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Effect of two commercial limestone sources with different solubility on the efficacy of two phytases in 0-21 d old broilers

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RESEARCH ARTICLE

POULTRY

Abstract

A study evaluated the effects of limestone sources with different solubility on the efficacy of two phytases in broilers. A 2×5 factorial arrangement was employed with two commercial limestone sources and five diet treatments; a positive control (PC) diet with 7.2 g/kg P and 9.6 g/kg Ca, and a negative control diet containing reductions of 1.87 g/kg available P, 1.99 g/kg Ca and 0.4 g/kg Na, supplemented with either *Buttiauxella* phytase (PhyB) or *Escherichia coli* phytase (PhyE) at 500 or 1000 FTU/kg diet. The two limestone sources were feed-grade commercial products with different particle sizes and solubility (fast-soluble (FS) 100%; slow-soluble (SS) 26% soluble after 30 minutes at pH 3) containing similar levels of Ca. Diets were fed to one-day-old Ross 308 males (n=2,400) with 30 birds/pen and eight pens/treatment in two phases (starter 0-10 d and grower 10-21 d). On d 21, ileal digesta was collected from 12 birds/pen to determine apparent ileal digestibility (AID) of P and Ca, and myo-inositol hexakisphosphate (IP6) disappearance, and tibias from four birds/pen for ash determination. The SS limestone improved body weight gain (BWG), feed intake (FI) and FCR vs FS limestone in starter/grower phases ($P<0.05$), and improved AID of P ($P<0.05$) and IP6 disappearance ($P<0.05$) at d 21. There was an interaction between limestone and phytase on BWG, FI and FCR in the grower phase ($P\leq0.05$) whereby FS (vs SS) limestone reduced BWG at either dose of PhyE, but only at 500 FTU/kg of PhyB. At an equivalent dose, PhyB had higher BWG and feed intake than PhyE ($P<0.05$). At 1000 FTU/kg, performance was equivalent (BWG and FI) or superior (FCR) to the PC, PhyB produced greater tibia ash, AID of P and IP6 disappearance ($P<0.05$). The findings showed that the effects of limestone particle size on phytase efficacy varied with phytase source and dose.

Keywords: broiler chickens, calcium, calcium carbonate, phosphorus, phytase

1. Introduction

Limestone is used as the major source of inorganic calcium (Ca) in poultry diets. The influence of limestone particle size and solubility on quality and performance outcome measures has been intensively studied in layers and broilers. Results from studies in layers indicated that large particle size (>1 mm diameter) can improve egg shell quality, Ca retention, bone mineral content and breaking force – effects which are thought to result from an increase in retention time in the gizzard, thus increasing the *in vivo* solubility and utilisation of Ca in laying hens (Araujo *et al.,* 2011; Cheng and Coon, 1990; Rao *et al.,* 1992; Roland, 1986; Zhang and Coon, 1997). Fewer studies are available for broilers, in which smaller particle size (<1 mm diameter) is commonly used in commercial diets. Guinotte *et al.* (1991, 1995) reported that large particles of Ca (>0.6 mm diameter) reduced Ca retention and bone mineralisation compared to fine particles of Ca (0.15 mm diameter) in broilers. Anwar *et al.* (2016, 2017) showed that limestone particle size and solubility had a marked effect on Ca digestibility, which was higher with coarse, less soluble limestone (1-2 mm diameter) than with fine, more soluble limestone (<0.5 mm). This was attributed to a slower Ca-release in the

gizzard with coarse, less soluble limestone, because of an increase in retention time.

Microbial phytase is commonly used in commercial broiler diets to improve P and Ca utilisation and to reduce P excretion into the environment. The efficacy of microbial phytase for degrading phytate has improved substantially since phytases were first developed for commercial use, but individual phytases can differ substantially in their ability to degrade phytate (myo-inositol hexakisphosphate (IP6)) in the gastrointestinal tract (GIT), dependent on dose, dietary composition (and phytate levels) and host genetics (Dersjant-Li *et al.*, 2015). The major site of activity for phytase in the GIT is the upper region (principally the proventriculus and gizzard), in which the pH is optimal for phytase activity (pH 2 to 5), although different phytases are known to have different optima pH (Menezes-Blackburn *et al.,* 2015). The effects of microbial phytase on phytate degradation and P and Ca digestibility have been well studied in broilers (Dersjant-Li *et al.,* 2015; Selle and Ravindran, 2007; Selle *et al.,* 2009) and decreased efficacy has been extensively reported with high dietary Ca (as limestone) levels (Amerah *et al.,* 2014; Plumstead *et al.,* 2008; Sebastian *et al.,* 1996; Tamim *et al.,* 2004). However, the effect of limestone particle size on phytase efficacy has been less studied.

The *in vitro* study by Manangi and Coon (2007), involving a 3-phytase, suggested that small Ca particle sizes with high solubility may limit the ability of phytase to hydrolyse P from phytate. More recently, Kim *et al.* (2018) studied the effect of limestone particle size and dietary Ca level in the presence or absence of *Buttiauxella* phytase (a 6-phytase) on the apparent ileal digestibility (AID) of P and Ca. The authors reported that coarse limestone (geometric mean diameter (GMD)=402 µm) from the same origin was less detrimental to AID P and phytase efficacy, compared to fine limestone GMD<75 µm (Kim *et al.* 2018).

The objective of the following study was to evaluate the effect of two commercial limestone sources with differing origin, particle size and solubility on the efficacy of two microbial phytases supplemented at two dose levels on performance, bone ash, ileal P and Ca digestibility and IP6 disappearance in broilers during 0 to 21 days of age.

2. Materials and methods

The Institutional Animal Care and Use Committee (Schothorst Feed Research, the Netherlands) approved the experimental protocol used in this study. The treatment, management, housing, husbandry and slaughtering conditions strictly conformed to European Union Guidelines (European Parliament, 2010).

Birds and husbandry

Ross 308 male broilers (n=2,400) were obtained on day of hatch from a local hatchery, where they had been vaccinated against infectious bronchitis, and assigned to floor-pens based by body weight, so that pens contained birds with approximately equal average bird weight. Bird stocking density was of 13.6 chicks/m^2 (30 birds per pen). Pens contained wood shavings as bedding material. Birds were maintained in an environmentally controlled broiler house, in which ambient temperature was maintained initially at 34.5 °C, and thereafter gradually decreased to 22 °C at 21 days of age, under a photoperiod of 23 h light (L):1 dark (D) during the first day and 4D:10L:2D:8L thereafter. Birds were given *ad libitum* access to feed and water during the experimental period.

Experimental design and diets

The experimental design was a 2×5 factorial arrangement with two limestone sources (fast and slow-soluble) and five dietary treatments. Treatments were arranged in a completely randomised block design. Each of the 10 treatment was replicated eight times in floor-pens containing 30 birds each. The two limestone sources differed in their particle size, solubility and origin. A fine and fast-soluble (FS) limestone (particle diameter range 0-90 µm, GMD<22 µm) was purchased from Carbocia (Louvil, France) and a coarse and slow-soluble (SS) limestone (particle diameter range 300-600 µm, GMD 564 µm) was purchased from KWM (Medembach, Germany) (Figure 1). The analysed Ca content was similar (38.2 vs 37.4% for FS and SS limestones, respectively) and a nominal Ca level of 38.0% was assigned for dietary formulation purposes. The positive control (PC) diet was based on corn-soybean meal and was formulated to meet the recommended requirements for nutrients set by the breeder for starter (d 0-10) and grower (d 10-21) birds (Aviagen, 2014). A negative control (NC) basal diet was formulated with a reduction of 1.87 g/kg total P, 1.87 g/kg available P, 1.99 g/kg Ca and 0.4 g/kg Na (Table 1) vs PC, in accordance with the manufacturer's recommended minerals matrix for diets formulated with 1000 FTU/kg *Buttiauxella* phytase (PhyB), by varying only monocalcium phosphate (MCP), limestone, salt and diamol (a filler).

A NC treatment without phytase supplementation was not include in the present study due to ethical reasons and main goal of the study, i.e. comparison of two commercial limestone products in commercial-like diets.

The NC diet was supplemented with either a *Buttiauxella*. 6-phytase (PhyB) expressed in *Trichoderma reesei* or an *Escherichia coli* 6-phytase expressed in *Pichia pastoris* (PhyE), at a dose level of 500 or 1000 FTU/kg (PhyB500, PhyB1000, PhyE500 or PhyE1000, respectively). Phytase was

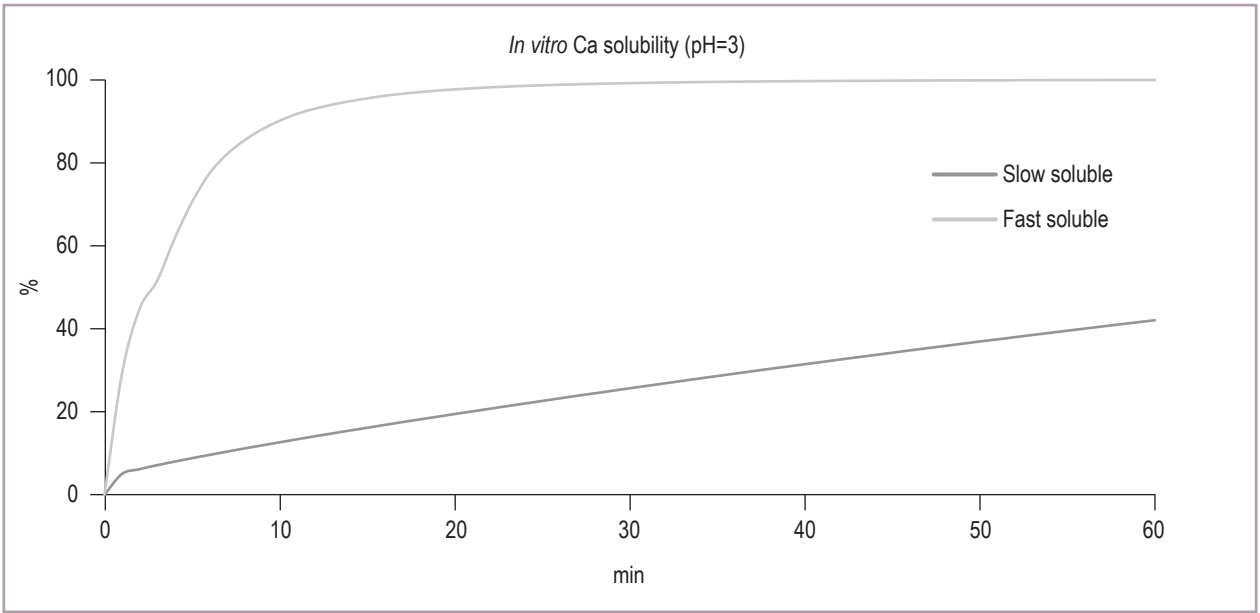

Figure 1. *In vitro* solubility of Ca from two limestone sources (fast-soluble/slow-soluble) at pH 3.1. Fast-soluble: Carbocia, France. Geometric mean diameter (GMD)<20 µm; Slow-soluble: KWM, Germany. GMD=564 µm.

dosed based on analysed product activity, as determined by an independent laboratory (LUFA, Nord West, Oldenburg, Germany). One phytase unit (FTU) was defined as the amount of enzyme that released 1 µmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37 °C (AOAC, 2000). Titanium dioxide (TiO_2) was added to all diets at a level of 3.5 g/kg as an inert marker. A common basal diet was produced for each limestone source and each dietary phase. Experimental diets were individually mixed by adding the corresponding amounts of MCP, limestone, salt, diamol and PhyB or PhyE. All diets were pelleted (3 mm diameter) with a target pellet temperature ≤80 °C, and starter diets were subsequently crumbled.

Sampling and measurements

Body weight and feed intake (FI) were measured per pen on d 0, d 10 and d 21 and used to determine average body weight gain (BWG) and feed conversion rate (FCR). Mortality was recorded daily and was used to correct FCR and FI.

On d 21, 12 birds per pen were randomly selected and anaesthetised by CO_2 asphyxiation. The left tibias from four birds were obtained and pooled for determination of de-fatted tibia ash content. Tibias were stripped of adjacent tissues and dried overnight initially at 40 °C and subsequently overnight at 70 °C. Fat was extracted from the tibias using a Soxhlet apparatus and 100% petroleum ether according to modified methods of Watson *et al.* (2006). Fat-extracted-tibias were dried for 4 h at 103 °C and ashed in a muffle furnace for 24 h at 700 °C to determine bone ash content.

Digesta was obtained from all 12 euthanised birds (per pen) by gentle squeezing from the last 25 cm of the ileum, as described by Rodehutschord *et al.* (2012). Ileal digesta samples were pooled, freeze-dried, finely ground to pass through a 1 mm sieve prior to nutrient analysis. Digestibility of nutrients was calculated using TiO_2 as the indigestible marker.

Chemical analysis

Samples were analysed in duplicate for all analyses. The dry matter content of diets and digesta was determined by drying overnight in a 103 °C force-draft oven, according to ISO 6496 (1999). Crude protein in the basal diets was analysed according to ISO 16634-2 (2016). Limestone sources, diets and ileal contents were digested with acid and analysed for Ca using inductively coupled plasma atomic emission spectrometry (ICP-AES; AOAC, 1999), whereas P was determined by a colorimetric method adapted from AOAC method 986.24 (1990). Microminerals (Fe, Mn, Zn and Cu) concentrations in limestone sources were analysed by ICP-AES based on AOAC 2011.14 and AOAC 985.01 (AOAC, 2006, 2011). Titanium (Ti) concentrations in diets and ileal digesta were determined by a colorimetric method adapted from Short *et al.* (1996). Sodium was analysed by atomic absorption spectrometry (AAS; dry ashing) method (ISO 6869, 2001). Phytate phosphorus (PP (inositol hexaphosphate IP6)) concentrations in diets and ileal digesta (blind samples) and phytase activity in diets were analysed

Table 1. Ingredient and chemical composition of the basal diets (g/kg, as-fed basis).[1]

Ingredient (g/kg, as-fed basis)	Starter (d 0-10)		Grower (d 11-21)	
	PC	NC	PC	NC
Corn	558.8	558.8	567.9	567.9
Soybean meal (48% crude protein)	331.9	331.9	267.0	267.0
Rapeseed meal	38.0	38.0	40.0	40.0
Sunflower seed meal	0.0	0.0	40.0	40.0
Lard	20.1	20.1	35.3	35.3
Soybean oil	4.7	4.7	4.7	4.7
Lysine HCl (79%)	2.3	2.3	2.6	2.6
DL-Met (99%)	2.7	2.7	2.4	2.4
L-Thr (L 98%)	0.9	0.9	0.8	0.8
Sodium bicarbonate	1.5	1.5	1.5	1.5
Vitamin-mineral premix[2]	5.0	5.0	5.0	5.0
Coccidiostat[3]	1.0	1.0	1.0	1.0
TiO_2	-	-	3.5	3.5
Monocalcium phosphate	14.2	5.9	11.8	3.5
Limestone (FS or SS; dependent on treatment)[4]	15.6	14.1	13.2	11.7
Salt	3.3	2.3	3.3	2.3
Mepregenol diacetate[5]	0.0	10.8	0.0	10.8
Calculated composition (g/kg)				
Apparent metabolizable energy (MJ/kg)	11.92	11.92	12.24	12.24
Crude protein	221.0	221.0	205.0	205.0
Lysine	13.6	13.6	12.5	12.5
Digestible Lys	12.0	12.0	11.0	11.0
Methionine + cysteine	9.8	9.8	9.1	9.1
Digestible methionine + cysteine	8.6	8.6	8.0	8.0
Total P	7.2	5.3	6.7	4.8
Phytate-P	2.5	2.5	2.7	2.7
Available P[6]	4.6	2.7	4.0	2.2
Ca	9.6	7.6	8.4	6.4
Na	1.8	1.4	1.8	1.4
Cl	3.1	2.5	3.2	2.5
K	9.8	9.8	9.0	9.0
Dietary electrolyte balance (meq/kg)	244	244	221	221
Analysed nutrient composition (g/kg)				
Crude protein	214.0	214.0	206.0	206.0
Total P	7.0	5.3	6.6	4.8
Phytate P	2.7	2.7	2.9	2.9
Available P[5]	4.3	2.6	3.7	1.9
Ca - FS limestone diets	9.7	8.2	8.0	6.5
Ca - SS limestone diets	9.7	7.7	8.1	6.1
Na	1.8	1.4	1.7	1.4

[1] NC = negative control; NC diets were supplemented with phytase, according to treatment. PC = positive control.

[2] Supplied per kilogram of diet: vitamin A, 10,000 IU; vitamin D3, 2,400 IU; vitamin E, 50 mg; vitamin K3, 1.5 mg; vitamin B1, 2.0 mg; vitamin B2, 7.5 mg; pantothenic acid, 12 mg; niacin, 35 mg; biotin, 200 mcg; vitamin B12, 20 mcg; folic acid, 1.0 mg; vitamin B6, 3.5 mg; choline chloride 460 mg; Fe, 80 mg (as $FeSO_4.H_2O$); Cu, 12 mg (as $CuSO_4.5H_2O$); Zn, 60 mg (as $ZnSO_4.H_2O$); Mn, 85 mg (as MnO); I, 0.8 mg (as KI); Se, 0.15 (as Na_2SeO_3); Co, 0.4 mg (as $CoSO_4.7H_2O$).

[3] Clinacox in Starter and Sacox in Grower diets.

[4] FS = fast-soluble; SS = slow-soluble.

[5] Diamol, inert filler.

[6] Concentration determined based on analysed total P minus analysed phytate-P.

by DuPont Laboratories (Brabrand, Denmark), using the methods described by Engelen *et al.* (2001).

The *in vitro* solubility of samples of the two limestone sources was determined, in duplicate, by a 60-minute pH-stat method (SFR in-house method). Briefly, HCl (1 M; 40 °C) was added to a water solution (500 ml; 40 °C) of each limestone (5.0 g) in order to maintain the solution at a constant pH 3. The HCl added was automatically recorded every 5 minutes and the pH of the solution was continuously monitored. This method assumed that the amount of acid required to maintain a constant pH was dependent on the amount of Ca entering the solution (Schothorst Feed Research in-house method). Blank samples were incorporated as a control.

To determine the particle size distribution of the two limestone sources, a set of sieves (Retsch, Haan, Germany) sized 1.4, 1, 0.6, 0.4 and 0.2 mm, and a sieve shaker were used. The samples were passed through the sieve stack for 12 minutes. The amount of sample retained on each sieve was determined and the GMD calculated for each sample.

Calculations

The *in vitro* limestone source solubility was calculated using the amount of HCl added at each time-point, according to the following formula:

$$\text{Solubility (\%)} = \frac{[(\text{ml HCl}_{test} - \text{ml HCl}_{blank}) \times \text{HCl molarity} \times 40.08 \times 100]}{[2 \times \text{weight (g)} \times 1000]}$$

where HCl_{test} was the amount of HCl needed to maintain the solution containing the test limestone source at constant pH 3, and HCl_{blank} the amount of HCl needed to maintain a constant pH 3 of the blank solution.

AID coefficients of P, Ca and phytate (IP6) were calculated using the ratio of TiO_2 in the diet and digesta, according to the following formula:

$$\text{AID (\%)} = (1 - (\frac{Ti_d}{Ti_i}) \times (\frac{N_i}{N_d})) \times 100$$

where Ti_d was the titanium concentration in the diet, Ti_i the titanium concentration in the ileal digesta, N_i the nutrient (P, Ca or IP6) concentration in the ileal digesta and N_d the nutrient concentration in the diet. All analysed values were expressed as g/kg DM.

Statistical analyses

Data were based on pen as the experimental unit. Data were analysed as a 2×5 factorial arrangement with two limestone sources and five levels of phytase treatments, using a two-way ANOVA in GenStat® software (19th edition; VSN Internation, Hemel Hempstead, UK). Fisher's exact test was used to separate means when the model was significant. Differences were considered significant at $P<0.05$ (two-way), and $P<0.10$ was considered a trend.

3. Results

Limestone composition, particle size distribution and Ca solubility

The analysed mineral concentrations, limestone particle size distribution and Ca solubility of samples of the two limestone sources are presented in Table 2 and in Figure 1. The Ca concentration in the FS and SS limestone was similar (38.2 and 37.4%, respectively). The SS limestone had a greater micromineral (Fe, Mn, Zn, Cu) content than the FS limestone. The *in vitro* solubility after 30 min at pH 3 was 100 and 26%, respectively. The analysed GMD were <22 µm for the FS limestone and 564 µm for the SS limestone.

Table 2. Mineral analysis and particle size distribution of limestone samples (as-received basis)[1].

	Fast-soluble	Slow-soluble
Origin	Carbocia, France	Medembach, Germany
Mineral contents		
Ca (%)	38.2	37.4
P (%)	0.01	0.01
Mg (%)	0.15	0.35
K (%)	0.01	0.01
Na (%)	0.01	0.04
Fe (mg/kg)	309	513
Mn (mg/kg)	24.3	313
Zn (mg/kg)	6.43	162
Cu (mg/kg)	0.01	25.2
Particle size distribution		
<0.20 mm	100%	6.6%
0.20-0.40 mm	-	21.7%
0.40-0.60 mm	-	28.7%
0.60-1.00 mm	-	41.9%
1.00-1.40 mm	-	0.96%
>1.40 mm	-	0.15%

[1] Mineral determination was conducted by Chemuniqué, South Africa, based on AOAC methods 2011.14 and 985.01, except Ca analyses which was determined by Schothorst Feed Research, the Netherlands, based on AOAC, 1999. Particle size distribution was determined by Schothorst Feed Research, the Netherlands.

Analysed nutrients

Analysed values of P, phytate-P, Ca and Na in the experimental diets were in close agreement with calculated values (Table 3). Analysed phytase activities in the PC diets were less than 50 FTU/kg, suggesting no cross contamination between phytase-supplemented and the control diets.

The phytase activity in the PhyB-supplemented diets was within 20% of target value, except for PhyB500 in FS limestone that was 27% above target value. The phytase activity in the PhyE-supplemented diets was more variable, especially when added into diets containing SS limestone where activity was 35-70% higher than target (Table 3).

Growth performance

The influence of particle size and phytase on bird performance in the starter (d 0-10), grower (d 10-21) and overall (d 0-21) phases is summarised in Table 4, 5, and 6, respectively. During the starter phase BWG, FI and FCR were affected by particle size and by phytase ($P<0.01$). Birds fed SS limestone exhibited higher BWG, FI and reduced FCR, compared with those fed the FS limestone in the diets ($P<0.05$). Among phytase treatments, performance was greatest in birds fed the PC and PhyB1000 diet, followed by those fed the PhyB500 diet and the PhyE1000 diet ($P<0.05$). The PhyE500 diet had the lowest BWG and FI compared to all other treatments ($P<0.05$, Table 4).

Table 3. Expected and analysed phytase activity, Ca and P content of the experimental diets.

Limestone source	Phytase treatment[1]	Starter (d 0-10)						Grower (d 11-21)				
		Phytase (FTU/kg)		Ca (%)		P (%)		Phytase (FTU/kg)	Ca (%)		P (%)	
		Expected	Analysed	Expected	Analysed	Expected	Analysed	Analysed	Expected	Analysed	Expected	Analysed
Fast-soluble	PC	–	<50	0.96	0.97	0.72	0.70	<50	0.84	0.80	0.67	0.65
Fast-soluble	PhyB	500	527	0.76	0.79	0.53	0.53	633	0.64	0.66	0.48	0.48
Fast-soluble	PhyB	1000	893	0.76	0.82	0.53	0.52	928	0.64	0.64	0.48	0.48
Fast-soluble	PhyE	500	686	0.76	0.83	0.53	0.53	450	0.64	0.65	0.48	0.48
Fast-soluble	PhyE	1000	1,028	0.76	0.83	0.53	0.52	1,026	0.64	0.65	0.48	0.48
Slow-soluble	PC	–	<50	0.96	0.97	0.72	0.71	<50	0.84	0.81	0.67	0.67
Slow-soluble	PhyB	500	589	0.76	0.74	0.53	0.53	542	0.64	0.62	0.48	0.49
Slow-soluble	PhyB	1000	1,115	0.76	0.80	0.53	0.53	983	0.64	0.60	0.48	0.48
Slow-soluble	PhyE	500	807	0.76	0.78	0.53	0.53	850	0.64	0.63	0.48	0.48
Slow-soluble	PhyE	1000	1,346	0.76	0.75	0.53	0.53	1,116	0.64	0.59	0.48	0.49

[1] PhyB: *Buttiauxella* phytase expressed in *Trichoderma reesei;* PhyE*: E. coli* phytase expressed in *Pichia pastoris;* Phytase products were added to negative control diet; Fast-soluble: Carbocia, France. GMD<20 µm; Slow-soluble: KWM, Germany. GMD=564 µm.

Table 4. Influence of limestone source (fast-soluble/slow-soluble) and phytase treatment on growth performance of broilers during Starter phase (d 0-10); factorial analysis (2×5 factorial arrangement with two levels of limestone source and five levels of phytase treatment).[1]

Limestone source	Phytase treatment[2]	Phytase dose (FTU/kg)	Body weight gain (g/bird)	Feed intake (g/bird)	Feed conversion ratio (g/g)
Comparison of treatment means					
Fast-soluble	PC	0	258	278	1.077
Fast-soluble	PhyB	500	250	274	1.092
Fast-soluble	PhyB	1000	260	281	1.082
Fast-soluble	PhyE	500	241	263	1.092
Fast-soluble	PhyE	1000	249	273	1.095
Slow-soluble	PC	0	267	286	1.075
Slow-soluble	PhyB	500	259	279	1.080
Slow-soluble	PhyB	1000	263	282	1.075
Slow-soluble	PhyE	500	251	273	1.086
Slow-soluble	PhyE	1000	253	277	1.091
SEM			1.99	2.10	0.0037
Main effect means					
Fast-soluble			252^{a}	274^{a}	1.088^{b}
Slow-soluble			258^{b}	279^{b}	1.081^{a}
SEM			0.89	0.94	0.0017
	PC	0	262^{c}	282^{c}	1.076^{a}
	PhyB	500	255^{b}	276^{b}	1.086^{b}
	PhyB	1000	261^{c}	282^{c}	1.078^{a}
	PhyE	500	246^{a}	268^{a}	1.089^{bc}
	PhyE	1000	251^{b}	275^{b}	1.093^{c}
	SEM		1.41	1.49	0.0026
Main effect P and LSD values					
P (Limestone×Diet)			0.274	0.252	0.702
LSD (Limestone×Diet)			5.634	5.941	0.011
P (Limestone)			<0.001	<0.001	0.010
LSD (Limestone)			2.519	2.657	0.005
P (Diet)			<0.001	<0.001	<0.001
LSD (Diet)			3.984	4.201	0.007

[1] Means within a column with different superscript differ (*P*<0.05).

[2] PhyB: *Buttiauxella* phytase expressed in *Trichoderma reesei;* PhyE*: E. coli* phytase expressed in *Pichia pastoris;* Phytase products were added to negative control diet; Fast-soluble: Carbocia, France. GMD<20 µm; Slow-soluble: KWM, Germany. GMD=564 µm.

In the grower phase (Table 5), there was an interaction between limestone source and phytase on BWG, FI and FCR (*P*<0.05). Compared with SS limestone, FS limestone reduced BWG in all treatments supplemented with phytase, except for PhyB1000. The BWG of birds fed PhyB1000 diet was similar across SS and FS limestone (829 vs 820 g/bird, respectively). There was no effect of particle size on FCR in the grower phase, except for birds fed the PhyE1000 diet, in which FCR was lower for birds receiving SS limestone compared with FS limestone (*P*<0.05). For the overall phase

Table 5. Influence of limestone source (fast-soluble/slow-soluble) and phytase treatment on growth performance of broilers during Grower phase (d 11 to 21); factorial analysis (2×5 factorial arrangement with two levels of limestone source and five levels of phytase treatment).[1]

Limestone source	Phytase treatment[2]	Phytase dose (FTU/kg)	Body weight gain (g/bird)	Feed intake (g/bird)	Feed conversion ratio (g/g)
Comparison of treatment means					
Fast-soluble	PC	0	822^{d}	1,044^{cd}	1.271^{abc}
Fast-soluble	PhyB	500	804^{bc}	1,018^{b}	1.266^{ab}
Fast-soluble	PhyB	1000	829^{d}	1,046^{cd}	1.262^{a}
Fast-soluble	PhyE	500	766^{a}	978^{a}	1.277^{c}
Fast-soluble	PhyE	1000	798^{b}	1,020^{b}	1.278^{c}
Slow-soluble	PC	0	831^{d}	1,059^{d}	1.275^{bc}
Slow-soluble	PhyB	500	827^{d}	1,045^{cd}	1.264^{a}
Slow-soluble	PhyB	1000	820^{d}	1,040^{c}	1.269^{abc}
Slow-soluble	PhyE	500	804^{bc}	1,021^{b}	1.271^{abc}
Slow-soluble	PhyE	1000	818^{cd}	1,036^{bc}	1.266^{ab}
SEM			5.36	6.52	0.0035
Main effect means					
Fast-soluble			804^{a}	1,021^{a}	1.271
Slow-soluble			820^{b}	1,040^{b}	1.269
SEM			2.40	2.92	0.0016
	PC	0	826^{d}	1,051^{d}	1.273^{bc}
	PhyB	500	815^{bc}	1,031^{bc}	1.265^{a}
	PhyB	1000	824^{cd}	1,043^{cd}	1.266^{ab}
	PhyE	500	785^{a}	1000^{a}	1.274^{c}
	PhyE	1000	808^{b}	1,028^{b}	1.272^{bc}
	SEM		3.79	4.61	0.0025
Main effect P and LSD values					
P (Limestone×Diet)			0.001	0.009	0.050
LSD (Limestone×Diet)			15.170	18.44	0.010
P (Limestone)			<0.001	<0.001	0.392
LSD (Limestone)			6.78	8.25	0.004
P (Diet)			<0.001	<0.001	0.030
LSD (Diet)			10.73	13.04	0.007

[1] Means within a column with different superscript differ (*P*<0.05).

[2] PhyB: *Buttiauxella* phytase expressed in *Trichoderma reesei;* PhyE*: E. coli* phytase expressed in *Pichia pastoris;* Phytase products were added to negative control diet; Fast-soluble: Carbocia, France. GMD<20 µm; Slow-soluble: KWM, Germany. GMD=564 µm.

(d 0-21), a similar interaction between limestone source and diet was observed for BWG and FI (*P*<0.01), but not for FCR (*P*=0.24). Across limestone sources, at the same phytase dose, FCR fore birds fed PhyE was greater compared to PhyB (*P*<0.05), although not different to the PC, whilst supplementation with PhyB1000 resulted in a lower FCR than seen in the PC group (*P*<0.05).

Tibia ash

Effects of treatment on tibia ash are presented in Table 6. No effect of particle size on tibial ash was observed. Tibial ash levels in birds fed PhyB1000 and PhyB500 were similar to that of the PC (505, 508, and 502 g/kg fat free DM, respectively). Tibia ash of birds fed PhyB500 was similar to that from birds fed PhyE1000 (502 and 498 g/kg), and tibia ash of birds fed PhyE500 (482 g/kg) was lower than that of all other treatments (*P*<0.05; Table 6).

Nutrient digestibility

Effects of treatment on AID of P, Ca, and IP6 disappearance are presented in Table 7. Significant main effects of limestone and phytase on AID of P, Ca and IP6 disappearance were observed at d 21 (*P*<0.05). Compared with FS limestone, the SS source increased AID P (64.4 vs 59.8%) and IP6 disappearance at d 21 (55.0 vs 51.8%; *P*<0.05). Across limestone sources, AID of P and IP6 disappearance were lowest in birds fed the PC diet, followed by birds fed PhyE500, PhyE1000, PhyB500 and PhyB1000, with significant (*P*<0.05) incremental increases being evident between each of these groups for both nutrient variables. The only exception was for the AID of P between PhyB500 and PhyE1000 groups, which did not differ (64.9 vs 64.3%, respectively).

Figure 2 shows that there was a linear relationship between ileal digestible IP6 and BWG from 0-21 d in both FS (R^2=0.74, *P*<0.05) and SS (R^2=0.47, *P*<0.05) limestone sources. The slope and residues of the two linear equations were significantly different (*P*<0.001), which was greater for FS than SS limestone.

There was an interaction between limestone and phytase treatment on AID of Ca at d 21 (*P*=0.03). Compared with FS, SS limestone reduced (*P*<0.05) AID Ca in all phytase treatments, except for the PC (37.7 vs 39.4%) and the PhyE1000 treatment (50.9 vs 53.8%).

Table 6. Influence of limestone source (fast-soluble/slow-soluble) and phytase treatment on growth performance and tibia ash content of broilers during the overall phase (d 0 to 21), determined on d 21; factorial analysis (2×5 factorial arrangement with two levels of limestone source and five levels of phytase treatment).[1]

Limestone source	Phytase treatment[2]	Phytase dose (FTU/kg)	Body weight gain (g/bird)	Feed intake (g/bird)	Feed conversion ratio (g/g)	Tibia ash (g/kg fat-free DM)
Comparison of treatment means						
Fast-soluble	PC	0	1,082^{de}	1,324^{de}	1.224	510
Fast-soluble	PhyB	500	1,054^{bc}	1,289^{b}	1.223	502
Fast-soluble	PhyB	1000	1,091^{e}	1,330^{de}	1.218	508
Fast-soluble	PhyE	500	1,007^{a}	1,241^{a}	1.233	479
Fast-soluble	PhyE	1000	1,047^{b}	1,293^{bc}	1.235	497
Slow-soluble	PC	0	1,097^{e}	1,345^{e}	1.226	506
Slow-soluble	PhyB	500	1,086^{de}	1,324^{de}	1.220	502
Slow-soluble	PhyB	1000	1,085^{de}	1,324^{de}	1.219	502
Slow-soluble	PhyE	500	1,055^{bc}	1,294^{bc}	1.227	485
Slow-soluble	PhyE	1000	1,072^{cd}	1,313^{cd}	1.225	500
SEM			6.43	7.96	0.0030	2.41
Main effect means						
Fast-soluble			1,056^{a}	1,295^{a}	1.227	499
Slow-soluble			1,079^{b}	1,320^{b}	1.223	499
SEM			2.87	3.56	0.0013	1.08
	PC	0	1,089^{c}	1,335^{c}	1.225^{bc}	508^{d}
	PhyB	500	1,070^{b}	1,306^{b}	1.221^{ab}	502^{bc}
	PhyB	1000	1,088^{c}	1,327^{c}	1.219^{a}	505^{cd}
	PhyE	500	1,031^{a}	1,268^{a}	1.230^{c}	482^{a}
	PhyE	1000	1,059^{b}	1,303^{b}	1.230^{c}	498^{b}
	SEM		4.54	5.63	0.0021	1.71
Main effect P and LSD values						
P (Limestone×Diet)			0.002	0.009	0.241	0.076
LSD (Limestone×Diet)			18.17	22.5	0.008	6.816
P (Limestone)			<0.001	<0.001	0.095	0.977
LSD (Limestone)			8.124	10.06	0.004	3.048
P (Diet)			<0.001	<0.001	<0.001	<0.001
LSD (Diet)			12.85	15.91	0.006	4.82

[1] Means within a column with different superscript differ (*P*<0.05).

[2] PhyB: *Buttiauxella* phytase expressed in *Trichoderma reesei;* PhyE*: E. coli* phytase expressed in *Pichia pastoris;* Phytase products were added to negative control diet; Fast-soluble: Carbocia, France. GMD<20 µm; Slow-soluble: KWM, Germany. GMD=564 µm.

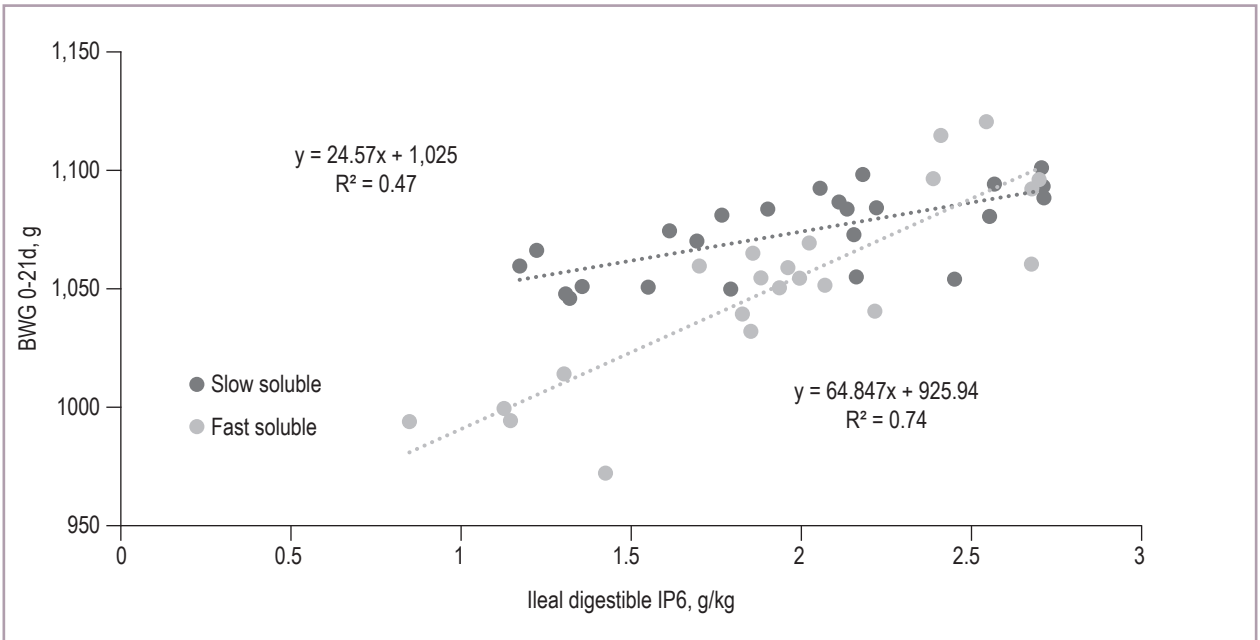

Figure 2. Relationship between ileal digestible myo-inositol hexakisphosphate (IP6) (g/kg) and overall 0-21 d body weight gain (BWG) in broilers (excluding PC due to different inorganic P levels between positive control and phytase treatments). Fast-soluble: Carbocia, France. GMD<20 µm; Slow-soluble: KWM, Germany. GMD=564 µm.

Table 7. Influence of limestone source (fast-soluble/slow-soluble) and phytase treatment on apparent ileal digestibility (AID) of P and Ca on ileal myo-inositol hexakisphosphate (IP6) concentrations and disappearance, at d 21.[1]

Limestone source	Phytase treatment[2]	Phytase dose (FTU/kg)	AID P (%)	dig P (g/kg diet)	AID Ca (%)	dig Ca (g/kg diet)	IP6 disappearance (%)	dig IP6 disappearance (g/kg diet)	Ileal IP6 content (g/kg DM)
Comparison of treatment means									
Fast-soluble	PC	0	48.3	3.23	37.7^{a}	3.05^{abc}	-1.0	-0.03	3.68
Fast-soluble	PhyB	500	61.3	2.96	52.7^{cde}	3.41^{d}	68.3	2.00	1.17
Fast-soluble	PhyB	1000	72.3	3.49	54.6^{de}	3.53^{d}	87.6	2.57	0.46
Fast-soluble	PhyE	500	54.8	2.65	55.8^{e}	3.61^{d}	40.3	1.18	2.04
Fast-soluble	PhyE	1000	62.3	3.01	53.8^{cde}	3.47^{d}	63.9	1.87	1.46
Slow-soluble	PC	0	52.4	3.51	39.4^{a}	3.18^{bc}	5.3	0.16	3.41
Slow-soluble	PhyB	500	68.5	3.31	48.8^{b}	2.96^{a}	73.0	2.14	0.98
Slow-soluble	PhyB	1000	76.8	3.71	49.2^{b}	2.99^{ab}	89.3	2.62	0.40
Slow-soluble	PhyE	500	58.0	2.80	52.6^{cd}	3.19^{c}	45.5	1.33	2.02
Slow-soluble	PhyE	1000	66.3	3.20	50.9^{bc}	3.09^{abc}	61.9	1.81	1.40
SEM			1.08	0.055	1.10	0.072	2.21	0.065	0.116
Main effect means									
Fast-soluble			59.8^{a}	3.07^{a}	50.9^{b}	3.41^{b}	51.8^{a}	1.52^{a}	1.76
Slow-soluble			64.4^{b}	3.31^{b}	48.2^{a}	3.08^{a}	55.0^{b}	1.61^{b}	1.64
SEM			0.48	0.024	0.49	0.032	0.99	0.029	0.052
	PC	0	50.3^{a}	3.37^{c}	38.6^{a}	3.11^{a}	2.2^{a}	0.06^{a}	3.54^{e}
	PhyB	500	64.9^{c}	3.14^{b}	50.8^{b}	3.18^{ab}	70.7^{d}	2.07^{d}	1.07^{b}
	PhyB	1000	74.6^{d}	3.60^{d}	51.9^{b}	3.26^{abc}	88.4^{e}	2.59^{e}	0.43^{a}
	PhyE	500	56.4^{b}	2.73^{a}	54.2^{c}	3.40^{c}	42.9^{b}	1.26^{b}	2.03^{d}
	PhyE	1000	64.3^{c}	3.11^{b}	52.3^{bc}	3.28^{bc}	62.9^{c}	1.84^{c}	1.43^{c}
	SEM		0.76	0.039	0.78	0.051	1.56	0.046	0.082
Main effect P and LSD values									
P (Limestone×Diet)			0.43	0.44	0.032	<0.001	0.354	0.354	0.801
LSD (Limestone×Diet)			3.08	0.155	3.13	0.204	6.318	0.185	0.331
P (Limestone)			<0.001	<0.001	<0.001	<0.001	0.028	0.028	0.106
LSD (Limestone)			1.38	0.069	1.40	0.091	2.83	0.083	0.148
P (Diet)			<0.001	<0.001	<0.001	0.004	<0.001	<0.001	<0.001
LSD (Diet)			2.18	0.110	2.21	0.144	4.47	0.131	0.234

[1] Means within a column with different superscript differ (*P*<0.05).

[2] PhyB: *Buttiauxella* phytase expressed in *Trichoderma reesei;* PhyE*: E. coli* phytase expressed in *Pichia pastoris;* Phytase products were added to negative control diet; Fast-soluble: Carbocia, France. GMD<20 µm; Slow-soluble: KWM, Germany. GMD=564 µm.

4. Discussion

Two commercial limestone products were used in the current study. Their analysed Ca content of the FS and SS limestone samples used in the experimental diets was similar (FS 38.2%; SS 37.4%). This was consistent with the value for Ca content reported by the NRC for limestone used in poultry diets (38%; NRC, 1994), although concentrations varying from 36.0 to 41.5% have been reported in the wider literature (Reid and Weber, 1976; Browning and Cowieson, 2014). The differences for *in vitro* solubility and *in vivo* parameters between limestone sources in the present study were mainly related to particle size, and could be related to the limestone composition (e.g. micromineral content, as presented in Table 2), geographical origin or a combination of these factors. The effect of limestone particle size, Ca solubility and its other characteristics on broiler growth performance and other measured parameters in this study could not be determined. However, it was unlikely that the greater micromineral contents in the SS limestone source could explain differences on growth performance, given the small micromineral concentration in limestone and the relatively bigger micromineral content from the vitamin-mineral premix and major feed ingredients.

The rate of *in vitro* solubility of limestone has been reported to be inversely related to its *in vivo* availability in layers, whereby larger limestone particles have a longer transit time in the gizzard of laying hens, thereby increasing *in vivo* Ca solubility and availability (De Witt *et al.,* 2006; Rao and Roland, 1989; Zhang and Coon, 1997). Recently, Anwar *et al.* (2016) demonstrated that a similar scenario existed for broiler chickens. Additional *in vitro* and *in vivo* studies in broilers by Manangi and Coon (2007) demonstrated an interaction between Ca particle size and solubility, which can limit the ability of phytase to hydrolyse phytate and release P for growth and bone mineralisation. These authors found that, compared with larger particle sizes which had low solubility, a smaller particle size (28 µm) with a high solubility (>70%) limited phytate-P hydrolysis by phytase. This, in turn, limited P availability for growth and bone formation, due to increased formation of phytate-Ca complexes. In the current study, the *in vitro* solubility of FS (<22 µm) limestone was evidently higher than that of SS limestone.

A number of recent *in vivo* studies have shown that reducing dietary limestone inclusion (e.g. reducing Ca level in the diet) improves phytase efficacy in broilers, as measured by better growth performance, ileal P and Ca digestibility and IP6 disappearance (Dersjant-Li *et al.,* 2018; Hamdi *et al.,* 2015; Li *et al.*, 2016, 2017). In the current study, SS limestone (GMD 300-600 µm) with a lower Ca solubility, had a clear benefit on growth performance (BWG, FI and FCR) during the starter, grower and overall phases, compared with FS limestone (GMD<22 µm). The greatest percentage improvements in these measures were evident in the starter phase (+2.4%, +1.8% and +6.4% vs FS limestone for each measure for BWG, FI and FCR, respectively). This may be explained because, in the starter phase, Ca requirements are higher and availability could be expected to have greater impact on performance and weight gain. These findings support those of Manangi and Coon (2007) who observed increased weight gains in young broilers (0-28 d) fed limestone particle sizes of 137-88 µm versus those fed particles sizes of 28 µm or 1,306 µm.

The SS limestone improved the AID of P and IP6 disappearance at day 21. In a recent study, Kim *et al.* (2018) reported that feeding fine limestone (GMD<75 µm) increased gizzard pH compared to feeding coarse limestone (GMD=402 µm), because less $CaCO_3$ was dissolved due to the larger particle size with lower surface area. There authors reported that fine limestone reduced AID P compared with coarse limestone in the absence or presence of phytase (Kim *et al.,* 2018). This is in agreement with the current study, whereby ileal IP6 disappearance and P digestibility were increased in SS versus FS limestone diets. Across all treatments, IP6 disappearance increased by 3.2 percentage points in SS versus FS diets. However, no beneficial effect of SS limestone on tibial ash was evident in the present study; and the differences among treatments on d 21 could only be explained by phytase treatment (i.e. source/dose).

The observed decrease in Ca digestibility in the SS limestone treatments was not consistent with the existing literature, and was in direct contrast to recent results from Anwar *et al.* (2016, 2017) who reported increased Ca digestibility among broilers fed coarser, SS limestone compared with fine, FS limestone (1-2 vs <0.05 mm). In the present study, the negative effect of the SS limestone on AID Ca was not observed in the PC diets. The Ca content of the PC diets was similar to those of Anwar *et al.* (2016; 2017), so it was unclear why the two studies produced this different result. The SS limestone used in the current study had a smaller particle size than the one used by Anwar *et al.* (2016; 2017), and different trace mineral content. It was expected that the low *in vitro* Ca solubility of the SS limestone (Figure 1) used in the present study could reduce the Ca available for absorption, thus resulting in a lower Ca digestibility.

The effects of phytase in the experimental diets that contained reductions of P from MCP, Ca, and Na, varied depending on the phytase source and dose-level. The only phytase treatment that maintained BWG, FI and tibia ash equivalent with the PC was PhyB1000; all other phytase treatments produced a lower BWG, FI and/or tibia ash compared to PC. These results confirmed that PhyB1000 compensated the mineral reduction and maintained performance. In addition, for the overall period (d 0-21), PhyB1000 improved FCR versus PC by 0.5%. This suggested an 'extra-phosphoric' effect, i.e. a beneficial effect of the phytase on the digestibility and utilisation of nutrients other than P. Other recent studies have suggested a similar extra-phosphoric effect of *Buttiauxella* phytase (Amerah *et al.*, 2014; Truong *et al.*, 2017). When comparing the two phytases at equivalent dose levels, it was evident that growth performance was superior with PhyB than with PhyE, as well as tibia ash content, AID of P and IP disappearance at day 21, independent of the limestone source.

Differences in efficacy between individual phytase products can be explained by multiple factors, including their intrinsic bioefficacy properties, or by their interaction with other dietary factors, such as limestone particle size and solubility. The intrinsic bioefficacy of a phytase product is dependent upon, amongst other factors, its pH activity profile, kinetic constants for phytate hydrolysis, stability under upper GIT pH conditions, affinity and specificity for phytate and its isomers (Menezes-Blackburn *et al.*, 2015). The upper GIT (i.e. the proventriculus and gizzard) is the primary site of action for most commercial microbial phytase, where the pH is typically around 2.5-3.5 (Selle and Ravindran, 2007). According to Li *et al.* (2016), the average reduction of IP6 in the gizzard by *Buttiauxella* phytase was 44% when supplemented at 500 FTU/kg and 62% when supplemented at 1000 FTU/kg, and ileal IP6 degradation was increased from 20% without added phytase to 86% at 1000 FTU phytase/kg feed (Li *et al.*, 2016). This is in agreement with the findings of the present study where, across limestone sources, ileal IP6 disappearance was 71% and 88% for PhyB, and 43% and 63% for PhyE at dose levels of 500 and 1000 FTU/kg, respectively. These data suggested clear differences in efficacy of the two phytases under the tested conditions. The difference in IP6 degradation rates between the two phytases may be explained by such activity in the upper GIT. Phytases were added to the diets based on the activity measured under standardised conditions at pH 5.5, however, the relative activity in the upper GIT among different phytases differs. Menezes-Blackburn *et al.* (2015) observed that the relative activity of *Buttiauxella* phytase at pH 3 was 235% of the activity at pH 5.5, which could lead to greater phytate degradation rate in the upper GIT, and resulted in greater IP6 disappearance rate at ileum, as observed in the current study.

It is known that limestone can increase the pH in the upper GIT due to its high acid-binding capacity, and an increased crop and gizzard pH may promote Ca, phytate, and P precipitation (complexation), thereby reducing Ca and P digestibility (Selle *et al.,* 2009; Walk *et al.*, 2012). In the present stud, the greater solubility of the fine limestone may have increased the pH in the upper GIT, which could have had a negative effect on IP6 hydrolysis. This could explain why the growth performance of birds fed the FS limestone was reduced compared with birds who received the SS limestone. As shown in Figure 2, IP6 digestibility was positively correlated to the BWG response during the overall period (0-21 d) for each limestone source. With both FS and SS limestone sources, a linear relationship was observed. The slope of both linear equations was significantly different (65 vs 25, for FS and SS limestone; $P<0.001$), indicating a 60 or 25 g BWG increase with each gram of digestible IP6 increase. So, the BWG response to increasing IP6 degradation was greater with FS limestone vs SS limestone. Using PhyB at the higher dose of 1000 FTU/kg, IP6 degradation was 87 and 88% respectively for FS and SS limestone, indicating that the *Buttiauxella* phytase broke down phytate more quickly and efficiently and thereby reduced the negative effect of FS limestone. This may explain why limestone source did not influence bird performance or nutrient digestibility in the diets supplemented with PhyB at 1000 FTU/kg.

In conclusion, a limestone source with larger particle size (300-600 µm diameter) and lower *in vitro* solubility had a beneficial effect on growth performance in starter and grower phases, as well as on ileal P digestibility and IP6 hydrolysis in broilers at 21 d of age when compared to limestone with a small particle size (<22 µm diameter) and high *in vitro* solubility. An interaction was found between limestone source and phytase treatment on performance (BWG and FI) during the grower and overall phases, where FS (vs slow-soluble) limestone reduced BWG for PhyE at both doses, PhyB at 500 FTU/kg but not at 1000 FTU/kg. Across limestone sources, PhyB produced greater overall BWG and FI than PhyE at an equivalent dose level, and, at a dose level of 1000 FTU/kg, the performance of birds supplemented with PhyB was equivalent to (BWG, FI) or greater (FCR) than that recorded for birds fed the PC. The findings suggested that the effects of limestone particle size on phytase efficacy vary with phytase source and dose.

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Conflict of interest

The author Y. Dersjant-Li is an employee of DuPont Animal Nutrition.

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