Modification of a limestone solubility method and potential to correlate with in vivo limestone calcium digestibility

S.-W. Kim,[*] W. Li,[*,1] R. Angel,[*] and P. W. Plumstead[†]

[*]*Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742; and* [†]*Chemunique PTY LTD, 330 Surrey Avenue, Ferndale, Randburg, 2194, South Africa*

ABSTRACT Work was done to modify a limestone solubility assay to improve predictions of in vivo apparent ileal digestibility of Ca (**AID Ca**) in broilers and impacts on AID P. Limestones (**LIME**) were obtained from 3 commercial sources. LIME-1 (0.633 mm mean diameter, **GMD**); LIME-2 (ground sub-sample of LIME-1, GMD = 0.063 mm); LIME-3 (GMD = 0.326 mm), and LIME-4 (GMD = 0.831 mm). Solubility was determined at 5, 15, and 30 min of incubation using either a 0.2 N HCl (**S1**) or a pH 3 HCl (pH = 0.26) solution buffered with 3 M glycine (**S2**) to mimic gizzard and proventriculus pH. An AID trial was conducted with 320 Ross 708 male broilers. Treatments (**Trt**) were no added LIME, or added LIME-1, 2, 3, and 4 to achieve 0.67% Ca, to a basal diet (no added inorganic P, 0.07% Ca) with or without 1,000 U phytase/kg (36 h, 23 to 24 D of age, n = 8, 4 birds/n). Distal ileal digesta was collected from all birds and pooled by pen. Irrespective of interaction, LIME solubilized quicker and more completely with S1 vs. S2 at all time points ($P < 0.05$). LIME-2 solubilized the quickest, while LIME-3 had the lowest solubility through all incubation times ($P < 0.05$). The AID Ca was 66.30, 47.46, 19.93, and 66.33% for LIME-1, 2, 3, and 4, respectively (0 U/kg, $P < 0.05$). Phytase inclusion increased AID Ca by 15% on average ($P < 0.05$). The AID P dig was highest in no LIME added diet (74.91%) and adding LIME reduced ($P < 0.05$) AID P to 23.14, 12.78, 65.47, and 37.40%, for LIME-1, 2, 3, and 4, respectively in the absence of phytase. Regression showed that GMD, 15- and 30-min solubility were critical for AID Ca (R^2 between 0.978 and 0.988). In conclusion, the solubility dynamics including speed and extend of solubilization, rather than a single timepoint, yield better predictions for in vivo Ca digestibility of LIME.

Key words: particle size, calcium and phosphorus digestibility, solubility, phytase, broiler

INTRODUCTION

Calcium (**Ca**) is an indispensable macro mineral needed to support the growth and maintenance functions as well as skeletal development and maintenance in vertebrate animals. Calcium from limestone (**LIME**) is normally added to poultry diets to meet or exceed Ca requirements. Depending on the growth stage and ingredients used, limestone can contribute more than 50% of total analyzable Ca in a broiler diet. However, limestones are not uniform either in source, mineral content, or physical characteristics and considerable variations on particle size and Ca concentration have been

reported (Zhang and Coon, 1997b; Mussini et al., 2019). In a recent survey of 47 US limestone samples commercially available for broilers, the average Ca concentration was 36.58% (range 34.67 to 38.42%) and geometric mean diameter (**GMD**) average of 0.318 mm, with a GMD range from 0.070 to 0.495 mm. Even though different concentrations were detected from various minerals (Mn, Zn, Fe et al.), there was no apparent correlation between the concentration of Ca and other minerals. In addition, the average solubility in an acid solution at 5 min was 59% but ranged from 32 to 91%, demonstrating that some limestones were much more soluble than others (Mussini et al., 2019). It was also noted from the survey that the mine the limestone samples came from, independent of the particle size or its distribution, also influenced the in vitro solubility profile.

In vitro solubility of limestone has been shown to be highly correlated to in vivo solubility and Ca digestibility in laying hens (Rao and Roland, 1989; Zhang and Coon, 1997a). In broiler studies, as limestone particle size increased, in vivo Ca digestibility increased (Anwar et al., 2016a; Kim et al., 2018). In addition,
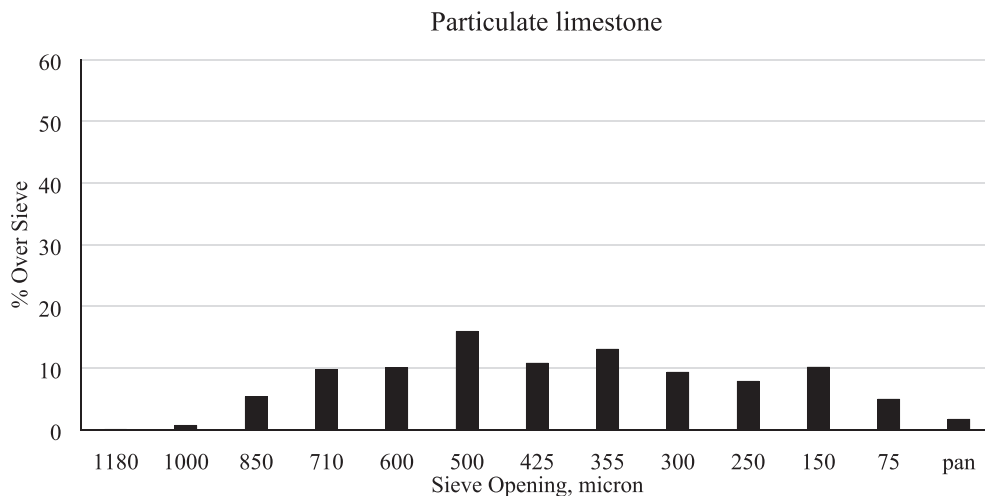
Particulate limestone

Figure 1. Particle size distribution of particulate limestone used in in vitro solubility assay (geometric mean diameter = 0.402 mm, geometric SD = 0.255 mm). Analyzed Ca concentration: 36.5%, analyzed Mg concentration: 0.62%.

highly soluble limestone may also be more detrimental to P digestibility and phytase efficacy. This occurs because highly soluble limestone results in greater amounts of ionizable Ca in solution and thus the potential for greater chelation between ionized Ca and phytic acid in the acidic gizzard and proventriculus (Manangi and Coon, 2007; Kim et al., 2018). Given the variation seen in limestone characteristics and its potential significance on Ca and P digestibility as well as phytase efficacy, an in vitro solubility method that predicts limestone Ca availability and its impact on diet P digestibility would be of great use for nutritionists by allowing them to optimize Ca and P diet formulation.

Several in vitro methods have been published for the determination of limestone solubility. Cheng and Coon (1990) developed an assay based on percentage weight loss and found the proton consumption and Ln(pH) changes were highly correlated with weight loss (R^2 = 0.93 and 0.95, for proton consumption and Ln(pH) change, respectively), suggesting the in vitro solubility measurement could be simplified and predicted by pH changes. Zhang and Conn (1997b) commented that the amount of H^+ (100 mL 0.1 N HCl solution with 2 g sample) could become limiting when highly soluble Ca sources were tested and further optimized the method to increase H^+. They recommended the use of 200 mL 0.2 N HCl solution and a 2 g sample. The procedure has been adopted/modified by several researchers for in vitro Ca source solubility determination (Manangi and Coon, 2007; de Witt et al., 2009; Saunders-Blades et al., 2009; Kim et al., 2018). Nevertheless, 0.2 N HCl solution is a very acidic solution (pH = 0.76) and solubility in the afore mentioned studies was determined at only one-time point (10 min).

We hypothesized that the use of a dynamic model with more than 1 solubility time point and the use of a solution that more closely reflected gizzard conditions (pH 3 and buffered) would result in greater separation between limestones with different solubilities and prediction equations that would more closely describe in vivo digestibilities. Therefore, the objectives of the current study were to: (1) modify an existing in vitro limestone solubility assay (Zhang and Coon, 1997b) to expand assay factors (time and solution type) and (2) determine if the use of a multifactor in vitro limestone solubility system could yield applicable predictions of Ca and P digestibility in vivo for broilers.

MATERIALS AND METHODS

In Vitro Solubility Assay

To determine the solubility differences between very acidic condition (0.2 N HCl) and a pH more relevant to gizzard condition (pH3 buffered), 2 limestone samples from the same origin were used in the solubility assay development. To ensure the 2 samples had the same composition, a large batch of commercially available limestone (defined as particulate, **PAR**) was purchased from Irving Materials, Inc. (#20, IMI Cal Pro, Greenfield, IN). A sub-sample was taken from each 25 kg bag, mixed and sub-sampled for particle size determination. An aliquot of 1 kg of the PAR limestone was carefully sub-sampled, to prepare the pulverized (**PUV**) limestone sample. The 1 kg subsample was ground (IKA, Model 211 basic, Germany) to pass through a 0.075 mm sieve. The GMD, determined by ANSI/ASAE method S319.4 (2008), was 0.402 mm, with 0.255 geometric SD (S_{gw}) for PAR limestone. The GMD of PUV limestone was <0.075 mm as all particles were ground to pass through a 0.075 mm sieve on to the pan. Particle size distribution for the PAR limestone is shown in Figure 1.

Solubility of PAR and PUV samples were determined both in 0.2 N HCl (modified from Zhang and Coon, 1997a) and a pH 3 HCl solution buffered with
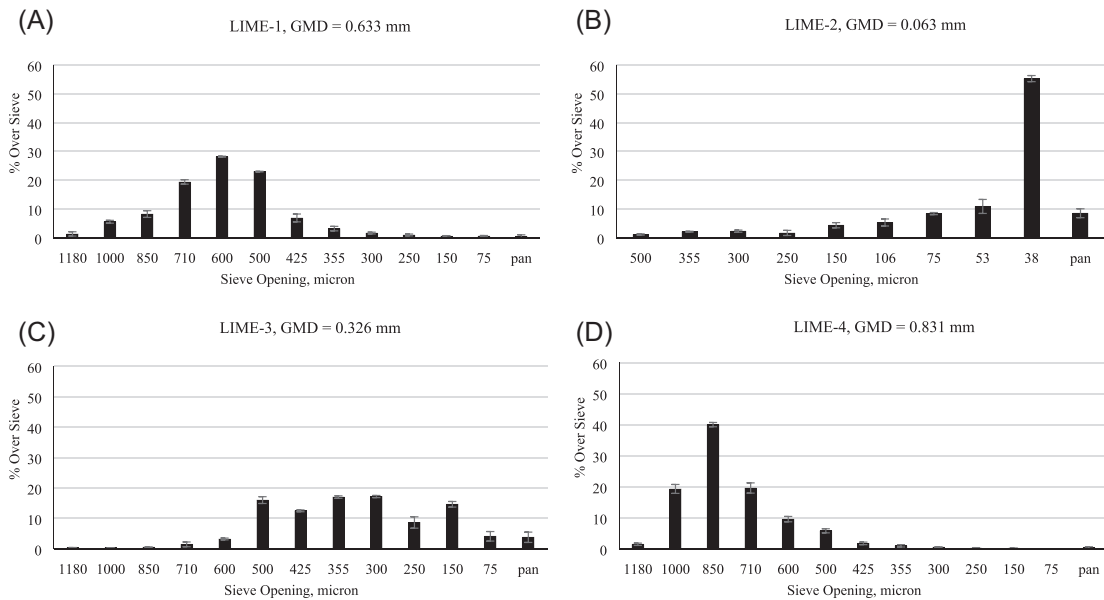
Figure 2. Particle size distribution of the 4 limestones used in the animal trial (mean±SD, n = 3), LIME-1, commercial limestone from US; LIME-2, pulverized from LIME-1, with same chemical characteristic as LIME-1 except particle size; LIME-3 and LIME-4 are commercial limestones from different sources.

3 M glycine (**pH3 Buffered**). The 0.2 N HCl solution was prepared with deionized distilled water **(dd)** (Zhang and Coon, 1997b) and stored until use. The pH3 Buffered solution was prepared by weighing 225.2 g glycine (VWR Life Science, Cat#: 97061-132, Purity ≥ 99%) into a 1 L Erlenmeyer flask and dissolving in 500 mL of dd water until all glycine was solubilized. After which 50 mL concentrated HCl (12.1 N, 37.1% v/v, specific gravity 1.19) were added with 430 mL dd water. The concentrated HCl was used to adjust the pH of the solution until a pH of 3 was reached and dd water was added to 1,000 mL. This solution was stored at 4°C until used.

To determine limestone solubility, a 1 g representative sample was weighed into a 250 mL Erlenmeyer flask. The flask was placed into a 42°C shaking water bath for 10 min and 138 mL of pre-warmed (42°C) 0.2 N HCl or pH3 Buffered solution was added into the flask that was then shaken vigorously, to maximize mixing without losing solution, for 1, 3, 5, 10, 20, and 30 min. Digestion was stopped by adding 100 mL ice-cold dd water into the flask and immediately pouring all content (liquid and remaining limestone) through a vacuum filtering system using a pre-weighed and pre-labelled Whatman No.40 filter (Whatman, GE Healthcare Bio-Sciences, Pittsburg, PA). Additional ice-cold dd water was added as needed to flush any remaining pieces of limestone in the Erlenmeyer flask. The filter was placed in a pre-weighed, pre-labelled aluminum weigh pan and then dried at 100°C for a minimum of 8 h. The assay was done in triplicate for every time point. Solubility at every time point was determined by weight loss using the calculation below:

$$
\begin{aligned}
&\text{Solubility of limestone} \quad (\%) \\
&= \left(1 - \frac{\text{dried remaining limestone}}{\text{dry initial limestone}}\right) \times 100
\end{aligned}
$$

Animal Trial

To determine correlations between in vitro limestone solubility and in vivo Ca digestibility of the limestone, a total of 3 limestone sources were obtained from various locations (Figure 2). LIME-1 was purchased from the US, with a GMD of 0.633 mm; LIME-2 was an aliquot sub-sample of LIME-1 ground to pass through a 0.075 mm sieve (GMD = 0.063 mm); LIME-3 (GMD = 0.326 mm) and LIME-4 (GMD = 0.831 mm) were obtained from commercial sources in South America. Particle size and distributions of the 4 limestone samples were determined by ANSI/ASAE method S319.4 (2008; Figure 2). The in vitro solubility of the 4 limestone samples was determined, using both 0.2 N HCl and pH3 Buffered solution at 5, 15, and 30 min.

All animal care procedures were approved by the University of Maryland Animal Care and Use Committee. Ross 708 male broilers were purchased from a local hatchery. On arrival, birds were placed in floor pens within temperature and light controlled rooms and allowed ad libitum access to feed and water. Birds were fed corn-SBM based pre-starter and starter diets from hatch to 8 and 9 to 22 D of age, respectively (Table 1) that met or exceeded all NRC (National

Table 1. PreStarter and starter diets fed prior to digestibility trials. Ingredients and nutrient composition used in animal trial.

Ingredients, %	PreStarter (0 to 8 D)	Starter (9 to 21 D)
Corn	54.43	59.81
SBM (47%)	38.01	33.57
Soy oil	2.92	3.72
Mono calcium phosphate (16.6% Ca, 21.7% P)	1.70	0.81
Limestone (38.5% Ca) (0.402 mm GMD)	1.59	0.91
NaCl	0.48	0.36
DL methionine	0.29	0.29
L-lysine	0.12	0.18
L-threonine	0.09	0.07
Choline chloride, 60%	0.16	0.05
Mineral premix[1]	0.08	0.08
Vitamin premix[2]	0.08	0.08
Coban 90	0.05	0.05
Total	100.00	100.00
Nutrient content, formulated (Analyzed)		
Crude protein, %	23.00 (23.1)	21.01 (21.22)
Ether extract, %	5.13 (5.01)	6.21 (6.09)
MEn, kcal/kg	3,000	3,120
dLys, %	1.23	1.15
Total Lys, %	1.32 (1.34)	1.26 (1.27)
dMet,%	0.59	0.58
dThr, %	0.84	0.75
dTSAA, %	0.92	0.86
dTrp, %	0.25	0.22
dIle	0.87	0.79
dVal	0.95	0.86
dArg	1.41	1.28
Ca, %	1.00 (1.05)	0.7 (0.74)
P, %	0.77 (0.78)	0.57 (0.57)
Non-phytate P (nPP), %	0.50 [0.50][3]	0.34 [0.33][3]
Phytate P, %	0.27 (0.28)	0.24 (0.25)

[1]Mineral premix supplied per kg of diet: zinc from zinc sulfate, 80 mg; manganese from manganese sulfate, 100 mg; iron from iron sulfate, 20 mg; copper from copper sulfate, 3 mg; iodine from calcium iodate, 3.9 mg; selenium from selenium sulfate, 0.3 mg.

[2]Vitamin premix supplied per kg of diet: vitamin A, 15,111 IU; vitamin D, 5,333 IU; vitamin E, 53.33 IU; vitamin B_{12}, 26.66 mg; riboflavin, 17.78 mg; niacin, 71.11 mg; pantothenic acid, 24.89 mg; vitamin K_3, 3.2 mg; folic acid, 2.13 mg; biotin, 0.142 mg; thiamine, 4.44 mg; pyridoxine, 6.22 mg.

[3]Non-phytate P was calculated by the difference between formulated (analysed) total P and phytate P.

Table 2. Basal mixes, ingredients, and nutrient composition for animal trial.

Ingredient	Basal
Corn	51.00
SBM	25.80
Jasmine rice	15.10
Celite	1.08
Soy oil	2.30
Mineral premix[1]	0.08
Vitamin premix[2]	0.08
NaCl	0.59
Choline chloride, 60%	0.20
Limestone and/or celite[3]	1.77
TiO_2 premix/corn[4]	2.00
Total	100.00
Nutrient content, formulated (Analyzed)	
Crude protein, (CP) %	16.88 (17.01)
MEn, kcal/kg	3062
Ca, %[5]	0.05 (0.06)
P, %	0.324 (0.301)
Phytate P[6], %	0.23 (0.22)

[1]Mineral premix supplied per kg of diet: zinc from zinc sulfate, 80 mg; manganese from manganese sulfate, 100 mg; iron from iron sulfate, 20 mg; copper from copper sulfate, 3 mg; iodine from calcium iodate, 3.9 mg; selenium from selenium sulfate, 0.3 mg.

[2]Vitamin premix supplied per kg of diet: vitamin A, 15,111 IU; vitamin D, 5,333 IU; vitamin E, 53.33 IU; vitamin B_{12}, 26.66 mg; riboflavin, 17.78 mg; niacin, 71.11 mg; pantothenic acid, 24.89 mg; vitamin K_3, 3.2 mg; folic acid, 2.13 mg; biotin, 0.142 mg; thiamine, 4.44 mg; pyridoxine, 6.22 mg.

[3]Celite and/or limestones were added at a concentration of 1.772% to the basal diet at the same inclusion level depending on treatment. Limestones were added to achieve 0.67% Ca.

[4]Titanium dioxide/corn mix contained 70% corn and 30% titanium dioxide (TiO_2). This mix was ground together 3 times through a small hammer mill without screen to remove any clumps of TiO_2 and to maximize mix ability of TiO_2 into complete diet.

[5]See Table 3 for analysed values.

[6]Analysed phytic acid x 28.18% = phytate P.

Research Council, 1994) recommendations as well as average nutrient use concentrations in the US for 2015 (AgriStats end of year summary, 2015). On day 22, 320 birds were grouped (4 birds/cage), such that between cage weight as well as within cage bird weight variations were minimized, and moved to battery cages (Petersime Incubator Co., Gettysburg, OH) previously assigned to treatments (**Trt**) randomly. Birds were fed the same starter diet and allowed to adjust to cages for 24 h, after which the experimental diets were fed ad libitum for 36 h (from 23 to 24 D of age) to avoid physiological adaptation to P deficient or Ca and P imbalanced diet (Li et al., 2014). Similar procedure (24 to 48 h digestibility trial) has also been used in studies reported by Li et al. (2017), Kim et al. (2018), and Tamim and Angel (2003). Each Trt was replicated 8 times with 4 replicate cages in each of 2 battery cage rooms. The room was considered a block. The wire-floored battery cages (width × depth × height; 99.7 cm × 68.6 cm × 29.2 cm) were equipped with a water nipple system (2 nipples per cage) and 2 feed troughs (width × depth × height; 63.5 cm × 8.9 cm × 5.67 cm). Photoperiod was 24 light (**L**):0 dark (**D**) from hatch to 3 D, 14L:10D from 4 to 7 D, 16L:8D from 8 to 12 D, and 18L:6D from 13 to 24 D of age. Room temperature was kept at an average of 31°C from hatch to 3 D and was lowered by 1°C every 2 to 3 D up to 21°C and maintained until 24 D. Mortality was checked twice a day.

A corn, SBM, and white rice basal diet (Table 2) was mixed in 1 batch to prepare a low phytate P diet (0.23% phytate P), which maximum inhibition on P digestibility can be expected with highly soluble limestone based on previous studies conducted in our lab (Li et al., 2017; Kim et al., 2018). The basal was subdivided equally into 5 batches and represented 98.4% of all final experimental diets. Based on the analyzed Ca and P concentrations in the basal diet, pre-analyzed limestone was added to the basal to achieve the desired Ca concentrations in the final diets. Celite (Celite, Celite Corp, Santa Barbara, CA) was included as a filler to achieve 100% of the final diet. Titanium dioxide (**TiO_2**) was added as an inert marker at 0.6%. The final dietary arrangement consisted of 10 Trt, of which 8 Trts resulted from a 4 limestone samples × 2 phytase dose (0

Table 3. Formulated (**FML**) and analyzed (**ANA**) calcium (**Ca**), phytase activity, and limestone information in treatment (**Trt**) diets (as is basis).[1]

Diet Ca, %			Limestone sample						Phytase, FTU/kg	
FML	ANA	% added	Source[2]	Ca, %	Mg, %	Fe, ppm	GMD[3], mm	S_{gw}[4], mm	FML	ANA
0.07	0.07	0	–	–	–	–	–	–	0	<50
0.07	0.06	0	–	–	–	–	–	–	1,000	1,037
0.67	0.66	1.6	LIME-1	37.22	0.60	547	0.633	0.235	0	<50
0.67	0.66	1.6	LIME-1	37.22	0.60	547	0.633	0.235	1,000	1021
0.67	0.67	1.6	LIME-2	37.22	0.60	547	0.063	0.043	0	<50
0.67	0.66	1.6	LIME-2	37.22	0.60	547	0.063	0.043	1,000	1038
0.67	0.63	1.6	LIME-3	35.03	1.19	142	0.326	0.197	0	<50
0.67	0.62	1.6	LIME-3	35.03	1.19	142	0.326	0.197	1,000	1070
0.67	0.69	1.6	LIME-4	37.40	0.68	96	0.831	0.253	0	<50
0.67	0.67	1.6	LIME-4	37.40	0.68	96	0.831	0.253	1,000	1087

[1]Diet Ca, and phytase concentrations for each treatment were analysed in triplicate. All diets analysed 0.226% phytate P and 0.319% P. No inorganic P was added.

[2]No limestone was added in 0.07% Ca diets. LIME-1, commercial limestone from US; LIME-2, pulverized from LIME-1, with same chemical characteristic as LIME-1 except particle size; LIME-3 and LIME-4 are 2 limestones from different sources.

[3]Geometric mean diameters.

[4]Geometric standard deviation.

and 1,000 U/kg) factorial arrangement, plus the basal with no added LIME with or without 1,000 U/kg phytase (Table 3). To prepare the final diets with phytase, 1 batch of the previously aliquoted basal was used to make each limestone diet and divided into 2 equal lots to minimize potential variability. A 6-phytase,[1] from *Buttiauxella* sp., expressed in *Trichoderma reesei*, was then added on top, at 0, and 1,000 FTU/kg (based on pre-analyzed batch phytase concentration), to 1 of the 2 lots and mixed so that the only difference among those 2 lots was the phytase concentration.

Weight gain and feed consumption were determined by cage for the 36 h trial. At the end of the 36 h trial, birds were anesthetized with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2 (2 to 3 min) and then euthanized with CO_2 (2 to 3 min). The distal half of the ileum was immediately removed (last half of the segment encompassed between the Meckel's diverticulum to 3 cm above the ileocecal junction). The content was gently squeezed by flushing with ice-cold dd water. Distal ileal contents were pooled by cage, frozen at −20°C, and freeze dried. Dried ileal samples were ground using a mortar and pestle to pass through a 0.25 mm sieve and stored in airtight containers at 4°C until analyzed.

Diet samples were ground to pass through a 0.5 mm screen before analysis. Diet and ileal samples were analyzed in duplicate except where specified otherwise. Dry matter was determined by drying overnight 100°C (Shreve et al., 2006), Ca and P were determined, in triplicate, after acid digestion and analyzed using inductively coupled plasma atomic emission spectrometry (ICP-AES; AOAC, 1999). Titanium (**Ti**) concentration was determined by a colorimetric method adopted from Short et al. (1996) where samples were first ashed at 580°C and then digested in 7.4 M H_2SO_4. Crude protein and ether extract in the basal diet were analyzed, according to AOAC methods 990.03 (2003) and

AOAC methods 920.39 (2003), respectively. Basal diet phytic acid concentration was analyzed according to the method described by Skoglund et al. (1997, 1998). A conversion factor of 28.18% was used to calculate phytate P from analyzed phytic acid concentration. Phytase activity in all Trt diets were determined in duplicate according to the ISO 30024 (2009) procedure where one phytase unit (**FTU**) is the amount of enzyme that releases 1μmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37°C.

The apparent ileal digestibility (**AID**) of nutrients was calculated based on the following formula using TiO_2 as the inert marker (**Ti**):

$$AID = \frac{(Nutrient/Ti)_d - (Nutrient/Ti)_i}{(Nutrient/Ti)_d} \times 100\%,$$

where $(Nutrient/Ti)_d$ is the ratio of nutrient (Ca and P) to Ti in the diet and $(Nutrient/Ti)_i$ is the ratio of nutrient (Ca, P) to Ti in the ileal contents.

Statistical Analysis

Digestibility and in vitro solubility data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., 2014). The in vitro solubility results were analyzed as a factorial design with limestone sample and solution type being the 2 factors. The effect of time point on solubility was also determined within each limestone sample and solution type using repeated measure. For in vivo digestibility, limestone samples, phytase dose and block (room) were considered as fixed effects whereas cage within a block (room) as a random effect. Tukey's test (Tukey, 1949) was used to separate means when the model was significant for both in vitro and in vivo results. Significance was declared at $P < 0.05$. Pearson correlation, separated by
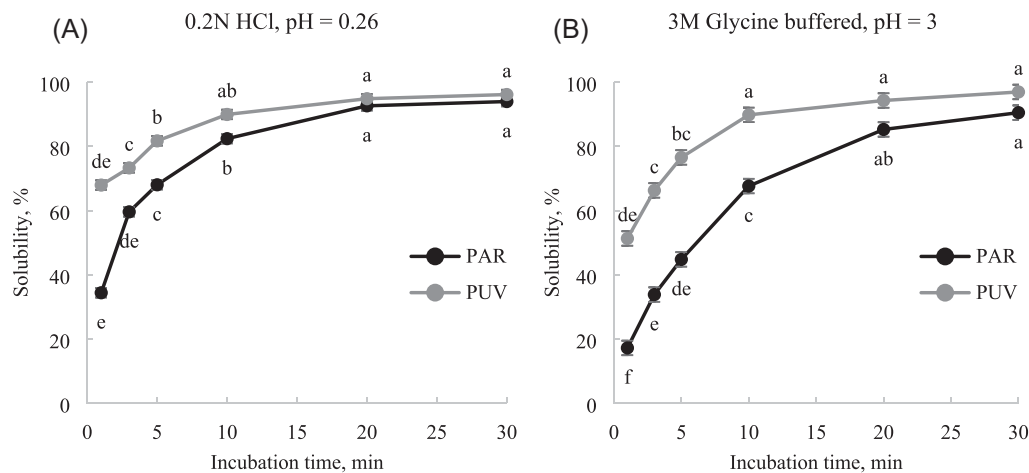
Figure 3. In vitro limestone solubility of 2 limestones with the same origin but different particle sizes in 2 different solutions, (A) 0.2 N HCl and (B) pH3 buffered solution (mean ± SEM). Measured starting pH was 0.26 and 3, for 0.2 N HCl and pH3 buffered solution, respectively. [a-f] Data points with different superscript letters differ ($P < 0.05$). PAR: particulate limestone, GMD = 0.402 nm; PUV: pulverized limestone, GMD < 0.075 nm, ground sample from PAR.

phytase (0 or 1,000 FTU/kg), was applied to illustrate the correlation between in vitro solubility and in vitro digestibility. Furthermore, a stepwise regression model selection (GLMSELECT) approach was used (SAS Institute Inc., 2014) to determine the most appropriate regression model to predict in vivo Ca and P digestibility from in vitro solubility results.

RESULTS AND DISCUSSION

In Vitro Assays and Limestone Solubilities

The in vitro solubility assay from Zhang and Coon (1997b) has been well adopted by the industry due to its ease of use. Because this assay uses a high concentration of HCl (0.2 N) to provide sufficient H$^+$ to neutralize Ca^{2+}, the pH on commencement of the assay is very acidic and much lower than values seen in the gizzard (Kim et al., 2018). The work described in the current trial was done to determine if the use of a more physiological relevant pH, by buffering at a 3 pH to maintain a stable pH would improve correlations between in vitro limestone solubility and in vivo Ca digestibility. In addition, different time points (from 1, 5, 15, 30, and 60 min to 120 min of incubation, data shown to 30 min) were used initially to determine the dynamics of different limestones and the most relevant time points that would yield the greatest separation between limestones were selected (Figure 3). Based on the initial observations, a 30-min incubation length was chosen for "broiler type" limestone solubility test, with intermediate solubility measurements at 5 and 15 min.

The PAR and PUV limestone samples were incubated in both solutions and solubility determined at different time points. In general, using pH3 Buffered solution yielded similar solubility dynamics as compared to 0.2 N HCl solution for both limestone samples (Figure 3). Solubility of PUV limestone was 68

and 96% at 1 and 30 min, respectively when using 0.2 N HCl solution, whereas the solubility was 51 and 97%, respectively at 1 and 30 min in pH3 Buffered solution. In addition, there was greater separation of solubilities between the 2 limestone samples at the 5 and 15 min ($P < 0.05$) time points with pH3 Buffered solution. The pH3 Buffered solution resulted in greater solubility separation between different limestone samples compared to the 0.2 N HCl solution.

Irrespective of solution used, limestone solubility was distinctively different at 5 min, with LIME-3 and LIME-2 having the lowest and highest solubility, respectively ($P < 0.05$, Table 4). The interaction between limestone sample and solution type was only significant at 5 min. At 30 min, there was no difference in solubility among LIME-1 (92.65%), LIME-2 (93.98%), or LIME-4 (92.01%), across the 2 solutions ($P > 0.05$).

The GMD or particle size of limestone, more often than other factors, has been suggested to be highly correlated with in vitro solubility (Guinotte et al., 1991; Zhang and Coon, 1997b; Saunders-Blades et al., 2009). The significance of GMD was also reinforced in the current study. LIME-1 and 2 were from the same origin and batch with the only difference being GMD. With reduced particle size, LIME-2 (GMD = 0.063 mm) solubilization in 0.2 N HCl occurred faster ($P < 0.01$) as compared to LIME-1 (GMD = 0.633 mm, 96.71 vs. 71.62%, respectively) at 5 min, even though their 30-min solubility did not differ (main effect means, 92.65% and 93.98% for LIME-1 and 2, respectively, $P > 0.05$).

The in vitro results showed that GMD alone did not adequately explain observed differences in limestone solubility. For example, the average particle size of LIME-3 (GMD = 0.326 mm) was smaller as compared to LIME-1 (GMD = 0.633 mm) and 4 (GMD = 0.831 mm). If GMDs were the only factor affecting solubility, speed (5 min) of solubilization would be higher

Table 4. In vitro solubility profile of limestones using different solubility assays (n = 3).

Solution	Limestone[1]	Particle size GMD, mm	Solubility, % 5 min	15 min	30 min	Time point SEM[2]	Time point P-value
0.2 N HCl	LIME-1	0.633	71.62[c,z]	89.95[y]	94.73[x]	0.766	<0.001
	LIME-2	0.063	96.71[a]	96.78	97.30	0.481	0.673
	LIME-3	0.326	20.58[f,z]	43.33[y]	63.25[x]	0.952	<0.001
	LIME-4	0.831	42.10[d,z]	72.26[y]	93.98[x]	0.960	<0.001
pH3 Buffered	LIME-1	0.633	67.75[c,z]	81.76[y]	90.57[x]	1.208	0.002
	LIME-2	0.063	81.61[b,y]	90.73[x]	90.67[x]	0.671	0.003
	LIME-3	0.326	13.65[g,z]	36.08[y]	53.96[x]	0.675	<0.001
	LIME-4	0.831	36.02[e,z]	64.57[y]	90.04[x]	0.802	<0.001
SEM			0.693	0.955	0.852		
			Main effect[2]				
Solution	0.2 N HCl		–	75.58[a]	87.31[a]		
	pH3 Buffered		–	68.28[b]	81.31[b]		
	SEM			0.426	0.477		
Limestone	LIME-1		–	85.86[b]	92.65[a]		
	LIME-2		–	93.75[a]	93.98[a]		
	LIME-3		–	39.70[d]	58.60[b]		
	LIME-4		–	68.41[c]	92.01[a]		
	SEM			0.603	0.675		
P-values							
	Solution		<0.001	<0.001	<0.001		
	Limestone		<0.001	<0.001	<0.001		
	Interaction		<0.001	0.648	0.071		

[1]LIME-1, commercial limestone from US; LIME-2, pulverized from LIME-1, with same chemical characteristic as LIME-1 except particle size; LIME-3 and LIME-4 are 2 testing limestones from different sources.

[2]Main effect within a time point was only compared when interaction was not significant. Treatment means were only compared when interaction was significant ($P < 0.05$).

[a–d]Means within a column with different superscript letters differ ($P < 0.05$).

[x,y,z]Means within a row with different superscript letters differ ($P < 0.05$).

for LIME-1 vs 4, but the opposite was true as seen from the current in vitro results. The low solubility of LIME-3 cannot be explained by the particle size or distribution, since more than 90% of the particles were smaller than 0.600 mm, whereas only 37 and 10% of the particles were smaller than 0.600 mm for LIME-1 and 4, respectively. This lack of correlation among limestone particle size, its distribution, and solubility suggested that intrinsic characteristics of limestone which is primarily determined by the geological origin and physical properties of the rock is critical in determining limestone solubility. In addition, even though Mg did not seem to impact solubility for LIME-1, 2, and 4 when its concentration was lower than 1.0%, the low solubility seen in LIME-3 suggests that it might interfere the limestone solubility when the concentration at higher concentration.

Impact of Limestone Particle Size and Origin on Apparent In Vivo Ca and P Digestibility

Analyzed dietary Ca concentrations were all close to formulated values (Table 3). Phytase activities in non-phytase Trt were all below the detection limit of the assay, demonstrating no cross-contamination between phytase and non-phytase Trt during mixing. Analyzed phytase activities in phytase Trt were close to formulated within 10% of formulated values (Table 3).

The 3 limestones originated from various locations and differed in GMD or particle size distribution, as well as Ca concentration. Analyzed limestone Ca concentration was 37.22, 35.03, and 37.40% for LIME-1 (and 2), 3, and 4, respectively (Table 3). By the chemical formula, pure $CaCO_3$ should contain 40.15% Ca. However, impurities with other trace and/or macro minerals are not uncommon in feed grade limestones (Mussini et al., 2019). Of the 3 limestone samples, the highest Ca concentration was seen in LIME-4 (37.40%), whereas LIME-3 was the lowest (35.03% Ca). Magnesium content was highest in LIME-3 (1.19%), which coincided with its lower Ca concentration. Sa and Boyd (2017) examined different agriculture grade limestone samples and found Mg content could be as high as 12.2% (19.8% Ca) and was negatively correlated with Ca concentration ($R^2 = 0.98$). Other trace minerals, such as Fe, S, Zn, and Cu can also be found in limestone samples (Robinson, 1980; Anwar et al., 2016b).

There was no interaction between limestone and phytase on AID Ca. Dietary AID Ca in birds fed diets containing no limestone without added phytase was 19.51%, increasing to 66.30, 47.46, 19.93, and 66.33%, in diets with LIME-1, 2, 3, and 4, respectively ($P < 0.05$, Table 5). Phytase inclusion at 1,000 FTU/kg increased AID Ca by an average of 6.7 percentage points regardless of dietary Ca concentration or limestone samples ($P < 0.05$). There were large differences ($P < 0.05$) in AID Ca in diets containing the different limestones, even though all limestone samples were added to the same

Table 5. Apparent Ileal Ca and P digestibility of broilers fed diets containing 0 or 0.6% Ca from different limestones from 22 to 23 D of age (n = 8).

Phytase	Limestone[1]	GMD, mm	Apparent digestibility, %	
			Ca	P
0 FTU/kg	No limestone	–	19.51	74.91[b]
	LIME-1	0.633	66.30	23.14[f]
	LIME-2	0.063	47.46	12.78[g]
	LIME-3	0.326	19.93	65.47[c]
	LIME-4	0.831	66.33	37.40[e]
1,000 FTU/kg	No limestone	–	26.64	89.32[a]
	LIME-1	0.633	72.07	66.04[c]
	LIME-2	0.063	55.32	45.76[d]
	LIME-3	0.326	28.44	89.57[a]
	LIME-4	0.831	70.61	90.70[a]
	SEM		0.843	0.806
	Main effect[2]			
Limestone	No limestone		23.07[c]	–
	LIME-1		69.19[a]	–
	LIME-2		51.39[b]	–
	LIME-3		24.19[c]	–
	LIME-4		68.47[a]	–
	SEM		0.596	
Phytase	0 FTU/kg		43.90[b]	–
	1,000 FTU/kg		50.62[a]	–
	SEM		0.377	
P-values				
	Limestone		<0.001	<0.001
	Phytase		<0.001	<0.001
	Interaction		0.106	<0.001

[1]LIME-1, commercial limestone from US; LIME-2, pulverized from LIME-1, with same chemical characteristic as LIME-1 except particle size; LIME-3 and LIME-4 are 2 testing limestones from different sources.

[2]Main effect within a time point was only compared when interaction was not significant. Treatment means were only compared when interaction was significant ($P < 0.05$).

[a–g]Means within a row with different superscript letters differ ($P < 0.05$).

batch of basal diet to reach 0.67% total Ca (analyzed 0.62 to 0.69%). The relationship between limestone particle size, in vitro solubility and AID Ca has been examined extensively with a focus on laying type poultry (laying hen, breeders) rather than meat type chickens (Guinotte et al., 1991; Zhang and Coon, 1997a,b; de Witt et al., 2009). Increasing limestone GMD has been associated with decreased in vitro solubility and increased in vivo Ca digestibility in laying hens (Zhang and Coon, 1997b; de Witt et al. 2009). Similar results were also reported in broiler chickens (Anwar et al., 2016a, Kim et al., 2018) when 2 limestone samples with the same origin but different GMD were compared. In the current trial, a similar effect of GMD was observed, where LIME-1 (GMD = 0.633 mm) and LIME-2 (GMD = 0.063 mm) from the same batch of limestone, but 2 particle sizes, differed in AID Ca. The smaller GMD limestone (LIME-2 GMD = 0.063 mm) had 26% lower AID Ca than that of LIME-1 (GMD = 0.633 mm, $P < 0.05$).

Calcium from limestone has traditionally been considered as a high available Ca source and variations of limestone Ca availability have been rarely taken into account when formulating poultry diets. Several recent

publications agreed with the observations seen in the current study that there were considerable variations in limestone Ca digestibility between sources of limestone (Saunders-Blades et al., 2009; Anwar et al., 2016a,b; Li et al., 2017; Kim et al., 2018). From the results of the current work, GMD alone can only explain less than 40% of (values presented in Table 8) variation in Ca availability. For example, the different AID Ca seen in birds fed diets containing LIME-1, LIME-3, or LIME-4, suggested other factors, such as the mine of origin, type of rock (geology) and the physical and chemical characteristics of the rock may also play an important role in how much Ca will be digested from these sources, by poultry.

In the absence of phytase, AID P was higher when no limestone was added (74.91%) as compared to AID P in birds fed limestone containing diets (Table 5, $P < 0.05$). This digestibility value of P was comparable to other published values that used similar procedures and corn/soy-based diets with no added limestone (Tamim et al., 2004; Kim et al., 2018), and confirms that in the absence of Ca from limestone and in very low Ca diets, phytate P from corn and soy is well utilized by broiler birds.

The detrimental effect of Ca on AID P has been well established (Sebastian et al., 1996; Plumstead et al., 2008; Anwar et al., 2016a; Kim et al., 2018; Li et al., 2018) but it is important to note that all this work was done using limestone as the Ca source. The AID P of the corn soy basal diet, in the absence of phytase were 23.14, 12.78, 65.47, and 37.40%, respectively, when LIME-1, 2, 3, and 4 were added (Table 5; $P < 0.05$). However, the degree of AID P reduction due to limestone inclusion vs. that seen in the basal diet without added limestone, differed between limestones. The biggest reduction on AID P was seen when LIME-2 was added to the basal diet, an 83% lower AID P than that of birds fed the no added limestone basal diet ($P < 0.05$) without phytase. Similarly, Kim et al. (2018) reported that fine particle size limestone (< 0.075 mm) which solubilized more completely within 10 min, was more detrimental to P digestibility as compared with the same limestone but with greater GMD (0.402 mm). On the other hand, LIME-3, with the lowest in vitro solubility at any given time point and AID Ca, had the smallest impact on AID P (65.47 vs. 74.91% in no LIME trt) when added to the basal corn soy diet.

When phytase (1,000 U phytase/kg) was added to the basal diet, AID P increased irrespective of Ca concentration or limestone source (Table 5). The AID P was 89.32, 66.04, 45.76, 89.57, and 90.70% for birds fed the basal diet containing no added LIME, LIME-1, 2, 3, and 4, respectively ($P < 0.05$). Noticeably, the AID P in birds fed diets containing either LIME-3 or 4 were not different from the birds fed no added LIME diet in the presence of phytase which was around 90%. Similar responses of AID P to phytase have been reported by Li et al. (2017), where 90% of phytate degradation

Table 6. Pearson correlation among in vitro solubility assay time point, pH, limestone particle size, and in vivo Ca and P digestibility.

	P digestibility	GMD	pH3 Buffered solution solubility[2]			0.2 N HCl solution solubility[3]		
			5	15	30	5[2]	15	30
			Phytase = 0 FTU/kg					
Ca digestibility	0.613[1]	−0.046	0.705	0.786	0.820	0.716	0.780	0.841
P digestibility		0.626	0.494	0.645	0.897	0.411	0.668	0.859
GMD			−0.346	−0.180	0.246	−0.444	−0.147	0.167
P-value								
Ca digestibility	<0.001	<0.001	0.005	<0.001	<0.001	0.022	<0.001	<0.001
P digestibility		0.804	<.0001	<0.001	<0.001	<0.001	<0.001	<0.001
GMD			0.056	0.332	0.183	0.012	0.429	0.368
			Phytase = 1,000 FTU/kg					
Ca digestibility	0.235	−0.569	0.862	0.790	0.512	0.887	0.777	0.563
P digestibility		0.586	0.564	0.701	0.915	0.485	0.722	0.884
GMD			−0.314	−0.149	0.259	−0.411	−0.117	0.185
P-value								
Ca digestibility	0.196	<0.001	0.001	<0.001	<0.001	0.005	<0.001	<0.001
P digestibility		0.001	<0.001	<0.001	0.003	<0.001	<0.001	0.001
GMD			0.080	0.415	0.153	0.020	0.522	0.312

[1]Denotes the strength of a linear association between 2 variables. Values closer to −1 or 1 indicate greater negative or positive linear association, respectively.

[2]3 pH Buffered solution solubility_5, 15, 30, limestone solubility measured at 5, 15, or 30 min, respectively, for limestone incubated in pH3 Buffered solution at starting pH = 3.

[3]5, 15, 30, limestone solubility measured at 5, 15, or 30 min, respectively, for limestone incubated in 0.2 N HCl solution at starting pH = 0.26.

was reported with 1,000 U/kg, *Buttiauxella* spp. in a low phytate diet, with no differences seen between 0.7 and 1.0% Ca treatments. In an earlier trial conducted by Tamim et al. (2004), it was reported that detrimental impact of Ca could be significantly reduced even with 500 FTU/kg first generation phytase. These studies suggested that maximum phytate degradation can be achieved if phytase dose can be used accordingly based on Ca source property and dietary phytate concentrations (Kim et al., 2018).

Nevertheless, the lack of apparent correlation between in vivo AID Ca or P and limestone GMD suggests that other factors such as geological origin, chemical and physical characteristics as well as particle size distribution are potentially also important to the availability of Ca from limestone and its impact on P digestibility. To date, most work has focused on either source/origin alone with or without processing all samples to similar particle size and distribution, or examining the particle size alone without considering origin, or particle size distribution differences (Guinotte et al., 1991; Ajakaiye et al, 1997; Manangi and Coon, 2007). From the current study, limestones have very different characteristics that resulted in different responses in vivo. Therefore, when examining the particle size effect of limestone, samples should be obtained from the same origin and batch otherwise the geological factors may confound the findings for particle size, and vice versa.

Correlation Between In Vitro Solubility and In Vivo Digestibility

Pearson correlation was used to illustrate the relationship between in vitro solubility and in vivo digestibility (Table 6). Because the efficacy of phytase

on Ca and P digestibility was a function of both dose and specific activity of phytase, the relationship may differ depending on type of phytase and its inclusion level. Therefore, it was more appropriate to separate the correlation by phytase addition. The GMD was correlated to 5 min but not to 15 or 30 min solubility in the presence and absence of phytase with 0.2 N HCl solution ($P < 0.05$). In addition, irrespective of solution (0.2 N HCl or pH3 Buffered) or phytase, solubilities at all time points were all significantly correlated to both AID Ca and P even though it was not clear based on the Pearson correlation alone, which time point was most important.

To better understand the correlations, solubility at different time points, GMD and their interactions were tested against AID Ca or P by GLMSELECT. Because of the potential collinearity among solubilities at different time points, values of variation inflation (**VIF**) were also evaluated. Using the stepwise selection, in combination with the adjusted-R^2, VIF and Akaike information criterion, the final models are listed in Table 7. In order to compare the differences between using multiple and single time point, the overall adjusted-R^2 and Root MSE of using a single parameter to predict AID Ca and P are also listed in Table 8. When using multiple time points, the prediction models were similar between the 2 solutions, both in the presence and absence of phytase, even though pH3 Buffered yielded slightly better adjusted-R^2 and smaller Root MSE as compared to 0.2 N HCl solution.

The GMD alone in almost all cases, had the lowest R^2 (0.0009 to 0.3885) and highest Root MSE (14.814 to 19.732), suggesting it is not an ideal parameter for predicting AID Ca or P in vivo. In addition, the variation (adjusted-R^2) explained by using single time points was overall poorer than using multiple time points

Table 7. Prediction models for ileal Ca and P apparent digestibilities based on in vitro limestone solubility profile and particle size, with or without added phytase.[1]

Phytase	Digestibility, %	Solution	Model[2]	Adjust-R^2	Root MSE
0 FTU/kg	Ca	0.2 N HCl	$= -43.174 + 0.906 \times \mathbf{Sol\_30} + 0.410 \times \mathbf{Sol\_15} \times \mathbf{GMD}$	0.988	2.093
		pH3 Buffered[3]	$= -28.964 + 0.817 \times \mathbf{Sol\_30} + 0.407 \times \mathbf{Sol\_15} \times \mathbf{GMD}$	0.988	2.085
	P	0.2 N HCl	$= 192.451 + 3.422 \times \mathbf{Sol\_5} - 5.343 \times \mathbf{Sol\_15} + 104.749 \times \mathbf{GMD}$	0.988	2.136
		pH3 Buffered	$= 106.499 + 0.274 \times \mathbf{Sol\_5} - 1.283 \times \mathbf{Sol\_15} + 4.653 \times \mathbf{GMD}$	0.988	2.136
1,000 FTU/kg	Ca	0.2 N HCl	$= -31.906 + 0.875 \times \mathbf{Sol\_30} + 0.353 \times \mathbf{Sol\_15} \times \mathbf{GMD}$	0.978	2.650
		pH3 Buffered	$= -18.293 + 0.790 \times \mathbf{Sol\_30} + 0.346 \times \mathbf{Sol\_15} \times \mathbf{GMD}$	0.979	2.631
	P	0.2 N HCl	$= 88.711 + 0.275 \times \mathbf{Sol\_5} - 0.007 \times \mathbf{Sol\_5} \times \mathbf{Sol\_5}$	0.966	3.478
		pH3 Buffered	$= -81.438 + 0.804 \times \mathbf{Sol\_5} - 0.015 \times \mathbf{Sol\_5} \times \mathbf{Sol\_5}$	0.990	1.863

[1]Only significant parameters ($P < 0.05$) were included in the model.
[2]GMD: geometric mean diameter, mm; Sol_5, Sol_15 and Sol_30, solubility (%) at 5, 15 and 30 min, respectively for the respective solution.
[3]3 M Glycine buffered pH 3 solution.

Table 8. Comparisons of linear prediction models for ileal Ca and P digestibilities based on geometric mean diameter (**GMD**) alone or in vitro solubility at a single point.

Phytase	Digestibility, %	Factor	Adjust-R^2	Root MSE
0 FTU/kg	Ca	GMD	0.3709	15.000
		0.2 N HCl, 5 min	0.1399	17.540
		0.2 N HCl, 15 min	0.4276	14.308
		0.2 N HCl, 30 min	0.7291	9.845
		pH3 Buffered[1], 5 min	0.2181	16.724
		pH3 Buffered, 15 min	0.3957	14.702
		pH3 Buffered, 30 min	0.7976	8.509
	P	GMD	0.0009	19.732
		0.2 N HCl, 5 min	0.9099	5.926
		0.2 N HCl, 15 min	0.9837	2.524
		0.2 N HCl, 30 min	0.8386	7.931
		pH3 Buffered, 5 min	0.9360	4.978
		pH3 Buffered, 15 min	0.9873	2.228
		pH3 Buffered, 30 min	0.7854	9.146
1,000 FTU/kg	Ca	GMD	0.3217	14.814
		0.2 N HCl, 5 min	0.2097	15.990
		0.2 N HCl, 15 min	0.5217	12.645
		0.2 N HCl, 30 min	0.7736	8.559
		pH3 Buffered, 5 min	0.2949	15.104
		pH3 Buffered, 15 min	0.4746	13.038
		pH3 Buffered, 30 min	0.8324	7.364
	P	GMD	0.3885	14.814
		0.2 N HCl, 5 min	0.9030	5.901
		0.2 N HCl, 15 min	0.6693	10.894
		0.2 N HCl, 30 min	0.3153	15.676
		pH3 Buffered, 5 min	0.8497	7.344
		pH3 Buffered, 15 min	0.6948	10.465
		pH3 Buffered, 30 min	0.2511	16.394

[1]3 M Glycine buffered pH 3 solution.

especially for AID Ca both in the presence and absence of phytase. The AID P predictions were better as compared to AID Ca using single time point but still not comparable to multiple time point predictions especially in the presence of phytase. The comparisons between single and multiple time points show that dynamics of solubilization rather than a static point would more accurately predict limestone AID Ca and the impact of limestone AID P of the diet.

Among those prediction models, it was apparent that solubility at 15 and 30 min were more relevant to AID Ca, which suggested that the extend of solubilization, or the amount of Ca^{2+} that would eventually be solubilized, was more important in determining Ca availability from limestone, both in the presence and absence of phytase. On the other hand, the speed of solubilization (5 and 15 min solubility) that is a measure of the amount of Ca^{2+} that was rapidly available for chelation with the phytic acid molecule, was most influential in predicting AID P especially without phytase.

The current trial demonstrated that using a multiple-time point solubility approach was advantageous for characterizing differences between limestones and more accurately predict AID Ca and the impact of the limestone on AID P as compared to single time point analysis and prediction. There were limitations from the

current trial, due to the small numbers of limestone samples, diet type and phytase (dose and type) tested. In addition, incubation time in current assay development was selected to reflect the average gizzard and proventriculus retention time for meat type poultry (ie. broilers). Incubation time will need to be modified when larger particle size limestones are tested for laying type poultry. Therefore, the prediction equations should be interpreted with care especially in cases beyond the scope of current study. Further studies involving a greater number of limestone samples are warranted to establish more accurate prediction equations. Validation of the prediction will also be necessary, using samples outside of those used to develop the prediction equations.

CONCLUSION

Limestone frequently is the single biggest contributor of Ca in feed for poultry. Particle size, distribution, and origin of limestone, as well as physical properties of the rock, geological origin, and its chemical composition influence: (1) in vitro solubility, (2) in vivo Ca digestibility, and (3) in vivo diet P digestibility and phytase efficacy. This work demonstrated that using a multiple-time point dynamic solubility assay model yields better correlations and predictions of apparent in vivo Ca digestibility than what is obtained using 1 time-point. Additionally, the use of the pH3 Buffer solution (3 molar glycine buffered 3 pH solution) results in greater separation between limestone solubilities at 5 and 15 min and yields slightly better correlations with in vivo Ca digestibility than using the 0.2 N HCl solution.

REFERENCES

AgriStats. 2015. End of Year Summary: 2016. Agri Stats, Inc., Fort Wayne, IN.

Ajakaiye, A., J. O. Atteh, and S. Leeson a. 1997. Effects of calcium source, particle size and time on in-vitro calcium solubility of some indigenous Nigerian mineral ingredients for poultry diets. Anim. Feed Sci. Technol. 64:293–298.

American Society of Agricultural and Biological Engineers. 2008. Method of Determining and Expressing Fineness of Feed Materials by Sieving (ANSI/ASAE S319.4). ASABE. St. Joseph, MI.

Anwar, M. N., V. Ravindran, P. C. H. Morel, G. Ravindran, and A. J. Cowieson. 2016a. Effect of limestone particle size and calcium to non-phytate phosphorus ratio on true ileal calcium digestibility of limestone for broiler chickens. Br. Poult. Sci. 57:707–713.

Anwar, M. N., V. Ravindran, P. C. H. Morel, G. Ravindran, and A. J. Cowieson. 2016b. Apparent ileal digestibility of calcium in limestone for broiler chickens. Anim. Feed Sci. Tech. 213:142–147.

Association of Official Analytical Chemists. 1999. Macro and micro elements in plant tissues by ICP-AES. Pages 110–116 in Analytical Techniques for Inorganic Contaminants. Assoc. Off. Anal. Chem., Arlington, VA.

Association of Official Analytical Chemists. AOAC method 920.39. 2003. Official Methods of Analysis. 2nd rev. 17th ed. Assoc. Off. Anal. Chem., Arlington, VA.

Association of Official Analytical Chemists. AOAC method 990.03. 2003. Official Methods of Analysis. 2nd rev. 17th ed. Assoc. Off. Anal. Chem., Arlington, VA.

Cheng, T. K., and C. N. Coon. 1990. Comparison of various methods for the determination of limestone solubility. Poult. Sci. 69:2204–2208.

de Witt, F. H., N. P. Kuleile, H. J. van der Merwe, and M. D. Fair. 2009. Effect of limestone particle size on egg production and eggshell quality of hens during late production, S. Afr. J. Anim. Sci. 39:37–40.

Guinotte, F., Y. Nys, and F. de Monredon. 1991. The Effects of particle size and origin of calcium carbonate on performance and ossification characteristics in broiler chicks. Poult. Sci. 70:1908–1920.

ISO 30024. 2009. Animal feeding stuffs − determination of phytase activity.

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, S.-W. Kim, E. Jiménez-Moreno, M. Proszkowiec-Weglarz, and P. W. Plumstead. 2014. Age and adaptation effects to Ca and P deficiencies: impact on P digestibility. Poultry Science Association Annual Meeting. July 14−17, Corpus Christi, Texas. (Abstr. 248).

Li, W., R. Angel, S.-W. Kim, K. Brady, S. Yu, and P. W. Plumstead. 2017. Impacts of dietary calcium, phytate, and phytase on inositol hexakisphosphate degradation and inositol phosphate release in different segments of digestive tract of broilers. Poult. Sci. 96:3626–3637.

Li, W., R. Angel, S.-W. Kim, E. Jiménez-Moreno, M. Proszkowiec-Weglarz, and P. W. Plumstead. 2018. Impacts of age and calcium on phytase efficacy in broiler chickens. Anim. Feed Sci. Tech. 238:9–17.

Manangi, M. K., and C. N. Coon. 2007. The effect of calcium particle size and solubility on the utilization of phosphorus from phytase for broilers. Int. J. Poult. Sci. 6:85–90.

Mussini, F., J. Wilson, J. Remus, L. Kim, and M. Hruby. 2019. Evaluation of different limestone sources used by the US poultry feed industry. International Production and Processing Expo. (Abstr. T195).

National Research Council, 1994. Nutrient Requirements of Poultry. 9th rev. ed. National Academy of Science, Washington, DC.

Plumstead, P. W., A. B. Leytem, and R. O. Maguire. 2008. Interaction of calcium and phytate in broiler diets. 1. Effects on apparent prececal digestibility and retention of phosphorus. Poult. Sci. 87:449–458.

Rao, K. S., and D. A. Roland. 1989. Influence of dietary calcium level and particle size of calcium source on in vivo calcium solubilisation by commercial Leghorns. Poult. Sci. 68:1499–1505.

Robinson, P. 1980. Determination of calcium, magnesium, manganese, strontium, sodium and iron in the carbonate fraction of limestones and dolomites. Chem. Geol. 28:135–146.

Sa, M. V. D. C. E., and C. E. Boyd. 2017. Variability in the solubility of agricultural limestone from different sources and its pertinence for aquaculture. Aquacult. Res. 48:4292–4299.

Saunders-Blades, J. L., J. L. MacIsaac, D. R. Korver, and D. M. Anderson. 2009. The effect of calcium source and particle size on the production performance and bone quality of laying hens. Poult. Sci. 88:338–353.

SAS Institute. 2014. Version 9.4. SAS Institute, Inc., Cary, NC.

Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Laguë. 1996. Efficacy of supplemental microbial phytase at different dietary calcium levels on growth performance and mineral utilization of broiler chickens. Poult. Sci. 75:1516–1523.

Short, F. J., P. Gorton, J. Wiseman, and K. N. Boorman. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim. Feed Sci. Tech. 59:215–221.

Shreve, B., N. Thiex, and M. Wolf. 2006. National forage testing association reference method: dry matter by oven drying for 3 hours at 105°C. NFTA Reference Methods. National Forage Testing Association, Omaha, NB.

Skoglund, E., N. G. Carlsson, and A. S. Sandberg. 1997. Determination of isomers of inositol mono- to hexaphosphates in selected foods and intestinal contents using high-performance ion chromatography. J. Agric. Food Chem. 45:431–436.

Skoglund, E., N. G. Carlsson, and A. S. Sandberg. 1998. High performance chromatographic separation of inositol phosphate isomers on strong anion exchange columns. J. Agric. Food Chem. 46:1877–1882.

Tamim, N. M., and R. Angel. 2003. Phytate phosphorus hydrolysis as influenced by dietary calcium and micro-mineral source in broiler diets. J. Agric. Food Chem. 5:4687–4693.

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.

Tukey, J. 1949. Comparing individual means in the analysis of variance. Biometrics. 5:99–114.

Zhang, B., and C. Coon. 1997a. The relationship of calcium intake, source, size, solubility *in vitro* and *in vivo*, and gizzard limestone retention in laying hens. Poult. Sci. 76:1702–1706.

Zhang, B., and C. Coon. 1997b. Improved *in vitro* methods for determining limestone and oyster shell solubility. J. Appl. Poult. Res. 6:94–99.