Performance and bone integrity of broilers receiving dietary calcium at lower concentrations than current recommendations

By

JC Venter

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Supervisor: Dr C Jansen Van Rensburg

Co-supervisor: Dr F Crots

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.

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Abstract

Calcium and phosphorus are the two major macro-minerals required for proper growth and bone mineralisation in broiler chickens. Typical feed ingredients in broiler rations are low in calcium and phosphorus and inorganic sources of calcium and phosphorus are therefore used to supplement the shortfall. The phosphorus present in the feed ingredients is mostly in phytate form which is unavailable to the chicken. The hydrolysis of phytate with exogenous phytase helps to decrease the required amount of inorganic calcium and phosphorus in the diet, reduce the environmental effect of broiler production and reduces costs. However, calcium and phytate-phosphorus are known to form complexes that are resistant to the hydrolytic effect of phytase and are indigestible. Thus, excess calcium will reduce the amount of phosphorus available to the broiler chicken thereby impairing broiler performance. The main objective of this study was to determine if calcium inclusion levels lower than currently recommended by Aviagen for Ross broilers, will enhance broiler production without impairing bone mineralisation. Current Ca:P recommendations from Aviagen is 2:1. In order to achieve the objective, Ross 308 day-old chicks were randomly assigned to eight different treatments varying in calcium and phosphorus inclusion levels. The trail was designed to evaluate the interaction between dietary calcium and non-phytate phosphorus. Broiler performance, bone ash percentage and bone breaking strength were the parameters used to evaluate the interaction.

In this trial, 64 pens were used to allow for eight replications for each of the eight treatments. Sixty unsexed chicks were placed in each pen. The Ca inclusion levels ranged from 0.70% to 0.95% and was tested with three different P inclusion levels. The Ca:P ratio ranged from 1.54:1 to 2.50:1. The treatments were fed through a starter, grower and finisher phase and no phytase was included in the diet. Thirty two chicks per treatment were euthanised on 14, 32 and 35 days of age when the tibias were removed for analysis of bone ash and bone breaking strength. From the performance data obtained from this trial, it was clear that an interaction between calcium and phosphorus existed. The treatment diet with a Ca:P ratio of 1.60:1 resulted in the highest body weight and lowest feed conversion ratio of broilers at 35 days of age. As the Ca:P ratio increased, the body weight and feed intake decreased. The bone ash percentage was linked to the amount of calcium and phosphorus in the diet. As the mineral content of the diet increased the bone ash percentage of broilers at 14 and 35 days of age also showed an increase. The bone breaking strength increased as the calcium concentration in the diet increased.

It can be concluded that reducing the amount of calcium in the diet below the current Aviagen recommendation for Ross broilers improves broiler performance without compromising bone mineralisation.

Contents

List of Abbreviations

aP	Available phosphorus
BW	Body weight
BWG	Body weight gain
Ca	Calcium
CaR	Ca-sensing receptor
CT	Calcitonin
CV	Coefficient of variation
FCR	Feed conversion ratio
FI	Feed intake
GIT	Gastro-intestinal tract
GLM	Generalised linear model
H_A	Alternative hypothesis
H_0	Null hypothesis
HSC	Highly soluble calcium
IB	Infectious bronchitis
kN	Kilonewton
MCP	Mono-calcium phosphate
ME	Metabolisable energy
Mg	Magnesium
Mn	Manganese
NCB	Newcastle disease
NIR	Near infrared spectroscopy
nPP	Non-phytate phosphorus
NRC	National Research Council
P	Phosphorus
PEF	Performance efficiency factor

pH	Potential of Hydrogen
PP	Phytate phosphorus
PTH	Parathyroid hormone
rH	Relative humidity
SAS	Statistical analysis system
SD	Standard deviation
SEM	Standard error of the mean
tP	Total phosphorus
VDR	Vitamin D receptor
Zn	Zinc

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Chapter 1

General Introduction

Feed costs make out 60% to 70% of the production costs in broiler production systems. Due to the increasing pressure on profit margins, the focus has been placed on reducing feed costs while maintaining optimal broiler performance. The efficiency of broiler production systems has also increased over the last 100 years. This increase in efficiency is mainly attributable to improved genetics and improved animal husbandry. The improvement in genetics led to the development of the modern broiler strains with improved growth rates and feed conversion (Cesar *et al*., 2008). The average chicken weight was 1.13 kg at 112 days in 1925. Through improved genetics, the average broiler weight increased to 1.78 kg at 53 days by 1980. In 2011, the broilers achieved an average weight of 2.63 kg in only 47 days (Proszkowiec-Weglarz & Angel, 2013). Improved broiler productivity is realised through improved efficiency with which dietary nutrients are converted into body protein and fat. Over the past few decades, research has focussed on reducing wastage caused by over or under supplying of nutrients. Ongoing research is done to quantify the requirements of the modern broiler and define feed ingredients more accurately as to reduce feed formulation costs while optimising broiler performance (Hamdi *et al.*, 2015). Feed enzymes have been introduced into broiler feed formulation in order to reduce feed costs by increasing the amount of nutrients available for absorption. Phytase is the feed enzyme used most extensively in modern broiler feed formulation (Qian *et al*., 2017). This enzyme hydrolyses phytate, which is the natural storage form of P in plants and feeds, and make P available to the broiler (Tamin & Angel, 2003).

With the improvement of broiler growth rate, soundness of the skeletal system to support the heavier body weight without compromising mobility, must be emphasised. Since calcium (Ca) is the most abundant mineral found in the skeletal system of broilers and plays an integral part in maintaining a sound skeletal system, Ca nutrition became more important (Hamdi *et al*., 2015). It is well known that a deficiency of dietary Ca will lead to a weak skeletal system but it is also important to note that an excess Ca will not only lead to poor skeletal conformation, but also to reduced broiler performance due to its interacting nature with other dietary minerals, protein and fat (Gautier *et al*., 2017). Inclusion of excess Ca in the diet also has economic implications. The Ca sources, such as limestone, are using space of other ingredients while increasing total feed costs. Ca also forms insoluble complexes with P, rendering both Ca and P unavailable to the broiler. The unutilised minerals are eliminated through faecal excretion, which contributes to environmental pollution (Gautier *et al*., 2017). Excess Ca will also reduce the energy content of the diet in that it chelates with lipids rendering them unavailable for absorption, by forming an insoluble complex (Driver *et al*., 2005).

Previous studies (Leytem *et al*., 2008; Hamdi *et al*., 2015) have shown that broiler performance may benefit from feeding lower dietary Ca levels than current commercial recommendations, without compromising skeletal health. Calcium levels included in the diets of modern broiler strains are rarely the same as those recommended by the National Research Council (NRC; 1984, 1994). The recommendations made by the NRC (1994) for Ca inclusion levels in the broiler diet are 1.00% for the starter, 0.90% for the grower and 0.80% for the finisher phase (Driver *et al*., 2005). According to Driver *et al*. (2005), who collected information on the Ca inclusion levels across 100 mills in the United States of America, Ca inclusion levels were 0.90% for pre-starter, 0.82% for starter, 0.77% for grower and 0.72% for finisher, which shows a lower overall Ca inclusion in modern broiler diets across all feeding phases compared to the NRC (1994) recommendations for Ca inclusion levels.

The extent to which Ca is reduced below the current recommendation to improve broiler performance varies among studies, because of different broiler strains and source of Ca and P used in the diets. Soluble sources of Ca increase the acid-binding capacity of the digesta which promotes formation of insoluble complexes between Ca and P. Phytase is also pH dependant and functions optimally at a pH of 3. By reducing the Ca inclusion level, the pH in the upper, more acidic part of the gastro-intestinal tract may not be affected to the extent where the phytase efficacy is affected (Li *et al*., 2016). By reducing the Ca inclusion level, the Ca:P ratio will decrease (Gautier *et al*., 2017). Due to the interactive nature between Ca and P in forming insoluble complexes and their influences on absorption efficiency of each of the elements, a lower Ca:P ratio will improve absorption efficiency of Ca and P due to reduced complex formation (Li *et al*., 2016; Gautier *et al*., 2017).

The aim of this study was to determine the effect of altering the Ca:P ratio in the diet by reducing the amount of Ca, with and without a reduction in the P level, on broiler performance, leg health and profitability. The primary objective was to measure broiler performance and leg health at Ca levels lower than current recommended Ca inclusion levels while P was kept at current recommeded levels. The second objective was to measure broiler performance and leg health at lower Ca and lower P inclusion levels with a higher Ca:P ratio than current recommendations.

Hypothesis of current study

The null hypothesis (H_0) of this study is that Ca inclusion levels and Ca:P ratio below the current Aviagen recommendation for Ross 308 broilers will have no effect or a negative effect on broiler performance with detrimental effects on skeletal health and bone integrity, whereas the alternative hypothesis (H_A) is that these lower levels of Ca and lower Ca:P ratio will improve broiler performance with no negative effect on skeletal health and bone integrity.

Chapter 2

Literature review

2.1 Introduction

Calcium and P are the most abundant minerals found in the body of broilers and bone serve as the major storage site for these minerals (Hamdi *et al*., 2015). Ca and P play a vital role in maintaining bone integrity and strength. Ca and P not located in bone are found in extracellular fluid where it functions in metabolic pathways, and therefore may affect general broiler performance as well (Proszkowiec-Welgarz & Angel, 2013). Ca and P are known to interact with each other to form insoluble complexes (Gautier *et al*., 2017). These two minerals have a low affinity for each other (Paiva *et al*., 2013), but since they are added to broiler diets in the greatest quantity of all minerals the interaction between them has a significant influence on broiler performance (Tamin *et al*., 2004). Excess dietary Ca may influence broiler performance because it forms complexes not only with other dietary minerals, but with protein and fat as well, reducing the digestibility of these nutrients (Li *et al*., 2016). Thus, by reducing the dietary Ca inclusion levels it may have a beneficial effect on broiler performance through increasing the availability of other nutrients while saving feed costs (Gautier *et al*., 2017).

The objective of the literature review is to provide an overall view on the importance of Ca and P in broiler nutrition and the effect of Ca on the availability of other nutrients by describing the interactive nature among Ca and nutrients. The negative effects of excess Ca on broiler performance and requirements of Ca and P for optimal broiler performance will also be discussed.

2.2 The role of calcium and phosphorus in the broiler

Due to the improvement in broiler growth rate, the requirement for nutrients has also increased. The improvement in growth rate was mainly manifested through increased rate of protein deposition. The requirement for dietary protein increased with the increased rate of muscle growth (Proszkowiec-Welgarz & Angel, 2013). Requirements for nutrients change with age and the potential growth performance of the strain (Williams *et al*., 2000). Along with the increased growth rate, the rate of bone growth and development, together with requirement for Ca and P, have increased.

Bones are the major storage site for Ca and 99% of Ca is found in the bones of the animal (Hamdi *et al*., 2015). It is mainly stored in the form of hydroxyapatite. Ca plays a vital role in development and mineralisation of bone which makes bone an important storage site for Ca (Proszkowiec-Welgarz & Angel, 2013). An excess in either Ca of P will impair bone development which will result in bone pathologies (Williams *et al*., 2000). Driver *et al.* (2005) stated that the Ca required for bone mineralisation is greater compared with the requirement for growth, because body

weight gain is not as sensitive of a measurement when quantifying Ca and non-phytate phosphorus (nPP) requirements (Gautier *et al*., 2017). Therefore, bone mineralisation depends to a greater extent on the dietary concentrations of nPP and Ca than body weight gain does (Gautier *et al*., 2017).

Calcium and P are the minerals found most abundantly in the body and are involved in many integral functions in metabolism and skeletal integrity (Gautier *et al*., 2017). Ca requirements are usually established based on parameters which include bone breaking strength and bone ash percentage. Bone breaking strength can be used as criteria for the amounts of Ca and P supplemented in the diet with relatively good accuracy. The correlation coefficient between bone breaking strength and bone ash percentage is 0.98 (Rowland *et al*., 1967). Bone breaking strength becomes important with the slaughtering of chickens. Stronger bones can resist the forces exerted on the bones during processing (Cesar *et al*., 2008). Poor bone breaking strength will result in broiler carcasses in the abattoir with multiple broken bones. These carcasses are discarded which leads to a loss of income for the producer (Lima *et al*., 1997; Selle *et al*., 2009). It was noted by Skinner *et al.* (1992) that Ca had a greater influence on bone breaking strength than P did. Therefore, a Ca imbalance during any stage of the production cycle of the broiler will result in lower bone strength (Mitchell & Edwards, Jr, 1996).

Bone mineralisation and development is not the only function of Ca, since the remaining Ca is located in the extracellular fluid, plasma, and within cells (Proszkowiec-Welgarz & Angel, 2013). The 10000-fold concentration gradient of ionised Ca between the extracellular fluid and the cytoplasma permits Ca to serve as a signalling molecule to activate intracellular processes. Here the Ca functions in metabolism, blood clotting, enzyme activation, neuromuscular function, muscle contraction, cell adhesion, intracellular signaling and hormonal secretion (Williams *et al*., 2000; Onyango *et al*., 2003). Phosphorus is the second most abundant mineral in the animal body. About 80% of P is found in the bones of the animal. The 20% of P not located in the bones are widely distributed throughout the body, and is found in nucleic acids, nucleotides, phospholipids, phosphorylated proteins, involved in enzymatic reactions as well as oxygen transport (Onyango *et al*., 2003). The P serves a fundamental function in growth, cellular membrane function, energy metabolism and acid-base balance (Proszkowiec-Welgarz & Angel, 2013). Through the involvement of P in these metabolic pathways, it is considered to be an essential nutrient to attain maximum genetic potential for growth and feed efficiency (Applegate & Angel, 2008).

2.3 Calcium and phosphorus homeostasis

Due to the interrelationship between Ca and P, the metabolism of these two minerals is influenced by one another. A deficiency or excess of either of these two minerals will cause an imbalance of the other one in many biological pathways because of the ability of these two minerals to interact with each other to form insoluble complexes. These insoluble complexes are indigestible and

is not absorbed through the digestive system for metabolism (Wilkinson *et al*., 2012). This imbalance will cause a decrease in growth rate, bone mineralisation and bone development. Furthermore, inadequate or oversupply of any of the two minerals leads to increased mortality and morbidity rates. This can be seen through increased skeletal abnormalities and lameness which manifests itself through rickets and tibial dyschondroplasia. Imbalances in dietary Ca and P lead to increased amounts of P excreted in the faeces of broilers. Undigested faecal P is excreted into the environment and ends up in water resources when the excreta from broilers are applied as fertilizer to the soil, which is a major environmental concern (Proszkowiec-Welgarz & Angel, 2013; Ghasemi *et al*., 2018).

Calcium and P are ingested with the feed and absorbed in the jejunum and ileum in the small intestine (De Vries *et al*., 2010). After absorption, Ca and P are rapidly transferred into the bones and soft tissue in the body. The main storage site of these two minerals is the bones (France *et al*., 2010). The structure of bone is kept intact by pyridinoline and deoxypyridinoline cross-links between fibrils in mature collagen and the cross-links are responsible for the tensile strength of the collagen (Williams *et al*., 2000). However, deposition of Ca and P into bone is not a static process. There is a continuous turnover of Ca and P in the bones through the process of resorption and these minerals are mobilised from the bones when needed (France *et al*., 2010).

A range of feedback mechanisms exist in the chicken to maintain the blood Ca concentrations within narrow physiological ranges. These feedback mechanisms involve the parathyroid hormone (PTH), active vitamin D_3 (1,25-dihydroxyvitamin D_3) and calcitonin (CT) (Williams *et al*., 2000). These hormones interact with specific receptors which are localised in the small intestine, bone and kidneys (Proszkowiec-Welgarz & Angel, 2013).

Factors which result in a decrease in blood Ca levels like feeding a Ca-deficient diet or an increase in Ca requirement trigger mechanisms which will increase blood Ca levels so that blood Ca concentration can be within its physiological range. One of the mechanisms, PTH, functions in activating 1α-hydroxylase in the kidney. 1α-hydroxylase, in turn, stimulates the release of Ca and P from bones (Proszkowiec-Welgarz & Angel, 2013). Another mechanism is the ability of the bird to compensate for a low dietary Ca concentration by increasing the absorption efficiency of Ca. At young ages, birds are able to adapt to low dietary Ca concentrations (Gautier *et al*., 2017) by up-regulating calbindin which improves the absorption of Ca to compensate for lower Ca intakes (Driver *et al*., 2005). In contrast to low dietary Ca concentrations, when dietary Ca concentrations are in excess, no mechanisms exist other than excretion to get rid of the excess Ca (Gautier *et al*., 2017). Excess Ca in the small intestinal digesta binds with other nutrients and render them indigestible (Wilkinson *et al*., 2012).

Vitamin D can be regarded as a pleiotropic hormone that exerts a variety of biological effects including the regulation of bone and mineral metabolism as well as the modulation of the immune

response (Rodriquez-Lecompte *et al*., 2016). Vitamin D_3 can be either synthesised from isomerisation of 7-dehydrocholesterol to Vitamin D_3 in the skin after exposure to UV light or it can be supplied in the diet in the form of vitamin D_2 or vitamin D_3. Broilers get minimal exposure to UV light in modern broiler production systems and therefore it is essential to supply vitamin D through the diet (Proszkowiec-Welgarz & Angel, 2013). Dietary vitamin D is absorbed by the small intestine and is readily taken up by the liver where it is hydrolysed by the enzyme 25-hydroxylase to 25-hydroxy-D_3, the storage form of vitamin D in the body, which is further converted by 1-α-hydroxylase in the kidney to its biologically active form, 1,25-dihydroxy-vitamin D_3. Immune cells such as macrophages and dendritic cells express 1-α-hydroxylase and therefore high concentrations of 1,25-dihydroxy-vitamin D_3 can be found in lymphoid microenvironments (Rodriquez-Lecompte *et al*., 2016). The fundamental role of 1,25-dihydroxy-vitamin D_3 is to regulate Ca and P homeostasis by acting directly on the intestine, kidney and bones by causing a down regulation of PTH production in the parathyroid glands (Proszkowiec-Welgarz & Angel, 2013). When Ca and P are deficient in the diet, vitamin D acts on the kidney and bone for new mineralisation. When Ca and P levels in the diet are sufficient, vitamin D acts on the intestine as a result of suppressed PTH due to the actions of both Ca and 1,25-dihydroxy-vitamin D_3 in the parathyroid gland thereby conserving bone resorption and bone Ca. (Rodriquez-Lecompte *et al*., 2016). 1,25-dihydroxy-vitamin D_3 production is also increased when the blood Ca is low which in turn leads to increased absorption from the small intestine and reabsorption of Ca from the kidney (Williams *et al*., 2000).

Vitamin D is an important element in these processes, functioning as a transcription factor inducing the expression of Ca transporters. Vitamin D receptors (VDR) has been documented in various chicken tissues, including the intestine, kidney, bone and parathyroid gland. VDR serves as a pathway through which most of the actions of vitamin D is carried out. VDR-like receptors associated with plasma membranes has an important function in that when it binds with 1,25-dihydroxy-vitamin D_3, it can increase Ca absorption from the small intestine within minutes (Proszkowiec-Welgarz & Angel, 2013).

PTH is synthesised in the chief cells of the parathyroid glands. Under physiological conditions, PTH is released in response to low blood Ca concentrations predominantly as the PTH molecule which then binds to the PTH membrane receptor. PTH responds both to acute, short-term changes in Ca concentration as well as sustained low Ca concentrations. In the case of sustained low Ca concentration, PTH stimulates the conversion of vitamin D_3 to 1,25-dihydroxyvitamin D_3 which increases intestinal Ca absorption through a mechanism that involves the protein kinase dependent pathways. Ca reabsorption from the renal tubes is also stimulated through the protein kinase pathways (Proszkowiec-Welgarz & Angel, 2013).

The Ca-sensing receptors (CaR) are located in the parathyroid chief cells and are responsible for sensing high blood Ca concentrations. Ca activates the CaR in the parathyroid gland which in turn activates Ca-dependent proteases which cleave and inactivate PTH. When blood Ca concentration is high the thyroid glands secrete calcitonin (CT) and of which its expression is regulated by 1,25-dihydroxy-vitamin D_3. CT functions also as an inhibitor to bone resorption leading to a decrease in plasma Ca concentration (Proszkowiec-Welgarz & Angel, 2013).

Plasma PTH concentration and plasma 1,25-dihydroxy-vitamin D_3 concentrations have opposing effects on plasma Ca concentrations through its respective feedback mechanisms, thereby controlling the plasma Ca concentration and causing it to return to normal (Proszkowiec-Welgarz & Angel, 2013). Dietary inclusion levels which exceed the physiological requirements of broilers for various metabolic functions will be eliminated through the kidneys which may pose environmental P pollution risks (Hamdi *et al*., 2015; Gautier *et al*., 2017). When Ca intake exceeds the physiological requirements of the bird, the parathyroid gland produces calcitonin to inhibit renal Ca reabsorption and therefore increases Ca excretion via the urine (Gautier *et al*., 2017). Reducing dietary Ca or available P result in increased efficiency of Ca retention. The chicken responds physiologically to the deficiency through up-regulating the nutrient transfer and deposition infrastructure (Browning *et al*., 2012).

2.4 Dietary sources of calcium and phosphorus

Calcium and P in broiler diets are present in plant-based feed ingredients and from inorganic sources. Vegetable feed ingredients are generally low in Ca content as illustrated in Figure 2.1 which rarely meets broiler requirements. Along with a low Ca content, the bioavailability is variable with Ca availability of between 20% and 33% in a typical maize soybean meal diet (Tamin & Angel, 2003). It is generally accepted that the availability is rather low due to the low Ca content or because the Ca present is bound to oxalate which reduces its availability (Angel, 2011). Therefore, the provision of adequate dietary Ca is almost completely achieved by using inorganic feed ingredients with Ca bioavailability of more than 60% (NRC, 1994). Figure 2.1 is a graphic representation of the difference in Ca content among vegetable ingredients typically used in broiler diets.

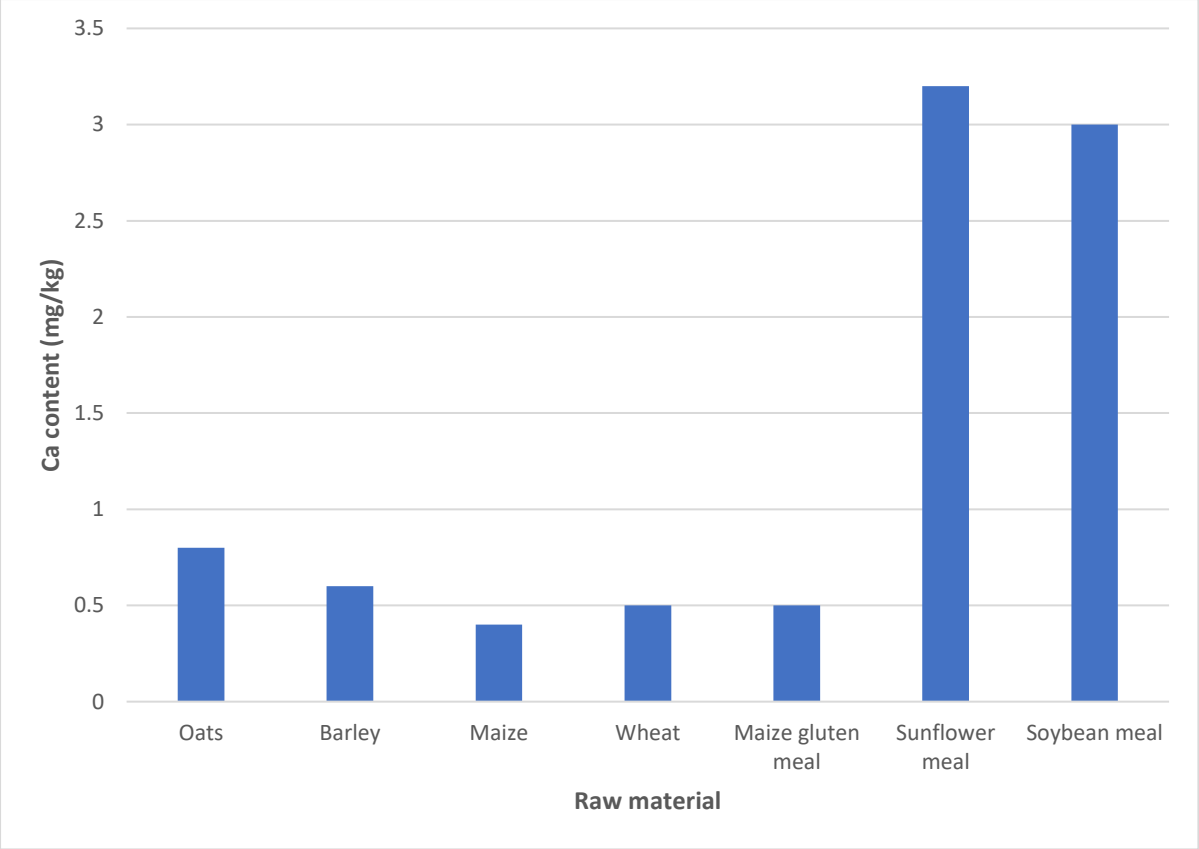

Figure 2.1 Comparison of the calcium content of different raw materials typically used in broiler diets (Hamdi *et al*., 2015)

The most commonly used inorganic sources of Ca and P in the poultry industry are limestone, oyster shell, mono-calcium phosphate and di-calcium phosphate (Walk *et al*., 2012). Dicalcium phosphate and monocalcium phosphate are commonly used as the inorganic source to supplement P. However, these two ingredients have a contribution towards inorganic Ca as well (Anwar *et al*., 2018). Ground limestone is the Ca source which is most accessible because more than 80% of the Ca in the earth's crust is in the form of limestone. Ca is present as calcium carbonate in limestone and oyster shell, but limestone is inorganic Ca of calcitic origin while oyster shell is organic Ca of marine origin (Anwar *et al*., 2017). According to a study done by Anwar *et al.* (2017), the Ca content differ between different sources of Ca and found that the Ca content of limestone and oyster shell were 420 and 370 g/kg respectively. Calcium content not only differ between different sources but differs among samples of the same source. The bioavailability of Ca from different sources of limestone is variable and has been reviewed extensively (Shafey, 1993; Walk *et al*., 2012). Ca concentration of limestone is influenced by the concentration of other minerals among it, such as iron and manganese. Impurities in limestone are influenced by the location at which limestone is sourced since more than 80% of the Ca in the earth's crust exist as limestone and the soil type and its mineral constitution differ among different regions (Anwar *et al*., 2016). The immature gastro-intestinal tract of young chicks could be more

sensitive to the level and properties of Ca in the diet, unlike that of adult birds, because older birds have a more developed GIT with the ability to regulate gut pH in a closer range through secretion of hydrochloric acid into the proximal GIT (Hamdi *et al*., 2015).

2.5 Consequences of excess dietary calcium levels on broilers

It is well known that Ca is an essential nutrient in broiler diets for both bone development and performance, but excess Ca will negatively affect broiler production as well as skeletal integrity (Angel *et al*., 2002). Excess Ca will have a negative impact on broiler production through its interactive nature with other nutrients making them indigestible (Gautier *et al*., 2017) and by reducing the efficacy of exogenous and mucosal phytase by increasing the digesta pH (Li *et al*., 2015).

2.5.1 Interaction among calcium and other nutrients

High dietary Ca levels are known to be responsible for reduced animal performance (Angel *et al*., 2002). Ca is a multivalent cation and has the ability to bind with other macro and micro minerals and form soap precipitations with free saturated fatty acids, thus decreasing the energy digestibility and indirectly overestimating the energy value of the diet leading to a drop in animal performance (Driver *et al*., 2005; Hamdi *et al*., 2015; Li *et al*., 2016; Gautier *et al*., 2017). Ca is one of the divalent cations with the lowest affinity for P (Paiva *et al*., 2013), but the interaction between P and Ca is of significance in broiler performance because Ca is the mineral added in the greatest quantities in the diet and therefore it has a greater impact than other minerals on the formation of calcium-phytate complexes (Tamin *et al*., 2004). One phytate molecule carries up to twelve negative charges which enable phytate to bind with 5.1 Ca atoms (Paiva *et al*., 2013).

As the Ca:P ratio increases, P bioavailability and retention decreases since a higher Ca:P ratio promotes Ca-phytate complex formation which is insoluble and are resistant to enzymatic hydrolysis by phytase. Faecal Ca and P excretion into the environment is increased (Hamdi *et al*., 2015; Gautier *et al*., 2017). The apparent precaecal bioavailability for Ca, P and phytate decreased as the dietary Ca inclusion level increased from 0.47% to 1.16% in a study conducted by Plumstead *et al*. (2008). Gautier *et al*. (2017) tested the effect of reducing the Ca inclusion levels at various P inclusion levels and found that body weight increased and feed conversion ratio decreased as the Ca inclusion level decreased at all the tested P inclusion levels, which confirms the effect of the interaction between Ca and P on broiler performance. Circulating Ca is found in three forms due to the ability of Ca to interact with other molecules: an ionised fraction, a protein-bound fraction and a fraction which is complexed with anions such as P (Williams *et al*., 2000). Ca reduces the digestibility and absorbability of phytate and inorganic P due to its capacity to bind phytate and inorganic P. Calcium forms a complex with phytin and phosphates which interfere with the availability of P and Zn (Hamdi *et al*., 2015). The high acid-binding capacity of limestone reduces the digestibility of protein in the diet, which also contributes to reduced

animal performance, suggesting that the buffering capacity of Ca reduces the efficacy of pepsin in the proventriculus and gizzard (Hamdi *et al*., 2015).

Calcium is known to bind directly with protein-phytate complexes, which reduces the solubility of protein. With an increase in dietary Ca the digestibility of amino acids also decreases (Li *et al*., 2015). Calcium may interact with soya protein via side-chain carboxyl groups of aspartic acid and glutamic acid. At pH levels below the isoelectric point of protein the polyanionic phytate molecule electrostatically binds with residues of the basic amino acids arginine, histidine and lysine which results in precipitation of amino acids. The phytate bound protein is refractory to pepsin digestion. Also, phytate may bind the peptide that activates pepsinogen and impede the conversion of the zymogen to pepsin. Phytate-protein complex formation operates in a narrow pH range of between 2 and 3. Calcium tend to increase the pH because of the high acid binding capacity which may counteract the protein-phytate complex formation (Selle *et al*., 2009).

2.5.2 Effect of excess dietary calcium on broiler leg health

Due to accelerated chicken growth during the last few decades, Williams *et al*. (2000) have noted lower bone ash content in these fast-growing strains, which may suggest that a higher requirement for Ca and P exist. In contrast to Williams *et al*. (2000), Letourneau-Montminy *et al*. (2008) stated that by increasing the efficiency of P utilisation, bone ash percentage will also increase. Increasing dietary Ca may aggravate a P deficiency for bone ash criteria, especially at low nPP levels, while decreasing the dietary Ca may improve P utilisation (Letouneau-Montminy *et al*., 2008; Gautier *et al*., 2017). With a decrease in dietary Ca, the efficiency of P absorption and utilisation is improved, which subside bone abnormalities and improve growth. This suggests that a balanced ratio between Ca and nPP is essential when formulating poultry diets (Gautier *et al*., 2017).

According to Onyango *et al*. (2003) bone-mineral content, bone mineral density and ash percentage increased as dietary Ca increased. The relationship is linear up to a point where the level of Ca interacts with nPP after which bone ash percentage decreases and bird growth is reduced due to less P available for bone mineralisation and metabolism (Hamdi *et al*., 2015; Gautier *et al*., 2017). According to results obtained by Gautier *et al*. (2017), bone breaking force increased from 12.805 N to 18.761 N when the Ca was increased from 0.40% to 1.00% after which the bone breaking force decreased to 18.171 N when the Ca inclusion level was further increased to 1.60%. The source of Ca may also influence bone characteristics as shown in a study done by Hamdi *et al*. (2015) where the tibia ash percentage increased from 50.75% to 51.29% when the Ca solubility decreased. Calcium sources which have a higher solubility also has a higher acid binding capacity. More soluble sources of Ca has the ability to increase the pH in the gastro-intestinal tract to a greater extend which favours the formation of insoluble complexes between Ca and phytate (Hamdi *et al*., 2015). Ultimately, bone density and bone

ash percentage depend on the efficacy with which Ca and P are absorbed from the intestines and incorporated into the bones (Hamdi *et al*., 2015).

2.5.3 Effect of excess calcium on broiler performance

Calcium requirements of broilers are sometimes established based on parameters such as performance data. The parameters measured to determine the performance of broilers includes growth rate, feed intake and feed conversion ratio (Driver *et al*., 2005). High concentrations of Ca and low concentrations of nPP have negative effects on broiler performance due to the interaction between these minerals. The absolute amounts of Ca and nPP are less important than its relative proportions (Selle *et al*., 2009; Hamdi *et al*., 2014; Wilkinson *et al*., 2014).

The average daily gain of broilers decreased linearly as dietary Ca increased from 0.67% to 1.33%. The average daily feed intake followed a similar pattern (Powell *et al*., 2011; Wilkinson *et al*., 2014). Wilkinson *et al*. (2014) showed that increased dietary Ca slowed digesta passage rate. Slowed digesta passage rate will decrease feed intake and average daily body weight gain will follow a similar pattern. Slower digesta transit time also creates favourable conditions for microbial growth and it is hypothesised that this may cause irritation to intestinal mucosa and impair absorption (Wilkinson *et al*., 2014). The same pattern was seen by Hamdi *et al*. (2015) where the broilers had a lower body weight gain and feed intake when dietary Ca was 0.90% vs 0.70%. However, when the nPP inclusion level increased from 0.25% to 0.45% at Ca inclusion level of 0.90% the body weight increased from 391 grams to 446 grams at 14 days of age (Hamdi *et al*., 2014). In a study conducted by Gautier *et al*. (2017) where the Ca:nPP ratio was increased from 1.33% to 5.33% the feed conversion ratio also increased from 1.42 to 1.52. Effects of Ca on mortality rates were non-significant although a trend was noted by Hamdi *et al.* (2015) that mortality rates decreased as dietary Ca increased up to a point where leg abnormalities started to appear.

2.5.4 Effect of excess dietary calcium on the efficiency of exogenous and mucosal phytase

In modern poultry production, phytase plays an integral role in meeting the P requirements of the broiler. Phytate is a natural storage form of P in plant material (Li *et al*., 2015) and between 60% and 90% of P in a maize and soybean meal based diet is present as phytate P. Phytate P is unavailable to poultry species and is dependent on an exogenous phytase source for the utilisation of P from phytate P due to insufficient production of endogenous phytase (Tamin & Angel, 2003; Li *et al*., 2016). The main purpose of supplementing poultry diets with exogenous phytase is to increase the availability of nutrients (Paiva *et al*., 2013). Walk *et al*. (2014) proved the efficacy of phytase on phytate hydrolysis through a study in which the phytate concentration was 98% lower in the gizzard where 500 U *E. coli* was added to the diet compared to the diet without phytase. Through the inclusion of phytase in the diet, hydrolysis of phytate may liberate up to 0.36% more Ca. This allows for significant reductions in dietary

Ca without decreasing broiler performance and bone mineralisation (Hamdi *et al*., 2015). Phytase increases the amount of Ca available due to the hydrolysis of phytate whereby less phytate is present for calcium-phytate complex formation (Paiva *et al*., 2013). Phytase is also able to increase the amount of Ca available and absorbed by the chicken through its hydrolytic effect on the Ca-phytate complexes (Qian *et al*., 1997). Phytase also improves the availability of other nutrients, such as proteins, amino acids and carbohydrates (Paiva *et al*., 2013) by preventing the formation of protein-phytate complexes through its hydrolytic effect on phytate (Li *et al*., 2015). It has been proposed by Selle *et al*. (2009) that phytate possibly complex with starch thereby reducing the digestibility of starch or that phytate reduces the efficacy of α-amylase, in which Ca-phytate interactions may play a role.

The efficiency of phytase is influenced by pH. The pH range where phytase functions optimally were found to be in the acidic GIT compartments where the pH was between 3 and 5. Limestone has a high acid binding capacity which increases digesta pH (Brejnholt *et al*., 2011). In a study done by Paiva *et al*. (2013), high levels of Ca were found to increase the digesta pH in the upper GIT and thereby lowered the phytase activities. Ca had a much more pronounced effect because it was added to the diet in large quantities (McGuaig *et al*., 1972). Qian *et al*. (1997) proposed that excess Ca also inhibited phytase activity by competing for the same active sites of the enzymes. Results are summarised in Figure 2.2 where the effect of increasing Ca:P ratio on the activity of four different phytase inclusion levels was tested. As the Ca:P ratio increased at any phytase inclusion level a decrease in phytase activity could be seen.

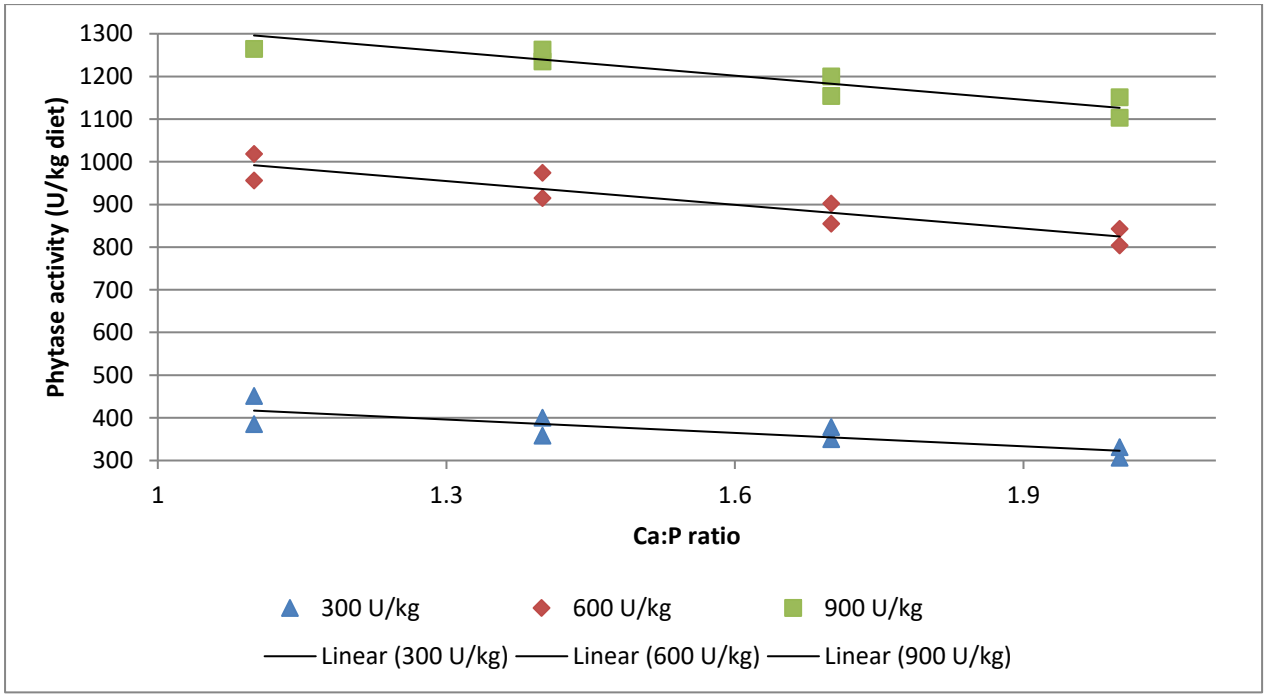

Figure 2.2 Effect of increasing Ca:P ratio on phytase activity of three different phytase inclusion levels (Qian *et al*., 1997)

Calcium sources which are more soluble reduced the activity of phytase to a greater extent than a less soluble source and different types of microbial phytases responded differently to Ca in the diet (Brejnholt *et al*., 2011; Li *et al*., 2018). Therefore, when feeding phytase supplemented diets, it is beneficial to include a source of Ca with a larger particle size with a lower solubility which minimises the Ca and P insoluble complex formation in the crop and the anterior portion of the gastro-intestinal tract. This increases the phytase enzyme contact time with phytic acid in the crop, proventriculus and gizzard and provides more available P from phytic acid hydrolysis in the broiler (Manangi & Coon, 2007).

2.6 Bioavailability of calcium and phosphorus

Research has been done to define the availability of P in various raw materials more accurately which have led to a shift from total P to available P. The value used for Ca in formulations is still on a total Ca basis which may lead to inaccurate formulation (Anwar *et al*., 2017). Various factors contribute to the variation in the amount of Ca digested in the gastro-intestinal tract of the bird which ultimately become available for absorption and digestion (Kim *et al*., 2017).

2.6.1 Definitions and terminology

There is inconsistency in the terms describing P and Ca, which creates confusion on which values to use during feed formulation. Over- or under supplementation of these minerals may result due to a misunderstanding of the definition used. There has been a shift from total Ca to available Ca in the past few years and extensive studies have been done on bioavailability of different Ca sources. Thus, accurate quantification of Ca concentration and bioavailability in different Ca sources used in poultry diets will become more important for efficient poultry production (Anwar *et al*., 2017). The following are definitions from literature describing Ca and P.

The first definition is total P (P) which includes any and all forms of P (Angel, 2011). Available P (aP) is calculated by subtracting the P found in the distal ileum digesta minus the P in the feed, thus it is the P which is absorbed by the animal and available for metabolism. There is confusion between aP and relative available P which are used interchangeably. These two values for P are not the same. Relative aP is determined by setting the availability of a standard P containing product as 100 and compares other P products in relation to this standard (Apke *et al.*, 1987; Soares, 1995). Relative aP does not provide absolute values on the P which are available to the animal but it can be useful when comparing products. This confusion in the term aP has led to the use of digestible P. Digestible P is determined by using a marker system in which the difference in concentration between feed P and ileal P are used to determine the bioavailability of P. Retained P is the P that is kept inside, or retained in the body. Inorganic P (iP) is the term used to distinguish the P not bound to an organic molecule. Phytate P (PP) is P bound to an organic molecule like six carbon molecules found in plant seeds (phytic acid).

Non-phytate P (nPP) is calculated by subtracting PP from total P in the feed that is not bound to the phytic acid molecule (Angel, 2011).

To determine aP, experiments are required which involves live chickens (Van der Klis & Versteegh, 1996). This process is time-consuming and is influenced by various factors such as the concentration of P relative to other nutrients which may interact with P as well as physiological, health and management factors. nPP is chemically determined using a relatively rapid process (Angel & Applegate, 2001). Importantly, nPP and aP are sometimes erroneously interchanged (Plumstead *et al*., 2007), as not all nPP is available. The key differences between aP and nPP are that aP includes absorbed inorganic as well as organic P, which includes PP. Non-phytate Phosphorus excludes any potentially available PP and includes any potentially unavailable iP.

The concept of total Ca is still used rather than available Ca (Hamdi *et al*., 2015). Due to environmental pressure and the need to reduce feed costs, the development of systems to determine digestible Ca and P values are needed. These will allow nutritionists to limit wastage and to formulate more accurately according to requirements (Angel, 2011).

Bone ash in the tibia of broilers is a valuable parameter to assess the availability of Ca and to detect an imbalance. The tibia in broilers has a high growth rate and is considered to be the fastest growing bone in the body. A common phenomenon when a Ca imbalance is present is tibial dysplasia (McLean & Urist, 1961). The three most common ways to express tibia ash is through milligram ash per volume of bone, ash percentage which is determined by dividing the weight of the ash by the weight of the defatted bone and lastly as ash percentage per unit of body weight which is determined by dividing the amount of ash by the body weight of the bird.

Tibia ash as a percentage of dry defatted bone weight has been used widely as response criterion for mineral deposition**,** because the amount of ash reveals the mineral content of the bone. Ca and P make up the majority of these minerals and therefore it is a sensitive parameter of the dietary Ca and P availability (Shastak *et al.*, 2012)**.** There is a correlation between the size of the bone and total ash because the amount of ash varies with the weight and length of the bone. By using tibia ash weight as a parameter to determine the effect of differences in mineral supplementation, differences in bone size and weight can be taken into consideration. With bone ash percentage, differences in bone size and weight will not be revealed in bone ash percentage values (Applegate & Lilburn, 2002; Shim *et al.*, 2012).

2.6.2 Digestion of calcium and phosphorus

At hatch, the chicks have immature digestive tracts and low endogenous enzyme production. As the bird ages, there is an increase in digestive mass and enzyme concentration. These changes are

associated with improved digestive efficiency (Li *et al*., 2015). For efficient digestion and absorption of nutrients, a healthy intestine is required. Various factors can affect the gut health of chicks (Dinev, 2011). For example, the colonisation of the intestine by pathogenic bacteria damages the intestinal lining and may reduce the absorption of Ca and P, which will influence performance (Paiva *et al*., 2013). Other factors that may affect gut health and therefore nutritional absorption and growth are the viscosity of the digesta in the GIT. High viscous digesta promotes the growth of unfavourable bacteria which causes damage to the GIT lining and reduces nutrient absorption (Abdollahi *et al*., 2019). Hypoxia, which can be a result of heat stress where blood flow is diverted to the periphery, and nutrient restriction, results in a shortage of glucose supply to the enterocytes which reduces nutrient absorption (Kvidera *et al*., 2016). Inflammation of the GIT may predispode the gut for invasion of *Clostridiae* which in turn may damage the intestinal lining and reduce nutrient absorption (Niewold *et al*., 2009).

Measurements of digestion and absorption of Ca and P over the total tract give little information on the site and extent of digestion and absorption of Ca and P, neither on the interactions which occur between Ca and P along the digestive tract. Identification of the site of digestion and absorption of Ca and P gives information on the dynamics of digestion and the interactions between these two minerals over the digestive tract (Mohammed *et al*., 1991). The affinity of Ca is higher for phytate than for inorganic P. Interaction between Ca and phytate P reduces the bioavailability of Ca and phytate P by 50% (Mohammed *et al*., 1991). Complex formation between Ca and phytate P occurs more rapidly when the pH increases. Therefore, because of the rapid increase of the pH of digesta as it moves from the acidic environment of the gizzard into the duodenum where it mixes with buffers, most insoluble Ca-phytate complexes form in the small intestine (Manangi & Coon, 2008). Ca and P not digested and absorbed through the gastro-intestinal tract of the bird are excreted into the environment, which lead to P polluting the environment (Kim *et al*., 2017).

The two main mechanisms by which intestinal Ca is transported is by means of active and passive transport. Active transport is transcellular and involves three steps: entry across the cell wall, diffusion through the cytoplasm and exit at the basolateral cell membrane. Passive transport is driven by a concentration gradient between the intestinal lumen and circulation which facilitates ion movement along the chemical gradient through spaces between cells (Bronner *et al*., 1986). Passive transport of Ca predominates when Ca intake is either high or at adequate concentrations due to inhibition of active Ca transport by high plasma Ca concentrations. Passive transport is not dependent on Vitamin D and occurs throughout the length of the small intestine (Buckley & Bronner, 1980) whereas active Ca transport has been associated with the upper duodenum and is vitamin D-dependent (Pansu *et al*., 1983). Active intestinal absorption can be regulated by dietary Ca concentration. Active absorption of Ca is increased when dietary Ca requirement is increased or in cases of low dietary Ca (Auchere *et al*., 1998). With active Ca transport the entry of Ca across the cell wall is facilitated by two epithelial Ca selective anion channels namely transient receptor potential channels, TRPV5 and TRPV6 (Proszkowiec-

Welgarz & Angel, 2013). These channels are localised in the brush border membrane of the intestine. TRPV5 and TRPV6 have different expression locations in the intestine, TRPV6 has the highest expression in the duodenum, caeca and colon and the lowest in the ileum. TRPV5 is predominantly expressed in the kidney with limited expression in the duodenum and caeca (Peng *et al*., 2003).

Calbindin are responsible for transcellular diffusion of Ca in the intestine and renal tissue, respectively. The function of calbindin is to bind Ca and move it from the brush border membrane to the basolateral membrane of duodenal cell (Gross & Kumar, 1990). A vitamin D-induced calbindin is the primary protein responsible for transcellular diffusion of Ca in the avian intestine. In the skeleton, 1,25-dihydroxyvitamin D_3 together with PTH promotes mobilisation of Ca from bone to maintain a constant blood Ca concentration. Bone resorption occurs through the actions of osteoclasts and osteoblasts. Osteoclasts do not have the PTH receptor and activation of bone resorption occurs indirectly through osteoblasts (Spencer *et al*., 1978). The mode of action of the parathyroid hormone is that it stimulates the expression of the receptor activator in osteoblasts which in turn induces the formation, activation and survival of osteoclasts. The receptor activator is regulated by 1,25-dihydroxyvitamin D_3 and PTH (Proszkowiec-Welgarz & Angel, 2013).

2.6.3 Factors affecting bioavailability of calcium and phosphorus

Not all the Ca and P in the diet are absorbed and available for metabolism through the gastro-intestinal tract of the chicken. One of the reasons that not all of the dietary Ca and P are absorbed through the gastro-intestinal tract is because of the interaction between Ca and P and Ca with other nutrients. The interactions lead to the formation of insoluble complexes which is unavailable for digestion by the broiler (Bar *et al*., 2003). Potential factors which have an influence on the bioavailability of Ca or phytate P include Ca source, Ca level, Ca:P ratio, pH of the digesta, phytase dose and adaptation to a deficiency of these nutrients (Li *et al*., 2016).

2.6.3.1 Source of calcium and phosphorus

The dietary sources of Ca may influence the bioavailability of Ca and has a significant influence on broiler performance mainly due to differences in solubilities between different sources. Solubility is affected by physic-chemical characteristics of the Ca source (Hamdi *et al*., 2015). The chemical structure and origin of P in the diet determine its availability to the chicken (Ajakaiye *et al*., 2003). Ca sources with a high solubility has a higher acid binding capacity compared to Ca sources with a lower Ca solubility which have a negative influence on broiler performance (Walk *et al*., 2012) and are more effective in increasing the digesta pH (Cesar *et al*., 2008), which may result in an increase in calcium-phosphate or calcium phytate precipitation (Paiva *et al*., 2013; Hamdi *et al*., 2015). The level and

properties of Ca in the diet is important for young broilers due to the immaturity of the gastro-intestinal tract (Hamdi *et al*., 2015). According to Paiva *et al*. (2013) there is a significant difference in body weight gain, feed conversion ratio and feed intake when more soluble and less soluble sources of Ca are compared to each other. Results from a study where limestone and a highly soluble calcium source in the diets of broilers on performance was compared, are summarised in Table 2.1.

Table 2.1 Effects of Ca sources of different solubilities on body weight gain (BWG), feed conversion ratio (FCR) and feed intake (FI) (Paiva *et al*., 2015)

	BWG (kg)		FI (kg)		FCR (kg:kg)	
Ca source	0-14	0-21	0-14	0-21	0-14	0-21
Limestone	0.4	0.84	0.48	1.14	1.22	1.38
Highly soluble Ca source	0.39	0.8	0.49	1.15	1.28	1.46

The solubility of dietary Ca sources is dependent on the pH in the gastro-intestinal tract (Hamdi *et al*., 2015). There is a natural tendency for the solubility of Ca in different Ca sources to decrease as the pH increases (Selle *et al*., 2000). Thus, as the pH increases from the acidic environment of the proventriculus and gizzard to the more neutral environment of the small intestine the solubility of Ca decreases and may precipitate as carbonate and phosphate before absorption occurs (Hamdi *et al*., 2015). The sources of Ca, P, amino acids and bile component influence the rate of neutralisation and modify the components of Ca absorption (Goss *et al*., 2010).

Different Ca sources may also influence feed intake and subsequent performance. Ca sources with lower solubility like limestone may allow better performance compared to highly soluble sources due to differences in feed intake. Mineral sources of P are higher in digestibility than vegetable sources regardless of the phytase dose (Hamdi *et al*., 2015).

2.6.3.2 Particle size of feed/calcium source

In modern poultry production, broiler diets lack structure and structural components with the only structure being the pellet texture. Introducing structural components in broiler diets have shown to partly restore the suboptimal gut health and functionality associated with feeding highly processed diets and to promote nutrient digestion. Mechanical stimulation of the proventriculus and gizzard are realised which in turn ensure provision of more time for the secretion of hydrochloric acid. Moreover, a gizzard with a well-developed muscle layer generates vigorous reverse peristaltic contractions which reduce digesta transit time to the lower GIT and promotes longer contact time between phytate and phytase and also re-exposing the digesta to hydrochloric acid which favours phytase activity. This will ensure more complete hydrolysis of phytate (Abdollahi *et al*., 2019).

Not only does particle size affect rate of solubilisation, it also influences the rate of which digesta move from the gizzard towards the lower gastro-intestinal tract. The slower passage rate allows longer contact time between the phytase enzyme and the phytate molecules, which may result in a more complete degradation of phytate and release of P. Ca sources with a larger particle size have a slower rate of solubilisation whereas a Ca source with a finer texture will have a faster rate of solubilisation (Hetland *et al*., 2002). Also, smaller particles may increase the pH in the upper part of the gastro-intestinal tract more rapidly, consequently reducing the efficacy of phytase and enhancing the formation of insoluble Ca phytate complexes (Hamdi *et al*., 2015). That explains why birds that were fed a diet containing 0.9% Ca had a higher body weight gain when the Ca was supplied by limestone with a particle size of between 137 µm and 388 µm compared to limestone with a finer particle size of 28 µm (Manangi & Coon, 2007).

However, the results of another study on the influence of a highly soluble Ca source, included in the diet from 0.45% to 0.90%, on broiler performance and bone mineralisation showed that feeding broiler chicks with a higher soluble source of Ca with phytase allowed for reductions in dietary Ca while maintaining broiler performance and bone ash (Walk *et al*., 2012). Again, it can be suggested from this results that the current recommendation of total Ca for broilers may be overestimated, which encourages the interest of quantifying Ca requirements on a digestible basis.

2.6.3.3 Intestinal pH

For Ca to be absorbed it must be in a soluble form. The solubility of Ca is closely related to small intestinal pH and phytate concentration (Paiva *et al*., 2013). Mineral-phytate complex formation is accelerated at a pH of five or higher (Plumstead *et al*., 2008; Paiva *et al*., 2013; Hamdi *et al*., 2015; Li *et al*., 2016), which is the approximate pH in the small intestine (Champagne, 1988), thus decreasing phytate dephosphorylation (Angel *et al*., 2002; Li *et al*., 2016). This corresponds with the findings of *in vitro* studies done by Grynspan & Cheryan (1983) in which the solubility of phytate decreases at a pH higher than four, which is also negatively affected by Ca concentration. Higher dietary Ca increases the pH, which reduce the solubility of the phytate and consequently accelerate Ca-phytate chelation (Adeola & Walk, 2013; Amerah *et al*., 2014).

Phytase is pH dependant and functions optimally at a pH of three (Baradaran *et al*., 2013). The areas in the chicken gastro-intestinal tract where these acidic conditions predominate are the upper part of the gastro-intestinal tract, which include the proventriculus, gizzard and possibly the crop (Li *et al*., 2016). When the pH in the upper part of the gastro-intestinal tract is increased, enzymatic phytate P hydrolysis through the action of phytase may be impaired. This leads to more phytate P reaching the lower gastro-intestinal tract where higher pH conditions predominate, which encourage Ca phytate P complex formation and where phytase activity is also reduced (Li *et al*., 2016).

With an increase in the Ca:P ratio by increasing dietary Ca, P and Ca retention is reduced. Ca-phytate formation is enhanced, which results in increased phytate reaching the small intestine which is indigestible. Phytate has the ability to drag excess Na into the small intestinal lumen, which impedes uptake of nutrients through the Na-dependant transport system (Ravindran *et al*., 2008). Li *et al*. (2016) noted that an increase in nPP had no influence on phytate degradation but as the dietary Ca increased the phytate concentration in the gizzard increased, partly due to the subsequent increase of the pH in the gizzard. Phytate disappearance in the ileal portion of the gastro-intestinal tract was also negatively affected by an increase in dietary Ca concentration (Li *et al*., 2016). Results from a study where the effect of different Ca inclusion levels on phytate disappearance along the gastro-intestinal tract were compared, is summarised in Table 2.2.

Table 2.2 The effects of dietary calcium (Ca), phytate phosphorus (PP), non-phytate phosphorus (nPP) and phytase on phytate concentration in the crop, stomach (proventriculus and gizzard) as well as ileal phytate disappearance (adapted from Li *et al*., 2016)

				Phytate concentration %		
Ca %	PP %	nPP %	Phytase	Crop	Proventriculus and gizzard	Ileal phytate disappearance %
0.7	0.23	0.28	0	0.548	0.323	18.4
0.7	0.34	0.45	0	0.819	0.361	26.1
0.7	0.23	0.28	0	0.577	0.303	32
0.7	0.34	0.45	0	0.842	0.386	26.4
1	0.23	0.28	0	0.565	0.326	16.7
1	0.34	0.45	0	0.881	0.369	20
1	0.23	0.28	0	0.63	0.346	30.7
1	0.34	0.45	0	0.874	0.369	26.1
0.7	0.23	0.28	500	0.345	0.191	78
0.7	0.34	0.45	500	0.55	0.25	71
0.7	0.23	0.28	500	0.371	0.173	76.6
0.7	0.34	0.45	500	0.497	0.196	66.2
1	0.23	0.28	500	0.33	0.157	78.8
1	0.34	0.45	500	0.556	0.168	66.3
1	0.23	0.28	500	0.379	0.152	67.9
1	0.34	0.45	500	0.562	0.267	57.6
0.7	0.23	0.28	1000	0.206	0.111	91.5
0.7	0.34	0.45	1000	0.452	0.132	87.7
0.7	0.23	0.28	1000	0.286	0.123	91.4
0.7	0.34	0.45	1000	0.44	0.135	82.1
1	0.23	0.28	1000	0.303	0.127	90.4
1	0.34	0.45	1000	0.43	0.153	75.4
1	0.23	0.28	1000	0.321	0.163	84.1
1	0.34	0.45	1000	0.533	0.185	76.3

2.7 Conclusion

Calcium inclusion level and source have an effect on broiler performance and bone characteristics. From the literature it can be concluded that it may be beneficial to reduce the Ca inclusion levels below the current recommendations for Ross broilers by Aviagen, not only to improve broiler performance, but to decrease feeding costs as well. Several studies have shown that, at any given P inclusion level, broiler performance decreased as the Ca inclusion level increased within a specific inclusion range. Thus, as the Ca:P ratio increased broiler performance decreased. This suggest that the Ca:P ratio is more important than the absolute inclusion levels of either of these minerals due to the interaction which exists between the two. Calcium has the ability to bind with phytate to form calcium-phytate molecules. These molecules are insoluble and phytate hydrolysis through the action of phytase are hindered. Furthermore, phytase is pH dependent and functions optimally between a pH of 3 and 5. The high acid binding capacity of limestone results in an increase in digesta pH when limestone is added at high levels to the diet. This higher pH reduces phytase activity, which in turn reduces phytate hydrolysis and promotes Ca-phytate complex formation.

Chapter 3

Materials and Methods

3.1 Birds and husbandry

Ethical clearance was obtained from the Faculty of Natural and Agricultural Sciences at the University of Pretoria (NAS388/2019). The test facilities at Daybreak Farms, Sundra, South Africa, were used for the trail. The facility was a standard open-sided broiler house fitted with tunnel ventilation and was divided into 64 identical pens of 3 m^2 each. Pine shavings were used as bedding material, which was placed at a depth of approximately 10 cm and was supplemented with more shavings throughout the trail when required. In each pen, there were two Auger feeders and six nipple drinkers. The birds had *ad libitum* access to feed and water throughout the duration of the trial and water were monitored daily to ensure that the water pressure was maintained at desired levels and that the feeders were refilled as required. Ross 308 chicks were purchased from Midway chicks. Upon arrival, the chicks were randomly selected and 60 chicks were placed mixed sexed in each pen at a stocking density of 20 birds per m^2. A total of three thousand eight hundred and forty (3840) chicks were placed in the house.

Temperature and humidity loggers were installed before the onset of trial in order to control the environment within the closest range possible. Heat was generated from a coal burner and hot air was blown into the house when needed. Side curtains were opened as required to get rid of excess heat and to allow fresh air to enter the house. Appendix 1 shows the temperature profile which was followed from two days pre-placement to day 35. The average house temperature was decreased every second day from placement up until 24 days of age to get rid of excess heat and to keep the chickens in its thermoneutral zone. The day length cycle of the broilers was controlled by the lighting program as illustrated in Appendix 2. From day 1 to day 3 the chicks were only allowed darkness for 1 hour in a 24 hour cycle. This was done to ensure the chicks consumed as much feed and water as possible during the first 24 hours upon arrival.

A standard vaccination program was followed. The chicks were vaccinated against Newcastle Disease virus (NDV) and infectious bronchitis (IB). The first vaccination took place at the hatchery before the chicks arrived at the trial facilities. From 10 days to 12 days of age the chicks received the vaccine against NDV and IB for the second time at the trial facility. The vaccine was added to the tank from where the water flowed through the water line and the vaccine was administered to the chicks through the nipple drinkers. At 16 days to 18 days of age the chicks received again vaccine against NDV which was also administered through the water. The vaccination program is shown in Appendix 3.

There were trial farm personnel present at all times for the duration of the trail to monitor the housing conditions in terms of temperature, ventilation, feed, water as well as bird health.

3.2 Experimental design and treatments

A complete randomised block design was used in this trial. There were eight treatments with eight replications for each treatment. The house was divided into eight blocks with a replication of each treatment per block. Appendix 4 shows the layout of the blocks within the house. The eight treatments contained varying levels of Ca and P. The Ca and P inclusion levels and Ca:P ratio recommended by Aviagen for Ross Broilers were used as the control. The Ca:P ratio was altered by lowering the Ca inclusion levels for all phases. Format © (Format International Limited, Woking, England. Version 1-May-1998/23.4) was the program used to formulate the feed for the different treatments. All conditions were kept constant and the only difference between the treatments were the Ca and P levels as illustrated in Table 3.1. The feed was a maize-soybean based diet for broilers and was made by the AFGRI Animal Feeds factory located at Isando. A three-phase feeding program comprised of a starter, grower and finisher was used. The starter was fed for 14 days, the grower was fed for 14 days and the finisher was fed for seven days. No phytase was used in the diets and limestone and monocalcium phosphate was used to reach the desired Ca and P concentrations in the diet. Each diet was formulated on least cost basis and is shown in Tables 3.2, 3.3 and 3.4 for the starter, grower and finisher diet, respectively.

Table 3.1 Formulated calcium (Ca) and phosphorus (P) levels (%) of the various diets used in the trial

	Starter			**Grower**			**Finisher**		
	0-14 days of age			15-28 days of age			29-35 days of age		
Treatment	Ca	P	Ca:P	Ca	P	Ca:P	Ca	P	Ca:P
Trt 1	0.80	0.50	1.60	0.60	0.44	1.60	0.54	0.38	1.60
Trt 2	0.85	0.50	1.70	0.70	0.44	1.70	0.61	0.38	1.70
Trt 3	0.90	0.50	1.80	0.79	0.44	1.80	0.68	0.38	1.80
Trt 4 (Control)	**1.00**	**0.50**	**2.00**	**0.88**	**0.44**	**2.00**	**0.76**	**0.38**	**2.00**
Trt 5	0.80	0.38	2.10	0.59	0.28	2.10	0.53	0.25	2.10
Trt 6	0.87	0.38	2.30	0.64	0.28	2.30	0.58	0.25	2.30
Trt 7	0.95	0.38	2.50	0.70	0.28	2.50	0.63	0.25	2.50
Trt 8	0.70	0.45	1.56	0.60	0.39	1.54	0.50	0.33	1.52

Table 3.2 Raw material inclusion (%) and calculated nutrient composition (%) for starter treatments

Raw material (%)	Trt 1	Trt 2	Trt 3	Trt 4	Trt 5	Trt 6	Trt 7	Trt 8
Maize yellow (fine)	57.73	57.43	57.17	56.60	57.80	58.17	57.73	57.97
Soya oilcake meal 46	35.80	35.87	35.87	36.00	34.67	35.73	35.80	36.27
Sunflower oilcake meal					1.53			
Soya oil	0.87	0.97	1.07	1.23	0.87	0.73	0.87	0.70
Blood meal	0.53	0.53	0.53	0.53	0.50	0.52	0.53	0.50
Synthetic methionine (DL-Methionine)	0.30	0.30	0.30	0.30	0.29	0.30	0.30	0.29
Lysine sulphate 70% (55% true lysine)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Synthetic lysine (L-Lysine)	0.12	0.12	0.12	0.12	0.14	0.12	0.12	0.11
Synthetic threonine (L-Threonine)	0.09	0.09	0.09	0.09	0.10	0.09	0.09	0.09
Synthetic valine (L-Valine)	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.06
Creamino (Evonik)	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Mono-dicalcium phosphate (Local)	2.10	2.10	2.10	2.10	1.37	1.37	1.37	1.80
Limestone	0.97	1.10	1.23	1.53	1.27	1.47	1.67	0.80
Salt (fine)	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47
Bicarbonate of soda	0.20	0.20	0.20	0.20	0.23	0.20	0.20	0.20
Olaquindox (Ceva)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mycofix select (Biomin)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Choline chloride liquid. Lm (Provimi)	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Salinomycin 12% (Phibro)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Hemicell ht (Elanco)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Nutrase Xylanase (Nutrex)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Formulated nutrient composition (%)								
Apparent ME (MJ/kg)	12.61	12.61	12.61	12.61	12.61	12.62	12.61	12.62
Crude protein	22.55	22.56	22.54	22.55	22.57	22.56	22.55	22.73
Total Lysine	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43
Total Methionine	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.62
Fat	3.67	3.76	3.85	3.99	3.68	3.55	3.67	3.52
Crude fibre	3.16	3.16	3.15	3.14	3.39	3.17	3.16	3.19
Ash	6.27	6.40	6.53	6.83	5.94	6.07	6.26	5.85
Calcium	0.80	0.85	0.90	1.00	0.80	0.87	0.94	0.70
Total phosphorus	0.83	0.83	0.83	0.83	0.68	0.68	0.68	0.77
Available phosphorus	0.50	0.50	0.50	0.50	0.50	0.38	0.38	0.45
Calcium:Available phosphorus	1.60	1.70	1.80	2.00	2.10	2.30	2.50	1.54

Table 3.3 Raw material inclusion (%) and calculated nutrient composition (%) for grower treatments

Raw material (%)	Trt 1	Trt 2	Trt 3	Trt 4	Trt 5	Trt 6	Trt 7	Trt 8
Maize yellow (fine)	54.76	55.00	52.64	53.82	55.78	55.76	55.30	55.02
Soya oilcake meal 46	28.88	28.48	28.80	29.00	28.80	29.00	29.04	29.04
Full fat germ	7.00	7.00	10.50	7.00	8.52	8.42	8.72	8.92
Sunflower oilcake meal	3.52	3.58	3.00	3.52	3.00	2.72	2.68	2.64
Soya oil	1.60	1.52	0.56	1.92	0.50	0.56	0.56	0.56
Synthetic methionine (DL-Methionine)	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27
Synthetic lysine (L-Lysine)	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31
Synthetic threonine (L-Threonine)	0.09	0.09	0.09	0.09	0.08	0.09	0.09	0.09
Synthetic valine (L-Valine)	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Creamino	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Mono-dicalcium phosphate (Local)	1.76	1.76	1.76	1.76	0.82	0.82	0.82	1.46
Limestone	0.86	0.99	1.12	1.36	0.94	1.08	1.26	0.70
Salt (fine)	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
Bicarbonate of soda	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Olaquindox (Ceva)	0.04	0.06	0.04	0.04	0.04	0.04	0.04	0.04
Choline chloride liquid. Lm (Provimi)	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Salinomycin 12% (Phibro)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Hemicell ht (Elanco)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Nutrase Xylanase (Nutrex)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Formulated nutrient composition (%)								
Apparent ME (MJ/kg)	13.30	13.29	13.30	13.30	13.29	13.29	13.29	13.29
Crude protein	20.32	20.33	20.37	20.31	20.36	20.34	20.35	20.34
Total Lysine	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Total Methionine	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
Fat	6.90	6.83	7.40	7.19	6.53	6.54	6.67	6.75
Crude fibre	4.12	4.20	4.26	4.10	4.18	4.13	4.13	4.13
Ash	5.52	5.26	5.75	6.02	4.70	4.83	5.00	5.06
Calcium	0.70	0.75	0.79	0.88	0.59	0.64	0.70	0.60
Total phosphorus	0.76	0.77	0.76	0.77	0.56	0.56	0.56	0.70
Available phosphorus	0.44	0.44	0.44	0.44	0.28	0.28	0.28	0.39
Calcium:Available phosphorus	1.60	1.70	1.80	2.00	2.10	2.30	2.50	1.54

Table 3.4 Raw material inclusion (%) and calculated nutrient composition (%) for finisher treatments

Raw material (%)	Trt 1	Trt 2	Trt 3	Trt 4	Trt 5	Trt 6	Trt 7	Trt 8
Maize yellow (fine)	51.16	52.02	50.82	50.44	52.34	52.10	51.86	52.00
Soya oilcake meal 46	26.36	26.48	26.44	26.52	26.16	26.20	26.24	26.20
Full fat germ meal	11.00	10.00	11.00	11.00	11.00	11.00	11.00	11.00
Sunflower oilcake meal	5.16	5.12	5.16	5.12	5.28	5.28	5.24	5.28
Soya oil (mixer)	2.66	2.64	2.76	2.90	2.26	2.34	2.42	2.38
Synthetic methionine (DL-Methionine)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Lysine sulphate 70% (55% true lysine)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Synthetic lysine (L-Lysine)	0.15	0.15	0.15	0.15	0.16	0.15	0.15	0.15
Synthetic threonine (L-Threonine)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Synthetic valine (L-Valine)	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Mono-dicalcium phosphate (local)	1.41	1.41	1.41	1.41	0.63	0.63	0.63	1.11
Limestone	0.76	0.86	0.96	1.18	0.86	1.00	1.14	0.58
Salt (fine)	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32
Bicarbonate of soda	0.12	0.10	0.12	0.12	0.12	0.12	0.12	0.12
Choline chloride liquid. Lm (Provimi)	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Hemicell ht (Elanco)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Nutrase Xylanase (Nutrex)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Formulated nutrient composition (%)								
Apparent ME (MJ/kg)	13.61	13.61	13.61	13.61	13.61	13.61	13.61	13.61
Crude protein	20.07	20.07	20.07	20.07	20.07	20.07	20.07	20.07
Total Lysine	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13
Total Methionine	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51
Fat	5.27	5.27	5.27	5.27	5.27	5.27	5.27	5.27
Crude fibre	3.73	3.73	3.73	3.73	3.73	3.73	3.73	3.73
Ash	4.29	4.29	4.29	4.29	4.29	4.29	4.29	4.29
Calcium	0.61	0.65	0.68	0.76	0.53	0.58	0.63	0.50
Total phosphorus	0.67	0.67	0.67	0.67	0.44	0.44	0.44	0.58
Available phosphorus	0.38	0.38	0.38	0.38	0.25	0.25	0.25	0.33
Calcium:Available phosphorus	1.60	1.70	1.80	2.00	2.10	2.30	2.50	1.52

In Treatments 1, 2, 3 and 4 the Ca inclusion levels were 0.80%, 0.85%, 0.90% and 1.00%, respectively, at a constant P inclusion level of 0.50% so that the Ca:P ratio also increased from 1.60 to 2.00. The P inclusion level was thus kept constant at the level recommended by Aviagen for Ross broilers.

In Treatments 5, 6 and 7, Ca were included at 0.80%, 0.87% and 0.95%, respectively, and the P inclusion level was decreased to 0.38%. In these treatments, the Ca:P ratio was increased up from 2.00 to 2.50. The Ca:P ratio was decreased (Treatments 1-3) or increased (Treatments 5-7) relative to the Aviagen for Ross broilers recommendation (2.00) in order to test the interaction between Ca and P. Insufficient supply of Ca or P interferes with the homeostasis of the other and therefore, the Ca:P ratio may be more influential than the individual mineral concentrations when formulating poultry diets.

In Treatment 8 the Ca:P ratio was decreased to 1.56. A narrow Ca:P ratio could be beneficial to broiler performance because less Ca is available to bind with phytate. In Treatment 8 the Ca inclusion level was at 0.70% and the P inclusion level was at 0.45%.

3.3 Measurements

3.3.1 Chemical analysis of feed samples

Representative samples were collected from each treatment of each phase. 100 g of feed was sampled for every 100 kg of feed produced. All the samples were mixed and split down to a 500 g representative sample. The representative sample of the eight different treatments for each production phase was analysed in duplicate at Labworld (Pty) Ltd, a division of Philafrica, for total Ca and total P. A Skalar Auto Analyser (Model number 1050) was used for Ca and total P analysis using the segmented flow method (AOAC 960.06). The formation of a yellow complex due to a reaction between P and molybdovanadate in a buffered acidic medium at a pH of 1.0 to 0.5 was used to indicate the total P. Infrared wavelength of 420 nm was used to determine the amount of yellow complex formation. Ca was determined by mixing the feed with an acid 8-hydroxyquinoline. An alkaline complex was used for the complex formation and measured at a wavelength of 580 nm. Formulated Ca and P levels (%) are presented in Table 3.1 and the analysed Ca and total P levels (%) are presented in Table 3.5. The chemical analysis of feed samples was done to determine the Ca and P levels included corresponded to the formulated Ca and P values.

Table 3.5 Analysed calcium (Ca) and total phosphorus (tP) levels (%) of the trial diets

	Starter			Grower			Finisher		
Treatment	**Ca**	**tP**	**Ca:tP**	**Ca**	**tP**	**Ca:tP**	**Ca**	**tP**	**Ca:tP**
Trt 1	0.78	0.84	0.93	0.65	0.80	0.81	0.61	0.70	0.87
Trt 2	0.82	0.84	0.98	0.81	0.80	1.01	0.70	0.70	1.00
Trt 3	0.90	0.84	1.07	0.77	0.80	0.96	0.65	0.70	0.93
Trt 4 (Control)	**1.05**	**0.84**	**1.25**	**0.95**	**0.80**	**1.19**	**0.83**	**0.70**	**1.19**
Trt 5	0.78	0.76	1.03	0.55	0.54	1.02	0.58	0.40	1.45
Trt 6	0.89	0.76	1.17	0.70	0.54	1.30	0.65	0.40	1.63
Trt 7	0.91	0.76	1.20	0.71	0.54	1.31	0.63	0.40	1.58
Trt 8	0.74	0.80	0.93	0.68	0.68	1.00	0.47	0.57	0.82

A representative sample for each treatment of each phase was analysed for protein, moisture and fat using near infrared spectroscopy (NIR) to ensure the feed contained the same nutrient composition as the formulated value. This was done before the feed was delivered to the experimental site. Table 3.6 shows the analysed NIR values obtained via scanning the samples on the NIR machine at the on site quality control laboratory for AFGRI Animal feeds at the feed mill situated in Isando.

Table 3.6 Analysed crude protein, moisture and fat levels (%) of the trial diets

	Starter			Grower			Finisher		
Treatment	**Protein**	**Moisture**	**Fat**	**Protein**	**Moisture**	**Fat**	**Protein**	**Moisture**	**Fat**
Trt 1	22.40	11.41	3.60	20.15	11.22	6.50	19.88	11.05	5.13
Trt 2	22.38	11.20	3.70	20.00	11.49	6.75	19.56	11.60	5.55
Trt 3	22.60	11.98	3.75	20.52	11.81	7.12	20.41	11.71	5.42
Trt 4 (Control)	**22.72**	**11.51**	**4.00**	**20.41**	**11.75**	**6.49**	**20.03**	**11.00**	**5.20**
Trt 5	22.50	11.65	3.72	19.95	12.02	6.88	20.43	12.03	5.03
Trt 6	22.49	11.33	3.41	20.81	11.69	6.28	20.52	12.15	4.98
Trt 7	22.61	11.50	3.55	20.63	11.55	6.81	20.11	11.82	5.55
Trt 8	22.81	10.99	3.49	20.40	11.05	6.55	19.63	11.79	5.32

3.3.2 Performance measurements

3.3.2.1 Body weight (BW)

Broilers were weighed on a weekly basis to obtain the average body weight for each individual pen. A crate was used to weigh all the broilers in the pen collectively. Before each weighing the weight of the crate was tarred. The collective weight was divided by the number of birds in the pen to obtain an average individual bird weight in the pen. The birds were weighed at placement (day-old) and then weekly on 7, 14, 21, 28 and 35 days of age.

3.3.2.2 Feed intake (FI)

Average feed intake per broiler per pen was obtained by weighing of a specific amount of feed at the beginning of each phase for each pen. At the end of each week, the left-over feed for each pen was weighed to determine the feed intake for that week. The weighing of the feed occurred on the same day as the birds were weighed. Cumulative feed intake was calculated by the sum of the weekly feed intakes.

3.3.2.3 Feed conversion ratio (FCR)

The weekly and cumulative feed conversion ratio per pen was determined by dividing the total FI of a pen by the BW gained of that pen over a specific period of time. Feed conversion ratio was corrected for mortalities by adding the weight of the dead bird to the weight of the total pen for that specific week.

3.3.2.4 Performance efficiency factor (PEF)

PEF is a value which gives an indication of overall performance and incorporates all the performance measurements of a pen. The birds with the higher PEF value were the better performing birds overall.

PEF = (Liveability % x Weight (kg))/(Slaughter age (days) x FCR) x100

3.3.2.5 Mortalities

The trial house was inspected twice daily to remove all mortalities. Correction for mortalities was done by weighing the dead birds and recording the mortalities per pen and per treatment.

3.3.3 Tibia ash

Four chicks per pen were sacrificed on 14 days of age. Chicks were randomly selected from the pen and weighed individually and sacrificed only if its body weight was within 20 g from the average body weight of the pen. Selected birds were euthanised by cervical dislocation and the right leg of each bird was removed. The removed legs were placed in appropriately marked sealable bags. All the muscular tissue and cartilage cap were removed, leaving a clean tibia. Ash % and mg ash per tibia were determined on a dry defatted basis. The clean tibias were dried in an oven overnight at 55°C. The dried tibias were then placed into a Soxhlet apparatus filled with petroleum ether for defatting. The petroleum ether was recycled for 12 hours, which removed the fat from the bones, where after the bones were placed under an exhaust fan to dry and to remove excess ether from the bones. Bones were weighed before placing them in a muffle furnace at 600°C for 12 hours. The initial weight of the bones was used in the ash % and mg ash per tibia calculations. On 35 days of age, four chicks per pen were selected

based on the same selection criteria than on day 14 and euthanised in the same way. The only difference was that both legs were removed. The tibias of these chicks were used to determine bone breaking strength prior to tibia ash determination

3.3.4 Bone breaking strength

To perform the bone breaking strength test, only the tibias of the 35 day-old chicks were used after removal of the muscular tissue and cartilage cap. A texture analyser (Lloyd Instron) was used to do a 3 point break test. The support distance was 36 mm, a 5 kN load cell was used which was descending at 4 mm per min. Parameters that were measured included maximum load (kN), maximum machine extension and the time that it took for the bones to break (Sec). Stress (kN/mm^2) was determined by dividing the load (kN) by the transverse area at which the break occurred.

3.4 Statistical analysis

Data were analysed statistically as a randomised block design with the GLM model (Statistical Analysis Systems, 2017) for the average effects over time. Repeated Measures Analysis of Variance with the GLM model was used for repeated week or period measures. Means and standard errors were calculated and significance of differences ($P<0.05$) between means was determined by Fischers test (Samuels, 1989).

The linear model used is described by the following equation:

$$Y_{ij} = \mu + T_i + B_j + e$$

Where Y = variable studied during the period

μ = overall mean of the population

T = effect of the i^{th} treatment

B = effect of the j^{th} block

e = error associated with each Y

Standard chi-square test was used for the mortalities frequency data, and the data were analysed with the frequency model of (2017). In all cases the level of statistical significance was $P<0.05$.

Chapter 4

Results

4.1 Effect of different levels of Ca and P and their interaction on the performance of young broilers

4.1.1 Body weight

The weekly body weight of chickens is shown in Table 4.1.

Table 4.1 The influence of various calcium and phosphorus concentrations in the diet of broilers on weekly body weights (g)

	Days of age					
Treatment	**Day-old**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**
T1	38.0	169.2^{b}	412.6^{b}	854.6^{ab}	1466.2^{ab}	2094.6
T2	38.1	169.3^{b}	412.7^{b}	856.5^{ab}	1465.2^{ab}	2087.8
T3	37.9	165.6^{bcd}	407.5^{bc}	846.9^{b}	1457.3^{ab}	2092.5
T4 (Control)	37.9	166.6^{bcd}	410.2^{bc}	853.0^{ab}	1461.2^{ab}	2085.6
T5	37.9	167.4^{bc}	410.0^{bc}	845.8^{b}	1457.8^{ab}	2067.3
T6	37.9	162.8^{d}	403.4^{c}	839.6^{b}	1446.7^{b}	2060.7
T7	38.0	163.5^{cd}	409.1^{bc}	850.3^{ab}	1473.3^{ab}	2077.0
T8	38.0	173.5^{a}	423.3^{a}	865.5^{a}	1480.4^{a}	2100.3
SEM	0.14	1.51	3.28	6.88	10.73	15.81

$^{a-d}$Values in the same column without common superscripts are significantly different (P<0.05) from each other.
SEM: Standard error of the mean
T1: Ca:P ratio = 1.60; T2: Ca:P ratio = 1.70; T3: Ca:P ratio = 1.80; T4: Ca:P ratio = 2.00, T5: Ca:P ratio = 2.10
T6: Ca:P ratio = 2.30; T7: Ca:P ratio = 2.50; T8: Ca:P ratio = 1.56

There were no significant differences between the body weight means of treatments (P>0.05) at day-old of the Ross 308 broilers.

Differences among chick body weight of T1 (Ca=0.80%), T2 (Ca=0.85%), T3 (Ca=0.90%) and T4 (Ca=1.00%) at a P inclusion of 0.50% at 7 and 14 days of age were of no significance (P>0.05). The differences in the means of treatments between the body weight of T5 (Ca=0.80%) and T6 (Ca=0.87%) at 7 days of age were significantly different (P<0.05) from each other, where the body weight of T5 was higher than T6. The body weight of T5 was higher than T7 as well but the difference was not significant (P>0.05). The differences between the body weight means of T5, T6 and T7 at 14 days of age were not significantly different (P>0.05) from each other, with T5 being the highest. T1 (Ca:P=1.6) and T2 (Ca:P=1.7) were significantly higher (P<0.05) than T6 (Ca:P=2.3) and T7 (Ca:P=2.5) at 7 days

of age, and only significantly higher (P<0.05) than T6 (Ca:P=2.3) at 14 days of age. T8, which had a dietary Ca inclusion level of 0.70% and a dietary P inclusion level of 0.45% with a Ca:P of 1.56, had the highest mean body weight at 7 and 14 days of age and was significantly higher (P<0.05) than T1 to T7 at 7 and 14 days of age.

The differences among T1 (Ca=0.70%), T2 (Ca=0.75%), T3 (Ca=0.79%) and T4 (Ca=0.88%) were non-significant (P>0.05) at a P inclusion level of 0.28% at 21 and 28 days of age. With T5, T6 and T7, T7 (Ca=0.70%) had the highest body weight but was not significantly different (P>0.05) from T5 (Ca=0.59%) or T6 (Ca=0.64%) at 21 and 28 days of age with a P inclusion level of 0.28%. There was no significant difference (P>0.05) in body weight among T1 to T7 at 21 and 28 days of age. T8 (Ca=0.60%; P=0.39%; Ca:P=1.54) had the highest body weight at 21 and 28 days of age. T8 was significantly higher (P<0.05) than T3, T5 and T6 at 21 days of age and was significantly higher (P<0.05) than T6 at 28 days of age.

None of the body weight means of T1 to T8 differed significantly (P>0.05) from one another at 35 days of age. Although no significant differences were seen among the treatment means of 35 day body weight, there was a significant (P<0.05) quadratic correlation between Ca:P ratio and 35-day body weight as graphically illustrated in Figure 4.1. The correlation between 35 day body weight and Ca:P ratio was significant (P<0.05) both, quadratically and linear, but the R-square value was higher for the quadratic correlation.

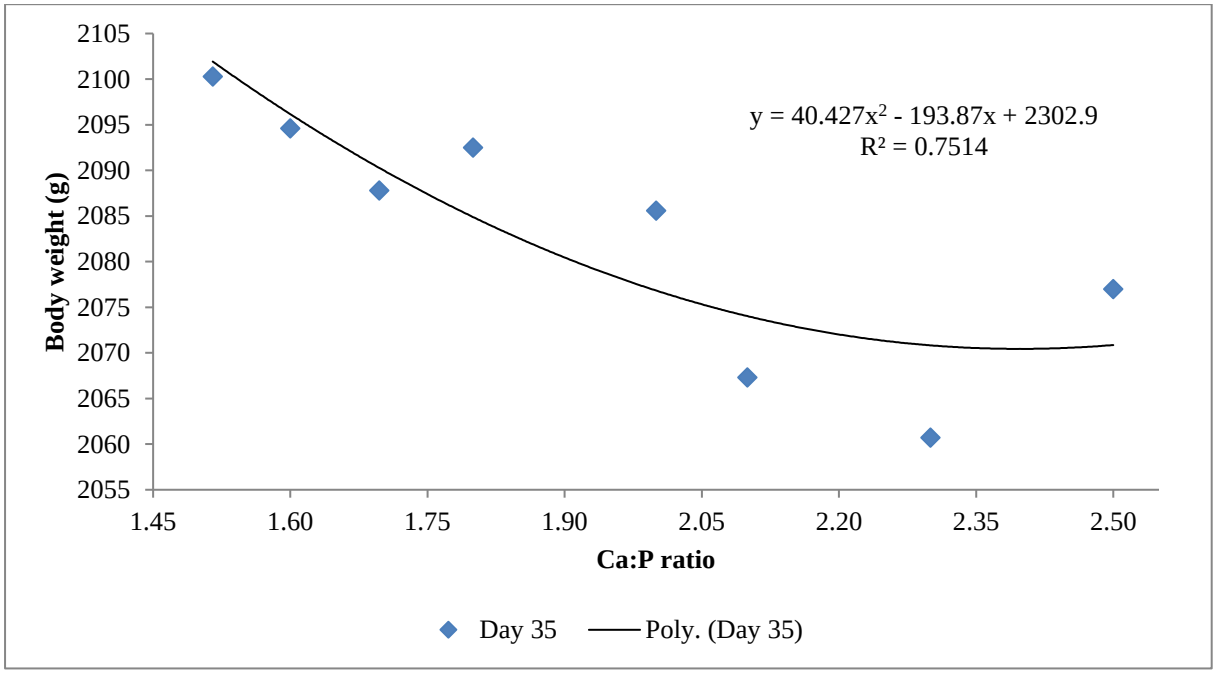

Figure 4.1 Effect of increasing Ca:P ratio on 35-day broiler body weight

4.1.2 Daily weight gain

The daily body weight gain (BWG) of chickens is shown in Table 4.2.

Table 4.2 The influence of various calcium and phosphorus concentrations in the diet of broilers on daily weight gain (g/bird/day)

	Days of age				
Treatment	**7 days**	**14 days**	**21 days**	**28 days**	**35 days**
T1	18.8^{b}	34.8^{b}	63.1	87.4	89.8
T2	18.7^{b}	34.8^{b}	63.4	87.0	88.9
T3	18.2^{bc}	34.6^{b}	62.8	87.2	90.7
T4 (Control)	18.4^{bc}	34.8^{b}	63.3	86.9	89.2
T5	18.5^{b}	34.7^{b}	62.3	87.4	87.1
T6	17.9^{c}	34.4^{b}	62.3	86.7	87.7
T7	17.9^{c}	35.1^{ab}	63.0	89.0	86.2
T8	19.4^{a}	35.7^{a}	63.2	87.8	88.6
SEM	0.20	0.28	0.61	0.82	1.33

$^{a\text{-}c}$Values in the same column without common superscripts are significantly different (P<0.05) from each other.
SEM: Standard error of the mean
T1: Ca:P ratio = 1.60; T2: Ca:P ratio = 1.70; T3: Ca:P ratio = 1.80; T4: Ca:P ratio = 2.00, T5: Ca:P ratio = 2.10
T6: Ca:P ratio = 2.30; T7: Ca:P ratio = 2.50; T8: Ca:P ratio = 1.56

No significant differences (P>0.05) were observed among T1 (Ca=0.80%), T2 (Ca=0.85%), T3 (Ca=0.90%) and T4 (Ca=1.00%) at 7 days of age for daily BWG, although T1 had the highest daily BWG. The differences among T1 to T4 at 14 days of age were non-significant (P>0.05), with T1, T2 and T3 having the highest daily BWG. At 7 days of age, T5 (Ca=0.80%) was significantly higher (P<0.05) than T6 (Ca=0.87%) and T7 (Ca=0.95%) at a P inclusion level of 0.38%. In T5, T6 and T7 no significant differences (P>0.05) were observed at 14 days of age among daily BWG of treatments at a P inclusion level of 0.38%. The daily BWG of T1 (Ca:P=1.60) was the highest among T1 to T7 and were significantly higher (P<0.05) than T6 (Ca:P=2.30) and T7 (Ca:P=2.50) at 7 days of age. At 14 days of age, there were no significant differences (P>0.05) among the daily BWG of T1 to T7 although T7 had the highest daily BWG at 14 days of age. The differences in daily BWG of T8 (Ca=0.70%; P=0.45%; Ca:P=1.56) was significantly higher (P<0.05) than T1 to T7 at 7 days of age. The significant (P<0.05) correlation between daily BWG and Ca:P ratio at 7 days of age is graphically illustrated in Figure 4.2. The same significant (P<0.05) correlation between 35-day BWG and Ca:P ratio is illustrated in Figure 4.3, but with a lower R-square value than 7-day BWG. The daily BWG of T8 was significantly higher (P<0.05) than T1 to T6 but was not significantly different (P>0.05) from T7 at 14 days of age.

The mean daily BWG of the chicks at 21 days of age was the highest for T2 (Ca=0.75%) among T1 (Ca=0.70%), T3 (Ca=0.79%) and T4 (Ca=0.88%), but the daily BWG did not differ significantly

(P>0.05) among these treatments at a P inclusion level of 0.44%. The means of daily BWG from day 21 to 28 days of age for T1 to T4 also did not differ significantly (P<0.05). T7 (Ca=0.70%) had the highest daily BWG among T5 (Ca=0.59%) and T6 (Ca=0.64%) for day 21 and 28 days of age with a P inclusion level of 0.28%. Although T7 had the highest mean daily BWG, it did not differ significantly (P>0.05) from T5 and T6.

None of the means of the treatments differed significantly (P>0.05) from each other at 28 and 35 days of age.

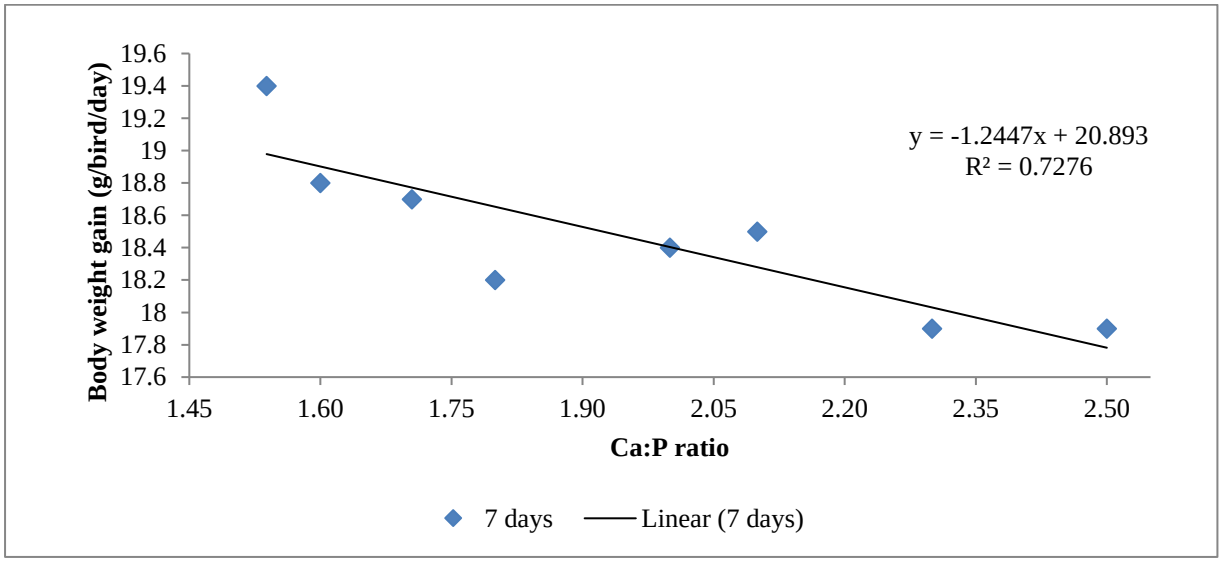

Figure 4.2 Effect of increasing Ca:P ratio on 7-day broiler body weight gain

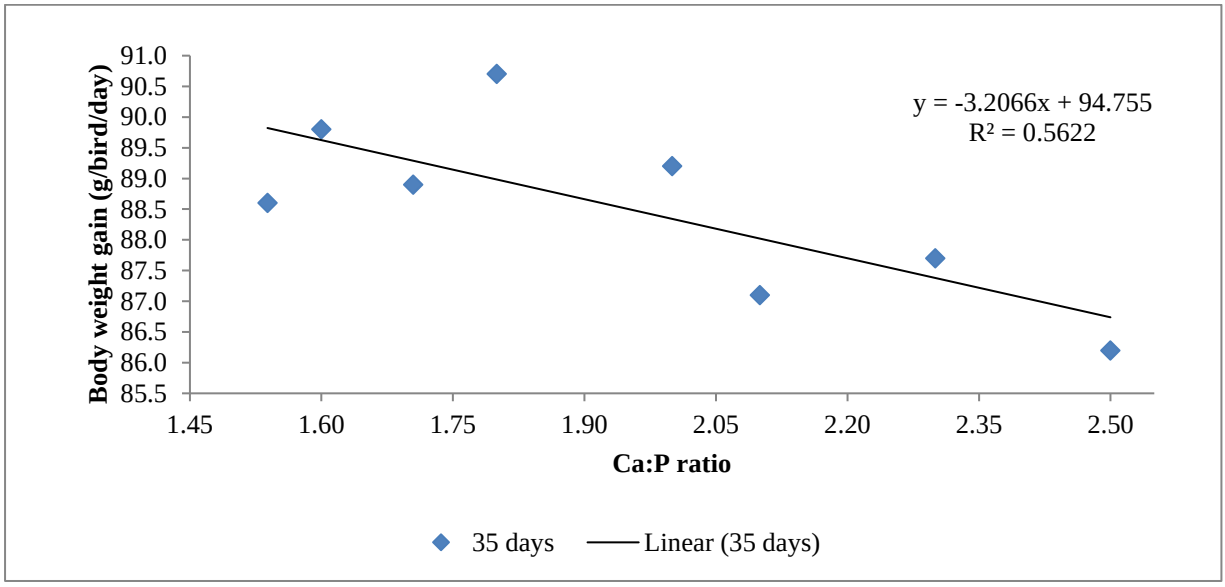

Figure 4.3 Effect of increasing Ca:P ratio on 35-day broiler body weight gain

4.1.3 Cumulative feed intake

The cumulative feed intake of chickens is shown in Table 4.3.

Table 4.3 The influence of various calcium and phosphorus concentrations in the diet of broilers on cumulative feed intake (g/bird)

	Days of age				
Treatment	**7 days**	**14 days**	**21 days**	**28 days**	**35 days**
T1	147.8^{b}	452.8^{c}	1012.3^{c}	1927.7^{b}	2944.2^{b}
T2	154.7^{a}	468.6^{a}	1032.9^{ab}	1979.2^{ab}	3019.3^{ab}
T3	156.7^{a}	460.0^{abc}	1015.5^{bc}	1949.2^{ab}	2975.6^{ab}
T4 (Control)	154.6^{a}	455.5^{c}	1016.1^{bc}	1964.6^{ab}	3005.7^{ab}
T5	155.1^{a}	463.2^{abc}	1029.6^{abc}	2017.7^{a}	3063.2^{a}
T6	152.0^{ab}	453.0^{c}	1018.4^{bc}	1985.2^{ab}	3020.8^{ab}
T7	152.7^{ab}	457.7^{bc}	1025.9^{abc}	1996.8^{ab}	3040.9^{a}
T8	153.6^{ab}	466.2^{ab}	1042.5^{a}	2016.7^{a}	3054.4^{a}
SEM	2.23	4.00	6.86	26.51	35.91

$^{a-c}$Values in the same column without common superscripts are significantly different (P<0.05) from each other.
SEM: Standard error of the mean
T1: Ca:P ratio = 1.60; T2: Ca:P ratio = 1.70; T3: Ca:P ratio = 1.80; T4: Ca:P ratio = 2.00, T5: Ca:P ratio = 2.10
T6: Ca:P ratio = 2.30; T7: Ca:P ratio = 2.50; T8: Ca:P ratio = 1.56

At 7 days of age T1 (Ca=0.80%) had a significantly (P<0.05) lower cumulative feed intake than T2 (Ca=0.85%), T3 (Ca=0.90%) and T4 (Ca=1.00%) at a P inclusion level of 0.50%. There were no significant differences (P>0.05) in cumulative feed intakes among T2, T3 and T4 at 7 days of age. At 14 days of age T1 still had the lowest cumulative feed intake. T1 was significantly (P<0.05) lower than T2 at a P inclusion level of 0.38%. The birds in T2 had significantly (P<0.05) higher cumulative feed intakes than T1 and T4 but not (P>0.05) T3 at 14 days of age at a P inclusion level of 0.38%. There were no significant differences (P>0.05) in cumulative feed intake among T5 (Ca=0.80%), T6 (Ca=0.87%) and T7 (Ca=0.95%) at 7 days of age or 14 days of age at a P inclusion level of 0.38%. At 7 days of age T1 (Ca:P=1.60) had significantly lower cumulative feed intakes than T2 (Ca:P=1.70) to T7 (Ca:P=2.50). At 14 days of age T1 (CaP=1.60) again had the lowest cumulative feed intake and was significantly lower (P<0.05) than T2 (Ca:P=1.70) only. T2 had the highest feed intake and was significantly higher (P<0.05) than T1 (Ca:P=1.60), T4 (Ca:P=2.00) and T6 (Ca:P=2.30). At 7 days of age there was no significant differences (P>0.05) between the cumulative feed intake of T8 (Ca=0.70%; P=0.45%; Ca:P=1.56) and the cumulative feed intake of T1 to T7. At 14 days of age T8 (Ca=0.70%; P=0.45%; Ca:P=1.56) had a significantly higher cumulative feed intake than T1 (Ca=0.80%; P=0.50%; Ca:P=1.60), T4 (Ca=1.00%; P=0.50%; Ca:P=2.00) and T6 (Ca=0.87%; P=0.38%; Ca:P=2.30).

At 21 days of age the cumulative feed intake of the birds fed the diet assigned to T1 (Ca=0.70%) was significantly lower (P<0.05) than the cumulative feed intake of the birds in T2 (Ca=0.75%) at a P inclusion level of 0.44%. At 28 days of age there were no significant differences (P>0.05) among T1 (Ca=0.70%), T2 (Ca=0.75%), T3 (Ca=0.79%) and T4 (Ca=0.88%) at a P inclusion level of 0.44%. At 21 day and 28 days of age there was no significant differences (P>0.05) between the cumulative feed intake of T5 (Ca=0.59%), T6 (Ca=0.64%) and T7 (Ca=0.70%) at a P inclusion level of 0.28%. At 21 days of age, there was no significance (P>0.05) in cumulative feed intake among T1 to T7. At 28 days of age T1 (Ca:P=1.60) had a significantly lower (P<0.05) cumulative feed intake than T5 (Ca:P=2.10). At 21 days of age the birds which received the diet assigned to T8 (Ca=0.60%; P=0.39%; Ca:P=1.54) had the highest cumulative feed intake. Cumulative feed intake in T8 was significantly higher (P<0.05) than T1 (Ca=0.70%; P=0.44%; Ca:P=1.60), T3 (Ca=0.79%; P=0.44%; Ca:P=1.80), T4 (Ca=0.88%; P=0.44%; Ca:P=2.00) and T6 (Ca=0.64%; P=0.28%; Ca:P=2.30). At 28 days of age T8 (Ca=0.60%; P=0.39%; Ca:P=1.54) had a significantly higher feed intake than T1 (Ca=0.70%; P=0.44%; Ca:P=1.60).

At 35 days of age the cumulative feed intake of T8 (Ca=0.50; P=0.33; Ca:P=1.52) was significantly higher (P<0.05) than T1 (Ca=0.61; P=0.38; Ca:P=1.60).

4.1.4 Cumulative feed conversion ratio

The cumulative feed conversion ratio (FCR) of chickens is shown in Table 4.4.

Table 4.4 The influence of various calcium and phosphorus concentrations in the diet of broilers on cumulative feed conversion ratio (kg/kg)

	Days of age				
Treatment	**7 days**	**14 days**	**21 days**	**28 days**	**35 days**
T1	1.13^{a}	1.21^{a}	1.24^{a}	1.35^{a}	1.43^{a}
T2	1.18^{b}	1.25^{b}	1.26^{abc}	1.39^{abc}	1.47^{abc}
T3	1.23^{c}	1.24^{b}	1.26^{abc}	1.37^{ab}	1.45^{ab}
T4 (Control)	1.20^{bc}	1.22^{ab}	1.25^{ab}	1.38^{abc}	1.47^{abc}
T5	1.20^{bc}	1.25^{b}	1.27^{bc}	1.42^{c}	1.51^{c}
T6	1.22^{bc}	1.24^{b}	1.27^{bc}	1.41^{bc}	1.49^{bc}
T7	1.22^{bc}	1.23^{b}	1.26^{abc}	1.39^{abc}	1.49^{bc}
T8	1.13^{a}	1.21^{a}	1.26^{abc}	1.40^{bc}	1.48^{bc}
SEM	0.02	0.01	0.01	0.02	0.02

$^{a-c}$Values in the same column without common superscripts are significantly different (P<0.05) from each other.
SEM: Standard error of the mean
T1: Ca:P ratio = 1.60; T2: Ca:P ratio = 1.70; T3: Ca:P ratio = 1.80; T4: Ca:P ratio = 2.00, T5: Ca:P ratio = 2.10
T6: Ca:P ratio = 2.30; T7: Ca:P ratio = 2.50; T8: Ca:P ratio = 1.56

At 7 days of age, the chickens in T1 had the lowest cumulative FCR among T1 to T4. T1 (Ca=0.80%) was significantly lower (P<0.05) than T2 (Ca=0.85), T3 (Ca=0.90%) and T4 (Ca=1.00) at a P inclusion level of 0.50%. The chicks in T2 (Ca=0.85%) had the second lowest cumulative FCR among T1 to T4 and was significantly lower (P<0.05) than T3 (Ca=0.90%) but not T4 (1.00%). There was no significance (P>0.05) between the cumulative FCR of T3 and T4 at 7 days of age. At 14 days of age T1 still had the lowest cumulative FCR and was significantly lower (P<0.05) than T2 and T3. At 14 days of age the differences in cumulative FCR among T2, T3 and T4 were non-significant (P>0.05) at a P inclusion level of 0.50%. At 7 and 14 days of age, there were no significant differences (P>0.05) in cumulative FCR between the chicks which received the diets assigned to T5 (Ca=0.80%), T6 (Ca=0.87%) and T7 (Ca=0.95%) at a P inclusion level of 0.38%. T1 (Ca:P=1.60) had the lowest cumulative FCR among T1 to T7 and was significantly lower (P<0.05) than T2 (Ca:P=1.70), T3 (Ca:P=1.80), T4 (Ca:P=2.00), T5 (Ca:P=2.10), T6 (Ca:P=2.30) and T7 (Ca:P=2.50) at 7 days of age. At 14 days of age T1 (Ca:P=1.60) still had the lowest cumulative FCR and was significantly lower (P<0.05) than T2 (Ca:P=1.70), T3 (Ca:P=1.80), T5 (Ca:P=2.10), T6 (Ca:P=2.30) and T7 (Ca:P=2.50). The cumulative FCR of T8 (Ca=0.70%; P=0.45%; Ca:P=1.56) was significantly lower (P<0.05) than T2 to T7 at 7 days of age. At 14 days of age T8 (Ca=0.70%; P=0.45%; Ca:P=1.56) had a significantly lower (P<0.05) cumulative FCR than T2, T3, T5, T6 and T7.

Although the cumulative FCR of T1 (Ca=0.70%) was the lowest among T2 (Ca=0.75%), T3 (Ca=0.79%) and T4 (Ca=0.88%) at a P inclusion level of 0.44%, the difference was not significant (P>0.05) at 21 and 28 days of age. At 21 and 28 days of age there were no significant differences among the cumulative FCR of T5 (Ca=0.59%), T6 (Ca=0.64%) or T7 (Ca=0.70%) at a P inclusion level of 0.28%. At 21 and 28 days of age T1 (Ca:P=1.60) had a significantly lower (P<0.05) cumulative FCR than T5 (Ca:P=2.10) and T6 (Ca:P=2.30). At 21 days of age the cumulative FCR of T8 (Ca=0.60%; P=0.39%; Ca:P=1.54) was not significantly different (P>0.05) than any of the other treatments. At 28 days of age T8 (Ca=0.60%; P=0.39%; Ca:P=1.54) had a significantly higher (P<0.05) cumulative FCR than T1 (Ca=0.70%; P=0.44%; Ca:P=1.60).

At 35 days of age the FCR of T1 (Ca=0.61%; P=0.38%; Ca:P=1.60) was significantly lower (P<0.05) than T5 (Ca=0.53%; P=0.25%; Ca:P=2.10), T6 (Ca=0.58%; P=0.25%; Ca:P=2.30), T7 (Ca=0.63%; P=0.25%; Ca:P=2.50) and T8 (Ca=0.50%; P=0.33%; Ca:P=1.52).

4.1.5 Mortalities

At 35 days of age, there were no significant (P>0.05) differences among T1 to T8 in terms of percentage mortality per treatment. Various Ca and P inclusion levels in the experimental diets of T1 to T8 fed to broiler chickens did not affect mortality rates. No significant (P>0.05) correlation between Ca and P inclusion levels and mortality could be seen.

4.1.6 Performance efficiency factor

The performance efficiency factor (PEF) of chickens is shown in Table 4.5.

Table 4.5 The influence of various calcium and phosphorus concentrations in the diet of broilers on performance efficiency factor (PEF)

	Days of age				
Treatment	**7 days**	**14 days**	**21 days**	**28 days**	**35 days**
T1	211.8^{ab}	238.7^{ab}	320.0^{a}	378.1^{a}	404.9^{ab}
T2	202.0^{bc}	231.5^{bc}	315.9^{ab}	366.5^{abc}	392.6^{abc}
T3	192.7^{cd}	232.2^{bc}	318.7^{ab}	374.7^{ab}	407.2^{a}
T4 (Control)	197.2^{cd}	237.2^{abc}	321.2^{a}	373.0^{abc}	396.0^{abc}
T5	198.9^{cd}	233.4^{bc}	313.4^{ab}	362.5^{bc}	386.4^{c}
T6	190.0^{d}	228.8^{c}	308.4^{b}	359.9^{c}	385.7^{c}
T7	190.0^{d}	232.9^{bc}	313.9^{ab}	370.4^{abc}	389.7^{bc}
T8	216.8^{a}	245.2^{a}	320.5^{a}	369.4^{abc}	394.5^{abc}
SEM	3.53	3.10	3.96	4.92	6.06

$^{a-d}$Values in the same column without common superscripts are significantly different (P<0.05) from each other.
SEM: Standard error of the mean
T1: Ca:P ratio = 1.60; T2: Ca:P ratio = 1.70; T3: Ca:P ratio = 1.80; T4: Ca:P ratio = 2.00, T5: Ca:P ratio = 2.10
T6: Ca:P ratio = 2.30; T7: Ca:P ratio = 2.50; T8: Ca:P ratio = 1.56

At 7 days of age the PEF of T1 (Ca=0.80%) was significantly higher (P<0.05) than T3 (Ca=0.90%) and T4 (Ca=1.00%) at a P inclusion level of 0.50%. At 14 days of age, there were no significant differences (P>0.05) among the PEF of T1 to T4. At 7 and 14 days of age, there were no significant differences (P>0.05) among the PEF of T5 (Ca=0.80%), T6 (Ca=0.87%) and T7 (Ca=0.95%) at a P inclusion level of 0.38%. At 7 days of age the PEF of T1 (Ca:P=1.60) was significantly higher (P<0.05) than T3 (Ca:P=1.80), T4 (Ca:P=2.00), T5 (Ca:P=2.10), T6 (Ca:P=2.30) and T7 (Ca:P=2.50). The PEF of T2 was significantly higher (P<0.05) than T6 and T7. At 14 days of age, statistical significance (P<0.05) in PEF only existed between T1 (Ca:P=1.60) and T6 (Ca:P=2.30) with T1 being the highest. At 7 days of age the PEF of T8 (Ca=0.70; P=0.45; Ca:P=1.56) was significantly higher (P<0.05) than T2 (Ca=0.85; P=0.50; Ca:P=1.70), T3 (Ca=0.90; P=0.50; Ca:P=1.80), T4 (Ca=1.00; P=0.50; Ca:P=2.00), T5 (Ca=0.80; P=0.38; Ca:P=2.10), T6 (Ca=0.87; P=0.38; Ca:P=2.30) and T7 (Ca=0.95; P=0.38; Ca:P=2.50). At 14 days of age the PEF of T8 (Ca=0.70; P=0.45; Ca:P=1.56) was significantly higher (P<0.05) than T2 (Ca=0.85; P=0.50; Ca:P=1.70), T3 (Ca=0.90; P=0.50; Ca:P=1.80), T5 (Ca=0.80; P=0.38; Ca:P=2.10), T6 (Ca=0.87; P=0.38; Ca:P=2.30) and T7 (Ca=0.95; P=0.38; Ca:P=2.50).

At 21 and 28 days of age, there were no significant differences (P>0.05) in PEF among T1 (Ca=0.70), T2 (Ca=0.75), T3 (Ca=0.79) and T4 (Ca=0.88) at a P inclusion level of 0.44%. At 21 and 28 days of age, there were no significant differences (P>0.05) in PEF among T5 (Ca=0.59), T6 (Ca=0.64) and T7 (Ca=0.70) at a P inclusion level of 0.28%. At 21 days of age T1 (Ca:P=1.60) and T4 (Ca:P=2.00) had a significantly higher (P<0.05) PEF than T6 (Ca:P=2.30). At 28 days of age the PEF of T1 (Ca:P=1.60) was significantly higher than T5 (Ca:P=2.10) and T6 (Ca:P=2.30). At 21 days of age T8 (Ca=0.60; P=0.39; Ca:P=1.54) was significantly higher than T6 (Ca=0.64; P=0.28; Ca:P=2.30). There were no significant differences (P>0.05) in PEF between T8 and any of the other treatments on 28 days of age.

At 35 days of age T3 (Ca=0.68; P=0.38; Ca:P=1.80) had the highest PEF among all treatments. T3 (Ca=0.68; P=0.38; Ca:P=1.80) was significantly higher (P<0.05) than T5 (Ca=0.53; P=0.25; Ca:P=2.10), T6 (Ca=0.58; P=0.25; Ca:P=2.30) and T7 (Ca=0.63; P=0.25; Ca:P=2.50). T1 (Ca=0.61; P=0.38; Ca:P=1.60) which had the second highest PEF among all treatments was significantly higher than T5 (Ca=0.53; P=0.25; Ca:P=2.10) and T6 (Ca=0.58; P=0.25; Ca:P=2.30).

4.2 Bone characteristics

Effect of different levels of Ca and P and their interaction on bone characteristics of young broilers are shown in Table 4.6.

Table 4.6 The influence of various calcium and phosphorus concentrations in the diet of broilers on bone breaking strength (kN), bone ash (%) are shown in Table 4.6

Treatment	Bone breaking strength		Bone ash percentage per fat free dry matter	
	Extension (mm)	Load (kN)	Bone ash 14 days (%)	Bone ash 35 days (%)
T1	4.57^{ab}	0.207^{ab}	47.23^{b}	48.77^{a}
T2	4.38^{b}	0.201^{ab}	47.25^{b}	47.74^{cd}
T3	4.52^{ab}	0.218^{a}	47.54^{ab}	48.68^{ab}
T4 (Control)	4.41^{b}	0.197^{ab}	48.14^{a}	48.80^{a}
T5	4.76^{a}	0.197^{ab}	47.30^{ab}	47.56^{cd}
T6	4.70^{ab}	0.198^{ab}	47.57^{ab}	48.16^{abc}
T7	4.82^{a}	0.209^{ab}	47.31^{ab}	47.87^{bcd}
T8	4.75^{a}	0.188^{b}	47.73^{ab}	47.10^{d}
SEM	0.12	0.01	0.3	0.3

a-dValues in the same column without common superscripts are significantly different (P<0.05) from each other.
SEM: Standard error of the mean
T1: Ca:P ratio = 1.60; T2: Ca:P ratio = 1.70; T3: Ca:P ratio = 1.80; T4: Ca:P ratio = 2.00, T5: Ca:P ratio = 2.10
T6: Ca:P ratio = 2.30; T7: Ca:P ratio = 2.50; T8: Ca:P ratio = 1.56

There were no significant differences (P>0.05) among the extension in the bones between T1, T2, T3 and T4 where different Ca inclusion levels were assigned at a P inclusion level of 0.38%. There was no significance (P>0.05) in the differences among the extension of the bones in T5, T6, T7 (P=0.25%) and T8 (P=0.33%) where different Ca inclusion levels were assigned to the treatments. Bones which was collected from the 35 day-old chicks assigned to T1, T2, T3 and T4 (P=0.38%) had a higher average extension than the 35 day-old chicks in T5, T6, T7 (P=0.25%) and T8 (0.33%). The bones of the chicks assigned to T2 (Ca:P=1.70) and T4 (Ca:P=2.00) had significantly lower extension values than the bones of the chicks assigned to T5 (Ca:P=2.10), T7 (Ca:P=2.50) and T8 (Ca:P=1.52).

There were no significant differences (P>0.05) in the load (kN) the bones could resist among T1, T2, T3 and T4 where different levels of Ca were assigned to the different treatments at a P inclusion level of 0.38% in the finisher phase. T5, T6 and T7 also had no significant differences in load (kN) among the treatments at a P inclusion level of 0.25% during the finisher phase. Although the average load (kN) of T1, T2, T3 and T4 were higher than the average of T5, T6 and T7, the difference was not significant (P>0.05). The only significance (P<0.05) was between T3 (Ca:P=1.80) and T8 (Ca:P=1.52) with the load (kN) of T8 being the lowest.

The bone ash percentage at 14 days of age of T4 (Ca=1.00%) was significantly (P<0.05) higher than the bone ash percentage of T1 (Ca=0.80%) and T2 (Ca=0.85%) at a P inclusion level of 0.50%. There were no significant differences (P>0.05) in bone ash percentage at 14 days of age among T5, T6 and T7 where different Ca inclusion levels were assigned to the treatments at a P inclusion level of 0.38%. There were no significant differences between the bone ash percentage at 14 days of age of T8 (Ca=0.70%; P=0.45%) and any of the other treatments.

The bone ash percentage of T1 (Ca=0.61%), T3 (Ca=0.68%) and T4 (Ca=0.76%) at 35 days of age were significantly (P<0.05) higher than the bone ash percentage of T2 (Ca=0.65%) at a P inclusion level of 0.38%. There were no significant (P>0.05) differences in the bone ash percentage at 35 days of age among T5 (Ca=0.53%), T6 (Ca=0.58%) and T7 (Ca=0.63%) at a P inclusion level of 0.25%. The bone ash percentage of T1, T3 and T4, which all had a P inclusion level of 0.38%, was significantly (P<0.05) higher than T5 which had a bone ash percentage of 0.25%. T8 (Ca=0.50%; P=0.33%), which had the lowest bone ash percentage at 35 days of age was significantly (P<0.05) lower than T1, T3, T4 and T6 at 35 days of age.

No significant (P>0.05) correlation was seen between Ca and P inclusion levels and bone parameters including, bone extension, load, 14 day bone ash percantage and 35 day bone ash percentage.

Chapter 5

Discussion

5.1 Performance data

It is evident from the data obtained from this trail that Ca and P are not only important for bone mineralisation, but bird performance as well. In this study, the treatment means for body weight showed significant differences within the first 7 to 28 days of age. The average body weight for the treatment means of treatments were higher where the P inclusion level was higher at 35 days of age. The 7 day weights of treatments were also higher where the P inclusion was higher. This finding is the same as studies done by Baradaran *et al*. (2013) who stated that a higher dietary P inclusion level during the first 7 days of age is beneficial to broilers due to the low and variable feed intakes. Another possible explanation for higher body weights at higher P inclusion levels is explained by Applegate & Angel, (2008) who emphasised the importance of P in metabolic pathways to attain maximum genetic potential. At any given P inclusion level at any age in this trial, there was a tendency for the body weight to decrease as the Ca inclusion level increased. Gautier *et al*. (2017) tested two different P inclusion levels, 0.45% and 0.30%, at a Ca inclusion range of 0.40% to 1.60%. At both P inclusion levels, the body weight of the chickens decreased as the Ca inclusion level increased. The chickens in the treatments with the higher P inclusion level had higher body weights (Gautier *et al*., 2017). In this particular study, the broilers that received the experimental diet with a Ca:P ratio of 2.50 had a 23.30 gram lower body weight than the broilers that received the diet in which the Ca:P ratio was 1.52 at the end of the trial. Ca is able to bind with amino acids, fats and P. This reduces the digestibility of amino acids and lowers the energy content of the diet which subsequently reduces broiler growth (Gautier *et al*., 2016). Ca also forms insoluble calcium-phytate bonds, rendering phytate unavailable to the action of enzymatic phytase and subsequently lowers the amount of P available to the bird (Hamdi *et al*., 2015). Phytase activity is pH dependent with the lowest activity observed at a higher pH. Limestone, which is the major source of Ca in poultry nutrition, has a high acid-binding capacity and may increase the pH in the proximal gastro-intestinal tract. This inhibits phytase activity and promotes the formation of calcium-phytate complexes (Adeola &Walk, 2013).

For a broiler to gain weight, feed intake must be as high as possible. Profitability of the broiler production system depends on how efficiently the broiler can convert feed into body weight. From the data obtained in this particular trial, it is evident that there was a difference in feed intake among the different treatments. One of the reasons for the variation in feed intake may be due to the difference in Ca and P inclusion levels. The chicks in treatments 1, 2, 3 and 4, which had a P inclusion level of 0.38%

had a feed intake of 42.90 grams lower than treatments 5, 6 and 7 where the P inclusion level was at 0.25%. A possible reason for this is explained by Wilkinson *et al*. (2012) who stated that broilers have an appetite for specific nutrients in the diet. Ca and P are nutrients that when deficient in the diet, broilers will up-regulate their intakes to meet their dietary requirements for the nutrients during the early stages of growth. Although there was a slight tendency for feed intake to decrease as Ca inclusion level increase at any P inclusion level, the correlation between feed intake and Ca or P inclusion level was non-significant ($P>0.05$) in this trial at any age. Thus, the variation seen in feed intake could have rather been influenced by external factors.

Although the feed intake of the chicks was 42.90 grams higher in treatments 5, 6 and 7, the chicks in treatments 1, 2, 3 and 4 gained 2.70 gram per day more. One reason for this may be due to the fact that the chicks in treatments 1, 2, 3 and 4 received a diet with a higher P inclusion level, thus lowering the Ca:P ratio. Less calcium-phytate complexes were formed and more P was available for absorption and metabolism. Bradbury *et al.* (2014) stated that the Ca:P ratio is more important than the absolute dietary concentration of either Ca or P and that P is more influential on performance metrics than total Ca. Exceeding the requirements of one of these minerals will cause an imbalance in the ratio (Guatier *et al*., 2017). During the grower phase, the birds in treatments 5, 6 and 7 had a higher daily body weight gain than the birds in treatments 1, 2, 3 and 4. This finding is in agreement with Powell *et al*. (2011) who stated that broilers that were fed low levels of P in the starter phase are better able to adapt and grow on low levels of P during the grower phase than those fed a higher P level during the starter phase.

The feed conversion ratio was 0.04 units lower in treatments 1, 2, 3 and 4, where a diet with a higher P level was fed, compared to treatment 5, 6 and 7. The reason for the higher feed conversion ratio in treatments 5, 6 and 7 is because the feed intakes were higher but the body weight gain was lower. There was a tendency for the feed conversion ratio to decrease as the Ca concentration of the diet increased due to the fact that the feed conversion ratio is influenced by the body weight gain and feed intake. The same reasons are applicable to the differences observed in the feed conversion ratio.

The chicks exposed to the diet of treatment 8 with a Ca inclusion level of 0.50% and a P inclusion level of 0.33% during the finisher phase performed the best in terms of body weight gain. This finding corresponds with the findings of Hamdi *et al*. (2015) and Driver *et al*. (2005) who found that a Ca inclusion level of 0.625% at a P inclusion level 0.45% performed the best in terms of body weight gain. According to the studies of Hamdi *et al*. (2015) higher Ca inclusion levels during the starter phase induced negative responses concerning body weight gain and feed intake. From this, it can be conducted that it is desirable to reduce the Ca inclusion level during the starter phase to improve weight gain and feed conversion ratio.

5.2 Bone characteristics

Calcium and phosphorus function in maintaining bone structure and integrity as bone is the major storage site of these two minerals. These two minerals functions in various other metabolic pathways in such a way that a deficiency of any of these two minerals will not only cause bone deformities but will result in reduced growth as well (Onyango *et al*., 2003). According to studies done by Onyango *et al*. (2003) there is an increase in the bone mineral content and thus bone ash percentage as the amount of calcium and phosphorus in the diet increase. Variations which exist in percentage bone ash, especially in the first 7 days of age, is a result of broiler breeder nutrition. The eggshell is the major calcium storage site for Ca and acts as a source of calcium for the developing embryo (Yair & Uni, 2011).

There was an increase in bone ash percentage as the amount of calcium in the diet increased at 35 days of age. The same was recorded at 14 days of age where the percentage tibia ash increased as the dietary calcium percentage increased from 0.70% to 1.00% in the starter phase. Early work done by Al-Masri (1995) showed that as the dietary calcium increased, the phosphorus retained in the tibia decreased due to the interaction between these two minerals. In correspondence with Al-Masri (1995), the tibia ash percentage was highest in the treatments where the phosphorus inclusion level was at 0.38% at 35 days of age. Ravindran *et al*. (1995) proved the same findings where the highest phosphorus levels at the highest calcium inclusion levels yielded the bones with the highest tibia ash percentage. Although a slight increase in bone ash percentage was seen as the Ca inclusion levels increased, the correlation between bone ash percentage and Ca inclusion levels at any P inclusion level was non-significant.

According to Gautier *et al*. (2017), as the dietary calcium increased the break force increased. Results from the present study also showed that there was an increase in the force required to break the bones as the calcium inclusion percentage increased, but the correlation was non-significant ($P>0.05$). Onyango *et al*. (2003) found that the shear force required to break the bones did not increase linearly or quadratically when the calcium and phosphorus inclusion levels were increased. Differences in results among studies may be due to variation in the time between bone removal from the bird and shear force analysis being done, freezing conditions, thawing time, defleshing procedure and crosshead speed (Rowland *et al*., 1968). These are all factors which contribute to variation in bone breaking strength values (Lott *et al*., 1980; Orban *et al*., 1993; Onyango *et al*., 2003).

Chapter 6

Conclusion

As broiler genetics have improved over the last few decades, the number of days required for a broiler to reach a desired body weight has decreased. More pressure is put on the skeletal system to account for the increased rate of muscle deposition. Therefore, Ca and P have become vitally important minerals in broiler nutrition firstly because of its function in bone mineralisation and secondly because of its effect on broiler performance. P plays an important role in broiler growth as it functions in energy metabolism and is prevalent in nucleic acids, nucleotides, phospholipids and phosphorylated proteins but excess dietary Ca will bind P in the gastro-intestinal tract of broilers, forming insoluble complexes, thereby rendering both P and Ca unavailable to the broiler. Research have shown that reducing the Ca inclusion levels in broiler diets will improve broiler performance without negatively affecting the skeletal integrity of broilers. Due to the lack of digestible values of Ca, nutritionists have cautiously over supplemented dietary Ca to prevent broiler morbidity which manifest itself through bone disorders. Evaluating the requirement of broilers for Ca and P are extremely challenging and will require continuous exploration.

The alternative hypothesis of this study was to show that lower levels of Ca and lower Ca:P ratios, below the current Aviagen recommendation for Ross 308 broilers, will improve broiler performance with no negative effect on skeletal health and bone integrity. It is evident from this study that performance parameters such as broiler growth, body weight gain, feed conversion ratio and performance efficiency factor were affected by lowering the dietary Ca inclusion levels. Body weight and body weight gain of the broilers that received the diet with the lowest Ca inclusion level and lowest Ca:P ratio was significantly higher during the starter phase with a significant correlation between the Ca:P ratio and body weight as well as Ca:P ratio and body weight gain. As the Ca:P ratio increased the body weight and body weight gain decreased, thus concluding that it is beneficial to feed a diet with a lower Ca inclusion level at a P inclusion level currently recommended by Aviagen for Ross 308 broilers during the early stages of broiler growth. Although significant differences were found for feed intake of broilers among treatment means, no significant correlation was found between feed intake and Ca inclusion level or Ca:P ratio. Nonetheless, the broilers which received the diets containing a lower P inclusion level had higher feed intake, concluding that the broilers increased feed intake to meet P requirements. Significant differences were found among treatment means for FCR at 35 days of age. The broilers which received the diets that had P inclusion levels at the current Avaigen recommendation for Ross 308 broilers had a lower feed conversion ratio than the broilers which had a lower P inclusion level and that the FCR increased as the Ca inclusion level increased at any given P inclusion level,

thereby concluding that FCR was improved by reducing the Ca inclusion levels. No significant differences were found among the treatment means of mortality. It can be concluded that the lower Ca inclusion levels did not affect mortality. Significant differences were found among the treatment means for PEF at 35 days of age where the broilers receiving the diets containing P inclusion levels at the current Aviagen recommendation for Ross 308 broilers had a higher PEF and that the PEF decreased as the Ca inclusion level increased, concluding that PEF was improved by reducing the Ca inclusion levels.

No significant correlation was found between bone breaking strength or bone ash percentage on 14 days and 35 days of age and Ca inclusion level in this particular study, although the results showed that bone ash percentage and bone breaking strength at 35 days of age was significantly lower in the treatment with the lowest Ca inclusion level. Thus, it can be concluded from the results in this trial that Ca inclusion levels can be reduced with caution and that Ca inclusion levels below the current Aviagen recommendation for Ross 308 broilers will not negatively affect bone breaking strength or bone ash percentage.

In conclusion, the best broiler performance in terms of body weight and feed conversion ratio were obtain from the treatments where the P inclusion level was at the current Aviagen recommendation for Ross 308 broilers and the Ca inclusion levels were reduced below the current Aviagen recommendation for Ross 308 broilers. Ca inclusion levels can be reduced by 20% below the current Aviagen recommendation for Ross 308 broilers without negatively affecting skeletal health and bone integrity while improving broiler performance.

Chapter 7

Critical Review and Recommendations

1. Research should be focussed on developing digestible calcium values as total calcium values are currently used. This lead to over estimation of calcium in broiler diets which influences broiler performance negatively and also increase formulation costs. By formulating on digestible calcium values, broiler requirement can be met more accurately and more space for protein and energy can be created in formulations.

2. Literature results from studies done in other countries on calcium and phosphorus inclusion levels had much lower calcium and phosphorus inclusion levels than what are currently used in South Africa. The difference may be in the solubility of calcium sources used among countries and research towards determining solubility values of the different calcium sources may allow for further reductions in calcium inclusion levels.

3. Water used in poultry production systems in South Africa is usually high in calcium and together with the dietary calcium inclusion levels may increase total calcium intake. This can contribute to reduced broiler performance by increasing digesta pH. Research can be conducted towards evaluating the influence of different water sources with different calcium content on broiler productivity.

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Appendix

Appendix 1. Temperature profile of the trial house from 2 days before placement to slaughtering at 35 days

Day	Target floor temperature (°C, 50 % rH[1])
-1 to 2	35.5
3 to 5	34.5
6 to 8	33.5
9 to 11	29.7
12 to 14	27.2
15 to 17	26.2
18 to 20	25.0
21 to 23	24.0
24 to 35	23.0

[1]rH=Relative Humidity

Appendix 2. Lighting program of the trial house from placement of the Ross broiler chicks to slaughter at 35 days of age

Day	Controller's set point			
	Lights on	Lights off	Hours of Daylight	Hours of Darkness
1 to 3	00:00	23:00	23	1
4 to 8	00:00	21:00	21	3
9 to 11	05:00	22:00	17	7
12 to 15	05:00	20:00	15	9
16 to 33	05:00	19:00	14	10
34 to 35	02:00	22:00	20	4

Appendix 3. Vaccination program (New Castle Disease and Infectious Bronchitis) of the Ross 308 broilers during the trail

Age (days)	Vaccination	Method	Trade name	Supplier
Hatchery	NCB[1]	Spray	Avinew	Merial South Africa (Pty) Ltd
Hatchery	IB[1]	Spray	Bioral H120	Merial South Africa (Pty) Ltd
10-12 days	NCB	Water	TAbic VH	Phibro Animal Health
10-12 days	IB	Water	TAbic MB	Phibro Animal Health
16-18 days	NCB	Water	Avinew	Merial South Africa (Pty) Ltd

[1]NCB=New Castle Disease; IB=Infectious Bronchitis

Appendix 4. Layout of the pens and blocks in the trail house with the random treatment allocations to each pen

Block 1	T5 T7 T4 T6 T2 T8 T1 T3	**Block 5**	T3 T6 T2 T8 T7 T1 T4 T5
Block 2	T6 T4 T1 T5 T2 T7 T3 T8	**Block 6**	T7 T3 T4 T6 T1 T5 T2 T8
Block 3	T7 T6 T2 T1 T4 T8 T5 T3	**Block 7**	T3 T1 T7 T2 T6 T8 T4 T5
Block 4	T3 T6 T4 T2 T1 T5 T8 T7	**Block 8**	T8 T4 T5 T6 T1 T3 T2 T7