Global survey of limestone used in poultry diets: calcium content, particle size and solubility

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Abstract

Limestone is used in poultry diets as a calcium (Ca) source. Feed formulation is often based on an estimate of limestone Ca content or, less frequently, wet chemistry analysis. However, limestone composition may vary, which has not previously been studied on a large scale. In this study, 641 limestone samples supplied to poultry feed mills in 40 countries across eight global regions were collected and analysed for macro- and micromineral content, particle size (geometric mean diameter, GMD), and *in vitro* solubility. Mean Ca content of fine limestone (GMD < 1000 µm, n = 566) was 37.8% (range 33.3–39.7%) and for coarse limestone (GMD > 1000 µm, n = 75) was 38.0% (range 34.7–40.0%). There was marked variation among and within regions in the concentration of other macro minerals and microminerals. Particle size (GMD) of fine limestone was 288 µm (range 37.7–991.9 µm) and 1,689 µm for coarse limestone (range 301.6–3,067.9 µm). *In vitro* solubility of fine limestone (5 min incubation, pH = 3.0) was 68.4% (range 18.8 to 99.4%). Particle size only explained 52% of the variation in 5 min incubation solubility ($R^2 = 0.52$). For coarse limestone, mean solubility (30 min incubation) was 65.5% (range 23.2–96%) which was not correlated ($R^2 = 0.09$) with particle size. Particle size and solubility rate of limestone have been shown to alter Ca and phosphorus utilisation in broilers and laying hens. Hence, better understanding of variation in mineral analysis, particle size and solubility rate would enable more accurate feed inclusion and subsequently digestibility to support health and performance.

Keywords: calcium, limestone characteristics, phosphorus digestibility, phytase

1. Introduction

Calcium (Ca) is an essential nutrient that is important for bone development and mineralisation, eggshell formation, muscle and neural functions (Klasing, 1998). Calcium metabolism is related to and influenced by the availability of phosphorus (P) and vitamin D (Proszkowiec-Weglarz and Angel, 2013) so that any inadequacy or excess of one may significantly affect the metabolism of the other. The utilisation of Ca and P depends on their concentration and availability in the feed and is modulated *via* intestinal, renal and skeletal mechanisms (Li *et al.*, 2017). Ca digestibility is affected by phytase enzymes that are commonly added to

poultry diets to improve the availability of phytate-bound P and reduce unwanted excretion into the environment (Dersjant-Li *et al.*, 2018; Selle and Ravindran, 2007). Phytase enzymes improve Ca availability by reducing the degree of phytate binding in the digestive tract, thereby increasing its availability for absorption and utilisation by the bird.

Ca requirements for poultry have historically been defined by the National Research Council (NRC, 1977), with various updates being published as data increased and the estimated nutrient requirements were refined (NRC, 1994). Additionally, other nutrient tables available from Centraal Veevoederbureau (CVB), Institut Nationale de la Recherche

Agronomique (INRA) and Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA) are available. Certain suppliers of poultry genetics have recommended specific ratios of Ca to available P in broiler (2:1) and layer (9:1 to 13:1) diets in order to ensure that these nutrients are in balance so that their metabolism and utilisation is optimised (Aviagen, 2019; Cobb, 2019; Joice and Hill Poultry, 2018). However, since poultry diets are still formulated using total Ca, and multiple factors can contribute to differences in its utilisation, providing diets that deliver the correct ratio of digestible Ca:P is not straightforward, and should ideally be based on knowledge of all factors that may influence availability *in vivo*. It has been reported that limestone in feed can contribute more than 50% of total dietary Ca consumed by broilers (Kim *et al.*, 2019). For all-vegetable based broiler diets containing no meat or bone meal, this may increase to 70%. In the case of layers, limestone can contribute more than 95% of Ca intake per hen per day. The remaining Ca comes from cereals, legumes and their by-products, bone meal, inorganic phosphates, mono-calcium phosphate or di-calcium phosphate, premixes and feed additives. Walk (2016) showed that analysed Ca content in finished feed were, on average, 22% higher than the calculated content, and that calculated dietary Ca content could vary considerably, depending on the reference used for ingredient Ca concentration. This implied that relying on calculated values only may fail to provide an accurate estimate of dietary Ca content or its ratio to P. Limestone variability can influence the analysed content of Ca in the final feed, although these factors are generally not considered in feed composition tables. For example, the Dutch feed tables (CVB, 2016) specify that the total Ca content of limestone is 38%, whereas in a survey of 192 fine limestone samples, Plumstead *et al.* (2020) reported that the analysed Ca content ranged from 30.4 to 40.0%. More recent literature has indicated that the Ca content of limestone can be as high as 42% (Anwar *et al.*, 2016; David *et al.*, 2019; 2020). Interestingly, the maximum Ca content in pure CaCO_3 is 40.04%, based on chemical composition and molecular weight of Ca, C and O. Levels of 42% could be due to analytical errors or, in rare instances, where crushing of limestone generates heat that converts CaCO_3 to CaO , where the content would be higher.

Limestone particle size, chemical composition and rate of solubilisation may influence dietary Ca availability *in vivo* and need to be taken into account during diet formulation. However, there is limited research regarding differences among limestone sources, especially in regional and global samples. Limestone particle size is known to be related to its *in vitro* solubility (Zhang and Coon, 1997), but is altered by differences in the physical structure of the limestone rock and geological origin (Plumstead *et al.*, 2020). Recent research has shown that limestone particle size and rate of solubility can affect Ca and P digestibility as well as phytase efficacy in complete feed (Davin *et al.*,

2020; Kim *et al.*, 2018, 2019; Li *et al.*, 2021). Knowledge in this area is still developing and a better understanding of the extent of variability in composition, particle size and solubility of limestone currently used in commercial feed production is required. Against this background, this study was undertaken to survey current limestone used in broiler and layer feed mills.

2. Materials and methods

Limestone sample collection

Limestone samples ($n=566$ for fine limestone geometric mean diameter (GMD) $<1000\ \mu\text{m}$) were obtained from limestone sources that supply commercial feed mills situated in eight geographical regions: African countries (AF, $n=11$, including Morocco, Malawi and other (non-described)); Australia and New Zealand (ANZ, $n=51$); Asia (India, Indonesia, Japan, Malaysia, Pakistan and Vietnam, $n=25$); European union including UK (EU, $n=256$, including Belgium, Czech Republic, Denmark, Finland, France, Germany, Ireland, Italy, Norway, Poland, Portugal, Spain, Sweden, the Netherlands, Ukraine and United Kingdom); Middle East (ME, $n=23$, including Jordan, Saudi Arabia and Turkey); North America (NA, $n=150$, including Canada, Costa Rica, Guatemala, Honduras, Nicaragua and USA); Russia (RU, $n=6$); South America (SA, $n=44$, including Argentina, Brazil and Colombia).

Additionally, total of 75 coarse limestone samples (GMD $>1000\ \mu\text{m}$) were collected from the EU (including UK) ($n=68$), RU ($n=3$) and ME ($n=4$).

Limestone collection and analysis

Limestone samples (500 g) were collected from commercial feed mills in each region and shipped to the laboratory (Chemuniqué Laboratory, Lanseria, South Africa). Upon arrival the samples were thoroughly mixed and halved using a Riffler sample splitter instrument (Lenton Laboratory and Scientific Equipment, Randburg, South Africa). One half was retained for repeat analysis if needed, and the other half (250 g) was analysed for mineral composition, particle size distribution and solubility.

Mineral analysis of limestone samples was done in duplicate using Inductively Coupled Plasma-optical Emission Spectroscopy (ICP-OES), according to the AOAC Method 985.01 (AOAC, 1996) to determine Ca, P, magnesium (Mg), iron (Fe), manganese (Mn), sodium (Na), potassium (K) and zinc (Zn).

Particle size distribution was determined by sieving using a shaker and a set of 14 sieves, ranging from 25–3,350 μm , plus a base pan according to method ANSI/ASAE S319.4 (American Society of Agricultural and Biological Engineers,

2008). The geometric diameter (mm) of particles was calculated by mass, according to the equations described by Wilcox *et al.* (1962). Samples with a GMD < 1000 µm were classed as 'fine', and those with a GMD > 1000 µm as 'coarse'.

Historically, limestone solubility has been measured under acidic conditions (0.2 N HCl) for 10 min using a method developed by Zhang and Coon (1997). However, this procedure was recently modified by Kim *et al.* (2019) using a pH more representative of conditions in the gizzard of chickens (pH=3.0, produced by using HCl solution buffered with 3 M glycine) and measured at three time points. The method of Kim *et al.* (2019) was used in this work. Briefly, the limestone solubility of 1 g from a representative sample of the limestone (for each time point) was measured at three incubation time points: 5, 15 and 30 min for the fine limestone and at 30, 90 and 150 min for the coarse limestone.

Statistical analysis

Data were analysed to determine mean, standard error (SE), standard deviation (SD) and coefficient of variance (CV) by region. One-way ANOVA was used to determine statistical differences ($P < 0.05$) and Tukey's HSD was used to compare means. Pearson correlation coefficients (R^2) were determined to evaluate the relationship between

quantitative variables. Correlations between the content of Ca and of other minerals in limestone were assessed by application of a bivariate fit curve and calculation of the R^2 value, where correlations were statistically significant ($P < 0.05$). Correlations between limestone mean particle size and solubility were assessed by non-linear curve fitting. All data analyses were performed in the Fit Model platform of JMP 15.0 (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

Limestone chemical composition

The mineral contents including Ca, copper (Cu), Fe, K, Mg, Mn, Na, P and Zn (percentage average) of the fine limestone was categorised by geographical region, and is presented in Figure 1 and Table 1 and 2.

Mean total Ca content of the 'fine' limestone was generally high, but was consistently below 40%, with African (AF) countries having consistently lower averages (typically <34%). Ca content varied significantly across regions as well as within a region (as shown by minimum and maximum values in tables and figure). Mean Ca content varied by 6.4% among regions when AF was included, and 2.2% when AF was excluded. Mean Ca content was highest for samples from the Middle East (ME; 39.7%). The number of samples

Figure 1. Calcium content in fine limestone from different global regions. Regions were defined as: AF = African countries Morocco, Malawi and other (non-described); ANZ = Australia and New Zealand; EU = European Union including UK (Belgium, Czech Republic, Denmark, Finland, France, Germany, Ireland, Italy, Norway, Poland, Portugal, Spain, Sweden, the Netherlands, Ukraine, United Kingdom; ME = Middle East Amman, Saudi Arabia and Turkey; NA = North America Canada, USA, Nicaragua, Honduras, Guatemala, Costa Rica; RU = Russia; SA = South America Argentina, Brazil, Columbia. SE = standard error. Different letters represent significant differences ($P < 0.05$).

Table 1. Macromineral content (percent) of fine limestone (geometric mean diameter <1000 mm) samples by geographical region (total n=566).¹

Region ²	n	Magnesium %				Phosphorus %				Potassium %				Sodium %			
		Mean	Min	Max	SE	Mean	Min	Max	SE	Mean	Min	Max	SE	Mean	Min	Max	SE
AF	11	1.56 ^a	0.08	6.25	0.62	0.02	0.01	0.05	0.00	0.02 ^{ab}	0.01	0.03	0.00	0.01	0.01	0.01	0.00
ANZ	51	0.31 ^{bc}	0.06	1.24	0.03	0.01	0.01	0.03	0.00	0.02 ^b	0.01	0.12	0.00	0.01	0.01	0.04	0.00
Asia	25	0.62 ^b	0.01	2.33	0.14	0.02	0.01	0.08	0.00	0.02 ^b	0.01	0.09	0.00	0.01	0.01	0.04	0.00
EU	256	0.28 ^c	0.02	2.86	0.02	0.22	0.01	34.71	0.15	0.06 ^b	0.01	8.31	0.03	0.04	0.01	5.05	0.02
ME	23	0.21 ^c	0.03	1.14	0.06	0.19	0.01	3.93	0.17	2.85 ^a	0.01	65.15	2.83	0.01	0.01	0.02	0.00
NA	150	0.31 ^c	0.07	2.21	0.02	0.03	0.01	0.72	0.01	0.02 ^b	0.01	0.22	0.00	0.03	0.01	0.51	0.00
RU	6	0.45 ^{bc}	0.20	1.33	0.18	0.01	0.01	0.01	0.00	0.01 ^{ab}	0.01	0.02	0.00	0.01	0.01	0.02	0.00
SA	44	0.39 ^{bc}	0.02	2.55	0.08	0.03	0.01	0.12	0.00	0.02 ^b	0.01	0.12	0.00	0.04	0.01	0.37	0.01

¹ Superscripts with different values differ significantly ($P<0.05$).
² Regions were defined as: AF = African countries Morocco, Malawi and other (non-described); ANZ = Australia and New Zealand; EU = European Union including UK (Belgium, Czech Republic, Denmark, Finland, France, Germany, Ireland, Italy, Norway, Poland, Portugal, Spain, Sweden, the Netherlands, Ukraine, United Kingdom; ME = Middle East Amman, Saudi Arabia and Turkey; NA = North America Canada, USA, Nicaragua, Honduras, Guatemala, Costa Rica; RU = Russia; SA = South America Argentina, Brazil, Columbia.

Table 2. Microminerals content (mg/kg) of fine limestone (geometric mean diameter <1000 mm) samples by geographical region (total n=566).¹

Region ²	n	Copper mg/kg				Iron mg/kg				Manganese mg/kg				Zinc mg/kg			
		Mean	Min	Max	SE	Mean	Min	Max	SE	Mean	Min	Max	SE	Mean	Min	Max	SE
AF	11	3.51 ^{ab}	0.01	10.00	1.30	4,463.3 ^a	120.69	26,897.30	2,283.00	625.4 ^a	12.59	4,197.42	364.41	11.79 ^{ab}	2.06	18.03	1.31
ANZ	51	3.58 ^{ab}	0.01	10.71	0.42	1,075.9 ^b	62.52	6,241.57	171.67	130.3 ^b	0.01	598.93	19.36	10.51 ^b	0.01	45.09	1.59
Asia	25	2.47 ^b	0.01	25.18	0.98	544.17 ^b	124.80	1,514.89	60.60	74.00 ^b	5.74	313.28	15.44	21.21 ^{ab}	0.01	161.75	6.57
EU	256	3.33 ^b	0.01	31.26	0.26	934.93 ^b	42.66	8,325.53	71.33	164.02 ^b	0.01	1,440.34	13.93	19.84 ^{ab}	0.01	93.92	1.04
ME	23	7.08 ^a	0.01	62.70	2.70	325.60 ^b	0.01	1,457.92	90.15	67.37 ^b	0.01	773.56	33.35	27.22 ^a	0.01	46.79	3.07
NA	150	2.45 ^b	0.01	39.58	0.40	817.57 ^b	143.64	5,246.49	68.75	114.47 ^b	0.01	1,012.53	11.79	26.10 ^a	0.01	239.00	2.52
RU	6	2.93 ^{ab}	0.70	7.52	1.10	1,761.4 ^b	703.92	4,982.76	654.01	163.77 ^b	30.70	345.44	50.81	14.24 ^{ab}	6.61	18.73	1.73
SA	44	2.46 ^b	0.01	24.37	0.63	966.89 ^b	0.01	7,680.44	204.25	96.99 ^b	0.01	1,724.43	40.39	17.29 ^{ab}	0.01	43.61	1.76

¹ Superscripts with different values differ significantly ($P<0.05$).
² Regions were defined as: AF = African countries Morocco, Malawi and other (non-described); ANZ = Australia and New Zealand; EU = European Union including UK (Belgium, Czech Republic, Denmark, Finland, France, Germany, Ireland, Italy, Norway, Poland, Portugal, Spain, Sweden, the Netherlands, Ukraine, United Kingdom; ME = Middle East Amman, Saudi Arabia and Turkey; NA = North America Canada, USA, Nicaragua, Honduras, Guatemala, Costa Rica; RU = Russia; SA = South America Argentina, Brazil, Columbia.

varied among regions which could explain some of the variation by region.

The analysed Ca content of limestone from different regions can be used to give an indication of the likely variation in dietary contribution. As an example, if a broiler grower diet was formulated to contain 1% limestone using NRC (1994) feed tables on an analysed basis, limestone from AF would be expected to contribute 0.33% of Ca in the diet, instead of 0.38% based on the NRC feed table (NRC, 1994). Whilst the difference between these estimated contributions is relatively small, it would impact the actual content of Ca in the final diet. Furthermore, these estimates reflect the differences between region, not the total extent of variation between individual samples from different sources within the same region. This variation was considerably greater, as suggested by the range (minimum 29.2% for AF limestone, maximum 42.2% for ME). It was evident that the analysed Ca content of the limestone from different regions did not

(with the exception of ANZ region) match the published value for limestone published by NRC (1994) of 38.0%, although this value is frequently used by feed nutritionists as a basis for dietary formulation. The average Ca content of limestone samples from AF, EU and ME regions were, respectively, 4.7% below, 0.3% below, and 1.7% above the NRC (1994) value, respectively.

Numerous *in vivo* studies have indicated that the analysed total Ca level in the diet can significantly alter growth performance outcomes, nutrient (Ca, P and phytate) digestibility and utilisation, and phytase efficacy (Dersjant-Li *et al.*, 2018; Li *et al.*, 2017; Powell *et al.*, 2011; Tamim *et al.*, 2004). Tamim *et al.* (2004) tested corn-soy diets containing no Ca compared with 0.5% Ca from limestone and reported a reduction in apparent ileal Ca (46.3 to 33.6%) and P (67.9 to 29.4%) absorption with increasing dietary Ca content. Studies by Kim *et al.* (2018) found no effect of increasing dietary Ca content from 0.6% to 1.0%

on its apparent ileal digestibility (AID), however, AID of P decreased from 66.9% (0.6% Ca) to 51.0% (1.0% Ca) P when pulverised limestone was used as the Ca source (75 vs 402 μm). This suggested that total dietary Ca along with particle size can influence AID of both Ca and P. The data for Ca content of limestone sources in Figure 1 and Table 3 suggested that analysis of limestone source prior to formulating is preferable to using estimated Ca content from feed composition tables. This would reduce uncertainty and improve accuracy in formulations for Ca, reducing the likelihood of Ca being limiting or in excess.

There are few studies that can be used to compare with the current results. Anwar *et al.* (2017) reported 42% Ca in fine (<0.5 mm) and coarse (1–2 mm) limestone sources from New Zealand (n=2) and Wilkinson *et al.* (2013) reported 40.1% for fine ('powdered') limestone from Australia (n=13). These values were slightly higher than the mean obtained for the ANZ region in the present study (38%), which may reflect analytical errors or real differences in composition or a product of the small sample sizes employed in earlier studies. Both studies used a standard ICP-OES method for Ca determination, however inter-lab variation is known to exist. Davin *et al.* (2020) measured 38.2% Ca in fine limestone (<22 μm , GMD) sourced from France and 37.4% for a coarse limestone (564 μm , GMD) from Germany using ICP-OES. Pelicia *et al.* (2009) reported 35.7% Ca for coarse (3.13 mm, GMD) and 36.5% for fine (0.18 mm, GMD) limestone, respectively, in a study in Brazil, which were somewhat lower than the average of 37.6% obtained for SA region in the present study. These limited comparisons supported the conclusion that there is considerable variation in Ca content of limestone within regions, between regions and across studies.

Other microminerals, such as Mg and K, were present in the fine limestone at low levels relative to Ca. However, AF region was significantly higher in Mg than other regions (Table 1). Similarly, EU and ME regional samples were lower in Mg. Levels of K were significantly higher in ME samples compared to other regions. The micromineral content of the fine limestone varied among regions (Table 2) but did not appear to be related to Ca. For example, total Ca was positively correlated with Fe ($R^2=0.21$, $P<0.001$)

and negatively correlated with Mg ($R^2=0.24$, $P<0.001$). There appeared to be some region-specific differences in micromineral content, which may have been due to differences in local geology of the limestone rock. For example, Cu in limestone from ME were significantly higher than in EU and North America (NA) samples; Fe and Mn were significantly higher in AF samples compared than other regions, although there were only a small number of samples. Fe in samples from other regions differed numerically ($P>0.05$). In contrast, Wilkinson *et al.* (2013) reported considerably lower Fe in samples from New Zealand than those reported currently for samples from the ANZ region (627 vs 1,075 mg/kg), although contents of Mn (129 vs 130.3 mg/kg) and Zn (7.2 vs 10.5 mg/kg) were comparable.

Coarse limestone (those with a GMD>1000 μm) are typically used in layer diets, which are fed in mash form rather than as pellets. The chemical composition of the 'coarse' limestone samples is presented in Table 3. The data were not separated by region, because there were fewer samples in total (n=75) and the majority were from one region, the EU (n=68), with the remaining samples originating from Russia (RU, n=3) and ME (n=4).

The mean Ca content of coarse limestone was in the upper range of values seen for fine limestone, which may have been due to the smaller sample size and origin of the sample. Nevertheless, variation in the Ca content of coarse limestone was evident (minimum and maximum values differed by >5%; Table 3). Given that, in layer diets, limestone can contribute in excess of 90% of total dietary Ca, such variation between individual coarse limestone types could significantly alter the total amount of Ca in the final diet if not taken into account during formulation.

The levels of microminerals in the coarse limestone showed substantial variability (Table 3), arguably greater than for the fine samples, with large CV (>90%) even when SE values were comparatively small. For example, for potassium, the CV% was 219.8 but the SE was only 0.01. Published data from other studies in coarse limestone are lacking, so no comparisons can be drawn. According to published work to date, limestone micromineral contents are not generally

Table 3. The chemical composition of coarse limestone (geometric mean diameter >1000 μm) samples from all regions combined (n=75).¹

	Calcium (%)	Phosphorus (%)	Magnesium (%)	Copper (mg/kg)	Iron (mg/kg)	Manganese (mg/kg)	Potassium (%)	Sodium (%)	Zinc (mg/kg)
Mean	38.0	0.02	0.28	2.29	929.1	205.5	0.02	0.02	23.26
SD	1.39	0.01	0.33	3.29	1,051	281.7	0.02	0.05	25.40
Min	34.7	0.01	0.01	0.01	47.17	3.41	0.01	0.01	1.77
Max	40.0	0.10	2.65	14.21	4,921	1,438	0.10	0.38	171.0
SE	0.16	0.00	0.04	0.39	123.9	33.20	0.00	0.01	2.99
CV (%)	3.65	91.74	119.0	143.7	113.1	137.1	96.34	219.8	109.2

¹ CV = coefficient of variation; SD = standard deviation of the mean; SE = standard error.

considered in diet formulation for poultry. The current data suggested that it may be beneficial to measure the micromineral contents on a regular basis to maintain levels in limits in finished feed. For example, Fe content ranged from 47.2 to 4,921 mg/kg. At a limestone inclusion level of 8.6% in a layer diet, this would contribute up to 42 mg Fe/kg of feed (compared to laying hens' requirement of 40 mg/kg of feed). It is important to mention that normally Fe is added in the premix to fulfil laying hens' requirements. Therefore, when selecting a limestone source, the purity and micromineral content should be considered.

Limestone particle size

The average GMD of the fine limestone samples (by region) and of the coarse limestone (all samples combined) is summarised in Table 4. Fine limestone exhibited considerable variation in particle size (GMD) both among, and especially within, geographic regions. Mean GMD was smallest in Asian samples (154 µm GMD) which were significantly lower than NA samples, which had the greatest GMD at 356 µm, and were broadly comparable amongst most other regions. However, the mean GMD of individual limestone samples within the same region exhibited high variation, as evident from the large SD values which, in some cases, were only marginally lower than the mean itself, and from the CV values which were universally high (minimum 45.7% for RU limestone, maximum 91.9% for AF limestone). Coarse limestone (mean GMD 1,689 µm) exhibited less variation in particle size among individual samples, as indicated by the lower CV% and SD value compared with the fine samples. It is possible that variation may increase with more samples (N) of coarse limestone.

In this survey, limestone samples were arbitrarily classified as 'fine' if they had a mean particle size (GMD) of <1000 µm, or 'coarse' if they had a mean particle size (GMD)

of >1000 µm. These classifications are commonly used to differentiate limestone used in diets for broilers and laying hens, respectively. However, in practice, broiler diet formulations frequently use limestone with a particle size <400 µm (GMD) because these feeds are generally pelleted. Smaller limestone particles result in less damage to feed pelleting equipment, minimises down time, and improves pellet quality.

Limestone *in vitro* solubility

The solubility of limestone in the fine limestone samples after 5, 15 and 30 minutes of incubation is shown by region in Figure 2A, 2B and 2C). The horizontal line in each box represents the median, symbol, x denotes to the mean value, the box limits around this mean represent the upper and lower percentile values either side of the mean, with the extremities of the vertical lines extending from each column represent the minimum and maximum values. Some outliers are shown as independent values. The average solubility of the limestone varied markedly even after only 5 min of incubation (Figure 2A), from as low as 56% for samples from AF region to as high as 83% for those from Asia and EU regions, with the percentile boundaries indicating a substantial amount of variation among individual samples around this mean value. Likewise, 5 min limestone solubility was significantly higher in samples from the EU compared to NA, SA and ANZ regions. Notwithstanding such individual variation, the rapid solubility of many of the fine samples was such that, *in vivo*, a large proportion of the Ca would be expected to have solubilised within the first 5 min of entering the proventriculus and gizzard. Such rapid solubilisation would result in a rise in pH due to the release of carbonate, while the rapid release of Ca cations can interact with phytate and other mineral cations, forming stable mineral-phytate complexes, which are less bioavailable and less accessible to phytase (Selle *et al.*, 2000).

Table 4. Mean particle size of fine (geometric mean diameter (GMD) <1000 µm) limestone by geographical region (total n=566) and of coarse (GMD >1000 mm) limestone for all regions combined (n=75).^{1,2}

Limestone type	Region ³	n	Mean GMD (µm)	SD	Min (µm)	Max (µm)	SE	CV (%)
Fine	AF	11	251.7 ^{ab}	231.2	58.2	895.9	69.7	91.9
	ANZ	51	275.7 ^{ab}	200.1	47.6	902.9	28.0	72.6
	Asia	25	153.9 ^b	131.4	45.9	521.1	26.3	85.3
	EU	258	269.6 ^b	234.3	38.5	988.2	14.6	86.9
	ME	23	261.5 ^{ab}	191.8	53.3	696.3	40.0	73.3
	NA	151	356.3 ^a	196.6	37.7	992.0	16.0	55.2
	RU	6	234.9 ^{ab}	107.3	127.1	442.0	43.8	45.7
	SA	46	258.1 ^{ab}	194.4	49.0	951.8	28.7	68.2
	P (ANOVA)	0.0002						
Coarse	EU+RU+ME	75	1,689	553.1	301.6	3,068	65.2	32.7

¹ Superscripts with different values differ significantly ($P<0.05$).
² CV = coefficient of variation; SD = standard deviation of the mean; SE = standard error.
³ Regions were defined as: AF = African countries Morocco, Malawi and other (non-described); ANZ = Australia and New Zealand; EU = European Union including UK (Belgium, Czech Republic, Denmark, Finland, France, Germany, Ireland, Italy, Norway, Poland, Portugal, Spain, Sweden, the Netherlands, Ukraine, United Kingdom; ME = Middle East Amman, Saudi Arabia and Turkey; NA = North America Canada, USA, Nicaragua, Honduras, Guatemala, Costa Rica; RU = Russia; SA = South America Argentina, Brazil, Columbia.

Figure 2. *In vitro* solubility of fine limestone (<1000 mm geometric mean diameter) after (A) 5 min, (B) 15 min and (C) 30 min at pH=3.0, by geographical region (n=566). Regions were defined as: AF = African countries Morocco, Malawi and other (non-described); ANZ = Australia and New Zealand; EU = European Union including UK (Belgium, Czech Republic, Denmark, Finland, France, Germany, Ireland, Italy, Norway, Poland, Portugal, Spain, Sweden, the Netherlands, Ukraine, United Kingdom); ME = Middle East Amman, Saudi Arabia and Turkey; NA = North America Canada, USA, Nicaragua, Honduras, Guatemala, Costa Rica; RU = Russia; SA = South America Argentina, Brazil, Columbia. Superscripts with different values differ significantly ($P<0.05$).

Thus, the wide variation in solubility of the samples both across, and especially within regions, would influence its bioavailability and potential to interact (beneficially or adversely) with other feed components.

After 15 min of incubation (Figure 2B), the mean solubility of fine limestone was further increased and by 30 min (Figure 2C) over 90% of the limestone had solubilised. The exception was the AF region (mean solubility after 30 min, 80%), in which there was greater variation in the solubility of individual samples and a wider margin of error around the mean. This may have been due to the lower purity of limestone from AF, reflected by the lower Ca

in limestone samples from the region. Similar to 5 min limestone solubility, 15 min solubilities for EU samples were significantly higher than ANZ, NA and SA regions but 30 min solubility was only significantly higher than AF and SA regions.

The solubility of coarse limestone after 30, 60 and 150 min of incubation is presented in Figure 3A, 3B and 3C. The small sample sizes from the ME and RU regions limited the interpretation of these results, but for the EU regional samples, mean solubility was ~70% after 30 min, ~95% after 60 min and >95% after 150 min. As observed with fine limestone, there was considerable variation in among

Figure 3. *In vitro* solubility of coarse limestone (>1000 µm geometric mean diameter) from the European Union (EU), Middle East (ME) and Russia (RU), after (A) 30 min, (B) 90 min and (C) 150 min at pH=3.0 (n=75).

individual coarse limestone from the EU at any given time point, but the shorter box plots in Figure 3, compared with Figure 2, suggested that the extent of this variability was less for coarse limestone.

Even though *in vitro* incubation attempted to mimic *in vivo* solubility in the proventriculus and gizzard (pH=3), how appropriate these assessments were is yet to be determined. The speed and extent of solubility in individual coarse or fine limestone samples *in vivo* have the capacity to influence Ca availability for absorption and to affect levels in the tissues which may impact on growth performance. Sinclair-Black (2019) recently observed that a coarse limestone (1.5 mm GMD, 30% solubility at 30 min) increased blood ionisable Ca concentration in laying hens overnight, when more calcium is needed for eggshell formation, in comparison to a fine limestone (0.2 mm GMD 86% solubility at 30 min). Zhang and Coon (1997) showed that limestone which was rapidly soluble *in vitro* (after 10 min incubation) were associated with reduced gizzard retention of limestone (after 72 h) *in vivo* among laying hens, compared to limestone that are slower to solubilise, which could affect eggshell formation. However, their reported *in vitro* solubility was measured under highly acidic conditions, so the results were unlikely to be representative of *in vivo* solubility. In addition, more recent studies by the same authors reported that slowly soluble limestone was associated with increased bone strength and breaking force compared with rapidly soluble limestone in layer hens, when solubility was measured at pH 3 (i.e. conditions more representative of the proventriculus and gizzard; Zhang *et al.*, 2017, 2020).

Correlation between limestone particle size and *in vitro* solubility

A few studies have demonstrated or suggested an inverse correlation between limestone particle size and solubility (Kim *et al.*, 2018, Plumstead *et al.*, 2020; Zhang and Coon, 1997), but it has been shown that different limestone sources with the same particle size can exhibit different solubilities (Plumstead *et al.*, 2020; Zhang and Coon, 1997). In the present study, after 5, 15 or 30 min of incubation, there was a weak negative correlation between mean particle size and *in vitro* solubility for fine limestone ($R^2=0.52$, 0.3 and 0.09, respectively). However, for the coarse limestone there was no correlation between particle size and solubility at any of the incubation time points (30, 90 or 150 min; $P>0.05$). A few examples from some regions are shown in Figure 4, which shows that similar particle sizes of 322 μm had 66 or 90% solubility at 5 min for the USA limestone samples. Likewise, samples from Brazil were almost 67% soluble at 5 min with contrasting 89 vs 435 μm particle sizes. A similar trend was seen in samples from other regions, like Poland. Properties other than particle size must therefore influence limestone solubility, which could include quality and purity, its Ca content in relation to that of other macro and micronutrients or other substances present. In addition, the unique particle size distribution of the limestone source will vary from source to source. Finally, differences in the geological formation of the limestone may influence the rate of solubilisation. Ultimately, these findings suggested that particle size cannot be used alone as a reliable predictor for the *in vitro* or, by inference, *in vivo* solubility of limestone. Measurement of the solubility characteristics of the limestone prior to its use in dietary formulation would be preferable.

Figure 4. *In vitro* solubility of coarse limestone (>1000 μm geometric mean diameter) from the European Union (EU), Middle East (ME) and Russia (RU), after (A) 30 min, (B) 90 min and (C) 150 min at pH=3.0 ($n=75$). Means not sharing a letter differ significant ($P<0.05$).

Practical implications of limestone solubility

The variation at both global and regional level in the *in vitro* solubility of limestone sources supplied to commercial poultry feed mills was only partially explained by particle size (GMD), which was likely to impact on mineral digestibility in poultry which has the potential to affect growth performance and production. This was seen in the results from several recent studies in broilers, which showed negative relationships between *in vitro* solubility and *in vivo* digestibility of P. Some, but not all studies, observed effects of limestone solubility on ileal Ca and P digestibility in broilers. A selection of these studies is summarised in Table 5, based on the effects of particle size and solubility on Ca and P digestibility. It was difficult to compare all these studies, due to differences in methodology used to measure limestone solubility, age of birds and total Ca in the test diets. However, these studies indicated that, in the absence of phytase supplementation, the effect of limestone solubility and particle size on AID or standardised ileal digestibility (SID) of Ca was inconsistent between studies, whilst the effect on AID or SID of P appeared to be clearer and more reliable. Limestone with smaller particle size and

higher solubility tended to reduce AID or SID of P (Table 5). Studies by Kim *et al.* (2018) and Davin *et al.* (2020) reported no effect of limestone particle size or solubility on AID Ca, independent of phytase addition. Kim *et al.* (2019) reported significant differences between limestone samples, and linear prediction models showed that GMD or solubility (5 min incubation) explained less than 40% and less than 25%, respectively, of the variation in AID of Ca. Differences in geology and/or other physical or chemical characteristics presumably accounted for the rest of the variation. Li *et al.* (2021) reported that fine limestone produced a lower SID of Ca than coarse samples.

It is problematic to try to capture the effects of limestone particle size/solubility on mineral digestibility in the presence of phytase, but each of the studies in Table 5 provided useful data, which suggested that the characteristics of limestone that alter P digestibility also alter phytase efficacy in broiler diets. What is not yet well understood is whether the effects of limestone are similar for different phytase enzymes or different dose rates of phytase. Davin *et al.* (2020) observed that a slowly-soluble limestone with larger particle size (~10% solubility at 5 min, 564 µm

Table 5. Summary of *in vivo* studies that have investigated the effect of limestone particle size/solubility in complete feed on mineral digestibility in broilers.¹

Publication	Limestone source ² and particle size (fine or coarse)	Limestone <i>In vitro</i> solubility at 5 minutes duration			Ca content (%, formulated)	Bird age (d)	AID Ca ⁴ (%)	AID P ⁴ (%)	AID P (coarse vs fine) ⁵
		(GMD, µm)	Method ³	Solubility (%)					
Kim <i>et al.</i> , 2018	A – fine	75	1	96.9	0.60	28	38.1	23.5	
					0.80	28	47.3	20.5	
					1.00	28	46.5	22.2	
	A – coarse	402	1	74.3	0.60	28	44.8	31.5	+8.0
					0.80	28	50.8	27.9	+7.4
Kim <i>et al.</i> , 2019	B – fine	63	2	81.6	0.67	22 to 23	47.5	12.8	
	C – coarse	326	2	13.7	0.67	22 to 23	19.9	65.5	+52.7
	B – coarse	633	2	67.8	0.67	22 to 23	66.3	23.1	+10.3
	D – coarse	831	2	36.0	0.67	22 to 23	66.3	37.4	+24.6
	E – fine	<20	3	~70.0 ⁶	0.76 (starter)	21	37.7	48.3	
Davin <i>et al.</i> , 2020	F – coarse	564	3	~10.0 ⁶	0.64 (grower)				
					0.76 (starter)	21	39.4	52.4	+4.1
Li <i>et al.</i> , 2021	G – fine	151	2	61.2	0.71	23	42.6	60.8	
	G – coarse	800	2	32.6	0.71	23	55.4	87.5	+26.7

¹ AID = apparent ileal digestibility; GMD = geometric mean diameter.

² Limestone bearing the same letter were from the same source (within each study), whilst those bearing different letters were from different sources.

³ 1 = modified weight loss method according to Zhang and Coon (1997); 0.2 N HCL, pH=0.7, incubated at 42 °C, post-filtered samples dried at 100 °C for 24 h and weighed to calculate weight loss. 2 = modified weight loss method according to Zhang and Coon (1997); pH 3.0 HCL buffered with 3 M glycine, incubated at 42 °C, post-filtered samples dried at 100 °C for >8 h and weighed to calculate weight loss. 3 = in-house method; 1 M HCL added to 500 ml water solution of limestone (5.0 g) at 40 °C, pH=3.0.

⁴ Values shown are for AID except for Li *et al.* (2021) where values are standardised ileal digestibility (SID).

⁵ Percentage point difference between coarse and fine limestone.

⁶ Approximate values obtained from Figure 1 in Davin *et al.* (2020).

GMD) reduced AID Ca in treatments containing either of two phytase sources (*Buttiauxella* spp. or *Escherichia coli* phytase added at 500 or 1000 phytase units (FTU)/kg) when compared with a rapidly-soluble, finer limestone (~70% soluble at 5 min, <20 µm). However, in the same study there was an interaction between limestone and phytase treatment, such that the improvement in AID Ca with phytase tended to be greater with the rapidly soluble, finer limestone. Meanwhile, Kim *et al.* (2018, 2019) and Li *et al.* (2021) observed no specific interactions between phytase and limestone on AID Ca. In terms of AID P, Kim *et al.* (2019) saw an interaction between phytase supplementation (1000 FTU/kg) and limestone type, so that effects of phytase on AID of P were not equal across all four limestone sources that differed in particle size. An earlier study by Kim *et al.* (2018) reported that AID of P in phytase supplemented treatments (1000 FTU/kg of a *Buttiauxella* spp. phytase) was affected by increasing the dietary Ca concentration only when fine limestone (75 µm GMD) was used, but not when coarse limestone (402 µm GMD) was used. These findings suggested that the influence of particle size and solubility on phytase efficacy in terms of Ca and P digestibility may vary for different limestone sources and may depend as well on other factors, such as dietary Ca and P content.

Davin *et al.* (2020) observed an interaction between limestone source (rapid vs slowly soluble) and phytase treatment on certain performance measures. Broiler bodyweight gain and feed intake during the grower and overall phases were reduced in rapid vs slowly soluble limestone supplemented with an *E. coli* phytase at 500 or 1000 FTU/kg, but only in rapid vs slowly soluble limestone supplemented with a *Buttiauxella* spp. phytase at 500 FTU/kg. At levels of 1000 FTU/kg of *Buttiauxella* spp. phytase, there was no longer any effect of solubility on performance. These findings suggested that, in order to mitigate the potential negative impact of limestone solubility on nutrient digestibility and growth performance, it may be necessary to increase phytase supplementation. In addition, the data implied that limestone solubility may be related to phytase source and enzymes that can break down phytate more rapidly in the upper GIT. This would, potentially reduce the formation of Ca-phytate complexes and mitigate the impact of rapidly soluble limestone.

4. Conclusion

This survey of limestone highlighted the extent of variation that currently exists in macro and micromineral content, particle size and *in vitro* solubility. All of these characteristics are important factors in understanding and predicting the availability of Ca from limestone in diets and its effect on P utilisation and phytase efficacy *in vivo*. The extent of variation in Ca content in both fine (<1000 µm GMD) and coarse (>1000 µm GMD) limestone suggested that the use of estimates of limestone Ca content from

published feed tables is not sufficient for accurate dietary formulation of Ca. Whilst broiler diets are currently often formulated with excess Ca and P in order to ensure that mineral requirements are met, high ingredient costs and sustainability considerations mean that there is a move towards diets closer to requirement levels and on a least-cost basis. Under this scenario, variations in limestone quality that may affect the Ca and P availability in the final diet and interactions with other feed components, such as phytase in the digestive tract, may become more important.

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Conflict of interest

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