# EFFECTS OF 25-HYDROXYVITAMIN $D_3$ ON GROWTH PERFORMANCE, BONE HEALTH, BLOOD CHEMISTRY, AND IMMUNE RESPONSE IN BROILERS EXPOSED TO DIFFERENT STRESS CONDITIONS

by

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(Under the Direction of CHONGXIAO CHEN)

## ABSTRACT

Vitamin D plays multiple physiological roles, including enhancing intestinal calcium and phosphate absorption, stimulating bone resorption, and promoting renal calcium reabsorption. 25-hydroxyvitamin D3 [25(OH)$D_3$] is a secondary metabolite, which is commercially available, that has been shown to have higher bioactivity than vitamin $D_3$ in poultry. Research has examined its role in bone health and performance in broilers, but little is known about the role of 25(OH)$D_3$ in broilers under stress, such as hot weather stress or coccidiosis. The current experiments were conducted to evaluate 25(OH)$D_3$ in broilers during hot weather and coccidiosis. Under elevated temperatures, supplementing different 25(OH)$D_3$ levels altered circulating metabolites, improved performance, and enhanced bone health. In coccidiosis-challenged broilers, infection reduced circulating 25(OH)$D_3$ concentrations, impaired growth performance, bone integrity, and immune response by altering cytokine and chemokine profiles.

Supplementing 25(OH)$D_3$ restored circulating levels, bone health, acid-base balance, and showed cytokine modulation effects during coccidiosis challenge while consequently reducing cecal lesions. Overall, 25(OH)$D_3$ supplementation benefited broilers beyond their classical roles, improving growth, bone health, and immune response under environmental or disease-related stresses.

INDEX WORDS: 25-hydroxyvitamin $D_3$, hot weather, vaccine, coccidiosis challenge, bone health, growth performances

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# DEDICATION

This thesis is dedicated to my parents, Rosario Abaunza and Jhon Mejía. Thank you for your unwavering support throughout my academic journey and for always motivating me to keep growing.

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# CHAPTER 1

# INTRODUCTION

Vitamins are low-molecular-weight organic compounds that promote and regulate essential biochemical reactions for the animal body, and normal physiological functions (Herrero et al., 2012). They are classified into fat and water-soluble vitamins: vitamin B complex and ascorbic acid belong to the water-soluble, and vitamins A, D, E, and K are fat-soluble vitamins. Among the fat-soluble vitamins, vitamin D, called the "sunshine vitamin," is frequently used to refer to a group of compounds with antirachitic activity, further classified as ergosterol (vitamin $D_2$) and cholecalciferol (vitamin $D_3$). However, vitamin $D_2$ is derived from fungi and yeast, which undergo a photochemical reaction caused by ultraviolet (UV) radiation from sunlight to form ergocalciferol. On the other hand, vitamin $D_3$ is produced only in animal skin following exposure to UV radiation. On its own, vitamin D is biologically inactive and must be converted to the active form through two hydroxylations. In poultry houses, windows or artificial light may be included to stimulate endogenous vitamin $D_3$ synthesis, although it is unlikely that they are able to provide the UVB required by birds (Ogbonna et al., 2023). Therefore dietary supplementation of vitamin $D_3$ is a common practice in commercial poultry production. The provitamin 7-dehydrocholesterol, derived from cholesterol, is synthesized in the body and is present in large amounts in the skin, the intestinal wall, and other tissues, but it lacks antirachitic activity. Following exposure to sunlight, 7-dehydrocholesterol is converted to previtamin $D_3$ and rapidly converted by a heat-dependent process to vitamin $D_3$ (Holick, 2010). Vitamin $D_3$ is then removed from the skin and transported to the blood, bound to its carrier protein, vitamin D-binding protein (DBP). The role of DBP is to

transport vitamin $D_3$ or their metabolites in the blood to the target organs. Additionally, DBP is important in the translocation of vitamin D from the skin into the blood circulation (Holick, 2010).

After vitamin $D_3$ binds to DBP, it is transported to the liver, where it is hydroxylated on C-25 to form 25-hydroxyvitamin D [$25(OH)D_3$]. This metabolite serves as the primary storage form in the blood and is commonly used as an indicator of vitamin D nutritional status. The hepatic conversion of vitamin $D_3$ and $25(OH)D_3$ is a minimally regulated step that occurs mainly in proportion to substrate availability (Henry, 2011). To become active, $25(OH)D_3$ is then transported to the kidney, where it is converted to 1,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$]. The second, and most important, hydroxylation occurs in the kidney mitochondria and results in the synthesis of the active hormone. Contrary to the previous steps, the conversion from $25(OH)D_3$ to $1,25(OH)_2D_3$ is highly regulated by endocrine factors that mostly respond to changes in plasma calcium and/or phosphorus (Pike & Christakos, 2017).

$1,25(OH)_2D_3$ is highly regulated by two hormones: parathyroid hormone (PTH) and fibroblast growth factor (FGF-23). Furthermore, high plasma $1,25(OH)_2D_3$ concentration inhibits renal 1α-hydroxylase and affects tissue 24-hydroxylase by the negative feedback, although the mechanism is not fully understood (Gil et al., 2018). Thus, the production and catabolism of the hormone $1,25(OH)_2D_3$ are tightly regulated before acting directly through the intestine, kidney, and bone mineral balance. When dietary calcium and phosphate are low, the effects of vitamin $D_3$ on the kidney and bone promote calcium and phosphate retention to support new mineralization. However, once calcium and phosphate requirements are met, the intestinal role of vitamin $D_3$ becomes dominant, as PTH hormone secretion is suppressed. The absence of PTH consequently inhibits bone resorption, conserving bone calcium. The FGF-23, a bone-derived hormone, downregulates 1α-hydroxylase activity in renal tubular epithelial cells, thereby reducing

$1,25(OH)_2D_3$ synthesis (Razzaque, 2022). The active form of vitamin $D_3$ stimulates calcium and phosphate mobilization from the skeleton by renewing osteoclast activity through stimulation of bone-reabsorbing osteoclasts and new osteoclast formation (Pike & Christakos, 2017).

The mechanism of action of $1,25(OH)_2D_3$ is mediated by the vitamin D receptors (VDRs). The VDRs are widely distributed in humans and animals, including cells of the intestinal epithelium, renal tubules, parathyroid gland cells, skin, mammary epithelium, pancreas, pituitary gland, skeleton, immune system, and germ tissues. Among these tissues, the highest VDR content is in the intestine, kidney, parathyroid gland, and bone (Wang et al., 2012). It is well known that vitamin $D_3$ plays different roles in the body, and it is also crucial to the immune system, regulating hormones that affect immune responses (Aslam et al., 1998; Vazquez et al., 2018). However, vitamin $D_3$ overdose can cause toxicity, leading to various problems in chickens, including reduced feed intake and increased feed conversion ratios. Additionally, toxicity is associated with hypercalcemia, twisted legs, and abnormal deposition of calcium affecting the kidneys due to an increase in calcium and phosphorus levels in the blood (Yarger, Quarles, et al., 1995). In contrast, when vitamin $D_3$ is deficient in the body, it can cause skeletal issues such as rickets in young birds, tibial dyschondroplasia, or osteoporosis in mature birds, especially in layers (Leeson & Summers, 2001). Vitamin D-deficient animals also showed lower growth performance, lower bone ash, and reduced cellular immune response (Aslam et al., 1998).

Findings regarding the effects of vitamin $D_3$ or their metabolites are inconsistent among published studies. In yellow-feathered broilers, supplementation with vitamin $D_3$ for 63 days at three different inclusion levels, low (1,200/1,000 IU/kg), medium (2,400/2,000 IU/kg), and high (3,600/3,000 IU/kg) during the starter, grower/finisher phases affected growth performance, carcass traits, bone characteristics, and intestinal morphology. Villus length and villus height-to-

crypt depth (V/C) ratio increased, which may enhance nutrient absorption and improve growth performance; this effect was not observed in diets without vitamin $D_3$ (Wei et al., 2024). Another study found that feeding cholecalciferol, 25(OH)$D_3$, or 1,25$(OH)_2D_3$ did not affect bone health in broilers. However, supplementation with 1α(OH)$D_3$ during any period (0–7, 8–21, or 22–42 days) reduced growth performance(Garcia et al., 2013). However, in broilers exposed to cyclic heat stress, supplementation with 69 μg/kg of 25(OH)$D_3$ mitigated weight loss, increased bone mineralization, and elevated serum 25(OH)$D_3$ levels. These discrepancies may be attributed to multiple factors, including rearing conditions, metabolite type, inclusion level, genetic line, or challenge conditions. This review will discuss vitamin D metabolism, requirements, and supplementation in poultry production, with a focus on broilers. Moreover, the roles of vitamin $D_3$ and 25(OH)$D_3$ under various stressors, including immunological and environmental stressors, are examined. In addition to highlighting the existing information on the non-classical functions of vitamin D, little is known about the use of vitamin $D_3$ or 25(OH)$D_3$ under hot weather conditions during the final week of production, or about the effects of coccidiosis vaccination followed by an *Eimeria* spp. challenge in broilers.

The main objective of this study was:

1. To explore the effect of 25-hydroxyvitamin $D_3$ on broiler growth performances and bone quality from 0-42 days during elevated rearing temperatures (36-42d).
2. To determine the effects of vitamin $D_3$ and 25-hydroxyvitamin $D_3$ on the growth performances, coccidiosis status, and bone health of coccidiosis-vaccinated chickens challenged with *Eimeria* spp.

3. To determine and understand the effect of vitamin $D_3$ and 25-hydroxyvitamin $D_3$ on blood chemistry and the immune response of coccidiosis-vaccinated chickens challenged with *Eimeria* spp.

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# CHAPTER 2

# LITERATURE REVIEW

## Vitamin D metabolism in chicken

Vitamin $D_3$ is important for broiler bone health and locomotor movement especially for fast growing breeds. Vitamin $D_3$ can be acquired in two ways: UV light synthesis or dietary supplementation. When the skin is exposed to sunlight, UV rays penetrate the dermis and epidermis, activating 7-dehydrocholesterol, which breaks the B ring to form the previtamin $D_3$, that is then rapidly converted by a heat-dependent process to vitamin $D_3$, and finally undergoes subsequent hydroxylation to become active. However, for optimal vitamin $D_3$ production in the skin, the UV energy must range from 290 to 315 nm (Holick, 2008). In modern production systems, either closed houses or windowed houses do not provide enough UV light to produce sufficient vitamin $D_3$. In addition, feathers act as a barrier to the skin, preventing UV rays from reaching the skin. For this reason, vitamin $D_3$ supplementation is important for poultry in intensive production systems. Once Vitamin $D_3$ is consumed by chicken and absorbed in the portal circulation from the intestinal tract by passive diffusion, it is transported to the liver, where the first hydroxylation occurs via the cytochrome P450 (CYP) family, specifically 25-hydroxylase (CYP2R1 and CYP27A1). In this step, vitamin $D_3$ is converted to 25-hydroxychocalciferol [25(OH)$D_3$] (Marques et al., 2022; Oviedo-Rondon et al., 2023). 25(OH)$D_3$ is biologically inactive and it is the main stored form of vitamin D in the body and the primary circulating form that is usually used to determine vitamin D status (Holick, 2008). Vitamin D-binding protein (DBP), is also produced by the liver, and has the greatest affinity for 25(OH)$D_3$, serving as the primary carrier protein for

$25(OH)D_3$. It is crucial in transporting $25(OH)D_3$ from the liver to the kidneys and in other organs (Bhan, 2014). Mainly in kidney, $25(OH)D_3$ is transformed into its bioactive form, 1,25-dihydroxychocalciferol [$1,25(OH)_2D_3$] by 1α-hydroxylase (CYP27B1). $1,25(OH)_2D_3$ interacts with its nuclear vitamin D receptor (VDR) and executes its function in different aspects of calcium and phosphorus metabolism, bone remodeling, and immune regulation (Khan et al., 2023). The production of active vitamin D is tightly regulated by PTH in response to blood calcium and phosphorus levels. According to Khundmiri et al. (2016), PTH regulates vitamin D metabolism primarily by affecting two renal enzymes. PTH increases the expression and activity of CYP27B1 in the proximal renal tubules, which catalyzes the conversion of 25-hydroxyvitamin D to its active form, $1,25(OH)_2D_3$. Additionally, PTH variably suppresses or increases CYP24A1, the enzyme responsible for degrading active vitamin D to 24,25-dihydroxyvitamin D. This downregulation of CYP24A1 favors the preservation of active vitamin D, reducing its catabolism and urinary loss. Finally, elevated levels of $1,25(OH)_2D_3$, in turn, activate CYP24A1 expression to self-limit its concentration and promote vitamin D inactivation and excretion, establishing a feedback loop that balances PTH action. In this way, PTH promotes vitamin D activation and decreases its excretion by stimulating CYP27B1 and suppressing CYP24A1. When PTH levels drop or FGF23 levels increase, CYP24A1 becomes dominant, leading to increased vitamin D breakdown to calcitroic acid, which is excreted in the uric urine, the main route of vitamin D disposal. However, when calcium and phosphorus concentrations are normal and no PTH is secreted, the kidneys convert 25-hydroxyvitamin $D_3$ to 24,25-dihydroxyvitamin $D_3$ and subsequently to 1,24,25-trihydroxyvitamin $D_3$. This metabolite was first isolated from chick kidneys and is approximately 60% as active as vitamin $D_3$ in curing rickets (Holick et al., 1973). Both $1,25(OH)_2D_3$ and $25(OH)D_3$ are metabolized by CYP24A1 (24-hydroxylase) in the liver or kidney to form

1,24,25(OH)$D_3$ or 24,25(OH)$D_3$. Ultimately, CYP24A1 initiates either the C24 or C23 oxidation pathway, serving as a precursor for multiple metabolic routes that regulate the excretion of 1,25$(OH)_2D_3$ (Veldurthy et al., 2016). The active form of vitamin D has a half-life of approximately 6 hours, 25(OH)$D_3$ has a half-life of about 20 days, and 24,25$(OH)_2D_3$ has a half-life of around 2 days (Leeson & Summers, 2019).

**Vitamin $D_3$ function**

Vitamin D regulates the absorption, transport, deposition, and mobilization of calcium (Ca) and phosphorus (P). Furthermore, the active metabolite works together with PTH, which is the major modulator of bone and mineral metabolism through its regulation of calcium and P homeostasis (Jacquillet & Unwin, 2019). Consequently, improper vitamin D supplementation could lead to hypocalcemia due to decreased calcium absorption from the diet. Therefore, increasing PTH would stimulate the synthesis of 1,25$(OH)_2D_3$ to elevate plasma Ca and P and restore the normal balance (Adhikari et al., 2020; Garcia et al., 2013; Oviedo-Rondon et al., 2023). Besides Ca and P absorption and reabsorption, the active form of vitamin $D_3$ has active functions in bone health, immune system modulation, muscle cell differentiation, and reproduction. In poultry, the relationships among Ca, P, and vitamin $D_3$ have been widely studied. Waldroup et al. (1963) found that adding extra vitamin $D_3$ increased body weight, bone ash percentage, and noted that chicks tolerated higher inclusion levels up to 360 IU/lb. Additionally, they reported that the response to increased vitamin $D_3$ levels decreased as the Ca:P ratio increased. Similarly, Shafey et al. (1990) reported that vitamin $D_3$ supplementation significantly increased plasma total calcium compared with diets lacking vitamin $D_3$. Calcium and phosphorus are absorbed in the small intestine. Calcium is absorbed through active transcellular transport or passive transport within enterocytes, and its movement depends on various Ca-binding proteins (CaBP-D28k and CaBP-

D9k), the sodium–calcium exchanger, and plasma membrane calcium ATPase 1b (Han et al., 2023). An experiment evaluating different dietary concentrations of vitamin $D_3$ (0, 125, 250, 500, 1,000, and 2,000 IU/kg) on growth performance, bone health, and gene expression of intestinal Ca and P transporters showed that vitamin $D_3$ supplementation increased mRNA expression of these transporters. This demonstrated that vitamin $D_3$ promoted the exchange of Ca and P between intestinal cells and the bloodstream, with the greatest increases (0.57- 1.74-fold) observed at 1,000–2,000 IU/kg (Han et al., 2023). These results highlight the benefits of supplementing adequate vitamin $D_3$ levels to improve growth performance, bone health, and increase Ca and P absorption in broiler production.

Vitamin D has been found to have immunomodulatory functions. In humans, vitamin D helps maintain intestinal homeostasis by preserving physical barriers, modulating gut microbiota, and regulating immune responses. At the intestinal barrier, VDR signaling strengthens mucus layers and epithelial tight junctions, preventing bacteria and toxins from crossing into tissues. Vitamin D deficiency results in a thinner mucus layer and increased intestinal permeability (Fakhoury et al., 2020). Similarly, VDRs are present in various immune cells and help regulate immune activity. Vitamin D reduces inflammation by suppressing Th1 and Th17 immune cells while promoting regulatory T cells. However, deficiency or alteration of vitamin D receptors can increase intestinal inflammation and worsen Crohn's disease and ulcerative colitis in humans (Fakhoury et al., 2020). In poultry, vitamin D induces similar responses, but poultry production faces greater stress factors and higher bacterial loads than humans. Heat stress is common in tropical climates and during hot summer periods. Elevated temperatures impair gut integrity and activate the innate immune system, leading to systemic inflammation (Zhang et al., 2021). In broilers, supplementation with 69 μg/kg of $25(OH)D_3$ prevented increases in intestinal

permeability and reductions in villus height, villus-to-crypt ratio, tight junction proteins, and *mucin*-2 transcription under hot weather stress, while decreasing B and T lymphocytes and proinflammatory cytokines IL-1β and TNF-α (Zhang et al., 2021). Chou et al. (2009) conducted three experiments to evaluate the effects of 25(OH)$D_3$ on growth performance, small intestine morphology, and immune response during challenge. During the third experiment, broilers were orally challenged with either Luria–Bertani broth or *Salmonella* Typhimurium E29 at 7 and 14 days of age to assess immune responses in birds supplemented with 25(OH)$D_3$. Although 25(OH)$D_3$ supplementation did not affect growth performance, it positively influenced villus length, crypt depth, and improved humoral immune responses during infection. These results demonstrate that vitamin D participates in several physiological processes beyond calcium and phosphorus regulation, contributing to better intestinal integrity and nutrient absorption under environmental or disease-induced stress. Moreover, vitamin D acts as an immunomodulator, regulating excessive immune responses to pathogenic bacteria.

**Vitamin $D_3$ requirements for poultry**

The vitamin $D_3$ requirement has been studied for the last 40 years. The NRC reported that 200 IU/kg is the minimum requirement (NRC, 1994). However, it is reported that inclusion levels of vitamin $D_3$ in the poultry industry typically exceed the NRC requirements (Applegate & Angel, 2014). FEFANA in 2015 recommends that 3,000 to 5,000 IU/kg is safe for use during all grower phases in broilers and broiler breeders, as well as in layer hens, where it can be used at 3,000 to 4,000 IU/kg (Blum et al., 2015). Similarly, Cobb reports that 5,000 IU/kg is an optimal supplementary level of vitamin $D_3$ in all dietary phases for the 500 genetic line (Cobb, 2022). Furthermore, Aviagen sets its requirements between 4,000 and 5,000 IU/kg vitamin D, depending on the dietary grain base (Aviagen, 2014).

Additionally, the Brazilian Tables for Poultry and Swine (Rostagno et al., 2024), mention higher vitamin $D_3$ inclusion in broiler feed for the first 8 days (3,378 mg/kg feed) while a lower inclusion at 49 days (1,915 mg/kg feed), calculated according to the body weight gain. For pullets, layer, and breeders, the vitamin $D_3$ recommendations vary between 2,500 and 3,150 mg/kg feed; this mainly depends on the phase and age of the layer (recommended levels of vitamin $D_3$ are summarized in Table 2.1).

$25(OH)D_3$ has higher bioactivity than regular vitamin $D_3$ due to efficient absorption in the upper portion of the intestine (P. Sakkas et al., 2019), when used at 75 µg/kg, was shown to improve performance, egg quality, and bone health. In layer hens, breeders, and broilers, $25(OH)D_3$ at 0.069 µg/kg is safe to improve production (Blum et al., 2015). Similarly, ten feed trials were conducted in broilers to evaluate the efficacy of $25(OH)D_3$, concluding that a safety range of 50 to 70 µg/kg is necessary to increase body weight and decrease adjusted feed efficiency (Yarger, Saunders, et al., 1995). Adequate vitamin D status requires plasma concentrations of at least 5 ng/mL in broilers and 10 ng/mL in laying hens(Goff, 1990).

In commercial practice, vitamin $D_3$ levels typically reach 5,000 IU/kg. However, these levels can increase; to select the most appropriate level of vitamin $D_3$ inclusion, factors such as genetics, production phase, health status, age, among others, must be considered. In Canada and the European Union, the use of vitamin D is regulated by law, and these levels vary according to the poultry species. In Canada, for broilers, breeders, and turkeys, inclusion levels of up to a maximum of 10,000 IU/kg are permitted (Canada, 2024). For laying hens, the maximum is 6,500 IU/kg (Canada, 2024). In contrast, the European Union has levels closer to commercial practice, with vitamin $D_3$ inclusion for broilers and turkeys at 5,000 IU/kg, while for laying hens it is 3,200 IU/kg (Council, 2003). Additionally, the use of $25(OH)D_3$ is also regulated, with a maximum

inclusion of 100 µg/kg in broilers and turkeys, and 80 µg/kg in layers (Council, 2003). An overview of the requirements in poultry is found in Table 1.1. Recently, P. Sakkas et al. (2019) found that different inclusion levels of vitamin $D_3$ (1,000/4,000/7,000 IU/kg) or a combination with 25(OH)$D_3$ (1,000 vitamin $D_3$ + 3,000 25(OH)$D_3$ IU/kg) did not improve performance due to optimal levels of Ca and P. However, offering a diet that includes 25(OH)$D_3$ improves bone mineralization, whereas providing high levels of vitamin $D_3$ did not improve bone health, and no statistical differences were observed between the medium and high inclusion levels.

**Vitamin D deficiency**

The primary disease caused by vitamin D deficiency in young animals is rickets, while in adults it is osteomalacia, which leads to weak bones, negatively affecting bone health and walking ability. This vitamin deficiency impairs the development of the bone (Long et al., 1984), limiting its capacity to express its full genetic potential. Compared to 25OH$D_3$, vitamin $D_3$ has a lower absorption rate; both compounds are absorbed in the duodenum and upper jejunum. Hens take approximately 6 to 7 weeks to deplete their body reserves of vitamin D and become dependent on dietary sources (Atencio et al., 2005). In the chicken embryo, Ca and P are mobilized from the eggshell to the yolk with the assistance of vitamin $D_3$ (Torres & Korver, 2018). The yolk primarily stores trace minerals and vitamin D in the form of 25(OH)$D_3$, which supports embryonic development (Chen et al., 2021). Therefore, in breeder hens, the administration of 25(OH)$D_3$ in drinking water reduces early embryonic mortality, protects the embryo, and may support performance during the broiler growth phase (Saunders-Blades & Korver, 2014). In line with these findings, supplementation with 100 mg/kg of 1,25$(OH)_2D_3$-G in breeders' diets also produced heavier broiler chickens at hatch, suggesting enhanced nutrient transfer from the breeders to the egg. Moreover, at day 21, the same broiler chickens showed higher levels of IL-1β, IL-10, and

CaBP-D28k (Andrade et al., 2025). Vitamin D supplementation from the breeders has a positive effect on the progeny, not only by improving hatch weight but also by supporting intestinal health, enhancing performance, and acting as an immunomodulator.

Since vitamin $D_3$ requires hydroxylation in the liver to become $25OHD_3$, young chicks do not possess an optimal supply of the enzyme 25-hydroxylase until approximately two weeks of age (Leeson & Summers, 2001). The limited presence of this enzyme may reduce the conversion of vitamin $D_3$ to $25OHD_3$. Furthermore, insufficient vitamin D levels in young broiler chickens can compromise the immune system (Aslam et al., 1998). In addition to its role in bone mineralization and calcium/phosphorus homeostasis, vitamin $D_3$ acts as a potent immunomodulator. Poor vitamin $D_3$ status may increase the CD4/CD8 ratio and impair the activation of T lymphocytes, such as CD8+ T cells (Teymoori-Rad et al., 2019). Vitamin $D_3$ enhanced cell- and antibody-mediated immune responses (Shojadoost et al., 2021). In laying hens, a vitamin $D_3$ diet deficiency results in lower productivity before and after a *Salmonella* challenge, compared to groups supplemented with 3,000 IU of vitamin $D_3$ per kilogram of feed. Thus, vitamin $D_3$ is associated with gut health and disease resistance through its anti-inflammatory and immunomodulatory effects (Akimbekov et al., 2020; Fangshen et al., 2022).

Vitamin $D_3$ deficiency reduces calcium and phosphorus concentrations, leading to bone disorders. Calcium absorption across the intestinal wall occurs via two pathways: transcellular and paracellular, both of which involve vitamin $D_3$ (Adedokun & Adeola, 2013). Similarly, reduced extracellular phosphorus, associated with vitamin $D_3$ deficiency, plays a significant role in the development of bone disorders (Pines & Reshef, 2015). In broilers, early signs include stunted growth and pronounced leg weakness. In laying hens, reduced egg production and smaller egg size are the first indications of a vitamin $D_3$ deficient diet. Additional clinical signs include poor

hatchability, softening of the beak, bones, and keel, reduced bone ash content, and elevated alkaline phosphatase activity, indicative of rickets. Lofton and Soares (1986) tested different levels of vitamin $D_3$ (0, 50, 100, 200, 400, 800, 1,000, 2,000, 4,000, and 8,000 IU/kg) in broiler diets up to day 56 and found that feeding less than 200 IU vitamin $D_3$/kg of diet resulted in reduced growth performance and an increased incidence of leg abnormalities, particularly rickets. Overall, the level of vitamin $D_3$ in the diet had a significant effect on body weight, body weight gain, FCR, tibial breaking strength, and bone ash percentage.

**Vitamin D Toxicity**

Unlike water-soluble vitamins, dietary excess of fat-soluble vitamins is retained in the body, making them potentially toxic. For this reason, different regulations exist to control the inclusion of vitamin $D_3$ or its metabolites and to prevent intoxication from excessive supplementation. Vitamin D intoxication is rare in broilers due to their high tolerance and short lifespan. However, excessive levels of vitamin $D_3$ or 25(OH)$D_3$ can induce pathological lesions such as muscular atrophy, soft tissue mineralization, renal damage, and mortality (Morrissey et al., 1977). Morrissey et al. (1977) reported that 25(OH)$D_3$ is approximately 100 times more toxic than vitamin $D_3$. Consequently, this first metabolite is considered 5 to 10 times more toxic than vitamin $D_3$ (Yarger, Quarles, et al., 1995). Doses exceeding 0.1 mg/kg have been shown to increase renal calcium concentrations, and 100 mg/kg has resulted in increased mortality (Morrissey et al., 1977). An increase in calcium levels due to an imbalance in vitamin D metabolism can damage the kidneys and impair bone mineralization in both broilers and layers. Moreover, elevated levels of dietary calcium and phosphorus may produce toxic effects similar to hypervitaminosis $D_3$ (Morrissey et al., 1977). Aside from renal damage, vitamin D intoxication can reduce feed intake, which negatively affects weight gain and feed conversion ratio (Yarger, Quarles, et al., 1995).

High doses of vitamin D also produced similar bone-related disorders in rats, leading to skeletal deformities and fractures (Leeson & Summers, 2019). In turkeys, 99 mg/kg of 25(OH)$D_3$ is a standard dietary dose and is considered safe at up to five times the recommended levels without affecting performance. Nevertheless, administration of doses 10 times higher increased mortality at 28 days from 5% to 19% compared to control groups (Lanenga et al., 1999). In laying hens, the recommended dose is 82.5 µg/kg of 25(OH)$D_3$. However, supplementation at 10 times [825 µg/kg of 25(OH)$D_3$] the recommended dose led to decreased performance and egg quality between 28 and 44 weeks of age. However, prolonged exposure (37 weeks) to this first metabolite did not appear to increase the severity of toxic effects with a 5x [412.5 µg/kg of 25(OH)$D_3$] safety margin (Terry et al., 1999). The lower incidence of toxicity symptoms during extended periods of exposure may be attributed to the higher physiological demand for calcium and vitamin $D_3$ in laying hens due to continuous egg production.

**Vitamin $D_3$ and 25(OH)$D_3$ supplementation on growth performances and bone health in broilers**

Based on current commercial guidelines, vitamin $D_3$ is supplemented in diets at levels 10 to 20 times the minimum recommended by the NRC (1994). This inclusion varies due to age, nutritional guidelines, micronutrient balance, diet type, the bird's health status, and slow- or fast-growing broilers. In the Chinese market, slow-growing chickens are more common due to consumer preferences. In yellow-feathered broilers, adding vitamin $D_3$ during the 63-day period at low-, medium-, or high-concentrations (S: 1,200/2,400/3,600, G and F: 1,000/2,000/3,000 IU/kg vitamin $D_3$, respectively) had a positive effect on growth performance, carcass traits, bone characteristics, and intestinal morphology compared to no supplementation of vitamin $D_3$. However, the optimal dose for a better physiological response is a high-concentration diet

(3,600/3,000 IU/kg vitamin $D_3$). The villus length and V/C ratio increased, which can increase nutrient absorption and improve growth performance. Furthermore, Ca and P correlated positively with dietary vitamin $D_3$ supplementation. This effect was not seen in diets without vitamin $D_3$ (Wei et al., 2024). In broilers, vitamin $D_3$-deficient diets resulted in a reduction of maternal $D_3$ by day 9, and the absence of any supplemental vitamin $D_3$ leads to reduced bone mineralization by day 17. This effect was reversed when increasing dietary vitamin $D_3$ levels (62.5, 125, 250, 500, and 1,000 IU/kg), with 1,000 IU/kg yielding the highest tibia bone ash (Leyva-Jimenez et al., 2018). Vitamin $D_3$ acts rapidly on bone mineralization, primarily by facilitating calcium and phosphorus mobilization. However, its non-classical functions may be delayed in young birds due to immature hepatic hydroxylation capacity, which limits conversion of cholecalciferol to 25(OH)$D_3$. This variability in metabolic activation may help explain the inconsistent effects of vitamin $D_3$ supplementation on feed intake and feed efficiency reported in the literature. While some studies describe improvements in performance with dietary vitamin $D_3$, others show no significant response.

In fast growing broilers, supplementing 25(OH)$D_3$ [0, 3.125, 6.25, 12.5, 25, and 50 µg/kg 25(OH)$D_3$] in basal diet without any vitamin D source for 21 days increases growth performance, bone mineralization, and promotes transcription of the intestinal Ca, thereby increasing Ca expression in the duodenum, jejunum, and ileum (Liu et al., 2023). Moreover, 12.5 µg/kg 25OH$D_3$ was the dose recommendation based on the expression of the intestinal Ca transporter gene (Liu et al., 2023). However, this dose inclusion is lower with previous mentioned [69 µg/kg 25(OH)$D_3$]. Since 25(OH)$D_3$ skips the first hydroxylation, and its bioactivity is higher than that of vitamin $D_3$, broilers require less 25(OH)$D_3$ than vitamin $D_3$ for growth (Liu et al., 2023). The beneficial effect of vitamin $D_3$ or 25(OH)$D_3$ in the diet is reflected in improved performance indices, dressing

percentage, bone mineralization, and Ca and P utilization. Yarger, Saunders, et al. (1995) conducted 10 feeding trials comparing dietary vitamin $D_3$ and $25(OH)D_3$ in broiler chickens. Their results suggest that supplementation with $25(OH)D_3$, instead of vitamin $D_3$, improves body weight and feed efficiency. Additionally, birds fed $25(OH)D_3$ showed a more rapid increase in serum $25(OH)D_3$ concentrations. In contrast, Garcia et al. (2013) reported that feeding cholecalciferol, $25(OH)D_3$, $1,25(OH)_2D_3$, or $1\alpha OHD_3$ doesn't affect bone health in broilers. However, adding $1\alpha OHD_3$ reduced growth performance. Additionally, during the first 3 weeks, $1,25(OH)_2D_3$ had the highest BW and BWG compared to $25(OH)D_3$ and vitamin $D_3$. During the starter period, birds lack a fully developed enzymatic system to perform the first hydroxylation in the liver, reducing the first hydroxylation. However, this effect did not persist at 42 days.

**The response to stress in poultry**

Stress is a condition in which an animal cannot maintain a normal physiologic state because various factors adversely affect its well-being (Asres & Amha, 2014). This condition can be associated with immunological challenges, whether viral, bacterial, or caused by environmental factors such as heat stress, leading to reduced egg or meat production and affecting other physiological processes. Additionally, stress impairs feed consumption, reducing performance and worsening gut health, thereby affecting growth rate. Modern broilers are exposed to multiple stimuli that increase the stress response. Common stressors include heat stress, high stocking density, poor nutrition, disease challenges, and immune disorders. A relevant aspect of chronic stress is the suppression of the immune system, which can cause significant damage to the animal's body and make it more susceptible to opportunistic or parasitic diseases. Stress has also been linked to impaired cell-mediated immunity, as elevated glucocorticoid levels are associated with decreased immune cell function (Asres & Amha, 2014). Stressors activate the sympathetic

adrenomedullary and hypothalamic-pituitary-adrenal (HPA) axes, releasing corticosterone. Prolonged or repeated exposure to stress can disrupt the HPA axis, leading to hyper- or hyposecretion of corticosterone (Blas, 2015), which can affect blood parameters, increasing the heterophil to lymphocyte ratio (Miah et al., 2025). In broilers, stress factors such as heat stress, transportation, and high stocking density significantly affect leukocyte profiles and serum biochemistry, particularly by inducing lymphocytopenia with heterophilia, elevating corticosterone levels, and disrupting liver, kidney, and lipid metabolism(Miah et al.).

**Heat stress**

Chronic HS damages the digestive system by reducing blood circulation and nutrient intake, increasing permeability and leaky gut, and increasing inflammation (Emami et al., 2020). During prolonged exposure to high temperatures, visceral blood circulation is reduced, while peripheral blood flow increases to mitigate the hot sensation and body heat. Consequently, nutrient intake in the intestine is affected by reduced blood flow, leading to an imbalance in supplementation and failure to transport nutrients to the body.

A healthy GI has multiple functions: digestion, absorption, and excretion. It also works in the immune system and has a broad range of bacteria and other microorganisms (Yegani & Korver, 2008). Additionally, a normal GI is important in growth performance and overall health. After blood flow is reduced, the reduction in oxygen causes an increase in tissues hypoxia of the GI. Yielding to oxidative and nitrosative stress while causing epithelial tight junction damage resulting in leaky gut and inflammation response (Hall et al., 2001; Rostagno, 2020). Elevated temperatures provoke an imbalance between reactive oxygen species (ROS) and antioxidants, which affect not only GI cells but also kidney, liver cells and muscle. ROS is formed during aerobic metabolism and is the leading cause of muscle protein hypercatabolism (Bhat & Ismail, 2015). Because

oxidative stress is closely linked to muscle degradation, nutritional strategies that reduce ROS production may help mitigate muscle protein catabolism. In rats, three groups were fed diets with different vitamin D inclusion levels for 18 weeks: a control group receiving adequate vitamin D, a second group fed a vitamin D-deficient diet, and a third group fed a vitamin D-deficient diet for 12 weeks and then switched to the control diet for 6 weeks. The authors found that vitamin D deficiency induced mild oxidative stress in rat muscles. In contrast, vitamin D supplementation corrected all oxidative stress–related parameters and reversed its effects on muscle protein catabolism (Bhat & Ismail, 2015). Similarly, inflammatory responses and immune stress are related to oxidative stress (Nong et al., 2023). Exposure to hot temperatures affects the entire chicken body, leading to systemic dysfunction and altered body metabolism. Therefore, heat stress induces metabolic changes during both long and short exposures, resulting in overall adverse effects on the body.

Multiple studies have been conducted to find the best way to mitigate HS in broilers (Abdel-Moneim, 2021; Goel, 2021). The most common nutritional strategies to mitigate heat stress are feed restriction to reduce the metabolic rate during hot weather peaks, wet feeding helps to recover water loss due to the higher respiratory ratio, and higher energy diets to help chickens reach the energy requirement and mitigate the effect of HS (Wasti et al., 2020). Vitamin and mineral supplementation also play an essential role in mitigating HS. An overview of heat stress and physiological responses supports a deeper understanding of poultry health and performance in hot weather (Table .1).

**25(OH)$D_3$ under heat stress**

Multiple reports show that 25(OH)$D_3$ can improve performance in layer hens and broiler chickens in egg and meat quality, respectively, immunological response, and gut health (Chou et al., 2009; Garcia et al., 2013; Soares et al., 1995). In layers hens raised under high stocking density and supplemented with 25(OH)$D_3$ at 69 µg/kg during 16 weeks, increased gut health was attributed to intestinal morphology, barrier function, antioxidant capacity, and microbiota composition (Wang et al., 2021).

On the other hand, broilers reared under heat stress supplemented with 25(OH)$D_3$ had higher body weight and body weight gain at 39 d, and walking ability was improved with increased mineral deposition and a lower fracture load compared to the control group. Primarily, administration of 25(OH)$D_3$ prevents increases in permeability and decreases in the villus height (V), villus height to crypt depth ratio (V/C ratio), tight junction proteins (TJPs), and mucin-2 transcription (Zhang et al., 2021). Similarly, substituting vitamin $D_3$ with 25(OH)$D_3$ in diets increased weight gain from 1 to 21d and serum concentrations of Ca and P (Marques et al., 2022). The bird's ability to convert vitamin $D_3$ to its active form can be reduced during heat stress (Leeson, 1986). 25(OH)$D_3$ can be an alternative to resolve this adverse transformation during hot seasonal periods. Moreover, better performance in bone quality and a lower incidence of leg abnormalities in broiler flocks have been reported with 25(OH)$D_3$ supplementation in broiler flocks reared under summer weather that consists of increased temperature and relative humidity (Nääs et al., 2012). The first metabolite could help with heat stress damage in the animal body, but a combination of vitamin D and Ca also supports immune activation led by heat stress during the hot season. In dairy cows, supplementation with high vitamin $D_3$ and Ca reduces hyperthermia, leaky gut, and markers of inflammation (Ruiz-González et al., 2023). Although few studies have been conducted

on the use of 25(OH)$D_3$ under heat stress, the inclusion of vitamin $D_3$ or its first metabolite has shown a positive effect on inflammation caused by environmental stress, as well as improvements in feed conversion. Since feed intake is reduced during periods of heat stress, alternative delivery methods should be considered, such as supplementing 25(OH)$D_3$ through drinking water. Further research should focus on supplementing 25(OH)$D_3$ through drinking water and comparing its effects with dietary supplementation under hot weather conditions.

**Coccidiosis**

Coccidiosis is a gastrointestinal infection caused by an intracellular intestinal protozoan parasite of the genus *Eimeria* spp. It is known for producing intestinal lesions, malabsorption syndrome, enteritis, depressed weight gain, uniformity issues, increased FCR, and mortality (Mathis et al., 2024). With the growing global demand for poultry meat, which is also the most affordable of the three major meats, poultry production is expected to continue to increase in the coming years (Kleyn & Ciacciariello, 2021). A recent study recalculated the economic impact of coccidiosis in poultry production and estimated that global losses from this parasite in chickens could reach approximately ~13 billion U.S. dollars in 2016. These costs are mainly associated with prophylaxis, treatments, and losses resulting from reduced growth performance and mortality (Blake et al., 2020). To our knowledge, nine *Eimeria* species are recognized as causing disease in broilers. However, the most prevalent species include *E. acervuline*, *E. maxima*, *E. tenella*, *E. necatrix*, *E. mitis*, *E. praecox*, and *E. brunetti*. Each species affects a specific region of the gastrointestinal tract, from the duodenum to the ceca. Among the seven species, E. acervuline, E. maxima, E. brunetti, E. necatrix, and E. tenella are the most pathogenic chicken-specific species (Mathis et al., 2024). Each species affects a different section of the GIT and has a genetically fixed, self-limiting life cycle. Therefore, the severity of each coccidiosis is positively correlated with the

number of infective oocysts ingested (Williams, 2005). *Eimeria* species are host-specific parasites; therefore, a coccidia species that infects broiler chickens cannot infect turkeys. However, it has been reported that a coccidia species that affects turkeys was also able to infect ducks under experimental conditions and in immunosuppressed birds (Yun et al., 2000). *Eimeria* is transmitted via the fecal–oral route, typically occurring when feed, litter, or drinkers become contaminated with feces containing oocysts. To complete its reproductive cycle, the parasite must go through several developmental stages, an exogenous phase that occurs in the environment, and an endogenous phase that occurs inside the birds, including three phases: sporogony, merogony, and gametogony (Mesa-Pineda et al., 2021; Yun et al., 2000).

Once the parasite enters the chicken, it travels to its target organ (which varies by species) and penetrates the intestinal mucosa, causing severe mucosal damage and triggering an immune response. The coccidia cycle is short, with an approximate duration of 4–6 days, depending on the species (Mesa-Pineda et al., 2021). It has been reported that the severity of *Eimeria* infections is influenced by several factors, including the animal's age, parasite species, the infection dose, host-parasite interactions, and the immune response. Among the *Eimeris* spp. *E. necatrix* and *E. tenella* are the most pathogenic in chickens, while *E. maxima*is is highly immunogenic and requires only a small number of oocytes to induce a complete immune response (Dalloul & Lillehoj, 2006; Yun et al., 2000).

**Control of coccidiosis**

Coccidiosis can also be controlled using coccidiostats, including ionophores and synthetic or chemical agents (Mathis et al., 2024). Polyether ionophores, natural compounds produced by bacteria of the family Streptomycetaceae, and synthetic coccidiostats such as guanidines, triazines,

quinolones, pyridines, alkaloids, or thiamine analogs (Ahmad et al., 2024). Ionophores eliminate parasites by preventing ions from passing through the cell membrane and altering osmotic equilibrium, while synthetic compounds interfere with specific metabolic processes of the parasite, inhibiting stages of its life cycle and effectively disrupting its development and preventing its cycle from continuing (Ahmad et al., 2024). However, these methods have some limitations, given the trend toward raising antibiotic-free animals which will limited the usage of ionophores, and with limited option in chemicals, there is a potential to for coccidia developing drug resistance. These changes in coccidiosis control strategies has led researchers to explore different alternatives to manage and reduce the disease on farms.

Alternatively, coccidiosis vaccines can be used at day old via spray or gel for coccidiosis control in poultry. Vaccination aims to produce immunity to *Eimeria* by increasing resistance and reducing lesion score. It also triggers an immune response by enhancing innate and adaptive immune responses. However, the immune response consumes energy and can reduce weight gain during the starter phase (Dalloul & Lillehoj, 2006). When chickens are vaccinated with *Eimeria* spp., their immune system secretes various cytokines, some pro-inflammatory and anti-inflammatory, including IFN-γ, IL-1β, IL-6, and IL-10. However, when this response becomes excessive, it can negatively affect the bird's health. Moreover, it is well known that *Eimeria* induces an increase in IL-10, which downregulates the immune response, allowing the parasite to evade the host's defenses and survive (Lee et al., 2022). Multiple strategies have been used to mitigate immune system overstimulation during infection challenges.

**Vitamin D under coccidiosis challenge**

Vitamin D has been studied for its non-classical role in modulating immune responses. In layers, a challenge with 1 x $10^5$ live coccidia oocysts at 21 day of age reduced BW and increased IL-10, $CD4^+CD25^+$ Tregs, while decreasing $CD8^+$ T cells. Supplementing with 6.25, 25, 50, or 100 μg/kg of $25(OH)D_3$ positively influenced immune regulation, with higher doses improving immune modulation and reducing production losses following coccidia challenge, suggesting a potential nutritional strategy to limit inflammation (Morris et al., 2015). In 11-day-old broilers challenged with 7,000 *E. maxima* oocysts, supplementation wiht 25 or 100 μg/kg of $25(OH)D_3$ increased plasma 25(OH)D and bone strength. However, the higher dose increased parasite load and compromised gut morphology, without affecting IL-10 or IFN-γ levels (Panagiotis Sakkas et al., 2019). In contrast to previous findings, supplementing 110 μg/kg of $25(OH)D_3$ in turkeys reduced fecal oocyst shedding by 41%, increased macrophage NO by 53%, increased IL-10 mRNA, and Treg IL-10 expression (Shanmugasundaram et al., 2019). This finding suggests that a high dose of $25(OH)D_3$ may be beneficial during a coccidiosis challenge, as vitamin D receptors are present in various cell types throughout the body.

Vitamin D plays an important role in immune responses, as vitamin D receptors (VDRs) are present in CD4 and CD8 T cells, B cells, neutrophils, and almost all immune cells (Baeke et al., 2010). The behavior of $1,25(OH)_2D_3$ in chicken macrophages stimulated with lipopolysaccharide (LPS) shows that it may have an inflammatory and immunomodulatory role. The active metabolite decreases the surface expression of MHC class II, CD86, CXCL8, and IL-1β (Shojadoost et al., 2015). Similarly, down-regulated T lymphocyte proliferation and the frequency of IFN-y producing cells (Boodhoo et al., 2016). Furthermore, 1 μl of $1,25(OH)_2D_3$ was added *in vitro* to intestinal epithelial cells. The study concluded that it could be used as an antibiotic

alternative to enhance chicken immunity, as potential Vitamin D Response Elements (VDREs) were found in the promoters of chicken avian β-defensins (Zhang et al., 2016). Antimicrobial peptides (AMPs) constitute a defense system similar to innate immunity and perform multiple antimicrobial functions (Silveira et al., 2021). AMPs and vitamin D are related, as vitamin D is known to synergize with 4-phenylbutyrate, a substance competent to induce the expression of AMPs (Golpour et al., 2019). $1,25(OH)_2D_3$ significantly enhances the innate immune system's ability to combat pathogens. Moreover, the modulation of antigen-presenting cells (APCs) is crucial in initiating and maintaining adaptive immune responses and promoting self-tolerance (Sassi et al., 2018). Due to T and B cells having the necessary machinery to synthesize and respond to 1,25 D, vitamin D may act in a paracrine or autocrine manner in an immune environment (Aranow, 2011) (Penna & Adorini, 2000). In layer hens, LPS decreases E2, LH, T, and PG in the serum levels. Subsequently, vitamin D deficient hens significantly reduced FSH and LH levels and remarkably increased E2 and PG. Thus, vitamin $D_3$ supplementation counteracts the negative feedback effect of immunological stress and $VD_3$ deficiency on reproductive hormone secretion, improving layer performance and reproductive hormones (Geng et al., 2018).

Vitamin $D_3$ and $25(OH)D_3$ have been studied under coccidiosis vaccine challenge or *Eimeria* challenge (Fatemi et al., 2021; Seyed Abolghasem Fatemi et al., 2022; S. A. Fatemi et al., 2022; Leyva-Jimenez et al., 2019; Morris et al., 2015; Shi et al., 2024; Suarez et al., 2023), yielding variable results depending on the metabolite used and the type of the challenge. The birds' response can differ when exposed to live Eimeria infections or to high-dose vaccine challenges. Vaccination provides early exposure to the pathogen to stimulate immunity; however, management errors or excessively high doses can elicit unintended immune stress. In broilers, a 20x coccidiosis vaccine challenge combined with in ovo injection of 2.4 μg of $25(OH)D_3$ improved performance, small-

intestinal morphology, and breast meat yield, while reducing nitric oxide concentrations and pro-inflammatory gene expression and increasing anti-inflammatory responses (Fatemi et al., 2021; Seyed Abolghasem Fatemi et al., 2022). Similarly, during a coccidiosis vaccine challenge, combining vitamin $D_3$ with 25(OH)$D_3$ was more effective than vitamin $D_3$ alone in supporting bone health and increasing plasma 25(OH)$D_3$ concentrations in young broilers (Leyva-Jimenez et al., 2019). In contrast, when the challenge involves field strains of Eimeria rather than vaccination, vitamin D3 alone appears less effective and may even enhance parasite replication (Panagiotis Sakkas et al., 2019). These differences emphasize that the birds' physiological response to vitamin D depends on both the form of the metabolite and the type or intensity of the challenge.

In conclusion, because chickens are unable to synthesize vitamin D through the skin in the absence of ultraviolet light, dietary supplementation is essential. Beyond its classical role in calcium and phosphorus metabolism, vitamin D also regulates functions in tissues expressing VDRs, including muscle and immune cells. These broader physiological effects make vitamin $D_3$ and 25(OH)$D_3$ valuable nutritional tools to mitigate the impact of stressors such as high environmental temperatures or parasitic infections like coccidiosis. Further research is warranted to elucidate the non-classical functions of vitamin D and to define optimal metabolite sources and inclusion levels under different production and challenge conditions. Several studies have investigated the classical and non-classical functions of vitamin D metabolites in poultry; however, significant knowledge gaps remain regarding the role of 25(OH)$D_3$ under common stress conditions, including high ambient temperatures and parasitic challenges. Understanding different approaches to mitigate the negative effects of stress through 25(OH)$D_3$ supplementation can provide insights into how this metabolite functions in poultry, particularly in broilers. Heat stress is a common issue during the rearing period, and multiple experimental models have been

developed to simulate this condition; however, most studies have focused on chronic heat stress rather than acute heat stress, which often occurs when broilers approach market age. Similarly, challenge models have investigated either coccidiosis vaccination or live *Eimeria* infection to mimic industry conditions. Nevertheless, no studies have evaluated the combined effects of these two stressors. Understanding the vitamin D response under different challenges can contribute to developing nutritional strategies to optimize broiler health under diverse stress conditions.

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## TABLES

Table 2.1 Recommended vitamin $D_3$ requirements in poultry

| Reference | Units | Broilers | Layer hens | Turkeys |
|---|---|---|---|---|
| NRC (Council, 1994) | ICU | 200 | 200-300 | 1,100 |
| FEFANA (Blum et al., 2015) | IU | 3,000-5,000 | 3,000-4,000 | 3,000-5,000 |
| Rostango (Rostagno et al., 2024) | IU | 3,378 | 2,500-3,150 | |
| Cobb (Cobb, 2022) | IU | 5,000 | | |
| Ross (Aviagen, 2014) | IU | 4,000-5,000 | | |
| Arbor Acres (Aviagen, 2022) | IU | 4,000-5,000 | | |
| Hy-line (Hy-Line, 2020) | IU | | 3,300 | |
| Hendrix (Genetics, 2020) | IU | | 2,500-3,000 | |
| Hybrid (Hybrid, 2016) | IU | | | 4,800-5,000 |
| Aviagen Turkeys (Aviagen, 2015) | IU | | | 2,500-4,000 |

Table 2.2 The effects of heat stress and 25(OH)$D_3$ supplementation on broiler performance, bone health, and physiological parameters

| Reference | Age (days) | Temp (°F) | HR% | Results |
|---|---|---|---|---|
| (Zhang et al., 2012) | 0-28<br>29-42 | **Raised:** 89.6 decreased gradually to research 73.4 by 28 d<br>**Cyclic**: 96.8 from 10 am to 4 pm and 75.2 from 4 pm to 10 am)<br>**Constant**: 93.2<br>**Control**: 73.4 | NTR | • Average BW decreases by 8.1% (cyclic) and 18.2% (constant high temp).<br>• Chicken breast from constant high temp had a higher moisture and fat content and a lower protein content.<br>• The breast had a higher pyruvate concentration in cyclic high temp and a higher lactic acid concentration in constant high temp.<br>• Meat quality was affected. |
| (Ruff et al., 2020) | 0-21<br>21-42 | **Raised**: 89.6 decreased gradually to research 75.2 by 21 d<br>**HS group**: 95<br>**Control**:75.2 | 55 +/- 5% for the first 21 d | • Heat stress caused a significant reduction in all performance parameters compared to the control group.<br>• No differences were observed in tibia break strength at 21 D before HS. At 42 D, a significant reduction in break strength was observed in heat-stressed chickens compared with control TN chickens. |
| (Awad et al., 2020) | 1-21<br>22-35 | **Raised**: 93.2 decreased gradually to research 73.4 by 21 d.<br>**Heat Treatment**: 93.2 for 6 h<br>**Control**: 73.6 | 70 – 80% | • Cobb 500 and Ross 308 broilers reported around 8%–9% reductions in feed intake (FI), 17% reductions in BWG, and around 9%–10% increments in feed conversion ratio (FCR) when birds were exposed to heat treatment.<br>• Heat treatment shows a higher immune response in IgY and IgM ($p<0.001$). |
| (Quinteiro-Filho et al., 2010) | 1-7,<br>7-21<br>21-35<br>35-42 | **Raised**:91.4, 82.4, 75.2<br>**Two cyclic**: 87.8 (1) and 96.8 (2) from 8 am to 6 pm, from 6 pm to 8 am was reduced to 69.8.<br>**Control**: 69.8 | NTR | • Lymphoid organs such as the thymus, bursa of Fabricius, and spleen are the primary immune organs that were found to decrease under heat stress.<br>• Broiler housing at 96.8 has a 43.33% mortality rate. |
| (Zhang et al., 2021) | 0-22<br>22-39d | **Raised:** 93.2 decreased gradually to research 71.6 by 22 d<br>**HS group:** 93.2 for 7 h daily and the rest of the day 78.8<br>**Control:** 71.6 | 50 – 60% | • HS directly induced a decrease in tibia material properties and bone mass. Increased intestinal permeability and expression of inflammatory cytokines, consequently, produce dysbiosis.<br>• Broilers fed with 25(OH)$D_3$ improve bone mass, strength, and health. Pro-inflammatory factors were modulated by dietary 25(OH)$D_3$ and prevented increases in permeability. |

# CHAPTER 3

# THE ROLE OF 25-HYDROXYVITAMIN $D_3$ IN BROILER GROWTH PERFORMANCE AND BONE QUALITY UNDER HOT WEATHER CONDITIONS UP TO 42 DAYS

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## ABSTRACT

25-hydroxyvitamin $D_3$ [25(OH)$D_3$] is the secondary metabolite of vitamin $D_3$, which has higher bioavailability and bioactivity than vitamin $D_3$. A study was conducted to evaluate two levels of 25(OH)$D_3$ on broiler performance. A total of 792 day-old off-sex male Cobb 500 chicks were housed in floor pens in a climate-controlled facility. They were randomly assigned to one of the following three dietary treatments with 22 birds/pen and 12 pens/treatment: T1, a control corn-soybean-based diet; T2, 34.5 µg/kg of 25(OH)$D_3$ (Smart D); and T3, 69 µg/kg of 25(OH)$D_3$ (2 x Smart D). Birds were subjected to cycling heat treatment from 9 am to 5 pm, starting at 30°C and lower it 21°C in the remaining hours during the last week of the production cycle (day 35-42). Blood was collected on d 28, 35 and 42 for serum 25(OH)$D_3$ test. Performance was recorded and calculated on d14, 28, 35, and 42. One bird/pen was used for whole-body composition analysis using dual x-ray absorptiometry at d 28, 35, and 42. Data were analyzed using one-way ANOVA and means separated using Tukey's in JMP 17 (significance level: $P<0.05$). The results indicated that serum 25(OH)$D_3$ concentrations were elevated in T3 and T2 compared to T1 on every collection day ($P<0.001$). Feed intake showed no significant differences across treatments. However, T3 had higher body weight and weight gain from d 0-14 ($P=0.017$; $P=0.018$), d 15-28 ($P=0.016$; $P=0.049$), and d 0-28 ($P=0.016$), as well as better feed efficiency from d 0-14 ($P=0.001$) than the control group. There were no differences between treatments in overall growth performance from d 0-35 and 0-42. T3 increased tissue lean mass ($P=0.015$) compared to the T1 at d 28 based on whole-body composition, and T2 at d 35 showed enhanced whole-body bone mineral content and bone mineral density ($P=0.001$; $P=0.001$). In conclusion, 69 µg/kg of 25(OH)$D_3$ supplementation in the first four weeks enhanced the growth performance and lean mass, and 34.5 µg/kg of 25(OH)$D_3$ can improve bone health at day 35.

# INTRODUCTION

Birds are homeotherms capable of regulating their core body temperature, particularly when the temperature is maintained in the thermoneutral zone (21°C-28°C) (Brugaletta et al., 2022) (Apalowo et al., 2024). However, when environmental temperatures rise above this range in poultry houses, broilers struggle to dissipate heat due to their lack of sweat glands, resulting in elevated core body temperatures (Khan, 2012). Prolonged heat stress (HS) triggers physiological changes, including alterations in behavior, metabolism, and digestive system functionality. Chronic HS damages the digestive system by reducing blood circulation and nutrient intake, increasing permeability and leaky gut, and increasing inflammation (Emami et al., 2020). After a constant hot temperature, visceral blood circulation is reduced while peripheral blood flow increases to dissipate the metabolic heat. Consequently, nutrient intake in the intestine is affected by lower blood flow, producing an imbalance in supplementation and failure to carry nutrients and transport them to different tissues. In addition, heat stress can also decreases bone health, particularly bone remodeling (Zhang et al., 2021), as well as fat transport and utilization (Yin et al., 2021).

Multiple studies have been conducted to find the best way to mitigate HS in broilers (Abdel-Moneim, 2021; Goel, 2021). The most common nutritional strategies to mitigate heat stress are feed restriction to reduce the metabolic rate during hot weather peaks, wet feeding helps to recover water loss due to the higher respiratory ratio, and higher energy diets to help chickens reach the energy requirement and mitigate the effect of HS (Wasti et al., 2020). Vitamin and mineral supplementation also play an important role in mitigating HS. In broilers under heat stress, adding selenium and vitamin E did not improve performance but increased selenium content in the breast meat and improved lipid oxidation while decreasing abdominal fat (Habibian et al., 2016).

Vitamins A, C, and D are also beneficial for broilers under stressful conditions, helping immune function and intestinal development while reducing oxidative stress in poultry (Akinyemi & Adewole, 2021).

Among these strategies, using 25(OH)$D_3$, caught our interests (Yin et al., 2021; Zhang et al., 2021). Vitamin D is a fat-soluble vitamin absorbed in the small intestine and must undergo two hydroxylation steps to become active: the first in the liver, where it is converted to 25(OH)$D_3$, and then in the kidney, where it becomes the active metabolite 1,25$(OH)_2D_3$. Both vitamin $D_3$ and 25(OH)$D_3$ are commercially available. Although both metabolites share the same absorption sites in the small intestine, vitamin $D_3$ requires the formation of micelles and bile salts for absorption, whereas 25(OH)$D_3$ is more polar and is partly independent of micelle formation. In this case, 25(OH)$D_3$ might have advantage of overcoming absorption issues when birds were subject to heat stress induced fat transport and utilization issues. Moreover, vitamin $D_3$ had a lower absorption ratio compared to 25(OH)$D_3$, on average, only 50% of an oral dose of vitamin $D_3$ is absorbed (Oviedo-Rondon et al., 2023). Different studies have indicated that supplementing 25(OH)$D_3$ increases circulation in birds, which also suggests more efficiency than vitamin $D_3$ (Chen et al., 2020; Wang et al., 2020; Yarger, Saunders, et al., 1995). Thus, we hypothesize that providing 25(OH)$D_3$ under the heat treatment could help birds maintain vitamin D status.

In terms of its effect on the growth performance, the results are inconsistent across studies. Some research indicates increased body weight (Fritts & Waldroup, 2003; Vazquez et al., 2018), while others show no impact compared to vitamin $D_3$ (Chou et al., 2009; Marques et al., 2022). Bone quality results are also mixed; some studies report increased tibia ash (Applegate et al., 2003; Fritts & Waldroup, 2003), while others show no effect on tibia bone quality (Bajwa et al., 2020). Under heat stress conditions, some studies have shown that adding 69 µg/kg of 25(OH)$D_3$, whether

in the feed or water, can improve bone mineral deposition and tibia bone quality and increase body weight in early stages. A more common situation for US broiler production is that the birds are more likely to be under a hot weather or mild cycling heat stress situation, especially during the late stage of production, which has not been exclusively studied (Marques et al., 2022; Nääs et al., 2012; Zhang et al., 2021). The present study aimed to evaluate the effects of two inclusion levels of 25(OH)$D_3$ on serum 25(OH)$D_3$ concentration, growth performance, and bone quality of broilers from 0-42 days under cycling heat stress, and to understand better how 25(OH)$D_3$ could mitigate the negative impact observed during the final production week (36-42 days).

## MATERIAL AND METHODS

The experiment was conducted at the Poultry Research Center at University of Georgia, following the guidelines of animal use protocol for research, prior approval by the University of Georgia Institutional Animal Care and Use Committee (Animal Use Protocol #A2023 04-013-Y1-A0).

### Bird management, diets, and experimental design

A total of 792 off-sex male Cobb 500-day-old chicks were obtained from a local hatchery (Cleveland, GA) and randomly allocated to 36-floor pens (121 x 146 $cm^2$) covered with pine shavings. Chicks were allocated to floor pens that were randomly assigned to three dietary treatments, with 22 chicks per pen and 12 replicates per treatment, in a completely randomized design. The corn-soybean diets (Table 3.1) were formulated to meet the nutrient specification for Cobb500$^{TM}$ Broiler guidelines (Cobb, 2022) with a vitamin premix providing 1,400 IU/kg of vitamin $D_3$ as the basal diet (C, Table 3.1). Treatments 2 (T2), C + 34.5 µg/kg of 25(OH)$D_3$ (Smart-

D®, Nutribins, Walnut, CA), and treatment 3 (T3), C + 69 µg/kg of 25(OH)$D_3$ (Smart-D®, Nutribins, Walnut, CA). Broilers were raised until d 42 (starter phase, 0-14d; grower phase, 15-28d; finisher 1, 29-35d; finisher 2, 36-42d). Feed and water were provided *ad libitum*. A sample of each experimental diet were ground and submitted for proximate analysis. The dietary 25-hydroxycholecalciferol [25(OH)$D_3$] concentration was determined by mass spectrometry (Heartland Assays, Ames, IA).

The house was equipped with a temperature-controlled environment. From days 1 to 35, environmental conditions followed Cobb's guidelines (33 ± 1°C, gradually reduced by 3°C until reaching 21 ± 1°C). From day 36 to the end of the trial, broiler chickens were exposed to daily heat stress from 9:00 a.m. to 5:00 p.m., with temperature beginning at 29+/- 1°C and dropping to 21+/- 1°C during the remaining hours. Two HOBO (MX2300) sensors (Onset, Bourne, MA 02532) recorded temperature and humidity to verify environmental conditions. Rectal temperatures were measured before and after the heat treatment to confirm adequate heat challenge (105.50 ± 0.110 and 107.75 ± 0.147, respectively). The measurements showed an average increase of 1.26°C from the start of the treatment. The light schedule was kept at 23L:1D for the first 3 days and 20L:4D for the rest of the trial.

**Performance parameters and sample collection**

Birds and feed were weighed by floor pen on days 0, 14, 28, 35, and 42 to determine group body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). Mortality was recorded daily and used to adjust FI and FCR. On days 28, 35, and 42, one bird per pen (12 birds per treatment) was randomly selected for sampling. Blood was collected via wing vein puncture into a 5-mL tube without any anticoagulant and stored at 4°C to allow clotting. After 24 hours at refrigeration temperature, samples were centrifuged at 1000 x g for 15 min (Allegra

X-30R centrifuge, Beckman Coulter, Brea, CA). Serum was then taken and stored at -80°C until further analysis. The same birds were euthanized by cervical dislocation following blood collection for additional sampling. Whole-body composition was assessed using a dual-energy X-ray absorptiometry (DEXA) scanner (GE Healthcare, Chicago, IL). The left femur and tibia were collected and kept at -20°C for further analysis.

**Serum 25(OH)$D_3$ content**

On days 28 and 42, one bird per pen was randomly selected for blood collection via the wing vein. In total, blood was collected from 36 birds (12 pens x 3 treatments). Serum 25(OH)$D_3$ level was determined using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) procedure (Heartland Assays, Ames, IA).

**Serum calcium and inorganic phosphorus concentration**

Analysis was carried out using an automated clinical chemistry analyzer (RX Daytona™; Randox Laboratories, Crumlin, County Antrim, UK). As standard practice, maintenance was performed before any samples were run in the analyzer, according to the manufacturer's instructions, and the analyzer's operation was verified by running calibrators and quality controls. All the values obtained from the standard control for the different biochemical parameters were always within the expected ranges. Reagents for blood serum analysis included calcium (Ca) and inorganic phosphorus (Pi). Serum samples were thawed and carefully mixed using a vortexer (Fisher Vortex Genie 2TM G560, 1764 FisherScientific, Hampton, NH). All samples were placed into the carousel, and the concentration of Ca and Pi was then measured.

**Whole bone composition**

On days 28, 35, and 42, broilers were individually weighed, euthanized by cervical dislocation, and scanned using a dual-energy x-ray absorptiometry (DEXA) scanner (GE Healthcare, Chicago, IL) to assess body composition, as described by Chen et al. (2020). Briefly, euthanized birds were placed in a standardized dorsoventral position on the scanner, ensuring that the wings were extended laterally to prevent overlap with the thoracic region, and both legs were extended. Proper positioning was visually verified prior to initiation each scan. The scanning mode was configured for small animals. The whole bird was defined as a region of interest. Following the scanning procedure, data were collected on whole-body bone density, mineral content, fat percentage, and lean muscle mass. The same trained operator performed all scans to minimize handling variation.

**Bone quality**

Following DEXA scanning, 36 left femurs and tibias were collected. Bone samples were stored at -20°C until further processing and analyses. The femur and tibia were cleaned from muscle and non-bone tissues. Bone ash content was measured according to (Kim et al., 2004) and (Shi et al., 2023). Tibia length was measured with a caliper, and volume was calculated assuming water's specific gravity of 1 g/cm³ at 22°C. Bones were dried at 100°C for 24 hours, defatted for 48 hours in a Soxhlet apparatus with hexane (Fisher Scientific Inc., MA), and redried at 100°C for 24 hours to obtain fat-free dry weight. Bones were then ashed at 600°C for 12 hours to obtain the ash weight.

Tibia ash concentration was calculated:

$$\frac{Ash\ weight}{Bone\ volume} \times 100$$

and the ash percentage was calculated

$$\frac{Ash\ weight}{Fat\ free\ bone\ weight} \times 100$$

Femurs were defleshed and analyzed for bone-breaking strength using a TA.HDPlus C Texture Analyzer (Texture Technologies Corp., South Hamilton, MA 01982) with Exponent Connect software (version 8.1.9.0). Femur weight, diameter, and length were recorded. Diameters were measured at the midpoint in both the medial-lateral and anterior-posterior directions using an INSIZE caliper (model 1169-150). A three-point bending test was performed with a 60-mm support span and (30 mm from the center on each side) a 250-kg load cell descending at 1 mm/s. The cross-sectional area at the fracture site was accurately measured with calipers. Fracture load, displacement, and energy were recorded to calculate breaking strength.

**Statistical Analysis**

All data were analyzed statistically using one-way ANOVA with dietary treatment as the main effect in JMP Pro software version 17.2.0 (SAS Institute Inc., Cary, NC, 1989-2024). Differences between means were determined using Tukey's HSD test, with significance assessed at $P \leq 0.05$.

## RESULTS

**Serum 25(OH)$D_3$**

Serum 25(OH)$D_3$ concentrations were measured on days 28, 35, and 42 days (Table 3.2). Broilers receiving any level of 25(OH)$D_3$ supplementation had significantly higher serum

25(OH)$D_3$ concentrations than the control group ($P < 0.001$). Furthermore, the T3 group [69 µg/kg of 25(OH)$D_3$] exhibited the highest serum levels throughout the experimental period ($P < 0.001$).

**Serum Ca and Pi concentration**

Serum Ca and Pi were assessed on days 28, 35, and 42 (Table 3.3). No significant differences were observed between dietary treatments at any sampling point ($P > 0.05$).

**Growth performances**

All birds were individually weighed and sorted into close weight ranges to minimize variation during placement. The initial pen weight across treatments was 43 g ± 0.0001. During the starter phase (Table 3.4), supplementation of 69 µg/kg of 25(OH)$D_3$ significantly increased body weight (BW, $P = 0.017$) and body weight gain (BWG, $P = 0.018$), whereas an inclusion of 34 µg/kg showed intermediate effects. Both levels of 25(OH)$D_3$ treatments improved feed efficiency ($P = 0.001$) while feed intake (FI) remained unaffected. Similar responses were observed in the grower phase (Table 3.4), where 69 µg/kg of 25(OH)$D_3$ improved BW ($P = 0.016$) and BWG ($P = 0.049$); however, no significant differences in FI or feed efficiency were detected at day 28.

The finisher phase was divided into pre- and post-heat treatment periods (day 29-35 and 36-42, respectively; Table 3.5). No significant differences were observed in the pre-heat treatment finisher phase. During the heat treatment, an average rectal temperature increase of 1.28 ± 0.129 °C was recorded. FCR significantly increased ($P$=0.008) with higher 25(OH)$D_3$ levels at day 42.

In overall growth performance (Table 3.6), broilers fed 69 µg/kg of 25(OH)$D_3$ exhibited the highest BWG ($P = 0.016$) from 0-14 and 0-28 days, but the effect did not persist into the

finisher phase (0-42 days). No significant differences in FI or FCR were observed across the full period, although the T3 group noted a trend toward improved FCR at 42 days ($P = 0.092$). Early supplementation with 25(OH)$D_3$ enhanced BWG, but its effect diminished after 28 days.

**Whole Body Composition**

Whole-body composition results are presented in Table 3.7-3.9. No differences were found at 35 or 42 days. Bone mineral content (BMC) and bone mineral density (BMD) were unaffected at 28 days, but tissue and lean muscle mass were greater in broilers fed 69 µg/kg of 25(OH)$D_3$ compared to those without supplementation ($P = 0.019$, and $P = 0.015$, respectively). At 35 days, the lower 25(OH)$D_3$ dose (34 µg/kg) significantly increased BMC and BMD ($P = 0.001$), with no effect on tissue or lean mass. No differences were found at 42 days.

**Bone Characteristics**

Tibia measurements are summarized in Tables 3.10-3.12. At 28 days, no significant differences were observed, except for an significantly increase ($P$=0.020) in tibia length in the high-dose group. At 35 days, 34 µg/kg of 25(OH)$D_3$ significantly improved fresh tibia weight ($P = 0.001$), bone volume ($P = 0.003$), fat-free dry weight ($P = 0.002$), and ash content ($P < 0.001$). No significant effects were observed at 42 days.

**Bone Breaking Strength**

Femur strength data are presented in Tables 3.13-3.14. Breaking strength increased with age. At 28 days, 25(OH)$D_3$ had no significant effect on femur strength. At 35 days, 34 µg/kg supplementation significantly increased femur weight ($P = 0.006$), though other traits did not differ significantly ($P > 0.05$). At 42 days, no treatment differences were found, but breaking strength

numerically increased from 30.0 kg (day 28) to 37.8 kg (day 42), with the 34 µg/kg group and consistently showing the highest values.

## DISCUSSION

During the last week of rearing (week 5), the mean ambient temperature increased, following the maximum ambient temperature (graph 3.1). The recorded temperatures were above the thermoneutral zone and remained elevated for eight hours (from 9:00 to 17:00). Most birds exhibited panting and other behavioral signs of heat stress. Additionally, under heat stress, feed consumption decreased, limiting nutrient, vitamin, and mineral intake (Zhang et al., 2021). This reduction in feed intake under heat stress could lead to an increased risk of vitamin D deficiency (Balducci et al., 2023) and disrupt acid-base balance (Abdel-Moneim, 2021). During early ages, broilers are incapable of hydroxylating vitamin $D_3$ due to an immature enzyme system in the liver, where 25(OH)$D_3$ can skip the first hydrolyzation step, resulting in more efficient utilization and greater bioavailability and bioactivity for younger birds (Marques et al., 2022). 25(OH)$D_3$ is a common biomarker for vitamin D status and is typically measured in serum following ingestion. Therefore, dietary supplementation directly influences serum concentration in poultry (Chen et al., 2020). In the current study, dietary supplementation with 25(OH)$D_3$ increased serum 25(OH)$D_3$ concentrations to a greater extent than supplementing vitamin $D_3$ alone (Chen et al., 2020; Wang et al., 2020; Yarger, Saunders, et al., 1995). This enhanced response is likely due to the ability of 25(OH)$D_3$ to bypass the initial hepatic conversion required for vitamin $D_3$, leading to higher circulating levels of 25(OH)$D_3$ (Chen et al., 2020).

Bone is a dynamic organ mainly formed by type 1 collagen, minerals, and cells of multiple lineages. 25(OH)$D_3$ supports skeletal homeostasis by enhancing intestinal absorption and renal reabsorption of calcium and phosphorus, ensuring their availability for bone formation and (Chen et al., 2020; Wang et al., 2020). However, no difference was observed in Ca and P levels in the serum by 25(OH)$D_3$. Previous studies have reported that birds fed diets with low or imbalanced Ca/P ratios exhibit reduced plasma calcium and poorer bone mineralization unless vitamin $D_3$ is included in the diet. These findings suggest that the vitamin $D_3$ requirement in broilers is higher when dietary calcium and phosphorus levels are low or unbalanced (Whitehead et al., 2004). In the current study, diets were formulated according to Cobb guidelines to ensure optimal nutrient intake. Calcium and phosphorus were not intended to be deficient which might be the reason why their concentrations were not affected by the different levels of 25(OH)$D_3$.

In the present study, supplementing 25(OH)$D_3$ improved body weight (BW), body weight gain (BWG), and feed efficiency during the starter phase, as well as BW and BWG during the grower phase. These findings align with previous studies reporting enhanced early growth performance (1–28 d) in broilers supplemented with 25(OH)$D_3$ (Fritts & Waldroup, 2003; Vazquez et al., 2018). The superior early performances could be attributed to the higher bioefficiency of 25(OH)$D_3$ compared with vitamin $D_3$, as it bypasses hepatic hydroxylation and is rapidly converted to 1,25$(OH)_2D_3$. In addition, 25(OH)$D_3$ is less dependent on dietary fat absorption, which is beneficial in young birds whose fat digestion and absorption are not yet fully developed. Zhang et al. (2021) reported that adding 25(OH)$D_3$ resulted in longer villus length in the duodenum at 21 and 28 d, and a similar effect was found in the jejunum at 14 and 28 d. It may have contributed to enhanced nutrient absorption and growth. These findings also agree with a previous study that chronic cyclic heat (34 °C for 7 h daily) from 22–39 days significantly

depressed broiler weight gain and feed intake. However, birds supplemented with 25(OH)$D_3$ had higher BW at 39 d and BWG than the birds fed without 25(OH)$D_3$ (Zhang et al., 2021).

However, these benefits are not seen at the later stages of production. On the contrary, under heat treatment, feed efficiency increased at the higher inclusion level. Similar to our results, other studies (Marques et al., 2022; Zhang et al., 2021) reported no significant differences in performance during the finisher period. It appears that the beneficial effects of 25(OH)$D_3$ on performance are primarily observed in younger birds. A recent study in broilers raised under normal conditions reported that dietary vitamin $D_3$, alone or in combination with two graded levels of 25(OH)$D_3$, improves performance, especially when extra 25(OH)$D_3$ is added. The positive effects were most evident during the starter (0-11 days) and grower (12-30 days) phases, while the finisher (31-40 days) phase showed less pronounced effects (Phutthaphol et al., 2025).

This may be related to the shift in nutrient partitioning from growth toward maintenance and fat deposition as birds age, reducing the need for additional vitamin D metabolites (Phutthaphol et al., 2025). Under heat stress, feed efficiency increased at the higher inclusion level, suggesting that the response to 25(OH)$D_3$ might be related to age and environmental conditions. The idea of toxicity from using 69 µg/kg of 25(OH)$D_3$ was discarded due to the low inclusion level previously reported (Yarger, Quarles, et al., 1995). In addition, a reduction in performance has only been observed when doses exceeded a 10x inclusion (690 µg/kg of 25(OH)$D_3$)(Yarger, Quarles, et al., 1995). The reason for the reduced feed efficiency at such 69 µg/kg of 25(OH)$D_3$ remains unclear. However, recent information suggests that vitamin D inclusion should be reduced during later phases of rearing (Rostagno et al., 2024). In summary, during growth phases, 25(OH)$D_3$ had a better response in broiler chickens by improving BW, BWG, and feed efficiency. During the heat

treatment and finisher stage, adding $25(OH)D_3$ increased serum $25(OH)D_3$ but did not affect the growth performance.

In the current study, the whole body composition and tibia bone quality were assessed at 28, 35, and 42 days. We found that at day 28, supplementing any level of $25(OH)D_3$ did not influence bone mineral content, bone mineral density, area, volume, fat-free dry weight, or ash percentage. Similar results were reported by Garcia et al. (2013), who compared vitamin $D_3$, $25(OH)D_3$, $1.25(OH)_2D_3$, and $1\alpha(OH)D_3$, and did not find differences in ash percentage at day 7, 21 and 42. (Garcia et al., 2013). This result varies depending on the vitamin D source, biological response, and age of the bird. At day 35, supplementing with a lower dose of $25(OH)D_3$, as opposed to a higher dose, increased both mineral content and bone mineral density as assessed by DEXA, and tibia bone ash. In agreement with the current findings, it was reported that promotion of bone resorption by $25(OH)D_3$ is four times as effective as vitamin $D_3$ (Soares et al., 1995).

Interestingly, the lower dose of $25(OH)D_3$ led to improvements in bone quality and mineral density at day 35, though these effects appeared to vanish by day 42. (Bajwa et al., 2020) found that supplementation with $25(OH)D_3$ or $1\alpha(OH)D_3$ improved tibial ash, calcium, and phosphorus contents compared with vitamin $D_3$ at both 21 and 35 days. In broilers, as they grow older, their nutritional requirements shift from supporting rapid growth and development to primarily meeting maintenance needs. Consequently, vitamin D may not have the same efficacy in later stages as in early stages. Additionally, another hypothesis is that heat stress could interfere with bone formation (Zhang et al., 2021). Increased panting may elevate $CO_2$ levels, altering acid-base balance and potentially causing Ca and P imbalances (Abdel-Moneim, 2021), potentially reducing bone mineral density. The stress itself might interact with the function of vitamin D. On the contrary, a previous study reported that heat stress impaired gut barrier and reduced feed intake

while reducing Ca intake, affecting bone health. However, supplementation of 25(OH)$D_3$ reversed the bone loss and restored tibia bone ash, density, and strength by restoring intestinal integrity, enhancing Ca absorption, and suppressing bone resorption (Zhang et al., 2021). Additionally, in a broiler study, reducing dietary Ca and P while supplementing with 25(OH)$D_3$ partially improved bone mineralization and strength (Zhang et al., 2020). Further research is needed to better understand the interaction between heat stress and vitamin D metabolites.

## CONCLUSION

In conclusion, dietary supplementation with 25(OH)$D_3$ effectively increased serum 25(OH)$D_3$ concentrations, with 69 μg/kg yielding the highest levels. However, serum Ca and Pi concentrations remained unaffected by any level of 25(OH)$D_3$ throughout the trial. Our findings indicate that 69 μg/kg 25(OH)$D_3$ significantly improved growth performance during the starter and grower phases (0–28 days). However, during the finisher phase under heat stress, higher 25(OH)$D_3$ levels led to an increased feed conversion ratio, with no overall effect on performance from 0–35 or 0–42 days. Bone quality improvements were more prominent at 35 days with 34 μg/kg 25(OH)$D_3$, particularly in tibia mineralization and femur strength. Although no significant effects were observed at 28 and 42 days. These results suggest that 25(OH)$D_3$ supports growth and bone development during the early stages but is less effective during the later stages under mild heat treatment. Further research is necessary to optimize 25(OH)$D_3$ inclusion based on age and environmental conditions.

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## TABLES

Tables 3.1 Basal composition of the experimental diets and calculated analysis

| Ingridients % | Starter (0-14 d) | Grower (15-28 d) | Finisher (29-42 d) |
|---|---|---|---|
| **Corn** | 53.75 | 61.84 | 67.00 |
| **Soybean meal** | 35.45 | 32.08 | 24.48 |
| **Corn DDGS** | 5.00 | 1.15 | 3.87 |
| **Monocalcium Phos** | 2.25 | 1.48 | 1.31 |
| **Limestone** | 1.21 | 1.15 | 1.28 |
| **Fat, vegetable** | 1.00 | 1.00 | 1.00 |
| **Salt, plain (NaCl)** | 0.34 | 0.35 | 0.36 |
| **DL-metheonine** | 0.34 | 0.32 | 0.23 |
| **L-lysine HCL** | 0.25 | 0.24 | 0.21 |
| **Vitamin Premix[1]** | 0.10 | 0.10 | 0.10 |
| **Trace Mineral Premix[2]** | 0.08 | 0.08 | 0.08 |
| **L-threonine** | 0.11 | 0.09 | 0.03 |
| **Choline Chloride 60%** | 0.04 | 0.06 | 0.03 |
| **L-valine** | 0.07 | 0.07 | 0.03 |
| **Calculated analysis** | | | |
| **ME (Kcal/kg)** | 2,952 | 3,050 | 3,075 |
| **CP (%)** | 22.50 | 20.50 | 18.00 |
| **CF (%)** | 3.48 | 3.31 | 3.32 |
| **Ash (%)** | 5.04 | 4.10 | 3.68 |
| **Digestible lysine** | 1.26 | 1.16 | 0.96 |
| **Available Phosphorus** | 0.58 | 0.40 | 0.38 |
| **Calcium** | 0.96 | 0.80 | 0.80 |
| **Kg:kg** | | | |
| **Met/Lys** | 51.43 | 52.17 | 50.98 |
| **(Met+Cys)/Lys** | 75.00 | 76.00 | 77.00 |
| **Trp/Lys** | 19.96 | 19.82 | 20.03 |
| **Thr/Lys** | 68.00 | 66.00 | 65.00 |

| | | | |
|---|---|---|---|
| **Arg/Lys** | 107.43 | 106.87 | 109.00 |
| **Val/Lys** | 76.00 | 76.00 | 77.00 |
| **Ile/Lys** | 66.24 | 65.33 | 67.22 |
| **Leu/Lys** | 136.86 | 136.01 | 151.05 |

Abbreviation: DDGS: Distiller Dried Grains with Soluble; ME: Metabolizable Energy; CP: Crude Protein; CF: Crude Fiber.

[1]Vitamins provided per kg of premix: vitamin A, 3,527,360 IU; vitamin $D_3$, 1,399,921 IU; vitamin E, 19,400 IU; vitamin B12, 8.8 mg; menadione, 1,102 mg; Riboflavin, 3,527 mg; α-pantothenic acid 5,467 mg; thiamine, 970 mg; niacin, 20,282 mg; vitamin B6, 1,455 mg; folic acid, 573 mg; biotin, 79 mg.

[2]Supplied per kilogram of diet: Ca; 3.2%; Mn; 13.40%; Zn; 10.70%; Mg; 2.68%; Fe; 2.63%, Cu, 4000 ppm; I, 1000 ppm; Se; 400 ppm

Table 3.2 Serum 25(OH)$D_3$ concentration at 28, 35, and 42 days measured by using MS/LC.

| Treatments | 28 d | 35 d | 42 d |
|---|---|---|---|
| | Serum 25(OH)$D_3$ concentration | | |
| | --------- ng/ml ---------- | | |
| **T1** | 12.85[c] | 10.51[c] | 10.22[c] |
| **T2** | 35.25[b] | 25.79[b] | 23.74[b] |
| **T3** | 46.75[a] | 41.02[a] | 36.28[a] |
| **SEM ±** | 1.70 | 1.25 | 1.18 |
| **Source of variation** | ---------- *P*-value ---------- | | |
| **Diet** | <0.001 | <0.001 | <0.001 |

Treatments: T1: Basal diet (BD) + 1,400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

[a–c] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 3.3 Serum Ca and Pi concentrations at 28, 35, and 42 days

| Treatments | 28 d | | 35 d | | 42 d | |
|---|---|---|---|---|---|---|
| | Ca | Inorganic P | Ca | Inorganic P | Ca | Inorganic P |
| | ---------- mmol/L ---------- | | | | | |
| **T1** | 2.62 | 2.65 | 2.23 | 2.39 | 1.81 | 1.44 |
| **T2** | 2.57 | 2.68 | 2.20 | 2.40 | 1.86 | 1.40 |
| **T3** | 2.52 | 2.64 | 2.19 | 2.31 | 1.88 | 1.34 |
| **SEM ±** | 0.051 | 0.070 | 0.058 | 0.100 | 0.082 | 0.051 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | |
| **Diet** | 0.396 | 0.931 | 0.916 | 0.796 | 0.819 | 0.375 |

Treatments: T1: Basal diet (BD) + 1,400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 3.4 The effect of supplementing 25(OH)$D_3$ on growth performances during the starter and grower phase

| | Starter (0-14d) | | | | Grower (15-28d) | | | |
|---|---|---|---|---|---|---|---|---|
| | **BW** | **BWG** | **FI*** | **FCR*** | **BW** | **BWG** | **FI*** | **FCR*** |
| | **---------- kg ----------** | | | **kg:kg** | **---------- kg ----------** | | | **kg:kg** |
| **T1** | 0.474[b] | 0.432[b] | 0.563 | 1.31[a] | 1.782[b] | 1.307[b] | 1.971 | 1.51 |
| **T2** | 0.483[ab] | 0.440[ab] | 0.563 | 1.27[b] | 1.819[ab] | 1.337[ab] | 2.026 | 1.50 |
| **T3** | 0.490[a] | 0.447[a] | 0.564 | 1.26[b] | 1.835[a] | 1.346[a] | 1.995 | 1.48 |
| **SEM ±** | 0.003 | | 0.003 | 0.007 | 0.012 | 0.012 | 0.019 | 0.014 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | | | |
| **Diet** | 0.017 | 0.018 | 0.996 | 0.001 | 0.016 | 0.049 | 0.158 | 0.395 |

Abbreviation: BW: body weight; BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio

Treatments: T1: Basal diet (BD) + 1,400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

*Average values of FI and FCR were used and adjusted for mortalities.

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 3.5 The effect of supplementing 25(OH)$D_3$ on growth performances under heat treatment during the last week of the finisher phase

| | Finisher 1 (29-35d) | | | | Finisher 2 (36-42d) | | | |
|---|---|---|---|---|---|---|---|---|
| | **BW** | **BWG** | **FI*** | **FCR*** | **BW** | **BWG** | **FI*** | **FCR*** |
| | **---------- kg ----------** | | | **kg:kg** | **---------- kg ----------** | | | **kg:kg** |
| **T1** | 2.616 | 0.816 | 1.432 | 1.76 | 3.524 | 0.89 | 1.466 | 1.65[b] |
| **T2** | 2.642 | 0.812 | 1.451 | 1.79 | 3.533 | 0.891 | 1.451 | 1.65[b] |
| **T3** | 2.633 | 0.798 | 1.446 | 1.81 | 3.490 | 0.84 | 1.475 | 1.76[a] |
| **SEM ±** | 0.019 | 0.010 | 0.010 | 0.017 | 0.029 | 0.016 | 0.023 | 0.026 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | | | |
| **Diet** | 0.617 | 0.437 | 0.427 | 0.085 | 0.551 | 0.07 | 0.756 | 0.008 |

Abbreviation: BW: body weight; BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio

Treatments: T1: Basal diet (BD) + 1,400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

*Average values of FI and FCR were used and adjusted for mortalities.

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 3.6 The effect of supplementing 25(OH)$D_3$ on overall growth performances

| Treatment | D0- 28 | | | D0-35 | | | D0-42 | | |
|---|---|---|---|---|---|---|---|---|---|
| | **BWG** | **FI*** | FCR* | **BWG** | **FI*** | FCR* | **BWG** | **FI*** | FCR* |
| | **------ kg ------** | | kg:kg | **------ kg ------** | | kg:kg | **------ kg ------** | | kg:kg |
| **T1** | 1.739[b] | 2.522 | 1.44 | 2.573 | 3.905 | 1.52 | 3.459 | 5.297 | 1.53 |
| **T2** | 1.776[ab] | 2.553 | 1.43 | 2.599 | 3.938 | 1.52 | 3.502 | 5.309 | 1.52 |
| **T3** | 1.792[a] | 2.556 | 1.43 | 2.59 | 3.964 | 1.53 | 3.425 | 5.378 | 1.57 |
| **SEM ±** | 0.013 | 0.021 | 0.01 | 0.019 | 0.03 | 0.017 | 0.024 | 0.049 | 0.018 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | | | | |
| **Diet** | 0.037 | 0.324 | 0.211 | 0.803 | 0.32 | 0.667 | 0.644 | 0.523 | 0.086 |

Abbreviations: BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio

Treatments: T1: Basal diet (BD) + 1400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

*Average values of FI and FCR were used and adjusted for mortalities.

Groups with different lowercase superscripts are significantly different from each other (P<0.05).

Table 3.7 The effect of two different levels of 25(OH)$D_3$ on whole-body composition using dual X-ray scanner (DEXA) at 28 days

| Treatment | BW | BMC | BMD | Area | Fat | Tissue | Fat | Lean |
|---|---|---|---|---|---|---|---|---|
| | g | | g/cm$^2$ | cm$^2$ | % | | g | |
| **T1** | 1808.17$^{b}$ | 27.95 | 0.181 | 154.33 | 15.68 | 1738.92$^{b}$ | 273.58 | 1465.33$^{b}$ |
| **T2** | 1921.0$^{a}$ | 29.79 | 0.183 | 162.25 | 16.26 | 1848.17$^{ab}$ | 300.92 | 1547.25$^{ab}$ |
| **T3** | 1932.5$^{a}$ | 29.82 | 0.183 | 162.58 | 15.92 | 1858.83$^{a}$ | 296.92 | 1561.92$^{a}$ |
| **SEM ±** | 32.11 | 0.787 | 0.002 | 2.829 | 0.449 | 31.540 | 11.365 | 23.884 |
| **Source of variation** | ---------- *P-value* ---------- | | | | | | | |
| **Diet** | 0.017 | 0.173 | 0.718 | 0.080 | 0.663 | 0.019 | 0.200 | 0.015 |

Abbreviations: BW: body weight; BMC: bone mineral content; BMD: bone mineral density

Treatments: T1: Basal diet (BD) + 1,400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 3.8 The effect of two different levels of 25(OH)$D_3$ on whole-body composition using dual X-ray scanner (DEXA) at 35 days

| Treatment | BW | BMC | BMD | Area | Fat | Tissue | Fat | Lean |
|---|---|---|---|---|---|---|---|---|
| | g | | g/cm$^2$ | cm$^2$ | % | | g | |
| **T1** | 2660.83 | 39.88$^b$ | 0.199$^b$ | 200.67 | 15.99 | 2566.92 | 409.00 | 2157.83 |
| **T2** | 2736.67 | 43.72$^a$ | 0.210$^a$ | 207.83 | 15.92 | 2634.17 | 420.42 | 2213.75 |
| **T3** | 2659.67 | 39.02$^b$ | 0.198$^b$ | 196.92 | 15.48 | 2566.42 | 397.67 | 2168.75 |
| **SEM ±** | 44.11 | 0.902 | 0.002 | 3.034 | 0.516 | 42.779 | 14.719 | 39.107 |
| **Source of variation** | ---------- *P-value* ---------- | | | | | | | |
| **Treatment** | 0.378 | 0.001 | 0.001 | 0.047 | 0.755 | 0.445 | 0.556 | 0.568 |

Abbreviations: BW: body weight; BMC: bone mineral content; BMD: bone mineral density

Treatments: T1: Basal diet (BD) + 1400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

$^{a,b}$ Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 3.9 The effect of two different levels of 25(OH)$D_3$ on whole-body composition using dual X-ray scanner (DEXA) at 42 days

| Treatment | BW | BMC | BMD | Area | Fat | Tissue | Fat | Lean |
|---|---|---|---|---|---|---|---|---|
| | --------- g -------- | | g/cm$^2$ | cm$^2$ | % | ---------- g ---------- | | |
| **T1** | 3505.82 | 51.20 | 0.207 | 246.91 | 14.23 | 3347.45 | 474.82 | 2872.55 |
| **T2** | 3524.50 | 52.09 | 0.209 | 248.67 | 14.70 | 3395.83 | 497.25 | 2898.50 |
| **T3** | 3504.00 | 51.73 | 0.208 | 248.17 | 15.60 | 3375.25 | 526.25 | 2849.33 |
| **SEM ±** | 75.33 | 1.36 | 0.002 | 5.00 | 0.638 | 72.91 | 21.05 | 71.60 |
| **Source of variation** | ---------- *P-value* ---------- | | | | | | | |
| **Diet** | 0.977 | 0.899 | 0.815 | 0.968 | 0.317 | 0.897 | 0.241 | 0.886 |

Abbreviations: BW: body weight; BMC: bone mineral content; BMD: bone mineral density

Treatments: T1: Basal diet (BD) + 1,400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 3.10 The effect of two different levels of 25(OH)$D_3$ on tibia bone quality evaluated at 28 days

| Treatment | Fresh Bone Weight | Length | Volume | Fat-free dry weight | Ash Bone | Bone Ash Concentration | Ash |
|---|---|---|---|---|---|---|---|
| | g | mm | $g/cm^3$ | ---------- g ---------- | | | % |
| **T1** | 10.05 | 81.63[ab] | 7.52 | 4.15 | 2.02 | 0.278 | 49.12 |
| **T2** | 10.33 | 81.43[b] | 7.71 | 4.34 | 2.07 | 0.276 | 48.85 |
| **T3** | 10.75 | 83.54[a] | 7.81 | 4.39 | 2.14 | 0.275 | 48.83 |
| **SEM ±** | 0.31 | 0.549 | 0.254 | 0.12 | 0.066 | 0.006 | 0.354 |
| **Source of variation** | ---------- *P-value* ---------- | | | | | | |
| **Diet** | 0.289 | 0.02 | 0.714 | 0.346 | 0.439 | 0.931 | 0.808 |

Treatments: T1: Basal diet (BD) + 1,400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 3.11 The effect of two different levels of 25(OH)$D_3$ on tibia bone quality evaluated at 35 days

| Treatment | Fresh Bone Weight | Length | Volume | Fat-free dry weight | Ash Bone | Bone Ash Concentration | Ash |
|---|---|---|---|---|---|---|---|
| | g | mm | g/cm$^3$ | g | g | | % |
| **T1** | 13.70$^b$ | 92.30 | 10.99$^b$ | 5.94$^b$ | 2.90$^b$ | 0.260 | 48.25 |
| **T2** | 14.76$^a$ | 92.64 | 11.83$^a$ | 6.38$^a$ | 3.12$^a$ | 0.262 | 48.94 |
| **T3** | 13.19$^b$ | 92.23 | 10.78$^b$ | 5.80$^b$ | 2.72$^b$ | 0.254 | 47.40 |
| **SEM ±** | 0.284 | 0.630 | 0.214 | 0.113 | 0.053 | 0.004 | 0.435 |
| **Source of variation** | ---------- *P-value* ---------- | | | | | | |
| **Diet** | 0.001 | 0.886 | 0.003 | 0.002 | <0.001 | 0.354 | 0.06 |

Treatments: T1: Basal diet (BD) + 1,400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

$^{a,b}$ Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 3.12 The effect of two different levels of 25(OH)$D_3$ on tibia bone quality evaluated at 42 days

| Treatment | Fresh Bone Weight | Length | Volume | Fat-free dry weight | Ash Bone | Bone Ash Concentration | Ash |
|---|---|---|---|---|---|---|---|
| | g | mm | g/cm$^3$ | g | g | | % |
| **T1** | 18.16 | 102.11 | 14.57 | 7.41 | 3.69 | 0.252 | 49.57 |
| **T2** | 18.42 | 103.39 | 14.66 | 7.63 | 3.81 | 0.261 | 49.94 |
| **T3** | 18.21 | 104.28 | 14.84 | 7.57 | 3.82 | 0.258 | 50.38 |
| **SEM ±** | 0.407 | 0.945 | 0.383 | 0.154 | 0.086 | 0.005 | 0.258 |
| **Source of variation** | ---------- *P-value* ---------- | | | | | | |
| **Diet** | 0.892 | 0.280 | 0.879 | 0.578 | 0.509 | 0.470 | 0.064 |

Treatments: T1: Basal diet (BD) + 1,400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 3.13 The effect of two different levels of 25(OH)$D_3$ on femur bone quality and breaking strength at 28 and 35 days on broiler chicken

| Treatment | 28 | | | | | 35 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Fresh Bone Weight** | **Length** | **Med/lat** | **Ant/Post** | **Peak Force** | **Fresh Bone Weight** | **Length** | **Med/lat** | **Ant/Post** | **Peak Force** |
| | g | mm | mm | mm | g | g | mm | mm | mm | g |
| **T1** | 8.77 | 67.35 | 8.36 | 9.01 | 31,127 | 9.03[b] | 69.92 | 9.78 | 10.22[b] | 33,897 |
| **T2** | 9.01 | 67.35 | 8.54 | 9.36 | 30,675 | 9.76[a] | 70.16 | 10.11 | 10.79[a] | 36,648 |
| **T3** | 9.33 | 68.39 | 8.73 | 9.31 | 29,643 | 8.96[b] | 69.50 | 9.85 | 10.24[b] | 33,068 |
| **SEM ±** | 0.234 | 0.662 | 0.130 | 0.147 | 1,642 | 0.184 | 0.555 | 0.130 | 0.141 | 1,521 |
| **Source of variation** | ---------- *P-value* ---------- | | | | | | | | | |
| **Treatment** | 0.254 | 0.451 | 0.147 | 0.211 | 0.808 | 0.006 | 0.699 | 0.180 | 0.010 | 0.234 |

Treatments: T1: Basal diet (BD) + 1400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

[a ,b] Groups with different lowercase superscript are significantly different from each other (P<0.05).

Table 3.14 The effect of two different levels of 25(OH)$D_3$ on femur bone quality and breaking strength at 42 days in broiler chickens

| Treatment | Fresh Bone Weight | Length | Med/lat | Ant/Post | Peak Force |
|---|---|---|---|---|---|
| | g | mm | mm | mm | g |
| **T1** | 11.36 | 76.38 | 10.72 | 11.09 | 37,263 |
| **T2** | 12.11 | 76.51 | 11.15 | 11.54 | 39,107 |
| **T3** | 12.02 | 77.24 | 10.75 | 11.45 | 36,707 |
| **SEM ±** | 0.390 | 0.713 | 0.196 | 0.264 | 1,645 |
| **Source of variation** | ---------- *P-value* ---------- | | | | |
| **Diet** | 0.342 | 0.660 | 0.240 | 0.460 | 0.564 |

Treatments: T1: Basal diet (BD) + 1,400 IU/kg vitamin $D_3$; T2: BD + 34 µg/kg 25(OH)$D_3$ (Smart D®); T3: BD + 69 µg/kg 25(OH)$D_3$ (Smart D®)

Groups with different lowercase superscripts are significantly different from each other (P<0.05).

**FIGURE**

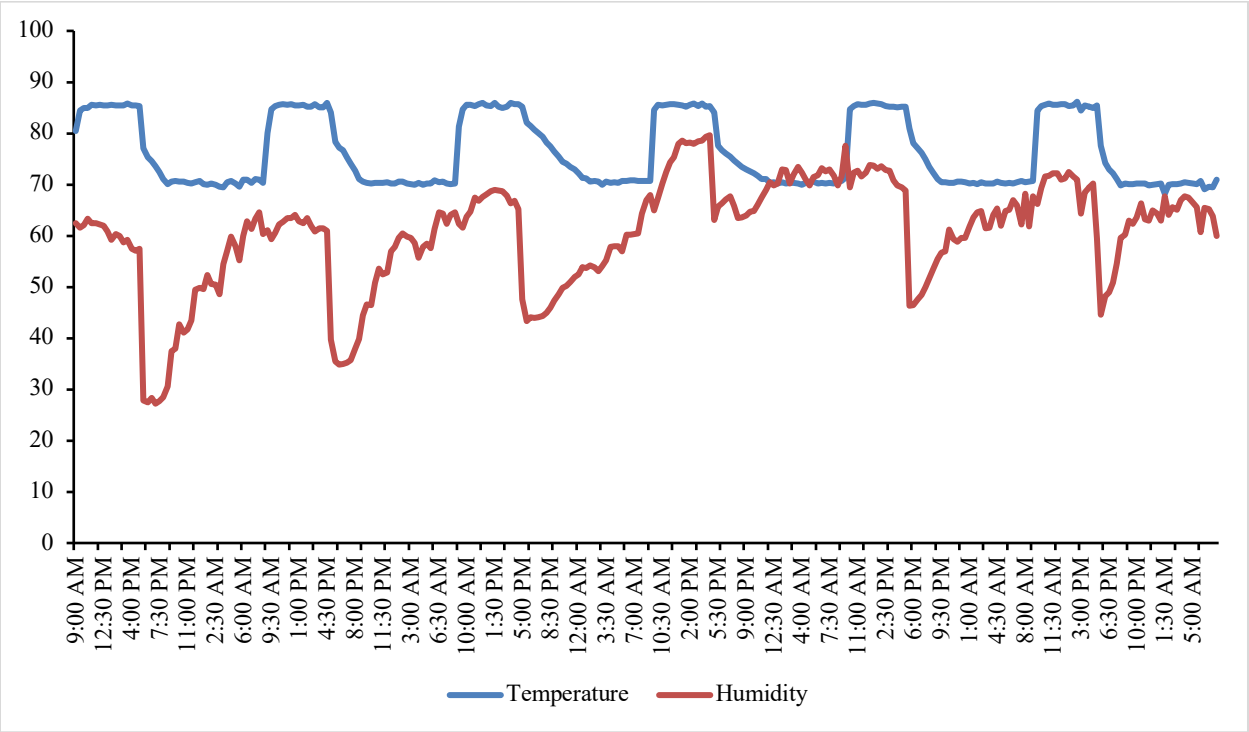


Figure 3.1 Temperature and humidity during days 36 to 42 to simulate hot weather conditions from 9 am to 5 pm

# CHAPTER 4

# EFFECTS OF DIETARY 25-HYDROXYVITAMIN $D_3$ ON THE GROWTH PERFORMANCE, CARCASS YIELD, BONE HEALTH, AND BLOOD CHEMISTRY IN BROILERS DURING COCCIDIOSIS CHALLENGE

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## ABSTRACT

Coccidiosis impacts intestinal and bone health of chickens and consequently growth performance. The objective of this study was to investigate the effects of 25-hydroxyvitamin $D_3$ ($25(OH)D_3$, Hy-D®, DSM-Firmenich, Switzerland) on performance, bone quality, and gut health in coccidiosis-vaccinated chickens challenged with *Eimeria* spp. A total of 900 one-day-old Cobb 500 male chicks vaccinated against coccidiosis were raised in floor pens and challenged on D22 with *E. acervulina* (250,000 oocysts/bird), *E. maxima* (50,000 oocysts/bird), and *E. tenella* (50,000 oocysts/bird). All the basal diets contained 1,400 IU/kg of Vitamin $D_3$. Birds were assigned to four treatments (25 birds x 9 replicates): NCC, Non-challenge control; CC, *Eimeria* challenge control DD, challenge + 2,760 IU/kg vitamin $D_3$; 25D, challenge + 69 μg/kg of $25(OH)D_3$ (bioequivalent to 2,760 IU/kg vitamin $D_3$). Serum $25(OH)D_3$ was measured on D28 and 42. Growth performance was assessed on D14, 28, and 42. At D28, oocyst shedding, gut lesion score, and permeability were evaluated. Tibia quality was measured at D28 and 42, and whole-body composition was evaluated at D42. Data were analyzed by one-way ANOVA and Tukey's test ($P<0.05$). The coccidiosis challenge reduced serum $25(OH)D_3$ compared to the NCC at D28 ($P<0.05$) but not at D42 ($P>0.05$). Meanwhile, DD and 25D increased serum $25(OH)D_3$ at D28 and 42, with the group 25D showing the highest value at D42. Coccidiosis challenge significantly impacted ($P<0.001$) BWG, FI, and FCR at D28, as well as BWG at D42 ($P<0.001$). Vitamin $D_3$ and $25OHD_3$ did not improve the performance, but the DD and 25D groups showed lower *E. tenella* lesion scores ($P<0.001$). At D28, coccidiosis challenge reduced bone-dry weight ($P=0.005$), fat-free bone-dry weight ($P=0.001$), and bone ash ($P=0.001$). Adding $25(OH)D_3$ increased fat-free dry weight ($P=0.001$) and ash content ($P=0.001$) in the tibia compared to CC, reaching a similar level as the NCC. Additionally, *Eimeria* challenge significantly increased gut lesions in the small intestine and

ceca ($P$<0.050). However, vitamin D supplementation did not reduce these negative effects, except that extra vitamin $D_3$ and 25(OH)$D_3$ reduced cecal lesion scores. In conclusion, the coccidiosis challenge induced gut lesions and negatively affected vitamin D status, bone quality, and growth performance 6 days post-challenge. These effects were partially alleviated by the additional inclusion of vitamin D, with more evident effects of supplemental 25(OH)$D_3$.

## INTRODUCTION

Coccidiosis is caused by *Eimeria* spp., a protozoan parasite that comprises several species, among which *E. maxima*, *E. acervulina*, and *E. tenella* are the most prevalent in broiler chickens (Dalloul et al., 2007). Currently, coccidiosis remains a significant cause of economic losses in broiler production. The global cost of coccidiosis in chickens is an estimated 13 billion US dollars, including production losses and expenses associated with prophylaxis and treatment (Blake et al., 2020).

The parasite has a complex life cycle that includes both exogenous and endogenous stages (López-Osorio et al., 2020). Once inside the host and digested, the oocyst initiates the asexual reproductive phase within intestinal epithelial cells. The intracellular replication of the parasite damages the intestinal mucosa, resulting in lesions. This tissue damage, along with the presence of the parasite itself, activates the innate immune system as the first line of defense, inducing inflammation (Madlala et al., 2021). Subsequently, the adaptive immune system is engaged, characterized by the recruitment of lymphocytes and the release of cytokines and chemokines. However, an excessive immune response can be detrimental, as it diverts energy from productive functions such as breast muscle growth. In addition to this metabolic cost, intestinal lesions impair nutrient absorption, leading to malabsorption syndrome, which reduces the availability of nutrients for systemic distribution. As a result, coccidiosis commonly leads to impaired growth performance and carcass yield, as well as lethargy, poor feather quality, and in severe cases, bloody diarrhea.

Various strategies have been investigated to control and reduce the impact of coccidiosis, including nutritional, pharmaceutical, and vaccination-based interventions (Mathis et al., 2024).

Vaccination stimulates a cascade of reactions in both the innate and adaptive immune systems, beginning with antigen recognition and the subsequent activation of macrophages and dendritic cells. This leads to localized inflammation and the release of cytokines and chemokines, which contribute to the immune response but also increase the bird's energy demands (Zaheer et al., 2022).

Cholecalciferol (vitamin $D_3$) is commonly supplemented in broiler diets and plays diverse physiological roles in broilers, including promoting growth performance, supporting bone integrity, modulating blood biochemical parameters, and regulating immune function through its hormone-like activity (Aslam et al., 1998; Vazquez et al., 2018). Broilers mainly rely on dietary vitamin $D_3$ supplementation to maintain adequate circulating vitamin D metabolites and support proper body function, especially calcium and phosphorus balances. After ingestion, vitamin $D_3$ is transported in the bloodstream bound to vitamin D-binding protein and is hydroxylated in the liver to form 25-hydroxyvitamin $D_3$ [$25(OH)D_3$], the major circulating and storage form. This metabolite is then transported to the kidneys, where it undergoes a second hydroxylation to become 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$], the biologically active form to execute its function (Bikle, 2014).

During a coccidiosis vaccine challenge in young birds, a combination of vitamin $D_3$ and $25(OH)D_3$ was more effective than vitamin $D_3$ alone in supporting bone health and increasing total plasma $25(OH)D_3$ levels (Leyva-Jimenez et al., 2019). Similarly, under a 20x coccidiosis vaccine challenge, *in ovo* injection of 2.4 μg of $25(OH)D_3$ improved performance, small intestinal morphology, and breast meat yield, while reducing nitric oxide levels and pro-inflammatory gene expression and increasing anti-inflammatory responses (Fatemi et al., 2021; Seyed Abolghasem Fatemi et al., 2022). This evidence could make vitamin $D_3$ or $25(OH)D_3$ supplementation as a

potential strategy to alleviate coccidiosis in broilers. In addition, in none-challenged models, research also showed adding 25(OH)$D_3$ improves body weight and feed efficiency compared to vitamin $D_3$ (Yarger, Saunders, et al., 1995). In broilers, supplementing vitamin $D_3$-deficient diets with increasing levels of 25(OH)$D_3$ (0, 3.125, 6.25, 12.5, 25, and 50 µg/kg) for 21 days enhanced growth performance, bone mineralization, and transcription of intestinal calcium transporters, thereby increasing calcium expression in the duodenum, jejunum, and ileum (Liu et al., 2023). These effects might improve the gut health, bone health and maintain growth performance when birds are under the challenge condition.

Vaccines provide early exposure to pathogens to encourage an immune response in case of a real challenge. However, this early exposure could bring harmful effects to young chicks. Given the beneficial effects of 25(OH)$D_3$ on growth and immune function during rearing, different research has been exploring combining 25(OH)$D_3$ level with other vaccines, such as Marek's disease, to improve performance, immunity, and bone development (Fatemi et al., 2024; Laboissiere et al., 2025). Fatemi et al. (2024) reported that the combination of *in ovo* Marek's disease vaccine with the injection of 1.2 or 2.4 µg of 25(OH)$D_3$ at 18 days of incubation did not affect hatchability but increased hatchling BW and immune response. Similarly, (Laboissiere et al., 2025) reported that *in ovo* injection of 1.25 µg of 25(OH)$D_3$ with Marek's disease vaccine did not alter hatchability or growth performances up to 35 days but improved early bone strength and reduced tibial dyschondroplasia at 7 days post-hatch. These studies suggest that during vaccine stress, 25(OH)$D_3$ can enhance BW, bone health, and boost immune response without compromising hatchability or performance.

Coccidia vaccination is a common practice in commercial production. However, limited research has examined the interaction between coccidiosis vaccine and vitamin $D_3$ or 25(OH)$D_3$.

Moreover, no studies have specifically focused on understanding how vitamin $D_3$ or 25(OH)$D_3$ interacts with vaccination prior to a live *Eimeria* challenge in broilers. Based on this evidence, we hypothesize that dietary supplementation with 25(OH)$D_3$ enhances the immune response to coccidiosis vaccination in broilers, thereby mitigating the adverse effects associated with *Eimeria* infection. Therefore, the objective of this study is to evaluate the impact of dietary vitamin $D_3$ or 25(OH)$D_3$ on growth performance, bone health, blood biochemistry, carcass yield, and immune response in broiler chickens vaccinated against coccidiosis and subsequently challenged with *Eimeria* spp.

## MATERIAL AND METHODS

The experiment was conducted at the Poultry Research Center at University of Georgia, following the guidelines of animal use protocol for research, prior approval by the University of Georgia Institutional Animal Care and Use Committee (Animal Use Protocol #A2023 06-003-Y1-A0).

### Bird management, diets, and experimental design

A total of 900 off-sex male Cobb 500-day-old chicks were obtained from a local hatchery (Cleveland, GA). All broilers were vaccinated with a coccidiosis vaccine (Coccivac®-B52) at day of hatch and randomly assigned to 36-floor pens (121.92 cm × 121.92 cm). All pens had concrete floors covered with new pine shavings. The facility was equipped with a temperature-controlled environment, negative pressure ventilation, and evaporative cooling pads. Brooding temperature and lighting programs followed Cobb management guidelines. The initial temperature was set at 33.8°C and reduced weekly by 2.8°C until the conclusion of the experiment. A lighting program

of 23 hours light and 1 hour dark (23L:1D) was implemented for the first three days, followed by 20L:4D for the remainder of the study. Light intensity was maintained at full brightness for the first nine days and then reduced to 5 lux. Two HOBO (MX2300) temperature and humidity sensors (Onset, Bourne, MA 02532) were used to monitor house conditions.

Corn-soybean diets were formulated using Bestmix software (version 4.20.2320901) to meet the nutrient specifications of the Cobb 500™ Broiler Guidelines (Cobb, 2022). The basal diet included a vitamin premix (University of Georgia poultry PX) containing 1,400 IU/kg of vitamin $D_3$ (Table 4.1). All diets contained the same ingredients to minimize variation between batches. Chicks were allocated to floor pens that were randomly assigned to non-challenged control and challenged groups. The challenge dose was determined based on a pilot trial before the infection day. The pilot trial consisted of three different *Eimeria* challenge doses: a 0.5x (*E. acervulina* 125,000 oocysts/bird, *E. maxima* 25,000 oocysts/bird, and *E. tenella* 25,000 oocysts/bird), a 1x (*E. acervulina* 250,000 oocysts/bird, *E. maxima* 50,000 oocysts/bird, and *E. tenella* 50,000 oocysts/bird) and 2x (*E. acervulina* 500,000 oocysts/bird, *E. maxima* 100,000 oocysts/bird, and *E. tenella* 100,000 oocysts/bird). Birds receiving the 1x dose develop mild to moderate intestinal lesion scores and measurable but not severe reductions in body weight gain and feed efficiency compared to the unchallenged control. Therefore, on day 22, broilers in the challenged groups were orally gavaged with 1 mL of a solution containing 1x dose to simulate a mild to medium level of coccidiosis infection. Birds were assigned to four treatments in a completely randomized design. All treatments received the same basal diet containing 1,400 IU/kg of vitamin $D_3$ from day 1. The treatments were as follows: (1) NC, non-challenged control; (2) CC, *Eimeria*-challenged control; (3) DD, challenged + an additional 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500, dsm-firmenich, Switzerland) added on top of the basal diet; and (4) 25D, challenged + an additional

2,760 IU/kg (69 µg/kg) of 25(OH)$D_3$ (Hy-D®, dsm-firmenich, Switzerland). The supplemental vitamin sources (vitamin $D_3$ or 25(OH)$D_3$) were included in the diets throughout the study, not only at the time of challenge.. Paper tray feeders were provided for the first seven days and placed under the waterlines. The birds were raised until 42 days of age and were provided with a crumble diet during the starter phase (0–14 days) and pelleted diets during the grower (15–28 days) and finisher phases (28–42 days). Feed and water were provided *ad libitum*.

**Growth performance**

Broilers and feed were weighed by pen and recorded on days 0, 14, 28, and 42 to calculate body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR= FI/BWG). Mortality and the weight of dead birds were recorded as they occurred to adjust FI and FCR.

**Serum 25(OH)$D_3$ levels**

At days 28 and 42, one bird per pen was randomly selected for blood collection from the wing vein into a 5-mL blood tubes without anticoagulant (greiner bio-one, Monroe, NC), and stored at 4°C to allow clotting. Then, the blood samples were centrifuged at 1000 × g for 15 minutes (Allegra X-30R Centrifuge, Beckman Coulter Life Sciences Headquarters, Indianapolis, IN). The serum was extracted and transferred into sterile microcentrifuge tubes. Samples were stored at -80°C until analysis. Serum 25(OH)$D_3$ levels were determined using a mass spectrometry procedure (Heartland Assays, Ames, IA)

**Coccidia lesion scores and oocysts shedding**

On day 28 (6 days post-infection), three birds per pen were randomly selected and euthanized by cervical dislocation. Lesion scoring was performed by an experienced avian veterinarian who was blind to treatment allocation. The intestines were opened and scored for lesions of coccidiosis at three sites: the duodenum (from the pyloric junction to the most distal point of insertion of the duodenal mesentery), the jejunum (from the most distal point of insertion of the duodenal mesentery to the junction with Meckel's diverticulum), and the ceca. Lesions were scored on a scale of 0 to 4, following the method described by Johnson and Reid (1970), where a score of 0 indicated the absence of gross lesions, and a score of 4 represented extensive hemorrhage or severe lesions, depending on the *Eimeria* species. The lesion scores were averaged across the three birds as one statistical unit for analysis. The total lesion score was determined as the sum of lesion scores for the duodenum, jejunum, and ceca.

On the same day, 10 g of fresh fecal samples were collected from each pen and stored in sample bags at 4°C for oocyst shedding analysis. The fecal sample was mechanically mixed until it became homogenous. 2.5 g of excreta was transferred into a conical tube, diluted with 22.5 mL of hypertonic salt water, and mixed using a vortexer (Fisher Vortex Genie 2$^{TM}$ G560, FisherScientific, Hampton, NH) to ensure uniform suspension. After mixing, a mesh was placed to separate the large particles and to allow the oocyst to float. A sample was immediately taken using a 1-mL pipette, and the final mixture was loaded into a McMaster chamber and examined microscopically. Oocyst counts were done 2 times in whole feces collected from individual pen, then averaged for each pen and expressed as oocysts per gram of excreta.

**Blood chemical analysis**

On day 28, one of the birds selected for necropsy was also subjected for blood collection using the same methods described above. Following the manufacturer's instructions, whole blood was analyzed for pH, sodium (Na), potassium (K), chloride (Cl), total carbon dioxide ($CO_2$), anion gap, ionized calcium (iCa), glucose, and hematocrit using the i-STAT Alinity system with the i-STAT CHEM8+ cartridge (600-9008-25, Abaxis, Union City, CA, United States). Aspartate aminotransferase (AST), uric acid, glucose, total calcium (Ca), phosphorus (P), total protein, bicarbonate (HCO3), albumin, and globulin were analyzed using the VetScan VS2 with the Avian/Reptilian Profile Plus cartridge (500-0041, Abaxis, Union City, CA). Total carotenoid concentration in whole blood was measured using the iCheck Carotene Photometer and test kit (BioAnalyt, Berlin, Germany).

**Intestinal permeability**

On day 29, intestinal permeability was assessed using the fluorescein isothiocyanate–dextran (FITC-d; Sigma-Aldrich Co., St. Louis, MO) assay, following the method described by Lowery et al. (2020). One bird per pen was randomly selected, and 1 mL of FITC-d solution (6.4 mg/ml) was administered via oral gavage. One hour after inoculation, blood was collected and kept in the dark until clotting. The blood samples were centrifuged at 1500 × g for 5 minutes to separate the serum. A standard solution was prepared using serum pooled from unchallenged birds (5 birds) and to dunite FITC-d and making standard curve. The FITC-d levels in the serum samples and the standard solution were measured using a microplate reader (Victor Nivo™, PerkinElmer, Springfield, IL) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

**Whole body composition**

On day 42, two birds per pen were randomly selected, individually weighed, euthanized via cervical dislocation, and scanned using a GE whole-body dual-energy X-ray absorptiometry (DEXA) scanner (GE Healthcare, Chicago, IL) for body composition analysis, following the method described by Chen et al. (2020). Birds were positioned in a dorsoventral projection on the scanner, with the scanning mode set for small animals. After scanning, data on whole-body bone density, bone mineral content, fat percentage, lean weight, and lean muscle mass were collected. The average value of two birds/pen were used for data analysis.

**Tibia bone quality analysis**

On days 28 and 42, one bird per pen was selected, and the left tibia bone was collected for bone ash analysis. Bone ash parameters were measured according to the methods described by Kim et al. (2004) and Shi et al. (2023). The length of each tibia was measured using a caliper, then weighed and suspended in water at room temperature. Bone volume was calculated assuming the specific gravity of water is 1 g/cm³ at 22°C. The bones were dried at 100°C for 24 hours, followed by 48 hours of refluxing in a Soxhlet apparatus with hexane (Fisher Scientific International Inc., MA) to remove fat. The fat-free dry bones were then dried at 100°C for another 24 hours and reweighed to determine fat-free dry weight. Finally, the fat-free dry bones were ashed at 600°C for 12 hours to obtain the ash weight.

Tibia ash concentration was calculated:

$$\frac{Ash\ weight}{Bone\ volume} \times 100$$

and the ash percentage was calculated

$$\frac{Ash\ weight}{Fat\ free\ bone\ weight} \times 100$$

**Cytokines/Chemokines panel**

On days 28 and 42, the whole blood from one bird per pen was used to evaluate avian cytokine and chemokine profiles. The blood samples were centrifuged at 1000 × g for 15 minutes (Allegra X-30R Centrifuge, Beckman Coulter Life Sciences Headquarters, Indianapolis, IN) to separate the serum from whole blood, and the separated serum was stored at -80°C. The MILLIPLEX® Chicken Cytokines/Chemokines panel (GCYT1-16K, EMD Millipore Corporation, Billerica, MA) was utilized to quantify 11 different analytes. Interferon-alpha (IFN-α), Interferon-gamma (IFN-γ), Interleukins 16, 10, 21, and 6 (IL-16, IL-10, IL-21, IL-6), Macrophage-Colony Stimulating Factor (M-CSF), chemokine C–C motif ligand 20 (CCL20; also known as MIP-3α), chemokine C–C motif ligand 4 (CCL4; also known as MIP-1β), CC-chemokine ligand 5 (CCL5; also known as RANTES), and Vascular Endothelial Growth Factor (VEGF). This assay uses Luminex xMAP® bead-based immunoassay technology to measure circulating cytokine and chemokine protein concentrations directly and does not assess mRNA expression. This assay was done in the USDA-ARS Southern Plains Agricultural Research Center (College Station, TX, United States). The assay was run according to the manufacturer's instructions with standards, samples, and quality control in duplicates. Premix antibody immobilized beads, quality control, wash buffer, and the serum were prepared prior to use. Overnight incubation with shaking at 4°C (16–18 h, 500 rpm) occurred, and a handheld magnetic separation block (EMD Millipore Corp.) was used during the plate washing steps. Samples were analyzed using the MagPix® System (Luminex Corporation, Austin, TX). Luminex xPonent 4.2 software was used for data acquisition. Five-PL regression curves were generated to plot standard curves for all analytes using the

Milliplex Analyst 5.1 (Merck Millipore, Darmstadt, Germany) software, with bead median fluorescence intensity (MFI) values used for calculation. Results are given in pg/ml.

**Carcass yield**

On day 44, 8 birds/pen were randomly selected and individually weighed after a 10-hour feed withdrawal. They were placed in crates and transported to the on-site commercial-style pilot poultry processing facility at the University of Georgia (Athens, GA). Broilers were processed on an automated shackle line, including electrical stunning, manual slaughter, and bleeding for approximately 1 minute and 40 seconds. They were scalded at 58°C for 1 minute and 20 seconds, feather-picked, and had their head, neck, and feet removed. Evisceration involved organ removal, and hot carcass weights were recorded. Carcasses were chilled in ice buckets in a walk-in cooler for 4 hours and then weighed to determine cold carcass weight. Carcass were then cut into wings, leg quarters, breast fillets (Pectoralis major), breast tenders (Pectoralis minor), and racks with skin, then weighed individually. Yields were calculated as dressing percentage (cold carcass weight / live weight × 100) and part percentages (part weight / cold carcass weight × 100). Each pen is a statistical unit.

**Statistical analysis**

All experimental data were analyzed statistically using one-way ANOVA, with feed treatment as the main effect, each pen as statistical unit, in JMP Pro software version 17.2.0 (SAS Institute Inc., Cary, NC, 1989–2024). Mean separations were determined using Tukey's HSD test, with significance assessed at $P \leq 0.05$.

# RESULTS

## Serum 25(OH)$D_3$

Serum 25(OH)$D_3$ concentration is an indicator of vitamin $D_3$ status in the birds. The result showed that at d 28 (6 dpi), the coccidiosis challenge significantly reduced serum 25(OH)$D_3$ compared to the non-challenge group ($P$<0.001; Table 4.2), indicating the challenge negatively impacted vitamin $D_3$ status. However, the chicken fed with higher dose vitamin $D_3$ and 25(OH)$D_3$ increased serum 25(OH)$D_3$ concentration ($P$<0.001). On D 42, there were no statistical differences between non-challenge and challenged treatments. But additional vitamin $D_3$ and 25(OH)$D_3$ supplementation had higher serum 25(OH)$D_3$ levels than the CC group, with 25(OH)$D_3$ having the highest values compared to the other treatments ($P$<0.001). These findings indicate that adding vitamin $D_3$ or 25(OH)$D_3$ can mitigate the adverse effect of coccidiosis challenge on serum 25(OH)$D_3$ concentration, with 25(OH)$D_3$ showing higher efficacy.

## Growth Performances

No significant differences ($P$ > 0.05) in body weight (BW), body weight gain (BWG), or adjusted feed intake (FI) between treatments during the starter phase (Table 4.3). However, CC group which has not been challenged with coccidia but showed an increased FCR than NC, which might be due to the variation during this phase.

During the grower period (Table 4.4), the coccidiosis challenge negatively impacted growth performance in the challenged groups ($P$ < 0.001) compared to NC. Adding additional vitamin $D_3$ and 25(OH)$D_3$ did not improve BW, BWG, FI, or FCR during the coccidiosis challenge. Although broilers fed 25(OH)$D_3$ exhibited the highest values for all BW and BWG. The

coccidiosis challenge damaged the small intestine, reducing nutrient absorption and increasing intestinal injury, producing malabsorption syndrome. Unlike vitamin $D_3$, 25(OH)$D_3$ is absorbed directly into the bloodstream via the portal system, bypassing the first hydrolyzation step, becoming more bioavailable than vitamin $D_3$ under challenge. When analyzing the main effects of the challenge ($P < 0.001$), CC broilers weighed 12% less and had 20% lower BWG than NC broilers (1.495 ± 0.016 kg vs. 1.703 ± 0.016 kg and 1.101 ± 0.017 kg vs. 1. 271 ± 0.017 kg, respectively). The FI reduction in the challenged treatments, caused by the coccidiosis challenge, resulted in lower BW, BWG, and increased FCR. Adding DD or 25(OH)$D_3$ throughout the grower phase did not mitigate the performance reduction during the challenged phase

During the finisher phase (Table 4.5) similar to the grower phase, broilers in the *Eimeria*-challenged group were unable to recover weight loss compared to NC. No statistical differences were found between challenged groups in BW, BWG, FI, or FCR. However, DD treatment broilers had the lowest BW than CC and 25D. Broilers fed a basal diet without a challenge achieved the highest live performance parameters compared to *Eimeria* challenge groups ($P$<0.001). The coccidiosis challenge reduced BW at 42 days regardless of vitamin D source, with no statistical differences in BWG, FI, or FCR among treatments.

For overall growth performance (Table 4.6), similar to the grower phase, the *Eimeria*-challenged group had the lowest BWG, FI, and FCR ($P < 0.001$) compared to the NC during days 0–28. In the CC treatment, FI was reduced by over 6% compared to NC, affecting overall BWG while increasing FCR. For overall FCR at day 28, *Eimeria* challenge increased FCR in CC, DD and 25D compared to the CC. Supplementing extra vitamin $D_3$ or 25(OH)$D_3$ did not mitigate growth performance losses six days post-infection. Furthermore, BWG at day 42 in *Eimeria*-challenged birds did not recover to NC levels ($P < 0.001$), though DD-treated broilers exhibited

the highest gain among challenged groups, it also showed a higher FCR. No statistical differences were found in FI or FCR among dietary treatments in the overall growth performances (0-42 d).

**Intestinal permeability, lesion score, and fecal oocyst shedding**

The results for intestinal permeability, lesion scores, and fecal oocyst shedding are presented in Table 4.7. The lesion score distribution is shown in Figure 4.1. Intestinal permeability was not affected by the *Eimeria* challenge. However, broilers in the CC treatment exhibited increased intestinal lesion scores in the duodenum, jejunum, and cecal ($P < 0.05$) at day 28 (6 dpi) compared to the non-challenge. No statistical differences were observed between CC, DD, and 25D for *E. acervulina* and *E. maxima*. Dietary supplementation with DD or 25(OH)$D_3$ mitigated the cecal lesions induced by *E. tenella* in vaccinated birds ($P < 0.001$). However, a reduction in E. *tenella* lesions did not prevent a decrease in growth performances during the challenge. The mean oocysts per gram of feces is shown in Table 4.7. The NC group excreted fewer oocysts per gram of feces than the challenged treatments ($P < 0.001$). However, the NC group was not infected and was carefully managed to avoid cross-contamination. Although no statistical differences were observed in the CC, DD, and 25D, supplementation with any source of vitamin D numerically reduced oocysts shedding. Vitamin D played a significant role during the coccidiosis challenge, reducing oocysts shedding and mitigating intestinal lesion scores in the ceca.

**Bone health**

At day 28 (Table 4.8), *Eimeria* challenge reduced bone dry weight, fat-free dry weight, volume, and bone ash compared to the NC; however, 25(OH)$D_3$ alleviated these adverse effects ($P < 0.05$). 25D treatment exhibited the highest volume values comparable to CC, whereas the DD group displayed the lowest values ($P < 0.05$). Furthermore, the challenge significantly impacted

bone dry and fat-free dry weights. However, the DD treatment exhibited no significant differences compared to the CC ($P < 0.001$ and $P = 0.001$, respectively). In contrast, 25(OH)$D_3$ did not exert adverse effects and demonstrated an improvement in these parameters. Furthermore, DD treatment showed significantly lower bone weights than other treatments. Supplementing 69 µg/kg of 25(OH)$D_3$ improved fresh bone weight, volume, bone dry weight, fat-free dry weight and ash weight to levels comparable to those of the NC group. Notably, 25(OH)$D_3$ resulted in the highest ash weight compared to CC and DD. The *Eimeria* challenge significantly compromised tibia bone quality; however, supplementation with 69 µg/kg of 25(OH)$D_3$ mitigated these negative effects, restoring bone parameters to levels comparable to those of the NC treatment during the challenge time. These findings highlight the beneficial effects of the 25(OH)$D_3$ supplementation on tibia bone quality under *Eimeria* challenge.

At day 42 (Table 4.9), ash concentration did not differ significantly among the CC, NC, and 25D groups; however, the DD treatment exhibited the lowest ash concentration compared to the other treatments ($P = 0.041$). No statistical differences were observed in fresh bone weight, length, volume, bone dry and fat-free weight, ash weight, and ash percentage. The DD treatment was the most adversely affected at day 28 post-challenge but recovered by day 42. Additionally, whole-body composition was assessed on day 42 (Table 4.10), with no significant differences observed in whole-body DEXA scanning metrics, including BMD, BMC, area, fat, tissue, and lean mass.

**Whole Carcass Yield**

The effects of dietary treatments on live body weight, hot carcass weight, cold carcass weight, and dressing percentage in poultry are summarized in Table 4.11. Consistent with the grower and finisher phase, significant differences were observed between the NC and CC treatments for live body weight ($P$ = 0.016), hot carcass weight ($P$ = 0.005), and cold carcass weight ($P$ = 0.006), with NC showing higher values compared to CC. Notably, supplementation with vitamin $D_3$ and 25(OH)$D_3$ partially alleviated the adverse effects of the challenge. No statistical differences were detected in dressing percentage among treatments. These parameters followed a similar trend to body weight on day 42, where broilers subjected to the *Eimeria* challenge experienced reduced body weight, leading to decreased performance and whole carcass yield ($P < 0.05$). However, supplementation with vitamin $D_3$ or 25(OH)$D_3$ mitigated the adverse effects, demonstrating a numerical improvement in live body weight, hot carcass weight, and cold carcass weight.

**Parts yield**

Similar to whole carcass yield, broilers under the *Eimeria* challenge without additional vitamin $D_3$ or 25(OH)$D_3$ supplementation exhibited the lowest treatment effects on cut-up parts yield on day 44 (Table 4.12). Significant dietary effects were observed for leg ($P$ = 0.022), wing ($P$ = 0.001), and *Pectoralis* major ($P$ = 0.002) weights, with the NC treatment demonstrating the highest values compared to the CC, DD, and 25D treatments. Supplementation with vitamin $D_3$, particularly in the form of 25(OH)$D_3$, partially mitigated weight loss in the legs and wings under challenge conditions. Interestingly, this trend was not observed in the *Pectoralis* major, where the CC and 25D groups showed the lowest weight and part percentage ($P$ = 0.005) compared to the

NC and DD groups. No statistical differences were found in *Pectoralis* minor and rack+skin weights.

**Blood chemical analysis**

*Eimeria* challenge negatively affected total carotenoids in all challenged treatments ($P < 0.001$) compared to the CC treatment, while supplementation with vitamin $D_3$ or 25(OH)$D_3$ did not reverse the adverse effect. (Table 4.13). The impact of vitamin $D_3$ and 25(OH)$D_3$ supplementation in vaccinated broilers under *Eimeria* challenge on blood chemistry is also presented in Table 4.13. The *Eimeria* challenge did not significantly affect AST, uric acid, glucose, or globulin concentrations on day 28. However, challenged broilers exhibited significantly lower total protein and albumin levels than non-challenge broilers ($P = 0.004$; $P < 0.001$, respectively), and vitamin $D_3$ or 25(OH)$D_3$ supplementation did not restore these reductions. The albumin/globulin ratio remained unaffected by the challenge. However, vitamin $D_3$ supplementation resulted in the lowest albumin/globulin ratio, whereas 25(OH)$D_3$ maintained similar levels to other treatments ($P = 0.006$). Na, iCa, Cl, anion gap, hematocrit, hemoglobin, and bicarbonate levels were unaltered during the challenge period or by vitamin $D_3$ metabolite supplementation. In contrast, *Eimeria* challenge reduced total Ca ($P < 0.001$), P ($P = 0.001$), and $CO_2$ ($P = 0.043$) compared to the CC, while DD and 25D treatments showed no difference from the non-challenge treatment. Additionally, 25(OH)$D_3$ supplementation increased blood pH compared to CC and DD, with no significant differences from NC ($P = 0.021$).

**Cytokines**

The mean concentrations of cytokines (IL-16, IFN-α, IFN-γ, IL-10, IL-21, IL-2, IL-6), chemokines (MIP-3α, MIP-1β, RANTES), M-CSF, and VEGF at day 28 and 42 are presented in Tables 4.14 and 4.15, respectively. At day 28, the *Eimeria* challenge did not significantly affect

IL-16, IFN-γ, IL-10, IL-2, IL-6, MIP-3α, MIP-1β, RANTES, M-CSF, and VEGF. However, it downregulated IFN-α compared to the NC, while adding 25(OH)$D_3$ partially increased IFN-α levels ($P$=0.016). Additionally, birds that received extra vitamin $D_3$ during the *Eimeria* challenge resulted in the highest levels of IL-10, IL-6, chemokines (MIP-3α, MIP-1β, RANTES), M-CSF, and VEGF compared to CC and 25D ($P$<0.05). In contrast, 25(OH)$D_3$ downregulated IL-10, MIP-1b, RANTES, M-CSF, and VEGF ($P$<0.05), reaching similar levels as the NC. No statistical differences were observed in IFN-γ levels among treatments; however, the DD group showed a tendency to upregulate IFN-γ, whereas the 25(OH)$D_3$ treatment exhibited the opposite trend (P = 0.051). At day 42 (Table 4.15), no statistical differences were observed in IL-16, IFN-g, IL-10, IL-21, IL-2, IL-6, MIP-3α, MIP-1β, RANTES, M-CSF and VEGF ($P$ > 0.05). IFN-α levels remained unaffected during the recovery period following the *Eimeria* challenge; however, adding 25(OH)$D_3$ significantly reduced the IFN-α during the same period, while the DD group exhibited intermediate levels compared to other treatments ($P$ = 0.046).

## DISCUSSION

This study aimed to induce a mild to medium level of coccidiosis infection in growing vaccinated broilers to test the hypothesis that dietary 25(OH)$D_3$ enhances coccidiosis vaccination in broilers, subsequently mitigating the adverse effects of the *Eimeria* challenges. We additionally hypothesize that a more pronounced impact of 25(OH)$D_3$ compared to vitamin $D_3$ would affect parasite replication and its effects on intestinal gut integrity during the coccidiosis acute and recovery phase.

**Serum 25(OH)$D_3$ levels**

Serum 25(OH)$D_3$ was considered for vitamin D status because it is the primary storage form of vitamin D in animals and the blood 25(OH)$D_3$ levels reflect dietary intake (Michael F. Holick, 2007; M. F. Holick, 2007; Hollis, 2005; Leyva-Jimenez et al., 2019; Yarger, Saunders, et al., 1995). Our results show that the coccidiosis challenge significantly reduced serum 25(OH)$D_3$ compared to the non-challenge group 6 dpi (6.01 vs 11.75 ng/ml, respectively), negatively impacting vitamin $D_3$ status. However, broilers fed higher doses of vitamin $D_3$, or 25(OH)$D_3$, increased serum 25(OH)$D_3$ concentration. Through the finisher period, broilers in the PC recovered vitamin D status, although serum concentration remained the lowest during the trial. Meanwhile, extra vitamin $D_3$ or 25(OH)$D_3$ increased the serum 25(OH)$D_3$ levels, with 25(OH)$D_3$ showing higher efficacy. This result was similar to previous studies, where infection with *E. maxim*a reduced serum 25(OH)$D_3$ concentration at 6, 10, and 14 dpi, with treatment supplemented with 25(OH)$D_3$ being higher at all testing points (Panagiotis Sakkas et al., 2019). On the contrary, Suarez et al. (2023) also reported serum 25(OH)$D_3$ concentrations were higher in broilers fed diets containing 25(OH)$D_3$ than those fed vitamin $D_3$, regardless of vaccination status. Additionally, no interaction between vaccination and vitamin D source at day 10 or day 21 were observed, indicating that the vaccine did not alter the birds' vitamin D status response. The higher efficiency of 25(OH)$D_3$ can be explained by its direct absorption into the blood circulation. During an *Eimeria* challenge, the small intestine is damaged, reducing nutrient absorption, particularly of lipids (Rochell et al., 2016). However, unlike vitamin $D_3$, which must first be incorporated into chylomicrons before absorption, 25(OH)$D_3$ being a more polar and water-soluble molecule can be

absorbed more readily, even when intestinal lesions are present. This allows it to enter the bloodstream directly and exert its biological effects more efficiently.

**Growth performances and carcass yield**

Coccidiosis affects the overall health of broilers, especially gut health, by impairing growth rate and feed consumption while decreasing body weight uniformity (Mathis et al., 2024). In the present study, birds were vaccinated on day 0 and challenged on day 22. During the starter period (0-14D) vaccinated boilers supplemented with extra vitamin $D_3$ or 25(OH)$D_3$ showed no differences in BW, BWG, and FI. However, feed conversion differs between NC and CC groups. Because all birds received the same all basal diets (starter, grower, and finisher) throughout the experiment, dietary composition was not a confounding factor Suarez et al. (2021) observed no differences in body weight, feed intake, and feed conversion during the 7-day and 21-day periods in broilers vaccinated with 2x vaccine. However, a vaccine effect was observed at day 14, with vaccine broilers having significantly lower body weight and increased feed conversion. Similarly, Shi et al. (2022) found that a single-dose vaccine had no effect on BW gain or feed conversion at 14 days, but reduced FI. Considering these reported effects from vaccination, the difference in NC and CC might be due to the birds variations, including the adaptive immune response caused by vaccination in birds with an immature immune system (Gaghan et al., 2022; Song et al., 2021). The replication cycle of the vaccine *Eimeria* within the host can also influence physiological responses. Moreover, young broiler chicks do not yet possess a fully developed immune system (Fatoba & Adeleke, 2018), and the allocation of nutrients toward supporting immune responses, rather than growth, may further contribute to performance variability during the starter phase.

In the present study, broilers challenged with coccidiosis significantly reduced BW, BWG, FI, and FCR during the grower and finisher phases. These findings are consistent with previous research, which reported that broilers challenged with *E. maxima* exhibited decreased average daily gain, daily feed intake, and impaired FCR at six days post-infection (dpi) (Panagiotis Sakkas et al., 2019). Similarly, higher doses of *E. acervulina* ($1.0 \times 10^6$ oocysts) have been associated with a 20% reduction in growth performance (Rochell et al., 2016). *Eimeria* infection induces villus atrophy in the intestine (Panagiotis Sakkas et al., 2019), which significantly impairs nutrient absorption. In addition, *Eimeria* activates various physiological and immunological pathways as part of the host's response to parasitic invasion (Yun et al., 2000). This immune activation reallocates nutrients normally used for muscle growth toward immune function and repair, increasing the metabolic cost in their effort to control coccidiosis. Meanwhile, there are different levels of effects observed in yield composition. Similar observations have been reported by Oikeh et al. (2019) and Panagiotis Sakkas et al. (2019), who found that muscle development was impaired during infection and did not fully recover in the later stages of production.

Studies involving vitamin $D_3$ or 25(OH)$D_3$ have shown varying effects on growth performance and carcass yield in broilers, both under challenging and non-challenging conditions. In vaccinated challenged broilers with *Eimeria*, supplementation with vitamin $D_3$ or a combination of vitamin $D_3$ and 25(OH)$D_3$ improved overall performance at 21 days compared to the control group receiving 5 μg/kg of vitamin $D_3$ (Leyva-Jimenez et al., 2019). In this study, treatments supplemented with vitamin $D_3$ or 25(OH)$D_3$ partially restore the carcass weight, as well as leg and wing yields, compared to the challenged group without additional vitamin D. This response supports the notion that vitamin D plays roles beyond calcium and phosphorus homeostasis and bone health. 25(OH)$D_3$ has been shown to stimulate satellite cell activity in the pectoralis major

muscle of chickens and likely enhances muscle growth and proliferation through activation of the mTOR-RPS6K signaling pathway (Hutton et al., 2014; Vignale et al., 2015). This beneficial effect on muscle cells may be related to the presence of VDRs, which enhance muscle activity and support muscle growth, even under a coccidiosis challenge.

**Bone health**

Coccidiosis penalizes homeostasis in Ca and P during the infection (Oikeh et al., 2019; Panagiotis Sakkas et al., 2019). An imbalance in Ca and P can affect bone mineralization during coccidiosis infection. In this study, coccidiosis reduced tibia ash at 6 dpi. However, broilers were available to recover the bone losses at 20 dpi. These results demonstrated that 25(OH)$D_3$ can mitigate the adverse effects of coccidiosis challenge on bone quality. Agree with current study, coccidosis can reduce feed intake cause malabsorption syndrome. 25(OH)$D_3$ could increase bone quality and reduce leg problems during coccidiosis challenge (Panagiotis Sakkas et al., 2019; Suarez et al., 2023). However, these effects might also be related to Ca and P levels in the diets. in marginally deficient Ca/P diets, it is reported that either vitamin $D_3$ or 25(OH)$D_3$ did not improve tibia ash and tibia ash percentage. but in normal Ca/P diets 25(OH)$D_3$ is more bioavailable compared to vitamin $D_3$ (Oikeh et al., 2019). There is no intended deficiency in Ca and P in the current study design, but coccidiosis might cause mild Ca, P absorption issues due to intestinal damage; however, it did not reach the level of marginal deficiency, so it agrees with previous findings that 25(OH)$D_3$ still showed higher bioefficiency in maintaining bone health.

**Intestinal permeability**

Intestinal permeability was not significantly affected by the coccidiosis challenge nor the treatment in the current study. In contrast to our findings, previous research has demonstrated that

broilers challenged with a mixture of *Eimeria* spp. exhibited increased gut leakage (Santos et al., 2020). Similarly, in laying hens during peak production, *Eimeria* infection increased intestinal permeability and negatively affected performance. Supplementation with 5,000 IU/kg 25(OH)$D_3$ partially alleviated gut damage and reduced lesion severity caused by *Eimeria* spp. (Sharma et al., 2024). It might be due to either vaccination protection or the mild challenges dosage in the current study, which did not result in significant gut damage.

**Coccidia lesion scores and oocyst shedding**

In terms of coccidiosis status, while we did not observe any differences in intestinal lesion scores between challenged birds in *E. maxima* and *E. acervulina*, adding vitamin $D_3$ or 25(OH)$D_3$ reduced ceca lesions caused by *E. tenella* in this study. It has been reported that replication of *E. tenella* is inhibited by nitric oxide (NO) (Lillehoj & Li, 2004). Vitamin D, particularly 25(OH)$D_3$, increases NO production during an inflammatory challenge (Morris & Selvaraj, 2014). Immune cells respond to 25(OH)$D_3$ by upregulating the expression of vitamin D binding protein and 1α-hydroxylase, thereby enhancing the conversion of this precursor to its active form (Morris et al., 2015). Elevated levels of 1,25-dihydroxycholecalciferol may therefore stimulate NO production, contributing to the reduction in *E. tenella* lesions observed in this study. Oocyst counts were measured as total oocysts per gram of feces and were not differentiated by *Eimeria* species. No significant differences among challenged birds were detected, although vitamin $D_3$ or 25(OH)$D_3$ supplementation numerically reduced total oocyst output. Oocyst shedding and lesion scores may also have been influenced by the coccidiosis vaccine, confirming that broilers in this study were appropriately immunized. In contrast to our findings, higher dietary vitamin $D_3$ levels or partial replacement with 25(OH)$D_3$ have been associated with greater E. maxima replication (Panagiotis

Sakkas et al., 2019). Similarly, in challenged broilers, the combination of phytase and 25(OH)$D_3$ supplementation increased *E. maxima* oocyst shedding, whereas 25(OH)$D_3$ alone did not influence parasite replication (Shi et al., 2024).

**Blood chemical analysis**

Evaluating blood chemistry can help people understand how a parasitic infection such as coccidiosis can alter nutrient transport. It is well studied that different *Eimeria* species reduce plasma carotenoids (Ruff et al., 1974) *E. tenella* can reduce them by 62 to 74%. However, the combination of different *Eimeria* species can result in an even greater reduction of plasma carotenoids (Ruff et al., 1974). In this study, a 55% reduction in plasma carotenoids was reported, and adding any source of vitamin D did not improve plasma carotenoid levels. This confirms that during the early stages of an infection by a mixture of *Eimeria spp*., fat absorption can be reduced, as reflected in the decrease of both carotenoids and plasma 25(OH)$D_3$.

Total Ca, P, and K were affected by the *Eimeria spp*. challenges. *E. acervulina* has been reported to reduce calcium concentration during the acute phase of infection (Turk, 1986). Potassium did not show any difference between challenged and non-challenged treatments. However, the form of vitamin D [vitamin $D_3$ or 25(OH)$D_3$] affected blood potassium levels: vitamin $D_3$ showed the highest value, while 25(OH)$D_3$ had the lowest potassium level. Since potassium is an intracellular ion, its appearance in the bloodstream is expected to coincide with periods of the greatest cell damage and may result from extensive cellular damage in the intestinal lining (Turk, 1986).

**Cytokines/ Chemokines response**

The immune systems of birds and mammals share similar mechanisms for combating infectious agents and parasites (Shanmugasundaram & Selvaraj, 2012). As the first line of defense, the innate immune system activates macrophages and dendritic cells, which release various cytokines and chemokines to recruit additional immune cells, triggering inflammation and facilitating the elimination of pathogens. Infections with *Eimeria acervulina* or *Eimeria tenella* have been shown to increase the expression of several cytokines, including IFN-α, IL-1β, IL-6, and IL-17. Additionally, IL-10, which inhibits the synthesis of pro-inflammatory cytokines, was moderately elevated during infection (Hong et al., 2006). Furthermore, *Eimeria maxima* has been reported to significantly increase IL-10 levels following infection (Rothwell et al., 2004).

In this study, infection with coccidiosis altered both pro-inflammatory and anti-inflammatory responses, specifically affecting IFN-α, IL-10, IL-6, MIP-3α, and M-CSF. IFN-α, which stimulates NK cells and T lymphocytes, was reduced during the acute phase of infection. Conversely, the increase in M-CSF may have triggered a response involving elevated IL-10 and MIP-3α levels. IL-10 acts as a regulator to prevent excessive damage to the intestinal tissue, while MIP-3α contributes to T-cell activity in the intestine (Dal Pont et al., 2023). IL-6 promotes inflammation to combat the parasite by stimulating B cells and supporting the development of T lymphocytes, which in turn activate monocytes and dendritic cells (Dal Pont et al., 2023; Giansanti et al., 2006).

In this experiment, 25(OH)$D_3$ reduced both pro- and anti-inflammatory cytokine activity compared to supplementation with vitamin $D_3$ alone. This result is consistent with findings by Abascal-Ponciano et al. (2022), who also reported reduced cytokine responses on days 18 and 21

when birds were fed diets supplemented with 25(OH)$D_3$, compared to diets supplemented only with vitamin $D_3$ under optimal conditions. The first hydroxylation of vitamin D occurs in the liver; however, under inflammatory conditions, liver function may be impaired, reducing the conversion of vitamin $D_3$ to 25(OH)$D_3$. Supplementation with 25(OH)$D_3$ bypasses this first hydroxylation step, making it more bioavailable for activation in extra-renal tissues or immune cells where the 1α-hydroxylase enzyme and vitamin D receptor are present (Morris et al., 2014; Shanmugasundaram & Selvaraj, 2012). These findings suggest that 25(OH)$D_3$ can enhance the host's immune resilience and may help mitigate productivity losses associated with immunosuppression during coccidial infection. However, considering the birds were vaccinated before the challenge, the immune interactions of vaccination, challenge, and vitamin D could be complicated with only two time points of immune marker were captured, it is hard to understand the whole picture, but the results show 25(OH)$D_3$ is trending to bring the cytokines level back to none-challenge control, which might contribute to the reduced lesion scores in the ceca, and indicated the certain levels of protection. However further research is needed to understand the role of 25(OH)$D_3$ on immune response during the coccidiosis in the birds with vaccination.

## CONCLUSION

In conclusion, this study demonstrates that coccidiosis challenges significantly impair 25(OH)$D_3$ serum concentrations, broiler performance, intestinal health, nutrient absorption, and altered immune and physiological responses. Supplementation with vitamin $D_3$, mainly in 25(OH)$D_3$, mitigated some of these adverse effects by improving serum 25(OH)$D_3$ concentrations, intestinal lesion scores, bone health parameters, and oocyst shedding. However, vitamin $D_3$ or

25(OH)$D_3$ did not improved growth performance during the coccidiosis challenge, 25(OH)$D_3$ consistently exhibited superior efficacy, particularly in relieving ileal lesions and maintaining tibia bone parameters at levels comparable to non-challenged birds. However, the challenge-induced growth performance losses were not fully recovered by day 42, regardless of supplementation. Nevertheless, extra vitamin $D_3$ or 25(OH)$D_3$ partially increases carcass yield and cut-up at day 44. Additionally, dietary vitamin D supplementation modulated immune responses, with 25(OH)$D_3$ reducing pro-inflammatory cytokines and chemokines more effectively than vitamin $D_3$. This suggests that 25(OH)$D_3$ supports growth and intestinal health and modulates inflammatory responses during coccidiosis. Although differences in carcass yield and blood biomarkers were observed, the numerical improvements in growth and carcass parameters feature the importance of vitamin D, particularly 25(OH)$D_3$, in enhancing resilience to coccidiosis challenges. These findings highlight the potential of 25(OH)$D_3$ supplementation as a strategic intervention to support broiler health and productivity under disease-challenge conditions. However, further research is necessary to understand the interaction between vaccines and vitamin D at different levels of inclusion.

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## TABLES

Table 4.1. Diet formulation and calculated nutrient composition for the rearing period (starter, grower and finisher).[1]

| Ingredients % | Starter (0-14 d) | Grower (15-28 d) | Finisher (29-42 d) |
|---|---|---|---|
| **Corn** | 55.12 | 61.68 | 67.17 |
| **Soybean meal 46%** | 35.83 | 31.64 | 24.47 |
| **DDGS** | 4.01 | 1.90 | 3.83 |
| **Dicalcium phosphate** | 2.57 | 1.67 | 1.49 |
| **Soybean oil** | 0.50 | 1.00 | 1.00 |
| **Limestone** | 0.67 | 0.81 | 0.97 |
| **Salt** | 0.36 | 0.34 | 0.36 |
| **L-lysine HCl** | 0.24 | 0.25 | 0.21 |
| **DL-methionine** | 0.34 | 0.32 | 0.23 |
| **L-threonine** | 0.11 | 0.09 | 0.03 |
| **L-Valine** | 0.07 | 0.07 | 0.03 |
| **Choline Chloride 60%** | 0.01 | 0.06 | 0.03 |
| **Vitamin premix[2]** | 0.10 | 0.10 | 0.10 |
| **Trace mineral premix[3]** | 0.08 | 0.08 | 0.08 |
| **Calculated analysis** | | | |
| **ME (Kcal/kg)** | 2,942 | 3,050 | 3,080 |
| **CP (%)** | 22.50 | 20.50 | 18.00 |
| **Crude fiber (%)** | 2.47 | 3.34 | 3.32 |
| **Ash (%)** | 5.36 | 4.30 | 3.86 |
| **Crude fat (%)** | 2.57 | 2.55 | 2.76 |
| **Digestible lysine** | 1.26 | 1.16 | 0.96 |
| **Available phosphorus** | 0.58 | 0.40 | 0.36 |
| **Calcium** | 0.96 | 0.80 | 0.80 |
| **Kg:kg** | | | |
| **Met/Lys** | 51.37 | 52.19 | 50.97 |
| **(Met+Cys)/Lys** | 75.00 | 76.00 | 77.00 |

| | | | |
|---|---|---|---|
| **Trp/Lys** | 20.07 | 19.69 | 20.02 |
| **Thr/Lys** | 68.00 | 66.00 | 65.00 |
| **Arg/Lys** | 108.00 | 106.24 | 109.00 |
| **Val/Lys** | 76.00 | 76.00 | 77.00 |
| **Ile/Lys** | 66.44 | 65.08 | 67.22 |
| **Leu/Lys** | 136.58 | 136.43 | 151.07 |

Abbreviation: DDGS: Distiller Dried Grains with Soluble; ME: Metabolizable Energy; CP: Crude Protein.

[1] Treatments were added on top of each basal diet: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

[2]Vitamins provided per kg of premix: vitamin A, 3,527,360 IU; vitamin $D_3$, 1,399,921 IU; vitamin E, 19,400 IU; vitamin $B_{12}$, 8.8 mg; menadione, 1,102 mg; Riboflavin, 3,527 mg; α-pantothenic acid 5,467 mg; thiamine, 970 mg; niacin, 20,282 mg; vitamin $B_6$, 1,455 mg; folic acid, 573 mg; biotin, 79 mg.

[3]Supplied per kilogram of diet: Ca, 3.2%; Mn; 13.40%; Zn, 10.70%; Mg, 2.68%; Fe, 2.63%, Cu, 4000 ppm, I, 1000 ppm, Se, 400 ppm

Table 4.2 The effect of dietary supplementation of vitamin $D_3$ and 25(OH)$D_3$ metabolite on serum 25(OH)$D_3$ concentration on days 28 and 42

| Treatment | D 28 | D 42 |
|---|---|---|
| | ----- (ng/ml) ----- | |
| **NC** | 11.75[b] | 11.47[bc] |
| **CC** | 6.01[c] | 10.33[c] |
| **DD** | 17.73[a] | 17.30[b] |
| **25D** | 16.22[ab] | 32.22[a] |
| **SEM ±** | 1.17 | 1.69 |
| **Source of variation** | ---- *P*-value ---- | |
| Diet | <0.001 | <0.001 |

[a–c] Groups with different lowercase superscripts are significantly different from each other (P<0.05).
Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

Table 4.3 The effect of dietary vitamin $D_3$ and 25(OH)$D_3$ on growth performances during the starter period (0-14)

| Treatments | BW | BWG | FI* | FCR* |
|---|---|---|---|---|
| | ---------- kg ---------- | | | Kg:kg |
| **NC** | 0.484 | 0.443 | 0.567 | 1.29[b] |
| **CC** | 0.464 | 0.423 | 0.566 | 1.33[a] |
| **DD** | 0.466 | 0.425 | 0.548 | 1.29[b] |
| **25D** | 0.472 | 0.431 | 0.562 | 1.31[ab] |
| **SEM ±** | 0.005 | 0.005 | 0.006 | 0.007 |
| **Source of variation** | ---------- *P*-value ---------- | | | |
| **Diet** | 0.072 | 0.068 | 0.137 | 0.002 |

Abbreviation: BW: body weight; BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

*Average values of FI and FCR were used and adjusted for mortalities

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 4.4 The effect of dietary vitamin $D_3$ and 25(OH)$D_3$ on growth performances during the grower period (15-28) under a coccidiosis challenge

| Treatments | BW | BWG | FI* | FCR* |
|---|---|---|---|---|
| | ---------- kg ---------- | | | Kg:kg |
| **NC** | 1.703[a] | 1.271[a] | 1.876[a] | 1.46[b] |
| **CC** | 1.495[b] | 1.013[b] | 1.747[b] | 1.67[a] |
| **DD** | 1.493[b] | 1.035[b] | 1.712[b] | 1.67[a] |
| **25D** | 1.510[b] | 1.053[b] | 1.715[b] | 1.64[a] |
| **SEM ±** | 0.016 | 0.017 | 0.013 | 0.018 |
| **Source of variation** | ---------- *P*-value ---------- | | | |
| **Diet** | <0.001 | <0.001 | <0.001 | <0.001 |

Abbreviation: BW: body weight; BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

*Average values of FI and FCR were used and adjusted for mortalities

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 4.5 The effect of dietary vitamin $D_3$ and 25(OH)$D_3$ on growth performances during the finisher period (29-42)

| Treatments | BW | BWG | FI* | FCR* |
|---|---|---|---|---|
| | ---------- kg ---------- | | | Kg:kg |
| **NC** | 3.373[a] | 1.689 | 2.843 | 1.69 |
| **CC** | 3.122[b] | 1.620 | 2.818 | 1.73 |
| **DD** | 3.111[b] | 1.620 | 2.746 | 1.71 |
| **25D** | 3.138[b] | 1.638 | 2.856 | 1.74 |
| **SEM ±** | 0.049 | 0.032 | 0.045 | 0.015 |
| **Source of variation** | ---------- *P*-value ---------- | | | |
| **Diet** | <0.001 | 0.379 | 0.372 | 0.065 |

Abbreviation: BW: body weight; BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

*Average values of FI and FCR were used and adjusted for mortalities

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 4.6 The effect of dietary vitamin $D_3$ and 25(OH)$D_3$ on overall growth performances (0-28 and 0-42)

| | D28 | | | D42 | | |
|---|---|---|---|---|---|---|
| **Treatments** | **BWG** | **FI*** | **FCR*** | **BWG** | **FI*** | **FCR*** |
| | ------ Kg ------ | | Kg:kg | ------ Kg ------ | | Kg:kg |
| **NC** | 1.663[a] | 2.427[a] | 1.44[b] | 3.351[a] | 4.905 | 1.47 |
| **CC** | 1.455[b] | 2.291[b] | 1.55[a] | 3.096[b] | 4.613 | 1.53 |
| **DD** | 1.452[b] | 2.238[b] | 1.55[a] | 3.133[b] | 4.709 | 1.59 |
| **25D** | 1.470[b] | 2.266[b] | 1.54[a] | 3.114[b] | 4.522 | 1.52 |
| **SEM ±** | 0.016 | 0.017 | 0.008 | 0.037 | 0.157 | 0.036 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | |
| **Diet** | <0.001 | <0.001 | <0.001 | <0.001 | 0.375 | 0.191 |

Abbreviation: BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

*Average values of FI and FCR were used and adjusted for mortalities

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 4.7 The effect on dietary vitamin $D_3$ and 25(OH)$D_3$ on intestinal permeability, intestinal lesion and oocysts shedding

| Treatment | Intestinal permeability | Lesion score | | | Oocysts/g of feces |
|---|---|---|---|---|---|
| | | *E. Acervulina* | *E. maxima* | *E. tenella* | |
| | µg/mL | % | | | g |
| **NC** | 111.90 | 0.33[b] | 0.07[b] | 0.037[b] | 20,799[b] |
| **CC** | 132.81 | 1.11[a] | 1.19[a] | 1.074[a] | 229,215[a] |
| **DD** | 170.70 | 1.03[a] | 1.11[a] | 0.407[b] | 181,530[a] |
| **25D** | 159.83 | 0.96[a] | 1.19[a] | 0.518[b] | 184,282[a] |
| **SEM ±** | 19.15 | 0.130 | 0.182 | 0.131 | 31,999 |
| **Source of variation** | *P*-value | | | | |
| **Diet** | 0.134 | 0.001 | 0.001 | <0.001 | 0.001 |

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® D3-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 4.8 The effect of dietary vitamin $D_3$ and 25(OH)$D_3$ on tibia bone quality of vaccinated broiler under *Eimeria* challenge at 28 days (6 dpi)

| Treatment | Fresh Bone Weight | Length | Volume | Bone dry weight | Fat-free dry weight | Ash weight | Ash Concentration | Ash |
|---|---|---|---|---|---|---|---|---|
| | g | mm | cm3 | g | g | g | g/cm3 | % |
| **NC** | 10.20[a] | 82.31 | 8.08[ab] | 4.49[a] | 4.34[a] | 1.97[a] | 0.245 | 45.48 |
| **CC** | 9.16[ab] | 80.82 | 7.31[ab] | 3.77[b] | 3.56[b] | 1.59[b] | 0.228 | 44.83 |
| **DD** | 8.41[b] | 80.54 | 6.84[b] | 3.70[b] | 3.51[b] | 1.57[b] | 0.226 | 44.78 |
| **25D** | 10.15[a] | 82.36 | 8.33[a] | 4.48[a] | 4.26[a] | 1.93[a] | 0.232 | 45.52 |
| **SEM ±** | 0.378 | 0.953 | 0.367 | 0.129 | 0.141 | 0.069 | 0.001 | 0.586 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | | | |
| **Diet** | 0.005 | 0.403 | 0.026 | <0.001 | 0.001 | 0.001 | 0.089 | 0.709 |

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 4.9 The effect of dietary vitamin $D_3$ and 25(OH)$D_3$ on tibia bone quality of vaccinated broiler under *Eimeria* challenge at 42 days

| Treatment | Fresh Bone Weight | Length | Volume | Bone dry weight | Fat-free dry weight | Ash weight | Ash Concentration | Ash |
|---|---|---|---|---|---|---|---|---|
| | g | mm | cm3 | g | g | g | g/cm3 | % |
| **NC** | 13.23 | 101.48 | 9.58 | 8.19 | 7.43 | 3.58 | 0.37[ab] | 48.29 |
| **CC** | 12.79 | 99.92 | 9.24 | 7.94 | 7.27 | 3.57 | 0.38[a] | 49.05 |
| **DD** | 12.85 | 99.48 | 9.19 | 7.90 | 6.97 | 3.33 | 0.36[b] | 47.83 |
| **25D** | 13.52 | 99.67 | 9.87 | 8.07 | 7.46 | 3.64 | 0.37[ab] | 48.78 |
| **SEM ±** | 0.498 | 0.899 | 0.307 | 0.252 | 0.273 | 0.137 | 0.005 | 0.537 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | | | |
| **Diet** | 0.701 | 0.787 | 0.381 | 0.836 | 0.574 | 0.407 | 0.041 | 0.399 |

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 4.10 The effect of dietary vitamin $D_3$ and 25(OH)$D_3$ on whole body composition of vaccinated broiler under *Eimeria* challenge at 42 days

| Treatment | Body weight | BMD | BMC | Area | Fat | Tissue | Fat | Lean |
|---|---|---|---|---|---|---|---|---|
| | **kg** | g/cm2 | g | cm2 | % | **g** | **g/cm3** | **%** |
| **NC** | 3392.78 | 0.2000 | 48.13 | 240.39 | 14.66 | 3266.33 | 477.56 | 2788.67 |
| **CC** | 3236.63 | 0.2008 | 47.02 | 233.83 | 14.42 | 3111.94 | 438.72 | 2670.63 |
| **DD** | 3141.33 | 0.1999 | 46.23 | 231.00 | 14.93 | 3020.39 | 450.50 | 2569.78 |
| **25D** | 3224.89 | 0.2013 | 47.73 | 236.94 | 14.19 | 3100.72 | 451.50 | 2649.22 |
| **SEM ±** | 66.590 | 0.002 | 1.123 | 3.508 | 0.408 | 64.350 | 14.374 | 58.900 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | | | |
| **Diet** | 0.073 | 0.9738 | 0.6517 | 0.2827 | 0.6234 | 0.0679 | 0.2916 | 0.0838 |

Abbreviation: BMD: bone mineral density; BMC: bone mineral content.

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

Table 4.11. The effect of dietary treatments on processing yields of Cobb 500 broilers on d 44

| Treatment | Live Body weight | Hot carcass weight | Cold carcass weight | Dressing |
|---|---|---|---|---|
| | ---------- kg ---------- | | | % |
| **NC** | 3.507[a] | 2.640[a] | 2.657[a] | 75.64 |
| **CC** | 3.185[b] | 2.353[b] | 2.368[b] | 74.81 |
| **DD** | 3.319[ab] | 2.467[ab] | 2.489[ab] | 74.27 |
| **25D** | 3.331[ab] | 2.460[ab] | 2.476[ab] | 74.25 |
| **SEM ±** | 66.58 | 0.053 | 0.054 | 0.391 |
| **Source of variation** | ---------- *P*-value ---------- | | | |
| **Diet** | 0.016 | 0.005 | 0.006 | 0.055 |

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® D3-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

[a ,b] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Dressing = (cold carcass weight / live body weight) * 100

Table 4.12. The effect of dietary treatments on cut-up parts yield of Cobb 500 on d 44.

| Treatment | *Pectoralis* major | | *Pectoralis* minor | | Legs | | Wing | | Rack + Skin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | g | % | g | % | g | % | g | % | g | % |
| **NC** | 770.19[a] | 28.82[a] | 136.51 | 5.15 | 745.81[a] | 28.16 | 271.07[a] | 10.24 | 719.83 | 27.11 |
| **CC** | 648.39[b] | 27.12[b] | 123.97 | 5.24 | 685.19[b] | 29.03 | 242.75[b] | 10.29 | 654.58 | 27.76 |
| **DD** | 695.26[ab] | 27.67[ab] | 128.56 | 5.16 | 710.72[ab] | 28.71 | 252.69[b] | 10.18 | 685.82 | 27.63 |
| **25D** | 676.21[b] | 27.14[b] | 128.42 | 5.19 | 714.88[ab] | 28.94 | 253.85[ab] | 10.27 | 689.11 | 27.90 |
| **SEM ±** | 21.02 | 0.353 | 3.36 | 0.085 | 13.00 | 0.266 | 4.69 | 0.094 | 16.67 | 0.337 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | | | | | |
| **Diet** | 0.002 | 0.005 | 0.084 | 0.871 | 0.022 | 0.113 | 0.001 | 0.855 | 0.072 | 0.392 |

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

[a ,b] Groups with different lowercase superscript are significantly different from each other ($P<0.05$).

Table 4.13 The effect of dietary treatment on whole blood biochemistry in broiler chickens under *Eimeria* challenge at d 28 (6dpi) (1/3)

| Treatment | Carotenoids | AST | Uric Acid | Glucose | Total protein | Albumin | Globulin | Albumin/ Globulin |
|---|---|---|---|---|---|---|---|---|
| | **g** | U/L | mg/dL | **mg/dL** | g/dL | g/dL | g/dL | |
| **NC** | 2.09[a] | 277.86 | 5.84 | 271.13 | 3.23[a] | 2.11[a] | 1.13 | 1.91[a] |
| **CC** | 0.93[b] | 233.78 | 10.83 | 263.78 | 2.78[b] | 1.72[b] | 1.07 | 1.62[ab] |
| **DD** | 0.94[b] | 216.50 | 8.35 | 263.78 | 2.77[b] | 1.62[b] | 1.14 | 1.45[b] |
| **25D** | 0.94[b] | 213.11 | 7.07 | 250.67 | 2.87[b] | 1.78[b] | 1.07 | 1.70[ab] |
| **SEM ±** | 0.120 | 16.708 | 1.373 | 6.197 | 0.094 | 0.061 | 0.058 | 0.087 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | | | |
| **Diet** | <0.001 | 0.052 | 0.0789 | 0.155 | 0.004 | <0.001 | 0.702 | 0.006 |

Abbreviation: AST: aspartate aminotransferase; Ca: calcium; P: phosphorus; Na: sodium; K: potassium; Cl: chloride; $CO_2$: total carbon dioxide; iCa: ionized calcium; $HCO_3$: bicarbonate

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

[a-c] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 4.13 The effect of dietary treatment on whole blood biochemistry in broiler chickens under *Eimeria* challenge at d 28 (6dpi) (2/3)

| Treatment | Total Ca | P | K | Na | iCa | Cl | $CO_2$ |
|---|---|---|---|---|---|---|---|
| | mg/dL | mg/dL | **mmol/L** | **mmol/L** | **mmol/L** | **mmol/L** | **mmol/L** |
| **NC** | 11.86[a] | 7.18[a] | 5.37[bc] | 145.00 | 1.42 | 109.00 | 27.11[a] |
| **CC** | 10.83[b] | 5.60[b] | 5.87[ab] | 142.89 | 1.44 | 110.00 | 23.88[b] |
| **DD** | 10.92[b] | 6.25[b] | 6.16[a] | 141.67 | 1.41 | 109.22 | 23.88[b] |
| **25D** | 10.68[b] | 5.70[b] | 5.25[c] | 141.00 | 1.40 | 106.67 | 26.55[ab] |
| **SEM ±** | 0.133 | 0.229 | 0.161 | 1.06 | 0.026 | 0.999 | 0.984 |
| **Source of variation** | ---------- *P*-value ---------- | | | | | | |
| **Diet** | <0.001 | 0.001 | 0.001 | 0.057 | 0.804 | 0.124 | 0.043 |

Abbreviation: AST: aspartate aminotransferase; Ca: calcium; P: phosphorus; Na: sodium; K: potassium; Cl: chloride; $CO_2$: total carbon dioxide; iCa: ionized calcium; $HCO_3$: bicarbonate

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 μg)/kg of 25(OH)$D_3$ (Hy-D®)

[a-c] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 4.13 The effect of dietary treatment on whole blood biochemistry in broiler chickens under *Eimeria* challenge at d 28 (6dpi) (3/3)

| **Treatment** | **Anion Gap** | **Hematocrit** | **Hemoglobin** | HCO3 | **pH** |
|---|---|---|---|---|---|
| | **mmol/L** | **%** | **g/dL** | **mmol/L** | |
| **NC** | 15.44 | 20.56 | 7.00 | 25.93 | 7.48[ab] |
| **CC** | 15.89 | 21.13 | 7.19 | 22.88 | 7.46[b] |
| **DD** | 15.33 | 21.11 | 7.19 | 23.12 | 7.46[b] |
| **25D** | 13.89 | 20.00 | 6.80 | 25.70 | 7.52[a] |
| **SEM ±** | 0.645 | 0.463 | 0.157 | 1.017 | 0.016 |
| **Source of variation** | ---------- *P*-value ---------- | | | | |
| **Diet** | 0.166 | 0.277 | 0.259 | 0.070 | 0.021 |

Abbreviation: AST: aspartate aminotransferase; Ca: calcium; P: phosphorus; Na: sodium; K: potassium; Cl: chloride; $CO_2$: total carbon dioxide; iCa: ionized calcium; $HCO_3$: bicarbonate

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

[a-c] Groups with different lowercase superscripts are significantly different from each other ($P<0.05$).

Table 4.14. The effect of dietary treatment on cytokines (IL-16, IFN-α, IFN-γ, IL-10, IL-2, IL-6), chemokines (MIP-3α, MIP-1β, RANTES), M-CSF, and VEGF at d 28 (6 dpi).

| **Treatment** | IL-16 | IFN-α | IFN-γ | IL-10 | IL-2 | IL-6 | MIP-3α | MIP-1 β | RANTES | M-CSF | VEGF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | pg/mL | | | | | | | | | | |
| **NC** | 267.95 | 15.02[a] | 141.49 | 85.95[b] | 651.26 | 180.72[b] | 181.75[b] | 46.12[b] | 61.03[b] | 580.22[b] | 27.27[b] |
| **CC** | 273.21 | 6.16[b] | 133.72 | 238.00[ab] | 752.85 | 318.55[ab] | 301.09[ab] | 66.19[ab] | 62.43[b] | 616.21[ab] | 36.02[ab] |
| **DD** | 230.43 | 7.05[b] | 338.67 | 439.06[a] | 621.22 | 836.19[a] | 571.40[a] | 110.37[a] | 146.92[a] | 643.58[a] | 112.37[a] |
| **25D** | 200.18 | 8.49[ab] | 75.88 | 167.29[b] | 807.46 | 233.35[ab] | 240.47[ab] | 53.03[b] | 53.65[b] | 571.49[b] | 27.23[b] |
| **SEM ±** | 32.05 | 2.03 | 65.59 | 63.67 | 72.57 | 158.04 | 97.26 | 14.59 | 16.73 | 14.6 | 19.95 |
| **Source of variation** | *P*-value | | | | | | | | | | |
| **Diet** | 0.336 | 0.016 | 0.051 | 0.004 | 0.2464 | 0.027 | 0.044 | 0.021 | 0.001 | 0.004 | 0.010 |

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

[a-c] Groups with different lowercase superscripts are significantly different from each other (P<0.05).

Table 4.15. The effect of dietary treatment on cytokines (IL-16, IFN-α, IFN-γ, IL-10, IL-21, IL-2, IL-6), chemokines (MIP-3α, MIP-1β, RANTES), M-CSF and VEGF at d 42

| **Treatment** | IL-16 | IFN-α | IFN-g | IL-10 | IL-21 | IL-2 | IL-6 | MIP-3α | MIP-1β | RANTES | M-CSF | VEGF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pg/mL | | | | | | | | | | | |
| **NC** | 349.50 | 10.85[ab] | 110.41 | 168.95 | 24.25 | 560.62 | 349.50 | 238.98 | 54.76 | 195.34 | 637.38 | 42.32 |
| **CC** | 303.29 | 14.59[a] | 111.19 | 235.71 | 39.14 | 612.17 | 303.29 | 330.79 | 62.43 | 185.75 | 630.31 | 43.52 |
| **DD** | 316.58 | 10.46[ab] | 312.25 | 273.43 | 22.71 | 542.74 | 316.58 | 252.36 | 71.66 | 188.48 | 640.73 | 52.59 |
| **25D** | 348.02 | 5.57[b] | 295.61 | 271.43 | 30.07 | 627.80 | 348.02 | 868.20 | 75.16 | 182.40 | 644.21 | 59.19 |
| **SEM ±** | 45.76 | 2.10 | 66.15 | 56.02 | 9.44 | 47.50 | 45.76 | 284.27 | 9.35 | 34.25 | 17.83 | 34.25 |
| **Source of variation** | *P*-value | | | | | | | | | | | |
| **Diet** | 0.858 | 0.046 | 0.053 | 0.518 | 0.606 | 0.541 | 0.858 | 0.359 | 0.417 | 0.994 | 0.953 | 0.736 |

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

[a-c] Groups with different lowercase superscripts are significantly different from each other (P<0.05).

## FIGURE

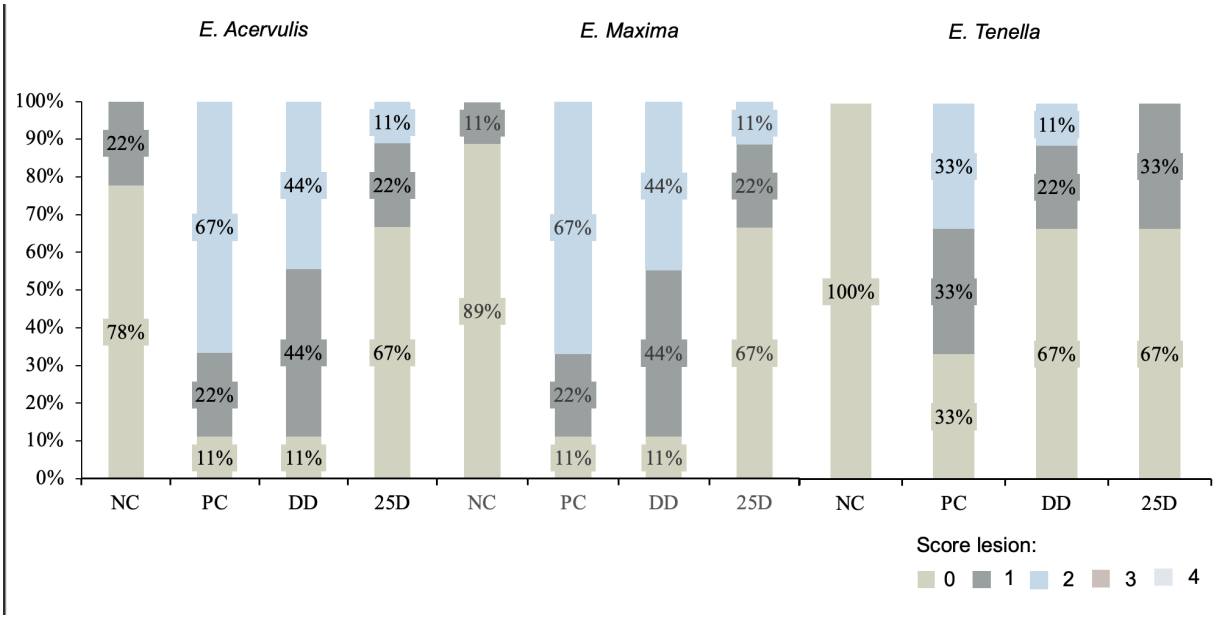


**Figure 1.** Effect of dietary vitamin $D_3$, or 25(OH)$D_3$ supplementation in cocci-vaccine broilers on percentage of lesion score in the duodenum, jejunum-ileum junction, and ceca of challenged broilers with *Eimeria* spp on d 28 (6 dpi)

Treatments: NC; non-challenge control + 1,400 UI/kg Vitamin $D_3$; CC: *Eimeria* challenge + 1,400 UI/kg Vitamin $D_3$; DD: CC + 2,760 IU/kg vitamin $D_3$ (Rovimix® $D_3$-500); 25D: CC + 2,760 IU (69 µg)/kg of 25(OH)$D_3$ (Hy-D®)

# CHAPTER 5

## USING A QUANTITATIVE ULTRASOUND MACHINE AS A NONINVASIVE BONE HEALTH ASSESSMENT TOOL IN BROILER CHICKENS

Mejía-Abaunza, Nicolás. To be Submitted to Poultry Science.

## ABSTRACT

Lameness is one of the major causes of mortality in heavy broiler production and results in economic losses and animal welfare concerns. Establishing a noninvasive, on-farm tool to assess bone health in broiler chickens is beneficial and could improve flock management. Quantitative ultrasound is used in humans to characterize bone density using attenuation and speed of sound (SOS) measurements. However, this method is not well studied in broilers. The current study aimed to explore using SOS measured with an ultrasound machine as an indicator of bone mineralization in broilers. Forty-eight randomly selected birds from a 42-day-old broiler flock were examined with an ultrasound device (HMD-A3) to obtain SOS in the tarsometatarsus (un-feathered). The tarsometatarsus length (L) and diameters (posterior to anterior (W1) and medial to lateral (W2) were measured using a caliper. The tarsometatarsus volume (VOL) is approximately calculated by L x W1 x W2. Whole body and tarsometatarsus bone mineral density (BMD) and bone mineral content (BMC) were determined by Dual-energy X-ray absorptiometry (DEXA) scanner. Additionally, SOS is used to assess bone density, and when combined with tarsometatarsus VOL, it can be converted into a parameter similar to bone mineral content, expressed as SOS x VOL. The correlation was obtained using a linear regression model in JMP 17.0. Significance level was considered at $P<0.05$. The results showed a positive linear relationship between SOS and tarsometatarsus BMD ($P = 0.0346$; r = 0.3231), as well as between SOS x VOL and tarsometatarsus BMC ($P = 0.0186$; r = 0.3386). The current results indicate that SOS could reflect the bone mineral density in the tarsometatarsus. No significant correlation was found in tarsometatarsus BMD and whole-body BMD ($P = 0.1875$; r = 1956). However, tarsometatarsus BMC is correlated to both whole-body BMD ($P < 0.001$; r = 0.6387) and BMC ($P < 0.001$; r =

0.7724). Ultimately, SOS x VOL is positively correlated with whole-body BMC ($P = 0.0264$; r = 0.3203) in relation to SOS x VOL. In conclusion, SOS could be used as an indicator of shank BMD, and the product of SOS with volumetric approximate parameter could be used as an indicator of tarsometatarsus BMC and, ultimately, to evaluate whole-body BMD and BMC in 42-day-old broilers. The current study shows the possibility of using quantitative ultrasound as a noninvasive bone mineralization assessment tool in broilers. However, a dataset with more observations at different ages is necessary to establish an accurate prediction of BMD and BMC in broilers.

## INTRODUCTION

Bone disorders remain among the most costly conditions associated with rapid growth and represent one of the most serious welfare concerns in commercial broiler production (Sanotra et al., 2003). Lameness is observed in approximately 53% of broiler chickens, with an additional 44% exhibiting slight abnormalities (Granquist et al., 2019). It constitutes a major welfare issue because locomotion is impaired by pain during walking attempts. Birds with leg problems tend to remain seated or lying down, which prevents them from eating and drinking, ultimately leading to starvation and reduced body condition (Sanotra et al., 2003). Economic losses are primarily related to culling, reduced feed efficiency, lower carcass weight at slaughter, and increased mortality during production. Furthermore, expenses associated with treatment and preventive measures contribute substantially to the overall economic burden, which has been estimated at approximately $4 billion annually (Ao, 2016).

Understand the bone quality is important to develop a strategy to reduce bone health issues. Currently, several methods are available for assessing bone quality. Traditional approaches include bone ash analysis (Donnelly, 2011), bone-breaking strength, and histological evaluation. However, these techniques are time-consuming, invasive, and primarily focused on planar morphology. More advanced tools are also available, such as dual-energy X-ray absorptiometry (DEXA) (Hester et al., 2004), quantitative computed tomography (QCT) (Korver et al., 2004), and micro-computed tomography (micro-CT) (Chen & Kim, 2020). Nevertheless, these methods present challenges, as results are influenced by bird positioning during scanning, and the equipment is bulky, expensive, and requires specialized expertise. Thus, less invasive methods for assessing bone health may represent more attractive alternatives.

QUS is an established diagnostic technique in human medicine for bone assessment involving clinical osteoporosis (Hans et al., 2022; Y. Xu et al., 2016) and premature bone diseases (Tarrell et al., 2023). QUS measures the speed of sound (SOS), which is determined by transmitting an ultrasonic signal along the cortex using a probe equipped with four ultrasonic transducers (two emitters and two receivers) (Yang Xu et al., 2016). QUS could be a potential tool to understand the chicken bone quality; it also has advantages compared to DEXA, computed tomography, and micro-computed tomography, as quantitative ultrasonography does not involve radiation, making it safer to use in the lab or field. Moreover, the equipment is more portable and less expensive. However, some limitations of the QUS include its limited current use and limited knowledge regarding its application in the poultry industry, as well as the feather coverage that makes sound conduction impossible for some parts of the chicken's body.

Early work on layer hens demonstrated QUS as a sustainable tool for detecting poor bone quality (Fleming et al., 2004; Martinez-Cummer et al., 2006). A study conducted by Fleming et al. (2004) compared different tools for assessing bone mineral density, including radiographic density, QCT, and ultrasound. The ultrasound measurement was taken at the distal end of the first phalanx of the third toe and correlated with QCT and radiographic density values. This correlation with the QCT was significant but not strongly correlated. On the other hand, correlations between 3rd toe ultrasound and 3rd toe radiographic density values were weak but significant, whereas those between 3rd toe ultrasound and 3rd toe shear energy to maximum load were stronger (Fleming et al., 2004). This finding suggests that the ultrasound machine could be an alternative to current bone quality assessment methods. Similarly, Martinez-Cummer et al. (2006) indicates the QUS is capable of detecting differences in the humeral SOS that are repeatable over time and correlate with classical in vivo measurement of bone density. In contrast, a more recently study

conducted by Crespo et al. (2024) reported no significant association between SOS and the biochemical properties of the bone.

Given the inconsistencies in the literature and the limited knowledge regarding QUS application in broilers, the current study is to aims to evaluate the use of QUS on bone quality assessment in broiler chickens. Different than previous studies, we chose the tarsometatarsus as the targeted measuring site, due to no feather coverage, and it is easy to assess which makes it possible to perform the measurement on live birds in the field. We hypothesized that the speed of sound is correlated with mineralization parameters in the tarsometatarsus. Additionally, the quality of the tarsometatarsus reflects whole-body bone mineralization, and SOS measurements may also represent overall bone quality. Therefore, the objective of this study was to explore the use of SOS measured by an ultrasound device as an indicator of bone mineralization in 42-day-old broilers.

## MATERIAL AND METHODS

### Birds' management, diets, and experimental design

The experiment was conducted following the guidelines of animal use protocol for research, prior approval by the University of Georgia Institutional Animal Care and Use Committee (Animal Use Protocol #A2023 04-013-Y1-A0).

A total of 792 male Cobb 500 days-old broilers were raised at the Poultry Research Center (University of Georgia, Athens, GA) 36-floor pens (121 x 146 $cm^2$) covered with pine shaving. Broilers were randomly assigned to 3 dietary treatments with 22 chicks per group and twelve replicates using a completely randomized design. The corn-soybean diets (Table 1) were formulated to meet the nutrient specifications for Cobb500 Broiler guidelines (Cobb, 2022), with

a vitamin premix containing 1,400 IU/kg vitamin $D_3$ as the basal diet (T1, Table 5.1). Treatments 2 (T2) and 3 (T3) included an additional 34.5 or 69 µg/kg of 25(OH)$D_3$ (Smart-D®, Nutribins, Walnut, CA), respectively. Broilers were raised until d 42 (starter phase, 0-14d; grower phase, 15-28d; finisher 1, 29-35d; finisher 2, 36-42), and feed and water were provided *ad libitum*.

The house was equipped with a temperature-controlled environment. From d 1 to 35, environmental conditions followed Cobb's guidelines (33+/- 1oC from 1 to 35, decreasing by 3°C until it reached 21+/- 1°C). From d 36 to the end of the trial, broiler chickens were exposed to hot weather conditions. The heat treatment was from 9 a.m. to 5 p.m. The temperature started at 29+/- 1°C and lowered to 21+/- 1°C during the remaining hours. Two HOBO (MX2300) temperature and humidity sensors (Onset, Bourne, MA 02532) recorded the heat stress period to ensure the management target. Rectal temperatures were also measured before and after the heat treatment to confirm that the birds were subjected to appropriate heat treatment (data not shown). The measurements showed an average increase of 1.26°C from the start of the treatment. The light schedule was kept at 23L:1D for the first 3 days and 20L:4D for the rest of the trial. Forty-eight broilers were selected for further bone analysis.

**Bone analysis**

On day 42, forty-eight broilers were randomly selected from the entire experimental population without considering treatments and euthanized by cervical dislocation. Following euthanasia, the broilers were then positioned chest down on the scanner. After scanning and getting the whole-body composition, the right tarsometatarsus was removed by cutting the articulation and was scanned again. Immediately following the measurement of DEXA, the tarsometatarsus was

stored at -20 °C until further analysis. The bone density was measured by GE dual X-ray absorptiometry (DEXA) scanner (GE Healthcare, Chicago, IL) and quantitative ultrasound (QUS) devices HMD-A3 (Healicon Medical Equipment Co Ltd.) devices recorded as the speed of sound (SOS). Each tarsometatarsus was measured in semilunar DEXA and a quantitative ultrasound device. The scanning mode was set for small animals in the DEXA. Each tarsometatarsus was thawed overnight in a cold room, and during analysis, samples were maintained at room temperature. The QUS device was calibrated at 21 ˚C according to the manufacturer's recommendations, and measurements were taken three times per tarsometatarsus and averaged to minimize variability among samples. The diameter of the tarsometatarsus was measured at mid-shaft with a caliper with a precision of 0.01mm, measuring the posterior to anterior and medial to lateral, and the length was measured from the tarsometatarsal joint to the palm. The data of whole-body bone mineral density (BMD) and bone mineral content (BMC) were collected, as well as tarsometatarsus bone mineral density and mineral content. The tarsometatarsus volume was obtained by multiplying the length by diameter one (posterior to anterior) and diameter two (medial to lateral).

**Statistical analysis**

Statistical analyses were performed using JMP Pro 17 (SAS Institute Inc., Cary, NC, USA). A simple linear regression model ($y = b_0 + b_1 x$) was used to examine the relationship between the variables of interest. The strength and direction of these relationships were evaluated using the correlation coefficient (r). Values of r were interpreted based on conventional thresholds: strong ($r \geq 0.8$), moderate ($0.5 \leq r < 0.8$), and weak ($r < 0.5$). Statistical significance was established at $P \leq 0.05$.

## RESULTS AND DISCUSSION

In this study, we evaluated quantitative ultrasound (QUS) as a tool to indicate bone mineralization and compared it with DEXA, the gold standard in bone quality analysis. The QUS equipment is more portable and less expensive, allowing for on-farm assessment of bone quality in broiler chickens. The speed of sound (SOS) varied between repeated measurements of the same tarsometatarsus; therefore, the average of three readings was used for statistical analysis. In studies conducted on laying hens, sampling different bones has been shown to affect the results. Fleming et al. (2004) also reported that the speed of sound depends on the type of bone, making it necessary to report the specific bone analyzed. On the other hand, Crespo et al. (2024) the speed of sound was measured in the humerus and tarsometatarsus of laying hens using two quantitative ultrasound devices and, after correcting for measurement differences, reported no significant differences in SOS values between the humerus and tarsometatarsus. In the present study, the tarsometatarsus was analyzed without removing any tissue, to mimic the situation of measuring the live birds at farm without removing any feathers. The results were compared with DEXA to understand the correlation between the two tools for assessing bone health.

In Figure 5.1a, a positive correlation was observed between BMD and the speed of sound (SOS) in the tarsometatarsus. This indicates that as the SOS increases, the bone mineral density of the tarsometatarsus also tends to increase, although the correlation coefficient is weak ($P = 0.034$; $r = 0.323$). as reflected by the positive slope of the regression line showing that higher SOS values are associated with greater bone density. Additionally, SOS is used to estimate bone density and

elasticity, since sound waves travel faster through denser bone. For this reason, SOS was multiplied by the tarsometatarsus volume to derive a parameter similar to BMC. In Figure 5.1b, a significant positive correlation was observed between BMC and SOSxVOL of the metatarsus. This correlation indicates that as BMC in the tarsometatarsus increases, SOSxVOL also increases ($P$ = 0.018; r = 0.338) These results confirm that the speed of sound is correlated with the mineral density and mineral content of the metatarsus, but in general the correlation is weak, which might due to the sample size, and the range of bone quality is narrow as no challenges, but difference levels of vitamin $D_3$ were used to create the range of qualities of bones.

No correlation was found between tarsometatarsal bone density and whole-body bone density in chickens ($P$>0.05; r = 0.2336; Figure 5.1c). However, the tarsometatarsal BMC showed a strong correlation with whole-body BMC ($P$< 0,001; r = 0.772; Figure 5.1d). This suggests that the mineral content of the metatarsus is strongly associated with, and may predict, total body mineral content. These findings may be relevant when using QUS, as SOS×VOL was also found to be associated with tarsometatarsus BMC. With this, an attempt was also made to correlate SOS in the tarsometatarsus with whole-body BMD; however, no relationship was found ($P$ = 0.118; r = 0.2336; Figure 5.1e). Neither the correlation analysis nor the linear regression supported SOS as a predictor of whole-body BMD. Interestingly, a negative slope was observed in the regression model, suggesting that higher BMD values were associated with lower SOS, and vice versa. Additionally, a linear regression model between whole-body BMC and SOS×VOL revealed a statistically significant relationship ($P$ = 0.026; r = 0.3203; Figure 5.1f), despite the weak correlation. This suggests that SOS×VOL may have potential as a predictor of whole-body BMC

in QUS applications, providing an alternative tool to use in broiler chickens or even in breeding programs aimed at checking bone problems with a non-invasive assessment.

To our knowledge, this is the first study to directly compare bone mineral density and bone mineral content by DEXA with the speed of sound measured by QUS. Due to the shortage of studies conducted in broiler chickens, human literature was reviewed to help interpret the results obtained. Tarrell et al. (2023) also compared DEXA with QUS in infants predisposed to bone diseases, finding that the two techniques measure different properties of bone. DEXA isolates mineral content, while QUS reflects a composite of bone qualities, including BMC, density, elasticity, cortical thickness, strength, and microarchitecture. This explains why their results may move in opposite directions during early life. These findings suggest that QUS may be more sensitive to early impairments in bone quality and could become a valuable tool in neonatal care. Since broilers are also in an early stage of life, this may likewise be relevant for broiler chickens. However, no definitive conclusions can yet be drawn, and these results should be considered preliminary, as they require a larger sample size for confirmation.

Crespo et al. (2024) after testing two different QUS devices in layer hens using the same bone, found that results from different QUS devices are not directly comparable due to differences in technology, frequency, and probe size. Operator skill and measurement errors also influence outcomes. In addition, SOS alone was shown to be unreliable, whereas combining it with biochemical measures provided a better predictor of bone density in the metatarsus. This is similar to our results, when SOS was used as a single evaluation parameter, it was not possible to correlate it with whole-body BMC. However, when SOS was combined with bone volume to estimate

mineral content, a correlation was observed between the tarsometatarsus and whole-body BMC, providing a stronger predictor of bone mineral content using the tarsometatarsus.

Based on the results obtained and the literature reviewed, multiple factors can influence the interpretation of findings, including production type, bone type, bird age, and the tools used to evaluate bone quality. Differences between devices can generate variable outcomes, potentially leading to false diagnoses. Furthermore, due to the variability in measurements, it is not recommended to compare results across different QUS devices (Crespo et al., 2024).

## CONCLUSION

The speed of sound is correlated with bone density in the tarsometatarsus, and the product of SOS and bone volume may serve as a predictor of BMC in the same region. Additionally, tarsometatarsus bone quality appears to reflect overall bone mineralization in the body. While tarsometatarsus BMC was significantly correlated with whole-body BMC, no such correlation was found between tarsometatarsus BMD and whole-body BMD. In conclusion, the SOS × volume parameter may serve as a useful indicator of both tarsometatarsus BMC and whole-body BMC in 42-day-old broilers. Quantitative ultrasound shows promise as a non-invasive tool for assessing bone health. However, this result was observed only in older birds. The present study highlights the potential of QUS as a practical method for evaluating bone mineralization in broilers. Further research is needed to validate this tool, particularly across a wider age range where bone development is still ongoing.

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## TABLE

Table 5.1 Basal composition of the experimental diet and calculated analysis

| Ingridients % | Starter (0-14 d) | Grower (15-28 d) | Finisher (29-42 d) |
|---|---|---|---|
| **Corn** | 53.75 | 61.84 | 67.00 |
| **Soybean meal** | 35.45 | 32.08 | 24.48 |
| **Corn DDGS** | 5.00 | 1.15 | 3.87 |
| **Monocalcium Phos** | 2.25 | 1.48 | 1.31 |
| **Limestone** | 1.21 | 1.15 | 1.28 |
| **Fat, vegetable** | 1.00 | 1.00 | 1.00 |
| **Salt, plain (NaCl)** | 0.34 | 0.35 | 0.36 |
| **DL-metheonine** | 0.34 | 0.32 | 0.23 |
| **L-lysine HCL** | 0.25 | 0.24 | 0.21 |
| **Vitamin Premix[1]** | 0.10 | 0.10 | 0.10 |
| **Trace Mineral Premix[2]** | 0.08 | 0.08 | 0.08 |
| **L-threonine** | 0.11 | 0.09 | 0.03 |
| **Choline Chloride 60%** | 0.04 | 0.06 | 0.03 |
| **L-valine** | 0.07 | 0.07 | 0.03 |
| **Calculated analysis** | | | |
| **ME (Kcal/kg)** | 2,952 | 3,050 | 3,075 |
| **CP (%)** | 22.50 | 20.50 | 18.00 |
| **CF (%)** | 3.48 | 3.31 | 3.32 |
| **Ash (%)** | 5.04 | 4.10 | 3.68 |
| **Digestible lysine** | 1.26 | 1.16 | 0.96 |
| **Available Phosphorus** | 0.58 | 0.40 | 0.38 |
| **Calcium** | 0.96 | 0.80 | 0.80 |
| **Kg:kg** | | | |
| **Met/Lys** | 51.43 | 52.17 | 50.98 |
| **(Met+Cys)/Lys** | 75.00 | 76.00 | 77.00 |
| **Trp/Lys** | 19.96 | 19.82 | 20.03 |

| | | | |
|---|---|---|---|
| **Thr/Lys** | 68.00 | 66.00 | 65.00 |
| **Arg/Lys** | 107.43 | 106.87 | 109.00 |
| **Val/Lys** | 76.00 | 76.00 | 77.00 |
| **Ile/Lys** | 66.24 | 65.33 | 67.22 |
| **Leu/Lys** | 136.86 | 136.01 | 151.05 |

Abbreviation: DDGS: Distiller's Dried Grains with Solubles; ME: Metabolizable Energy; CP: Crude Protein; CF: Crude Fiber.

[1]Vitamins provided per kg of premix: vitamin A, 3,527,360 IU; vitamin D3, 1,399,921 IU; vitamin E, 19,400 IU; vitamin B12, 8.8 mg; menadione, 1,102 mg; Riboflavin, 3,527 mg; α-pantothenic acid 5,467 mg; thiamine, 970 mg; niacin, 20,282 mg; vitamin B6, 1,455 mg; folic acid, 573 mg; biotin, 79 mg.

[2]Supplied per kilogram of diet: Ca, 3.2%; Mn; 13.40%; Zn, 10.70%; Mg, 2.68%; Fe, 2.63%, Cu, 4000 ppm, I, 1000 ppm, Se, 400 ppm

FIGURE

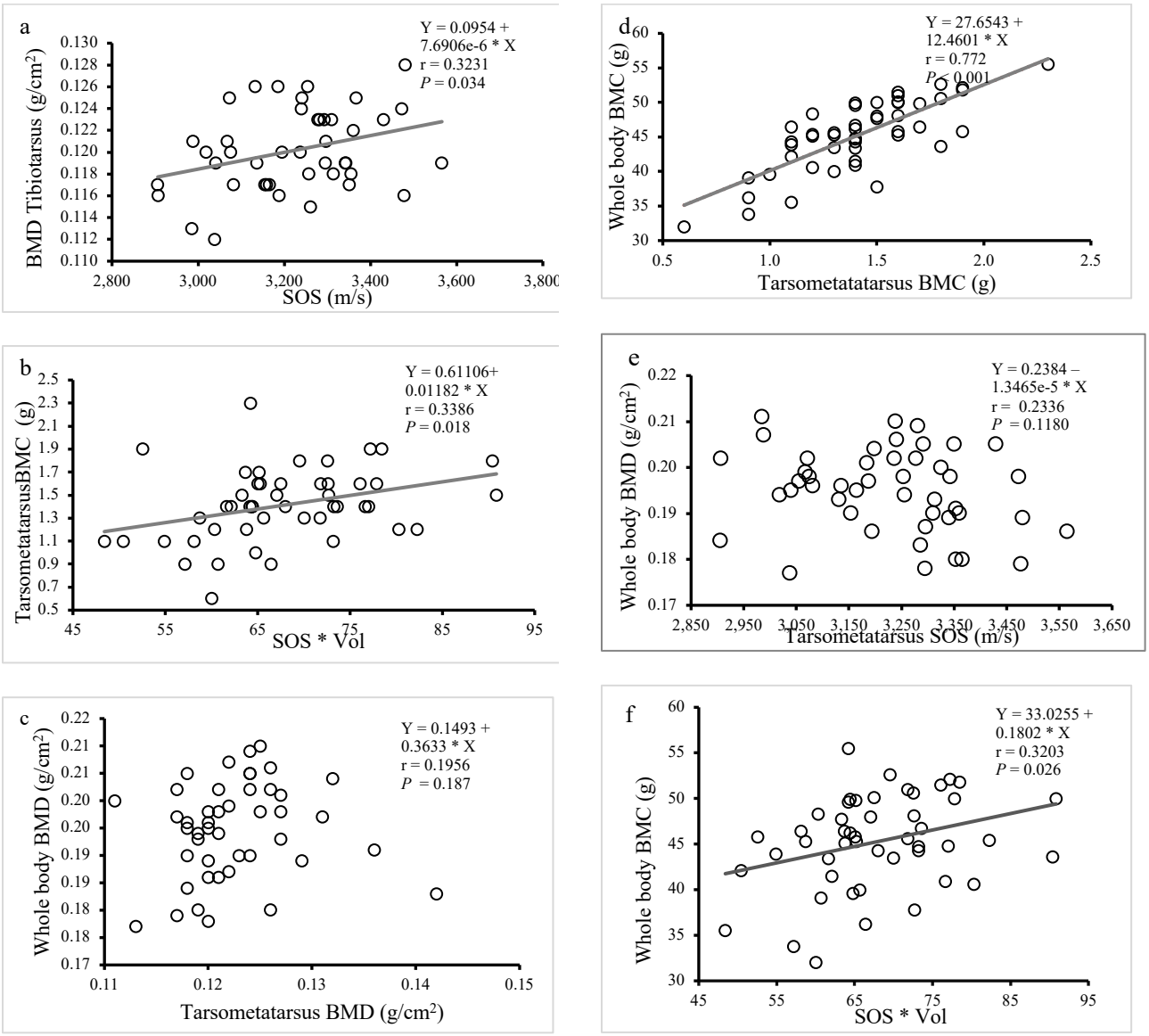


Figure 5.1 Linear regression model to predict bone mineral content and bone mineral density using a quantitative ultrasound machine as a non-invasive tool.

Abbreviations: BMC, bone mineral content; BMD, bone mineral density; SOS, speed of sound; Vol, volume.

[#] The tarsometatarsus was measured three time with the QUS to increase sample size, and the average was used for the statistically analysis.

Statistical significance was established at $P \leq 0.05$.

# CHAPTER 6
# CONCLUSION

Vitamin $D_3$ supplementation in broiler chickens cannot be overlooked. Since broilers have an accelerated growth rate, they require a balanced diet to meet all their nutritional needs and to prevent potential issues during the growing period, while also reaching their target weight within the expected timeframe. Nutritionists and researchers often rely on literature-based references such as the NRC to establish poultry nutrient requirements. However, the last NRC revision was published in 1994, and many of the cited studies date back to the 1960s. Therefore, in many cases, the NRC recommendations no longer reflect the nutritional needs of modern poultry strains.

In this study, two forms of vitamin D, cholecalciferol and 25-hydroxyvitamin $D_3$ were used at higher doses than those recommended by the NRC. Several factors influence vitamin $D_3$ utilization, particularly when birds are exposed to stress conditions such as high temperatures or parasitic infections. In general, birds do not obtain the minimum required levels of vitamin D in poultry houses because these facilities lack windows that allow sunlight to enter. For this reason, supplementation should be provided from the time chicks arrive.

In the first experiment, comparing different levels of 25(OH)$D_3$ showed improvements in growth performance during the starter and grower phases, with increases in body weight, weight gain, and a reduction in feed conversion ratio. The inclusion of this metabolite also influenced serum 25(OH)$D_3$ concentrations in a dose-dependent manner. These results indicate that supplementing higher doses than those recommended by the NRC does not negatively affect broiler performance when raised without exposure to UV light. Supplementation at 34.5 µg/kg of 25(OH)$D_3$ resulted in higher bone mineral density and bone mineral content, likely due to

improved absorption and the bypassing of the first hydroxylation step. This level also enhanced bone health. Moreover, even half of the commonly used dose yielded positive results in bone quality, suggesting that precise dose adjustments at different growth stages may optimize 25(OH)$D_3$ supplementation and improve overall performance and skeletal health in broilers.

Beyond its benefits for bone health, a second experiment was conducted in which birds were challenged with a cocktail of *Eimeria* spp. containing the most prevalent species in poultry production, *E. maxima*, *E. acervulina*, and *E. tenella*. These species are well known for causing damage to different sections of the digestive tract, leading to malabsorption syndrome and subsequent reductions in body weight. Supplementation with vitamin $D_3$ did not improve growth performance, and coccidiosis was observed to reduce fat absorption, resulting in lower vitamin D uptake in the duodenum and jejunum. However, supplementation with 25(OH)$D_3$ helped counteract this reduced absorption, increasing vitamin D levels even beyond those observed in the unchallenged control group.

In addition to the intestinal lesions caused by *Eimeria* spp., the infection also disrupts acid–base balance and reduces mineral absorption. However, supplementation with vitamin $D_3$ or 25(OH)$D_3$, particularly the first metabolite, helped restore acid–base balance, improve kidney function, reduce cecal lesions, and decrease oocyst excretion. Once all hydroxylation steps are completed and vitamin D reaches its active form, it can act on different cells in the body through vitamin D receptors. These receptors allow vitamin D to perform functions beyond bone health, enhancing various systems, including the immune and muscular systems. In this experiment, 25-hydroxyvitamin $D_3$ modulated both pro- and anti-inflammatory cytokines, reaching levels comparable to the non-challenged control group. However, further research is needed to clarify the mode of action of vitamin D metabolites under a coccidiosis challenge. Another important

finding was that, although the challenge reduced bird body weight, carcass yield improved in birds supplemented with additional vitamin D, particularly 25(OH)$D_3$. This study demonstrates that vitamin D can exert physiological effects beyond bone health, serving as a valuable nutritional strategy to support broilers during coccidiosis infection.