



# Microbial phytase and 25-hydroxy-vitamin D<sub>3</sub> fed to growing pigs increase digestibility of calcium and phosphorus and influence plasma vitamin D metabolites and serum bone biomarkers, but effects are not always additive

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## Abstract

The objective was to test the hypothesis that calcifediol [25(OH)D<sub>3</sub>] and microbial phytase have additive effects on the standardized total tract digestibility (STTD) of Ca and P, serum bone biomarkers, and plasma vitamin D<sub>3</sub> metabolites when fed to growing pigs. Sixty barrows (initial body weight: 25.98 ± 2.01 kg) were housed individually in metabolism crates and assigned to a randomized complete block design with three blocks, 5 diets, and 12 replicate pigs per diet. The positive control (PC) diet was formulated to meet Ca and P requirements of growing pigs. Four additional diets contained 75% of the required Ca and P and were used in a 2 × 2 factorial design with 0 or 50 µg/kg of 25(OH)D<sub>3</sub> and 0 or 500 units of phytase per kg diet. Pigs were fed experimental diets for 13 d that included a 5-d adaptation period and a 5-d fecal collection period. Fecal samples were analyzed for Ca and P, and STTD of Ca and P was calculated. Blood samples were collected on days 1 and 13 to measure bone alkaline phosphatase, osteocalcin, type 1 collagen, and fibroblast growth factor 23. Analyzed plasma vitamin D<sub>3</sub> metabolites included 25(OH)D<sub>3</sub>, 24,25 dihydroxycholecalciferol [24,25(OH)<sub>2</sub>D<sub>3</sub>], and 1,25 calcitriol [1,25(OH)<sub>2</sub>D<sub>3</sub>]. Results indicated that the STTD of P was greater (*P* < 0.05) in the PC diet than in the diet containing 75% of the required Ca and P and no microbial phytase or 25(OH)D<sub>3</sub>. The STTD of Ca and P increased (*P* < 0.001) in pigs fed diets containing phytase, and STTD of Ca and P tended to increase if 25(OH)D<sub>3</sub> was added to the diet, but only in the absence of phytase (interaction; *P* < 0.10). On day 13, osteocalcin, which is a biomarker for bone tissue synthesis, was increased (*P* < 0.05) if 25(OH)D<sub>3</sub> or phytase was added to diets, but the other bone biomarkers did not differ among treatments. Plasma 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> increased (*P* < 0.05) if diets contained 25(OH)D<sub>3</sub> and/or phytase, indicating increased metabolic activity of vitamin D<sub>3</sub>. Plasma 1,25(OH)<sub>2</sub>D<sub>3</sub> was greater (*P* < 0.001) in pigs fed the diet with 75% of the required Ca and P and no phytase or 25(OH)D<sub>3</sub> than in pigs fed the PC diet, but microbial phytase decreased (*P* < 0.001) plasma 1,25(OH)<sub>2</sub>D<sub>3</sub>. In conclusion, microbial phytase and 25(OH)D<sub>3</sub> increased Ca and P digestibility and serum osteocalcin, and vitamin D<sub>3</sub> status was improved with the addition of 25(OH)D<sub>3</sub> to the diet, but effects of 25(OH)D<sub>3</sub> and microbial phytase were not always additive.

## Lay Summary

An experiment was conducted to test the hypothesis that effects of microbial phytase and calcifediol [25(OH)D<sub>3</sub>] on Ca and P digestibility and metabolism in growing pigs are additive. Sixty growing barrows were allotted to five diets that included a diet in which Ca and P were at the requirements and four diets that contained 75% of the requirements of Ca and standardized total tract digestible P with either 0 or 50 µg/kg of 25(OH)D<sub>3</sub> and either 0 or 500 units of microbial phytase. Pigs were housed individually in metabolism crates, and feces were collected for 5 d after 5 d of adaptation. Blood samples were collected to determine bone biomarkers and vitamin D<sub>3</sub> metabolites. Results indicated that microbial phytase improved Ca and P digestibility, but increased digestibility of Ca and P by 25(OH)D<sub>3</sub> was observed only if no phytase was included in the diet. Both supplements increased serum osteocalcin, indicating increased bone tissue synthesis, and dietary 25(OH)D<sub>3</sub> elevated plasma vitamin D<sub>3</sub> metabolites. In conclusion, both phytase and 25(OH)D<sub>3</sub> may increase the digestibility of Ca and P by growing pigs, but effects are not always additive.

**Key words:** 25(OH)D<sub>3</sub>, calcium, phosphorus, phytase, pig, vitamin D

**Abbreviations:** 25(OH)D<sub>3</sub>, 25 calcifediol; 24,25(OH)<sub>2</sub>D<sub>3</sub>, 24,25 dihydroxycholecalciferol; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,25 calcitriol; ATTD, apparent total tract digestibility; BAP, bone alkaline phosphatase; CTX-I, carboxy-terminal cross-linked telopeptide of type I collagen; DM, dry matter; FGF23, fibroblast growth factor; FTU, phytase units; NC, negative control; OC, osteocalcin; PC, positive control; STTD, standardized total tract digestibility; VMR, vitamin D metabolites ratio.

## Introduction

Vitamin D is important in bone development, prevention of tetany, and in Ca and P homeostasis, and has other physi-

ological functions related to growth, maintenance, and health (DeLuca, 2004). Cholecalciferol (i.e. vitamin D<sub>3</sub>) is the main source of vitamin D in animal diets (Stein, 2024),

Received May 27, 2025 Accepted August 11, 2025.

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but vitamin D<sub>3</sub> is inactive and must be hydroxylated to 25-hydroxycholecalciferol [25(OH)D<sub>3</sub>] in the liver, and then a second hydroxylation occurs in the kidney to produce 1,25 dihydroxycholecalciferol [1,25(OH)<sub>2</sub>D<sub>3</sub>], which is the active form of vitamin D<sub>3</sub> (Combs and McClung, 2017). Supplementation of 25(OH)D<sub>3</sub> to diets for sows in late gestation increased the digestibility of P and Ca, increased bone mineralization, and improved blood vitamin D<sub>3</sub> status (Lauridsen et al., 2010). In laying hens, long-term supplementation of 25(OH)D<sub>3</sub> resulted in improved bone growth, increased bone volume, and improved bone quality (Chen et al., 2020).

In diets for pigs based on cereal-grains and oilseed meals, most P is unavailable because it is bound to phytate, resulting in low P digestibility in grain-based diets (Liao et al., 2005). However, the use of exogenous microbial phytase in diets for pigs results in the hydrolysis of the ester bonds between P and phytate, which results in increased digestibility of Ca and P by pigs with a subsequent increase in absorption of P (Pallauf et al., 1994; Almeida et al., 2013). Inclusion of 25(OH)D<sub>3</sub> in diets for gestating sows increased the apparent total tract digestibility (ATTD) and retention of Ca and P, and it appeared that the increase in digestibility of Ca and P caused by 25(OH)D<sub>3</sub> was independent of the increase in digestibility caused by phytase (Lee et al., 2022). These results were obtained in diets containing vitamin D<sub>3</sub> well above the requirement for gestating sows, and it was, therefore, concluded that sows may not be efficient in converting vitamin D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub> (Lee et al., 2022). However, it is not known if growing pigs also have difficulty converting vitamin D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub>, but if that is the case, it is expected that addition of 25(OH)D<sub>3</sub> to the diets will have a positive effect on the digestibility of Ca and P, but there is limited information about a possible additive effect of 25(OH)D<sub>3</sub> and microbial phytase. Therefore, the hypothesis for this experiment was that effects of 25(OH)D<sub>3</sub> and microbial phytase on ATTD and standardized total tract digestibility (STTD) of Ca and P in diets fed to growing pigs are additive and that both 25(OH)D<sub>3</sub> and microbial phytase will result in increased serum biomarkers for bone synthesis and resorption and optimal vitamin D<sub>3</sub> metabolite status in plasma.

## Materials and Methods

The Institutional Animal Care and Use Committee at the University of Illinois reviewed and approved the protocol for the experiment before the animal work was initiated. Pigs used in the experiment were the offspring of L800 males mated to Camborough sows (PIC, Hendersonville, TN).

### Animals, housing, diets, feeding, and sample collection

Sixty growing male pigs with a body weight of 25.98 ± 2.01 kg were allotted to one of five diets in three blocks with weaning group as the blocking factor. Each block had 20 pigs (4 pigs per diet) for a total of 12 replicate pigs per treatment. Pigs were housed in metabolism crates (0.81 m × 1.52 m) that were equipped with a self-feeder, a nipple drinker, and a slatted floor.

Five corn-soybean meal-based diets were formulated (Table 1). The positive control (PC) diet contained total Ca and digestible P at the recommended levels for 25 to 50 kg growing pigs (NRC, 2012). A negative control (NC) diet was formulated with 75% of the requirement of Ca and digestible P,

and this diet, therefore, contained 0.17 percentage units total Ca and 0.08 percentage units STTD P, less than the PC diet. Three additional diets were formulated by supplementing NC with either 50 µg/kg of 25(OH)D<sub>3</sub> (Hy-D®; DSM, Parsippany, NJ, USA), 500 phytase units (FTU) per kg (HiPhorius®; DSM, Parsippany, NJ, USA), or both 50 µg/kg 25(OH)D<sub>3</sub> and 500 FTU/kg. The Ca to digestible P ratio was maintained at 2.13:1 in all diets. All vitamins and minerals other than Ca and P were included in all diets to meet or exceed current nutrient requirements (NRC, 2012). The daily feed allowance was calculated as 3.2 times the maintenance requirement for metabolizable energy based on the initial body weight of pigs (i.e. 197 kcal metabolizable energy/kg body weight<sup>0.60</sup>; NRC, 2012). Feed allotments were provided in two daily meals that were fed at 0730 and 1530 h. Water was available at all times.

Experimental diets were fed for 13 d. The initial 5 d were considered the adaptation period to the diets, and the adaptation period was followed by 5 d of fecal collection using the marker-to-marker procedure (Adeola, 2001). The start marker was fed in the morning meal on day 6, and the stop marker was fed in the morning meal on day 11. Fecal collection was initiated when the first marker (i.e. indigo carmine) appeared in the feces and ceased when the second marker (i.e. ferric oxide) appeared (Adeola, 2001). Fecal samples were stored at -20 °C as soon as collected, and at the conclusion of the experiment, samples were dried at 65 °C in a forced air oven (Heratherm OMH750; Thermo Fisher 1873 Scientific Inc., Waltham, MA, USA) and finely ground through a 0.5-mm screen using a hammermill (model: MM4; Schutte, Buffalo, NY, USA).

On days 1 and 13, one blood sample was collected via venipuncture 3 h after the morning meal to determine bone biomarker concentrations. Samples were collected in a 10-mL red-top vacutainer, which did not contain any coagulant. The blood in the tube was allowed to clot, and serum was then harvested. Additionally, on day 1, two pigs per diet were randomly selected, and a blood sample was collected in a 10-mL purple-top vacutainer containing the anticoagulant ethylenediaminetetraacetic acid (EDTA). After 13 d on experimental diets, a second sample was collected from 5 randomly selected pigs per diet (25 in total). Blood samples were immediately centrifuged, and plasma and serum were harvested and stored at -20 °C. Samples were stored at -80 °C until analysis.

### Chemical analysis

Before the animal part of the experiment was initiated, the concentration of 25(OH)D<sub>3</sub> in diets was analyzed at Technical Marketing Analytical Service (dsm-firmenich; Belvidere, NJ, USA) using liquid chromatography with tandem mass spectrometry (Aronov et al., 2008; Table 2). Concentrations of Ca and P in feed ingredients, diets, and dried fecal samples were analyzed (method 985.01 A, B, and C; AOAC Int., 2019) using inductively coupled plasma-optical emission spectrometry (Avio 200, PerkinElmer, Waltham, MA; Table 2). Sample preparation included dry ashing at 600 °C for 4 h according to method 942.05 (AOAC Int., 2019) and wet digestion with nitric acid according to method 3050 B (U.S.-EPA, 2000). Corn and soybean meal were analyzed for phytate (Ellis et al., 1977), and phytate in diets was calculated based on the analyzed phytate in corn and soybean meal and the inclusion rates of corn and soybean meal in each diet. Phytase activity in diets was also analyzed according to method 2000.12 (AOAC Int., 2019). Corn, soybean meal, diets, and fecal

**Table 1.** Ingredient composition of experimental diets, as-fed basis

Item Ca and P:	Normal <sup>1</sup>	75% of requirements			
	PC <sup>2</sup>	NC <sup>2</sup>	NC	NC	NC
25(OH)D <sub>3</sub> :	–	–	+	–	+
Phytase:	–	–	–	+	+
Corn	70.89	71.55	71.05	71.05	70.55
Soybean meal	25.50	25.50	25.50	25.50	25.50
Soybean oil	0.40	0.40	0.40	0.40	0.40
Dicalcium phosphate	0.98	0.48	0.48	0.48	0.48
Calcium carbonate	0.84	0.69	0.69	0.69	0.69
L-Lys-HCl, 78.8% Lys	0.30	0.30	0.30	0.30	0.30
DL-Met, 99% Met	0.10	0.10	0.10	0.10	0.10
L-Thr, 99% Thr	0.09	0.09	0.09	0.09	0.09
Sodium chloride	0.40	0.40	0.40	0.40	0.40
Corn-25(OH)D <sub>3</sub> premix <sup>3</sup>	–	–	0.50	–	0.50
Corn-phytase premix <sup>4</sup>	–	–	–	0.50	0.50
Vitamin-mineral premix <sup>5</sup>	0.50	0.50	0.50	0.50	0.50

<sup>1</sup>Normal = diets containing Ca and P at 100% of the requirement (NRC, 2012).

<sup>2</sup>PC = positive control; NC = negative control.

<sup>3</sup>The corn-25(OH)D<sub>3</sub> premix was prepared by mixing 362.84 g of 25(OH)D<sub>3</sub> concentrate (137.8 mg/kg; Hy-D®; DSM, Parsippany, NJ, USA) and 1,387.16 g ground corn. At 0.50 % inclusion, the corn-25(OH)D<sub>3</sub> premix provided 50 µg/kg of 25(OH)D<sub>3</sub> to the complete diet.

<sup>4</sup>The corn-phytase premix was prepared by mixing 333.33 g of phytase concentrate (1,500 phytase units per g; HiPhorius; DSM, Parsippany, NJ, USA) and 1,416.67 g ground corn. At 0.50 % inclusion, the corn-phytase premix provided 500 units of microbial phytase to the complete diets.

<sup>5</sup>The vitamin-micromineral premix provided the following quantities of vitamins and micro minerals per kg of complete diet as following: vitamin A as retinyl acetate, 10,622 IU; vitamin D<sub>3</sub> as cholecalciferol, 1,660 IU; vitamin E as DL- $\alpha$ -tocopherol acetate, 66 IU; vitamin K as menadione nicotinamide bisulfate, 1.40 mg; thiamin as thiamine mononitrate, 1.08 mg; riboflavin, 6.49 mg; pyridoxine as pyridoxine hydrochloride, 0.98 mg; vitamin B<sub>12</sub>, 0.03 mg; D-pantothenic acid as calcium pantothenate, 23.2 mg; niacin, 43.4 mg; folic acid, 1.56 mg; biotin, 0.44 mg; Cu, 20 mg as copper chloride; Fe, 123 mg as iron sulfate; I, 1.24 mg as ethylenediamine dihydroiodide; Mn, 59.4 mg as manganese hydrochloride; Se, 0.27 mg as sodium selenite and selenium yeast; and Zn, 124.7 mg as zinc hydroxychloride.

samples were analyzed for dry matter (DM; method 930.15; AOAC Int., 2019) and ash (method 942.05; AOAC Int., 2019). Limestone and dicalcium phosphate were analyzed for ash as well. Diets, corn, and soybean meal were also analyzed for gross energy using bomb calorimetry (Model 6400, Parr Instruments, Moline, IL, USA) and for insoluble dietary fiber and soluble dietary fiber using the Ankom Dietary Fiber Analyzer (Ankom Technology, Macedon, NY, USA; method 991.43, AOAC Int., 2019). Total dietary fiber was calculated as the sum of soluble and insoluble dietary fiber. Crude protein was calculated as analyzed nitrogen  $\times$  6.25, and nitrogen in diets, corn, and soybean meal was analyzed by combustion (method 990.03; AOAC Int., 2019) using a LECO FP628 Nitrogen Analyzer (LECO Corp., Saint Joseph, MI, USA). Serum osteocalcin (OC) was determined using an N-MID® Osteocalcin Enzyme-Linked Immunosorbent Assay Kit (ImmunodiagnosticSystemsLtd,TheBoldons,UK).Serumcarboxy-terminal cross-linked telopeptide of type I collagen (CTX-I) was determined using a Pig Cross-Linked C-Telopeptide of Type I Collagen Enzyme-Linked Immunosorbent Assay Kit (Abbeva Ltd., Cambridge, UK). Serum bone-specific alkaline phosphatase (BAP) was determined using Ostase® BAP

Enzyme Immunoassay Kit (Immunodiagnostic Systems Ltd, The Boldons, UK). Serum fibroblast growth factor (FGF23) concentration was determined using My BioSource® FGF23 competitive enzyme immunoassay Kit (Biotech, Life Sciences, Manufacturing, San Diego, CA, USA). Plasma 1,25(OH)<sub>2</sub>D<sub>3</sub>, 24,25 dihydroxycholecalciferol [24,25(OH)<sub>2</sub>D<sub>3</sub>], and 25(OH)D<sub>3</sub> were determined using liquid chromatography–mass spectrometry (LC/MS/MS; AOAC, 2019) by Heartland Assays (Ames, IA, USA).

### Calculations and statistical analysis

Concentrations of phytate-bound P were calculated by multiplying the analyzed phytate by 0.282 (Tran and Sauvant, 2004), and non-phytate P was calculated by subtracting phytate-bound P from total P. Feed intake was calculated by subtracting the weight of dried orts from feed provisions. The ATTD of Ca and P in diets was calculated (Almeida and Stein, 2010), and the STTD of P was calculated by correcting ATTD for the basal endogenous loss of P (i.e. 190 mg/kg DM intake; NRC, 2012). The ratio between 24,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub>, known as the vitamin D metabolite ratio (VMR), was used to assess optimal functional vitamin D<sub>3</sub> status (Cavelier et al., 2020).

Normality and homogeneity of data were verified using the UNIVARIATE and MIXED procedures (SAS Inst. Inc., Cary, NC, USA). Outliers were detected using Internally Studentized Residuals (Tukey, 1977). Pig was the experimental unit for all analyses. Data were analyzed using MIXED procedures of SAS. The statistical model included diet as a fixed effect and block and replicate pig within block as random effects. For analyzing data for serum biomarkers on day 13, concentrations of the biomarkers on day 1 were used as a covariate in the model. Plasma vitamin D<sub>3</sub> metabolites analyzed in samples from day 1 were assumed to be representative for all pigs, and changes in plasma vitamin D<sub>3</sub> metabolites from day 1 to 13 were analyzed. Least squares means were calculated using the LSMmeans statement in SAS. Contrast coefficients were used to determine the effects of 25(OH)D<sub>3</sub>, phytase, and the interaction between 25(OH)D<sub>3</sub> and phytase when Ca and P were low. Differences between PC and NC diets containing no 25(OH)D<sub>3</sub> or phytase were also analyzed using a contrast statement. Statistical significance and trends were considered at  $P < 0.05$  and  $P < 0.10$ , respectively.

### Results

Pigs remained healthy during the experiment, and limited feed refusals were observed. Diet analysis confirmed correct mixing.

#### Standardized total tract digestibility of Ca

Results indicated that feed intake was not different between pigs fed the PC and NC diets (Table 3). Fecal excretion, Ca intake, Ca excretion in feces, and absorbed Ca were greater ( $P < 0.05$ ) for the PC diet than the NC diet, but the ATTD of DM was greater ( $P < 0.05$ ) in the NC diet than in the PC diet. However, there were no differences in the ATTD of Ca, the basal endogenous loss of Ca, or the STTD of Ca between PC and NC.

Supplementation of 25(OH)D<sub>3</sub> to the NC diet did not affect feed intake, fecal excretion, or the ATTD of DM in pigs. Calcium intake and absorbed Ca increased ( $P < 0.01$ ) if 25(OH)D<sub>3</sub> was added to the NC diet, but Ca excretion in feces was not affected by supplemental 25(OH)D<sub>3</sub>.

**Table 2.** Analyzed nutrient composition of diets and feed ingredients, as-fed basis<sup>1</sup>

Item Ca and P:	Normal <sup>2</sup>		75% of requirements			Feed ingredients			
	PC <sup>3</sup>	NC <sup>3</sup>	NC	NC	NC	Corn	Soybean meal	Limestone	Dicalcium phosphate
25(OH)D <sub>3</sub> :	-	-	+	-	+				
Phytase:	-	-	-	+	+				
Dry matter, %	91.41	91.07	91.35	91.51	91.7	86.3	86.28	-	-
Gross energy, kcal/kg	4,011	4,013	4,010	4,035	4,013	3,918	3,937	-	-
Crude protein, %	16.88	16.81	16.94	16.93	17.31	7.11	45.44	-	-
Ash, %	4.30	3.81	3.92	3.78	3.70	4.87	4.32	99.84	88.26
Ca, %	0.63	0.49	0.51	0.49	0.50	0.03	0.33	38.12	19.96
P, %	0.58	0.49	0.52	0.49	0.51	0.31	0.71	0.22	18.96
Phytate <sup>4</sup> , %	0.97	0.97	0.97	0.97	0.97	0.76	1.68	-	-
Phytate-P <sup>5</sup> , %	0.27	0.27	0.27	0.27	0.27	0.21	0.47	-	-
Non-phytate P <sup>6</sup> , %	0.31	0.22	0.25	0.22	0.24	0.10	0.24	-	-
Total dietary fiber <sup>7</sup> , %	13.70	14.20	13.70	14.10	13.70	8.60	18.10	-	-
Soluble dietary fiber, %	2.00	2.00	2.00	1.80	1.60	0.40	2.20	-	-
Insoluble dietary fiber, %	11.70	12.20	11.70	12.30	12.10	8.20	15.90	-	-
Phytase, units/kg	<100	<100	<100	424	426	-	-	-	-
25(OH)D <sub>3</sub> µg/kg	ND <sup>8</sup>	ND	43	ND	46	-	-	-	-

<sup>1</sup>Calculated metabolizable energy was 3,302 kcal/kg in the PC diet and 3,324 kcal/kg in the other diets. The calculated concentrations of standardized ileal digestible Lys, Met, and Thr were 0.98, 0.31, and 0.60%, respectively. The calculated concentrations of Ca and standardized total tract digestible P were 0.66 and 0.31 in the PC diet and 0.495 and 0.233% in all other diets.

<sup>2</sup>Normal = diets containing Ca and P at 100% of the requirement (NRC, 2012).

<sup>3</sup>PC = positive control; NC = negative control.

<sup>4</sup>Phytate in all diets was calculated based on the analyzed concentration of phytate in corn and soybean meal and the inclusion rate in each diet.

<sup>5</sup>Phytate-P was calculated by multiplying the analyzed phytate by 0.282 (Tran and Sauvant, 2004).

<sup>6</sup>Non-phytate-P was calculated as total P minus phytate-P.

<sup>7</sup>Total dietary fiber was calculated as the sum of soluble dietary fiber and insoluble dietary fiber.

<sup>8</sup>ND = not detectable.

Feed intake, fecal excretion, and the ATTD of DM were not affected by supplemental phytase. Calcium intake and Ca excretion in feces were reduced ( $P < 0.01$ ) if phytase was included in the diet, whereas absorbed Ca, the ATTD of Ca, and the STTD of Ca increased ( $P < 0.001$ ) if phytase was used. Both 25(OH)D<sub>3</sub> and phytase increased the ATTD and STTD of Ca, but the increase caused by 25(OH)D<sub>3</sub> was less if phytase was used than if no phytase was used (tendency for interaction;  $P = 0.064$ , and  $P = 0.072$ , respectively).

### Standardized total tract digestibility of P

Phosphorus intake was less ( $P < 0.001$ ) for pigs fed the NC diet than for pigs fed the PC diet. Phosphorus excretion in feces and the basal endogenous loss of P were not different between PC and NC diets, but absorbed P, the ATTD of P, and the STTD of P were less ( $P < 0.05$ ) for pigs fed the NC diet than the PC diet.

Phosphorus intake increased ( $P < 0.001$ ) if 25(OH)D<sub>3</sub> was added to the diet, whereas phytase tended ( $P = 0.083$ ) to reduce P intake. Excretion of P was reduced if 25(OH)D<sub>3</sub> was added to the diet without phytase, but that was not the case if the diet also contained phytase (interaction,  $P < 0.01$ ). However, phytase reduced ( $P < 0.001$ ) P excretion in feces regardless of the level of 25(OH)D<sub>3</sub> in the diet. Likewise, phytase increased ( $P < 0.001$ ) absorbed P, ATTD of P, and STTD of P both in the diet without and with 25(OH)D<sub>3</sub>, whereas 25(OH)D<sub>3</sub> increased absorption of P, ATTD of P, and STTD of P in diets that did not contain phytase, but not in diets with phytase (interaction,  $P < 0.01$ ).

### Bone biomarkers

On day 13, there were no differences in serum levels of OC, BAP, CTX-I, OC to CTX-I ratio, or FGF23 between PC and NC diets (Table 4). Serum OC was increased ( $P < 0.01$ ) by dietary 25(OH)D<sub>3</sub>, but BAP, CTX-I, OC to CTX-I ratio, or FGF23 were not influenced by supplemental 25(OH)D<sub>3</sub>. Regardless of 25(OH)D<sub>3</sub>, supplemental phytase increased ( $P < 0.001$ ) serum OC, but serum levels of BAP, CTX-I, OC to CTX-I ratio, or FGF23 were not affected by phytase.

### Blood vitamin D biomarkers

On day 13, pigs fed PC had greater ( $P < 0.05$ ) plasma 24,25(OH)<sub>2</sub>D<sub>3</sub> and VMR than pigs fed NC, whereas plasma concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> was greater ( $P < 0.001$ ) in pigs fed NC than in pigs fed PC (Table 5). Addition of 25(OH)D<sub>3</sub> or microbial phytase to the diet increased ( $P < 0.01$ ) plasma concentrations of 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub>, whereas 1,25(OH)<sub>2</sub>D<sub>3</sub> in plasma was reduced ( $P < 0.001$ ) if phytase was added to the diet. The VMR increased if 25(OH)D<sub>3</sub> was added to the diet without phytase, but that was not the case if phytase was included in the diet (interaction,  $P < 0.05$ ). However, phytase increased ( $P < 0.01$ ) VMR regardless of the level of 25(OH)D<sub>3</sub> in the diet.

Changes from day 1 to 13 in plasma 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> were not affected by dietary Ca and P, but compared with PC, plasma VMR was reduced ( $P < 0.01$ ) and 1,25(OH)<sub>2</sub>D<sub>3</sub> increased ( $P < 0.01$ ) from day 1 to 13 in pigs fed the NC diet (Table 6). Supplementing 25(OH)D<sub>3</sub> to the diet increased ( $P < 0.001$ ) plasma 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> from day 1 to 13, but the change in plasma 1,25(OH)<sub>2</sub>D<sub>3</sub>

**Table 3.** Apparent total tract digestibility (ATTD) of dry matter (DM), and ATTD and standardized digestibility (STTD) of Ca and P in experimental diets fed to growing pigs<sup>1</sup>

Item Ca and P:	Normal <sup>2</sup>		75% of requirements			SEM	Contrast P-value <sup>4</sup>			
	PC <sup>3</sup>	NC <sup>3</sup>	NC	NC	NC		PC vs. NC	25(OH)D <sub>3</sub>	Phytase	Interaction
25(OH)D <sub>3</sub> :	-	-	+	-	+					
Phytase:	-	-	-	+	+					
Feed intake, kg/d	1.31	1.31	1.29	1.28	1.30	0.03	0.814	0.796	0.226	0.073
Fecal excretion, kg/d	0.13	0.12	0.12	0.12	0.13	0.005	0.023	0.242	0.278	0.112
ATTD of DM, %	89.86	90.92	91.07	91.00	90.19	0.34	0.030	0.342	0.244	0.170
Ca intake, g/d	8.01	6.41	6.61	6.25	6.47	0.18	<0.001	<0.001	0.001	0.872
Ca excretion in feces, g/d	2.53	2.10	1.79	1.25	1.27	0.13	0.005	0.157	<0.001	0.108
Absorbed Ca, g/d	5.47	4.31	4.79	4.99	5.19	0.13	<0.001	0.006	<0.001	0.226
ATTD of Ca, %	68.40	67.20	72.92	79.96	80.26	1.45	0.550	0.041	<0.001	0.064
BEL <sup>5</sup> of Ca, mg/d	518	515	512	506	515	13.00	0.550	0.441	0.509	0.092
STTD <sup>5</sup> of Ca, %	74.87	75.23	80.65	88.07	88.23	1.45	0.857	0.056	<0.001	0.072
P intake, g/d	7.62	6.41	6.66	6.28	6.60	0.18	<0.001	<0.001	0.083	0.547
P excretion in feces, g/d	3.46	3.30	2.87	1.93	2.19	0.15	0.313	0.439	<0.001	0.003
Absorbed P, g/d	4.16	3.11	3.76	4.34	4.39	0.12	<0.001	0.007	<0.001	0.019
ATTD of P, %	54.64	48.53	56.78	69.24	66.68	1.57	0.008	0.078	<0.001	0.001
BEL of P <sup>5</sup> , mg/d	227	226	225	222	226	6.00	0.550	0.442	0.509	0.093
STTD of P <sup>5</sup> , %	57.62	52.05	60.15	72.78	70.11	1.57	0.015	0.093	<0.001	0.001

<sup>1</sup>Each least squares mean represents 12 observations except for PC, NC, and the diet containing phytase and no 25(OH)D<sub>3</sub> ( $n = 11$ ).

<sup>2</sup>Normal = diets containing Ca and P at 100% of the requirement (NRC, 2012).

<sup>3</sup>PC = positive control; NC = negative control.

<sup>4</sup>Four contrasts were used: PC vs. NC = effects of Ca and P levels in diets without 25(OH)D<sub>3</sub> or microbial phytase; 25(OH)D<sub>3</sub> = effects of 25(OH)D<sub>3</sub>; phytase = effects of microbial phytase; and interaction = the interaction between 25(OH)D<sub>3</sub> and microbial phytase.

<sup>5</sup>BEL = basal endogenous loss; BEL was calculated by multiplying the daily DM intake of pigs by BEL of Ca or P. Values for the STTD of Ca were calculated by correcting the ATTD of Ca with the average BEL of Ca (i.e. 433 mg/kg DM intake, Lee and Stein, 2023); values for the STTD of P were calculated by correcting the ATTD of P with the average BEL of Ca (i.e. 190 mg/kg DM intake; NRC, 2012).

from day 1 to 13 was not affected by supplemental 25(OH)D<sub>3</sub>. Adding 25(OH)D<sub>3</sub> to the diet also increased VMR from day 1 to day 13, but more so in diets without phytase than in diets with phytase (tendency for interaction,  $P = 0.057$ ). Supplementation of phytase did not change plasma 25(OH)D<sub>3</sub>, 24,25(OH)<sub>2</sub>D<sub>3</sub>, or VMR, but reduced ( $P < 0.01$ ) plasma 1,25(OH)<sub>2</sub>D<sub>3</sub> on day 13 compared with day 1.

## Discussion

### Calcium and phosphorus digestibility

Analyzed concentrations of ash, Ca, and P in corn, soybean meal, limestone, and dicalcium phosphate were in agreement with published values (NRC, 2012), and analyzed diet concentrations of crude protein, gross energy, total dietary fiber, Ca, and P agreed with calculated values. Analyzed 25(OH)D<sub>3</sub> in the two diets that contained this metabolite were 43 and 46 µg/g, respectively, which corresponded to 86% and 92% of the expected value. The analyzed phytase was 424 and 426 FTU per kg in diets with phytase, which was 85% of the expected concentration.

The increase in ATTD and STTD of Ca and P when microbial phytase was added to diets agrees with results of previous experiments (Veum and Ellersieck, 2008; Almeida et al., 2013; Blavi et al., 2019), and the responses are within the range of results obtained for corn-soybean meal diets (Arredondo et al., 2019; Gebhardt et al., 2021). These results confirmed the efficiency of the phytase used in the experiment and are a result of the increased release of Ca and P from the phytate complex

when phytase is added to the diet. The tendency for an increase in STTD of Ca in diets containing 25(OH)D<sub>3</sub> is likely a result of the increased concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> in the plasma of pigs fed 25(OH)D<sub>3</sub>, which may have increased transcellular absorption of Ca from the small intestine (Lagos et al., 2019). Likewise, the increase in absorbed P and the tendency for increased STTD of P that was observed when 25(OH)D<sub>3</sub> was added to the diet is likely a result of the increased plasma concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> (Katai et al., 1999; Stein, 2024), because increased 1,25(OH)<sub>2</sub>D<sub>3</sub> results in increased expression of Ca channel proteins and Ca binding proteins in the enterocytes (González-Vega et al., 2016; Lagos et al., 2019). It therefore appears that although all diets contained vitamin D<sub>3</sub> in quantities that were believed to be well above the requirement for growing pigs (NRC, 2012), provision of the 25(OH)D<sub>3</sub> metabolite in addition to the dietary vitamin D<sub>3</sub> was efficient in increasing plasma 1,25(OH)<sub>2</sub>D<sub>3</sub> and thus increase absorption of Ca and P. This observation is in agreement with results from a recent experiment with gestating sows (Lee et al., 2022) and indicates that growing pigs are not able to fully convert dietary vitamin D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub>. Theoretically, it is also possible that the requirement for vitamin D<sub>3</sub> is much greater than previously thought, which might have been the reason why the addition of 25(OH)D<sub>3</sub> to the diets tended to increase STTD of Ca and P if no phytase was used. However, because the inclusion of vitamin D<sub>3</sub> to diets used in this experiment was more than eight times greater than the requirement (NRC, 2012), it is unlikely that the added vitamin D<sub>3</sub> was below the requirement and it is, therefore, more likely

**Table 4.** Concentration of osteocalcin (OC), bone alkaline phosphatase (BAP), carboxy-terminal cross-linked telopeptide of type I collagen (CTX-I), and fibroblast growth factor (FGF23) in serum of growing pigs fed experimental diets for 13 d<sup>1</sup>

Item Ca and P:	Normal <sup>2</sup>		75% of requirements			SEM	Contrast P-value <sup>4</sup>			
	PC <sup>3</sup>	NC <sup>3</sup>	NC	NC	NC		PC vs. NC	25(OH)D <sub>3</sub>	Phytase	Interaction
25(OH)D <sub>3</sub> :	-	-	+	-	+					
Phytase:	-	-	-	+	+					
OC, µg/L	38.08	36.17	39.20	40.17	42.90	1.47	0.150	0.005	<0.001	0.880
BAP, µg/L	64.90	62.64	66.02	61.49	60.77	6.18	0.618	0.702	0.356	0.542
CTX-I, µg/L	0.14	0.13	0.14	0.13	0.12	0.04	0.735	0.926	0.817	0.645
OC to CTX-I ratio <sup>5</sup>	358	313	348	351	391	67	0.264	0.229	0.182	0.932
FGF23, µg/L	0.42	0.42	0.44	0.41	0.43	0.04	0.994	0.485	0.796	0.855

<sup>1</sup>Each least squares mean represents 12 observations except for PC, NC, and the diet containing phytase and no 25(OH)D<sub>3</sub> (*n* = 11).

<sup>2</sup>Normal = diets containing Ca and P at 100% of the requirement (NRC, 2012).

<sup>3</sup>PC = positive control; NC = negative control.

<sup>4</sup>Four contrasts were used: PC vs. NC = effects of Ca and P levels in diets without 25(OH)D<sub>3</sub> or microbial phytase; 25(OH)D<sub>3</sub> = effects of 25(OH)D<sub>3</sub>; phytase = effects of microbial phytase; and interaction = the interaction between 25(OH)D<sub>3</sub> and microbial phytase.

<sup>5</sup>OC to CTX-I ratio was calculated as the mean value of OC for the treatment divided by the mean value of CTX-I for the treatment (Lee et al., 2020).

**Table 5.** Vitamin D<sub>3</sub> metabolites in plasma of growing pigs fed experimental diets for 13 d<sup>1</sup>

Item Ca and P:	Normal <sup>2</sup>		75% of requirements			SEM	Contrast P-value <sup>4</sup>			
	PC <sup>3</sup>	NC <sup>3</sup>	NC	NC	NC		PC vs. NC	25(OH)D <sub>3</sub>	Phytase	Interaction
25(OH)D <sub>3</sub> :	-	-	+	-	+					
Phytase:	-	-	-	+	+					
25(OH)D <sub>3</sub> , ng/mL	17.50	13.38	44.98	19.36	49.90	1.80	0.121	<0.001	0.007	0.771
24,25(OH) <sub>2</sub> D <sub>3</sub> , ng/mL	3.47	1.57	8.55	3.97	10.49	0.63	0.011	<0.001	<0.001	0.636
VMR <sup>5</sup>	19.76	11.89	18.87	20.30	21.02	2.16	0.001	0.016	0.002	0.041
1,25(OH) <sub>2</sub> D <sub>3</sub> , pg/mL	166.10	270.18	291.38	211.60	218.88	15.30	<0.001	0.363	<0.001	0.654

<sup>1</sup>Each least squares mean represents 5 observations.

<sup>2</sup>Normal = diets containing Ca and P at 100% of the requirement (NRC, 2012).

<sup>3</sup>PC = positive control; NC = negative control.

<sup>4</sup>Four contrasts were used: PC vs. NC = effects of Ca and P levels in diets without 25(OH)D<sub>3</sub> or microbial phytase; 25(OH)D<sub>3</sub> = effects of 25(OH)D<sub>3</sub>; phytase = effects of microbial phytase; and interaction = the interaction between 25(OH)D<sub>3</sub> and microbial phytase.

<sup>5</sup>VMR = vitamin D metabolite ratio; 24,25(OH)<sub>2</sub>D<sub>3</sub>/25(OH)D<sub>3</sub> × 100 (Zelzer et al., 2020).

**Table 6.** Changes of vitamin D<sub>3</sub> metabolites in plasma of growing pigs from day 1 to day 13<sup>1</sup>

Item Ca and P:	Normal <sup>2</sup>		75% of requirements			SEM	Contrast P-value <sup>4</sup>			
	PC <sup>3</sup>	NC <sup>3</sup>	NC	NC	NC		PC vs. NC	25(OH)D <sub>3</sub>	Phytase	Interaction
25(OH)D <sub>3</sub> :	-	-	+	-	+					
Phytase:	-	-	-	+	+					
25(OH)D <sub>3</sub> , ng/mL	40.1	46.4	250.7	118.8	299.5	39.5	0.902	<0.001	0.160	0.780
24,25(OH) <sub>2</sub> D <sub>3</sub> , ng/mL	61.1	18.4	359.3	130.1	378.7	40.1	0.411	<0.001	0.136	0.287
VMR <sup>5</sup>	13.6	-26.1	31.0	9.5	20.7	10.8	0.009	0.007	0.281	0.057
1,25(OH) <sub>2</sub> D <sub>3</sub> , pg/mL	-33.2	8.5	23.4	-7.1	-12.4	7.8	0.001	0.540	0.009	0.225

<sup>1</sup>Each least squares mean represents 10 observations on day 1, and 5 observations on day 13.

<sup>2</sup>Normal = diets containing Ca and P at 100% of the requirement (NRC, 2012).

<sup>3</sup>PC = positive control; NC = negative control.

<sup>4</sup>Four contrasts were used: PC vs. NC = effects of Ca and P levels in diets without 25(OH)D<sub>3</sub> or microbial phytase; 25(OH)D<sub>3</sub> = effects of 25(OH)D<sub>3</sub>; phytase = effects of microbial phytase; and interaction = the interaction between 25(OH)D<sub>3</sub> and microbial phytase.

<sup>5</sup>VMR = vitamin D metabolite ratio; 24,25(OH)<sub>2</sub>D<sub>3</sub>/25(OH)D<sub>3</sub> × 100 (Zelzer et al., 2020).

that pigs are not able to efficiently convert dietary vitamin D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub>.

An additive effect on Ca and P digestibility of using both 25(OH)D<sub>3</sub> and phytase in diets fed to sows was observed by Lee et al. (2022), but such an effect was not observed in the current experiment. This observation is in agreement with data from growing-finishing pigs fed low P diets containing 25(OH)D<sub>3</sub> or microbial phytase (O'Doherty et al., 2010). It therefore appears that for growing pigs, 25(OH)D<sub>3</sub> only increases STTD of Ca and P if no phytase is used in the diets, whereas the effects are different in sows. It is possible that this is a result of sows being less efficient than growing pigs in converting vitamin D<sub>3</sub> into 1,25(OH)<sub>2</sub>D<sub>3</sub> (Stein, 2024). Nevertheless, the hypothesis that effects of 25(OH)D<sub>3</sub> and phytase on STTD of Ca and P are additive was rejected. It is speculated that the reason for the lack of additivity between microbial phytase and 25(OH)D<sub>3</sub> may be that when phytase was used, the availability of Ca and P was sufficient to meet the requirements of the pigs, which then resulted in down regulation of the transcellular absorption of Ca and P, and thus resulted in no measurable effect of 25(OH)D<sub>3</sub> on Ca and P digestibility. In contrast, when no microbial phytase was used, expression of Ca channel proteins and intracellular transport proteins was stimulated by 25(OH)D<sub>3</sub>, which resulted in increased absorption of Ca and P. If this hypothesis is correct, it is possible that the NC diet needs to be more deficient in Ca and P to demonstrate the additive effects of microbial phytase and 25(OH)D<sub>3</sub>.

### Bone turnover biomarkers and FGF23

Bone turnover biomarkers in serum were analyzed to provide an assessment of the bone tissue status in pigs, as they may reflect changes in bone integrity, especially when dietary Ca and P levels are changed (Liesegang et al., 2002). Greater absorption of Ca and P stimulates osteoblast activity and increases bone tissue formation and serum OC levels (Sørensen et al., 2018). Thus, the increase in OC that was observed when 25(OH)D<sub>3</sub> or phytase was added to the diet indicates that osteoblast activity, and therefore bone tissue synthesis, was stimulated by dietary 25(OH)D<sub>3</sub> and microbial phytase. Low or high concentrations of Ca and P in diets for weanling or growing-finishing pigs are believed to increase BAP, and in low Ca and P diets, CTX-I increases as well (Liesegang et al., 2002; Sørensen et al., 2018; Lee et al., 2020). Therefore, the observation that BAP and CTX-I did not increase when diet Ca and P were reduced is in contrast with the results of previous experiments (Lee et al., 2020). It is possible that the length of the experiment was too short to detect changes in BAP and CTX-I, as it may take more than 13 d for these biomarkers to exhibit measurable changes in serum (Carter et al., 1996; Liesegang et al., 2002; Sørensen et al., 2018). Thus, the hypothesis that dietary 25(OH)D<sub>3</sub> and/or phytase increase biomarkers for bone tissue synthesis and reduce biomarkers for bone resorption was only partially accepted because only OC was increased by dietary treatments.

Plasma FGF23 is a hormone involved in negative feedback with 1,25(OH)<sub>2</sub>D<sub>3</sub> to downregulate reabsorption of phosphate in the kidneys, which increases P excretion in urine and decreases P retention (Agoro and White, 2023; Vötterl et al., 2023). Therefore, it was expected that plasma FGF23 would be greater in pigs fed the PC diet than in pigs fed the NC diet, and that FGF23 would be increased when 25(OH)D<sub>3</sub>, phytase, or both 25(OH)D<sub>3</sub> and phytase were supplemented

to the NC diet because of increased Ca and P availability in the body (Hasan et al., 2022). However, the observation that plasma FGF23 was not affected by dietary Ca and P or by supplementation of 25(OH)D<sub>3</sub> or phytase was in agreement with previous data (Oster et al., 2016), and it is possible that because FGF23 is a hormone, the concentration is tightly regulated, which is the reason for the lack of impact of diet on plasma FGF23. However, further research is needed to understand the regulatory mechanisms of plasma FGF23 in response to dietary treatments in pigs.

### Vitamin D<sub>3</sub> plasma metabolites

Vitamin D<sub>3</sub> metabolites were analyzed in plasma to determine effects of dietary Ca and P concentration and addition of 25(OH)D<sub>3</sub> or phytase on the concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub>. The observation that plasma 25(OH)D<sub>3</sub> on day 13 was greater in pigs fed diets containing 25(OH)D<sub>3</sub> than in pigs fed no 25(OH)D<sub>3</sub> indicates that more 25(OH)D<sub>3</sub> was available for conversion to 1,25(OH)<sub>2</sub>D<sub>3</sub> (Cavelier et al., 2020; Upadhaya et al., 2021), which may explain the increased absorption of Ca and P by pigs fed 25(OH)D<sub>3</sub> although no significant increase in plasma 1,25(OH)<sub>2</sub>D<sub>3</sub> was caused by 25(OH)D<sub>3</sub>. This observation is in agreement with data from weanling pigs, which also indicated that serum 25(OH)D<sub>3</sub> increased when pigs were fed a diet containing 25(OH)D<sub>3</sub> (Becker et al., 2024). However, it is acknowledged that plasma concentrations of the metabolites, which were measured in this experiment, may not always be representative of metabolic flux and substrate conversion due to the big difference in half-life between 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>.

The observation that inclusion of either 25(OH)D<sub>3</sub> or phytase in the diets resulted in increased plasma 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> is in agreement with the increases in serum OC that were observed and supports the hypothesis that both metabolites may be needed for bone plate growth and mineralization (Boyan et al., 2001; Zelzer et al., 2020; Becker et al., 2024). The observation that 1,25(OH)<sub>2</sub>D<sub>3</sub> was reduced in plasma of pigs fed phytase indicates that the increased intestinal availability of Ca and P that was caused by phytase reduced the need for 1,25(OH)<sub>2</sub>D<sub>3</sub> to aid in absorption of Ca and P (Cavelier et al., 2020; Dugar et al., 2023). Because 25(OH)D<sub>3</sub> increased VMR, it is likely that 25(OH)D<sub>3</sub> partly prevented the negative impact of low Ca and P in diets without microbial phytase. The greater level of plasma 1,25(OH)<sub>2</sub>D<sub>3</sub> in pigs fed the NC than PC diets indicates that more vitamin D<sub>3</sub> was activated to 1,25(OH)<sub>2</sub>D<sub>3</sub> in pigs fed the NC diet, which may have aided in increasing absorption of Ca and P to maintain homeostasis (DeLuca, 2004).

The observation that 1,25(OH)<sub>2</sub>D<sub>3</sub> was reduced from day 1 to day 13 in PC indicates that if diets meet the requirements for Ca and P, the need for activating vitamin D receptors is reduced over time, which may be a result of the reduced requirement for Ca and P as pigs grow. The metabolite 24,25(OH)<sub>2</sub>D<sub>3</sub> is generated after degradation of 1,25(OH)<sub>2</sub>D<sub>3</sub> or 25(OH)D<sub>3</sub> to reduce the concentration of active vitamin D (Lida et al., 1995). The increase in plasma 24,25(OH)<sub>2</sub>D<sub>3</sub> that was observed when diets were supplemented with 25(OH)D<sub>3</sub> or phytase was expected because pigs fed these diets had a reduced need for synthesizing 1,25(OH)<sub>2</sub>D<sub>3</sub>. It thus seems that 24,25(OH)<sub>2</sub>D<sub>3</sub> is an indicator of vitamin D<sub>3</sub> catabolism and overall vitamin D<sub>3</sub> metabolic activity in pigs.

Limitations to this experiment include that for some of the blood analyses, we had to reduce the replications due to the

high cost of these analyses. It is also acknowledged that the length of the experiment was short, and it is possible that some of the blood measurements would be different if diets were fed for a longer time. It is also possible that if bone strength had been measured using a DEXA scan, additional information about the impact of 25(OH)D<sub>3</sub> and phytase on bone formation might have been obtained.

## Conclusion

Supplementation of diets for growing pigs with microbial phytase and/or 25(OH)D<sub>3</sub> resulted in increased STTD of Ca and P. However, 25(OH)D<sub>3</sub> only had a positive effect on STTD of Ca and P if no phytase was used, indicating that the effects of 25(OH)D<sub>3</sub> and phytase are not additive in growing pigs. It is, however, possible that diets with lower concentrations of Ca and P than those used in this experiment are needed to demonstrate additive effects between microbial phytase and 25(OH)D<sub>3</sub>. The increased STTD of Ca and P caused by microbial phytase or 25(OH)D<sub>3</sub> was reflected in elevated serum OC levels, which indicated increased bone tissue synthesis. Inclusion of 25(OH)D<sub>3</sub> or phytase in the diets also improved plasma 24,25(OH)<sub>2</sub>D<sub>3</sub>, which indicates increased vitamin D<sub>3</sub> metabolism.

## Author contributions

Bibiana Jaramillo (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing—original draft), Jessica Acosta (Methodology, Resources, Writing—review & editing), Su A Lee (Investigation, Methodology, Resources, Supervision, Writing—review & editing), Michael Murphy (Methodology, Supervision, Writing—review & editing), and Hans H. Stein (Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing—review & editing)

**Conflict of interest statement.** The authors have no real or perceived conflicts of interest.

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