Effect of phytase and limestone particle size on mineral digestibility, performance, eggshell quality, and bone mineralization in laying hens

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ABSTRACT The effect of microbial phytase and limestone particle size (**LmPS**) was assessed in Lohmann Tradition laying hens from 31 to 35 wk of age. Seventy-two hens were used in a completely randomized trial according to a 2 × 2 factorial arrangement with 2 levels of phytase/basal available P (***a*P**); 0 FTU/kg with 0.30% *a*P or 300 FTU/kg with 0.15% *a*P, and 2 limestone particle sizes; fine particles (**FL**, <0.5 mm) or a mix (**MIX**) of 75% coarse limestone (**CL**, 2−4 mm) and 25% FL. Diets contained equivalent levels of Ca (3.5%), phytic P (**PP**; 0.18%), and *a*P (0.30%) considering the P equivalency of phytase. Thus, dietary treatments were **FL0** and **MIX0** without phytase, and **FL300** and **MIX300** with 300 FTU/kg phytase. Performance were recorded daily and eggshell quality (eggshell weight proportion, weight, thickness, and breaking strength) was measured weekly. At the end of the trial, bone parameters (tibia breaking strength, elasticity, and ash) and the apparent precaecal digestibility (**APCD**) of P and Ca were determined. No differences were observed between treatments in feed intake, FCR and bone parameters. Addition of MIX increased the eggshell proportion, weight and thickness in groups receiving no phytase (+6.5, +6.9, and +4.5%, respectively) while no effect was observed in groups receiving phytase (Phytase × LmPS, $P < 0.05$). In hens receiving FL, the APCD of P was lower in diets supplemented with phytase (−14 percentage points; Phytase × LmPS, $P < 0.001$). A higher phytate disappearance was observed in hens fed diets with phytase in combination with MIX (Phytase × LmPS, $P = 0.005$). Phytase and MIX together increased the APCD of Ca by 7.3 percentage points (Phytase × LmPS, $P < 0.001$). In conclusion, addition of CL could limit the formation of Ca-phytate complex thus improving the response of the birds to phytase compared to FL.

Key words: calcium, phosphorus, coarse particles, solubilization

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INTRODUCTION

Around 60 to 70% of seed phosphorus (**P**) is complexed to myo-inositol to form phytic acid, which drastically reduces its bioavailability to the animal (Abbasi et al., 2019). Thus, phosphates are added to the diet to meet the daily requirements of poultry. The use of phytase enhances the bioavailability of P from phytic acid, and reduces P excretion (Selle and Ravindran, 2007). The positive effects of phytase on digestive performance, especially on precaecal digestibility of P are well known in broilers (Bournazel et al., 2018; Babatunde and Adeola, 2020; Ravindran et al., 2020). However, less studies measuring P digestibility at the precaecal level have been performed in laying hens (van der Klis et al., 1997; Javadi et al., 2021).

Another nutritional concern in laying hens is the high requirement for Ca occurring during the night to produce the eggshell. The diet provides approximately 60% of the Ca required (Nys, 2017), however, because of a desynchronization between dietary Ca availability during the light period and the high Ca requirement to produce the eggshell during the dark period (Nys and Leroy, 2018), additional Ca is mobilized from medullary bone (Kim et al., 2012). To reduce the gap between the supply and the demand for Ca, coarse limestone (**CL**) is commonly added to the diet (Molnár et al., 2018). Compared to fine limestone (**FL**), CL is retained for longer in the gizzard (Rao et al., 1992). The acidic content of the gizzard and a longer retention time of CL in this organ allow a slower solubilization of Ca and provide a longer diffusion of available Ca in the duodenum during the night, reducing bone Ca mobilization (Fleming, 2008). Moreover, CL has been shown to increase eggshell

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quality, without influencing production performance parameters (Hervo et al., 2022).

Phytic acid has the capacity to chelate some divalent cations to form phytic acid salts: phytate (Maenz et al., 1999; Angel et al., 2002). Due to the high Ca concentration in laying hen's feed, phytic acid can easily bind Ca to form unavailable complexes. This process depends on pH (Grynspan and Cheryan, 1983) and Ca-to-phytic acid ratio (Wise, 1983). In broilers, FL shows a higher capacity than CL to form insoluble complexes with phytate, thus reducing P digestibility, growth performance and bone mineralization (Manangi and Coon, 2007; Bradbury et al., 2016; Majeed et al., 2020). However, no data are available in laying hens. The objective of the current study was to assess the effect of microbial phytase, limestone particle size and their interaction on mineral digestibility in laying hens. The hypothesis was that FL could form insoluble complexes with phytic acid thus reducing phytase efficiency and P digestibility.

MATERIALS AND METHODS

The experiment was conducted under the guidelines of the French Ministry of Agriculture for Animal Research at the Experimental Poultry Unit of Tours, INRAE, Nouzilly, France (PEAT INRAE 1295, doi.org/10.15454/1.5572326250887292E12). The experimental design was approved by the Regional Ethics Committee on Animal Experimentation (Tours, France) and the French Ministry of Higher Education and Research (Paris, France; authorization: APAFIS #32000-202 1061008462021 v3).

Animal Housing and Management

Seventy-two laying hens (Lohmann Tradition, Lohmann, Saint-Fulgent, France) were housed in groups of 3 hens per cage from 18 to 25 wk of age. During this period, animals were fed according to breeder recommendations (Lohmann Breeders, 2021) to cover all nutritional requirements, that is, 3.4% Ca and 0.34% available P (***a*P**). At 26 wk of age, hens were transferred to individual cages for a 5-wk adaptation period. This was followed by a 4-wk experimental period from 31 to 35 wk of age. Temperature was maintained between 18 and 20°C with 16 h of light per day (from 6 am to 10 pm). On each day of the 4-wk experimental period, eggs were collected and weighed to obtain egg production, egg weight and to calculate egg mass. Cumulative weekly refusals were weighed to determine the average daily feed intake (**ADFI**) and feed conversion ratio (**FCR**). Animals were weighed at the beginning and end of the experiment.

Experimental Treatments and Diets

Diets contained corn and wheat and were offered as mash form. Experimental diets were formulated to meet all nutrient requirements, with 3.5% Ca and 0.34% *a*P, according to breeder recommendations (Lohmann Breeders, 2021) (Table 1). At 31 wk of age, 72 laying hens were assigned to 1 of the 4 experimental treatments, with 18 replicates per treatment. A 2 × 2 factorial arrangement was used with 2 limestone particle sizes (**LmPS**), 100% fine limestone (**FL**) or a mix (**MIX**) of FL (25%) and coarse limestone (**CL**, 75%), and 2 levels of phytase, 0 FTU/kg or 300 FTU/kg. The mean diameter was below 0.5 mm for FL and between 2 and 4 mm for CL. Since the origin of limestone is known to influence the solubilization of Ca in the digestive tract of poultry (Kim et al., 2019), the same source of CL was used and CL particles were ground to obtain FL.

Table 1. Ingredients, calculated, and analyzed nutrient levels, and phytase activities of experimental diets.[1]

	0 FTU		300 FTU	
Ingredients (g/kg)	FL	MIX	FL	MIX
Corn	532	532	537	537
Wheat	100	100	100	100
Soybean meal (46% CP)	240	240	240	240
Soybean oil	20	20	18.4	18.4
Corn gluten meal	5.3	5.3	5.3	5.3
Fine limestone[2]	83.7	21	87.5	22
Coarse limestone[3]	0	62.7	0	65.5
Monocalcium phosphate[4]	10.3	10.3	3.1	3.1
Phytase[5]	0	0	0.0091	0.0091
NaCl	3	3	3	3
Layer premix[6]	4	4	4	4
DL-Methionine	1.7	1.7	1.7	1.7
Nutrient composition (%)[7]				
Calculated values[8]				
DM	89	89	89	89
ME (kcal/kg)	2,749	2,749	2,751	2,751
CP	17.1	17.1	17.1	17.1
Dig. Lys	0,73	0,73	0,73	0,73
Dig. Met	0,25	0,25	0,25	0,25
Ca	3.50	3.50	3.50	3.50
*t*P	0.56	0.56	0.39	0.39
NPP	0.34	0.34	0.18	0.18
PP	0.22	0.22	0.16	0.16
*a*P	0.30	0.30	0.30	0.30
Analyzed values				
DM	89.7	89.7	89.9	89.9
CP	17.3	17.8	17.8	17.7
Ca	3.52	3.68	3.58	3.49
*t*P	0.55	0.52	0.38	0.39
PP	0.20	0.16	0.19	0.19
Phytase activity (FTU/kg)	85	45	373	293

[1]FL, fine limestone (<0.5 mm); MIX, mix of 25% fine limestone and 75% coarse limestone (2−4 mm); 0, diet without phytase and 0.34% NPP; 300, diet with 300 FTU/kg phytase and 0.18% NPP

[2]Fine limestone was <0.5 mm (geometric mean diameter = 0.21 ± 0.17 mm) and was obtained by grinding coarse limestone particles.

[3]Coarse limestone was between 2 and 4 mm (geometric mean diameter = 2.76 ± 0.13 mm). Calcium content in limestone was 38% Ca, according to producer.

[4]Monocalcium phosphate contained 17.0% Ca, and 22.7% P, according to producer.

[5]Phytase was a novel biosynthetic bacterial 6-phytase produced by *Trichoderma reesei* (Rovabio PhyPlus, Adisseo France SAS, Commentry, France).

[6]Layer premix contains the following, expressed per kilogram: vitamin A = 2,500,000 UI; vitamin D3 = 860,000 UI; vitamin E = 20,000 UI; vitamin K3 = 1,000 mg; vitamin B1 = 1,000 mg; vitamin B6 = 1,600 mg; pantothenic acid = 5,000 mg; vitamin B6 = 1,400 mg; vitamin B12 = 5.2 mg; vitamin PP = 20,000 mg; folic acid = 600 mg; biotin = 60 mg; Cu = 3,200 mg; Fe = 10,000 mg; Zn = 14,000 mg; Mn = 16,000 mg; I = 400 mg; Se = 40 mg.

[7]DM, dry matter; ME, metabolizable energy; CP, crude protein; *t*P, total P; NPP, nonphytate P; *a*P, available P; PP, phytic P.

[8]Calculated values of nutrients based on INRA-AFZ tables (2017).

Microbial phytase (a novel biosynthetic bacterial 6-phytase produced by *Trichoderma reesei*, Adisseo France SAS, Commentry, France) was added to 2 diets at 300 FTU/kg to provide 0.15% *a*P (only a matrix value for *a*P was considered). To summarize, **FL0** contained fine limestone without phytase; **MIX0** contained coarse and fine limestone without phytase; **FL300** contained fine limestone with 300 FTU/kg phytase; and **MIX300** contained coarse and fine limestone with 300 FTU/kg phytase. Feed and water were provided ad libitum except for the last day of the experiment. For the last 10 d of the experiment, hens received a diet supplemented with titanium dioxide (**Ti**) at 5.0 g/kg to determine the apparent precaecal digestibility (**APCD**) of P and Ca.

Sample Collection and Analysis

Limestone Particle Size Distribution. The particle size distribution of limestone was determined by sifting the limestone through a series of sieves. Sieve sizes were according to the US standard mesh chart as follows: #100 (150 μm), #50 (300 μm), #30 (600 μm), #12 (1,700 μm), #10 (2,000 μm), #8 (2,360 μm), and #6 (3,350 μm). The geometric mean diameter (mm) and the geometric standard deviation (mm) were calculated for CL, FL, and mixed limestones, according to the method described by Wilcox et al. (1970). Limestone contained 38% Ca according to the manufacturer.

Eggshell Quality

Once per week, eggs were collected to evaluate eggshell thickness and breaking strength using a DET 6000 (Nabel Co., Kyoto, Japan). Shells were then cleaned and dried at 110°C for 2 h. Dried eggshells were weighed, and the eggshell proportion (%) was calculated.

Feed and Digesta Analysis

At 35 wk of age, all birds were euthanized by injection of sodium pentobarbital at the wing-vein. To synchronize hens, they were fasted from 10 pm the previous evening to the time of laying on the day of sampling. From the moment of laying (t lay = t0), hens were fed and euthanized 4 h later. As eggshell formation had not started, hens were considered to be in a steady state. The digesta of the distal half of the ileum were collected. Digesta were frozen at −20°C, freeze-dried for 72 h, and stored in order to determine the APCD of P and Ca and the PP disappearance. Diets and digesta of the distal ileum were analyzed to determine the Ca, *t*P, and Ti concentration using inductively coupled plasma optical emission spectrometry (ICP OES Thermo Scientific iCAP 7200, Courtaboeuf, France; method 990.08; AOAC International, 2006). Phytate concentration was determined in diets and digesta using a kit based on the modification of the ammonium molybdate method (Phytic Acid Assay Kit; NEOGEN Europe Ltd., Auchincruive, Scotland). Diets were analyzed for phytase activity (Adisseo France SAS, Commentry, France) and for dry matter and crude protein content by near-infrared spectroscopy, with a Büchi NIRFlex N-500 spectrometer (Büchi SARL, Vellibon-sur-Yvette, France).

Bone Parameters

The right tibia was collected for measurement of breaking strength (N) and elasticity (mm) with the 3-point destructive bending test using an Instron compression machine (Model 4411, Instron Corp., Canton, MA) loaded with a 200-N load cell. A 6-cm distance between the 2 fixed points supporting the tibia and a crosshead speed of 5.0 mm/min were held constant throughout each measurement. Tibia ash content was determined on dried defatted bones according to the method described by the Association of Official Analytical Chemists (AOAC, 1990). Briefly, soft tissues were gently removed before bones were dried at 100°C for 24 h. Tibias were defatted by immersion in a diethyl-ether solution for 24 h before ashing at 600°C overnight.

Calculations and Statistical Analysis

The apparent precaecal digestibility of P and Ca was determined using Ti as an indigestible marker. The same method was applied to determine the phytic P (**PP**) disappearance at the ileum level. The apparent precaecal digestibility and the PP disappearance were calculated as follows:

$$\text{APCD (\%)} = \left[1 - \left(\frac{\text{Ti}_{\text{feed}}}{\text{Ti}_{\text{digesta}}} \times \frac{\text{mineral}_{\text{digesta}}}{\text{mineral}_{\text{feed}}}\right)\right] \times 100$$

where $\text{Ti}_{\text{digesta}}$ and $\text{mineral}_{\text{digesta}}$ are the Ti and mineral content (P, Ca, or PP; g/kg) in the freeze-dried digesta, respectively, and Ti_{feed} and $\text{mineral}_{\text{feed}}$ are the Ti and mineral content (P, Ca, or PP; g/kg) in the feed, respectively.

A randomized block design was used (with a total of 18 blocks of 4 cages) with a 2 × 2 factorial arrangement: 2 limestone particle size and 2 levels of phytase resulting in 4 treatments, each with 18 replicates. Statistical analysis was performed using R open-source software version 3.02 (R Foundation for Statistical Computing, Vienna, Austria). All data were tested for normality according to a Shapiro test. Analysis of variance was conducted to determine the effect of phytase, limestone particle size, and their interaction. For each dependent variable, the experimental unit was the individual. The block effect of the experimental design, representing the position of the cage into the building and considering the potential variability due to the environment, was considered as a fixed effect. For egg weight and bone parameters body weight was also introduced as a covariable. Tukey's range test was used for multiple means comparisons when $P < 0.05$. As egg production did not follow normality, a non-parametric Kruskal-Wallis test was performed to

Table 2. Proportion of particle size distribution for fine limestone, coarse limestone, and mixed limestone (75% coarse and 25% fine).

Sieve diameter (mm)	FL[1]	CL[2]	MIX[3]
>3.35	0	29.5	22.3
>2.36−3.35	0	34.5	23.9
>2.00−2.36	0	22.6	18.2
>1.70−2.00	0	13.2	10.2
>0.60−1.70	2.3	0.2	0.8
>0.30−0.60	26.3	0	5.8
>0.15−0.30	30.8	0	7.9
<0.10	40.6	0	10.9
Geometric mean diameter (mm)	0.21	2.76	1.36
Geometric standard deviation (mm)	0.17	0.13	0.32

[1]FL, fine limestone.
[2]CL, coarse limestone.
[3]MIX, mixed limestone particles with 75% CL and 25% FL.

determine the effect of phytase, limestone particle size, and their interaction. A Dunn test with a Bonferroni correction for the P value was used for multiple means comparisons. Statistical significance was considered when $P < 0.05$, and a trend was reported when $0.05 \leq P < 0.10$.

RESULTS

Particle Size Distribution and Phytase Activity

As expected, fine limestone was characterized by a main proportion of particles smaller than or equal to 0.30 mm (71%) and coarse limestone showed a high proportion of particles larger than 2.36 mm (64%; Table 2). Thus, the geometric mean diameter of FL and CL was 0.21 ± 0.17 and 2.76 ± 0.13 mm, respectively. It is noteworthy that in the MIX, due to the combination of 75% CL and 25% FL, the geometric mean diameter of limestone was 1.36 ± 0.32 mm. Phytase activity in adequate P level diets was 85 and 45 FTU/kg for FL0 and MIX0, respectively. The analyzed phytase activity of FL300 and MIX300 was 373 and 293 FTU/kg, respectively (Table 1).

Production Performance

There was no mortality during the experimental period. No significant effect was observed on ADFI, body weight, or egg production (Table 3). Diets with FL increased FCR by 3.2% compared with MIX ($P < 0.05$). Phytase increased egg weight by 3.7% in diets containing FL but had no effect in MIX diets (Phytase × LmPS, $P < 0.05$).

Eggshell Quality and Bone Mineralization

The addition MIX increased the eggshell proportion ($P < 0.001$), weight ($P = 0.005$) and thickness ($P = 0.003$) in diets without phytase (respectively, 6.5, 6.9, and 4.5%; Phytase × LmPS; Table 4). Phytase incorporation increased eggshell breaking strength in a lower extent in hens fed MIX than in hens fed FL (Phytase × LmPS, $P = 0.002$): +3.2 and +6.5%, respectively. No significant effect was observed on the tibia elasticity, ash weight, ash relative content or breaking strength (Table 5). However, tibia breaking strength of hens fed with phytase tended to be lower compared with hens fed without phytase ($P = 0.082$).

Apparent Precaecal Digestibility

As expected, laying hens receiving exogenous phytase ingested significantly less total P than others (−25%, $P < 0.001$; Table 6). No effect of dietary treatments was observed on Ca intake. Incorporation of MIX increased the APCD of Ca in a greater extent in hens fed phytase

Table 3. Effect of phytase and limestone particle size on average daily feed intake, feed conversion ratio, egg production, egg weight, and egg mass.[1]

Treatment[4]	ADFI[2] (g/d)	FCR[3]	Final body weight (g)	Egg production (%)	Egg weight (g)
FL0	101.9 ± 1.05	1.64 ± 0.019	1,785 ± 49.6	94.1 ± 8.6	62.8 ± 0.46[b]
MIX0	101.8 ± 0.91	1.57 ± 0.021	1,796 ± 43.4	95.4 ± 7.5	64.0 ± 0.55[ab]
FL300	103.8 ± 0.88	1.60 ± 0.022	1,780 ± 39.3	96.1 ± 7.3	65.1 ± 0.54[a]
MIX300	101.3 ± 1.09	1.57 ± 0.021	1,882 ± 57.7	93.6 ± 8.9	63.9 ± 0.41[ab]
Phytase					
0	101.8 ± 0.69	1.60 ± 0.015	1,790 ± 32.6	94.7 ± 8.1	63.4 ± 0.36[b]
300	101.9 ± 0.70	1.58 ± 0.015	1,835 ± 36.7	94.8 ± 8.4	64.5 ± 0.34[a]
LmPS[5]					
FL	102.8 ± 0.69	1.62 ± 0.015[a]	1,782 ± 31.9	95.1 ± 8.0	63.9 ± 036
MIX	101.2 ± 0.71	1.57 ± 0.015[b]	1,840 ± 36.6	94.5 ± 8.5	63.9 ± 0.34
Source of variation	P value				
Phytase	0.976	0.332	0.356	0.276	0.015
LmPS	0.179	0.023	0.235	0.151	0.888
Phytase × LmPS	0.275	0.077	0.392	0.231	0.021

[1]Mean values of 18 replicates of 1 hen once per week during 4 wk for each treatment ± standard error of the mean (SEM).
[2]ADFI, average daily feed intake.
[3]FCR, feed conversion ratio.
[4]FL, fine limestone (<0.5 mm); MIX, mix of 25% fine limestone and 75% coarse limestone (2−4 mm); 0, diet without phytase and 0.34% NPP; 300, diet with 300 FTU/kg phytase and 0.18% NPP.
[5]LmPS, limestone particle size.
[a−b]Means within a column with different superscripts differ significantly ($P < 0.05$), based on Tukey's HSD means separation. Except for egg production, where different superscripts differ significantly ($P < 0.05$), based on Dunn test (with a Bonferroni correction for the P value) means separation.

Table 4. Effect of phytase and limestone particle size on eggshell proportion, eggshell weight, eggshell thickness, and eggshell breaking strength.[1]

Treatment[2]	Eggshell proportion (%)	Eggshell weight (g)	Eggshell thickness (mm)	Eggshell breaking strength (kg)
FL0	9.18 ± 0.086[b]	5.72 ± 0.055[b]	0.357 ± 0.0030[b]	4.72 ± 0.099[b]
MIX0	9.78 ± 0.088[a]	6.12 ± 0.073[a]	0.373 ± 0.0033[a]	5.07 ± 0.100[ab]
FL300	9.47 ± 0.087[bc]	6.13 ± 0.057[a]	0.366 ± 0.0029[ab]	5.03 ± 0.105[ab]
MIX300	9.54 ± 0.064[ab]	6.09 ± 0.045[a]	0.365 ± 0.0021[ab]	5.23 ± 0.077[a]
Phytase				
0	9.50 ± 0.067	5.92 ± 0.050[b]	0.366 ± 0.0024	4.89 ± 0.072[b]
300	9.49 ± 0.051	6.10 ± 0.036[a]	0.366 ± 0.0017	5.15 ± 0.063[a]
LmPS[3]				
FL	9.34 ± 0.063[b]	5.94 ± 0.044[b]	0.362 ± 0.0021[b]	4.89 ± 0.074[b]
MIX	9.67 ± 0.053[a]	6.11 ± 0.039[a]	0.369 ± 0.0018[a]	5.17 ± 0.061[a]
Source of variation			*P* value	
Phytase	0.960	<0.001	0.884	0.005
LmPS	<0.001	0.002	0.025	0.006
Phytase × LmPS	<0.001	0.005	0.003	0.002

[1]Mean values of 18 replicates of 1 hen once per week during 4 wk for each treatment ± standard error of the mean (SEM).
[2]FL, fine limestone (<0.5 mm); MIX, mix of 25% fine limestone and 75% coarse limestone (2−4 mm); 0, diet without phytase and 0.34% NPP; 300, diet with 300 FTU/kg phytase and 0.18% NPP.
[3]LmPS, limestone particle size.
[a−b]Means within a column with different superscripts differ significantly ($P < 0.05$), based on Tukey's HSD means separation.

Table 5. Effect of phytase and limestone particle size on tibia breaking strength, tibia elasticity, tibia ash, and tibia ash weight.[1]

Treatment[2]	Tibia breaking strength (N)	Elasticity (mm)	Tibia ash (%)	Ash weight (g)
FL0	94.1 ± 3.53	1.73 ± 0.066	49.8 ± 0.49	2.95 ± 0.073
MIX0	91.8 ± 3.02	1.73 ± 0.057	49.6 ± 0.54	2.77 ± 0.052
FL300	88.1 ± 2.78	1.74 ± 0.066	49.2 ± 0.66	2.77 ± 0.063
MIX300	87.9 ± 2.76	1.61 ± 0.030	49.4 ± 0.41	2.82 ± 0.063
Phytase				
0	92.7 ± 2.63	1.73 ± 0.043	49.7 ± 0.35	2.87 ± 0.046
300	88.0 ± 1.92	1.68 ± 0.039	49.3 ± 0.38	2.79 ± 0.044
LmPS[3]				
FL	90.8 ± 2.51	1.73 ± 0.046	49.5 ± 0.41	2.86 ± 0.050
MIX	90.1 ± 2.06	1.67 ± 0.034	49.5 ± 0.33	2.79 ± 0.041
Source of variation		*P* value		
Phytase	0.082	0.354	0.113	0.207
LmPS	0.785	0.241	0.443	0.201
Phytase × LmPS	0.339	0.300	0.197	0.277

[1]Mean values of 18 replicates of 1 hen for each treatment ± standard error of the mean (SEM).
[2]FL, fine limestone (<0.5 mm); MIX, mix of 25% fine limestone and 75% coarse limestone (2−4 mm); 0, diet without phytase and 0.34% NPP; 300, diet with 300 FTU/kg phytase and 0.18% NPP.
[3]LmPS, limestone particle size.

Table 6. Effect of phytase and limestone particle size on P and Ca intake, apparent precaecal digestibility of P and Ca, and digestible P and Ca of feed.

	Mineral intake (g/d)		APCD (%)[3]		
Treatment[5]	Ca[2]	P[1]	Ca	P	PP disappearance (%)[4]
FL0	3.71 ± 0.09	0.55 ± 0.012	75.6 ± 1.89[b]	48.6 ± 2.32[a]	49.58 ± 9.47[b]
MIX0	3.87 ± 0.13	0.53 ± 0.021	80.9 ± 2.21[ab]	45.6 ± 2.93[a]	50.57 ± 5.12[b]
FL300	3.71 ± 0.09	0.41 ± 0.010	75.9 ± 1.87[b]	34.9 ± 2.62[b]	59.72 ± 8.93[b]
MIX300	3.71 ± 0.10	0.41 ± 0.013	82. 9 ± 2.19[a]	41.5 ± 2.84[ab]	80.64 ± 8.37[a]
Phytase					
0	3.78 ± 0.066	0.55 ± 0.012[a]	78.2 ± 1.56	47.3 ± 1.86[a]	50.04 ± 5.53[b]
300	3.71 ± 0.082	0.41 ± 0.008[b]	78.9 ± 1.62	39.1 ± 1.98[b]	71.14 ± 6.38[a]
LmPS[6]					
FL	3.71 ± 0.079	0.47 ± 0.016	74.5 ± 1.32[b]	41.4 ± 2.09	53.98 ± 6.55
MIX	3.78 ± 0.070	0.53 ± 0.017	80.3 ± 1.54[a]	43.7 ± 2.05	66.26 ± 5.85
Source of variation			*P* value		
Phytase	0.317	<0.001	0.735	<0.001	0.004
LmPS	0.342	0.945	<0.001	0.341	0.085
Phytase × LmPS	0.329	0.491	0.002	<0.001	0.005

[1]Mean values of 17, 17, 16, and 14 replicates of 1 hen for treatment FL0, MIX0, FL300, and MIX300, respectively ± standard error of the mean (SEM).
[2]Mean values of 14, 15, 17, and 14 replicates of 1 hen for treatment FL0, MIX0, FL300, and MIX300, respectively ± standard error of the mean (SEM).
[3]APCD, apparent precaecal digestibility.
[4]Mean values of 7, 6, 5, and 6 replicates of 1 hen for the treatment FL0, MIX0, FL300, and MIX300, respectively ± standard error of the mean (SEM).
[5]FL, fine limestone (<0.5 mm); MIX, mix of 25% fine limestone and 75% coarse limestone (2−4 mm); 0, diet without phytase and 0.34% NPP; 300, diet with 300 FTU/kg phytase and 0.18% NPP.
[6]LmPS, limestone particle size.
[a−b]Means within a column with different superscripts differ significantly ($P < 0.05$) based on Tukey's HSD means separation.

compared to hens fed without phytase: +9.5 and 7.0%, respectively (Phytase × LmPS, $P = 0.002$; Table 6). The P APCD of hens was decreased with phytase addition with a higher magnitude in hens receiving FL than MIX diets (−28 and −9%; Phytase × LmPS, $P < 0.001$; Table 6). The incorporation of microbial phytase increased PP disappearance by 30 compared with 10 percentage points in hens fed MIX and FL diets, respectively (Phytase × LmPS, $P = 0.005$; Table 6).

DISCUSSION

Only a few studies have been performed regarding the effect of phytase on the APCD of P and Ca in laying hens. Moreover, there is no information on the effect of limestone particle size together with phytase on these parameters in laying hens; only data in broilers are available. As a result, part of this discussion is based on data issued from broiler trials. Phytase incorporation is known to increase APCD of P in the laying hen (van der Klis et al., 1997; Keshavarz, 2000). For instance, Bello and Korver (2019) showed that 32-wk-old hens fed 0.18% NPP with 300 FTU/kg exhibited an 18% higher APCD of P than hens fed with 0.38% NPP without phytase. Additionally, Pongmanee et al. (2020) showed no differences in APCD of P between 37-wk-old laying hens fed with 0.35% PNP without phytase or with 0.16% NPP with 300 FTU/kg, in accordance with the present results. The discrepancies between authors concerning phytase effect may be related to age of the laying hen (Bello and Korver, 2019) or to the length of the experimental period (Javadi et al., 2021). In the current experiment, it is noteworthy than hens fed FL exhibited a lower APCD of P in the diet formulated with phytase compared to other groups ($P < 0.001$; Table 6). Calcium can form insoluble complexes with phytate when the pH is above 5.0 (Nolan and Duffin, 1987), and this pH condition is found in the crop of the laying hen (Denbow, 2015; Classen et al., 2016). These complexes limit mineral solubilization, which in turn limits the capacity of phytase to liberate P from phytate (Selle et al., 2009). Consequently, the formation of Ca−phytate complex could reduce the APCD of P (Tamim et al., 2004). Results of the current experiment support this hypothesis: the positive impact of microbial phytase on PP disappearance was more important when using the mix of CL and FL compared to FL alone. The formation of the Ca−phytate complex is greatly influenced by limestone particle size in broilers: Manangi and Coon (2007) have shown that FL facilitates the formation of these complexes. In an in vitro assay, at pH = 6.5 and after 30 min, FL (0.03 mm) reduced PP degradation by phytase by 15% compared to CL (1.31 mm). Fine limestone is highly soluble compared to CL and rapidly provides a high quantity of soluble Ca in the gizzard (Kim et al., 2019). This soluble Ca can easily bind phytate and also decrease the APCD of P and Ca (Li et al., 2021). For example, the same authors found that, in broilers, the APCD of P was decreased from 87 to 61% in diets supplemented with phytase when limestone particle size decreased from 0.80 mm to 0.15 mm. To reinforce the significance of limestone particle size in Ca−phytate complex formation, an increase in dietary Ca in broilers did not affect the APCD of P when limestone was supplied as CL but decreases it by 16% with FL (Kim et al., 2018). Phytase inclusion allows to reduce the quantity of nonabsorbed P at the precaecal level (by 16% in the current study) decreasing in turn the P excretion (Lim et al., 2003; Abudabos, 2011). For instance, Martinez Rojas et al. (2018) showed that the use of phytase in a low P diet allows a reduction of the total amount of P present in the excreta by approximately 24% compared to a control diet receiving an adequate P level.

The level of APCD of Ca observed in this study was high (around 79%). This could be partly explained by the experimental design: laying hens were fasted from the evening (10 pm) until they laid the next day (8:30 am in average) to favor feed ingestion and ensure the presence of content in the ileal compartment. After laying, Ca and P are required to finalize medullary bone replenishment (Etches, 1987). The Ca deposition into medullary bone occurs mainly during the first 6 h after laying (Hurwitz, 1964; Clunies et al., 1993). Fasting may have induced hypocalcaemia and enhanced intestinal Ca absorption by the 1,25-OH_2-D_3 pathway (van der Klis et al., 1990; Bar, 2008) in order to meet the mineral demand. To reinforce this hypothesis, observed APCD of P was quite high when comparing to data of Keshavarz (2000) with values of 29 and 33% in hens fed 0.30% NPP without or with 300 FTU/kg, respectively. Thus, it cannot be excluded that Ca and P absorption has been increased in the current experiment due to physiological regulations. CL increased the APCD of Ca in a greater extent in hens fed diets with phytase. By favoring P absorption in phytase supplemented diets, CL decreases the propensity of Ca to complex phosphate (Hurwitz and Bar, 1965) and phytate (Selle et al., 2009) in the small intestine. Consequently, Ca would be more available for absorption.

In laying hens, P is an essential mineral involved in many functions such as bone development, energy metabolism, cell membrane structure and egg formation (Suttle, 2010). As mentioned earlier, during eggshell formation, bone resorption is stimulated to provide sufficient Ca (Dacke et al., 1993). Through this process, P is also released and excreted in the urine (Nys and Leroy, 2018). Eggshell production consequently increases the P requirement more than predicted by the small amount of P in the egg (~120 mg). In commercial feeds, microbial phytase is commonly used to save phosphate and limit P excretion (Humer et al., 2015). In the current study, 300 FTU/kg of phytase were added, saving 7.2 g of phosphate per kg of feed with a replacement of 1.5 g *a*P without affecting feed intake or growth performance. These results corroborate those of Pongmanee et al. (2020), who reported an equivalency of 1.9 g *a*P in terms of ADFI and FCR for 300 FTU of phytase per kg in a diet containing 1.9 g *a*P in 37-wk-old laying hens.

It is worth noting that hens were individualized during the experimental period which was relatively short compared to practical conditions. So, the current results are probably not completely representative of commercial flocks. Egg production was maintained in the current experiment. A low dietary nonphytic P (**NPP**) level is known to decrease egg production (Teng et al., 2020). In their meta-analysis, Ahmadi and Rodehutscord (2012) showed that the effect of phytase on egg production is greater with low levels of NPP. Based on their results, 300 FTU of microbial phytase per kg feed containing 1.7 g NPP/kg did not significantly decrease the production performance of laying hens compared to a diet formulated with 3.4 g NPP/kg. The current study showed that phytase inclusion increased egg weight in the presence of FL, allowing to reach the same egg weight than MIX300 (Table 3). Concerning the effect of phytase on egg weight, phytase mainly acts on P utilization, which is not a major constituent of the egg as stated before. Meanwhile, the amino acids constituting proteins represent 11% of the egg white (Nys and Guyot, 2011). Phytic acid can form insoluble complexes with proteins (Kies et al., 2006; Selle et al., 2012), affecting amino acid availability. Phytase, by hydrolyzing phytate, can release amino acids and thus increase their digestibility in broilers (Rutherfurd et al., 2002). In accordance with our results, Skrivan et al. (2018) observed that inclusion of phytase at 300 FTU/kg increased egg weight by 2%, in 36-wk-old laying hens receiving 1.8 g NPP/kg. However, in the MIX diets, no effect of phytase was observed on egg weight. In the current study, CL was 2.76 mm and Scott et al. (1971) specified that particles greater than 1.0 mm were specifically retained in the gizzard. Coarse limestone has been shown to increase the gizzard grinding capacity (Roche and Martinoli, 1974) and decrease the gizzard pH (Singh et al., 2014). It is thus possible that CL improved amino acid digestibility and consequently increased egg weight compared to FL. By this mechanism, CL may hide the positive effect of phytase on egg weight.

Coarse limestone also improved eggshell weight and thickness in diets without phytase by providing solubilized Ca for an extended period compared to FL (Rao and Roland, 1990). A recent meta-analysis already described the positive effect of CL on eggshell thickness (Hervo et al., 2022): addition of CL (1.36 mm) increased eggshell thickness by 1.5% compared to FL (0.21 mm). Phytase addition and MIX together increased eggshell breaking strength. Bello et al. (2020) observed that phytase addition (600 FTU/kg) in 60-wk-old laying hen during 10 wk increased the eggshell breaking strength. Eggshell breaking strength is a synthetic indicator resulting from Ca and proteins deposition and arrangement. Indeed, proteins of the eggshell are known to play a major role in the ultrastructure (Gautron and Nys, 2007).

No effect of dietary treatment was observed on bone characteristics. Phosphorus is a major constituent of bone together with Ca in the form of hydroxyapatite (Whitehead, 2004). Even if the mineral P level was low, the extra P provided by phytase was sufficient to reach equivalent levels of bone ash content and mechanical properties compared to control diets (Table 5). Hens receiving phytase used P more efficiently for bone metabolism, as illustrated by the tibia ash weight to *t*P intake ratio: 5.3, 5.5, 6.6, and 7.1 for FL0, MIX0, FL300, and MIX300, respectively (Phytase: $P < 0.001$; data not shown). No effect of the MIX was observed on bone strength in the current study. However, a reduction of bone resorption was previously observed using CL (Guinotte and Nys, 1991; Gloux et al., 2020). Indeed, by diffusing soluble Ca into the intestine longer during the eggshell calcification period, CL limits bone mobilization. Because the structural bone content declines with age and leads to bone weakness (Whitehead, 2004), the effect of CL would be more pronounced in aged laying hens. In this way, de Witt et al. (2009) did not observe an effect of CL (2.9 mm) on bone strength before 66 wk of age. Thus, in the current study, the absence of an effect may have been due to the young age of the birds.

To conclude, this study demonstrates that using CL as a Ca source compared with FL improves the digestive utilization of P and Ca in phytase formulated diets maintaining performance and bone characteristics in a short-term. Further studies are needed to elucidate the underlying mechanisms in a dynamic way and the overall P excretion. Then, mineral requirements can be refined and nutritional strategies based on precision feeding implemented (i.e., "chrono-nutrition").

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DISCLOSURES

The authors (Hervo et al.) declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in the present study.

REFERENCES

Abbasi, F., T. Fakhur-un-Nisa, J. Liu, X. Luo, and I. H. R. Abbasi. 2019. Low digestibility of phytate phosphorus, their impacts on the environment, and phytase opportunity in the poultry industry. Environ. Sci. Pollut. Res. Int. 26:9469–9479.

Abudabos, A. M. 2011. Phytate phosphorus utilization and intestinal phytase activity in laying hens. Ital. J. Anim. Sci. 11:1e8.

Ahmadi, H., and M. Rodehutscord. 2012. A meta-analysis of responses to dietary nonphytate phosphorus and phytase in laying hens. Poult. Sci. 91:2072–2078.

Angel, R., N. M. Tanim, T. J. Applegate, A. S. Dhandu, and L. E. Ellestad. 2002. Phytic acid chemistry: influence on phytin-

phosphorus availability and phytase efficacy. J. Appl. Poult. Res. 11:471–480.
AOAC. 1990. Official Methods of Analysis. 15th ed. Association of Official Analytical Chemists, Arlington, VA.
AOAC International. 2006. Official Methods of Analysis, 18th ed. Association of Official Analytical Chemists, Arlington, VA.
Babatunde, O. O., and O. Adeola. 2020. Additivity of apparent and standardized ileal digestibility of phosphorus in corn and canola meal mixed diets; basal endogenous loss of phosphorus responses to phytase and age in broiler chickens. Br. Poult. Sci. 62:244–250.
Bar, A. 2008. Calcium homeostasis and vitamin D metabolism and expression in strongly calcifying laying birds. Comp. Biochem. Physiol. Mol. Amp. Integr. Physiol. 151:477–490.
Bello, A., Y. Dersjant-Li, and D. R. Korver. 2020. Effects of dietary calcium and available phosphorus levels and phytase supplementation on performance, bone mineral density, and serum biochemical bone markers in aged white egg-laying hens. Poult. Sci. 99:5792–5801.
Bello, A., and D. R. Korver. 2019. Long-term effects of *Buttiauxella* sp. phytase on performance, eggshell quality, apparent ileal Ca and P digestibility, and bone properties of white egg layers. Poult. Sci. 98:4848–4859.
Bournazel, M., M. Lessire, S. Klein, N. Même, C. Peyronnet, A. Quinsac, M. J. Duclos, and A. Narcy. 2018. Phytase supplementation in diets rich fiber from rapeseed enhances phosphorus and calcium digestibility but not retention in broiler chickens. Poult. Sci. 97:1627–1640.
Bradbury, E. J., S. J. Wilkinson, G. M. Cronin, C. L. Walk, and A. J. Cowieson. 2016. Effects of phytase, calcium source, calcium concentration and particle size on broiler performance, nutrient digestibility and skeletal integrity. Anim. Prod. Sci. 58:271–283.
Classen, H. L., J. Apajalahti, B. Svihus, and M. Choct. 2016. The role of the crop in poultry production. Worlds Poult. Sci. J. 72:459–472.
Clunies, M., R. J. Etches, C. Fair, and S. Leeson. 1993. Blood, intestinal and skeletal calcium dynamics during egg formation. Can. J. Anim. Sci. 73:517–532.
Dacke, C. G., S. Arkle, D. J. Cook, I. M. Wormstone, S. Jones, M. Zaidi, and Z. A. Bascal. 1993. Medullary bone and avian calcium regulation. J. Exp. Biol. 184:63–88.
Denbow, D. 2015. Chapter 14: Gastrointestinal anatomy and physiology. Pages 337−366 in Sturkie's Avian Physiology. C. G. Scanes, ed. 6th ed. Springer, New York, NY.
de Witt, F. H., N. P. Kuleile, H. J. van der Merwe, and M. D. Fair. 2009. Effect of limestone particle size on bone quality characteristics of hens at end-of-lay. S. Afr. J. Anim. Sci. 39:41–44.
Etches, R. J. 1987. Calcium logistic in the laying hen. J. Nutr. 117:619–628.
Fleming, R. H. 2008. Nutritional factors affecting poultry bone health. Proc. Nutr. Soc. 67:177–183.
Gautron, J., and Y. Nys. 2007. Function of eggshell matrix proteins. Pages 109−115 in Bioactive Egg Compounds. Huopalahti R., R. López-Fandiño, M. Anton and R. Schade, eds. Springer, New York, NY.
Gloux, A., N. Le Roy, J. Ezagal, N. Même, C. Hennequet-Antier, M. L. Piketty, D. Prié, G. Benzoni, J. Gautron, Y. Nys, A. Narcy, and M. J. Duclos. 2020. Possible roles of parathyroid hormone, 1.25(OH)2D3, and fibroblast growth factor 23 on genes controlling calcium metabolism across different tissues of the laying hen. Domest. Anim. Endocrinol. 72:106407.
Grynspan, F., and M. Cheryan. 1983. Calcium phytate: effect of pH and molar ratio on in vitro solubility. J. Am. Oil Chem. Soc. 60:1761–1764.
Guinotte, F., and Y. Nys. 1991. Effects of particle size and origin of calcium sources on eggshell quality and bone mineralization in egg laying hens. Poult. Sci. 70:583–592.
Hervo, F., A. Narcy, Y. Nys, and M. P. Létourneau-Montminy. 2022. Effect of limestone particle size on performance, eggshell quality, bone strength, and in vitro/in vivo solubility in laying hens: a meta-analysis approach. Poult. Sci. 101:101686.
Humer, E., C. Schwarz, and K. Schedle. 2015. Phytase in pig and poultry nutrition. J. Anim. Physiol. Anim. Nutr. 99:605–625.
Hurwitz, S. 1964. Bone composition and Ca retention in fowl as influenced by egg formation. Am. J. Physiol. 206:198–204.
Hurwitz, S., and A. Bar. 1965. Absorption of calcium and phosphorus along the gastrointestinal tract of the laying fowl as influenced by dietary calcium and eggshell formation. Nutrition 86:433–438.
Javadi, M., J. J. Pascual, M. Cambra-López, J. Macías-Vidal, A. Donadeu, J. Dupuy, L. Carpintero, P. Ferrer, and A. Cerisuelo. 2021. Effect of dietary mineral content and phytase dose on nutrient utilization, performance, egg traits and bone mineralization in laying hens from 22 to 31 weeks of age. Animals 11:1495.
Keshavarz, K. 2000. Nonphytate phosphorus requirement of laying hens with and without phytase on a phase feeding program. Poult. Sci. 79:748–763.
Kies, A. K., L. H. de Jonge, P. A. Kemme, and A. W. Jongbloed. 2006. Interaction between protein, phytate, and microbial phytase. In vitro studies. J. Agric. Food Chem. 54:1753–1758.
Kim, W. K., S. A. Bloomfield, T. Sugiyama, and S. C. Ricke. 2012. Concepts and method for understanding bone metabolism in laying hens. Worlds Poult. Sci. J. 68:71–82.
Kim, S. W., W. Li, R. Angel, and P. W. Plumstead. 2019. Modification of a limestone solubility method and potential to correlate with in vivo limestone calcium digestibility. Poult. Sci. 98:6837–6848.
Kim, S. W., W. Li, R. Angel, and M. Proszkowiec-Weglarz. 2018. Effects of limestone particle size and dietary Ca concentration on apparent P and Ca digestibility in the presence or absence of phytase. Poult. Sci. 97:4306–4314.
Li, W., R. Angel, P. W. Plumstead, and H. Enting. 2021. Effects of limestone particle size, phytate, calcium source, and phytase on standardized ileal calcium and phosphorus digestibility in broilers. Poult. Sci. 100:900–909.
Lim, H., H. Namkung, and I. Paik. 2003. Effects of phytase supplementation on the performance, egg quality, and phosphorous excretion of laying hens fed different levels of dietary calcium and nonphytate phosphorous. Poult. Sci. 82:92–99.
Lohmann Breeders, Lohmann Tradition, Management Guide, 2021. Accessed Nov. 2022. https://lohmann-breeders.com/files/downloads/MG/Cage/LB_MG_Cage_Tradition_FR.pdf.
Maenz, D. D., C. M. Engele-Schaan, R. W. Newkirk, and H. L. Classen. 1999. The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in a slurry of canola meal. Anim. Feed Sci. Technol. 81:177–192.
Majeed, S., R. Qudsieh, F. W. Edens, and J. Brake. 2020. Limestone particle size, calcium and phosphorus levels, and phytase effects on live performance and nutrients digestibility of broilers. Poult. Sci. 99:1502–1514.
Manangi, M. K., and C. N. Coon. 2007. The Effect of calcium carbonate particle size and solubility on the utilization of phosphorus from phytase for broilers. Int. J. Poult. Sci. 6:85–90.
Martinez Rojas, I. Y., E. Ávila González, J. Acre Menocal, T. T. Dos Santos, J. Rubio Arguello, and C. López Coello. 2018. Assessment of a phytase included with lactic acid on productive parameters and on deposition of phosphorus, calcium, and zinc in laying hens fed with sorghum-soybean-meal-based diets. J. Appl. Anim. Res. 46:314–321.
Molnár, A., L. Maertens, B. Ampe, J. Buyse, J. Zoons, and E. Deleize. 2018. Effect of different split-feeding treatments on performance, egg quality, and bone quality of individually housed aged laying hens. Poult. Sci. 97:88–101.
Nolan, K. B., and P. A. Duffin. 1987. Effects of phytate on mineral bioavailability. In vitro studies on Mg^{2+}, Ca^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} (also Cd^{2+}) solubilities in the presence of phytate. J. Sci. Food Agric. 40:79–85.
Nys, Y. 2017. Chapter 2 - Laying hen nutrition: optimising hen performance and heath, bone and eggshell quality. Pages 47−74 in Achieving Sustainable Production of Eggs, Volume 2: Animal Welfare and Sustainability. J. R. Roberts, 1st ed. Burleigh Dodds Science Publishing, London, United Kingdom.
Nys Y. and Guoyt N., Egg formation and chemistry, In: Nys Y., Bain M., and van Immersel F. Pages 83−132 in Improving the Safety and Quality of Eggs and Egg Products. Volume 1: Egg Chemistry, Production and Consumption, 2011, Woodhead Publishing Ltd, 2011, Woodhead Publishing Series in Food Science, Technology and Nutrition, London, United Kingdom.
Nys, Y., and N. Leroy. 2018. Chapter 22: Calcium homeostasis and eggshell biomineralization in female chicken. Pages 361−382 in Vitamin

D. Volume 1: Biochemistry, Physiology, and Diagnostics. D. Feldman, ed. 4th ed. Academic Press, London, United Kingdom.

Pongmanee, K., I. Kühn, and D. R. Korver. 2020. Effects of phytase supplementation on eggshell and bone quality, and phosphorus and calcium digestibility in laying hens from 25 to 37 kw of age. Poult. Sci. 99:2595–2607.

Rao, K., S. D. Roland, J. Adams, and W. Durboraw. 1992. Improved limestone retention in the gizzard of commercial leghorn hens. J. Appl. Poult. Res. 1:6–10.

Rao, K. S., and D. A. Roland, Sr. 1990. In vivo limestone solubilization in commercial Leghorns: role of dietary calcium level, limestone particle size, in vitro limestone solubility rate, and the calcium status of the hen. Poult. Sci. 62:2170–2176.

Ravindran, V., L. S. David, M. R. Abdollahi, and M. R. Bedford. 2020. True ileal calcium digestibility in soybean meal and canola meal, and true ileal phosphorus digestibility in maize-soybean meal and maize-canola meal diets, without and with microbial phytase, for broiler growers and finishers. Br. Poult. Sci. 62:293–303.

Roche, M., and J. L. Martinoli. 1974. Motricite gastro-intestinale chez le poulet [in French]. Ann. Med. Vet. 5:295–309.

Rutherfurd, S. M., T. K. Chung, and P. J. Moughan. 2002. The effect of microbial phytase on ileal phosphorus and amino acid digestibility in the broiler chicken. Br. Poult. Sci. 43:598–606.

Scott, M. L., S. J. Hull, and P. A. Mullenhoff. 1971. The calcium requirement of laying hens and effects of dietary oyster shell upon eggshell quality. Poult. Sci. 50:1055–1063.

Selle, P. H., A. J. Cowieson, N. P. Cowieson, and V. Ravindran. 2012. Protein-phytate interactions in pig and poultry nutrition: a reappraisal. Nutr. Res. Rev. 25:1–17.

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.

Selle, P. H., and V. Ravindran. 2007. Microbial phytase in poultry nutrition. Anim. Feed Sci. Technol. 135:1–41.

Singh, Y., V. Ravindran, T. J. Wester, A. L. Molan, and G. Ravindran. 2014. Influence of pelleting inclusion of whole corn on performance, nutrient utilization, digestive tract measurements, and cecal microbiota of young broilers. Poult. Sci. 93:3073–3082.

Skrivan, M., M. Englmeairova, and V. Skrivanova. 2018. Negative effect of phytase superdosing in laying hens. Czech J. Anim. Sci. 63:182–187.

Suttle, N. F. 2010. Chapter 6: Phosphorus. Pages 122−167 in Mineral Nutrition of Livestock. N. F. Suttle, ed. 4th ed. CABI Publishing, Wallingford, United Kingdom.

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.

Teng, X., W. Zhang, D. Xu, Z. Liu, N. Yang, D. Luo, H. Wang, M. Ge, and R. Zhang. 2020. Effects of low dietary phosphorus on tibia quality and metabolism in caged laying hens. Prev. Vet. Med. 181:105049.

van der Klis, J. D., H. A. J. Versteegh, P. C. M. Simons, and A. K. Kies. 1997. The efficacy of phytase in corn-soybean meal-based iets for laying hens. Poult. Sci. 76:1535–1542.

van der Klis, J. D., M. W. A. Verstegen, and W. de Witt. 1990. Absorption of minerals and retention time of dry matter in the gastrointestinal tract of broilers. Poult. Sci. 69:2185–2194.

Whitehead, C. C. 2004. Overview of bone biology in the egg-laying hen. Poult. Sci. 83:193–199.

Wilcox, R. A., C. W. Deyoe, and H. B. Pfost. 1970. A method for determining and expressing the size of feed particles by sieving. Poult. Sci. 49:9–13.

Wise, A. 1983. Dietary factors determining the biological activity of phytates. Nutr. Abstr. Rev. Clin. Nutr. 53:791–806.