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Effect of calcium and phosphorus specifications and limestone solubility on mineral utilisation and performance on modern broilers

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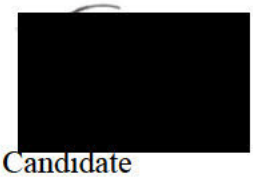

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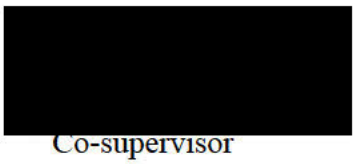

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Abstract

Modern broilers exhibit extremely rapid weight gain that needs to be supported by concomitant increases in the size and structural integrity of the skeleton. Calcium (Ca) and phosphorus (P) are vital minerals for metabolism, bone formation, and regeneration and in order to support the required rate of skeletal mineralization, an understanding of Ca and P requirements that matches the continuously changing genetic potential of modern broiler genetics is required. Poultry diets are formulated to total Ca (tCa) and either available P (AvP) or retained P (oP) using fixed estimates of the availability, or retention of dietary P. However, recent research has shown that characteristics of limestone included in diets such as the geology, purity, particle size and solubility speed can profoundly alter phytate P utilisation, Ca and P digestibility and phytase efficacy. These effects of limestone on Ca and P digestibility can therefore also potentially alter dietary recommendations for these nutrients. The study objectives were to assess the effects of three Ca and P recommendations (REC) on broiler performance, bone mineralisation and production costs, and mineral excretion and evaluate the effect of limestone solubility in broiler response to the Ca and P recommendations. The Ca and P REC were from a Dutch Nutrition Group (NL), University of Maryland (UMD), and Commercial (COM) following breed company recommendations. Further, the solubility profile of two commercially available limestones was identified, and these were utilised as either fast soluble (FS-LS) or slow soluble (SS-LS).

A commercial type of corn-soy diet was fed *ad libitum* to 3600 as-hatched Cobb-500-day-old chicks for 32d, which were randomly allocated to six treatments with twelve replicates and 50 birds/pen in 72 pens. The study had a 2x3 factorial design with treatments arranged in a completely randomized-blocking design. All diets of all phases were supplemented with 2000 FTU/kg feed of a 6-phytase. Performance parameters were recorded at placement and further at 7, 10, 14, 21, 28, and 32 days of broiler age. Five birds/pen were sampled for tibia ash at 10 and 32d. Litter sampling for mineral excretion, and foot pad (FPD) and hock burn (HB) was done at 32d. Each pen was regarded as an experimental unit. The data were analysed using JMP (V.15) (SAS Inst. Inc., Cary, NC, 2016) in a two-way ANOVA in a mixed model. Differences between means were analysed using a protected Tukey's test, and significant differences were reported at $P < 0.05$ level of significance. The FPD and HB data were analysed using a Rao-Scott Chi-square method.

The REC had significant effect on BW performance throughout the duration of the study. The lower Ca and P contained in the NL-REC depressed 7d body weight (BW) by 1.6% and 2.5% compared to the COM and UMD REC and by 2.6% and 2.8% compared to the UMD and COM REC at 32d, respectively ($P < 0.001$). Feeding Ca and P (UMD-REC) improved the FCR by 0.02 and 0.03

compared to the COM and NL REC at 7 and 32d, respectively ($P < 0.001$). Low dietary Ca and P (NL-REC) were detrimental to bone mineralisation at 10 and 32d, irrespective of LS solubility ($P < 0.05$). Dietary treatments influenced the incidence of FPD and HB. Increased dietary Ca and P (COM) promoted high P excretion in litter over 32d irrespective of LS solubility ($P < 0.001$). A cost saving of ZAR0.17 and ZAR0.21 cents can be made in feed cost/kg broiler harvested at 32d on the UMD REC compared to the COM and NL REC, respectively ($P < 0.05$). These results suggest that Ca and P REC, and LS used in commercial broiler diets can impact live performance, bone mineralisation and production costs.

Key words: bone mineralisation; broiler; limestone particle size; solubility, performance

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List of abbreviations

1,25-(OH)2-D3	1,25 dihydroxycholecalciferol / Vitamin D_3
AF	African Countries
AID	Apparent ileal digestibility
ANZ	Australia and New Zealand
AOAC	Association of Analytical Chemists
ATP	Adenosine triphosphate
AvP	Available phosphorus
BPP	B-Propeller phytase
BWG	Body weight gain
Ca	Calcium
$Ca(OH)_2$	Calcium hydroxide
$Ca_{10}(P)_4)_6OH)_2$	Hydroxyapatite
$CaCO_3$	Calcium carbonate
$CaMg(CO_3)_2$	Dolomite
COM	Commercial
CP	Cysteine phosphatase
Cu	Copper
CVB	Centraal Veevoederbureau
DCP	dicalcium phosphate
DFP	defluorinated phosphate
EU	European Union
FCR	Feed conversion ratio
Fe	Iron
FGF23	Growth factor 23
FI	Feed intake
FPD	Foot pad dermatitis
FS-LS	Fast soluble limestone
FTU/FYT	Phytase units
GDP	Gross domestic product
GLiM	Global lithological map
GMD	Geometric mean diameter
H_3PO_4	Phosphoric acid
HAP	Histidine acid phosphate
HB	Hock burn
HCL	Hydrochloric acid
Hz	Hertz
IP1	Inositol monophosphate
IP2	Inositol diphosphate
IP3	Inositol triphosphate
IP4	Inositol tetraphosphate
IP5	Inositol pentaphosphate
IP_6	Inositol hexakisphosphate

K	Potassium
Kg	Kilogram
LS	Limestone
MCP	Monocalcium phosphate
MDCP	Monodicalcium phosphate
ME	Middle East
Mg	Magnesium
mL	Millilitre
Mn	Manganese
Mt	Metric tonne
Na	Sodium
NA	North America
NC	Negative control
NL	Dutch nutrition group
NLR	Neuro livestock research
nPP	Non-phytate phosphorus
oP	Digestible calcium
P	Phosphorus
PAP	Purple acid phosphatase
PC	Positive control
pH	Power of hydrogen
PSD	Particle size distribution
PTH	Parathyroid hormone
REC	Recommendations
RU	Russia
SA	South America
Se	Selenium
SFA	Substance flow analysis
SS-LS	Slow soluble limestone
t	Tonne
tCa	Total calcium
TCP	Tricalcium phosphate
tP	Total phosphorus
Trt	Treatment
UMD	University of Maryland
US	United States
USGS	United State Geological Survey
UV	Ultraviolet rays
UVB	Ultraviolet B rays
VDR	Vitamin D receptor
ZAR	South African Rand
Zn	Zinc

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Chapter 1

General background

For the past 60 years, broiler production has been focused on optimising feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR). Rapid growth rate has rendered broilers susceptible to leg weakness (Beane *et al.*, 1962; Wideman Jr *et al.*, 2013), and often display high incidence of leg culls due to lameness and poor bone mineralisation. In addition, fast growing modern broilers with high live weights may also suffer from leg deformities and skeletal disorders primarily due to inadequate dietary mineral provision and utilisation, namely calcium (Ca) and phosphorus (P) for bone mineralisation (Williams *et al.*, 2000). It has been 30 years since the National Research Council (NRC, 1994) last gave recommendations for dietary Ca and P in poultry. During this time, broiler genetics has advanced dramatically; meaning that dietary Ca and P recommendations may not be adequate to meet the demands of the modern broiler. Current breeder standard Ca and P recommendations are lower than what was recommended by the (NRC, 1994). Which means the modern broiler Ca and P requirements might be met with lower specifications compared to the ones prescribed in the past.

Calcium is essential for bone mineralisation, formation and development, growth, and bone regeneration (Fallah *et al.*, 2018). A deficiency in Ca hinders skeletal development, growth and regeneration, therefore; promoting the severity of leg disorders (Proszkowiec-Weglarz *et al.*, 2013). Contrarily, high dietary Ca concentrations can reduce the availability of the mineral and, antagonize the uptake and utilisation of P (Sommerfeld *et al.*, 2018), as a consequence of the formation of insoluble Ca-phytate complexes (Angel *et al.*, 2002), which reduces P availability in the intestinal lumen (Plumstead *et al.*, 2008).

Phosphorus is an essential mineral that has vital functions in nucleic acid synthesis, as the energy currency of the cell, signal transduction, metabolism, and bone mineralisation (Berndt *et al.*, 2009; Lin *et al.*, 2017). A P deficiency could result in porous cortical bones, skeletal abnormalities, tibial dyschondroplasia, poor performance, and high mortalities (Dinev, 2012; Shim *et al.*, 2012; Webster, 2004). Additionally, sound nutritional strategies are essential to reduce P excretion in litter, and minimising eutrophication. One economical strategy to reduce the cost of production is to exclude the use of any form of inorganic P source in finisher phase diets (Ribeiro *et al.*, 2019). This is because finishing broilers have high FI therefore finisher diets would require high supplemental quantities of

P than earlier phases of production, also because high FI would contribute to high P excretion (Rousseau *et al.*, 2012).

Differences amongst the available Ca and P recommendations in the literature, breeder companies, and the NRC reveal a contrariety regarding the recommendations necessary to meet the modern broiler requirements for the minerals. The Ca and P recommendations by the NRC (1994) proposes that broilers at different ages be fed 1.00% Ca and 0.45% nPP (non-phytate phosphorus) (0-3 weeks), 0.90% Ca and 0.35% nPP (3-6 weeks), and 0.80% Ca and 0.30% nPP (6-8 weeks) (NRC, 1994). Based on the nutritional evaluation, the Centraal Veevoederbureau (CVB) recommends feeding 0.88-0.92% Ca and 0.40% oP (digestible phosphorus) (0-10d), 0.68-0.71% Ca and 0.31% oP (10-31d), 0.62-0.64% Ca and 0.28% oP (30-40d), and 0.59-0.62% Ca and 0.27% oP (40-50d) (CVB, 2018). Contrarily, breeder companies such as Hubbard recommend 0.98% Ca and 0.48% AvP (0-10d), 0.88% Ca and 0.45% AvP (10-22d), and 0.85% Ca and 0.42% AvP (22d to end) (Hubbard, 2014). Cobb-Vantress recommends feeding 0.90% Ca and 0.45% AvP (0-8d), 0.84% Ca and 0.42% AvP (9-18d), 0.76% Ca and 0.37% AvP (19-28d), and 0.76% Ca and 0.38% AvP (>29d) (Cobb500, 2018). Aviagen recommends feeding 0.96% Ca and 0.48% AvP (0-10d), 0.87% Ca and 0.43% AvP (11-24d), and 0.81% Ca and 0.40% AvP (>25d) (Aviagen, 2019). Multiple Ca and P recommendations have been reported in literature in over half a century. Twining *et al.*, (1965) published that for optimum broiler performance, it would be advisable to feed 0.80% Ca and 0.60% tP (0-4 weeks), and 0.70-0.80% Ca and 0.50% tP (6-8 weeks). Delezie *et al.*, (2012) reported that the Aviagen nutrient recommendations of 2009 can be lowered, where reducing dietary Ca by 15-20% and tP by 20-30% across all phases of growth can have no detrimental effects of broiler performance; the study found positive response when reducing these recommendations 0.90% Ca and 0.67% tP (starter, 1-13d), 0.82% Ca and 0.62% tP (grower, 13-26d), and 0.74% Ca and 0.54% tP (finisher, 26-39d). Ceylan *et al.*, (2020) reported optimised broiler performance when feeding 0.90% Ca and 0.45% nPP (starter, 0-10d), 0.75% Ca and 0.38% nPP (grower, 11-24d), and 0.60% Ca and 0.30% nPP (finisher, 25-41d) recommendations, which were reduced by 20% for both Ca and nPP compared to the Aviagen 2014 and 2019 recommendations. In a 21d trial, Kop-Bozbay *et al.*, (2021) fed starter diets reduced by 10% in both Ca and AvP compared to the commercial specifications of 0.96% Ca and 0.45% AvP, these recommendations 0.85% Ca and 0.42% AvP, 0.77% Ca and 0.38% AvP, 0.68% Ca and 0.34% AvP, and 0.61% Ca and 0.31% did not negatively affect final BW performance. Due to these discrepancies in having a consensus in Ca and P recommendations, there is a need to revise the Ca and P specifications to meet the broiler needs at different growth phases.

Limestone (LS) solubility due to particle size has been shown to alter Ca and P digestibility. Kim *et al.*, (2019) showed that fast soluble limestone (FS-LS) compared to slow soluble limestone (SS-LS) hydrolyses faster in the proventriculus and gizzard, which results in the formation of soluble Ca-phytate complexes, resulting in increased chelation of ionizable Ca and a decrease in P digestibility. In a study with laying hens, Zhang *et al.*, (1997a), showed that large particle-sized limestone has a longer retention time in the gizzard and using large particle limestone at low dietary Ca level increased *in vivo* solubility. Slow solubilising limestone with large particle size has been shown to improve broiler performance compared to fast solubilising limestone, showing performance benefits at low Ca concentrations (Lee *et al.*, 2021). Majeed *et al.*, (2020) reported significantly higher body weight gain (BWG) in broilers fed coarse (slow solubilising) limestone (1724g, 0-28d) compared to broilers fed fine (fast solubilising) limestone (1675g, 0-28d) at low Ca (0.72%, 0.58%; starter and grower) and AvP (0.30%, 0.235; starter and grower) concentrations, but the particle size effect was not significant between 0-35d. However, the study showed that fine limestone results in significantly greater BWG when fed in conjunction with high Ca (0.90%, 0.76%; starter and grower) and AvP (0.45%, 0.38%, starter and grower) levels compared to coarse limestone.

The lack of consensus on the birds Ca and P requirements in the published literature hinders the ability of the nutritionist from formulating and feeding Ca and P to meet the birds' requirements, which can have welfare, environmental and economic implications. Furthermore, the impact of limestone characteristics on the availability and digestibility of Ca and P is a further consideration that can alter the response of broilers to Ca and P recommendations. Therefore, the aim of the current study was to evaluate the effects of three dietary Ca and P recommendations and two limestone solubilities on productive performance, bone mineralisation, and Ca and P excretion in litter. The objectives of the study was to determine the individual and interactive effects of feeding three dietary Ca and P recommendations, and the recommendations with either fast or slow solubilising limestone on performance (BW, FI, mortality, leg culls) at pre-starter (0d – 8d), starter (9d – 13d), grower (14d – 22d), and finisher (23d – 32d); tibia ash at 10d and 32d; Ca and P excretion on litter at 32d; hock burn and food pad lesions at 32d; and quantify the cost of feeding a 32d broiler. Hence the study evaluated the following hypothesis.

H0: The Ca and P recommendation will not have an effect on broiler performance, bone mineralisation, and mineral excretion.

H1: The Ca and P recommendations will have an effect on broiler performance, bone mineralisation, and mineral excretion.

H0: Limestone solubility will not have an effect on broiler performance, bone mineralisation, and mineral excretion.

H1: Limestone solubility will have an effect on broiler performance, bone mineralisation, and mineral excretion.

Chapter 2

Literature Review

2.1 Introduction

Modern broiler genetics has advanced in increasing growth rates, which consequently led to broilers being produced in shorter production cycles. However, rapid growth rates are also often associated with a high incidence of leg abnormalities and sub-optimal bone mineralisation. Calcium is essential for bone mineralisation, formation and development, growth, and regeneration (Fallah *et al.*, 2018). A deficiency in Ca promotes the severity of leg disorders (Proszkowiec-Weglarz *et al.*, 2013). Phosphorus is vital in nucleic acid synthesis, as an energy currency of the cell, signal transduction, metabolism, and bone mineralisation (Lin *et al.*, 2017); a deficiency could result in porous cortical bones, skeletal abnormalities, tibial dyschondroplasia, rickets, poor performance, and high mortalities (Dinev, 2012; Shao *et al.*, 2019; Shim *et al.*, 2012). Jointly through nucleation of the matrix vesicles, Ca and P are stored in the skeletal structure as hydroxyapatite ($(Ca_{10}(P)_4)_6(OH)_2$) with an abundance of 99% Ca and 80% P (Veum, 2010). Sound nutritional strategies are essential for efficient Ca and P utilisation, reducing P excretion in the litter minimizing eutrophication.

Despite the importance of these minerals and possible antagonistic effects, there is variability in published Ca and P recommendations, both by the (CVB, 2019) and the (NRC, 1994), in the literature, and by breeder companies (Aviagen, 2022; Cobb500, 2022; Hubbard, 2014). This range of recommendations could result in an erroneous supply of the minerals with severe consequences, such as poor performance, poor bone mineralisation, and increased mineral excretion (Hamdi *et al.*, 2015; Rousseau *et al.*, 2016; Valable *et al.*, 2018). The inability to meet a consensus on the birds' Ca and P requirements hinders nutritionists from formulating and feeding Ca and P recommendations to meet the birds' requirements.

The birds' ability to digest Ca in limestone depends on particle size and solubility speed (Kim *et al.*, 2019), therefore potentiating the capability to alter the dietary levels needed to meet the broiler requirements for the mineral. Reports in the literature have demonstrated the possibility of improving broiler performance when feeding below breeder standard Ca and P and with a slow solubilising limestone. Hence reducing dietary Ca concomitantly with dietary P can improve broiler performance and bone mineralisation. This chapter aims to review the impact of Ca and P recommendations and limestone solubility on broiler performance, bone mineralization, and mineral excretion.

2.2 Calcium and phosphorus defined

Calcium (Ca) is an essential macro mineral, and apart from being abundant in the body, it is cardinal for bone mineralisation, formation and development, growth, and regeneration (Fallah *et al.*, 2018); also for muscle contractions, signal transduction, and blood coagulation (Bradbury *et al.*, 2002). About 99% of Ca is deposited in the skeletal structure to form hydroxyapatite ($((Ca_{10}(P)_4)_6(OH)_2$), while the remainder plays a crucial role in metabolism and enzyme activation (Veum, 2010). A deficiency in Ca hinders skeletal development, growth, and regeneration, therefore; promoting the irreversible leg disorders (Proszkowiec-Weglarz *et al.*, 2013). In contrast, high dietary Ca concentrations can impair the availability of the mineral, as well as the uptake and utilisation of P (Sommerfeld *et al.*, 2018) because of the formation of Ca insoluble complexes with phytate (Angel *et al.*, 2002), as well as inorganic phosphate in the intestinal lumen (Plumstead *et al.*, 2008). This is often perpetuated by using limestone as a carrier in premixes resulting in excess supplementation of Ca in poultry diets (Kim *et al.*, 2018).

About 85% of the total body P is stored in the bones as hydroxyapatite. It has vital functions in nucleic acid synthesis, as an energy currency of the cell, for signal transduction, metabolism, and bone mineralisation (Berndt *et al.*, 2009; Lin *et al.*, 2017). A P deficiency could result in porous cortical bones, skeletal abnormalities, tibial dyschondroplasia, poor performance, and high mortalities (Dinev, 2012; Shim *et al.*, 2012; Webster, 2004). Asimov, (1975), the author of "Asimov on Chemistry", mentioned that we might be able to substitute nuclear power for coal or plastic for wood, but we neither can substitute nor replace P; hence he said, "Life can multiply until all the P is gone and then there is an inexorable halt which nothing can prevent". Therefore, sound nutritional strategies for effective use of P as a finite resource and to reduce P excretion in litter and minimize eutrophication are essential.

Modulation and utilisation of Ca and P depend on the relative amount of each in a diet. However, the availability of each mineral for their respective metabolic functions also depends on intestinal absorption, and endogenous loses, glomerular filtration and renal reabsorption, and the rate of transfer from the blood to the bone (Van der Klis *et al.*, 1996; Vitti *et al.*, 2010). The efficiencies of these processes are regulated by the endocrine system, which modulates various hormones, primarily the parathyroid hormone (PTH) and calcitriol (the hormonal form of vitamin D3 or 1,25 dihydroxycholecalciferol) (Vitti *et al.*, 2010). The control of P metabolism prior to P homeostasis differs from Ca metabolism and homeostasis, with P being more regulated in the kidneys than in the gastrointestinal tract (Fleet, 2017; Vitti *et al.*, 2010).

Definition of terminology

It is necessary to explain terminologies before summarising the Ca and P requirements because it is essential to understand the different forms and quantisation of the minerals despite the system being used in feed formulation. Organic phosphorus refers to P obtained from all plant sources used as feed ingredient, while inorganic phosphorus (iP) refers to the P obtained from supplemental sources such as MCP and MDCP which undergo processing and lack carbon atoms. Total phosphorus (tP) or commonly abbreviated as P can be referred to as all form of P (Angel, 2011), which would be organic and/or inorganic. While available P (avP/aP) can be defined as the P both organic and inorganic in the diet available to the animal for absorption. However, it is worth mentioning that the level of P in feed ingredients used in feed formulation can be variable (Nelson *et al.*, 1968). Phytin is dominantly found in seeds used as ingredients in diets (Ravindran, 1995), and its role is to preserve P resulting in PP (phytate phosphorus). While nPP (non-phytate phosphorus) is P which is not chelated to phytate or phytin, and this can be quantified by subtracting analysed PP from the overall analysed P (Angel, 2013). While dP/oP is digestible P which is the portion of P which is digested and absorbed by the animal (Dilger *et al.*, 2006).

2.2.1 Vitamin D biosynthesis

The effect of vitamin D on the regulation and uptake of Ca in birds has been studied by numerous researchers (Proszkowiec-Weglarz *et al.*, 2013; Shanmugasundaram *et al.*, 2012). Vitamin D is vital for Ca and P absorption and homeostasis, but it needs activation to its hormonal form (calcitriol) post ingestion as vitamin D or from 7-dehydrocholesterol isomerization post-skin exposure to ultraviolet B rays (Fleet, 2017).

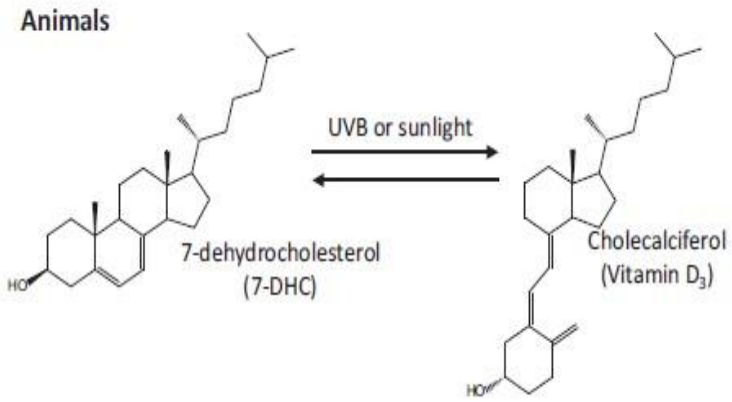

Figure 2.1. Thermal isomerization of vitamin D precursors (adapted from (Warren *et al.*, 2021)

Cholecalciferol (vitamin D_3) is synthesised de novo in animals through thermal isomerization of 7-dehydrocholesterol in a non-enzymatic process for animals with direct ultraviolet violet (UV) exposure (Figure 2.1), or it can be supplemented in the diet. When provided through dietary means, vitamin D_3 is transported from the intestinal lumen into the blood to the liver by the chylomicrons and vitamin D_3 Binding Protein (Jäpelt *et al.*, 2013). The amount of vitamin D_3 available for hydroxylation in the liver might be low depending on the availability of fats and bile salts in the intestinal lumen; as it is a fat-soluble vitamin (Dawson-Hughes *et al.*, 2015). Once vitamin D_3 is in the liver, its hydroxylation is facilitated by the enzyme 25-hydroxylase at C-25, resulting in Calcifediol ($25OHD_3$), the major circulating form of vitamin D_3 in vertebrates (Figure 2.2). In the kidneys, $25OHD_3$ is hydroxylated at the α-point at C-1 by the enzyme 1α-hydroxylase resulting in the active hormonal form metabolite 1α,25-dihydroxycholecaliceferol ($1,25-(OH)_2-D_3$) (calcitriol) (Figure 2.2). The hydroxylation of calcifediol is stringently controlled by circulating serum Ca and P levels and parathyroid hormone (PTH) activity (Prosser *et al.*, 2004).

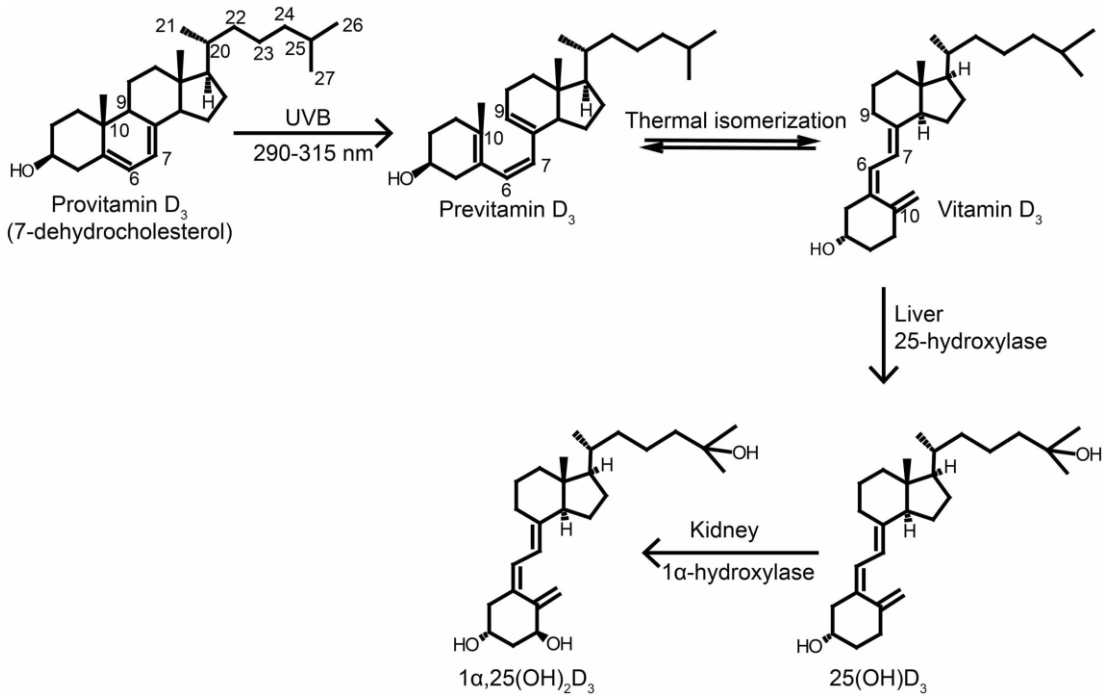

Figure 2.2. Biosynthesis of calcitriol through the hydroxylation of vitamin D_3 (schematic presentation by (Jäpelt *et al.*, 2013))

2.2.2 Calcium and Phosphorus metabolism

The metabolism of Ca and P is highly regulated to ensure that the biological demands for Ca and P are met. Calcium metabolism is controlled in response to plasma levels of ionised Ca, regulated by PTH, calcitonin, and calcitriol through a feedback mechanism involving receptors in the small intestines, the bone, and the kidneys (Veum, 2010). The absorption of Ca in the mucosa of the small intestines occurs by passive diffusion (paracellular) or by active transport (transcellular) mechanisms (Bronner, 2009; Veum, 2010). The absorption of Ca depends on the vitamin D_3 receptor (VDR) activated by calcitriol (Gallagher, 2013; Veum, 2010; Whiting, 2010), and this transcellular mechanism upregulates the absorption of Ca on low or moderate Ca intake (Ross *et al.*, 2011).

Phosphorus metabolism differs from that of Ca because it is highly controlled in the kidney (Li *et al.*, 2016). A reduction in serum Ca signals for upregulated secretion of PTH by the parathyroid glands, which results in bone resorption and renal Ca reabsorption, stabilising the Ca levels but upregulating P renal excretion (Figure 2.3). Also, in the kidney, PTH upregulates the synthesis of calcitriol through signals from *CPY27B1* (cytochrome P450) protein which activates 1α-hydrolase resulting in $25OHD_3$ hydroxylation (Hurst *et al.*, 2020), which therefore upregulates Ca^{2+} absorption (Figure 2.3). Furthermore, a negative feedback mechanism ensures homeostasis by reducing plasma PTH concertation, and should hypercalcaemia occur, the parathyroid gland secretes the hormone calcitonin, which inhibits bone resorption. When phosphate concentrations are high, the Growth Factor 23 (FGF23) hormone is secreted from the bones to reduce renal reabsorption of P and renal synthesis of calcitriol, resulting in P balance.

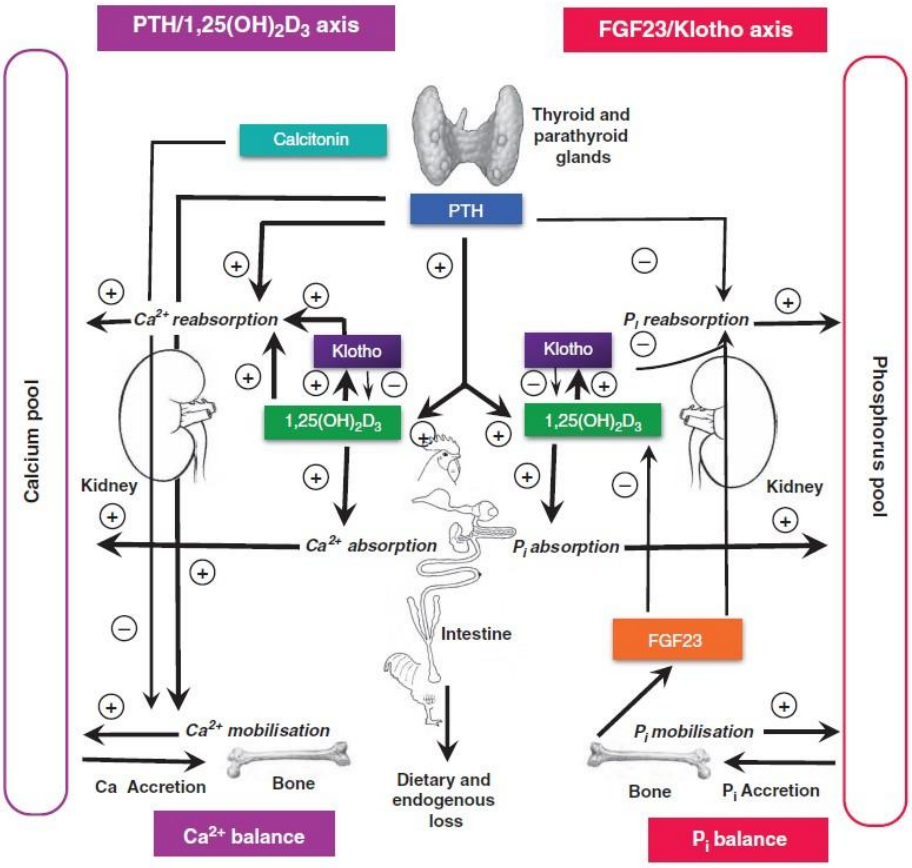

Figure 2.3. Coordinated regulatory mechanism of the intestines, kidneys and bone in Ca and P modulation and homeostasis (source: (Li *et al.*, 2017)

2.2.3 Calcium and phosphorus interactions

Calcium source has been shown to impact performance and birds' liveability. A study by Paiva *et al.*, (2013) found a significantly high mortality rate when the birds were fed 0.9% Ca from a highly soluble calcified marine seaweed compared to feeding 0.9% and 0.6% Ca from limestone. As a divalent cation, Ca has a low affinity for phytate, meaning chelating with phytates would occur at a low rate compared to other minerals. Because Ca is added in high amounts in broiler diets, it is more available compared to other minerals, therefore allowing it to chelate with phytates. The deleterious effects of Ca resulting in low performance may be explained by the capability of Ca to form insoluble-phytate complexes reducing Ca absorption and P utilisation due to precipitates of Ca-phosphates (Plumstead *et al.*, 2008; Walk *et al.*, 2012). Also, low performance can be attributable to inadequate dietary P provision, in which protein synthesis is taxed by inadequate Adenosine triphosphate (ATP) synthesis to promote body mass accretion. Further, Ca has also been shown to form insoluble soaps

with fatty acids in the intestinal lumen, reducing the energy derived from lipids (Stroebinger *et al.*, 2021).

2.3.2 Bone formation and the role of Ca and P on skeletal mineralization

An essential part of bone growth is played by the epiphyseal growth plate. Two opposing processes govern the thickness of the growth plate: chondrocyte death and vascular invasion of the growth plate followed by conversion into primary bone spongiosa on the one hand, and chondrocyte proliferation and hypertrophy on the other; in which Ca and P deficiency can ward off mineralization of the growth plate (Hunter *et al.*, 1991).

Endochondral bone formation of the tibia and femur requires angiogenesis which is mediated by a variety of cells and factors involving signals generated by either endochondral or intramembranous ossification, and osteogenic cells are responsible for osteogenesis for bone formation (Ribatti *et al.*, 2023). In young growing animals or adult mammals with persisting growth plates, endochondral bone formation proceeds from growth plate chondrocyte proliferation, maturation, and hypertrophy to the mineralization of cartilaginous matrix to form an osseous tissue (Wongdee *et al.*, 2012). Because angiogenesis and osteogenesis are closely related, their interaction is necessary to ensure the production of new bone and to preserve bone homeostasis.

Unlike most other tissues, the bone is a unique type of connective tissue that has undergone physiological mineralisation. The components of a bone include the marrow space, the mineralized cortical and trabecular bone structures, the calcified cartilage of the growth plate (during skeletal growth only), and cartilaginous joints (Bilezikian *et al.*, 2008; Burr *et al.*, 2019). This main ossification core forms at the centre of each growing bone in the diaphysis, similar mechanisms cause secondary ossification centres to form in the epiphyses a little later, in which the epiphyseal plate divides the primary and secondary ossification centres (Breeland *et al.*, 2022).

Calcium phosphate is a solid mineral abundant within the skeletal tissue, and it is crucial to understand that the Ca phosphate mineral phase is deposited in live tissue and that the activity of living cells constantly synthesises, resorbs, and replaces this substance which results in a highly crystallised hydroxyapatite structure (Glimcher, 2006). Calcium phosphate has been identified within the mitochondrial granules of the osteoblast and intracellular vesicles, which transport the material to the extracellular matrix (Boonrungsiman *et al.*, 2012). The apatite crystals grow on the collagenous extracellular matrix secreted by osteoblasts, forming the nano-composite structure of bone. The inorganic mineral matrix called hydroxyapatite is responsible for the maintenance and regeneration of the skeletal structure, in which ionised Ca post digestion and absorption becomes bound to

coagulation proteins which, through blood circulation, reach the bone matrix, therefore depositing the needed Ca to the hydroxyapatite for bone formation (Talmage *et al.*, 2003). During the mineralisation of the extracellular matrix, hydroxyapatite production depends on two primary ionic components, one of which is inorganic P (Magne *et al.*, 2003). The majority of the phosphate in the skeletal structure is complexed with Ca as hydroxyapatite crystals; the residual phosphate manifests as amorphous Ca phosphate (Farrow *et al.*, 2010).

2.3 Requirements for calcium and phosphorus

2.3.1 Age and sex

Sexual dimorphism has been reported in broilers of different ages within a strain (Cygan-Szczegielniak *et al.*, 2021). As early as the embryonical stage, differences between male and female broilers have been identified, which influence post-hatch performance (Henry *et al.*, 1998). Figure 2.4 as presented by Gous *et al.*, (1999), shows the growth rate and potential of male and female strains overtime. This clearly shows the need for dietary changes in nutrients provided and, on the Ca and P provision to meet the needs for growth and bone mineralisation at different stages of life. Fernandes *et al.*, (2013) reported the impact of sex differences in performance amongst different strains. Similarly, Trocino *et al.*, (2015) reported higher BW performance in males compared to females, irrespective of the genotype at 22 and 46 days of age.

Dhandu *et al.*, (2003) identifying male broiler nPP requirements, fed (1% Ca, 0.43% nPP) 0 - 18d, (0.81% Ca, 0.31% nPP) 18 - 32d, and (0.71% Ca, 0.25% nPP) 32 - 42d, which were below the NRC (1994) recommendations. The Ca levels were progressively reduced and could be one reason for lower nPP requirement compared to the NRC (1994) recommended levels. The author reported that for male broiler weighing between 2300g and 3000g would require 0.16 +/-0.02% nPP. Experimental data showing female broiler requirements for Ca and P is scarce, as most studies use male broilers. But Mello *et al.*, (2012) reported that female broilers would need 5.12g/kg Ca and 2.56g/kg AvP at finisher phase.

A study by Valable *et al.*, (2018) investigated the effect of Ca and P deficiency and recovery in a 37d period using male broilers which were fed a starter diet meeting the NRC Ca and P recommendation from 0-10d. The authors reported that the Ca and P recommendations can be lowered without detrimental effects on broiler performance. The fed a grower control diet (C) (0.90% Ca, 0.39% nPP), medium level diet (M) (0.71% Ca, 0.35% nPP) or a low Ca and nPP diet (0.60% Ca, 0.30% nPP) from 11d – 21d; and a finisher diet C, M or L containing, respectively, 0.85%, 0.57% or 0.48% Ca and 0.35%, 0.29% or 0.24% nPP from 22d – 37d. Also, findings by authors who reared

broiler chicks as hatched (Ceylan *et al.*, 2020), and mixing equal males and females per treatment (Kop-Bozbay *et al.*, 2021,) reported that lower dietary Ca and P that what the breeders and the NRC recommended had no detrimental effects on broiler performance.

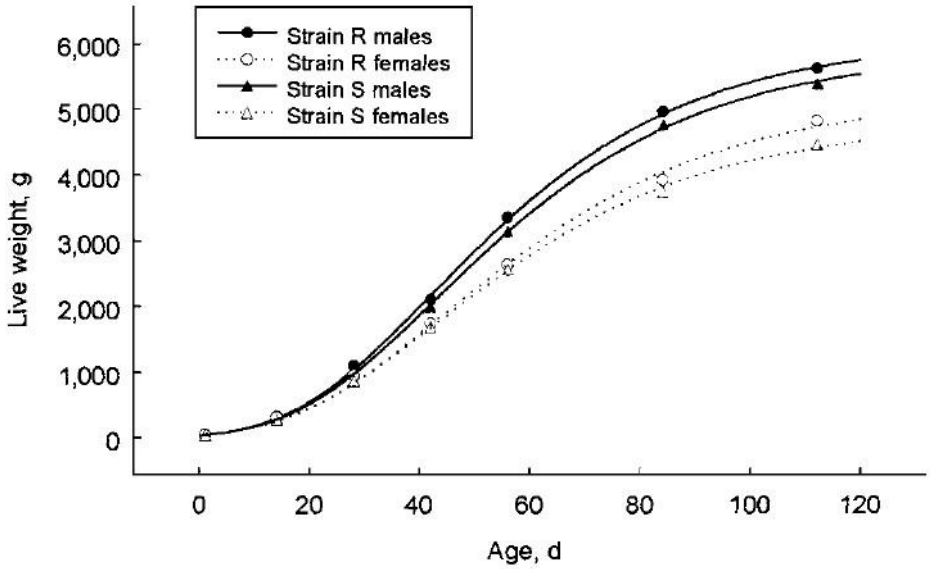

Figure 2.4. Growth curve showing the live weight of two strain crosses of males and females from 0-120 days (source (Gous *et al.*, 1999)

2.3.2 Dietary calcium and phosphorus recommendations by age

Multiple studies have been conducted at different broiler ages to determine the suitable Ca and P dietary recommendations. Over the years, research has been undertaken in an attempt to determine the Ca and P recommendations appropriate to obtain optimal broiler performance, bone mineralisation, and mineral digestibility while avoiding either an excess or deficiency of these minerals (Adeola *et al.*, 2013; Angel, 2011; Anwar *et al.*, 2016; Cowieson *et al.*, 2020; Li. *et al.*, 2021; Plumstead *et al.*, 2008). A consensus on the broiler Ca and P recommendations is yet to be reached; hence differences on the Ca and P recommendations can be noted based on different sources **(**Table 2.1).

Table 2.1 Calcium and Phosphorus recommendations grouped by phase from starter to finisher as recommended by different organisations

Phase	Period	Recommendations[1]	Calcium (%)	Available Phosphorus (%)
Starter	0 – 10 days	Aviagen	0.96	0.48
	0 – 8 days	Cobb	0.90	0.45
	0 – 10 days	Hubbard	0.98	0.48
	0 – 10 days	CVB	0.88-0.92	(oP)[2],0.40
	0 – 3 weeks	NRC	1.00	(nPP)[3],0.45
Grower	11 – 24 days	Aviagen	0.87	0.44
	9 – 18 days	Cobb	0.84	0.42
	10 – 22 days	Hubbard	0.88	0.45
	10 – 30 days	CVB	0.68	(oP)[2],0.31
	3 – 6 weeks	NRC	0.90	(nPP)[3],0.35
Finisher	25 – market	Aviagen	0.79	0.40
	19 – market	Cobb	0.76	0.38
	22 – market	Hubbard	0.85	0.42
	30 – 40 days	CVB	0.62-0.64	(oP)[2],0.28
	6 – 8 weeks	NRC	0.80	(nPP)[3],0.30

[1]Recommendations: Aviagen ref; Cobb; Hubbard; CVB; and NRC.
[2]Phosphorus system: (oP) Digestible phosphorus
[3]Phosphorus system: (nPP) non-phytate phosphorus

Differences in BW have been noted when feeding varying levels of Ca and P due to the antagonistic effect Ca has on P utilisation (Rao *et al.*, 2006). The study showed that feeding 9g/kg Ca in a diet containing 3g/kg of nPP, at 14,28, and 42 days of age broilers showed reduced performance in BWG and FI. The study further showed that increasing dietary nPP levels to 3.5g/kg and above alleviated the depressed performance, and improved performance could be achieved when dietary Ca and P are adjusted concomitantly. Different researchers have demonstrated that a reduction in dietary Ca by 10 to 15% , and AvP by 20 to 25% compared to the breeder Ca and P recommendations (Aviagen, 2019; Cobb500, 2018) had no deleterious effects on performance, especially when done concomitantly with P (Akter *et al.*, 2016; Delezie *et al.*, 2012; Gautier *et al.*, 2017).

A study evaluating the growth performance amongst other parameters reported on the effects of two Ca levels (adjusted to meet a 2:1 ratio or variable Ca levels relative to the dietary AvP levels), and six graduated levels of AvP (0.13%, 0.23%, 0.33%, 0.43%, 0.53%, and 0.63%) in male broilers from 8 to 21 days of age (Díaz-Alonso *et al.*, 2019). The male broilers fed adjusted Ca: AvP (0.26% Ca: 0.13% AvP, and 0.46% Ca: 0.23% AvP) showed a higher FI than broilers fed the variable AvP levels in the study. Therefore, indicating that FI can be maintained with lower AvP levels, provided dietary Ca remains sufficient for better P utilisation. Feeding variable levels of AvP between 0.13% to 0.33% were detrimental to weight gain, while feeding adjusted Ca: AvP levels yielded significantly

higher weight gain when dietary AvP levels were at 0.43%. Fallah *et al.*, (2018) investigated the Ca requirements of male broilers when fed seven levels of dietary Ca levels (0.40%, 0.55%, 0.70%, 0.85%, 1.0%, 1.15% and 1.30%) at a constant nPP (0.40%) from two sources of Ca from 1-21 days of age. The authors concluded that performance was significantly influenced by dietary calcium concentration rather than calcium source, where performance worsened when dietary Ca levels exceeded 0.85% at a fixed nPP level of 0.40%.

Two experiments were conducted investigating the Ca requirements of male and female broilers between 19-42 (study one), and 0-16 (study two) days of age feeding six levels of Ca 0.325%, 0.4%, 0.475%,0.55%, 0.625%, and 0.9%) with 0.45% nPP (Driver *et al.*, 2005). In the first study there were no significant differences in performance due to the different Ca levels. But for the second study the authors reported that feeding up to 0.625% Ca with 0.45% nPP resulted in an increase in BWG and an improvement in FCR in both female and male broilers between 0 and 16 days of age. In this study it was found that male broilers between 0-16d have a lower Ca requirement (0.49%) than females who exhibited a high Ca requirement of 0.62%, all being fed 0.45% nPP. Therefore, the NRC recommendation for 1.0% Ca in broiler starter diets might be more than what young broilers need for performance, hence performance was optimized when feeding up to 0.625% Ca.

The NRC (1994) recommendation of 0.90% Ca for the grower phase, seems to be exceeding what the broilers need for optimal FCR and BWG performance. Mello *et al.*, (2012) investigated the requirements for AvP fed in increments of 0.7g/kg for female broilers aged between 1-46d. The study found that the AvP and Ca required for female broilers to optimize performance were 4.59 and 9.18 g/kg between 1-10d; 3.88 and 7.66 g/kg between 11-21d; 3.58 and 7.16 g/kg between 22-33d; and 2.56 and 5.12 g/kg between 34-46d. Also, Kop-Bozbay *et al.*, (2021) reported that male and female broiler Ca and P recommendations suitable to optimize performance do not significantly differ by sex, but it is within the phase of growth where these recommendations need evaluation and these have been reported to be lower than what values reported by the NRC (1994). Also, Kop-Bozbay *et al.*, (2021) reported improved feed efficiency in broiler fed dietary Ca and P below the commercial recommendations from starter to finisher phase, further issues of leg abnormalities were not noted due to a reduction in dietary Ca and P recommendations.

Akter *et al.*, (2016) showed that increasing Ca from 0.60% to 1.0% at a low while feeding low nPP of 0.30% depressed broiler FI and BWG between d1-d24. But increasing nPP from 0.30% to 0.40% showed an improvement in FI and BWG. Furthermore, the study showed that reducing dietary Ca to 0.80% while feeding nPP at 0.30% increased FI by 244 g. In addition, feeding 0.40% nPP with 0.80% Ca resulted in high FI (1624 g) between d1-d24 compared to when feeding 0.40% nPP with

either 0.60% or 1.0% Ca. The authors also reported a reduction in BWG when increasing dietary Ca from 0.60% to 1.0%, but an increase in BWG was reported when elevating the nPP level from 0.30% to 0.40% between 1-10d and 1-24d. There were no significant effects of Ca and P levels on FI, BWG, and FCR between 1 – 35d.

Research by Hamdi *et al.*, (2015) feeding isocaloric (2960 ME, kcal/kg) and isonitrogenous (22% crude protein) diets showed that a wide range between Ca and P could be deleterious for BW and FI. The study investigated an interaction between 3 levels of Ca (0.5, 0.7, or 0.9%), and 4 levels of nPP (0.25, 0.31, 0.38, or 0.45%) fed to male broilers between 1-14d. The study found that elevating nPP from 0.25% to 0.38% improved BW, while 0.38% nPP at fixed Ca level 0.90% improved FI, but FCR was not improved. Further, reducing Ca to 0.70% with 0.38% nPP significantly improved FI between 1-14d. Liu *et al.*, (2017) explored the possible nPP recommendations for a 21-day-old broiler when feeding a constant Ca level at 1.00% with varying levels of nPP graduated by 0.05% from 0.18% nPP to 0.58% nPP. The authors reported high daily weight gain, FI, and improved FCR when feeding 0.43% nPP as opposed to either higher or lower nPP levels than 0.43%. A study by Ceylan *et al.*, (2020) showed that the breeder standard Ca and P levels as per the 2019 recommendations might be high, hence increased BW and FCR in a 11d to 41d period were obtainable in birds fed below the breeder recommendations.

Bai *et al.*, (2022) investigated the broiler Ca and nPP dietary recommendations while only feeding levels of Ca from 0.60% to 1.20% graduated by 0.10% while keeping nPP levels fixed at 0.39% for 21 days. At 21d of age, Abor Acres male broilers fed 0.60% Ca and 0.39% nPP had a higher BW (825 g) and ADG (37.13 g) performance compared to broilers of the same strain and sex who were fed above 0.60% Ca and above with 0.39% nPP. Also, the study showed a linear decrease in ADG when increasing dietary Ca level from 0.70% to 1.20% in broilers of the same age. Though the high performing broilers were below the Abor Acres breed standard according to male performance objective (Aviagen, 2022), the study demonstrated the impact of Ca and P on broiler performance and age.

In a 2023 study, Wu *et al.*, (2023) investigated the interactions between Ca and nPP to identify the possible recommendations for the minerals with respect to performance and bone mineralisation. The comparison was amongst five levels of Ca (0.60%, 0.70%, 0.80%, 0.90%, 1.00%, respectively), and three levels of nPP (0.35%, 0.40%, 0.45%, respectively) in broilers of 21 days of age. The study reported high ADG in broilers fed 0.60% Ca and 0.35% nPP above all Ca and nPP interactions above these two levels, therefore indicating that concomitantly reducing dietary Ca and nPP has no deleterious effects on growth performance.

An earlier study by Mello *et al.*, (2012) investigated the broiler recommendations for Ca and AvP at different stages of growth for female broiler of ages 0d to 46d showed that better performance in BW, FI, FCR can be achieved with lesser dietary Ca and AvP (Cobb500, 2012). Using the Linear Response Plateau model (LRP) the authors reported that these were the ideal Ca and AvP recommendations 1d-10d (0.92% Ca, 0.46% nPP), 11-21d (0.78% Ca, 0.39% nPP), 22-33d (0.72% Ca, 0.36% nPP), and 34-45d (0.51% Ca, 0.26% nPP). Comparable to Mello *et al.*, (2012) 11-21d period, Hamdi *et al.*, (2015) reported that d14 broilers achieved the highest FI and BWG when feeding 0.38% nPP, and feeding 0.70% Ca with a nPP ranging between 0.31% to 0.38% was beneficial for broiler FI.

2.4.1 The effect of dietary Ca and P concentration on bone mineralisation

The dietary levels of Ca and P can influence bone mineralisation of growing broilers as can be represented by tibia ash percentage and bone other bone quality measurements such as bone mineral density and bone mineral content. A sixteen-day trial by Walk *et al.*, (2012) showed a reduction in tibia ash percent relative to a dietary decrease in Ca from 1.03% to 0.64%. However, an improvement in tibia parameters has been reported by several authors (Ceylan *et al.*, 2020; Cowieson *et al.*, 2020; Liu *et al.*, 2017; Rousseau *et al.*, 2016) who demonstrated the benefits of concomitantly reducing dietary Ca and P levels. An increase in tibia ash percent has been reported when feeding 0.45% nPP irrespective of dietary Ca concentration (Rousseau *et al.*, 2016). Liu *et al.*, (2017) showed that at a constant dietary Ca of 1.0%, increasing dietary nPP from 0.18% to 0.38% significantly increased tibia ash percentage in 21d broilers. Yang *et al.*, (2020) showed that due to dietary Ca or P deficiency, tibia ash from finishing broilers was greatly reduced when 0.90% Ca and 0.18% nPP or 0.30% Ca and 0.18% nPP were fed. An earlier study by Hamdi *et al.*, (2015) showed that the breeder Ca recommendations could be lowered to 0.70% at 0.38% dietary nPP for improved tibia ash weight and percent, and mentioned that a Ca level of 0.90% can be detrimental for bone mineralisation in young broilers. A further investigation by Hamdi *et al.*, (2017) found improved tibia ash weight and bone mineral content when dietary nPP was fed at 0.40% and 0.45% as opposed to 0.30% and 0.35%, in agreement with authors who fed above 0.35% dietary P in the earlier phases of broiler life. However, it should be noted that concomitantly decreasing dietary Ca and P has been reported not to have detrimental effects on bone mineralisation (Kim *et al.*, 2017). Nevertheless, investigations by Rousseau *et al.*, (2016) and Valable *et al.*, (2018) showed the adaptive response of broilers to dietary Ca and P reduction at finisher phase, indicating the possibility of reducing Ca and P levels below the breeder standard and to possibly eliminate inorganic sources of P at finisher phases.

The digestibility of Ca and P does not only depend on LS solubility, but the dietary concentration of Ca and P plays also a role. A sixteen-day research by Walk *et al.*, (2012) investigated broiler performance and ileal digestibility of Ca and P based on the high Ca (1.03%) and low Ca (0.64%) at fixed dietary tP (0.61%). The study showed that reducing dietary Ca to 0.64% improved AID (apparent ileal digestibility) of P. The study also found a reduction in tibia ash percent relative to a dietary decrease in Ca from 1.03% to 0.64%. However, an improvement in tibia parameters has been reported by several authors (Ceylan *et al.*, 2020; Cowieson *et al.*, 2020; Liu *et al.*, 2017; Rousseau *et al.*, 2016) who demonstrated the benefits of concomitantly reducing dietary Ca and P levels. A study investigating broiler response to dietary Ca and P restrictions, irrespective of limestone solubility, found an improvement in AID P when dietary Ca was reduced to 0.60% from 1.0% with either 0.30% or 0.45% nPP (0.55% or 0.70% total P). The tibia ash percent was high at 0.45% nPP, irrespective of dietary Ca concentration (Rousseau *et al.*, 2016). Concurring with Rousseau *et al.*, (2016), Liu *et al.*, (2017) showed that reducing dietary nPP below 0.38% with dietary Ca kept constant at 1.0% markedly reduced the tibia ash percentage and bone-related parameters, but increasing dietary nPP above 0.40% improved tibia ash percentage therefore demonstrating better bone mineralisation.

2.4.2 The effect of dietary Ca and P concentration on foot pad dermatitis and hock burn in broilers

Litter quality plays a significant role in broiler production and performance, health, carcass quality, and welfare (Garcês *et al.*, 2013). Broilers spend their lifetime being continuously in contact with litter, from the time of placement to the end of a production cycle before slaughter at the abattoir (de Jong *et al.*, 2012; de Toledo *et al.*, 2020; Esmail, 2011; Muthusamy, 2021; Ritz *et al.*, 2009). Optimum genetic performance in broilers can be supported by a nutritious diet and suitable housing and litter conditions with good management (de Toledo *et al.*, 2020). Therefore, good litter quality should be maintained to avoid economic losses due to disease, foot pad dermatitis (FPD) and hock burns (HB) (De Jong *et al.*, 2014; Esmail, 2011; Marušić *et al.*, 2019). Both FPD and HB lesions can be identified as cutaneous discolouration coupled with hyperkeratosis of foot pads and hock skin which can progress to deep ulcers, epidermal necrosis, and inflammation (Michel *et al.*, 2012).

Elevated dietary Ca levels in broiler diets has been associated with poor litter quality (Collett, 2012), which would result to cases of FPD (Shepherd *et al.*, 2010). Findings by Delezie *et al.*, (2015) showed that when feeding treatment 1 Ca and P (0.65% Ca, 0.30% AvP), or treatment 3 and 4 Ca with low P (0.65% Ca, 0.18% AvP), or treatment 2, 5, and 6 Ca and P (0.50% Ca, 0.18% AvP) found

that the incidences of FPD and HB at slaughter age were due to treatment effects because of varying dietary Ca and P levels. Significant incidence and severity of FPD increased with broiler age and were influenced by the dietary treatments. The authors reported high incidences of FPD reaching a score of 3 when feeding 0.65% Ca and 0.18% AvP, and the same treatment exhibited high scores of 2 and 3 for HB incidences for broilers aged 39d than broilers fed treatment 1, 2, 5, and 6. Interestingly the study also showed reduced score 2 and 3 for both FPD and HB when feeding treatment 4 which had similar 0.65% Ca and 0.18% AvP levels to treatment 3, and this occurrence was not explained by the authors, however, this could be due to the different levels of phytase used in the study. There was a higher incidence of HB in birds fed high dietary Ca with low AvP where more broilers showed scores 2 and 3 which were regarded as severe.

Research has shown that dietary Ca levels above 0.80% fed with dietary P below 0.40% can be result in high incidences of FPD. Rousseau *et al.*, (2016) reported high FPD prevalence in broilers aged 35d when fed grower (1.0% Ca, 0.30% nPP) and finisher (0.90% Ca, 0.30% nPP) levels. Kim *et al.*, (2017) noted a linear increasing trend on FPD prevalence and mentioned that it could be related to graduated levels of dietary Ca from 0.60% to 1.0% at constant dietary nPP (0.35%) level. It is suggested that high Ca intake may induce wet litter problems because poultry has a limited capacity of reabsorbing Ca in the kidney, and therefore, high Ca intake increases urinary Ca loss, leading to a polyuria (Collett, 2012; Wideman Jr *et al.*, 1985). Apart from dietary Ca and P influence on leg lesion, studies by (Broom *et al.*, 2005; Kjaer *et al.*, 2006) reported on HB lesions which were said to be positively related to an increase in BW.

2.5 Sources of Calcium and Phosphorus

Broiler diets are supplemented with limestone (calcium carbonate/$CaCO_3$) as a means to provide dietary Ca, but the physical properties of the Ca source often affect the availability of the nutrient (Lee *et al.*, 2021; Li *et al.*, 2021; Majeed *et al.*, 2020)**.** Amongst Ca sources, besides being widely available on the market globally, limestone is preferred because it has less contamination with sand, consistent Ca concentration, and is unlikely to contain marine contaminants (Sahraei *et al.*, 2017). According to reports, limestone provides more than 50% Ca in broiler diets, whereas it provides 90% Ca in layer hen diets (Kim *et al*., 2019; Plumstead *et al*., 2020).

Inorganic sources of P used to meet the broiler nutritional needs are available on the market globally; these include monocalcium phosphate (MCP), dicalcium phosphate (DCP), mono-dicalcium phosphate (MDCP), tricalcium phosphate (TCP), and defluorinated phosphate (DFP) which are all manufactured from the finite phosphate rock (Edixhoven *et al.*, 2014; Liu *et al.*, 2019;

Sauvant *et al.*, 2004; Walan *et al.*, 2014)**.** Phosphates are crucial in providing dietary P, mainly available P. Knowledge of available or digestible P per source of inorganic P enables animal nutritionists to formulate feeds close to the bird's requirements avoiding either under or over supplying dietary P (Trairatapiwan *et al.*, 2018).

2.5.1 Properties of Limestone

2.5.1.1 Limestone composition and origin

Limestone is a naturally occurring sedimentary rock which is predominantly composed of Ca carbonate ($CaCO_3$). Limestone chemical and physical properties differ due to geology, which influences the level of impurities (Douglas, 1969) and trace minerals (Davin *et al.*, 2020). Lamar, (1961) described the geological differences of limestone, where the high Ca limestone contains more than 97% CaCO3 while the dolomite rock has more than 42% magnesium carbonate. Feed-grade limestone fine in particle size were analysed by Sa *et al.*, (2017), as were found to have Mg levels which were negatively correlated to Ca concentration.

By molecular weight, $CaCO_3$ has 40% Ca in total. However, a high of 42% Ca concentration from limestone has been reported (Anwar *et al.*, 2016; Browning *et al.*, 2013; Reid *et al.*, 1976). Contrary to CVB, (2018) which reports limestone to have a concentration of 38% Ca, Plumstead *et al.*, (2020) investigated 255 limestone samples and found a Ca range of 30.36% to 39.99% indicating the impact of geology even from limestone sourced from the same supplier.

Apart from being the least expensive source of Ca used in poultry diets; limestone is a cost-efficient acid-neutralization material for acid mine drainage (Hammarstrom *et al.*, 2003). Limestone contains a variety of chemical components. After oxygen, silicon, aluminium, and iron, Ca is the fifth most prevalent element in the Earth's crust. It was removed from early igneous rocks by the combined actions of weather erosion and corrosion by acidic gases (Oates, 1998). Compared to phosphate rock which is arguably depleting both, in reserves and quality, limestone resources are commonly well distributed globally. According to the USGS reports, limestone reserves are indefinitely sufficient; hence of the mined and crushed stones, about 70% on average of the crushed product is limestone and dolomite between the year 2013 and 2021 (USGS, 2014; USGS, 2021; USGS, 2022). A study investigating potentiating limestone as an ocean alkalinity enhancer stipulated a conservative 25-metre excavation on outcrop rock to produce pure carbonates approximated up to 70,800 Gt (Caserini *et al.*, 2022). In agreement with the notion that limestone is nowhere close to depletion, Hartmann *et al.*, (2012) reported that pure carbonate represents 33% of the 64% of sedimentary rock identified

using the global lithological map (GLiM). This would justify the low cost of limestone for both the agriculture and construction industries.

2.5.1.2 Classification of limestone by particle geometric mean diameter

Limestone particle size distribution (PSD) quantisation has been performed using the sieve methodology resulting in gradation of particle sizes, which identifies if a sample is fine or coarse (Gilani *et al.*, 2022; Wilcox *et al.*, 1970; Yang *et al.*, 2019). The geometric diameter and the geometric mean diameter (GMD) of particles in each sieve can be calculated as per the equations described by (Wilcox *et al.*, 1970). Plumstead *et al.*, (2020) analysed 255 limestone samples for particle size distribution, mineral analysis, and for solubility. The study categorised the sample to be either fine of coarse limestone based on GMD; where GMD < 1000 µm was regarded as fine limestone, and GMD > 1000 µm as coarse limestone. The authors reported a Ca concentration between 30.36% - 39.99% in both fine and coarse limestone, which is 1.16% higher compared to Gilani *et al.*, (2022) who reported a minimum Ca concentration of 29.2%, and the coarse limestone exhibited a Ca concentration between 34.67% and 39.99%. Other microminerals that form part of the rock are a vital constituency which should be considered as these impurities might have an effect on Ca availability. This would explain the wide range in Ca concentrations amongst the limestones irrespective of particle size as impacted by the contaminants microminerals in a sample (Table 2.2). Gilani *et al.*, (2022) quantified the PSD of 641 limestone samples from global poultry feed mills. The study found variations within feed mill regions and outside of feed mill regions, where 566 samples were classified as fine limestone (GMD < 1000 µm) and 75 samples were classified as coarse limestone (GMD > 1000 µm). Both investigations indicated how geology affects limestone purity; as a result, there were notable differences in the Ca concentration between the regional samples (Table 2.2); Also, in the examined samples, Ca concentration shows a deviation from the reported limestone levels in feed tables which nutritionists use to formulate (Gilani *et al.*, 2022; Plumstead *et al.*, 2020). A distinct difference in Ca concentration between fine (GMD < 1000 µm) and coarse (GMD > 1000 µm) limestone exist. Gilani *et al.*, (2022) showed a Ca range between 29.2% - 39.7% in fine limestone and 34.7% - 40% in coarse limestone (Table 2.2).

Table 2.2. Average limestone mineral analysis (source: (Gilani *et al.*, 2022)

Mineral GMD < 1000 mm[1]	K	Na	Cu	Fe	Mg	Mn	Zn
Region[2]	%	%	mg/kg	mg/kg	%	mg/kg	Mg/kg

AF	0.02^{ab}	0.01	3.51^{ab}	4463.3^{a}	1.56^{a}	625.4^{a}	11.79^{ab}
ANZ	0.02^{b}	0.01	3.58^{ab}	1075.9^{b}	0.31^{bc}	130.3^{b}	10.51^{b}
Asia	0.02^{b}	0.01	2.47^{b}	544.17^{b}	0.62^{b}	74.00^{b}	21.21^{ab}
EU	0.06^{b}	0.04	3.33^{b}	934.93^{b}	0.28^{c}	164.02^{b}	19.84^{ab}
ME	2.85^{a}	0.01	7.08^{a}	325.60^{b}	0.21^{c}	67.37^{b}	27.22^{a}
NA	0.02^{b}	0.03	2.45^{b}	817.57^{b}	0.31^{c}	114.47^{b}	26.10^{a}
RU	0.01^{ab}	0.01	2.93^{ab}	1761.4^{b}	0.45^{bc}	163.77^{b}	14.24^{ab}
SA	0.02^{b}	0.04	2.46^{b}	966.89^{b}	0.39^{bc}	96.99^{b}	17.29^{ab}

Mineral GMD > 1000 mm[1]	K	Na	Cu	Fe	Mg	Mn	Zn
	%	%	mg/kg	mg/kg	%	mg/kg	Mg/kg
All regions	0.02	0.02	2.29	929.1	0.28	205.5	23.26

[1] Average mineral analysis from limestone sourced from various regions: (GMD) Geometric Mean Diameter; (K) potassium; (Na) sodium; (Cu) copper; (Fe) iron; (Mg) magnesium; (Mn) manganese; (Zn) zinc.
[2] Regions identified as: (AF) African countries (Morocco, Malawi, and others not disclosed); (ANZ) Australia and New Zealand; (EU) European Union including UK countries (Belgium, Czech Republic, Denmark, Finland, France, Germany, Ireland, Italy, Netherlands, Norway, Poland, Portugal, Spain, Sweden, Ukraine, United Kingdom); (ME) Middle East Amman, Saudi Arabia, Turkey; (NA) North America, Costa Rica, Guatemala, Honduras, Nicaragua, United State of America; (RU) Russia; (SA) South America, Argentina, Brazil, Columbia.

2.5.1.3 Impact of limestone solubility on Ca and P digestibility

Limestone PSD measured as GMD in microns (µm) has been shown to have a significant impact on limestone solubility, broiler performance, and Ca and P digestibility (Gilani *et al.*, 2022; Kim *et al.*, 2019; Lee *et al.*, 2021; Li. *et al.*, 2021; Zhang *et al.*, 1997b). Kim *et al.*, (2019) showed that fast soluble limestone compared to slow soluble limestone solubilises faster resulting in high insoluble Ca-phytate complexes, which leads to low Ca and P digestibility (Figure 2.7). Interestingly, Kim *et al.*, (2019) found unexpected results where a fine limestone with 326 µm solubilised slower than a large particulate limestone of GMD 633 µm and 831 µm. The unanticipated solubility rate could be due to unaccounted-for intrinsic characteristics of limestone, such as impurities and other factors because of geology.

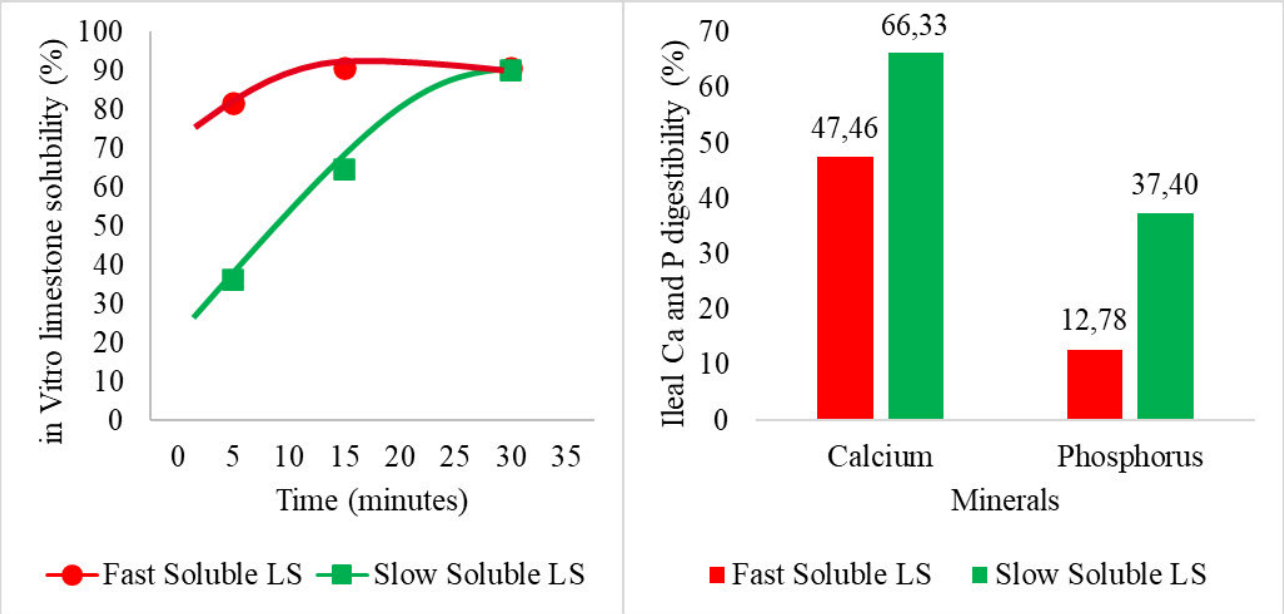

Figure 2.5 Dynamic effect of limestone solubility on calcium and phosphorus digestibility (adapted from Kim *et al.*, (2019))

A study by Guinotte *et al.*, (1991), demonstrated improved broiler FCR when feeding a finely ground limestone (<0.15mm) compared to when feeding limestone with particle size of 1.18mm and 4.75mm. Bradbury *et al.*, (2016) investigating the effect of calcium source, particle size, concentration, and phytase supplementation found that particle size and Ca concentration can have overlapping effects. The authors reported that feeding 3.5g/kg AvP and 6.0g/kg Ca with LS particle size of <0.5mm improved FI and live weight gain greater than when feeding 3.5g/kg AvP with 9.0g/kg Ca, irrespective of LS particle size. While feeding either LS particle size <0.5mm or >0.5mm with 3.5g/kg AvP with 9.0g/kg Ca concentration depressed both FI and live weight gain between 1-13 days of age. Though there were no significant differences in FCR, feeding a coarser particle LS resulted in high FI and high final live weight gain between 1-28 days of age. Contrarily, (Majeed *et al.*, 2020) reported no significant differences in FI, BW, FCR, and mortality rate between 0-35d when feeding either fine or coarse LS. The study showed that feeding coarse LS with either high or low Ca and P concentration, or fine LS with high Ca and P concentration resulted in high FI, BW, while particle size with either low or high Ca and P concentrations showed no differences in FCR performance. Concurringly, Lee *et al.*, (2021) showed that particle size alone does not yield differences in performance. Feeding coarse LS irrespective of Ca and P level and feeding fine LS with high Ca and P level can improve FI, BW, and FCR between 1-21 days of age. This demonstrate the impact of limestone, because of its particle size affecting the solubility can change the rate at

which Ca is released into the gastrointestinal tract at various Ca concentrations, which in turn can impair broiler performance.

2.5.2 Phosphorus sources

Like water, oxygen, carbon, and nitrogen, P is vital for life and impacts plant and animal production; and most importantly these elements and molecules have no substitutes. Phosphates are an integral part of poultry diets; with inorganic P sources being used to supplement poultry diets to meet the birds' requirements (Applegate *et al.*, 2008; Shastak *et al.*, 2012). Supplementation occurs even though the raw materials used in poultry diets contain P (Adedokun *et al.*, 2013; Nelson *et al.*, 1968); as it is not available to the birds due to being bound into inositol hexaphosphate. The inorganic phosphates (IP) production differs depending on the intended final inorganic source of P (ISP). Commercially available ISP, amongst others, include monocalcium phosphate (MCP), dicalcium phosphate (DCP), mono-dicalcium phosphate (MDCP), tricalcium phosphate (TCP), and defluorinated phosphate (DFP). Mineral content in inorganic feed phosphates differs, which determines the availability of P and Ca to the birds (Lamp *et al.*, 2020). Numerous characteristics, including variations in pH, particle size, crystallinity, chemical structure, source of the substances utilised, the production method, and the concentration of contaminants, all contribute to large variability in final IP sources resulting in performance differences (Dilworth, & Day, 1964; DilworthDay *et al.*, 1964; Kleyn, 2013; Lima *et al.*, 1999; Souza *et al.*, 2009).

Beneficiation of the phosphate rock deposits to release the gangue materials involves intricate steps with stringent methodologies. The lowest possible level of fluorine, arsenic, and heavy metals is essential for an inorganic source of P to be suitable as an ingredient in animal feed. Micronutrients such as fluorine, can result in fluorosis when present in high concentration in feed phosphates resulting in immunotoxicity (Deng *et al.*, 2013; Wang *et al.*, 2018). The production of DFP has been conducted through a thermal method which consumed high energy (1400°C – 1500°C) and resulted in hydrogen fluoride, which is environmentally detrimental; thus, a low thermal and environmentally friendly MCP and DCP became the point of interest (Figure 2.8) (Wzorek *et al.*, 2001). The production of DCP and MCP is through a chemical reaction. Where phosphoric acid, limestone ($CaCO_3$) or dolomite ($CaMg(CO_3)_2$), and $Ca(OH)_2$ are homogenised. Furthermore, mixing lime, phosphoric acid and hydrochloric acid results in MCP and hydrated DCP (Kleyn, 2013).

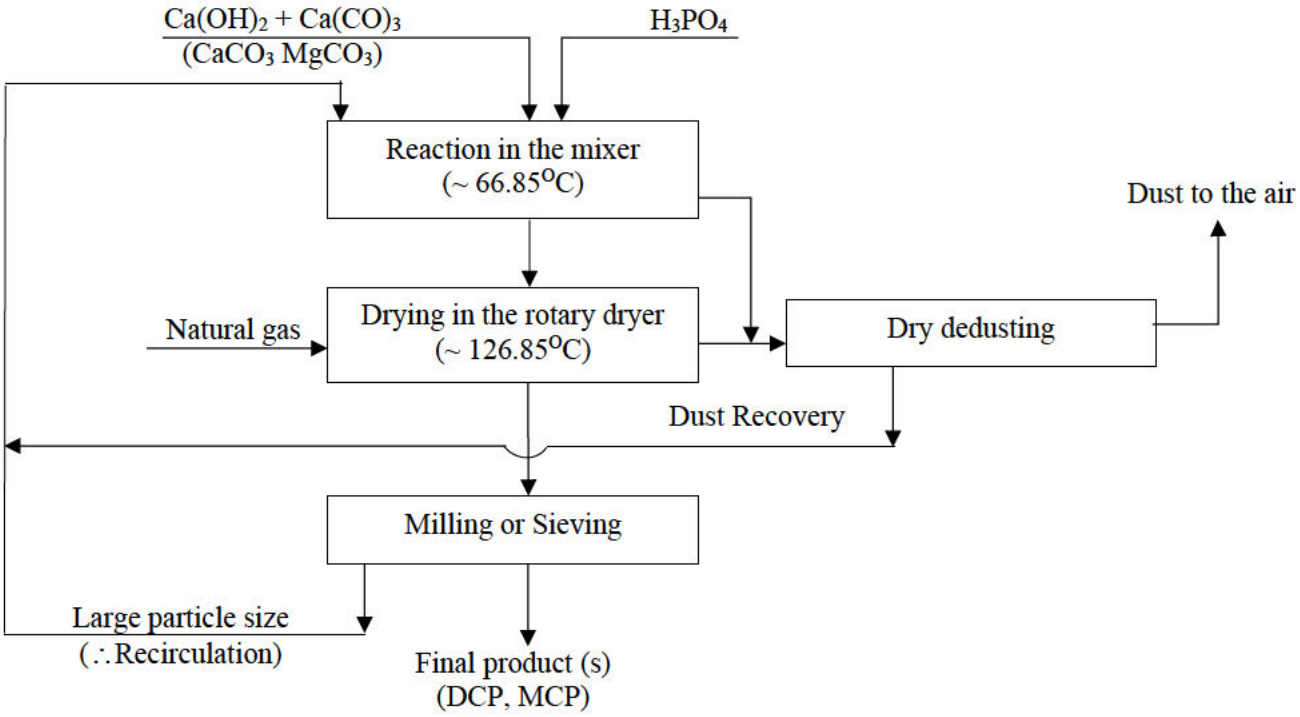

Figure 2.6. Inorganic feed phosphate production utilising a low thermal methodology (Wzorek *et al.*, (2001))

A eutectic bond of MCP and DCP results in MDCP. The use of MDCP in animal feed could be more beneficial as it provides highly digestible and absorbable P compared to other feed phosphates, especially to broilers (Lamp *et al.*, 2020), it is economical and is less environmentally unfriendly (Zhou *et al.*, 2021). However, Zhang *et al.*, (2017) reported that even though MDCP is advantageous in animal production, its production and use, like other feed phosphates, has environmental consequences. Zhou *et al.*, (2021) reported that beneficiation is highest on the cost of MDCP production due to taxing on the power grid, and the cost increases more when low-grade phosphate ore is purified. Thus, sustainable methods are needed to produce feed phosphates with minimal environmental impact. This deduce the high prices of feed phosphates, so producers who have not innovatively developed their production technologies produce at a loss (Hoffmann *et al.*, 2009); contributing to broiler production cost from inorganic P sources. Mew, (2016) reported on multiple factors which result to high phosphate prices, in which these would escalate to farmers and eventually to the consumer. Two scenarios have been reported by said to have occurred in 1975 and 2008, where phosphate rock prices were reported to be the highest ever seen till this day, which resulted in drastic food price changes (Mew, 2016) highlighting the need to accurately supplement dietary P to minimise diet cost and to lower unnecessary mineral excretion.

2.5.2.1 Phosphate rock global reserves

Literature reports the phosphate rock to be a finite resource depicting dispersed availability on Earth currently and for the future and warns of an impending scarcity leading to a gradual decrease in phosphorus production (Cordell *et al.*, 2009; De Bruijne *et al.*, 2009; Edixhoven *et al.*, 2014; Sverdrup *et al.*, 2011). Phosphate rock depletion through modelled projections has been reported in the literature (Rosmarin, 2004; Steen, 1998), and some reported the P depletion to be around the next excavation activity (Cordell *et al.*, 2009; Herring *et al.*, 1993). Despite the looming crisis as reported in the literature, Schipper, (2014) mentioned more than the total depletion of the phosphate rock; it is the decrease in quality of phosphate rock one should be concerned about resulting in the use of contaminated phosphate sources. Last decade excavations reported high tonnes of phosphate rock production being available globally from 16000 Mt to 65000 Mt from 2010 to 2011 therefore indicating that Earth may still have substantial reserves (USGS, 2012); further, the USGS, (2014) reported a 4-fold increase in phosphate rock production globally in metric tonnes from 16000 Mt to 67000 Mt between 2010 and 2014, said to due to an upgrade post beneficiation. A modelling study by Herring *et al.*, (1993) projected an extension of the phosphate rock reserves by a further 50 years post 2025, and possibly the reserves can extent to the 23rd century based on mindful utilisation and recycling (Cooper *et al.*, 2011; Koppelaar *et al.*, 2013).

The geological availability of the phosphate rock reserves not only influences the pricing of the commodity but influences food and nutrition security and geopolitics globally. Countries such as Morocco, China, Syria, Algeria, and South Africa hold about 89% of the world's phosphate rock reserves (Cordell, 2016; USGS, 2015). The war between Ukraine and Russia and the sanctions posed in response to the conflict resulted in market changes concerning fertiliser and feed-grade P, causing a shift in supply and demand. Global reports on phosphate rock mining and reserves based on government and company reports as presented by the USGS demonstrate a dynamic change, therefore showing a possibility of new excavation sites, which then serve to object to the comments on the depletion of the phosphate rock (USGS, 2022).

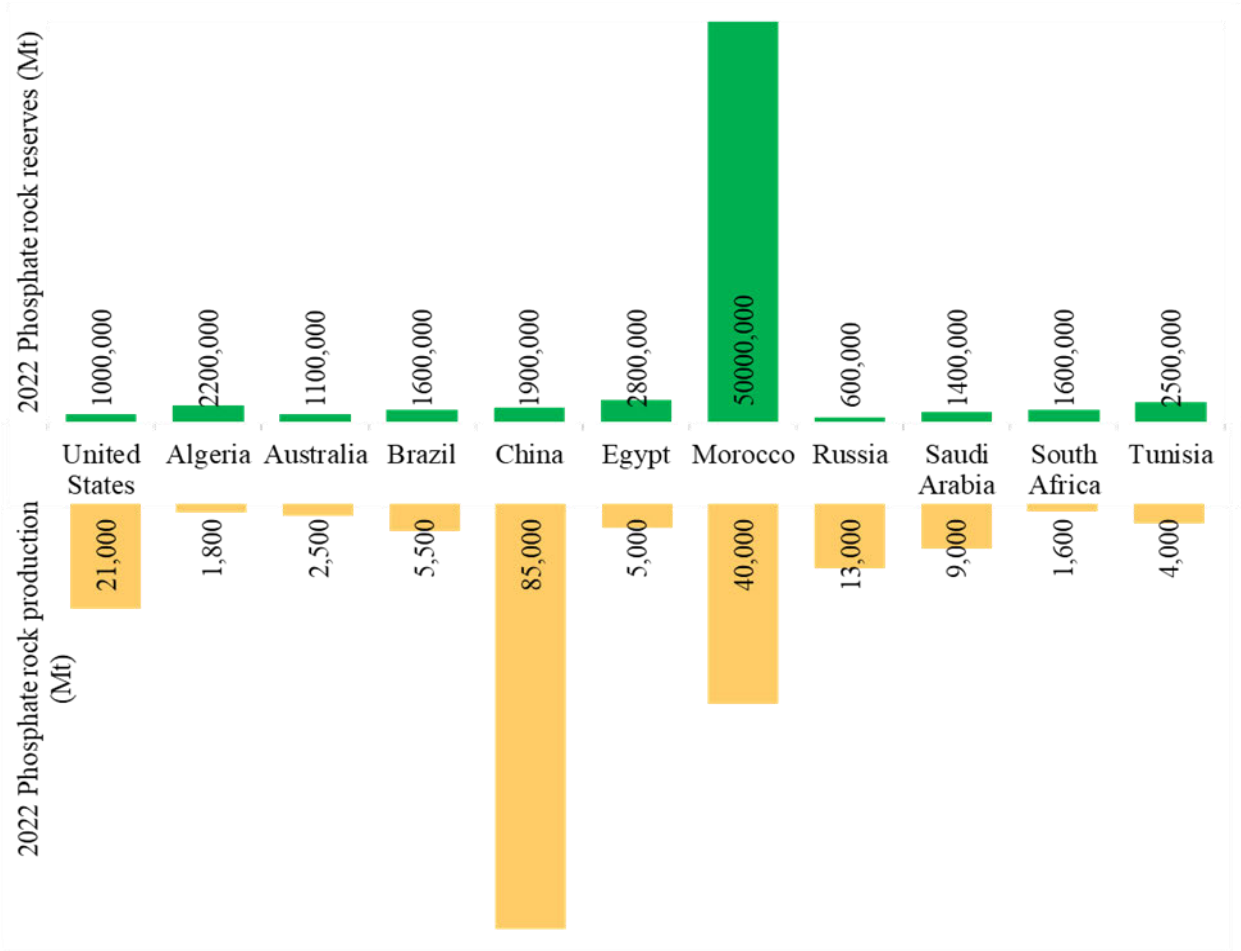

Figure 2.7. Graph showing the global phosphate rock mining production and reserves as of year 2022 (adapted from USGS, (2022))

The US mineral commodity geological survey has shown a global decrease in marketable phosphate rock production from 2018 to 2022 (Ash, 2019), supporting the statement that it is the quality of the mined phosphate rock that should be of major concern as opposed to the availability of the rock itself. Based on the USGS data (USGS, 2022), Figure 2.9 shows that there is no imminent shortage of the phosphate rock.

An update showing positive tonnes in reserves was noted from countries which were shown to have low tonnes of sedimentary rock (Vaccari, 2009). This shows how the lack of data and data collection, and proper research could have caused a panic resulting in significant market ripples, which would negatively affect the consumer and food production entirely. Literature reported by Cordell *et al.*, (2011) justifies concerns related to phosphate rock depletion and reserves due to the lack of independent bodies responsible for providing accurate and transparent data on the matter. In addition, there is a lack of a standardised mathematical methodology to quantify what constitutes a phosphate rock reserve across countries and companies, as noted by the USGS (USGS, 2021). This

therefore contributes to a lack of consensus on phosphate availability as a source of P, resulting in possibly unnecessary multiple pricing paradigms.

2.5.2.2 Phosphate rock cost and resource interaction

A large quantity of P is used in fertiliser production, and a small quantity is used to produce feed-grade inorganic P sources for animal consumption. As the quality of the phosphate rock decreases, there will be an excessive spending on energy requirements for beneficiation and refining of the phosphate rock to a usable state. The production of MDCP from a decreasing phosphate ore grade from 25% to 15% has been shown to result in increased cost of production from $300.76/t to $332.35/t from mining to the final product (Zhou *et al.*, 2021). More than the cost of P source-related production, the market price fluctuation has an impact on fertiliser and animal production, increasing gross domestic product (GDP). Despite the 800% phosphate rock price hike observed between 1975 and the 2008, Mew, (2016) reported that the phosphate rock prices increased less than the inflation rate over the past three decades globally.

Increasing phosphate rock prices translates to increasing fertiliser prices for farmers and animal producers. Farmers need high-quality phosphate fertilisers to produce high-yielding crops, which would become food for humans and raw materials used in broiler diets. An increase in soybean yield has been reported due to P fertiliser measured by the amount of leaf P concentration of 0.2% to 0.3% by weight (Reich *et al.*, 2010). The opposite also occurs, Cordell *et al.*, (2015) demonstrated that P deficient soils contribute to poor crop yield.

Ragnarsdóttir *et al.*, (2011) identified the increase in population size as the primary influence on P consumption and demand in their system dynamics model measuring P supply. Price changes in 2022 were hiking closely to the 2008-2010 fertiliser and phosphate rock price hikes. This could be partly due to the war between Ukraine and Russia (Baffes *et al.*, 2022). Notably, accompanying the Ukraine and Russia war was the COVID-19 pandemic which led to multiple disruptions, especially in the agricultural supply chain. This shows the P-related market volatility between the mining and agricultural industry due to direct and indirect factors which could hamper food and nutrition security. Amongst other reasons, the increase in meat consumption globally has contributed to the rise in phosphate rock prices. Pending the projected 9 billion population size by the year 2050 (DeSA, 2013), the demand for meat consumption is projected to rise to 14% by 2030 and poultry consumption by 17.5%; therefore, its production is estimated to be higher than all other meat forms making 41% protein to be sourced from just poultry (OECD *et al.*, 2021). Compared to the 1960s, meat consumption has quadrupled (Ritchie *et al.*, 2017), and between 2000 and 2019 alone rose to 14.8

kg/capita from 9.8 kg/capita, respectively (Whitton *et al.*, 2021). China is projected to have the highest global meat consumption as driven by population size. Not only will this affect target slaughter average body weight but crop production and feed formulation, and how sources of P are utilised to meet the birds' requirements while minimising negative environmental impacts.

2.6 Environmental impact of mineral runoff and livestock mineral excretion

Eutrophication is the process through which a whole body of water, or sections of it, gets gradually enriched with minerals and nutrients, mainly nitrogen (N) and P. It has been demonstrated to have detrimental effects on water usage for drinking, industry, agriculture, recreation, and fisheries (Carpenter *et al.*, 1998). Predominantly, eutrophication is driven by human activities such as industrial runoffs, application of P fertilisers in crop fields, and supplementation of livestock feed and sewage (Bennett *et al.*, 1999; Khan *et al.*, 2014; Schröder *et al.*, 2004). It has been reported that N and P cause eutrophication, resulting in dead zones due to algal biomass outgrowth which leaches oxygen in water (Dodds *et al.*, 2016; Lihepanyama *et al.*, 2022; Smith, 2003). Algal overgrowth or algal bloom is the exponential increase in algae population size in water (Anderson *et al.*, 2002). Furthermore, the consequence of algal bloom is toxic and anoxic water conditions (Anderson *et al.*, 2002; Duan *et al.*, 2017). Lihepanyama *et al.*, (2022) reported that water transparency declined as the dissolved oxygen declined inversely to algal bloom. Although the study did not find significant differences in water pH, it showed a decrease in water quality which is essential for livestock production and for human health. From a poultry nutrition perspective, more focus is directed to P eutrophication due to broiler diets being highly supplemented with inorganic sources of P such as MCP, DCP, and MDCP (Adedokun *et al.*, 2013; Lamp *et al.*, 2020). Dietary Ca and P imbalance can lead to excess P excretion resulting in P eutrophication and environmental degradation (Sharpley, 1999). Inorganic P sources are cardinal for sustainable broiler production, however; adverse environmental effects arise from excess P excretion to the body of water from the manure.

2.6.1 Mitigation strategies to lower P excretion and recycle P

Strategies to mitigate P eutrophication are vital for environmental preservation and sustainable livestock and broiler production (Kumar *et al.*, 2015). Implementation needs to be from two fronts which would need to lower P release through manure to soils and water and rehabilitate the environment from the existing P (Lin *et al.*, 2017). If eutrophication is to be well controlled, a system of understanding P flow from mining and manufacturing, crop and livestock production, and the construction industry to the environment is essential (Tonini *et al.*, 2019; Wu *et al.*, 2012). Wu *et al.*,

(2012) used the substance flow analysis (SFA) methodology and showed that in China, the largest pool of P lost into the water was about 5334 t and 5247 t from crop production and livestock manure used on fields, respectively greater than other P flow sources.

2.6.1.1 Resourceful waste

Recovery of P from wastewater for agricultural usage has been attempted, thus preserving untainted water for human and animal consumption. A study by Nedelciu *et al.,* (2019) reported a 34% P recovery by 2016 prior to the targeted 40% by 2018 from wastewater in Stockholm, Sweden. In this study, P recovery was achieved through sludge from wastewater used for crop production post digestion for hazardous heavy metals such as Cadmium, Iron, Lead, and Mercury. The study further compared P recovery from Budapest, Hungary, which is comparable to Stockholm of Sweden in population size, thus projecting relative P runoff to wastewater. Although Budapest uses sludge for landscaping recultivation projects, about 42% of sludge gets utilised on land, and 17% is mixed with other compost. The application of sludge in European countries for agricultural use has been done for over a decade at a large scale (Lloret *et al.,* 2016). In a 10-year study, Melo *et al.,* (2018) showed that the sludge could substitute P fertiliser with no loss in grain yield and dry matter. Compared to temperate European environmental conditions (Lloret *et al.,* 2016), the investigation by Melo *et al.,* (2018) showed that using sludge to recycle P is possible and can be beneficial in tropical climatic conditions. A report by Huygens *et al.,* (2019) mentioned that P recovery from wastewater would add value in fertilising the fields and is projected to replace between 17% and 31% of the phosphate rock used to produce P fertiliser by the year 2030 in Europe.

2.7 Conclusion

Extensive research has been done to understand the functions and utilisation of Ca and P in the body and the responses in animal nutrition. Literature has reported Ca and P as abundant macro minerals in broiler diets and are essential for metabolism, growth, development, and bone mineralisation. It is, therefore, cardinal for nutritionists to formulate the dietary levels of the minerals to optimise performance and avoid detrimental and antagonistic effects. However, there is a probability that commercial diets are either deficient or over-supplemented with sources of Ca and P, resulting in adverse Ca and P interactions. Research has demonstrated the detrimental effects on the performance and welfare of broilers when dietary Ca and P are either deficient, in excess, or imbalanced. Furthermore, research has shown outstanding broiler performance, bone mineralisation, and ileal digestibility of Ca and P when these are fed below the breed standard 2:1 (Ca: AvP) ratio.

Since the digestibility of Ca has been shown to vary considerably, the application of a fixed ratio of Ca: AvP as recommended by breed companies can likely also contribute to some of the observed leg problems / skeletal issues since large variation in Ca digestibility would inevitably lead to variable delivery of digestible Ca: digestible P to the bird. Literature has shown that over the decades, the P system has been redefined multiple times, resulting in better formulations, while less work has been done on Ca. Hence the poultry industry continues to utilise the total Ca system, in which the recommendations from breeder companies (Aviagen, Cobb, Hubbard), NRC, CVB, and the Brazilian tables are similar, and all still use the total Ca system.

While limestone is abundantly available and cheap to supplement in broiler diets, sources of P are expensive and pose adverse environmental impacts if not monitored how they flow from mining to livestock feeding and excretion into manure. Literature and statistics have shown that phosphate rock depletion is not of major concern, but the quality of the rock used to produce feed-grade sources warrants consideration. Poultry production waste in the form of manure in more ways than one will end in nature, contributing to environmental degradation in the form of eutrophication. Literature has evidence that not all limestones are similar; and particle size has been reported to impact performance as well as to impact Ca and P digestibility in vivo and in vitro leading to variable responses in broiler performance and bone mineralisation.

Chapter 3

Material and Methods

3.1 Animal Husbandry

All animal work done in this study was approved by the Neuro Livestock Research (NLR) ethics committee (NLR 2107/2021) and by the University of KwaZulu Natal (UKZN) Animal Research Ethics Committee (AREC) with protocol reference number AREC/00004433/2022. A total of 3600 as-hatched day-old Cobb 500 chicks were randomly allocated to 72 pens installed within a large commercial broiler house. A total of 50 birds were randomly allocated to each pen. Birds with deformities were not selected for placement into the pens. The chicks were reared under artificial light and temperature-controlled environmental conditions in accordance with Aviagen brooding standards (Aviagen, 2018). The initial brooding temperature was set at 35^{o}C and was gradually reduced to 22^{o}C by 22d of age until the end of the study. The photoperiod was set at 23L: 1D from 0-7d. It was progressively reduced to 16L: 8D by 7d and maintained until the end of the study at 32d. Each wired floor pen (2.0m X 1.2m) was provided with a supplemental bell drinker, feeder tray, nipple drinkers connected to a 20-litre carboy for each pen, and a tube feeder from 0 – 7d. Feed and water were provided *ad libitum*.

3.2 Litter

Prior to placement, unused dry wooden shavings were weighed to 4 Kg and allocated to each of the 72 pens. The litter was evenly spread within each pen to a depth between 2 – 5 cm (Aviagen, 2018). Litter acidifying agents were not used post allocation per pen to regulate the litter pH. A fine protective mesh was installed in and around each pen to avoid litter spillage to adjacent pens. Wet litter was not changed, and no further top-ups were done for the remainder of the study. Periodical litter turning was done following Neuro Livestock Research (NLR) protocols. Following the protocol, litter was observed daily for clumping and caking and was turned for all pens when needed. Post 14d of the study, litter turning was done every two days until the end of the study. At 32d, all the litter from each pen was shovelled into a weighed 45-litre container, weighed the container with litter, and then mixed thoroughly before being evenly spread on a tarpaulin to be further homogenised. Two representative samples of 1 kg per pen were collected from the litter spread on the tarpaulin. The collected litter samples were kept at room temperature before being oven dried. Each of the 144x1 kg samples were spread in 32.5x26.5x5.5 (l x b x h) cm aluminium foil containers. Sample and container weights were recorded before oven drying. The samples were individually oven dried for dry matter analysis at 90^{o}C for 48hrs. The dried samples were weighed straight out of the oven. Before milling,

the dried samples were stored at room temperature in sealed bags. Before mineral analysis, the litter samples were milled to a fine powder using the Miller 3500 Series milling machine to pass through a 1-mm screen. The milled litter was sent for Ca, P, and moisture analysis using the method 935.13 (AOAC, 1999).

3.3 Experimental Diets

Corn-soybean meal based diets that either met or exceeded the nutritional recommendations (except for Ca and P) for broilers (NRC, 1994) except Ca and P using a four-phase diet regimen were used in the study from 0 to 32d of age. Table 3.1 shows the Ca and P specifications for each dietary phase with the limestone solubility profile of each treatment, while Table 3.2 and Table 3.3 show the dietary treatments which were provided *ad libitum* in four phases (pre-starter, starter, grower, and finisher). All dietary treatments (Trt) were fed in crumbs form at pre-starter (0 – 8d) and starter phase (9 – 13d), while pellets were fed from grower (14 – 22d) to finisher phase (23 – 32d). A 6-Phytase from Danisco Animal Nutrition, DuPont Industrial Biosciences, Marlborough (Axtra® PHY Gold, *Buttiauxella* sp., expressed in *Trichoderma reesei*) providing 2000 FTU/Kg in each treatment for all dietary phases. Tables 3.2 and 3.3 show the dietary ingredients used with the calculated nutrient composition for the four phases of the study, including the formulated Ca and P with the analysed Ca and P values of the treatment diets. The experimental feeds were mixed, pelleted, and packed at a feed mill specialising in experimental diet mixing. To ensure diets contained the correct Ca and P levels, a large batch of basal diet was first mixed containing all macro ingredients. The basal diet was then used to formulate the dietary treatments by adding limestone and monocalcium diphosphate (MDCP) to obtain the correct Ca and P level for each dietary treatment. To avoid cross contamination, treatments with fast solubilising limestone were mixed first, followed by treatments with slow solubilising limestone within the dietary Ca and P recommendations (REC) main effects i.e., NL (Dutch Nutrition Group), UMD (University of Maryland), and COM (Commercial). A total of 28 dietary samples were analysed in duplicate for Ca and P (Table 3.2 and 3.3) using the method 935.13 (AOAC, 1999).

Table 3.1. Recommendations for Ca, P, and limestone solubility for each treatment per dietary phase

Phase	Pre-Starter (0-9)								Starter (10-15)					
Recommendations	NL		UMD				COM		NL		UMD		COM	
Treatments and limestone solubility[1]	T1 SS[2]	T2 FS[3]	T3.1 SS	T3.2 SS	T4.1 FS	T4.2 FS	T5 SS	T6 FS	T1 SS	T2 FS	T3 SS	T4 FS	T5 SS	T6 FS
Total Ca (%)*	0.84	0.84	0.94	0.89	0.94	0.89	0.96	0.96	0.75	0.75	0.89	0.89	0.87	0.87
Av Phosphorus (%)	0.35	0.35	0.51	0.38	0.51	0.38	0.48	0.48	0.29	0.29	0.38	0.38	0.44	0.44

oP**	0.38	0.38	0.53	0.40	0.53	0.40	0.51	0.51	0.31	0.31	0.40	0.40	0.45	0.45
Phase	Grower (16-23)										Finisher (24-32)			
Recommendations	NL				UMD		COM		NL		UMD		COM	
Treatments and limestone solubility[1]	T1.1 SS	T1.2 SS	T2.1 FS	T2.2 FS	T3 SS	T4 FS	T5 SS	T6 FS	T1 SS	T2 FS	T3 SS	T4 FS	T5 SS	T6 FS
Total Ca (%)*	0.75	0.75	0.75	0.75	0.80	0.80	0.87	0.87	0.75	0.75	0.69	0.69	0.79	0.79
Av Phosphorus (%)	0.29	0.28	0.29	0.28	0.28	0.28	0.44	0.44	0.28	0.28	0.28	0.28	0.40	0.40
oP**	0.31	0.28	0.31	0.28	0.31	0.31	0.46	0.46	0.28	0.28	0.31	0.31	0.42	0.42

[1] Treatments structured as (**Pre-Starter diets**): Dutch Nutrition Group (NL), T1 (Slow Solubilizing limestone) and T2 (Fast solubilizing limestone); University of Maryland (UMD), T3.1 pre-starter1 (Slow solubilizing limestone), T3.2 pre-starter2 (Slow solubilizing limestone) and T4.1 pre-starter1 (Fast solubilizing limestone), T4.2 pre-starter2 (Fast solubilizing limestone); Commercial (COM), T5 (Slow solubilizing limestone) and T6 (Fast solubilizing limestone). **Starter diets:** Dutch Nutrition Group (NL), T1 (Slow Solubilizing limestone) and T2 (Fast solubilizing limestone); University of Maryland (UMD), T3 (Slow solubilizing limestone) and T4 (Fast solubilizing limestone); Commercial (COM), T5 (Slow solubilizing limestone) and T6 (Fast solubilizing limestone). **Grower diets**: Dutch Nutrition Group (NL), T1.1 grower1 (Slow Solubilizing limestone), T1.2 grower2 (Slow Solubilizing limestone) and T2.1 grower1 (Fast solubilizing limestone), T2.2 grower2 (Fast solubilizing limestone); University of Maryland (UMD), T3 (Slow solubilizing limestone) and T4 (Fast solubilizing limestone); Commercial (COM), T5 (Slow solubilizing limestone) and T6 (Fast solubilizing limestone). **Finisher diets**: Dutch Nutrition Group (NL), T1 (Slow Solubilizing limestone) T2 (Fast solubilizing limestone); University of Maryland (UMD), T3 (Slow solubilizing limestone) and T4 (Fast solubilizing limestone); Commercial (COM), T5 (Slow solubilizing limestone) and T6 (Fast solubilizing limestone).

[2]SS (Slow Solubilizing limestone).

[3]FS (Fast solubilizing limestone).

*Total Ca formulated to includes a 0.189% Ca contribution from 2000 FTU of AxtraPhy Gold phytase.

** Digestible phosphorus (oP)

The pre-starter diets were in overall fed from 0 – 8d. The NL treatments (Trt 1 and 2) were fed one pre-starter diet in the phase. The COM and UMD treatments were fed as pre-starter1 (Trt 3.1, 0-6d), pre-starter2 (Trt 3.2, 7-8d); and pre-starter1 (Trt 4.1, 0-6d), and pre-starter2 (Trt 4.2, 7-8d), respectively. While the COM treatments (Trt 5 and 6) were fed one pre-starter diet in the phase. One starter diet was fed from 9 – 13d to all treatments. The grower diets were fed from 14 – 22d. The NL treatments were fed as grower1 (Trt 1.1, 14-18d), grower2 (Trt1.2, 19-22d). The UMD treatments (Trt 3 and 4), and the COM treatment (Trt 5 and 6) were all fed from 14d to 22d. The finisher diets for all treatments (Trt 1 to 6, irrespective of recommendations) were fed from 23d to 32d (Table 3.1).

Table 3.2. Ingredient composition and nutrient content of the treatment diets (g/Kg, as fed basis unless otherwise stated) for pre-starter and starter phases

Ingredient (% as fed)			Pre-Starter (0-8)						Starter (9-13)					
	NL		UMD				COM		NL		UMD		COM	
Treatments[1]	T1	T2	T3.1	T.32	T4.1	T4.2	T5	T6	T1	T2	T3	T4	T5	T6
Yellow corn (8.5%)	53.66	53.66	53.09	53.09	53.09	53.09	53.13	53.13	58.37	58.37	57.00	57.00	56.73	56.73
Full fat soya	9.80	9.80	10.00	10.00	10.00	10.00	10.00	10.00	8.00	8.00	8.00	8.00	8.00	8.00
Soyabean meal (44%)	29.43	29.43	29.36	29.36	29.36	29.36	29.36	29.36	26.97	26.97	27.23	27.23	27.28	27.28
Soya oil	0.50	0.50	0.65	0.65	0.65	0.65	0.64	0.64	0.96	0.96	1.42	1.42	1.51	1.51
Sunflower cake (38%)	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Sodium Chloride	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
Sodium Bicarbonate	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.18	0.18	0.18	0.18	0.18	0.18
Ca free Premix[2]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
L-Lysine (HCL) (%)	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.28	0.28	0.27	0.27	0.27	0.27
L-Methionine (%)	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.33	0.33	0.33	0.33	0.33	0.33
L-Threonine (%)	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.16	0.16	0.16	0.16	0.16	0.16
L-Valine (%)	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.05	0.05	0.05	0.05	0.05	0.05
Limestone (%)	1.28	1.28	1.13	1.35	1.13	1.35	1.26	1.26	1.22	1.23	1.36	1.36	1.14	1.15
MDCP[3]	0.36	0.36	1.24	0.48	1.24	0.48	1.10	1.10	-	-	0.52	0.52	0.86	0.86
Choline Chloride (60%)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Cycostat (Robenidine 6.6%) 0.05%	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Zinc Bacitracin 15%	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Nutrient composition[4]														
Metabolisable Energy (MJ/Kg)	11.66	11.66	11.66	11.66	11.66	11.66	11.66	11.66	12.00	12.00	12.00	12.00	12.00	12.00
Crude Protein (%)	23.27	23.27	23.27	23.27	23.27	23.27	23.27	23.27	21.82	21.82	21.82	21.82	21.82	21.82
Crude fibre (%)	4.29	4.29	4.29	4.29	4.29	4.29	4.29	4.29	4.24	4.24	4.21	4.21	4.20	4.20
Crude fat (%)	5.05	5.05	5.20	5.20	5.20	5.20	5.20	5.20	5.31	5.32	5.72	5.72	5.80	5.80
TSAA[5] (%)	0.72	0.72	0.71	0.71	0.71	0.71	0.71	0.71	0.68	0.68	0.68	0.68	0.68	0.68
Total Ca (%) *	0.84	0.84	0.94	0.89	0.94	0.89	0.96	0.96	0.75	0.75	0.89	0.89	0.87	0.87
Analysable Ca (%)	0.65 (0.63)[5]	0.65 (0.67)	0.75 (0.80)	0.70 (0.74)	0.75 (0.72)	0.70 (0.68)	0.77 (0.72)	0.77 (0.82)	0.56 (0.53)	0.56 (0.56)	0.70 (0.67)	0.70 (0.67)	0.68 (0.73)	0.68 (0.72)
Total Phosphorus (%)	0.43 (0.47)	0.43 (0.44)	0.62 (0.57)	0.46 (0.42)	0.62 (0.65)	0.46 (0.47)	0.59 (0.62)	0.59 (0.64)	0.34 (0.37)	0.34 (0.35)	0.45 (0.40)	0.45 (0.49)	0.52 (0.49)	0.52 (0.52)
Av Phosphorus (%)	0.35	0.35	0.51	0.38	0.51	0.38	0.48	0.48	0.29	0.29	0.38	0.38	0.44	0.44
Digestible phosphorus	0.38	0.38	0.53	0.40	0.53	0.40	0.51	0.51	0.31	0.31	0.40	0.40	0.46	0.46

[1] Treatments structured as **(Pre-Starter diets):** Dutch Nutrition Group (NL), T1 (Slow Solubilizing limestone) and T2 (Fast solubilizing limestone); University of Maryland (UMD), T3.1 pre-starter1 (Slow solubilizing limestone), T3.2 pre-starter2 (Slow solubilizing limestone) and T4.1 pre-starter1 (Fast solubilizing limestone), T4.2 pre-starter2 (Fast solubilizing limestone); Commercial (COM), T5 (Slow solubilizing limestone) and T6 (Fast solubilizing limestone). **Starter diets:** Dutch Nutrition Group (NL), T1 (Slow Solubilizing limestone) and T2 (Fast solubilizing limestone); University of Maryland (UMD), T3 (Slow solubilizing limestone) and T4 (Fast solubilizing limestone); Commercial (COM), T5 (Slow solubilizing limestone) and T6 (Fast solubilizing limestone).[2] Provided the following vitamins and minerals per kilogram of diets: vitamin A, 1333333.333 IU (retinyl acetate); cholecalciferol, 555555.555 IU; vitamin E, 6666.666 mg; vitamin B12, 1.111 mg; menadione, 222.222 mg; B2 riboflavin, 555.555 mg; B1 thiamine, 222.222 mg; B5 pantothenic acid, 1333.333 mg; B3 niacin, 5555.555 mg; folic acid, 222.222 mg; biotin, 11.111 mg; pyridoxine, 333.33 mg. Antioxidant, 13888.889 mg; calcium (Ca), 0 mg; manganese (Mn2(OH)3Cl), 12222.222 mg; zinc (Zn5(OH)8Cl2), 11111.111 mg; iron (FeSO4•7H2O), 4444.444 mg; copper (Cu2(OH)3Cl), 1111.11 mg; selenium (Na2SeO3), 33.333 mg; iodine (Ca(IO3)2), 222.222 mg; cobalt (Co), 55.555 mg; choline (C5H14NO), 38888.889 mg. phytase 2000 FTU/kg (0.189 Ca & P contribution), Syncra AVI 101 (1000 xylanase).

[3] MDCP: monocalcium diphosphate.

[4] Values in parenthesis represent analysed nutrient contents.

[5] TSAA: total Sulphur amino acids.

*Total Ca formulated to includes a 0.189% Ca contribution from 2000 FTU of AxtraPhy Gold phytase.

Table 3.3. Ingredient composition and nutrient content of the treatment diets (g/Kg, as fed basis unless otherwise stated) for the grower and finisher phases

Ingredient (% as fed)	Grower (14-22)								Finisher (23-32)					
	NL				UMD		COM		NL		UMD		COM	
Treatments[1]	T1.1	T1.2	T2.1	T2.2	T3	T4	T5	T6	T1	T2	T3	T4	T5	T6
Yellow corn (8.5%)	62.53	62.53	62.51	62.51	62.24	62.24	61.96	61.96	67.14	67.14	62.26	62.26	60.97	60.97
Full fat soya	9.30	9.30	9.32	9.32	10.00	10.00	10.00	10.00	7.50	7.50	12.00	12.00	12.00	12.00
Soyabean meal(44%)	21.87	21.87	21.85	21.85	21.33	21.33	20.65	20.65	17.90	17.90	18.89	18.89	19.13	19.13
Soya oil	0.70	0.70	0.70	0.70	0.70	0.70	0.91	0.91	1.28	1.28	1.31	1.31	1.75	1.75
Sunflower cake(38%)	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Sodium Chloride	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
Sodium Bicarbonate	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Ca free Premix[2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
L-Lysine (HCL) (%)	0.27	0.27	0.27	0.27	0.27	0.27	0.29	0.29	0.44	0.44	0.29	0.29	0.29	0.29
L-Methionine (%)	0.28	0.28	0.28	0.28	0.28	0.28	0.29	0.29	0.28	0.28	0.29	0.29	0.29	0.29
L-Threonine (%)	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.14	0.17	0.17	0.14	0.14	0.14	0.14
L-Valine (%)	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.06	0.09	0.09	0.06	0.06	0.06	0.06
Limestone (%)	1.23	1.25	1.24	1.26	1.39	1.39	1.16	1.16	1.27	1.21	1.09	1.09	1.06	1.06
MDCP[3]	0.03	-	0.03	-	-	-	0.88	0.88	-	-	-	-	0.65	0.65
Choline Chloride (60%)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Cycostat (Robenidine 6.6%) 0.05%	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Zinc Bacitracin 15%	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Nutrient composition[4]														
Metabolisable Energy (MJ/Kg)	12.20	12.20	12.20	12.20	12.20	12.20	12.20	12.20	12.50	12.50	12.50	12.50	12.50	12.50
Crude Protein (%)	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Crude fibre (%)	4.18	4.18	4.18	4.18	4.18	4.18	4.13	4.13	4.04	4.04	4.16	4.16	4.13	4.13
Crude fat (%)	5.36	5.36	5.36	5.36	5.47	5.47	5.65	5.65	5.71	5.71	6.37	6.37	6.76	6.76
TSAA[5] (%)	0.64	0.64	0.64	0.64	0.64	0.64	0.63	0.63	0.59	0.59	0.63	0.63	0.62	0.62
Total Ca (%)*	0.75	0.75	0.75	0.75	0.80	0.80	0.87	0.87	0.75	0.75	0.69	0.69	0.79	0.79
Analysable Ca (%)	0.56 (0.57)[5]	0.56 (0.55)	0.56 (0.60)	0.56 (0.59)	0.61 (0.64)	0.61 (0.61)	0.68 (0.66)	0.68 (0.69)	0.56 (0.57)	0.56 (0.55)	0.50 (0.49)	0.50 (0.55)	0.60 (0.64)	0.60 (0.58)
Total Phosphorus (%)	0.33 (0.33)	0.32 (0.27)	0.33 (0.34)	0.32 (0.32)	0.33 (0.34)	0.33 (0.33)	0.51 (0.52)	0.51 (0.48)	0.32 (0.32)	0.32 (0.32)	0.32 (0.32)	0.32 (0.32)	0.46 (0.50)	0.46 (0.43)
Av Phosphorus (%)	0.29	0.28	0.29	0.28	0.28	0.28	0.44	0.44	0.28	0.28	0.28	0.28	0.40	0.40
Digestible phosphorus	0.31	0.28	0.31	0.28	0.31	0.31	0.46	0.46	0.28	0.28	0.31	0.31	0.42	0.42

[1] Treatments structured as **(Grower diets):** Dutch Nutrition Group (NL), T1.1 grower1 (Slow Solubilizing limestone), T1.2 grower2 (Slow Solubilizing limestone) and T2.1 grower1 (Fast solubilizing limestone), T2.2 grower2 (Fast solubilizing limestone); University of Maryland (UMD), T3 (Slow solubilizing limestone) and T4 (Fast solubilizing limestone); Commercial (COM), T5 (Slow solubilizing limestone) and T6 (Fast solubilizing limestone). **Finisher diets:** Dutch Nutrition Group (NL), T1 (Slow Solubilizing limestone) T2 (Fast solubilizing limestone); University of Maryland (UMD), T3 (Slow solubilizing limestone) and T4 (Fast solubilizing limestone); Commercial (COM), T5 (Slow solubilizing limestone) and T6 (Fast solubilizing limestone).

[2] Provided the following vitamins and minerals per kilogram of diets: vitamin A, 1600000 IU (retinyl acetate); cholecalciferol, 666666.67 IU; vitamin E, 8000 mg; vitamin B12, 1.3332 mg; menadione, 266.6668 mg; B2 riboflavin, 666.6668 mg; B1 thiamine, 266.6668 mg; B5 pantothenic acid, 1600 mg; B3 niacin, 6666.6668 mg; folic acid, 266.6668 mg; biotin, 13.3332 mg; pyridoxine, 400 mg. Antioxidant, 16666.667 mg; calcium (Ca), 0 mg; manganese (Mn2(OH)3Cl), 14666.667 mg; zinc (Zn5(OH)8Cl2), 13333.333 mg; iron (FeSO4•7H2O), 5333.3332 mg; copper (Cu2(OH)3Cl), 1333.3332 mg; selenium (Na2SeO3), 40 mg; iodine (Ca(IO3)2), 266.6668 mg; cobalt (Co), 66.6668 mg; choline (C5H14NO), 46666.667 mg. phytase 2000 FTU/kg (0.189 Ca & P contribution), Syncra AVI 101 (1000 xylanase).

[3] MDCP: monocalcium diphosphate.

[5] TSAA: total Sulphur amino acids.

*Total Ca formulated to includes a 0.189% Ca contribution from 2000 FTU of AxtraPhy Gold phytase.

3.4 Tibia Ash

At 10d and 32d of age, the body weight from each pen was recorded, and treatment means were determined. Five birds within 305 and 332 grams of the average BW were selected from each replicate pen and euthanised with cervical dislocation, totalling 360 birds. On day 32, five birds within 1969 and 2160 grams of the average BW were selected totalling 360 birds sampled for tibia analysis.

The bird's right leg was used to sample the right tibia for ash analysis. Post thawing, all adhering flesh on the right tibias was removed. Post flesh and cartilaginous cap removal, pooled tibia weights (five tibias) were recorded before oven drying and defatting for dry matter calculations. The tibias were dried in an oven at 70^{o}C for 72hrs before defatting with a refluxing petroleum ether (Li *et al*., 2015). Using the Soxhlet fat extractor, tibias were placed in the apparatus using thimbles. Each batch was fully immersed in the petroleum ether and underwent fat extraction for seven days at 30^{o}C. On the fourth day of the extraction, thimbles at the top of the Soxhlet would be rotated to the bottom. The apparatus was switched off daily for 15hrs during knock off hours as a safety measure. The bones were ashed in a muffle furnace as per AOAC (2000) method 4.1.10 (942.05). The tibia Ash weight was determined for the tibias sampled at 10 and 32 days of age.

3.5 Limestone Solubility

Table 3.4. Limestone solubility profile

		Time in minutes		
Sample	Particle size & Solubility	T5	T15	T30
Sample A	Slow solubilizing	43.5	62.5	96.4
Sample B	Fast solubilizing	95.0	95.6	95.8

Two commercially available limestones (LS) were used, namely, fast solubilising (FS-LS) or slow solubilising (SS-LS). Ten samples were collected from the two limestone batches. Five were from the SS batch of limestone bags, and the other five were from the FS batch of limestone bags. Sampling was done at three different points of each bag. The solubility profile of the samples was determined (Table 3.4) at 5, 15, and 30 minutes of incubation using a pH 3 HCL buffered solution with 3M of glycine or 140 mL of 0.2N HCL (modified from Zhang *et al.*, (1997a). The 0.2N HCL solution was prepared with ionized water (modified from Zhang *et al.*, (1997b), thereby mimicking the pH level of the proventriculus and the ventriculus. The pH was elevated with NaOH, where it fell below the pH of 3. Each sample solubility profile was run in duplicate; the coefficient of variation and the percentage of limestone solubility were determined. Limestone solubility for each of the 10 samples was determined by taking a representative sub-sample of 1 g per sample, which was

separately weighed into 250 mL Erlenmeyer flasks. The flasks were then placed in a shaking water bath at 42^{o}C for 5, 10, and 30 minutes. Digestion was stopped by adding 100 mL of ice-cold distilled water into the flask. All contents in the flask were immediately filtered using a pre-weighed MN 640w ash-less filter paper equivalent to Whatman No.40 filter paper. Further ice-cold distilled water was used to wash out the remaining limestone particles in the flask. The filter papers were placed in pre-weighed Petri dishes and then oven dried at 70^{o}C for 12hrs. Post drying, the undigested limestone was weighed, and the disappearance gave the solubility profile of the samples (Kim *et al.*, 2019).

3.6 Data Collection

Performance data were collected throughout the experimental period of the study. The birds were weighed per pen at 0, 7, 10, 14, 21, 28 and 32d. Cumulative FI was calculated at 0 – 7, 0 – 14, 0 – 21, 0 – 28, and 0 – 32d. The average body weight (BW; g), feed conversion ratio (FCR; feed; g/ BWG; g)/mortality corrected FCR (cFCR); feed; g/BWG; g + total mortality; g), and FI (g feed/bird) were cumulatively calculated from 0 – 7, 0 – 14, 0 – 21, 0 – 28, and 0 – 32 days. Cumulative FI was calculated as the Total feed added into bins minus feed taken out of the bins (of which was the weight of bin & feeder with feed minus the empty bin & feeder weight). The FCR was calculated as cumulative FI divided by BWG (g/g), where the mortality correct FCR (cFCR) was calculated as cumulative FI divided by BWG plus total mortality weight (g/g) and was used to represent all result and discussion related to feed conversion. Daily mortalities were recorded, which included culling for runts and birds with leg deformities, and cumulative leg culls and mortalities were quantified. Both dead and culled birds were weighed, and no necropsy was performed.

The P consumed was the sum product calculated from the P analysed values of each treatment per phase from the total feed allocated in each pen. This was calculated as: P consumed = (pre-starter total feed allocation x analysed P%) + (starter total feed allocation x analysed P%) + (grower total feed allocation x analysed P%) + (finisher total feed allocation x analysed P%). The same methodology was followed to calculate the Ca consumed, where Ca consumed = (pre-starter total feed allocation x analysed Ca%) + (starter total feed allocation x analysed Ca%) + (grower total feed allocation x analysed Ca%) + (finisher total feed allocation x analysed Ca%). Also, diets which had 2 diet allocations per phase (e.g. pre-starter1 and 2) were incorporated in the formula for both Ca and P consumed.

Footpad dermatitis (FPD) and hock burn (HB) scoring were conducted by visual and palpation inspection at 32d by selecting 15 birds per pen at random. The lesions were scored using a five-point scale system with a 0 - 4 rating based on the scales described by (Kaukonen *et al.*, 2016). While the

HB were rated on a three-point scale of 0 – 2, where 0 (had no burns), 1 (red hock without black marks/scars), and 2 (severe hock burn with black marks and scars) (Welfare, 2009).

The economic analysis in the current study should not be mistaken for cost of production which would consider cost of chick price, environmental conditioning, labour, medication amongst others. The economics of the study with regards the feed cost per bird and feed cost per kilogram harvested at 32 days of age was conducted using raw material prices of December 2021. Both feed cost/bird and feed cost/kg harvested were quantified based on live birds per pen at 32 days of age. Feed cost per pen was calculated as [(total FI of all phases/pen ÷ total tons produced for each Trt)*Trt diet cost in Rands (ZAR). While feed cost/bird (ZAR) was calculated as (feed cost/pen ÷ live bird count/pen at 32d). Kilograms harvested per pen were quantified as total pen weight/Trt at 32d. Therefore, feed cost/kg harvested (ZAR) was calculated as (feed cost/pen ÷ kg harvested at 32d).

3.7 Experimental Design

The experimental design was a 2x3 factorial design with two different solubilising limestone (slow and fast soluble) and three Ca and P REC with six treatments, each with 12 replicates. Treatment allocation was arranged in a completely randomised blocking design.

3.8 Statistical Analysis

Each pen represented an experimental unit. The data were analysed as a 2x3 factorial design, where each of the six treatments combinations with 12 replicates were randomly allocated to 12 blocks treated as random effects. Where the slow soluble limestone and the fast-soluble limestone were the 2 factors in the design, and the 3 factors were the Ca and P recommendations namely NL, UMD, and COM recommendations. Statistical software JMP (V.15) (SAS Inst. Inc., Cary, NC, 2016) on a two-way ANOVA in a mixed model was used to analyse performance data. A protected Tukey's test (Tukey, 1949) was run for differences in means, where significant differences were regarded at $P \leq 0.05$ significance level. Factors affecting FPD and HB were analysed using a Rao-Scott Chi-square (Lavassani *et al.*, 2009).

Chapter 4

Results

4.1 Growth Performance

The analysed dietary values for Ca and P were within acceptable ranges as per the dietary formulation (Tables 3.2 and 3.3). There were no significant interactions (P > 0.05) for BW, FI, FCR, leg deformities and mortality between the Ca and P REC and LS solubility throughout the study period of 32 days (Table 4.1). At 7d of age, the NL (197g) REC resulted in lighter birds (P < 0.01) than UMD (202g) and COM (200g) REC, respectively. At 14d, UMD (537g) birds were heavier (P < 0.05) than COM (531g) and NL (528g) REC, respectively (P < 0.05). At 21d, both the COM (1039g) and UMD (1039g) birds were heavier (P < 0.05) than birds reared as per the NL (1018g) REC. The BW performance was higher (P < 0.01) on COM at 28d and 32d (1688g; 2037g) REC and on UMD (1682g; 2032g), respectively, than on the NL (1642g; 1979g) REC at the same period (P < 0.01) (Table 4.1). The limestone solubility effect was only observed at 32d, where the slow soluble LS improved (P < 0.05) the 32d BW (2028g vs. 2005g) compared with birds fed the fast-soluble LS (Table 4.1).

Table 4.1. Influence of dietary calcium level and limestone solubility on broiler body weight performance and feed conversion efficiency

Main effects			Body weight (g)[1]						cFCR (g:g)[2]				
REC[3]			0d	7d	14d	21d	28d	32d	7d	14d	21d	28d	32d
	NL		40.7	197^{b}	528^{b}	1018^{b}	1642^{b}	1979^{b}	1.06^{a}	1.11^{ab}	1.21	1.30^{a}	1.36^{a}
	UMD		40.7	202^{a}	537^{a}	1039^{a}	1682^{a}	2032^{a}	1.02^{b}	1.10^{b}	1.20	1.27^{b}	1.33^{c}
	COM		40.9	200^{a}	531^{ab}	1039^{a}	1688^{a}	2037^{a}	1.05^{a}	1.12^{a}	1.20	1.28^{a}	1.34^{b}
	SEM		0.11	0.87	2.66	5.11	8.23	10.2	0.005	0.003	0.002	0.002	0.003
	P-value		0.18	< .001	0.04	0.003	< .001	< .001	<.001	0.03	0.09	<.001	<.001
LS[4]													
	Fast		40.8	199	531	1030	1664	2005^{b}	1.04	1.11	1.21	1.29	1.35
	Slow		40.7	201	533	1034	1678	2028^{a}	1.04	1.10	1.20	1.28	1.34
	SEM		0.10	0.72	2.24	4.25	7.14	8.66	0.004	0.003	0.002	0.002	0.002
	P-value		0.40	0.16	0.61	0.54	0.10	0.04	0.94	0.51	0.06	0.06	0.12
Trt[5]	REC	LS											
T1	NL	Slow	40.5	197	530	1017	1646	1989	1.07	1.11	1.21	1.29	1.35
T2		Fast	40.8	197	527	1018	1638	1969	1.05	1.10	1.21	1.30	1.36
T3	UMD	Slow	40.8	203	535	1040	1691	2038	1.02	1.09	1.20	1.27	1.33
T4		Fast	40.6	202	538	1038	1674	2026	1.03	1.10	1.20	1.27	1.33
T5	COM	Slow	40.8	202	533	1044	1696	2056	1.04	1.11	1.20	1.28	1.34
T6		Fast	40.9	199	529	1034	1680	2019	1.05	1.11	1.21	1.29	1.35
SEM			0.14	1.21	3.63	7.10	10.8	14.0	0.007	0.005	0.004	0.004	0.004
(*P*-values) REC X L			0.24	0.39	0.54	0.76	0.88	0.64	0.07	0.36	0.51	0.67	0.63

[1]Body weight: Quantified cumulatively from day 0 to day 7, 14, 21, 28, and 32.

[2]cFCR: Mortality corrected feed conversion ratio (FCR), quantified cumulatively from day 0 to day 7, 14, 21, 28, and 32.
[3]REC: Ca and P Recommendations (Dutch Nutrition Group (NL), University of Maryland (UMD), Commercial (COM).
[4]LS: Limestone solubility (Fast or Slow).
[5]Trt: Denotes dietary treatments.
[a,b,c] Column means with same superscript do not differ significantly (P < 0.05)

There were no significant differences on placement BW at 0d (*P* > 0.05) (Table 4.1). Birds on UMD REC were more efficient (*P* < 0.001) compared to COM and NL (1.02, 1.05, and 1.06, respectively) at 7d of age (Table 4.1). There were significant differences in FCR at 14d amongst the three Ca and P REC, namely UMD REC, NL REC, and COM REC (*P* < 0.05) (1.10, 1.11, and 1.12, respectively) (Table 4.1). There was no effect of the three Ca and P REC on FCR *(P* > 0.05) at 21d of age (Table 4.1). The UMD (1.27) Ca and P REC resulted in low FCR (*P* < 0.001) at 28d compared to both COM (1.28) and NL (1.30). At 32 days of age; compared to both COM REC (1.34) and NL (1.36), the UMD REC (1.33) resulted in lower FCR (P <0.001) (Table 4.1).

Compared to COM and NL REC, birds on UMD REC showed the lowest *(P* < 0.05) FI (166g, 166g, and 168g, respectively) (Table 4.2) at 7d. Feeding birds as per the NL REC reduced *(P* < 0.05) cumulative FI compared to UMD and COM at 14d (595g, 604g, and 607g, respectively), at 21d (1243g, 1267g, and 1275g, respectively), and at 28d (2173g, 2176g, and 2206g, respectively) (Table 4.2). No significant differences *(P* > 0.05) were observed at 32d in cumulative FI for COM, UMD, and NL REC (Table 4.2).

Table 4.2. Impact of dietary calcium level and limestone solubility speed on broiler feed intake

Main effects			Feed Intake (g)[1]				
REC[2]			7d	14d	21d	28d	32d
	NL		166[ab]	595[b]	1243[b]	2173[b]	2774
	UMD		166[b]	604[ab]	1267[a]	2176[ab]	2770
	COM		168[a]	607[a]	1275[a]	2206[a]	2792
	SEM		0.84	2.70	5.54	9.83	14.8
	P-value		0.04	0.008	< .001	0.03	0.55
LS[3]							
	Fast		166	601	1260	2181	2769
	Slow		167	603	1264	2189	2789
	SEM		0.72	2.21	4.62	8.09	12.2
	P-value		0.09	0.56	0.55	0.50	0.24
Trt[4]	REC	LS					
T1	NL	Slow	167	601	1245	2177	2795
T2		Fast	165	589	1242	2169	2754
T3	UMD	Slow	166	601	1268	2173	2770
T4		Fast	165	608	1266	2179	2770
T5	COM	Slow	169	608	1277	2216	2801
T6		Fast	167	607	1272	2196	2782

SEM	1.08	3.89	7.67	13.3	20.7
(*P*-values) REC X L	0.62	0.05	0.97	0.60	0.60

[1] Feed Intake: Quantified cumulatively from day 0 to day 7, 14, 21, 28, and 32.
[2]REC: Ca and P Recommendations (Dutch Nutrition Group (NL), University of Maryland (UMD), Commercial (COM).
[3]LS: Limestone solubility (Fast or Slow).
[4]Trt: Denotes dietary treatments.
[a,b] Column means with same superscript do not differ significantly (P < 0.05).

There were no significant differences *(P* > 0.05) observed in the Ca and P REC for the first 7 days of age with regards to leg culls. However, there were significant differences *(P* < 0.05) amongst the Ca and P RECs at 14d and 21d. Feeding as per the NL REC greatly increased *(P* < 0.05) the incidences of leg culls at 14d compared to the UMD and COM REC (0.37%, 0.08%, and 0.04%, respectively) (Table 4.3). At 21d, the NL REC showed the highest *(P* < 0.05) incidences of leg culls compared to UMD and COM REC (0.41%, 0.08%, and 0.08%, respectively) (Table 4.3). Furthermore, dietary Ca and P REC had no significant effect *(P* > 0.05) on leg weakness post the 21d to 32d period. Limestone solubility only had an impact *(P* < 0.05) on leg health at 7d, where leg culls due to FS-LS (0.25%) were higher than SS-LS (0.02%) (Table 4.3), which after that LS solubility had no effect *(P* > 0.05) on leg culls for the remainder of the study.

The Ca and P REC accentuated the mortality rate from 28d of age (Table 4.3). Feeding as per the COM (1.08%) REC at 28d improved *(P* < 0.05) the mortality rate compared to the UMD (1.29%) REC, while the NL (1.91%) REC increased *(P* < 0.05) the mortality rate during the same period (Table 4.3). A similar trend was observed at 32d where the NL (2.62%) REC had a high *(P* < 0.05) mortality rate compared to UMD (1.87%) and COM (1.54%) REC. Limestone solubility throughout the study period of 32d had no impact *(P* > 0.05) on mortality rate.

Table 4.3. Effect of dietary calcium and phosphorus recommendation and limestone solubility speed on broiler leg deformities and mortality rate in broiler chickens from 0 to 32 days of age

Main effects		Cumulative leg culls (%)[1]					Mortality rate (%)[2]				
REC[3]		7d	14d	21d	28d	32d	7d	14d	21d	28d	32d
	NL	0.29	0.37[a]	0.41[a]	0.45	0.54	0.26	0.70	1.29	1.91[a]	2.62[a]
	UMD	0.08	0.08[ab]	0.08[b]	0.16	0.25	0.37	0.41	0.77	1.29[ab]	1.87[ab]
	COM	0.04	0.04[b]	0.08[b]	0.20	0.25	0.21	0.54	0.68	1.08[b]	1.54[b]
	SEM	0.09	0.09	0.09	0.11	0.12	0.10	0.14	0.17	0.20	0.26
	P-value	0.14	0.04	0.03	0.17	0.16	0.62	0.48	0.05	0.03	0.02
LS[4]											
	Fast	0.25[a]	0.25	0.27	0.30	0.38	0.22	0.50	0.86	1.36	1.94
	Slow	0.02[b]	0.08	0.11	0.25	0.30	0.35	0.60	0.97	1.50	2.08
	SEM	0.07	0.07	0.07	0.09	0.09	0.08	0.10	0.13	0.15	0.20
	P-value	0.04	0.15	0.16	0.68	0.56	0.35	0.58	0.60	0.59	0.67

Trt[5]	REC	LS										
T1	NL	Slow	0.00	0.16	0.16	0.25	0.33	0.13	0.65	1.33	2.00	2.75
T2		Fast	0.58	0.58	0.66	0.66	0.75	0.39	0.75	1.25	1.83	2.50
T3	UMD	Slow	0.08	0.08	0.08	0.16	0.25	0.50	0.58	1.05	1.33	1.91
T4		Fast	0.08	0.08	0.08	0.16	0.25	0.25	0.25	0.50	1.25	1.83
T5	COM	Slow	0.00	0.00	0.08	0.33	0.33	0.41	0.58	0.54	1.16	1.58
T6		Fast	0.08	0.08	0.08	0.08	0.16	0.01	0.50	0.83	1.00	1.50
SEM			0.13	0.13	0.14	0.16	0.17	0.15	0.22	0.25	0.30	0.38
(*P*-values) REC X L			0.07	0.30	0.15	0.14	0.23	0.14	0.65	0.32	0.98	0.97

[1]Cumulative leg culls: Quantified daily and totalled by phase at day 7, 14, 21, 28, and 32.
[2]Mortality rate: Quantified daily and totalled by phase at day 7, 14, 21, 28, and 32.
[3]REC: Ca and P Recommendations (Dutch Nutrition Group (NL), University of Maryland (UMD), Commercial (COM).
[4]LS: Limestone solubility (Fast or Slow).
[5]Trt: Denotes dietary treatments.
[a,b] Column means with same superscript do not differ significantly (P < 0.05).

4.2 Bone Mineralisation

There was a significant interaction *(P* < 0.05) between the Ca and P REC and LS solubility on tibia ash weight at 10d and 32d (Table 4.4). While the COM Ca and P REC irrespective of LS solubility resulted in the highest tibia ash weight (*P* < 0.05), birds fed as per the UMD REC with a FS-LS had reduced tibia ash weight compared to UMD REC with SS-LS and COM REC at 10d (Table 4.4). Irrespective of LS solubility speed, NL treatments resulted in lower (*P* < 0.05) tibia ash weight than the UMD and COM RECs at 10d (Table 4.4). At 32d, the NL treatments, irrespective of LS solubility continued to reduce *(P* < 0.05) tibia ash weight compared to the UMD and COM treatments (Table 4.4).

Table 4.4. Tibia Ash as an indicator for bone mineralisation during early and late growth stages

Main effects			Tibia Ash weight (g)[1]	
REC[2]			D10 (g)	D32 (g)
	NL		1.58[c]	1.91[c]
	UMD		1.68[b]	2.13[b]
	COM		1.73[a]	2.20[a]
	SEM		0.009	0.01
	P-value		< .001	< .001
LS[3]				
	Fast		1.652[b]	2.10[a]
	Slow		1.68[a]	2.06[b]
	SEM		0.008	0.01
	P-value		0.002	0.03
Trt[4]	REC	LS		
T1	NL	Slow	1.59[bc]	1.86[d]
T2		Fast	1.57[c]	1.96[c]
T3	UMD	Slow	1.72[a]	2.13[ab]
T4		Fast	1.64[b]	2.12[b]
T5		Slow	1.73[a]	2.19[ab]

T6	COM	Fast	1.73[a]	2.21[a]
SEM			0.01	0.02
(*P*-values) REC X L			0.004	0.03

[1]Tibia Ash weight: quantified from tibias pooled per experimental unit.
[2]REC: Ca and P Recommendations (Dutch Nutrition Group (NL), University of Maryland (UMD), Commercial (COM).
[3]LS: Limestone solubility (Fast or Slow).
[4]Trt: Denotes dietary treatments.
[a,b,c,d] Column means with same superscript do not differ significantly (P < 0.05).

4.3 Mineral Excretion in Litter and Foot Health

An interaction ($P < 0.05$) was observed for the total P consumed (g) (calculated based on final BW). The NL treatments irrespective of LS solubility had the lowest P consumption ($P < 0.01$) compared to UMD and COM treatment. The UMD treatment with a SS-LS had lower ($P < 0.01$) P consumption compared to the UMD treatment with a FS-LS and compared to the COM treatments (Table 4.5). The COM treatment with SS-LS had high ($P < 0.01$) P consumption (g) compared to the COM treatment with FF-LS, and compared to both UMD and NL treatments, irrespective of LS solubility. There was an interaction in P excreted (g) per kg harvested where the COM treatments resulted in high ($P < 0.01$) P excretion. In contrast, both the NL treatments and the UMD treatment with SS-LS had reduced mineral P excretion ($P < 0.01$, Table 4.5).

Table 4.5. Analysed calcium and phosphorus excretion in litter

Main effects			Mineral excretion in litter			
REC[1]			P consumed (g)	P excreted per Kg harvested (g/kg)	Ca consumed (g)	Ca excreted per Kg harvested (g/kg)
	NL		401[c]	4.83[c]	670[c]	8.07[c]
	UMD		445[b]	5.16[b]	715[b]	8.30[b]
	COM		603[a]	6.93[a]	800[a]	9.20[a]
	SEM		2.37	0.03	3.59	0.04
	P-value		<.001	<.001	<.001	<.001
LS[2]						
	Fast		480[b]	5.64	720[b]	8.47
	Slow		486[a]	5.64	736[a]	8.57
	SEM		2.01	0.02	3.05	0.03
	P-value		0.01	0.87	<.001	0.08
Trt[3]	REC	LS				
T1	NL	Slow	401[e]	4.83[d]	663[d]	7.99[c]
T2		Fast	401[e]	4.83[d]	676[cd]	8.15[c]
T3	UMD	Slow	432[d]	5.00[d]	740[b]	8.56[b]
T4		Fast	458[c]	5.33[c]	690[c]	8.03[c]
T5	COM	Slow	625[a]	7.10[a]	806[a]	9.16[a]
T6		Fast	580[b]	6.75[b]	794[a]	9.24[a]
SEM			3.22	0.04	4.87	0.06

(*P*-values) REC X L	<.001	<.001	<.001	<.001

[1]REC: Ca and P Recommendations (Dutch Nutrition Group (NL), University of Maryland (UMD), Commercial (COM).
[2]LS: Limestone solubility (Fast or Slow).
[3]Trt: Denotes dietary treatments.
[a,b,c,d,e] Column means with same superscript do not differ significantly (P < 0.05).

An interaction ($P < 0.01$) on Ca consumed (g) was observed (Table 4.5). Both the COM treatments had high ($P < 0.01$) Ca consumption (g) compared to UMD and NL treatments (Table 4.5). The interaction on Ca excreted exhibited an increased ($P < 0.01$) Ca excretion per kg harvested for the COM treatments, irrespective of LS solubility. In contrast, both the NL treatments had the lowest Ca excreted ($P < 0.01$); while the UMD treatment with FS-LS reduced Ca excretion (g) per kg harvested compared to the UMD treatment with SS-LS and to both the COM treatments ($P < 0.01$, Table 4.5).

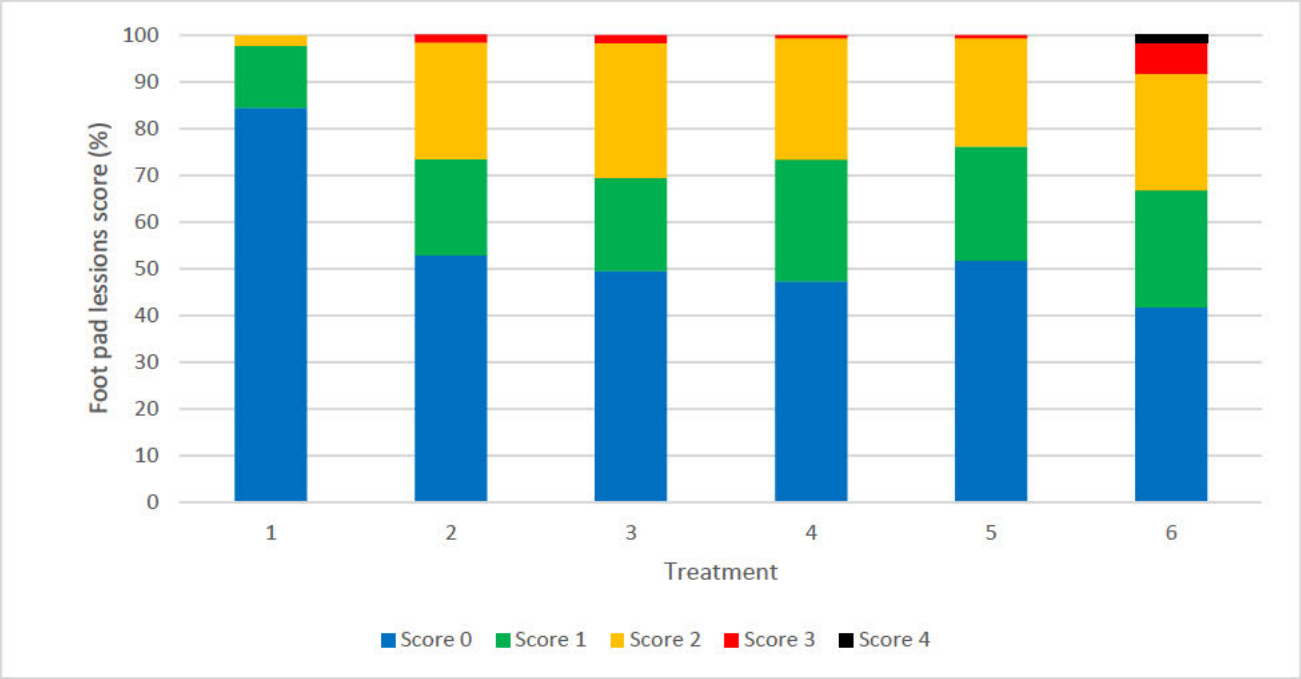

Figure 4.1. Foot pad lesions prevalence score of broiler chickens at 32d. T1 (Dutch Nutrition Group, Slow Solubilizing limestone: NL-SS-LS) and T2 (Fast solubilizing limestone: NL-FS-LS); T3 (University of Maryland, Slow solubilizing limestone: UMD SS-LS) and T4 (Fast solubilizing limestone: UMD FS-LS); T5 (Commercial, Slow solubilizing limestone: COM SS-LS) and T6 (Fast solubilizing limestone: COM FS-LS)

Figures 4.1 and 4.2 show significantly different ($P < 0.001$) prevalence of FPD and HB lesion scores amongst the dietary treatments examined at 32 days of age. The dietary treatments influenced the prevalence of FPD and HB lesions. Regarding the FPD incidence, Trt 1 (NL-SS-LS) resulted in 84.4% of birds having a score of 0, while Trt 6 (COM-FS-LS) had a high prevalence of FPD score of

3 and 4 (6.7% and 1.7%, respectively) compared to all the dietary treatments ($P < 0.001$). Higher incidences of score 1 were observed from Trt 2 to Trt 6 compared to Trt 1, where low incidences of score 1 were observed. A similar trend was observed from Trt 2 to Trt 6 for FPD score 2. A high prevalence of severe HB lesions was observed in the NL and COM treatments (Trt 1 and 2, and Trt 5 and 6, respectively) ($P < 0.001$) (Figure 4.2). While a high incidence of HB lesion score 1 was observed in the UMD treatments (Trt 3 and 4).

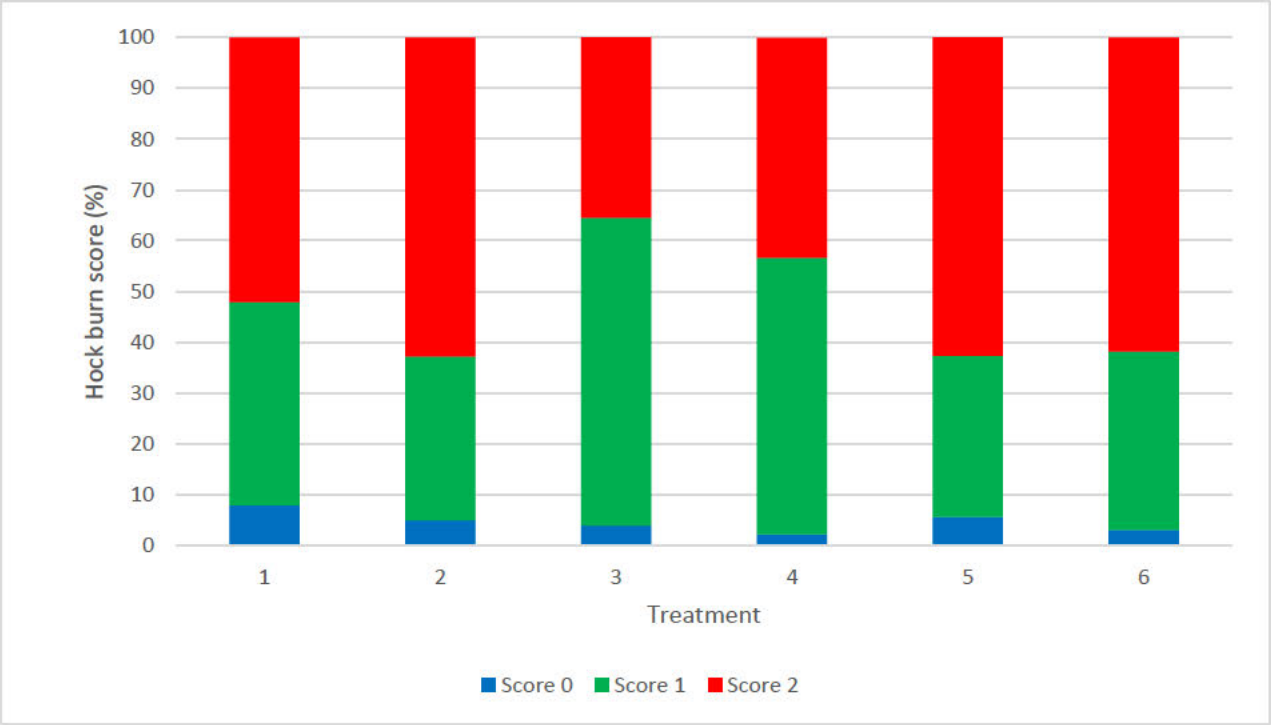

Figure 4.2. Hock burn prevalence score per treatment in broiler chickens at 32d. T1 (Dutch Nutrition Group, Slow Solubilizing limestone: NL-SS-LS) and T2 (Fast solubilizing limestone: NL-FS-LS); T3 (University of Maryland, Slow solubilizing limestone: UMD SS-LS) and T4 (Fast solubilizing limestone: UMD FS-LS); T5 (Commercial, Slow solubilizing limestone: COM SS-LS) and T6 (Fast solubilizing limestone: COM FS-LS)

4.4 Economic analysis

The Ca and P REC, irrespective of LS solubility had an effect ($P > 0.05$) on the cost of feeding a 32d broiler (Table 4.6). Furthermore, LS had no effect ($P > 0.05$) on the cost of feeding a 32-day broiler (Table 4.6). The feed cost/bird of rearing a 32-day broiler was reduced ($P < 0.001$) by feeding the birds as per the NL and UMD REC (15.9 ZAR, 16.3 ZAR respectively), compared to rearing birds as per the COM REC (16.9 ZAR) (Table 4.6). The UMD REC (8.01 ZAR) showed a lower feed cost/kg broiler harvested ($P < 0.05$) compared to the NL and COM REC (8.18 ZAR, 8.23 ZAR respectively) at 32 days of age (Table 4.6).

Table 4.6. Cost of production and of rearing each bird in a 32-day production period

Main effects			Feed cost	
REC[1]			Feed cost/bird (ZAR[2])	Feed cost/kg harvested (ZAR)
	NL		15.9[b]	8.18[a]
	UMD		16.3[b]	8.01[b]
	COM		16.9[a]	8.23[a]
	SEM		0.12	0.04
	P-value		< 0.001	0.003
LS[3]				
	Fast		16.3	8.16
	Slow		16.4	8.12
	SEM		0.09	0.03
	P-value		0.24	0.48
Trt[4]	REC	LS		
T1	NL	Slow	16.0	8.20
T2		Fast	15.8	8.17
T3	UMD	Slow	16.2	7.99
T4		Fast	16.3	8.04
T5	COM	Slow	17.1	8.18
T6		Fast	16.7	8.27
SEM			0.17	0.06
(*P*-values) REC X L			0.45	0.57

[1]REC: Ca and P Recommendations (Dutch Nutrition Group (NL), University of Maryland (UMD), Commercial (COM).
[2]ZAR: South African Rand (Currency)
[3]LS: Limestone solubility (Fast or Slow).
[4]Trt: Denotes dietary treatments.
[a,b] Column means with same superscript do not differ significantly ($P < 0.05$).

Chapter 5

Discussion

This study aimed to determine whether feeding three different dietary Ca and P recommendations coupled with two limestone solubilities would affect broiler growth performance, bone mineralisation, and mineral excretion over a 32d period. The improved BW performance of broilers at 32d fed SS-LS compared to FS-LS can be attributed to the differences in the rate of solubilization between the FS-LS and SS-LS, where the former solubilizes at a faster rate in the proventriculus and gizzard compared to SS-LS (Majeed *et al.*, 2020). Thus, the SS-LS would have resulted in less interaction of free Ca with phytate, thereby increasing the availability of both Ca and P to the bird (Kim et al., 2019). In contrast, Majeed *et al.*, (2020), found no significant effect of LS particle size on broiler BW from 0-35d of age. Likewise,Bradbury *et al.*, (2016) in a 28d study did not find effects of LS particle size on broiler BW . The lack of effect of LS particle size on live performance in the latter studies can potentially be attributed to differences in Ca and P levels fed in the respective studies, or differences in particle size between the fine and coarse LS not having been different enough to elicit an effect on BW performance. In this study, the effect of LS solubility on BW performance was only seen at 32d. In contrast, Lee *et al.*, (2021) reported significant differences in BW performance in 21d broilers fed fine and coarse LS at 0.96% and 0.60% dietary Ca. Perhaps treatments differences noted by Lee *et al.*, (2021) were due to the 0.36% difference between the treatments dietary Ca of the study which was much higher than what the current study was formulated to in terms of Ca. Nonetheless, effects of limestone on broiler performance observed in the present study were small and only significant at 32d of age and not observed in younger broilers.

Feeding Ca and P below the recommend NRC levels (NRC, 1994) has been reported to yield better broiler performance (Ceylan *et al.*, 2020; Ghobadi *et al.*, 2011; NRC, 1994). Other researchers have further reported either an improvement or no loss in performance when feeding below the commercially recommended Ca and P levels (Delezie *et al.*, 2012; Hamdi *et al.*, 2015; Kop-Bozbay *et al.*, 2021; Valable *et al.*, 2018). Likewise, in the currently study reducing pre-starter dietary Ca by 4% and AvP by 3.5%, increasing starter dietary Ca by 2% and reducing AvP by 14%, reducing grower dietary Ca by 10% and AvP by 14%, and finisher dietary Ca by 13% and AvP by 30% in broilers fed the UMD REC compared to the commercially recommended level (COM REC) resulted in improved performance. But the currently study established that reducing pre-starter Ca by 12% and AvP by 13%, starter and grower dietary Ca by 14% and AvP by 34%, and finisher dietary Ca by 5% and AvP by 30% in broilers fed as per the NL-REC compared to the COM REC proved to be detrimental for BW performance in the current 32d study. Also, the low BW performance of the NL REC can be

attributable to inadequate dietary P provision, in which protein synthesis is taxed by inadequate Adenosine triphosphate (ATP) synthesis to promote body mass accretion. Xu *et al.*, (2021) reported poor BWG, ADG, and FI in broiler rear between 0-7d and 0-9d. The author mentioned that this was attributable to a diet high in Ca and deficient in P, hence P is a component of creatine-phosphate which is an energy buffer to regenerate ATP.

The FCR at 7d and 28d was worse for both COM REC and NL REC while at 14d and 32d all REC were significantly different. However, at 21d, the P-value of treatment differences was *P*=0.07 that is not considered significant. The smaller impact of REC at 21d can be attributed to smaller differences in Ca and P levels between treatments fed at this age vs. other dietary phases. Birds fed as per the UMD REC showed an improved FCR compared to both NL REC and COM REC indicating that the UMD Ca and P REC were the closest to what the bird's require for growth and development. A 21d study by Kop-Bozbay *et al.*, (2021) investigating the impact on performance of a 10% step wise decrement in both dietary Ca and AvP from the Aviagen 2014 recommendations of 0.96% Ca and 0.45% AvP (Aviagen, 2014). An improved broiler FCR in the decrement treatments was noted compared to the commercial recommendation, also there were no differences reported amongst the treatment Ca and AvP recommendations (Trt1: 0.85% Ca and 0.42% AvP), (Trt2: 0.77% Ca and 0.38% AvP), (Trt3: 0.68% Ca and 0.34% AvP) and (Trt4: 0.61% Ca and 0.31% AvP). Ceylan *et al.*, (2020) in a 41d study showed a high FCR in broilers fed as per the Aviagen Ca and AvP recommendations of 2019 (Aviagen, 2019); while feeding lower Ca and AvP than what the breeder recommended improved the FCR, and these were not different from each other. In the current study the UMD REC were found to be efficacious in improving FCR in all stages of growth, except at 21d where no differences were found. While feeding Ca and P below the UMD REC such as the NL REC or above the UMD REC such as the COM REC had no benefits in improving FCR.

Hamdi *et al.*, (2015) showed that a dietary increase of nPP from 0.25% to 0.38% resulted in high FI. Furthermore, the study showed that a dietary Ca level of 0.70% with 0.38% nPP can improve BWG and FI beyond a dietary inclusion of 0.90% Ca and 0.38% nPP, an effect observed in the current study on BW and FI. A study by Ceylan *et al.*, (2020) composed of 11200 broilers found that the breeder company's modern broiler Ca and P REC might be high; hence an improvement in BW, FI, and FCR in an 11d-41d period was noted on reduced Ca and P levels. Similar results were found in the current study where the high Ca and P COM REC which promoted high FI did not result in significantly more efficient broilers to 32 days of age.

In the current study, there were no differences in mortality rate between treatments up to 21 days but were only seen between 28d and 32d. Feeding 0.75% Ca and 0.28% AvP as per the NL REC at finisher phase resulted in high mortality rate between 28d to 32d. A decrease to 0.69% Ca at 0.28% AvP on the UMD REC, and a decrease to 0.79% Ca and 0.40% AvP from 0.44% (at grower phase) reduced mortality. The high mortality rate at finisher phase can possibly be due to mineral imbalance and feeding Ca and P below requirements between 0d-32d. A 21d broiler study by (Powell *et al.*, 2011) showed a 14% mortality rate when feeding high dietary Ca at 1.13% with 0.20% nPP, mortality rate improved to 3% when Ca was decreased to 1.00% at 0.20% nPP, and was reduced to zero at 0.45% nPP and 1.00% Ca. Which was what occurred between the NL and the UMD REC but increasing AvP in the COM REC resulted in much lower mortality. Contrarily to this study, (Powell *et al.*, 2011) reported a 6% mortality rate when feeding lower dietary Ca at 0.67% which is similar to the UMD REC in this study. However, feeding 0.69% Ca did not show an increase in mortality rate. This shows the sensitivity of the modern broiler to a deficiency in dietary Ca and P, which was demonstrated by (Majeed *et al.*, 2020), where a negative control (0.58% Ca, 0.23% AvP) resulted in 1.91% mortality rate compared to 0.64% of the positive control (0.76% Ca, 0.38% AvP) between 0-35d.

Due to low Ca and P provision on the NL treatments, poor bone mineralisation occurred irrespective of LS solubility speed. Furthermore, these birds could not recover post 10 days; hence the tibia ash weight at 32 days irrespective of limestone was low for the NL REC. This could be attributable to bone turnover due to inadequate dietary supply of P in the NL treatments to minimize risks of hypocalcaemia. The UMD and COM REC, irrespective of LS solubility resulted in improved bone mineralisation indicating that these recommendations availed Ca and P fed close to or above the broiler requirements for Ca and P. Research by Liu *et al.*, (2017) investigated the impact of dietary non-phytate phosphorus in broilers over 21 days of age. The study found that decreasing dietary nPP below 0.38% was detrimental for tibia ash when Ca was kept constant at 1.0%. Nevertheless, an increase in nPP without a change in Ca level did not result in tibia ash differences, indicating the importance of mineral balance for beneficial broiler performance and mineralisation. While in the current study tibia ash of birds fed as per the UMD REC with SS-LL at d10 and d32 fed 0.80% Ca and 0.28% AvP grower, 0.69% Ca and 0.28% AvP finisher respectively were not different from the COM REC with SS-LS 0.87% Ca and 0.44% AvP grower, 0.79% Ca and 0.40% AvP finisher. Therefore, indicating the importance of concomitantly adjusting dietary Ca and P for better mineral balance.

Concurring results to the UMD REC were found in a study by Ceylan *et al.*, (2020), who reported that a reduction in dietary Ca and P below 0.80% and 0.40%, respectively, after the starter phase did not have significant effect on tibia ash weight and percent. Also, Valable *et al.*, (2018) did indicate that reducing dietary Ca from 0.90% to 0.60% (grower, 11-21d) and from 0.85% to 0.48% (finisher, 22-37d) though it may be sufficient for performance, it was detrimental for bone mineralisation. Contrarily to Valable *et al.*, (2018), in the current study, it was noted that it was the high reduction in dietary AvP in the NL treatments which resulted to low bone mineralisation.

Dietary Ca and P levels can be changed several times, but these can be at the expense of the birds and the environment; hence understanding P utilisation is vital for sustainable broiler production. In the current study, the dietary treatments significantly affected the Ca and P consumed and excreted between 0 and 32d. The grams of Ca and P consumed increase with the level of Ca and AvP provisioned in the diets; hence high Ca and P intake were observed in the COM treatments irrespective of LS-solubility. Indeed, P consumption and excretion was improved by reducing dietary P; hence P consumed reduced by 170g and 145g (UMD SS-LS, and UMD FS-LS); and was even lower on the NL diets where it was reduced by 202g irrespective of LS-solubility compared to the COM diets. High dietary Ca and P REC adversely affected P excretion. Irrespective of LS-solubility, the COM diets resulted in high mineral excretion because of reduced intestinal Ca and P digestion and absorption due to likely formation of insoluble Ca-phytate complexes. Likewise, Rousseau *et al.*, (2012) found that increasing dietary Ca also increased Ca intake linearly from 0.68g/d in low Ca diets to 0.93g/d in medium Ca diets and to 1.24g/d in high Ca diets. Also, the study found an increase in P consumption from 0.71g/d in low nPP diets to 0.88g/d in high nPP diets, respectively. The low P consumption and excretion in the NL treatments might have been because of a regulatory mechanism being stimulated in the birds to cope with low dietary P. Also, the low P consumption and excretion could be due to the exclusion of MDCP in the starter, grower and finisher diets as a source in inorganic P. Consequently, P deficiency is susceptible to poor growth performance than Ca deficiency (Shao *et al.*, 2019). Xu *et al.*, (2021) reported on broiler sensitivity to P deficient diets which resulted in low broiler performance, including low FI and lameness as a response to in adequate dietary P and mineral imbalance between Ca and AvP.

Litter quality has been identified as one of primary contributors to pododermatitis (Collett, 2012; Shepherd *et al.*, 2010). Nevertheless, high dietary Ca has been mentioned to result in poor litter quality (Collett, (2012); which would increase the prevalence of foot pad dermatitis and hock burn. The current study established the high prevalence of FPD and HB to be the result of dietary treatments. Feeding high Ca and AvP with COM REC-FS-LS (Trt 6) increased the prevalence of

severe FPD lesions, while feeding low Ca and P with NL REC-SS-LS (Trt 1) improved the incidences of FPD (Figure 4.1). While feeding close to the bird requirements for Ca and AvP as per the UMD REC (Trt 3 and 4) resulted in more birds being distributed to score 0 than score 2 and 3. Furthermore, feeding low Ca and AvP (NL-FS-LS) was comparable to the UMD REC treatments and Trt 5 (COM REC-SS-LS) which showed the positive effect of SS-LS compared to FS-LS. High cases of FPD and HB were noted on birds reared as per the COM REC, possibly because of high dietary Ca and live weight compared to birds on NL and UMD REC, irrespective of limestone solubility profile. Dietary provision of either low or high dietary Ca and P was detrimental to HB health; hence severe lesions were observed in Trt 1 and 2 (NL REC) and Trt 5 and 6 (COM REC). This is due to BW differences, where the heavier COM REC birds would walk on the hocks as a support structure. Also, the NL birds were visually observed spending time lying on the litter, possibly due to weak legs. Lethargy and lameness because of weak legs in broilers has been reported due to P deficiency (Xu *et al.*, 2021), which could contribute to an increase in HB cases. The high HB severity of COM REC birds can be attributable to high litter moisture due to high water intake as influenced by high dietary Ca, BW and FI, resulting in wet excreta. On the contrary, Delezie *et al.*, (2015) found no lesions in birds fed balanced Ca and P levels (0.65% Ca, 0.30% AvP), while mild to severe FPD were noted on treatments receiving high Ca and low P (0.65% Ca, 0.18% AvP) in the finisher phase at 39 days. Also, in this study, HB results were comparable to the FPD data, where high Ca and low P increased the incidences of HB. Broom *et al.*, (2005) and Kjaer *et al.*, (2006) found an increase in HB incidences, which were positively related to BW as opposed to FPD cases. Ziaei *et al.*, (2011) reported that feeding either 15% higher or lower Ca and AvP than the NRC recommendations led to high FI and water intake in broilers aged between 11-21d. Furthermore, a study in laying hens by Smith *et al.*, (2000) found that increasing dietary P increased water intake and wet excreta. This could be the case in the current study where birds were fed Trt 5 and 6; hence excess dietary P gets excreted through the kidneys resulting in wet excreta due to polydipsia and polyuria. Xu *et al.*, (2021) reported poor BWG, ADG, and FI in broiler rear between 0-7d and 0-9d. The author mentioned that this was attributable to a diet high in Ca and deficient in P, hence P is a component of creatine-phosphate which is an energy buffer to regenerate ATP.

Feed ingredient prices are volatile; hence strategic and cost-effective feed formulation is essential. Expensive diets increase the cost of production, which could result in economic losses to the producer. The COM diets were more expensive per kilogram/feed in a 32d period than the UMD and NL diets. Possibly because in combination with phytase, the latter diets did not require the use an inorganic P source at later phases. The Ca and P REC strongly influenced the cost of production,

making the Ca and P specifications a relevant factor requiring special attention in broiler diets. A cost saving of ZAR 0.17 and ZAR 0.21 cents in feed cost/kg harvested was achieved when feeding as per the UMD REC compared to the NL and COM REC, respectively. While the NL and UMD diets at later phases did not have an inorganic source of P, the UMD REC possibly resulted in a low feed cost/Kg broiler harvested because of feeding the birds close to their requirements. Thus, when metabolic needs for Ca and P are met, more was directed for production at minimal feed intake and possible feed waste. A linear programming study evaluating diet optimisation through ingredient substitution demonstrated the benefits of ingredient switching within a production cycle (Alqaisi *et al.*, 2017). In this study, the inorganic P source was not included in later phases of the UMD and NL diets, which therefore showed to be cheaper than the COM diets. Nevertheless, it is time for nutritionists to formulate more with digestible forms of P sources in the early phase to reduce costs thereafter. Furthermore, the phosphate rock is a finite and non-renewable resource globally (Scholz *et al.*, 2013); and its use should be optimised to lower eutrophication.

The current study showed that a 32d broiler can be fed below the breeders recommend for dietary Ca and P without risking performance, bone mineralisation and mineral excretion; and endangering the welfare of the birds. Also, in combination with 2000 FTU phytase, the current study showed it is possible to rear high performing 32d old broilers without supplementation with a source of inorganic P in grower and finisher phases thereby reducing feed cost and minimising negative environmental impacts.

Chapter 6

Conclusion and recommendations

Based on the current study, it is suggested that when the UMD recommendations for Ca and P are followed in pre-starter and starter diets, Ca and P levels can be reduced at the grower and finisher phases, and the inorganic source of P can be eliminated from the ration during the same period without negatively affecting broiler performance. This can further be used strategically with slow solubilising limestone to reduce the cost of production. This provides an opportunity not only for economic gains for producers, but to improve broiler production and environmental sustainability. In the present study, the Dutch NL recommendations that are aimed at reducing P excretion and environmental sustainability of broiler production appeared to undersupply the birds Ca and P and resulted in loss of broiler performance and poorer economic return vs. UMD Rec.

Further research needs to be conducted on the impact of limestone particle size on broiler performance and bone mineralisation on diets supplemented at graduated phytase FTUs both below and above 2000 FTUs, as opposed to the current study which focused only supplementing all diets with 2000 FTU/kg in all dietary phases. Furthermore, the impact of limestone particle size combined with the different levels of Ca and P needs in the current study along with phytase supplementation could be evaluated if there would be an optimal dose of phytase supplementation depending on the limestone solubility. Though no fractures were observed on the tibias prior drying and ashing, it would be beneficial to measure other bone quality parameters such as bone mineral density and bone breaking strength and evaluate the correlation with tibia ash if any. Additionally, because this study is aimed to contribute to broiler production globally, it would be beneficial to investigate the impact of the Ca and P recommendations and limestone solubility with the exclusion of an inorganic P source under various broiler production periods beyond 32 days of age.

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