Digestibility of dietary calcium and phosphorus in young and growing broilers supplemented with 1α-hydroxycholecalciferol in combination with phytase

by

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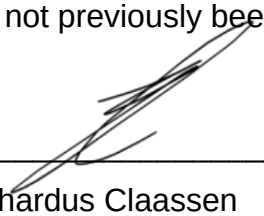
Declaration

I, Gerhardus Claassen hereby declare that this thesis, submitted for the MSc (Agric) Animal Science: Animal Nutrition degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other University



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Abstract

1α-Hydroxycholecalciferol (**1α-OH-D_3**) is a synthetic cholecalciferol derivative that is rapidly hydroxylated into 1α,25-dihydroxycholecalciferol, the biologically active form of cholecalciferol. 1α,25-Dihydroxycholecalciferol plays a critical role in several biological processes and is important for the regulation of several genes involved in calcium (**Ca**) and phosphorus (**P**) absorption. Phytase is an enzyme that hydrolyses phytate and improves the digestibility of Ca and P in the small intestine. The objective of this study was to establish the effects of 1α-OH-D_3 in combination with phytase on apparent ileal digestibility (**AID**) of Ca and AID P. To achieve this a digestibility trial was conducted on broilers at two different ages, days 10 and 23 (**D10** and **D23**), respectively. Treatments were arranged in a 3 × 3 factorial with three 1α-OH-D_3 doses (0, 2.5 and 5 µg 1α-OH-D_3/kg feed) × three phytase doses (0, 500, 1000 FTU phytase/kg feed). Treatment diets were prepared by adding 0, 2.5 or 5 µg 1α-OH-D_3/kg and/or a 6-phytase, which is a novel consensus bacterial 6-phytase variant, expressed in *Trichoderma reesei*, at 0, 500 or 1000 FTU phytase/kg feed. Each treatment was replicated 11 times on both D10 and D23 with 8 birds/replicate for D10 and 6 birds/replicate for D23. On the respective sampling days, ileal digesta was collected, frozen, freeze-dried and ground to pass through a 0.25mm screen before being analysed for dry matter, Ca, P, and titanium. The data was analysed with a full factorial using the JMP 16.0 statistical package where block was included as a random effect and means were separated using a protected Tukey HSD test at $P<0.05$. Both 1α-OH-D_3 and phytase improved AID Ca, AID P, digestible Ca (**dCa**) and digestible P (**dP**). There was no difference in birds that were fed diets that were supplemented with 2.5 µg and those supplemented with 5 µg 1α-OH-D_3/kg feed for any of the parameters measured on D10 and D23, suggesting that there is no added benefit on Ca and P digestibility when increasing the supplementation of 1α-OH-D_3 from 2.5 µg to 5 µg 1α-OH-D_3/kg feed. Phytase improved AID Ca, AID P, dCa and dP in a stepwise fashion on both D10 and D23 when supplemented into the diet of birds at 500 or 1000 FTU phytase/kg. There was no interaction between 1α-OH-D_3 and phytase for any of the parameters measured on D10 or D23.

Contents

List of Abbreviations

µg	Microgram
1α,25-$(OH)_2$-D_3	1α,25-Dihydroxycholecalciferol
1α-OH-D_3	1α-Hydroxycholecalciferol
25-OH-D_3	25-Hydroxycholecalciferol
AID	Apparent ileal digestibility
ATP	Adenosine triphosphate
BW	Bodyweight
C	Carbon
Ca	Calcium
CaSR	Calcium sensing receptor
CF	Crude fibre
CP	Crude protein
dCa	Digestible calcium
DM	Dry matter
dP	Digestible phosphorus
EE	Ether extract
FCR	Feed conversion ratio
FGF23	Fibroblast growth factor-23
FTU	Phytase units
g	Grams
GIT	Gastrointestinal tract
IP6	1,2,3,4,5,6-Hexakis dihydrogen phosphate
kg	Kilogram
KO	Knockout
l	Litre
mEq	Milliequivalent
mmol	Millimole
mRNA	Messenger ribonucleic acid

Na	Sodium
NCX	Sodium/calcium-exchanger
nm	Nanometer
P	Phosphorus
Pi	Inorganic phosphorus
pH	Potential hydrogen
PMCA1b	Plasma membrane calcium ATPase 1b
PTH	Parathyroid hormone
RXR	Retinoid X receptor
TD	Tibial dyschondroplasia
TRPV	Transient receptor potential of the vanilloid type
UV	Ultraviolet
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VDRE	Vitamin D response element

List of Tables

List of Figures

Chapter 1

General Introduction

1.1 Introduction

Cholecalciferol (vitamin D_3) is a fat-soluble vitamin that is supplemented into broiler and layer diets due to the lack of ultraviolet (UV) exposure (Soares Jr *et al.*, 1995). Cholecalciferol is important for the expression of genes involved in the regulation of calcium (Ca) and phosphorus (P) absorption and metabolism, bone mineralisation, and the preservation of skeletal integrity (Bikle, 1994; Garcia *et al*., 2013). Target genes on a wide variety of cell types and tissues throughout the body contain vitamin D receptors (VDR), which is the receptor to which vitamin D binds to initiate its effects (Pike *et al.*, 2017). The complete scope of vitamin D target genes has not been defined in animals; however, it has been shown that approximately 3% of the human genome is regulated by the VDR (Caprio *et al.*, 2017), which includes numerous cells and tissues such as those of the bone (Goltzman, 2018), kidney (Yang *et al.*, 2018), intestine (Wang *et al.*, 2012), immune system (Colotta *et al.*, 2017; Koivisto *et al.*, 2020; Martens *et al.*, 2020), cardiovascular system (Norman & Powell, 2014), and brain (Eyles *et al.*, 2014). Although vitamin D is used by the body for a variety of purposes and in many tissues, its role in the absorption of Ca and P is one of its most vital functions and is of the greatest importance to the animal nutrition industry.

Several strategies to limit the use of limestone and inorganic phosphate (Pi) sources are being applied in industry. This is due to the adverse effect of limestone on the digestibility of minerals, proteins, fats, the expensive nature of Pi sources, and the adverse impact of mineral excretion on natural ecosystems (Mallin, 2000; Jorquera *et al.*, 2008; Selle *et al.*, 2009; Li *et al.*, 2017; Majeed *et al.*, 2020; Li *et al.*, 2021). The application of enzymes such as phytase and the use of alternative sources of Ca and P are currently being utilised as partial solutions. Although the application of enzymes and the use of alternative sources of Ca and P have proven effective, cholecalciferol supplementation could make a beneficial contribution to already existing approaches. One analogue of cholecalciferol, 1α-hydroxycholecalciferol (1α-OH-D_3), has been demonstrated to improve mineral digestibility

in laying hens and laying quails (Bar *et al.*, 1976; Bar *et al.*, 1978). However, very few studies have examined the effect of 1α-OH-D_3 supplementation on mineral digestibility in broilers. Regardless, several authors have noted a beneficial effect of 1α-OH-D_3 supplementation on blood-ionised calcium status, the incidence of tibial dyschondroplasia (TD), bone mineralisation, performance, as well as the regulation and expression of genes responsible for Ca and P absorption (Ebrahimi *et al.*, 2016; Han *et al.*, 2016; Han *et al.*, 2017; Han *et al.*, 2018; Yang *et al.*, 2019; Landy *et al.*, 2020; Warren *et al.*, 2020; Ibrahim *et al.*, 2022).

Phytase has been used commercially for decades due to its beneficial effects on nutrient digestibility, bone mineralisation and performance (Selle *et al.*, 2009; Dersjant-Li *et al.*, 2022). Phytase increases nutrient digestibility by hydrolysing phytate to lower inositol esters (Laird, 2016). In doing this, phytase increases the amount of Pi available for absorption in the small intestine while simultaneously reducing the capacity of phytate to bind divalent cations such as Ca (Selle *et al.*, 2009). Several studies have suggested that a synergistic effect exists between phytase and vitamin D (Biehl *et al.*, 1995; Mitchell & Edwards Jr, 1996; Snow *et al.*, 2004). It can be hypothesised that the synergism that exists between phytase and vitamin D can be attributed to the different modes of action of phytase and vitamin D. Phytase increases the amount of Ca and P available for absorption in the small intestine. Vitamin D then acts by increasing the expression of genes involved in the absorption of Ca and P across the apical membrane of the epithelial cells of the small intestine and kidney (Han *et al.*, 2009; Han *et al.*, 2018). It can be hypothesised that 1α-OH-D_3 supplementation can increase the apparent ileal digestibility (AID) of Ca and P in the small intestine of broilers in the presence and absence of phytase. Furthermore, the addition of phytase could synergistically act with 1α-OH-D_3 to further increase the digestibility of Ca and P in the small intestine. The combined use of 1α-OH-D_3 and phytase could make a valuable contribution to the efforts of the poultry industry to reduce the use of non-renewable sources of minerals and support the goal of a more sustainable future.

1.2 Aim

This study aims to investigate the effects of 1α-hydroxycholecalciferol in combination with phytase on several digestibility parameters in broilers at two different ages. The objectives of this study were:

(1) To establish the effects of different levels of 1α-hydroxycholecalciferol (0, 2.5 and 5 µg 1α-OH-D_3/kg feed) on the apparent ileal digestibility of Ca and P in combination with three different levels of phytase (0, 500 and 1000 FTU phytase/kg feed) on broilers at 10 days-of-age and 23 days-of-age

(2) To determine digestible calcium (dCa) and digestible phosphorus (dP) values for 1α-hydroxycholecalciferol in the presence and absence of phytase, for the use in feed formulation

1.3 Hypotheses

H0 = 1α-Hydroxycholecalciferol will not influence ($p>0.05$) AID Ca, AID P, dCa or dP of broilers at 10 or 23 days-of-age

H1 = 1α-Hydroxycholecalciferol will influence ($p<0.05$) AID Ca, AID P, dCa or dP of broilers at 10 or 23 days-of-age

H0 = Phytase will not influence ($p>0.05$) AID Ca, AID P, dCa or dP of broilers at 10 or 23 days-of-age

H1 = Phytase will influence ($p<0.05$) AID Ca, AID P, dCa or dP of broilers at 10 or 23 days-of-age

H0 = 1α-Hydroxycholecalciferol in combination with phytase will not have an interaction effect on any of the parameters measured at 10 or 23 days-of-age ($p>0.05$).

H1 = 1α-Hydroxycholecalciferol in combination with phytase will have an interaction effect on any of the parameters measured at 10 or 23 days-of-age ($p<0.05$)

Chapter 2

Literature Review

2.1.1. Introduction

South Africa is the largest producer of poultry products on the African continent, followed by Egypt, Morocco, Algeria, and Nigeria (Nkukwana, 2018). South Africa is also one of the largest consumers of poultry meat in the world with per capita consumption being as high as 38.93 kg per capita/year in 2020 (Nkukwana, 2018; SAPA, 2020). Poultry meat forms one of the most important sources of protein in the typical South African diet. When all locally produced protein sources are considered, 65.6% can be attributed to the consumption of poultry products (SAPA, 2020). The poultry industry is also a large contributor to the economy of the country. At the end of 2019, 41% of the gross value from animal-derived products came from the poultry industry, making it the single largest contributor to the agricultural sector in the country (Makgopa, 2020). Currently, 25% of South Africans live below the poverty line, and 40% of the population is thought to be unable to afford a basic food basket due to the country's high unemployment rate of over 34.4% (The World Bank, 2018; Makgopa, 2020). This emphasises the importance of more affordable animal protein production in South Africa. To add to this, the poultry industry will play an important role in providing food security for a nation, while also providing jobs in the economic recovery of the country following the devastation caused by the Covid-19 pandemic.

Globally there is a constant increase in the demand for poultry meat. To support this growing demand, advances in reproductive technologies and genetics have been made. These genetic advances, with the aid of short generation intervals, have rapidly accelerated the performance of broilers. While performance traits of broilers were improved by genetic selection, skeletal development did not improve proportionally. This has caused susceptibility in modern-day broilers to skeletal defects such as rickets and tibial dyschondroplasia (Garcia *et al.*, 2013). Along with this, the poultry industry is facing several challenges of sustainable, environmental, ethical, and economical concern. Calcium and phosphorus are two of the most abundant and important minerals that are involved in several biological processes throughout the animal’s body (Wu, 2022). The bulk of Ca (99%) and P (80%) in the body is retained in bone mineral, primarily in the form of hydroxyapatite crystals (Veum, 2010; Wu, 2022). The remainder of Ca has an important role in the regulation of several physiological processes in

the body, such as muscle contraction, blood coagulation, as well as the normal functioning of the nervous and immune systems and is present in the interstitial fluid, the intracellular space and blood serum (Portale & Perwad, 2009; Veum, 2010). The P which is not part of bone is found in cell contents and cell walls, where it performs vital metabolic functions. Phosphorus is found in nucleic acids, as well as in nucleotides, such as ATP, which are involved in energy metabolism. Phosphorus also plays a role in blood and bodily fluid acid–base buffer systems, cell development, and maintenance of cellular integrity (Veum, 2010).

The main source of calcium in the majority of South African commercial broiler and layer diets is limestone, while phosphorus is obtained from phosphate rock sources, both of which are non-renewable sources of minerals. Therefore, researchers and nutritionists should actively pursue minimising the use of non-renewable sources of nutrients and explore more sustainable strategies and alternatives. From an environmental perspective, problems such as eutrophication is a direct result of unabsorbed P being excreted by animals that subsequently end up contaminating water resources. As much as 70% of the P present in animal feed has the potential to be excreted as a consequence of inefficient uptake (Jorquera *et al.*, 2008). This causes the undesired growth of aquatic flora, decreasing the oxygen content of the water, and ultimately making it unviable for use by other species (Mallin, 2000). The broiler industry also faces several welfare-related problems such as lameness, TD, and rickets. As much as 27% of commercially grown broilers show signs of locomotive problems, while 3% are completely unable to walk (Knowles *et al.*, 2008). While a range of factors contributes to these numbers, dietary-related factors that influence bone mineralisation cannot be dismissed and therefore nutritionists have an important role to play in improving overall bird welfare. Due to limited resources and increased demand for poultry meat as a direct result of an ever-growing population, there is a need to explore more sustainable solutions. The combination of phytase and a potentially more efficacious vitamin D source may provide a partial solution to the challenges faced by the poultry industry, bringing the industry one step closer to a more sustainable future.

2.1.2. Overview of 1α-hydroxycholecalciferol

A vitamin that plays an integral role in the homeostatic regulation of Ca and P in the body is vitamin D. Vitamin D was discovered by Sir. Edward Mellanby, a British scientist, after he became concerned about the high incidence of rickets in the United Kingdom. He intuitively

suggested that rickets might be due to a deficiency of a dietary element. Through a range of hypothesis testing and subsequent experiments, he suggested that a deficiency in a new vitamin, vitamin D, was responsible for the high incidence of rickets in the UK (DeLuca, 2016). Since the discovery of vitamin D, significant progress has been made in our understanding of the role of vitamin D and its metabolites and their functions performed in humans and animals.

Vitamin D is a fat-soluble vitamin that is commercially available in several analogue forms including cholecalciferol, 25-hydroxycholecalciferol (25-OH-D_3), 1α-hydroxycholecalciferol (1α-OH-D_3) and 1α,25-dihydroxycholecalciferol (1α,25-$(OH)_2$-D_3). Vitamin D plays a critical role in several biological processes in the birds' bodies. Among others, vitamin D is important for the expression of genes involved in the regulation of calcium and phosphorus absorption and metabolism, bone mineralisation, skeletal development, as well as for the regulation of the secretion of parathyroid hormone (PTH) (Bikle, 1994; Garcia *et al.*, 2013). Vitamin D and its analogues are often supplemented in poultry diets and are the main source of vitamin D for birds in both broiler and layer diets rather than relying on cutaneous synthesis (Soares Jr *et al.*, 1995). This is due to birds in most modern commercial systems being kept entirely indoors, in environmentally controlled housing with limited exposure to UV light (Soares Jr *et al.*, 1995). One of the analogues of vitamin D is 1α-hydroxycholecalciferol. During the early 1970s, 1α-OH-D_3 was first released to support patients with renal complications that were unable to effectively convert vitamin D into its physiologically active form. Due to a lack of cycling active vitamin D, Ca absorption from the small intestine is limited and hence these patients suffered hypocalcemia (Morii *et al.*, 1997). Since the development of 1α-OH-D_3, the metabolite has been adopted for use in the poultry industry. Producers include 1α-OH-D_3 with the goal to either replace cholecalciferol or to supplement cholecalciferol and in the process benefit from the potential effects the metabolite has to offer on mineral digestibility, bone mineralisation and performance (Biehl *et al.*, 1995; Snow *et al.*, 2004; Han *et al.*, 2009; Wang *et al.*, 2015; Ebrahimi *et al.*, 2016; Han *et al.*, 2018; Yang *et al.*, 2019). Regardless, very few studies have examined the effects of 1α-OH-D_3 on mineral digestibility in recent years. The use of 1α-OH-D_3 in combination with phytase could potentially have economic benefits while also supporting the goal of a more sustainable future.

2.2. Metabolism of cholecalciferol

Cholecalciferol that is subcutaneously synthesised or supplemented into the diet is unable to bind to the vitamin D receptors of target tissues and is thus biologically inactive. The

same is true for other supplemental analogues of cholecalciferol such as 25-OH-D_3 and 1α-OH-D_3. Cholecalciferol and its analogues first need to be converted into the active metabolite, 1α,25-$(OH)_2$-D_3, before being able to bind to the VDR of target genes. This conversion involves two hydroxylation steps that are mediated by different enzymes that are concentrated within different locations in the body.

2.2.1. Cholecalciferol synthesis and metabolism

Cholecalciferol is a non-essential vitamin that can be naturally synthesised in the skin from cholesterol upon radiation by UV light. Cholecalciferol can also be obtained by supplementation into the diet (Jäpelt & Jakobsen, 2013). The synthesis of cholecalciferol in the skin of animals starts with 7-dehydrocholesterol. 7-Dehydrocholesterol is an intermediate produced during the *de novo* synthesis of cholesterol from acetyl-COA and is transformed into cholecalciferol in two steps (Bikle, 2014). The first step takes place in the skin where pre-cholecalciferol is formed from 7-dehydrocholesterol in an enzyme-independent process upon radiation by UV-B light with a wavelength of 280-320 nanometer (Bikle, 2014). However, this step is limited by the indoor housing of poultry in modern production systems and feather covering, especially in older birds, which likely reflects or absorbs a large proportion of UV light, preventing the penetration of UV light to the skin (Burkhardt, 1989; Warren & Livingston, 2021). Nevertheless, this process causes an alteration in the chemical structure of the 7-dehyrdrocholesterol molecule in the 9th Carbon (C9) - C10 position (Figure 2.1) (Tian & Holick, 1995). By means of a thermo-sensitive process, pre-cholecalciferol is transformed into cholecalciferol (Tian & Holick, 1995; Bikle, 2014). However, pre-cholecalciferol can also be thermally transformed into tachysterol-3 and lumisterol-3, however, since the thermal isomerisation of pre-cholecalciferol to cholecalciferol requires the least amount of energy, the majority of the pre-cholecalciferol that is present in the skin is transformed into cholecalciferol (Webb *et al.*, 1989; Warren & Livingston, 2021). Cutaneous cholecalciferol synthesis is regulated by the amount of UV exposure, the concentration of available 7-dehydrocholesterol and the activity of 7-dehydrocholesterol reductase. The enzyme 7-dehydrocholesterol reductase converts cholesterol into 7-dehydrocholesterol and is the primary regulator in the cutaneous biosynthesis of cholecalciferol (Saponaro *et al.*, 2020). Cholecalciferol has a half-life of four days and first needs to undergo two subsequent hydroxylation steps before becoming a physiologically active molecule (Mawer *et al.*, 1969; DeLuca, 2004).

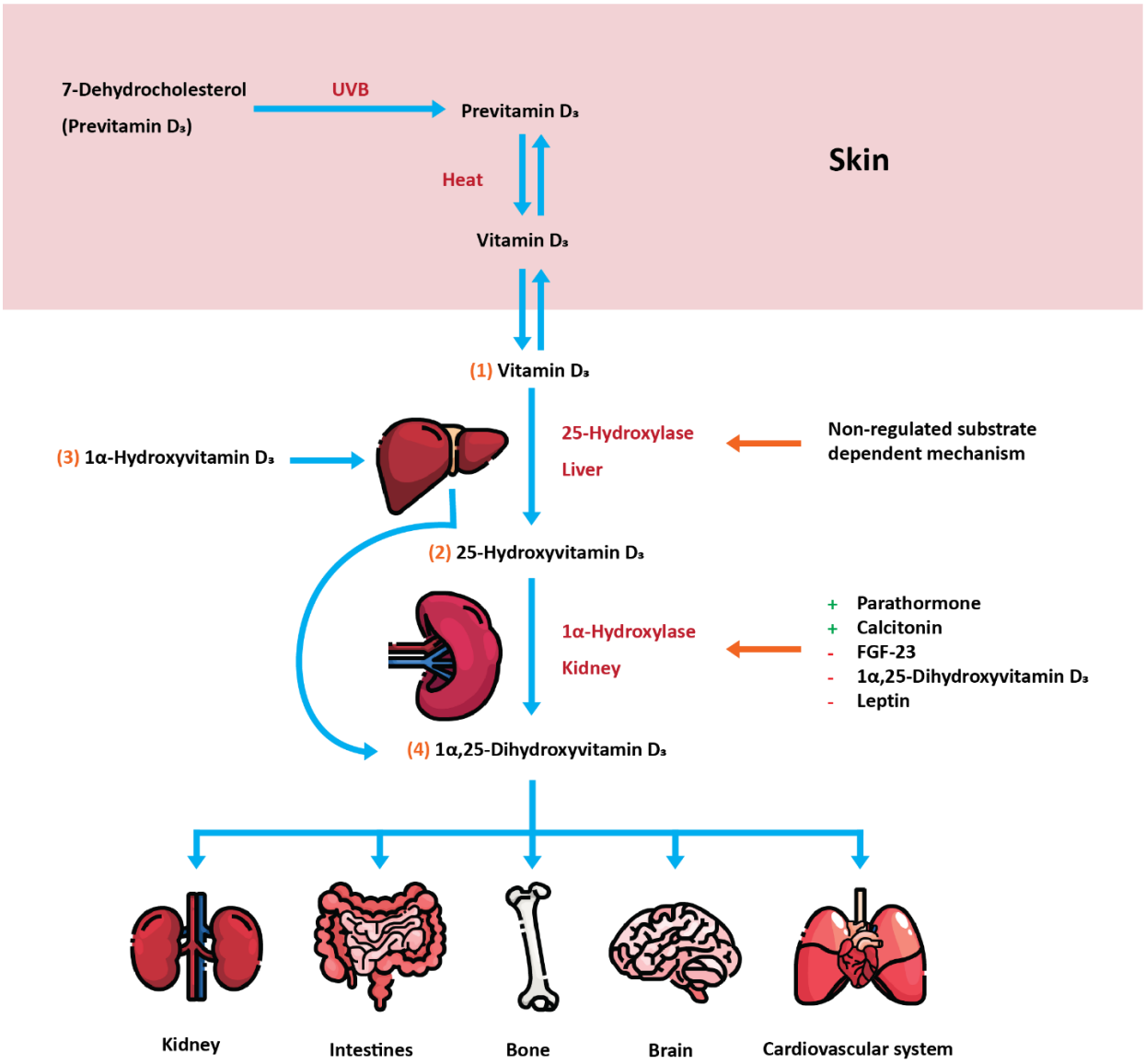

Figure 2.1 The synthesis and metabolism of vitamin D (adapted from Jäpelt & Jakobsen, 2013; Saponaro *et al.*, 2020)

2.2.2. 25-Hydroxycholecalciferol

25-Hydroxycholecalciferol is an alternative metabolite that is commonly used in the poultry industry. The 25-OH-D_3 metabolite can be chemically or naturally synthesised and included in poultry diets or can be obtained by the hydroxylation of endogenously produced cholecalciferol to 25-OH-D_3 in the liver (Figure 2.2). 25-Hydroxycholecalciferol is the major circulating form of vitamin D and is often used as an indication of the vitamin D status of humans and animals (Christakos *et al.*, 2016). The hydroxylation of cholecalciferol to 25-OH-D_3 is the first of two hydroxylation steps required to transform cholecalciferol into 1α,25-$(OH)_2$-D_3 (DeLuca, 2004).

The hydroxylation of cholecalciferol to 25-OH-D_3 is mediated by several 25-hydroxylase enzymes that belong to the hepatic microsomal P450 fraction including CYP2R1, CYP2D11, and CYP2D25 (Cheng *et al.*, 2003; Bikle, 2014). The 25-hydroxylase enzyme, CYP2R1, is considered the enzyme predominantly responsible for the conversion of cholecalciferol to 25-OH-D_3. This was first suggested by Cheng *et al.* (2004) who discovered that a mutation in the CYP2R1 gene caused low circulating levels of 25-OH-D_3 and typical symptoms of vitamin D deficiency. This was later confirmed by Zhu *et al.* (2013) who found that the circulating 25-OH-D_3 levels of CYP2R1 knockout (KO) mice were reduced to 50% the level of that found in wild-type mice. The enzyme CYP2R1 is largely unregulated and dependent on the delivery of cholecalciferol to 25-hydroxylase enzymes. However, recently it was discovered that hepatic CYP2R1 messenger ribonucleic acid (mRNA) and protein content gradually decreased with ageing in male mice from 26 to 49 weeks (Roizen *et al.*, 2018). It was also found that CYP2R1 activity is depressed with extended periods of fasting, and in patients suffering from obesity and type two diabetes (Roizen *et al.*, 2018; Aatsinki *et al.*, 2019). This suggests a possible influence of age and the metabolic environment on the regulation of CYP2R1. However, these findings are suggestive and further investigation regarding the influence of the metabolic environment of poultry on vitamin D metabolism is required.

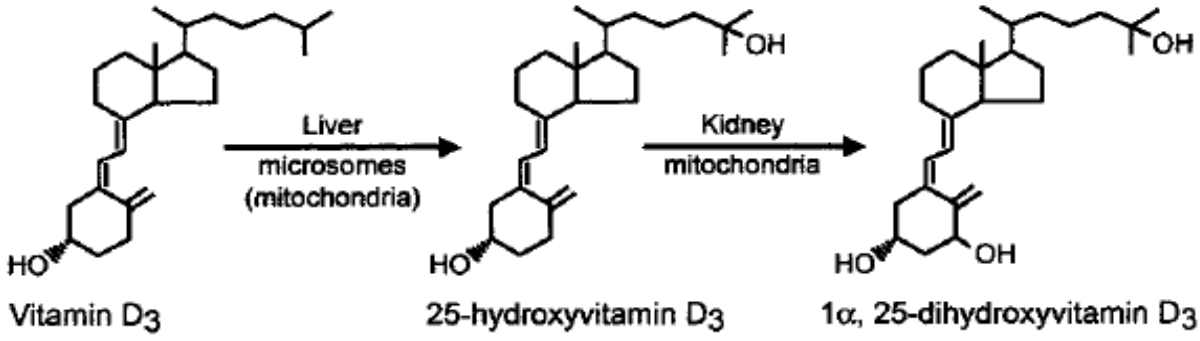

Figure 2.2 The hydroxylation of inert vitamin D_3 to physiologically active 1α,25-dihydroxyvitamin D_3 (DeLuca, 2004)

2.2.3. 1α,25-Dihydroxycholecalciferol

The second hydroxylation step involves the hydroxylation of 25-OH-D_3 to 1α,25-$(OH)_2$-D_3, the physiologically active and functional metabolite of vitamin D. This step is mediated by the 1α-hydroxylase enzyme, CYP27B1, in the kidney (Torres *et al.*, 2016). Tissues of the skin and

those relating to the immune system also express low levels of CYP27B1, however, extrarenal sources of CYP27B1 are more focused on the local production of 1α,25-$(OH)_2$-D_3 for autocrine functions unrelated to Ca and P homeostasis (Bikle, 2011; Adams & Hewison, 2012). Renal CYP27B1 is substrate dependent and is tightly hormonally regulated (Saponaro *et al.*, 2020). Parathyroid hormone and calcitonin have been shown to induce renal CYP27B1 while fibroblast growth factor-23 (FGF23), 1α,25-$(OH)_2$-D_3, and leptin inhibit the enzyme via a negative feedback mechanism (Tsuji *et al.*, 2010; Jones *et al.*, 2014; Takahashi *et al.*, 2014; Jones *et al.*, 2017). Because of the tight hormonal regulation of renal CYP27B1, this step is often regarded as the rate-limiting step in the activation of cholecalciferol to 1α,25-$(OH)_2$-D_3 (Portale & Miller, 2000; Ringe *et al.*, 2005). Vitamin D that is supplied in the form of 1α-OH-D_3 requires 25-hydroxylation by CYP2R1 only and is therefore not subject to the tight regulation of CYP27B1 (Figure 2.3), improving the bio-efficacy of the analogue on a cellular level and therefore its ability to positively influence Ca and P absorption and bone mineralization when added into poultry diets.

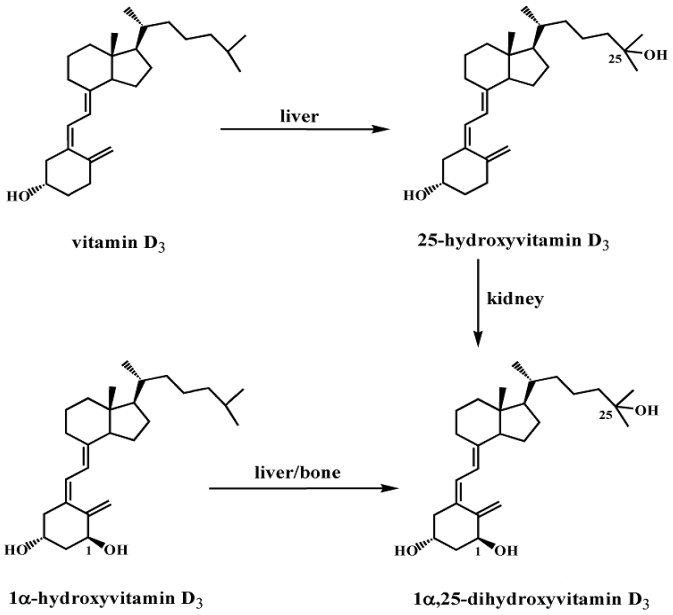

Figure 2.3 The hydroxylation steps involved in the formation of 1α,25-dihydroxyvitamin D_3 from vitamin D_3 and 1α-hydroxyvitamin D_3 (adapted from Takahashi *et al.*, 2014)

2.2.4. Vitamin D catabolism and transport

Circulating vitamin D metabolites can be catabolized into a range of biologically inactive products that are excreted in the bile or urine (Saponaro *et al.*, 2020). The hydroxylase enzyme, CYP24A1, belongs to the mitochondrial P450 fraction and is predominantly responsible for the

catabolism of vitamin D metabolites into biologically inert derivatives (Jones *et al.*, 2014). The enzyme, CYP24A1, is present in the majority of tissues expressing VDR, and primarily functions by halting the biological activity of vitamin D and is therefore important for the prevention of hypercalcemia and hyperphosphatemia (Jones *et al.*, 2012). The enzyme, CYP24A1, is hormonally regulated by the same factors that activate CYP27B1, however, in the opposite direction (Jones *et al.*, 2014). This includes several hormones related to Ca and P homeostasis such as 1α,25-$(OH)_2$-D_3, FGF23, and PTH (Jones *et al.*, 2012). In a species-dependent manner, the enzyme CYP24A1 can hydroxylate 25-OH-D_3 and 1α,25-$(OH)_2$-D_3 in the C23 and C24 position into 24R,25-$(OH)_2$-D_3 and 1α,24,25-$(OH)_3$-D_3, respectively, yielding biologically inactive metabolites (Jones *et al.*, 2012). The products are then further broken down by CYP24A1 to produce a range of 23- and 24- hydroxylated derivatives which are then transformed into the final inactive products 26,23-lactone or calcitroic acid (Jones *et al.*, 2012; Bikle, 2014; Saponaro *et al.*, 2020). Several minor metabolic pathways in addition to CYP24A1 have been described, one of them being the C3-epimerisation pathway which yields several C3 epimer metabolites in which the C3-hydroxyl group takes the α-orientation in space rather than the β-orientation (Al-Zohily *et al.*, 2020). In humans, this pathway is highly expressed in neonates and young children, however, further research is required to establish the physiological role of this pathway in both humans and animals (Al-Zohily *et al.*, 2020; Saponaro *et al.*, 2020).

Vitamin D is present in the blood in the free form or bound to a transporter protein such as vitamin D-binding protein (VDBP) Vitamin D is transported in the blood to vitamin D-target tissues mainly by vitamin D-binding protein. Vitamin D-binding protein is produced in the liver and is present in the blood in high concentrations, resulting in 97% of VDBP binding sites being unutilized (Bouillon & van Baelen, 1980). More than 80% of circulating vitamin D is transported by VDBP, however, vitamin D can also be bound to lipoprotein particles and albumin (Haddad *et al.*, 1993; Bouillon *et al.*, 2020). Vitamin D-binding protein is capable of binding all vitamin D metabolites, however, has a lower affinity for cholecalciferol and 1α,25-$(OH)_2$-D_3 and the highest affinity for 25-OH-D_3 (Bouillon *et al.*, 1980). However cellular receptors have a higher affinity for 1α,25-$(OH)_2$-D_3 than for 25-OH-D_3 (Bouillon *et al.*, 1980). Vitamin D that is not bound to VDBP can passively diffuse into cells, whereas vitamin D bound to VDBP enters the cell via active-receptor-mediated uptake (Chun, 2012).

2.3. Vitamin D requirements of broilers

Vitamin D_3 requirements of poultry are not well defined and there are some discrepancies in the true vitamin D requirements of broilers. Aviagen recommends that Ross broilers are fed 5 000 IU vitamin D_3/kg feed in the starter phase which gradually decreases to 4 000 IU vitamin D_3/kg in the finisher phase (Aviagen, 2022), while the recommendation of Cobb is 5 000 IU vitamin D_3/kg feed throughout the rearing cycle (Cobb-Vantress, 2022). The NRC recommendation for vitamin D_3 in broiler feed is 200 IU vitamin D_3/kg (NRC, 1994), however, this number is likely outdated as the genetic makeup of broilers have changed significantly during the last nineteen years. Whitehead *et al.* (2004) found that up to 10 000 IU vitamin D_3/kg is required to maximise bodyweight (BW) and tibia-breaking strength on day 14, however, 5 000 IU vitamin D_3/kg was sufficient to reduce TD to very low levels and maximise tibia ash. Fritts & Waldroup (2003) found 2 000 - 4 000 IU vitamin D_3/kg was adequate to maximise tibia ash and to reduce TD incidence and severity to its lowest level. A similar conclusion was reached by Sun *et al.* (2013) that found 2 000 - 4 000 IU vitamin D_3/kg improved birds' walking ability, tibia quality, and reduced footpad or hock dermatitis. Bodyweight and feed conversion ratio were improved when 2 000 IU vitamin D_3/kg were added to the diet of day 1 to 21 broilers (Gómez-Verduzco *et al.*, 2013). Rao *et al.* (2006) showed that tibia ash, feed efficiency and BW gain were improved to the level of the positive control when 2 400 IU vitamin D_3/kg was added to the diet of broilers from day 2 to 42, however, bone mineralisation only improved to the level of the positive control when 3 600 IU vitamin D_3/kg was added to the diet. Later, Rao *et al.* (2009) showed that 1 000 IU vitamin D_3/kg was adequate to maintain bone mineralisation, BW gain, feed intake and leg abnormality score of birds that were fed diets containing 5 grams (g) Ca/kg and 2.5 g non-phytate P/kg up to 5 weeks of age. Colet *et al.* (2015) concluded that there is no added benefit in increasing vitamin D supplementation above 3 500 IU vitamin D_3/kg on performance, TD incidence, bone quality parameters or carcass yield in day 1-21 broilers. From the studies referenced it can be concluded that the vitamin D requirement of broilers is in the range of 1 000 - 4 000 IU vitamin D_3/kg and that the 200 IU vitamin D_3/kg recommended by the NRC can be treated as the minimum vitamin D requirement to prevent pre-mature mortality. However, most research on vitamin D requirements as well as the factors that influence vitamin D requirements were performed more than a decade ago, with none being conducted in a South African commercial setting using South African diet types. New research is needed to establish the exact requirement of modern broilers.

2.4. Calcium absorption

In mineral homeostasis, vitamin D functions by increasing Ca absorption from the small intestine (Li *et al.*, 1997; Yoshizawa *et al.*, 1997). Calcium absorption in the small intestine occurs through two general pathways including the energy-dependent transcellular pathway and the energy-independent paracellular pathway (Christakos *et al.*, 2011).

2.4.1. Transcellular calcium absorption

The transcellular pathway is a saturable process and is the predominant mechanism of Ca absorption for low Ca diets. When the luminal Ca concentration is high, the majority of Ca is absorbed via the paracellular pathway through tight junctions (Figure 2.4) (Khanal & Nemere, 2008). The transcellular pathway occurs predominantly in the duodenum and jejunum as well as in the proximal colon and renal distal convoluted and connecting tubules (Alexander *et al.*, 2014).

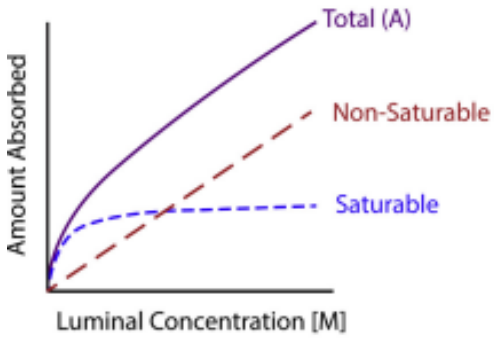

Figure 2.4 Kinetic modelling of the dynamics of intestinal mineral absorption via the saturable transcellular- and non-saturable paracellular pathways with increasing luminal concentration of calcium and phosphorus (Fleet, 2018)

Transcellular Ca absorption (Figure 2.5) involves three basics steps: entry of Ca into the cell, facilitated diffusion of Ca from the apical membrane to the basolateral membrane and finally, extrusion of Ca into the extracellular fluid (Proszkowiec-Weglarz & Angel, 2013). Entry of Ca into the cell and movement across the apical membrane is driven by a strong electrochemical gradient and facilitated by channel proteins, transient receptor potential of the vanilloid type (TRPV) 5, which is limited to the kidney, and TRPV6, which is present in the intestine and kidney (Montell, 2001; Pedersen, 2021). Once Ca has entered the cell, it binds to a transporter protein, calbindin 9k in mammals or calbindin 28k in avian species, forming a

calbindin-Ca complex, which mediates the diffusion from the apical membrane to the basolateral membrane before extrusion (Gross & Kumar, 1990; Nemere *et al.*, 1991). After the calbindin-Ca complex reaches the basolateral membrane, Ca is extruded into the circulatory system via the plasma membrane Ca-ATPase 1b (PMCA1b) and the sodium-calcium exchanger I (NCX1) (Huybers, 2009). The plasma membrane Ca-ATPase 1b transporter is localised in both the intestine and kidney whereas the NCX1 exchanger is limited to the tissues of the kidney (Van de Graaf *et al.*, 2004; Khanal & Nemere, 2008; Christakos *et al.*, 2011). Vitamin D is involved in the expression of proteins involved in the absorption of Ca into the cell as well as those involved in transcellular transport. The mechanism by which vitamin D influences the expression of these proteins is primarily mediated by the VDR. Studies in VDR KO mice have shown that expression of TRPV 6 decreases to 5 to 10% of the levels found in wild-type mice, while calbindin mRNA levels reduce by more than 50% the level of that found in the wild-type, indicating the importance of vitamin D for the expression of the proteins involved in Ca absorption (Wasserman & Taylor, 1968; Van Cromphaut *et al.*, 2001). Studies in TRPV6 KO mice have shown that when dietary Ca intake is normal, Ca absorption is similar in both wild-type and KO mice, suggesting that TRPV6 is redundant for Ca absorption and possibly compensated for by another protein (Kutuzova *et al.*, 2008). However, when the Ca supply is restricted, Ca absorption is less efficient in KO mice than in wild-type mice suggesting that TRPV6 might be important for Ca absorption under conditions of restricted supply (Benn *et al.*, 2008; Kutuzova *et al.*, 2008; Lieben *et al.*, 2010). Han *et al.* (2018) found 1α-OH-D_3 increases the expression of VDR in the small intestine and kidney of broilers. By increasing the expression of VDR in the duodenum of broilers, 1α-OH-D_3 indirectly increases the expression of TRPV proteins thereby increasing the absorption of calcium across the apical membrane.

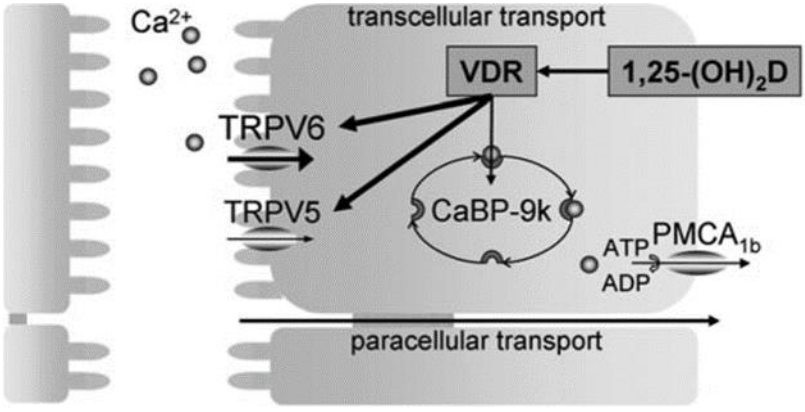

Figure 2.5 Vitamin D receptor mediated transcellular calcium absorption (Bouillon *et al.*, 2020)

2.4.2 Paracellular calcium absorption

Tight junctions are specialised membrane domains made up of complex structures composed of several interacting transmembrane and peripheral membrane proteins. Tight junctions are required for the normal functioning of epithelial cells and mediate the adhesion between them (Shin *et al.*, 2006; Christakos *et al.*, 2011). Tight junctions are located between the apical and basolateral membranes and act as a facilitator for paracellular transport (Tsukita *et al.*, 2001). Paracellular transport occurs throughout the entire length of the small intestine and involves the energy-independent transport of small molecules and ions from the intestinal lumen into the extracellular matrix across tight junctions (Khanal & Nemere, 2008; Lambert *et al.*, 2014). When dietary Ca intake is normal, the paracellular mechanism is responsible for the bulk of the Ca absorption from the intestine and renal tubule (Alexander *et al.*, 2014). For birds that receive diets with normal Ca concentrations, the ileum is the primary site of Ca absorption (Ravindran, 2013).

The intraluminal electrochemical gradient, the permeability of tight junctions, intestinal sojourn time, and the solubility of the Ca in the diet will dictate the rate of Ca absorption from a given segment of the digestive tract (Bronner, 1998). The transepithelial voltage potential is negative for all segments of the digestive tract, this means that the energy required for the paracellular movement of Ca must be derived from the chemical concentration gradient (Alexander *et al.*, 2014). The chemical concentration gradient required for the paracellular diffusion of Ca is generated by the movement of sodium (Na) with the purpose to create an osmotic gradient for water reabsorption from the small intestine. This allows Ca to diffuse across tight junctions down its concentration gradient or be driven across tight junctions along with water through solvent drag (Figure 2.6) (Alexander *et al.*, 2014).

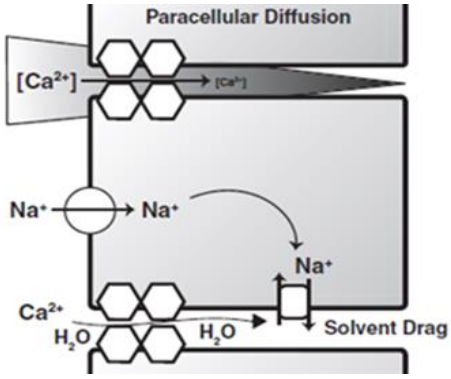

Figure 2.6 The paracellular movement of calcium across tight junctions via diffusion and solvent drag (Alexander *et al.*, 2014)

Tight junction permeability is not constant and is regulated under different physiological conditions. Tight junction permeability can be altered by growth factors, cytokines, bacterial toxins, serine-threonine kinases, and hormones, including 1α,25-$(OH)_2$-D_3 (Christakos *et al.*, 2011). Paracellular Ca transport is facilitated by transmembrane proteins claudin-2 and claudin-12. Active vitamin D, 1α,25-$(OH)_2$-D_3, has been shown to increase the expression of these genes, thereby increasing the permeability of tight junctions, and increasing Ca absorption (Fujita *et al.*, 2008). In a study conducted by Zhang *et al.* (2015) on VDR KO mice, the authors have shown that the CLDN2 gene that encodes claudin-2 is a direct target of VDR and therefore under the direct influence of 1α,25-$(OH)_2$-D_3. The results of Zhang *et al.* (2015) and Fujita *et al.* (2008) indicate the importance of vitamin D for the paracellular absorption of Ca across the intestinal tight junctions. The transit times or intestinal sojourn times of digesta through different segments of the digestive tract is another important factor influencing the amount of Ca that will be absorbed via the paracellular pathway. In poultry, the ileum has the longest average digestive transit time and feed can reside within the ileum for 50 to 70 minutes, hence the ileum is the site where the majority of Ca is absorbed paracellularly (Ravindran, 2013).

2.4.3. Hormonal regulation of calcium homeostasis

Calcium homeostasis involves the regulation of the concentration of Ca ions present in the extracellular fluid within very specific narrow limits (Mundy & Guise, 1999). Calcium is present in several distinct forms in the blood serum of animals, which includes free ionised Ca, Ca bound to proteins such as albumin and other biomolecules as well as Ca that is bound to other anions such as bicarbonate, citrate and phosphate (Stanford, 2006). Ionised Ca is the physiologically active fraction of serum Ca and is responsible for the physiological actions of Ca (Parsons & Combs Jr, 1981). Normal ionised Ca values for broilers range from ±1.5 mmol/l in young birds to ±1.4 mmol/l in older birds (Kölling *et al.*, 1992) with total Ca values comparable to those found in mammals (Parsons & Combs Jr, 1981). The regulation of ionised Ca is controlled by several tissues such as the intestines, bone and kidneys and regulated by endocrine hormones secreted from several organs such as the parathyroid gland, kidney, and thyroid gland. The main hormones involved in the regulation of Ca are PTH, 1α,25-$(OH)_2$-D_3 and calcitonin. Other hormones that also play a role in Ca and P homeostasis include fibroblast growth factor-23 and klotho, however, research suggests that only PTH, 1α,25-$(OH)_2$-D_3 and

calcitonin are regulated by direct negative feedback control (Mundy & Guise, 1999; Taylor & Bushinsky, 2009).

2.4.4. The role of vitamin D in calcium homeostasis

The classical genomic pathway is the main pathway by which 1α,25-$(OH)_2$-D_3 exerts its effects and involves the binding of 1α,25-$(OH)_2$-D_3 to VDR. This allows VDR to reach the nucleus and form a heterodimer with retinoid X receptor (RXR) and interact with gene response elements (Saponaro *et al.*, 2020). The interaction of VDR with RXR is a crucial step in the formation of transcriptional machinery at specific DNA sequences called vitamin D response elements (VDRE) at the promoters regions of 1α,25-$(OH)_2$-D_3 target genes (Rowland-Goldsmith *et al.*, 1999; Proszkowiec-Weglarz & Angel, 2013; Saponaro *et al.*, 2020). Vitamin D response elements have been discovered in the promoter regions of numerous genes involved in Ca homeostasis such as the genes of PTH, calbindin, TRPV5, TRPV6, NCX1, osteoclastin and osteoporin (Liu *et al.*, 1996; Lu *et al.*, 2000; Pike *et al.*, 2007; Saponaro *et al.*, 2020). The interaction of VDR and RXR with the VDRE initiates a series of events that involve the recruitment of several coactivator or corepressor complexes that alter the transcriptional output of 1α,25-$(OH)_2$-D_3 target genes (Pike *et al.*, 2007). Although the classical genomic pathway seems to be the pathway of preference by which 1α,25-$(OH)_2$-D_3 exerts its effects, an alternative pathway characterised by a rapid response to an increased concentration of 1α,25-$(OH)_2$-D_3 has been suggested (Haussler *et al.*, 2011). This rapid VDR-mediated response (15 to 45 minutes) is uncharacteristic of the slow response time of the classical genomic pathways which can take hours to days before a measurable response can be detected. Haussler *et al.* (2011) suggested that a classical steroid receptor allows a variety of ligand shapes to bind to the receptor and to initiate either a quick or a genomic response. The primary function of 1α,25-$(OH)_2$-D_3 is to maintain Ca and P balance in the body by acting on VDR-regulated genes located in tissues across the GIT and bones of broilers (Pike *et al.*, 2007). The hydroxylation of 25-OH-D_3 to active 1α,25-$(OH)_2$-D_3 in the kidney is determined by plasma ionised Ca concentration (Liao, 2018). When the blood ionised Ca concentration reaches its homeostatic threshold, 25-OH-D_3 is inactivated by 24-hydroxylation to 24R,25-$(OH)_2$-D_3 which is then further catabolised to 26,23-lactone or calcitroic acid and excreted in the bile or urine. When the ionised Ca concentration in the blood is below the homeostatic threshold, 1α-hydroxylase is activated in

the kidney and physiologically active 1α,25-$(OH)_2$-D_3 is synthesised, stimulating Ca absorption from the small intestine or resorption from the bone (Proszkowiec-Weglarz and Angel, 2013).

2.4.5. The role of parathyroid hormone, the calcium-sensing receptor and calcitonin in calcium homeostasis

The chief cells of the parathyroid gland produce parathyroid hormone, which is an 84-amino-acid peptide that directly influences Ca homeostasis (Nemere & Norman, 1986). Parathyroid hormone secretion is largely reliant on ionised Ca concentrations, forming a straightforward negative feedback loop, meaning that as ionised Ca levels increase, the concentration of PTH in the blood decreases. The primary function of PTH is to ensure adequate blood-ionised Ca levels for the normal physiological functioning of cellular processes and tissues. When blood ionised Ca levels are low, PTH increases osteoclastic activity to promote the reabsorption of Ca and P from the bone (Mundy & Guise, 1999; Proszkowiec-Weglarz & Angel, 2013). When an animal is in a state of hyperphosphatemia or the Ca:P ratio in the blood is suboptimal, PTH restricts phosphate reabsorption from the renal tubules and stimulates Ca absorption by increasing the renal synthesis of the physiologically active form of vitamin D (Mundy & Guise, 1999; Proszkowiec-Weglarz & Angel, 2013). Parathyroid hormone is secreted in response to both short-term fluctuations in serum Ca concentrations, and sustained low serum Ca concentrations, of which the latter stimulates the conversion of cholecalciferol to 1α,25-$(OH)_2$-D_3 (Mundy & Guise, 1999; Hoenderop *et al.*, 2005; Schröder & Breves, 2006). The calcium-sensing receptor (CaSR) is the molecular mechanism by which the chief cells of the parathyroid gland detect alterations in blood-ionised Ca concentration and regulate PTH secretion to keep serum Ca levels within a tight physiological range (Brown *et al.*, 1993; Chen & Goodman, 2004; van Abel, 2006). The Ca-sensing receptor stimulates the increased release of pre-synthesised, stored PTH when the ionised Ca concentration drops below the normal physiological range, increasing the uptake of Ca from the gastrointestinal tract (GIT) (Taylor and Bushinsky, 2009). Under hypercalcemic conditions, CaSRs stimulate the release of Ca from within cells and activate certain Ca-dependent proteases that cleave active PTH to its inactive form, thereby reducing the PTH-induced increase in ionised Ca concentration (Potts Jr & Jüppner, 1998; van Abel *et al.*, 2005; Taylor & Bushinsky, 2009). Calcium-sensing receptors have also been identified in thyroid tissue, where it regulates

calcitonin secretion (Bar *et al.*, 1972). Calcitonin is a peptide hormone consisting of 32 amino acids that is produced by the c-cells of the thyroid gland (Murayama *et al.*, 1999; Felsenfeld & Levine, 2015). Calcitonin plays an important role in Ca homeostasis and protects the skeleton by acting as a potent inhibitor of osteoclast-mediated bone resorption (Brandi & Brown, 2015). Calcitonin is mainly secreted in response to elevated ionised Ca concentrations, although several gastro-intestinal hormones have also been found to stimulate the secretion of calcitonin (Mundy & Guise, 1999; Davey & Findlay, 2013). Calcitonin secretion is inhibited by severe decreases in ionised Ca concentrations. It has been suggested that the ability of calcitonin to lower blood ionised Ca, is because of the effects of the hormone on Ca efflux rather than influx (Davey & Findlay, 2013). This means that calcitonin restricts the resorption of Ca from the bone rather than promoting bone formation.

2.5. Phosphorus absorption

2.5.1 Transcellular and paracellular phosphorus absorption

Like Ca absorption, P absorption occurs via transcellular and paracellular mechanisms in the small intestine of broilers. Paracellular phosphate transport is a passive, non-saturable and sodium-independent process by which inorganic phosphorus is absorbed across tight junctions down their electrochemical gradient (Borowitz & Ghishan, 1989). Paracellular Pi absorption seems to be hormonally unregulated, however, is dependent on the electrochemical gradient, the permselectivity of the tight junctions, as well as the concentration gradient across the intestinal epithelium (Saurette & Alexander, 2019). The paracellular mechanism transports the majority of Pi from the small intestine into the blood when the concentration of Pi in the intestinal lumen is normal, however, when the concentration of Pi in the intestinal lumen is low, transcellular Pi absorption pre-dominates (Saurette & Alexander, 2019; Hill Gallant & Vorland, 2021). Transcellular Pi transport relies pre-dominantly on type II sodium-dependent Pi transporters (NaPi-II) in the small intestine and kidney (Sabbagh *et al.*, 2009). The type IIb NaPi transporter has the highest expression in the duodenum followed by the jejunum and the ileum and is considered the primary transporter responsible for the absorption of Pi from the small intestine (Yan *et al.*, 2007). The expression of the type IIb NaPi transporter in the small intestine is regulated by hormonal and dietary factors (Saurette & Alexander, 2019). Hormones that regulate NaPi-IIb include 1α,25-$(OH)_2$-D_3, FGF23 and PTH. When serum Pi levels are low, the

production of 1α,25-$(OH)_2$-D_3 is upregulated, which in turn increases the expression of NaPi-IIb and the uptake of Pi from the jejunum (Segawa *et al.*, 2004). When serum Pi increases above the homeostatic threshold, FGF23 is secreted from osteocytes and osteoblasts. The secretion of FGF23 restricts the synthesis of 1α,25-$(OH)_2$-D_3 thereby decreasing the expression of the VDR-dependent NaPi-IIb transporter, subsequently lowering the absorption of Pi from the small intestine (Bergwitz & Jüppner, 2010; Takahashi *et al.*, 2014). Parathyroid hormone is released in response to elevated serum Pi levels and stimulates the kidney to secrete Pi into the urine, thereby preventing hyperphosphatemia (de Francisco *et al.*, 1998; Lee *et al.*, 2017). The secretion of PTH when serum Ca is low also increases 1α,25-$(OH)_2$-D_3 production which increases NaPi-IIb expression and therefore PTH has a dual effect on Pi absorption (Tatsumi *et al.*, 2016; Jacquillet & Unwin, 2019; Saurette & Alexander, 2019). Dietary factors also influence transcellular Pi absorption via its influence on Na-IIb expression. Segawa *et al.* (2004) found that VDR-KO rats fed a P-deprived diet, upregulated the expression of NaPi-IIb. This suggests that low P diets can increase the expression of NaPi-IIb independently of 1α,25-$(OH)_2$-D_3. Yan *et al.* (2007) also showed that reducing dietary P from 0.50% to 0.25% led to a 2.3-fold increase, on average, in the expression of NaPi-IIb across all segments of the small intestine. The relative contribution of the transcellular pathway to total phosphorus absorption is dictated by the relative abundance of Pi transporters in the small intestine and the Pi concentration in the lumen (Saurette & Alexander, 2019). Hu *et al.* (2018) suggested that inorganic Pi transporters (PiT 1 and PiT 2) may also play a role in Pi absorption in the small intestine of broilers. The protein expression level of the inorganic PiT 2 transporter and NaPi-IIb transporter increased when dietary non-phytate P increased (Hu *et al.*, 2018). This suggests a possible role of inorganic Pi transporters in Pi absorption in the small intestine of broilers, however requires further investigation. While NaPi-IIb is the predominant transcellular Pi transporter in the small intestine, NaPi-IIa and NaPi-IIc are the predominant Pi transporters in the proximal tubule of the kidney (Sabbagh *et al.*, 2011). In the kidney, 70 - 80% of Pi reabsorption can be attributed to the action of NaPi-IIa (Lederer, 2014). The NaPi-IIa transporter is regulated by numerous factors, including 1α,25-$(OH)_2$-D_3, PTH, FGF23, dopamine, glucocorticoids, acid-base balance, insulin, and dietary Pi intake (Bourgeois *et al.*, 2013; Manghat *et al.*, 2014). While the NaPi-IIc transporter is regulated by PTH, FGF23 and dietary Pi intake (Bourgeois *et al.*, 2013; Manghat *et al.*, 2014). The supplementation of 1α-OH-D_3 did not increase the mRNA expression of type IIb Na-Pi cotransporter in the duodenum of day 21 broilers. However, 1α-OH-D_3 supplementation increased the expression of the type IIb- and IIa Na-Pi cotransporters

in the jejunum and ileum, and proximal renal tubule, respectively (Han *et al.*, 2009; Han *et al.*, 2018).After Pi have been absorbed across the apical membrane of the epithelial cells of the small intestine and kidney by NaPi-IIa, NaPi-IIb and NaPi-IIc, Pi is extruded across the basolateral membrane into the extracellular fluid. The mechanism by which Pi is transported across the cell and extruded across the basolateral membrane is not clear (Manghat *et al.*, 2014).

2.6. The role of phytate and phytase on calcium and phosphorus digestibility

The majority of P (60-90%) present in conventional ingredients that are used for the production of poultry diets, is present in the form of myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (Phytate; IP6) (Figure 2.7), also known as phytic acid or its salt form, phytate (Nelson & Ferrara, 1968). Phytate is a polyanionic molecule that forms the principal storage form of P in many plant tissues. Phosphorus stored in the form of phytate needs to be released from the phytate molecule to be utilised by monogastric animals. Phytate is capable of chelating different divalent and trivalent cations and has a strong binding affinity to many minerals present in poultry diets such as Ca, copper, cobalt, magnesium, zinc, manganese, and iron (Jatuwong *et al.*, 2020). These mineral-phytase complexes precipitate into insoluble mineral-phytate complexes in the neutral to slightly basic pH environment of the small intestine (Taylor, 1965; Schlemmer *et al.*, 2001; Selle *et al.*, 2009; Proszkowiec-Weglarz & Angel, 2013). Additionally, when more than one type of cation is present, as is the case in the digestive tract of birds, it further promotes the formation of insoluble complexes (Simpson & Wise, 1990; Kryukov *et al.*, 2021).

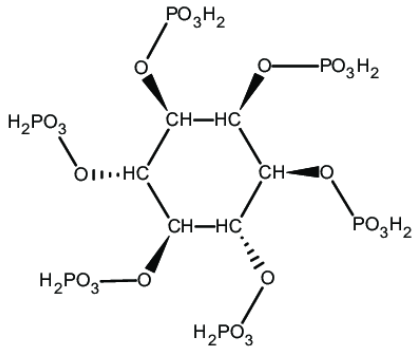

Figure 2.7 The chemical structure of 1,2,3,4,5,6-hexakis dihydrogen phosphate (Lesjak & Srai, 2019)

The pH determines the solubility of the formed phytate-mineral complex, with most phytate-mineral complexes being insoluble at pH values ranging from 4 - 7. This creates a challenge as the pH range of insoluble mineral-phytate complex formation corresponds to the pH of the small intestine of birds, where most of the mineral absorption occurs (Tamim & Angel, 2003; Selle *et al.*, 2009). Phytate can form binary and ternary protein-phytate complexes. Binary protein phytate complexes are formed at low pH values where proteins and amino acids such as histidine, lysine and arginine are below their isoelectric point and carry a net positive charge that can bind directly to the negative charge of the phytate molecule under acidic conditions (Selle *et al.*, 2012). Ternary protein-phytate complexes are produced in the more alkaline environment of the small intestine where proteins and amino acids exceed their isoelectric point and carry a net negative charge, which can indirectly bind to phytate through the formation of a cationic bridge with a phytate-bound cation, such as Ca, resulting in the production of a protein-mineral-phytate complex (Selle *et al.*, 2012).

2.6.1 Phytase classification and action

Phytate P as well as minerals, proteins and amino acids bound to phytate in the form of phytate-mineral and phytate-protein complexes are poorly digested and absorbed in the small intestine (Selle *et al.*, 2009; Selle *et al.*, 2012; Dersjant-Li *et al.*, 2015). However, the ability of phytic acid to bind cations is significantly reduced after the hydrolysis of phytate to lower phosphate inositol esters. This reduced ability to bind cations is due to a decrease in the negative charge of the phytic acid molecule with the removal of every phosphate group (Schlemmer *et al.*, 2001; Kryukov *et al.*, 2021). Phytases used in commercial feed formulation belong to the group of enzymes called histidine-acid phosphatases and are classified according to their dephosphorylation starting point on the inositol ring (Lei *et al.*, 2013). Currently, three forms of phytases exist including 3-phytases, 5-phytases and 6-phytases (Zeller *et al.*, 2015). The majority of commercially available phytases are in the form of 6-phytases with only a few being 3-phytases (Menezes-Blackburn *et al.*, 2015). Phytases can be further classified into alkaline phytases and acidic phytases based on their pH optimum (Jorquera *et al.*, 2008).

Phytase hydrolyses phytate into lower inositol-phosphate esters i.e., IP5, IP4, IP3 and IP2. The hydrolyses occurs in a stepwise fashion, releasing inorganic P that is available for absorption. Phytases first cleave the phosphate in the 3rd or 6th position, depending on whether it is a 3 or

6- phytase, respectively. Phytase enzymes give the highest priority to IP6 with the affinity decreasing for every subsequent phosphate molecule that is released from the inositol structure. This means that phytase has the highest affinity for IP6 followed by IP5 and so forth, and the lowest affinity for IP1 (Wyss *et al.*, 1999; Yu *et al.*, 2012). The progressive elimination of each succeeding phosphate group from the inositol structure improves the solubility of the produced molecule, thereby increasing the accessibility of the formed molecule to phytase enzymes (Schlemmer *et al.*, 2001; Kryukov *et al.*, 2021). For instance, only 2% of IP6 is soluble in the small intestine which increases to 7% for IP5, 8% for IP4, 31% for IP3 and 75% for IP2 (Schlemmer *et al.*, 2001). The ideal would be for phytase enzymes to successfully hydrolyse and make every bound phosphate group available to the animal, however, *in vivo*, the degree of hydrolysis is often incomplete and as a result, IP6 is hydrolysed to a mixture of lower inositol-phosphate esters (Dersjant-Li *et al.*, 2015).

2.6.2. The relationship between calcium and phosphorus

Leytem *et al.* (2008) found that broilers can effectively utilise more than 75% of phytate-P in cereal-based diets in the absence of phytase when the diets did not contain added Ca and P. Similarly, Tamim *et al.* (2004) found that 68% of phytate-P in a corn-soybean meal based diet is effectively hydrolysed by broilers in the absence of added Ca and phytase. However, this number declines rapidly to 21% when 5g Ca/kg is added. Plumstead *et al.* (2008) also demonstrated that phytate P digestibility linearly declines by 71% as Ca inclusion increases from 4.7 g Ca/kg to 11.6 g Ca/kg. Plumstead *et al.* (2008) calculated that the optimal Ca: non-phytate P ratio to maximise P retention and minimise P excretion for broilers given diets containing 2.8, 2.4, and 1 g phytate-P/kg is 2.53:1, 2.40:1 and 2.34:1, respectively. Therefore, as the Ca level in the diet increases P digestibility decreases. Three reasons have been proposed to explain the decline in P digestibility with increasing levels of Ca and are as follows: (1) The formation of indigestible Ca-phytate complexes, (2) Increased pH of the GIT which influences the solubility properties of phytate mineral-complexes and (3) the competition for the active site of phytase by Ca (Tamim *et al.*, 2004).

2.6.3 Calcium-complex formation

Phytate is a polyanionic molecule that carries a total of twelve negative charges when in a completely deprotonated state. Calcium has a positive charge of two and can readily bind to phytate until the Ca-phytate complex is in an electrochemically stable state. In the process of achieving electrochemical stability, phytate can bind a maximum of six Ca ions (Selle *et al.*, 2009). Divalent cations have differing affinities for phytate, however, the cation most susceptible to phytate binding is Ca. Although Ca has a relative binding affinity lower than most divalent cations, it is present in the diet at much greater concentrations than other minerals and hence the greatest effect is observed in Ca digestion and absorption (Tamim & Angel, 2003; Tamim *et al.*, 2004). Mineral-phytate complexes such as Ca-phytate complexes are insoluble and resistant to hydrolysis by phytate enzymes (Taylor, 1965), consequently, the Ca and P bound to phytate is not absorbed in the small intestine and is ultimately excreted, contributing to environmental contamination. The Ca concentration of most cereal grains and by-products are relatively low. When considered together with the intrinsic chemical structure of phytate, it restricts the extent to which mineral-phytate complexes are present in feed ingredients (Selle *et al.*, 2009). The majority of insoluble Ca-phytate complexes are formed when ingested Ca and phytate progress along the GIT of monogastric animals. According to Champagne (1988), the main factors influencing the solubility of mineral-phytate complexes are (1) the specific mineral, (2) the pH, (3) the phytate-to-mineral molar ratio and (4) the presence of other minerals. Ca-phytate complexes precipitate into insoluble complexes at a pH of 4-6. The pH of the GIT of poultry ranges from 2.5 to 3.5 in the proventriculus and gizzard to as high as 8 in the caecum, with the pH of the small intestine, the location where mineral absorption takes place, ranging from 5 to 7.5 (Ravindran, 2013). Therefore, the pH of the small intestine favours the formation of Ca-phytate complexes and limits the extent to which phytase enzymes can operate. Except for a few studies that indicate that Ca-phytate complexes can form at a low pH or over a wide range of pHs (Graf, 1983; Wise, 1983; Marini *et al.*, 1985; Champagne, 1988), it is generally agreed upon that a pH of 5 it the pivotal pH for Ca-phytate complex formation (Evans & Pierce, 1981; Grynspan & Cheryan, 1983; Martin & Evans, 1986; Oberleas & Chan, 1997). With regards to the molar ratio, the Ca-to-phytate molar ratio in a typical Ca-phytate complex is 4.93:1 (Marini *et al.*, 1985). This indicates that on average, every phytate molecule that forms part of a Ca-phytate complex is bound to 5 Ca atoms. Therefore, Ca will directly influence P

digestibility through its interaction with phytate and subsequent Ca-complex formation, hindering the hydrolysis of phosphate groups from the insoluble Ca-phytate-complex.

2.6.4 The influence of calcium on pH and phosphorus digestibility

Limestone serves as the primary source of Ca in most poultry diets. The acid binding capacity of limestone is 15044 mEq/kg at a pH of 3 (Lawlor *et al.*, 2005). This is remarkably high and consequently Ca in the form of limestone will tend to increase the pH of digesta along the gut. This alkalising property of ingested limestone has a profound effect on both the formation of Ca-phytate complexes, the activity of phytase enzymes and protein digestion. By increasing the concentration of Ca in the diet, it favours the formation of Ca-phytate complexes due to an increase in the molar ratio of Ca: phytate as well as an increase in the pH of the gut in favour of Ca-phytate complex formation (Selle *et al.*, 2009). According to Liebert (1993), the crop, proventriculus and gizzard are the epicentres of phytase activity, this is due to the phosphate groups of phytic acid being in a protonated state at lower pHs allowing hydrolysis by phytase enzymes. Additionally, Ca can directly compete for the active sites of phytase, thereby directly influencing phytase activity and hence phytate P hydrolysis, the exact mechanism by which this happens is still unclear and further research regarding the binding of Ca to the active sites of phytase is required (Wise, 1983; Pointillart *et al.*, 1989; Qian *et al.*, 1996; Walk, 2016). Calcium supplied in the form of limestone also indirectly influences P digestibility through its effects on phytase enzymes themselves, by increasing the pH of the gut, it decreases the activity of most commercial phytase enzymes, the extent of which will be determined by the optimal pH range of the phytase product (Dersjant-Li *et al.*, 2015).

2.7. Conclusion

From the reviewed literature it can be concluded that vitamin D is important for the optimal metabolism of calcium and phosphorus, especially when dietary Ca and P are restricted. Vitamin D_3 must undergo two hydroxylation steps before becoming biologically active. The second hydroxylation, which is mediated by renal 1α-hydroxylases, is highly regulated and therefore regarded as the rate-limiting step in the synthesis of the active metabolite, 1α,25-$(OH)_2$-D_3. The vitamin D analogue, 1α-OH-D_3, bypasses this rate-limiting regulatory step and is transformed into 1α,25-$(OH)_2$-D_3 after hydroxylation by hepatic 25-

hydroxylase enzymes. Once in the active form, vitamin D upregulates the expression of proteins involved in the transcellular absorption of Ca and P. Phytase increases Ca and P digestibility by the detailed mechanisms outlined in this review and could potentially synergistically act with vitamin D to further increase the digestibility of Ca and P. The hypothesised mechanism for the synergism can be ascribed to the different modes of action by which the two supplements exert their effects. Phytase can increase the amount of Ca and P available for absorption in the small intestine while vitamin D directly stimulates the expression of proteins that are responsible for the absorption of Ca and P. The extent to which 1α-OH-D_3 can influence Ca and P digestibility is not well defined in literature and requires further investigation. Moreover, more research is needed to clarify and validate the existence of a possible synergistic effect when cholecalciferol and phytase are jointly supplemented into the diets of poultry as well as the extent to which it can alter Ca and P digestibility. These intricacies are those that this project aims to address. The use of 1α-OH-D_3 and phytase in poultry diets could potentially make a valuable contribution to the future sustainability of the poultry industry while also decreasing the environmental footprint and feed cost.

Chapter 3

Materials and Methods

3.1. Digestibility trial

3.1.1 Site of study

This study was conducted in the broiler facilities and metabolic facility of the Innovation Africa experimental farm at the University of Pretoria. The University of Pretoria's Animal Ethics Committee gave its approval to all the methods employed in this study (NAS040/2022).

3.2.2 Animal rearing and metabolic facility placement

A total of 1500 male Ross 308 broilers were purchased from a local hatchery and reared in floor pens in an environmentally controlled broiler house (Innovation Africa, Hillcrest, South Africa). The rearing facility consisted of 34 large floor pens (3m x 2m) containing two tube feeders and two bell drinkers per pen. Before placement, the house was disinfected, and the floor of each pen was covered with clean pine shavings. The house was then pre-heated to 36°C to ensure optimal chick comfort upon chick arrival. Birds were randomly selected and weighed before being placed into pens. Birds were placed in such a manner that every pen contained 45 birds. After placement, automatic heaters controlled by SKOV controllers regulated minimum and maximum temperatures to provide the ideal temperature to ensure birds are comfortable at all times. Automatic ventilation was used to provide an ideal oxygen supply and to prevent the build-up of unwanted gasses. Following Aviagen (2018) recommendations, a standard lighting regime was followed that promotes healthy eating and drinking habits as well as optimal organ development. This consisted of one-hour darkness followed by 23 hours of light for the first seven days followed by six hours of darkness and 18 hours of light for the remainder of the period. All birds had *ad libitum* access to feed and water. All further housing and care not specified in this document were done according to the breed standard (Aviagen, 2018). Birds were fed a nutritionally adequate pre-starter, starter, a grower diet as part of the rearing program (Table 3.3).

This study consisted of two experimental periods conducted over two age ranges. The first experimental period ranged from days 6 to 10 (D6-10), while the second experimental period ranged from days 19 to 23 (D19-23). In preparation for the digestibility trial to be concluded on D10, on day 6, a hundred random birds were weighed to determine the mean and standard deviation as a representation of the group. On the same day, directly after weighing a hundred birds and determining the mean and standard deviation of the BW, every bird was weighed individually and grouped according to one of the following groups:

(1) within one standard deviation above the mean
(2) within one standard deviation below the mean
(3) more than one standard deviation above the mean but less than two standard deviations above the mean
(4) more than one standard deviation below the mean but less than two standard deviations below the mean
(5) more than two standard deviations above or below the mean

After weighing and grouping the birds into various groups based on BW, four birds from group (1) and four birds from group (2) were randomly selected and put together and the weight of the eight birds was recorded. The eight birds were then transferred to one of 99 battery cages (9 treatments x 11 replicates per treatment = 99 cages) that have been prepared in the metabolic facility (Innovation Africa, Hillcrest, South Africa), forming an experimental unit. This process was repeated until all 99 cages have been filled with eight broilers. The ±708 birds that were not selected to form part of the first experiment to be conducted from D6-10 were redistributed equally into the 34 pens of the rearing facility for further rearing until preparations for the next sampling day commenced. On day 19, in preparation for the second digestibility experiment, the same methodology was used to group birds as done previously in preparation for the first experiment. However, six birds were used instead of eight and therefore three birds from group (1) and three birds from group (2) were randomly selected and put together and the weight of the six birds was recorded. The six birds were then transferred to one of 99 battery cages in the metabolic facility until all 99 cages were filled. This process ensured that each cage had a similar mean BW and BW distribution before treatment diets were administered. This process minimises variation that could arise due to differences in BW. Selecting and

transferring birds to the metabolic facility four days before being sampled, allowed birds to adapt to the cages as well as to feeding and drinking in their new environment, minimising variation in feed and water intake. The birds that have been transferred to the metabolic cages then received an adaptation diet (Table 3.3), which was formulated to supply neither an excess nor a deficit of Ca and P. Adaptation diets were fed for 2.5 days before treatment diets were administered. Treatment diets were administered in a staggered manner exactly 36h before the scheduled sampling of the pen. This prevented any physiological changes in the birds' bodies that could result in the up-or-down regulation of Ca and P absorption, potentially influencing the results (Imondi & Bird, 1966; Uni *et al.*, 2001). Fresh treatment feed was provided in a staggered manner 6 hours before the scheduled sampling to ensure adequate ileal content at sampling.

3.2.3 Treatment blocking, bird allocation and pen design

The metabolic facility was set up to contain 99 battery cages (90 cm x 52 cm x 34 cm) which allowed for eleven replicates of nine treatments, allowing adequate statistical power to prove significance ($p<0.05$). The cages were arranged in tiers, with each tier containing six cages. The treatments were arranged in a completely randomised block design as shown in Figure 3.1. The metabolic facility was pre-heated to the desired temperature before the birds were transferred into the cages. Temperature and ventilation were further regulated automatically by the installed SKOV system to ensure optimal comfort. The lighting regiment was consistent with what was done during rearing. All birds had continuous access to fresh feed and water. All other management practices were aligned with the breed standard (Aviagen, 2018).

Index:

—— Wall

—— Door

---- Block division

Figure 3.1 Blocked arrangement of pens in the metabolic facility

3.2.4 Experimental design and treatments

A digestibility trial was conducted at two different ages at days 10 (D10) and 23 (D23), respectively. The treatments were arranged in a 3 x 3 factorial design (Table 3.1). Dietary treatments were formulated to contain either 0, 2.5 or 5 µg/kg 1α-OH-D_3 (Premex, Medellín, Colombia) (AlphaD3™) and 0, 500 or 1000 FTU/kg phytase (IFF Nutrition & Biosciences, Leiden, Netherlands) (Axtra® PHY GOLD, novel consensus bacterial 6-phytase variant., expressed in *Trichoderma reesei*). The 1α-OH-D_3 and phytase levels were based on the manufacturer's recommendations and what is done commercially in

broiler integrations, respectively. The experimental design remained identical for both ages.

Table 3.1 Formulated and analysed values of 1α-hydroxycholecalciferol (µg/kg) and phytase (FTU/kg) of treatment diets used for the determination of calcium and phosphorus digestibility of broilers at 10 and 23 days-of-age

Treatment (T)	D10		D23	
	Formulated (analysed) 1α-OH-D_3* (µg/kg)	Formulated (analysed) Phytase# (FTU/kg)	Formulated (analysed) 1α-OH-D_3 (µg/kg)	Formulated (analysed) Phytase (FTU/kg)
T1	0 (<0.48)	0 (46)	0 (<0.48)	0 (0)
T2	2.5 (2.71)	0 (44)	2.5 (2.45)	0 (79)
T3	5 (6.78)	0 (2)	5 (7.91)	0 (84)
T4	0 (<0.48)	500 (513)	0 (<0.48)	500 (513)
T5	2.5 (2.8)	500 (425)	2.5 (2.21)	500 (661)
T6	5 (4.50)	500 (457)	5 (5.11)	500 (512)
T7	0 (0.05)	1000 (922)	0 (<0.48)	1000 (1289)
T8	2.5 (2.80)	1000 (1009)	2.5 (2.52)	1000 (1052)
T9	5 (4.75)	1000 (863)	5 (4.67)	1000 (1091)

*1α-OH-D_3: 1α-hydroxycholecalciferol (AlphaD3™; Premex, Medellín, Colombia)

#Phytase: Axtra® PHY GOLD (IFF Nutrition & Biosciences, Leiden, Netherlands)

3.2.5 Diets and mixing

The diets used in this study can be grouped according to three distinct phases i.e., rearing, adaptation and experimental (Table 3.2). All diets used in this study were formulated using Spesfeed Express software (Spesfeed consulting, Broederstroom, South Africa). The rearing phase involved a specially formulated pre-starter, starter, and grower diet. Rearing diets were administered until birds were moved to the metabolic cages on days 6 and 19, respectively. The ingredient inclusion levels of rearing diets are displayed in Table 3.3**.** After birds were moved to metabolic cages on days 6 and 19, an

adaptation diet, formulated to provide Ca and P in neither an excess nor a deficit, was fed for 72 hours.

Table 3.2 The description, phase, age (days) and pellet size (mm) of diets used during rearing, adaptation, and the experimental period

Phase	Diet description	Age (days)	Pellet size (mm)	Facility
Rearing	Pre-starter	0 – 6	3	Rearing
	Starter	7 – 14	3	
	Grower	15 – 19	5	
Adaptation	Adaptation 1	6 – 8.5	3	
	Adaptation 2	19 – 21.5	5	
Experimental	T1-9 (D10)[1]	8.5 – 10	3	Metabolic
	T1-9 (D23)[2]	21.5 – 23	5	

[1]T1-9 (D10): Treatments 1 – 9 for day 10 experimental period
[2]T1-9 (D23): Treatments 1 – 9 for day 23 experimental period

Experimental diets were mixed in two steps. First, two separate basal diets were mixed for each of the two 36-hour experimental periods i.e., days 8.5 - 10 (D8-10) and 21.5 - 23 (D21-23) (Table 3.4). A 40:60 mixture of titanium dioxide (TiO_2) and maize was prepared by adding the TiO_2 and maize proportionally into a 25 kg Hobart mixer and mixing it for 3 minutes. After completion, the TiO_2-maize blend was milled through a 1 mm screen to allow for better distribution throughout the feed before being mixed into the two respective basals to allow for final treatment diets to contain 5 g TiO_2/kg.

A set amount of 1α-OH-D_3 and/or phytase was weighed out separately using an analytical balance (0.0001 g) for every treatment, to allow for final treatment diets to contain 1α-OH-D_3 and/or phytase at levels specified in Table 3.1. A blend of 1α-OH-D_3 and/or phytase was then first mixed in a small blender with 400 g of maize meal and 10 g of soy oil to improve the distribution of tested substances throughout the final mixture. A pre-determined weight of either the D8-10 or D21-23 basal was then weighed out separately for each treatment and added together with the 1α-OH-D_3 and/or phytase blend in a Hobart mixer and mixed for 3 minutes to give the final treatment diets. The

maize and soy oil added to 1α-OH-D_3 and/or phytase and TiO_2 blends were accounted for in the formulation of the final diets. Rearing, adaptation, and basal diets were mixed using a two-tonne fountain blender at SimpleGrow (Centurion, South Africa). All feed was pellet as specified in Table 3.2.

Table 3.3 Raw material composition of rearing and adaptation diets on a dry matter basis

Feed ingredient (%)	Rearing			Adaptation	
	Pre-starter	Starter	Grower	Adaptation 1	Adaptation 2
Yellow maize	50.74	54.33	60.57	60.44	60.26
Soybean oilcake, 46%	30.93	29.00	23.64	23.64	23.66
Full-fat soya	10.00	10.00	10.00	10.00	10.00
Gluten 60	2.00	0.00	0.00	0.00	0.00
Soya oil	1.13	2.07	1.83	1.83	1.37
Limestone	1.06	0.93	1.01	1.08	0.81
MDCP[1]	2.23	1.68	0.99	1.05	0.53
Salt	0.34	0.35	0.34	0.34	0.35
Choline chloride 60%	0.20	0.20	0.20	0.20	0.20
Sodium Bicarbonate	0.18	0.18	0.18	0.18	0.18
Lysine HCL, 79%	0.24	0.23	0.25	0.25	0.25
L-Methionine, 99%	0.34	0.34	0.32	0.32	0.30
L-Threonine, 98.5%	0.16	0.18	0.16	0.16	0.16
L- Isoleucine, 90%	0.04	0.07	0.08	0.08	0.08
L-Valine, 98%	0.03	0.06	0.05	0.05	0.05
L-Tryptophan, 98%	0.01	0.01	0.01	0.01	0.01
Vitamin premix[2]	0.10	0.10	0.10	0.10	0.1
Mineral premix[3]	0.15	0.15	0.15	0.15	0.15
Robenidine, 6.6%	0.05	0.05	0.05	0.05	0.05
Zinc Bacitracin, 15%	0.07	0.07	0.07	0.07	0.07
Formulated (analysed) nutrients					
ME[4], MJ/kg	11.50	11.90	12.20	12.20	12.20
Crude Protein %	23.00 (23.59)	22.01 (22.08)	20.03 (20.48)	20.03 (20.48)	20.06 (21.10)
Crude Fibre %	(0.44)	(0.38)	(0.31)	(0.31)	(0.29)
Crude Fat %	(0.60)	(0.72)	(0.71)	(0.67)	(0.67)
Total Ca, %	0.90 (0.79)	0.75 (0.68)	0.68 (0.69)	0.68 (0.68)	0.50 (0.50)
Total P %	0.77 (0.76)	0.65 (0.66)	0.50 (0.52)	0.50 (0.52)	0.40 (0.43)
oP[5] %	0.48	0.39	0.28	0.28	0.20

[1]Monodicalcium phosphate
[2]Supplied per kilogram of diet: vitamin A, 13 000 IU; vitamin D, 2750 IU; vitamin E, 70 IU; vitamin B12, 0.02 mg; riboflavin, 8.6 mg; niacin, 65 mg; pantothenic acid, 20 mg; vitamin K_3, 4 mg; folic acid, 2.2 mg; biotin, 0.15 mg; thiamine, 3.2 mg; pyridoxine, 4.3 mg
[3]Supplied per kilogram diet: zinc supplied as zinc amino acid chelate, 60 mg; manganese supplied as manganese sulfate, 110 mg; iron supplied as iron amino acid complex, 30 mg; copper supplied as copper sulphate, 10 mg; iodine supplied as calcium iodate, 2 mg; selenium supplied as zinc-L-selenomethionine, 0.3 mg
[4]Metaboliseable energy
[5]Digestible P (CVB, 1997)

Table 3.4 Raw material composition of basal diets on a dry matter basis

Feed ingredient (%)	Day 8-10 basal	Day 21-23 basal
Yellow maize	55.70	61.70
Soybean oilcake (46%)	28.56	23.19
Full-fat soya	10.00	10.00
Soya oil	1.26	1.04
Limestone	1.30	0.93
Salt	0.34	0.34
Sodium Bicarbonate	0.18	0.18
Lysine HCL, 79%	0.24	0.26
L-Methionine, 99%	0.34	0.30
L-Threonine, 98.5%	0.18	0.16
L- Isoleucine, 90%	0.08	0.08
L-Valine, 98%	0.06	0.05
L-Tryptophan, 98%	0.01	0.02
Vitamin Premix[1]	0.10	0.10
Mineral Premix[2]	0.15	0.15
TiO_2 – maize blend[3]	1.50	1.50
Formulated (analysed) nutrients		
ME[4], MJ/kg	11.90	12.20
Crude Protein, %	22.00 (22.23)	20.00 (20.35)
Crude Fibre, %	(3.05)	(2.72)
Crude Fat, %t	(6.19)	(6.12)
Total Ca, %	0.58 (0.62)	0.43 (0.42)
Total P, %	0.35 (0.32)	0.33 (0.29)
oP[5], %	0.12	0.11

[1]Supplied per kilogram of diet: vitamin A, 13 000 IU; vitamin D, 2750 IU; vitamin E, 70 IU; vitamin B12, 0.02 mg; riboflavin, 8.6 mg; niacin, 65 mg; pantothenic acid, 20 mg; vitamin K_3, 4 mg; folic acid, 2.2 mg; biotin, 0.15 mg; thiamine, 3.2 mg; pyridoxine, 4.3 mg.
[2]Supplied per kilogram diet: zinc supplied as zinc amino acid chelate, 60 mg; manganese supplied as manganese sulfate, 110 mg; iron supplied as iron amino acid complex, 30 mg; copper supplied as copper sulphate, 10 mg; iodine supplied as calcium iodate, 2 mg; selenium supplied as zinc-L-selenomethionine, 0.3 mg.
[3]A premix containing a mixture of maize and TiO_2 in a ratio of 60:40 (maize: TiO_2)
[4]Metaboliseable energy
[5]Digestible P (CVB, 1997)

Limestone

The limestone solubility profile of the limestone used during this study is displayed in figure 2.6.1. The mineral composition of the limestone used during this study is displayed in table 2.6.1. The limestone used for this study had a geometric mean diameter of 128.55 ± 45.26 µm.

Figure 2.6.1 The limestone solubility profile of the limestone used during this study

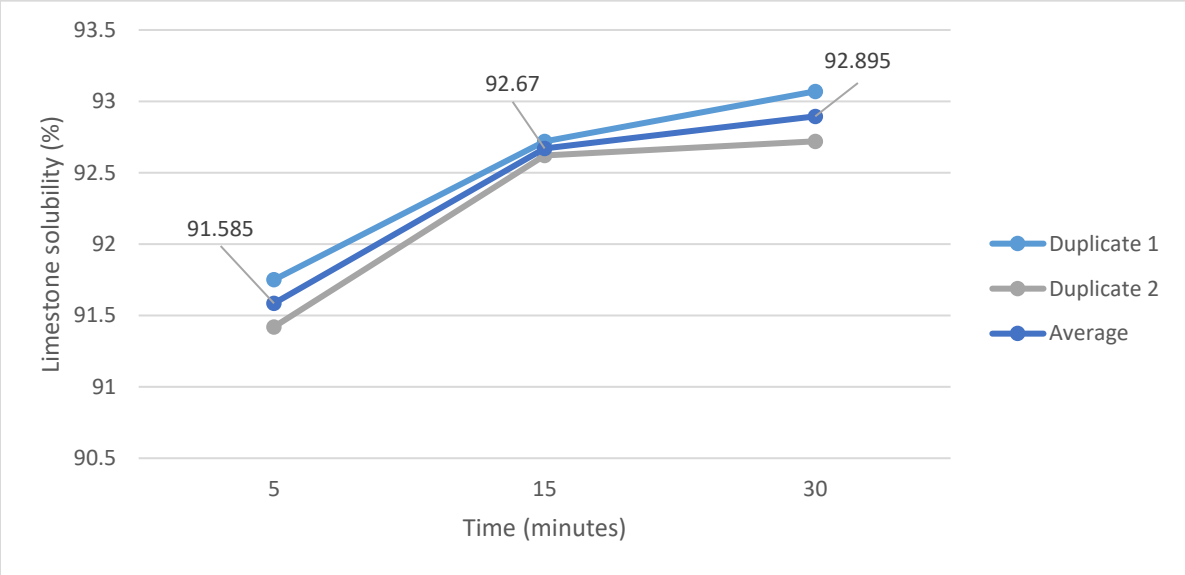

Table 2.6.1 Mineral composition (%) of the limestone used in this study.

Analysed nutrient	
Dry Matter (%)	98.16
Moisture (%)	1.84
Calcium (%)	37.75
Phosphorus (%)	0.01
Magnesium (%)	0.36
Potassium (%)	0.01
Sodium (%)	0.04
Iron (mg/kg)	2309.54
Manganese (mg/kg)	30.58
Zinc (mg/kg)	1.12
Copper (mg/kg)	2.85

3.2.6 Ileal sample collection

On days 10 and 23, eight and six birds were sampled per cage, respectively. On the respective sampling days, birds were removed from their cages, the weight of the pen was recorded, and all birds were euthanised by intravenous injection of sodium pentobarbital into the wing vein. The euthanisation of birds was done by a veterinarian on the respective sampling days. The use of sodium pentobarbital induces a rapid pain-free death and prevents involuntary smooth muscle contraction and wing flapping that could potentially lead to losses in digestive material. Scalpels were used to remove the digestive segment from Merkel's diverticulum to 5 cm distal of the ileocaecal junction and thereafter the ileal contents were flushed by pushing triple distilled water through the ileum with a syringe (Ravindran *et al.*, 1999). The ileum was flushed on ice-cold granite slabs with ice surrounding the granite to prevent any additional microbial fermentation that could influence the results. The ileal content of all birds from the same cage was pooled, labelled and frozen at -20°C before being freeze-dried for seven days. After freeze drying, all samples were finely ground using a pestle and mortar to pass through a 0.25 mm screen and stored in airtight containers at 4°C until being analysed in duplicate for dry matter (DM), Ca, P and titanium (Ti).

3.2.7 Digestibility calculations

The equations used to calculate digestibility parameters are described in this section. Equation 3.1 was used to calculate the apparent ileal digestibility of Ca and P. Digestible Ca and P were calculated using Equation 3.2.

Equation 3.1 Apparent ileal digestibility of Ca or P using TiO_2 as a marker

$$AID\ (\%) = [\frac{[(\frac{[Ca]\ or\ [P]}{TiO_2})_d - (\frac{[Ca]\ or\ [P]}{TiO_2})_i]}{(\frac{[Ca]\ or\ [P]}{TiO_2})_d}] \times 100$$

Where:

$\left(\frac{[Ca]\ or\ [P]}{TiO_2}\right)_d$ = The ratio of the analysed Ca or P to TiO_2 in the specific basal diet

$\left(\frac{[Ca]\ or\ [P]}{TiO_2}\right)_i$ = The ratio of the analysed Ca or P to TiO_2 in the ileal digesta of the pen

Equation 3.2 The percentage of Ca or P in the complete diet that is digestible

$$Digestible\ Ca/P\ (\%) = \frac{AID(\%)}{100} \times [Ca/P]_d$$

Where:

$[Ca/P]_d$= The analysed dietary Ca or P concentration of the specific basal diet

3.3 Chemical analysis

A single sample of each of the rearing, adaptation, and experimental feed was sampled for analysis of relevant nutrients. Rearing and adaptation diets were analysed for DM, moisture, crude fibre, crude fat, crude protein, Ca and P, while experimental diets and basals were analysed for DM, moisture, crude fibre, crude fat, crude protein, Ca, P, Ti, phytase and 1α-OH-D_3. All analyses were performed in duplicate. The official methods as described by the Association of Official Analytical Chemists (AOAC, 2000) were used to determine the content of the specific nutrients from the feed samples (Table 3.6). These methods have been scientifically proven to be highly accurate and reliable and can produce credible results repeatedly. All mineral analyses were conducted by an independent laboratory, ChemNutri Analytical (Pty) Ltd (Centurion, South Africa). Enzyme analysis was done by Chemunique (Pty) Ltd (Lanseria, South Africa). 1α-OH-D_3 analysis was conducted by Eurofins Scientific food integrity & innovation laboratory (Madison, Wisconsin, United States of America).

Table 3.6 AOAC official methods of analysis used during the course of this study

Nutrient analysed	Official AOAC method
DM	942.05
Moisture	943.01
Crude Fibre	962.09
Crude Fat	920.39
Crude Protein	988.05
Ca[2]	935.13
P[2]	935.13
Ti[1]	985.01 / 2006.03

[1]Titanium analysis method was adapted from AOAC 985.01 and AOAC 2006.03 (AOAC, 2006)
[2]Ca and P were analysed using inductively coupled plasma atomic emission spectroscopy (ICP) based on AOAC method 935.13 (AOAC, 1999).

3.4 Statistical analysis

The data were analysed with a full factorial using the JMP 16.1 Pro statistical package (SAS, Cary, North Carolina, United States of America), where 1α-OH-D_3 dose and phytase dose was inserted into the model as the main effects. In the model, "block" was included as a random effect and the means for AID Ca, AID P, digestible Ca and digestible P were separated using a protected Tukey HSD test set at $P<0.05$.

Chapter 4

Results

4. Digestibility of calcium and phosphorus in broilers at 10 and 23 days-of-age

The results for the apparent ileal digestibility of Ca, apparent ileal digestibility of P, digestible Ca and digestible P of broilers at 10 days-of-age and 23 days-of-age fed diets with different levels of 1α-OH-D_3 and phytase are summarised in Table 4.1 and Table 4.2, respectively. There was no interaction ($p>0.05$) observed when diets were supplemented with 1α-OH-D_3 and phytase on AID Ca on D10 or D23 when individual treatments were compared. The interaction between 1α-OH-D_3 and phytase on D23 AID Ca approaches significance ($p<0.1$). The AID Ca of birds was independently influenced ($p<0.05$) by 1α-OH-D_3 and phytase, on both D10 and D23. The supplementation of 1α-OH-D_3 improved ($p<0.05$) AID Ca when included at 2.5 or 5 µg 1α-OH-D_3/kg in the diet. The AID Ca of birds that were fed diets supplemented with 2.5 or 5 µg 1α-OH-D_3/kg increased ($p<0.05$) from 43.47% in the control to 49.49 and 53.34% on D10, respectively. There was an increase ($p<0.05$) in AID Ca on D23 from 64.01% when birds were fed diets without 1α-OH-D_3 supplementation to 70.83 and 72.61% when diets were supplemented with at 2.5 and 5 µg 1α-OH-D_3/kg, respectively. There was no difference observed in D10 or D23 AID Ca between birds that were fed diets supplemented with 2.5 µg and those supplemented with 5 µg 1α-OH-D_3/kg feed. Broilers fed diets supplemented with phytase obtained higher ($p<0.05$) AID Ca when supplemented with 500 or 1000 FTU phytase/kg on both D10 and D23. There was a stepwise increase ($p<0.05$) in AID Ca, with increasing phytase dose on both D10 and D23. Birds that received diets without supplemental phytase achieved an AID Ca of 40.41% on D10, which improved ($p<0.05$) to 49.43% and 56.46% when diets were supplemented with 500 and 1000 FTU phytase/kg, respectively. On D23 the AID Ca of birds fed diets without supplemental phytase was 57.52% and improved in a stepwise manner ($p<0.05$) to 72.20% and 77.73% in birds that were fed diets with 500 and 1000 FTU phytase/kg feed.

Table 4.1 The apparent ileal digestibility of calcium (%), apparent ileal digestibility of phosphorus (%), digestible calcium (%) and digestible phosphorus (%) of broilers at 10 days-of-age fed diets containing different levels of 1α-hydroxycholecalciferol (µg/kg) and phytase (FTU/kg)

Treatment (T)	1α-OH-D_3[1] dose (µg/kg)	Phytase dose (FTU/kg)	AID Ca[2] (%)	AID P[3] (%)	dCa[4] (%)	dP[5] (%)
T1	0	0	36.60	22.18	0.227	0.071
T2	2.5	0	37.92	25.61	0.235	0.082
T3	5	0	46.69	26.34	0.290	0.084
T4	0	500	43.20	41.69	0.268	0.133
T5	2.5	500	52.71	46.93	0.327	0.150
T6	5	500	52.39	52.17	0.325	0.167
T7	0	1000	50.61	53.44	0.314	0.171
T8	2.5	1000	57.84	58.17	0.359	0.186
T9	5	1000	60.93	62.66	0.378	0.201
Main effects						
1α-OH-D_3	0		43.47^{B}	39.10^{B}	0.270^{B}	0.125^{B}
	2.5		49.49^{A}	43.57^{A}	0.307^{A}	0.139^{A}
	5		53.34^{A}	47.06^{A}	0.331^{A}	0.151^{A}
	SEM		2.44	1.89	0.017	0.006
Phytase		0	40.41^{C}	24.71^{C}	0.251^{C}	0.079^{C}
		500	49.43^{B}	46.93^{B}	0.306^{B}	0.150^{B}
		1000	56.46^{A}	58.09^{A}	0.350^{A}	0.186^{A}
		SEM	2.44	1.85	0.017	0.006
P-values						
1α-OH-D_3			<0.001	<0.001	<0.001	<0.001
Phytase			<0.001	<0.001	<0.001	<0.001
Interaction			0.373	0.670	0.373	0.670

[1]1α-OH-D_3: 1α-Hydroxycholecalciferol
[2]AID Ca: Apparent ileal digestibility of calcium
[3]AID P: Apparent ileal digestibility of phosphorus
[4]dCa: Digestible calcium
[5]dP: Digestible phosphorus
[A,B] Column means with the same superscript do not differ significantly (p> 0.05)

Diets that contained either 2.5 or 5 µg 1α-OH-D_3/kg and 1000 FTU phytase/kg had a numerically higher AID Ca when compared to treatments that contained a lower dose of phytase or no phytase. The same was observed at a lower dose of phytase. Treatments that contained 2.5 or 5 µg 1α-OH-D_3/kg along with 500 FTU phytase/kg had a numerically higher AID Ca when compared to the control and treatments that contained only 1α-OH-D_3 at 2.5 or 5 µg 1α-OH-D_3/kg.

Table 4.2 The apparent ileal digestibility of calcium (%), apparent ileal digestibility of phosphorus (%), digestible calcium (%) and digestible phosphorus (%) of broilers at 23 days-of-age fed diets containing different levels of 1α-hydroxycholecalciferol (µg/kg) and phytase (FTU/kg)

Treatment (T)	1α-OH-D_3[1] dose (µg/kg)	Phytase dose (FTU/kg)	AID Ca[2] (%)	AID P[3] (%)	dCa[4] (%)	dP[5] (%)
T1	0	0	50.47	38.76	0.212	0.112
T2	2.5	0	58.68	46.06	0.246	0.134
T3	5	0	63.40	51.07	0.266	0.148
T4	0	500	68.87	68.79	0.289	0.199
T5	2.5	500	74.25	71.12	0.312	0.206
T6	5	500	73.49	73.35	0.309	0.213
T7	0	1000	72.68	77.86	0.305	0.226
T8	2.5	1000	79.56	84.14	0.334	0.244
T9	5	1000	80.93	83.82	0.340	0.243
SEM			0.78	1.77	0.007	0.006
Main effects						
1α-OH-D_3	0		64.01^{B}	61.80^{B}	0.269^{B}	0.179^{B}
	2.5		70.83^{A}	67.10^{A}	0.297^{A}	0.195^{A}
	5		72.61^{A}	69.42^{A}	0.305^{A}	0.201^{A}
	SEM		0.96	1.12	0.004	0.004
Phytase		0	57.52^{C}	45.30^{C}	0.242^{C}	0.131^{C}
		500	72.20^{B}	71.09^{B}	0.303^{B}	0.206^{B}
		1000	77.73^{A}	81.94^{A}	0.326^{A}	0.238^{A}
		SEM	0.96	1.11	0.004	0.004
P-values						
1α-OH-D_3			<0.001	<0.001	<0.001	<0.001
Phytase			<0.001	<0.001	<0.001	<0.001
Interaction			0.079	0.142	0.079	0.142

[1]1α-OH-D_3: 1α-Hydroxycholecalciferol
[2]AID Ca: Apparent ileal digestible calcium
[3]AID P: Apparent ileal digestible phosphorus
[4]dCa: Digestible calcium
[5]dP: Digestible phosphorus
[A,B] Column means with the same superscript do not differ significantly (p> 0.05)

There was no interaction (p>0.05) between 1α-OH-D_3 and phytase on AID P on D10 or D23 when comparing individual treatments with each other. There was an effect of 1α-OH-D_3 (p<0.05) on AID P on D10 and D23. However, there was no difference (p>0.05) observed in AID P on D10 or D23 in birds that were fed diets supplemented with 2.5 and 5 µg 1α-OH-D_3/kg. The supplementation of 1α-OH-D_3 into the diet at 2.5 or 5 µg

1α-OH-D_3/kg increased ($p<0.05$) the AID P from 39.10 to 43.57 and 47.06% on D10, respectively. When compared to birds that were fed diets without any supplemental 1α-OH-D_3 on D23, the AID P improved ($p<0.05$) from 61.80% to 67.10 and 69.42% when 2.5 or 5 µg 1α-OH-D_3/kg was supplemented into the diet of birds, respectively. When supplemented, there was a stepwise effect ($p<0.001$) of phytase on AID P on D10 and D23. The AID P of birds as a result of phytase supplementation increased from 24.71% in non-supplemented diets to 46.93% when 500 FTU phytase/kg was supplemented and 58.09% when 1000 FTU phytase/kg was supplemented into the diet on D10. When diets were supplemented with phytase at 500 or 1000 FTU/kg on D23 there was an increase ($p<0.05$) in AID P from 45.30% in birds without any supplemental phytase, to 71.09 and 81.94%, respectively. The AID P was higher ($p<0.05$) in birds that were fed diets supplemented with 1000 FTU/kg phytase compared to those that were fed diets supplemented with 500 FTU/kg phytase on both D10 and D23.

There was no interaction between 1α-OH-D_3 and phytase on either D10 dCa ($p>0.05$) and dP ($p>0.05$) or D23 dCa ($p>0.05$) and dP ($p>0.05$) when comparing individual treatments with each other. When 1α-OH-D_3 was supplemented into the diet fed to birds, it had an effect ($p<0.001$) on dCa and dP on D10 and D23. The dCa and dP were higher ($p<0.05$) in birds that were fed diets that were supplemented with 1α-OH-D_3 compared to birds that were fed diets without any 1α-OH-D_3 supplementation. 1α-Hydroxycholecalciferol supplementation into the diet of birds at 2.5 or 5 µg/kg caused an increase ($p<0.05$) in the dCa of birds from 0.270 to 0.307 and 0.331% on D10, respectively. There was an increase ($p<0.05$) in dCa from 0.269% in birds that were fed diets without 1α-OH-D_3 supplementation to 0.297 and 0.305% when 1α-OH-D_3 was supplemented at 2.5 or 5 µg/kg on D23, respectively. The dP was 0.125% in birds that were fed diets without any supplemental 1α-OH-D_3 on D10 and increased ($p<0.05$) to 0.139 and 0.151% in birds that were fed diets supplemented with 2.5 or 5 µg/kg 1α-OH-D_3, respectively. There was an increase ($p<0.05$) in dP on D23 from 0.179% when birds were fed diets without 1α-OH-D_3 supplementation to 0.195 and 0.201% when diets were supplemented with 2.5 and 5 µg/kg 1α-OH-D_3, respectively There was no difference ($p>0.05$) in dCa or dP of birds that were fed diets supplemented with 2.5 or 5 µg 1α-OH-D_3/kg on either D10 or D23. Phytase had an effect ($p<0.05$) on dCa when supplemented into the diet of birds on D10 and D23. The dCa of birds increased ($p<0.05$) in a stepwise fashion as phytase supplementation in the diet increased from 0 to 1000 FTU/kg. Birds

that were fed diets supplemented with 500 or 1000 FTU/kg phytase had a higher ($p<0.05$) dCa compared to birds that were fed diets without any supplemental phytase. The dCa of birds that were fed diets supplemented with 0, 500 or 1000 FTU phytase/kg was 0.251, 0.306 and 0.350% on D10, respectively. The dCa of birds that were fed diets supplemented with 0, 500 or 1000 FTU phytase/kg was 0.242, 0.303 and 0.326% on D23, respectively. Phytase supplementation increased ($p<0.05$) dP of birds in a stepwise fashion on D10 and D23. Phytase supplementation into the diet of birds at 500 or 1000 FTU/kg caused an increase ($p<0.05$) in the dP of birds from 0.079 to 0.150 and 0.186% on D10, respectively. There was an increase ($p<0.05$) in dP from 0.131% in birds that were fed diets without phytase supplementation to 0.206 and 0.238% when phytase was supplemented at 500 or 1000 FTU/kg on D23, respectively.

Chapter 5

Discussion

5.1. Calcium absorption

Analysed concentrations of Ca and P in the basal diet (Table 3.4) as well as analysed concentrations of 1α-OH-D_3 and phytase activities in treatment diets (Table 3.1) were all close to formulated values. There was no interaction observed when diets were supplemented with 1α-OH-D_3 and phytase on either AID Ca or AID P on both D10 ($p>0.05$) and D23 ($p>0.05$) when individual treatments were compared with each other. There was an effect ($p<0.05$) of 1α-OH-D_3 when fed to birds on AID Ca and AID P on D10 and D23. The AID Ca of birds fed diets supplemented with 2.5 or 5 µg 1α-OH-D_3 /kg were higher ($p<0.05$) on both D10 and D23 compared to the control. There was no difference ($p>0.05$) in AID Ca in birds that were fed diets supplemented with 2.5 µg 1α-OH-D_3/kg and those supplemented with 5 µg 1α-OH-D_3/kg on either D10 or D23. The AID Ca of birds supplemented with 0, 2.5, and 5 µg 1α-OH-D_3/kg was 43.47, 49.49 and 53.34% on D10 and 64.01, 70.83, and 72.61 on D23, respectively. This indicates an increase in Ca absorption in response to 1α-OH-D_3 supplementation. Ca absorption in the small intestine happens via two pathways: the active transcellular, energy-dependent system and the passive, energy-independent, paracellular pathway in which Ca is absorbed through tight junctions (Proszkowiec-Weglarz & Angel, 2013). The transcellular pathway is vitamin D dependent and is a saturable process which is the predominant mechanism of Ca absorption when Ca intake is restricted (Khanal & Nemere, 2008). Very few studies have examined the effects of 1α-OH-D_3 on the AID Ca of broilers. However previous studies have shown that 1α-OH-D_3 can increase AID Ca in Japanese laying quails and laying hens (Bar *et al.*, 1976; Bar *et al.*, 1978), while others have demonstrated that vitamin D can increase total tract Ca retention in poultry and swine (Edwards Jr, 2002; Han *et al.*, 2018; Lee & Stein, 2021). Tibia ash data from various studies also indicate an increase in the % Ca of the tibia with increasing doses of 1α-OH-D_3 (Ebrahimi *et al.*, 2016; Han *et al.*, 2017). The manner by which 1α-OH-D_3 promotes Ca absorption is mediated by the

VDR. Han *et al.* (2018) found 1α-OH-D_3 can increase the expression of VDR in the small intestine and kidney of broilers. Transient receptor potential channels, of the vanilloid subtype 5 and TRPV6 control the absorption of Ca across the apical membrane of enterocytes and are transcriptionally regulated by 1α,25-$(OH)_2$-D_3 via the VDR (Van Cromphaut *et al.*, 2001; Hoenderop *et al.*, 2005). By increasing the expression of VDR in the duodenum of broilers, 1α-OH-D_3 indirectly increases the expression of TRPV proteins thereby increasing the absorption of calcium across the apical membrane. The efficacy of 1α-OH-D_3 on AID Ca and P was age-dependent in this study. The response in AID Ca or P as a result of 1α-OH-D_3 supplementation was greater in D23 birds than in D10 birds. This can likely be attributed to the better-developed digestive system present in older birds allowing for better digestion and absorption of nutrients (Noy & Sklan, 1997; Uni *et al.*, 1999; Li *et al.*, 2018). Increased Ca levels have been shown to negatively impact the 1α-OH-D_3 efficacy in D23 broilers (Han *et al.*, 2012). By increasing the dietary Ca concentration from 0.40% to 1.20%, Han *et al.* (2012) found a decrease in total tract Ca and P retention with maximum retention occurring when Ca was included at 0.40%. The analysed Ca concentration of the basal for D23 birds was 0.41% and therefore the negative effects of dietary Ca on 1α-OH-D_3 efficacy were mitigated in this study, however, may still be present under commercial conditions. Therefore, to achieve the maximum benefit of 1α-OH-D_3 supplementation, dietary Ca inclusion in commercial diets need to be reduced.

There was no interaction between 1α-OH-D_3 and phytase ($p > 0.05$) on AID Ca on either D10 or D23, however, the interaction approaches significance ($p<0.1$). Birds that were fed diets supplemented with 2.5 or 5 µg 1α-OH-D_3/kg, along with 500 or 1000 FTU/kg phytase had a numerically higher AID Ca compared to birds that received diets without any supplementation or birds that were fed diets that were supplemented with 1α-OH-D_3 or phytase only. The AID Ca was numerically the highest when a combination of 2.5 or 5 µg 1α-OH-D_3/kg and 1000 FTU phytase/kg was supplemented into the diet of birds on D10 and D23. This suggests that a possible complementary effect exists between phytase and 1α-OH-D_3, that when combined, it improves Ca absorption from the small intestine above the level each product can achieve in isolation. The possible complementary effect between 1α-OH-D_3 and phytase acting together to improve Ca

absorption from the small intestine can likely be attributed to the two different modes of action of the two additives in different parts of the digestive tract. Phytate P as well as minerals, proteins and amino acids bound to phytate in the form of phytate-mineral and phytate-protein complexes are poorly digested and absorbed in the small intestine (Selle *et al.*, 2009; Selle *et al.*, 2012; Dersjant-Li *et al.*, 2015). Although minerals have varying affinities for phytate, Ca is the mineral most sensitive to phytate binding. Ca has a lower relative binding affinity than most minerals, however, it is present in considerably higher amounts in feed than other minerals, and hence phytate has the largest effect on Ca digestion and absorption (Tamim & Angel, 2003). The capacity of phytic acid to bind cations, such as Ca, is significantly reduced after hydrolysis of phytate by exogenous phytase to lower phosphate inositol esters. This is due to a decrease in the negative charge of the phytic acid molecule with the removal of every subsequent phosphate group, allowing more Ca to reach the small intestine. The solubility of lower phosphate inositol esters (IP4, IP3 and IP2) is also higher in the small intestine than IP5 and IP6 (Schlemmer *et al.*, 2001). The action of exogenous phytase in the proximal parts of the GIT results in the increased solubility and proportion of lower phosphate inositol esters reaching the small intestine. The increased solubility and proportion of lower phosphate inositol esters, allow intestinal phytase, present in the intestinal mucosa of the small intestine, to access and further hydrolyse these phosphate inositol esters. In doing this, it further reduces the capacity of the phytic acid molecule to bind Ca and thereby increases the amount of Ca available for absorption in the small intestine. 1α-Hydroxycholecalciferol then acts by stimulating the expression of VDR-dependent Ca transporter proteins, which increases the abundance of transporter proteins able to transcellularly absorb Ca from the intestinal lumen across the enterocytes of the small intestine.

There was an effect ($p<0.05$) of phytase on AID Ca on D10 and D23. Phytase supplementation into the diet of broilers improved ($p<0.05$) the AID Ca and AID P in a stepwise manner on D10 and D23. The AID Ca when 0, 500 and 1000 FTU/kg phytase was supplemented into the diet of broiler were 40.41, 49.43 and 56.46% on D10 and 57.52, 72.20 and 77.73% on D23. Several studies have found an increase in AID Ca with increasing phytase doses (Li *et al.*, 2018; Majeed *et al.*, 2020; Li *et al.*, 2021). Phytase

acts by hydrolysing phytic acid, or its salt form, phytate, into lower inositol-phosphate esters in the proximal parts of the GIT of broilers (Kryukov *et al.*, 2021). With the removal of every subsequent phosphate group from the inositol structure of phytic acid, the capacity of the compound to bind Ca decreases, while the solubility of the formed compound increases (Luttrell, 1993; Schlemmer *et al.*, 2001). Due to the reduced capacity of phytic acid to bind Ca, the amount of unbound Ca flowing into the small intestine increases. It is therefore suggested that the increase in AID Ca seen on D10 and D23 in response to phytase supplementation are due to an increased flow of unbound Ca into the small intestine rather than an alteration of the gut morphology or gene expression patterns in favour of Ca absorption.

5.2 Phosphorus absorption

There was an effect ($p<0.05$) of 1α-OH-D_3 on AID P when supplemented into the diet of broilers on D10 and D23. Birds that were fed diets supplemented with 2.5 or 5 µg 1α-OH-D_3/kg had an improved ($p<0.05$) AID P compared to the control. There was no difference ($p>0.05$) in AID P of birds that were fed diets supplemented with 2.5 and those supplemented with 5 µg 1α-OH-D_3/kg on either D10 or D23. The AID P of birds supplemented with 0, 2.5, and 5 µg 1α-OH-D_3/kg was 39.10, 43.57 and 47.06% on D10 and 61.80, 67.10 and 69.42 on D23, respectively. This agrees with previous work suggesting 1α-OH-D_3 and other vitamin D derivatives increase the absorption of P in the small intestine of birds (Edwards Jr *et al.*, 2002; Snow *et al.*, 2004; Han *et al.*, 2009; Liem *et al.*, 2009; Wang *et al.*, 2015; Yang *et al.*, 2019). The type IIb sodium-dependent phosphate cotransporter (NaPi-IIb) has the highest expression in the duodenum followed by the jejunum and ileum and is the predominant transporter of Pi in the small intestine of chickens (Hilfiker *et al.*, 1998; Yan *et al.*, 2007). In the kidney, the type IIa and IIc NaPi cotransporters regulate the reabsorption of Pi back into the blood in the proximal renal tubules. Both NaPi-IIa and NaPi-IIc transporters are involved in Pi reabsorption in the kidney, however, the extent of Pi reabsorption is determined predominantly by the abundance of NaPi-IIa cotransporters (Segawa *et al.*, 2005). The supplementation of 1α-OH-D_3 does not increase the mRNA expression of the type IIb NaPi cotransporter in the

duodenum of day 21 broilers (Han *et al.*, 2009; Han *et al.*, 2018). However, when 1α-OH-D_3 was supplemented it increased the expression of the type IIb- and IIa Na-Pi cotransporters in the jejunum and ileum, and proximal renal tubule, respectively (Han *et al.*, 2009; Han *et al.*, 2018). This suggests 1α-OH-D_3 increases the absorption of Pi in the distal segments of the small intestine and reabsorption in the kidney by increasing the expression of related Na-dependent Pi transporters. There was an effect of phytase ($p<0.05$) on AID P on D10 and D23. There was a stepwise improvement in AID P when phytase was supplemented into the diet of broilers. The AID P when 0, 500 and 1000 FTU phytase/kg was supplemented into the diet of broilers were 24.71, 46.93 and 58.09% on D10 and 45.30, 71.09 and 81.94% on D23. The effects and mode of action by which phytase increases AID P, as well as the factors that influence phytase efficacy, are well documented and in agreement with the results of this study (Selle *et al.*, 2009; Dersjant-Li *et al.*, 2015; Li *et al.*, 2017; Li *et al.*, 2018). The primary site of phytase action is localised in the crop, proventriculus and gizzard with very little phytate P hydrolysis occurring after digesta have entered the small intestine (Angel *et al.*, 2002; Selle & Ravindran, 2007). The suitability of a phytase as a feed additive is dependent on the pH optimum at which a phytase functions, its intrinsic thermostability and its resistance to intestinal degradation by intestinal proteases (Angel *et al.*, 2002). The phytase used in this study was a variant of a novel consensus-6 bacterial phytase expressed in *Trichoderma reesei*. In an *in vitro* simulation, this phytase variant was able to hydrolyse IP6 rapidly and completely to IP3 at PHs comparable to those found in the proximal parts of the small intestine (Christensen *et al.*, 2020). This was replicated *in vivo* by Marchal *et al.* (2021) and Dersjant-Li *et al.* (2022) in which inorganic phosphorus was totally replaced by supplementation of a variant novel consensus phytase for the entirety of the 42-day rearing cycles. This highlights the impact of the phytase source used in this study on AID Ca and AID P of young and growing broilers.

Chapter 6

Conclusion

Based on the findings of this study, it can be stated that 1α-OH-D_3 can increase Ca and P digestibility independent of phytase in young and growing broilers. There was however no benefit observed when 1α-OH-D_3 supplementation into the diet increased from 2.5 to 5 µg 1α-OH-D_3/kg. This suggests that 2.5 µg 1α-OH-D_3/kg is an adequate dose to improve Ca and P digestibility, regardless of age. The mechanism by which 1α-OH-D_3 increases Ca and P digestibility is by its effects on the expression of transporter proteins directly related to Ca and P absorption in the small intestine and kidney of broilers and is primarily mediated by VDR. The magnitude of the effect of 1α-OH-D_3 on Ca and P digestibility is age dependent. This is likely due to a better-developed digestive system at an older age allowing for improved mineral absorption, highlighting the importance of age when formulating diets for broilers. Phytase also increased Ca and P digestibility, and although this result has been established in previous years, this gives insight into the efficacy of the phytase source used in this study. The numerical increase in Ca digestibility because of the combined supplementation of 1α-OH-D_3 and phytase propose the existence of a possible complementary effect in older birds. Due to space constraints of the facility used in this study, the existence of a complementary effect could not be confirmed and could have resulted in a type 1 error due to the lack of adequate replication. Nevertheless, this study highlights the importance of vitamin D and phytase for the improvement of Ca and P digestibility in broilers. However, further research is required to establish the efficacy of 1α-OH-D_3 under different dietary conditions and at increased doses of phytase. The use of 1α-OH-D_3 has the potential to decrease the reliance of the poultry industry on non-renewable sources of Ca and P, contributing to the sustainability of the industry.

Chapter 7

Critical Review

For a broader understanding of the effects of 1α-OH-D_3 and phytase supplementation in broilers, future research into this subject should consider the following changes and suggestions to improve the trial design and increase the value of the study to industry professionals:

(1) Future research should consider including higher doses of phytase (1500, 2000 FTU phytase/kg) in the trial design. Higher doses of phytase are currently being implemented globally as a result of the increased prices of Pi sources. By including higher doses of phytase in the trial design, the results will have greater relevance and improved application in the industry. Additionally, higher phytase doses could potentially amplify the effects of 1α-OH-D_3 on Ca and P digestibility that were observed in this study due to an even greater flow of absorbable forms of Ca and P into the small intestine.

(2) Future research should also consider including indirect measures of Ca and P digestibility such as bone parameters. This will allow researchers to deduce whether the increase in Ca and P digestibility translate into improved bone mineralisation and performance, indirectly validating the results of this study.

(3) Prospective research can also consider including different vitamin D analogues in the trial design, without compromising on replication. This would allow researchers to evaluate which vitamin D analogue is the most efficacious in improving Ca and P digestibility.

(4) It would also benefit researchers to investigate the effects of 1α-OH-D_3 and phytase on performance over an entire rearing cycle. This will allow researchers to understand the full impact of 1α-OH-D_3 and phytase over an entire rearing cycle.

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