Journal of Animal Science, 2021, Vol. 99, No. 12, 1–13

https://doi.org/10.1093/jas/skab333
Advance Access publication November 8, 2021
Received: 28 August 2021 and **Accepted:** 3 November 2021
Non Ruminant Nutrition

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Increased microbial phytase increased phytate destruction, plasma inositol, and feed efficiency of weanling pigs, but reduced dietary calcium and phosphorus did not affect gastric pH or fecal score and reduced growth performance and bone ash

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Abstract

An experiment was conducted to test two hypotheses: 1) reducing dietary Ca and P reduces gastric pH and diarrhea in weanling pigs; 2) negative effects of low Ca and P on pig growth performance may be overcome if phytase is added to the diets. A total of 320 weanling pigs (6.35 ± 0.87 kg) were allotted to eight corn-soybean meal-based diets in a randomized complete block design with five pigs per pen. Two phase 1 (days 1 to 14) control diets containing 100 or 50% of total Ca and digestible P relative to the requirement, and six diets in which 500, 2,000, or 16,000 units of phytase/kg feed (FTU) were added to each control diet were formulated. Phytase was assumed to release 0.16% total Ca and 0.11% digestible P. Common diets were fed in phases 2 (days 15 to 27) and 3 (days 28 to 42). Growth performance data were recorded within each phase. Data for fecal scores and gastrointestinal pH were recorded for phase 1. Colon content (day 14), the right femur (days 14 and 42), and blood samples (days –1, 14, 27, and 42) were collected from one pig per pen. In phase 1, reducing Ca and P did not reduce gastric pH or fecal score, but pigs fed the 50% diets had reduced ($P < 0.05$) average daily gain (ADG) and average daily feed intake (ADFI) compared with pigs fed the 100% diets. In both 50% and 100% diets, phytase above 500 FTU increased ($P < 0.05$) gain:feed ratio (G:F) and tended ($P < 0.10$) to reduce gastric pH of pigs. From days 1 to 42, pigs fed the 50% diets tended ($P < 0.10$) to have reduced ADG and ADFI compared with pigs fed the 100% diets, but among the 100% diets, pigs tended ($P < 0.10$) to have a linear increase in G:F as phytase level increased. Pigs fed the 50% diets had reduced ($P < 0.05$) concentrations of inositol phosphate esters (IP) in the colon and reduced bone ash (days 14 and 42) compared with pigs fed the 100% diets. Phytase did not affect bone ash or most blood metabolites. Concentrations of IP in the colon decreased, whereas plasma inositol increased (d 14; $P < 0.05$) in pigs fed diets with phytase (≥ 500 FTU). In pigs fed the 100% diets, IP in the colon linearly decreased ($P < 0.05$), but plasma inositol linearly increased ($P < 0.05$) with increasing levels of phytase. In conclusion, reducing Ca and P in diets for weanling pigs did not influence gastric pH or fecal score, but compromised growth performance and bone ash. However, regardless of dietary Ca and P, high doses of phytase increased phytate degradation and inositol absorption, which consequently increased G:F of pigs.

Key words: bone ash, calcium, inositol, pigs, phosphorus, phytase

Abbreviations

AEE	acid hydrolyzed ether extract
ADFI	average daily feed intake
ADG	average daily gain
BUN	blood urea nitrogen
BW	body weight
ELISA	enzyme-linked immunosorbent assay
FTU	phytase units
G:F	gain to feed ratio
IgA	immunoglobulin A
IP	inositol phosphate
IFNγ	interferon γ
IL	interleukin
STTD	standardized total tract digestible
TNF-α	tumor necrosis factor-α

Introduction

During the post-weaning period, pigs are stressed due to environmental, nutritional, physiological, and immunological changes that increase morbidity and mortality (Pluske et al., 1997). This situation is exacerbated with the restriction in the use of antibiotic growth promoters (Casewell et al., 2003). Thus, alternatives to antibiotic growth promoters such as direct-fed microbials, prebiotics, plant extracts, and acidifiers have been studied (Liu et al., 2018). The reason for the use of acidifiers is that it is believed that weanling pigs are unable to secrete enough HCl in the stomach to provide an appropriate pH for pepsin to efficiently digest plant and animal proteins (Kil et al., 2011). However, the inability to reach a low pH in the stomach can also be attributed to the inclusion of limestone and monocalcium phosphate in nursery diets, because these ingredients have high acid binding capacity at pH 3 and 4 (Lawlor et al., 2005). Therefore, it is possible that reducing limestone and monocalcium phosphate in weaning diets results in decreased stomach pH.

Inclusion of microbial phytase in diets for pigs also contributes to lowering Ca and P in the diets due to increased release of digestible Ca and P from phytate, which results in reduced need for dietary limestone and monocalcium phosphate (Zeng et al., 2016). Additionally, high doses of phytase may partially or fully alleviate the negative effects of lowering Ca and P in diets because elevated levels of phytase result in further degradation of lower phytate esters and greater digestibility of Ca and P in diets fed to young pigs (Almeida et al., 2013). Increasing the concentration of phytase also results in increased concentration of inositol in plasma of pigs from the complete destruction of phytate (Cowieson et al., 2017). Inositol is a cyclic sugar that plays an important role in several cellular functions and is believed to have a growth promoting effect in broiler chickens (Lee and Bedford, 2016).

Data from broiler chickens indicate that reducing dietary Ca reduces tibia ash and gizzard pH, whereas inclusion of high levels of phytase results in increased tibia ash and gizzard pH (Walk et al., 2012). However, limited data evaluating the interaction between dietary concentrations of Ca and P and increasing levels of phytase on growth performance, bone ash, and gastric pH of weanling pigs are available. Therefore, the objective of this experiment was to test the hypothesis that reducing Ca and P in diets for weanling pigs reduces diarrhea because of lower stomach pH. The second hypothesis was that inclusion of high doses of phytase in diets for pigs fully or partly overcomes the negative effects of low Ca and P due to phytate degradation, increased plasma inositol, improved protein utilization, and increased immune response.

Materials and Methods

The Institutional Animal Care and Use Committee at the University of Illinois reviewed and approved the protocol for this experiment. Pigs that were the offspring of Line 359 boars and Camborough females (Pig Improvement Company, Hendersonville, TN) were used.

Animals, housing, and diets

Three hundred twenty newly weaned pigs with an initial average body weight (BW) of 6.35 ± 0.87 kg were randomly allotted to eight diets and two blocks in a randomized complete block design. Pigs were blocked based on weaning group and BW and sex within weaning group. There were five pigs per pen (three gilts and two castrates) and four replicate pens per diet in each block (weaning group). Pigs were housed in pens with fully slatted floors equipped with a feeder and a nipple drinker. Water was available at all times.

The experiment was conducted for 6 wk after weaning. A 3-phase feeding program was used with days 1 to 14 as phase 1, days 15 to 27 as phase 2, and days 28 to 42 as phase 3. Pigs were fed one of eight diets in phase 1, whereas a common diet was fed in phases 2 and 3. Therefore, a total of 10 diets were formulated (Table 1). A representative sample of 2 kg of diets and ingredients was collected.

In phase 1, the eight diets were based on corn and soybean meal (Table 2) and included two control diets that contained 100% or 50% of total Ca and standardized total tract digestible (STTD) P relative to the requirement (i.e., 0.85% and 0.45%, respectively; NRC, 2012). The two control diets were formulated based on the requirement for total Ca and STTD P (NRC, 2012) without inclusion of microbial phytase. Three diets were formulated to be identical to the 100% control diet with the exception that 500, 2,000, or 16,000 phytase units (FTU; Quantum Blue; AB Vista Feed Ingredients, Marlborough, UK) per kilogram of feed were included and provisions of total Ca and STTD P were reduced by 0.16 and 0.11 percentage units, respectively, to account for the amount of Ca and P that was assumed to be released by phytase. Three additional diets were formulated to be identical to the 50% control diet, with the exception that 500, 2,000, or 16,000 FTU of microbial phytase were included and provisions of total Ca and STTD P were reduced as explained for the diets formulated at the requirement for Ca and STTD P. All phase 1 diets were formulated to have similar concentrations of standardized ileal digestible amino acids, crude protein, net energy, Na, Cl, K, and vitamin D. Phase 2 and 3 diets were formulated to meet requirements for total Ca (0.80% and 0.70%, respectively) and STTD P (0.40% and 0.33%, respectively; NRC, 2012).

Feeding, sample collection, and bone measurements

Pigs were allowed ad libitum access to feed and water throughout the experiment. The amount of feed offered was recorded daily and the amount of feed in the feeders was recorded at the end of each phase. On the day before weaning (day –1) and at the conclusion of each phase (days 14, 27, and 42), all pigs were weighed and two blood samples were collected from one pig per pen by jugular venipuncture. Pigs from which blood was collected were selected in such a way that there were two gilts and two castrates per treatment in each block, and blood was

Table 1. Ingredient composition, calculated values for Ca and P, and analyzed values of experimental diets[1,2]

Item	Phase 1									
Ca and P requirement	100% of requirement				50% of requirement				Phase 2	Phase 3
Phytase, FTU	0	500	2,000	16,000	0	500	2,000	16,000		
Ingredient, %										
Corn	52.20	53.45	53.40	52.92	55.19	56.38	56.34	55.87	49.78	54.89
Soybean meal, 48% crude protein	15.05	15.05	15.05	15.05	15.00	15.00	15.00	15.00	26.00	29.00
Lactose	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50	–	–
Whey powder	–	–	–	–	–	–	–	–	11.50	10.00
Potato protein concentrate	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00	–	–
Enzyme treated soybean meal	–	–	–	–	–	–	–	–	6.00	–
Spray dried plasma protein	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–
Soybean oil	2.89	2.38	2.40	2.60	1.70	1.22	1.23	1.42	3.00	3.00
Limestone	1.22	1.08	1.08	1.08	0.66	0.51	0.51	0.51	1.21	1.10
Monocalcium phosphate	1.75	1.14	1.14	1.14	0.57	–	–	–	0.89	0.67
L-Lys HCL	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.40	0.36
DL-Met	0.14	0.14	0.14	0.14	0.13	0.13	0.13	0.13	0.17	0.14
L-Thr	–	–	–	–	–	–	–	–	0.10	0.09
Sodium chloride	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.80	0.60
Vitamin-mineral premix[3]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Phytase concentrate[4]	–	0.01	0.04	0.32	–	0.01	0.04	0.32	–	–
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Calculated values, %										
Total Ca	0.83	0.68	0.68	0.68	0.42	0.26	0.26	0.26	0.80	0.70
Total P	0.64	0.52	0.52	0.51	0.40	0.28	0.28	0.28	0.63	0.56
STTD P	0.44	0.33	0.33	0.33	0.23	0.12	0.12	0.12	0.40	0.33
Analyzed values										
Gross energy, kcal/kg	4,084	3,991	4,029	4,120	4,098	4,089	4,080	4,109	4,030	4,010
Dry matter, %	88.43	88.39	88.25	88.34	88.09	88.00	87.80	88.14	89.14	88.85
Ash, %	5.46	4.57	4.66	4.41	3.66	3.03	3.15	2.95	6.16	6.22
Crude protein, %	21.37	21.28	21.46	21.49	21.83	21.00	21.23	21.55	19.63	18.30
AEE, %	4.11	3.35	3.25	3.50	3.20	2.93	2.71	2.61	4.43	4.81
Amino acids, %										
Arg	1.25	1.15	1.17	1.19	1.26	1.18	1.18	1.11	1.32	1.23
His	0.54	0.51	0.52	0.53	0.55	0.52	0.52	0.51	0.53	0.51
Ile	1.12	1.06	1.10	1.09	1.14	1.09	1.08	1.05	0.97	0.92
Leu	2.10	2.03	2.07	2.08	2.16	2.07	2.04	2.05	1.75	1.67
Lys	1.66	1.55	1.62	1.60	1.73	1.65	1.57	1.65	1.49	1.38
Met	0.52	0.54	0.53	0.55	0.52	0.54	0.57	0.51	0.44	0.43
Phe	1.27	1.22	1.25	1.25	1.32	1.24	1.22	1.23	1.03	0.97
Thr	1.03	0.97	1.03	1.03	1.07	1.04	1.02	1.05	0.93	0.83
Trp	0.28	0.27	0.28	0.30	0.29	0.27	0.28	0.28	0.26	0.24
Val	1.30	1.24	1.27	1.28	1.34	1.27	1.24	1.26	1.02	0.97
Ca, %	0.87	0.71	0.72	0.66	0.42	0.26	0.25	0.25	0.84	0.65
P, %	0.66	0.51	0.52	0.51	0.40	0.28	0.27	0.27	0.69	0.58
Phytate[5], %	0.61	0.59	0.58	0.59	0.60	0.59	0.58	0.58	0.80	0.75
Phytate bound-P, %	0.17	0.17	0.16	0.17	0.17	0.17	0.16	0.16	0.22	0.21
Non-phytate P[6], %	0.49	0.34	0.36	0.35	0.23	0.11	0.11	0.11	0.47	0.37
Phytase activity, FTU	< 50	700	2,660	19,100	< 50	609	2,560	20,200	< 50	< 50

[1]Phase 1, phase 2, and phase 3 diets were formulated to have the following quantities of net energy (NE; kcal/kg) and amino acids (expressed as standardized ileal digestible; %): NE, 2,696, 2,488, and 2,465; Lys, 1.41, 1.35, and 1.23; Met, 0.50, 0.46, and 0.41; Thr, 0.86, 0.80, and 0.73; Trp, 0.23, 0.24, and 0.22, respectively.

[2]AEE, acid hydrolyzed ether extract; FTU, phytase units; STTD, standardized total tract digestible.

[3]The vitamin-micromineral premix provided the following quantities of vitamins and micro minerals per kg of complete diet: vitamin A as retinyl acetate, 11,150 IU; vitamin D_3 as cholecalciferol, 2,210 IU; vitamin E as $_{DL}$-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 1.10 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 1.00 mg; vitamin B_{12}, 0.03 mg; $_{D}$-pantothenic acid as $_{D}$-calcium pantothenate, 23.6 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate; Fe, 125 mg as ironsulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganous sulfate; Se, 0.30 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate.

[4]The phytase concentrate contained 5,000 units of phytase/g (Quantum Blue, AB Vista, Marlborough, UK).

[5]Phytate was calculated by dividing the concentration of phytate-bound P by 0.282 (Tran and Sauvant, 2004).

[6]Non-phytate P was calculated as the difference between total P and phytate-bound P.

Table 2. Analyzed composition of ingredients

Item	Corn	Soybean meal	Potato protein concentrate	Spray-dried plasma protein	Calcium carbonate	Monocalcium phosphate
Gross energy, kcal/kg	3,833	4,194	5,333	4,815	–	–
Dry matter, %	85.38	88.10	91.13	90.70	99.96	93.58
Ash, %	1.20	6.76	0.81	7.36	91.32	80.55
Crude protein, %	6.94	45.94	81.93	78.88	–	–
AEE[1], %	3.32	1.84	0.66	0.22	–	–
Ca, %	0.02	0.29	0.02	0.12	38.91	17.31
P, %	0.26	0.63	0.08	1.49	0.04	20.81
Phytate[2], %	0.61	1.40	0.18	–	–	–
Phytate-bound P, %	0.17	0.39	0.05	–	–	–
Non-phytate P[3], %	0.09	0.24	0.03	–	–	–

[1]AEE, acid hydrolyzed ether extract.
[2]Phytate was calculated by dividing the concentration of phytate-bound P by 0.282 (Tran and Sauvant, 2004).
[3]Non-phytate P was calculated as the difference between total P and phytate-bound P.

collected from the same pigs throughout the experiment. Blood samples were collected in vacutainers that contained spray-coated silica or ethylenediaminetetraacetic acid to yield blood serum or blood plasma, respectively, after blood samples had been centrifuged at 1,500 × *g* at 4 °C. Serum and plasma samples were frozen at −20 °C until used for analysis. During the initial 14 d, fecal scores were assessed visually every other day using a score from 1 to 5 (1 = normal feces; 2 = moist feces; 3 = mild diarrhea; 4 = severe diarrhea; and 5 = watery diarrhea). On the last day of phase 1, one pig per pen was euthanized via captive bolt stunning. Pigs were chosen so that there were four gilts and four castrates per treatment. The abdominal cavity was opened and pH was measured twice in situ by making a small incision for a pH electrode in the stomach, duodenum, and ileum. All stomach contents were then collected and mixed and the ex situ pH was measured twice. The right femur and colon contents were also collected. At the conclusion of the experiment (day 42), the pig that was used for blood collection throughout the experiment was euthanized and blood samples and the right femur were collected. Femurs were autoclaved at 125 °C for 55 min and the muscles attached to the bones were removed. Femurs were broken, dried overnight at 105 °C, and soaked for 72 h in petroleum ether under a chemical hood to remove marrow and fat. Bones were then dried at 135 °C for 2 h and ashed at 600 °C for 16 h.

Sample analysis

Samples of colon content were lyophilized and diet, ingredient, and colon content samples were finely ground prior to analysis. Diets and ingredients were analyzed for dry matter by oven drying at 135 °C for 2 h (Method 930.15; AOAC Int., 2019) and for ash by heating to 600 °C in a muffle furnace for 2 h (Method 942.05; AOAC Int., 2019). Calcium and P were also analyzed in these samples by inductively coupled plasma-optical emission spectrometry (Method 985.01 A, B, and C; AOAC Int., 2019) after dry ash preparation (Method 942.05; AOAC Int., 2019) followed by wet digestion with nitric acid (Method 3050 B; US-EPA, 2000). Corn, soybean meal, potato protein concentrate, spray-dried plasma protein, and diets were analyzed for gross energy using an isoperibol bomb calorimeter (Model 6400, Parr Instruments, Moline, IL) and for N (Method 990.03; AOAC Int., 2019) using a LECO FP628 (LECO Corp., Saint Joseph, MI) and crude protein was calculated as N × 6.25. These samples were also analyzed for acid hydrolyzed ether extract (AEE; Method 2003.06; AOAC Int., 2019) using an Ankom^{HCl} followed by an Ankom^{XT15} (Ankom Technology, Macedon, NY). Corn, soybean meal, potato protein concentrate, and diets were also analyzed for phytate-bound P by wet chemistry using the Megazyme kit (Megazyme Inc., Chicago, IL). Diet samples were analyzed for amino acids (Method 982.30 E [a, b, c]; AOAC Int., 2019) using a Hitachi Analyzer (Model No. L8800; Hitachi High Technologies America, Inc., Pleasanton, CA) and for phytase activity by the enzyme-linked immunosorbent assay (ELISA) method using Quantiplate Kits for Quantum Blue (AB Vista, Plantation, FL). Serum samples were analyzed for blood urea nitrogen (BUN), total protein, and albumin using a Beckman Coulter Clinical Chemistry AU analyzer (Beckman Coulter, Inc., Brea, CA), and for Ca by inductively coupled plasma-optical emission spectrometry after wet ash preparation [Method 975.03 B(b); AOAC Int., 2019]. Plasma samples were analyzed for concentrations of immunoglobulin A (IgA) using an ELISA kit (Bethyl Laboratories, Inc., Montgomery, TX) and for cytokines including tumor necrosis factor-α (**TNF-α**), interferon gamma (**IFNγ**), interleukin (**IL**) -6, IL-1β, IL-8, and IL-10 using the MILLIPLEX MAP Porcine Cytokine Magnet Bead panel (MilliporeSigma, Burlington, MA). Plasma samples were also analyzed for inositol after deproteination (Mesina et al., 2019) by high-performance ion chromatography-based techniques described by Walk et al. (2018). Colon samples were also analyzed for inositol and inositol phosphate (IP) esters by high-performance ion chromatography. Deproteinated plasma samples and colon samples were analyzed at the University of East Anglia, School of Biological Sciences, UK. The pH of the gastrointestinal tract was measured using a portable cheese pH meter (HANNA instruments, Woonsocket, RI) that was mounted with a FC2423 pH electrode for semi-solid samples. The pH meter was calibrated using two buffer solutions (4.01 and 7.01 pH).

Calculations and statistical analyses

The concentration of phytate in corn, soybean meal, potato protein concentrate, and diets was calculated by dividing the analyzed concentration of phytate-bound P by 0.282 (Tran and Sauvant, 2004) and non-phytate P was calculated by subtracting the amount of phytate-bound P from total P. Average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F) were calculated for each phase and for the overall experiment. Bone ash percentage was calculated by dividing the quantity of bone ash by the quantity of fat-free dried bone and multiplying by 100. Data for pH were transformed into H^+ concentration by raising the negative pH value to the power of 10

before statistical analysis to reduce the relative error defined as the absolute value of the difference between pH mean and $-\log_{10}(H^+)$ mean (Murphy, 1982). Diarrhea frequency as a percentage was calculated by dividing the number of days with fecal score ≥ 3 by the total number of scoring days (i.e., 8) times 100.

Normality of residuals and homogeneity of variances were tested using the INFLUENCE, GPLOT, and UNIVARIATE procedures of SAS (SAS Inst. Inc., Cary, NC). However, because data for the H^+ concentration did not meet the assumptions of the model, data were analyzed as pH values. On the contrary, data for cytokines in plasma were Log10 transformed before analysis because the residuals from the data were not normally distributed. Outliers were determined by plotting the residuals in a quantile-quantile plot against the normal distribution and values that were beyond ± 2.5 standard deviations were removed.

Data for growth performance, gastrointestinal pH, fecal evaluation, concentration and percentage of bone ash, concentrations of phytate esters in the colon, and blood metabolites were analyzed using the PROC MIXED procedure of SAS with the experimental unit being the pen. The model included the fixed effect of treatment and the random effect of block. Contrast statements were used to determine effects of dietary concentration of Ca and P and inclusion level of phytase. Contrasts included 1) 50% Ca and P diets vs. 100% Ca and P diets (including control and phytase diets); 2) 100% diets: control diet vs. 500 FTU diet; 3) 100% diets: control diet vs. 2,000 + 16,000 FTU diets; 4) 50% diets: control diet vs. 500 FTU diet; and 5) 50% diets: control diet vs. 2,000 + 16,000 FTU diets. Contrast statements were also used to determine linear effects of inclusion level of phytase (i.e., from 500 to 16,000 FTU) at each concentration of Ca and P. Coefficients for the unevenly spaced linear contrasts were obtained using the PROC IML procedure of SAS. Data for blood metabolites obtained on day −1 were used as a covariate to analyze blood data from day 14. Repeated measures were used to analyze the effect of time on the concentration of blood metabolites using an unstructured variance based on the likelihood ratio test. The model included the main effects of dietary treatment and day and the interaction between dietary treatment and day. The time effect was day, the random effect was block, and the experimental unit was the pig. If the interaction was not significant, only main effects were included in the final model. Treatment means were calculated using the LSMEANS statement in SAS. Statistical significance and tendency were considered at $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

Results

Four pigs died during the experiment and three pigs were removed from their pens due to bad condition. The seven removed pigs were from pens fed five different diets and data for ADFI in these pens were adjusted (Lindemann and Kim, 2007). The remaining pigs consumed their diets without apparent problems and no health problems were observed.

During phase 1, there was a reduction ($P < 0.05$) in ADG and ADFI of pigs fed diets with 50% Ca and P diets compared with pigs fed diets with 100% Ca and P diets (Table 3). Among diets with 100% Ca and P, there was a tendency ($P < 0.10$) for pigs fed the diet with 500 FTU to have greater ADG and ADFI than pigs fed the control diet. Likewise, ADG was greater ($P < 0.05$) and ADFI tended ($P < 0.10$) to be greater for pigs fed diets with phytase doses above 500 FTU compared with pigs fed the control

Table 3. Growth performance of pigs fed diets formulated with 100% or 50% of the requirement for Ca and P and 0 (control), 500, 2,000, or 16,000 units of microbial phytase per kilogram of feed (FTU)[1]

	100% of Ca and P requirement				50% of Ca and P requirement					
		FTU				FTU				
Item, kg	Control	500	2,000	16,000	Control	500	2,000	16,000	SEM	Contrasts[2,3]
Phase 1, days 1 to 14										
Initial BW	6.39	6.39	6.35	6.34	6.33	6.33	6.35	6.33	0.51	–
ADG	0.134	0.162	0.169	0.171	0.134	0.135	0.141	0.146	0.01	a**, b, c**
ADFI	0.181	0.205	0.210	0.200	0.188	0.179	0.178	0.184	0.01	a*, b, c
G:F	0.740	0.783	0.797	0.860	0.714	0.755	0.788	0.789	0.03	c*, d, f*
Final BW	8.27	8.65	8.72	8.73	8.20	8.23	8.32	8.37	0.63	–
Phase 2, days 15 to 27										
ADG	0.485	0.482	0.466	0.466	0.481	0.438	0.450	0.436	0.02	–
ADFI	0.635	0.621	0.623	0.605	0.636	0.551	0.591	0.584	0.03	a, e*
G:F	0.761	0.779	0.749	0.770	0.756	0.797	0.762	0.745	0.04	–
Final BW	14.63	14.81	14.83	14.80	14.63	13.79	14.17	14.00	0.70	–
Phase 3, days 28 to 42										
ADG	0.622	0.641	0.664	0.628	0.620	0.592	0.651	0.623	0.03	–
ADFI	0.982	1.030	1.036	0.967	0.967	0.970	1.019	0.977	0.03	d
G:F	0.633	0.622	0.642	0.650	0.641	0.609	0.641	0.638	0.02	e*
Final BW	23.96	24.43	24.79	24.13	24.07	22.66	24.06	23.34	1.04	–
Overall, days 1 to 42										
ADG	0.418	0.429	0.439	0.424	0.423	0.389	0.422	0.405	0.01	a
ADFI	0.608	0.628	0.633	0.599	0.605	0.577	0.606	0.591	0.02	a
G:F	0.688	0.683	0.694	0.707	0.698	0.673	0.694	0.685	0.01	d, e*

[1]Data are least squares means of 8 observations.
[2]Contrasts that were significant ($P < 0.05$) or tended ($P < 0.10$) to be significant were expressed as follows: a = 100% vs. 50%; b = 100%: control vs. 500 FTU; c = 100%: control vs. 2,000 + 16,000 FTU; d = 100%: linear effect of phytase; e = 50%: control vs. 500 FTU; f = 50%: control vs. 2,000 + 16,000 FTU; g = 50%: linear effect of phytase.
[3]No *$P < 0.10$; *$P < 0.05$; **$P < 0.01$.

diet. A tendency ($P < 0.10$) for a positive linear effect of phytase on the G:F of pigs was also observed in the 100% Ca and P diets, but regardless of the dietary concentration of Ca and P, pigs fed diets with 2,000 or 16,000 FTU of phytase had greater ($P < 0.05$) G:F than pigs fed the control diet. During phase 2, pigs fed the 50% Ca and P diets in phase 1 tended ($P < 0.10$) to have reduced ADFI compared with pigs fed the 100% Ca and P diets, and pigs fed the diet with 50% Ca and P and 500 FTU of phytase had lower ($P < 0.05$) ADFI than pigs fed the 50% control diet. In phase 3, pigs fed the 100% Ca and P diets in phase 1 tended ($P < 0.10$) to have a linear reduction in ADFI as dietary phytase increased from 500 to 16,000 FTU in phase 1. Pigs fed the 50% control diet during phase 1 had greater ($P < 0.05$) G:F in phase 3 than pigs fed the 50% Ca and P diet with 500 FTU of phytase in phase 1. For the overall experimental period, there was a tendency ($P < 0.10$) for pigs fed the 50% diets in phase 1 to have reduced ADG and ADFI compared with pigs fed the 100% diets. For the 100% Ca and P diets, a tendency ($P < 0.10$) for a positive linear effect of phytase in phase 1 on G:F of pigs was observed, and the G:F was greater ($P < 0.05$) for pigs fed the 50% control diet during phase 1 than for pigs fed the 50% Ca and P diet with 500 FTU of phytase.

At the end of phase 1, there was a reduced ($P < 0.05$) concentration and percentage of bone ash in pigs fed the 50% Ca and P diets compared with pigs fed the 100% Ca and P diets (Table 4). Among the 50% Ca and P diets, there was a tendency ($P < 0.10$) for pigs fed the control diet to have a greater percentage of bone ash than pigs fed diets with 2,000 or 16,000 FTU of phytase. Among the 100% Ca and P diets, there was a tendency ($P < 0.10$) for lower gastric pH (in situ) in pigs fed diets containing phytase doses above 500 FTU than in pigs fed the control diet. Likewise, pigs fed diets containing 500 FTU of phytase had lower ($P < 0.05$) gastric pH measured ex situ than pigs fed the control diet. Among the 50% Ca and P diets, a tendency ($P < 0.10$) for a reduced gastric pH (ex situ) in pigs fed diets containing phytase above 500 FTU was observed compared with pigs fed the control diet. However, regardless of the dietary concentration of Ca and P, pigs fed diets with 2,000 or 16,000 FTU of phytase tended ($P < 0.10$) to have lower gastric pH (average) than pigs fed the control diet. A tendency ($P < 0.10$) for a reduced duodenal pH in pigs fed the 50% Ca and P diets as phytase inclusion increased was also observed. However, there was no effect of dietary treatment on ileal pH, fecal score, or diarrhea frequency of pigs. At the end of phase 3, the concentration of bone ash in pigs fed the 50% Ca and P diets in phase 1 was reduced ($P < 0.05$) compared with pigs fed the 100% Ca and P diets, but no differences in percentage of bone ash were observed among treatments.

On the last day of phase 1, pigs fed diets with 100% Ca and P had increased concentrations of IP6, IP5, and IP4 in colon contents compared with pigs fed the 50% Ca and P diets (Table 5). Regardless of the concentration of dietary Ca and P, pigs fed diets with phytase had reduced ($P < 0.05$) concentrations of IP6, IP5, and IP4 in the colon compared with pigs fed the control diets. Concentrations of IP6, IP5, and IP4 in the colon content of pigs linearly decreased ($P < 0.05$) as the dietary level of phytase increased from 500 to 16,000 FTU in diets with 100% Ca and P. In all samples, the concentration of IP3 and inositol was undetectable.

At the end of phase 1 (day 14), pigs had reduced ($P < 0.05$) concentrations of Ca and albumin in serum if fed the 50% Ca and P diets compared with the 100% Ca and P diets (Table 6). The concentration of BUN in serum of pigs was not influenced by dietary treatments, but pigs fed diets containing 500 FTU of phytase and 50% Ca and P had greater ($P < 0.05$) concentration of total protein in serum than pigs fed the 50% control diet.

Regardless of the concentration of Ca and P in the diet, on day 14, pigs fed diets with phytase had increased ($P < 0.05$)

Table 4. Bone mineralization, gastrointestinal pH, and fecal evaluation of pigs fed diets formulated with 100 or 50% of the requirement for Ca and P and 0 (control), 500, 2,000, or 16,000 units of microbial phytase per kilogram of feed (FTU)

	100% of Ca and P requirement				50% of Ca and P requirement					
		FTU				FTU				
Item	Control	500	2,000	16,000	Control	500	2,000	16,000	SEM	Contrasts[1,2]
End of phase 1, day 14										
Bone mineralization[3]										
Ash, g	5.03	4.77	5.13	4.99	4.23	4.29	4.05	4.13	0.27	a***
Ash, %	54.5	53.4	53.7	53.0	51.6	50.9	50.4	50.3	0.65	a***, f
Gastrointestinal pH[4]										
Stomach *In-situ*	3.70	3.50	3.40	3.20	3.85	3.41	3.41	3.54	0.20	c
Stomach *Ex-situ*	3.68	3.19	3.48	3.33	3.60	3.50	3.37	3.22	0.16	b*, f
Stomach average	3.69	3.34	3.44	3.26	3.73	3.54	3.39	3.38	0.16	c, f
Duodenum *In situ*	5.92	5.93	5.69	5.77	5.93	6.04	5.40	6.13	0.15	g
Ileum *In situ*	6.75	6.66	6.48	6.63	6.68	6.72	6.77	6.62	0.13	–
Fecal evaluation[4]										
Fecal score	2.18	2.29	2.29	2.02	2.16	2.22	2.04	2.02	0.14	–
Diarrhea frequency, %	33.9	34.0	35.1	26.1	25.0	33.9	25.0	26.8	7.78	–
End of phase 3, day 42										
Bone mineralization[3]										
Ash, g	12.11	11.25	11.60	11.41	10.56	10.21	11.67	9.96	0.62	a*
Ash, %	50.7	49.9	49.4	49.8	48.9	49.8	49.8	48.8	1.09	–

[1]Contrasts that were significant ($P < 0.05$) or tended ($P < 0.10$) to be significant were expressed as follows: a = 100% vs. 50%; b = 100%: control vs. 500 FTU; c = 100%: control vs. 2,000 + 16,000 FTU; d = 100%: linear effect of phytase; e = 50%: control vs. 500 FTU; f = 50%: control vs. 2,000 + 16,000 FTU; g = 50%: linear effect of phytase.
[2]No *$P < 0.10$; *$P < 0.05$; ***$P < 0.001$.
[3]Data are least squares means of 8 observations.
[4]Data are least squares means of 7 or 8 observations.

Table 5. Concentration of inositol phosphate (IP) esters (nmol/g dry matter) in colon content from pigs fed diets formulated with 100% or 50% of the requirement for Ca and P and 0 (control), 500, 2,000, or 16,000 units of microbial phytase per kilogram of feed (FTU)[1,2]

	100% of Ca and P requirement				50% of Ca and P requirement					
		FTU				FTU				
Item	Control	500	2,000	16,000	Control	500	2,000	16,000	SEM	Contrasts[3,4]
IP6	3,715	1,925	509	140	1,423	0	0	0	437.4	a***, b*, c***, d*, e*, f*
IP5	956	495	111	6	337	0	0	0	93.7	a***, b**, c***, d*, e*, f*
IP4	1,700	1,079	711	305	793	86	69	33	134.1	a***, b**, c***, d***, e***, f***

[1]Values for IP3 and inositol were undetectable in all samples.
[2]Data are least squares means of 7 or 8 observations.
[3]Contrasts that were significant ($P < 0.05$) were expressed as follows: a = 100% vs. 50%; b = 100%: control vs. 500 FTU; c = 100%: control vs. 2,000 + 16,000 FTU; d = 100%: linear effect of phytase; e = 50%: control vs. 500 FTU; f = 50%: control vs. 2,000 + 16,000 FTU; g = 50%: linear effect of phytase.
[4]*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 6. Indicators of protein utilization in serum from pigs fed diets formulated with 100 or 50% of the requirement for Ca and P and 0 (control), 500, 2,000, or 16,000 units of microbial phytase per kilogram of feed (FTU)[1]

Item	100% of Ca and P requirement				50% of Ca and P requirement					
		FTU				FTU				
	Control	500	2,000	16,000	Control	500	2,000	16,000	SEM	Contrasts[2,3]
Ca, mg/dL[4]										
d –1	10.8	10.7	10.6	10.5	10.9	10.3	10.9	10.4	–	
d 14	9.7	10.1	9.1	10.0	9.4	9.2	9.0	9.2	0.27	a*
Blood urea nitrogen,[5] mg/dL										
d -1	6.75	5.75	7.13	6.50	6.38	6.75	5.46	5.63	–	
d 14	5.22	5.65	7.24	7.17	6.40	8.35	6.90	8.37	2.06	–
Total protein,[4] g/dL										
d -1	4.55	4.39	4.39	4.59	4.55	4.49	4.63	4.26	–	
d 14	4.39	4.48	4.34	4.38	4.13	4.50	4.22	4.41	0.14	e*
Albumin,[4] g/dL										
d -1	3.05	2.95	2.94	2.91	2.98	2.71	2.94	2.74	–	
d 14	2.51	2.54	2.43	2.48	2.34	2.33	2.24	2.30	0.11	a**

[1]Data from day –1 were used as a covariate for data obtained on day 14.
[2]Contrasts that were significant ($P < 0.05$) were expressed as follows: a = 100% vs. 50%; b = 100%: control vs. 500 FTU; c = 100%: control vs. 2,000 + 16,000 FTU; d = 100%: linear effect of phytase; e = 50%: control vs. 500 FTU; f = 50%: control vs. 2,000 + 16,000 FTU; g = 50%: linear effect of phytase.
[3]*$P < 0.05$; **$P < 0.01$.
[4]Data are least squares means of 8 observations.
[5]Data are least squares means of 7 or 8 observations.

concentration of inositol in plasma compared with pigs fed the control diet (Table 7). The concentration of inositol in plasma tended ($P < 0.10$) to linearly increase in pigs fed diets with 50% Ca and P, and linearly increased ($P < 0.05$) in pigs fed 100% Ca and P diets, as the level of phytase increased in the diet. Pigs fed diets with 100% Ca and P and 500 FTU of phytase tended ($P < 0.10$) to have reduced concentration of IgA compared with pigs fed the 100% control diet. There was a tendency ($P < 0.10$) for pigs fed the 100% Ca and P diets to have a linear increase in plasma concentration of INFγ as phytase increased from 500 to 16,000 FTU in diets. Pigs fed diets with 50% Ca and P tended ($P < 0.10$) to have reduced concentration of IL-1β in plasma compared with pigs fed diets with 100% Ca and P. In pigs fed diets with 50% Ca and P, plasma IL-6 and IL-10 tended ($P < 0.10$) to be reduced, whereas plasma IL-8 was reduced ($P < 0.05$) if diets contained 500 FTU of phytase compared with the control diet. However, dietary treatments had no influence on the concentration of TNF-α in plasma of pigs.

There was no interaction between dietary treatment and day for serum Ca, BUN, total protein, or albumin or for IgA or cytokines in plasma of pigs (Table 8). No effect of dietary treatment on BUN, total protein, IgA, IL-8, and IL-10 was observed. However, pigs fed phase 1 diets with 100% Ca and P had greater ($P < 0.05$) concentrations of Ca and albumin in serum than pigs fed phase 1 diets with 50% Ca and P. Concentrations of INFγ and IL-6 in plasma of pigs fed the control diet with 50% Ca and P were greater ($P < 0.05$) than in plasma of pigs fed diets with 500 FTU of phytase. The concentration of plasma IL-1β, IL-6, and TNF-α was greater ($P < 0.05$) in pigs fed the 50% Ca and P control diet than in pigs fed diets with 50% Ca and P and 2,000 or 16,000 FTU of phytase. Concentrations of BUN and IgA linearly increased ($P < 0.05$) and the concentration of IgA also tended to increase (quadratic; $P < 0.10$) from days –1 to 42. Likewise, there was an increase (quadratic; $P < 0.05$) in the concentration of Ca, total protein, and albumin in serum of pigs from days –1 to 42. In contrast, the concentration of INFγ and IL-8 linearly decreased

Table 7. Plasma metabolites in pigs fed diets formulated with 100% or 50% of the requirement for Ca and P and 0 (control), 500, 2,000, or 16,000 units of microbial phytase per kilogram of feed (FTU)[1]

Item	100% of Ca and P requirement				50% of Ca and P requirement					
		FTU				FTU				
	Control	500	2,000	16,000	Control	500	2,000	16,000	SEM	Contrasts[2],[3]
Inositol,[4] μM										
d –1	38.0	57.3	43.5	49.0	36.1	52.8	49.2	39.8	–	
d 14	15.3	43.6	55.9	63.6	19.2	41.7	49.7	51.0	7.72	b**, c***, d*,e**, f***, g
Immunoglobulin A,[4] mg/mL										
d –1	0.159	0.197	0.187	0.192	0.145	0.162	0.195	0.160	–	
d 14	0.316	0.226	0.308	0.254	0.316	0.276	0.269	0.291	0.0393	b
Interferon γ,[5],[6] ng/mL										
d –1	15.8	26.5	5.2	10.4	40.9	7.2	27.9	7.3	–	
d 14	6.24	3.85	5.43	7.62	5.71	3.44	6.09	5.89	1.948	d
Interleukin 1β,[5],[6] pg/mL										
d –1	234	546	93	222	596	179	491	99	–	
d 14	250	261	199	163	392	320	232	390	85.9	a
Interleukin 6,[5],[6] pg/mL										
d –1	164	339	43	110	330	127	286	52	–	
d 14	97	89	98	67	186	65	92	134	36.4	e
Interleukin 8, [5],[6] pg/mL										
d –1	48	58	34	56	91	39	91	44	–	
d 14	40	41	35	39	57	29	33	42	8.6	e*
Interleukin 10,[5],[6] pg/mL										
d –1	875	1528	282	713	1,551	593	1,343	318	–	
d 14	446	508	506	296	744	345	472	490	152.5	e
Tumor necrosis factor-α, [5],[6] pg/mL										
d –1	251	350	158	172	494	165	343	161	-	
d 14	151	176	141	150	162	111	91	159	43.0	-

[1]Data from day –1 were used as a covariate for data obtained on day 14.
[2]Contrasts that were significant ($P < 0.05$) or tended ($P < 0.10$) to be significant were expressed as follows: a = 100% vs. 50%; b = 100%: control vs. 500 FTU; c = 100%: control vs. 2,000 + 16,000 FTU; d = 100%: linear effect of phytase; e = 50%: control vs. 500 FTU; f = 50%: control vs. 2,000 + 16,000 FTU; g = 50%: linear effect of phytase.
[3]No *$P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[4]Data are least squares means of 7 or 8 observations.
[5]Data are least squares means of 8 observations.
[6]Values were Log10 transformed before analysis to obtain a normal distribution, but data are shown as back-transformed least square means.

($P < 0.05$) with time, and pigs had a reduction (quadratic; $P < 0.05$) in the concentration of IL-6, IL-10, and TNF-α in plasma from days –1 to 42. The concentration of IL-1β also tended to decrease (quadratic; $P < 0.10$) from days –1 to 42 post-weaning.

There was an interaction ($P < 0.05$) between dietary treatment and day for plasma inositol (Figure 1). At days –1, 28, and 42, there were no differences in the concentration of inositol in plasma among treatments, but on day 14, pigs fed the two control diets had reduced ($P < 0.05$) concentration of inositol in plasma compared with pigs fed diets with phytase.

Discussion

The effect of reducing the concentration of Ca and P in diets without or with increasing levels of dietary phytase was determined in the present study to evaluate potential nutritional strategies to reduce weaning stress and improve post-weaning growth performance. The reason this nutritional strategy was only used in phase 1 was to avoid bone development issues in later phases. Requirements for Ca and P by pigs to maximize bone ash are greater than requirements to maximize growth performance, and this difference becomes wider as pigs get heavier (NRC, 2012; Lagos et al., 2019).

The observed reduction in ADG and ADFI of pigs fed the 50% Ca and P diets compared with pigs fed the 100% diets during 2 wk after weaning is in contrast with published data indicating that the level of Ca and P in phase 1 diets had no effect on these 2 variables (Létourneau-Montminy et al., 2010; Lagos et al., 2021). The observed reduction in ADG and ADFI in phase 2 and for the overall period for pigs fed diets with 50% Ca and P compared with pigs fed adequate diets in phase 1 indicates that pigs were not able to recover from reduced concentrations of Ca and P in phase 1 diets. However, it is possible that a different result would have been obtained if the period with restriction in dietary Ca and P had been less than 14 d, or if phase 2 and 3 diets had contained Ca and P above requirements.

The observed increase in ADG and ADFI of pigs fed diets with 100% Ca and P by the inclusion of phytase concurs with published data indicating that ADG and ADFI of weanling pigs linearly increased as phytase increased in phase 1 diets (Moran et al., 2017). These data indicate that phytase, in addition to the release of Ca and P, provides benefits to pigs fed diets with adequate levels of Ca and P, likely as a result of a reduction in the anti-nutritional effects of phytate and phytate esters. The observed increase in G:F of pigs fed diets with 2,000 or 16,000 FTU of phytase compared with pigs fed the control diet, regardless of the concentration of Ca and P, is in agreement with results by

Table 8. Effect of treatment and day on blood metabolites from pigs fed diets formulated with 100 or 50% of the requirement for Ca and P and 0 (control), 500, 2,000, or 16,000 units of microbial phytase per kilogram of feed (FTU)[1,2]

Item	100%				50%											
		FTU				FTU										
	Control	500	2,000	16,000	Control	500	2,000	16,000		–1	14	27	42		L	Q
Serum																
Ca[3]	10.9	10.8	10.7	10.8	10.7	10.5	10.7	10.7	0.13	10.6	9.5	11.1	11.7	0.17	< 0.001	< 0.001
BUN[4]	7.26	7.06	8.42	8.18	7.78	8.37	7.37	7.72	0.70	6.30	6.86	8.52	9.41	1.01	< 0.001	0.704
Protein[4]	4.79	4.68	4.64	4.68	4.65	4.70	4.83	4.60	0.07	4.48	4.36	4.64	5.30	0.05	< 0.001	< 0.001
Albumin[3]	3.02	2.89	2.87	2.80	2.82	2.63	2.86	2.70	0.09	2.90	2.40	2.74	3.26	0.05	< 0.001	< 0.001
Plasma																
IgA[4]	0.48	0.49	0.52	0.50	0.46	0.48	0.50	0.49	0.028	0.17	0.28	0.64	0.86	0.024	< 0.001	0.065
INFγ[5,6]	3.44	5.86	1.25	2.18	8.52	1.68	5.81	1.68	0.196	13.86	5.38	1.65	0.72	0.096	< 0.001	0.470
IL-1β[5,7]	173	273	143	141	342	226	146	157	65.5	246	264	101	199	42.3	0.108	0.089
IL-6[5,6,7]	70	113	47	52	178	51	77	37	29.6	142	98	27	59	14.8	< 0.001	< 0.001
IL-8[4,5]	33	43	31	37	42	29	37	33	9.1	54	39	31	23	5.3	< 0.001	0.586
IL-10[4,5]	396	660	285	297	706	346	348	221	151.5	755	460	223	263	89.8	< 0.001	0.016
TNF-α[5,7]	127	145	142	125	163	156	114	123	21.8	350	121	66	55	23.3	< 0.001	0.004

[1]There was no interaction between treatment and day.
[2]BUN = blood urea nitrogen (mg/dL); Protein = total protein (g/dL); Albumin (g/dL); IgA = immunoglobulin A (mg/mL); TNF-α = tumor necrosis factor alpha (pg/mL); INF-γ = interferon gamma (ng/mL); IL = interleukin (pg/mL).
[3]Contrast 100% vs. 50% ($P < 0.05$).
[4]None of the contrasts analyzed were significant.
[5]Values were Log10 transformed before analysis to obtain a normal distribution, but data are shown as back-transformed least square means.
[6]Contrasts 50%: control vs. 500 ($P < 0.05$).
[7]Contrast 50%: control vs. 2,000 + 16,000 ($P < 0.05$).

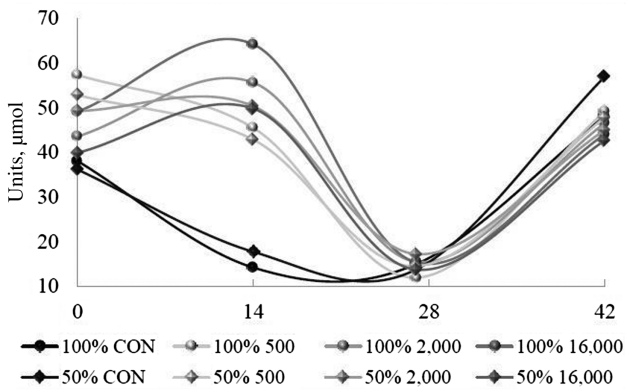

Figure 1. Concentration of inositol in plasma of pigs fed diets formulated with 100 or 50% of the requirement for Ca and P and 0 (CON), 500, 2,000, or 16,000 units of microbial phytase during days −1 to 42.

Holloway et al. (2019) and Moran et al. (2019), who demonstrated that inclusion of 2,500 FTU of phytase increased feed efficiency of newly weaned pigs. These observations indicate that inclusion of phytase at ≥ 2,000 FTU not only released the amount of Ca and P that was assumed in diet formulations, but also provided additional benefits to pigs undergoing the stress of weaning.

The reason phytase appeared to be less efficient in the diets with 50% Ca and P compared with diets with 100% Ca and P may be that due to the reduced concentration of limestone in these diets, there was less potential for Ca to bind to phytate or P and form un-digestible complexes. If this was the case, there would have been less room for phytase to counteract the negative effects of these complexes, which may explain the reduced effect of phytase on the diets with 50% Ca and P.

The observed reduction in bone ash as a result of Ca and P deficiencies in phase 1 diets is in agreement with previous data (Létourneau-Montminy et al., 2010; Lagos et al., 2021). This response was expected because diets were formulated below the requirement for optimal growth performance and requirements for Ca and P to maximize bone mineralization are greater than to maximize growth performance (NRC, 2012; Lagos et al., 2019). Data from day 42 indicate that early bone mineralization is important because pigs were not able to recover in terms of bone ash from diets deficient in Ca and P, which also concurs with previous data (Létourneau-Montminy et al., 2010). Pigs fed diets containing 70% of the Ca requirement for 28 d were able to recover from reduced bone mineral content and density after a repletion period of 42 d during which pigs were fed diets that contained 50% more Ca than the requirement (Aiyangar et al., 2010). These observations indicate that pigs can recover from low Ca and P diets only if the repletion diets contain Ca and P above the requirement. The lack of differences in bone ash of pigs fed the 100% Ca and P diets between the control diet and the diets with phytase indicates that the release values for Ca and P assumed for phytase in diet formulations were accurate and that the positive effect of phytase on growth performance is beyond Ca and P release. However, because phytate is composed of 6 phosphate molecules, but can only bind 5 Ca molecules (Selle et al., 2009), it is also possible that supplementation of high levels of phytase results in a reduced amount of Ca absorbed relative to P, and the Ca to P ratio in plasma may be less than that required for bone tissue synthesis. However, more research is needed to confirm this hypothesis.

Values for stomach pH obtained in this experiment concur with previous data (Radcliffe et al., 1998; Rice et al., 2002) and are within the range of gastric pH reported from pigs, which varies from 1.0 to 4.5 depending on feeding time and site of measure (Chesson, 1987; Lee et al., 2018). Although for this experiment the site was maintained constant, the time of pH measure related to feeding was much harder to standardize. The observation that reducing the concentration of Ca and P in diets did not influence gastrointestinal pH or diarrhea occurrence is in agreement with data from Lagos et al. (2021), but in contrast with data from broiler chickens (Walk et al., 2012). Although it was hypothesized that reducing the concentration of limestone and monocalcium phosphate reduces the buffering capacity of the diet and consequently decreases stomach pH and diarrhea incidence (Jasaitis et al., 1987; Bolduan et al., 1988; Lawlor et al., 2005), data from Lagos et al. (2021) and from the present experiment reject this hypothesis. It is possible that this is because diets used in this experiment and in the experiment by Lagos et al. (2021) contained lactose, which through bacterial fermentation produces lactic acid and contributes to a reduced stomach pH (Zhao et al., 2021). Lactic acid is an acidifier that can be included in diets for weanling pigs (Suiryanrayna and Ramana, 2015) and appears to counter the high acid binding capacity of limestone and monocalcium phosphate in weaning diets.

The observed tendency for phytase to reduce stomach pH, regardless of dietary concentrations of Ca and P, concurs with published data from weanling pigs (Lee et al., 2018; Lagos et al., 2021; Lee et al., 2021); however, the mode of action is not well understood. Inclusion of phytase in diets for broiler chickens (Walk et al., 2012) and pigs (Radcliffe et al., 1998; Rice et al., 2002) resulted in increased stomach pH as a consequence of the reduction of the acidogenic effect of phytate (Józefiak et al., 2016). Thus, additional research is needed to elucidate how phytase influences gastric pH.

The reduced concentrations of phytate and IP esters in colon contents of pigs fed diets with phytase demonstrate the role of phytase in removing phosphate from phytate and IP esters. However, these values do not solely reflect the effect of dietary phytase, but also the phytase synthesized by intestinal microbes in the large intestine of pigs (Mesina et al., 2019). Phytate esters were measured in colon contents instead of ileal digesta samples because of the difficulty associated with collecting sufficient quantities of ileal digesta from small pigs. Nevertheless, the observed influence of dietary levels of Ca and P on concentrations of IP6, IP5, and IP4 in colon contents was not expected because the concentration of dietary phytate was constant among treatments. Therefore, these results indicate that reducing the concentration of dietary Ca and P from inorganic sources results in less phytate-Ca-P complexes and greater phytase efficacy in the large intestine. This hypothesis is supported by in vitro (Sommerfeld et al., 2017) and in vivo (Tamim et al., 2004) data from poultry indicating that increasing levels of Ca and P in diets with phytase results in reduced degradation of phytate and phytate esters.

The analyzed concentrations of serum metabolites are in agreement with previous data (Lagos et al., 2021) and indicate that neither concentrations of dietary Ca and P nor phytase level influenced protein utilization as indicated by the lack of differences among treatments in serum BUN. However, dietary Ca influenced the concentrations of Ca and albumin in serum of pigs, which may be because after absorption, around 32% of total Ca that circulates in blood is bound to albumin (Bazydlo et al., 2014). Therefore, it appears that reduced dietary Ca reduced the concentration of Ca in serum and the need of albumin as carrier. However, the concentration of Ca in serum was maintained within the physiological range of 8 to 12 mg/dL (Amundson et al., 2017), which is likely because the ionized Ca, which comprises

50% of the total circulating Ca, remains unchanged regardless of dietary treatment because of hormonal regulation (Bazydlo et al., 2014). Results also indicate that the effect of dietary Ca and P on serum Ca and albumin observed in phase 1 is maintained after a nutritionally adequate diet is fed in phases 2 and 3.

The observed increase in the concentration of plasma inositol upon phytase supplementation supports the data for phytate and IP esters in colon contents, and indicates that some dietary phytate was fully degraded if at least 500 FTU of phytase was included in the diet. These results concur with data indicating that inclusion of phytase above 1,000 FTU results in increased concentration of plasma inositol in pigs (Guggenbuhl et al., 2016; Cowieson et al., 2017). Inositol is the end product of phytate degradation and plays an important role in several metabolic processes through cell signaling (Huber, 2016). Inositol is believed to have an insulin-like effect due to its role in protein B kinase activation, and increased inositol release results in increased concentration of glucose transporter type 4 in muscle of weanling pigs (Lu et al., 2019). This result concurs with the observed linear increase in G:F of pigs with increasing levels of phytase in diets formulated to have Ca and P at the requirement, and indicates that the increased plasma inositol concentration may be the main reason for the observed positive effect of phytase on pig growth performance immediately after weaning. This hypothesis is supported by data indicating that supplementation of inositol or inclusion of high doses of phytase in phase 1 diets resulted in increased plasma inositol and improved G:F of pigs (Moran et al., 2019).

The observation that dietary Ca and P did not influence plasma inositol indicates that the observed reduction or elimination of phytate and IP esters in colon contents of pigs fed diets containing 50% Ca and P is related to the efficacy of phytase synthesized by microbes in the hindgut. Pigs are unable to absorb P beyond the ileum (Mesina et al., 2019), and inositol released in the colon is likely metabolized by the microbes; therefore, this effect is nutritionally irrelevant for pigs.

The observed interaction between dietary treatments and day for inositol in plasma illustrates the importance of phytase on inositol release. The observation that regardless of the diet provided in phase 1, the concentration of inositol in plasma decreased to 15 μM on day 27 and at day 42 increased back to around 45 μM, indicates that pigs are only able to increase plasma inositol back to the concentration observed before weaning after week 6. Therefore, inositol may be a conditionally essential nutrient during the immediate post-weaning period because it appears that pigs are not able to synthesize sufficient quantities of inositol during the initial weeks post-weaning.

Cytokines are small proteins secreted by immune cells that play an important role in the regulation of the immune and inflammatory response (Zhang and An, 2007). The lack of a pattern in the results for plasma IgA and cytokines due to dietary treatments indicates that the concentration of Ca and P or phytase did not influence blood indicators for the immune or inflammatory response of weanling pigs. Results from the analysis of blood metabolites over time indicate that as pigs grow, concentrations of IgA in plasma and Ca, BUN, total protein, and albumin in serum increase, but cytokine concentrations in plasma decrease.

Conclusions

Reducing the concentration of Ca and P in diets for 2 wk after weaning did not reduce gastric pH or diarrhea incidence of pigs, but decreased growth performance and bone ash. Pigs did not recover from the negative effects of low Ca and P in phase 1 diets during the following 4 wk. A 50% reduction in the provision of Ca and P during the initial 2 wk post-weaning is, therefore, not recommended. Phytase inclusion had limited impact on growth performance of pigs fed Ca and P deficient diets, which is likely because diets with phytase were formulated with reduced Ca and P. However, inclusion of high doses of phytase in diets formulated to be adequate in Ca and P improved growth performance of pigs, which was likely a result of phytate degradation and increased inositol release and absorption. Phytase tended to reduce gastric pH, but did not affect incidence of diarrhea or concentrations of blood indicators for protein utilization or inflammatory response. Therefore, it appears that the positive effect of phytase on weanling pigs is related to the reduced anti-nutritional effect of phytate and the role of inositol in metabolism during the post-weaning period.

Acknowledgments

Financial support for this research from AB Vista, Marlborough, UK, is appreciated.

Conflict of interest statement

M.R.B. is an employer of AB Vista, Marlborough, UK, which is a global supplier of microbial phytase. The other authors have no real or perceived conflicts of interest.

Literature Cited

Aiyangar, A. K., A. G. Au, T. D. Crenshaw, and H. L. Ploeg. 2010. Recovery of bone strength in young pigs from an induced short-term dietary calcium deficit followed by a calcium replete diet. *Med. Eng. Phys.* **32**:1116–1123. doi:10.1016/j.medengphy.2010.08.001.

Almeida, F. N., R. C. Sulabo, and H. H. Stein. 2013. Effects of a novel bacterial phytase expressed in *Aspergillus oryzae* on digestibility of calcium and phosphorus in diets fed to weanling or growing pigs. *J. Anim. Sci. Biotechnol.* **4**:8. doi:10.1186/2049-1891-4-8.

Amundson, L. A., L. L. Hernandez, and T. D. Crenshaw. 2017. Serum and tissue 25-OH vitamin D3 concentrations do not predict bone abnormalities and molecular markers of vitamin D metabolism in the hypovitaminosis D kyphotic pig model. *Br. J. Nutr.* **118**:30–40. doi:10.1017/S0007114517001751

AOAC Int. 2019. *Official methods of analysis of AOAC Int.* 21st ed. Rockville (MD): AOAC Int.

Bazydlo, L. A. L., M. Needham, and N. S. Harris. 2014. Calcium, magnesium, and phosphate. *Lab. Med.* **45**:e44–e50. doi:10.1309/lmglmz8ciymfnogx

Bolduan, G., H. Jung, E. Schnabel, and R. Schneider. 1988. Recent advances in the nutrition of weaner piglets. *Pig News Inf.* **9**:381–385.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J. Antimicrob. Chemother.* **52**:159–161. doi:10.1093/jac/dkg313.

Chesson, A. 1987. Supplementary enzymes to improve utilization of pig and poultry diets. In: W. Haresign, and D. J. A. Cole, editors, *Recent advances in animal nutrition*. London (UK): Butterworths. p. 71–89.

Cowieson, A. J., F. F. Roos, J. P. Ruckebusch, J. W. Wilson, P. Guggenbuhl, H. Lu, K. M. Ajuwon, and O. Adeola. 2017. Time-series responses of swine plasma metabolites to ingestion of diets containing myo-inositol or phytase. *Br. J. Nutr.* **118**:897–905. doi:10.1017/S0007114517003026.

Guggenbuhl, P., E. P. Calvo, and F. Fru. 2016. Effect of a bacterial 6-phytase on plasma myo-inositol concentrations and P and Ca utilization in swine. *J. Anim. Sci.* **94**(Suppl. 3):243–245. doi:10.2527/jas.2015-9803

Holloway, C. L., R. D. Boyd, D. Koehler, S. A. Gould, Q. Li, and J. F. Patience. 2019. The impact of "super-dosing" phytase in pig diets on growth performance during the nursery and grow-out periods. *Transl. Anim. Sci.* **3**:419–428. doi:10.1093/tas/txy148.

Huber, K. 2016. Cellular *myo*-inositol metabolism. In: C. L. Walk, I. Kühn, H. H. Stein, M. T. Kidd, and M. Rodehutscord, editors, *Phytate destruction—consequences for precision animal nutrition*. Wageningen (The Netherlands): Wageningen Academic Publishers. p. 53–60.

Jasaitis, D. K., J. E. Wohlt, and J. L. Evans. 1987. Influence of feed ion content on buffering capacity of ruminant feedstuffs in vitro. *J. Dairy Sci.* **70**:1391–1403. doi:10.3168/jds.S0022-0302(87)80161-3

Józefiak, D., R. M. Engberg, and A. Ptak. 2016. Relevance of gut microbiome and endogenous phosphatases on phytate breakdown in poultry – implications on phosphorus utilisation. In: C. L. Walk, I. Kühn, H. H. Stein, M. T. Kidd, and M. Rodehutscord, editors, *Phytate destruction—consequences for precision animal nutrition*. Wageningen (The Netherlands): Wageningen Academic Publishers. p. 33–43.

Kil, D. Y., W. B. Kwon, and B. G. Kim. 2011. Dietary acidifiers in weanling pig diets: a review. *Rev. Colomb. Cienc. Pecu.* **24**:231–247.

Lagos, L. V., S. A. Lee, M. R. Bedford, and H. H. Stein. 2021. Reduced concentrations of limestone and monocalcium phosphate in diets without or with microbial phytase did not influence gastric pH, fecal score, or growth performance, but reduced bone ash and serum albumin in weanling pigs. *Transl. Anim. Sci.* **5**:txab115. doi:10.1093/tas/txab115

Lagos, L. V., S. A. Lee, G. Fondevila, C. L. Walk, M. R. Murphy, J. J. Loor, and H. H. Stein. 2019. Influence of the concentration of dietary digestible calcium on growth performance, bone mineralization, plasma calcium, and abundance of genes involved in intestinal absorption of calcium in pigs from 11 to 22 kg fed diets with different concentrations of digestible phosphorus. *J. Anim. Sci. Biotechnol.* **10**:47. doi:10.1186/s40104-019-0349-2

Lawlor, P. G., P. B. Lynch, P. J. Caffrey, J. J. O'Reilly, and M. K. O'Connell. 2005. Measurements of the acid-binding capacity of ingredients used in pig diets. *Ir. Vet. J.* **58**:447–452. doi:10.1186/2046-0481-58-8-447

Lee, S. A., and M. R. Bedford. 2016. Inositol—an effective growth promotor? Worlds Poult. *Sci. J.* **72**:743–760. doi:10.1017/S0043933916000660

Lee, S. A., J. Dunne, E. Febery, P. Wilcock, T. Mottram, and M. R. Bedford. 2018. Superdosing phytase reduces real-time gastric pH in broilers and weaned piglets. *Br. Poult. Sci.* **59**:330–339. doi:10.1080/00071668.2018.1440379.

Lee, S. A., E. Febery, P. Wilcock, and M. R. Bedford. 2021. Application of creep feed and phytase super-dosing as tools to support digestive adaption and feed efficiency in piglets at weaning. *Animals*. **11**:2080. doi:10.3390/ani11072080

Létourneau-Montminy, M. P., A. Narcy, M. Magnin, D. Sauvant, J. F. Bernier, C. Pomar, and C. Jondreville. 2010. Effect of reduced dietary calcium concentration and phytase supplementation on calcium and phosphorus utilization in weanling pigs with modified mineral status. *J. Anim. Sci.* **88**:1706–1717. doi:10.2527/jas.2008-1615.

Lindemann, M. D., and B. G. Kim. 2007. Technical note: a model to estimate individual feed intake of swine in group feeding. *J. Anim. Sci.* **85**:972–975. doi:10.2527/jas.2006-412.

Liu, Y., C. D. Espinosa, J. J. Abelilla, G. A. Casas, L. V. Lagos, S. A. Lee, W. B. Kwon, J. K. Mathai, D. M. D. L. Navarro, N. W. Jaworski, *et al.* 2018. Non-antibiotic feed additives in diets for pigs: a review. *Anim. Nutr.* **4**:113–125. doi:10.1016/j.aninu.2018.01.007.

Lu, H., I. Kühn, M. R. Bedford, H. Whitfield, C. Brearley, O. Adeola, and K. M. Ajuwon. 2019. Effect of phytase on intestinal phytate breakdown, plasma inositol concentrations, and glucose transporter type 4 abundance in muscle membranes of weanling pigs. *J. Anim. Sci.* **97**:3907–3919. doi:10.1093/jas/skz234

Mesina, V. G. R., L. V. Lagos, R. C. Sulabo, C. L. Walk, and H. H. Stein. 2019. Effects of microbial phytase on mucin synthesis, gastric protein hydrolysis, and degradation of phytate along the gastrointestinal tract of growing pigs. *J. Anim. Sci.* **97**:756–767. doi:10.1093/jas/sky439.

Moran, K., R. D. Boyd, C. Zier-Rush, P. Wilcock, N. Bajjalieh, and E. van Heugten. 2017. Effects of high inclusion of soybean meal and a phytase superdose on growth performance of weaned pigs housed under the rigors of commercial conditions. *J. Anim. Sci.* **95**:5455–5465. doi:10.2527/jas2017.1789.

Moran, K., P. Wilcock, A. Elsbernd, C. Zier-Rush, R. D. Boyd, and E. van Heugten. 2019. Effects of super-dosing phytase and inositol on growth performance and blood metabolites of weaned pigs housed under commercial conditions. *J. Anim. Sci.* **97**:3007–3015. doi:10.1093/jas/skz156

Murphy, M. R. 1982. Analyzing and presenting pH data. *J. Dairy Sci.* **65**:161–163. doi:10.3168/jds.S0022-0302(82)82165-6

NRC. 2012. *Nutrient requirements of swine*. 11th rev. ed. Washington, D.C.: Natl. Acad. Press.

Pluske, J. R., D. J. Hampson, and I. H. Williams. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livest. Prod. Sci.* **51**:215–236. doi:10.1016/S0301-6226(97)00057-2

Radcliffe, J. S., Z. Zhang, and E. T. Kornegay. 1998. The effects of microbial phytase, citric acid, and their interaction in a corn-soybean meal-based diet for weanling pigs. *J. Anim. Sci.* **76**:1880–1886. doi:10.2527/1998.7671880x.

Rice, J. P., R. S. Pleasant, and J. S. Radcliffe. 2002. The effect of citric acid, phytase, and their interaction on gastric pH, and Ca, P, and dry matter digestibilities. In: *Purdue University Swine Research Report*. West Lafayette (IN): Purdue University. p. 36–42.

Selle, P. H., A. J. Cowieson, and V. Ravindran. 2009. Consequences of calcium interactions with phytate and phytase for poultry and pigs. *Livest. Sci.* **124**:126–141. doi:10.1016/j.livsci.2009.01.006

Sommerfeld, V., M. Schollenberger, L. Hemberle, and M. Rodehutscord. 2017. Modification and application of an in vitro assay to examine inositol phosphate degradation in the digestive tract of poultry. *J. Sci. Food Agric.* **97**:4219–4226. doi:10.1002/jsfa.8297.

Suiryanrayna, M. V., and J. V. Ramana. 2015. A review of the effects of dietary organic acids fed to swine. *J. Anim. Sci. Biotechnol.* **6**:45. doi:10.1186/s40104-015-0042-z.

Tamim, N. M., R. Angel, and M. Christman. 2004. Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. *Poult. Sci.* **83**:1358–1367. doi:10.1093/ps/83.8.1358.

Tran, G., and D. Sauvant. 2004. Chemical data and nutritional value. In: D. Sauvant, J.-M. Perez, and G. Tran, editors, *Tables of composition and nutritional value of feed materials: pigs, poultry, cattle, sheep, goats, rabbits, horses and fish*. Wageningen (The Netherlands): Wageningen Academic Publishers. p. 17–24.

US-EPA. 2000. *Acid digestion of sediments, sludges, and soils*. U.S. Environmental Protection Agency. https://www.epa.gov/sites/production/files/2015-12/documents/3050b.pdf Accessed March, 2021.

Walk, C. L., M. R. Bedford, and A. P. McElroy. 2012. Influence of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. *Poult. Sci.* **91**:1371–1378. doi:10.3382/ps.2011-01928

Walk, C. L., M. R. Bedford, and O. A. Olukosi. 2018. Effect of phytase on growth performance, phytate degradation and gene expression of myo-inositol transporters in the small

intestine, liver and kidney of 21 day old broilers. *Poult. Sci.* **97**:1155–1162. doi:10.3382/ps/pex392

Zeng, Z. K., Q. Y. Li, P. F. Zhao, X. Xu, Q. Y. Tian, H. L. Wang, L. Pan, S. Yu, and X. S. Piao. 2016. A new phytase continuously hydrolyzes phytate and improves amino acid digestibility and mineral balance in growing pigs fed phosphorous-deficient diet. *J. Anim. Sci.* **94**:629–638. doi:10.2527/jas.2015-9143

Zhang, J. M., and J. An. 2007. Cytokines, inflammation, and pain. *Int. Anesthesiol. Clin.* **45**:27–37. doi:10.1097/AIA.0b013e318034194e

Zhao, J., Z. Zhang, S. Zhang, G. Page, and N. W. Jaworski. 2021. The role of lactose in weanling pig nutrition: a literature and meta-analysis review. *J. Anim. Sci. Biotechnol.* **12**:10. doi:10.1186/s40104-020-00522-6