For example, Kiarie *et al.* (2015) observed that increasing the phytase dose supplemented in a diet significantly improved ($p<0.05$) performance and bone parameters linearly, such that a phytase dose of 2000FTU/kg realised a 20%, 7.4% and 8% improvement in body weight gain, FCR and tibia ash, respectively. In contrast, observations by Junqueira *et al.* (2011) show that an increasing phytase dose proportionally reduced performance parameters. A possible explanation is that the Ca:avP ratio was 1.88, whereas in Kiarie *et al.* (2015), the Ca:avP ratio was 2.38.

The cereal grains and oilseeds incorporated into the diet is another factor that influences the efficacy of a phytase enzyme and, thus, the growth and development of the broilers (Dersjant-Li *et al.*, 2015). The location, solubility of the phytate and IP6 content within the raw material, as well as the type of processing the grains and oilseeds have undergone influences the accessibility of phytase to the phytate (Hidvégi & Lásztity, 2002; Dersjant-Li *et al.*, 2015). Approximately 80% of the phytate in maize is located in the germ, compared to wheat, barley and rice where phytate is predominantly found in the aleurone layer (Hidvégi & Lásztity, 2002). Liu *et al.* (2014) determined the influence of cereal type and phytase on broiler performance. It was observed that supplemented phytase to maize-based diets improved ($p<0.05$) broiler performance to a greater extent compared to sorghum and wheat-based diets. The theory is that the aleurone layer, which contains more fibre, is more resistant to degradation by endogenous carbohydrases, and therefore restricts the access of the exogenous phytase to phytate.

In terms of this study, the diet formulated was maize-based. However, the starter diet contained full-fat soya and soya oil, whereas the finisher diet contained only soya oil. The differences in their inherent susceptibility to phytate could be a reason for the surge in growth rate after day 21. Morgan *et al.* (2016) conducted a study to compare the effects of a high and low susceptible phytate diet on broiler performance and bone development. The outcomes show

that a diet high in susceptible phytate results in significantly improved calcium and phosphorous content in the tibia, body weight and FCR compared to a diet that consist of wheat bran and soya oil. Morgan *et al.* (2016) concluded that diets should be formulated to contain a high susceptible phytate content and that highly susceptible grains are those that have a high total phytate content. In contrast Dersjant-Li *et al.* (2015) reviewed a study that showed the IP6 dephosphorylation in soybean meal to be greater than that of canola meal (37.5% compared to 19%), even though canola meal has a greater total phytate content than soybean meal. The reason is the type of processing (solvent vs mechanical extraction, cooking temperature, soaking etc.) that the grains and oilseeds undergo.

Chapter 7

Conclusion

In conclusion the modified new generation *E.coli* 6-phytase product supplemented at 500FTU/kg diet is the optimal dose for this product as no significant ($p>0.05$) improvements in broiler performance and bone development was observed between 500FTU/kg and 1000FTU/kg supplementation. Performance and bone development results of broilers fed diets containing the modified new generation *E.coli* 6-phytase product were comparable to those of boilers fed diets containing other heat-stable phytase products.

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Appendix 1

Diet composition

(A) Starter Diet

1. Basal diet composition

Raw Materials	NC1		NC2		PC	
	Kg	%	Kg	%	Kg	%
Maize (Finely Milled)	1409.36	59.02	197.93	58.10	194.51	57.15
Soya Oilcake	621.08	26.01	83.61	24.54	78.49	23.06
Sunflower Oilcake	119.44	5.00	17.06	5.01	17.06	5.01
Full-fat Soya	83.6	3.50	18.77	5.51	25.59	7.52
Soya Oil	71.66	3.00	10.24	3.01	10.24	3.01
Limestone	28.66	1.20	3.24	0.95	2.56	0.75
MCP	14.34	0.60	4.1	1.20	6.14	1.80
Premix	39.89	1.67	5.70	1.67	5.73	1.68
Total	2388.03	100.00	340.65	100.00	340.32	100.00

2. Premix composition (kg)

	NC1	NC2	PC
Salt	8.36	1.19	1.19
Lysine	5.49	0.79	0.79
MHA	6.21	0.89	0.92
Threonine	0.72	0.10	0.10
BSGF	7.17	1.02	1.02
Zinc Bacitracin	1.19	0.17	0.17
Ancoban	1.19	0.17	0.17
Sodium Bicarbonate	4.78	0.68	0.68
Kembind	4.78	0.68	0.68
Total	39.89	5.70	5.73

3. Phytase addition (g) to NC1 diet

	T4	T5	T6	T7	T8	T9
Modified *E.coli*	17	34	0	0	0	0
Product X	0	0	34	68	0	0
Product Y	0	0	0	0	17	34

4. Nutritional analysis (%)

	Formulated			Analysed		
	NC1	NC2	PC	NC1	NC2	PC
Moisture	11.47	11.43	11.39	11.31	11.52	11.32
Crude protein	20.02	19.98	19.93	21.17	21.13	20.27
Fat (EE)	6.65	6.96	7.27			
Fat (AH)	7.02	7.34	7.66	8.49	8.1	8.19
Fibre	3.63	3.68	3.72	4.18	4.17	4.14
Ash	4.79	5.05	5.36	5.16	5.13	5.32
Starch	38.39	37.87	37.34			
ME (Kcal)	2585.11	2587.22	2587.78			
ME (MJ)	11.90	11.91	11.91			
Calcium	0.70	0.77	0.85	0.87	0.91	0.92
Total phosphorus	0.53	0.64	0.75	0.58	0.68	0.82
Ca:Tot P	1.33	1.20	1.14	1.50	1.34	1.12
Available phosphorus	0.23	0.33	0.43			
Ca: avP	3.07	2.34	1.99	3.79	2.76	2.14
Sodium	0.20	0.20	0.20			
Chloride	0.32	0.32	0.31			
Dig. Lys	1.07	1.07	1.06			
Dig. Met	0.51	0.51	0.51			
Dig. Met + Cys	0.77	0.77	0.77			
Dig. Thr	0.65	0.65	0.65			
Dig. Trp	0.20	0.20	0.20			
Dig. Arg	1.23	1.23	1.23			
Dig. Ile	0.74	0.74	0.74			
Dig. Val	0.84	0.83	0.83			

* avP = available phosphorus

(B) Finisher Diet

1. Basal diet composition

Raw Materials	NC1		NC2		PC	
	Kg	%	Kg	%	Kg	%
Maize (Finely Milled)	2649.9	65.61	374.43	65.09	370.3	64.57
Soya Oilcake	907.65	22.47	129.66	22.54	129.66	22.61
Sunflower Oilcake	288.78	7.15	41.26	7.17	41.26	7.19
Soya Oil	82.5	2.04	11.79	2.05	11.79	2.06
Limestone	51.57	1.28	6.19	1.08	4.72	0.82
MCP	0	0.00	3.54	0.62	7.37	1.29
Premix	58.59	1.45	8.37	1.46	8.37	1.46
Total	4038.99	100.00	575.24	100.00	573.47	100.00

2. Premix composition (kg)

	NC1	NC2	PC
Salt	12.38	1.77	1.77
Lysine	10.31	1.47	1.47
MHA	9.49	1.36	1.36
Threonine	1.65	0.24	0.24
BSGF	8.25	1.18	1.18
Zinc Bacitracin	2.06	0.30	0.30
Ancoban	2.06	0.30	0.30
Sodium Bicarbonate	4.13	0.59	0.59
Kembind	8.25	1.18	1.18
Total	58.58	8.37	8.37

3. Phytase addition (g) to NC1 diet

	T4	T5	T6	T7	T8	T9
Modified *E.coli*	29	59	0	0	0	0
Product X	0	0	59	118	0	0
Product Y	0	0	0	0	29	59

4. Nutritional analysis (%)

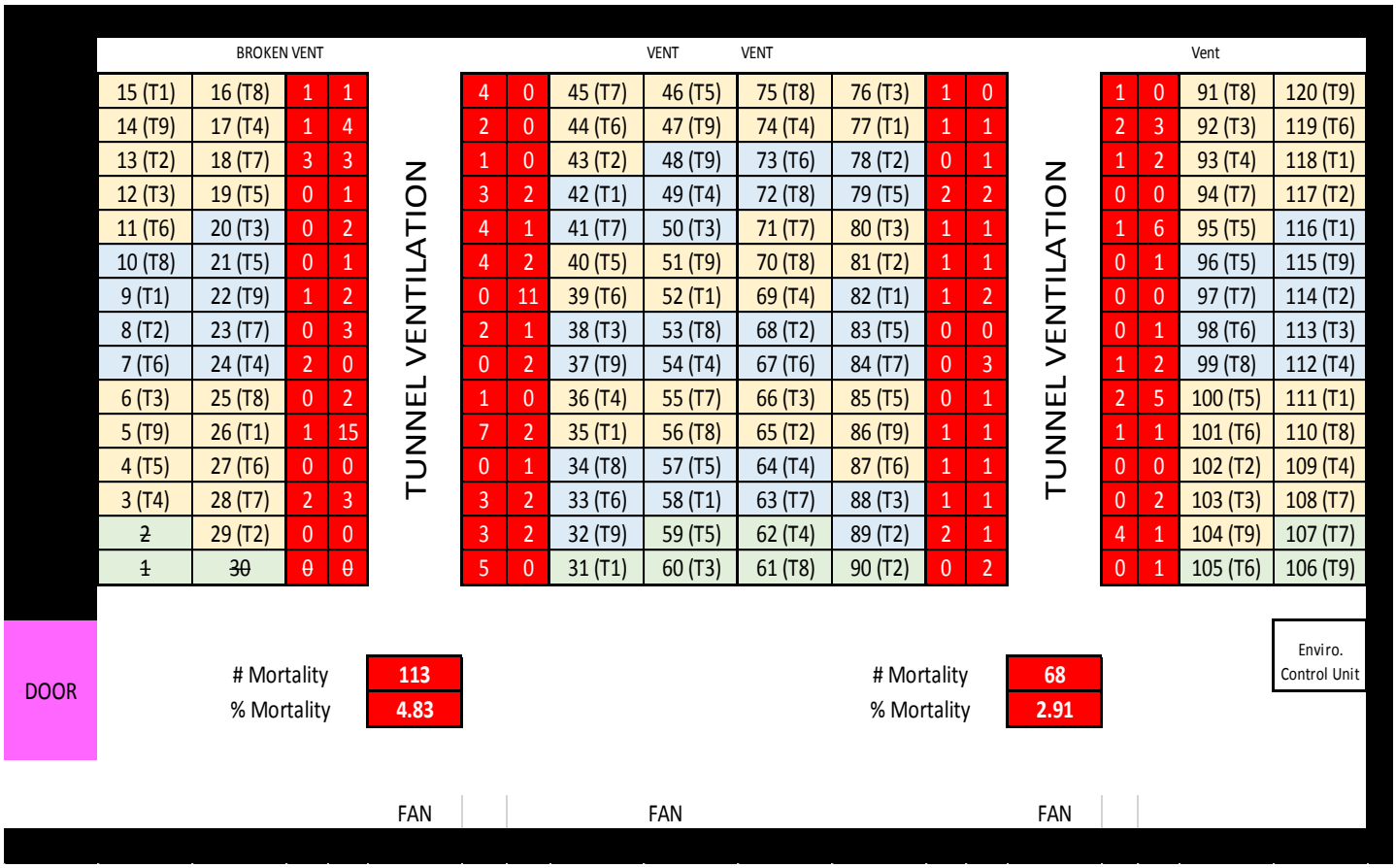
	Formulated			Analysed		
	NC1	NC2	PC	NC1	NC2	PC
Moisture	11.33	11.26	11.18	10.76	10.27	10.07
Crude protein	18.02	17.97	17.91	18.34	18.72	19.29
Fat (EE)	7.39	7.66	7.93			
Fat (AH)	7.77	8.04	8.31	6.77	7.13	6.67
Fibre	3.79	3.77	3.75	3.65	3.57	3.62
Ash	3.99	4.28	4.57	4.67	4.65	4.81
Starch	41.54	41.10	40.67			
ME (Kcal)	2592.92	2570.35	2547.78			
ME (MJ)	12.39	12.41	12.42			
Calcium	0.55	0.63	0.71	0.78	0.85	0.79
Total phosphorus	0.41	0.51	0.63	0.44	0.56	0.72
Ca:Tot. P	1.36	1.23	1.13	1.77	1.52	1.10
Available phosphorus	0.12	0.22	0.32			
Ca: avP	4.52	2.89	2.18	6.38	3.88	2.44
Sodium	0.15	0.15	0.15			
Chloride	0.29	0.29	0.29			
Dig. Lys	0.95	0.95	0.95			
Dig. Met	0.46	0.46	0.46			
Dig. Met + Cys	0.70	0.70	0.70			
Dig. Thr	0.59	0.59	0.59			
Dig. Trp	0.17	0.17	0.17			
Dig. Arg	1.09	1.09	1.09			
Dig. Ile	0.66	0.65	0.65			
Dig. Val	0.75	0.75	0.75			

* avP = available phosphorus

Appendix 2

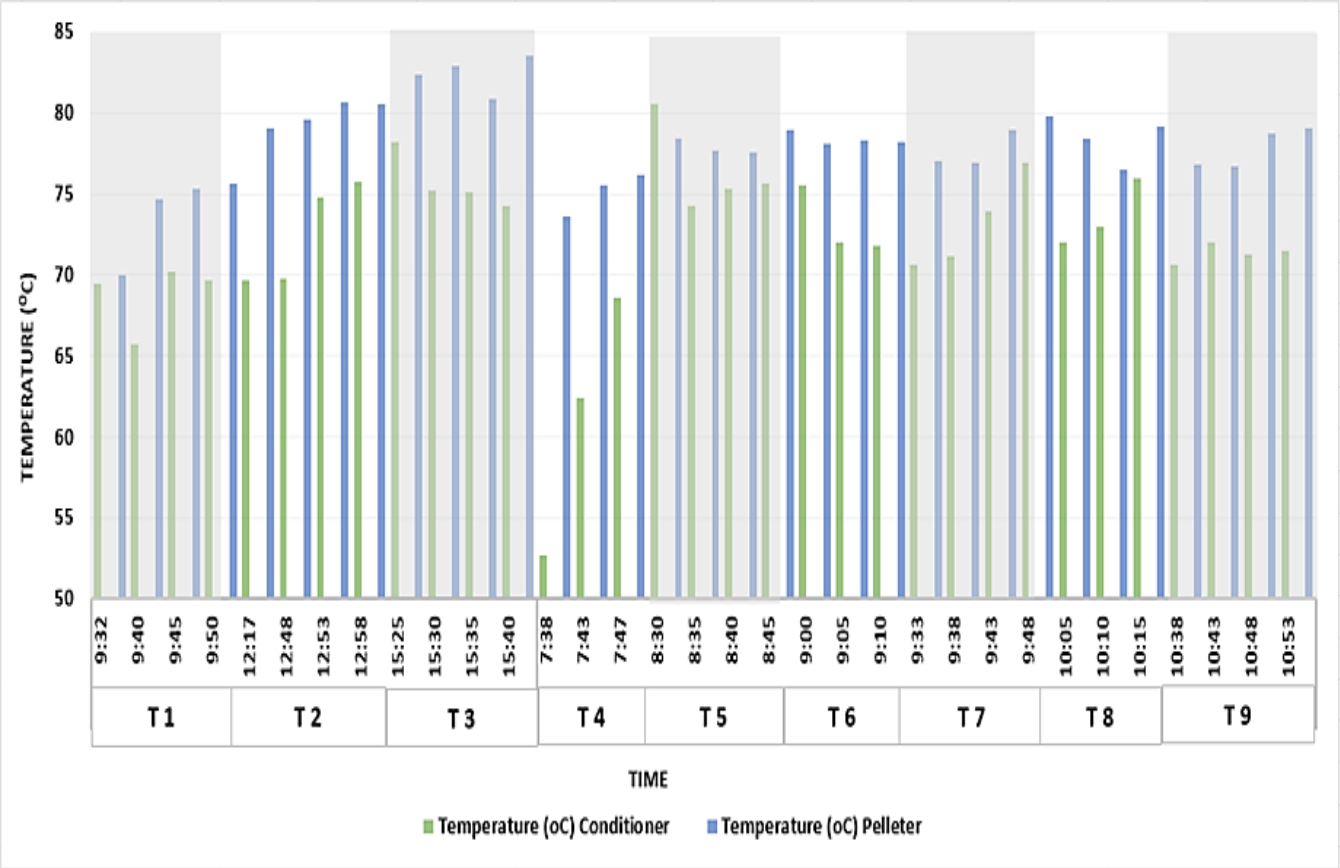
Allocation of treatment to pens

Schematic of the broiler house with the total number of mortalities at each pen by day 35:



Appendix 3

Pelleting temperatures

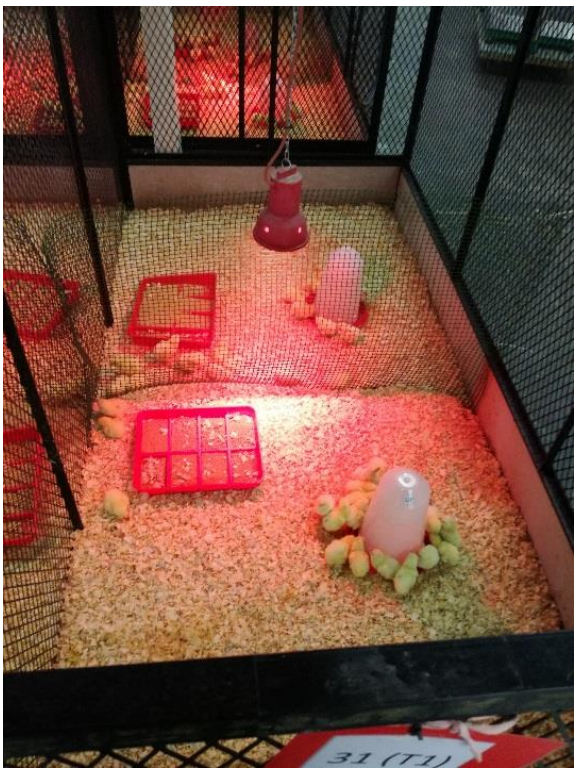


Appendix 4

Pictures

Picture 1: Mixing the diet at Simple Grow Agricultural Services (SGAS)

Picture 2: Phytase enzymes to be added to diets



Picture 3: Placement of chicks on day 0

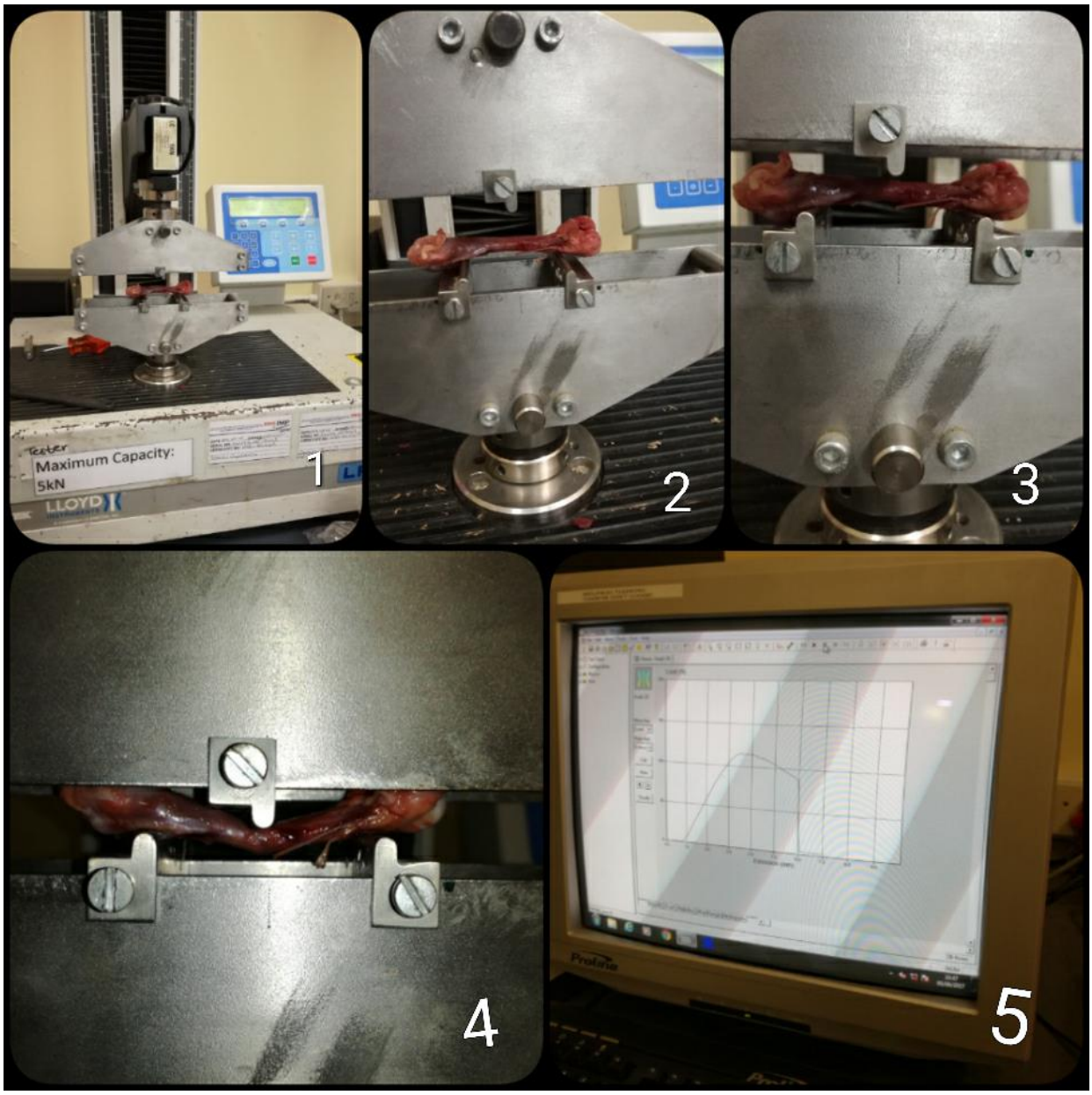
Picture 4: Biosecurity foot powder

Picture 5: Chicken with a broken foot euthanised

Picture 6: Mold growing on feed bag

Picture 7: Moldy feed removed from feed bag

Picture 8: Tibia collection technique



Picture 9: Tibia breaking strength analysis at the University of Pretoria, Civil Engineering Department.

* Cartilage caps on the bones were removed on the right tibia's for tibia ash and mineral analysis.