Final Report

Project code: 20-218

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Date: 30 December 2022

Ileal Ca digestibility

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Project Summary

Project Title	Ileal Ca digestibility
Project No.	PHA Project 20-218 Li
Date	Start: 1 July 2020 End: 10 February 2023
Project Leader(s)	Xiuhua Li, Wayne L. Bryden, Dagong Zhang, and Stuart J. Wilkinson
Organisation	The University of Queensland; Feedworks, Australia
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Project Aim	1. Validate/consolidate the method for ileal digestible Ca determination in meat chickens. 2. Modify the method for Australian poultry diets.
Background	The nutritional requirements for Ca and P are intricately linked, and divergence from the dietary ratio of these minerals adversely affects bone formation, shell quality, and overall bird performance. In our previous research on Ca and P requirements, we were able to show that concentrations of these nutrients in both broiler and layer diets could be substantially reduced. **The challenge** in reducing dietary P concentrations is to decide what dietary availability values to use for Ca and P in diet formulation. This is due to a lack of biologically determined data for available Ca in feed ingredients used for poultry. Up until now there has been no agreement on an evaluation system for Ca availability. However, there is general agreement that ileal digestibility is an appropriate method for estimating nutrient availability. The availability of Ca in different limestones, the main source of Ca in poultry diets, is considered uniform. However, recent USA and South African data have shown that the ileal Ca digestibility of limestone varies considerably. Unfortunately, there are currently no data on the Ca digestibility of Australian limestones, and there is an urgent need for these data to be collected. When collecting this data, it is important to remember that Ca digestibility in broilers can vary dramatically depending on the Ca source, source of phytate, and dietary addition of phytase. Previously we found that P is oversupplied in Australian poultry diets, with implications for both diet cost, and P pollution. Moreover, to reduce dietary P concentration accurately, knowledge of Ca digestibility is critical. In addition, Ca and P homeostasis requires an optimal ratio of digestible Ca: digestible P.
Research Outcome	The method evaluated in this project is suitable for the determination of ileal Ca digestibility of limestone. The particle size of limestone and diet composition affect ileal Ca digestibility of limestone. Phytase improves ileal Ca digestibility of limestone and ileal P digestibility of diets.
Impacts and Outcomes	Ileal digestibility can be used to develop a digestible Ca database for Australian limestone samples and other inorganic sources of Ca. This

	approach will provide nutritionists with essential information to formulate diets that meet the requirements for Ca and P, thus reducing dietary safety margins, which leads to more efficient nutrient utilisation, reduced poultry production cost and the risk of environmental pollution caused by excessive P excretion.
Publications	Li X, Zhang D, Pan LY, Wilkinson SJ and Bryden WL (2022) Determination of calcium and phosphorus digestibility in a short-term bioassay with broilers. *Australian Poultry Science Symposium Proceedings,* **33**: 141.

Executive Summary

Calcium (Ca) is the major mineral in poultry diets. Limestone provides the major portion of the total dietary Ca requirement, contributing over 50% for broilers and 90% for laying hens. The availability of Ca from different limestone sources varies considerably and presents a great challenge for nutritionists to decide what Ca concentrations should be used in poultry diets. This challenge would be overcome if estimates of availability values for Ca were determined. The accepted method for estimating Ca availability is digestible Ca. However, there is no agreed method for determining Ca digestibility. The current project evaluated and adapted the method developed by Professors Roselina Angel (University of Maryland, USA) and Peter Plumstead (Chemuniqué, South Africa) for determining ileal Ca digestibility of limestone. In the project, the digestibility of Ca in limestone with different particle sizes was determined in typical Australian broiler diets with or without supplemental phytase. Five experiments were conducted.

The method, which used 22 day-old broilers that have been fed the bioassay diet for 36 hours, is suitable to determine ileal Ca digestibility of limestone samples. The particle size of limestone and the composition of diets fed (maize, wheat, sorghum or wheat/sorghum blend) affect ileal Ca digestibility of limestone. Phytase improves ileal Ca digestibility of limestone and ileal Phosphorus (P) digestibility of diets.

Determination of ileal Ca digestibility of limestone samples from around Australia and other inorganic sources of Ca and P is required to establish digestible Ca database. This approach will provide nutritionists with essential information to formulate diets to meet the exact requirements of Ca and P for broilers. It will also allow the reduction of safety margins for both minerals leading to increased nutrient utilisation, reduced costs of poultry production and a reduced risk of environmental pollution caused by excessive P excretion.

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Introduction

Calcium (Ca) is an essential element for bone and eggshell formation, blood clotting, muscle contraction and transmission of nerve impulses. Phosphorus (P) is the second most significant mineral after Ca. The nutritional requirements for Ca and P are intricately linked, and Ca and P homeostasis requires an optimal dietary ratio of Ca to P. P research has attracted more attention than Ca, especially since the introduction of the feed enzyme, phytase. The majority of feed phosphate is sourced from phosphate rock, which is a non-renewable resource and is estimated to be depleted within 50–100 years (Cordel et al. 2009). The production of feed phosphate from raw phosphate resources will be limited, and the price of phosphate will substantially increase (Shastak et al. 2012). Phytase, however, increases the availability of P in plant phytate sources. Importantly, in our previous work on Ca and P requirements of poultry (Li et al. 2017) we were able to show that concentrations of these nutrients in both broiler and layer diets could be substantially reduced. This was not unexpected as numerous authors have found excessive dietary P concentrations in poultry diets (Summers 1997; Angel et al. 2000a, 2000b; Waldroup et al. 2000; Leske & Coon 2002; Applegate et al. 2003; Persia & Saylor 2006). We (Li et al. 2017) found that P is oversupplied in Australian poultry diets, with implications for both diet cost and P pollution.

To reduce dietary P concentrations accurately, knowledge of Ca digestibility is critical. Our previous results showed that as dietary Ca increased, ileal P digestibility and weight gain decreased (Li et al. 2017). Walk et al. (2011) found that elevated dietary Ca reduced ileal crude protein digestibility. The detrimental effects of high Ca in poultry diets have been reviewed by Li et al. (2016). In addition, Ca and P homeostasis requires an optimal ratio of digestible Ca: digestible P, not the ratio of total Ca: AvP supplied in the diet.

The challenge in reducing dietary P concentrations is to decide what dietary availability values to use for Ca and P in diet formulation. This is due to a lack of biologically determined data for available Ca in feed ingredients used for poultry. It should be remembered that the requirements for Ca and P are tightly integrated, and divergence from this ratio adversely affects bone formation, shell quality, and overall performance. It is common practice to maintain a ratio of total Ca to available P (AvP) when specifying requirements of these elements. The bottleneck for further refinement of requirements is how to determine available Ca. Up until now there has been no agreement on an evaluation system for available Ca. However, there is general agreement that ileal digestibility is an appropriate method for estimating nutrient availability and this has been demonstrated with amino acids (Bryden & Li 2010) and P (Mutucumarana et al. 2015).

The ileal methodology we used to measure the ileal digestibility of Ca in limestone has been developed by Professors Angel and Plumstead over the past 5 years as described by Li et al. (2021). The new and novel methodology allows for the accurate determination of Ca and P digestibility from a single source of inorganic Ca or phosphate that is absorbed in the terminal ileum of 22 day-old broilers. **What makes the method novel** is that by feeding the test diets for a period of only 36 hours (most other methods use 3 to 5 days), overcomes confounding due to physiological adaptation/compensation that occurs when longer feeding periods are used. Thus the evaluation of the test source and the digestibility values obtained are not dependent on previous diets fed, or dietary Ca and P concentrations.

The availability of Ca in different limestones, the main source of Ca in poultry diets, is considered uniform. However, recent USA and South African data (Plumstead et al. 2020) have shown that ileal Ca digestibility of limestone varies considerably. Unfortunately, there are currently no data on the Ca digestibility of Australian limestones and there is an urgent need for these data to be collected, especially as limestone is the main source of Ca in plant-based poultry diets, and contributes over 50% and 90% of the total Ca for broilers and laying hens, respectively. Moreover, when collecting these data it is important to remember that Ca digestibility in broilers can vary dramatically depending on the Ca source, source of phytate, and dietary addition of phytase (Anwar et al. 2016; Angel 2019; Kim et al. 2019, Taylor et al. 2019; Plumstead et al. 2020).

Objectives

1. Collaborate with international researchers (South Africa and USA) to validate/consolidate the method for ileal digestible Ca determination in meat chickens.
2. Modify the method for Australian broiler diets.

Methodology

The work undertaken included physical characterisation of USA and Australian limestone samples and ileal digestibility studies.

1. Limestone samples

A limestone sample was imported from the USA (PureCal^{tm} 12-40, manufactured by Cerne Calcium Company, a subsidiary of Fort Dodge Fine & Granular Products, Ft Dodge, IA, hereafter referred to as USA limestone), and was suggested as a 'control' sample for the establishment of the Ca ileal digestibility procedure by researchers in South Africa and the USA.

Fine grit limestone (250–1000µm) was obtained by South Queensland Lime Pty Ltd., hereafter referred to as Australian limestone. This limestone was used in studies to determine the effects of limestone particle size and diet composition on ileal Ca digestibility.

2. Determination of limestone particle size

Representative limestone samples (200 g) were shaken in a sieve shaker for 10 min in duplicate. Each fraction was weighed, and the percentage retained was calculated.

The USA limestone sample was sieved with screen pore sizes of 106, 180, 250, 355, 425, 500, 600, 710, 1000 and 2000 µm.

The Australian limestone sample was sieved using screen pore sizes of 106, 180, 250, 355, 425, 500, 600, 710 and 1000 µm.

3. Determination of limestone solubility

Solubility of limestone samples was determined in 0.2 N HCl (Kim et al. 2019).

The 0.2 N HCl solution was prepared with deionised distilled water (dd) (Zhang & Coon 1997). A 1 g representative sample of limestone was weighed into a 250 mL Erlenmeyer flask. The flask was placed in a 42°C shaking water bath for 10 min and 138 mL of pre-warmed (42°C) 0.2 N HCl solution was added to the flask, which was then shaken vigorously, to maximise mixing without losing solution, for 0, 5, 15, 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. 540 filter (8 µm). Additional ice-cold dd water was added as needed to flush any remaining pieces of limestone in the Erlenmeyer flask. The filter papers with the limestone residues were placed in a pre-weighed, pre-labelled crucible and then dried at

105°C overnight. The assay was repeated in triplicate for every time point. Solubility at every time point was determined by weight loss.

Solubility of limestone (%) = (1 − dried remaining limestone/dry initial limestone) × 100

4. Ileal Ca digestibility of limestone protocol

The methodology used to determine the ileal Ca digestibility of limestone was described by Li et al. (2021). A series of experiments was conducted using this experimental protocol. The experiments were approved by the University of Queensland Animal Ethics Committee, and were in accordance with the Australian code for the care and use of animals for scientific purposes.

1.1. Birds

Day old, Ross 308 male chicks were purchased from the Aviagen Hatchery, Goulbourn, NSW for all experiments.

1.2. Pre-experimental diet for all experiments

A typical Australian wheat-sorghum-soybean meal broiler diet was fed as the pre-experimental diet (Appendix 1). The diet contained 0.65% Ca and 0.25% ileal digestible P with supplemental phytase at 500 FTU (AXTRA® PHY TPT 10,000). A combination of xylanase (with enzyme activity: 12200 U/g) and beta-glucanase expressed in *Trichoderma reesei* (with enzyme activity: 1520 U/g) (AXTRA® XB 201 TPT) was added to the diet as recommended by the manufacturer. Both enzymes were supplied by Feedworks, Australia. The diet was fed to chicks from day 1 to 20 post-hatch.

1.3. Basal experimental diets

The composition of the basal diets used in the different experiments is shown in Table 1. A maize-soybean meal diet with a low Ca concentration was used as the basal diet for Experiments 1, 2 and 3. A sorghum and wheat (50:50) basal diet was for Experiment 4. In Experiment 5, three basal diets were used: a sorghum, a wheat, and a combination of sorghum and wheat (50:50).

Table 1 Composition of basal diets (g/kg)

Ingredient (g/kg)	**Maize**	**Sorghum**	**Wheat**	**Sorghum + wheat**
Maize	769			
Sorghum		613		320
Wheat			625	310
Soybean meal	165	310	291	290
Canola oil	18.4	23.8	32	27
Celite	20	20	20	20
Lysine. HCl	1.99	2.56	1.96	2.4
DL Methionine	2.8	3.53	2.49	3.06
L-Threonine	0.5	1.11	0.7	0.97
L-Tryptophan			0.42	
Salt	2.24	2.44	1.98	2.17
Sodium bicarbonate	2.59	2.35	3.06	2.77
Vitamin & minerals Premix[1]	0.5	0.5	0.5	0.5
Choline chloride 60% dry	1.06	0.96	0.66	0.83
Xylanase (500 g/t)			0.5	0.5
Ingredient total	1000	1000	1000	1000
Nutrient calculation (%)				
AME (MJ/kg)	13.61	13.05	13.05	13.05
Crude protein	14.72	20.8	22.2	21.3
Dig Lysine	0.75	1.1	1.1	1.1
Dig Met + Cys	0.71	0.84	0.84	0.84
Dig Threonine	0.53	0.73	0.73	0.73
Dig Arginine	0.81	1.141	1.263	1.188
Dig Tryptophan	0.13	0.205	0.277	0.218
Ca	0.15	0.122	0.123	0.122
Total P	0.311	0.409	0.388	0.397
Avail. P	0.093	0.094	0.118	0.105
Phytate P	0.2177	0.315	0.27	0.292
Na	0.17	0.17	0.17	0.17
Cl	0.23	0.23	0.23	0.23
Salt	0.4	0.4	0.4	0.4
Choline (mg/kg)	1283	1500	1500	1500
Linoleic acid (18:2)	2.74	2.138	2.367	2.224

[1] The premix was specially made without Ca and P.

1.4. Experimental treatments

Limestone (USA or Australia) was the only source of inorganic Ca added to the respective basal diets in the different experiments to provide a dietary Ca concentration of 0.65% (0.66% analysed). The experimental diets were prepared in a mash form with or without supplemental phytase. An indigestible marker, celite, was added at 20 g/kg as a source of acid-insoluble ash (AIA) to all diets for the calculation of digestibility coefficients.

Experiment 1: Apparent ileal Ca digestibility of the USA limestone sample in birds fed a maize-soybean meal diet

There were 4 dietary treatments in Experiment 1:

Diet 1: Low Ca (0.15%) maize-soybean meal basal diet without addition of limestone and phytase.

Diet 2: Diet 1 supplemented with phytase (1000 FTU/kg diet).

Diet 3: Diet 1 supplemented with USA limestone to increase the dietary Ca concentration to 0.65% (0.66% analysed) without phytase.

Diet 4: Diet 3 supplemented with phytase (1000 FTU/kg diet).

Experiment 2: Repeat of Experiment 1 – Apparent ileal Ca digestibility of the USA limestone in birds fed a maize-soybean meal diet

Treatments of Experiment 2 as per Experiment 1.

Experiment 3: Apparent ileal Ca digestibility of Australian limestone with different particle sizes in birds fed a maize-soybean meal diet

Two particle size ranges (250–355 µm and 600–710 µm) from the same batch of Australian limestone were selected according to the particle size distribution of the limestone. The samples were tested in a maize-soybean meal diet. There were 4 experimental treatments in Experiment 3:

Diet 1: Maize-soybean meal basal diet with addition of limestone (limestone fraction of 250–355 µm) to increase the dietary Ca concentration to 0.65% (0.66% analysed) without phytase.

Diet 2: Diet 1 supplemented with phytase (1000 FTU/kg).

Diet 3: Maize-soybean meal basal diet with addition of limestone (limestone fraction of 600–710 µm) to increase the dietary Ca concentration to 0.65% (0.66% analysed) without phytase.

Diet 4: Diet 3 supplemented with phytase (1000 FTU/kg).

Experiment 4: Apparent ileal Ca digestibility of Australian limestone with different particle sizes in birds fed a typical Australian broiler diet

Two particle size ranges (250–355 µm and 600–710 µm) of the same batch of Australian limestone as described in Experiment 3 were tested in a sorghum and wheat (50:50) basal diet.

There were 4 dietary treatments in Experiment 4:

Diet 1: Sorghum and wheat (50:50) basal diet with addition of limestone (limestone fraction with particle size of 250–355 µm) to increase the dietary Ca concentration to 0.65% (0.66% analysed) without phytase.

Diet 2: Diet 1 supplemented with phytase (1000 FTU/kg).

Diet 3: Sorghum and wheat (50:50) basal diet with addition of limestone (limestone fraction with particle size of 600–710 µm) to increase the dietary Ca concentration to 0.65% (0.66% analysed) without phytase.

Diet 4: Diet 3 supplemented with phytase (1000FTU/kg).

Experiment 5: Apparent ileal Ca digestibility of Australian limestone in broilers fed typical Australian diets

The same batch of Australian limestone used in Experiments 3 and 4 was tested in typical Australian broiler diets without regard to particle size distribution.

There were 6 dietary treatments in Experiment 5:

Diet 1: Sorghum basal diet (analysed Ca of 0.13%) with addition of the limestone to increase the dietary Ca concentration to 0.65% (0.66% analysed) without phytase.
Diet 2: Diet 1 supplemented with phytase (1000 FTU/kg).
Diet 3: Wheat basal diet (analysed Ca of 0.13%) with addition of the limestone to increase the dietary Ca concentration to 0.65% (0.66% analysed) without phytase.
Diet 4: Diet 3 supplemented with phytase (1000 FTU/kg).
Diet 5: Sorghum and wheat (50:50) basal diet (analysed Ca of 0.13%) with addition of the limestone to increase the dietary Ca concentration to 0.65% (0.66% analysed) without phytase.
Diet 6: Diet 5 supplemented with phytase (1000 FTU/kg).

1.5. Experiment and sampling

On day 20, the birds were weighed and those with similar body weights were selected and randomly allocated into cages, with 8 birds per cage. The experimental diets were offered to 8 replicate cages per diet from day 20 post-hatch (Kim et al. 2019). After 36 hrs, birds were euthanised by cervical dislocation. The contents of the distal half of the ileum, measured half-way from Merkel's diverticulum to 3 cm anterior to the ileo-ceacal junction, were gently flushed out using deionised water. Ileal samples were pooled per replicate, freeze dried and ground to pass through a 0.25 mm screen. Diet samples were ground to pass through a 0.5 mm screen. Feed and freeze dried digesta were analysed in duplicate for Ca and the AIA marker. Ileal Ca and P digestibility coefficients were calculated.

1.6. Chemical analysis and calculations

Ca and P contents of diets and ileal digesta were analysed by inductively coupled plasma atomic emission spectrometry (axial viewing) (ICP-AES, Spectro Flame Modula E, SPECTRO® Analytical Instruments, Kleve, Germany) after acid digestion.

The method of Li et al. (2006) for determining AIA content in diets and ileal digesta samples was followed. Briefly, a sample containing approximately 100 mg of AIA was weighed into a pre-weighed sintered glass crucible (Pyrex, porosity 4), dried at 105°C for 24 h and re-weighed to determine dry matter (DM) content. The dried sample was ashed at 550°C for 8 h, boiled with 4 M hydrochloric acid in a crystallising dish for 30 min and then thoroughly washed with purified water. The processes of drying, ashing, boiling and washing were repeated. The crucible was then dried and re-weighed. The AIA content was expressed as % of dry matter.

Apparent ileal digestibility of Ca and P was calculated using AIA as an indigestible marker.

The calculation of apparent ileal Ca digestibility is as follows:

$$\text{Apparent ileal Ca digestibility (\%)} = [(Ca/AIA)d - (Ca/AIA)i]/(Ca/AIA)d \times 100$$

Where (Ca/AIA)d = ratio of Ca and AIA in the diet, and (Ca/AIA)i = ratio of Ca and AIA in ileal digesta.

1.7. Statistical analysis

The general linear model and Minitab program version 18.1 (2017) were used to analyse all the data. One-way ANOVA was used to compare ileal Ca digestibility between diets, or particle sizes of limestone, or with and without phytase supplementation. Two-way ANOVA was also used where applicable. The treatment interactions were described in the respective sections. The significant difference level is $P < 0.05$.

Discussion of results

1. Limestone particle size

The Australian and USA limestone samples have different particle size distributions. The distribution of particle size is shown in Tables 2 and 3 for USA and Australian limestone samples, respectively. Approximately 89% of USA limestone was retained between screens of 710 and 1000 µm, while 84% of the Australian limestone was retained between screens of 250 and 710 µm.

Table 2 Particle size distribution of USA limestone

Sieve size (µm)	Mean (% retained)	SD
<106	2.6	0.24
106	1.1	0.06
180	0.5	0.05
250	0.6	0.05
355	0.6	0.03
425	0.8	0.06
500	1.2	0.01
600	2.6	0.03
710	17.8	0.53
1000	71.9	0.81
2000	0.4	0.08

SD: Standard deviation.

Table 3 Particle size distribution of Australian limestone

Sieve size (µm)	Mean (% retained)	SD
<106	6.2	0.06
106	1.6	0.10
180	8.1	0.41
250	17.5	0.51
355	12.8	0.07
425	12.6	0.33
500	12.8	0.04
600	14.4	0.91
710	14.1	0.48
1000	0	0.00

SD: Standard deviation.

2. Limestone solubility

In general, limestone of smaller particle size is more soluble. Solubility is increased as incubation time in 0.2 N HCl at 42°C is extended. The solubility of USA and Australian limestone samples is shown in Tables 4 and 5.

Table 4 Solubility of the USA limestone (%; mean±SD) at 1, 5, 15 and 30 min

Particle size (µm)	Time (min, at 42°C)			
	1	5	15	30
<106	97.3±1.1	99.7±0.3	99.6±0.4	100±0.3
106–180	83.9±1.0	98.0±0.2	99.8±0.3	100±0.1
500–600	14.6±2.1	61.1±5.1	71.9±3.8	92.2±2.7
600–710	17.2±1.6	60.5±3.0	73.9±2.6	90.6±2.3
710–1000	15.4±0.7	54.7±1.6	75.8±4.4	92.4±5.0
1000–2000	15.4±0.5	55.3±2.6	69.2±1.3	88.2±2.1
Whole	17.4±1.0	61.2±2.7	71.1±1.7	84.7±2.4

SD: Standard deviation.

The proportion of USA limestone with particle size between 180 and 500 µm was only 2.5%. Therefore when limestone solubility was determined, not all particle sizes were tested because of the small proportion.

Table 5 Solubility of Australian limestone (%, mean±SD) at 1, 5, 15 and 30 min

Particle size (µm)	Time (min, at 42°C)			
	1	5	15	30
<106	94.0±2.3	99.5±0.2	99.2±0.1	98.9±1.1
106–180	39.8±1.7	81.8±5.9	89.8±1.5	90.9±2.2
180–250	23.0±1.6	59.5±3.2	69.4±8.4	80.6±4.3
250–355	15.2±1.8	66.3±5.7	72.0±9.8	81.7±7.3
355–425	12.7±1.6	61.9±4.1	65.5±6.4	72.1±2.7
425–500	11.7±1.3	63.1±3.0	71.0±4.3	83.6±3.5
500–600	10.5±1.3	64.2±2.1	68.9±2.6	82.3±6.0
600–710	8.7±0.7	62.5±0.6	63.6±1.8	78.0±4.3
710–1000	12.6±5.2	63.1±3.2	72.1±2.6	84.0±3.0
Whole	16.8±0.4	55.7±0.9	69.9±2.8	83.7±1.2

SD: Standard deviation.

3. Ileal Ca digestibility

Experiment 1: Apparent ileal Ca digestibility of the USA limestone sample in birds fed a maize-soybean meal diet

The results of Experiment 1 are shown in Table 6. The apparent ileal digestibility of Ca and P of the maize-soybean basal diet without adding both limestone and phytase (Diet 1) was much higher than when limestone was added (Diet 3). Phytase improved apparent ileal Ca and P digestibility (Diet 2) but this effect was reduced with the dietary addition of limestone (Diet 4).

Table 6 Apparent ileal Ca digestibility (%) of USA limestone with or without supplemental phytase in Experiment 1

	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**
	-Limestone-phytase	-Limestone+phytase	+Limestone-phytase	+Limestone+phytase
Apparent ileal Ca digestibility	**56.32**^{c}	**72.41**^{a}	**47.01**^{d}	**67.35**^{b}
SD	1.91	3.21	4.33	3.05
SEM	0.67	1.13	1.53	1.08
Apparent ileal P digestibility of diet	**70.06**^{c}	**88.93**^{a}	**31.77**^{d}	**79.92**^{b}
SD	1.94	1.55	4.45	2.31
SEM	0.69	0.55	1.57	0.82

^{a,b,c,d} means within the rows with different superscripts differ ($P < 0.05$).
SD: Standard deviation.
SEM: Standard error of mean.

The results of the current evaluation (Table 6) are in accord with the results expected for both characterisation and digestibility for similar USA limestone products (Li et al. 2021). Importantly, the negative impact of dietary Ca concentration on ileal Ca and P digestibility and phytase efficacy is clearly evident in Table 6. This is in agreement with our previous studies (Li et al. 2016, 2017) and with the work of Angel and Plumstead (Kim et al. 2019; Li et al. 2021).

The USA limestone has a larger particle size than the limestone used in Australian poultry diets (see Survey 2, by Feedworks, 2020). Nutritionist Cameron Wright from BEC Feed Solutions recommended approximate 500 (µm) (personal comm.). It is possible that a larger particle size may increase variation in ileal Ca digestibility. As a commercial product there would be batch to batch variation in the USA limestone. The paper by Li et al. (2021) is the only other paper that we have found that has evaluated the USA limestone used in this study. In that paper, standardised ileal Ca and P digestibility was reported (see Table 7). They did not report apparent ileal Ca and P digestibility, as we did.

The standardised ileal Ca digestibility of the limestone sample was calculated in the current experiment using the value for endogenous loss of Ca (105.6 mg/kg DM intake) reported by Li et al. (2021) and the following equation by Lemme et al. (2004).

Standardised ileal Ca digestibility (%) = apparent ileal Ca digestibility (%) + ((basal endogenous Ca losses, as g/kg DM intake) / (Ca content of the raw material, as g/kg DM x 100)

The standardised ileal Ca digestibility value is approximately 0.02% unit greater than the apparent ileal Ca digestibility (Table 7) and therefore within the rounding error of the calculation. This is consistent with Walk et al. (2021), who found no difference in apparent and standardised ileal Ca digestibility. The comparison of our data with that of Li et al. (2021) is shown in Table 7.

Table 7 UQ determined apparent, and calculated standardised ileal Ca digestibility (%) of USA limestone (Experiment 1), compared to reported standardised ileal Ca digestibility with or without supplemental phytase

	USA limestone	
	-phytase	**+phytase**
UQ-Apparent Ca digestibility (maize-soybean meal diet)	47.01	67.35
SD	4.33	3.05
SEM	1.53	1.08
UQ-Calculated standardised ileal Ca digestibility (%)*	47.02	67.37
SD	4.33	3.05
SEM	1.53	1.08
Reported standardised ileal Ca digestibility (%)**		
Maize diet	43.00	55.36
Maize-soybean meal diet	49.34	62.50

* Standardised ileal Ca digestibility of the limestone sample was calculated using the endogenous Ca loss value reported by Li et al. (2021) and the equation published by Lemme et al. (2004).

** From Li et al. (2021).

SD: Standard deviation.

SEM: Standard error of mean.

The main concern when comparing the standardised ileal Ca digestibility values derived in this way is the different variables involved in the experiments as listed in Table 8. In addition, the ingredients that made up the diets were grown in the different locations under different environmental conditions. The importance of these differences is not known. Moreover, as noted above, the impact of dietary Ca concentration on Ca digestibility is clear evident in our study (Table 6) and in the study of Li et al. (2021) where their maize diet and maize plus soybean diet had different Ca concentrations (Table 7). With regard to diets, our diet contained 16%, whereas the diet of Li et al. (2021) contained 25% SBM and different phytases (from the same company) were used.

Table 8 Differences in experimental protocol between UQ (Experiment 1) and USA (Li et al. 2021)

	UQ	**USA**
Birds (male)	Ross 308	Ross 708
Experimental diet	Maize-soybean meal	Maize Maize-soybean meal
Body weight (day 20)	1000 g	724 g
Euthanasia method	Cervical dislocation	Mixture of gases
Digestibility marker	Celite	Titanium oxide

There are inherent difficulties in determining ileal P and Ca digestibility. This was very evident from an international ring-test for ileal P digestibility that was used in leading international laboratories (Rodehutscord et al. 2017). The results showed significant variations in P digestibility values among the different labs, although the same protocol was followed; the same diets were tested and one lab analysed all the samples. Nevertheless, labs were able to successfully rank samples for ileal digestibility of P.

Experiment 2: Repeat of Experiment 1 – Apparent ileal Ca digestibility of the USA limestone in birds fed a maize-soybean meal diet

Experiment 2 was a repeat of Experiment 1 to determine the repeatability of the ileal digestibility protocol for Ca. The results of Experiment 2 showed very similar responses to Experiment 1 as indicated in Table 9. Again, the apparent ileal Ca and P digestibility of the basal diet without added limestone or phytase (Diet 1) was much higher than when limestone was added without phytase (Diet 3) (Table 9). Phytase improved apparent ileal Ca and P digestibility to a greater extent without additional dietary limestone (Diet 2) than with added limestone (Diet 4).

Table 9 Apparent ileal Ca digestibility (%) of USA limestone with or without supplemental phytase in Experiment 2

	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**
	-Limestone-phytase	-Limestone +phytase	+Limestone-phytase	+Limestone+phytase
Apparent ileal Ca digestibility	**54.7^c**	**71.0^a**	**47.3^d**	**63.6^b**
SD	6.78	5.12	5.12	3.63
SEM	2.40	1.81	1.81	1.28
Apparent ileal P digestibility	**82.4^b**	**92.1^a**	**37.8^c**	**84.4^b**
SD	2.49	1.22	4.94	3.37
SEM	0.88	0.43	1.75	1.19

^{a,b,c,d,} means within the rows with different superscripts differ ($P < 0.05$).
SD: Standard deviation.
SEM: Standard error of mean.

The standardised ileal Ca digestibility of the limestone sample was calculated using the endogenous loss of Ca (105.6 mg/kg DM intake) value reported by Li et al. (2021) and the following equation by Lemme et al. (2004).

Standardised ileal Ca digestibility (%) = apparent ileal Ca digestibility (%) + ((basal endogenous Ca losses, as g/kg DM intake) / (Ca content of the raw material, as g/kg DM x 100)

Despite efforts to minimise the differences in the conduct of Experiments 1 and 2, some differences were inevitable as shown in Table 10.

Table 10 Experimental differences between Experiments 1 and 2

Variables	**Experiment 1**	**Experiment 2**
Chicks/ingredients	Batch 1	Batch 2
Numbers of chicks per replicate	8	10
	Ca content (%)	
Basal experimental diet	0.15	0.10
Experimental diet	0.66	0.67
Season	December	August

Comparisons of the apparent ileal Ca digestibility between Experiments 1 and 2 are shown in Table 11. There were no significant differences in apparent ileal Ca digestibility between the two experiments for diets 1 to 3 ($P > 0.05$), whereas a significant difference was found with Diet 4 (+limestone + phytase). The complicated interrelationship between phytate-Ca impacts on phytase efficacy and may explain the variation (Li et al. 2016).

To determine how closely the results of Experiment 1 and Experiment 2 are, reproducibility was calculated (Table 11). **Reproducibility**, also known as replicability and repeatability, is a measure of precision under a defined set of conditions

In this report, reproducibility is determined as the standard deviation of the results from the two experiments. The variations between the two experiments were smaller than those within the treatments of each experiment (Table 11).

Table 11 Comparison of the Ca digestibility results of Experiments 1 and 2

	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**
	-Limestone-phytase	-Limestone +phytase	+Limestone-phytase	+Limestone+phytase
Apparent ileal Ca digestibility				
Experiment 1	56.32	72.41	47.01	67.35^{a}
SD	1.91	3.21	4.33	3.05
SEM	0.67	1.13	1.53	1.08
Experiment 2	54.66	70.96	47.31	63.58^{b}
SD	6.78	5.12	5.12	3.63
SEM	2.40	1.81	1.81	1.28
P value	0.574	0.555	0.900	0.041
Reproducibility*	1.17	1.03	0.21	2.67

^{a,b,} means within the column with different superscripts differ ($P < 0.05$).
SD: Standard deviation.
SEM: Standard error of mean.
* Reproducibility explained as the standard deviation (SD) of the mean values from the two experiments.

The results of Experiment 2 were consistent with Experiment 1. There were no significant differences in apparent ileal Ca digestibility between the two experiments for Diets 1 to 3 ($P > 0.05$), whereas significant differences were found between two experiments for Diet 4 (+limestone + phytase). The effect of the negative impact of dietary Ca concentration on ileal Ca and P digestibility and phytase efficacy is clearly evident again (Table 11). This is in agreement with the results of Experiment 1 and our previous studies (Li et al. 2016, 2017) and with the work of Angel and Plumstead (Kim et al. 2019; Li et al. 2021).

Published information on reproducibility of ileal Ca digestibility is very limited. Walk et al. (2021) reported that average ileal Ca digestibility of limestone was 53 ± 12 % from seven published papers. With such a large variation, it was not surprising that significant differences were not detected by Walk et al. (2021) between method (AID vs SID; $P = 0.724$), basal diet (semi-purified vs maize-based; $P = 0.185$), location (USA, New Zealand, or South America; $P = 0.715$), and phytase supplementation (0 vs 1000 FTU/kg; $P = 0.270$). This demonstrates that the impact of inherent ingredient variability and particle sizes, differences in dietary Ca to P ratios, the presence or absence of phytase, and the age of the bird need to be determined with a reproducible method and ideally, in the same laboratory.

Similar factors affect amino acid digestibility, but these have been accounted for by careful experimentation using a reproducible method (Bryden & Li 2010).

There is much industry interest in moving to a digestible Ca system for poultry nutrition. Our results indicate that the method used in the current project generated reproducible ileal Ca digestibility values.

Experiment 3: Apparent ileal Ca digestibility of Australian limestone with different particle sizes in birds fed a maize-soybean meal diet

The influence of limestone particle size on ileal digestibility of Ca and P of broiler fed maize-soybean meal diet is shown in Table 12. There was a significant ($P < 0.05$) interaction between limestone particle size and phytase supplementation on ileal Ca digestibility.

Table12 Ileal Ca digestibility of limestone with different particle sizes with or without phytase supplementation; Experiment 3; maize-soybean meal diet

Limestone particle size (µm)	Phytase	Ileal Digestibility (%)	
		Ca	P
250–355	-	44.5	37.2
250–355	+	48.0	77.7
600–710	-	40.3	44.4
600–710	+	54	81.5
Main effect			
Particle size			
SEM		1.15	1.06
P value		0.549	0.001
Phytase			
SEM		1.15	1.06
P value		0	0
Particle size x phytase			
SEM		1.62	1.50
P value		0.003	0.255

SEM: Standard error of mean.

Further statistical analysis of the effect of limestone particle sizes and the phytase supplementation on ileal Ca and P digestibility was performed and details are shown in Tables 13a and 13b.

Ileal Ca digestibility of the same source of limestone with different particle sizes showed significant differences. Without phytase supplementation feeding diets with limestone of larger particle size (600–710 µm) resulted in a lower ($P < 0.05$) Ca digestibility value (40.3%) compared with smaller particle size limestone (250–355 µm, 44.5%) (Table 13a), whereas ileal P digestibility was higher in the diet containing larger limestone particle size than those containing smaller limestone particle size, which agrees with the report of Kim et al. (2019).

Phytase significantly ($P < 0.05$) improved ileal digestibility of Ca (Table 13a) and P (Table 13b) when bioassay diets containing either particle size of limestone were fed. Feeding limestone of a larger particle size (600–710 µm) resulted in a higher ($P < 0.05$) Ca digestibility value (54%) compared with smaller particle size limestone (250–355 µm, 48%) when phytase supplemented in the diets (Table

13b). Dietary addition of phytase essentially doubled P digestibility irrespective of limestone particle size.

It should be remembered that to date, there are only seven published papers that report ileal Ca digestibility of limestones in broiler chickens (Walk et al. 2021). Moreover, limestone particle size was not examined in all the papers. The effect of limestone particle sizes on ileal Ca digestibility was reported by Anwar et al. (2016) and Kim et al. (2019). Anwar et al. (2016) reported that feeding small particle size limestone (< 500 µm) resulted in a lower ($P < 0.05$) apparent ileal Ca digestibility value (42%) compared to limestones with larger particle size (1000–20000 µm, 70%) without phytase supplementation. Kim et al. (2019) reported significant differences in the apparent ileal Ca digestibility values of the same limestone source ground at 60 or 600 µm (GMD), 47% vs. 66% without phytase and 55% vs 72% with phytase. It is difficult to make direct comparisons between our results with the publications due to differences in the ranges of particle sizes tested and sources of limestone, methods used, bird source, basal diet, Ca:P ratio, phytase and geological location. Therefore, more research is required on the bioavailability of Ca in Australian limestones, including studies with limestones sourced from different locations and different particle sizes.

Table 13a Ileal Ca digestibility of Australian limestone with different particle sizes with or without phytase supplementation in Experiment 3; maize-soybean meal diet

Limestone particle size (µm)	-phytase	+phytase	SEM	P value
250–355	44.5^{aB}	48^{bA}	0.94	0.02
600–710	40.3^{bB}	54.2^{aA}	2.09	0.00
SEM	1.28	1.9		
P value	0.035	0.037		

^{a,b,} means within column with different superscripts differ ($P < 0.05$).
^{A,B,} means within row with different superscripts differ ($P < 0.05$).
SEM: Standard error of mean.

Table 13b Ileal P digestibility of diet in Experiment 3 with or without phytase supplementation; maize-soybean meal diet

Limestone particle size (µm)	-phytase	+phytase	SEM	P value
250–355	37.2^{bB}	77.7^{A}	1.46	0.0
600–710	44.4^{aB}	81.5^{A}	1.53	0.0
SEM	1.36	1.62		
P value	0.002	0.126		

^{a,b,} means within column with different superscripts differ ($P < 0.05$).
^{A,B,} means within row with different superscripts differ ($P < 0.05$).
SEM: Standard error of mean.

Experiment 4: Apparent ileal Ca digestibility of Australian limestone with different particle sizes in birds fed a typical Australian broiler diet

Ileal Ca and P digestibility of birds fed sorghum-wheat basal diets containing limestone of different particle sizes with or without phytase supplementation is summarised in Table 14. The effect of limestone particle size on ileal Ca ($P = 0.0567$) and P ($P = 0.964$) digestibility did not show significant differences. Phytase significantly increased ($P < 0.05$) ileal Ca and P digestibility (Table 14). There are significant interactions between limestone particle size and phytase supplementation ($P < 0.05$) (Table 14).

Table 14 Influence of limestone particle size, with or without phytase supplementation, on ileal Ca digestibility of limestone and ileal P digestibility of the diet with or without phytase supplementation of a sorghum/wheat diet: Experiment 4

Limestone particle size (µm)	Phytase	Ileal digestibility (%)	
		Ca	P
250–355	-	50.1	42.7
250–355	+	52.2	76.5
600–710	-	43.9	41.7
600–710	+	53.0	77.3
Main effect			
Particle size			
SEM		0.95	1.20
P value		0.057	0.964
Phytase			
SEM		0.95	1.20
P value		0.000	<0.001
Particle size x phytase			
SEM		1.34	1.70
P value		0.016	0.609

SEM: Standard error of mean.

Further statistical analysis on the effect of limestone particle size on ileal Ca and P digestibility with or without phytase supplementation was performed and are detailed in Tables 15a and 15b.

Table 15a Influence of limestone particle size, with or without phytase supplementation, on ileal Ca digestibility of limestone of broilers a fed sorghum/wheat diet in Experiment 4

Limestone particle size (µm)	-phytase	+phytase	SEM	P value
250–355	50.1^{a}	52.2	1.54	0.342
600–710	43.9^{bB}	53^{A}	1.11	0
SEM	1.42	1.27		
P value	0.009	0.667		

^{a,b,} means within column with different superscripts differ ($P < 0.05$)
^{A,B,} means within row with different superscripts differ ($P < 0.05$)
SEM: Standard error of mean

Table 15b Ileal P digestibility of sorghum/wheat diet containing different limestone particle sizes with or without phytase supplementation in Experiment 4

Limestone particle size (µm)	-phytase	+phytase	SEM	P value
250–355	42.7^{B}	76.5^{A}	1.98	0
600–710	41.7^{B}	77.3^{A}	1.38	0
SEM	1.91	1.46		
P value	0.729	0.703		

^{A,B,} means within row with different superscripts differ (P < 0.05).
SEM: Standard error of mean.

Ileal Ca digestibility of limestone with different particle sizes from the same source, showed significant differences, which agrees with the results of Experiment 3 (without phytase supplementation) in which higher ileal Ca digestibility was observed with limestone of smaller particle size than limestone of larger particle size. Phytase supplementation significantly improved Ca digestibility of limestone with a particle size of 600–710 µm (P < 0.05), but not with the smaller particle size of 250–355 µm (P > 0.05). Limestone particle size did not have a significant effect on ileal P digestibility in the current study when a blended wheat/sorghum diet was used as basal experimental diet. That is in contrast to the results of Kim et al. (2019) and our previous experiment (Experiment 3), when maize-soybean meal diets were used.

As discussed in Experiment 3, more research is required on the bioavailability of Ca in Australian limestones, including studies with limestones sourced from different locations and different particle sizes. It is also possible that there is an optimal limestone particle size range to be determined.

Experiment 5: Apparent ileal Ca digestibility of Australian limestone in broilers fed typical Australian diets

Ileal Ca digestibility of Australian limestone in broilers fed typical diets with or without phytase supplementation is summarised in Table 16. Both main factors of diet and phytase had a significant effect on ileal Ca digestibility (P < 0.05). The effect of diet on ileal P digestibility did not show a statistically significant difference (P = 0.068). There were interactions (P < 0.05) between diet and phytase supplementation (P < 0.05) (Table 16).

Table 16 Effects of diet and phytase on ileal Ca digestibility of Australian limestone; Ileal P digestibility of diet with or without phytase supplementation in Experiment 5

Diet	**Phytase**	**Ileal digestibility**	
		Ca	**P**
Sorghum	-	50.2	44.7
Sorghum	+	63.8	82.3
Wheat	-	46.1	44.4
Wheat	+	70.2	82.4
Sorghum/wheat	-	56.5	52.8
Sorghum/wheat	+	70.2	80.8
Main effect			
Diet			
SEM		1.36	1.16
P value		0.005	0.068
Phytase			
SEM		1.11	0.944
P value		0.000	0.000
Diet x Phytase			
SEM		1.93	1.63
P value		0.013	0.005

SEM: Standard error of mean

Separate statistical analyses of the diet effect with or without phytase supplementation on ileal Ca and P digestibility were performed and are shown in Tables 17a and 17b.

Diet composition had a significant effect on ileal Ca digestibility of limestone. Without supplemental phytase, feeding combinations of sorghum and wheat diets had higher ileal Ca digestibility than wheat alone ($P < 0.05$). Ileal Ca digestibility in the sorghum diet did not show significant differences ($P > 0.05$) compared to either wheat or combinations of sorghum and wheat ($P > 0.05$) (Table 17a). Sorghum had the lowest response to phytase supplementation compared to the other two diets, that is significantly lower ileal Ca digestibility than either wheat or sorghum/wheat combination diets ($P < 0.05$) with phytase supplementation (Table 17a). There were no significant differences in ileal Ca digestibility between wheat and the sorghum/wheat combination.

Ileal P digestibility was similar in sorghum or wheat diets ($P > 0.05$) and significantly lower than in the dietary combination of sorghum and wheat ($P < 0.05$) without phytase supplementation (Table 17b). All diets responded to phytase supplementation in a similar manner, with significantly ($P < 0.05$) higher ileal P digestibility than without supplemental phytase, with no significant differences ($P > 0.05$) in P digestibility between diets (17a and 17b).

Table 17a Effects of diet with or without phytase supplementation on ileal Ca digestibility of Australian limestone in Experiment 5

	Ileal Ca digestibility (%)			
Diet	**-phytase**	**+phytase**	**SEM**	**P value**
Sorghum	50.2^{abB}	63.8^{bA}	1.73	0.00
Wheat	46.1^{bB}	70.2^{aA}	1.82	0.00
Sorghum+ Wheat	56.5^{aB}	70.2^{aA}	2.21	0.01
SEM	2.11	1.74		
P value	0.008	0.024		

^{a,b,} means within column with different superscripts differ ($P < 0.05$).
^{A,B,} means within row with different superscripts differ ($P < 0.05$).
SEM: Standard error of mean.

Table 17b Effects of diet with or without phytase supplementation on ileal P digestibility in Experiment 5

	Ileal P digestibility (%)			
Diet	**-phytase**	**+phytase**	**SEM**	**P value**
Sorghum	44.7^{bB}	82.3^{A}	1.34	0.00
Wheat	44.4^{bB}	82.4^{A}	1.32	0.00
Sorghum+ Wheat	52.8^{aB}	80.8^{A}	2.11	0.00
SEM	1.19	1.98		
P value	<0.001	0.825		

^{a,b,} means within column with different superscripts differ ($P < 0.05$).
^{A,B,} means within row with different superscripts differ ($P < 0.05$).
SEM: Standard error of mean.

Implications

The results of the present evaluation demonstrate that an ileal digestibility assay using 22 day-old broilers that have been fed the bioassay diet for 36 hours is suitable for determining Ca digestibility of tested limestone samples. Our results indicate that the method evaluated in this project is reproducible and suitable for **ranking** samples. The results also imply that both limestone particle size and the diet being fed to broilers are important digestibility variables:

- Particle size is an important characteristic of limestone that affects Ca and P digestibility, and should be considered when formulating diets. The particle size of limestone significantly affected ileal Ca digestibility. However, the effect of limestone particle size on ileal P digestibility was found in maize-soybean meal diet (Experiment 3), not in sorghum/wheat diet (Experiment 4). Phytase improved ileal Ca digestibility in diet containing larger limestone particle size (600–710 µm), and ileal P digestibility regardless of limestone particle size.
- The diet (maize, wheat, sorghum, wheat/sorghum) to which limestone is added impacts on Ca digestibility, but phytase supplementation increased ileal Ca and P digestibility regardless of diet composition.

The ileal digestibility method assessed in this project can be successfully applied to determine ileal Ca digestibility of Australian Ca sources, and provides industry with a **ranked** database suitable for commercial application.

Recommendations

The method evaluated in the project is suitable for determining ileal Ca digestibility in broilers at 22 days of age.

The same limestone samples with a range of particle sizes should be tested in birds of different ages fed the same basal experimental diet to determine the optimal particle size of limestone for birds.

A wide range of limestone samples and inorganic Ca sources from various locations should be tested to establish a digestible Ca database.

Many factors affect Ca digestibility, and these should also be examined in future research.

After establishing an ileal Ca digestibility database, it would be interesting to formulate diets based on digestible Ca and P values, and feed birds to day 21 or 49/56, and measure their performance and bone characteristics. This would permit the determination of digestible Ca and P requirements and their optimal ratios.

Acknowledgments

We are grateful for support by the Poultry Hub Australia, the School of Agriculture and Food Sciences, the University of Queensland and Feedworks, Australia.

Professor Peter Plumstead provided the experimental protocol and he and Professor Rosalina Angel were most helpful with initial discussions.

Valuable comments and suggestions from Australian leading poultry nutritionists, in particular, Drs David Cadogan and Kim H. J. Huang are greatly appreciated.

We thank our colleagues, Katherine Raymont and Peter Isherwood and our postgraduate students who helped with bioassays, collection and analysis of the samples.

A great team effort was required to complete the project successfully due to the impact and restrictions imposed the Covid-19 pandemic.

Media and Publications

Li X, Zhang D, Pan LY, Wilkinson SJ, Bryden WL (2022) Determination of calcium and phosphorus digestibility in a short-term bioassay with broilers. *Australian Poultry Science Symposium,* **33**:141.

Intellectual Property Arising

Not applicable.

References

Angel CR (2019) Update on ingredient calcium digestibility: impact of presence and source of phytate and source of calcium. *Australian Poultry Science Symposium,* **30:** 51-56.

Angel CR, Applegate TJ, Christman M (2000a) Effect of dietary non-phytate phosphorus (nPP) on performance and bone measurements in broilers fed on a four-phase feeding system. *Poultry Science,***79** (Suppl. 1), 21-22.

Angel CR, Applegate TJ, Christman M, Mitchell AD (2000b) Effect of dietary non-phytate phosphorus (nPP) level on broiler performance and bone measurements in the starter and grower phase. *Poultry Science,***79** (Suppl. 1), 22.

Anwar MN, Ravindran V, Morel PCH, Ravindran G, Cowieson AJ (2016) Effect of limestone particle size and calcium to non-phytate phosphorus ratio on true ileal calcium digestibility of limestone for broiler chickens. *British Poultry Science*, **57**: 707–713.

Applegate TJ, Angel R, Classen HL (2003) Effect of dietary calcium, 25-hydroxycholecalciferol, or bird strain on small intestinal phytase activity in broiler chickens. *Poultry Sci*ence, **82:** 1140–1148.

Aviagen (2014) Ross 308 broiler nutrition specifications. 0814-AVNR-035.

Bryden WL, Li X (2010) Amino acid digestibility and poultry feed formulation: Expression, limitations, and application. *Revista Brasileira de Zootecnia* **39** suppl.: 279- 287.

Cobb (2018) *Cobb broiler performance and nutrition supplement*. Cobb-Vantress.com.

Cordel D, Drangert J, White S (2009) The story of phosphorus: Global food security and food for thought. *Global Environment Change*, **19:** 292-305.

Feedworks Pty Ltd (2020) Australian Limestone Survey 2.

Kim SW, Li W, Angel R, Plumstead PW (2019) Modification of a limestone solubility method and potential to correlate with in vivo limestone calcium digestibility. *Poultry Science*, **98**: 6837–6848.

Lemme A, Ravindran V, Bryden WL (2004). Ileal digestibility of amino acids in feed ingredients for broilers. World's Poultry Science Journal. 60:423-437.

Leske K & Coon CN (2002) The development of feedstuff retainable phosphorus values for broilers. *Poultry Science,* **81*:***1681-1693.

Li W, Angel R, Plumstead PW, Enting H (2021) Effects of limestone particle size, phytate, calcium source, and phytase on standardized ileal calcium and phosphorus digestibility in broilers. *Poultry Science*, 100: 900-909.

Li X, Higgins TJV, Bryden WL (2006). Biological response of broiler chickens fed peas (*Pisum sativum* L.) expressing the bean (*Phaseolus vulgaris* L.) α-amylase inhibitor transgene. *Journal of the Science of Food and Agriculture,***86**: 1900-1907.

Li X, Zhang D, Yang TY, Bryden WL (2016) Phosphorus bioavailability: A key aspect for conserving this critical animal feed resource with reference to broiler nutrition. *Agriculture* **6**, 25.

Li X, Zhang D & Bryden WL (2017) Calcium and phosphorus metabolism and nutrition of poultry: Are current diets formulated in excess? *Animal Production Science,* **57:** 2304- 2310.

Mutucumarana RK, Ravindrana V, Ravindran G, Cowieson AJ (2015) Measurement of true ileal phosphorus digestibility in maize and soybean meal for broiler chickens: Comparison of two methodologies. Animal Feed Science and Technology **206**: 76–86.

Persia ME, Saylor WW (2006) Effects of broiler strain, dietary nonphytate phosphorus, and phytase supplementation on chick performance and tibia ash. *Journal of Applied Poultry Research,***15**, 72-81.

Plumstead PW, Sinclair-Black M & Angel CR (2020) The benefits of measuring calcium digestibility from raw materials in broilers, meat breeders, and layers. *Australian Poultry Science Symposium,* **31**:8-15.

Rodehutscord *et al*. (2017) Results of an international phosphorus digestibility ring test with broiler chickens. *Poultry Science*, **96**:1679–1687.

Shastak Y, Witzig M, Hartung K, Rodehutscord M (2012) Comparison of retention and prececal digestibility measurements in evaluating mineral phosphorus sources in broilers. *Poultry Science,***91:** 2201–2209.

Summers JD (1997) Precision phosphorus nutrition. *The Journal of Applied Poultry Research,* **6**:495-500.

Taylor S, Sinclair Black M, Angel R, Jansen van Rensburg C & Plumstead PW (2019) *Proceedings of IPSF*, Atlanta GA, 10-11 Feb. 2019, pp 77.

Waldroup P W, Kersey JH, Saleh EA, Fritts CA, Yan F, Stilborn HL, Crum RC Jr, Raboy V (2000) Nonphytate phosphorus requirement and phosphorus excretion of broilers fed diets composed of normal or high available phosphate corn with and without microbial phytase. *Poultry Science,***79:** 1451-1459.

Walk CL, Bedford MR, McElroy AP (2011) Influence of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. *Poultry Science,* **91**: 1371-1378.

Walk CL, Romero LF, Cowieson AJ (2021) Towards a digestible calcium system for broiler chicken nutrition: A review and recommendations for the future. *Animal Feed Science and Technology*, **276:** 114930. https://doi.org/10.1016/j.anifeedsci.2021.114930.

Zhang B & Coon C (1997) Improved *in vitro* methods for determining limestone and oyster shell solubility. *Journal of Applied Poultry Research*, **6**: 94–99.

Appendices

Appendix 1 Composition of the pre-experimental diet fed from day 1 to 20 post-hatch for all experiments

Ingredient	**(g/kg)**
Wheat	270
Sorghum	269
Soybean meal	351
Canola meal	30
Meat and bone meal	20
Canola oil	35.5
Lysine. HCl 78	2.0
DL-Methionine	3.2
L-Threonine	0.8
Australian limestone	7.0
MDCP Biophos	1.47
Salt	2.2
Sodium bicarbonate	2.5
Vitamin & minerals Premix	5.0
Choline chloride 60% dry	0.5
Ingredient total	1000
Nutrient calculation (%)	
AME (MJ/kg)	12.65
Crude protein	24.7
Crude fat	5.55
Lysine	1.43
Met + Cys	1.04
Methionine	0.67
Cystine	0.37
Threonine	0.96
Arginine	1.57
Isoleucine	0.99
Valine	1.09
Leucine	1.90
Tryptophan	0.29
Dig Lysine	1.27
Dig Met + Cys	0.94
Dig Methionine	0.64
Dig Cystine	0.30
Dig Threonine	0.83
Dig Arginine	1.42
Dig Isoleucine	0.88
Dig Valine	0.95

Ingredient	**(g/kg)**
Dig Leucine	1.69
Dig Tryptophan	0.25
Ca	0.65
Total P	0.55
Avail. P	0.25
Na	0.18
Cl	0.23
K	0.95
Choline (mg/kg)	1592.2
Linoleic acid (18:2)	2.10