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Non Ruminant Nutrition

Influence of added 1,25(OH)$_2$D$_3$-glycoside on nursery pig growth performance, bone measurements, and cytokine concentrations

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Abstract

A total of 2,268 crossbred pigs (L337 × 1050, PIC; initially 5.5 ± 0.18 kg) were used in a 42-d growth study to evaluate the effects of 1,25(OH)$_2$D$_3$-glycoside provided from a plant extract on growth performance, bone characteristics, and serum criteria of nursery pigs. Pigs were weaned at approximately 21 d of age and randomly assigned to 1 of the 3 dietary treatments in a randomized complete block design. A total of 84 pens were used with 27 pigs per pen and 28 replications per treatment with pens blocked by BW and date of entry into the facility. Treatment diets were corn–soybean meal-based and consisted of a control diet (1,653 IU/kg of vitamin D$_3$), or the control diet with 1.2 or 2.0 μg of 1,25(OH)$_2$D$_3$-glycoside/kg. Blood samples were collected from 25 gilts/treatment on days 21 and 42 to assess 25(OH)D$_3$, cytokine concentrations, and antibody titers. At the end of the study, 10 pigs per treatment were euthanized and the right fibula, metacarpal, second and 10th ribs were collected to determine bone density, breaking strength, and percentage bone ash. Overall, there was a tendency (linear, $P = 0.067$) for a reduction in G:F as added 1,25(OH)$_2$D$_3$-glycoside increased, but no significant effects on final BW, ADG, ADFI, or mortality were observed. There were no treatment × bone interactions for bone breaking strength and bone ash. Percentage bone ash increased (linear, $P = 0.030$) across all bones as 1,25(OH)$_2$D$_3$-glycoside increased. Treatment did not affect bone ash weight and breaking strength. Metacarpals and 10th ribs had the greatest bone ash weight followed by the fibula with the second ribs having the lowest ($P < 0.05$). Metacarpals had greater breaking strength compared to all other bones, followed by the fibula and 10th rib, with the second rib having the lowest ($P < 0.001$). There was a bone × treatment interaction for bone density, where increasing 1,25(OH)$_2$D$_3$-glycoside increased bone density for the second rib ($P = 0.012$), but there was no treatment difference for other bones. There was no difference between treatments for antibody titers, 25(OH)D$_3$ status, or circulating cytokine concentrations except for IL-8 concentrations which decreased (linear, $P = 0.037$) as 1,25(OH)$_2$D$_3$-glycoside increased. In summary, adding 1.2 or 2.0 μg 1,25(OH)$_2$D$_3$-glycoside/kg provided from a plant extract to a diet already containing 1,653 IU/kg of vitamin D$_3$ had no effect on growth or the evaluated serum parameters; however, increasing 1,25(OH)$_2$D$_3$-glycoside increased percentage bone ash.

Lay Summary

After being consumed, vitamin D$_3$ must undergo a two-step hydroxylation process to be converted to the bioactive form, 1,25-dihydroxicholecalciferol [1,25(OH)$_2$D$_3$]. The dietary addition of this bioactive form directly allows pigs to bypass the hydroxylation steps and provides them with a readily available metabolite of vitamin D$_3$. This active vitamin D$_3$ metabolite plays an important role in Ca and P absorption influences bone development and mineralization and alters immune function. The objective of this study was to determine the response to supplementation of 1,25(OH)$_2$D$_3$-glycoside provided from a plant extract on nursery pig growth performance, mortality, bone characteristics, and blood measurements. Overall, supplementation of 1,25(OH)$_2$D$_3$-glycoside had minimal impact on growth or serum parameters; however, increasing 1,25(OH)$_2$D$_3$-glycoside increased the percentage of bone ash.

Key words: bone characteristics, cytokine, growth, nursery pig, vitamin D

Introduction

Vitamin D is a lipophilic vitamin that is required for growth, bone development and mineralization, and immune function. The two major forms of vitamin D are ergocalciferol (vitamin D$_2$), which is synthesized in plants, and cholecalciferol (vitamin D$_3$), which can be synthesized in the skin of many animals and humans (Baeke et al., 2010). Vitamin D$_3$ must undergo a two-step hydroxylation process to become the bioactive form. After absorption in the small intestine, vitamin

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D_3 is stored in the liver where it is hydroxylated to produce 25-hydroxyvitamin D_3 [25(OH)D_3], which is the major circulating metabolite of vitamin D (DeLuca, 2008). After hydroxylation in the liver, 25(OH)D_3 is transported to the kidney and undergoes a second hydroxylation process in the proximal tubules to become 1,25-dihydroxicholecalciferol [1,25(OH)$_2$D$_3$], which is the most bioactive form of vitamin D in the body (Norman, 2008). Under disease challenges, low feed intake situations, or situations where liver or kidney conversions are less than sufficient, direct supplementation of dietary 1,25(OH)$_2$D$_3$ may be beneficial (Bachmann et al., 2012).

In recent years, experiments have been conducted with different concentrations of dietary vitamin D_3 and 25(OH)D_3 (Flohr et al., 2014; Duffy et al., 2018; Williams et al., 2023). The absorption rate of 25(OH)D_3 is approximately 20% higher than that of vitamin D_3 (Applegate and Angel, 2005; Garcia et al., 2013). Previous research has indicated that serum 25(OH)D_3 concentrations are not always maintained above recommendations (Lauridsen, 2014; Arnold et al., 2015) when supplementing with vitamin D_3 alone in the feed even when fed at levels well above the NRC (2012) requirements (Flohr et al., 2014). However, supplementation of 25(OH)D_3 allows pigs to elevate serum 25(OH)D_3 concentrations (Zhang et al., 2021; Williams et al., 2023) but still requires renal metabolism to be converted to the bioactive form. The 1,25(OH)$_2$D$_3$ metabolite does not require renal metabolism, but little research has been conducted on it in swine diets (Schlegel et al., 2017; Trautenmüller et al., 2021). Direct addition of 1,25(OH)$_2$D$_3$ will provide the bioactive form to the pig to be readily utilized. An experiment conducted by Alves et al. (2018) in broilers observed a reduction in growth performance when 1,25(OH)$_2$D$_3$ completely replaced vitamin D_3 in the diet. Thus, this data may suggest that 1,25(OH)$_2$D$_3$ may need to be added to diets containing a basal level of vitamin D_3. Basal levels of vitamin D_3 are commonly provided at much greater levels than the requirement described by the NRC (2012) as summarized by Faccin et al. (2023), although research by Williams et al. (2023; 2024) has indicated that even with basal levels of vitamin D well above NRC (2012) requirements, supplementing bioactive forms of vitamin D may be needed to reach desired levels of circulating vitamin D metabolites. Researchers suggest that serum concentrations of 25(OH)D_3 below 10 to 15 ng/mL are speculated to be deficient in swine (Lauridsen, 2014). Levels of 25(OH)D_3 in circulation need to be maintained in order to support bone mineralization.

The 1,25(OH)$_2$D$_3$-glycoside used in this study is derived from a plant mixture of herbal origin. Bacteria with glycosidase activity in the colon metabolize 1,25(OH)$_2$D$_3$-glycoside before it can be absorbed (Zimmerman et al., 2015). This results in a slow release of 1,25(OH)$_2$D$_3$ and decreases the risk of toxicity due to a lower plasma peak concentration and a longer half-life (Mathis et al., 2016). In addition, there has been unpublished evidence suggesting that the pure form of 1,25(OH)$_2$D$_3$ is not stable during thermal processing, specifically pelleting. The glycoside stabilizes the 1,25(OH)$_2$D$_3$ and allows it to withstand the high temperatures associated with pelleting. Therefore, this study aimed to evaluate the effects of dietary 1,25(OH)$_2$D$_3$-glycoside in diets containing levels of vitamin D_3 representative of current formulation practices on growth performance, bone characteristics, and serum criteria of nursery pigs in diets containing standard industry vitamin D_3 concentrations (Faccin et al., 2023). We hypothesized that while 1,25(OH)$_2$D$_3$-glycoside bypasses the hydroxylation steps in the liver and kidneys, it would be a more readily available metabolite of vitamin D and thus increase growth performance and bone mineralization.

Materials and Methods

General

The Kansas State University Institutional Animal Care and Use Committee approved the protocol used in this experiment. The study was conducted at the Hord Farms West nursery research facility located in Pipestone, MN. The experiment utilized two identical nursery rooms that were completely enclosed, environmentally controlled, and mechanically ventilated. Each pen contained a six-hole, dry self-feeder, and a pan waterer to provide ad libitum access to feed and water. Feed additions were accomplished using a robotic feeding system (FeedPro, FeedLogic Corp., Wilmar, MN).

Animals and Housing

A total of 2,268 mixed-sex pigs (L337 × 1050, PIC; initially 5.5 ± 0.18 kg) were used in a 42-d growth study to evaluate the effects of 1,25(OH)$_2$D$_3$-glycoside on growth performance, bone characteristics, and serum criteria of nursery pigs. Pigs were weaned at approximately 21 d of age and assigned to one of the three dietary treatments in a randomized complete block design. A total of 84 pens were used with 27 pigs per pen and 28 replications per treatment across two rooms with pens blocked by BW and weaning date. Treatment diets were corn–soybean meal-based, fed in three phases, and consisted of a control diet (1,653 IU/kg of added vitamin D_3 provided from vitamin premix), or the control diet with 1.2 or 2.0 µg of added 1,25(OH)$_2$D$_3$-glycoside/kg diet (Table 1). The 1,25(OH)$_2$D$_3$-glycoside was provided by a plant extract (Herbal Active D, Phytobiotics, Cary, NC) that contained 10 mg 1,25(OH)$_2$D$_3$ per kg. Phase 1 diets were fed from approximately days 0 to 7 (5.5 to 5.9 kg BW). Phase 2 diets were fed from approximately days 7 to 21 (5.9 to 10.2 kg BW). Phase 3 diets were fed from approximately days 21 to 42 (10.2 to 20.0 kg BW).

During the experiment, the pens of pigs were weighed and feed disappearance was recorded every 7 d to determine ADG, ADFI, and G:F. Pigs that died or were removed during this study because of sickness or injury were recorded. Removals were defined as any pig removed from a test pen and placed into a treatment-specific off-test pen where they remained on treatment diets for the duration of the study. Any pig that died while in a test pen or a pig that died from an off-test pen was defined as mortality.

Blood Sampling

Individual gilts in 25 pens per treatment were randomly selected and bled on days 21 and 42 via jugular venipuncture and circulating cytokine concentrations, antibody titers, and vitamin D_3 levels were determined. The same average BW gilt in each pen was sampled for both blood collection days. Blood was collected in tubes without anticoagulant to obtain serum. Blood was allowed to clot before centrifuging for 15 min at 1,500 × *g* and then serum was stored at

Table 1. Diet composition (as-fed basis)

Ingredient, %	Phase 1[*]	Phase 2[†]	Phase 3[‡]
Corn	38.28	54.07	57.10
Soybean meal, dehulled	25.14	26.13	29.57
Dried distillers grains with solubles	—	—	10.00
Whey powder	12.50	10.00	—
Whey permeate	11.25	—	—
Microbial-enhanced soy protein[ǁ]	6.25	5.00	—
Choice white grease	—	1.00	—
Soybean oil	3.00	—	—
Calcium carbonate	0.62	0.70	0.90
Monocalcium P, (21% P)	0.88	0.95	0.65
Salt	0.35	0.60	0.50
L-Lys-HCl	0.40	0.38	0.45
DL-Met	0.25	0.20	0.12
L-Thr	0.18	0.19	0.19
L-Trp	0.02	0.03	0.03
L-Val	0.10	0.07	0.05
Vitamin and trace mineral premixes[$]	0.40	0.45	0.45
Zinc oxide	0.39	0.25	—
1,25(OH)$_2$D$_3$-glycoside[¶]	+/–	+/–	+/–
Total	100	100	100
Calculated analysis			
SID AA, %			
Lys	1.40	1.35	1.30
Ile:Lys	61	62	59
Leu:Lys	114	122	130
Met:Lys	38	37	33
Met and Cys:Lys	56	56	56
Thr:Lys	64	64	63
Trp:Lys	20.4	20.2	19.2
Val:Lys	77	71	70
Total Lys, %	1.55	1.50	1.47
ME, kcal/kg	3,485	3,338	3,258
NE, kcal/kg	2,612	2,471	2,388
SID Lys:ME, g/Mcal	5.36	5.46	5.44
CP, %	22.1	22.3	22.3
Ca, %	0.68	0.68	0.65
STTD P, %	0.54	0.52	0.45

[*]Phase 1 diets were fed from approximately days 0 to 7 (5.5 to 5.9 kg BW).
[†]Phase 2 diets were fed from approximately days 7 to 21 (5.9 to 10.2 kg BW).
[‡]Phase 3 diets were fed from approximately days 21 to 42 (10.2 to 20.0 kg BW).
[ǁ]Me-Pro, Prairie AquaTech, Brookings, SD.
[$]Provided per kg of diet: 4,134 IU vitamin A; 1,653 IU vitamin D; 44 IU vitamin E; 3 mg vitamin K; 0.03 mg vitamin B12; 50 mg niacin; 28 mg pantothenic acid; 8 mg riboflavin; 110 mg Zn from zinc sulfate; 110 mg Fe from iron sulfate; 33 mg Mn from manganese oxide; 17 mg Cu from copper sulfate; 0.30 mg I from calcium iodate; 0.30 mg Se from sodium selenite. Ronozyme HiPhos (DSM, Parsippany, NJ) included in phase 1 diets at 1,250 FTU/kg provided an estimated release of 0.13% STTD P. Optiphos 2,500 G (Huvepharma; Peachtree City, GA) included in phase 2 and 3 diets provided an estimated release of 0.13% STTD P with 1,251 FTU/kg.
[¶]1,25(OH)$_2$D$_3$-glycoside derived from a plant extract (Herbal Active D, Phytobiotics, Cary, NC) was diluted with wheat middlings and added to provide 1.2 or 2.0 μg of 1,25(OH)$_2$D$_3$-glycoside/kg in the final diet.

−80 °C until analyzed using a panel testing for 13 cytokines (GM-CSF, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, and TNFα; Eve Technologies, Calgary, AB Canada) via 13-Plex Discovery Assay (MilliporeSigma, Burlington, MA, USA). Serum samples also were sent to the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA) to determine 25(OH)D$_3$ (day 42 samples only; via liquid chromatography with tandem mass spectrometry) and antibody titers of porcine circovirus type 2 (PCV2; INgezim Circovirus IgG/IgM, Ingenasa, Madrid, Spain), porcine reproductive and respiratory syndrome virus (PRRSV; PRRS X3 Ab Test, IDEXX Laboratories, Westbrook, Maine), and *Mycoplasma hyopneumoniae* (*M. hyo* Ab Test, IDEXX Laboratories, Westbrook, Maine).

Bone Characteristics

At the end of the study, 10 gilts per treatment (weighing closest to the average BW of the 10 pens) were euthanized and the right fibula, metacarpal, second rib, and 10th rib were collected to determine bone density, bone-breaking strength, and percentage bone ash by utilizing the de-fatted processing method (Wensley et al., 2020). After removal, bones were stored at −20 °C until analysis. Extraneous soft tissues and cartilage were removed from the bones prior to assessment. Bone density was measured on each bone based on Archimedes principle (Williams et al., 2023). A dry bone weight was collected, and then bones were submerged in ultra-purified water under a negative pressure vacuum with 1.06 kg per cubic centimeter for a minimum of 4 h. Bones were then weighed while suspended in a vessel of ultra-purified water, and the weight was used to calculate bone density. Bone breaking strength is reported as the maximum compressive load on each bone via an Instron (Instron 5569, NV Lab, Norwood, MA). Briefly, each bone was held by two supports spaced 30 mm apart and were broken by a wedge lowered on the center of the bone at a speed of 100 mm per min and a maximal pressure of 5,000 kg. The force was measured by a pressure-sensitive cell, and peaks of maximum force were recorded (Williams et al., 2023). For de-fatted bone ash, all bones were placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were dried at 105 °C for 7 d in a drying oven and then ashed in a muffle furnace at 600 °C for 24 h to determine total bone ash weight and percentage ash relative to dried bone weight (Wensley et al., 2020).

Additionally, the left 10th rib was processed for histopathology examination. Bones were fixed in 10% neutral buffered formalin for at least 24 h and decalcified using DeltaCAL (Delta Medical, Inc., Aurora, IL, USA) for at least 4 to 6 h. The costochondral junction and the body of the 10th rib were embedded in paraffin blocks, sectioned at 4 µm thickness, and stained with hematoxylin and eosin. The microscopic examination was performed by blinded assessment from three pathologists at Iowa State University Veterinary Diagnostic Laboratory (Ames, IA). Histopathology grading of each bone consisted of physis grading, infractions/fracture lines, and fibrosis grading as described by Williams et al., 2023. A full description of the scoring system is presented in Supplementary Table 1.

Statistical Analysis

Growth performance, vitamin D status, bone characteristics, and histopathology data were analyzed as a randomized complete block design using the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC). Pen was considered the experimental unit. Treatment was used as the fixed effect and block was used as the random effect. Initial pen average BW and date of entry into the facility were incorporated within the blocking structure. Linear and quadratic contrasts were evaluated within increasing $1,25(OH)_2D_3$-glycoside considering the control diet as no added $1,25(OH)_2D_3$-glycoside. For bone characteristics and histopathology, treatment, bone, and associated interactions were considered fixed effects, with block and pig serving as random effects. Antibody titers and cytokines were analyzed as repeated measures representing multiple observations on each pen over time. Treatment, day, and the associated interactions were considered fixed effects. A Log_2 transformation was used for PCV2 antibody titers. Results were considered significant with $P \le 0.05$ and were considered a tendency with $P \le 0.10$.

Results

Growth Performance

From days 0 to 7, ADG and G:F increased (linear, $P \le 0.048$) as $1,25(OH)_2D_3$-glycoside increased (Table 2). There was a tendency (linear, $P = 0.056$) for an increase in d 7 BW as $1,25(OH)_2D_3$-glycoside increased. Treatment diets had no effect on ADFI. From days 7 to 21, increasing $1,25(OH)_2D_3$-glycoside decreased (linear, $P \le 0.050$) d 21 BW, ADG, and G:F. No differences were observed in ADFI. Overall (days 0 to 42), there was a tendency for a decrease (linear, $P = 0.067$) in G:F was observed as $1,25(OH)_2D_3$-glycoside increased. Overall, treatment diets had no significant effect on final BW, ADG, or ADFI. No statistical differences in mortality, removals, or mortality of the removed pigs were observed.

Bone Characteristics

A linear $1,25(OH)_2D_3$-glycoside × bone interaction ($P = 0.021$) was observed for bone density (Figure 1). The interaction was the result of a linear ($P = 0.012$) increase in bone density as $1,25(OH)_2D_3$-glycoside increased in the second rib, but no effects for the other bones. A main effect of bone was observed with 10th ribs having the greatest bone density followed by fibulas, second ribs, and metacarpals having the least ($P < 0.001$). No $1,25(OH)_2D_3$-glycoside × bone interactions were observed for bone-breaking strength (Table 3). However, a main effect of bone ($P < 0.001$) was observed with metacarpals having the highest values for breaking strength and second ribs having the lowest values (Table 4). Treatment diets had no effect on bone-breaking strength.

For percentage bone ash, no $1,25(OH)_2D_3$-glycoside × bone interactions were observed. The percentage of bone ash increased (linear, $P = 0.030$) as $1,25(OH)_2D_3$-glycoside increased. Additionally, a main effect of bone was observed where fibulas had the greatest percentage of bone ash, followed by the 10th rib, with the second rib and metacarpal having the lowest ($P < 0.05$) percentage of bone ash. For bone ash weight, no $1,25(OH)_2D_3$-glycoside × bone interactions were observed. However, a main effect of bone ($P < 0.001$) was observed with metacarpals and 10th ribs having the greatest bone ash weight followed by the fibula with the second rib having the lowest ($P < 0.05$) bone ash weight. Treatment diets had no effect on bone ash weight.

For histopathologic evaluation of the physis of the 10th ribs, there tended to be a greater probability of having a higher score (indicating abnormal bone architecture) as $1,25(OH)_2D_3$-glycoside increased (linear, $P < 0.052$; Table 5). For infraction and fibrosis scores, no effect of $1,25(OH)_2D_3$-glycoside was observed.

Blood Analysis

No treatment × day interactions were observed for any of the blood measurements collected on days 21 and 42 (Table 6). Treatment diets had no effect on PCV2, PRRS,

Table 2. Influence of 1,25(OH)$_2$D$_3$-glycoside on nursery pig growth performance*

	μg of 1,25(OH)$_2$D$_3$-glycoside/kg†				P =	
	0	1.2	2.0	SEM	Linear	Quadratic
BW, kg						
Day 0	5.5	5.5	5.5	0.18	0.924	0.639
Day 7	5.8	5.9	5.9	0.18	0.056	0.855
Day 21	10.2	10.2	10.1	0.27	0.050	0.615
Day 42	20.1	20.1	19.7	0.42	0.115	0.300
Days 0 to 7						
ADG, g	46	54	58	5.0	0.030	0.921
ADFI, g	113	116	120	5.0	0.224	0.731
G:F, g/kg	395	466	482	36.3	0.048	0.626
Days 7 to 21						
ADG, g	296[a]	292[ab]	281[b]	8.2	0.015	0.361
ADFI, g	354	359	358	7.6	0.448	0.641
G:F, g/kg	835[a]	809[b]	783[c]	12.0	<0.001	0.536
Days 21 to 42						
ADG, g	459	459	447	9.9	0.304	0.420
ADFI, g	695	697	680	14.2	0.306	0.371
G:F, g/kg	660	660	658	6.4	0.825	0.912
Days 0 to 42						
ADG, g	327	329	320	7.1	0.359	0.289
ADFI, g	472	477	470	8.9	0.891	0.352
G:F, g/kg	693	689	681	5.1	0.067	0.589
Removals, %	11.87	9.90	10.03	1.331	0.221	0.545
Mortality, %	3.69	2.90	3.29	0.705	0.613	0.493
Total mortality, %‡	5.09	4.32	4.07	0.896	0.322	0.889
Total removals and mortality, %ǁ	15.54	12.77	13.30	1.528	0.177	0.371

*A total of 2,268 pigs (initially 5.5 ± 0.18 kg) were used with 27 pigs per pen and 28 replications per treatment. Treatment diets were fed in all 3 phases.
†1,25(OH)$_2$D$_3$-glycoside derived from a plant extract (Herbal Active D, Phytobiotics, Cary, NC).
‡Percentage of pigs that died in original pen or off-test pen after being removed.
ǁPercentage of pigs that were removed from an original pen or died in the original pen.

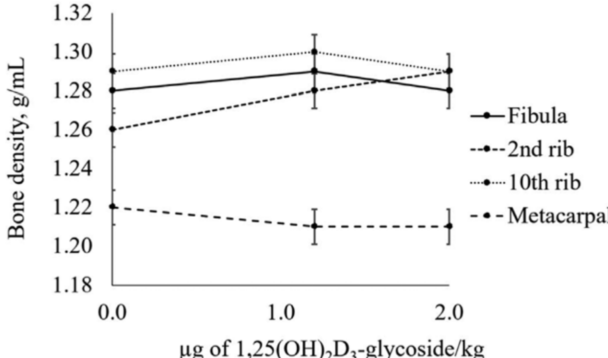

Figure 1. Influence of 1,25(OH)$_2$D$_3$-glycoside on nursery pig bone density. Density was measured on each bone based on Archimedes principle. Linear 1,25(OH)$_2$D$_3$-glycoside × bone interaction, $P = 0.021$. Linear effect of 1,25(OH)$_2$D$_3$-glycoside for second rib, $P = 0.012$. Linear effect of 1,25(OH)$_2$D$_3$-glycoside for all other bones, $P > 0.10$. Error bars represent +/− 1 SEM.

and *Mycoplasma hyopneumoniae* antibody titers, vitamin D status, or most circulating cytokine concentrations. However, IL-8 concentrations decreased (linear, $P = 0.037$) as 1,25(OH)$_2$D$_3$-glycoside increased. Furthermore, a main effect of the day was observed where pigs had increased ($P < 0.001$) PCV2, PRRS, and *Mycoplasma hyopneumoniae* antibody titers, and lower circulating cytokine concentrations on day 42 compared to day 21. However, a main effect of the day was not observed for IL-1ra and IL-8 ($P \geq 0.473$).

Discussion

Vitamin D$_3$ requirement estimates set by the NRC (2012) are 220 IU/kg of a complete diet for nursery pigs weighing 5 to 11 kg, and 200 IU/kg for those weighing 11 to 25 kg. Nevertheless, commercial swine diets typically contain concentrations of vitamin D$_3$ that are 5 to 7 times higher (Reese and Hill, 2010). Faccin et al. (2023) surveyed nutritionists in commercial production and found a wide range of vitamin D$_3$ levels in weanling pig diets from 1,389 to 10,494 IU/kg with a weighted average of 2,397 IU/kg. Our diets provided 1,653 IU/kg of vitamin D$_3$, falling within the 25th percentile according to the survey (Faccin et al., 2023). Additionally, the diets in the current experiment were formulated to contain levels of STTD P that exceed the requirement estimate for nursery pigs (NRC 2012). The total Ca levels were established by utilizing a 1.1:1 total Ca:P ratio.

Table 3. Influence of $1,25(OH)_2D_3$-glycoside on nursery pig bone characteristics*

	µg of $1,25(OH)_2D_3$-glycoside/kg†			
Item	0	1.2	2.0	SEM
Bone breaking strength, kg‡				
Fibula	15.5	14.9	17.7	1.50
Second rib	5.9	6.7	6.2	1.50
10th rib	13.3	13.8	15.0	1.50
Metacarpal	40.1	38.9	38.9	1.50
Bone ash, %ǁ				
Fibula	63.1	63.7	63.5	0.43
Second rib	58.3	59.1	59.1	0.43
10th rib	59.0	60.5	60.5	0.43
Metacarpal	58.4	58.6	59.4	0.43
Bone ash, g$				
Fibula	1.02	1.03	1.14	0.078
Second rib	0.77	0.83	0.82	0.078
10th rib	1.45	1.50	1.54	0.078
Metacarpal	1.50	1.51	1.55	0.078

*A total of 2,268 pigs (initially 5.5 ± 0.18 kg) were used with 27 pigs per pen and 28 replications per treatment. Ten pigs per treatment were euthanized and the right metacarpal, fibula, second rib, and 10th rib were collected. Values reported are the averages within each bone.
†$1,25(OH)_2D_3$-glycoside derived from a plant extract (Herbal Active D, Phytobiotics, Cary, NC).
‡Bone breaking strength is reported as the maximum compressive load on each bone via an Instron (Instron 5569, NV Lab, Norwood, MA). Linear and quadratic $1,25(OH)_2D_3$-glycoside × bone interaction, $P > 0.10$. Main effect of $1,25(OH)_2D_3$-glycoside, $P > 0.10$. The main effect of bone, $P < 0.0001$.
ǁBone ash was measured on each bone based on utilizing the de-fatted processing method. Bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were dried at 105 °C for 7 d in a drying oven and then ashed in a muffle furnace at 600 °C for 24 h. Linear and quadratic $1,25(OH)_2D_3$-glycoside × bone interaction, $P > 0.10$. Linear effect of $1,25(OH)_2D_3$-glycoside, linear $P = 0.030$. The main effect of bone, $P < 0.0001$.
$Bone ash weight was measured on each bone based. Linear and quadratic effect of $1,25(OH)_2D_3$-glycoside, $P > 0.10$. The main effect of bone, $P < 0.0001$.

Table 4. Main effects of $1,25(OH)_2D_3$-glycoside and bone on nursery pig bone characteristics*

	µg of $1,25(OH)_2D_3$-glycoside/kg†				Bone‡				
Item	0	1.2	2.0	SEM	Fibula	Second rib	10th rib	Metacarpal	SEM
Bone density, g/mLǁ	1.262	1.270	1.270	0.0066	1.286[a,b]	1.276[b]	1.292[a]	1.216[c]	0.0056
Bone breaking strength, kg$	18.7	18.6	19.4	1.03	16.0[b]	6.3[c]	14.0[b]	39.3[a]	1.07
Bone ash, %¶	59.7	60.5	60.6	0.30	63.4[a]	58.8[c]	60.0[b]	58.8[c]	0.26
Bone ash, g**	1.19	1.22	1.26	0.071	1.06[b]	0.81[c]	1.50[a]	1.52[a]	0.066

*A total of 2,268 pigs (initially 5.5 ± 0.18 kg) were used with 27 pigs per pen and 28 replications per treatment. Ten pigs per treatment were euthanized and the right metacarpal, fibula, second rib, and 10th ribs were collected.
†$1,25(OH)_2D_3$-glycoside derived from a plant extract (Herbal Active D, Phytobiotics, Cary, NC). Values reported represent the main effect of treatment averaged across all 4 bones (fibula, second rib, 10th rib, and metacarpal).
‡Values reported represent the main effect of bone averaged across all treatments (0, 1.2, and 2.0 µg of $1,25(OH)_2D_3$-glycoside/kg).
ǁBone density was measured on each bone based on Archimedes principle. Linear and quadratic effect of $1,25(OH)_2D_3$-glycoside, $P > 0.10$. The main effect of bone, $P < 0.0001$.
$Bone breaking strength is reported as the maximum compressive load on each bone via the Instron machine (Instron 5569, NV Lab, Norwood, MA). Linear and quadratic effect of $1,25(OH)_2D_3$-glycoside, $P > 0.10$. The main effect of bone, $P < 0.0001$.
¶Bone ash was measured on each bone based on utilizing the de-fatted processing method. Bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were dried at 105 °C for 7 d in a drying oven and then ashed in a muffle furnace at 600 °C for 24 h. Linear effect of $1,25(OH)_2D_3$-glycoside, $P = 0.030$. The main effect of bone, $P < 0.0001$.
**Bone ash weight was measured on each bone based on utilizing the de-fatted processing method. Linear and quadratic effect of $1,25(OH)_2D_3$-glycoside, $P > 0.10$. The main effect of bone, $P < 0.0001$.

Dietary vitamin D_3 undergoes a two-step hydroxylation process for activation. The first hydroxylation step primarily occurs in the liver and produces $25(OH)D_3$, the primary circulating metabolite of vitamin D (DeLuca, 2008). The enzyme responsible for catalyzing the initial hydroxylation step is 25-hydroxylase (Hewison et al., 2000). Vitamin D_3 must undergo further hydroxylation to form $1,25(OH)_2D_3$, the most biologically active form of vitamin D_3 (Norman, 2008). The second hydroxylation step takes place in the kidney, facilitated by the enzyme 1α-hydroxylase (Hewison et al., 2000). This active metabolite of vitamin D_3 plays an important role in the absorption of calcium and phosphorus, bone development and mineralization, and immune function (Hewison et al., 2000).

Table 5. Influence of 1,25(OH)$_2$D$_3$-glycoside on nursery pig bone histopathology measurements*

Item	µg of 1,25(OH)$_2$D$_3$-glycoside/kg†		
	0	1.2	2.0
Physis score frequency, %‡			
0: no abnormalities	50.0	23.3	30.0
1: mild abnormalities	36.7	36.7	30.0
2: moderate abnormalities	13.3	33.3	20.0
3: extensive abnormalities	0.0	6.7	20.0
Infraction score frequency, %ǁ			
0: no infractions	93.3	60.0	73.3
1: small infractions	6.7	23.3	16.7
2: large infractions	0.0	16.7	10.0
3: cortical fracture	0.0	0.0	0.0
Fibrosis score frequency, %$			
0: no fibrosis	90.0	90.0	93.3
1: fibrosis	10.0	10.0	6.7

*A total of 2,268 pigs (initially 5.5 ± 0.18 kg) were used with 27 pigs per pen and 28 replications per treatment. The left 10th rib was processed for a histopathology examination. Microscopic examination was performed by blinded assessment from three pathologists at Iowa State University Veterinary Diagnostic Laboratory (Ames, IA).
†1,25(OH)$_2$D$_3$-glycoside derived from a plant extract (Herbal Active D, Phytobiotics, Cary, NC).
‡Physis score consisted of: (0) no histologic findings of significance, (1) multifocal small tongues or islands of viable cartilage extend into the primary spongiosa, (2) moderate-sized tongues or islands of viable cartilage extend into the primary and secondary spongiosa, and (3) extensive areas of the zone of hypertrophy are expanded and extend down into the primary and secondary spongiosa. Linear 1,25(OH)$_2$D$_3$-glycoside effect, $P = 0.052$.
ǁInfractions scoring consisted of: (0) no evidence of infractions, (1) small infractions covering <50% of the diameter of the bone, (2) large infractions covering >50% of the diameter of the bone, and (3) cortical fracture. No effect of 1,25(OH)$_2$D$_3$-glycoside was observed, $P > 0.10$.
$Fibrosis score consisted of: (0) no evidence of fibrosis, and (1) fibrosis in the medullary bone. No effect of 1,25(OH)$_2$D$_3$-glycoside was observed, $P > 0.10$.

Table 6. Main effects of 1,25(OH)$_2$D$_3$-glycoside on nursery pig serum parameters*

Item	µg of 1,25(OH)$_2$D$_3$-glycoside/kg†				P =		Day			
	0	1.2	2.0	SEM	Linear	Quadratic	21	42	SEM	P =
Porcine circovirus type 2										
S/P ratio	0.47	0.44	0.42	0.035	0.373	0.997	0.31	0.58	0.031	<0.001
Porcine reproductive and respiratory syndrome										
S/P ratio	0.78	0.77	0.78	0.050	0.972	0.870	0.08	1.48	0.057	<0.001
Mycoplasma hyopneumoniae										
S/P ratio	0.20	0.19	0.20	0.039	0.949	0.825	0.08	0.31	0.034	<0.001
25-hydroxyvitamin D$_3$, ng/mL‡	20.0	20.1	19.1	0.93	0.514	0.600	—	—	—	—
Cytokine										
GM-CSF, pg/mL	40	43	72	27.6	0.447	0.633	86	17	27.7	0.006
IFNγ, pg/mL	8,945	9,984	9,048	1,806.5	0.925	0.651	15,211	3,441	1,841.7	<0.001
IL-1α, pg/mL	93	95	96	14.1	0.873	0.960	124	66	13.2	0.001
IL-1β, pg/mL	599	554	579	79.2	0.824	0.736	737	417	73.5	0.001
IL-1ra, pg/mL	1,201	1,356	1,213	102.7	0.824	0.247	1,253	1,261	97.0	0.957
IL-2, pg/mL	657	621	622	98.5	0.774	0.895	872.7	394	93.7	<0.001
IL-4, pg/mL	3,239	2,929	2,939	543.6	0.677	0.845	4,542	1,529	540.6	<0.001
IL-6, pg/mL	294	265	266	56.7	0.699	0.853	390	160	56.8	<0.001
IL-8, pg/mL	365	329	189	64.1	0.037	0.315	311	278	54.2	0.473
IL-10, pg/mL	1,648	1,644	1,516	243.5	0.707	0.793	2,207	998	238.7	<0.001
IL-12, pg/mL	1,127	1,192	1,124	68.5	0.954	0.430	934	1,361	70.3	<0.001
IL-18, pg/mL	4,155	3,920	3,416	625.5	0.407	0.782	4,997	2,664	603.5	0.001
TNFα, pg/mL	159	211	178	58.5	0.759	0.572	317	48.5	64.2	<0.001

*A total of 2,268 pigs (initially 5.5 ± 0.18 kg) were used with 27 pigs per pen and 28 replications per treatment. Serum samples were collected from 1 average weight gilt from 25 pens per treatment. Blood was collected from the same gilts on days 21 and 42. Antibody titers and vitamin D concentration were analyzed at Iowa State Veterinary Diagnostic Lab (Ames, IA). Cytokine analysis was conducted at Eve Technologies (Calgary, AB Canada). No treatment × day interactions were observed, $P > 0.10$.
†1,25(OH)$_2$D$_3$-glycoside derived from a plant extract (Herbal Active D, Phytobiotics, Cary, NC).
‡Samples were only analyzed on day 42.

Weaning is a stressful period for pigs and can lead to increased hepatic oxidative stress that may result in liver abnormalities (Luo et al., 2016). Direct supplementation of $1,25(OH)_2D_3$ enables pigs to bypass the hydroxylation steps in the liver and kidney, providing them with a readily available source of the bioactive form of vitamin D. Basal levels of vitamin D_3 in swine diets are commonly provided at much greater levels than recommended by the NRC (2012) as summarized by Faccin et al. (2023), although research by Williams et al. (2023; 2024) has indicated that even with basal levels of vitamin D well above NRC (2012) requirements, supplementing bioactive forms of vitamin D may be needed to reach desired levels of circulating vitamin D metabolites. In the current study, $1,25(OH)_2D_3$-glycoside was added to a diet containing 1,653 IU/kg of vitamin D_3. Previous research in broilers indicated that $1,25(OH)_2D_3$ cannot replace basal levels of vitamin D_3 otherwise a decrease in performance occurs (Alves et al., 2018). It may be necessary to provide basal levels of vitamin D_3 in the diet because the metabolite after the first hydroxylation step, $25(OH)D_3$, is used for other metabolic processes in the body and is not completely hydroxylated to $1,25(OH)_2D_3$. The $25(OH)D_3$ can also be converted to 24,25-dihydroxycholecalciferol $[24,25(OH)_2D_3]$ which plays an important role in bone mineralization (Boyan et al., 2001). Given this need for circulating $25(OH)D_3$, the current study aimed to determine the effects of adding $1,25(OH)_2D_3$-glycoside into a diet that already contained 1,653 IU/kg of vitamin D_3.

Several studies have evaluated the effects of adding $25(OH)D_3$, the vitamin D_3 metabolite produced after the first hydroxylation step, on growth performance and bone characteristics. When $25(OH)D_3$ was added to a diet that contained vitamin D_3, at or above NRC (2012) recommendations, no differences were observed in growth performance or bone characteristics (O'Doherty et al., 2010; Sandoval et al., 2022; Williams et al., 2023). Although the addition of $25(OH)D_3$ bypasses the first hydroxylation step, the addition of this metabolite is not translated into increased growth performance.

Serum or plasma $25(OH)D_3$ is considered the best biomarker of vitamin D status in mammals (Jones, 2012). The hydroxylation process from vitamin D_3 to $25(OH)_2D_3$ and $1,25(OH)_2D_3$ is not reversible. Therefore, we did not anticipate increased levels of serum $25(OH)D_3$ in pigs fed supplemental $1,25(OH)_2D_3$-glycoside. Additionally, serum concentrations of $25(OH)D_3$ below 10 to 15 ng/mL are speculated to be deficient in swine (Lauridsen, 2014); however, we are not aware of any data confirming this threshold. Arnold et al. (2015) reported reference values for serum $25(OH)D_3$ concentrations between 18 and 30 ng/mL in 2- to 4-wk-old pigs. The serum concentrations of $25(OH)D_3$ were between 19 and 20 ng/mL in the current study indicating that these pigs were not deficient in vitamin D_3. These values were greater than observed by Williams et al. (2023) who observed serum $25(OH)D_3$ levels between 10.9 and 14.1 ng/mL in nursery pigs when fed diets containing the same level of added vitamin D_3 as in our study. This may be a result of the duration of the study and amount of vitamin D_3 consumption as Williams et al. (2023) fed experimental diets for 28 d before measuring serum $25(OH)D_3$, whereas in our study status was measured after 42 d of feeding.

Calcium and P are essential for multiple physiological roles in the body including growth, development, and maintenance of the skeletal system. Vitamin D is also an important factor in the absorption and retention of Ca and P for bone mineralization (O'Doherty et al., 2010). Schlegel et al., (2017) observed no differences in bone density or bone ash when 10.0 or 20.0 μg/kg of plant-based $1,25(OH)_2D_3$ was added to a diet already containing 2,000 IU/kg of vitamin D_3 in pigs. In broilers, the addition of 0.5 μg/kg plant-based $1,25(OH)_2D_3$-glycoside had no effect on bone density, bone-breaking strength, or percentage of bone ash (Alves et al., 2018; Castro et al., 2018). In contrast, our study observed a linear increase in the percentage of bone ash as plant-based $1,25(OH)_2D_3$-glycoside/kg increased. The high concentrations of 10 or 20 μg/kg of added $1,25(OH)_2D_3$ fed by Schlegel et al. (2017) may have been in excess of nearing toxicity levels. Evidence of toxicity appears with plasma Ca concentrations above 3.0 mmol/L which is slightly above the upper normal limit of 2.9 mmol/L (Kaneko et al., 2008). Excessive intake of $1,25(OH)_2D_3$ promotes intestinal absorption of Ca which can cause soft tissue calcification when chronically high plasma Ca concentrations are experienced. Conversely, providing 0.5 μg/kg of $1,25(OH)_2D_3$ in previous research may not have provided enough of the active metabolite to observe differences in bone characteristics (Alves et al., 2018; Castro et al., 2018) compared to the 1.2 or 2.0 μg $1,25(OH)_2D_3$-glycoside/kg used in the current experiment.

Histopathology can also be used to evaluate metabolic bone disease in pigs (Williams et al., 2023). Based on the findings of Williams et al. (2023), tenth ribs were sampled for histopathology because they are more sensitive in response to P- or vitamin D-deficiency compared to second ribs and have the potential to show failure of endochondral ossification and infraction. In the current experiment, increasing $1,25(OH)_2D_3$-glycoside increased the likelihood of having abnormal histopathology findings. This response was not expected because previous research observed no differences in physeal score or infraction score with the addition of a vitamin D_3 metabolite $25(OH)D_3$ (Williams et al., 2023). Further research is needed on the effect of vitamin D_3 and its metabolites on histopathology because few data exist in the literature.

Beyond being important for growth performance and bone characteristics, vitamin D_3 also plays an important role in immune function because vitamin D receptor signaling occurs in several immune cells (Yang and Ma, 2021). In the current experiment, the addition of 1.2 or 2.0 μg $1,25(OH)_2D_3$-glycoside/kg to a diet containing 1,635 IU/kg vitamin D_3 had no effect on antibody titers and circulating cytokines, with the exception of IL-8. Thirteen cytokines were measured to get an understanding of how vitamin D modulates the immune system and as a biomarker for inflammation. The addition of $1,25(OH)_2D_3$-glycoside downregulated IL-8, which is a pro-inflammatory cytokine. Vitamin D inhibits the secretion of pro-inflammatory cytokines and promotes the production of more anti-inflammatory cytokines. This was observed in humans where $1,25(OH)_2D_3$ inhibited the production of type 1 pro-inflammatory cytokines including IL-8, and downregulated type 1 helper cells, part of the adaptive immune system (Bui et al., 2021). A downregulation of IL-8 can result in more severe diseases because IL-8 plays a key role in the control of bacteria translocation (Mahanty et al., 2001). However, this downregulation of IL-8 was not observed in pigs supplemented with $25(OH)D_3$ (Madsen et al., 2023), although age at sampling differed between these experiments. The reasoning behind the observed reduction in

IL-8 is not fully understood and requires more research to further elucidate.

In the current experiment, several cytokines (CM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-18, and TNFα) decreased between days 21 and 42 postweaning. A reduction in pro-inflammatory cytokines concentrations indicates an improvement in immune status suggesting that these pigs can spend less energy on immune overexpression (Gessner et al., 2017). Weaning is a stressful period for pigs and can result in inflammation due to an increase in cytokine production (Gessner et al., 2017). Although there are no reference values for cytokine concentrations based on a pigs' age, we expected the cytokine concentrations to decrease as the pigs became older because feed intake increased and the weaning stressors are reduced. de Groot et al., (2021) also observed a decrease in cytokines (IFN-γ, IL-1α, and TNFα) between days 30 and 45 postweaning in pigs' jejunum, ileum, or colon tissues. However, Rao et al., (2023) observed an increase in cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12) between days 10 and 42 postweaning in pigs. This variation in the literature suggests that the environment in which pigs are raised plays a role in cytokine concentrations due to different stressors or immune challenges.

In conclusion, although 1,25$(OH)_2D_3$-glycoside bypasses the hydroxylation steps and provides a readily available metabolite of vitamin D_3, its addition to a diet already containing vitamin D_3 did not translate to changes in growth, bone density, or bone-breaking strength. However, the addition of 1.2 or 2.0 μg 1,25$(OH)_2D_3$-glycoside/kg to a diet containing 1,653 IU/kg vitamin D_3 increased the percentage of bone ash.

Supplementary Data

Supplementary data are available at *Translational Animal Science* online.

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Conflict of interest statement

The authors declare no conflict of interest; however, Murat Devlikamov is an employee of Phytobiotics (Cary, NC, USA) who contributed partial financial support for this project.

Author Contributions

Larissa Becker (Data curation, Formal analysis, Investigation, Writing—original draft), Mike Tokach (Conceptualization, Project administration, Supervision, Validation, Writing—review & editing), Jason Woodworth (Conceptualization, Funding acquisition, Methodology, Writing—review & editing), Robert Goodband (Conceptualization, Funding acquisition, Methodology, Writing—review & editing), Joel DeRouchey (Conceptualization, Funding acquisition, Methodology, Writing—review & editing), Murat R. Devlikamov (Funding acquisition, Writing—review & editing), Michael C. Rahe (Data curation, Investigation, Methodology, Writing—review & editing), Christopher L. Siepker (Data curation, Investigation, Methodology, Writing—review & editing), Panchan Sitthicharoenchai (Data curation, Investigation, Methodology, Writing—review & editing), and Jordan Gebhardt (Conceptualization, Funding acquisition, Project administration, Supervision, Writing—review & editing)

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