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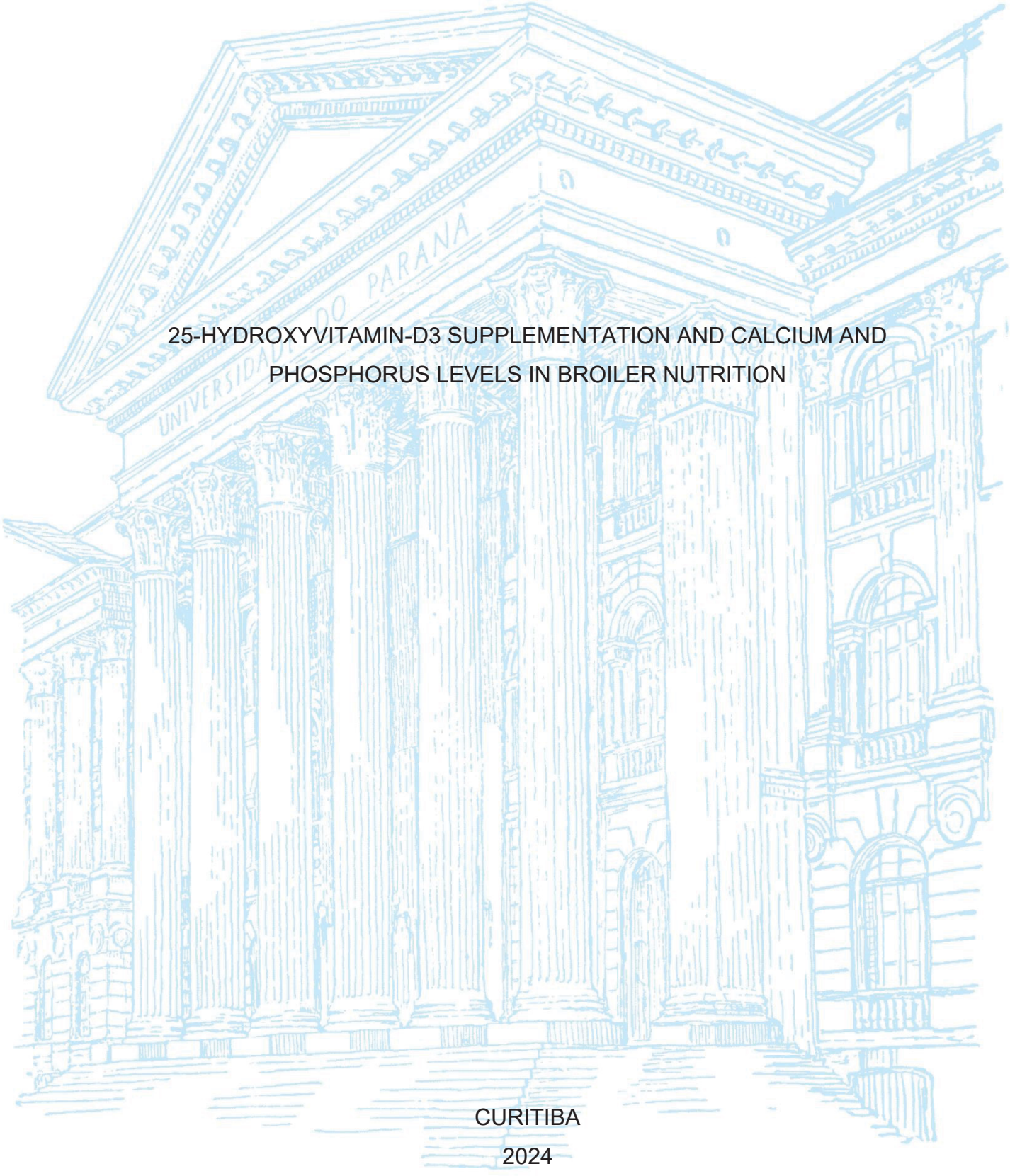
LUCAS SCHMIDT BASSI



25-HYDROXYVITAMIN-D3 SUPPLEMENTATION AND CALCIUM AND PHOSPHORUS LEVELS IN BROILER NUTRITION

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LUCAS SCHMIDT BASSI

25-HYDROXYVITAMIN-D3 SUPPLEMENTATION AND CALCIUM AND PHOSPHORUS LEVELS IN BROILER NUTRITION

Tese apresentada ao Curso de Pós-graduação em Zootecnia, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito para a obtenção do Título de Doutor em Zootecnia.

Orientador: Prof. Dr. Alex Maiorka

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Assinatura Eletrônica
29/02/2024 16:31:44.0
ALEX MAIORKA
Presidente da Banca Examinadora

Assinatura Eletrônica
01/03/2024 06:20:04.0
FABIANO DAHLKE
Avaliador Externo (INSTITUTO POLITéCNICO DE SANTARéM)

Assinatura Eletrônica
29/02/2024 17:18:57.0
CLÁUDIA CASSIMIRA DA SILVA MARTINS
Avaliador Externo (DSM PRODUTOS NUTRICIONAIS)

Assinatura Eletrônica
01/03/2024 09:45:43.0
CHAYANE DA ROCHA
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

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“I think that self-limitation is the major limiting factor for most people in the world. People could do far more things than they believe they can.”

- Frank Herbert

RESUMO

O calcidiol (25-OH-D_3) é um metabólito da vitamina D_3 (VitD) cuja alta biodisponibilidade lhe confere um potencial de aumento do status de VitD quando incluso na dieta de frangos, resultando em otimização da digestibilidade e absorção de Ca e P e permitindo a redução dos níveis dietéticos destes minerais. Três experimentos foram conduzidos com o objetivo de avaliar a suplementação de 69 µg/kg de 25-OH-D_3 em dietas para frangos de corte formuladas com níveis reduzidos de P (Capítulo 2), níveis reduzidos de Ca e P (Capítulo 3), e com diferentes doses de fitase exógena (Capítulo 4), avaliando-se o efeito dos tratamentos sobre o aproveitamento de Ca e P, desempenho e integridade óssea. No primeiro experimento, 560 frangos de 1 a 21 dias de idade foram distribuídos aleatoriamente em um delineamento fatorial 4x2, com 4 níveis de P disponível (0,45, 0,42, 0,39, e 0,36%), com ou sem 25-OH-D_3, totalizando 8 tratamentos de 7 repetições com 10 aves cada. Todas as dietas continham níveis regulares de VitD (100 µg/kg) e fitase (1,500 unidades [FYT]/kg). Aves alimentadas com 25-OH-D_3 apresentaram maior ganho de peso (GP) e melhor conversão alimentar (CA), maior digestibilidade ileal de Ca e P, e maior teor de cinzas na tíbia ($P<0.05$). Níveis decrescentes de P na dieta aumentaram linearmente a digestibilidade ileal de Ca e P, porém reduziram a concentração de P na tíbia ($P<0.05$). A inclusão de 25-OH-D_3 em dietas de 0.36 a 0.42% de P resultou em maior nível circulante do metabólito ($P<0.05$). No segundo experimento, utilizou-se o mesmo número de aves, tratamentos e repetições, cujo delineamento fatorial distinguiu-se por uma redução mais acentuada e concomitante dos níveis dietéticos de Ca + P (0,9+0,45; 0,8+0,4; 0,7+0,35; e 0,6+0,3% de Ca total + P disponível), mantendo-se relação Ca:P em 2:1. Novamente, aves suplementadas com 25-OH-D_3 obtiveram melhor CA no período e aumento da digestibilidade ileal de Ca e P e dos níveis séricos de 25-OH-D_3, Ca, P, e fosfatase alcalina, resultando em maior resistência óssea e maior deposição de cinzas e minerais ($P<0.05$). A redução mais acentuada de Ca+P em comparação ao primeiro experimento comprometeu o desempenho, piorando a CA. Níveis decrescentes de Ca+P novamente aumentaram a digestibilidade ileal dos minerais, porém os níveis séricos de Ca, P e fosfatase alcalina foram reduzidos linearmente, levando a um menor teor de cinzas e Ca e menor resistência óssea ($P<0.05$). No terceiro experimento, 1200 frangos de 1 a 42 dias de idade foram distribuídos em um delineamento fatorial 2x2, com 2 doses de

fitase exógena (600 e 2000 FYT/kg), com ou sem 25-OH-D_3. A inclusão de 25-OH-D_3 resultou em maior GP e melhor CA no período de 1 a 21 dias, e maior GP no período total de 1 a 42 dias, com aumento do nível sérico de 25-OH-D_3 aos 21 e 42 dias. O aumento da dose de fitase de 600 para 2000 FYT levou a menor CA no período de crescimento (22 a 42 dias) e período total, além de melhorar a expressão gênica de mTOR quinase, indicativo de incremento no metabolismo amino ácidos e desenvolvimento muscular. Os resultados encontrados evidenciam que a suplementação de 25-OH-D_3 em conjunto com VitD acentua o status de VitD do organismo e sugerem que o metabólito pode modificar a forma de transporte de Ca e P através das células epiteliais para um transporte predominantemente ativo mediado por VitD, de forma a impulsionar a absorção intestinal destes minerais independentemente de seus níveis dietéticos ou da inclusão de uma alta dosagem de fitase. Por conseguinte, o metabólito propiciou melhora no desempenho e fortalecimento ósseo.

Palavras-chave: 25-hidróxivitamina D, cálcio, digestibilidade, fitase, frangos de corte, fósforo, mineralização óssea, vitamina D.

ABSTRACT

Calcidiol (25-OH-D3) is a metabolite of vitamin D3 (VitD) whose high bioavailability grants it a potential for increasing VitD status when included in the diet of chickens, resulting in the optimization of Ca and P digestibility and absorption and allowing for the reduction of dietary levels of these minerals. Three experiments were conducted to evaluate the supplementation of 69 µg/kg of 25-OH-D3 in diets for broiler chickens formulated with reduced levels of P (Chapter 2), reduced levels of Ca and P (Chapter 3), and with different doses of exogenous phytase (Chapter 4), assessing the effect of treatments on Ca and P utilization, performance, and bone integrity. In the first experiment, 560 chickens from 1 to 21 days old were randomly distributed in a 4x2 factorial design, with 4 levels of available P (0.45, 0.42, 0.39, and 0.36%), with or without 25-OH-D3, totaling 8 treatments with 7 replicates of 10 birds each. All diets contained regular levels of VitD (100 µg/kg) and phytase (1,500 units [FYT]/kg). Birds fed with 25-OH-D3 showed higher body weight gain (BWG) and lower feed conversion ratio (FCR), higher ileal digestibility of Ca and P, and higher ash content in the tibia ($P<0.05$). Reducing levels of P in the diet linearly increased the ileal digestibility of Ca and P but reduced P concentration in the tibia bone ($P<0.05$). The inclusion of 25-OH-D3 in diets ranging from 0.36 to 0.42% of P resulted in a higher circulating level of the metabolite ($P<0.05$). In the second experiment, the same number of birds, treatments, and replicates were used, with a factorial design distinguished by a more pronounced and simultaneous reduction in dietary levels of both Ca + P (0.9+0.45; 0.8+0.4; 0.7+0.35; and 0.6+0.3% of total Ca + available P), while maintaining a Ca:P ratio of 2:1. Again, supplemented birds had lower FCR during the period and higher ileal digestibility of Ca and P, and serum levels of 25-OH-D3, Ca, P, and alkaline phosphatase, thus resulting in greater bone strength and higher bone ash and mineral deposition ($P<0.05$). The more pronounced reduction of Ca+P compared to the first experiment compromised performance, increasing FCR. Reducing levels of Ca+P again increased the ileal digestibility of minerals, but serum levels of Ca, P, and alkaline phosphatase were linearly reduced, leading to lower ash content and Ca and lower bone strength ($P<0.05$). In the third experiment, 1200 chickens from 1 to 42 days old were distributed in a 2x2 factorial design, with 2 doses of exogenous phytase (600 and 2000 FYT/kg), with or without 25-OH-D3. The inclusion of 25-OH-D3 resulted in higher BWG and lower FCR in the period from 1 to 21 days and higher BWG in the total period

of 1 to 42 days, with an increase in serum levels of 25-OH-D3 at 21 and 42 days. Increasing the dose of phytase from 600 to 2000 FYT resulted in worse FCR in the growth period (22 to 42 days) and total period, as well as higher gene expression of mTOR kinase, indicating an increase in amino acid metabolism and muscle development. The results demonstrate that 25-OH-D3 supplementation together with VitD accentuates the broilers' VitD status and suggest that the metabolite can shift the transport form of Ca and P through epithelial cells to a predominantly active transport mediated by VitD, therefore boosting the intestinal absorption of these minerals regardless of their dietary levels or the inclusion of a high dose of phytase. As a result, the metabolite led to improvements in performance and bone strength.
Key-words: 25-hydroxyvitamin D, calcium, digestibility, phytase, broilers, phosphorus, bone mineralization, vitamin D.

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GENERAL INTRODUCTION

Calcium (Ca) and Phosphorus (P) stand as pivotal macrominerals in poultry nutrition due to their extensive involvement across various metabolic processes: formation of bone, nervous and muscular tissues, transmission of nerve impulses, formation of cell membrane phospholipids, energy utilization and transfer; as well as enzymatic activation, among others. The appropriate availability of Ca and P for broilers is crucial to ensure metabolic homeostasis, proper development and maintenance of the skeletal system, and optimal animal performance/growth. Moreover, P holds a significant financial impact in broiler feed production following protein and energy components, and its inefficient utilization contributes to soil contamination and groundwater pollution due to an excessive presence of the mineral in organic fertilizers made from poultry manure (TOOR et al., 2005).

A significant aspect of Ca and P supplementation is their highly intertwined metabolism. Deficiencies, excesses, or imbalances in Ca and P supply affect the absorption, utilization, and overall homeostasis of both minerals together (Amerah et al., 2014). In the gastrointestinal tract (GIT), Ca and P interplay and interact with other substances, collectively influencing the intestinal absorption of nutrients. As highlighted by Amerah et al. (2014), dietary excess of Ca can stimulate the formation of insoluble Ca-Phytate complexes in the small intestine of birds, reducing diet solubility and the absorption of phytic P, whereas insufficient Ca supply can impair bone mineralization and animal growth. Therefore, formulating diets with adequate levels of both minerals, considering not only separate Ca and P levels but also their Ca:P ratio in the diet has become a standard practice in poultry nutrition.

Vitamin D (VitD) plays a crucial role in the metabolism of Ca and P. Its supplementation in poultry diets is intrinsically related to an improved Ca and P absorption that can lead to better bone mineralization conditions, resulting in better growth performance and carcass yield (GARCIA et al., 2013; SAKKAS et al., 2019). The dietary inclusion of VitD is typically done through vitamin premixes in the form of vitamin D_3 (cholecalciferol); once absorbed in the intestine, cholecalciferol is metabolized into 25-hydroxycholecalciferol (25-OH-D_3 or calcidiol) in the liver and subsequently transformed into 1,25-dihydroxyvitamin D3 (1,25-OH_2-D_3) calcitriol) in the kidneys, which is the metabolic active form of cholecalciferol. Recent studies have underscored the direct supplementation of 25-OH-D_3 in the feed of broilers and laying

hens, either partially replacing or in combination with cholecalciferol (FATEMI et al., 2020; HAN et al., 2016; OIKEH et al., 2019), showing that in both cases the metabolite led to positive outcomes regarding higher mineral and VitD status, higher bone mineralization and body weight gain (BWG), and lower feed conversion ratio (FCR). These investigations report that 25-OH-D_3 has greater bioavailability and intestinal absorption rate, which promptly leads to a higher vitamin D status (measured by the serum concentration of 25-OH-D_3). This implies that the direct dietary inclusion of 25-OH-D_3 can further enhance the metabolism of Ca and P and contribute to the strengthening of the skeletal system, improving growth performance and allowing for the reduction of non-phytic dietary Ca and P levels.

Another nutrition strategy increasingly adopted to improve the utilization of Ca and P is the use of exogenous phytase at higher doses, roughly above 1000 phytase units/kg of diet (although the exact dose is set by the manufacturer; Boney and Moritz, 2017).This has been shown to intensify the hydrolysis of phytate in the intestinal lumen, increasing the rate at which the 6 phosphates (IP_{1-6}) surrounding the inositol molecule are released. As supported by studies (COWIESON et al., 2017; WALK and RAMA RAO, 2020), by mitigating the antinutritional effects of phytase, a high phytase dosage can increase the availability of Ca and other phytate-bound nutrients besides P, which is referred to as extra-phosphoric effect. Given the significance of both phytase and VitD on Ca and P metabolism, emerging research suggests synergetic effects between both components in broiler chicken diets (KERMANI et al., 2023; TAHERI and MIRISAKHANI, 2020) that may bolster the utilization of dietary minerals.

The first chapter of this Dissertation is a literature review aimed at comprehensively describing the overall metabolism of Ca, P, VitD and its metabolites, and the mechanisms behind the balance of such components. The remaining three chapters encapsulate the three experiments conducted to assess the effects of dietary 25-OH-D_3 supplementation on the performance, nutrient digestibility, and bone status of broiler chickens fed diets formulated with varying levels of Ca and P and with a high dose of exogenous phytase.

CHAPTER I – LITERATURE REVIEW

1. CALCIUM AND PHOSPHORUS IN BROILER NUTRITION

1.1 CALCIUM

Calcium is the most prevalent macro mineral in the body, of which 99% is stored in the skeletal system as hydroxyapatite crystals, $Ca_{10}(PO_4)_6(OH)_2$, that calcifies and grants structural function to the connective tissue in the bone, making Ca the major component associated with the normal growth and development of bone strength and health. In laying hens, Ca is exceptionally important during eggshell formation, accounting for a high mobilization of Ca from the bone (SINCLAIR-BLACK et al., 2023). The remaining 1% of Ca can be found in the plasma, as a free ion, bound to proteins, or in muscle and other tissues, as part of a pool of Ca whose concentration is tightly controlled by the organism. This serum ionized Ca is a physiologically active form used for muscle contraction, vascular contraction and vasodilatation, nerve impulse transmission, blood coagulation, and control of hormone secretions, especially vitamin D_3 and parathormone (MATOS, 2008; VANNUCCI et al., 2018).

In poultry diets, the main sources of Ca are typically inorganic rocks like calcitic limestone but may include organic sources such as oyster shell and bone meal. The availability of Ca in these ingredients is often rated at 100%, relative to a standard Ca carbonate, rendering Ca as a rather inexpensive nutrient in feed formulation and one of the reasons why Ca requirements for poultry are commonly defined in a total basis (ANWAR et al., 2017). Nonetheless, distinct sources of Ca or those from different origins may exhibit varying bioavailability and solubility that will impact dietary Ca utilization by the bird (MANANGI and COON, 2007). The particle size of Ca source has also been reported to influence Ca availability and affect bone mineralization and growth (ANWAR et al., 2017; BASSI et al., 2022).

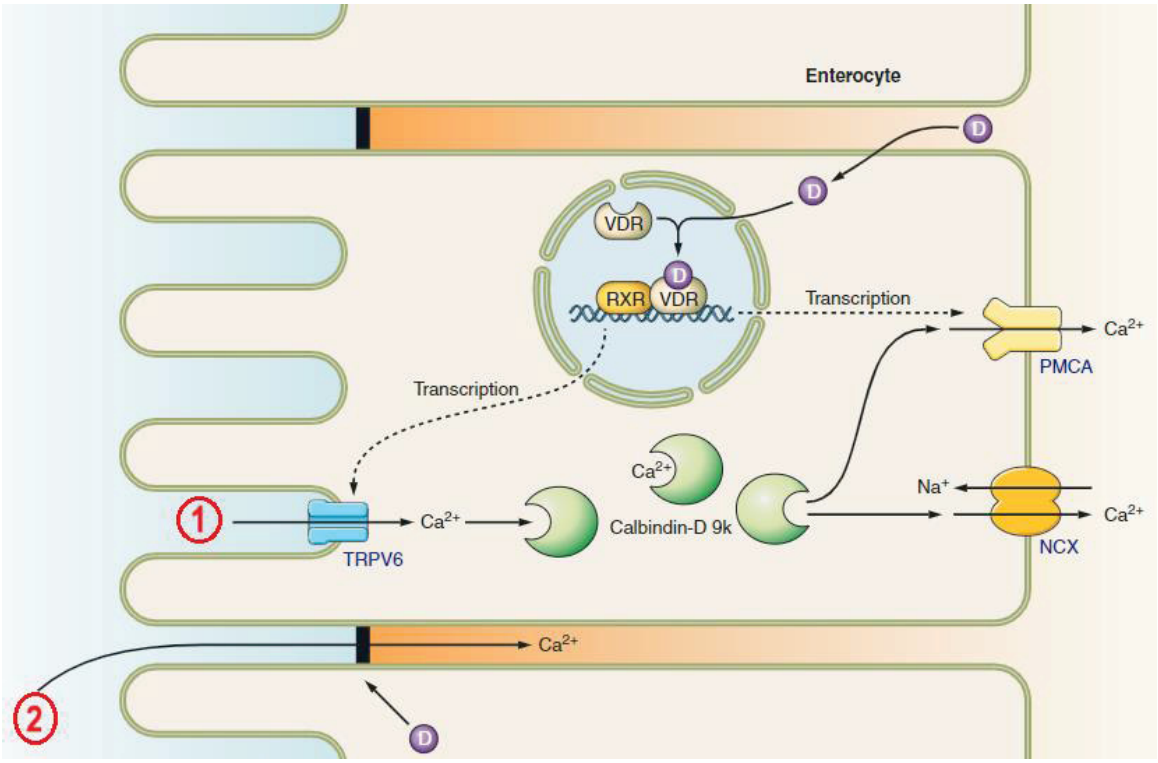
1.1.1 Calcium absorption and metabolism

It’s important to stress that minerals like Ca are not exactly digested per se, as no enzymatic hydrolysis is involved; once ingested, it must first be solubilized and dissociated from its ligands prior to absorption across the epithelium, and the rates of

solubilization and dissolution in the GIT are pH- and particle size-dependent. At lower pH, Ca salts become more soluble (BRUGGENCATE et al., 2011), so the pH of the tract is important. Ca sources ground at finer particles are more rapidly dissolved than coarse particles, leading to a faster passage of Ca through the GIT – which does not necessarily translate into a higher digestibility (KIM et al., 2018)

The absorption of Ca takes place in the small intestine. In the lumen, Ca can be transported through the endothelial cells by active (transcellular, against the gradient) and passive (paracellular, between cells) means (Figure 1). The transcellular transport commences with the entry of Ca across the membrane, mediated by a Ca channel protein called TRPV6; inside the cell, Ca binds to calcium-binding proteins such as calbindin and is diffused in the cytoplasm; finally, Ca exits the cell into the extracellular space via plasma membrane Ca ATPase (PMCA), at the direct expense of ATP, or at a minor rate via a sodium (Na)-Ca exchanger (NCX), at the expense of Na. The paracellular passive transport, conversely, is simply characterized by the movement of Ca ions along a chemical gradient between the lumen and plasma, through spaces between enterocytes.

FIGURE 1 –TRANSCELLULAR VITAMIN D-MEDIATED (1) AND PARACELLULAR (2) ABSORPTION OF CALCIUM IN THE INTESTINAL LUMEN



SOURCE: KOPIC and GEIBEL (2013)

In rats, transcellular transport mechanisms were largely located in the duodenum and upper jejunum (BRONNER, 2003), but Mutucumarana et al. (2014) identified the jejunum and ileum as major sites of active Ca absorption in broiler chickens, whereas passive absorption occurs throughout all small intestine segments. The transcellular system is mediated by VitD, more specifically by its metabolically active form, 1,25-OH_2-D_3. Once reaching the intracellular space of enterocytes, 1,25-OH_2-D_3 interacts with vitamin D receptors (VDR), forming a heterodimer that enter the nucleus and binds to DNA, promoting the transcription of the genes involved in intestinal Ca absorption, such as calbindin, TRPV6, and parathormone (PTH) (PROSZKOWIEC-WEGLARZ and ANGEL, 2013). The activation of 1,25-OH_2-D_3 is regulated by its own production and by PTH and plasma Ca concentrations: at low Ca concentrations, the hydrolysis of VitD into 1,25-OH_2-D_3 is increased, reinforcing the transcellular system to increase Ca absorption in the GIT (VAN DER VELDE et al., 2014). Moreover, Ca levels also directly affect mRNA expression of VDR, PTH, and calbindin (HSIAO et al., 2018; LI et al., 2012). In short, low Ca intake triggers a VitD-mediated active transport of Ca in an attempt to maximize Ca absorption and adjust plasma Ca levels. Noticeably, this means of transport can become saturated, as both the number and turnover of the Ca transport proteins are limited (KOPIC and GEIBEL, 2013). A more comprehensive description of the metabolism of VitD and its metabolites is presented further below in this review.

Paracellular transport of Ca, on the other hand, is a concentration-dependent process, where ions of Ca move from the lumen to the circulatory system through spaces between cells (PROSZKOWIEC-WEGLARZ and ANGEL, 2013). At high plasma Ca concentrations, the active transcellular transport is downregulated, and paracellular transport predominates (BRONNER, 2003). In contrast to active transport, the passive absorption of Ca occurs continuously along the GIT and is not sensitive to 1,25-OH_2-D_3 or low Ca intake (VAN DER VELDE et al., 2014). Notably, studies with rats demonstrated that the diffusion of Ca through paracellular transport in the GIT is usually low due to barriers imposed by the epithelial tight junctions between two cells (DUFLOS et al., 1995). It is outlined, though, despite limiting ion movement between cells, tight junctions are not a static complex and can be regulated by various physiological conditions to change permeability to water and ions.

1.1.2 Parathyroid hormone and calcitonin

The parathyroid glands, found in pairs (1 to 2), are located next to the thyroid glands, consisting of “chief cells” that produce and release the parathyroid hormone, or parathormone (PTH). This hormone is one of the major calciotropic hormones alongside 1,25-OH_2-D_3 and calcitonin (CT). The avian PTH is composed of 88 amino acids, PTH(1-88), and is majorly produced in response to a state of hypocalcemia, i.e. low plasma Ca concentrations (DACKE, 2000). It shares a reciprocal feedback mechanism with VitD, as it can stimulate 1,25-OH_2-D_3 synthesis while its own gene expression is regulated by 1,25-OH_2-D_3 and its secretion is controlled by plasma Ca concentration (GIL et al., 2018; PROSZKOWIEC-WEGLARZ e ANGEL, 2013).

Once released, PTH binds to PTH receptors located in the bones and kidneys where it exerts direct effects to increase plasma Ca levels. In the kidneys, PTH augments glomerular filtration rate and urine flow rate, leading to a rapid increase of Ca reabsorption in the renal tubules and a decrease in Ca excretion via urine (DACKE, 2000). Indirectly, PTH also increases intestinal Ca absorption by stimulating the conversion of VitD to 1,25-OH_2-D_3 in the kidneys (GIL et al., 2018). In the skeletal system, PTH has numerous effects, as described by Silva and Bilezikian (2015) and Vannucci et al. (2018): By connecting to PTH receptors PTH1R in the surface of osteoblasts and osteocytes (cells responsible for mineralization and bone growth), PTH stimulates the expression of a protein known as receptor activator of nuclear factor kappa beta ligand (RANKL). RANKL, in turn, binds to the surface of osteoclasts (cells responsible for breaking down old bones and creating space for newer bone tissue), promoting their differentiation and stimulating their activity; this increased osteoclastogenesis leads to bone resorption, i.e. the dissolution of old bone tissues, resulting in a transfer of Ca from the skeleton to the blood. The RANKL-osteoclast binding is also fostered by a protein called monocyte chemoattractant protein-1 (MCP-1), whose mRNA expression is increased by PTH.

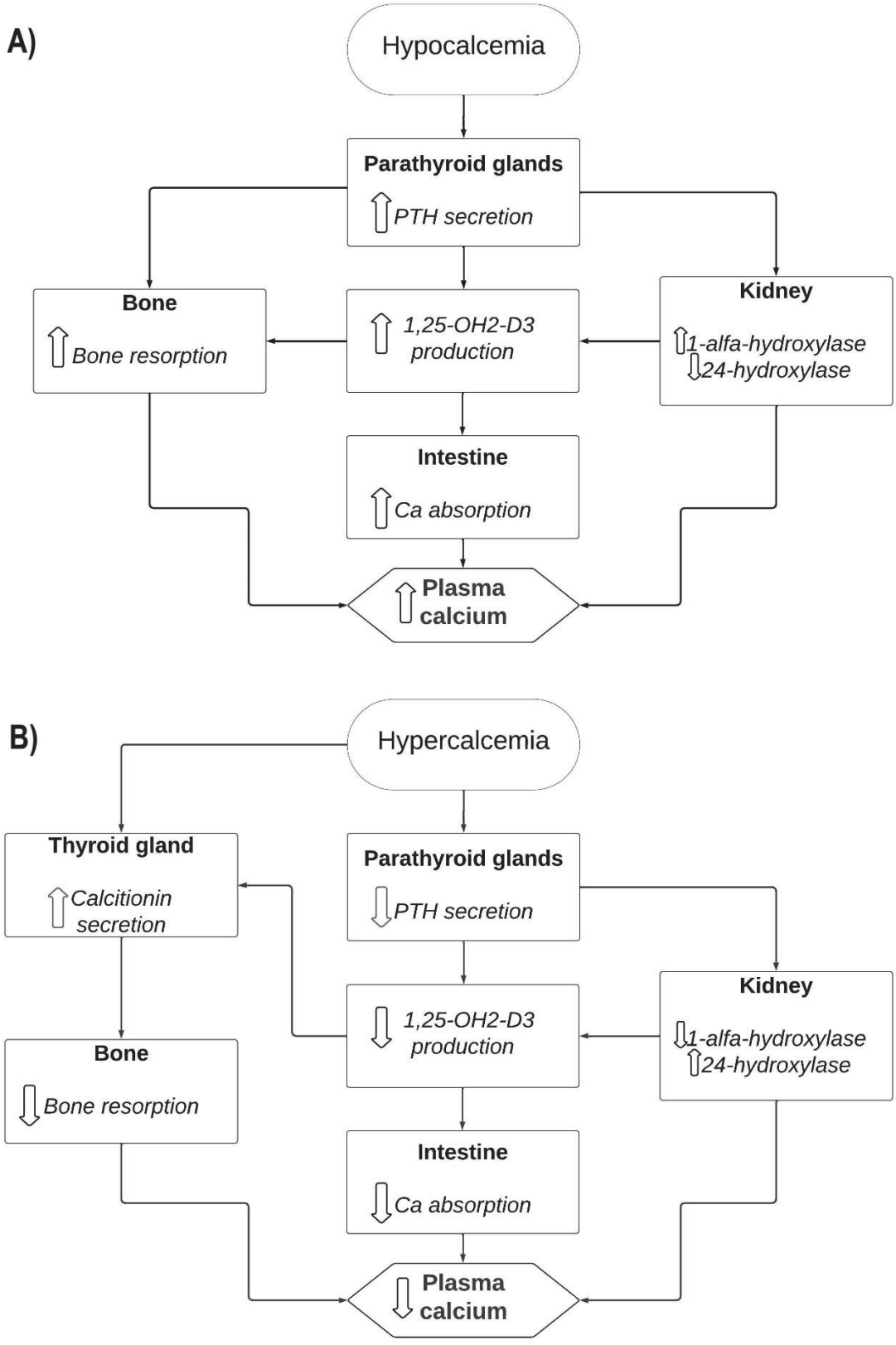
Silva and Bilezikian (2015) states that PTH can also have an anabolic effect in bone formation by acting directly on osteoblasts, promoting osteoblastogenesis, and stimulating the expression of genes that signal bone formation. According to the authors, whether PTH trigger catabolic or anabolic effects on the skeleton depends on duration and periodicity of exposure to PTH, as a continuous exposure leads to bone resorption, and a more intermittent low-dose exposure leads to bone formation. From

this fact, it may be assumed that the organism resorts to dissolution of Ca from bones only when facing a more prolonged situation of hypocalcemia.

Poultry seems to remarkably sensitive to PTH action compared to mammals, as reviewed by Dacke (2000). While the hypercalcemic effect in response to PTH is slow in mammals, poultry birds respond rapidly to PTH administration, particularly laying hens. As observed by Yasuoka et al. (2001), there is an increase in the binding capacity of bone and renal PTH receptors of layers after puberty, perceived as an adaptation to a higher Ca demand for egg-laying. Conversely, roosters develop a higher binding capacity of CT receptors at puberty, coinciding with an increased skeletal maturation during this period.

Calcitonin is the third calciotropic pillar, consisting of a 32 amino acid peptide synthesized in the C cells of thyroid glands. While VitD and PTH actions increase plasma Ca levels to correct a state of hypocalcemia, CT is secreted in response to hypercalcemia, high plasma Ca concentration (MATSUDA et al., 2006). The main target tissue of CT is the bone, where it binds to CT receptors in osteoclasts, restricting their secretory activity and functioning as an inhibitor of bone resorption to decrease plasma Ca levels, in contrast to the action of PTH (INZERILLO et al., 2004). At high doses, CT may also act on kidneys by increasing Ca excretion in the urine (MATOS, 2008). In laying hens, CT serum levels were found to be higher immediately before and after oviposition, indicating the role of CT in preventing excessive Ca resorption at a moment when Ca was not being removed from the blood for eggshell formation (MATOS, 2008; YASUOKA et al., 2001). The action of calciotropic hormones during hypocalcemia and hypercalcemia is summarized in Figure 2.

FIGURE 2. CONTROL OF PLASMA CALCIUM AND REGULATION OF CALCIOTROPIC HORMONES DURING HYPOCALCEMIA (A) AND HYPERCALCEMIA (B)



SOURCE: ADAPTED FROM MATOS (2008)

1.2 PHOSPHORUS

Following Ca, P is the second most abundant mineral in the body, 80% of which is stored in combination with Ca in hydroxyapatite crystals in the bone and 20% is distributed in fluids and other body tissues as dihydrogen phosphate, $H_2PO_4^-$ and hydrogen phosphate, HPO_4^{2-}. Phosphorus itself is highly reactive with oxygen and only exists in the body as the ion phosphate (WAGNER, 2023). Not only crucial as a structural component in bones and cell membranes, but P also has a pivotal role in fundamental physiological processes. These include the formation of muscular tissue, nutrient absorption, activation of enzymatic processes, osmotic regulation, acid-base balance, adenosine triphosphate (ATP) molecule formation, DNA formation, and is also a co-factor of various enzymes (PEACOCK, 2021).

The source of P in the diet significantly influences the mineral's bioavailability and utilization, and it comes as organic or inorganic phosphates. Phosphorus derived from inorganic sources like mono-, di-, or triphosphates are obtained from phosphate rocks and exhibits a near 100% bioavailability akin to inorganic Ca, although P content differs between sources (HAMDI et al., 2017). Unlike Ca, though, these sources are finite, costly, and expected to be extinguished by 2050 (KUMAR et al., 2016). Furthermore, an indiscriminate dietary inclusion of rock phosphates may contribute to excessive amounts of non-absorbed P in manure, increasing the pollution of lakes and water sources and aggravating surface water eutrophication (TOOR et al., 2005). Animal meals such as meat and bone meals have a relatively high content of bioavailable P, and while their use in pig and poultry diets was banned in European countries due to the occurrence of Bovine Spongiform Encephalopathy, the ban was recently partially lifted (BONNEY, 2021).

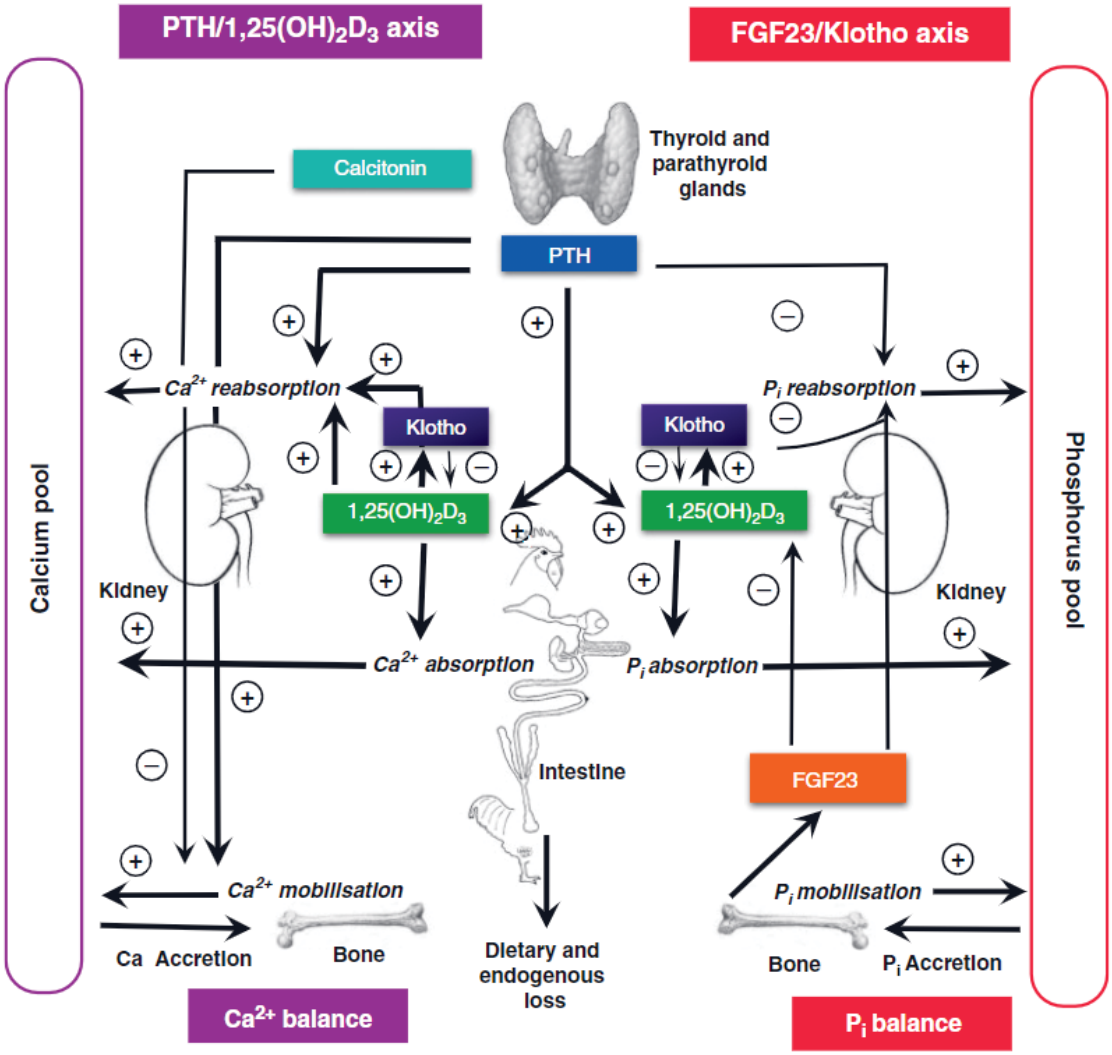
Cereal grains and other plant feedstuffs are the main ingredients of monogastric animals’ diets and are also accounted for dietary P supply, which is stored in the seeds. However, most of the P present in these feedstuffs is in the form of phytic P, which is unavailable for digestion and represents a major obstacle in P and overall mineral nutrition of poultry (COWIESON et al., 2016; HUMER et al. (2015).

1.2.1 Phosphorus absorption and metabolism

The absorption mechanisms and overall metabolism of P are intimately related to Ca metabolism. Homeostasis of P and regulation of plasma P concentration, like

Ca, occurs through a coordinated endocrine action on intestinal, renal, and skeletal systems (Figure 3). Both minerals share VitD metabolites and PTH as their main mediating hormones, with a few particularities: CT, for instance, takes part exclusively in Ca balance, while P balance is also regulated by a hormonal pathway involving Fibroblast Growth Factor 23 (FGF23) and Klotho protein in bone and kidney, in addition to the PTH/1,25-OH_2-D_3 pathway (MARTIN et al., 2012).

FIGURE 3. ENDOCRINE REGULATION OF CALCIUM AND PHOSPHORUS IN BIRDS.



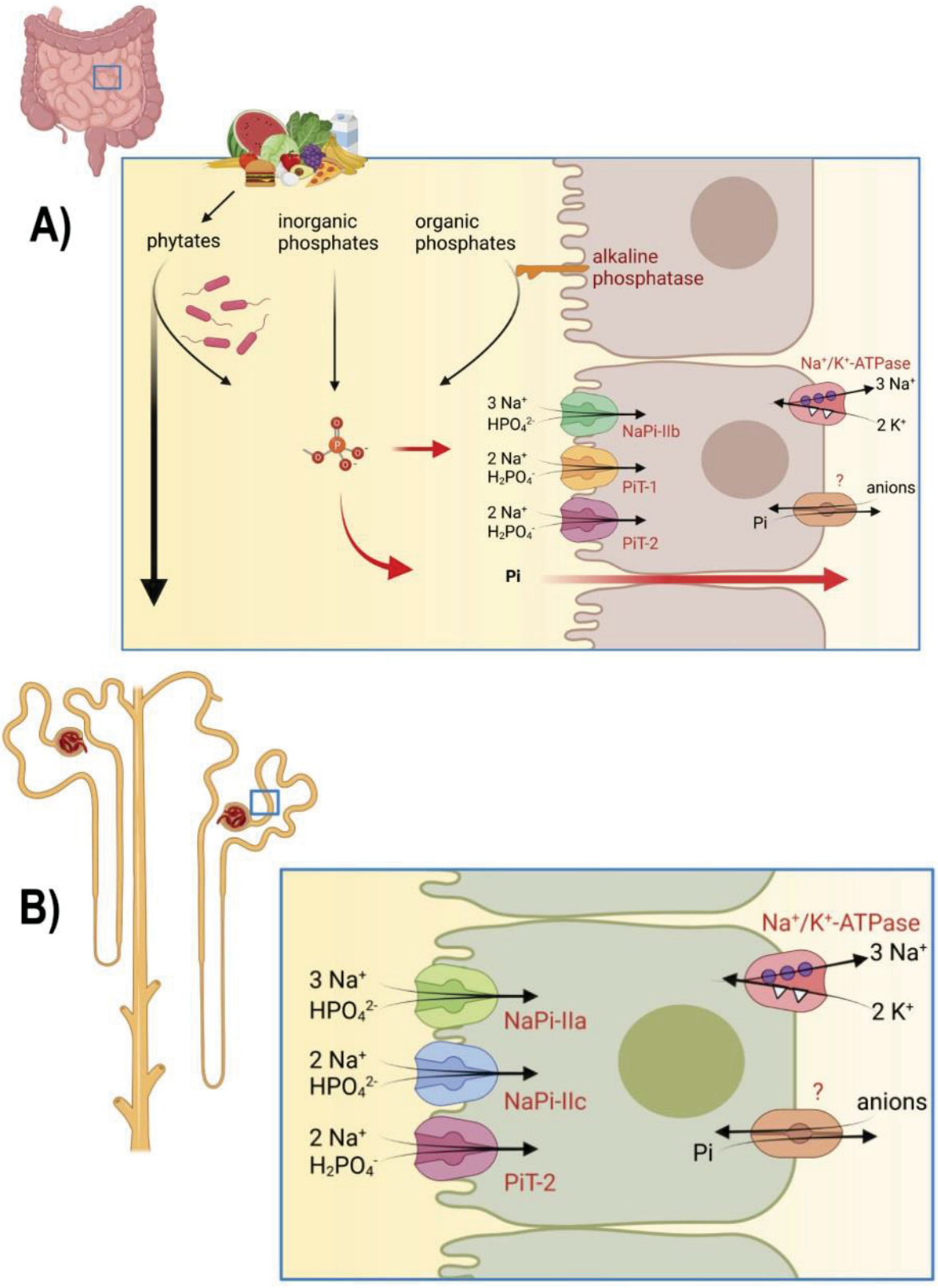
SOURCE: LI et al. (2017)

Intestinal and renal phosphate transport mechanisms, while fundamentally similar to Ca uptake, are also slightly different, with a more prominent participation of Na in as a component of transport proteins. Active transcellular transport of phosphates in the intestinal lumen and kidneys is mediated by Na–phosphate cotransporters, while passive phosphate transport is driven by diffusion through a gradient (Figure 4).

The basic process of P transport across the brush border membranes of the renal proximal tubular and intestines is very similar but involve specific transporters. The Na-phosphate co-transporters are divided into three classes (TAKEDA et al., 1999): type I (NaPi-I) is predominantly expressed in the renal tubes but has a lesser role in phosphate homeostasis, serving as a carrier of organic anions without significant contributions to phosphate transport; type II (NaPi-II, SLC34 gene family), which consists of 3 carriers, IIa (SLC34A1 gene), IIb (SLC34A2 gene), and IIc (SLC34A3 gene), expressed in renal and intestinal brush borders; and type III (SLC20 gene family) consisting of 2 carriers, PiT1 and PiT2, expressed in various cells and generally considered to be responsible for supplying P to individual cells functioning. Transporters type II and III are the ones substantially involved in P transport and homeostasis. NaPi-IIa and NaPi-IIc are the major regulators of renal phosphate transport, and NaPi-IIb is exclusively responsible for intestinal P transport, while PiT1 and PiT2 take part of both renal and intestinal transport to a lesser extent (SABBAGH et al., 2011).

The NaPi-IIb co-transporter is found in the apical membrane of the enterocyte, and its activity is regulated by 1,25-OH_2-D_3 and VDR. Under low P intake and low plasma P conditions, the parathyroid glands signal the secretion of PTH to increase the formation of 1,25-OH_2-D_3, which in turn upregulates the gene expression of NaPi-IIb in the small intestine and Na to enhance the active P absorption system (LI et al., 2012; SHAO et al. (2019). In the kidneys, both NaPi-IIa and NaPi-IIc are located in the brush border of the proximal tubules, and their expression is regulated directly by PTH, FGF23, and Kloth proteins (WAGNER, 2023). The type III transporters PiT1 and PiT2 have been localized in enterocytes, whereas PiT2 has also been found in proximal tubules (HERNANDO and WAGNER, 2018).

FIGURE 4. TRANSCELLULAR SODIUM-DEPENDENT AND PARACELLULAR TRANSPORT OF PHOSPHORUS IN THE INTESTINAL LUMEN (A) AND TRANSCELULLAR TRANSPORT OF PHOSPHORUS IN THE KIDNEYS (B).



SOURCE: WAGNER (2023)

The transport mode of phosphate through enterocytes and proximal tubules is described by Hernando and Wagner (2018): phosphate ions exhibit a strong affinity for cations such as Na^+, forming a Na^+:HPO_4^{2-} substrate and using the downhill Na^+ concentration gradient to traverse Na/Pi co-transporters into the intracellular space. Werner et al. (2001) further clarify that the coupling to Na^+ is obligatory due to the negative electrochemical potential of phosphate hindering the molecule of entering the cytosol by diffusion. The release of phosphate into the blood is then carried out by Na+/K+-ATPases pumps. The different co-transporters differ as to the stoichiometry of substrates they are capable of transporting (FENOLLAR-FERRER and FORREST, 2019): NaPi-IIa and NaPi-IIb are electrogenic and transport three Na ions per phosphate (3 Na^+: 1 HPO_4^{2-}), while NaPi-IIc is electroneutral and transport divalent Na/Pi bonds (2 Na^+: 1 HPO_4^{2-}). Type III PiT1 and PiT2 prefer monovalent phosphates, but their contribution to renal and intestinal phosphate transport is less defined, with seemingly no significant effect on mineral balance (WAGNER, 2023).

Regarding paracellular transport, it is predominant at high P intake, where the phosphate ions then move along the concentration gradient through tight junctions between cells without the aid of Na-cotransporters, akin to paracellular transport of Ca (FOUQUE et al., 2018). One important aspect of paracellular P transport, though, is that it is affected not only by phosphate intake, but also by Ca intake. At high concentrations of Ca in the lumen, pH is increased and Ca loses solubility, fostering a complexation between Ca and phosphate that renders both minerals unavailable for digestion and absorption (LIU et al., 2013; SINCLAIR-BLACK et al., 2023).

Notably, control of P metabolism is tighter in the kidneys than in the gut. Together, NaPi-IIa and NaPi-IIc are responsible for 80% of the filtered P in the proximal tubular (MARKS et al., 2010). Like NaPi-IIb, their activity is upregulated by 1,25-OH_2-D_3 but the action of PTH is inversed, also differing from Ca homeostasis: While PTH directly increases renal Ca reabsorption, it simultaneously displays a phosphaturic effect, i.e. reduces the capacity of the kidneys to reabsorb phosphate by triggering the degradation of NaPi-IIa and NaPi-IIc (BERGWITZ and JÜPPNER, 2010). PTH also mobilizes phosphate from the bone by enhancing activity of osteoclasts (RAZZAQUE, 2009). This phosphaturia induced by PTH aims to hinder the aforementioned formation of salts between phosphate and Ca in the serum that are insoluble and result in decreased plasma Ca (MATUSZEWSKI et al., 2020). Therefore, PTH is given the task of balancing serum Ca and P, and by controlling phosphate serum levels, calcemia is

also managed. In addition to PTH, phosphoturic effects are also shared by phosphatonins and Klotho protein.

1.2.2 Phosphatonins and Klotho protein

To fully understand P metabolism, the role of phosphatonins and Klotho protein must be addressed. Phosphatonins are hormones part of the fibroblast growth factor (FGF) family and that have been identified as important regulators of P homeostasis, especially FGF23 (MASI, 2011). The skeletal system is the main source of production and secretion FGF23, synthesized primarily in osteoblasts and osteocytes, appointing the bone tissue as an important endocrine organ involved in phosphatemia, but FGF23 can also be found in endothelial cells, small intestine, and lymphonodes (BERGWITZ and JÜPPNER, 2010; Martin et al., 2012). Once released, FGF23 targets the kidney, where it regulates renal phosphate reabsorption and counterbalances the effects of 1,25-OH_2-D_3.

To properly function, FGF23 requires binding with αKlotho. This protein, located in cell membranes, is part of a cofactor that activates FGF receptors responsible for binding FGF23 to its target organs, mainly the kidneys (MARTIN et al., 2012). The Klotho-FGF receptor complex binds FGF23 to target cells with much higher affinity than to FGF receptor alone, and the absence of Klotho may result in an organ resistance to FGF23, thus Klotho is essential to FGF23 signaling (MARTIN et al., 2012; MASI, 2011).

The expression of FGF23 us upregulated by high serum phosphate and 1,25-OH_2-D_3, which signals the release of FGF23 into circulation to exert hyphosphatemic effects: FGF23 inhibits renal reabsorption and increases excretion of phosphate by reducing the expression and activity of both NaPi-IIa and NaPi-IIc in the proximal tubule (MASI, 2011). Moreover, FGF23 mediates the reduction of VitD availability by suppressing the expression of 1α-OH-hydroxylase, the enzyme responsible for converting 25-OH-D_3 into 1,25-OH_2-D_3, thus indirectly reducing P absorption in the small intestines (BERGWITZ and JÜPPNER, 2010; MASI, 2011). In addition, Krajisnik et al. (2007) demonstrated that FGF23 downregulates the expression of PTH. Even though both substances have phosphaturic effects, Razzaque (2009) postulates that this suppression of PTH by FGF23 occurs to counterbalance the increased production of 1,25-OH_2-D_3 driven by PTH, further establishing the role of FGF23 in controlling both

phosphatemia and calcemia. As thoroughly reviewed by Sinclair-Black et al. (2023) and Poorhemati et al. (2023), FGF23 expression in poultry birds is increased with age; this was observed for both broiler chickens and laying hens and indicate an adjustment of Ca and P homeostasis attributable to rapid mass gain in broilers and oviposition in layers.

1.2.3 Phytic phosphorus and its antinutritional effects

In plant ingredients globally used as primary energy and protein sources in poultry diets, notably corn and soybean meal, most of the organic P exists in the form of phytic P, partially unavailable to the animal (Table 1). This designation refers to P when it complexes with inositol, forming phytic acid, also known as phytate when in salt form, formed by 6 phosphates linked to 12 hydrogens on an inositol ring (myo-inositol-1,2,3,4,5,6-hexaphosphate; IP_6). Phytate found in cereals and seeds is associated to intracellular reserves to be mobilized during germination, representing up to 80% of the total P content (HUMER et al., 2015).

TABLE 1. AVERAGE CONTENTS OF TOTAL AND PHYTIC PHOSPHORUS (P) IN PLANT INGREDIENTS.

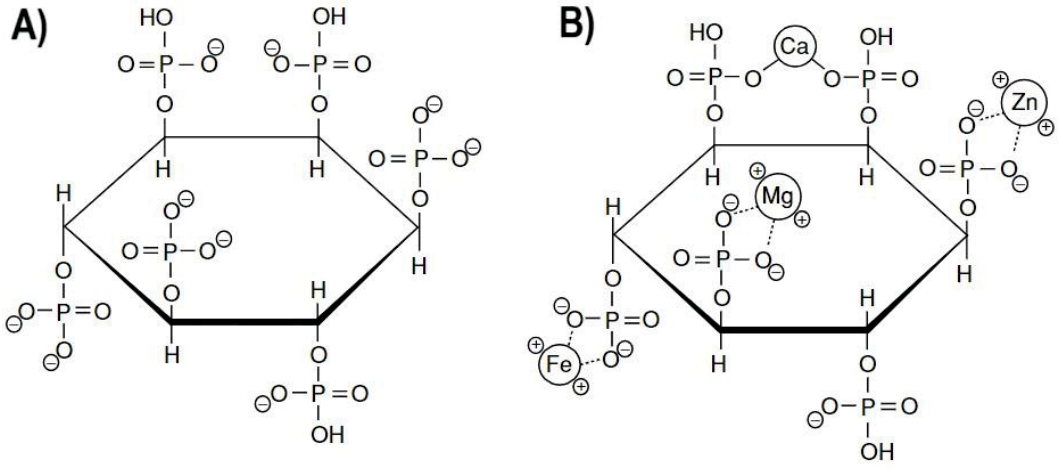
Ingredients	Total P (%)	Phytic P (%)	Phytic P/ Total P (%)
Corn	0.28	0.20	71.4
Wheat	0.37	0.24	64.9
Rice bran	1.50	1.28	85.3
Oats	0.27	0.22	81.5
Barley	0.32	0.20	63.1
Sorghum	0.28	0.21	73.1
Soybean meal	0.65	0.38	58.5
Cottonseed meal	0.97	0.75	77.3
Rapeseed meal	1.07	0.65	60.7
Peanut bran	0.63	0.50	79.4

SOURCE: ADAPTED FROM NRC (1994).

Because it’s a polyanionic molecule - negatively charged - phytate can readily form insoluble salts with cations, notably minerals like Ca+, K+, Mg++, Zn+, Fe+, and Mn++; additionally, phytate can form chemical associations with dietary polysaccharides, proteins, and amino acids (COWIESON et al., 2009). Although phytic

acid and phytate are commonly referred to as synonymous, an accepted definition is that phytic acid is the given denomination before chelation, while phytate is the chelated salt, as illustrated in Figure 5.

FIGURE 5. PHYTIC ACID (A) AND PHYTATE CHELATE (B).



SOURCE: ZITTERMAN (2003)

One of the most influential factors in the interaction of phytate with diet components and is the pH of the intestinal tract (Amerah et al., 2014). In the gizzard and proventriculus of birds, where pH ranges from 2.5 to 5.0, most of phytate chelates formed with other nutrients can become more soluble and prone to hydrolysis by phytate-degrading enzymes whose peak activity occurs at more acidic pH (LIEBERT et al., 1993). However, at an intestinal pH range closer to neutral, phytate chelation is fostered. When at high luminal concentrations, Ca in particular is a major concern, as it will further increase pH and strengthen the complexation with phytate, creating an insoluble and indigestible molecule (LIU et al., 2013; SELLE et al., 2009). Other cations like magnesium (Mg) and zinc (Zn) are also likely to form phytate chelates at higher intestinal pH. As exemplified by Konietzny and Greiner (2003), a synergetic effect is triggered at the presence of two cations reacting with phytate, as Ca enhances the adsorption of Zn to form a Ca-Zn phytate, or similarly Mg was shown to increase precipitation of Zn with phytate – to a lesser extent than Ca. The higher the Ca level and Ca:P ratio, the more extensive is its precipitation with other ions.

In addition to minerals, proteins and amino acids can also react with phytate. Protein losses through phytate complexation have been noted to occur at both low pH, where phytic acid directly react with charged protein groups, or at high pH, where the

reaction is mediated by the presence of adsorbed cations like Ca, Zn, or Mg (COWIESON et al., 2004: HUMER et al., 2015). The presence of phytate in the GIT also induces an excess of HCl release to the intestinal lumen, an organism's reaction aimed at compensating for reduced protein digestibility (COWIESON et al., 2004), albeit leading to greater irritation of the intestinal mucosa. Due to mucosal irritation, there is an increase in mucin production, as observed by Onyango et al. (2009), leading to the loss of endogenous amino acids. Liu et al. (2014) explains that phytate also targets starch, either by a direct reaction or indirectly through complexation with the protein matrix surrounding starch granules, hence restricting energy utilization by the animal.

The consequences of low phytic P availability and antinutritional effects of phytate have been thoroughly documented in the literature (HUMER et al., 2015; Walk et al., 2016; WALK and RAMA RAO, 2020; WOYENGO and NYACHOTI, 2013). Through an increase in endogenous losses of minerals, amino acids, and even starch – detailed in Figure 6 -, phytate leads to a reduced dietary energy utilization, reduced growth performance, lower egg production in laying hens, impaired bone development and health, higher occurrence of skeletal problems, and others. As put by Walk et al. (2016), the destruction of phytate is imperative to meliorate its effects in the GIT and aim for a more precise nutrition.

For the phytate-bound nutrients to become available and be absorbed in the GIT, the myo-inositol ring must first be broken down, releasing phosphate groups through the action of phytase and other phosphatases. In monogastric animals, the endogenous production of these enzymes is insufficient to efficiently hydrolyze phytate, thus limiting nutrient utilization (DERSJANT-LI et al., 2015). To compensate for low phytic P availability, the oversupply of inorganic phosphates in non-ruminant diets was a common practice. However, even though P availability in inorganic sources is highly available, such feed additives are produced from a finite material, phosphate rock, in a process greatly demanding of energy (WALAN et al., 2014), thus adversely driving up feed production and formulation costs. Over the years, the use of exogenous phytase has become the main strategy to counteract phytate, increasing phytic P availability, and reducing the need for inorganic phosphate sources. Recent advances show that high doses of phytase can trigger extra-phoshoric effects that increase the digestibility of phytate-bound nutrients beyond P (BASSI et al., 2021; LEE et al., 2017;

TAHERI and MIRISAKHANI (2020). Use of exogenous phytase in poultry diets – and its potential synergetic interactions with VitD - is detailed further in this review.

FIGURE 6. ENDOGENOUS NUTRIENT LOSSES CAUSED BY DIETARY PHYTIC ACID.

SOURCE: WOYENGO AND NYACHOTI (2013)

1.3 CALCIUM AND PHOSPHORUS REQUIREMENTS AND RATIO

The intensive genetic selection of broiler chickens toward breast muscle deposition over the years has led to a significant increase in body weight gain. This fast growth was met with inadequate development of the skeletal system, unable to carry such a heavy body and inevitably raising the occurrence of locomotion problems and skeletal disorders (LIU et al., 2023; MATUSZEWSKI et al., 2020). Despite the negative correlation to weight gain, long-term selection for improving bone growth and bone health have been carried out so that bone growth can match the increment in

muscle mass and reduce leg diseases (LIU et al., 2023). As a consequence, the homeostatic system of Ca and P has been strained and dietary Ca and P needs – as well as VitD - of fast-growing birds have been increasing, calling out for an ongoing reassessment of mineral and vitamin requirements of modern broilers (ANGEL, 2011; SAKKAS et al., 2019; WALDENSTEDT, 2006).

To discuss P requirements, it's important to first define the terms associated with its bioavailability and digestibility rates. Earlier formulation practices were based on total P, but the high presence of phytic P in plant ingredients and the high variability of P digestibility in inorganic sources urged the need to shift from a total P basis to meet the dietary P requirements for poultry. Angel (2011) defines the terms used to refer to the many forms of P in animal nutrition:

- Total P: Refers to all and any forms of P present in the diet without considering differences in availability.
- Phytic P: The P that exists in the diet complexed with phytate salts, poorly available for digestion.
- Non-phytic P: Any P present in the diet that is not bound to phytate, and that can be determined by subtracting phytic P from total P.
- Available P: The amount of P that is effectively absorbed from the diet into the organism, obtainable by feed P minus P in the distal ileum.
- Digestible P: similar to available P, often used from a determination standpoint.
- Retained P: refers to P that stays in the body, obtainable by feed P minus excreta P.

Recent discussions by David et al. (2023a) highlight the prevailing challenges in Ca nutrition for poultry, particularly the issue of oversupply of Ca in commercial diets. In contrast to P, Ca requirements are still largely addressed in a total Ca basis, as the bioavailability of inorganic Ca is often deemed to near 100%. However, in addition to the low price and abundance of Ca sources, the lack of assessment of nutritional quality and particle size of ingredients, issues with mixing, and inadequate Ca digestibility measurements all contribute to an excess of dietary Ca that will invertedly reduce nutrient availability. The authors further emphasize that, as happened to P nutrition, Ca nutrition in poultry over time ought to shift toward a digestible Ca basis to

adequately meet the birds' requirements and enable the reduction of formulated levels with no harm done to bone development and growth.

In a series of publications, David et al. (2021, 2022, 2023b) establish the requirements of digestible Ca at different dietary levels of digestible P for broiler starters, grower, and finishers. The authors present standardized ileal digestible values (calculated equivalent values based on total Ca and available P) for both Ca and P, as well as their ratio, based on achieving maximum body weight gain or tibia ash (Table 2). A noteworthy aspect here is how requirements of digestible Ca for maximum bone ash deposition surpass those for maximum body weight gain, while for P, requirements for growth and bone mineralization are equal. This agrees with previous calculations such as those proposed by Bar et al. (2003) and Driver et al. (2005) and showcases the more prominent role of Ca on skeletal growth. Furthermore, the authors draw attention to the fact that Ca and P requirements were greater in starter broilers and later decline during grower and finisher phases, representing the higher mineral needs – particularly for Ca - of a skeletal system under development in younger birds (FLEMING, 2008).

TABLE 2. STANDARDIZED ILEAL DIGESTIBLE (SID) REQUIREMENTS OF CALCIUM AND PHOSPHORUS OF BROILER CHICKENS ACCORDING TO AGE.

Parameter	Starter (1 to 10 d)	Grower (11 to 24 d)	Finisher (25 to 35 d)
Body weight gain			
SID Ca (g/kg)	3.32	3.05	3.50
SID P (g/kg)	5.00	3.50	3.50
SID Ca: SID P	0.66	0.87	1.00
Tibia ash			
SID Ca (g/kg)	4.51	3.69	3.00 – 3.50
SID P (g/kg)	5.00	3.50	3.50
SID Ca: SID P	0.9	1.05	0.86 – 1.00

SOURCE: DAVID ET AL. (2021, 2022, 2023B).

1.3.1 Calcium and phosphorus ratio

In the GIT, the collective interplay of Ca and P to each other and to other substances significantly influences the intestinal nutrient absorption (LIU et al., 2013). As highlighted by Selle et al. (2009), an excessive dietary intake of Ca can result in the formation of insoluble Ca-phytate complexes in the upper gut. This, in turn, reduces diet solubility and limit the absorption of P and other phytate-bound nutrients. Conversely, an inadequate supply of Ca may impair bone mineralization and compromise the animal's growth. Diet formulation must not only consider individual

levels of Ca and P; instead, attention must be directed towards the dietary Ca:P ratio. This approach has become a standard practice in poultry nutrition.

Historically, Ca:P ratio recommendations were defined on a total Ca and total P basis and were later changed to the currently adopted total Ca and available P ratio. Recommendations for broiler chickens described by the NRC (1994) ranged from 2.22 to 2.67:1 depending on the growth stage. A more recent proposal by Rostagno et al. (2017) shortens the range and sets a steadier Ca:P ratio of 2.1:1 on average for medium performance birds, with low variation throughout starter to finisher phases. Layers and broiler breeders remarkably demand a much higher ratio given their extraordinary needs for Ca during oviposition (Table 3). In general, a 2:1 ratio is the normally adopted value in commercial broiler diets worldwide (DAVID et al., 2023a). Noteworthy, when working on a digestible Ca to digestible P basis, suitable values may range from 0.66 to 0.9:1 for starter and around 1:1 for finisher broilers, as summarized by DAVID et al. (2021, 2022, 2023b).

High Ca:P ratios can be detrimental to growth and bone health. Gautier et al. (2017) evaluated increasing Ca:P ratios starting from 1.3:1 up to 5.3:1 and reported a linear reduction on overall growth performance, bone ash, and P retention; Xu et al. (2021) observed higher occurrences of hypophosphatemic rickets on young broilers with a Ca:P ratio of 3.08:1, an indication of lower P availability. Nonetheless, studies indicate that broilers are able to sustain lower (BASSI et al., 2022) or higher (IMARI, 2022) ratios within a certain range without adversely affecting growth performance or compromising the digestibility of Ca and P. In that case, such adjustments may not only prove economically advantageous but also contribute to environmental sustainability.

TABLE 3. RECOMMENDATIONS OF TOTAL CALCIUM AND AVAILABLE PHOSPHORUS AND CALCIUM:PHOSPHORUS RATIO FOR POULTRY BIRDS.

Species	Total Ca	Available P (AvP)	Ca:AvP
Broilers (21-d-old)	0,92	0,44	2.1:1
Laying hens	4,3	0,37	11.6:1
Broiler breeders	2,61	0,25	10.4:1
Turkeys (8 weeks-old)	1,2	0,6	2:1

SOURCE: ADAPTED FROM ROSTAGNO ET AL. (2017).

2. VITAMIN D

Vitamins are compounds with various developmental and metabolic functions, playing an indispensable role in energy production mechanisms while optimizing overall health and growth. According to Nelson and Cox (2006), one defining attribute of vitamins is their status as organic substances essential in minute quantities to uphold vital physiological processes, exerting influence over metabolic functions, mineral balance, and the preservation of bodily structures and tissues.

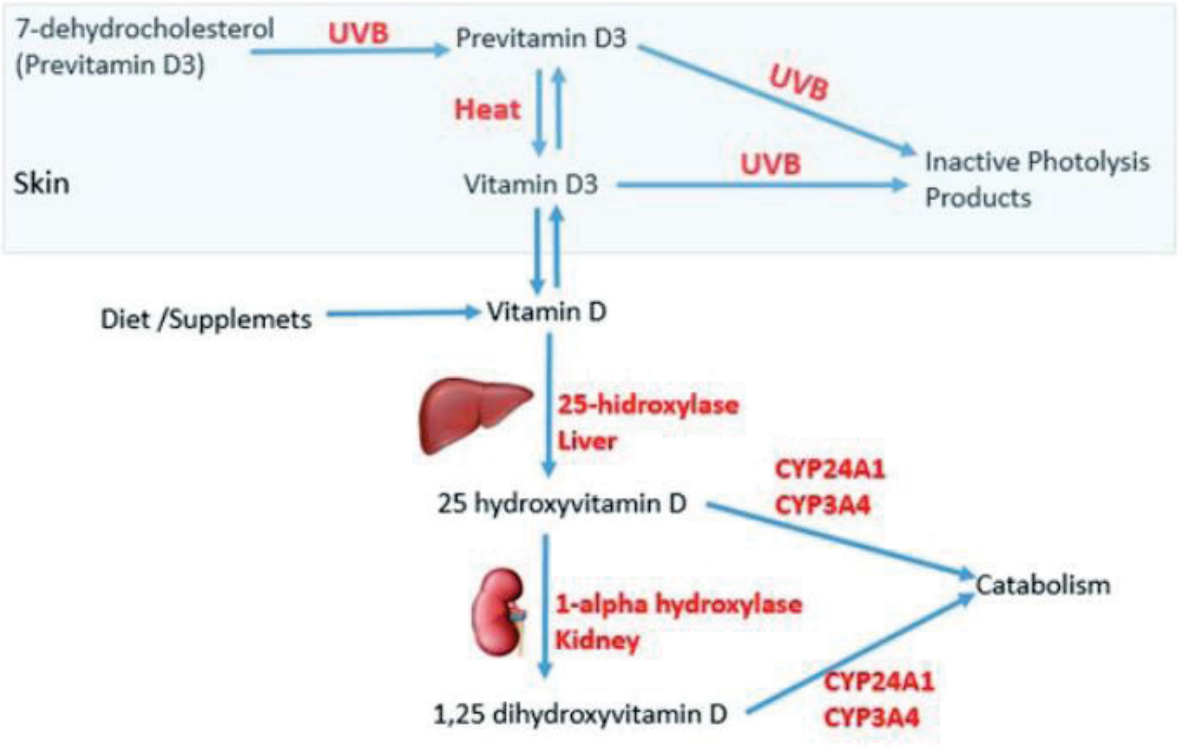
In cereals used as basis for poultry diets, there exists significant variation in vitamin levels attributable to factors such as crop origin, genetic variations, employed fertilizers, drying conditions, storage, and processing methodologies, among others (Mcdowell and WARD, 2009). Consequently, the vitamin requirements of broilers, as with other monogastric production animals, are met through the inclusion of vitamin supplements or premixes within their diets. These supplements typically constitute a mere 0.1 to 0.5% of the entire formula yet may represent up to 3% of the total feed cost (TOLEDO et al., 2006). Among the key vitamins often integrated into poultry diet supplements, the fat-soluble vitamins A, D, E, and K feature prominently, linked to the development and maintenance of various bodily tissues and substances (FÉLIX et al., 2009). Particularly, vitamin D plays pivotal roles in the metabolism of Ca and P and will be the focus of this review.

2.1 VITAMIN D: FORMATION AND METABOLISM

Vitamin D is a fat-soluble vitamin closely associated with the absorption and metabolism of Ca and P It is a group of compounds with a molecular structure similar to steroids. The two most common forms of the vitamin are ergocalciferol (vitamin D_2), derived from plants and fungi, formed through the action of radiation on ergosterol — a precursor sterol of vitamin D2; and cholecalciferol (vitamin D_3, VitD), produced photochemically by animal cells in lower layers of the epidermis when exposed to ultraviolet light acting on VitD's precursor 7-dehydrocholesterol. In other words, it is synthesized endogenously by the skin in a process activated by direct exposure to sunlight (BIKLE and CHRISTAKOS, 2020). VitD can also be obtained via diet and absorbed through diffusion, primarily in the small intestine, in an absorptive process similar to fatty compounds, involving the formation of micelles and the participation of bile salts (BAR et al., 1980).

To become a functional molecule in catabolic processes, VitD must undergo conversion into its active forms, as described by Acar and Özkan (2021) and illustrated in Figure 7: Ultraviolet light exposure on the epidermis leads to the photolysis of 7-dehydrocholesterol into pre-VitD and subsequently into VitD, which is then converted in the liver to 25-hydroxycholecalciferol (25-OH-D_3 or 25-hydroxyvitamin D_3 or calcidiol) by a specific 25-hydroxylase. Subsequently, 25-OH-D_3 undergoes further hydroxylation in the kidneys carried out by 25-hydroxycholecalciferol-1-hydroxylase, becoming 1,25-dihydroxycholecalciferol (1,25-OH_2-D_3 or calcitriol), the biologically active form of VitD. 1,25-OH_2-D_3 is transported in the bloodstream until it reaches VDR's located on the membranes of target tissues and organs.

FIGURE 7. VITAMIN D3 METABOLISM.



SOURCE: ACAR AND ÖZKAN (2021)

2.1.1 Vitamin D3 action mechanisms

VitD metabolism is interconnected to PTH and to serum Ca and P concentrations. Both hypocalcemia and hypophosphatemia urges the 1-α-hydroxylation of 25-OH-D_3 into 1,25-OH_2-D_3 to correct imbalances on the plasma pool

of Ca and P. PTH also stimulates 1,25-OH_2-D_3 formation, which in turn has a negative feedback on PTH secretion (DACKE, 2000).

The intestines, bones, and kidneys are the main sites of action for VitD and its metabolites, whose primary function is to regulate the absorption and metabolism of Ca and P — acting along PTH, calcitonin, FGF23 and Klotho in a clockwork regulated balance. In the intestines, 1,25-OH_2-D_3 enhances the active transport of Ca and P through epithelial cells by fostering the synthesis of transport proteins that participate on transcellular transport, such as calbindin, TRPV6, PMCA, and type II and III Na-phosphate cotransporters (HSIAO et al., 2018; SHAO et al., 2019). Once 1,25-OH_2-D_3 binds to VDR in intestinal cells, it then binds to the vitamin-D-responsive element (VDRE) of TRPV6 to enhance its expression and increase the uptake of Ca into the cells (BRONNER, 2003).

In the kidneys, 1,25-OH_2-D_3 minimizes the loss of Ca by increasing renal tubule reabsorption and influencing the entrance of Ca through apical membrane of renal cells. 1,25-OH_2-D_3 stimulates the expression of calbindin and PMCA in renal cells that increase PTH-dependent transport of Ca (BERGWITZ and JÜPPNER, 2010). Conversely, 1,25-OH_2-D_3 directly inhibits phosphate reabsorption by increasing α-Klotho expression in the kidneys and indirectly by inducing FGF23 expression in the osteocytes (MARTIN et al., 2012).

In bone tissue, 1,25-OH_2-D_3 regulates the action of PTH via a bone-kidney-parathyroid pathway and increases bone resorption of Ca into the extracellular fluid (CAO et al., 2021). When serum Ca levels are low, PTH-activated 1,25-OH_2-D_3 interacts with VDR in bone cells and increases the plasma membrane expression of RANKL, which in turn finishes the conversion of preosteoclasts into osteoclasts, responsible for metabolizing Ca and P stores from the bones into circulation (Laird et al., 2010). In contrast, 1,25-OH_2-D_3 actions in the bone can also suppress bone resorption and stimulate bone formation. As reported by Nakamichi et al. (2018), the binding of 1,25-OH_2-D_3 to VDR in osteoblasts can mediate the secretion of osteoprotegerin (OPG) a decoy receptor that inactivates the resorptive activities of RANKL. Furthermore, the increased Ca and P plasma pool enabled by VitD metabolites can be used by the body to promote the renovation of bone tissue. Skeletal muscle development is also closely dependent on VitD; studies have shown that diets deficient in VitD reduce the proliferation of FGF and myogenic regulatory factors responsible for the differentiation and proliferation of muscle cells, whereas

supplementary treatment with VitD metabolites reinforces this process (Montenegro et al., 2019). Alkaline phosphatase, an important biomarker and indicator of bone tissue development in growing broilers - reaching peak serum levels at around 25 days of age (SENANAYAKE et al., 2015) - also has its activity regulated by 1,25-OH_2-D_3 (VIMALRAJ, 2020).

Adequate supplementation of VitD followed by the formation of its metabolites is therefore essential for the optimal performance of birds and the reduction of skeletal disorders caused by Ca and P deficiency or rapid animal growth. Additionally, VitD can act as an important immunomodulator, given the presence of VDR in B and T lymphocytes, monocytes, macrophages, and other immune cells that can locally convert 25-OH-D_3 into 1,25-OH_2-D_3 (SASSI et al., 2018).

2.1.2 Vitamin D3 requirements for broilers

The biological efficacy of vitamin D_2 in avian organisms is considerably lower than that of vitamin D_3, and its binding to vitamin D transport proteins and VDR is inefficient, emphasizing the greater metabolic importance of vitamin D_3 in poultry nutrition. Broilers face challenges in endogenously synthesizing sufficient VitD through the conversion of 7-dehydrocholesterol by UV irradiation to meet their physiological needs, especially due to their feather-covered bodies and limited sunlight exposure in intensive production environments. To address possible deficiencies, VitD is provided through the diet, typically in the synthetic crystalline form present in the vitamin premix along with other essential vitamins, or as VitD metabolites. Félix et al. (2009) point out that formulation practices adopted by the broiler industry often relies on supplementing vitamins (including VitD) with safety margins exceeding the minimum recommended levels outlined by major reports, such as the National Research Council (NRC, 1994), Brazilian Tables for Poultry and Swine (ROSTAGNO et al., 2017), and other research sources summarized in Table 4. These recommendations vary due to factors such as reduced dietary mineral levels, the use of exogenous enzymes, poultry strains, and bone characteristics (ATENCIO et al., 2005; SAKKAS et al., 2019; SOUZA et al., 2013). The rationale for using higher vitamin levels, as per the authors, is to account for variations in feed consumption, the bioavailability of vitamins in ingredients and diets, and anti-quality factors in the feed, e.g. phytic acid, that may hinder the utilization of vitamins, among other considerations. In younger birds, the practiced vitamin levels

can be up to 15 times higher than the actual needs of the animals (SOUZA and VIEITES, 2014).

TABLE 4. MINIMUM RECOMMENDATIONS OF VITAMIN D3 IN BROILER CHICKEN DIETS.

Reference	Days of age	Recommendation of vitamin D_3 (IU/kg feed)
Brazilian Tables for Poultry and Swine (Rostagno et al., 2017)	1 – 7	3385
	8 – 21	3054
	22 – 33	2409
	34 – 42	1968
	43 - 49	1763
Fundación Española para el Desarollo de la Nutrición Animal (FEDNA, 2008)	0 – 18	3500
	22 – 33	2800
	35 – market	2000
Vitamin Supplementation Guidelines (DSM, 2011)	1 – 10	3000 - 5000
	11 – 24	
	25 – market	
National Research Council (NRC, 1994)	1 - market	200

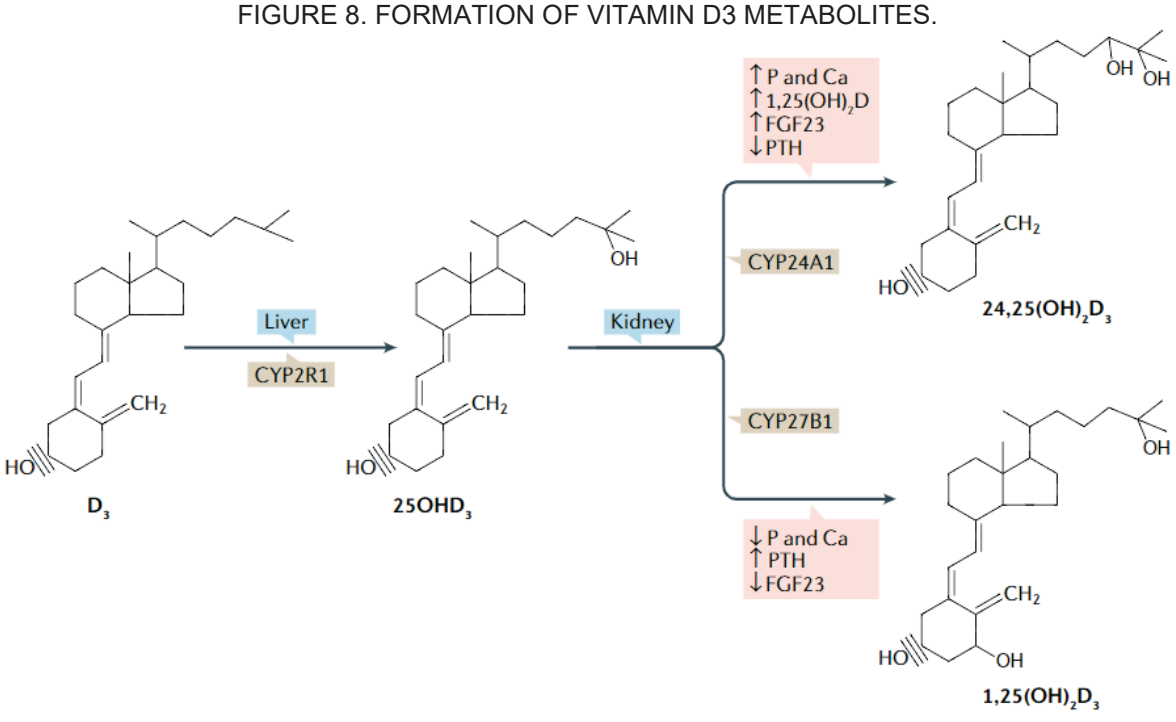
SOURCE: SOUZA AND VIEITES (2014)

As discussed by Sakkas et al. (2019), the selection of modern broiler genotypes has sustained a rise on Ca, P, and consequently, VitD requirements for optimal growth performance and bone mineralization. To overcome this growing need, the use of VitD metabolites in the diet has been progressively gaining attention of the industry. Currently, 25-OH-D_3 and 1,25-OH_2-D_3 are the primary active metabolites of vitamin D3 produced artificially by the industry, granting them significant academic and economic interest for in-feed supplementation for farm animals, either totally or partially replacing VitD. Studies indicate that the relative bioavailability of these metabolites compared to VitD is considerably higher (HAN et al., 2016; WU et al., 2022). According to Han et al. (2016), the increasing order of bioavailability of VitD metabolites would go as: VitD < 25-OH-D_3 < 1,25-OH_2-D_3. Therefore, the dietary use of these metabolic compounds would be advantageous compared to solely addition of VitD, in terms of improving Ca and P utilization of Ca and P, as well as growth performance and bone mineralization in broilers (BRITO et al., 2010; COLET et al., 2015; NÄÄS et al., 2012; OIKEH et al., 2019).

2.2 VITAMIN D3 METABOLITES

When ingested via diet, VitD absorbed in the enterocytes by diffusion in the form of micelles, similar to other hydrophobic compounds, and must then be converted into is active forms to exert its homeostatic effects on Ca and P metabolism. It is transported by specific vitamin-D-binding proteins (VDBP) and upon reaching the liver, it undergoes hydroxylation by the enzyme D-25-hydroxylase (CYP2R1) that adds a OH group into position 25, thus converting to 25-OH-D_3. Subsequently, 25-OH-D_3 is transported to the kidneys by carrier proteins, where it undergoes further hydroxylation by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) at position 1, generating 1,25-OH_2-D_3, which is the metabolically active form of vitamin D3 (BIKLE and SCHWARTZ, 2019). In the kidneys, 25-OH-D_3 can also be converted into 24,25-dihydroxycholecalciferol (24,25-OH_2-D_3) by 24-hydroxylase (CYP24A1), primarily serving as the main form for eliminating excess VitD from the body, although Seo et al. (1997) reported its involvement in bone development with functions analogous to 1,25-OH_2-D_3. As it seems, during hypocalcaemia, hydroxylation at position 1 is promoted to increase 1,25-OH_2-D_3-mediated absorption of Ca and P, but at normal or hypercalcemia, hydroxylation at position 24 is up-regulated to form 24,25-OH_2-D_3 which targets tissues undergoing calcification. The conversion of VitD into its active forms is depicted in Figure 8.

FIGURE 8. FORMATION OF VITAMIN D3 METABOLITES.



SOURCE: BIKLE AND CHRISTAKOS (2020)

2.2.1 Absorption of vitamin D3 metabolites

The 25-OH-D_3 and 1,25-OH_2-D_3 are the main active forms of VitD produced by the industry and commercially available for supplementation in diets for poultry birds. 25-OH-D_3 is the primary storage form of VitD in the animal's body, with a half-life of approximately 2 to 3 weeks, whereas 1,25-OH_2-D_3 has a shorter half-life of 4 to 6 hours (CASTRO, 2011). As a result, 25-OH-D_3 is also the most common form of VitD in the bloodstream, and its concentration is assessed as a measurement of the VitD serum status in animals (SASSI et al., 2018).

The primary objective behind using VitD metabolites in animal nutrition is to enhance the vitamin’s availability more efficiently. Compared to the supplementation of VitD, including 25-OH-D3 directly offers a distinct advantage due to its higher absorption efficiency. In the proximal portion of the jejunum of broiler chickens, the absorption of 25-OH-D3 can reach 83%, as opposed to 66% for VitD (BAR et al., 1980). This heightened and faster absorption rate, as elucidated by Teegarden et al. (2000), seems to be mainly attributed to a greater affinity of intestinal VDBP for 25-OH-D_3 – up to a thousand times greater than for other vitamin D3 metabolites – in addition to the presence of specific receptors for 25-OH-D3 in the intestinal epithelium.

Bar et al. (1980) further explains that the more polar the molecule of a fat-soluble component, the less it relies on the action of bile salts for intestinal absorption. Therefore, the high polarity of 25-OH-D3 grants its molecule greater ease of endocytosis. Due to these distinctive characteristics, Han et al. (2016) clarifies that in broiler chicken diets, the biological value or relative bioavailability of 25-OH-D_3, compared to VitD is notably higher. Specifically, it reaches up to 185%, 245%, 252%, and 205% for criteria such as body weight gain, femur mineralization, tibia mineralization, and metatarsal mineralization, respectively. Another important aspect is that in younger broiler chickens, the intestinal absorption of VitD is low due to an underdeveloped digestive enzyme system and still immature tract, in addition to a limited hepatic hydroxylation capacity to form 25-OH-D_3 from VitD molecules (NOY and SKLAN, 1995). However, it has been shown that this limitation can be overcome with the direct dietary supply of 25-OH-D_3 via the diet as it bypasses the need for hepatic conversion of VitD, thus increasing VitD status (HUTTON et al., 2014).

The physiologically active form of VitD *de facto*, 1,25-OH_2-D_3, is derived from 25-OH-D_3, and it's an enhanced activity of 1,25-OH_2-D_3 that is the essence of providing VitD to the animal. Therefore, a direct supply of 1,25-OH_2-D_3 in poultry diets has been the target of investigations. Some studies suggest that the inclusion of 1,25-OH_2-D_3 in broiler diets may yield more favorable results compared to 25-OH-D_3 concerning improvements in BWG and FCR, and amelioration of bone disorders (GARCIA et al., 2013; SOUZA et al., 2013; WU et al., 2022). A specific outcome of direct 1,25-OH_2-D_3 supplementation outlined by Hsiao et al. (2018) and Wu et al. (2022) seems to be a more effective regulation of Ca and P homeostasis-related gene expression, including calbindin, TRPV6, and Na-P II cotransporters. Han et al. (2017) noted that the bioavailability of 1,25-OH_2-D_3 compared to 25-OH-D_3 in diets for broiler chickens is up to 234%, 253%, 202%, 263%, and 267%, respectively, for BWG, bone ash weight, bone Ca content, tibia mineralization, and metatarsal mineralization. However, direct supplementation of 1,25-OH_2-D_3 is accompanied by higher risks of toxic effects caused by very small doses included in the diet, with a recommended dose of only 5 µg/kg compared to 69 µg/kg for 25-OH-D_3 (HSIAO et al., 2018). This may hinder or limit the use of this metabolite in formulations.

As the most potent element of VitD, elevated levels of free 1,25-OH_2-D_3 can induce toxicity by causing an excess of serum Ca, leading to a drastic removal of Ca from bones, excessive soft tissue calcification, and triggering inflammatory responses (SOARES et al., 1995). The conversion of 25-OH-D_3 to 1,25-OH_2-D_3, involving hydroxylation at position 1 in the kidneys, is regulated by plasma Ca levels. This process is promoted during hypocalcemia and restrained during hypercalcemic conditions (ACAR and ÖZKAN, 2021). Despite this regulatory mechanism, direct ingestion of 1,25-OH_2-D_3 can rapidly result in hypervitaminosis in broiler chickens (KUMAR et al., 2017). This risk also extends to analogous compounds commercially available for poultry diets, such as alpha-calcidiol (1α-OH-D3) and 1,25-OH2-D3-glycoside derived from plant extracts. 1α-OH-D3 is a synthetic analogue to 1,25-OH_2-D_3 that is converted in the liver to active VitD without requiring renal hydroxylation. Because it bypass the regulatory system for 1,25-OH_2-D_3 production, 1α-OH-D3 supplementation causes a swift increase in free 1,25-OH_2-D_3 concentration (HAN et al., 2017; WARREN et al., 2020). Moreover, its transformation is not dependent on serum Ca, hence 1α-OH-D3 shows a higher risk of toxicity than other VitD metabolites.

Conversely, there is scant evidence that balanced supplementation of 25-OH-D_3 poses any toxicity issues. In a study by Yarger et al. (1995), adverse effects of 25-OH-D_3 on the BWG of broiler chickens were observed only with inclusions ten times higher than the recommended 69 µg/kg. Both VitD and 25-OH-D_3 supplementation prevent peaks in 1,25-OH_2-D_3 production because VDBP exhibit a higher affinity for 25-OH-D_3, surpassing that for 1,25(OH)2D3 by up to tenfold (Bikle and Schwartz, 2019; Teegarden et al., 2000). Here, the "Free Hormone" hypothesis, elucidated by Bikle & Schwartz (2019), becomes relevant, explaining that only free hormones, detached from their carrier proteins, can enter cells to perform biological functions. When 25-OH-D_3 is bound to VDBP, a stoichiometric restriction controls the formation and presence of free 1,25-OH_2-D_3, acting as a safeguard against hypervitaminosis. Consequently, inclusion of 25-OH-D_3 in poultry formulations proves to be significantly more advantageous than 1,25-OH_2-D_3 supplementation.

2.2.2 Effects of vitamin D3 and 25-OH-D3 in poultry nutrition

The beneficial impacts of VitD and its metabolites on skeletal system development, bone mineralization and health, improved BWG, and enhanced FCR are notably the primary goal of incorporating these compounds into poultry diets, as evidenced by several studies (BASSI et al., 2023; BRITO et al., 2010; HSIAO et al., 2018; OIKEH et al., 2019; SANTIAGO et al., 2016; WANG et al., 2020; ZHANG et al., 2020). These positive outcomes derive primarily from a better utilization of Ca and P, which become more available for the animal’s growth. Even in diets containing sufficient Ca and P levels, elevated doses of VitD – within a safe range - have been shown to further improve the utilization of dietary minerals, enhancing bone mineralization and overall performance (OIKEH et al., 2019: SAKKAS et al., 2019).

Optimizing VitD metabolism through dietary inclusion offers the potential to reduce Ca and P levels for broiler chickens without compromising their development, as highlighted by Rama Rao et al. (2006). Their observations indicated that doses of VitD up to 3,600 IU/kg maintained optimal performance and bone mineralization in broilers fed lower levels of Ca and non-phytic P (0.5% and 0.25%, respectively). Notably, VitD metabolites may prove more efficient than VitD in enhancing the absorption of Ca and P, even at comparable or lower inclusion levels, further allowing

a reduction in the formulated levels of these minerals (LANDY et al., 2020; OIKEH et al., 2019).

Research indicates that the inclusion of VitD and its metabolites in the diet of broiler chickens leads to a significant upregulation of gene expression associated with regulating proteins of Ca and P homeostasis. Noteworthy genes include calbindin, beta-glucuronidase, TRPV6, and Type II and III Na-P co-transporters (Na/Pi IIa and IIb), which play crucial roles in regulating Ca and phosphate intestinal absorption and renal reabsorption/excretion (HSIAO et al., 2018; SHAO et al., 2019; WU et al., 2022). As pointed out by the authors, intestinal gene expression of these proteins could be directly regulated by VDR in response to binding to dietary VitD metabolites, with 1,25-OH2-D3 binding apparently being more effective in upregulating this expression than 25-OH-D_3, albeit both metabolites more effective than dietary VitD per se. Han et al. (2017) also observed an increased mRNA expression of VDR in the small intestine and kidneys of broilers directly supplemented with 1α-OH-D3.

Reducing the incidence of skeletal disorders and improving bone health is another valuable objective of VitD supplementation. The rapid genetic selection of broiler chickens has enabled accelerated growth and greater deposition of lean mass and muscle tissue, but at the expense of bone tissue development. Abnormalities in the legs, bone deformities, and diseases such as rickets, tibial dyschondroplasia, and femoral degeneration, as well as changes in bone composition, are common in modern poultry farming (LIU et al., 2023). Adequate supplementation of VitD suppress the incidence of these disorders, and the use of 25-OH-D_3 is reportedly more effective in improving bone characteristics. Fritts and Waldroup (2003) found higher bone ash content and lower incidence of tibial dyschondroplasia in broilers supplemented with increasing doses of 25-OH-D_3 (from 125 to 4,000 IU/kg) compared to equivalent doses of VitD. Santiago et al. (2016) observed higher Ca content in the tibia of chickens supplemented with 25-OH-D3 in addition to regular VitD premix. Wang et al. (2020) reported an increase in tibia strength and serum concentrations of PTH and calcitonin in laying hens fed with a standard dose of 69 μg/kg of 25-OH-D3 along with 5,000 IU of VitD in the premix, demonstrating the potential of the metabolite to enhance Ca and P metabolism and bone quality even on top of regular VitD levels.

The supplementation of dietary 25-OH-D3 can be conducted either in combination with VitD present in the vitamin premix, partially replacing VitD, or as the sole source of vitamin D in the diet. Fritts and Waldroup (2005) assessed the supply

of 1,000, 2,000, or 4,000 IU/kg of vitamin D from the inclusion of VitD or an equivalent amount of 25-OH-D3 in chicken diets. The authors observed that increasing the dose of both vitamin D sources equally improved BWG, possibly because the dosages utilized were already above commercial practices. In a previous publication, though, they noticed a greater efficacy of using 25-OH-D3 as a substitute for VitD in reducing the incidence of severe tibial dyschondroplasia and increasing BWG when supplementing lower levels of up to 500 IU/kg (FRITTS and WALDROUP, 2003). As the levels increased, both vitamin D sources were similar, indicating that the use of 25-OH-D3 allows the reduction of vitamin D levels. Likewise, Atencio et al. (2005) compared the use of 25-OH-D3 as a substitute for VitD in diets for broiler breeders and observed greater effects of the metabolite in increasing egg production rate, hatchability, and body ash of the offspring when both sources were used at a lower dose (3,125 ng/kg). There is evidence that the combined supply of 25-OH-D3 and VitD can be equally beneficial to the animal in terms of improved utilization of Ca and P and optimization of performance and bone mineralization characteristics (BRITO et al., 2010; GARCIA et al., 2013; LANDY et al., 2020; SAKKAS et al., 2019; WANG et al., 2020). These results, as well as those of the other studies described so far, are linked back to the fact that the 25-OH-D_3 molecule has a higher and faster absorption rate and greater affinity for binding to VDR and VDBP in the GIT (BAR et al., 1980; TEEGARDEN et al., 2000). Hutton et al. (2014) emphasizes that the direct ingestion of 25-OH-D_3 or 1,25-OH_2-D_3 can also reduce energy expenditure by bypassing the hydrolysis stages of VitD necessary for obtaining these metabolites.

3. PHYTASE

Phytase is an enzyme that acts on the phytic acid molecule present in the feed, catalyzing its degradation to release nutrients complexed in the structure of IP_6 for absorption. The breakdown of phytic acid primarily releases P, Ca, and trace minerals, along with other nutrients such as proteins and amino acids, carrying various other beneficial effects resulting off the elimination of phytate from the animal's digestive tract. Conversely to ruminants, the inclusion of exogenous phytase in non-ruminant diets is a routine practice given their limited endogenous activity (produced and secreted by the intestinal microbiota) incapable of harnessing the high amounts of Ca and P commonly present in commercial diets (DERSJANT-LI et al., 2015).

Phytase promotes the dephosphorylation of the IP_6 molecule, characterized by the stepwise removal of phosphate groups attached to the myo-inositol ring. The complete degradation pathway of phytic acid, as characterized by Kempapidis et al. (2020), is IP6 → IP5 → IP4 → IP3 → IP2 → IP1 → free inositol, ultimately generating inositol as the final product – which can be absorbed in the GIT (BELLO et al., 2019) - along with six phosphorus portions. After hydrolysis, the nutrients bound to the phosphates become available for absorption in the animal's digestive tract. However, Hirvonen et al. (2019) states that no single phytase is able to completely dephosphorylate all 6 phosphate groups in cereals, yet their combined activity with other endogenous phosphatases secreted in the mucosa can aid towards a near-complete breakdown of phytate. Phytase is also naturally occurring in plants, although in corn and soybean, the most used cereals in poultry diet formulation, the presence of phytase in the grain is almost negligible. Other feedstuffs such as wheat, barley, and rice bran are richer in phytase, but also contain greater concentrations of phytic P (LOTT et al., 2000).

While there is no universally standardized international unit for measuring phytase activity, Engelen et al. (1994) introduced the concept of phytase activity in phytase units (FTU). In this context, one FTU is defined as the quantity of enzyme that releases 1 µmol of inorganic orthophosphate per minute from a 0.0051 mol L-1 Na phytate under standardized conditions - specifically, at pH 5.5 and a temperature of 37°C. Alternate abbreviations for phytase units, such as FTY and U, have been adopted for various commercial phytases, all established under a similar set of parameters.

The commercialization of exogenous phytase began in the 1990s in response to new Dutch legislation establishing a limit on environmental pollution by P (SELLE and RAVINDRAN, 2007). Most phytases available for animal diet supplementation are sourced from microorganisms such as fungi, yeasts, bacteria, and protozoa, which produce the enzyme to utilize phosphorus from the soil and plant decomposition (SIMON and IGBASAN, 2002). Therefore, industrial phytases are typically obtained through the control of fermentation processes that replicate the enzyme on a large scale, performed in liquid or solid medium (KUMAR et al., 2016). Fungi of the genus *Aspergillus* are the most used microorganisms in phytase production. Phytases of bacterial origin are also common, e.g. from genera *Enterobacter*, *Pseudomonas*,

Bacillus, and *Streptomyces*, as well as yeasts *Saccharomyces* and *Schawnniomyces* (SIMON and IGBASAN, 2002).

The pH level in the avian GIT is a crucial factor influencing both phytate degradation and the activity of phytase. Optimal deprotonation of phytate occurs in an acidic environment, while a shift towards a more neutral pH can result in the formation of complexed phytate salts, particularly with cations like Ca (AMERAH et al., 2014; SHANMUGAN, 2018). The upper portion of the avian gastrointestinal tract, including the crop, proventriculus, and gizzard, exhibits a more acidic pH, typically ranging from 2.5 to 5.0, which renders phytate chelates more susceptible to dephosphorylation (LIEBERT et al., 1993), thereby potentiating the enzymatic activity of phytase in this region. Yu et al. (2012) have shown in their research that the binding capacity of IP_6 to dietary proteins surpasses that of its respective esters (IP_5-IP_1), a consequence of the dephosphorylation process. Additionally, the binding of phytate to Ca is contingent on the ester composition present in the digestive tract. For instance, IP_3 has approximately 11% of the binding capacity to Ca compared to IP_6, i.e. isomers IP_{1-4} exhibit a limited ability to bind to proteins and minerals, indicating that the intermediate and final products resulting from phytate dephosphorylation are less effective in forming complexes with nutrients. In correlation with phytate susceptibility at more acidic pH levels, extracting the maximum amount of IP_6 and IP_5 before the digesta reaches the lower, more neutral pH portions of the GIT, becomes pivotal to mitigate the antinutritional effects.

3.1 PHYTASE IN BROILER DIETS

Phytase is widely used in the feeding of monogastric animals, particularly poultry and swine, to mitigate the detrimental effects of phytate on nutrient utilization. Improvements on growth performance and bone mineralization of broiler chickens resulting from exogenous phytase have been well substantiated in the literature (COWIESON et al., 2009; DERSJANT-LI et al., 2015; SELLE and RAVINDRAN, 2007; SHANMUGAM, 2018). As phytase breaks down phytic acid and increases the digestibility while also reducing endogenous losses of P, Ca, and other nutrients bound to phosphate groups (COWIESON et al., 2009), a cascade of positive effects is prompted: increased bone ash and bone resistance stemming from a greater availability and deposition of Ca and P (KACZMAREK et al., 2016; LEE et al. (2017);

reduced incidence of skeletal disorders, higher bone resistance, and overall higher bone quality (BRADBURY et al., 2018; Kim et al., 2017); overall better performance BONEY and MORITZ, 2017; KACZMAREK et al., 2016; SENS et al., 2021) and increased carcass yield (KRISELDI et al., 2021). Furthermore, while phytic acid can downregulate the mRNA expression of Na-P contransporters, expression of mTOR kinase (related to bone growth), and intestinal activity of amylase and proteolitic enzymes, supplementation with phytase has been shown to counteract this effect (LIU et al., 2008, 2009).

As Selle and Ravindran (2007) explains, responses in growth performance and bone mineralization to phytase supplementation are influenced by nutrient specifications, as increments in BWG and lower FCR are more significant in less nutritionally dense diets, specially with lower non-phytate P levels. Dersjant-Li and Kwakernaak (2019) further reinforced that phytase addition was more effective in improving growth performance and tibia ash compared to increasing non-phytate P levels because of a higher digestibility of amino acids and Na enabled by phytase in addition to higher P digestibility. These extra-phosphoric effects are more likely to take place when increasing the utilized doses of phytase.

Evidences have been sought in recent years for the so-called extra-phosphoric effect that may be caused by supplementing higher doses of phytase in the diet, beyond the standard industry levels, typically established as above 1,500 units/kg (BONEY and MORITZ, 2017). The concept is based on targeting the highest number of phytate molecules in the GIT as possible to enhance the breakdown of phytic acid down to lower esters, therefore promoting the release of nutrients beyond P, halting the complexation of phytate salts, and speeding up the elimination of phytate from the tract (WALK et al., 2013). The benefits of higher doses of phytase super-dosing have been indicated in studies with poultry (Bassi et al., 2021; Beeson et al., 2017; Cowieson et al., 2017; Walk and Olukosi, 2019; Walk and Rama Rao, 2020). Some investigations characterize the greater intensity of phytate degradation using high phytase doses (WALK and OLUKOSI, 2019; BEESON et al., 2017; WALK and OLUKOSI, 2019), observed through a reduction of IP_6 in the tract and an increase in circulating inositol, a product derived from the complete dephosphorylation of phytic acid. The higher the concentration of phytate in the diet, the more pronounced the antinutritional effects, and higher doses of phytase should be supplemented to counteract these effects. Walk and Rama Rao (2020) evaluated the effects of 3 levels of phytic P in the diet of

chickens (0.24; 0.345; 0.45%) with 4 phytase doses (0, 500, 1000, and 2000 FTU/kg). The maximum BWG was obtained with 1285 FTU/kg and 0.29% phytic P, while for FCR, it was not possible to reach the lowest value even with a higher dose than 2000 FTU/kg. In turkey poults, Bassi et al. (2021) observed a linear response to up to 4,000 FYT/kg in higher BWG, higher ileal digestibility of P, total tract retention of CP, Ca, and AME, as well as higher plasmatic myo-inositol.

Some studies delve into the interaction between phytase and vitamin D, noting synergistic effects when both substances are included in the diet of broiler chickens (BROWNING et al., 2012; KERMANI et al., 2023; MCGRATH et al., 2010). Elevated Ca levels in the diet can form chelates with phytate, potentially diminishing phytase efficacy, particularly under conditions of high luminal pH and excessive P concentrations (AMERAH et al., 2014). Consequently, by optimizing the absorption of Ca and P in the GIT, the inclusion of VitD can hinder their potential complexations with phytate and enhance the enzymatic activity of phytase, thereby optimizing the overall utilization of the diet.

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CHAPTER II - EFFECT OF 25-HYDROXYCHOLECALCIFEROL SUPPLEMENTATION WITH DIFFERENT DIETARY AVAILABLE PHOSPHORUS LEVELS FOR BROILERS

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ABSTRACT

Based on the hypothesis that 25-hydroxycholecalciferol (25-OH-D3) inclusion would optimise dietary mineral digestibility and ameliorate growth performance and bone mineralisation in available phosphorus (AvP) deficient-fed broilers, a trial was conducted to evaluate its effect on diets with different levels of AvP. Broilers aged 1–21 d were randomly assigned one of the eight treatments, consisting of four dietary levels of AvP (0.45%, 0.42%, 0.39%, and 0.36%) and with or without supplementation with 25-OH-D3 at 69 µg/kg of feed. All diets contained 100 µg/kg of vitamin D3 (cholecalciferol). The addition of 25-OH-D3 to the diets resulted in greater feed intake and body weight gain, and better FCR ($P < 0.05$) compared to non-supplemented diets, whereas AvP levels had a quadratic effect only on feed intake, with no interaction between factors. Reducing AvP levels linearly increased the ileal digestibility of Ca and P ($P < 0.01$) and supplementing 25-OH-D3 increased both Ca and P ileal digestibility ($P < 0.05$), without any interactions observed for ileal digestibility. There was an interaction, whereby 25-OH-D3 inclusion increased serum metabolites in broilers fed 0.36% to 0.42% AvP compared to the non-supplemented diets ($P < 0.001$), whereas, at 0.45% AvP, diets with or without 25-OH-D3 had similar results. The P content in bone was linearly reduced in line with AvP levels ($P < 0.05$), and the supplementation of 25-OH-D3 increased ash bone content ($P < 0.001$). Broilers can benefit from 25-OH-D3 supplementation combined with cholecalciferol with regard to Ca and P utilization and vitamin D status, allowing for a reduction of dietary AvP levels down to 0.36% without impairing growth performance or bone status.

Keywords: 25-hydroxycholecalciferol, broiler, bone mineralization, nutrient digestibility, vitamin D.

CAPÍTULO II - EFEITO DA SUPLEMENTAÇÃO DE 25-HIDROXICOLECALCIFEROL COM DIFERENTES NÍVEIS DIETÉTICOS DE FÓSFORO DISPONÍVEL PARA FRANGOS DE CORTE

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RESUMO

Com base na hipótese de que a inclusão de 25-hidroxicolecalciferol (25-OH-D_3) otimizaria a digestibilidade de minerais e melhoraria o desempenho e mineralização óssea em frangos de corte alimentados com dietas deficientes em fósforo disponível (Pdisp), foi realizado um ensaio para avaliar o efeito de 25-OH-D_3 em dietas com diferentes níveis de Pdisp. Frangos de corte com idade de 1 a 21 dias foram aleatoriamente atribuídos a 1 de 8 tratamentos, consistindo em 4 níveis dietéticos de Pdisp (0,45%, 0,42%, 0,39% e 0,36%) e com ou sem suplementação de 25-OH-D_3 a 69 µg/kg de ração. Todas as dietas continham 100 µg/kg de vitamina D_3 (colecalciferol). A adição de 25-OH-D3 às dietas resultou em maior consumo de ração e ganho de peso corporal, e melhor conversão alimentar ($P < 0,05$) em comparação com dietas não suplementadas, enquanto os níveis de Pdisp tiveram um efeito quadrático apenas no consumo de ração, sem interação entre os fatores. A redução nos níveis de Pdisp aumentou linearmente a digestibilidade ileal de Ca e P ($P < 0,01$), enquanto a suplementação de 25-OH-D_3 aumentou a digestibilidade ileal de ambos Ca e P ($P < 0,05$) independente dos níveis de Pdisp. Houve uma interação, em que a inclusão de 25-OH-D_3 aumentou o nível sérico do metabólito em frangos alimentados com 0,36% a 0,42% de Pdisp em comparação com as dietas não suplementadas ($P < 0,001$), enquanto, ao nível 0,45% de Pdisp, dietas com ou sem 25-OH-D_3 tiveram resultados semelhantes. O teor de P no osso decaiu linearmente de acordo com os níveis de Pdisp ($P < 0,05$) e a suplementação de 25-OH-D3 resultou em aumento do teor de cinzas ósseas ($P < 0,001$). Frangos de corte podem se beneficiar da suplementação de 25-OH-D_3 combinada com colecalciferol em relação à utilização de Ca e P e a melhoria do metabolismo de vitamina D, permitindo uma redução dos níveis dietéticos de Pdisp para 0,36% sem prejudicar o desempenho ou qualidade óssea.

Palavras-chave: 25-hidroxicolecalciferol, frango de corte, mineralização óssea, digestibilidade de nutrientes, vitamina D.

1. INTRODUCTION

Vitamin D3 (VitD) is a fat-soluble vitamin with substantial importance to the absorption and metabolism of calcium (Ca) and phosphorus (P), and its use in poultry nutrition is strongly related to bone mineralization, health, growth and stimulation of the immune system (HAN et al. 2017; SHOJADOOST et al. 2021; SOUZA and VIEITES 2014). The endogenous production of VitD in animal cells is induced by the photochemical conversion of ergosterol and 7-dehydrocholesterol into cholecalciferol (vitamin D3), which is metabolized in the liver into 25-hydroxycholecalciferol (25-OH-D3) and subsequently into 1,25-dihydroxycholecalciferol (1,25-OH2-D3), which is the active form of VitD (Bikle and Schwartz 2019). The 1,25-OH2-D3 is carried by binding proteins *via* the bloodstream onto VitD membrane receptors (VDR) on target organs and tissues, notably the intestines, muscle and bone cells, and kidneys (Sakkas et al. 2019). Its main function is to optimize absorption and metabolism of dietary Ca and P. In the intestines and kidney, 1,25- OH2-D3 increases the absorption rate and minimizes Ca and P losses when circulating levels are low, while upregulating the synthesis of Ca and P protein cotransporters such as calbindin and Na/Pi IIb (HSIAO et al. 2018; SHAO et al. 2019). In bone tissues, 1,25-OH2-D3 manages the withdrawal of Ca from the bone along with parathormone to maintain adequate Ca serum levels (NÄÄS et al. 2012).

The selection of modern broiler chicken strains for faster growth rate and breast muscle deposition has long been known to lead to higher incidences of skeletal disorders and an increase in the nutritional requirements for Ca, P, and VitD levels (WALDENSTEDT, 2006). Because broilers do not receive enough solar radiation for a satisfactory endogenous synthesis of VitD (OGBONNA et al. 2022), these increased requirements must be met through the diet. Many studies have assessed the dietary inclusion of VitD metabolites for poultry, especially 25-OH-D3, in combination with or as a replacement, and have determined that 25-OH-D3 can enhance Ca and P utilisation due to its greater intestinal absorption rate (83% *vs*. 66% for VitD; BAR et al. 1980) and to the higher affinity of binding proteins for 25-OH-D3 (TEEGARDEN et al. 2000). Improvements in bone mineralisation, body weight gain (BWG), feed conversion ratio (FCR) and mineral and VitD status have been reported when supplementing with 25-OH-D3 (BOZKURT et al., 2017; GARCIA et al., 2013; OIKEH et al., 2019; SANTIAGO et al., 2016; ZHANG et al., 2020).

Supplementation with 25-OH-D3 has the potential to increase P availability in broiler diets. Most studies have tested its use in broiler diets which were marginally deficient at both Ca and P levels (FRITTS and WALDROUP 2005; OIKEH et al. 2019; ZHANG et al. 2020), but the current study proposes a steeper reduction of available P (AvP) to investigate possible interactions with 25-OH-D3 in commercial diets for starter broilers (21-d-old) containing a high dose of phytase and regular VitD levels.

2. MATERIALS AND METHODS

2.1 BIRDS, FACILITIES, AND EXPERIMENTAL DESIGN

All experimental procedures complied with the Animal Use Ethics Committee of Federal University of Paraná (Annex I). A total of 560, one-d-old Ross® 308 male broiler chicks from a commercial hatchery were randomly assigned to a completely randomized design with 8 treatments and 7 cage replicates containing 10 birds each. A 4 × 2 factorial design was used, with four dietary levels of AvP (0.45%, 0.42%, 0.39%, and 0.36%) and with or without 25-OH-D3 supplementation. Birds were housed from 1 to 21 d of age in metabolic battery cages. Each battery consisted of four floors with two cages per floor. Each cage measured 0.98 m length × 0.90 m width × 0.50 m height and was equipped with gutter feeders and nipple drinkers. The initial room temperature was set to 30°C and gradually reduced by 1°C every 3 days to 22°C until d 21. During the first week, incandescent light was provided for 23 h with 1 h of dark, and thereafter a lighting program of 4 h of dark per day was applied until the end of the experiment. Pens were checked daily for removal of dead birds (mortality rate throughout the experiment was 3.1%), and the causes were found to be unrelated to dietary treatments.

2.2 EXPERIMENTAL DIETS

The experimental diets were based on maize and soybean meal and offered in mashed form (Table 1), feed and water were offered *ad libitum* throughout the experimental period. Representative feed samples were collected during manufacturing. The source of 25-OH-D3 was Hy-D® premix (DSM, Kaiseraugst, Switzerland), included at 250 g/ton of feed (providing 69 µg/kg of feed) as per the

manufacturer's recommendation. All diets were supplied with commercial levels of vitamin D3 (100 µg/kg) containing 1,500 units of phytase (FYT)/kg. The phytase used was Ronozyme® (Ronozyme® Hiphos GranulatedThermostable – DSM, Kaiseraugst, Switzerland), a 6-phytase generated from *Citrobacter braakii* and expressed in *Aspergillus oryzae*, with a minimum activity of 20 000 FYT/g of product and non-phytate P and Ca matrix values of 1.5 and 1.8 g/kg, respectively. The target total Ca level of all experimental diets was kept at 0.9%, hence Ca:P ratios varied across diets, ranging from 2:1 (with 0.45% AvP) up to 2.5:1 (with 0.36% AvP).

TABLE 1. INGREDIENTS AND COMPOSITION OF EXPERIMENTAL DIETS.

Ingredients (g/kg)	Dietary available P (%)							
	Without inclusion of 25-OH-D_3				With inclusion of 25-OH-D_3			
	0.36	0.39	0.42	0.45	0.36	0.39	0.42	0.45
Maize	551	550	549	548	551	550	549	548
Soybean meal	386	386	386	386	386	386	386	386
Soybean oil	22	22.3	22.7	23	22	22.3	22.7	23
Limestone	13.3	12.3	11.1	10.1	13.3	12.3	11.1	10.1
Dicalcium phosphate	5	6.6	8.4	10	5	6.6	8.4	10
Sodium chloride	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9
L-Lysine HCl	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
L-Threonine	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
DL-Methionine	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8
Choline chloride	1	1	1	1	1	1	1	1
Vitamin premix[1]	1	1	1	1	1	1	1	1
Mineral premix[2]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Phytase[3]	0.075	0.075	0.075	0.075	0.075	0.075	0.075	0.075
25-OH-D_3 [4]	0	0	0	0	0.25	0.25	0.25	0.25
Indigestible marker[5]	10.25	10.25	10.25	10.25	10	10	10	10
Calculated chemical composition								
Metabolizable energy (MJ\kg)	12.75	12.75	12.75	12.75	12.75	12.75	12.75	12.75
Calcium (g/kg)	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Sodium (g/kg)	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1
Chlorine (g/kg)	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6
Digestible lysine (g/kg)	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2
Digestible methionine (g/kg)	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Methionine + cysteine (g/kg)	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Digestible threonine (g/kg)	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2
Analysed chemical composition								
Crude protein (g/kg)	229	233	235	233	231	238	230	233
Calcium (g/kg)	8.7	8.9	8.6	9.2	9.0	8.7	8.6	8.6
Total phosphorus (g/kg)	4.7	5.1	5.7	5.9	5.1	5.2	5.4	5.8
Ash (g/kg)	71.5	69.4	71.9	71.3	70.2	71.8	69.8	75.4

[1] Supplied per kg of diet: retinol, 3.3 mg; cholechalciferol, 0.10 mg; α-tocopherol, 55 mg; menadione, 3 mg; thiamine, 2.3 mg; riboflavin, 7 mg; pantothenic acid, 12 mg; pyridoxine, 4 mg; cyanocobalamin, 0.025 mg; nicotinic acid, 60 mg; folic acid, 2 mg; biotin, 0.25 mg; selenium, 0.3 mg.
[2] Supplied per kg of diet: copper, 10 mg; iron, 50 mg; iodine, 1 mg; manganese, 65 mg; zinc, 65 mg.
[3] RONOZYME® HiPhos GT with 20,000 FYT/g (DSM Nutritional Products - Kaiseraugst, Switzerland). Nutrient matrix values (1.5 g/kg non-Phytate P and 1.8 g/kg Ca) were considered.
[4] Hy-D® (DSM Nutritional Products – Kaiseraugst, Switzerland), providing 69 mg 25-OH-D_3/ton of feed.
[5] Celite® Insoluble marker (Celite® 400 - Celite Corp., Lompoc, US).

Table 2 shows the recovery analysis of phytase and 25- OH-D3 in the experimental diets. The activity of phytase was measured at Biopract GmbH (Berlin, Germany) using the PHY-101/05E method, in accordance with ISO30024:2009 as described by the International Organisation for Standardisation (ISO 2009). The quantification of 25-OH-D3 in the diets was performed by high performance liquid chromatography according to method SOP01464 by Biomin Holding GmbH (Tulln, Donau, Austria).

TABLE 2. EXPECTED AND ANALYSED PHYTASE ACTIVITY AND DIETARY CONCENTRATION OF 25-OH-D3 IN FEED SAMPLES.

Dietary treatment	Phytase[2] (FYT[3]/kg)		25-OH-D_3 (µg/kg)	
	Declared	Analysed	Declared	Analysed
0.36% AvP[1]	1,500	1,643	0	<LOD[4]
0.39% AvP	1,500	1,725	0	<LOD
0.42% AvP	1,500	1,578	0	<LOD
0.45% AvP	1,500	2,147	0	<LOD
0.36% AvP + 25-OH-D_3	1,500	1,705	69	59.3
0.39% AvP + 25-OH-D_3	1,500	2,028	69	62.5
0.42% AvP + 25-OH-D_3	1,500	1,600	69	65.3
0.45% AvP + 25-OH-D_3	1,500	2,054	69	58.0

Analyses performed by BioPract GmbH, Berlin, Germany.
[1] Available phosphorus.
[2] RONOZYME® HiPhos GT with 20,000 FYT/g (DSM Nutritional Products - Kaiseraugst, Switzerland). Enzyme activity is expressed as the quantity of product added in the feed.
[3] Phytase units.
[4] Limit of detection.

2.3 GROWTH PERFORMANCE

Broilers were weighed by cage at d 1 and 21 to determine mean body weight and to calculate BWG. Feed allowance and feed refusals (as weigh backs) were measured on d 1 and 21 to calculate feed intake (FI). Feed conversion ratio (FCR) was calculated as the ratio between FI and BWG, corrected for the weight of any dead birds.

2.4 DIGESTIBILITY ASSAY

At 21 d of age, five birds per replicate were euthanized by cervical dislocation. The birds were eviscerated, and the ileum was separated for contents removal, defined as 4 cm below Meckel's diverticulum and 4 cm above the ileum-caecum-colonic junction. The ileal content from all five birds was pooled, placed in identified plastic containers and frozen at −18°C. Samples were subsequently thawed to room

temperature and dried in a force-ventilation oven at 55°C for 48 h. Feed and ileal samples were ground to 0.5 mm particle size. The dry matter (DM) content was obtained by oven drying the samples at 105°C for 16 h, and crude protein (CP; method 954.01), ash (method 942.05), calcium (Ca; method 927.02), and phosphorus (P; method 965.17) contents were analyzed according to AOAC methodology (1995). Gross energy (GE) of the samples was determined in a calorimetric bomb (Ika Werke C2000 Control Oxygen Bomb Calorimeter – Ika-Werke GmbH&Co, Staufen, Germany). Acid-insoluble ash (AIA) was used as the insoluble marker for digestibility calculations and the AIA content in the samples was determined according to methodology by Scott and Boldaji (1997). The coefficient of apparent ileal digestibility (CAID) was determined according to the following calculation:

$$CAID = \frac{(Nutrient\ in\ the\ diet) - (Nutrient\ in\ the\ ileal\ digesta \times IF)}{Nutrient\ in\ the\ diet}$$

Where IF (indigestibility factor) was the ratio between AIA content in the diet and AIA in the excreta or ileal digesta. Ileal digestible energy (IDE) was determined according to the following calculation:

$$IDE\ (kcal/kg\ DM) = GE\ of\ the\ diet - (GE\ of\ the\ ileal\ digesta\ \times IF)$$

2.5 25-OH-D_3 SERUM CONCENTRATION

At 21 d of age, two birds per replicate were randomly chosen and blood samples were taken by puncturing the ulnar vein. A drop of blood from each bird was then immediately blotted and dried on filter paper, which was used to analyze serum concentration of 25-OH-D3 *via* the Dried Blood Spots method using liquid chromatography-tandem mass spectrometry as described by Zakaria et al. (2020).

2.6 BONE MINERAL COMPOSITION AND BREAKING STRENGTH

Two broilers per replicate, from the same five broilers used for ileal content collection at 21 d of age, had both legs excised and the tibial bones removed. The left

tibia were then cleaned with ether to remove any remnants of fat and muscle and oven-dried at 105°C for 12 h. Bone length and weight measurements were made using a digital calliper and a digital scale to the nearest 0.0001 g and used in the calculation of the Seedor Index (SI), by dividing the bone weight by its length, to determine bone mineral density (SEEDOR, 1993). Dried bones were ashed in a muffle furnace at 600°C and ash (method 942.05), Ca (method 927.02) and P (method 965.17) contents were determined, according to AOAC (2005).

Bones from the right leg were used to determine breaking strength (BBS), measured using a 3-point method with a universal testing machine (PMPA – Stable Micro Systems, Surrey, UK). The tibia was rested on two points with a gap of 50 mm, and pressure was applied with a pressure sensitive load cell of 500 kilogram-force (kgf) at the centre of both points, coinciding with the centre of the bone at a speed of 10 mm/s. The BBS represented the highest force-load supported by the bone during the test.

2.7 STATISTICAL ANALYSES

All data were tested by the Shapiro–Wilk test, and after normal distribution was confirmed, data were used in a two- way ANOVA using the linear model of ExpDes package (Experimental Designs Package, E. B. Ferreira et al., Belo Horizonte, Minas Gerais, Brazil), including two main factors and their interaction, using the R program (R Foundation for Statistical Computing, Vienna, Austria). When significant, the effect of the four dietary AvP levels was assessed through linear and quadratic regression. When significant interactions were observed, they were subject to the Tukey test for mean comparison. Significance was assigned when $P < 0.05$.

3. RESULTS

3.1 GROWTH PERFORMANCE

No interaction between the factors was observed ($P > 0.05$) for growth performance from 1 to 21 d of age (Table 3). The effect of the available P (AvP) levels on FI was quadratic ($P < 0.05$), as it increased linearly from 0.36% to 0.42%, but was reduced at 0.45% AvP. Body weight gain and FCR were not affected by AvP levels.

The addition of 25-OH-D3 to the diets resulted in greater FI and BWG and lower FCR ($P < 0.05$) in comparison to the non-supplemented diets.

TABLE 3. EFFECT OF 25-OH-D3 SUPPLEMENTATION ON GROWTH PERFORMANCE OF BROILERS FED DIETS WITH DIFFERENT LEVELS OF AVAILABLE PHOSPHORUS FROM 1 TO 21 DAYS OF AGE.

Available P (%)	25-OH-D_3 (µg/kg)[1]	FI (g)	BWG (g)	FCR (g/g)
Interaction				
0.36	0	1,094	818	1.338
0.39		1,139	851	1.339
0.42		1,093	818	1.336
0.45		1,105	820	1.347
0.36	69	1,135	866	1.311
0.39		1,160	876	1.324
0.42		1,158	882	1.314
0.45		1,131	867	1.305
Pooled SEM		11.44	10.84	0.011
Effect of available P				
0.36		1,114[b]	842	1.324
0.39		1,149[a]	863	1.331
0.42		1,125[ab]	850	1.325
0.45		1,118[b]	846	1.326
Effect of 25-OH-D_3				
	0	1,107	826	1.340
	69	1,146	872	1.313
P-values				
Available P		0.015	0.192	0.921
P-linear		0.696	0.793	0.946
P-quad		0.010[2]	0.084	0.697
25-OH-D_3		<0.001	<0.001	0.001
Interaction		0.226	0.366	0.659

Data represents the mean of 7 replicates per treatment (10 birds per replicate).
FI, feed intake; BWG, body weight gain; FCR, feed conversion ratio; SEM, standard error of the mean.
[1] Hy-D® (DSM Nutritional Products – Kaiseraugst, Switzerland).
[2] Quadratic equation: $y = -11{,}962\ x^2 + 9{,}641.6\ x - 802.22$; $R^2 = 0.62$.

3.2 ILEAL NUTRIENT DIGESTIBILITY

No effect or interaction of diet was seen for apparent ileal nutrient digestibility or IDE (Table 4). Increasing dietary AvP levels had a reducing linear effect on the ileal digestibility of Ca ($P < 0.001$) and P ($P < 0.01$). Supplementation with 25-OH-D3 increased both Ca and P ileal digestibility ($P < 0.05$). Apparent digestibility of DM, CP, as well as IDE were not affected by dietary treatments.

TABLE 4. EFFECT OF 25-OH-D3 SUPPLEMENTATION ON APPARENT NUTRIENT ILEAL DIGESTIBILITY AND ILEAL DIGESTIBLE ENERGY OF 21-D-OLD BROILERS FED DIETS WITH DIFFERENT LEVELS OF AVAILABLE PHOSPHORUS.

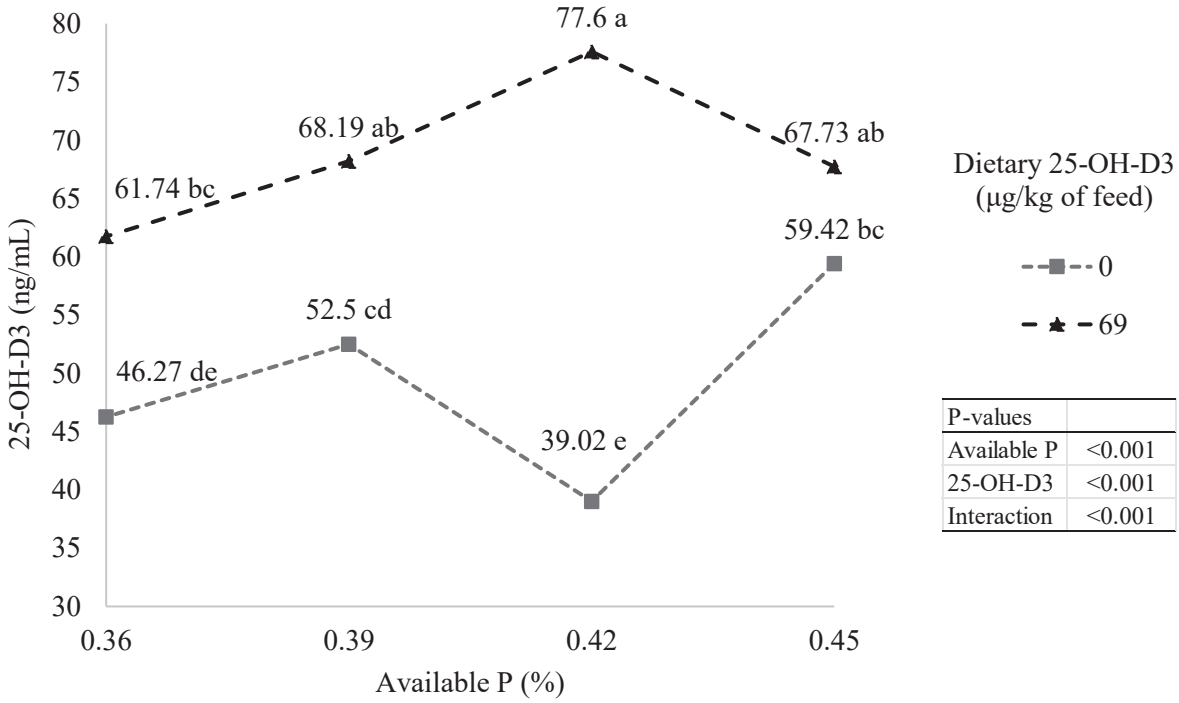
Available P (%)	25-OH-D_3 (µg/kg)[1]	Coefficient of apparent ileal digestibility				IDE (MJ)
		DM	CP	Ca	P	
Interaction						
0.36	0	0.70	0.83	0.58	0.72	14.15
0.39		0.69	0.83	0.55	0.70	13.67
0.42		0.68	0.82	0.50	0.68	13.50
0.45		0.69	0.81	0.52	0.64	13.68
0.36	69	0.69	0.83	0.62	0.74	13.84
0.39		0.69	0.83	0.57	0.71	13.96
0.42		0.70	0.83	0.56	0.72	14.09
0.45		0.68	0.83	0.53	0.72	13.73
Pooled SEM[4]		0.014	0.009	0.016	0.015	0.163
Effect of available P						
0.36		0.70	0.83	0.60	0.73	13.99
0.39		0.69	0.83	0.56	0.70	13.82
0.42		0.69	0.83	0.53	0.70	13.80
0.45		0.69	0.82	0.52	0.68	13.70
Effect of 25-OH-D_3						
	0	0.69	0.82	0.54	0.68	13.75
	69	0.69	0.83	0.57	0.72	13.91
P-values						
Available P		0.529	0.586	<0.001	0.049	0.353
P-linear		0.176	0.270	<0.001[2]	0.008[3]	0.093
P-quad		0.588	0.411	0.143	0.731	0.723
25-OH-D_3		0.440	0.116	0.006	0.002	0.172
Interaction		0.100	0.322	0.391	0.176	0.065

Data represents the mean of 7 replicates per treatment (5 birds per replicate).
DM, dry matter; CP, crude protein; IDE, ileal digestible energy; SEM, standard error of the mean.
[1] Hy-D® (DSM Nutritional Products – Kaiseraugst, Switzerland).
[2] Linear equation: $y = 87.25 x + 38.21$; $R^2 = 0.92$.
[3] Linear equation: $y = 0.46 x + 0.61$; $R^2 = 0.94$.

3.3 25-OH-D_3 BLOOD CONCENTRATION

Figure 1 shows an interaction, whereby supplementation with 25-OH-D3 increased the concentrations of its metabolites in the serum of broilers fed diets with 0.36% to 0.42% AvP compared to non-supplemented diets ($P < 0.001$). The diets containing 0.45% AvP with or without 25-OH-D3 had similar results.

FIGURE 1. EFFECT OF 25-OH-D3 SUPPLEMENTATION ON 25-OH-D3 SERUM CONCENTRATION OF 21-D-OLD BROILERS FED DIFFERENT LEVELS OF AVAILABLE P. A-E: MEANS FOLLOWED BY DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P < 0.05). POOLED STANDARD ERROR OF THE MEAN: 2.13.



SOURCE: THE AUTHOR (2023)

3.4 BONE MINERAL COMPOSITION AND BREAKING STRENGTH

No interactions were seen for bone mineralization and BBS (Table 5). Only the P content of bone was affected by AvP levels ($P < 0.05$), linearly increasing as AvP levels were raised from 0.36% to 0.45%. Supplementation with 25-OH-D3 increased ash bone content ($P < 0.001$), although Ca and P contents were not affected. There was no effect of treatments on Ca bone content, SI or BBS.

TABLE 5. EFFECT OF 25-OH-D3 SUPPLEMENTATION ON BONE MINERALIZATION AND BREAKING STRENGTH OF 21-D-OLD BROILERS FED DIFFERENT LEVELS OF AVAILABLE P.

Available P (%)	25-OH-D_3 (µg/kg)[2]	Ash (g/kg)	Ca (g/kg)	P (g/kg)	SI (mm/mg)	BBS (kgf)
Interaction						
0.36	0	473	172	83.4	31.7	17.4
0.39		476	173	83.3	33.2	17.6
0.42		479	176	84.4	33.9	17.7
0.45		477	172	85.0	34.7	18.2
0.36	69	485	171	84.1	32.8	17.9
0.39		486	176	84.7	33.6	18.1
0.42		486	175	83.8	33.8	18.4
0.45		485	177	87.7	34.4	18.7
Pooled SEM[4]		3.71	2.45	1.05	1.01	0.64
Effect of available P						
0.36		479	172	83.7	32.2	17.6
0.39		481	174	84.0	33.4	17.8
0.42		483	176	84.1	33.9	18.1
0.45		481	175	86.4	34.6	18.5
Effect of 25-OH-D_3						
	0	476	173	84.0	33.37	17.72
	69	485	174	85.1	33.65	18.26
P-values						
Available P		0.759	0.482	0.040	0.142	0.614
P-linear		0.429	0.219	0.019[2]	0.231	0.211
P-quad		0.477	0.344	0.183	0.762	0.812
25-OH-D_3		<0.001	0.357	0.158	0.704	0.243
Interaction		0.900	0.517	0.469	0.909	0.998

Data represents the mean of 7 replicates per treatment (2 birds per replicate).
SI, Seedor Index; BBS, bone breaking strength; SEM, standard error of the mean.
[1] Hy-D® (DSM Nutritional Products – Kaiseraugst, Switzerland).
[2] Linear equation: y = 0.027 x + 7.93; R^2 = 0.72.

4. DISCUSSION

When raising dietary AvP levels from 0.36% to 0.42%, broilers increased FI, but this was reduced at 0.45% AvP. It has been shown that dietary P deficiency – as well as an inadequate Ca:P ratio – leads to a decrease in voluntary FI (ADERIBIGBE et al. 2022; LIU et al. 2013). Delezie et al. (2015) and, more recently, Aderibigbe et al. (2022) explained that reduced intake at low levels of P (or, equally, high levels of Ca) is an attempt to balance Ca intake. Hence, the quadratic response seen in this study indicates that FI is altered to ensure an adequate AvP level. Reducing the supply of P can be detrimental to performance (ADERIBIGBE et al. 2022; IMARI, 2022), yet BWG and FCR were not affected by dietary AvP. This was likely due to the high dose of phytase included in the diets (1,500 FYT/kg), which was able to sustain growth performance, even in P-deficient-fed broilers (LEE et al. 2017; WALK and OLUKOSI, 2019).

Some studies have reported a lack of effect of 25-OH-D3 on growth performance in broilers (BOZKURT et al. 2017; FRITTS and WALDROUP, 2005; SAKKAS et al. 2019). Other studies, where 25-OH-D3 supplementation was shown to increase BWG and/or reduce FCR, related this effect to the source of 25- OH-D3, low levels of VitD or Ca- and P-deficient diets (KHAN et al. 2010; ZHANG et al. 2020). In the current study, 25- OH-D3 inclusion combined with adequate VitD levels (100 µg/kg) improved growth performance from 1 to 21 d, regardless of dietary AvP, which agreed with other reports (BRITO et al. 2010; FRITTS and WALDROUP, 2003; VAZQUEZ et al. 2018) and was most likely due to Ca and P availability.

The amount of Ca and P present in the intestinal lumen dictates their digestibility and absorption rates (PROSZKOWIEC-WEGLARZ and ANGEL, 2013). Studies have shown that high levels of Ca can impair pre-cecal P utilization, due to the formation of insoluble Ca-phytate complexes and concomitant reduced availability of Ca, P, N and other nutrients (IMARI 2022; LIU et al. 2013). Contrary to these observations, reducing dietary AvP in the current study, *i.e*., increasing the Ca:P ratio, led to a linear increase in ileal Ca and P digestibility, with no negative effects on DM, CP or energy availabilities. According to a review by Proszkowiec-Weglarz and Angel (2013), the Type III Na-P cotransporters (PiT1 and PiT2) located in the renal and intestinal brush borders are upregulated by low dietary P and are not influenced by Ca levels. It may be that reducing AvP levels triggers a need to maximise P digestibility in conditions of a low P supply, thus increasing absorption *via* the Na-dependent system. Following the lower presence of P in the lumen, Ca digestibility increased as well. Reducing AvP to the lowest level was akin to increasing Ca:P ratio from 2 to 2.5:1, and Imari (2022) reports that nutrient retention was only impaired with ratios higher than 3:1. Other factors to be considered include particle size and solubility of different Ca sources (BASSI et al. 2022; MANANGI and COON, 2007), but, in general terms, the increase in Ca:P ratio caused by lowering AvP in this study was perhaps not high enough to instigate such adverse effects.

The role of vitamin D3 to ensure adequate rates of intestinal Ca and P digestibility and absorption in broilers is undoubted (SHOJADOOST et al. 2021; SOUZA and VIEITES, 2014), but the effect of VitD metabolite supplementation on Ca and P uptake is often seen indirectly through improvements in bone health and mineralisation (BOZKURT et al. 2017; CASTRO et al. 2018; COLET et al. 2015). In the current study, 25- OH-D3 directly improved the ileal apparent digestibility of Ca and

P, which was increased regardless of dietary AvP levels. The active transport of both Ca and P through absorptive cells in the lumen is dependent on VitD, more specifically on 1,25-OH2-D3, which binds onto VDR to promote transcellular absorption when plasmatic Ca and P are low. Once the plasmatic concentrations of Ca and P are high, a passive paracellular transport system prevails (PROSZKOWIEC-WEGLARZ and ANGEL, 2013). Yet, even at higher dietary AvP levels, 25-OH-D3 effectively increased Ca and P ileal digestibility. Supplementing the feed with 25-OH-D3 improved VitD status, as seen in higher serum concentration, which was observed by Bozkurt et al. (2017), possibly by ramping up 1,25-OH2-D3 activation. What may have occurred was a shift from a predominantly passive transport of Ca and P to active 1,25-OH2-D3-mediated transport, enabled by greater 25-OH-D3 status. This is a rather noteworthy effect, considering that requirements for Ca and P in the modern broiler have increased, along with a greater need for VitD and active transport to meet adequate serum mineral levels (ANGEL, 2011; DAVID et al. 2023; SAKKAS et al. 2019).

Bone P deposition was linearly increased with higher dietary levels of AvP, denoting that P supply was propitious to bone formation, even though ileal digestibility of minerals was lower at these levels. Bone ash content was increased with 25-OH-D3 supplementation, regardless of AvP levels, indicating greater mineral deposition. Although VitD stimulates the resorption of Ca from the bone (DITTMER and THOMPSON, 2011), the greater Ca and P absorption and availability obtained with an enhanced 25-OH-D3 status helped increase mineral deposition in the tibia while still maintaining an adequate Ca and P plasmatic pool without Ca bone resorption. Improved bone quality with 25-OH-D3 supplementation for broilers has been reported. Zhang et al. (2020) observed higher tibial Ca content, breaking strength and better femoral bone density in 42-d-old broilers fed 69 µg/ kg 25-OH-D3. Bozkurt et al. (2017) reported higher weight and improved mineral profile of the sternum in 38-d-old broilers fed 25-OH-D3, indicating bone strengthening on flat bones as well. Heightened bone mineral composition helps reduce lameness and common skeletal disorders in broilers (NÄÄS et al. 2012; OGBONNA et al. 2022; WALDENSTEDT, 2006).

The effects of 25-OH-D3 can be additive to VitD, whereas adjusting dietary VitD levels alone may bring no further benefit to mineral utilisation by broilers. Khan et al. (2010) evaluated four different levels of VitD in 21 and 42- d-old broilers, and, while birds fed a low level of 5 µg/kg had worse FCR and bone mineralisation, no differences were seen for VitD levels ranging from 37.5 to 75 µg/kg. Likewise, Taheri and

Mirisakhani (2020) saw no improvement in bone characteristics, growth performance or serum P in broilers when increasing dietary VitD from 50 to 100 µg/kg. However, the combination or partial replacement of VitD by 25-OH-D3 has been known to enhance Ca and P utilisation and bone status (BOZKURT et al. 2017; OIKEH et al. 2019; VAZQUEZ et al. 2018; ZHANG et al. 2020). Moreover, studies such as Garcia et al. (2013) and Marques et al. (2022), indicated that 25-OH-D3 is a feasible substitute for VitD, due to its higher bioavailability (HAN et al. 2016). In conclusion, the results of this study demonstrated that 25-OH-D3 supplementation at 69 µg/kg had beneficial effects on Ca and P utilisation, bone ash content and VitD status which are complementary to a recommended dietary VitD inclusion for 21-d-old broilers. The lack of interaction with AvP indicated that 25-OH-D3 may change epithelial transport of minerals to increase Ca and P uptake, regardless of dietary levels or Ca:P ratio.

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CHAPTER III - 25-HYDROXYCHOLECALCIFEROL IN BROILER DIETS WITH DIFFERENT LEVELS OF CALCIUM AND PHOSPHORUS

ABSTRACT

The effect of including 25-OH-D_3 in diets for broilers with different levels of Ca and available (av) P was evaluated. A total of 560 broilers were reared from 1 to 21 days and distributed in a completely randomized design with a 4 x 2 factorial arrangement: 4 levels of total Ca+avP (0.9+0.45, 0.8+0.4, 0.7+0.35, 0.6+0.3%), and with or without the inclusion of 69 µg/kg feed of 25-OH-D_3, totaling 8 treatments and 7 replicates of 10 birds. All diets contained 100 µg/kg of vitamin D_3. No interactions were found between treatments for any of the analyzed variables. Reducing Ca+P levels linearly reduced BW gain and worsened feed conversion ratio, while supplementing 25-OH-D_3 improved feed conversion ratio ($P < 0.05$). Reducing Ca+P levels linearly increased ($P < 0.05$) ileal digestibility of Ca and P, but serum Ca, P, and alkaline phosphatase concentrations were linearly reduced, leading to lower bone ash, bone Ca, and bone breaking strength ($P < 0.05$). Dietary inclusion of 25-OH-D_3 led to greater ileal digestibility and serum concentrations of Ca and P, as well as increased serum alkaline phosphatase and 25-OH-D_3 ($P < 0.05$) compared to non-supplemented birds, which resulted in greater bone ash, Ca, P, and breaking strength ($P < 0.05$). It was concluded that a reduction of dietary Ca+P down to 0.6 + 0.3% was detrimental to growth performance and bone mineralization; dietary supplementation of 25-OH-D_3 combined with regular vitamin D3 levels improves performance and increases ileal digestibility, absorption, and bone deposition of Ca and P, along with increased bone strength and vitamin D status regardless of dietary Ca + avP levels. Calculated equivalence of 25-OH-D_3 were 0.10% avP and 0.20% Ca to achieve an average 52.5% tibia ash, and 0.08% avP and 0.16% Ca for 1.283 average feed conversion ratio from 1 to 21 d.

Key-words: 25-hydroxycholecalciferol, broilers, calcium, phosphorus, vitamin D.

CAPÍTULO III - 25-HYDROXICOLECALCIFEROL EM DIETAS PARA FRANGOS DE CORTE COM DIFERENTES NÍVEIS DE CÁLCIO E FÓSFORO

RESUMO

O efeito da inclusão de 25-OH-D_3 em dietas para frangos de corte com diferentes níveis de Ca e P disponível (Pdisp) foi avaliado. Um total de 560 frangos foram alojados de 1 a 21 dias de idade e distribuídos em um delineamento completamente ao acaso com um arranjo fatorial 4 x 2: 4 níveis de Ca+P (0.9+0.45, 0.8+0.4, 0.7+0.35, 0.6+0.3%), e com ou sem a inclusão de 69 µg/kg de 25-OH-D_3, totalizando 8 tratamentos e 7 repetições de 10 aves. Todas as dietas continham 100 µg/kg de unidades de vitamina D_3. Não foram encontradas interações entre os tratamentos para nenhuma das variáveis analisadas. A redução dos níveis de Ca+P linearmente reduziu o ganho de peso corporal e piorou a conversão alimentar, enquanto a suplementação de 25-OH-D_3 melhorou conversão alimentar no período ($P < 0,05$). A redução dos níveis de Ca+P aumentou linearmente ($P < 0,05$) a digestibilidade ileal de Ca e P, mas as concentrações séricas de Ca, P e fosfatase alcalina reduziram linearmente, resultando em menor teor de cinza óssea, Ca ósseo e resistência óssea ($P < 0,05$). A inclusão de 25-OH-D_3 resultou em maior digestibilidade ileal e concentrações séricas de Ca e P, assim como aumento dos níveis séricos de fosfatase alcalina e 25-OH-D_3 ($P < 0,05$) em comparação com aves não suplementadas, o que resultou em maior cinza, Ca, e P ósseos e resistência óssea ($P < 0,05$). Concluiu-se que a redução do Ca+P da dieta para 0,6 + 0,3% foi prejudicial ao desempenho e mineralização óssea; a suplementação de 25-OH-D_3 combinada com níveis regulares de vitamina D_3 melhora o desempenho e aumenta a digestibilidade ileal, absorção e deposição óssea de Ca e P, juntamente com o aumento da resistência óssea e do metabolismo de vitamina D, independentemente dos níveis dietéticos de Ca+P. As equivalências calculadas para 25-OH-D_3 foram 0,10% Pdisp e 0,20% Ca para se atingir uma média de 52,5% de cinzas ósseas, e 0,08% Pdisp e 0,16% Ca para uma média de 1,283 de conversão alimentar dos 1 aos 21 dias de idade.

Palavras-chave: 25-hidroxicolecalciferol, frangos de corte, cálcio, fósforo, vitamina D.

1. INTRODUCTION

The significance of Ca and P to poultry nutrition is paramount, as both minerals are involved in several vital metabolic processes related to bone health and mineralization, cellular functions, electrolytic balance, and overall growth and development (Delezie et al., 2015; Gautier et al., 2017; Imari, 2022). However, over-supplementation of dietary Ca and P levels in poultry diets can be detrimental to digestibility and performance (Driver et al., 2005; Li et al., 2017; Proszkowiec-Weglarz and Angel, 2013) instigated by the unavailability of phytate-P in plant ingredients and the formation of Ca-phytate insoluble complexes that is fostered with a high presence of Ca in the lumen (Cowieson et al., 2016; Humer et al., 2015). Additionally, the environmental impact caused by Ca and P excretion in the litter is a recurrent issue (Li et al., 2016; Liu et al., 2019). Nutritional and formulation strategies that optimize Ca and P utilization by broilers and enable the reduction of their dietary levels are continuously sought-after. To that end, dietary supplementation of 25-hydroxycholecalciferol (25-OH-D_3) has become increasingly exploited.

The 25-OH-D_3, also known as calcidiol, is a metabolite formed in the liver from the hydroxylation of vitamin D_3 (VitD) and is the precursor of 1,25-dihydroxycholecalciferol (1,25-OH_2-D_3) or calcitriol, which is the active hormonal form of VitD (Bikle and Schwartz, 2019). Once activated, 1,25-OH_2-D_3 acts at the target tissues (mainly intestine, kindey, muscle and bone cells) as a core component of Ca and P homeostasis, stimulating their intestinal absorption, renal reabsorption, and bone resorption (Gil et al., 2018). Both 25-OH-D_3 and 1,25-OH_2-D_3 are commercially available as supplements to animal diets, but studies demonstrate that the affinity of vitamin-D-binding proteins in the epithelium for 25-OH-D_3 is superior to other metabolites (Han et al., 2017, 2016). The presence of specific receptors to 25-OH-D_3 (Teegarden et al., 2000) and a longer half-life of 25-OH-D_3 compared to 1,25-OH_2-D_3 (Han et al., 2016) makes the supplementation of 25-OH-D_3 more advantageous. 25-OH-D_3 has been shown to improve growth performance, bone health, bone mineralization, and meat quality in poultry birds (Bassi et al., 2023; Garcia et al., 2013; Santiago et al., 2016; Vazquez et al., 2018), substantiated by higher serum levels of VitD and 25-OH-D_3 after supplementation (Bozkurt et al., 2017; Fatemi et al., 2020).

In light of the interconnected metabolism of Ca, P, and VitD and the remarkable effect that VitD metabolites have on Ca and P availability, studies have investigated the interaction between these nutrients at different dietary levels in broilers. While

some studies report no interaction between 25-OH-D_3 and changing dietary Ca and P (Marques et al., 2022; Oikeh et al., 2019), there has been cases like Zhang et al. (2020) who demonstrated that 25-OH-D_3 can improve bone mineralization and density in broilers fed low Ca+P diets. In a previous study (Bassi et al., 2023), we investigated the reduction of only dietary available (Av.) P levels for 21-d-old broiler chickens down to 0.35% while keeping constant Ca levels but found no interaction with 25-OH-D_3, which improved performance and mineral digestibility regardless of Av. P levels. The current study proposes a more acute reduction of both Ca and P levels altogether to ascertain its effects and possible interactions with 25-OH-D_3 supplementation in starter broiler diets containing commercial VitD levels. Additionally, a calculation of Ca and P equivalence for 25-OH-D_3 is presented.

2. MATERIAL AND METHODS

2.1 BIRDS AND HUSBANDRY

All experimental procedures were approved by the Animal Use Ethics Committee of Federal University of Paraná (Annex II). A total of 560 Ross® 308 broiler chicks obtained from a commercial hatchery were housed from 1 to 21 days in metabolic battery cages with 10 broilers per cage. Each cage measured 0.98 m length x 0.90 m width x 0.50 m height (11.3 broilers/m^2) and was equipped with gutter feeders and nipple drinkers. The initial room temperature was set to 30ºC and weekly reduced to 22ºC until day 21. During the first week, incandescent light was provided for 23 h with 1 h of dark, and after that a lighting program of 4 h of dark per day was applied until the end of the experiment. Pens were checked daily for removal of dead birds; the mortality rate throughout the experiment was 3.35%, and the causes were unrelated to dietary treatments.

2.2 EXPERIMENTAL DESIGN AND DIETS

Birds were randomly assigned to a completely randomized design with 8 treatments and 7 replicates of 10 birds each. A 4 x 2 factorial arrangement was conducted, including 4 dietary levels of total Ca + available (av) P: 0.9 + 0.45%, 0.8 + 0.4%, 0.7 + 0.35%, and 0.6 + 0.3%; and with or without the supplementation of 25-OH-

D_3 at 69 µg/kg of feed. The Ca+P levels were altered together while keeping a constant 2:1 ratio. The experimental diets were mashed, based on corn and soybean meal (Table 1), and feed and water were offered ad libitum throughout the experimental period. Representative feed samples were collected during manufacturing.

TABLE 1. INGREDIENTS AND COMPOSITION OF EXPERIMENTAL DIETS.

Ingredients (g/kg)	Calcium + Available phosphorus levels							
	Without inclusion of 25-OH-D_3				With inclusion of 25-OH-D_3			
	0.6 + 0.3	0.7 + 0.35	0.8 + 0.4	0.9 + 0.45	0.6 + 0.3	0.7 + 0.35	0.8 + 0.4	0.9 + 0.45
Corn	614	608	601	594	614	608	601	594
Soybean meal	334	334	335	336	334	334	335	336
Soybean oil	17.7	20.1	22.4	24.7	17.7	20.1	22.4	24.7
Limestone	6.8	7.6	8.4	9.2	6.8	7.6	8.4	9.2
Dicalcium phosphate	1.6	4.3	7.0	9.8	1.6	4.3	7.0	9.8
Sodium chloride	5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3
L-Lysine HCl	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9
L-Threonine	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
L-Valine	0.7	0.8	0.8	0.8	0.7	0.8	0.8	0.8
DL-Methionine	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Choline Chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin Premix[1]	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Mineral Premix[2]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Phytase[3]	0.075	0.075	0.075	0.075	0.075	0.075	0.075	0.075
25-hydroxycholecalciferol[4]	0	0	0	0	0.25	0.25	0.25	0.25
Celite[5] (Marker)	10.25	10.25	10.25	10.25	10	10	10	10
Calculated chemical composition (g/kg)								
Metabolizable energy (MJ/kg)	12.8	12.8	12.8	12.8	12.8	12.8	12.8	12.8
Available phosphorus	3.0	3.5	4.0	4.5	3.0	3.5	4.0	4.5
Sodium	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1
Chlorine	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
Digestible lysine	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2
Digestible methionine	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Methionine + cysteine	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Digestible threonine	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2
Digestible valine	9.1	9.1	9.1	9.1	9.1	9.1	9.1	9.1
Analyzed chemical composition (g/kg)								
Crude protein	215	226	210	210	221	219	212	216
Total calcium	5.9	6.8	7.6	8.5	6.0	6.9	7.8	8.6
Total phosphorus	3.9	4.5	5.1	5.5	4.1	4.5	5.1	5.6
Ash	52.1	57.4	57.7	61.8	55.7	54.5	58.0	61.4

[1] Supplied per kg of diet: retinol, 3.3 mg; cholechalciferol, 0.10 mg; α-tocopherol, 55 mg; menadione, 3 mg; thiamine, 2.3 mg; riboflavin, 7 mg; pantothenic acid, 12 mg; pyridoxine, 4 mg; cyanocobalamin, 0.025 mg; nicotinic acid, 60 mg; folic acid, 2 mg; biotin, 0.25 mg; selenium, 0.3 mg.
[2] Supplied per kg of diet: copper, 10 mg; iron, 50 mg; iodine, 1 mg; manganese, 65 mg; zinc, 65 mg.
[3] RONOZYME® HiPhos GT with 20,000 FYT/g (DSM Nutritional Products - Kaiseraugst, Switzerland). Nutrient matrix values (1.5 g/kg non-Phytate P and 1.8 g/kg Ca) were considered.
[4] Hy-D® (DSM Nutritional Products – Kaiseraugst, Switzerland), providing 69 mg 25-OH-D_3/ton of feed.
[5] Celite® Insoluble marker (Celite® 400 - Celite Corp., Lompoc, US).

The source of 25-OH-D_3 was Hy-D® premix (DSM, Kaiseraugst, Switzerland), included at 250 g/ton of feed (providing 69 mg of 25-OH-D_3/ton of feed) following the manufacturer's recommendation. All diets contained commercial levels of vitamin D_3

(100 µg/kg) added via premix. Phytase was supplied to all diets at a dose of 1,500 units (FYT)/kg of Ronozyme® Hiphos GT (Ronozyme® Hiphos GranulatedThermostable - DSM, Kaiseraugst, Switzerland), a 6-phytase originated from *Citrobacter braakii* and expressed in *Aspergillus oryzae*, with a minimum activity of 20,000 FYT/g of product.

The recovery of phytase and 25-OH-D_3 in the diets is presented in Table 2. Phytase activity was measured at Biopract GmbH (Berlin, Germany) using the PHY-101/05E method, in accordance with ISO30024:2009 as described by the International Organization for Standardization (ISO, 2009). The quantification of 25-OH-D_3 was performed via high performance liquid chromatography according to method SOP01464 by Biomin Holdin GmbH (Tulln, Donau, Austria).

TABLE 2. EXPECTED AND ANALYZED PHYTASE ACTIVITY AND DIETARY CONCENTRATION OF 25-OH-D3 IN FEED SAMPLES.

Dietary treatment	Phytase[1], FYT[2]/kg		25-OH-D_3 (µg/kg)	
	Declared	Analyzed	Declared	Analyzed
0.3 P and 0.6 Ca	1,500	1,787	0	<LOD[3]
0.35 P and 0.7 Ca	1,500	1,755	0	<LOD
0.4 P and 0.8 Ca	1,500	1,608	0	<LOD
0.45 P and 0.9 Ca	1,500	1,987	0	<LOD
0.3 P and 0.6 Ca + 25-OH-D_3	1,500	1,540	69	69.5
0.35 P and 0.7 Ca + 25-OH-D_3	1,500	1,573	69	71.4
0.4 P and 0.8 Ca + 25-OH-D_3	1,500	1,977	69	75.9
0.45 P and 0.9 Ca + 25-OH-D_3	1,500	2,110	69	63.4

Analyses performed by BioPract GmbH, Berlin, Germany.
[1] RONOZYME® HiPhos GT with 20,000 FYT/g (DSM Nutritional Products - Kaiseraugst, Switzerland). Enzyme activity is expressed as the quantity of product added in the feed.
[2] FYT = phytase units.
[3] LOD = limit of detection.

2.3 GROWTH PERFORMANCE

Broilers were weighted by cage on d 1 and 21 to determine mean body weight and calculate body weight gain (BWG). Feed allowance and feed refusal were weighted on d 1 and 21 to calculate feed intake (FI). Feed conversion ratio (FCR) was calculated as the ratio between FI and BWG, corrected to the weight of dead birds. Intake of Ca and avP in the period were calculated by multiplying dietary levels of the minerals by FI.

2.4 DIGESTIBILITY ASSAY

At 21 d, 5 birds per replicate were sacrificed by cervical dislocation and eviscerated. Ileum was separated, defined as 4 cm below Meckel's diverticulum and 4 cm above the ileum-cecum-colon junction, and ileal content of all five birds from each replicate was collected by gently stripping, pooled, placed in identified plastic containers, and frozen at -18ºC. Samples were subsequently thawed to room temperature and dried in a force-ventilation oven at 55ºC until constant weight. Feed and ileal samples were then grounded to 0.5 mm particle size. The dry matter (DM) content was obtained by oven drying the samples at 105ºC for 16 h, and crude protein (CP; method 954.01), Ca (method 927.02), and P (method 965.17) contents were analyzed according to methodology by AOAC (2005). Gross energy (GE) of the samples was determined in a calorimetric bomb (Ika Werke C2000 Control Oxygen Bomb Calorimeter – Ika-Werke GmbH&Co, Staufen, Germany). Acid-insoluble ash (AIA) was used as an insoluble marker compound, and AIA content in the samples was determined according to Scott and Boldaji (1997). The coefficient of apparent ileal digestibility (CAID) was calculated according to the following equation:

$$CAID = \frac{(Nutrient\ in\ the\ diet) - (Nutrient\ in\ the\ ileal\ digesta \times IF)}{Nutrient\ in\ the\ diet}$$

Where IF (indigestibility factor) is the ratio between diet AIA and ileal AIA. Ileal digestible energy (IDE) was calculated according to the equation:

$$IDE\ (kcal/kg\ DM) = GE\ of\ the\ diet - (GE\ of\ the\ ileal\ digesta\ \times IF)$$

2.5 CALCIUM, PHOSPHORUS, ALKALINE PHOSPHATASE, AND 25-OH-D_3 SERUM CONCENTRATION

At 21 d, two birds per replicate were randomly chosen for collection of blood by puncturing of the ulnar vein. Samples were held in 5 mL tubes with sodium heparin (Vacutainer Plus, BD, Franklin Lakes, NJ) and serum concentration of Ca, P, and alkaline phosphatase (ALP) were determined colorimetrically using commercial kits (Quibasa-Bioclin, Belo Horizonte, Brazil): Calcium Arsenazo III (K051), Phosphorus (K020), and Alkaline Phosphatase Kinetic (K021). A drop of blood was collected from each bird and immediately blotted and dried on filter paper to analyze serum

concentration of 25-OH-D_3 via the Dried Blood Spots (DBS) method using liquid chromatography-tandem mass spectrometry as described by Zakaria et al. (2020).

2.6 BONE MINERAL COMPOSITION AND BREAKING STRENGTH

Two broilers per replicate, randomly selected from the same 5 broilers sacrificed for ileal content collection at 21 d of age, had both legs manually removed. Tibial bones were severed from the legs without boiling and cleaned of litter and excrement. Tibiae from the left leg were cleaned with ether to remove remnants of fat and muscle and oven-dried at 105ºC for 12 hours. Dried bones were ashed in a muffle furnace at 600ºC and ash (method 942.05), Ca (method 927.02), and P (method 965.17) content were analyzed according to AOAC (2005). Tibiae from the right leg were used to measure breaking strength (BBS) using a three-point method with a universal testing machine (PMPA - Stable Micro Systems, Surrey, UK). The bone was rested on 2 points with a gap of 50 mm and pressure was applied with a pressure sensitive load cell of 500 kilogram-force at the center of both points at 10 mm/s speed. The BBS (expressed in kg) represented the highest force-load supported by the bone during the test.

2.6 STATISTICAL ANALYSES AND EQUIVALENCE CALCULATION

All collected data were tested for residue normality by Shapiro-Wilk test and analyzed via a two-way ANOVA including 2 main factors and their interaction ($P < 0.05$). When significant interactions were observed, their deployment was submitted to mean comparison by Tukey test. Linear and quadratic analyses of regression were carried out to assess the effect of Ca+P levels, and a linear equation was fitted: $Y = a + b \times X$, in which: Y = response variable; a = intercept, representing the value of y when x = 0; b = the line slope; and X = dose variable (Ca+P). The Ca and P equivalence of 25-OH-D_3 was then determined by plotting bone ash and FCR in supplemented diets against the respective concentrations of Ca (0.9, 0.8, 0.7, and 0.6%) or P (0.45, 0.4, 0.35, and 0.3%) and comparing it with values obtained from non-supplemented diets. Because effects were linear with no detectable plateau, the equivalence of Ca or P from the metabolite was calculated using the average value of bone ash and FCR from

the 4 plotted levels of Ca and P. All statistical procedures were conducted using a linear model on R program (R Foundation for Statistical Computing, Vienna, Austria).

3. RESULTS

3.1 GROWTH PERFORMANCE

No interaction between factors was observed (P > 0.05) for growth performance variables from 1 to 21 d (Table 3). Average FI was not affected by any treatments but reducing Ca+P levels linearly reduced (P < 0.001) BWG, which led to a linear increase of FCR (P < 0.05). Total Ca and avP intakes in the period were also both linearly reduced (P < 0.001) with lower dietary concentrations. Inclusion of 25-OH-D_3 improved FCR (P < 0.05) compared to non-supplemented diets.

TABLE 3. EFFECT OF DIETARY LEVELS OF TOTAL CA AND AVAILABLE PHOSPHORUS (CA+P) AND INCLUSION OF 25-OH-D3 ON FEED INTAKE, BODY WEIGHT GAIN, FEED CONVERSION RATIO, AND INTAKE OF CA AND P OF BROILERS FROM 1 TO 21 DAYS OF AGE.

Ca+P (%)	25-OH-D_3 (µg/kg)[1]	FI (g)	BWG (g)	FCR (g/g)	Ca intake (g)	Available P intake (g)
Interaction						
0.9+0.45	0	1,194	920	1.297	10.07	5.37
0.8+0.4		1,142	882	1.295	8.84	4.57
0.7+0.35		1,124	838	1.34	7.64	3.93
0.6+0.3		1,191	866	1.374	6.9	3.51
0.9+0.45	69	1,161	934	1.243	9.92	5.19
0.8+0.4		1,196	950	1.261	9.2	4.78
0.7+0.35		1,119	854	1.312	7.72	3.92
0.6+0.3		1,123	851	1.318	6.74	3.41
Pooled SEM		25.01	17.46	0.020	0.15	0.08
Effect of Ca+P						
0.9+0.45		1,177	927	1.27	10	5.28
0.8+0.4		1,169	916	1.278	9.02	4.68
0.7+0.35		1,121	846	1.326	7.68	3.93
0.6+0.3		1,157	858	1.346	6.82	3.46
Effect of 25-OH-D_3						
	0	1,163	877	1.326	8.36	4.35
	69	1,150	897	1.283	8.40	4.33
P-values						
Ca+P		0.137	<0.001	0.002	<0.001	<0.001
Linear		0.203	<0.001[2]	0.001[3]	<0.001[4]	<0.001[5]
Quadratic		0.263	0.319	0.894	0.600	0.236
25-OH-D_3		0.469	0.098	0.006	0.761	0.757
Interaction		0.110	0.311	0.869	0.271	0.113

[1] Hy-D® (DSM Nutritional Products – Kaiseraugst, Switzerland).
[2] Linear equation: y = 548.01 x + 681.49; R^2 = 0.78.
[3] Linear equation: y = -0.57 x + 1.52; R^2 = 0.96.
[4] Linear equation: y = 21.73 x + 0.23; R^2 = 0.98.
[5] Linear equation: y = 12.43 x - 0.32; R^2 = 0.98.
SEM, Standard error of the mean; FI, feed intake; BWG, body weight gain; FCR, feed conversion ratio.
Data represents the mean of 7 replicates per treatment (10 birds per replicate).

3.2 ILEAL NUTRIENT DIGESTIBILITY AND DIGESTIBLE ENERGY

No interaction was detected for apparent ileal nutrient digestibility or IDE (Table 4). Reducing dietary Ca+P had an increasing linear effect on ileal digestibility of both Ca ($P < 0.05$) and P ($P < 0.001$). Supplementation of 25-OH-D_3 increased Ca and P ileal digestibility ($P < 0.05$). Apparent ileal digestibility of DM, CP, as well as IDE were not affected by any of the dietary treatments.

TABLE 4. EFFECT OF DIFFERENT DIETARY LEVELS OF TOTAL CA AND AVAILABLE PHOSPHORUS (CA+P) AND INCLUSION OF 25-OH-D3 ON APPARENT NUTRIENT ILEAL DIGESTIBILITY AND ILEAL DIGESTIBLE ENERGY OF 21-D-OLD BROILERS.

Ca+P (%)	25-OH-D_3 (µg/kg)[1]	Coefficient of apparent ileal digestibility				IDE (kcal)
		DM	CP	Ca	P	
Interaction						
0.9+0.45	0	0.65	0.81	0.63	0.66	3,331
0.8+0.4		0.67	0.82	0.67	0.70	3,336
0.7+0.35		0.66	0.82	0.70	0.73	3,267
0.6+0.3		0.66	0.81	0.71	0.75	3,236
0.9+0.45	69	0.66	0.82	0.72	0.74	3,342
0.8+0.4		0.65	0.82	0.7	0.74	3,304
0.7+0.35		0.65	0.81	0.73	0.77	3,275
0.6+0.3		0.63	0.80	0.74	0.8	3,198
Pooled SEM		0.009	0.008	0.018	0.018	48.72
Effect of Ca+P						
0.9+0.45		0.66	0.81	0.67	0.70	3,336
0.8+0.4		0.66	0.82	0.69	0.72	3,320
0.7+0.35		0.66	0.81	0.71	0.75	3,271
0.6+0.3		0.65	0.80	0.73	0.78	3,217
Effect of 25-OH-D_3						
	0	0.66	0.81	0.68	0.71	3,293
	69	0.65	0.81	0.72	0.76	3,280
P-values						
Ca+P		0.109	0.264	0.024	<0.001	0.087
Linear		0.281	0.544	0.025[2]	<0.001[3]	0.093
Quadratic		0.253	0.325	0.934	0.781	0.723
25-OH-D_3		0.350	0.548	<0.001	<0.001	0.715
Interaction		0.185	0.635	0.316	0.716	0.934

[1] Hy-D® (DSM Nutritional Products – Kaiseraugst, Switzerland).
[2] Linear equation: $y = -0.37 x + 0.84$; $R^2 = 0.98$.
[3] Linear equation: $y = -0.52 x + 0.93$; $R^2 = 0.98$.
SEM, Standard error of the mean; DM, dry matter; CP, crude protein; IDE, Ileal digestible energy.
Data represents the mean of 7 replicates per treatment (5 birds per replicate).

3.3 SERUM MINERALS AND 25-OH-D_3

No interaction was observed for any of the parameters determined in the serum at 21 d of age (Table 5). Reducing dietary Ca+P linearly reduced serum Ca, P, and ALP ($P < 0.05$), but had no effect on serum 25-OH-D_3. Broilers fed diets

supplemented with 25-OH-D_3 showed greater concentrations of 25-OH-D_3, Ca, P, and ALP in comparison to the non-supplemented treatment.

TABLE 5. EFFECT OF DIFFERENT DIETARY LEVELS OF TOTAL CALCIUM AND AVAILABLE PHOSPHORUS (CA+P) AND INCLUSION OF 25-OH-D3 ON SERUM CONCENTRATION OF 25-OH-D3, ALKALINE PHOSPHATASE, CA, AND P OF 21-D-OLD BROILERS.

Ca+P (%)	25-OH-D_3 (µg/kg)[1]	Serum 25-OH-D_3 (ng/mL)	Alkaline phosphatase (U/L)	Calcium (mg/dL)	Phosphorus (mg/dL)
Interaction					
0.9+0.45	0	50.4	12,648	12.1	9.14
0.8+0.4		51.2	11,281	12.2	8.65
0.7+0.35		49.4	9,927	11.7	8.53
0.6+0.3		46.8	9,238	11.2	8.32
0.9+0.45	69	95	14,604	12.5	9.36
0.8+0.4		85.2	13,991	12.4	9.31
0.7+0.35		82.9	13,026	12.3	9.06
0.6+0.3		78.2	12,165	12.1	8.99
Pooled SEM		6.362	1,020	0.161	0.236
Effect of Ca+P					
0.9+0.45		72.7	13,660	12.4	9.25
0.8+0.4		68.2	12,636	12.3	8.98
0.7+0.35		66.2	11,477	12.0	8.79
0.6+0.3		62.5	10,702	11.6	8.65
Effect of 25-OH-D_3					
	0	49.4	10,773	11.8	8.66
	69	85.3	13,467	12.4	9.18
P-values					
Ca+P		0.100	0.002	<0.001	0.008
Linear		0.111	0.002[2]	<0.001[3]	0.009[4]
Quadratic		0.952	0.881	0.136	0.707
25-OH-D_3		<0.001	0.003	<0.001	<0.001
Interaction		0.308	0.599	0.121	0.490

[1] Hy-D® (DSM Nutritional Products – Kaiseraugst, Switzerland).
[2] Linear equation: y = 20067 x + 4593.8; R^2 = 0.94.
[3] Linear equation: y = 4.47 x + 10.43; R^2 = 0.89.
[4] Linear equation: y = 3.98 x + 7.42; R^2 = 0.97.
SEM, Standard error of the mean.
Data represents the mean of 7 replicates per treatment (2 birds per replicate).

3.4 BONE MINERALIZATION AND CA AND P EQUIVALENCE

No interactions were detected for bone mineralization or BBS (Table 6). A decreasing linear effect ($P < 0.05$) on bone ash and Ca were observed with reducing dietary Ca+P, as well as on BBS, although bone P was not affected. Tibiae from broilers fed 25-OH-D_3-supplemented diets had greater contents of ash, Ca, and P, and greater BBS ($P < 0.05$).

TABLE 6. EFFECT OF DIFFERENT DIETARY LEVELS OF TOTAL CA AND AVAILABLE PHOSPHORUS (CA+P) AND INCLUSION OF 25-OH-D3 ON BONE MINERAL COMPOSITION AND BREAKING STRENGTH OF 21-D-OLD BROILERS.

Ca+P (%)	25-OH-D_3 (µg/kg)[1]	Ash (%)	Ca (%)	P (%)	BBS (N)
Interaction					
0.9+0.45	0	52.1	20.8	9.19	222
0.8+0.4		51.5	19.7	9.06	206
0.7+0.35		51.1	19.1	8.65	165
0.6+0.3		49.9	18.9	8.54	161
0.9+0.45	69	54.1	21.1	9.39	236
0.8+0.4		53.1	20.9	9.37	233
0.7+0.35		51.8	20	9.13	176
0.6+0.3		51.2	19.4	9.26	171
Pooled SEM		0.743	0.452	0.231	8.81
Effect of Ca+P					
0.9+0.45		53.1	20.9	9.29	229
0.8+0.4		52.3	20.3	9.21	219
0.7+0.35		51.4	19.5	8.89	171
0.6+0.3		50.5	19.2	8.9	166
Effect of 25-OH-D_3					
	0	51.1	19.6	8.86	189
	69	52.5	20.4	9.29	204
P-values					
Ca+P		0.009	0.021	0.189	0.001
Linear		0.007[2]	0.001[3]	0.449	0.001[4]
Quadratic		0.972	0.656	0.785	0.687
25-OH-D_3		0.008	<0.001	0.011	0.014
Interaction		0.859	0.316	0.686	0.783

[1] Hy-D® (DSM Nutritional Products – Kaiseraugst, Switzerland).
[2] Linear equation: y = 17.03 x + 45.45; R^2 = 0.99.
[3] Linear equation: y = 12.35 x + 15.37; R^2 = 0.98.
[4] Linear equation: y = 477.99 x + 17.51; R^2 = 0.89.
SEM, standard error of the mean; BBS, bone breaking strength.
Data represents the mean of 7 replicates per treatment (2 birds per replicate).

3.5 CA AND P EQUIVALENCE OF 25-OH-D3

The linear equations generated for bone ash and FCR were used to estimate the equivalence of Ca and P from 25-OH-D_3. Bone ash and FCR were plotted and the average equivalence for Ca and P was obtained from the difference between estimated values for supplemented and non-supplemented treatments. Results are summarized in Table 7. Broilers from the supplemented group required 0.75% Ca and 0.37% avP to achieve an average tibia ash content of 52.5% compared to 0.95% Ca and 0.47% avP in non-supplemented groups, indicating an average avP and Ca equivalence of 25-OH-D_3 of 0.1 and 0.2% respectively. For an average FCR (1 to 21 d) of 1.283, supplemented broilers required 0.74% Ca and 0.37% avP in relation to 0.9% Ca and 0.45% avP for non-supplemented birds, meaning a respective dietary Ca and avP release of 0.16 and 0.08% from 25-OH-D_3 inclusion.

TABLE 7. TOTAL CALCIUM AND AVAILABLE PHOSPHORUS EQUIVALENCE OF 25-OH-D3 VITAMIN FOR 21-D-OLD BROILER CHICKENS.

Evaluated variable	25-OH-D_3 (µg/kg)[1]	Regression	Equation	R^2	Estimated nutrient (%)[2]	Equivalence (%)[3]
Available P						
Tibia ash 21 d[1]	0	Linear	y = 14.16x + 45.82	0.980	0.47	0.10
	69	Linear	y = 19.90x + 45.08	0.956	0.37	
Feed conversion ratio 1 to 21 d	0	Linear	y = -0.555x + 1.534	0.885	0.45	0.08
	69	Linear	y = -0.592x + 1.505	0.931	0.37	
Total Ca						
Tibia ash 21 d[1]	0	Linear	y = 7.08x + 45.82	0.980	0.95	0.20
	69	Linear	y = 9.95x + 45.08	0.956	0.75	
Feed conversion ratio 1 to 21 d	0	Linear	y = -0.277x + 1.534	0.889	0.90	0.16
	69	Linear	y = -0.296x + 1.505	0.931	0.74	

[1] Hy-D® (DSM Nutritional Products – Kaiseraugst, Switzerland).
[2] Dietary available P or total Ca level required to achieve an average 52.5% of tibia ash and 1.283 feed conversion ratio.
[3] Difference of estimated nutrient between 25-OH-D_3-supplemented and non-supplemented treatments.

4. DISCUSSION

High dietary levels of Ca can impair nutrient utilization by fostering the formation of Ca-phytate complexes (Imari, 2022) and consequently affecting growth performance, especially when associated with low levels of P, i.e. a high Ca:P ratio. Effectively, Ca:P ratio is a foremost aspect to be considered in diet formulation rather than Ca and P concentrations alone, and its impact on growth performance has been highlighted throughout the literature (Akter et al., 2016; Gautier et al., 2017; Imari, 2022). In the current study, Ca:P ratios were kept at a constant 2:1, but the dietary concentrations of Ca and P per se affected growth performance, as reducing Ca+P linearly reduced BWG and worsened FCR. Delezie et al. (2015) have shown that neither normal nor low levels of Ca and total P were detrimental to growth performance of broilers at different phases when the ratio between them was balanced; the reduction was not so sharp (from 0.85 to 0.7% Ca and 0.68 to 0.54% avP) compared to this study. Hamdi et al. (2015) indicated that 0.7% Ca and 0.38% avP were able to maintain performance of starter broilers combined with high phytase, but still, the current observations suggest that a greater reduction of Ca+P down to 0.6+0.3% was not sufficient to meet Ca and avP requirements even with phytase supplementation, thus causing the drop on performance.

Although the supplementation of 25-OH-D_3 had no significant effect on FI or BWG, FCR was reduced in broilers fed the metabolite on top of regular VitD levels. This agrees with Vazquez et al., (2018) and Abascal-Ponciano et al. (2022), both showing that 25-OH-D_3 improved broiler performance in relation to supplying VitD

alone, much owed to the increased absorption and enhanced utilization of Ca and P. Although Garcia et al. (2013) implies that there is no difference between supplementing VitD, 25-OH-D_3, or 1,25-OH_2-D_3 regarding improvements on growth performance or even bone mineralization, the current study supports a combining effect of 25-OH-D_3 with VitD, and, as was suggested by Atencio et al. (2005), one should consider the potentiality of 25-OH-D_3 inclusion at lower levels of VitD.

Reducing Ca+P levels linearly increased CAID of both minerals, in agreement with current literature. As it is known, a high presence of Ca in the lumen fosters the complexation with phytate, restricting the action of phytases upon it and rendering P, N, and other phytate-bound minerals unavailable for absorption (Kim et al., 2018; Proszkowiec-Weglarz and Angel, 2013). Although, again, this negative effect on digestibility – or rather, solubility - is commonly assigned to higher Ca:P ratio (Imari, 2022; Liu et al., 2013), these results evidence that a marked reduction on both Ca and P inclusion does affect their AID. An increase on N ileal digestibility and IDE was also expected in this sense, given that lowering Ca levels has the potential to improve energy and diet utilization in general (Mutucumarana et al., 2014), but it was not observed.

A riveting observation was that broilers fed normal Ca+P presented greater serum concentrations of Ca, P, and ALP despite ileal digestibility being lower. When Ca and P intakes are high, intracellular active transport system can get saturated and shift to an intercellular passive transport (Na-independent in the case of P), as the ions move through spaces between cells (Proszkowiec-Weglarz and Angel, 2013). The dietary Ca+P levels of 0.9 + 0.45 % and 0.8 + 0.4% might have led to the saturation of active transport systems, or it simply indicated that a threshold on the Ca and P plasma pool was attained because there were more ions available for absorption in the lumen (David et al., 2023), hence AID was reduced. This complies with the observed intakes of Ca and P from 1-to-21-d, which were linearly increased with higher dietary Ca+P – given that average FI was not affected by Ca+P - and thus contributed to their increment in the serum. The same was reported by Zhang et al. (2020), where Ca+P deficiency led to lower serum Ca, P, and ALP, the latter being downregulated by low Ca and P as a sign of bone formation suppression (Vimalraj, 2020).

The mode of action of VitD and its metabolites in the intestine, succinctly, starts with the binding of 1,25-OH_2-D_3 to vitamin-D-receptors (VDR) located in the basolateral membrane of enterocytes, which promotes calbindin- and NaPi-IIb-mediated active

transport systems, thus bolstering Ca and P absorption, respectively (Gil et al., 2018; Shao et al., 2019). Most studies indirectly report the effect of 25-OH-D_3 on increasing Ca and P digestibility by showing its impact on bone mineralization and reduced incidence of skeletal disorders (Colet et al., 2015; Landy et al., 2020; Santiago et al., 2016), but this study evinced that ileal digestibility of Ca and P is improved by 25-OH-D_3 supplementation, alongside greater serum Ca and P, even when the metabolite is added on top of regular VitD levels. This most likely occurred due to the higher affinity of vitamin-D-binding-proteins in the intestine that will quickly absorb 25-OH-D_3 than other VitD forms (Han et al., 2017, 2016), more effectively boosting the formation of 1,25-OH_2-D_3 and its action upon Ca and P absorption.

According to Sassi et al. (2018), the blood measurement of 25-OH-D_3 is a marker of VitD status. The increased serum 25-OH-D_3 in the supplemented group indicates an enhanced VitD status resulting from a more efficient absorption of 25-OH-D_3 via epithelium, as has been demonstrated in other studies (Fatemi et al., 2020; Zhang et al., 2020). The fact that Ca and P digestibility and serum concentrations were higher in 25-OH-D_3-fed birds regardless of dietary Ca+P also denotes that VitD metabolite supplementation can potentially change how Ca and P are absorbed to a predominantly active VitD-mediated transport that can enhance mineral transport even at higher dietary intakes. Studies have shown how supplementing VitD metabolites can upregulate the mRNA expression of not only Ca- and P-homeostasis-related genes such as calbindin and Na/P cotransporters at the small intestine of broilers, but also VDR, overall increasing the efficacy of VitD metabolism (Han et al., 2018; Hsiao et al., 2018).

The linear increase on tibia ash and Ca contents and BBS when increasing Ca+P levels matches the higher serum Ca and P observed in these treatments, meaning a larger mineral pool was available for bone mineralization. A more prominent osteoblastic activity is also related to the higher serum ALP concentration, as ALP expression in bone cells is fostered by higher Ca and phosphate intake to step up the bone formation process (Vimalraj, 2020). Broilers in this study were at their starter-to-grower phase, when requirements for skeletal growth are relatively high (Driver et al., 2005), which were then met by the regular levels of dietary Ca+P. Again, detrimental effects in bone quality of poultry are more strongly linked to an imbalanced Ca:P ratio rather than the levels of minerals per se (Bassi et al., 2022; Driver et al., 2005; Gautier et al., 2017), and in the current study Ca:P ratio was constant.

Supplementation of 25-OH-D_3 improved overall bone mineralization as seen by greater bone ash, Ca, and P, and BBS, which has been previously reported (Bozkurt et al., 2017; Colet et al., 2015; Fritts and Waldroup, 2003). VitD is crucial to skeletal growth not only by increasing Ca and P provision, but by taking roles in several actions such as enabling the proliferation and differentiation of bone morphogenetic proteins and other bone formation markers – including ALP (Vimaraj, 2020) - and increasing VDR expression in bone and muscle cells (Montenegro et al., 2019). Therefore, it is implied that supplementation of 25-OH-D_3 promotes bone growth by enhancing VitD status, tying together with increments on digestibility and absorption of Ca and P, serum ALP, and serum 25-OH-D_3 found in the supplemented broilers.

5. CONCLUSION

The results of this study show that a steep reduction of dietary Ca and avP levels (kept to a constant 2:1 ratio) down to 0.6 and 0.3%, respectively, can be detrimental to growth performance, bone mineralization, and bone strength of 21-d-old broiler chickens even when fed diets containing phytase at 1,500 FYT/kg. Supplementation of 25-OH-D_3 at 69 µg/kg improved performance and increased Ca and P digestibility, mineral and 25-OH-D_3 status, and bone quality regardless of dietary Ca and avP concentration. The results suggest that inclusion of 25-OH-D_3 in combination with commercial vitamin D_3 levels (100 µg/kg) is beneficial to vitamin D status and utilization of Ca and P for growing broilers, but reduction of formulated levels of total Ca and avP must be considered warily. The proposed calculated equivalence of mineral release from the metabolite were 0.10% avP and 0.20% Ca to achieve an average 52.5% tibia ash, and 0.08% avP and 0.16% Ca for 1.283 average feed conversion ratio from 1 to 21 d.

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CHAPTER IV - SUPPLEMENTATION OF BROILER CHICKEN DIETS WITH 25-HYDROXYCHOLECALCIFEROL AND PHYTASE ON GROWTH PERFORMANCE, VITAMIN D STATUS, BONE MINERALIZATION, AND MTOR KINASE GENE EXPRESSION

ABSTRACT

A study was conducted to investigate possible interactions between supplementation of 25-hydroxycholecalciferol (25-OH-D_3) and high doses of phytase for broiler chickens fed Ca and P-deficient diets. A total of 1200 one-d-old male broiler chicks were randomly allocated from 1 to 42 d to 1 of 4 dietary treatments in a 2 x 2 factorial arrangement: 600 or 2,000 phytase units (FYT)/kg and with or without the inclusion of 25-OH-D_3 at 69 µg/kg, with 12 replicates of 25 broiler chickens each. All diets contained commercial levels vitamin D_3 (100 µg/kg) and total Ca and available P were set to 0.6 and 0.3%, respectively. Supplementation with 25-OH-D_3 increased body weight gain (BWG) and reduced feed conversion ratio (FCR) from 1 to 21 d ($P < 0.05$), as well as increased BWG from 1 to 42 d ($P < 0.05$). Serum 25-OH-D_3 levels at 21 and 42 d were increased with 25-OH-D_3 ($P < 0.001$). Phytase did not affect growth performance from 1 to 21 d, but a higher dose (2,000 FYT/kg) reduced feed intake and FCR from 22 to 42 d compared to 600 FYT/kg, also reducing FCR in the total period ($P < 0.05$). Expression of mTOR kinase mRNA in breast muscle assessed at 42 d was enhanced with 2,000 FYT/kg ($P < 0.001$). Bone weight, bone contents of ash, Ca, and P, and bone breaking strength of tibia bone measured at 42 d were not affected by any dietary treatment. Although both additives are known to improve dietary Ca and P utilization, there were no detected additive or synergic effects. The results suggested that inclusion of 25-OH-D_3 and phytase combined with regular vitamin D_3 levels can enable lower Ca and P without impairing growth performance and bone mineralization of broiler chickens. Furthermore, vitamin D status is refined with dietary 25-OH-D_3 and potential improvements on breast meat yield can be obtained with a higher phytase dose of 2,000 FYT/kg.

Key-words: 25-hydroxycholecalciferol, broiler chickens, phytase, vitamin D.

CAPÍTULO IV – EFEITO DA SUPLEMENTAÇÃO DE DIETAS DE FRANGOS DE CORTE COM 25-HIDROXICOLECALCIFEROL E FITASE SOBRE DESEMPENHO, STATUS DE VITAMINA D, MINERALIZAÇÃO ÓSSEA, E EXPRESSÃO GÊNICA DE MTOR QUINASE

RESUMO

Um estudo foi conduzido para investigar possíveis interações entre a suplementação de 25-hidroxicolecalciferol (25-OH-D_3) e altas doses de fitase para frangos de corte alimentados com dietas deficientes em Ca e P. Um total de 1200 pintos de corte machos de um dia de idade foram aleatoriamente alocados dos 1 aos 42 dias à 1 de 4 tratamentos em um arranjo fatorial 2 x 2: com 600 ou 2.000 unidades de fitase (FYT)/kg e com ou sem a inclusão de 25-OH-D_3 a 69 µg/kg, com 12 repetições de 25 frangos cada. Todas as dietas continham níveis comerciais de vitamina D_3 (100 µg/kg) e os níveis de Ca e P disponível foram fixados em 0,6 e 0,3%, respectivamente. A suplementação com 25-OH-D_3 aumentou o ganho de peso (GP) e reduziu a conversão alimentar (CA) de 1 a 21 dias ($P < 0,05$), bem como aumentou o GP de 1 a 42 dias ($P < 0,05$). Os níveis séricos de 25-OH-D_3 aos 21 e 42 dias foram aumentados com inclusão de 25-OH-D_3 ($P < 0,001$). A fitase não afetou o desempenho de 1 a 21 dias, mas uma dose mais alta (2.000 FYT/kg) reduziu o consumo de ração e CA de 22 a 42 dias em comparação com 600 FYT/kg, reduzindo também a CA no período total ($P < 0,05$). A expressão de mRNA da enzima quinase mTOR no músculo peitoral avaliada aos 42 dias foi aumentada com 2.000 FYT/kg ($P < 0,001$). O peso ósseo, os teores de cinzas, Ca e P, e a resistência óssea da tíbia medida aos 42 dias não foram afetados pelos tratamentos. Embora ambos os aditivos sejam conhecidos por melhorar a utilização de Ca e P na dieta, não foram detectados efeitos aditivos ou sinérgicos. Os resultados sugeriram que a inclusão de 25-OH-D_3 e fitase combinadas com níveis regulares de vitamina D3 pode permitir níveis menores de Ca e P sem prejudicar o desempenho de crescimento e a mineralização óssea de frangos de corte. Além disso, o metabolismo de vitamina D é refinado com 25-OH-D_3 dietética e potenciais melhorias no rendimento do peito podem ser obtidas com uma dose mais alta de fitase de 2.000 FYT/kg.

Palavras-chave: 25-hidroxicolecalciferol, frangos de corte, fitase, vitamina D.

1. INTRODUCTION

Calcium and P are the two most essential macro minerals for poultry, and the interplay between both molecules and phytate (myo-inositol- 1,2,3,4,5,6-hexaphosphate; $InsP_6$) inside the avian gastrointestinal tract (GIT) remains a distinguished topic in poultry research to this day (David et al., 2023). Phosphorus in plant ingredients is majorly bound to phytate and rendered unavailable to non-ruminants. This chelation effect can extend to other nutrients (Cowieson et al., 2009) and it is intensified in the presence of high levels of Ca in the GIT due to the affinity of phytate for cations, thus making it imperative to strive for adequate dietary Ca:P ratio (Amerah et al., 2014). Although inorganic Ca is deemed inexpensive, the use of mineral P to overcome P deficiency is onerous to feed formulation costs, hastens the depletion of nonrenewable sources, and increases the excretion of P with adverse impacts on the environment (Liu et al., 2019). Among the nutritional strategies that countermeasure the oversupply of mineral P and aim to increase the utilization of both P and Ca, exogenous phytase and vitamin D_3 (VitD) supplementation have been the most prominent in poultry nutrition (Dersjant-Li et al., 2015; Selle and Ravindran, 2007; Świątkiewicz et al., 2016).

Phytase catalyzes the hydrolysis of phytate, cleaving the phosphate groups from the molecule and releasing P. As the breakdown of phytate nears completion, minerals such as Ca, Zn, Fe, and amino acids are also released, granting extra-phosphoric effects, and making these nutrients more available for absorption (Cowieson et al., 2009). To this purpose, higher doses of phytase have been increasingly supplemented to poultry diets. Investigations report on how high inclusions of phytase can foster a complete dephosphorylation of phytate, increasing the digestibility of nutrients beyond P and enhancing diet utilization and growth performance of poultry birds (Bassi et al., 2021; Walk et al., 2013; Walk and Rama Rao, 2020). In addition to phytase, dietary supplementation of VitD is absolute to a proper Ca and P metabolism. Ingested VitD is absorbed and carried by vitamin-D binding proteins to the liver and hydroxylated to 25-hydroxycholechalciferol (25-OH-D_3), which undergoes a second hydroxylation in the kidneys into 1,25-dihydroxycholechalciferol (1,25-OH_2-D_3), the active form of VitD. Once formed, 1,25-OH_2-D_3 reaches target tissues such as intestine, kidneys, and bones to perform a series of biological functions intricately related to improving Ca and P absorption,

retention, and utilization, as described by Chen et al. (2021). In this regard, it has been shown that the direct supplementation of 25-OH-D_3 – either replacing or in combination with VitD – can increase Ca and P availability and improve growth performance and bone mineralization in a more effective manner (Bassi et al., 2023; Bozkurt et al., 2017; Marques et al., 2022; Zhang et al., 2020), mainly due to its greater relative biological value and higher affinity with VitD-binding proteins (Bar et al., 1980; Han et al., 2016).

Given the significance of phytase and VitD on Ca and P metabolism, emerging research suggests synergetic effects between both components in broiler chicken diets (Green and Persia, 2012; Kermani et al., 2023; Taheri and Mirisakhani, 2020). However, the literature lacks information on potential interactions when using VitD metabolites. It is postulated that the greater Ca and P absorption rates obtained through 25-OH-D_3 supplementation can optimize phytase activity by reducing the potential of phytate to form insoluble complexes in the GIT, further improving dietary mineral utilization for broiler chickens. This effect could be reinforced with higher doses of the enzyme. With these aspects in mind, this study investigated the inclusion of 25-OH-D_3 in low Ca and P diets containing regular or high dose of phytase on growth performance, bone mineral composition and strength, mineral and VitD status, and relative mTOR kinase expression in breast muscle of broiler chickens from 1 to 42 d of age.

2. MATERIALS AND METHODS

2.1 ANIMAL HUSBANDRY

All experimental procedures were approved by the Animal Use Ethics Committee of the Innovation and Applied Science (CEUA-I&AS) department of dsm-firmenich (Annex 3). A total of 1200 one-d-old male broiler chicks (Cobb 500; Cobb Brazil Ltda, São Paulo, Brazil) were obtained from a commercial hatchery and randomly allocated to 48 floor pens (1.4 x 1.4 m) in groups of 25 birds per pen (12.7 birds/m^2) with wood shavings as litter, equipped with nipple drinkers and tubular feeders providing free access to water and feed. During the first day, incandescent light was provided uninterrupted, followed by a lighting program of 6 h of dark per day. Room temperature was set to 34ºC on d 0 and weekly reduced by 2 ºC to attain 25ºC

until the end of the trial period. Pens were checked daily for temperature, humidity, and bird mortality.

2.2 EXPERIMENTAL DIETS AND TREATMENTS

Dietary treatments were arranged in a completely randomized 2 x 2 factorial design, comprising 2 phytase doses (600 or 2,000 phytase units [FYT]/kg) and with or without the supplementation of 25-OH-D_3. Each pen with 25 birds was assigned to 1 of the 4 treatments, totaling 12 pens per treatment. The experimental diets (Table 1) were mash, based on corn-soybean meal, and divided into 2 phases: grower (1-to-21-d) and finisher (22-to-42-d). Target formulated levels of Ca and available P across all diets were 0.6 and 0.3% (2:1 ratio). Particle size distribution of diets was determined by dry sieving and geometrical mean diameter (GMD) and geometrical standard deviation (GSD) were calculated using GRANUCALC® software (Embrapa Poultry and Swine, Concórdia, Brazil). The average GMD and GSD were respectively 911 µm and 2.3 for grower diet, and 953 µm and 2.4 for finisher diet. Feed samples were collected after manufacturing, oven-dried at 105°C for 24h, ground to 0.5 mm particle size, and analyzed for crude protein (method 954.01), total Ca (method 927.02), and total P (method 965.17) according to the Association of Official Analytical Chemists (AOAC, 2005). Phytate phosphorus was determined in feed by near infrared reflectance spectroscopy, as described by Aureli et al. (2015).

The 25-OH-D_3 source was Hy-D® premix (DSM, Kaiseraugst, Switzerland), included at 250 g/ton of feed to provide 69 µg 25-OH-D_3/kg of feed, and all diets also contained commercial levels of VitD (4,000 IU/kg) added via vitamin premix. The utilized phytase was Ronozyme® HiPhorius (DSM, Kaiseraugst, Switzerland), a 6-phytase originated from *Citrobacter braakii* and expressed in *Aspergillus oryzae*, with a minimum activity of 20,000 FYT/g of product. One FYT is defined as the quantity of enzyme required to produce 1 mmol of inorganic P/min from 5.1 mmol/L sodium phytate at 5.5 pH and 37°C. The assessed doses of 600 and 2,000 FYT were respectively considered a regular and high dose according to commercial practices.

TABLE 1. INGREDIENTS AND NUTRIENT COMPOSITION OF THE EXPERIMENTAL DIETS (DRY-MATTER BASIS).

Ingredients (%)	Grower phase (1-to-21-d)				Finisher phase (22-to-42-d)			
	T1	T2	T3	T4	T1	T2	T3	T4
Corn	62.5	62.3	62.8	62.7	68.8	68.7	69.2	69.0
Soybean meal	33.3	33.4	33.3	33.3	26.1	26.1	26.0	26.0
Soybean oil	2.00	2.05	1.90	1.95	3.00	1.65	2.85	2.95
Limestone	0.62	0.62	0.69	0.69	0.63	0.63	0.72	0.72
Sodium chloride	0.51	0.51	0.51	0.51	0.46	0.46	0.46	0.46
Dicalcium phosphate	0.24	0.24	0.01	0.01	0.31	0.31	0.06	0.06
DL-Methionine	0.30	0.30	0.30	0.30	0.26	0.26	0.26	0.26
L-Lysine	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
Threonine	0.07	0.07	0.07	0.07	0.05	0.05	0.05	0.05
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix[1]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mineral premix[2]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
B.H.T.	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
25-OH-D_3[3]	-	0.025	-	0.025	-	0.025	-	0.025
Phytase[4]	0.0015	0.0015	0.005	0.005	0.0015	0.0015	0.005	0.005
Calculated chemical composition (%)								
ME (kcal)	3,050	3,050	3,050	3,050	3,180	3,180	3,180	3,180
Calcium	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Total phosphorus	0.50	0.50	0.50	0.50	0.48	0.48	0.48	0.48
Available phosphorus	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Digestible lys	1.17	1.17	1.17	1.17	1.00	1.00	1.00	1.00
Digestible met	0.59	0.59	0.59	0.59	0.52	0.52	0.52	0.52
Digestible thr	0.76	0.76	0.76	0.76	0.65	0.65	0.65	0.65
Sodium	0.22	0.22	0.22	0.22	0.20	0.20	0.20	0.20
Analyzed chemical composition (%)								
Crude protein	19.8	20.6	20.6	20.7	17.6	17.5	18.0	17.4
Calcium	0.56	0.54	0.57	0.56	0.49	0.51	0.50	0.50
Total phosphorus	0.36	0.36	0.34	0.34	0.45	0.46	0.47	0.47
Phytate phosphorus[5]	0.24	0.25	0.22	0.23	0.28	0.29	0.27	0.27

T1 = 600 units of phytase (FYT)/kg without 25-OH-D_3; T2 = 600 FYT/kg with 25-OH-D_3; T3 = 2,000 FYT/kg without 25-OH-D_3; T4 = 2,000 FYT/kg with 25-OH-D_3.

[1] Provided per kilogram of diet: vitamin A, 11,000 IU; vitamin D3, 4,000 IU; vitamin E, 55 IU; vitamin K3, 43 mg; vitamin B1, 2.3 mg; vitamin B2, 7 mg; pantothenic acid, 12 mg; vitamin B6, 4 mg; vitamin B12, 0.025 mg; nicotinic acid, 60 mg; folic acid, 2 mg; biotin, 0.25 mg.

[2] Provided per kilogram of diet: copper, 10 mg; iron, 50 mg; iodine, 1 mg; manganese, 65 mg; zinc, 65 mg; selenium, 0.15 mg.

[3] Hy-D Premix (DSM Nutritional Products, Kaiseraugst, Switzerland) providing 69 µg of 25-OH-D_3/kg of feed.

[4] Ronozyme HiPhorius (DSM Nutritional Products, Kaiseraugst, Switzerland) with 20,000 FYT/g of product.

[5] Determined via near infrared reflectance spectroscopy.

Enzyme activity in experimental diets is presented in Table 2. Phytase activity was measured at Biopract GmbH (Berlin, Germany) using the PHY-101/05E method, in accordance with ISO30024:2009 as described by the International Organization for Standardization (ISO, 2009).

TABLE 2. EXPECTED AND ANALYZED PHYTASE ACTIVITY RECOVERED IN FEED SAMPLES.

Treatment	Phase	Phytase[1], FYT/kg	
		Expected	Analyzed
600 FYT without 25-OH-D_3	Grower	600	727
600 FYT without 25-OH-D_3	Finisher	600	647
600 FYT with 25-OH-D_3	Grower	600	732
600 FYT with 25-OH-D_3	Finisher	600	584
2,000 FYT without 25-OH-D_3	Grower	2,000	2,564
2,000 FYT without 25-OH-D_3	Finisher	2,000	1,928
2,000 FYT with 25-OH-D_3	Grower	2,000	2,202
2,000 FYT with 25-OH-D_3	Finisher	2,000	1,902

[1] Ronozyme HiPhorius (DSM Nutritional Products, Kaiseraugst, Switzerland) with 20,000 FYT/g of product. Enzyme activity is expressed as the quantity of product added in the feed.
FYT = phytase units.
Recovery analysis performed by BioPract GmbH, Berlin, Germany.

2.3 DATA COLLECTION AND ANALYSES

On days 1, 21, and 42, broilers and feed leftovers were weighted by pen to determine body weight gain (BWG), feed intake (FI), and mortality-corrected feed conversion ratio (FCR) in each period.

On days 21 and 42, two birds per replicate were randomly chosen for collection of blood samples. Blood was collected from the ulnar vein into heparin tubes (Vacutainer® Plus – BD, New Jersey, US) and the serum was separated by centrifuging at 3,000 × g for 10 min and stored at -80ºC prior to analyses. Serum concentration of Ca and P were determined by colorimetry using commercial kits (Quibasa-Bioclin, Belo Horizonte, Brazil): Calcium Arsenazo III (K051) and Phosphorus (K020). Additionally, a drop of blood from each bird was collected, blotted, and dried on filter paper; serum concentration of 25-OH-D3 was then determined via the Dried Blood Spots method using liquid chromatography-tandem mass spectrometry as described by Zakaria et al. (2020).

On day 42, 8 broilers per replicate were randomly chosen and sacrificed and eviscerated to collect the cranial portion of the breast muscle (Pectoralis major). Approximately 100 mg of breast tissue was homogenized, and total RNA was extracted with commercial kits (MVXA-PU16 FAST, Loccus, São Paulo, Brazil). The quality and integrity of RNA was determined spectrophotometrically by OD 260/280 nm absorption ratio >1.95. Afterwards, 1 µg of extracted RNA was reverse transcribed into cDNA using Superscript II Plus RNase H–Reverse Trancriptase (Gibco BRL Life Technologies, Gaithersburg, MD). The mRNA expression level of mTOR kinase was then measured by Real-time qPCR using a Quantinova Sybr Green PCR Kit (QIAGEN

Biotecnologia Brazil; São Paulo, Brazil). Beta-actin and glyceraldehyde-3-phosphate dehydrogenase were used for internal normalization. Cycling conditions were set as 95°C for 10 min, 40 cycles of 95°C for 5s, and 60°C for 1 min. Relative mRNA expression level of mTOR kinase was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Real-time qPCR was conducted by Imunova Análises Biológicas LTDA (Curitiba, Brazil).

Two broilers per replicate were randomly chosen from the 8 broilers sacrificed for collection of breast muscle on d 42 and had both legs manually removed. Tibial bones were excised from the legs without boiling and cleaned of litter and surrounding soft tissue, and immediately frozen at -80ºC. Left bones were cleaned, oven-dried at 105ºC for 12 hours, and ashed in a muffle furnace at 600ºC to determine ash (method 942.05), Ca (method 927.02), and P (method 965.17) contents according to AOAC (2005). Right bones were weighted and used to measure breaking strength (BBS) using a three-point method with a universal testing machine (PMPA - Stable Micro Systems, Surrey, UK). The bone was rested on 2 points with a gap of 50 mm and pressure was applied with a pressure sensitive load cell of 500 kilogram-force at the center of both points at 10 mm/s speed. The force required to fracture the bone was recorded as BBS and expressed in kilogram-force (kgf).

2.4 STATISTICAL ANALYSIS

Each pen with 25 birds per pen was considered an experimental unit. Data were tested for homoscedasticity by Bartlett's test, residual normality by Shapiro-Wilk test, and submitted to a two-way ANOVA to assess the 2 main factors and their interaction using General Linear Model (GLM) of SAS (SAS Enterprise Guide 8.3, SAS Version 9.4; SAS Institute Inc., Cary, NC, 2012).

3. RESULTS

3.1 GROWTH PERFORMANCE

Growth performance during grower (1-to-21-d), finisher (22-to-42-d), and total (1-to-42-d) periods is presented in Table 3. Diet supplementation with 25-OH-D_3 increased BWG by an average 3.17% and reduced FCR by 1.62% in the grower phase

(P < 0.05), as well as increased BWG by 1.82% in the total period (P < 0.05) compared to non-supplemented broiler chickens, although performance during finisher phase was not affected. Phytase did not affect growth performance of grower broiler chickens, but a higher dose (2,000 FYT/kg) reduced FI by 2.3% and FCR by 2.48% in the finisher phase compared to lower dose (600 FYT/kg), which led to a 1.87% reduction of FCR in the total period (P < 0.05).

TABLE 3. GROWTH PERFORMANCE OF BROILER CHICKENS FED DIETS SUPPLEMENTED WITH 25-OH-D3 AND DIFFERENT DOSES OF PHYTASE AT DIFFERENT GROWTH PHASES.

Phytase[1] (FYT/kg)	25-OH-D_3 (µg/kg)	1 to 21 d			22 to 42 d			1 to 42 d		
		FI (kg)	BWG (kg)	FCR	FI (kg)	BWG (kg)	FCR	FI (kg)	BWG (kg)	FCR
Interaction										
600	0	1.15	0.93	1.233	3.80	2.33	1.633	4.95	3.26	1.519
2,000	0	1.17	0.94	1.247	3.71	2.32	1.600	4.88	3.26	1.497
600	69	1.17	0.96	1.226	3.81	2.35	1.625	4.99	3.30	1.509
2,000	69	1.19	0.97	1.223	3.74	2.37	1.578	4.93	3.34	1.475
Pooled SEM		0.014	0.010	0.018	0.031	0.022	0.013	0.037	0.023	0.010
Effect of phytase										
600		1.16	0.94	1.229	3.81	2.34	1.629	4.97	3.28	1.514
2,000		1.18	0.95	1.235	3.72	2.35	1.589	4.91	3.30	1.486
Effect of 25-OH-D_3										
	0	1.16	0.93	1.244	3.76	2.32	1.617	4.92	3.26	1.508
	69	1.18	0.96	1.224	3.78	2.36	1.601	4.96	3.32	1.492
P-values										
Phytase		0.151	0.189	0.206	0.018	0.744	0.004	0.105	0.384	0.009
25-OH-D_3		0.165	0.010	0.023	0.560	0.123	0.251	0.298	0.010	0.131
Interaction		0.788	0.737	0.509	0.785	0.492	0.605	0.882	0.432	0.537

Data represents the mean of 12 replicates (25 broiler chickens per replicate).
FYT = Phytase units; FI = Feed intake; BWG = Body weight gain; FCR = Feed conversion ratio; SEM = Standard error of the mean.
[1] Ronozyme HiPhorius (DSM Nutritional Products, Kaiseraugst, Switzerland) with 20,000 FYT/g of product.
[2] Hy-D Premix (DSM Nutritional Products, Kaiseraugst, Switzerland) providing 69 µg of 25-OH-D_3/kg of feed.

3.2 BLOOD ANALYSIS AND MTOR KINASE MRNA EXPRESSION

No treatment effects were observed for serum Ca and P at 21 or 42 d nor interactions between treatments for blood measurements and relative mTOR gene expression (Table 4). The 25-OH-D_3-supplemented broiler chickens had a respective increase of 26% and 28% on serum 25-OH-D_3 at 21 and 42 d compared to the non-supplemented group (P < 0.001), which was not affected by phytase doses. Expression of mTOR kinase mRNA assessed at 42 d of age was increased by 48% when increasing phytase dose from 600 to 2,000 FYT/kg (P < 0.001).

TABLE 4. SERUM CONCENTRATION OF CA, P, AND 25-OH-D3 AND RELATIVE GENE EXPRESSION OF MTOR KINASE IN BREAST MUSCLE OF 21-D AND 42-D-OLD BROILER CHICKENS FED DIETS SUPPLEMENTED WITH 25-OH-D3 AND DIFFERENT DOSES OF PHYTASE.

Phytase (FYT/kg)[1]	25-OH-D_3 (µg/kg)[2]	21 d			42 d			mTOR RNA[3]
		Ca (mg/dL)	P (mg/dL)	25-OH-D_3 (ng/mL)	Ca (mg/dL	P (mg/dL)	25-OH-D_3 (ng/mL)	
Interaction								
600	0	10.2	6.69	76.1	9.17	5.96	32.6	1.04
2,000		10.3	6.49	74.4	8.76	5.85	33.6	1.68
600	69	10.7	6.84	96.6	9.26	6.08	45.1	1.05
2,000		10.5	6.85	98.8	9.31	6.10	42.9	1.78
Pooled SEM		0.251	0.172	6.593	0.301	0.142	2.502	0.032
Effect of phytase								
600		10.4	6.76	86.3	9.21	6.02	38.8	1.05
2,000		10.3	6.67	86.6	9.04	5.98	38.2	1.73
Effect of 25-OH-D_3								
	0	10.2	6.55	75.2	8.97	5.91	33.1	1.36
	69	10.5	6.84	97.7	9.13	6.06	44.0	1.41
P-values								
Phytase		0.813	0.703	0.949	0.290	0.638	0.819	<0.001
25-OH-D_3		0.171	0.078	<0.001	0.609	0.286	<0.001	0.149
Interaction		0.556	0.664	0.686	0.818	0.764	0.545	0.200

Data represents the mean of 12 replicates (two broiler chickens per replicate).
FYT = Phytase units; SEM = Standard error of the mean.
[1] Ronozyme HiPhorius (DSM Nutritional Products, Kaiseraugst, Switzerland) with 20,000 FYT/g of product.
[2] Hy-D Premix (DSM Nutritional Products, Kaiseraugst, Switzerland) providing 69 µg of 25-OH-D_3/kg of feed.
[3] Fold change of mTOR kinase gene expression relative to reference genes.

3.3 BONE MINERAL COMPOSITION AND BREAKING STRENGTH

No interaction or effect of phytase and 25-OH-D_3 supplementation were found for bone weight, bone contents of ash, Ca, and P, or BBS at 42-d-old (Table 5).

TABLE 5. BONE WEIGHT, MINERAL COMPOSITION, AND BREAKING STRENGTH OF 42-D-OLD BROILER CHICKENS FED DIETS SUPPLEMENTED WITH 25-OH-D3 AND DIFFERENT DOSES OF PHYTASE.

Phytase (FYT/kg)[1]	25-OH-D_3 (µg/kg)[2]	Weight (g)	Ash (%)	Ca (%)	P (%)	BBS (kgf)
Interaction						
600	0	8.42	41.9	17.1	9.00	41.4
2,000		8.32	42.3	16.9	8.89	40.6
600	69	8.31	42.0	16.8	8.98	42.5
2,000		8.14	41.4	17.7	9.22	42.3
Pooled SEM		0.250	0.512	0.283	0.112	1.397
Effect of phytase						
600		8.37	42.0	16.9	8.99	41.9
2,000		8.23	41.8	17.3	9.05	41.4
Effect of 25-OH-D_3						
	0	8.37	42.1	17.0	8.94	41.0
	69	8.23	41.7	17.2	9.10	42.4
P-values						
Phytase		0.105	0.757	0.213	0.563	0.719
25-OH-D_3		0.794	0.448	0.380	0.164	0.328
Interaction		0.893	0.349	0.075	0.127	0.822

FYT = Phytase units; BBS = Bone breaking strength; SEM = Standard error of the mean.

[1]Ronozyme HiPhorius (DSM Nutritional Products, Kaiseraugst, Switzerland) with 20,000 FYT/g of product.
[2]Hy-D Premix (DSM Nutritional Products, Kaiseraugst, Switzerland) providing 69 µg of 25-OH-D_3/kg of feed.

4. DISCUSSION

No interactions were observed between 25-OH-D_3 and the phytase dose for any of the evaluated variables, so only the main effects will be discussed further on.

Grower broiler chickens (1-to-21-d-old) had higher BWG and lower FCR when supplemented with 25-OH-D_3. Although some studies report a lack of effect of 25-OH-D_3 on growth performance of broiler chickens (Bozkurt et al., 2017; Sakkas et al., 2019), improvements on BWG and FCR with 25-OH-D_3 have been observed by Zhang et al. (2020), as well as on a previous study (Bassi et al., 2023) when assessing inclusion of the metabolite with low dietary levels of Ca and P. Ca and P deficiencies can adversely impact performance due to a limited Ca and P pool available for bone and skeletal muscle development, hindering the animal's growth (Valable et al., 2018; Xu et al., 2021). Moreover, a state of hypocalcemia and hypophosphatemia can stress the VitD-regulated metabolic pathway and increase the need for VitD supplementation (Omotoso et al., 2023). Presumably, 25-OH-D_3 supplementation along with regular VitD inclusion (4,000 UI/kg) was able to meet VitD needs of Ca and P-deficient broiler chickens and overcome the loss of performance during grower phase. In contrast, 25-OH-D_3 had no effect on growth performance of finisher broiler chickens (22-to-42-d-old), which could be related to the lower requirements for Ca and P at an older age (David et al., 2023). Additionally, Imari et al. (2020) suggests that broiler chickens can adapt to low Ca and P diets fed during starting phases, helping them in using Ca and P more efficiently at a later age. Applying this theory and the remarks of Omotoso et al. (2023) to the current study, it could be assumed there was an adaptive capacity of the body to maintain a greater production of 1,25-OH_2-D_3 in response to a long exposure to low Ca and P intakes, soothing out the effect of dietary 25-OH-D_3 supplementation.

Increasing the phytase dose from 600 to 2,000 FYT/kg did not affect performance during growing phase, contrary to observations by other studies where high doses of phytase (> 1,000 units/kg) led to greater performance in 21-d-old broiler chickens (Beeson et al., 2017; Lee et al., 2017; Walk et al., 2013). It has been outlined by Li et al. (2018) and Babatunde et al. (2019) that age and duration of feeding of low Ca and P diets can influence phytase efficacy, with both studies evidencing that the impact of mineral deficiencies and benefits of phytase inclusion are greater in younger

birds whose endogenous enzyme apparatus is underdeveloped. Both studies were limited to assessing broilers up to 21 d of age, though. In contrast, FI was increased and FCR reduced by increasing the phytase dose during finisher phase in this study, agreeing with other reports on improved performance of older broiler chickens fed high doses of phytase (Lee et al., 2017; Taheri et al., 2015). FCR was reduced by higher phytase dose but not affected by 25-OH-D_3 during finisher phase. This might indicate the improvement on performance was derived not from greater Ca and P utilization (already achieved with 25-OH-D_3), but from extra-phosphoric effects, i.e. a greater availability of phytate-bound nutrients other than P, such as amino acids, starch, and other minerals (Cowieson et al., 2009; Walk and Rama Rao, 2020), enabling a higher BWG at lower FI, thus reducing FCR.

The VitD metabolite supplementation increased serum 25-OH-D_3 during both phases, in line with a previous study (Bassi et al., 2023) and other research (Bozkurt et al., 2017; Hutton et al., 2014; Sakkas et al., 2019). Serum 25-OH-D_3 is a marker of VitD status, and its increment in broiler chickens fed dietary 25-OH-D_3 can be related to a greater affinity of vitamin D-binding proteins for the metabolite, optimizing its intestinal absorption (Fatemi et al., 2020). An improved VitD status was then expected to directly increase Ca and P absorption, leading to greater serum Ca and P, but no effect was observed during neither grower nor finisher phase. This is contrary to the findings of Tizziani et al. (2019) who found higher serum P in 42-d-old broilers fed diets with 25-OH-D_3 compared to inclusion of VitD only, and Marques et al. (2022) who observed an increase in both serum Ca and P with dietary 25-OH-D_3 for finishing broiler chickens. It can be speculated in this case that low dietary Ca and P stressed the PTH/1,25-OH_2-D_3 pathway to a threshold where dietary supplementation exerted no further effect on Ca and P transport, or that regular dietary VitD levels added in the premix were sufficient to maintain Ca homeostasis in the extracellular fluid.

Increasing the dose of phytase from 600 to 2,000 FYT/kg had no effect on serum minerals. Phytase effects on increasing Ca and P digestibility for poultry are well-stablished (Dersjant-Li et al., 2015; Selle and Ravindran, 2007). Moreover, the use of phytase doses above 1,000 units/kg can further enhance ileal digestibility of Ca and P (Bassi et al., 2022; Walk et al., 2013; Walk and Olukosi, 2019). A heightened digestibility could lead to greater mineral serum concentrations, which was not observed in this study. It is possible that a regular dose of 600 FYT/kg was enough to achieve an adequate rate of Ca and P absorption into circulation, or rather that mineral

concentration in the extracellular fluid was tightly regulated by PTH/1,25-OH_2-D_3 pathway (Bergwitz and Jüppner, 2010). Increasing phytase dose had no influence on serum 25-OH-D_3 levels possibly for the same reason.

The mTOR kinase, also referred to as the mechanistic target of rapamycin, is a protein kinase that regulates processes related to protein synthesis and cell differentiation and proliferation. The mTOR pathway, as thoroughly described by Xu and Velleman (2023) is crucial for skeletal muscle physiology in poultry birds, having a major role in hypertrophic growth and deposition of muscle mass. Receptors for VitD (VDR) have been identified in myoblasts of rats, directly linking the metabolism of VitD to muscle hypertrophy mediated by mTOR pathway (Oku et al., 2016). Moreover, studies indicated how dietary VitD metabolites can affect mTOR expression in poultry birds (Prokoski et al., 2021; Vignale et al., 2015). Vignale et al. (2015) reported an increase in the expression of VDR and mTOR in the breast muscle of 42-d-old broiler chickens supplemented with 25-OH-D_3, along with greater serum 25-OH-D_3 and breast meat yield. Likewise, Prokoski et al. (2021) saw an increased mTOR gene expression and breast protein deposition in 46-d-old broiler chickens fed 25-OH-D_3-diets, to which the authors refer as non-classical effects alongside the classical effects of improved growth performance and bone quality. Although both referred studies used the same 25-OH-D_3 vitamin and dose, no effects on mTOR kinase expression were detected in the current study.

Phytase supplementation can benefit carcass and breast muscle yields (Kriseldi et al., 2021; Taheri and Mirisakhani, 2020), an effect that, besides resulting from higher nutrient availability, can be indirectly linked to an enhanced mTOR pathway. Józefiak et al. (2010) observed that phytase supplementation up-regulated insulin receptors and led to an increased sensitivity of liver cells to insulin, which would stimulate insulin-dependent pathways, including those related to protein turnover and cell growth. Schmeisser et al. (2017) similarly found a greater mRNA expression of phosphatidylinositide-3-phosphate kinase and insulin-like growth factor (whose pathways link to mTOR pathway) in breast muscle of phytase-fed broiler chickens, leading to greater breast meat yield. As further construed by Kriseldi et al. (2021), this higher activation of protein synthesis pathways is achieved by *myo*-inositol; by breaking down phytate and increasing *myo*-inositol uptake and plasma concentration, phytase supplementation indirectly bolsters muscle mass accretion. A greater availability of *myo*-inositol through higher doses of phytase in poultry diets has been

supported by the literature (Bassi et al., 2022; Bello et al., 2019; Hirvonen et al., 2019). Conceivably, increasing the phytase dose from 600 to 2,000 FYT/kg in this study enhanced breast muscle mRNA expression at 42-d-old, likely related to *myo*-inositol release.

Vitamin D is synonymous with bone mineralization and health. The effects of dietary supplementation of VitD metabolites to poultry diets on enhancing Ca and P bone deposition and increasing bone strength are widely recognized (Colet et al., 2015; Garcia et al., 2013castro 2018; Świątkiewicz et al., 2016). Phytase supplementation has the same approach, where a greater phytic-P availability can be utilized for bone tissue accretion, and higher doses of the enzyme can boost the provision of dietary Ca and other nutrients for skeletal growth (Bassi et al., 2021; Beeson et al., 2017; Taheri et al., 2015; Walk et al., 2013).

Low dietary levels of Ca and P are detrimental to bone density and strength, which can then be ameliorated by 25-OH-D_3 (Zhang et al., 2020) and phytase (Catalá-Gregori et al., 2007). However, in the current study, neither supplying 25-OH-D_3 nor increasing phytase dose in Ca and P-deficient broiler chickens influenced bone mineral composition and BBS at 42-d-old. At this age, requirements of Ca and P for broiler chickens are lower than at a starter phase and the skeletal system is well-developed (Fleming, 2008), hence why 25-OH-D_3 and phytase had no accrual effects on bone mineralization.

5. CONCLUSION

The lack of interaction between treatments rejects the hypothesis that 25-OH-D_3 supplementation would have additive or synergic effects with a high dose of phytase in broiler chicken diets with low levels of Ca and P, yet individual effects were noteworthy. Dietary supplementation with 25-OH-D_3 combined with regular vitamin D_3 levels can improve growth performance of growing broilers and enhance vitamin D_3 throughout grower and finisher phases. Increasing the dose of phytase from 600 to 2,000 FYT/kg led to a lower feed conversion ratio by reducing average feed intake while maintaining weight gain, and a greater mRNA expression of mTOR kinase in breast muscle showcases potential benefits to skeletal muscle and breast meat yield.

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FINAL CONSIDERATIONS

The importance of calcium (Ca) and phosphorus (P) to the various metabolic process of the animal body makes them undisputedly the most nutritionally and economically relevant minerals in poultry diet formulations. To ensure that Ca and P intakes meet the animal needs and that both minerals are appropriately absorbed, metabolized, and consolidated into the plasmatic pool is to ensure an optimal growth performance, bone quality, health, and welfare during its lifetime.

The intensive genetic selection process of broiler chickens over the years albeit leading to a fast growth and significantly higher body weight gain has resulted in a faulty development of the skeletal system, and modern broilers were faced with a higher incidence of locomotion problems and skeletal disorders. For bone development to match muscle mass accretion, dietary Ca and P needs were magnified, equally increasing the requirements for vitamin D, one of the crucial components behind Ca and P homeostasis. 25-hydroxycholecalciferol (25-OH-D_3), one of the metabolites of vitamin D_3 has been identified as having a greater bioavailability and intestinal absorption rate compared to its predecessor molecule, raising interest among industry and academy alike on investigating the inclusion of 25-OH-D_3 in poultry diets. The experiments carried out in this dissertation provided valuable insight into understanding how 25-OH-D_3 enhances dietary Ca and P utilization and consequently influences growth performance and bone mineralization.

The first experiment hypothesized an interaction between 25-OH-D_3 and available P levels, anticipating that a reduction of available P down to 0.36% (akin to increasing Ca:P ratio) would stress P homeostasis and reinforce the action of 25-OH-D_3. Despite a lack of interaction, the results proved the beneficial effects of combining a commercial recommendation of 69 µg of 25-OH-D_3 per kg with regular vitamin D_3 levels on directly increasing Ca and P digestibility and vitamin D status, which prompted greater bone ash content and improved growth performance. The same premise was adopted on the second experiment, this time reducing both dietary Ca and P altogether even further (down to 0.6% Ca and 0.3% P). Still no interaction was triggered, but the previously observed effects of 25-OH-D_3 remained, i.e. improved vitamin D status, growth performance, greater Ca and P digestibility, higher bone ash and breaking strength. The most interesting aspect of the second experiment was how 25-OH-D_3 resulted in greater serum concentrations of Ca and P regardless of their

dietary levels, implying that the metabolite induced a predominantly active transport of the minerals in the intestinal epithelium. Although active transport systems of Ca and P can be saturated at high mineral intake, it may be assumed that 25-OH-D_3 intensified the capacity of such systems by, among other factors, stimulating the expression of vitamin D receptors and transport proteins in intestinal and renal cells.

In the third experiment, phytase was incremented into the factorial. Because of the enzyme's role in breaking down phytate and increasing the availability of Ca and P, an interaction with 25-OH-D_3 was expected, which would signal additive/synergic effects. The interaction hypothesis was rejected, but individual effects were noteworthy. While the first and second studies lasted up to 21 days of age, the third experiment was conducted up to 42 days. Vitamin D status was again enhanced by 25-OH-D_3 at both growing (1 to 21 days) and finishing (22 to 42 days) broilers, although effects of the metabolite on growth performance were more prominent in the growing phase. Increasing the dose of phytase (from 600 to 2,000 FYT/kg) improved performance of finishing broilers (22 to 42 days of age), likely related to extra-phosphoric effects of higher doses, i.e. higher availability of phytate-bound nutrients beyond P, given that serum Ca and P remained unaltered. The heightened relative expression of mTOR kinase in breast muscle, induced by higher phytase dose, reflects an increased capacity of skeletal muscle growth. This aligns with the idea of an increased nutrient availability enabled by extra-phosphoric effects.

The low-cost of Ca sources often leads to their overuse, and an oversupply of Ca is harmful to nutrient availability; equally harmful is an undersupply of Ca, not unusual in the industry due to a lack of proper ingredient analysis and formulation. In contrast, P sources are onerous to nutrition and feed production costs, in addition to the excess of dietary being highly detrimental to the environment. Supplementation of poultry diets with vitamin D metabolites and higher doses of phytase is surely essential to prevent said issues with Ca and P availability. The outcome of all three studies helps deepen the knowledge on the direct incorporation of 25-OH-D_3 into broiler chicken diets, further establishing the metabolite as an important tool for an optimal mineral nutrition of poultry birds.

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ANNEX I APPROVAL BY THE ANIMAL ETHICS COMMITTEE (Nº 037/2020)

UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS AGRÁRIAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o protocolo número 037/2020, referente ao projeto de pesquisa **"Avaliação da interação entre vitamina D e fósforo disponível em dietas para frangos de corte contendo doses altas de fitase"**, sob a responsabilidade de **Alex Maiorka** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DO SETOR DE CIÊNCIAS AGRÁRIAS DA UNIVERSIDADE FEDERAL DO PARANÁ - BRASIL, com grau 2 de invasividade, em 02/10/2020.

Finalidade	Pesquisa
Vigência da autorização	Outubro/2020 até Outubro/2020
Espécie/Linhagem	*Gallus gallus domesticus* (ave)/Ross 308
Número de animais	560
Peso/Idade	0,045 kg/1 dia
Sexo	Macho
Origem	Incubatório comercial, Castro/PR, Brasil.

*A autorização para início da pesquisa se torna válida a partir da data de emissão deste certificado.

CERTIFICATE

We certify that the protocol number 037/2020, regarding the research project **"Evaluation of the interaction between vitamin D and available phosphorus in broiler diets with high levels of phytase"** under **Alex Maiorka** – which includes the production, maintenance and/or utilization of animals from Chordata phylum, Vertebrata subphylum (except Humans), for scientific or teaching purposes – is in accordance with the precepts of Law nº 11.794, of 8 October 2008, of Decree nº 6.899, of 15 July 2009, and with the edited rules from Conselho Nacional de Controle da Experimentação Animal (CONCEA), and it was approved by the ANIMAL USE ETHICS COMMITTEE OF THE AGRICULTURAL SCIENCES CAMPUS OF THE UNIVERSIDADE FEDERAL DO PARANÁ (Federal University of Paraná, Brazil), with degree 2 of invasiveness, in session of 10/02/2020.

Purpose	Research
Validity	October/2020 until October/2020
Specie/Line	*Gallus gallus domesticus* (poultry)/Ross 308
Number of animals	560
Weight/Age	0.045 kg/1 day
Sex	Male
Origin	Commercial farm, Castro/PR, Brazil.

*The authorization to start the research becomes valid from the date of issue of this certificate.

Curitiba, 06 de outubro de 2020

Simone Tostes de Oliveira Stedile

Coordenadora CEUA-SCA

ANNEX II APPROVAL BY THE ANIMAL ETHICS COMMITTEE (Nº 020/2021)

UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS AGRÁRIAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o protocolo número 020/2021, referente ao projeto de pesquisa **"Efeito dos níveis de cálcio e fósforo disponível e inclusão de 25-hidróxi-vitamina D em dietas para frangos de corte",** sob a responsabilidade de **Alex Maiorka** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DO SETOR DE CIÊNCIAS AGRÁRIAS DA UNIVERSIDADE FEDERAL DO PARANÁ - BRASIL, com grau 2 de invasividade, em 09/04/2021.

Finalidade	Pesquisa
Vigência da autorização	Abril/2021 a Maio/2022
Espécie/Linhagem	*Gallus gallus domesticus (*galinha*)*
Número de animais	560
Peso/Idade	45g/1 dia
Sexo	Macho
Origem	Incubatório comercial

*A autorização para início da pesquisa se torna válida a partir da data de emissão deste certificado.

CERTIFICATE

We certify that the protocol number 020/2021, regarding the research project **"Effect of calcium and available phosphorus levels and inclusion of 25-hydroxy-vitamin D in broiler diets"** under **Alex Maiorka** – which includes the production, maintenance and/or utilization of animals from Chordata phylum, Vertebrata subphylum (except Humans), for scientific or teaching purposes – is in accordance with the precepts of Law nº 11.794, of 8 October 2008, of Decree nº 6.899, of 15 July 2009, and with the edited rules from Conselho Nacional de Controle da Experimentação Animal (CONCEA), and it was approved by the ANIMAL USE ETHICS COMMITTEE OF THE AGRICULTURAL SCIENCES CAMPUS OF THE UNIVERSIDADE FEDERAL DO PARANÁ (Federal University of Paraná, Brazil), with degree 2 of invasiveness, on 2021, April 9th.

Purpose	Research
Validity	April/2021 until May/2021
Specie/Line	*Gallus gallus domesticus (*hens*)*
Number of animals	560
Weight/Age	45g/1 day old
Sex	Male
Origin	Commercial hatchery

*The authorization to start the research becomes valid from the date of issue of this certificate.

Curitiba, 09 de abril de 2021

Maity Zopollatro

Coordenadora pro-tempore

CEUA/AG/UFPR

ANNEX III APPROVAL BY THE ANIMAL ETHICS COMMITTEE (Nº004/22)

DSM	IDENTIFICAÇÃO DO PROJETO E APROVAÇÃO CEUA	F-ANC-001

CEUA – I&AS
ACESSO RESTRITO

Segundo a Comissão de Ética no Uso de Animais do *Innovation and Applied Science* (CEUA-I&AS) o projeto de ID "BR 220402", com a espécie aves, alocado no galpão experimental de Frangos de Corte, foi submetido à apreciação, tendo sido o mesmo considerado **APROVADO** sob o parecer de Nº 004/22, estando desta forma, liberada a execução do projeto desde que respeitada a descrição da metodologia apresentada a CEUA.

Período de vigência do projeto

Início 03/05/2022 Término 14/06/2022

Pesquisador Responsável: Claudia Silva

Telefone para Contato: 11 92125-1856

Coordenador do CEUA: Letícia Cardoso

Telefone para Contato:

Mairinque, 21/03/2022.

É obrigatório que esta identificação seja fixada em local visível durante arealização do(s) experimento(s)