The impact of limestone source and particle size on the digestibility of dietary Ca and P in broilers

By

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I, Stuart John Taylor declare that the dissertation, which I hereby submit for the degree MSc (Agric) Animal Production and Nutrition at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:……

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Abstract

Standard industry practice is to formulate broiler diets to a total calcium (Ca) and available or digestible phosphorous (P). The limitation with this is that inherent characteristics of the limestone source (LS) and particle size (PS) of limestone added to feed may affect phytate P utilisation and dietary Ca and P digestibility. The aim of the experiment was to evaluate the limestones in South Africa in order to give nutritionists a better understanding of the Ca and P digestibility associated with South African limestones. The study included both *in vitro* and *in vivo* experiments. Laboratory work was conducted in order to determine the particle size of the limestones and the solubility of the limestone used in the experiment. Limestones were evaluated based on particle size and solubility and compared to one another. To achieve this, two broiler digestibility trials were conducted, both including different limestone sources and two particle sizes. The aim of the first digestibility trial was to determine the effects of LS, limestone PS and the inclusion of phytase on Ca and P digestibility. Digestibility trial 2 aimed at examining the effects of different levels of phytase on Ca and P digestibility associated with limestone at two different particle sizes.

In Digestibility Trial 1 two groups of 600 Ross 308 male broilers were fed experimental diets in two sets (blocks) from days 19-21 and 23-25, respectively, in order to determine the effects of LS, PS, and phytase on apparent ileal digestibility (AID) of Ca and P. A randomised block design was applied with a 3x2x2 arrangement of treatments. The twelve treatments included three limestone sources, each with two particle sizes (0.8 mm and commercial) and two phytase levels (0 and 1000 FTU/kg feed). The experiment was repeated twice (block) with five cages per treatment per block. Each block consisted of 100 cages with 6 birds/cage. Digesta from the distal half of ileum was collected, freeze dried, and analyzed for Ca, P, and Cr marker to determine the apparent ileal digestibility (AID) of Ca and P. Data was analysed as a full factorial using Proc Mixed (SAS Institute 2012) with block included as a random effect. Results showed a tendency for AID of Ca to be altered by LS and phytase ($P<0.01$), with no interaction of LS, PS and phytase. A significant three-way interaction of LS, PS, and phytase was observed for AID of P. In the absence of phytase, a larger limestone PS consistently increased AID of P. With phytase added, the effect of PS on AID of P differed between LS. The increment in AID of P from added phytase was also dependent on the LS ($P<0.01$).

Digestibility Trial 2: The optimal phytase dose for broiler chickens using the *Buttiauxella* phytase strain has previously been determined, although the optimal dose of phytase using different particle sizes of limestone has not been quantified. Using only one source of limestone, the objective of the second digestibility trial was to determine whether or not the optimal phytase dose for broiler chickens changes when using different limestone particle sizes. Two groups of 600 Ross 308 male birds were fed from days 19-21 and 23-25, respectively, in order to determine the effects of limestone particle size and phytase concentrations on Ca and P digestion. The experiment had a randomised block design and 2x4 arrangement of treatments using a single limestone source with two particle

sizes (0.8 mm and Commercial) and four phytase (0, 250, 1000 and 2000 FTU/kg) concentrations. The experiment was repeated twice (block) with five replications/treatment per block. Each block consisted of 100 cages with six birds per cage. Digesta from the distal ileum was sampled in order to determine disappearance of Ca and P on days 21 and 25 of age. Data collected showed a highly significant ($P<0.0001$) positive response in P digestibility when a 0.8 mm particle size was used. Increasing the concentration of phytase in the diets using the same limestone source showed a positive response ($P<0.001$) for both Ca and P digestibility. The use of phytase up to 2000 FTU demonstrated that the negative effects of fine limestone can be overcome by increasing the phytase dose above that which is currently recommended (1500 FTU).

Digestibility Trial 1 showed that the use of limestone in broiler diets would have to be evaluated according to the source of the limestone being used as well and the particle size of the limestone being used. Data from Digestibility Trial 2 will enable nutritionists and scientists to more accurately calculate the optimal phytase dose for differing limestone particle sizes being used in broiler feed.

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List of abbreviations

$1,25(OH)_2D_3$ – 1,25-dihydroxyvitamin D_3

AID – Apparent ileal digestibility

AOAC – Association of Analytical Chemists

aP – Available phosphorous

Arg – Arginine

Ca – Calcium

$CaCO_3$ – Calcium carbonate

CaR – Calcium sensing receptor

Comm – Commercial

Cr – Chromium

CT – Calcitonin

dCa – Digestible calcium

d_{gw} – Geometric mean diameter of particles by mass

DM – Dry matter

dP – Digestible phosphorous

Fe – Iron

FTU – Phytase units

GMD – Geometric mean diameter

H_2S – Hydrogen sulphide

HCL – Hydrochloric acid

HID – Percentage hydrogen ion disappearance method

Hz – Hertz

Ile–- Isoleucine

iP – Inorganic phosphorous

Kcal/kg – Kilocalories per kilogram

kg – Kilogram

LS – Limestone

Met – Methionine

Mg – Magnesium

mg/kg – Milligrams per kilogram

mins – Minutes

mL – Millilitre

Mn – Manganese

mRNA – Mitochondrial ribonucleic acid

NaCl – Sodium chloride

nPP – Non-phytate phosphorous

NRC – National research council

oP – Opnembare phosphor (retained phosphorous)

P – Phosphorous

PP – Phytate phosphorous

PMCA – Plasma membrane calcium pump

PKA – Protein kinase A

PKC – Protein kinase C

PPT – pH plateau time

PS – Particle size

PTH – Parathyroid hormone

RANKL – Receptor activator of NF-ƙB ligand

SA – South Africa

SAPA – South African Poultry Association

SBM – Soya bean meal

SEM – Standard error of means

SWLM – Scott weight loss method

tCa – Total Ca

Thr – Threonine

Trp – Tryptophan

TRPV5 – Transient receptor potential channel 5

TRPV6 – Transient receptor potential channel 6

Trt – Treatment

TSAA – Total sulphur amino acids

USA – United States of America

UV – Ultraviolet

Val – Valine

VDR – Vitamin D receptor

WLA – Percentage weight loss (Auburn) method

WLM – Percentage weight loss (Minnesota) method

ZC – Zhang and Coon method

Zn – Zinc

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Chapter 1

Introduction

According to statistics released by the South African Poultry Association (SAPA) the projected poultry production for 2018 in South Africa was ± 1 billion birds per annum and the annual broiler feed production for the same period was ± 3 million tonnes of broiler feed (SAPA, 2018). The price and inclusion on raw materials in a broiler diet plays a pivotal role in the price of a complete broiler feed. It is of the utmost importance that the correct raw materials are selected when formulating a broiler diet to ensure that the diet meets the nutritional requirement of the bird in order for the bird to perform optimally. A challenge which nutritionists face is the problem of either under or over supplying a nutrient to the bird; an oversupply of a nutrient results in a monetary loss through wastage of feed raw materials, and an undersupply results in a monetary loss through decreased bird performance. It is therefore important that every ton of feed which is produced is done so accurately and for this to be achieved the nutritionist must know the nutrient content of their raw materials to accurately calculate the correct amount of each ingredient to meet the nutritional requirements of the rapidly growing modern broiler. Part of this is knowing the digestibility of the ingredients since certain ingredients are digested better than others while some will need additives to aid in the digestion. Limestone is included as a Ca source in all commercial poultry feeds and contributes the largest percentage of dietary Ca to meet the nutritional requirements of the birds. All of the poultry feeds in South Africa are formulated to a basis of total dietary Ca level. Knowledge on the digestibility of Ca from Ca sources in feed is limited and this has resulted in an over- or undersupply of Ca in the diet, worsening by the fact that there are differences in the sources and quality of limestone. The implications of over- or undersupply of Ca to birds manifests itself in the form of bone mineralisation and leg problems. The impact of this on the SA poultry industry supplying ± one billion broilers per annum (SAPA, 2018), is potentially far reaching.

Kim *et al*. (2018) showed the effects of limestone source, particle size and phytase dose on the digestibility of Ca and P. Since limestone quality and composition differ widely between sources within the same country it was important for us to conduct this research in SA under our conditions and with limestone sources from South African origin. The aim of the research conducted in South Africa was to determine the Ca and P digestibility coefficients from 3 leading limestone suppliers in SA (Agrilime, Prolime and Rossmin). The research focused on the variability of Ca digestion between limestone sources as well as between limestone of the same source at two different particle sizes (0.8 mm and commercial) and the effect thereof on P digestibility. The commercial particle size is that which was determined by particle size separation of the limestone collected directly from the mine and which is being supplied to the SA animal feed industry as the commercial standard. A separate

experiment was run to determine the effects of increasing levels of phytase in the feed to counteract the negative effects which fine limestones had on P digestibility. All limestone in the study were further evaluated in terms of *in vitro* solubility. The variation in solubility observed is a function of the source of the limestone as well as the particle size of the particular limestone which is being assessed. Collaborating with the University of Maryland in a global project, the ultimate aim was to establish digestibility coefficients of Ca and P for limestones characterised by particle size, limestone source and solubility. The methodology used to determine digestibility of Ca from limestone sources was developed by the University of Maryland over the past 5 years. The same University developed an *in vitro* limestone solubility assay with revised methodology (Angel, 2019a) which, when sufficient *in vivo* data is available, will aid in the estimation of Ca digestibility coefficients for limestone from *in vitro* data.

The aim of the study was to determine the effects of limestone source, particle size and phytase on Ca and P digestibility in broiler diets. To do this two separate, but concurrent, digestibility trials were conducted. The objective with Digestibility trial 1 was to determine the effects of three different limestone sources at two different particle sizes either with or without the use of phytase on the Ca and P digestibility. With this objective in mind the following hypotheses were formed:

Ho: Limestone sources does not affect Ca and P digestibility values
H_A: Limestone source does effect Ca and P digestibility values

Ho: Limestone particle size does not affect Ca and P digestibility values
H_A: Limestone particle size does affect Ca and P digestibility values

Ho: Phytase added to the diets does not affect Ca and P digestibility values
H_A: Phytase added to the diets does affect Ca and P digestibility values

The objective with Digestibility trial 2 was to determine the effect of graded levels of phytase on Ca and P digestibility values using a single source of limestone with two different particle sizes but four graded levels of phytase. With this objective in mind the following hypotheses were formed:

Ho: Increasing the levels of phytase added to the feed above what is commercially recommended has no effect on Ca and P digestibility values
H_A: Increasing the levels of phytase added to the feed above what is commercially recommended has a positive effect on Ca and P digestibility values

H_O: Fine limestone does not negatively affect Ca and P digestibility
H_A: Fine limestone does affect Ca and P digestibility

Chapter 2
Literature Review

2.1 Introduction

This literature review is a compilation of the existing published data on the use of Ca in commercial broiler nutrition, the different Ca sources which are available to the South African feed industry as well as the formation and quarrying of limestone. The current formulation practices and the current Ca recommendations in terms of total Ca are covered. To allow nutritionists to more accurately formulate to digestible Ca it is imperative that there is an understanding of what current practices are and how to move in the direction of digestible Ca values in feed formulations. The mechanisms of digestion and absorption of Ca and P in the broiler are explored in order to better understand the factors influencing these actions and how this research can assist in making these processes more effective or to utilise the existing processes to their full potential. Previous work is explored in terms of the published values of Ca digestibility and limestone solubility and a critical review of the previous results. The new *in vitro* limestone solubility assay which was used in this study is discussed and the effect of limestone solubility on Ca and P digestibility values. An analysis on the Ca levels in limestone is presented. The information contained in this literature review is the backbone of the study and the factors at play which are discussed herein are some of those which were studied in order to better understand the function of limestone in broiler diets, how it affects other nutrients and how to improve the use of limestone in broiler diets to benefit the bird.

2.2 Calcium sources in commercial broiler nutrition

Ca is an important mineral which is added to animal feed due to the high Ca requirement of food producing animals. The most widely used and most effective way of adding Ca to the diet of these animals is via the addition of limestone, that typically contributes ~50% of total dietary Ca, the remainder is contributed by inorganic phosphate sources, and Ca contained in plant or animal derived raw materials.

Limestone is a sedimentary rock which is composed of more than 50% carbonate minerals of which the most common is calcite (pure Ca carbonate, $CaCO_3$) or dolomite (Ca-magnesium carbonate, $CaMg[CO_3]_2$) or both (Douglas, 1969; Bliss *et al.*, 2008). The process of limestone formation can be divided in five classes of formation, (i) abiotic chemical precipitation (Douglas, 1969; Castanier *et al.*, 1999; Bliss *et al.*, 2008); (ii) from the internal and external skeletal structures of animals in the environment where the limestones forms (Douglas, 1969; Castanier *et al.*, 1999; Bliss *et al.*, 2008); (iii) fungal mediation of carbonate formation (Castanier *et al.*, 1999); (iv) through the autotrophic

processes of photosynthesis and methanogenesis (Castanier *et al.*, 1999) and (v) through heterotrophic action in the environment (Castanier *et al.*, 1999).

The abiotic formation of limestone does not involve the use of any organisms, it is a simple chemical reaction that takes place when the conditions are right and the correct constituents are available. The constituents that need to be available for such a reaction to take place are Ca (Ca^{++}) and hydrogen carbonate (HCO_{3-}) ions (Castanier *et al.*, 1999; Bliss *et al.*, 2008) and both must be present in sea water under conditions of saturation due to evaporation, temperature increase and/or a decrease in pressure (Castanier *et al.*, 1999). The reaction that takes place is as follows: $\mathbf{Ca^{++} + 2\ HCO_{3-} \leftrightarrow CaCO_3 + CO_2 + H_2O}$. The ions/constituents of the reaction may initially be produced by bacteria in the ocean but the reaction that forms the Ca carbonate precipitate does not involve the use of these bacteria and/or microorganisms. Once this reaction has taken place this precipitate can either form a limestone bed of its own by being deposited onto rocks or other structures. Alternatively, it may combine with other Ca sources as a carbonate cement, helping to bind Ca rich shells and other Ca carbonate particles together. The biotic processes that take place are far more complex and require the involvement of bacteria as well as some geological forces to form the limestone (Douglas, 1969). The biotic formation of limestone follows one of two pathways, either autotrophic or heterotrophic (Castanier *et al.*, 1999).

Autotrophic formation of Ca carbonate can follow one of three pathways: (i) non-methylotrophic methanogenesis (Marty, 1983); (ii) anoxygenic photosynthesis and (iii) oxygenic photosynthesis (Castanier *et al.*, 1999). All of these pathways under the autotrophic formation of Ca carbonate utilise CO_2 as their source of carbon (Castanier *et al.*, 1999) and as a result of this, the environment in which these bacteria find themselves becomes depleted of CO_2. When this takes place in an environment where there are Ca^{++} present it forces the precipitation of Ca carbonate (Castanier *et al.*, 1999). This is the point at which the abiotic and biotic pathways can overlap; the environment where the chemical formation for the Ca carbonate can take place is produced by biotic factors/pathways and in this case specifically the autotrophic pathways.

The heterotrophic pathways on the other hand are classed into only two categories: (i) passive precipitation and (ii) active precipitation (Castanier *et al.*, 2000). Passive precipitation of Ca carbonate in a medium works by producing carbonate or bicarbonate ions which will result in the precipitation of Ca carbonate. This can be done through the nitrogen cycle where either in the presence or absence of oxygen or in the presence of urea or uric acid, with Ca and organic matter present (in all three cases), the bacteria form an environment that allows Ca carbonate to precipitate. It can also be achieved through the sulphur cycle where-by the dissimilatory reduction of sulphate (Castanier *et al.*, 2000) in the presence of organic matter and Ca ions the bacteria produce Ca carbonates which depend

on the chemical action of the hydrogen sulphide (H_2S) present. If the H_2S increases the pH, Ca carbonate will precipitate, however if it decreases the pH no precipitate will form in the medium.
On the other hand, active precipitation, as its name suggests, is an active process that does not involve the use of either the nitrogen or sulphur cycles but rather makes use of the ion exchange across cell membranes. This ion exchange activates the Ca and magnesium channels which makes these ions available for the precipitation of Ca carbonate.

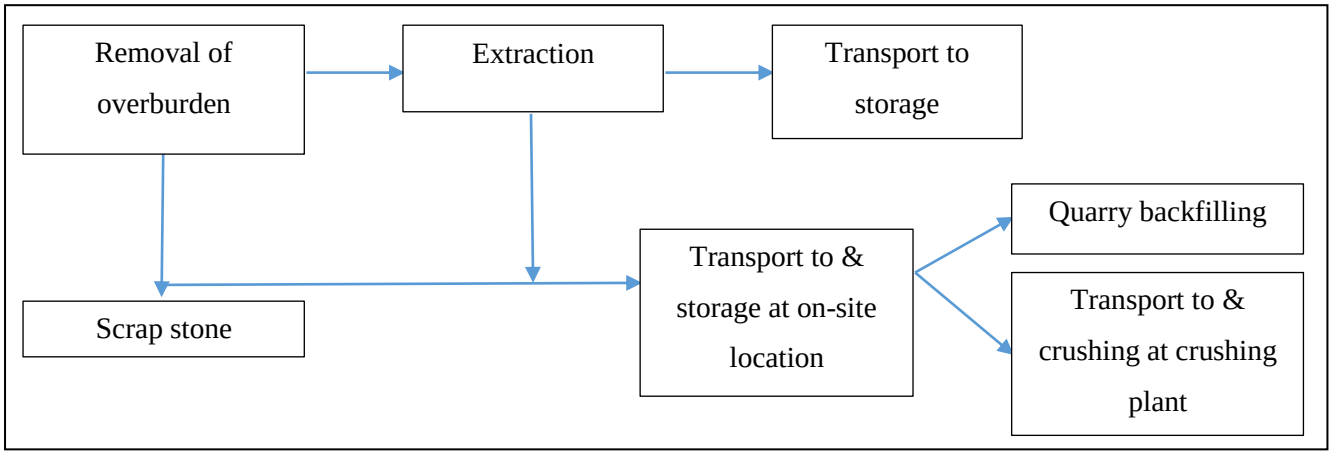
The formation of the actual rock which is called limestone is a much longer but relatively simple process that involves the products of all of the above mentioned processes of Ca carbonate formation. The most common limestone found is that which forms in shallow ocean waters (Douglas, 1969). This is called sedimentary limestone, it forms by the accumulation of the skeletal structures of both vertebrates and invertebrates which live in the ocean and have died. These skeletons and shells are rich sources of Ca. Once it has accumulated in an area it begins to compact under its own weight and the Ca carbonate particles which have formed either through abiotic or biotic process then bind to these shells and skeletons, filling the gaps and forming a calcite cement. After some time this site of accumulation will be covered with more sediment and the weight of this will compact it further, eventually turning it into rock and in some cases the pressure may also change the crystalline structure and form other Ca based rocks such as marble (Douglas, 1969).

The composition of limestone is another very vital aspect one needs to assess when looking at it as a Ca source as impurities may be present in the limestone which can alter the availability of the Ca to the birds. As previously mentioned, limestone is considered to be any rock which is composed of more than 50% carbonate minerals. With this in mind, based on the area in which the limestone formed, the rate at which it formed and which processes were involved in the limestone formation, it will contain varying amounts of impurities, such as magnesium carbonate, iron oxide, alumina and silica (Douglas, 1969). Apart from the mineral impurities which can play a role in the availability of the Ca there are other impurities which can play a role in the shape, form and hardness of the rock, including: particles of clay minerals, siderite, feldspar, pyrite and quartz (Angello, 2005).

For limestone to be ready for use in the feed industry, two distinct processes need to take place, (i) quarrying and (ii) processing (Figure 2.1). The process of quarrying/ mining the limestone first involves the process of identifying an area where a limestone bed/ deposit is situated and then very simply, removal of overburden which is all the vegetation and soils that may be on top of the deposit. This is done so that the limestone can be easily accessed by the machinery which will be used to mine it. The limestone is either blasted or cut into large blocks and loaded onto trucks which are transported to the processing plant. Any broken rock that is not taken to the processing plant can be used as hardening material for the mine roads and for backfilling of mined areas which are no longer in use.

Since limestone can be used in so many applications the processing techniques differ for each use and even the quality of the limestone taken out of the ground plays a role in the final use of that particular limestone. For the feed industry this involves the transport of the limestone to a crushing plant where the rock is crushed into very small particles and sieved so that the correct particle size can be achieved.

Figure 2.1. Flow diagram showing the process of limestone quarrying and transport to crushing plant



(Adapted from: Limestone Quarrying and processing report, 2008)

Pure $CaCO_3$ contains 40.04% Ca, with the molar mass of Ca being 40.078 g and the molar mass of $CaCO_3$ being 100.086. The Ca levels found in limestone would depend on the purity of the limestone (level of $CaCO_3$ in the rock). Factors affecting this include the age of the rock, the rate and method of deposition/ formation and the structure of the rock to the impurities present in the rock. The impurity which is most commonly found in limestone is magnesium (Mg) and is found as magnesium carbonate ($MgCO_3$). Huang *et al.* (2008) categorised the different types of limestone which are found.

1) Calcite: this is pure $CaCO_3$ and contains 40% Ca
2) Calcitic limestone: > 30% Ca and < 5% Mg
3) Dolomitic limestone: < 30% Ca and >5% Mg
4) The fourth category is very specific and refers to this limestone as dolomite. Dolomite contains both $CaCO_3$ and $MgCO_3$, although the ratio must be 22% Ca to 13% Mg for the rock to be classified as dolomite.

Given that there are differing types of rock which are called limestone and with each type having a different level of Ca present, it is important to examine the relative bioavailability of this Ca from the different rocks. Reid and Weber (1976) showed the bioavailability of Ca for broilers from ground calcitic limestone was 89% when graded levels of Ca was fed to broilers in the diet ranging from 0.4% – 1.0%. Waldroup *et al.* (1964) reported that the relative bioavailability of Ca for broilers from ground calcitic limestone was 100% when graded levels of Ca was fed to broilers in the diet ranging

from 0.3% – 0.7%. However, a separate study conducted by Stillmak and Sunde (1971) showed the relative bioavailability of Ca for broilers from dolomitic limestone to be between 64% – 68% when graded levels of limestone ranging from 0.5% – 1.1% in the diet, were fed.

2.3 Feed formulation and recommendations for Ca and P in broiler diets

Over the years there have been numerous changes to the approach used to define Ca and P in poultry diets. There have been many changes made to the National Research Council (NRC) recommendations in terms of the Ca to P ratio. NRC (1950) published a total Ca (tCa) to total P (tP) ratio, NRC (1954) published values for a tCa to inorganic P (iP) ratio followed by a further change in the NRC (1984) to a tCa to available P (aP) ratio. There have also been changes made to the NRC (1950, 1954, 1977, 1984, 1994) in terms of the recommendations of the P and the amount of the tP which must be made available from organic or inorganic sources. In the NRC (1994), recommendations are made on a tCa to non phytin P (nPP) basis instead of aP (Angel, 2013). The digestibility of P from plant sources is inherently low (Van Der Klis and Versteegh, 1996; Coon and Leske, 1998; Angel *et al.,* 2002; Tamim and Angel, 2003; Tamim *et al.,* 2004) and the fact that digestibility of P from animal and inorganic sources is highly variable (Van Der Klis and Versteegh, 1996; Coon and Leske, 1998) led to changes in the use of P from tP to aP, nPP, dP or retainable P, which has better shown the availability of P in dietary sources (Angel, 2013).

Although there have been numerous changes made to the ratios, definitions and levels of P in broiler feeds there have been little to no changes made to the Ca requirement or the way in which nutritionists need to be looking at Ca availability (Angel, 2013). The fact that it is extremely difficult to measure Ca availability/ digestibility of individual feed ingredients has forced the use of a tCa formulation as opposed to digestible Ca (dCa) formulation. Because of this difficulty and the lack of data in this area, there has been a general assumption that Ca has a 100% digestibility in feed ingredients (Angel, 2013). Tamim and Angel (2003) and Tamim *et al.* (2004) calculated the availability of Ca in a maize-soybean meal diet to be 20 to 33% by using two diets, one with zero inorganic Ca and P added and the same diet with added Ca and P. They also calculated that the Ca contribution from limestone is between 60 and 70%. The Ca contribution from maize and soybean meal (SBM) makes up between 0.17 and 0.21% of total Ca in the diet (Angel, 2013). In the past, the Ca supplied by maize and SBM has not been a concern for nutritionists as it only represents approximately 20% of the Ca in a diet containing 1% tCa (broiler starter diet), but when one examines diets that contain 0.6 to 0.5% tCa (typical withdrawl diets) the amount of Ca supplied by the maize and SBM makes up a much higher proportion of the tCa in the diet (Angel, 2013).

For the above mentioned reasons, nutritionists formulate to a tCa level in broiler diets. It is however important that nutritionists move towards a dCa system in order to better meet the Ca requirements of

the broiler in the different growth stages. This dCa system will have to include the contributions of Ca and P when a phytase is used in the diet (Angel, 2013).

Data presented in tables 2.1 – 2.5 show the differences in the nutrient recommendations for Ca and P based on the different systems and breeder companies used.

Table 2.1 Ca and P requirements of broilers (adapted from CVB, 2009)

Age	Ca (%)	oP* (%)
0 -10 Days	0.88 - 0.92	0.40
10 - 30 Days	0.68 - 0.71	0.31
30 - 40 Days	0.62 - 0.74	0.28
40 - 50 Days	0.59 - 0.62	0.27

* Opnembare P or retained P

Table 2.2 Ca and P requirements of broilers (adapted from Rostagno *et al.*, 2011)

Age	Ca (%)	aP* (%)	dP** (%)
1 - 7 Days			
Males	0.920	0.470	0.395
Females	0.920	0.470	0.395
8 - 21 Days			
Males	0.819	0.391	0.343
Females	0.809	0.386	0.339
22 - 33 days			
Males	0.732	0.342	0.313
Females	0.683	0.319	0.292
34 - 42 days			
Males	0.638	0.298	0.273
Females	0.566	0.264	0.242
43 - 46 days			
Males	0.576	0.26	0.247
Females	0.506	0.236	0.217

* Available P; ** Digestible P.

Table 2.3 Ca and P requirements of broilers (adapted from NRC, 1994)

Age	Ca (%)	nPP* (%)
0 - 3 Weeks	1.0	0.45
3 - 6 Weeks	0.9	0.35
6 - 8 Weeks	0.8	0.30

*Non Phytate P (NPP)

Table 2.4 Ca and P requirements of broilers (adapted from Aviagen, 2014)

Age	Ca (%)	aP (%)
0 - 10 Days	0.96	0.480
11 - 24 Days	0.87	0.435
25 days - Market	0.79	0.395

Table 2.5 Ca and P requirements of broilers (adapted from Cobb-Vantress recommendations, 2015)

Age	Ca (%)	aP (%)
0 - 10 Days	0.90	0.45
11 - 22 Days	0.84	0.42
23 - 42 Days	0.76	0.38
43 + Days	0.76	0.38

2.4 Ca and P digestion and absorption and Ca regulation in the body

When broaching the subject of Ca and P digestion and absorption one needs to take into consideration the effect that vitamin D_3 has on these actions in the gut of the bird. Ca and P interact both before and after absorption into the gastro-intestinal tract (GIT) (Angel, 2013). Vitamin D_3 affects both Ca and P requirements, and generally the requirements for Ca and P increase as the level of vitamin D_3 decreases and *vice versa* (Angel, 2013). Keeping this in mind, attending to the Ca needs of poultry can be a complex issue. Apart from the interactions between Ca, P and vitamin D_3, Ca:P ratio in the diets must also be taken into account during feed formulation. While trying to obtain the optimal ratio of Ca:P ratio one must keep in mind that the availability of P in grains is inherently low (Van Der Klis and Versteegh, 1996; Coon and Leske, 1998; Angel *et al.*, 2002; Tamim and Angel, 2003; Tamim *et al.*, 2004). Plants store P in the form of a phytate molecule, phytate is a 6-carbon ring which has six P groups bound to it, also known as myo-inositol hexakisphosphate or IP6.

Most poultry diets are grain-based and grains have inherently low levels of Ca but high levels of P which has a low bioavailability. Although the P has a low biological activity it can be released using phytase. For this reason, there is a need to feed a source of Ca to the birds in order to keep the Ca:P in

a desirable range. With regards to the Ca:P ratio, Mohammed *et al.* (1991) found that the wider the Ca:P ratio is, the more negative an impact it has on the birds' utilisation of both Ca and P, while a positive impact can be found when the Ca:P ratio is narrower.

Ca and P are two of the most abundant minerals in the body with 99% of the body's Ca and 80% of the body's P being stored in the bones of the bird as hydroxyapatite (Hemati Matin *et al.*, 2013; Proszkowiec-Weglarz and Angel, 2013). Both of these minerals therefore play a critical role in the development and mineralisation of bone (Veum, 2010). The outstanding 1% of the Ca in the bird is held within the extracellular fluid and the cells, and in the plasma where it plays very important roles in blood clotting, cellular metabolism, neuromuscular function, enzyme activation (Soares, 1995; Veum, 2010), cell adhesion, muscle contraction and intercellular signalling (Veum, 2010). In 1995, an average broiler chick increased its body weight by 15 times in the first three weeks of its life (Soares, 1995). This growth performance has increased to 22 times (Aviagen, 2014) and for the bird to achieve this, it must have 350 mg Ca/kg body weight available as opposed to the dairy cow who needs 50 mg Ca/kg body weight during peak lactation (Soares, 1987).

The 20% of the P which is not held in the bone can be found in nucleic acids, phospholipids, nucleotides and phosphorylated proteins. All of these play major roles in cellular and membrane function, growth, acid-base balance and energy metabolism (Wardlaw and Kessel, 2001; Berndt *et al.*, 2007; Veum, 2010).

The levels of Ca and P in the plasma are regulated by feedback mechanisms which consist of parathyroid hormone (PTH), calcitonin (CT), the active form of vitamin D_3 [1,25-dihydroxyvitamin D_3; $1,25(OH)_2D_3$], as well as all their receptors in the kidneys, small intestine and the bone (Veum, 2010). Vitamin D_3 can be made from the isomerisation of 7-dehydrocholesterol in the skin after the skin is exposed to UV light as well as the supplementation of vitamin D_2 and vitamin D_3 in the diet (Dusso *et al.*, 2005). Norman (1987) found that the biological activity of vitamin D_3 is in fact ten times higher than that of vitamin D_2. Once the formation of vitamin D_3 has taken place in the skin it must bind to vitamin D-binding proteins, it is then either stored in the fat or transported to the liver (Holick, 1981).

In order for vitamin D_2 and vitamin D_3 to be activated it must both undergo a double hydroxylation reaction (Dusso *et al.*, 2005), first taking place in the liver where it becomes $25(OH)D_3$ and then the second hydroxylation reaction in the kidneys forming $1,25(OH)_2D_3$ and is facilitated by 1α-hydrolase (Jones *et al.*, 1998). The main function of this activated form of vitamin D_3 is to regulate the levels of Ca and P homeostasis. This is accomplished through the direct action of the vitamin D_3 on the kidney, intestine and bones by working through feedback inhibition of the PTH production in the parathyroid

glands (Pike *et al.,* 2007). The activation of vitamin D_3 depends on the amount of Ca in the plasma; if the amount of Ca in the plasma is low then the 1α-hydroxylase is activated and $1,25(OH)_2D_3$ is synthesised. Conversely, if the amount of Ca in the plasma is sufficient then the $25(OH)D_3$ is hydrolysed at the 24^{th} carbon and not the first, forming $24,25(OH)_2D_3$, an inactive form of vitamin D_3 (Jones *et al.,* 1998). The active form of D_3 also controls its own production through the feedback mechanism, halting the production of 1α-hydroxylase and stimulates the production of 24-hydroxylase (Dusso *et al.,* 2005). Renal 1α-hydroxylase is also regulated by PTH and CT (Brenza *et al.,* 1998; Murayama *et al.,* 1998; Zhong *et al.,* 2009).

The effect of vitamin D on Ca regulation in the bird has been studied broadly. A study conducted by Gonnerman *et al.* (1975) showed the importance of vitamin D in the regulation of Ca levels in the body as well as the responsiveness to PTH in the bird. The lack of vitamin D decreases the Ca-mobilising response to injections of PTH (Gonnerman *et al.,* 1975). As a result of this, the bird was not able to maintain the standard levels of Ca in the plasma (Hurwitz, 1989). The birds that were not able to respond positively to an injection of PTH invariably showed a decrease in bone ash as well as a drop in plasma Ca (Ramp *et al.,* 1974). It was also shown by Forte *et al.* (1982) that a deficiency in vitamin D decreased the concentration of renal PTH receptors and their activity. It has been demonstrated in experiments by Russell *et al.* (1993) that there is a clear interaction between vitamin D_3 and Ca in the regulation of the function of the parathyroid gland. "Ca and $1,25(OH)_2D_3$ can regulate PTH and vitamin D receptor (VDR) mRNA expression in chicken parathyroid glands." (Russell *et al.,* 1993). This was shown in the results when a vitamin D deficient chick was fed a high Ca diet (1.8% Ca) and an increase was noted in plasma Ca concentration and an increase in VDR mRNA expression in the parathyroid gland. While a decrease in the PTH mRNA were also noted (Russell *et al.,* 1993). When exploring the effect that Ca and P have on bone mineralisation in rapidly growing chickens, Bar *et al.* (2003) found that when the birds were fed a Ca (Ca was restricted to 0.19%) and P deficient diet from day 0 – 11 of age, the birds showed signs of mild hypocalcemia, an increase in renal 1α-hydroxylase activity, an increase in duodenal calbindin D_{28k} concentration and a decrease in bone ash. Work done by Henry and Norman (1978) showed that layer hens that were given only the $1,25(OH)_2D_3$ form of the vitamin D_3 were able to lay eggs but the eggs never hatched, however, when the birds were supplied with both $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ they were able to lay eggs and the eggs hatched. This indicates that both of these dihydroxylated metabolites are needed for the bird to produce hatchable eggs.

In terms of the VDR mRNA, its expression was found to be unaffected by the presence or absence of vitamin D but rather affected by the concentration of dietary Ca. When the dietary Ca was low, the VDR mRNA was found to decrease (Meyer *et al.,* 1992). Most of the action of vitamin D is achieved through genomic pathways where the action of $1,25(OH)_2D_3$ is attributed to its binding to DNA

sequences (Pike *et al.*, 2007). This mechanism takes time though and a study conducted by Huhtakangas *et al.* (2004) revealed that rapid responses to active vitamin D_3 cannot be linked to the genomic pathways. Nemere (1995) and Pedrozo *et al.* (1999) identified a binding protein in the chicken's intestinal basal membrane that is not linked to the normal VDR. These findings are supported by Huhtakangas *et al.* (2004) who found evidence of a close association between Caveolae-enriched plasma membrane and VDR. The enriched plasma membranes are able to bind $1,25(OH)_2D_3$ with a high affinity. Adding to this, Nemere (1995) explored the actions of binding protein kinase C and A. Nemere (1995) was able to deduct that quick activation of protein kinase A (PKA) and protein kinase C (PKC) was observed within a few minutes when $1,25(OH)_2D_3$ was incubated with intestinal cells, but fast activation of only PKA occurred when incubated with bovine PTH.

Parathyroid hormone (PTH) is synthesised in the parathyroid glands, it takes the form of a prepro-peptide which undergoes cleavage to release the full length PTH which is made up of 84 amino acids (PTH(1-84)) (Juppner *et al.*, 2006). Parathyroid hormone must bind to the PTH membrane receptor in order for its action to be initiated (Strader *et al.*, 1994). Under normal conditions PTH is released as a response to a low Ca concentration and it is released mainly as PTH(1-84) (Hendy *et al.*, 1981). Parathyroid hormone, however, also has a shorter form which is made up of the first 34 amino acids (PTH(1-34)) of the longer PTH molecule, and this shorter version is the version of PTH which is the "native bioactive circulating form of PTH" (Hock *et al.*, 1997). Parathyroid hormone responds to severe short-term changes in [Ca] as well as long-term low [Ca] (Proszkowiec-Weglarz and
, 2013). When there is a case of long-term low [Ca] in the bird the PTH then stimulates the hydroxylation of vitamin D_3 to its active form, which, as a result of this conversion increases intestinal Ca absorption (Hoenderop *et al.*, 2005; Schröder and Breves, 2007) using a doubleheader mechanism which involves PKA- and PKC-dependant pathways (Friedman *et al.*, 1996; Hoenderop *et al.*, 1999a). The activation of both of these protein kinase pathways results in an increased reabsorption of Ca from the renal tubules (Forster *et al.*, 2006). According to research conducted by Nemere and Norman (1986), PTH has a direct effect on intestinal Ca transport. Additionally, Bar *et al.* (1972) showed that both the size of the parathyroid gland and its activity increased in vitamin D-deficient chicks when they respond to hypocalcaemia. However, an increase in Ca concentration was found to decrease the secretion of PTH (Feinblatt *et al.*, 1975). Due to the identification of the vitamin D response element (VDRE) in the promoter region of the PTH gene (Liu *et al.*, 1996), one can hypothesise that as with mammals, the chickens' parathyroid hormone gene expression can be linked to vitamin D.

The sensing of the Ca concentration in the body is carried out via the Ca-sensing receptor (CaR) while in the parathyroid chief cells the CaR detects high Ca concentrations (Hofer and Brown, 2003; Hoenderop *et al.*, 2005). Under conditions where the CaR in the parathyroid chief cells are activated,

it triggers the release of intercellular Ca and activates Ca-dependent proteases which cleave and inactivate PTH (Juppner *et al.* 2006). According to Yarden *et al.* (2000) the CaR protein is expressed in the chief cells of the parathyroid gland, these are the same cells that store PTH in the chicken. They also found that the expression of the CaR protein is subject to dietary vitamin D_3 deficiency and plasma Ca concentration. To support this, Yarden *et al.* (2000) revealed that birds fed a diet without vitamin D (1% Ca, 0.72% P and 0% vitamin D) had the lowest levels of CaR expression and this led to a lower level of plasma Ca when compared to birds which had been supplied with vitamin D in their diets. The greatest expression of CaR was seen in the birds with high plasma Ca concentration after an injection of PTH or after the vitamin D depleted diet had been replaced with a diet that contained vitamin D (Yarden *et al.,* 2000). In the chicken, an elevation of plasma Ca concentration was recorded by Yarden *et al.* (2000) to induce the expression of the CaR gene; this was in contrast to the findings in rats (Rogers *et al.,* 1995; Brown *et al.,* 1996). Yarden *et al.* (2000) also found that there was an inverse relationship between the expression of the CaR gene and the amount of PTH in the parathyroid gland. Thus, chickens have functional CaR that has similar traits to the CaR found in mammals (Proszkowiec-Weglarz and Angel, 2013).

Calcitonin (CT) is another very important protein that needs to be explored. Calcitonin is a peptide made up of a chain of 32 amino acids long and is produced in the thyroid gland. Calcitonin is produced in response to high plasma Ca concentration and its production is controlled by $1,25(OH)_2D_3$ (Silver *et al.*, 1985; Silver *et al.*, 1986; Cote *et al.*, 1987; Naveh-Many and Silver, 1988; Okazaki *et al.,* 1988). The function of calcitonin is to inhibit bone resorption, which in turn leads to a drop in the plasma Ca concentration (Matsuda *et al.,* 2006). Research conducted by Eliam *et al.* (1988) showed that birds who were fed a Ca-deficient diet had lower plasma Ca and CT levels than birds who were fed a diet containing Ca.

The transport of Ca in the intestinal tract can be split up into two distinctive pathways: (i) active (saturable) and (ii) passive (unsaturable) pathways (Proszkowiec-Weglarz and Angel, 2013). The active and metabolically dependant transport involves three steps: (i) entry into the cell across the cell wall, (ii) diffusion through the cell cytoplasm and (iii) leaving the intestinal cell through the basolateral membrane. The passive transport is more simple and involves the movement of the ions, moving with the concentration gradient out of the intestinal lumen into the circulatory system by moving through the spaces between the cells (Bronner *et al.,* 1986; Bronner, 1998; Bronner and Pansu, 1999). In times when the Ca concentration is high or is at normal levels, passive transport is the preferred primary pathway for Ca uptake due to the fact that active transport is inhibited by high plasma Ca concentrations (Buckley and Bronner, 1980). Buckley and Bronner (1980) also recorded that passive transport of Ca takes place along the entire small intestine. According to Rogers *et al.* (1995), the passive transport of Ca does not depend on vitamin D, although there have been reports

that passive transport does depend on vitamin D (Wasserman, 2004). Conversely, active transport is definitely vitamin D dependant and is localised to the duodenum (Pansu *et al.*, 1983).

The absorption of Ca in the intestine by means of the active pathway is regulated by the dietary Ca levels; when there are low levels of Ca in the diet or when the demand for Ca is high then active absorption of Ca is increased (Auchere *et al.*, 1998; Bronner and Pansu, 1999). Active transport involves three main steps, the first of which is entry into the cell across the cell wall and this step is accomplished through the use of two specific Ca sensitive anion channels (Hoenderop *et al.*, 2000; *et al.*, 2003). These are part of the group of transient receptor potential channels (TRPV5 and TRPV6) (Montell, 2001), both of these channels are found in a few tissues including the intestine where they are concentrated on the brush border membrane (Hoenderop *et al.*, 2000; Zhuang *et al.*, 2002). It has been hypothesised by Hoenderop *et al.* (2002) that these two channels constitute the rate-limiting step in transcellular Ca uptake, in the kidneys and intestine specifically (Hoenderop *et al.*, 1999a; Hoenderop *et al.*, 2002; Peng *et al*., 2003). Having found that these transport channels are expressed in various tissues within the bird, Nijenhuis *et al.* (2003) showed that the highest concentration of TRPV6 is in the duodenum and the caeca, followed by the colon and that the lowest concentration is in the ileum. They also revealed that the highest concentration of TRPV5 is found in the kidney with small amounts in the duodenum and caeca.

In terms of the transport of Ca through the cell cytoplasm, Wasserman and Taylor (1966) found that vitamin D-dependant calbindin D_{28k} is the chief protein responsible for this movement in the bird's intestine. This is unlike what has been found in mammalian tissues where there are two calbindin proteins (calbindin D_{9k} and D_{28k}) responsible for the diffusion through the intestinal and renal tissue cells, respectively (Gross and Kumar, 1990; Nemere *et al.*, 1991). It is also apparent from the research conducted by Taylor and Wasserman (1972), Bar *et al.* (1976) and Fullmer *et al.* (1976) that the calbindin D found in avian kidneys and avian intestine are exactly the same as each other, having the same molecular weight, immunogenicity and amino acid sequence. In support of this, it was found that there are three different codes for calbindin D in the intestine and kidneys of birds, all of which code for the exact same calbindin D_{28k} (Bar, 2009). Prior to 1978, the belief was that for Ca absorption to take place a very specific set of events needed to play out, namely: (i) synthesis of active vitamin D, (ii) increase in the expression of intestinal transporters, (iii) an increase in intestinal calbindin D_{28k} and finally (iv) an increase in Ca uptake (Proszkowiec-Weglarz and Angel, 2013). In spite of this, Spencer *et al.* (1978a) postulated that the synthesis of the calbindin D_{28k} is a result of activated vitamin D_3 mediated Ca absorption and not a mediator itself. Emtage *et al.* (1974 a, b) showed the ability of the bird's intestinal mucosa to synthesise calbindin D_{28k} in response to vitamin D. Spencer *et al.* (1978b) fed chickens a low Ca diet with an adequate supply of vitamin D and the results showed that there was an increase in Ca absorption and expression of calbindin D_{28k}. These authors also

observed that the same response was not present in birds that were raised on a vitamin D-deficient diet. Support for this research was published by Spencer *et al.* (1978b) and Bar *et al.* (1990) suggesting that the same was true in the cases that they studied. There is evidence showing chickens that have been deprived of vitamin D have lower plasma Ca concentrations, calbindin D_{28k} mRNA and protein expression in the kidneys and intestines (Bar *et al.*, 1990). The intestinal calbindin D_{28k}, which is widely accepted to be a measure of Ca absorption, has been found to be higher in fast growing broiler chicks when compared to the levels found in slower growing laying fowl when dietary Ca was supplied at a level of 1% (Bar and Hurwitz, 1981; Hurwitz, 1987; Hurwitz *et al.*, 1995).

The movement of Ca across the basolateral membrane of the intestinal cells is managed by the plasma membrane Ca pump (PMCA) (Carafoli, 1991) as well as the sodium or Ca exchanger (Bindels *et al.*, 1992; van Baal *et al.*, 1996). When the PMCA for chickens was characterised by Cai *et al.* (1993), it was shown that the protein shared a 96% similarity with the PMCA1b which is found in mammals. Adding to this similarity, the PMCA1b is the main isomer found in chicken intestine (Melancon and DeLuca, 1970; Davis *et al.*, 1987) and the kidney (Qin and Klandorf, 1993; Quinn *et al.*, 2007). The mRNA for the PMCA is found in the duodenum, jejunum and ileum and its expression was shown to be increased after the birds was treated with vitamin D or $1,25(OH)_2D_3$ when compared to chickens who were on a vitamin D depleted diet (Cai *et al.*, 1993). Melancon and DeLuca (1970) found that the number of intestinal Ca pump units increased in chickens which were adapted to diets low in Ca and P. Under the same conditions, Hurwitz *et al.* (1987) found that the mRNA concentration of PMCA reacted in a similar manner. The sodium or Ca exchanger was shown to play a small role in the transport of intestinal Ca (Hoenderop *et al.*, 2005), despite the fact that it is expressed in the basolateral membrane of the intestinal enterocytes (Kikuchi *et al.*, 1988; Van Abel *et al.*, 2003). However, it was found by Bindels *et al.* (1992) and Van Baal *et al.* (1996) that the sodium or Ca exchanger is the main driver of Ca removal in the kidney. Again, as shown in mammals, homologs of the chicken sodium or Ca exchanger were produced in the intestine and were found to be activated in response to a deficiency in Ca (Centeno *et al.*, 2004).

As previously mentioned, 99% of the Ca in the body is stored in the bones (Proszkowiec-Weglarz and Angel, 2013). This means that the skeletal system is the biggest pool of Ca in the bird. For a constant plasma Ca level to be maintained it is essential that Ca can be mobilised from the bones and this is achieved through the action of $1,25(OH)_2D_3$ and PTH (Dittmer and Thompson, 2011). Bone osteoclasts do not have receptors for PTH and as a result of this, bone resorption is activated through the osteoblasts (Proszkowiec-Weglarz and Angel, 2013). PTH enhances the expression of the receptor activator of NF-κB ligand (RANKL) in the osteoblasts (Kousteni and Bilezikian, 2008). The RANKL

in turn then induces the formation, activation and survival of bone resorbing osteoclasts (Lacey *et al.*, 1998).

2.5 Factors affecting Ca and P digestion and absorption

One of the biggest contributing factors to the bioavailability of nutrients in the GIT is the pH of the digesta (Pang and Applegate, 2007) as well as the intestinal microbiota population and health (Hajati and Rezaei, 2010). For this reason, it is extremely important to keep the pH of the GIT at a constant level for optimal digestion and absorption, with the gizzard pH at 1.2 - 4 and the duodenum at pH 5.7 - 6.5 (Pang and Applegate, 2007; Jimenez-Moreno *et al.*, 2009; Walk *et al.*, 2012). Even very small changes in the pH of the gizzard and duodenum can have considerable negative effects on the digestion and absorption of minerals (Bristol, 2003).

The interaction of other feed ingredients with Ca and/or Ca ions can also have a profound effect on the digestion and absorption of Ca. This is particularly apparent when one examines the interaction of Ca and phytate. By forming Ca-phytate complexes it is thought that high concentrations of Ca in the diet will reduce the hydrolysis of P from phytate molecules by endogenous and exogenous phytase enzymes (Tamim and Angel, 2003; Tamim *et al.*, 2004). In addition, Hurwitz and Bar (1971) showed that when there is a high ratio of Ca to nonphytate P (nPP) in the diet there is a formation of insoluble Ca:P complexes. Plumstead *et al.* (2008) hypothesised that 1% phytate has the ability to chelate with 0.36% Ca, and due to this reaction would decrease the amount of Ca available to be absorbed. Due to the nature of phytate (Figure 2.2) and the chelates which can form with mineral ions boasting a positive charge (Zn^{2+}, Fe^{2+}, Mn^{2+}, Fe^{3+}, Ca^{2+} and Mg^{2+}), it is of interest to nutritionists to have the phytate molecule hydrolysed as soon as possible in order for the P to become available for absorption (Tamim and Angel, 2003). The longer this process takes the higher the likelihood that available ions will chelate with the phytate forming a molecule known as phytin (Angel *et al.*, 2002). Although the P can still be cleaved from the main body of the phytate molecule it will not be utilised by the bird as P due to the fact that it is chelated with a mineral ion. To support this argument, Davis *et al.* (1959) reported that excess Ca in the diet can lead to the formation of Ca-phosphate salts which are insoluble and leads to decreased availability of P. Furthermore, Applegate *et al.* (2003) found that reducing dietary Ca from 9 g/kg to 4 g/kg resulted in an increase in apparent ileal phytate P (PP) hydrolysis of 24% and 12% in two separate experiments. Similar results were reported by Mohammed *et al.* (1991) where a decrease in dietary Ca from 1.0% to 0.5% resulted in a PP increase of 15%.

Since Ca is the mineral which is found to be most abundant in the diet, it stands to reason that it would be the mineral which causes phytic acid chelates to form. This is in spite of the fact that in terms of affinity to form phytic acid chelates Ca has one of the lowest affinities to do so (Tamim and Angel, 2003). Results of Tamim and Angel (2003) supported early studies conducted by Maenz *et al.*

(1999) who proposed the following order of phytate P hydrolysis resistance for six minerals when they chelated with phytate: Zn^{2+} >> Fe^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+} > Mg^{2+}.

The enzyme, phytase is used to combat the issue of Ca and P being bound in the phytin molecule (Figure 2.2). Phytase is a *myo*-inositol hexaphosphate hydrolase, and this enzyme allows the phytate P (PP) which is bound in the phytin molecule to be hydrolysed (Biochemistry, 1972). The first phytase was found by Patwardhan (1937) in the intestines of rats but since then it has been found in the intestines of chickens, pigs, cows, sheep and humans (Bitar and Reinhold, 1972). With the hydrolysis of the PP from the phytin molecule it allows the P to be utilised as such by the bird.

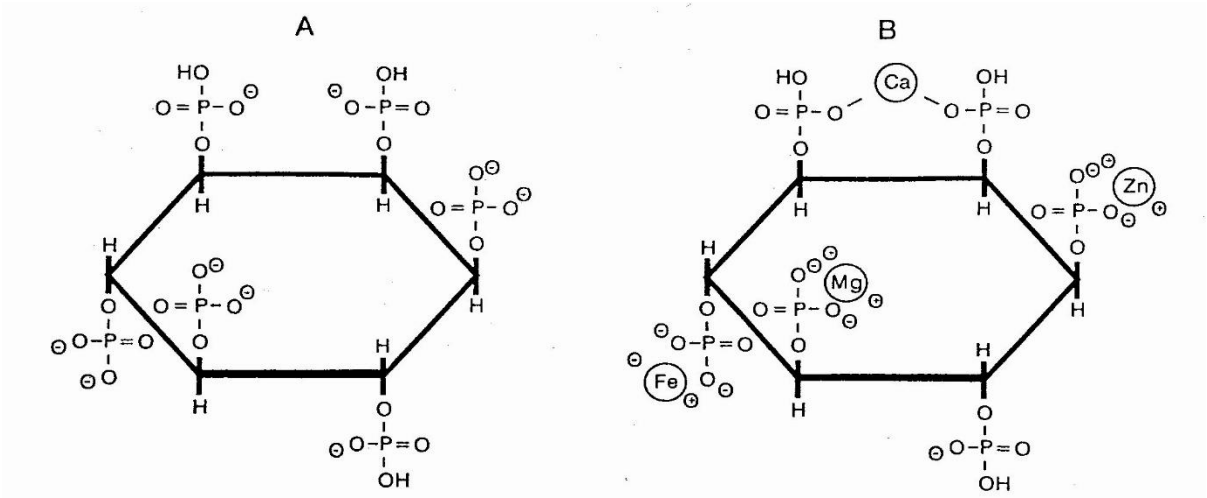


Figure 2.2. Image showing the structure of a phytate molecule (A) and the phytic acid chelates (B) it is able to form with mineral cations
(Image Source: http://www.ansc.purdue.edu/courses/ansc443/Class_notes/Nutrition.html)

Anwar *et al.* (2016a) examined the apparent ileal digestibility of Ca in limestone for broilers using three different limestone sources (LM-1, LM-2, LM-3) and two levels of P (0 and 0.45%) in the feed. The particle size of the limestone in this study was standardised to anything which passed through a 0.2 mm sieve. The study showed that there was no significance (P>0.05) between differences in digestibility coefficients for LM-1 (0.58) and the other two limestones, but there was significance (P<0.05) between the digestibility coefficients for LM-2 (0.61) and LM-3 (0.54). In another study published by Anwar *et al.* (2016b) the authors examined the effect of particle size and Ca:P ratio on Ca digestibility. Three different Ca:nPP ratios (1.5, 2.0, 2,5) and 2 different particle sizes (<0.5 and 1-2 mm) were used. The results from the study are represented in Table 2.6.

Table 2.6. Effect of Ca:nPP ratio and particle size on apparent and true Ca digestibility coefficients (Adapted from Anwar *et al.*, 2016b)

Ca:nPP ratio	**Particle size**	**Apparent ileal Ca digestibility coefficient**	**True ileal Ca digestibility coefficient[1]**
1.5	<0.5	0.52	0.54
	1.0-2.0	0.75	0.77
2	<0.5	0.40	0.42
	1.0-2.0	0.72	0.73
2.5	<0.5	0.34	0.35
	1.0-2.0	0.62	0.63
SEM[2]		0.02	0.02
Main Effects			
Ca:nPP ratio			
1.5		0.63[a]	0.65[a]
2		0.56[b]	0.57[b]
2.5		0.48[c]	0.49[c]
SEM[2]		0.01	0.01
Particle Size			
<0.5		0.42[b]	0.43[b]
1.0-2.0		0.70[a]	0.71[a]
SEM[2]		0.01	0.01
Probabilities (P-Values)			
Ca:nPP ratio		<0.001	<0.001
Particle size		<0.001	<0.001
Ca:nPP ratio x Particle size		0.15	0.14

[a,b,c]Values in the same column without a common superscript differ significantly.

[1]Ileal endogenous Ca losses were determined to be 127 ± 12 mg/kg of DM intake and were used to determine the true Ca digestibility.

[2]Pooled standard error of the mean (SEM).

In vitro limestone solubility can give a good indication of what the digestibility values for Ca and P will be. This can be attributed to the way in which the phytate molecule is structured (Figure 2.2). As previously explained, the ability of mineral cations to chelate with phytate is high and the different mineral cations have differing affinities for chelation (Maenz *et al.*, 1999). Tamim and Angel (2003) explained that the Ca cation has one of the lowest affinities for chelation with the phytate molecule. However, because it is the most predominant mineral in the diet it will have the most marked effect on P digestion.

For a limestone source with a high solubility very early on in the assay it stands to reason that in solution there will be a large amount of Ca^{2+} ions. If these are not bound in the form of $CaCO_3$ then they are available to bind to phytate forming phytic acid chelate and rendering the P in the phytate unavailable. The initial rate of solubility of limestone in the *in vitro* assay can therefore probably be correlated to P digestibility. The alternative to this is to have limestone which has a low solubility in the early stages of the assay. This would mean that phytate in the bird would be hydrolysed releasing the P before the Ca cations are available for chelation with the phytate and thus having a positive effect on the digestibility of P. Walk *et al.* (2012) found that feeding reduced Ca levels in the presence of phytase, increased the P digestibility and the P found in the bones of the broilers. By adding phytase to the diet an increase in Ca and P digestibility was demonstrated, which was attributed to a decrease in the number of Ca-P and Ca-phytate chelates forming (Walk *et al.*, 2012). Although this work was done *in vivo* a correlation is possible between the effects of *in vitro* solubility and the digestibility values because of the effect of free Ca^{2+} ions on the phytate molecule.

Addition of phytase to the diet will allow for the rapid hydrolysis of PP ensuring P is released from the phytate molecule before chelation of the phytate and a mineral cation can take place, thereby increasing P digestibility (Angel *et al.*, 2002).

2.6 Determination of limestone solubility

Over the years there have been numerous limestone solubility assays developed. *In vitro* assays have progressed with the advances in technology and the advancement of the understanding of Ca and its interactions within the birds' body and other nutrients.

The following summary of Cheng and Coon (1990a) described different methods of determining limestone solubility in an effort to compare the different methods available.

1) *Percentage Weight Loss (Minnesota) Method (WLM).* This method used a 400 mL beaker filled with 100 mL of 0.1N hydrochloric acid (HCl) which was warmed for 15 mins to 42°C in a water bath that oscillated at 60 Hz. A 2 g of sample was used and after 10 mins. the solution in the beaker was filtered through a "Whatman number 42 ashless filter paper", where-after the beaker and remaining sample were rinsed out with deionised water. The sample was then dried for 10 hours at 70°C. Six blanks were made for each run of the assay by filtering the acid solution alone through the filter paper. The mean decrease in weight of the filter papers from these blanks was used to adjust the dried weight of the samples. The solubility of the limestone was then expressed as a percentage weight loss of the limestone. One of the problems that was encountered with this method is that the amount of H^+ ions in solution may be a limiting factor in the reaction when a highly soluble limestone was tested using this assay. According to Zhang and Coon (1997) the number of H^+ ions in 100 mL of 0.1N HCl

was only be sufficient to dissolve 1.0 g of pure $CaCO_3$ which in turn would equate to a maximum solubility of 50%. Zhang and Coon (1997) have, however, offered solutions which could possibly eliminate these problems. The sensitivity of the method could be increased by increasing the molarity of the solution, decreasing the sample size, decreasing the reaction time of the assay and/or increasing the volume of the solution.

2) *pH Change Method.* This method used the same procedure as described above in the weight loss method. The difference between the two methods was that pH readings were taken at the beginning and end of the 10 min. digestion period. To ensure that a pH reading was taken in a homogenous solution the pH electrode was introduced into the solution 30 seconds before the intended time of the reading. The change in pH over the digestion time was recorded and subsequently used to develop a model which was used to predict the solubilities achieved in the weight loss method. Since this method uses pH which is a measure of the H^+ ions in solution this method would too have the same limitations as the WLM above (Zhang and Coon, 1997).
3) *Proton Consumption Method (PC).* This method worked in conjunction with the pH change methods as it used the pH values obtained at the beginning and the end of the experiment. These values were then converted to their respective hydrogen ion concentrations by using the following equation: pH = -log (hydrogen ion concentration). However, this expression is temperature dependent and will change slightly from 25ºC to 42ºC but no effort was made to account for this. On a theoretical basis the number of moles of proton consumption (PC) was calculated by difference. Savage (1982) stated that the Ca released was directly proportional to the PC. Again a model was developed in order to predict the solubility of the limestone using the values from the weight loss method.
4) *Percentage Hydrogen Ion Disappearance Method (HID).* This method used the pH change over the 10 min. test assay and converted this to a percentage of H^+ disappearance (HID). This process was described by Savage (1982). The original method used 10 g of limestone for the assay but Cheng and Coon (1990b) modified this to use 2 g of sample in 100 mL of 0.1M HCl.
5) *pH Plateau Time (PPT).* This method was described by Cheng and Coon (1990b) but their original information originated from personal communication they had with Charles and Whittle (1985). The original proposal for the assay used of 10 g of sample in 90 mL 0.1M HCl. This was modified to use 2 g of the sample in 100 mL 0.1M HCl. Data was collected using a pH electrode which was left in the solution for the entire assay and readings were taken every hour until a plateau was reached. The solubility was then expressed as time in hours taken to reach plateau.
6) *Percentage Weight Loss (Auburn) Method (WLA).* Rabon and Roland (1985) reported this method and its details. Using this method all the samples were treated at a ratio of 1:100

wt/vol with HCl at a pH of 1.5. The samples were allowed to react for 15 mins. The sample was dried and weighed. Half of the dry sample was used to run this procedure again. After the second run, half of the dry sample from the second run was taken and used in a third run. The solubility results from this assay were then expressed as an average percentage limestone weight loss per 15 min reaction.

7) *Scott Weight Loss Method (SWLM).* This method developed by Scott (1991) used the exact same method as the WLM. However, this method used a smaller sample size in that it used 0.5 g of samples and not 2.0 g. The reason for this change was to try and eliminate the possibility of an H^+ deficiency during the reaction (Zhang and Coon, 1997). However, the limitation of this method was a high risk of weighing inaccuracy when samples of limestone with large particle sizes were being tested.
8) *Zhang and Coon Method (ZC).* This *in vitro* method is widely used as the standard method for determining Ca solubility. ZC was described by Zhang and Coon (1997) after examining the other available methods and development of ZC was based on what they found to be the best attributes of other methods. ZC involved the addition of 200 mL of 0.2N HCL to a 400 mL beaker. The beaker was then warmed in an oscillating water bath at 80 Hz for 15 mins. or until the solution reaches 42°C. The water in the bath was at such a level that it was at least 1.0 cm above the level of the HCl in the beakers. A 2.0 g of sample was added to the beakers and the reaction was allowed to run for 10 mins. The supernatant was poured off without losing any undissolved sample and the reaction in the beaker was stopped using about 250 mL of deionised water. The undissolved sample was then filtered on filter paper which had been pre-weighed and dried, this was done using excess deionised water. The weight of the undissolved sample was determined by drying the sample at 70°C for 10 hours or until a there was no longer a change in the weight. This was used to determine the actual weight loss of the sample which can be reported as a solubility percentage.

There have been recent developments of a new assay for Ca solubility (Angel, 2019a, b). This method, which is described in Chapter 3, was used to determine solubility of the limestones in the present study.

2.7 Conclusion

In conclusion, it is evident that there was a very deep understanding and extensive research had been conducted on the function of Ca and P in the body and the uptake thereof. Literature has shown that over the years changes had been made to the definitions of P in the broiler diet, however, the definition of Ca in the diet has remained the same and the poultry industry continues to use a total Ca system. Because of this, the recommendations from breeder companies as well as the Brazilian

Tables, CVB and NRC are very similar, all providing a ratio of Ca:P in the diet. There had also been recent research on phytate and phyatse inclusion level in poultry diets. Since the modern broiler is changing so rapidly it is important that the optimal dose of phytase be adapted continuously. It was evident through the literature researched that limestone sources differ from one another in composition as well as in the way that it was formed. The Ca digestibility from limestone and the associated P digestibility has been studied, however, research on the effect of different limestone sources on Ca and P digestibility needed to be explored, also those available in South Africa. Particle size of limestone was shown to play a role and have an effect on Ca and P digestibility. Accompanying this was the fact that the dissolution or solubility rate of the limestone plays a role in Ca and P digestibility as it affects the formation of Ca-phytate chelates and complexes which render the Ca from limestone and P in phytate unavailable to the bird. The methods used for the determination of limestone solubility have been developed through much experimentation and improvements on older methods. The limitations of some of the older methods have been addressed with and the newer methods better simulate the environment in the broiler to which the limestone would be exposed to.

Chapter 3
Materials and Methods

All procedures used in this study were approved by the Animal Ethics Committee of the University of Pretoria (Project number: EC 047-17).

3.1 Limestone sourcing

Limestone was sampled from three limestone quarries, namely: Agrilime (Northam, North-West Province), Prolime/ SA Kalk en Gips (Bhurmansdrift, North-West Province) and Rossmin (Port Shepstone, Kwa-Zulu Natal). In the study the limestone from the different sources were referred to as limestone A, B and C for confidentiality purposes.

The mines were visited personally to ensure correct sampling of the limestone. Care was taken to ensure collection of a good representative sample from all the mines. The procedure for sample collection from the mines differed from mine to mine as each mine had different processes which the limestone underwent in order to reach the final product. However, samples were collected from the same point in the production line at all three mines. Two types of samples were collected from each mine: large rock before crushing and crushed limestone ready for shipping. Samples of raw material (large rocks) were collected from the area above the main crusher from each mine. The rocks are typically delivered to the crusher via trucks; once the rock truck had delivered its load to the crusher, large pieces of rock ± 20-25 cm diameter were collected and placed into a large sack (± 100 kg).

Crushed samples of the limestone destined for the broiler feed industry were collected from the conveyor belts just before the limestone dropped off the end of the conveyor into the collection pile.

1. Samples were collected off the conveyor using a cup, the cup was dragged in a diagonal line across the conveyor from one side to another to ensure a good distribution of the limestone sampled.
2. To ensure that the final sample was representative of what was produced in the mine that day, several samples were taken at 10 min intervals between each sampling.
3. The sample was poured into bags and sealed (± 50 kg).
4. Sealed bags were brought back to the laboratory (Chemunique, Ferndale, Johannesburg) and the particle size distribution was evaluated for each sample from all three of the mines.

For the study two particle sizes were used, the first being a commercial particle size, which was the particle size of the crushed limestone sampled at the mines. A second particle size was then manually

produced by crushing the large rock collected from the mines down to the desired particle size; this was then referred to as standardised limestone.

3.2 Splitting of limestone samples

Once the samples were at the laboratory each was divided into three parts. This was to accommodate the following: (i) evaluation of particle size distribution, (ii) evaluation of *in vitro* solubility and for the, (iii) *in vivo* digestibility. From each of the three samples a subsample of 10 g was taken from each and pooled into a single sample and sent away for mineral analysis at an independent laboratory (Chem Nutri Analytical, Cedar Lake Industrial Park).

Samples were divided as follows:

1. The samples were first mixed within the sample bag but tipping the bag end on end and rolling the bag on the counter. This ensured that the sample was mixed properly and that no separation of particle sizes had occurred.
2. The samples were then spread on top of a clean counter top in the laboratory and using gloved hands, the samples were split into three equal samples.
3. The three split samples on the counter were then placed into separate bags so that they could be used for the respective analysis.

3.3 Standardisation of limestone particle size

Since the limestone needed to be evaluated on a basis where particle size is removed as a variable, the particle size of the various limestone sources was standardised. This enabled the comparison of the digestibility and solubility of these limestone to be evaluated where the main determining factor of differences recorded was the geology of the limestone.

The way in which this was done is described in the following steps:

1. The raw rock samples from the different mines were taken to the Rossmin mine in Port Sheptone, Kwa-Zulu Natal. The company owns a mechanical jaw crusher which is able to crush rock to a pre-set size. The rocks were crushed to a size between 4 mm and 5 mm.
2. The crushed samples were then brought back to the laboratory (Chemunique) where it was further crushed by hand using a 4 lb hammer.
3. The rocks were crushed in a plastic box with the hammer and put through the particle size separator.
4. Any rocks that were still in excess of 2 mm in diameter were then further crushed on a plastic tray with the hammer and again put through the particle size separator.
5. This process continued until all of the rock was crushed to a particle size of 2 mm or less.

6. After each separation was conducted the limestone in each sieve was poured into a bag which was marked with that particular sieve size.
7. After all the rock was crushed and had been separated into its respective particle sizes it was mixed to make a 2.5 kg sample of limestone with a theoretical geometric mean diameter of particles by mass (d_{gw}) of 0.8 mm.
8. The 2.5 kg sample of theoretical 0.8 mm d_{gw} was then put through the particle size separator to ensure that the d_{gw} was in fact 0.8 mm. This procedure was conducted in triplicate.
9. This sample (2.5 kg) was then split into three parts each weighing (± 850 g), for the three different analyses.

3.4 Limestone particle size determination

Particle size of the limestones used in the diets was determined by using a DuraTap particle size separator with a set of 15 sieves, each 8-inch in diameter, half height, woven wire US standard test sieves. Sieve opening size ranged from 3.35 mm to the pan. A 100 g of sample was weighed out and poured into the top sieve. The sieve shaker was set to shake for 10 mins, where-after all sieves were then removed and weighed after shaking and weights recorded, after which weight of the limestone in each sieve was calculated. The particle size of each limestone was determined in triplicate. Data was then used to calculate the geometric mean diameter of particles by mass (d_{gw}**)** of each limestone using the procedure previously described (Baker and Herrman, 1995).

The sieve test was conducted on all samples collected from the mines. The method used was as follows, (Angel, 2019b):

1. Sieves were all cleaned using a soft brush to ensure that there was no residual left in the sieves.
2. Following this the sieves were stacked on top of one another in the correct order (Table 3.1).
3. The sieves were weighed and the empty sieve weight recorded.
4. A 100.00 g of a limestone sample was weighed out on a Mettler Toledo balance.
5. The stacked sieves were then placed in the sieve shaker and the 100.00 g of limestone added to the top sieve.
6. The sieve shaker was set to 10 minutes and allowed to finish the time.
7. Sieves were then removed from the shaker and placed on the counter top next to the balance.
8. Each sieve was gently removed from the stack of sieves and placed on the balance and the weight recorded and the weight of the limestone in each sieve calculated.
9. The d_{gw} was calculated using Equation 3.1 and the standard deviation of the particle by mass (S_{gw}) using Equation 3.2.

This process was repeated in triplicate for each of the samples.

Table 3.1. Sieve settings used in particle size determination

U.S Sieve Number	Opening Size (Micron)
6	3350
8	2360
12	1700
14	1400
16	1180
18	1000
20	850
25	710
35	500
50	300
70	212
140	106
200	75
270	53
Pan	25

Equation 3.1: Geometric mean diameter of particles by mass (d_{gw})

$$d_{gw} = log^{-1}\left[\frac{\sum_{i=1}^{n} W_i \; log \; \bar{d}_i}{\sum_{i=1}^{n} W_i}\right]$$

where:

d_i is diameter of the *i*th sieve: which is calculated using the equation $\bar{d}_i = (d_i \times d_{i+1})^{0.5}$

d_i is nominal sieve aperture size in ith sieve

$d_{i+1}$is nominal sieve aperture size in next larger than *i*th sieve (just above in a set)

W_i is mass on ith sieve, g

n is number of sieves + 1 (pan)

Equation 3.2: Standard deviation of particles by mass (S_{gw})

$$S_{gw} \approx \frac{1}{2} \; d_{gw} \left[log^{-1} S_{log} - \; (log^{-1} S_{log})^{-1}\right]$$

$$S_{log} = \left[\frac{\sum W_i (\log \bar{d}_i - log d_{gw})^2}{\sum W_i}\right]^{0.5}$$

Where:

S_{gw} is geometric standard deviation of particle diameter by mass, mm.

S_{log} is geometric standard deviation of log-normal distribution by mass in ten-based logarithm, dimensionless d_{gw}.

3.5 Limestone solubility

Solubility of all limestone samples used in the study was determined by an assay developed at University of Maryland (Angel, 2019a), which is an adaption of the method descrided by Zhang and Coon (1997). This modified method makes use of a pH 3 glycine buffered HCl solution to calculate solubility:

1. Water baths were filled (one oscillating bath and one still bath) with distilled water to 3 cm below the top to ensure that HCl solution in Erlenmeyer flask was below the surface of the water.
2. Water baths were turned on without shaking and temperature set to 42ºC, and allowed to warm up.
3. Water temperature was checked with a thermometer ensuring that the temperature was 42ºC.
4. HCl and HCl-Glycine solution was warmed in the still water bath at 42ºC.
5. Filter papers and small aluminum pans for drying were labelled numbered and weighed.
6. A 1.000 g limestone sample was weighed and transferred into a 250 mL Erlenmeyer flask and secured in oscillating water bath.
7. Oscillations of the water bath were started and water temperature checked that it was 42ºC.
8. Using a re-pipette 140 mL of HCl-glycine solution (pH 3 – buffered) was dispensed into the flask and the timer started.
9. When multiple samples are analysed at a time multiple timers were used to time the intervals at which the solubility was measured (5, 15 and 30 mins).
10. Once the allotted time had elapsed in oscillating bath the Erlenmeyer flask was removed and the reaction stopped using distilled water.
11. Erlenmeyer flask was rinsed with distilled water removing all contents and pouring through vacuum filtration system.
12. Filter paper was dried with remaining limestone on for 8 hours at 100ºC, as well as extra filter paper for determination of the dry matter of the filter papers.
13. The remaining weight was subtracted from the starting weight and calculated as a percentage of the starting sample weight.
14. This procedure was repeated for as many replicates as were needed and for as many time points that were needed.

3.6 Digestibility Trial 1: The effect of limestone source, limestone particle size and phytase inclusion on Ca and P digestibility

3.6.1 Birds and management

1400 fast-feathering Ross 308 males were placed on day zero in floor pens containing clean wood shavings in an environmentally controlled broiler house with artificial lighting. Each pen contained two bell drinkers and two tube feeders allowing adequate space for all birds to feed and drink. Feed and water were supplied *ad libitum.* The lighting programs used were as follows: days 0 – 5: 23 hours light (L) and 1 hour dark (D); days 6 – 11: 14L and 10D; days 12 – 25: 16L and 8D. The house was divided into 28 pens, each with a floor area of 4.5m^3, with 50 birds placed per pen. Due to space constraints in the metabolism facility, birds were divided into two sets to measure digestibility. Set one were moved to the metabolism facility on 18 days-of-age and set two were moved to the metabolism facility on 22 days-of-age. Previous research has shown that there is very little variance in Ca and P digestibility between these two ages in broilers (Li *et al.*, 2018). These two sets of birds were regarded as "blocks" during statistical analysis to minimize variability because of the possible age effect (see section 3.6.5: Statistical analysis). A common maize-soybean meal based pre-starter was fed for 0-10 days and starter diet was fed from 11 days until commencement of the digestibility trial. Birds were weighed at 7, 14 and 21 days of age. On day 17, 700 birds were weighed individually and the birds split into 6 groups according to body weight (group 1 – first quartile, group 2 – top half of second quartile, group 3 – bottom half of second quartile, group 4 – top half of third quartile, group 5 bottom half of third quartile and group 6 – fourth quartile). The heaviest 7.15% and the lightest 7.15% were removed from the population leaving 100 birds in each of the respective six groups, these 600 birds formed the first set. This process was repeated again on day 21 for the second set of birds in the experiment. Birds were moved to metabolism cages in an environmentally controlled and artificially lit house. Each cage was equipped with two trough drinkers and one trough feeder. The dimensions of the cages was 52 cm high x 90 cm long x 34 cm wide. Each of the 100 metabolism cages housed one bird from each of the six groups, and average weights of the birds per cage were checked to ensure that there were no cages that were significantly heavier or lighter than the average at the commencement of the digestibility trial.

3.6.2 Experimental design and diets

All diets fed throughout growing and experimental phases were fed as a mash. The diets were all maize-soybean meal based diets of which all major constituents were analysed for dry matter (DM), crude protein (CP), crude fat, crude fibre, ash, Ca and P before formulation.

Pre-starter diet: 0 – 10 days. The diet formulated for the period from placement on day zero until 10 days was formulated to contain 23% CP, 1.28% digestible lysine, 12.45 MJ/kg metabolisable energy (ME), 1.0% Ca and 0.799% tP (Table 3.2).

Starter diet: 10 days – commencement of digestibility trial. The diet formulated for the period between 10 days and the time the birds were fed the experimental diet was formulated to contain 20.91% CP, 1.15% digestible lysine, 12.45 MJ/kg ME, 0.86% Ca and 0.707% tP (Table 3.2).

For the experiment, a randomised block design was implemented and had a 3 x 2 x 2 factorial arrangements of treatments. Factors were limestone source (A, B, C); limestone particle size (0.8 mm or commercial), and phytase inclusion level (0, or 1000 FTU/kg feed). This experimental structure resulted in 12 treatments that were each replicated five times during the first set and five times during the second set. One cage containing six male broilers was one replicate. Research has shown that after a period of 36h on a new diet broilers are able to alter their Ca and P metabolism to meet their requirements (Li *et al.*, 2018). To prevent this from happening the birds were only allowed to eat the test diets for 34h before sampling took place.

The twelve experimental diets were made by first blending a basal feed of 450 kg without any limestone or inorganic phosphate.

The basal diet mix was bagged into 20 bags of 20 kg each. Treatment diets were mixed by adding one of three sources of South African limestone. The iinclusion levels of the different limestones differed between sources based on the Ca analysis of limestone sources (Table 3.4), ensuring the same level of Ca in each diet coming from the limestone. Limestone was from either commercial or 0.8 mm particle size and diets either contained zero added phytase or 1000 units of phytase (Table 3.3). Phytase used was a 6-phytase of *Buttiauxella spp.* (Danisco Animal Nutrition, DuPont Industrial Biosciences, Marlborough, UK). Ca and P analysis was conducted according to AOAC (2000) official method 985.01 (3.2.06) and AOAC (2016) official method 2011.14 (50.1.37) adapted for inductively coupled plasma optical emission spectrometry (ICP-OES) at an independent laboratory. Phytase analysis was conducted by the Chemunique laboratory using the methods developed and described by Du Pont Industrial Biosciences.

Table 3.2 Raw material composition and formulated nutrient content of diets used in the experiment

Ingredient	Pre-starter	Starter	Basal Diet
	%		%
Maize	48.53	54.46	76.42
Soybean Oilcake 47%	33.02	27.61	17.11
Full Fat Soya Expanded	10	10	-
Crude Vegetable Oil	2.95	3.05	2.34
MDCP (16.37% Ca, 19.0% P)	1.85	1.51	-
Limestone (32.43% Ca) (0.475 mm d_{gw})	1.5	1.32	*
NaCl	0.40	0.40	-
DL Methionine	0.36	0.30	-
Lysine HCL 78%	0.22	0.22	-
Threonine	0.14	0.13	-
L - Valine 99%	0.04	0.02	-
Choline Chloride 60%	0.20	0.20	0.1514
Non-Medicated Vitamin and Mineral Premix	0.20	0.20	0.25
Sodium Carbonate	0.08	0.09	0.61
Zinc Bacitracin 15%	0.0067	0.0067	-
Cycostat	0.50	0.50	-
Marker Premix[1]	-	-	3.06
Nutrient Content (DM basis): Formulated, (Analysed)			
Crude Protein, %	23	20.89	14.44 (13.72)
Crude Fat, %	6.74	7.02	5.31 (6.18)
ME Poultry (MJ/kg)	12.45	12.45	12.3
Total Lys, %	1.45	1.30	0.67
Dig. Lys, %	1.28	1.15	0.57
Dig. Met, %	0.66	0.58	0.22
Dig. TSAA, %	0.95	0.85	0.43
Dig. Thr,%	0.86	0.77	0.47
Dig. Trp,%	0.24	0.22	0.14
Dig. Ile, %	0.86	0.77	0.53
Dig. Val, %	0.96	0.85	0.42
Dig. Arg, %	1.39	1.22	0.71
Ca, %	1	0.86	0.11 (0.09)
P, %	0.8	0.71	0.34 (0.23)

[1]Marker premix mixed using maize (86.67%) and Cr_2O_3 (13.33%).

*****Shows that no limestone was added to the basal diet which was used to mix experimental diets. Limestone was added to individual treatment as they were mixed. Limestone source was either A, B or C and particle size was either commercial or 0.8mm.

The analysis (Table 3.3) of the experimental diets used in the experiment show that the analysed phytase in the diets was within the acceptable range of what was expected.

Table 3.3 Ca (%), P(%) and Phytase (FTU) analysis of the experimental diets for Digestibility trial 1

Treatment	Limestone Source	Particle Size	Phytase Dose (FTU)	Analysed Ca (%)	Analysed P (%)	Analysed Phytase (FTU)
1	A	Commercial	0	0.64	0.26	116
2	A	Commercial	1000	0.72	0.26	1306
3	A	0.8 mm	0	0.72	0.26	100
4	A	0.8 mm	1000	0.74	0.26	1209
5	B	Commercial	0	1.08	0.25	155
6	B	Commercial	1000	0.79	0.26	1414
7	B	0.8 mm	0	0.80	0.27	151
8	B	0.8 mm	1000	0.78	0.28	1420
9	C	Commercial	0	0.72	0.25	136
10	C	Commercial	1000	0.79	0.26	1466
11	C	0.8 mm	0	0.74	0.25	316
12	C	0.8 mm	1000	0.76	0.27	1459

Prior to diet formulation all three limestone sources were analysed by an independent laboratory for mineral composition (Table 3.4). The mineral analysis of the limestone was conducted according to AOAC (2000) official method 985.01 (3.2.06) and AOAC (2016) official method 2011.14 (50.1.37) adapted for ICP-OES. The mineral analysis of the limestone was a crucial part of the diet formulation as each limestone was included in the diet at a level which ensured that all diets supplied the same amount of Ca. Each limestone was included at a different level to ensure this.

Table 3.4 Mineral composition of limestone sources used in the study

	Units	Limestone A	Limestone B	Limestone C
Ca	%	33.72	36.76	34.7
P	%	0.01	0.01	0.01
Cu	mg/kg	2.29	1.81	1.03
Fe	mg/kg	4675.05	2484.08	264
Mg	%	1.2	0.36	0.81
Mn	mg/kg	323.12	599.47	13.2
K	%	0.01	0.03	0.06
Na	%	0.01	0.01	0.01
Zn	%	11.7	18.31	0.001

3.6.3 Sample collection and analysis

To determine apparent ileal digestibility all six of the birds from each cage were euthanised using carbon dioxide asphyxiation. A mixture of three commonly occurring gasses (35% CO_2, 30% O_2, and 35% N_2) was used to render the birds unconscious; the mixture was administered for approximately 5 mins. Once unconscious, carbon dioxide was administered for approximately 2 mins until the birds were dead. This method of euthanasia refrains the birds from flapping their wings and struggling, making it acceptable for ileal digestibility studies as there is very little movement of the digesta within the birds' GIT. Other methods such as cervical dislocation causes smooth muscle contraction in the GIT and might cause inaccuracies in digestibility calculations. Once birds had been euthanised the distal half of the entire ileum (defined as extending from Merkel's diverticulum to the ileocaecal junction) was removed (Rodehutscord *et al.,* 2012). The segments of distal ileum were immediately placed on ice cold granite slabs to aid in quick termination of digestion. The contents of the ileal segment were removed by gently flushing the segment with cold distilled water. The ileal contents of all six birds were flushed into a container to form a pooled sample. The sample was then immediately placed into a freezer to ensure the cold chain was kept. Samples were then freeze-dried at the University of Pretoria. Freeze-dried samples were ground using mortar and pestle and sent to a laboratory at the University of Arkansas for analysis in duplicate of Ca, P and chromium. Samples were analyzed according to AOAC (2000) official method 985.01 (3.2.06) and AOAC (2016) official method 2011.14 (50.1.37) adapted for ICP-OES.

3.6.4 Calculations

The apparent ileal disappearance of Ca and P from the distal ileum was calculated using Equation 3.3.

Equation 3.3. Apparent ileal digestibility (AID) coefficient using Cr as a marker

$$\frac{\left[\left(Nutrient / Cr_2O_3\right)_d - \left(Nutrient / Cr_2O_3\right)_i\right]}{\left(Nutrient / Cr_2O_3\right)_d} x100\%$$

Where $(nutrient/Cr_2O_3)_d$ is the ratio of the analysed nutrient to Cr_2O_3 in the diet and where $(nutrient/Cr_2O_3)_i$ is the ratio of the analysed nutrient to Cr_2O_3 in the ileal digesta.

Digested and undigested nutrient were calculated using Equations 3.4 and 3.5.

Equation 3.4. Digested nutrient (%) = (AID) coefficient × diet nutrient

Equation 3.5. Undigested nutrient (%) = (diet nutrient) – (digested nutrient)

Where diet nutrient is the analysed nutrient concentration in the diets fed to animals.

Nutrient disappearance was calculated using Equation 3.6.

Equation 3.6. Nutrient disappearance (%) = (AID coefficient * analysed nutrient in feed) / 100

3.6.5 Statistical analysis

The experiment was analysed as a randomised complete block design, using block, trial and random effects. Blocks were designated “Block 1” and “Block 2”. Each block consisted of one hundred (100) experimental cages where each treatment was replicated five times within the block. All data were analyzed in SAS (2012) using a mixed model in PROC mixed. Statistical probability for main and interaction effects was set at $P<0.05$, where the main or interaction effects were significant, means separation was done using LS means at $P<0.05$.

3.7 Digestibility Trial 2: Phytase dose response

Running concurrently with the above experiment a second experiment was conducted to examine the effect of graded levels of phytase on Ca and P digestibility. The birds were part of the above experiment therefore materials and methods remain the same for this experiment. For this trial a randomised complete block design was implemented with a 4 x 2 factorial treatment arrangement. Four levels of phytase (0, 250, 1000 and 2000 FTU) and two different particle size limestones (commercial or 0.8 mm) of the same source (Limestone B) made up the eight treatments in this trial. Each treatment was replicated five times in the first set and five times during the second set. Each replication was defined as one cage of six male broilers. Treatment structure and experimental diets are shown in Table 3.5 below.

Bird management, sample collection and analysis, basal diets and statistical analysis are exactly the same as previously described Digestibility trial 1 above. Only the experimental treatments differed in terms of limestone source, limestone particle size and phytase inclusion levels.

Table 3.5 Ca, P and phytase analysis of the diets used in Digestibility Trial 2

				Analysed Ca	**Analysed P**	**Analysed Phytase**
Treatment	**Limestone Source**	**Particle Size**	**Phytase Dose (FTU**	**(%)**		**(FTU)**
5	B	Commercial	0	1.08	0.25	155
6	B	Commercial	1000	0.79	0.26	1414
7	B	0.8 mm	0	0.80	0.27	151
8	B	0.8 mm	1000	0.78	0.28	1420
13	B	Commercial	250	0.67	0.24	433
14	B	Commercial	2000	0.60	0.27	2569
15	B	0.8 mm	250	0.85	0.26	465
16	B	0.8 mm	2000	0.84	0.28	2380

Chapter 4
Results

4.1 Particle size distributions

The particle size distribution was determined for each limestone sources in triplicate. The following figures show the particle size distributions of the limestone used in the study at either the commercial particle size or the 0.8 mm standardised particle size. Each bar in the graph represents one of the particle size distributions indicating that for each limestone source the particle size was determined three times (in triplicate) in order to reach the average particle size for each limestone used in the two digestibility trials. Tables 4.1 to 4.6 and Figures 4.1 to 4.6 show the results from the particle size distributions.

Table 4.1 confirms that the average particle size of this limestone was in fact 0.8 mm.

Figure 4.1 shows a normal particle size distribution around a mean of 0.8 mm particle size for standardised Limestone A.

Table 4.1 Mean particle size (d_{gw}) and standard deviation (s_{gw}) of standardised Limestone A.

	Particle Size (d_{gw})	Standard Deviation (s_{gw})
Average (µm)	809.05	535
Standard deviation	14.77	5.438123
Coefficient of variation (%)	1.83	1.02

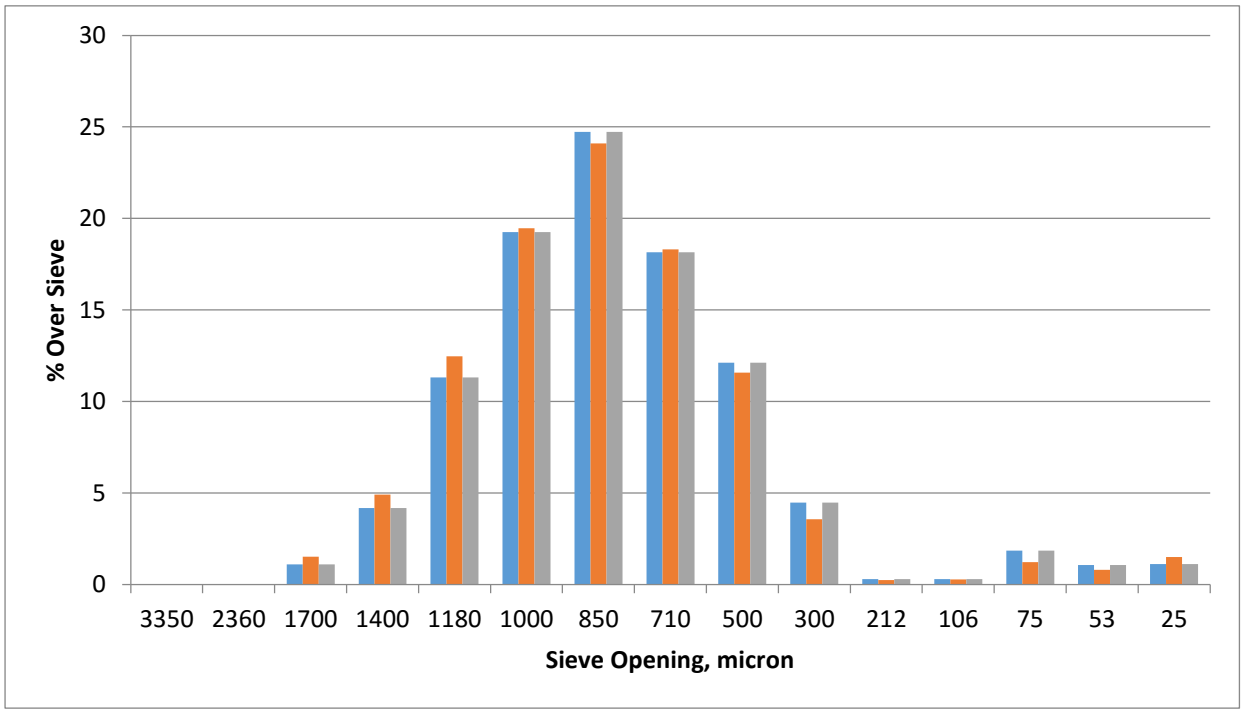


Figure 4.1 Particle size distribution in triplicate of standardised Limestone A.

Table 4.2 confirms that the average particle size of this limestone was in fact 0.8 mm.

Figure 4.2 shows a normal particle size distribution around a mean of 0.8 mm particle size for standardised Limestone B.

Table 4.2 Mean particle size (d_{gw}) and standard deviation (s_{gw}) of standardised Limestone B.

	Particle Size (d_{gw})	Standard Deviation (s_{gw})
Average (µm)	800.50	548
Standard deviation	8.96	12.6765
Coefficient of variation (%)	1.12	2.31

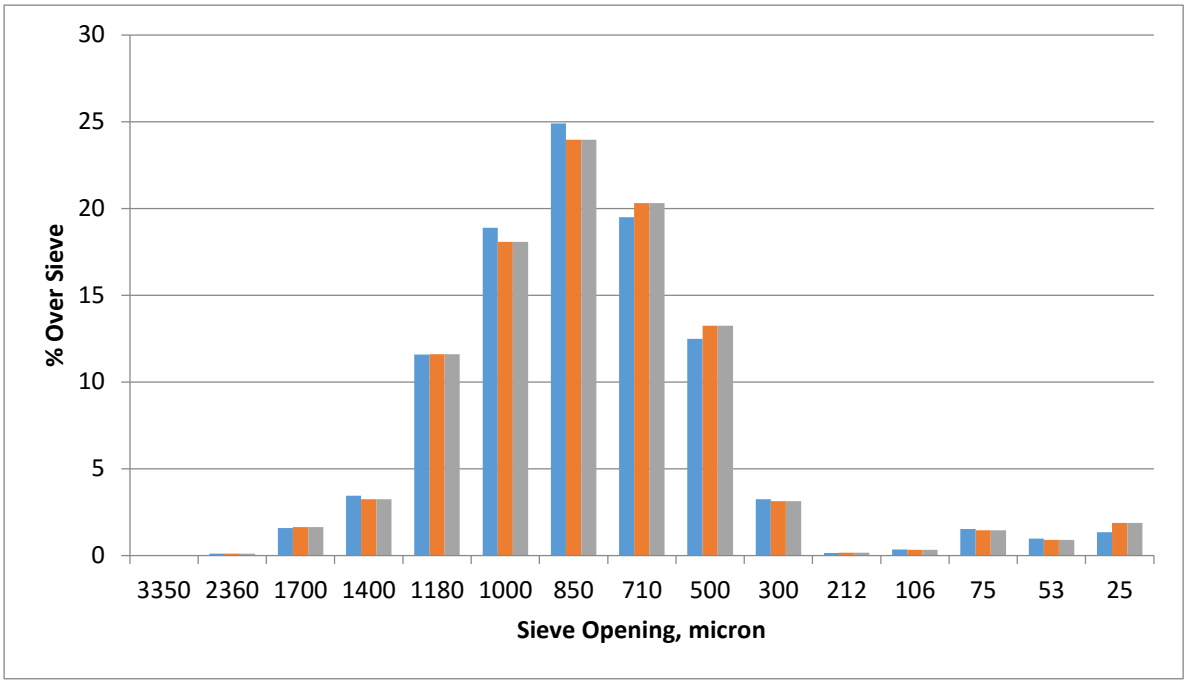


Figure 4.2 Particle size distribution in triplicate of standardised Limestone B.

Table 4.3 confirms that the average particle size of this limestone was in fact 0.8 mm.

Figure 4.3 shows a normal particle size distribution around a mean of 0.8 mm particle size for standardised Limestone C.

Table 4.3 Mean particle size (d_{gw}) and standard deviation (s_{gw}) of standardised Limestone C.

	Particle Size (d_{gw})	Standard Deviation, (s_{gw})
Average (µm)	802.19	559
Standard deviation	1.76	25.8541
Coefficient of variation (%)	0.22	4.62

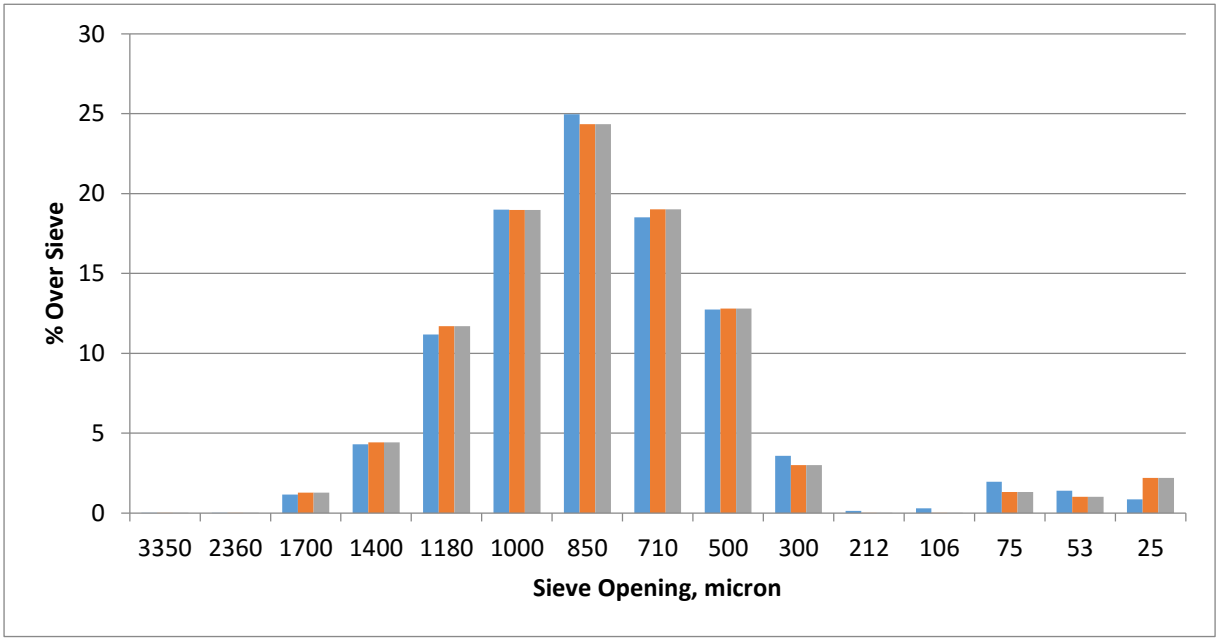


Figure 4.3 Particle size distribution in triplicate of standardised limestone C.

Table 4.4 confirms that the average particle size of this limestone was in fact 0.47 mm.

Figure 4.4 shows a bimodal particle size distribution of commercial Limestone A.

Table 4.4 Mean particle size (d_{gw}) and standard deviation (s_{gw}) of commercial Limestone A.

	Particle Size (d_{gw})	Standard Deviation (s_{gw})
Average (µm)	475.49	977
Standard deviation	25.72	77.65975
Coefficient of variation (%)	5.41	7.95

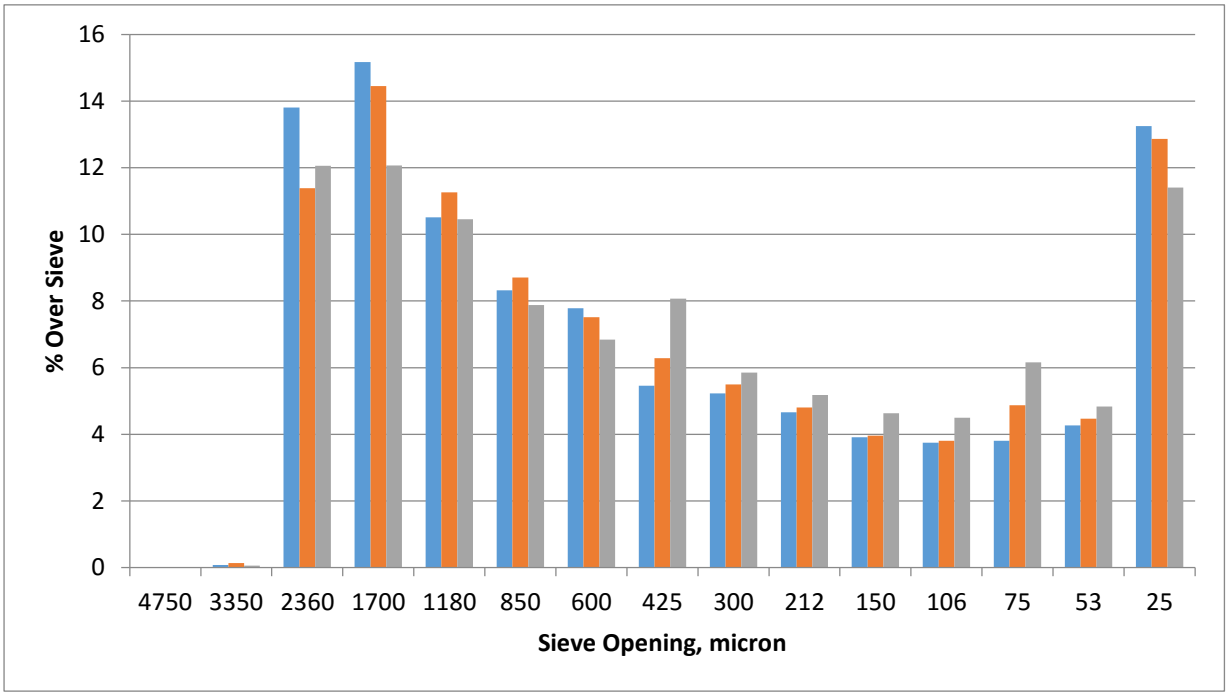


Figure 4.4 Particle size distribution in triplicate of commercial Limestone A.

Table 4.5 confirms that the average particle size of this limestone was in fact 0.39 mm.

Figure 4.5 shows a bimodal particle size distribution of commercial Limestone B.

Table 4.5 Mean particle size (d_{gw}) and standard deviation (s_{gw}) of commercial Limestone B.

	Particle Size (d_{gw})	Standard Deviation (s_{gw})
Average (µm)	398.43	488
Standard deviation	12.58	16.159867
Coefficient of variation (%)	3.16	3.31

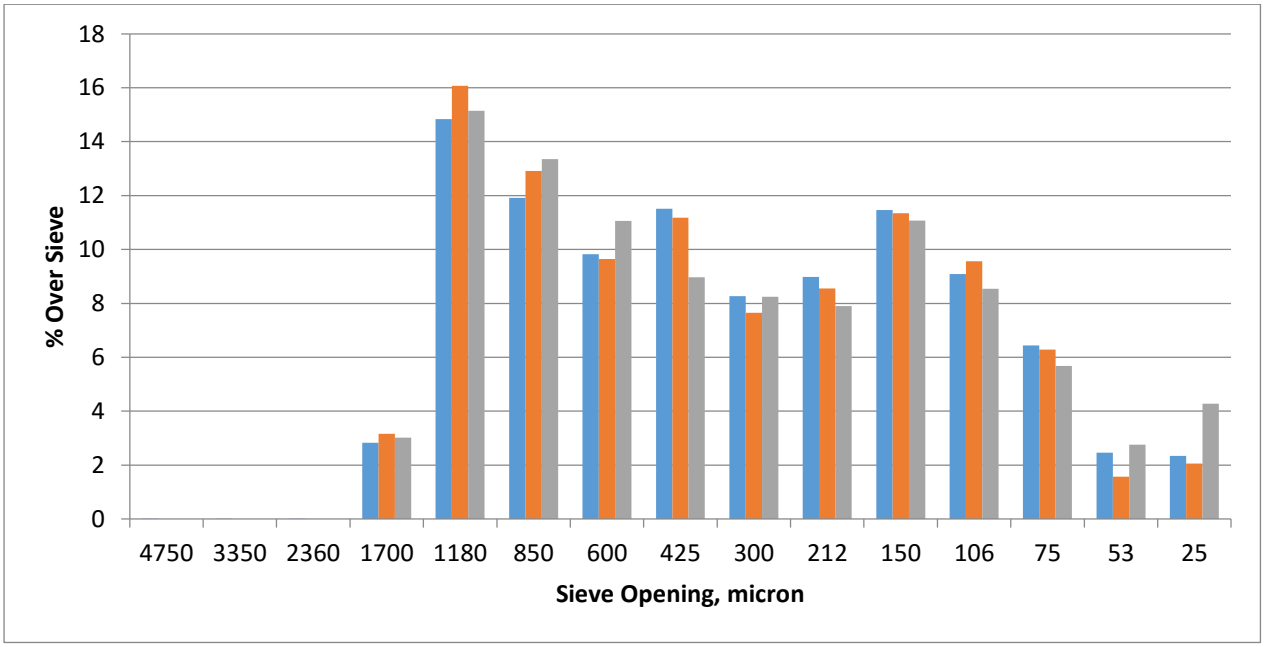


Figure 4.5 Particle size distribution in triplicate of commercial Limestone B.

Table 4.6 confirms that the average particle size of this limestone was in fact 0.34 mm.

Figure 4.6 shows a normal particle size distribution of commercial limestone C.

Table 4.6 Mean particle size (d_{gw}) and standard deviation (s_{gw}) of commercial Limestone C.

	Particle Size (d_{gw})	Standard Deviation (s_{gw})
Average (µm)	345.43	338
Standard deviation	16.05	17.0602
Coefficient of variation (%)	4.65	5.05

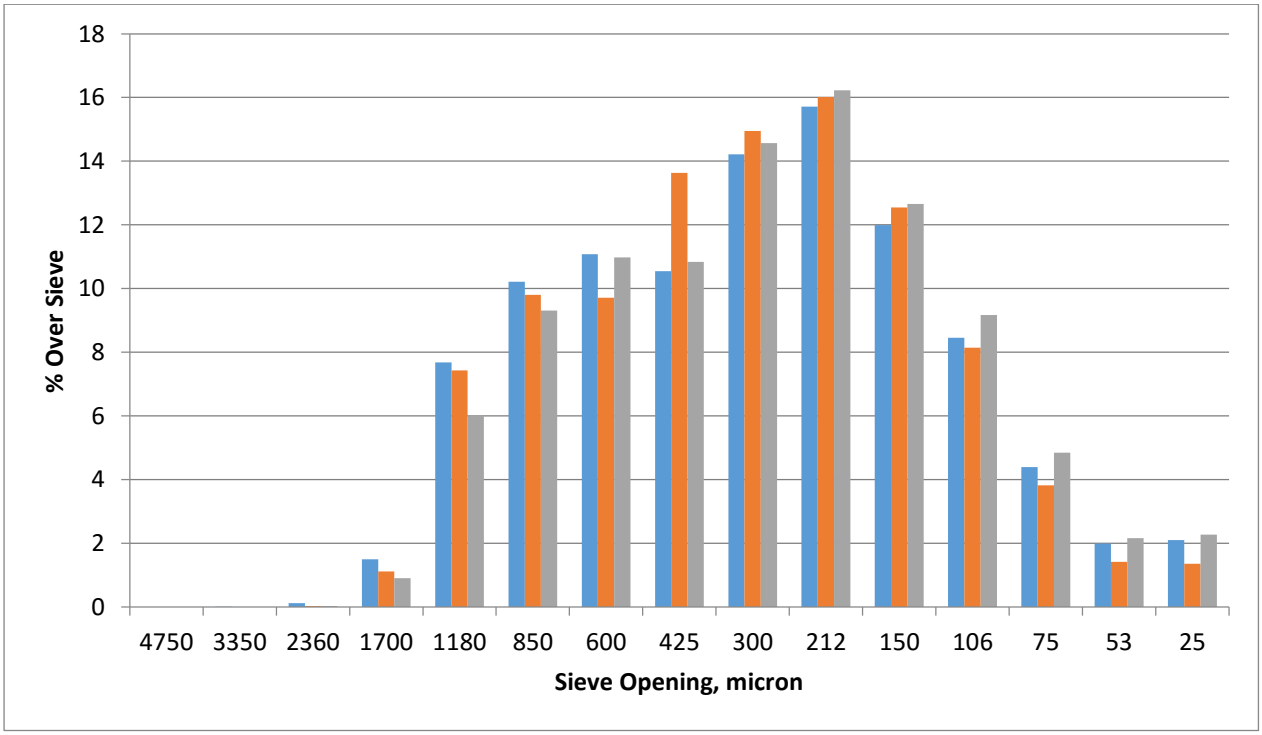


Figure 4.6 Particle size distribution in triplicate of commercial Limestone C.

4.2 Limestone solubility (*in vitro)*

Limestone solubility analysis of the 0.8 mm standardised samples showed clear differences between limestone sources (Figure 4.7). Limestone A achieved a solubilisation rate at time interval (T) 1 of 61.55% vs. 47.55% and 19.05% for limestones B and C, respectively. The rate of solubilisation from T1 to T3 and T6 was also different between limestone sources. Limestone A increased in solubility to 74.69% at T3, with only very small further increment in solubility to T6 (77.07%). In contrast, Limestone B and C continued to solubilise very consistently from T1 to T6, with a peak solubility of limestone B of 84.85%, while this was only 41.79% for limestone C at T6.

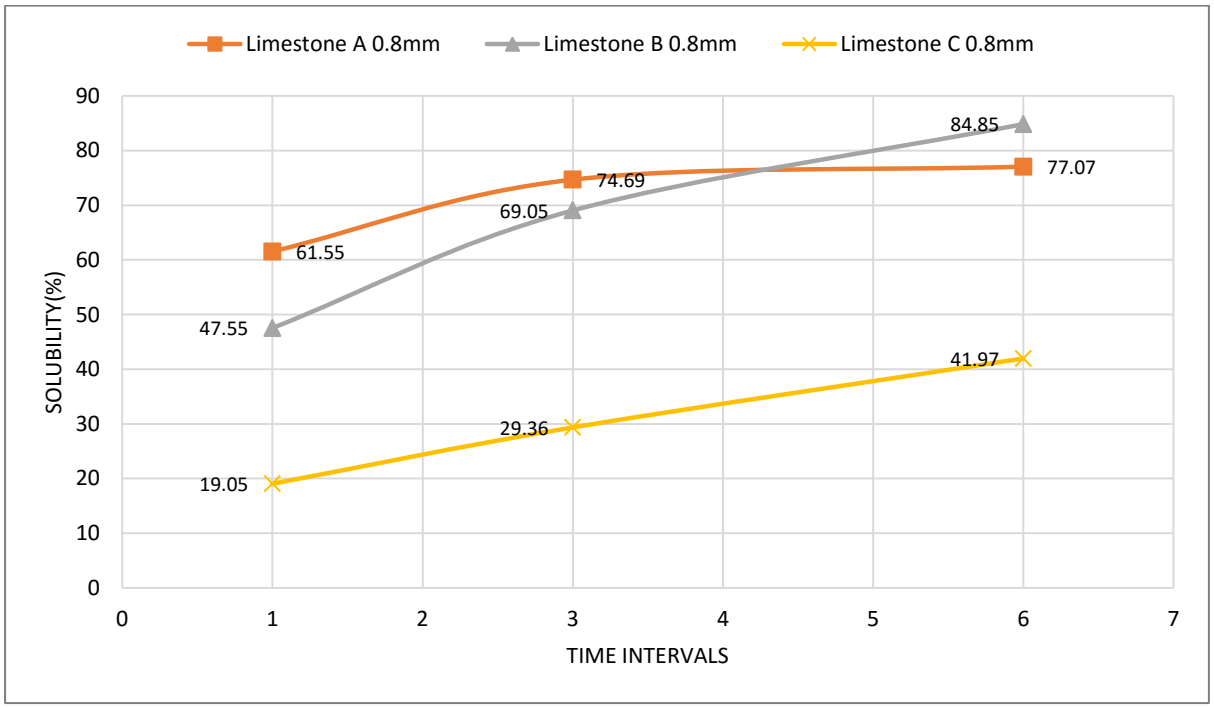


Figure 4.7 Limestone solubility (*in vitro*) of the three 0.8 mm d_{gw} standardised limestones used in this study.

The solubility of the three commercial particle sizes of the limestone used in the experiment are shown in Figure 4.8. Differences in solubility rates between the commercial limestone samples were found. Limestone A achieved the highest solubility at T1 of 63.02% followed by limestone B (52.47%) and Limestone C (26.52%). Similar to the solubilisation rates of the 0.8 mm sample, Limestone A tended to have a very small increment in solubility from T3 to T6, while limestone B and C continued to solubilised up to T6. The peak solubility achieved was 70.07%, 71.98%, and 62.58% for limestones A, B, and C, respectively at T6.

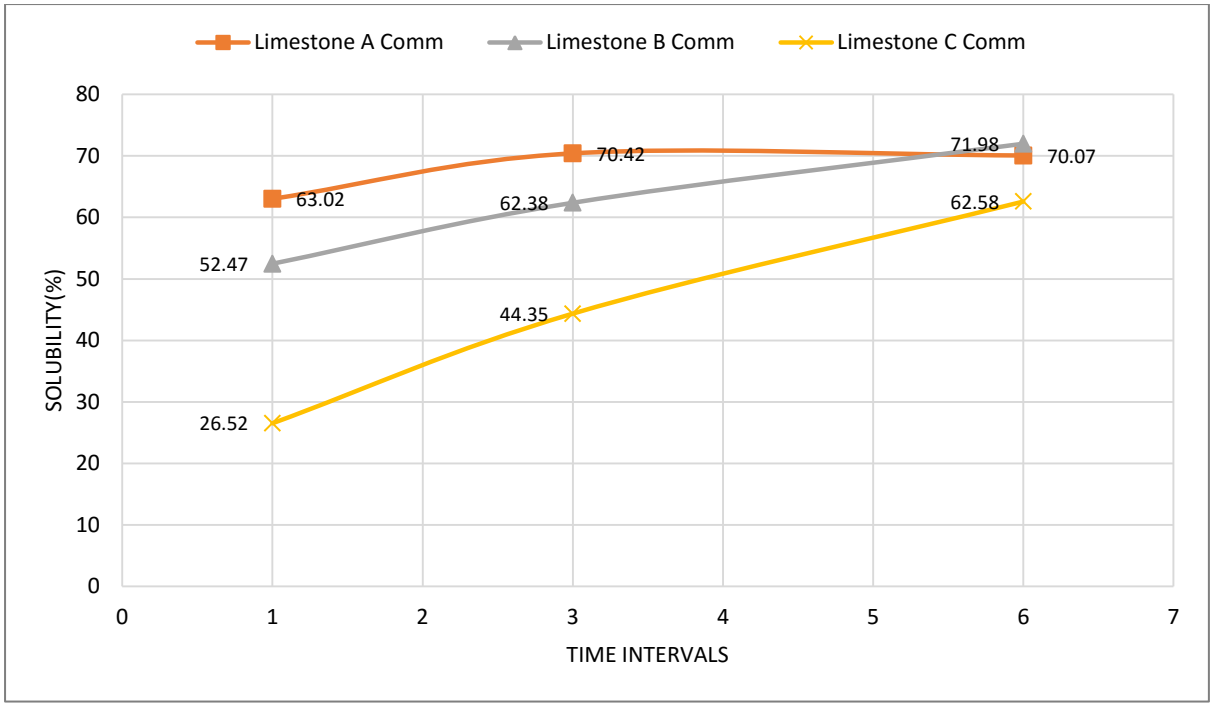


Figure 4.8. Limestone solubility (*in vitro*) of the three commercial particle size limestones used in this study.

4.3 Digestibility Trial 1: Effect of limestone source, limestone particle size and phytase inclusion on Ca and P digestion

4.3.1 Ca and P digestibility (*in vivo*)

There were significant main effects of limestone source, and phytase on Ca digestibility and disappearance, and significant effects of limestone source, particle size, and phytase on P digestibility and P disappearance. In addition, a significant three-way interaction of these factors was observed for P digestibility.

Table 4.7 Significance of main and interaction effects of block, limestone source, limestone particle size and phytase inclusion on digestibility (*in vivo*) and disappearance of Ca and P in broilers.

	Ca digestibility	**Ca disappearance[1]**	**P digestibility**	**P disappearance[1]**
Effect	**Pr > F**	**Pr > F**	**Pr > F**	**Pr > F**
Block	0.1372	0.1330	0.1298	0.1287
Limestone source	0.0024	0.1752	<0.0001	<0.0001
Particle size	0.3194	0.0484	<0.0001	<0.0001
Limestone source*Particle size	0.6523	0.0029	0.0829	<0.0001
Phytase	<0.0001	<0.0001	<0.0001	<0.0001
Limestone source*Phytase	0.7582	0.0524	0.2542	<0.0001
Particle size*Phytase	0.9515	0.4104	0.8349	0.1668
Limestone source*Particle size*Phyatse	0.7777	0.1060	0.0353	<0.0001

[1]Disappearance (%) was calculated by multiplying the digestibility x the analyzed nutrient level of the test diets and represents the amount of dietary Ca or P absorbed by the bird.

4.3.2 Main effect of limestone source

Limestone source had a highly significant effect on Ca (P<0.01) and P (P<0.0001) digestibility, while the effect on P disappearance was also highly significant (P<0.0001) (Table 4.8). Limestone source altered both Ca and P digestibility of the test diets (Table 4.8). The *in vivo* (ileal) Ca digestibility of diets containing Limestone A of 60.67% was significantly greater than for limestone B (52.54%) and C (54.98%). Due to small differences in analyzed dietary Ca, there were no differences between limestone sources on Ca disappearance from the diets. In contrast to Ca digestibility results, Limestone C had the highest *in vivo* (ileal) P digestibility (48.77%) vs. 39.89% and 39.39% for Limestones A and B, respectively. P disappearance was significantly different between limestone sources ranging from 0.124% for Limestone C to 0.098% and 0.086% for Limestones B and A, respectively.

Table 4.8 Main effect of limestone source on the mean apparent ileal digestibility (%) and disappearance[1] (%) of Ca and P in broilers.

Limestone Source	**Ca digestibility**	**P digestibility**	**Ca disappearance[1]**	**P disappearance[1]**
Limestone A	60.77[a]	39.89[b]	0.428[a]	0.087[c]
Limestone B	52.54[b]	39.39[b]	0.447[a]	0.098[b]
Limestone C	54.98[b]	48.78[a]	0.414[a]	0.124[a]
SEM[2]	1.682	1.787	0.013	0.004
$P_r > F$				
Limestone Source	0.0024	<0.0001	0.1752	<0.0001

[a-c]Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

[1]Disappearance (%) was calculated by multiplying the digestibility x the analyzed nutrient level of the test diets and represents the amount of dietary Ca or P absorbed by the bird.

[2]SEM: Standard error of the mean.

4.3.3 Main effect of particle size

Particle size (Table 4.9) had a significant effect on Ca disappearance (P<0.05). It also highly significant effect on both the digestibility and the disappearance of P (P<0.0001). An increase in P digestibility from 37.97% to 47.40% when using commercial and 0.8 mm particle sizes respectively showed the positive effect of using a slightly larger particle size (0.8 mm). A similar trend was seen on P disappearance whereby the P disappearance increases from 0.088% to 0.118%, when a larger particle size (0.8 mm) was used. In contrast to this, the Ca digestibility was not significantly affected when the particle size was increased, from the commercial limestone (57.05%) to the 0.8 mm limestone (55.15%). However, Ca disappearance was affected (P<0.05) when the particle size was increased to 0.8 mm (0.415% vs. 0.444%).

Table 4.9 Main effect of particle size on the mean apparent ileal digestibility (%) and disappearance[1] (%) of Ca and P in broilers.

Particle size	**Ca digestibility**	**P digestibility**	**Ca disappearance[1]**	**P disappearance[1]**
0.8 mm[2]	55.15[a]	47.40[a]	0.415[b]	0.118[a]
Commercial[3]	57.05[a]	37.97[b]	0.444[a]	0.088[b]
SEM[4]	1.390	1.656	0.011	0.004
$P_r > F$				
Particle size	0.3194	<0.0001	0.0484	<0.0001

[a-b]Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

[1]Disappearance (%) was calculated by multiplying the digestibility x the analyzed nutrient level of the test diets and represents the amount of dietary Ca or P absorbed by the bird.

[2]0.8 mm standardized particle size.

[3]Commercial limestone with unaltered particle size.

[4]SEM: Standard error of the mean.

4.3.4 Main effect of phytase

The addition of phytase (Table 4.10) to the diet, independent of the limestone source or particle size significantly increased the digestibility and disappearance of Ca (P<0.0001) and P (P<0.0001). The diets containing phytase showed, on average, an 11.16% increase in Ca digestibility and a 46.65% increase in P digestibility.

Table 4.10 Main effect of phytase dose on apparent ileal digestibility (%) and disappearance[1] (%) of Ca and P in broilers.

Phytase dose (FTU)	**Ca digestibility**	**P digestibility**	**Ca disappearance[1]**	**P disappearance[1]**
0	50.52[b]	19.36[b]	0.392[b]	0.046[b]
1000	61.68[a]	66.01[a]	0.468[a]	0.160[a]
SEM[2]	1.389	1.655	0.011	0.004
$P_r > F$				
Phytase dose	<0.0001	<0.0001	<0.0001	<0.0001

[a-b]Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

[1]Disappearance (%) was calculated by multiplying the digestibility x the analysed nutrient level of the test diets and represents the amount of dietary Ca or P absorbed by the bird.

[2]SEM: Standard error of the mean.

4.3.5 Interaction effect of limestone source x particle size x phytase interaction

Ca digestibility and disappearance was not affected by the interaction between limestone source, particle size and phytase dose (P>0.05) (Table 4.11). There was a significant 3-way interaction for P digestibility (P<0.05). Increasing particle size from the commercial limestone to the 0.8 mm limestone significantly increased P digestibility in diets containing phytase when limestones B (56.65% vs. 67.11%) and C (65.45% vs. 81.91%) were used. Altering the particle size in diets containing limestone A had no significant effect (62.23% vs 62.74%) on P digestibility values from this limestone source. However, P digestibility and disappearance both showed significance (P<0.05 and P<0.0001 respectively). The data showed a range in P digestibility between 11.49% and 81.91% taking into account the different sources, particles sizes and phytase levels in the feed. Examining a single level of phytase (1000 FTU) in the feed across limestone sources at the standardised particle size (0.8 mm), Table 4.11 shows that there was a significant source effect (P<0.0001) for P digestibility: Limestone A: 62.23%; Limestone B: 67.11% and Limestone C: 81.91%. The source effect was supported by the results obtained from the three limestone sources using the standardised

particle size but with zero phytase present in the feed; for this, P digestibility was 22.22%, 22.34% and 28.1% for sources A, B and C, respectively. These results were further supported by the values obtained for P disappearance; whereby an increase in digestibility values was reflected by an increase in P disappearance values. Diet with Limestone A, B and C at standardised particle size with 1000 FTU phytase added, showed P disappearance values of 0.135%, 0.171% and 0.226%, respectively. The standardised particle size with 0 FTU phytase, showed P disappearance values of 0.05%, 0.056% and 0.07%, for limestone sources A, B, and C respectively. Limestones A, B and C with a 1000 FTU dose of phytase and the commercially available particle sizes showed significant differences ($P<0.05$) in P digestibility (A: 62.23%, B: 56.65% and C: 65.45%). When no phytase was added to the diets, P digestibility was much lower at 12.38%, 11.49% and 19.65%, for the commercial limestone particle sizes respectively. P disappearance using the commercial particle size, and with added phytase, independent of limestone source was: 0.136%, 0.139% and 0.154% for Limestone A, B and C, respectively, and those with no added phytase, 0.028%, 0.028% and 0.045%, respectively. These findings support results that phytase inclusion not only assists with digestibility of P, but also the utilization or absorption thereof.

Table 4.11. Three-way interaction effects of limestone source, particle size and phytase dose on apparent ileal digestibility (%) and disappearance[1] (%) of Ca and P in broilers.

Limestone source	Particle size	Phytase dose (FTU)	Ca digestibility	P digestibility	Ca disappearance[1]	P disappearance[1]
Limestone A	0.8 mm[2]	0	56.69^{abcd}	22.22^{de}	0.409^{bcd}	0.050^{f}
Limestone A	Commercial[3]	0	55.61^{bcde}	12.38^{f}	0.354^{d}	0.028^{g}
Limestone A	0.8 mm[2]	1000	65.46^{a}	62.74^{bc}	0.483^{ab}	0.135^{d}
Limestone A	Commercial[3]	1000	65.32^{a}	62.23^{bc}	0.467^{ab}	0.136^{d}
Limestone B	0.8 mm[2]	0	45.21^{f}	22.34^{de}	0.360^{d}	0.056^{ef}
Limestone B	Commercial[3]	0	47.19^{def}	11.49^{f}	0.509^{a}	0.028^{g}
Limestone B	0.8 mm[2]	1000	56.77^{abcd}	67.11^{b}	0.441^{abc}	0.171^{b}
Limestone B	Commercial[3]	1000	61.01^{ab}	56.65^{c}	0.479^{ab}	0.139^{d}
Limestone C	0.8 mm[2]	0	46.65^{ef}	28.10^{d}	0.343^{d}	0.070^{e}
Limestone C	Commercial[3]	0	51.79^{cdef}	19.65^{e}	0.374^{cd}	0.045^{f}
Limestone C	0.8 mm[2]	1000	60.13^{abc}	81.91^{a}	0.454^{ab}	0.226^{a}
Limestone C	Commercial[3]	1000	61.37^{ab}	65.45^{b}	0.484^{ab}	0.154^{c}
SEM[4]			3.297	2.702	0.026	0.006
$P_r > F$						
Limestone source			0.0024	<0.0001	0.1752	<0.0001
Phytase dose			<0.0001	<0.0001	<0.0001	<0.0001
Particle size			0.3194	<0.0001	0.0484	<0.0001
Limestone source x Particle size x Phytase dose			NS [5]	0.0353	NS [5]	<0.0001

[a-g]Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

[1]Disappearance (%) was calculated by multiplying the digestibility x the analyzed nutrient level of the test diets and represents the amount of dietary Ca or P absorbed by the bird.

[2]0.8 mm Standardised particle size.

[3]Commercial limestone with unaltered particle size.

[4]SEM: Standard error of the mean.

[5] No significance.

4.4 Digestibility Trial 2: Phytase dose response

4.4.1 Main effect of particle size

Table 4.12 shows that there was a significant particle size effect on all parameters measured (P<0.05). Data from Table 4.12 supports data from Digestibility Trial 1 in Table 4.9. The 0.8 mm particle size had a significant positive effect on P digestibility (P<0.0001) and a negative effect on Ca digestibility (P<0.05) as compared to the smaller commercial particle size.

Table 4.12. Main effect of particle size on mean apparent ileal digestibility (%) and disappearance[1] (%) of Ca and P in broilers.

Particle size	Ca digestibility	P digestibility	Ca disappearance[1]	P disappearance[1]
0.8 mm[2]	45.11[b]	52.24[a]	0.298[b]	0.131[a]
Commercial[3]	50.46[a]	44.68[b]	0.342[a]	0.108[b]
SEM[4]	2.248	1.774	0.0161	0.0044
$P_r > F$				
Particle size	0.0189	<0.0001	0.0114	<0.0001

[a-b]Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

[1]Disappearance (%) was calculated by multiplying the digestibility x the analyzed nutrient level of the test diets and represents the amount of dietary Ca or P absorbed by the bird.

[2]0.8 mm Standardised particle size.

[3]Commercial limestone with unaltered particle size.

[4]SEM: Standard error of the mean.

4.4.2 Main effect of phytase dose

The response of Ca digestibility to increasing phytase dose was less clear with a 1000 FTU not having a significant impact on Ca digestibility when compared to either 250 or 2000 FTU, but 2000 FTU resulted in an improved Ca digestibility compared to 250 FTU. However, there is a linear increase in Ca digestibility in response to an increase in phytase dose as illustrated in Table 4.13. The P digestibility in Table 4.13 showed a clear and significant positive response when graded levels of phytase were added to the diets across all parameters (P<0.05). As the phytase dose increased so too did the P digestibility.

Table 4.13 Main effect of graded levels of phytase on apparent ileal digestibility (%) and disappearance[1] (%) of Ca and P in broilers.

Phytase dose (FTU)	**Ca digestibility**	**P digestibility**	**Ca disappearance[1]**	**P disappearance[1]**
0	40.24^{c}	17.12^{d}	0.347^{a}	0.042^{d}
250	46.12^{bc}	35.79^{c}	0.305^{ab}	0.091^{c}
1000	51.08^{ab}	61.89^{b}	0.340^{a}	0.155^{b}
2000	53.68^{a}	79.12^{a}	0.287^{b}	0.191^{d}
SEM[2]	2.748	2.132	0.020	0.0053
$P_r > F$				
Phytase dose	0.0003	<0.0001	0.0426	<0.0001

[a-d]Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

[1]Disappearance (%) was calculated by multiplying the digestibility x the analysed nutrient level of the test diets and represents the amount of dietary Ca or P absorbed by the bird.

[2]SEM: Standard error of the mean.

4.4.3 Interaction effects of particle size x phytase dose

Table 4.14 shows that there was significant interaction (P<0.01) between limestone particle size and graded levels of phytase for P digestibility and Ca disappearance. This demonstrated a clear positive response of P digestibility to increasing levels of phytase. The data from Table 4.14 also showed that increasing the phytase dose to 2000 FTU negated the negative effects finer limestone had on P digestibility particularly (P<0.01). Data in table 4.14 shows there was a significant (P<0.01) two-way interaction between particle size and phytase dose on P digestibility. There was a clear increase in P digestibility as there was an increase in phytase inclusion. It is possible that beyond 2000 FTU/kg of added phytase in the diet there could be further increases in both Ca and P digestibility.

Table 4.14 Two-way interaction effects of particle size and graded levels of phytase on apparent ileal digestibility (%) and disappearance[1] (%) of Ca and P in broilers.

Particle Size	Phytase dose (FTU)	Ca digestibility	P digestibility	Ca disappearance[1]	P disappearance[1]
0.8 mm[2]	0	39.3891[e]	22.5141[f]	0.2821[c]	0.0566[f]
Commercial[3]	0	41.0908[de]	11.5804[g]	0.4114[a]	0.0279[g]
0.8 mm[2]	250	43.4397[cde]	42.1013[d]	0.2936[c]	0.1061[d]
Commercial[3]	250	48.7914[abcd]	29.4700[e]	0.3155[bc]	0.0755[e]
0.8 mm[2]	1000	46.5482[bcde]	66.9532[b]	0.2978[c]	0.1704[b]
Commercial[3]	1000	55.6175[ab]	56.8271[c]	0.3828[ab]	0.1397[c]
0.8 mm[2]	2000	51.0468[abc]	77.3778[a]	0.3186[bc]	0.1926[a]
Commercial[3]	2000	56.3203[a]	80.8563[a]	0.2563[c]	0.1885[a]
SEM[4]		3.545	2.707	0.0262	0.0068
$P_r > F$					
Particle size		0.0189	<0.0001	0.0114	<0.0001
Phytase dose		0.0003	<0.0001	0.0426	<0.0001
Particle size x Phytase dose		0.7211	0.0032	0.0009	0.0690

[a-g]Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

[1]Disappearance (%) was calculated by multiplying the digestibility x the analysed nutrient level of the test diets and represents the amount of dietary Ca or P absorbed by the bird.

[2]0.8 mm Standardised particle size.

[3]Commercial limestone with unaltered particle size.

[4]SEM: Standard error of the mean.

Chapter 5
Discussion

5.1 Digestibility Trial 1: Effect of limestone source, limestone particle size and phytase on Ca and P digestibility

The results of this study showed that Ca digestibility of diets fed to broilers are affected by both limestone source and phytase inclusion. Previous research has frequently shown that phytase supplementation can significantly increase Ca digestibility (Walk *et al.,* 2012; Kim *et al.,* 2018; Li *et al.,* 2018). The mechanism whereby this occurs was thought to be due to the hydrolysis of dietary phytate by phytase, thus reducing the extent of Ca-phytate formation, allowing for increased Ca available for absorption in the small intestine (Angel *et al.,* 2002; Leytem *et al.,* 2008; Plumstead *et al.,* 2008; Li *et al.,* 2016). The extent of the increase in Ca digestibility by 1000 FTU phytase in the present study was from 50.52% to 61.67%. This effect of phytase on Ca digestibility was comparably small, compared to the over 46% point increase in P digestibility (19.36% vs. 66.01%). These results are supported by research published by Anwar *et al.* (2016b), who tested Ca, but not P, digestibility, using different limestone sources. Anwar *et al.* (2016b) recorded that the P levels in the feed were 0.45% and Ca digestibility values ranged between 57% and 62%, with limestone source having a significant effect ($P<0.05$). Therefore, both the range of Ca digestibility values reported as well as the significance of limestone source as a main effect ($P<0.01$) in the current study is supportive to the findings of Anwar *et al*. (2016b).

5.1.1 Solubility and particle size of South African limestone sources and effects thereof on Ca and P digestibility

The effects of limestone source in the present study may be related to differences between limestone sources in the rate of limestone solubilisation. Limestone A had the fastest solubilisation rate at time point 1 for both the 0.8 mm and commercial particle sizes whereas Limestone B had the highest solubilisation rate at time point 6 for both the 0.8 mm and commercial particle sizes (Tables 4.1 – 4.6 and Figures 4.1 – 4.8). Limestone C showed much slower solubilisation rates than Limestones A and B at all time points and this was reflected in the P digestibility (Tables 4.8) data collected in this study. Since the limestones where tested against each other at a standard particle size a conclusion can be drawn that the differences in the initial solubility at T1 and rate of solubilisation from T1 to T6 was caused by the inherent properties of the rock itself, these properties being: the age of the rock, the crystalline structure of the rock and impurities present in the rock.

Particle size of the limestone in this experiment was set as a fixed effect. Figures 4.1 to 4.3 represent graphically the particle size distributions of the standardized 0.8 mm limestone which was used in the

experiment. This particle size of limestone was determined by Kim *et al.* (2018) to provide excellent digestibility results. A standardized particle size was also used to eliminate particle size as a variable when measuring the effects of limestone source on Ca and P digestibility values. Figures 4.4 to 4.6 depict the particle size distributions of the commercial limestone that were used in the experiment. These particle distributions represented the particle sizes of the limestone used in broiler diets from the three different South African limestone suppliers. These limestone sources were not standardised in terms of particle size and were sampled from what was commercially available on the market. Limestone A had a mean particle size of 0.475 mm, Limestone B had a mean particle size of 0.398 mm while Limestone C had a mean particle size of 0.345 mm.

In vivo solubility tests were conducted on all limestones which were used in the study. From data collected in this experiment (Figures 4.7 and 4.8) and data shared by Angel *et al.* (2017), a prediction can be made as to whether or not the Ca and P digestibility would be high or low by studying the solubility curves. This is possible due to the fact that the hydrolysis of phytate has been studied extensively (Huber *et al.*, 2015; Li *et al.*, 2016; Beeson *et al.*, 2017). It could be hypothesised that the smaller the particle size of the limestone, the faster it will dissolve in the proventiculus and gizzard. If the limestone dissolved rapidly then the Ca^{2+} ions become available to bind to the phytate molecules in the GIT, forming a Ca-phytate chelate. This renders the PP unavailable to the bird thus decreasing the P digestibility.

Limestone A and B (Figure 4.7 and 4.8) had very similar *in vivo* digestibility values at time-point 1 for the commercial and 0.8 mm particle sizes. From this, it was expected that the P digestibility values would be similar but would be higher for the 0.8 mm limestones than the commercial limestone based on the particle size. This has been confirmed in the current study (Table 4.9). An explanation for the difference in solubilities at time-point 1 between limestones would be that commercial Limestone A had an extremely high percentage of very fine particles in the sample (>12% vs < 4% for Limestone B) and these would contribute to the higher solubility value at time-point 1 for this limestone source (Figures 4.7 and 4.8). Continuing this trend, Limestone C had solubility values of 26.25% and 62.58% at time-points 1 and 3 respectively. These translated into a P digestibility of 19.65% with a commercial particle size and 0 FTU phytase added, thus demonstrating the point that a lower solubility at time-point 1 was beneficial for P digestibility. When comparing the 0.8 mm with the smaller commercial particle size data it was expected to record a decrease in time-point 1 solubility and an increase in P digestibility (Kim *et al.*, 2018) even with 0 FTU phytase. The data obtained from this study supported this, whereby a decrease in limestone solubility at time-point 1 for all three limestones, 63.02% to 61.5% for Limestone A, 52.47% to 47.55% for Limestone B and 26.52% to 19.05 for Limestone C. Inverse to the decrease in limestone solubility, the three limestone sources all showed an increased P digestibility when the particle size was increased to 0.8 mm without the use of

phytase. Limestone A increased from 12.38 % to 22.22%, Limestone B increased from 11.49% to 22.34% and Limestone C increased from 19.65% to 28.1%. The reason for this being that the finer the particle size the faster the Ca ions dissociate and bind to the phytate molecules in the upper GIT. This rendered the PP unavailable (Tamin and Angel, 2003). Although Ca^{2+} does not have the highest affinity for binding to phytate (Maenz *et al.,* 1999; Tamim and Angel, 2003) the sheer number of Ca ions which will be present in the GIT after the dissociation occurs means that free Ca ions provoked the largest negative effect on P digestibility.

However, in the presence of phytase at a dose of 1000 FTU the results showed an interaction between limestone source, particle size and phytase dose on P digestibility. This has not been reported before. Although Kim *et al.* (2018) did report a significant interaction between Ca level, particle size and phytase (P=0.001) for P digestibility, source was not taken into account.

5.1.2 Particle size affecting phytase response

Data collected in the experiment revealed that the particle size of limestone in the diet affected the response of phytase for a specific limestone source. The phytase response was decreased for the smaller particle size limestone, independent of the specific source. This was supported by research which suggested that an increase in dietary Ca would decrease the ability of phytase to hydrolyse phytate (Huber *et al.,* 2015; Li *et al.,* 2016; Beeson *et al.,* 2017). Although this was true, the mechanism by which this occurs was not simply an increase in Ca concentration in the feed but rather an increase in Ca ions present in solution when the feed was ingested (Li *et al.,* 2016). The increase of Ca ions in the upper GIT allowed many more Ca-phytate complexes to form rendering the PP unavailable to the bird. The reason for this is the changing rate of limestone solubility as the particle size changes. Kim *et al.* (2018) showed that different particle sizes of limestone solubilise at different rates. The researchers used similar methodology for limestone solubility analysis as those used in the present study and showed that a decrease in particle size resulted in a radical increase in limestone solubility. Results showed that by using a limestone with a d_{gw} of 0.402 mm the solubility at 1 min was 39.4% and by using a limestone with a d_{gw} of <0.075 mm the solubility after 1 min increased markedly to 94.6 % (Kim *et al.,* 2018). Data collected in the present study is supportive of the data collected by Kim *et al.* (2018), whereby a decrease in particle size of the limestone from 0.8 mm to what is commercially available for each source resulted in a marked increase in solubility values at time-point 1. The efficacy of phytase can be affected by Ca^{2+} bound to phytate. Previous studies showed that an increase in available Ca increase the incidence of Ca-phytate chelates forming (Wise, 1983; Qian *et al.,* 1997). With an increase in initial limestone solubility there was an increase in Ca available to form the Ca-phytate chelates. The formation of these chelates rendered the phytate-P insoluble. As a result of this, the P digestibility would decrease since the P molecules are not able to be released. Another mechanism explored by Hurwitz and Bar (1971), which explained the decrease

in P digestibility when a high initial rate of solubilisation is observed, was the fact that Ca-P precipitates formed in time when there was excess Ca relative to inorganic P. The rapid solubilisation of the limestone at time-point 1 ensured that there were high levels of Ca present which in-turn was be detrimental to the efficacy of phytase and subsequently decrease P digestibility. It stands to reason that if we were able to decrease the initial rate of dissolution of limestone in the upper GIT then we would effectively increase the digestibility of P in the bird by preventing the formation of Ca-phytate chelates and Ca-P precipitates, as well as other cation-phytate chelates and precipitates. Due to the relatively high concentration of Ca ions compared to other cations in the diet, Ca ions are the cations which have the greatest negative effect on phytase activity.

5.1.3 Effects of limestone source on Ca and P digestibility values

There are several reports of dietary Ca level impacting Ca digestibility. Anwar *et al.* (2016b) reported similar results in research where they increased the dietary Ca:non-phytate P ratio from 1.5 to 2.5 and noted a decrease in AID Ca. The study by Anwar *et al.* (2016b) showed that Ca digestibility values ranging between 34% and 75% depending on the level of Ca in the diet. For example, Plumstead *et al*. (2008) showed that increasing dietary Ca from 0.47% to 1.16%, reduced the digestibility of dietary Ca from 50.8% to 33.81%. In that study, no description of the source or particle size of limestone was given. During the present study significant effects were observed when limestone source was examined as a main effect. To the best of our knowledge there have been no reported values for P digestibility with limestone source as a main effect. However, Anwar *et al.* (2016b) did report Ca digestibility coefficients for three limestone sources out of New Zealand's North Island, however, not taking particle size into account. The Ca digestibility coefficients were 0.62, 0.60 and 0.57 for limestone sources LM-1, LM-2 and LM-3, respectively with 4.5 g/kg dietary P ($P<0.05$) (Anwar *et al.*, 2016b). In the current study the Ca digestibility of the three limestone used from South African mines were 60.77%, 52.54% and 54.98% for Limestones A, B and C, respectively (Table 4.8). Furthermore, the current study examined the effects of limestone source on P digestibility, whereby the P digestibility values from the three limestone sources were 39.89%, 39.39% and 48.78% for Limestone A, B and C respectively (Table 4.8). Although Kim *et al.* (2018) did report apparent P digestibility values, there was only a single limestone source used in the study and alternatively the authors used differing Ca levels in feed as a main effect. Differences between limestone sources and the way they are digested would be a function of the rock itself. This includes the geological region where the rock originated, the age of the rock, the formation process of that particular rock and the impurities present in that rock. However, which one of these variables affects the efficacy of phytase or the Ca and P digestibility primarily is uncertain; and further research is needed to determine which physical characteristics of limestone affect the efficacy of phytase. However, from this study it is clear that the geology of the limestone plays a role in phytase response. When limestone of a standardised particle size (0.8 mm) (Angel *et al.*, 2019a, b) was used, a clear difference between limestone sources

was observed. These differences were observed regardless of the use of phytase in certain diets, thus indicating that the inherent differences in digestibility lay within the rock.

5.1.4 Ca and P digestibility response to phytase

The response in Ca and P digestibility to the addition of phytase (Table 4.10) in the diet was significant. The use of phytase to enhance Ca and P digestibility by eliminating the negative effect that Ca has on phytate hydrolysis (Amerah *et al.,* 2014) has been examined in the past and the data from the current study seems to be supportive to previous findings. An increase of Ca digestibility from 50.52% to 61.68% ($P<0.0001$) and an increase in P digestibility from 19.36% to 66.01% ($P<0.0001$) was recorded when phytase was added to the diets at 1000 FTU regardless of the limestone source and for particle size (Table 4.10). The P digestibility increased by 46.65% by using phytase. At a 0.8% dietary Ca level, Kim *et al.* (2018) found that by adding phytase to the diet with a limestone which had a particle size of 0.402 mm, the P digestibility increased from 27.9% to 62.8% ($P<0.001$) and Ca digestibility remained exactly the same (50.8% with no phytase vs 50.8% when 1000 FTU *Buttiauxella* phytase was added). However, when the particle size decreased to < 0.075 mm, the increase in Ca digestibility with the addition of phytase did show a significant ($P<0.001$) response. The increase in both Ca and P digestibility values after the addition of phytase is explained by the fact that the rapidly acting phytase liberates phytate P which directly increases the P digestibility (Walk *et al.,* 2012). It also indirectly increases Ca digestibility by greatly decreasing the possibility of Ca-phytate chelates forming, thus allowing Ca to be absorbed (Amerah *et al.,* 2014).

5.1.5 Three way interaction between limestone source, limestone particle size and phytase

The response of P digestibility to the three-way interaction between limestone particle size, limestone source and phytase dose was significant ($P<0.05$) (Table 4.11). This showed that the effect of phytase on P digestibility was dependent on the limestone source and the particle size of that particular limestone. The three way interaction response demonstrated was the highest in Limestone C with a 0.8 mm particle size, where the P digestibility increased from 28.1% with 0 FTU phytase to 81.91% with 1000 FTU phytase, a 53.81% increase in P digestibility. Limestones A and B showed increases in P digestibility of 40.52% and 44.77% respectively with the addition of phytase. In all instances the 0.8 mm particle sizes showed higher P digestibility values than the commercial particle size limestone from the same source.

Without phytase addition Limestone A showed (Table 4.11) an increase in P digestibility from 12.38% to 22.22% when the particle size of the limestone was increased from what is commercially available to 0.8 mm d_{gw}. Limestone B and C showed the same trend with P digestibility increasing from 11.49% to 22.34% and from 19.65% to 28.1%, respectively. The exact same trend was seen when phytase was added to the diet where the commercially available (finer) limestone resulted in

lower P digestibility than the standardized 0.8 mm d_{gw} for all sources. This demonstrates the negative effect which finer limestones have on the P digestibility (Amerah *et al.*, 2014). However, the differences between P digestibility for the commercial and 0.8 mm limestones for each source differed when 1000 FTU phytase was added. Limestone A had a difference of 0.51%, Limestone B showed a difference of 10.46% and Limestone C showed a difference of 16.52% in P digestibility when phytase was added, thus clearly demonstrating the effect which limestone geology has on phytase efficacy. If the source of limestone did not have an effect on the phytase efficacy we would have expected to see a uniform percentage increase across all sources when the particle size was increased to 0.8 mm d_{gw}.

5.2 Digestibility Trial 2: Phytase dose response

The response of AID Ca and P to increasing levels of phytase in the feed was measured to examine if higher levels of phytase in the diet would improve AID of Ca and P. In this case it was found that the particle size of the limestone significantly affected the AID P from 44.68% to 52.24% ($P<0.05$) with the commercial and 0.8 mm particle size respectively while the ileal Ca digestibility improved ($P<0.05$) from 45.11% to 50.46% with an increase of particle size from commercial to 0.8 mm (Table 4.12). The reduction in P digestibility and the increase in Ca digestibility with the increase in particle size independent of phytase inclusion, could be explained by the differences these two particle sizes showed in the solubility results. When considering phytase dose as a main effect, the significant increase in AID of both P (17.12% to 79.12%) and Ca (40.24% to 53.68%) with increasing levels of phytase in feed from 0 FTU/kg to 2000 FTU/kg was a direct result of the increasing levels of phytase in the diet independent of particle size. There has been research in both poultry and pigs which mimics these results, where an increase in phytase dose to 2000 FTU/kg increased digestibility of Ca and P (Bento *et al.*, 2013; Dersjant-Li and Kwakernaak, 2017) or where an exponential increase in P digestibility was measured up to 1000 FTU/kg of phytase after which it plateaued (Almeida *et al.*, 2017). However, the two-way interaction between particle size of this limestone and increasing doses of phytase up to 2000 FTU/kg, revealed that the inclusion of phytase at the higher levels seemed to counteract the negative effect on Ca and P digestibility expected from the finer limestone, increasing the digestibility of the commercial particle size limestone above that of the 0.8 mm limestone (80.86% vs. 77.38%). This was uncharacteristic and converse to what the results show until the 2000 FTU/kg point. Data suggests that with a finer particle limestone it was possible to overcome the negative effects of the finer limestone on P digestibility, by increasing the dose of the fast acting phytase to a point where it negates these negative effects on both Ca and P digestibility.

Chapter 6
Conclusion and Recommendations

6.1 Conclusion

In conclusion a number of the hypotheses have been proven correct. It was firstly shown that limestone source had an effect on the AID of Ca and P. It was also shown that the limestone particle size influenced AID of P. The particle size did not affect AID of Ca in this study, with possible reasons for this being discussed in Chapter 7. It was also evident that the efficacy of the phytase enzyme was affected by both the limestone source and particle size, shown by higher AID of Ca and P in some limestone sources vs others at the same particle size and phytase dose. The fact that different particle sizes of limestone had an effect on the liberation of Ca and P at the same dose of phytase supports the hypothesis that limestone source affects Ca and P digestibility

The data collected from the phytase dose response trial, although showing a positive response when phytase was added up to 2000 FTU/kg, will need to be confirmed using a validation study, and this too are discussed in Chapter 7.

Ultimately the aim is to move towards formulation of broiler diets without the addition of inorganic P and formulating to a digestible Ca value rather that a total Ca value. This will mean less waste P output in commercial broiler production and therefore improve the sustainability of this farming practice. Another benefit would be to allow nutritionists to better meet the Ca requirements of the bird. The interactions which were observed between the limestone particle size and limestone source on P digestibility means that one is unable to define an ideal particle size of a limestone source without taking into consideration the geology and mineral content of that specific rock. This means that each limestone source will have an ideal particle size and due to the variation seen between limestone sources, therefore the ideal particle size will be different for each different limestone source in South Africa. Furthermore, the optimal use of phytase with limestone in broiler diets will differ between limestone sources and phytase enzymes. It will also be a function of the speed of the phytase, dose of the phytase, speed of limestone solubilisation and the level of phytate in the feed. Since the data is part of a wider and global study, this research is ongoing and will be used to model the effects of limestone, phytase and phytate on Ca and P contributions from phytase and ultimately formulate using a digestible Ca value for broiler diets. In the future it is plausible that nutritionists will, formulate diets with no added inorganic P after 14 days of age and achieve 90% P digestibility for phytate P from plants sources in the diet alone.

Current data suggest that Limestone source and phytase can alter the utilization of total dietary Ca by broilers. Further, since limestone source and limestone particle size altered the increment in AID P from added phytase, the characteristics of limestone should be considered when assigning matrix values to phytase in practical broiler diets.

6.2 Recommendations

Examining the data from these studies recommendations were made on the following:

1. The use of limestone in broiler diets should be done under the consideration of the particle size, the Ca content, impurities and solubilisation rate of the limestones which are being used. Every limestone source need to be assessed as its own entity and the view that all limestones are equal needs to change in order for nutritionists to more accurately meet the requirements of the bird and to take full advantage of this affordable feed ingredient.
2. The use of a rapidly acting phytase will improve the P and Ca digestibility of the diet by hydrolyzing phytate molecules before ionized Ca has an opportunity to form Ca-Phytate chelates which decreases the digestibility of both Ca and P. Early liberation of P from phytate in the digestive tract will result in increased Ca and P digestibility.
3. There may be room to increase the dose of phytase to 2000 FTU/kg and possibly higher doses with very fine limestones, improving the P digestibility value associated with the finer limestones.

Chapter 7
Critical review

The study was run with the utmost accuracy and measurements were taken meticulously. Feed mixing, weighing of birds, sample collection, sample analysis and statistical analysis were constantly being double checked by at least two qualified personnel at all stages of the study.

Particle size of the limestone did not have the expected effect on AID of Ca in the broilers. A possible reason for this could be the fact that commercial Limestone A, although having a GMD of 0.475 mm, had a large percentage of particles over the size of 1 mm and a large percentage of particles below 0.053 mm. The problem with this is that the very fine fractions solubilised rapidly which in turn decreased P digestibility. The large fractions solubilised slowly and released Ca later in the digestive tract so we did not encounter Ca digestibility values typical of a limestone with this GMD (Figure 4.4). Therefore, if only the GMD is taken into account without considering the bimodal particle size distribution, incorrect conclusions might be drawn on effect of particle size of limestone on Ca digestibility.

Another point which needs to be explored was the fact that when 2000 FTU/kg of phytase was added in the trial, finer limestone with the commercial particle size of 0.398 mm outperformed the 0.8 mm limestone in terms of P digestibility. There are two distinct interpretations for this and neither should be overlooked. The first being that for Limestone B at the commercial particle size, phytase dosed 2000 FTU/kg was been enough to overcome the negative effects of the smaller particle size on P digestibility. This interpretation may hold true but has to be validated by further studies using different limestones at different particle sizes with varying levels of phytase; this would confirm that the optimal phytase dose for each of the different particle sizes would differ, giving a clear representation that each different limestone has an optimal phytase dose at each different particle size. The second possibility could be the differences in analysed Ca value of the two diets at 2000 FTU/kg, the commercial particle size diet having 0.6% Ca and the standardised particle size diet having 0.84% Ca. The implication of the differing Ca levels in the diet was that higher Ca levels in a broiler diet are expected to yield lower P digestibility (Leytem *et al.,* 2008; Plumstead *et al.,* 2008; Kim *et al.,* 2018), but due to the high level of phytase in the diet we cannot be certain that this would have played as big a role as was expected.

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