MEASUREMENT OF TRUE ILEAL CALCIUM DIGESTIBILITY OF FEED INGREDIENTS FOR BROILER CHICKENS

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in

Animal Science

at Institute of Veterinary, Animal and Biomedical Science (IVABS)

Massey University, Palmerston North,

New Zealand

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2017

ABSTRACT

The recent interest towards the use of digestible phosphorus (P) in poultry feed formulations necessitates the measurement of true calcium (Ca) digestibility of feed ingredients because of the close relationship between these two minerals for their absorption and post absorptive utilisation. When this thesis research was initiated, no published data were available on Ca digestibility of feed ingredients for broiler chickens. The major objective of the studies reported in this thesis was to determine the true Ca digestibility of feed ingredients for broiler chickens. In total, nine studies were conducted.

The first study (Chapter 4) was conducted to determine the effect of methodology on ileal endogenous Ca losses. Three methods, namely feeding a Ca- and P-free diet, maize gluten meal based diet and egg albumen based diet, were used. Ileal endogenous Ca losses differed among different methodologies. The highest ileal endogenous losses of 125 mg/kg dry matter intake (DMI) were recorded on the Ca- and P-free diet, followed by 77 and 43 mg/kg DMI on maize gluten meal and egg albumen diets, respectively.

In the second and third studies (Chapters 5 and 6), regression and direct methods, respectively, were used to determine the true Ca digestibility of meat and bone meal (MBM). The true Ca digestibility coefficient of MBM samples were ranged from 0.41 to 0.60. No difference was observed between true Ca digestibility coefficients of MBM determined by regression and direct methods. Since the direct method is less laborious and cost effective compared to regression method, this method was used in subsequent studies (Chapters 7 to 10) to determine the true Ca digestibility of a range of Ca sources.

In fourth and fifth studies (Chapters 7 and 8), the influence of dietary P, particle size and Ca to non-phytate P ratio was investigated on the true Ca digestibility of limestone for broiler chickens. The true Ca digestibility of three limestone samples varied from 0.56 to 0.62. Supplementation with recommended dietary P (4.5 g/kg) increased the true Ca digestibility of limestone when compared to diets without P. An increase in particle size from <0.5 to 1-2mm improved the true ileal Ca digestibility of

limestone. Widening the Ca to non-phytate P ratio reduced the true Ca digestibility of limestone for broiler chickens.

The sixth study (Chapter 9) was conducted to determine the effect of Ca source and particle size on the true Ca digestibility and total tract retention. Limestone and oyster shell were used as Ca sources. No difference was observed between the true Ca digestibility of limestone and oyster shell. An increase in particle size from <0.5 to 1-2 mm increased both the Ca digestibility and retention of both Ca sources, and increased the Ca concentration of gizzard contents.

The study reported in Chapter 10 was conducted to determine the true Ca digestibility of dicalcium phosphate (DCP), monocalcium phosphate (MCP), canola meal, poultry by-product meal and fish meal, and to compare the effect of dietary adaptation length on true Ca digestibility of DCP and MCP. The true Ca digestibility coefficients of these feed ingredients were lower than MBM, limestone and oyster shell, and ranged from 0.24 to 0.33. It was speculated that the length of adaption to the assay diets may be responsible for the lower than expected estimates. The effect of dietary adaptation length (24, 48 or 72 hrs) was subsequently examined, but had no effect on true Ca digestibility of DCP and MCP.

In the final study (Chapter 11), the true Ca digestibility of DCP was determined using different methodologies (regression, difference and direct methods). The true Ca digestibility coefficients of DCP were 0.34 and 0.21 with direct and different methods, respectively. A very low digestibility coefficient of 0.13 was determined by the regression method.

In conclusion, the true Ca digestibility coefficient of major Ca sources (limestone, oyster shell and MBM) is not high and varied from 0.40 to 0.70. Particle size of limestone and oyster shell influenced Ca digestibility, with coarser particles having higher digestibility. The direct method appears to be suitable for the determination of true Ca digestibility of limestone, oyster shell and MBM, but may not be appropriate for other Ca sources with intrinsic imbalance of Ca and P.

Acknowledgements

First of all, I would like to express my deepest gratitude to my major supervisor Professor Ravi Ravindran for his patience, constructive ideas and guidance in planning and conduct of studies and thesis writing. It was really an honour for me to work with such an amazing person and one of the best poultry nutritionist in the world. For me, completion would have been an impossible goal without the help of Professor Ravindran.

I am immensely thankful to my co-supervisors, Dr Rana Ravindran, Professor Patrick Morel and Professor Aaron Cowieson for guiding me in every step of my research and writing process. Their support and guidance was helpful to complete my journey of Ph.D.

I appreciate the help and support of staff at the Poultry Research Centre, especially to Don Thomas, Colin Naftel, Edward James and Shaun de Malmanche for their technical support and assistance in diet mixing, conduct of studies and help in sample collections. I am thankful to Fliss Jackson and the laboratory staff at the Nutrition lab, and Food Chemistry laboratory for their assistance in the analysis of samples.

I am really thankful to HEC (Higher Education Commission of Pakistan) for giving me this scholarship to provide financial assistance for my studies. I really appreciate the Claude McCarthy Fellowship New Zealand that sponsored me to present my research work in 20th European Symposium on Poultry Nutrition, 2015 in Prague and 25th Wold Poultry Congress, 2016 in Beijing.

I appreciate the help and guidance of my colleagues and friends from Massey University, especially Reza Abdollahi, Fifi Zaefarian, Ruvini Mutucumarana, Nipuna and Khaldoun, for guidance and encouragement during my study.

It was a dream of my late father Muhammad Anwar that I should get a doctorate degree from abroad. He struggled throughout his life to provide motivation and good education to us. I have no words to express my feelings for my Mother Balqees Anwar who always remembers me in her prayers and I know that it was impossible to achieve this goal without her prayers. I am thankful to my brothers, sisters and cousins for

supporting and encouraging me to attain this goal. Especially my elder brothers, Hafeez Anwar and Shakeel Anwar for their support and the way they tried to fill the gap left by my late Father.

Finally big thanks to my wife Huma, my daughter Aleesha and my son Arham who were with me in New Zealand and supported me to achieve this milestone. They suffered a lot because of my busy schedule during the PhD programme.

Publications

Studies completed during candidature, some of which are reported in this thesis have been presented in the following communications:

Referred scientific papers

Anwar, M. N., Ravindran, V., Morel, P. C. H., Ravindran, G. and Cowieson, A. J. (2017). Effect of calcium source and particle size on the true ileal digestibility and total tract retention of calcium in broiler chickens. *Animal Feed Science and Technology*, **224**:39-45.

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calcium digestibility of limestone for broiler chickens. *The Proceedings of 25th World Poultry Congress.* pp. 17 (Abstract). Beijing, China.

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List of Abbreviations

%	Percent
µg	Micro gram
µl	Micro litre
1, 25-$(OH)_2D_3$	1, 25-dihyroxycholicalciferol
AAFCO	Association of American Feed Control Officials
AIDC	Apparent ileal digestibility coefficient
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
Ca	Calcium
Ca_E	Calcium in excreta
Ca_I	Calcium in diet
Ca_O	Calcium in digesta
CM	Canola meal
CO_2	Carbon dioxide
CP	Crude protein
CT	Calcitonin
Cu	Copper
DCP	Dicalcium phosphate
DM	Dry matter
DMI	Dry matter intake
Fe	Iron
FM	Fish meal
g	Gram
g/b/d	Gram per bird per day
GLM	General linear model
GMD	Geometric mean diameter
GSD	Geometric standard deviation

H_2O	Water
HCl	Hydrochloric acid
I	Iodine
IECaL	Ileal endogenous calcium losses
IU	International unit
kg	Kilogram
M	Molar
MBM	Meat and bone meal
MCP	Monocalcium phosphate
mg	Milligram
MJ	Mega joule
mm	Millimetre
Mn	Manganese
Mo	Molybdenum
N	Nitrogen
N_2	Nitrogen gas
NRC	National Research Council
NS	Non-significant
O_2	Oxygen
ºC	Degree centigrade
P	Phosphorus
P	Probability
PBPM	Poultry by-product meal
PTH	Parathyroid hormone
SAS	Statistical analysis software
SE	Standard error
Se	Selenium
SEM	Standard error of mean
Ti	Titanium dioxide

TIDC	True ileal digestibility coefficient
Ti_E	Titanium dioxide in excreta
Ti_I	Titanium dioxide in diet
Ti_O	Titanium dioxide in digesta
UV	Ultra violet
WPSA	World's Poultry Science Association
Zn	Zinc

CHAPTER 1

General introduction

Calcium (Ca) and phosphorus (P) are two of the important minerals for poultry. Calcium is the mineral present in highest concentration in the animal body and, plays an important role in the skeletal development and proper growth of poultry. It is also necessary for egg shell formation in laying hens. Almost 99% of Ca is present in the skeleton and the balance (1%) is involved in a large array of metabolic and physiological functions in the body. It plays an important role in blood clotting, muscle contraction, transmission of nerve impulse, hormone secretion, and the regulation of heart beat (Coon *et al*., 2002). Deficiency of Ca can cause poor growth, rickets and, tibial dyschondroplasia, especially in birds with a fast growth rate.

Different organic and inorganic Ca sources are used in poultry diets to meet the Ca requirements, with inorganic Ca sources providing most of the Ca. Major inorganic Ca sources used in poultry diets are limestone, dicalcium phosphate and monocalcium phosphate, while the important animal-based organic Ca sources are meat and bone meal and oyster shell. These sources contain 380, 220, 160, 103 and 380 g/kg Ca, respectively (NRC, 1994). Limestone is the most commonly used source of Ca in poultry diets. Availability of Ca from inorganic sources varies widely depending on their origin and particle size (McNaughton *et al*., 1974; Hilman *et al*., 1976; Zhang and Coon, 1997a; Saunders-Blades *et al*., 2009). A number of studies have investigated the effect of particle size of limestone on egg production and shell quality in laying hens, but corresponding studies in broilers are scant.

The absorption and utilisation of Ca is controlled by hormonal factors, which regulate plasma and tissue Ca concentrations by increasing or decreasing its absorption. The hormones involved are parathyroid hormone, vitamin D_3 and calcitonin. When plasma Ca concentrations are low, parathyroid hormone and vitamin D_3 increase the intestinal absorption of Ca. On the other hand, if plasma Ca concentration is too high, calcitonin tends to reduce the plasma concentration of Ca by reducing intestinal absorption, reducing resorption of Ca from bones and increasing the secretion of Ca by the kidneys (Veum, 2010). Most of the Ca in poultry diets is provided by inorganic

sources as plant-based feed ingredients generally contain low amounts of Ca (Kiarie and Nyachoti, 2010). The contribution of organic Ca to total Ca in a typical maize soybean meal based broiler starter diet, which contains 10g/kg Ca, is around 20%. Thus the contribution of organic Ca resources to poultry diets cannot be ignored, but a relevant issue is that no published values are available for the availability of Ca from these sources.

In recent years, there is increasing attention of the P nutrition of intensively-reared animals due to excess P excretion into the environment and the skyrocketing price of inorganic phosphate supplements. Currently three measurements, namely non-phytate P, available P and retainable P are used to describe the availability of P and it is accepted that a well-defined criterion is required to enable greater efficiency of P utilisation. It is also recognised that, of the different possibilities, digestible P may be the most suitable option to assess P availability to poultry (Rodehutscord, 2009). Although there is increasing interest in the industry moving to a digestible P system in feed formulations, only limited published data are available on the digestible P contents in feed ingredients for poultry (Dilger and Adeola, 2006a; Mutucumarana *et al*., 2013; 2014a,b.: 2015a,b; Mutucumarana and Ravindran, 2016).

The negative effect of high dietary Ca concentrations on P availability and the need to maintain proper ratios between Ca and P have been known for a long time. It is well known that the availability of P is lowered at high dietary Ca concentrations (Ballam *et al*., 1984; Tamim and Angel, 2003; Plumstead *et al*., 2008). Calcium also reduces the availability of other minerals such as zinc, magnesium and iron (Shafey *et al*., 1991), and may reduce the energy value of diets through chelation of lipids (Edwards *et al*., 1960). On the other hand, deficiency of Ca is also not desirable as it interferes with skeletal integrity and growth performance of the birds and can cause rickets, tibial dyschondroplasia and high mortality rate in poultry.

If the industry is moving towards a digestible P system, then owing to the negative impact of high dietary Ca on P availability, the development of a digestible Ca system is urgently needed to ensure that the Ca and P requirements of birds are precisely met. Currently there are no published values available on the digestibility of Ca in feed ingredients. It is generally assumed that Ca in feed ingredients is 100% digestible, but this needs to be confirmed.

There is no proper methodology for the determination of Ca digestibility in feedstuffs for poultry. However, it is possible to use the methods that are used for the measurement of amino acid digestibility for poultry. Three methods are used for the determination of amino acid digestibility - direct method, substitution method and regression method.

To determine the ileal digestibility of nutrients, ileal digesta samples are collected from the lower ileum. Indigestible markers are added in the diets, and diets and digesta samples are analysed for concentration of nutrients and markers to determine the digestibility. In the direct method, the test ingredient represents the only a source of specific nutrient in the diet. In the substitution or difference method, the basal and test diets are formulated; basal diet provides the sole essay nutrient while test diet comprises a mixture of both basal diet and test ingredient (usually 50:50). The digestibility of the desired nutrient in the test ingredients is measured by difference between the two digestibility measures and the concentration of specific nutrient in the test diet (Lemme *et al*., 2004).

Regression method is based on establishing a linear relationship between nutrient output in ileal digesta and their dietary inputs, expressed in g/kg of dry matter of digesta and diet, respectively. In this method, diets with graded concentrations of the specific nutrient from the specific assay ingredient are formulated. Theoretically, the digestibility estimates determined by the regression method are automatically corrected for endogenous losses and represent true digestibility values. Some studies have used the regression method for the determination of P digestibility in poultry (Dilger and Adeola, 2006a; Mutucumarana *et al*., 2013; 2014a,b; 2015a,b).

The digestibility values determined by using the direct and difference methods are apparent values and need to be corrected for endogenous losses. Thus, to calculate true Ca digestibility, endogenous Ca losses needs to be determined. Only one published study is available on the determination of total tract endogenous Ca losses in broiler chickens (Cowieson *et al*., 2004). This study determined the endogenous Ca losses by collecting the excreta of broiler chickens given an aqueous glucose solution after 24 hours of fasting. The other possible way to measure endogenous Ca losses is by feeding a Ca-free diet.

There are several animal and dietary factors that may affect the digestibility of Ca. Dietary factors include dietary P concentrations and supplementation of vitamin D_3, phytase or organic acids. Animal factors of importance include age, sex and class of animal. These factors should also be considered during the estimation of Ca digestibility.

So the main objectives of this thesis were

1. To develop a methodology for the determination of true ileal Ca digestibility in feed ingredients for broilers.
2. To evaluate the factors affecting Ca digestibility e.g., dietary Ca, P and Ca:non-phytate P ratio.
3. To determine the effect of particle size and *in vitro* solubility on true Ca digestibility of limestone and oyster shell.

CHAPTER 2

Literature review

2.1. Importance of calcium in poultry diets

Calcium (Ca) is the most important mineral present in the animal body. Calcium, phosphorus (P) and vitamin D_3 are the key nutrients required for skeletal development. Almost 99% of body Ca is present in the skeleton and the balance (1%) is involved in a large array of metabolic and physiological functions in the body. It plays important roles *inter alia* in blood clotting, muscle contraction, transmission of nerve impulses, hormone secretion, and regulation of heart function (Coon *et al*., 2002).

For proper growth and development of chickens, it is necessary to provide adequate concentrations of Ca in their diets. These dietary concentrations should support maximum growth performance without interfering with the availability and utilisation of other minerals. Recommended dietary Ca concentrations for broiler chickens are 10, 9 and 8 g/kg of diets for the starter, grower and finisher phases, respectively (NRC, 1994). Plant-based feed ingredients are deficient in Ca and supplemental organic and inorganic Ca sources have to be used in poultry diets to meet these requirements. Inorganic Ca sources provide most of the Ca in poultry diets. Major inorganic Ca sources used in poultry diets are limestone, dicalcium phosphate and mono-dicalcium phosphate, while the important animal based organic Ca sources are meat and bone meal and oyster shell. A deficiency of Ca in the diets of the chicken can cause bone abnormalities, disturbance in normal body functions, and impair growth performance. On the other hand, high dietary Ca can cause reduction in availability of several important nutrients such as P, manganese, zinc, lipids and energy.

2.2. Calcium sources for poultry

Commonly used inorganic and organic Ca sources for poultry diets are limestone, dicalcium phosphate, monocalcium phosphate; and meat and bone meal and oyster shell, respectively. These sources contain 380, 220, 160, 103 and 380 g/kg Ca, respectively (NRC, 1994). However, the Ca contents are reported to vary by origin of the sample. Wilkinson *et al*. (2013a) analysed the limestone sources used in the

Australian poultry industry and reported that the Ca concentration ranged from 392 to 411 g/kg. However, variation in Ca contents was greater in organic Ca sources. Sulabo and Stein (2013) reported a range of Ca in meat and bone meal samples from 51 to 110g/kg. Poultry diets are currently formulated on the basis of total Ca contents, but it must be recognised that the availability of Ca may vary between sources. Ajakaiye *et al*. (2003a) evaluated the apparent Ca availability of different Ca sources by total collection method and reported that the availability was 25.7, 35.2, 43.1, 26.7, 28.1 and 30.2% in calcium carbonate, bivalve shell, periwinkle shell, oyster shell, marble dust and snail shell, respectively in broilers at 2 weeks of age. These findings highlight the need to measure and utilise the digestibility and availability of Ca from different source so that exact Ca requirements can be met in the broiler's diet. The concentration of Ca in common feed ingredients used in poultry feed formulations is summarised in Table 2.1.

2.3. Need for calcium digestibility measurement

Skyrocketing prices of inorganic phosphate sources and concerns over environmental P pollution has attracted the attention of researchers to work on P digestibility of feed ingredients. In this context, determination of Ca digestibility also becomes relevant because incorrect Ca to P ratios will influence the availability of P and bird performance. Excess dietary Ca is known to form Ca-phytate complexes in the intestinal environment and lower the availability of P (Driver *et al*., 2005; Selle *et al*., 2009; Walk, 2016). Reductions in the availability of phytate P and digestibility of P has also been reported in studies where high dietary Ca concentrations were fed (Tamim and Angel, 2003; Tamim *et al*., 2004; Plumstead *et al*., 2008).

Plumstead *et al*. (2008) observed that apparent ileal digestibility of total P reduced from 64.3 to 50.5% and that of phytate P from 20.2 to 5.9% when dietary Ca concentration increased from 4.7 to 11.6g /kg of diet, respectively. Excess dietary Ca is known to causes reduction in the availability of P, iron, magnesium and zinc and also increases the digesta pH in the digestive tract (Shafey *et al*., 1991). It has also been shown to reduce the availability of lipids and energy (Edwards *et al*., 1960). On the other hand, Ca deficiency can cause poor growth performance, rickets and tibial dyschondroplasia in broiler chickens. To avoid these negative effects, it is necessary to have knowledge of Ca digestibility from different sources.

Table 2.1. Calcium concentration in common feedstuffs used in poultry diets[1]

Ingredients	Ca content (g/kg)
Cereals	
Maize	0.2
Wheat	0.5
Rice	0.8
Sorghum	0.4
Barley	0.3
Cereal By-products	
Wheat bran	1.4
Corn gluten feed	4.0
Corn gluten 60%	
Rice bran	7.0
Vegetable protein sources	
Soybean meal	2.7-2.9
Sunflower meal	2.1
Rapeseed/canola meal	6.8
Animal Protein sources	
Fish meal 62% CP	51.1
Fish meal 65% CP	37.3
Fish meal 70% CP	22.9
Blood meal	5.0
Feather meal	3.3
Meat and bone meal	103.0
Poultry by-product meal	30.0
Inorganic Sources	
Limestone	380
Dicalcium phosphate	220
Monocalcium phosphate	160
Oyster shell	380

[1] Source: NRC (1994).

2.4. Absorption of calcium

Calcium is present in three major forms in the body of chicken; almost 99% of Ca in present in bones in the form of hydroxyapatite, with the remaining 1% is either present in intracellular or extracellular spaces. Extracellular Ca is only 0.1% of the total body Ca and present in three different forms, namely ionised Ca, Ca bound to proteins, and Ca bound to anions. Of these, ionised Ca is the only physiologically active form of Ca in birds (Coon *et al*., 2002).

Intestinal Ca absorption, in ionic form (Ca^{+2}), is an important process for the maintenance of Ca homeostasis in the body. In poultry, Ca is reported to be absorbed mainly in the duodenum and jejunum (Hurwitz and Bar, 1969, 1970; Van der Klis *et al*., 1990; Larbier *et al*., 1994). However, Ca absorption in different intestinal segments varies with age, physiological stage and dietary Ca concentration. Calcium absorption in duodenum and jejunum of three-week old broilers fed 11.7 g/kg of Ca was 46 and 47%, respectively. Calcium absorption in hens during laying period fed 38.3 g/kg Ca has been reported to be 25, 46, 6 and 7% in the duodenum, upper jejunum, lower jejunum and upper ileum, respectively (Hurwitz *et al*., 1973). The corresponding values during non-laying period were 19, 18, 23 and 0.8% of total Ca consumed, respectively.

2.4.1. Mechanisms of calcium absorption

Ca is absorbed from the intestine by two mechanisms, namely active or transcellular which occurs through mucosal cells and passive or paracellular absorption which occurs between mucosal cells. Passive absorption is dependent on a concentration-gradient and occurs throughout the intestinal tract, whereas active absorption occurs mainly in the duodenum and upper jejunum and plays only a minor role in distal jejunum and ileum (Bronner, 1987).

2.4.1.1. Active absorption

Active Ca absorption is a three step process consisting of entry into the epithelial cells from the lumen; transit through the cytosol from the apical to basolateral pole and extrusion from the cell, across the basolateral membrane, to the vascular supply in the lamina propria (Fullmer, 1992). Active absorption is regulated by vitamin D_3 and Ca binding proteins (Bronner, 1987; 1992). Dietary Ca concentrations may also affect the active Ca absorption. An increase in dietary Ca intake causes a down-regulation of

active Ca absorption while a decrease in Ca intake results in an up-regulation in rats (Pansu *et al*., 1981).

The sequence of events involved in the active absorption of Ca is illustrated in Figure 2.1. Vitamin D_3 plays an important role in this mechanism and regulates all three steps of absorption by inducing changes in the structure and function of the intestinal epithelium (Perez *et al*., 2008). In the first step, vitamin D_3 stimulates the entry of Ca from the lumen into the mucosal cells through epithelial Ca channels called transient receptors potential of vanilloid family (TRPV 5 and 6). Secondly, it increases the synthesis of Ca binding proteins which binds with Ca ions in the cytosol and thirdly it causes the extrusion of Ca from mucosal cells to intestinal spaces through the basolateral membrane by using plasma membrane Ca ATPase or simply by sodium and Ca ion exchange (Hoenderop *et al*., 2005).

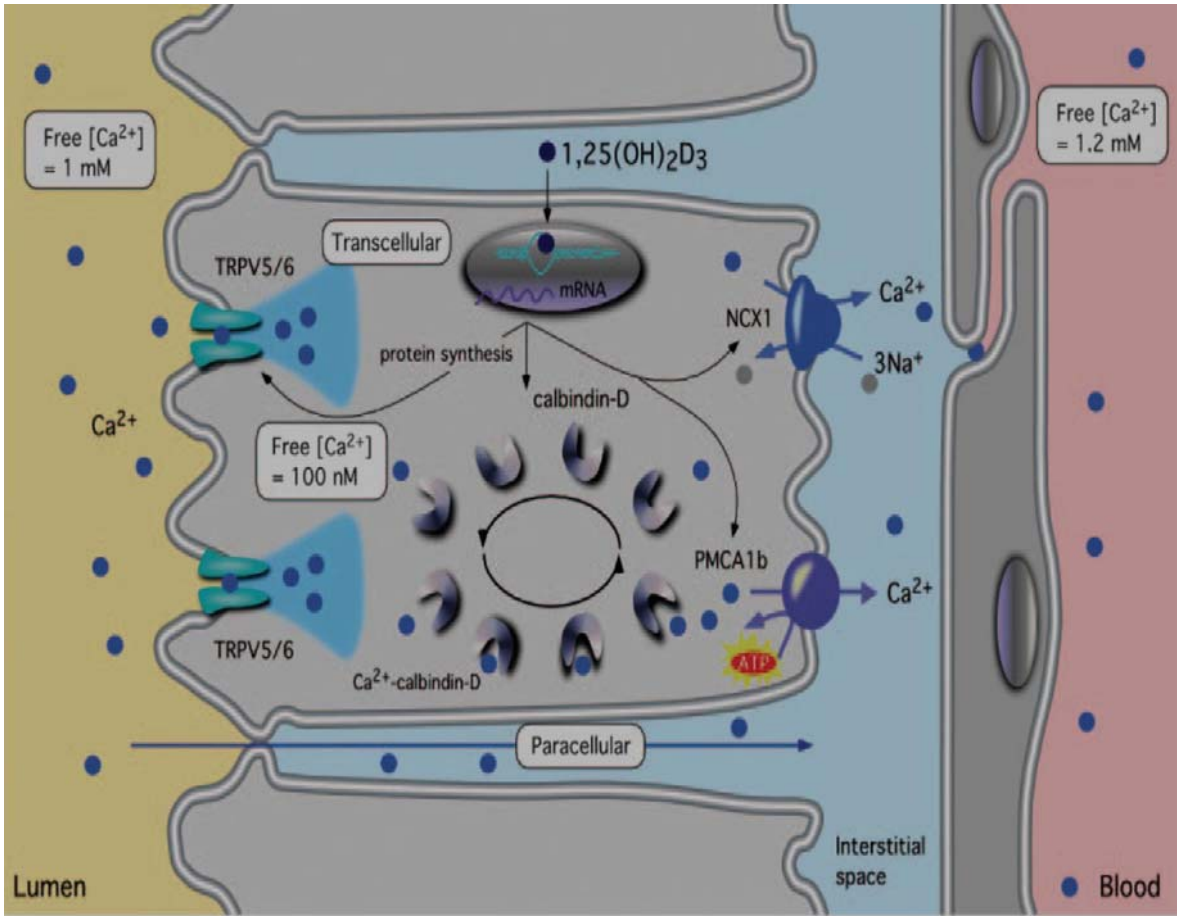

Figure 2.1. Steps involved in the active and passive absorption of Ca (Hoenderop *et al*., 2005)

Almost 20% of Ca extrusion from the cells occurs through sodium-Ca ion exchange, while the remainder is through plasma membrane Ca ATPase (Perez *et al*., 2008). Extrusion occurs against the electrochemical gradient, while overall movement of Ca is downhill from higher concentration in the intestinal lumen to lower concentration in the body fluid. Extrusion is affected mainly by the Ca ATPase, while contribution of sodium/calcium ion exchange is less important (Bronner, 1992).

The effect of vitamin D_3 on different steps involved in the active Ca absorption is shown in Table 2.2. Vitamin D_3 is reported to enhance the Ca absorption from the intestine at every step; it increases the entry of Ca at the brush border by 20-30%, binding to cellular sites by 100%, intracellular movement up to 100 fold and Ca/Mg-ATPase activity by two to three folds (Bronner *et al*., 1987).

Table 2.2. Effect of vitamin D3 on Ca absorption[1]

Step	Path or structure	Effect of vitamin D	Mechanism
Entry across brush border	Down the electrochemical gradient of Ca^{2+} via channel or carrier	Enhance by 20-30%	Possibly by plasma membrane-bound CaBP (Mr = 15,000)
Binding to fixed cellular sites (buffering)	Golgi apparatus, rough endoplasmic reticulum, mitochondria	Enhance by 100%	Unknown
Intracellular movement	Diffusion	Facilitates diffusion up to 100-fold	Biosynthesis of soluble CaBP (Mr = 8,000) which act as Ca ferry
Extrusion	Pumping against a gradient by the Ca/Mg-ATPase	Increase CA/Mg-ATPase activity two- to threefold	Unknown

[1]Adapted from Bronner *et al*. (1987).

Perez *et al*. (2008) reported that a dietary deficiency of vitamin D_3 decreases the expression and activity of plasma membrane Ca ATPase, causing reduction in Ca extrusion from the cells. While the initiation of intestinal Ca absorption by vitamin D_3

appears to be due to an increase in Ca efflux rate from the basolateral membrane rather than its influx at brush border membrane, indicating that the Ca extrusion mechanism in the mucosal cells starts first followed by entry into the cells (Takito *et al*., 1990).

Calcium binding proteins are also important for intestinal Ca absorption by active absorption. Calcium binding proteins are not only involved in the movement of Ca from the apical to basolateral pole, but also help in the buffering of Ca ions to protect against toxic Ca concentrations during high influx of Ca (Perez *et al*., 2008).

About 90% of cytosolic Ca is bound with proteins, while only 10% is free, as synthesis of Ca binding proteins depends upon vitamin D_3. Thus the deficiency of vitamin D_3 can cause a 90% reduction in Ca entry due to a reduction in the synthesis of Ca binding proteins (Bronner, 2003). Synthesis of Ca binding proteins is directly proportional to the active metabolite of vitamin D_3 {1,25-$(OH)_2D_3$}, and this metabolite is enzymatically synthesised in the kidney as a result of low plasma Ca concentrations. Bar and Wasserman (1973) reported an increase in the concentration of duodenal Ca binding proteins and Ca absorption in chickens supplemented with vitamin D_3.

Dietary intake of Ca affects the active Ca absorption in the duodenum. High dietary Ca intake is reported to reduce the binding protein content and Ca absorption in the duodenum (Morrissey and Wasserman, 1971). On the other hand, low dietary Ca intakes enhance Ca uptake and Ca extrusion activities by the cells through plasma membrane Ca ATPase or sodium/calcium ion exchange (Centeno *et al*., 2004).

2.4.1.2. Passive absorption

Passive absorption contributes a significant proportion to the total amount of Ca absorbed because it occurs throughout the intestinal tract, and secondly, the retention time of digesta is longer in the jejunum where the passive mechanism is dominant (Adedokun and Adeola, 2013). During the passive absorption, Ca moves through the micro-spaces between adjacent enterocytes of the epithelial membrane from high concentration in the intestinal lumen to lower concentration in the intestinal space (Wasserman, 2004). As shown in Figure 2.1, Ca enters through the tight junctions between the cells, passes through the intermediate junction and then to the blood from basolateral region (Hoenderop *et al*., 2005).

The structure of tight and intermediate junctions can limit absorption of Ca. However, the space between the two junctions can be manipulated to increase Ca absorption; potential modifications include use of substances such as lactose, medium chain triglyceraldehydes and amino acids (L-lysine and L-arginine) (Bronner, 1987).

Increasing dietary Ca concentrations increase absorption through the passive pathway while reducing its absorption through active pathway because of the down regulation of binding proteins (Perez *et al*., 2008). However, although high Ca intake increases the total amount of Ca absorbed, it reduces the percentage of Ca absorption (Hurwitz and Bar, 1969). Proportions of Ca absorbed by these two mechanisms vary depending on the dietary intake. At low intakes, active Ca absorption is up regulated and is more dominant, while at high Ca intakes passive Ca absorption is dominant (Bronner, 2003).

2.4.2. Hormonal regulation of calcium metabolism

Ca metabolism is controlled by three hormones, namely parathyroid hormone (PTH), calcitonin (CT), and 1, 25-$(OH)_2D_3$. Secretion of parathyroid hormone is regulated by ionic Ca concentrations in the extracellular fluid. Decreases in plasma Ca concentrations trigger the parathyroid gland and increases the secretion of PTH, resulting in increased reabsorption of Ca from the kidney and resorption of Ca from the bones (Taylor and Dacke, 1984). This action of PTH ultimately leads to an increase in body Ca concentrations.

High plasma Ca concentrations suppress the secretion of PTH and stimulate the ultimobranchial glands to secrete CT. Calcitonin helps to reduce plasma Ca concentrations by reducing its resorption from the bones and reabsorption from the kidneys.

2.4.2.1. Regulation of hypocalcaemia

During hypocalcaemia, there is increased secretion of PTH which increases the plasma Ca concentrations by the following mechanisms (Figure 2.2)

1. action on the kidney to reduce Ca excretion, by increasing 1α-hydroxylase and reducing 24-hydroxylase secretion which enhances 1,25-$(OH)_2$-D_3 production and increases Ca resorption from the kidneys which ultimately causes an increase in plasma Ca concentration,

2. increasing the production of 1,25-$(OH)_2$-D_3 which increase the intestinal absorption of Ca
3. increasing the Ca resorption from the bones.

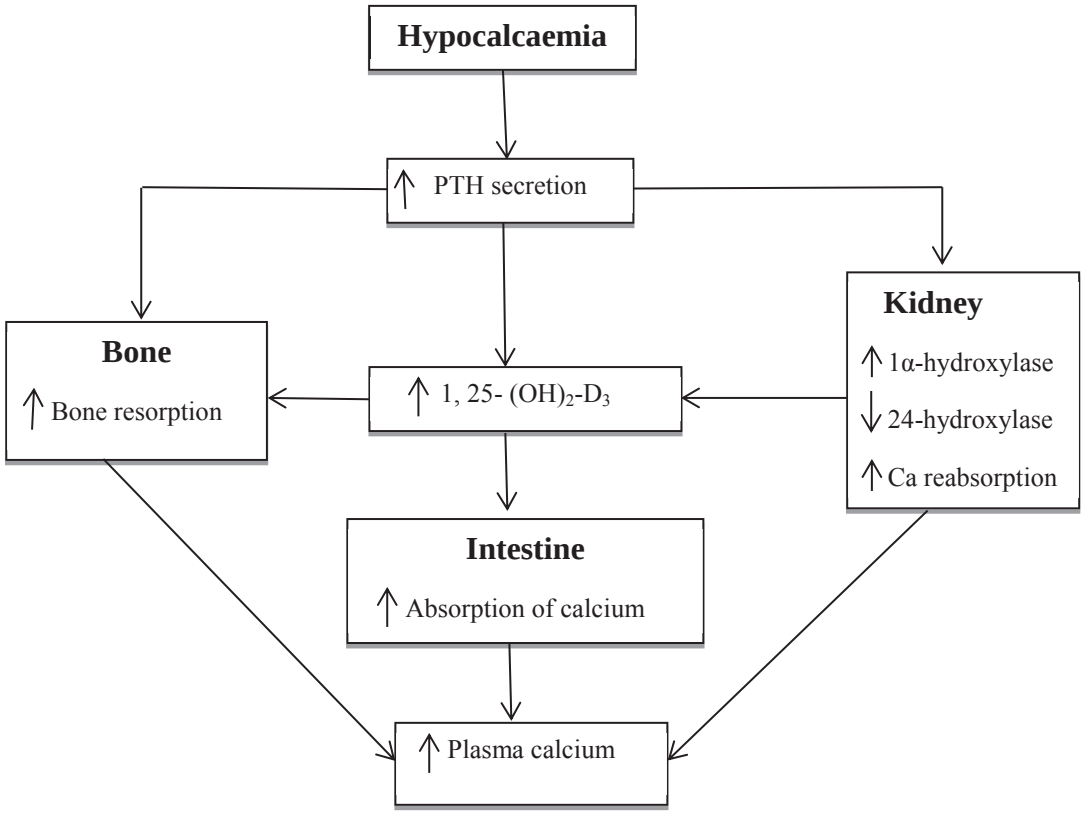

Figure 2.2. Regulation of hypocalcaemia (de Matos, 2008).

2.4.2.2. Regulation of hypercalcaemia

Hypercalcaemia is regulated by both PTH and CT. During hypercalcaemia, secretion of PTH is reduced which lowers plasma Ca concentrations by decreasing reabsorption from the kidney and absorption from the intestine. On the other hand, higher plasma Ca concentrations also stimulate the secretion of CT which reduces Ca resorption from bones which leads to low plasma Ca concentrations.

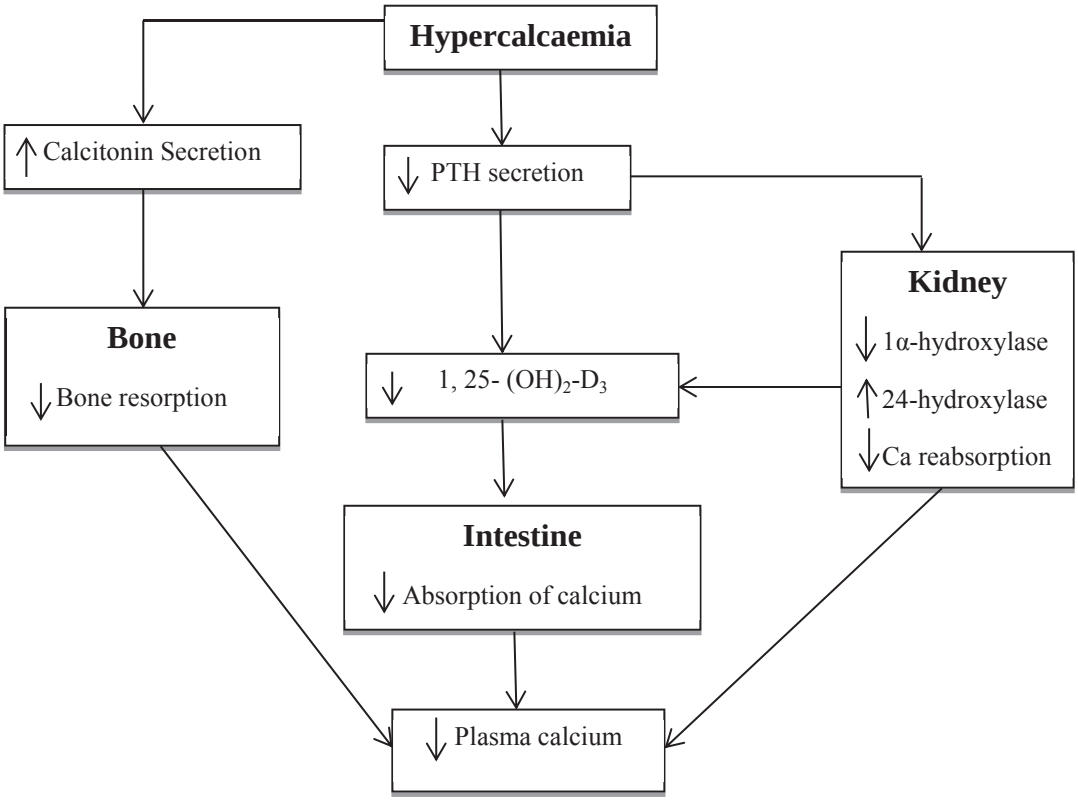

Figure 2.3. Regulation of hypercalcaemia (de Matos, 2008)

2.5. Calcium availability

Although no published data is available regarding the digestible Ca contents of feedstuffs for poultry, the term biological availability (bioavailability) has been used extensively for the measurement of Ca availability for chickens. Bioavailability of nutrients is a measure of the degree to which a nutrient source can support the physiological processes of an animal. Calcium availability from feedstuffs is considered to be very high, however no nutrient can be absorbed and utilised completely as some of it is lost during normal digestive and metabolic processes (Peeler, 1972).

Different approaches have been used to determine the availability of Ca and P. These approaches are divided into three main categories; namely qualitative measurements using the bone parameters and growth as parameters of Ca availability, quantitative measurement using retention and *in vitro* methods by using solubility (Shastak and Rodehutscord, 2013).

In the qualitative approach, the slope ratio method has been widely used to compare the availability of Ca from different ingredients. In these studies, bone criteria (bone ash, bone breaking strength, bone density) or growth responses are plotted against the dietary Ca intake and compared with standard source which is presumed to be 100% available (Kiarie and Nyochoti, 2010). Calcium carbonate has been used in studies as the standard source to determine the relative bioavailability of Ca from different sources (Hurwtiz and Rand, 1965; Blair *et al*., 1965). A summary of the relative bioavailability of Ca from different sources is shown in Table 2.3.

Retention is measured by total excreta collection, while the ileal digestibility is assessed by collecting the ileal digesta and using indigestible marker ratios. Compared to the slope ratio and retention methods, ileal digestibility does not consider the post-absorptive utilisation of Ca. True Ca digestibility can be measured by correcting the apparent digestibility for endogenous Ca losses. *In vitro* measurement of Ca bioavailability is achieved by determining the solubility of the Ca source. *In vitro* methods to assess Ca availability are time saving and less expensive as compared to the other two approaches. Cheng and Coon (1990a) have proposed different methodologies to determine the solubility of limestone and have shown that the *in vitro* solubility of limestone decreased with increasing particle size. *In vitro* solubility has been reported to be inversely related to *in vivo* solubility. Zhang and Coon (1997a) reported that larger particles with lower *in vitro* solubility (30-50%) stayed longer in the gizzard of hens and increased the *in vivo* solubility to 94%.

2.6. Factors affecting calcium availability

A number of factors influence the absorption of Ca along the intestinal tract and these can be broadly divided into two categories, animal and dietary factors. Animal factors includes age, sex and strain of the birds while dietary factors include dietary Ca concentration, Ca to P ratio, dietary P, dietary fat, supplementation of vitamin D_3, organic acid, enzymes (phytase) and the source and particle size of Ca.

Table 2.3. Relative bioavailability of calcium from different sources for young chicks[1]

Calcium source	Relative bioavailability of calcium (%)						
	Motzok *et al.*(1965)	Hurwitz and Rand (1965)	Dilworth and Day (1964)	Blair *et al.* (1965)	Stillmak and Sunde (1971)	Reid and Weber (1976)	Augspurger and Baker (2004)
Calcium Carbonate[2]	100	100	100	100	100	100	100
Limestone	-	-	-	102	-	73-109	77
Oyster shell	-	-	-	-	-	87-100	95
Dicalcium phosphate	100	-	-	113	-	-	-
Bone meal	-	-	-	109	-	-	-
Soft phosphate	70	-	68	-	-	-	-
Tricalcium phosphate	-	-	-	115	-	-	-
Gypsum	-	90	-	-	66	-	-

[1] Adapted from Peeler (1972).

[2] Considered as the standard source with 100% availability.

2.6.1. Dietary factors

2.6.1.1. Effect of dietary calcium concentration and calcium to phosphorus ratio

Broilers and layers have specific Ca requirements depending on their growth or production stage. Calcium requirements of broiler chickens are 10, 9 and 8 g/kg of diet for the starter, grower and finisher stages (NRC, 1994). High dietary Ca reduces the availability of both Ca and P by forming insoluble Ca phosphate in the digestive tract (Shafey *et al*., 1990).

Hurwitz and Bar (1969) reported that an increase in dietary Ca reduced absorption in laying hens. Calcium absorption was determined to be 80.8, 76.3 and 62.0% in hens fed diets with 5.9, 17.6 and 39.4 g/kg Ca, respectively. Sebastian *et al*. (1996a) similarly observed reductions in Ca retention in broilers with increasing dietary Ca concentrations. Calcium retention was 65.9, 49.0 and 43.5% on diets with 6.0, 10.0 and 12.5 g/kg of dietary Ca, respectively. Pintar *et al*. (2005) also found that Ca retention in broilers was decreased when dietary Ca concentrations increased from 6.0 to 10 g/kg of diet. The above findings are also consistent with those of Plumstead *et al*. (2008), where the apparent ileal digestibility of Ca decreased from 46.8 to 38.6% when dietary Ca concentration increased from 4.7 to 11.6 g/kg in broiler diets. Walk *et al*. (2012a) observed that the apparent ileal Ca digestibility of a maize-soybean meal based diet for broiler chickens reduced from 66% to 52% when dietary Ca was increased from 6.4 to 10.03 g/kg of diet.

Ca to P ratio is also important for absorption and utilisation of both minerals. NRC (1994) recommends a Ca:non-phytate phosphorus ratio of about 2:1 (weight to weight basis) as favourable for broilers. Widening or narrowing of this ratio has been reported to affect the Ca absorption and retention (Hurwitz and Bar, 1969; Sebastian *et al*., 1996a, b; Pintar *et al*., 2005; Plumstead *et al*., 2008). A wider Ca:P ratio represents either an increase in dietary Ca concentration or decrease in dietary P. Sebastian *et al*. (1996b) reported that Ca retention was reduced from 40.7 to 31.7% when the Ca:total P ratio increased from 2:1 to 2.6:1 in broiler diets. Similar results were observed in a subsequent study, where Ca retention was reduced from 65.9 to 49 and 43.5% when the Ca:total P ratio was increased from 1:1 to 1.74:1 and 2.22:1, respectively (Sebastian *et al*., 1996a). Moreover, reduction in the retention of Ca was observed from 58.4% to

42.6% when the dietary Ca:total P ratio was increased from 1.1:1 to 2:1 in broiler diets (Qian *et al*., 1997).

In contrast, Tamim and Angel (2003) reported an increase in Ca absorption from 44.7 to 52.3% when dietary Ca concentration was increased from 1.8 to 6.8g/kg of broiler diets. The possible reason for this finding may be that the Ca in the 1.8g/kg diet originated only from organic sources. It would have been bound to oxalates and phytate and thus less likely to be absorbed. Another possible reason could be that even the higher Ca concentration was much below the Ca requirements of the birds.

2.6.1.2. Effect of dietary phosphorus concentration

Balance between Ca and P is critical for the effective utilisation of these minerals for growth and skeletal development. Recommended dietary available P requirements are 5.0, 4.5 and 4.2 g/kg diet for starter, grower and finisher broiler chickens, respectively (Ross, 2007). Changes in the concentration of dietary P will influence the availability of Ca. On the other hand, the concentration of phytate P is also important, because it forms complexes with Ca reducing the availability of Ca and P (Selle *et al*., 2009). Adverse effects of high dietary P concentrations on Ca availability have been reported previously (Viveros *et al*., 2002; Plumstead *et al*., 2008).

Viveros *et al*. (2002) reported a reduction in Ca retention from 52.8 to 37.0% in broilers at 3 weeks of age when dietary non-phytate P concentration was decreased from 4.5 to 2.2g/kg of diet. This reduction was caused by increase in phytate P concentration, relative to non-phytate P, in the diets. Similar observations were made by Plumstead *et al*. (2008) that apparent Ca retention determined by excreta collection method was higher (59.4 vs. 47.9%) in broilers at 16-17 days of age fed diets containing soybean meal with a low phytate concentration (1.0 g/kg of diet) as compared to soybean meal with high phytate concentration (2.8 g/kg of diet).

Some researchers, on the other hand have reported contradictory results. Bar and Wasserman (1973) reported that a low P diet (10.5 g/kg Ca, 3.6 g/kg P) significantly improved the absorption of Ca from 33.1 to 49.5% and increased the concentration of Ca binding proteins from 81.6 μg/mg to 189.2 μg/mg as compared to a balanced diet (10.5 g Ca kg, 7.5 g/kg P). It was speculated that the increase in the concentration of Ca binding proteins ultimately increased the Ca absorption in the intestine by providing more space for Ca attachment and movement across the intestine.

2.6.1.3. Effect of phytase supplementation

Most of the P in plants is present in the form of phytate P. Phytic acid or phytin form complexes with other minerals such as Ca, manganese, zinc, copper, nickel, iron and cobalt (Angel *et al*., 2002), which reduces the availability of these minerals and phytate P, reducing the digestibility of protein and amino acids (Ravindran *et al*., 2000) and AME (Ravindran *et al*., 2006). Phytase enzymes are important, because they enhance the breakdown of these complexes and increase the availability of these minerals in broiler chickens. These phytases may come from different sources as they are present in feed ingredients such as wheat and barley; can be produced by microorganism present in the gastro intestinal tract; and may be exogenous and added in the feed (Angel *et al*., 2002).

Improvements in Ca availability by phytase supplementation have been extensively studied and reported (Sebastian *et al*., 1996b; Qian *et al*. 1997; Zanini and Sazzad, 1999; Viveros *et al*., 2002; Brenes *et al*., 2003: Ravindran *et al*., 2006; Ravindran *et al*., 2008; Chung *et al*., 2013). Sebastian *et al*. (1996b) demonstrated a 12.2% increase in Ca retention in broilers when phytase (600 units/kg of diet) was added in their diets. In another study, Ca retention was reported to improve from 965 to 1123 mg/bird/day when 500 units/kg of phytase was added to the diets of broiler chickens at 21 days of age (Zanini and Sazzad, 1999).

These findings are also supported by Viveros *et al*. (2002), indicating that the supplementation of phytase (500 units/kg of diet) improved the Ca retention in broilers at 3 weeks of age from 39.6 to 49.2% at 3.5 g/kg dietary non-phytate P concentrations while this improvement was 37.0 to 44.3% at 2.2 g/kg of non-phytate P concentration. Addition of phytase has been reported to enhance the Ca retention by 9% in broiler chickens at 18 days of age (Brenes *et al*., 2003). Calcium retention was 54% without phytase and 61% with supplementation of 600 phytase units/kg of diet.

Ravindran *et al*. (2006) offered four diets with phytase concentration of 0, 500, 750 and 1000 FTU/kg of diet to broiler chickens at 21 days of age and found that the apparent Ca digestibility values were 35.2, 38.1, 38.0 and 40.6%, respectively. In another study, apparent ileal availability of Ca was reported to improve from 25.9 to 32.9% by supplementation of 500 units of phytase/kg of diet to broiler chickens at 21 days (Ravindran *et al*., 2008). Chung *et al*. (2013) found that Ca retention was 50.4,

51.5 and 54.7% on diets without phytase, with 500 U/kg and 1000 U/kg of phytase, respectively, in broiler chickens from 18-21 days of age.

However, efficacy of phytase varies depending on the commercial source and its inclusion level. These variations sometimes give contradictory results regarding their effect on Ca availability. Pintar *et al*. (2005) reported that there was no effect of phytase supplementation (500 and 1000 units/kg of diet) on Ca excretion and retention in broiler chickens at two and three weeks of age. Similarly Chung *et al*. (2013) observed that there were differences between commercial phytases on their effect on Ca retention in broiler chickens. Walk *et al*. (2012a) found that supplementation of phytase (500 and 5000 units) had no effect on the Ca digestibility of maize-soybean meal based diet containing limestone and DCP as Ca sources for broiler chickens.

2.6.1.4. Effect of vitamin D_3 supplementation

Vitamin D exists in two major forms, namely ergocalciferol (D_2) and cholecalciferol (D_3). Vitamin D_3 ($1,25(OH)_2D_3$) influence the absorption of Ca from the intestine and its homeostasis in the body (Proszkowiec-Weglarz and Angel, 2013). Calcium absorption in the duodenum through active pathway is regulated by vitamin D_3 (Bronner, 1987; Bronner, 1992: Fullmer, 1992). During active Ca absorption, vitamin D_3 enhances the Ca entry into the cells by 20-40%, increases the production of Ca binding proteins and extrusion of Ca from the cells by 200-300% through plasma membrane Ca ATP-ase (Bronner, 1992).

On the other hand, deficiency of vitamin D_3 decreases the expression of plasma membrane ATP-ase and thus reduces Ca extrusion from the cells, which ultimately reduces the Ca absorption (Perez *et al*., 2008). Vitamin D_3 is also important for Ca reabsorption through kidneys. Qian *et al*. (1997) reported that Ca retention was 58.4, 60.5 and 63.4% when vitamin D_3 was supplemented at a rate of 66, 660 and 6600 μg/kg of diet, respectively, at a Ca:total P ratio of 1.1:1. The corresponding values were 42.6, 44.4 and 48.7%, respectively, when the Ca:total P ratio was 2.0:1.

2.6.1.5. Effect of organic acid supplementation

High intestinal pH reduces the solubility of minerals which causes a reduction in their availability (Shafey *et al*., 1991). Supplementation of organic acids lowers the intestinal pH which can cause an increase solubility and availability of minerals. Improvement in

Ca availability by supplementation of different organic acids has been reported in various studies (Shafey *et al*., 1991; Brenes *et al*., 2003; Islam *et al*., 2012).

Brenes *et al*. (2003) reported that citric acid supplementation of 20g/kg enhanced the plasma Ca concentrations and Ca retention by 10% and 3%, respectively, in broiler chickens. Findings of Islam *et al*. (2012) also support the outcomes of the above study. These researchers reported that the apparent total tract Ca digestibility in broiler chickens were 43, 48, 49 and 45% on diets supplemented with 0, 2.5, 7.5 and 12.5 g/kg of citric acid, respectively.

2.6.1.6. Effect of fat supplementation

High dietary fat forms insoluble soaps when combined with Ca in the intestine which reduces intestinal Ca absorption. Calcium absorption in the intestine is affected by dietary fat concentration and type (saturated or unsaturated) (Van der Klis, 1993). Supplementation of saturated fats has more adverse effect on Ca absorption as compared to unsaturated fatty acids (Atteh *et al*., 1989). The possible reason may be that the soaps formed with unsaturated fatty acids are absorbed from the intestine while soaps made from saturated fatty acids are not absorbable. The adverse effect of excessive dietary fat and fat type has been investigated in broiler chickens (Whitehead *et al*., 1971, 1972; Atteh *et al*., 1989).

Whitehead *et al*. (1971) investigated the effect of different fat sources with various dietary concentrations in two-week old broilers. Calcium retention was 38, 28, 24, 22 and 14% on diets without fat, and with 50 g maize oil, 100 g maize oil, 100 g tallow and 100 g lard/kg of diets, respectively. The lowest Ca retention was determined with the saturated fat sources, lard and tallow. In a follow-up study, Ca retention was approximately 55, 51 and 50% for dietary tallow concentrations of 0, 5 and 10 g/kg, respectively (Whitehead *et al*., 1972). Atteh *et al*. (1989), however, observed that Ca retention was reduced from 60 to 53% when an unsaturated dietary fat source (soybean oil) was replaced by a saturated fat (Animal and vegetable fat blend) in three-week old broilers. This reduction in Ca retention was reduced from 61 to 53% with increase in dietary fat concentration from 50 to 100 g/kg of diet irrespective of fat type.

2.6.1.7. Effect of calcium source and particle size

As cereals and other plant ingredients are low in Ca, different inorganic (limestone, mono-calcium phosphate, di-calcium phosphate) and organic Ca sources (meat and

bone meal, oyster shell, snail shell) are added in poultry diets to meet their requirements. Calcium availably is reported to be variable between these sources and even within a source depending on the origin and particle size (McNaughton *et al*., 1974; Hilman *et al*., 1976; Guinotte *et al*., 1991; Zhang and Coon, 1997a; Lichovnikova, 2007; Saunders-Blades *et al*., 2009; Oso *et al*., 2011).

Oso *et al*. (2011) found that Ca retention was 39, 38, 30 and 39% in broiler chickens fed diets supplemented with oyster shell, snail shell, wood ash and limestone as Ca source, respectively. McNaughton *et al*. (1974), using tibia ash as the indicator of Ca availability, examined the influence of various particle sizes (2.38-3.36, 0.84-1.19, 0.30-0.42, 0.11-0.15 and 0.05 mm) and found that tibia ash contents were higher in birds supplemented with diets containing medium particle size (0.30-0.42 mm) as compared to diets with fine and coarse particle sizes.

Several studies have been performed to determine the effect of different particle sizes of Ca sources on egg production, shell quality, bone mineral content and density in layers. Saunders-Blades *et al*. (2009) conducted an experiment on layers (74 weeks old) and used bone mineral density and bone mineral contents as indicators of Ca retention. It was observed that supplementation of diets with mixed particle sizes of limestone (70% fine and 30% large) improved the bone mineral density from 618 to 695 mg/cm^3 and bone mineral contents from 216 to 244 mg/mm of bone as compared to diets with 100% fine particle size. Whereas Lichovnikova (2007) reported that Ca retention was higher (57.8%) in diets with 50:50 combinations of fine and large (1 and 2 mm) limestone particles size as compared to 50.6% in diets with 29:71 combinations.

Prolonged retention of Ca in the gizzard may enhance its utilisation in layer hens during egg shell formation. Large Ca particles have been reported to be retained longer in the gizzard as compared to small particles (Zhang and Coon, 1997a). Layer hens were fed experimental diets for three days with limestone particle size of 4, 2.38, 1.41 and 0.65 mm, and the amount of Ca retained in the gizzard on different particles size after three days of experimental period was 7.87, 6.24, 4.52 and 0.72 g/hen, respectively. Calcium retention in the gizzard was also different (5.90 and 3.81g/hen) for two different limestone sources. These data show that, the Ca retention in the gizzard varies with Ca source and particle size and this deviation can cause variations in Ca availability.

In contrast, Guinotte *et al*. (1991) reported an increase in Ca retention in broiler chickens fed diets with fine Ca carbonate (0.15 mm) as compared to medium (0.6-0.8 mm) and coarse Ca carbonate (>1.18 mm).

2.6.2. Birds factors affecting calcium availability

2.6.2.1. Effect of genotype and age

Data on the effect of bird factors on availability and retention of Ca are limited. However, some studies indicate that Ca digestion, absorption and excretion may vary from breed to breed and even between strains within the same breed. Shafey *et al*. (1990) reported that Ca retention and excretion were different between two strains of broiler chickens, with relative Ca retention being 24.0% in strain 1 and 17.4% in strain 2.

Thomas and Ravindran (2010) reported that Ca retention was highest at five days of age and reduced at day seven and remained constant in maize based diets up to day 14 in broiler chickens. Calcium retention was 47, 35 and 44% at days five, seven and 14 in a wheat-based diet, while it was 45, 40 and 40% in a maize based-diet, respectively.

2.7. Methodology to determine the calcium digestibility

Currently there is no method available for determination of Ca digestibility in poultry. However, three different methods, namely direct, difference and regression, are used for determination of amino acid and P digestibility in feed ingredients and modifications of these approaches may be used for the measurement of Ca digestibility.

In the direct method, a test ingredient serves as the sole source of nutrient in the diet, while in the substitution or difference method, the test ingredient is substituted for specific proportion of a reference diet or replaces the specific ingredient. The digestibility of the desired nutrients in the test ingredient is determined by difference between the two digestibility measures and the concentration of the specific nutrient in the test diet (Nalle *et al*., 2007, Lemme *et al*., 2004). The regression method has been used in some studies for the determination of true ileal P digestibility in poultry (Dilger and Adeola, 2006a; Mutucumarana *et al*., 2013; 2014a, b, 2015a, b). Recently, Working Group No 2 (Nutrition) of the European Federation of Branches for WPSA (2013) has

proposed the regression method as a standard protocol for the estimation of ileal P digestibility in poultry. The regression method is based on establishing a linear relationship between nutrient output in ileal digesta and nutrient dietary input, expressed in g/kg of dry matter of digesta and diet, respectively. In this method, diets with graded concentrations of the specific nutrient from the specific assay ingredient are formulated. Theoretically, the digestibility estimates determined by the regression method are automatically corrected for endogenous losses and represent true digestibility values.

The digestibility values determined using the direct and difference methods are apparent values and need to be corrected for endogenous losses. Thus to calculate true Ca digestibility endogenous Ca losses need to be determined.

2.8. Endogenous calcium losses

Endogenous Ca originates from saliva, bile, pancreatic juice, gastric juice and damaged cells from the intestinal cell lining. Currently there are no published data on endogenous Ca losses in poultry. However, some studies have determined the endogenous P losses in pigs (Fan *et al*., 2001; Ajakaiye *et al*., 2003b; Dilger and Adeola, 2006b; Petersen and Stein, 2006; Pettey *et al*., 2006; Stein *et al*., 2006; Almeida and Stein, 2010; Sulabo and Stein, 2013) and poultry (Dilger and Adeola, 2006a; Liu *et al*., 2013; Mutucumarana *et al*., 2013; 2014a,b; 2015a,b). Two approaches have been used in these studies to determine the endogenous P losses, feeding a P-free diet (Petersen and Stein, 2006; Stein *et al*., 2006; Almeida and Stein, 2010; Liu *et al*., 2013; Sulabo and Stein, 2013; Mutucumarana and Ravindran, 2016) or the regression method (Fan *et al*., 2001; Ajakaiye *et al*., 2003b; Dilger and Adeola, 2006a,b; Pettey *et al*., 2006; Mutucumarana *et al*., 2013; 2014a,b; 2015a,b).

Some studies with pigs have used these approaches for the estimation of endogenous Ca losses, theoretical estimation by feeding a Ca free diet (Gonzalez-Vega *et al*., 2013a; 2014; 2015a,b; Merriman *et al*., 2016), and using the regression method (Gonzalez-Vega *et al*., 2013b).

CHAPTER 3

General materials and methods

Experimental procedures were approved by the Massey University Animal Ethics Committee and were conducted according to the New Zealand Revised Code of Ethical Conduct for the use of live animals for research, testing and teaching.

3.1. Birds and housing

For all studies, day-old male broilers (Ross 308) were obtained from a commercial hatchery (New Plymouth) and were raised on floor pens in an environmentally controlled room. Temperature was maintained at 31ºC on day one and gradually reduced to 22 ºC by 21 days of age. The birds were fed commercial broiler starter crumbles till day 14. On day 15, birds were moved to colony cages to provide acclimatisation period. During this period, the diet was gradually changed to a mash diet as experimental diets were in mash form. Before starting on the experimental diets, birds were individually weighed and allocated to cages on weight basis so that the average bird weight per cage was similar. The experimental diets were then randomly allotted to each cage. The diets were offered *ad libitum* and the birds had free access to water. Group body weights and feed intake were recorded at the start and end of the experimental period. Mortality was recorded on daily basis.

3.2. Digesta collection and processing

For ileal digesta collection, birds were euthanised by intravenous injection (1 ml per 2 kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand) and the contents of the lower half of the ileum were collected by gently flushing the contents with distilled water into plastic containers. The samples were frozen immediately and subsequently lyophilised. Lyophilised samples were ground to pass through a 0.5 mm sieve and stored in air-tight containers at 4ºC until chemical analysis.

3.3. Chemical analysis

3.3.1. Dry matter

Dry matter content was determined using the standard AOAC procedure (method 930.15; AOAC, 2005). Samples were weighed and placed in a drying oven for 24 hours at 105ºC and the weight was recorded after two hours of cooling in a desiccator.

3.3.2. Crude protein

Nitrogen content was determined by combustion (method 968.06; AOAC, 2005) using a CNS-200 carbon, nitrogen and sulphur analyse (LECO® Corporation, St, Joseph, MI). Pre-weighed samples were placed into a furnace at 850ºC with excess oxygen (O_2) and totally combusted. The combustion products, mainly carbon dioxide (CO_2), water (H_2O), nitrous oxide (NO_x) and nitrogen gas (N_2) were passed through a series of columns to remove H_2O, convert NO_x to N_2 and to remove the remaining oxides and excess O_2. The gaseous N_2, carried by helium, was then measured by thermal conductivity and expressed as percentage of the sample. Crude protein contents of the samples were determined by multiplying the nitrogen (N) content of the samples by 6.25.

3.3.3. Fat

Fat content was determined by the Soxhlet method (method 991.36; AOAC, 2005) using HT-1043 soxtec extraction unit (Tecator, Hoganas, Sweden). Samples were weighed and placed in extraction thimble. Crude fat was extracted with petroleum ether using soxtec an extraction unit. Ether was recovered and the flask containing the residue was dried at 125ºC in an oven for 30 minutes. After cooling down in a desiccator, the weight of the flask with the fat was recorded and fat content was calculated.

3.3.4. Ash

Ash content of the samples was determined gravimetrically by the standard AOAC procedure (method 942.05; AOAC, 2005). Samples were weighed in crucibles and ignited in the furnace at 550ºC for three hours to burn off all organic matter. Crucibles were then transferred to a desiccator, allowed to cool and then weighed to determine the ash content.

3.3.5. Titanium dioxide

Titanium dioxide was determined by the procedure of Short *et al.* (1996). The samples were ignited at 580ºC for 13 hours to burn off all organic materials and the ash was digested by 10 ml 7.4 M sulphuric acid to release the titanium which was then determined by using the calorimetric assay at 410 nm.

3.3.6. Calcium

For the measurement of Ca, samples were prepared by the standard AOAC procedure (method 968.08D; AOAC, 2005). Samples (1g) were first ignited at 550 ºC to burn off all organic matter and the ash was digested with 10 ml 6 M hydrochloric acid (HCl) to release the Ca. Digested samples were transferred to 25 ml volumetric flasks and distilled water was added to make the volume 25 ml. To make the standard solution of Ca (5 mmol/l), 0.05 g calcium carbonate was dissolved in 20 ml 6 M HCl and the volume was made up to 100 ml by adding RO water. This standard solution was used to prepare 5, 4, 3, 2 and 1 mmol/l of standard solutions to produce the calibration curve.

After the preparation step, Ca contents were determined by the dye-binding assay of Gitelman (1967) which uses o-cresolpthalein complexone in alkaline solution to develop colours. During this process, 650 µl of reagent A (ethanolamine buffer, 1mol/l, 10.6 pH) was added to 20µl of sample, blank and standards (5, 4, 3, 2 and 1 mmol/L) in cuvettes and mixed well for 25 seconds. 250 µl of reagent B (Chromogen containing o-cresolpthalein complexone: 0.3 mmol/l; 8-hydroxyquinoline: 13.8 mmol/l and HCl: 122 mmol/L) was then added to each sample. After 2 minutes, the absorbance was measured by using spectrophotometer (Flexor E, Vital Scientific NV, Spankeren/Dieren, the Netherlands) at 578 nm wavelength. The absorbance for each sample was measured in duplicate. A calibration curve was developed by regressing the absorbance against the calcium concentrations in the standard solutions. The concentration of Ca in the highest standard solution (5mmol/l) was around 20 mg/100 ml of solution. A linear trend of absorbance was observed against the Ca concentrations in the solutions. The Ca concentration in the sample solutions were determined using the following formula

$Y = ax$

Where,

Y= Absorbance

a = Slope

x = Concentration of Ca (mg/l of solution)

Calcium concentration in mg/g of diet or digesta samples was calculated as follows:

Calcium (mg/g) = (C×V×DF) / W

Where,

C = calcium concentration in sample solution (mg/litre)

V = Volume of sample solution (0.025 litre)

DF = Dilution factor (if any)

W = Weight of the sample (1 g)

3.3.7. Phosphorus

To determine the phosphorus (P) content, samples were prepared using the standard AOAC procedure (method 968.08D; AOAC, 2005). Phosphorus content was measured using the procedure of ISO 6491 (1998) by using ammonium molybdate reagent and amino-napthol sulphuric acids reagent to develop colour. The absorbance was measured by spectrophotometer (Shimadzu UV mini-1240, Shimadzu Corporation, Tokyo, Japan) at wavelength of 680 nm.

3.4. Determination of particle size

To determine the particle size of calcium sources, a set of sieves (Endocott, London, UK) sized 2, 1, 0.5, 0.212, 0.106 and 0.075 and a Tyler To-Tap sieve shaker were used as described by Baker and Herman (2002). The samples were passed through the sieve stack on shakers for 10 minutes. The amount of sample retained on each sieve was determined and, the geometric mean diameter (GMD) and geometric standard deviation (GSD) were calculated for each sample. These calculations were based on the assumption that the weight distribution of the samples is logarithmically normal. Two

replicates per sample were analysed. The following equations were used to calculate the GMD and GSD.

$$di = (du \times do)^{0.5}$$

$$GMD = \log^{-1} \{\sum (Wi \log di) / \sum Wi\}$$

$$GSD = \log^{-1} \{\sum Wi (\log di - \log GMD)^2 / \sum Wi\}^{0.5}$$

Where,

di = diameter of i^{th} sieve on stack

du = diameter opening through which particles will pass (sieve proceeding i^{th})

do = diameter opening through which particles will not pass (i^{th} sieve)

Wi = Weight fraction of sample on i^{th} sieve

CHAPTER 4

Measurement of ileal endogenous calcium losses in broiler chickens

4.1. Abstract

An experiment was conducted to determine the ileal endogenous calcium (Ca) losses in four-week old broiler chickens using three assay methodologies. Three experimental diets were used; a Ca and phosphorus-free (P-free) diet and two other diets by substituting 200g/kg dextrose (w/w) with maize gluten meal or dried egg albumen as Ca-free protein sources. Each of the three experimental diets was randomly allotted to six replicate cages (six birds/cage). Digesta samples were collected to measure the ileal endogenous Ca losses. The feed intake of birds fed diets containing the two protein sources was higher ($P < 0.05$) than the Ca and P-free diet. Body weight loss was observed in birds on the Ca and P-free diet and maize gluten meal based diets. Ileal endogenous Ca losses were influenced ($P < 0.05$) by the assay methodology. Ileal endogenous Ca losses were determined to be 125, 77 and 46 mg/kg dry matter intake in birds fed the Ca and P-free diet, maize gluten meal diet and dried egg albumen diet, respectively. Ileal endogenous P losses were determined in birds fed the Ca and P-free and dried egg albumen based diets. There was no effect ($P > 0.05$) of methodology on ileal endogenous P losses. Ileal endogenous P losses were determined to be 133 and 110 mg/kg of DMI on Ca and P-free and dried egg albumen based diets, respectively.

4.2. Introduction

Currently there is a move towards the use of digestible P in feed formulation for poultry diets (Dilger and Adeola, 2006a; Mutucumarana *et al*., 2014a, b; 2015a, b; Mutucumarana and Ravindran, 2016). Maintaining proper ratios between dietary calcium (Ca) and phosphorus (P) is critical for the absorption and utilisation of both minerals, estimation of true Ca digestibility is therefore required. For the estimation of true Ca digestibility, it is necessary to determine the endogenous Ca losses. Endogenous Ca originates from saliva, bile, pancreatic juice, gastric juice and damaged intestinal cell lining. Studies have been conducted with pigs to determine the endogenous Ca losses and two approaches, namely feeding of Ca free diet (Traylor *et al*., 2001; Gonzalez-Vega *et al*., 2013a, 2014), and the regression method (Gonzalez-Vega *et al*., 2013b)

have been used. Data on endogenous Ca losses in poultry, however, is scant. The purpose of this study was to determine the endogenous Ca losses in broiler chickens by three different methods, namely feeding a Ca and P-free diet, and diets containing protein sources (maize gluten meal and dried egg albumen) that have negligible amounts of Ca.

4.3. Materials and methods

The experiment was conducted according to the New Zealand Revised Code of Ethical Conduct for the use of live animals for research, testing and teaching, and approved by the Massey University Animal Ethics Committee.

4.3.1. Diets and experimental design

Three semi-purified diets were developed: a Ca and P-free diet and two other diets by substituting 200 g/kg dextrose with maize gluten meal (CGM) or dried egg albumen (Table 4.1). Maize gluten meal and dried egg albumen are protein sources that contain negligible amounts of Ca. All diets contained 3 g/kg of titanium dioxide as an indigestible marker.

4.3.2. Birds

One hundred and eight day-old male broilers (Ross 308) were obtained from a local hatchery and were raised according to the procedures described in Chapter 3, section 3.1. On day 26, birds were individually weighed and allocated to 18 cages (six birds/cage) on weight basis so that the average bird weight per cage was similar. The three experimental diets were then randomly allotted to six replicate cages each. The diets, in mash form, were offered *ad libitum* and the birds had free access to water. Group body weights and feed intake were recorded on days 26 and 28.

4.3.3. Digesta collection and processing

On day 28, all birds were euthanised by intravenous injection (1 ml per 2 kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand) and ileal digesta samples were collected, and processed as described in Chapter 3, section 3.2.

Table 4.1. Ingredient composition and analysis (g/kg as-fed basis) of experimental diets

	Calcium and phosphorus free	Maize gluten meal	Dried egg albumen
Dextrose	928.7	728.7	728.7
Maize gluten meal	-	200.0	-
Dried egg albumen	-	-	200.0
Cellulose	40.0	40.0	40.0
Soybean oil	20.0	20.0	20.0
Sodium chloride	4.0	4.0	4.0
Sodium bicarbonate	2.0	2.0	2.0
Titanium dioxide	3.0	3.0	3.0
Trace mineral-vitamin premix[1]	2.3	2.3	2.3
Calculated analysis			
Metabolisable energy (MJ/kg)	15.3	15.3	15.3
Crude protein	-	12.4	16.48
Calcium	-	0.06	0.18
Total phosphorus	-	1.0	0.18
Non-phytate phosphorus	-	0.28	0.18
Analysed values			
Calcium	<0.1	<0.1	<0.1
Phosphorus[2]	0.1	-	0.3

[1]Supplied per kilogram of diet: vitamin A, 12,000 IU; cholecalciferol, 4,000 IU; thiamine, 3 mg; riboflavin, 9 mg; pyridoxine, 10 mg; folic acid, 3 mg; biotin, 0.25 mg; cyanocobalamin, 0.02 mg; dl-α-tochopherol acetate, 80 mg; niacin, 60 mg; Ca-D pentothenate, 15 mg; menadione, 4 mg; choline chloride, 600 mg; Co, 0.25 mg; I, 1.5 mg; Mo, 0.25 mg; Se, 0.26 mg; Mn, 100 mg; Cu, 10 mg; Zn, 80 mg; Fe, 60 mg; antioxidant, 100 mg.

[2] Dietary phosphorus concentration of maize gluten meal based diet was not analysed and used for ileal endogenous P losses determination, as it was calculated to contain 1.0 g/kg total P (NRC, 1994 was used as a reference for P concentration in maize gluten meal).

4.3.4. Chemical analysis

Representative samples of test diets, and digesta were analysed for dry matter, Ca, P and titanium dioxide as described in Chapter 3, section 3.3.

4.3.5. Calculations

Ileal endogenous Ca losses were calculated by using the following Equation.

Ileal endogenous Ca losses = Ca in digesta × (Titanium in diet/Titanium in digesta)

All analysed values were expressed as mg/kg DM and ileal endogenous Ca losses were determined as mg/kg DMI.

4.3.6. Statistical analysis

Data were analysed using the analysis of variance procedure (SAS, 2004). Cages served as the experimental unit and an alpha level of 0.05 was used. Differences between means were separated by the Least Significance Difference test.

4.4. Results

Analysed dietary Ca concentrations are given in Table 4.1. All experimental diets contained less than 0.1 g Ca/kg of diet. Feed intake and body weight gain of the birds during the experimental period (26-28 days of age) are presented in Table 4.2. Weight loss was observed in birds fed Ca and P-free diet and the diet supplemented with 200 g/kg of maize gluten meal. This loss was higher ($P < 0.05$) in birds on the Ca and P-free diet compared to those on the maize gluten meal based diet. Birds fed diets supplemented with 200 g/kg of dried egg albumen gained weight. Feed intake of the birds was lowest ($P < 0.05$) on the Ca and P-free diet, followed by the maize gluten meal based diet and was highest ($P < 0.05$) on the dried egg album based diet.

Ileal endogenous Ca losses in 28-days old broilers determined by the three assay methodologies are presented in Table 4.3. Ileal endogenous Ca losses differed ($P < 0.05$) among the three methods. Ileal endogenous Ca losses determined for the Ca and P-free diet, maize gluten meal diet and dried egg albumen diet were 125, 77 and 46 mg/kg DMI, respectively. Ileal endogenous Ca losses were significantly higher ($P < 0.001$) in birds fed the Ca and P-free diet as compared to the maize gluten diet and dried

egg albumen based diet. Corresponding losses were higher ($P < 0.001$) in the maize gluten based diet as compared to the dried egg albumen based diet. Ileal endogenous P losses were also determined in birds fed the Ca and P-free and dried egg albumen based diets. The Ca and P-free and dried egg albumen based diets contained negligible amount of P, enabling the determination of endogenous P flow. The maize gluten meal based diet was calculated to contain 1.0 g/kg total P, so was not used for estimation of endogenous P losses. Ileal endogenous P losses were 133 and 110 mg/kg DMI on Ca and P-free and dried egg albumen based diets, respectively. There was no effect ($P > 0.05$) of either methodology on ileal endogenous P losses in broiler chickens.

Table 4.2. Body weight gain and feed intake (g/bird/day) of the birds fed the three experimental diets (26-28 days of age)[1]

	Body weight gain	Feed intake
Ca and P-free diet	-36[a]	47[a]
Maize gluten meal based diet	-15[b]	62[b]
Dried egg albumen based diet	25[c]	79[c]
SEM[2]	4.13	3.20
Probability, *P* ≤	0.001	0.001

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

[1] Each value represents the mean of six replicates (six birds per replicate).

[2] Pooled standard error of mean.

Table 4.3. Ileal endogenous calcium and phosphorus losses (mg/kg DMI) in broilers[1]

	Ileal endogenous calcium losses	Ileal endogenous phosphorus losses
Ca and P-free diet	125[a]	133
Maize gluten meal based diet	77[b]	-
Dried egg albumen based diet	43[c]	110
SEM[2]	10.26	12.31
Probability, *P* ≤	0.001	0.21

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

[1] Each value represents the mean of six replicates (six birds per replicate).

[2] Pooled standard error of mean.

4.5. Discussion

Body weight losses and lower feed intake in birds fed the Ca and P-free diet was as expected as the diet did not contain protein. Birds fed the maize gluten meal based diet also lost body weight as the diet was still deficient in CP than the recommended level of 190-230 g/kg (Ross, 2007). However, the weight loss on the maize gluten meal based diet was comparatively less than on Ca and P-free diet; this difference might be due the higher feed intake and supplementation of 120 g/kg CP in maize gluten diet. The increase in dietary protein (160 g/kg) and feed intake stimulated the body weight gain observed in birds on the dried egg albumen based diet as compared to the maize gluten meal based and Ca and P-free diets.

Saliva (Tryon and Bibby, 1966), gastric secretions (Moore and Tyler, 1955), bile, pancreatic secretions and cellular debris (Bronner, 1997) are all sources of endogenous Ca. In present study, ileal endogenous Ca losses differed among methodology used and observed to be 125, 77 and 46 mg/kg DMI on the Ca and P-free diet, maize gluten diet and dried egg albumen diet, respectively. No comparative data are currently available for the ileal endogenous Ca losses in poultry. Endogenous Ca losses in pigs are reported to be 349 mg/kg DMI (Traylor *et al*., 2001) and 420 mg/kg DMI (Gonzalez-Vega *et al*., 2013a), where casein and maize gluten and potato protein were supplemented as Ca-free protein sources, respectively. Digestive enzyme secretions vary with changes in the nutrient composition of diet and feed intake of the birds. Increases in dietary protein (Zhao *et al*., 2007) and feed intake (Sklan, 2001) have been reported to increase the digestive enzyme secretions that can contribute towards higher endogenous Ca. In the current study, findings were contrary to expectations and difficult to explain because the feed intake of the birds and dietary protein concentration of dried egg albumen and maize gluten meal diets were higher while respective endogenous Ca losses were lower than the bird on the Ca and P-free diet. A negative trend was observed between ileal endogenous Ca losses and body weight gain of the birds. A possible reason might be that the birds gaining weight on the dried egg albumen based diet reabsorbed part of the endogenous Ca more efficiently than birds on the Ca and P-free and maize gluten diets. A similar trend was observed for the maize gluten diet where ileal endogenous Ca losses were lower due to a lower body weight loss than birds on the Ca and P-free diet.

Ileal endogenous P losses were only measured for the Ca and P-free and dried egg albumen based diets, as the maize gluten meal based diet was calculated to contain 1.0 g/kg total P that could have caused an overestimation of endogenous P values. Variable results have been reported previously regarding ileal endogenous P losses in broiler chickens. Ileal endogenous P losses have been reported to be 272 and 446 mg/kg DMI in broiler chickens fed P-free synthetic amino acid-based diets in two different studies (Rutherfurd *et al*., 2002; 2004). In the current study lower ileal endogenous P losses were determined in birds fed the Ca and P-free diet (133 mg/kg DMI) and egg albumen based diet (110 mg/kg DMI) as compared to the values described previously. These differences can be attributed to the differences in diet composition between the previous and currents studies.

4.6. Conclusions

The present study demonstrated that estimates of ileal endogenous Ca losses in broiler chickens differed depending on the assay methodology used. Ileal endogenous Ca losses in broiler chickens were highest on the Ca and P-free diet, intermediate on the maize gluten meal based diet and lowest on the dried egg albumen based diet. No differences were observed for ileal endogenous P losses in birds fed the Ca and P-free diet or dried egg albumen based diet.

CHAPTER 5

Measurement of true ileal calcium digestibility in meat and bone meal for broiler chickens

5.1. Abstract

The objective of the present study was to determine the true ileal calcium (Ca) digestibility in three samples of meat and bone meal (coded as MBM-1, MBM-2 and MBM-3) for broiler chickens. Four experimental diets, containing graded concentrations of Ca, were formulated from each MBM sample with inclusion levels of 20, 40, 60 and 80 g/kg diet. Each experimental diet was randomly allotted to four replicate cages (six birds per cage) and fed from days 28-31 post-hatch. The apparent ileal digestibility coefficient of Ca was measured by the indicator method and linear regression analysis was used to determine the true Ca digestibility coefficient. Apparent Ca digestibility was unaffected ($P > 0.05$) by increasing Ca concentrations. The average apparent digestibility coefficients in MBM-1, MBM-2 and MBM-3 were 0.50, 0.44 and 0.45, respectively. Strong linear ($P < 0.001$) relationships were observed between dietary Ca intake and digesta Ca output in all MBM samples. The true ileal digestibility coefficients of Ca in MBM-1, MBM-2 and MBM-3 were determined to be 0.60, 0.46 and 0.50, respectively. The corresponding ileal endogenous Ca losses were 292, 123 and 174 mg/kg dry matter intake, respectively.

5.2. Introduction

Determination of calcium (Ca) digestibility in feed ingredients for poultry has not received any attention in the past due to the cheap availability of limestone, the major inorganic Ca source, and the low Ca content in plant feed ingredients. However, the recent interest in the determination of phosphorus (P) digestibility in feed ingredients necessitates the measurement of Ca digestibility because of the close relationship between P and Ca metabolism. High dietary Ca concentrations have been reported to reduce the availability of P in broiler chickens (Tamim and Angel, 2003; Tamim *et al*., 2004; Plumstead *et al*., 2008).

Currently there is no established method available for the determination of Ca digestibility in poultry. However, three different methods, namely direct, difference and regression, are used for the determination of amino acid digestibility in feed ingredients. Some recent studies have used the regression method for the determination of P digestibility in poultry (Dilger and Adeola, 2006a; Mutucumarana *et al*., 2014a, b; 2015 a, b). In the regression method, diets containing graded concentrations of the specific nutrient from the assay ingredient are formulated and fed to birds. This method is based on establishing a linear relationship between dietary nutrient input and their output in ileal digesta, expressed as g/kg dry matter of diet and digesta, respectively. The digestibility estimate determined as the slope of the regression is automatically corrected for endogenous losses and represents the true digestibility value.

Meat and bone meal (MBM) is an important organic Ca source in poultry diets and contains an average of 103 g/kg Ca (NRC, 1994). But wide variations have been found in the Ca concentration of MBM from different sources. The Ca concentration of MBM is reported to range from 40 to 150 g/kg (Waldroup, 1999; Sulabo and Stein, 2013). The apparent total tract digestibility of Ca in eight MBM samples for pigs has been recently reported (Sulabo and Stein, 2013), but there is no published data available for poultry. The purpose of the present study was to determine the true ileal digestibility of Ca in three MBM samples for broiler chickens.

5.3. Materials and methods

The experiment was conducted according to the New Zealand Revised Code of Ethical Conduct for the use of live animals for research, testing and teaching, and approved by the Massey University Animal Ethics Committee.

5.3.1. Diets and experimental design

Meat and bone meal samples from three commercial rendering plants (coded as MBM-1, MBM-2 and MBM-3) were obtained, and representative samples were analysed in triplicate for dry matter (DM), crude protein (CP), crude fat, ash, Ca and P, particle size distribution and, meat and bone fractions. For each MBM sample, four semi-purified diets were formulated with graded inclusions of MBM (20, 40, 60 and 80 g/kg) to maintain graded dietary Ca concentrations (Table 5.1). Inclusion levels of MBM were maintained below 80 g/kg to ensure that the dietary Ca concentrations were under the recommended Ca requirement for broiler finishers (Ross, 2007). Meat and bone meal

served as the sole source of Ca in all diets and the Ca: total P ratio was calculated to be around 2:1. Titanium dioxide (3 g/kg) was incorporated in the diets as an indigestible marker.

5.3.2. Birds

A total of 288 day-old male broilers (Ross 308) were obtained from a local hatchery and raised as described in Chapter 3, section 3.1. On day 28, the birds were individually weighed and allocated to 48 cages (six birds/cage) on a weight basis so that the average bird weight per cage was similar. The 12 experimental diets were then randomly allotted to four replicate cages each. The diets, in mash form, were offered *ad libitum* from day 28 to day 31 post-hatch and the birds had free access to water. Group body weight and feed intake were recorded on days 28 and 31.

5.3.3. Digesta collection and processing

On day 31, all birds were euthanised by intravenous injection (1ml per 2 kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand) and, ileal digesta were collected and processed as described in Chapter 3, section 3.2.

5.3.4. Chemical analysis

Representative samples of diets and ileal digesta were analysed for DM, CP, crude fat, ash, Ca, P and titanium as described in Chapter 3, section 3.3. The particle size distribution of MBM samples was determined as describe in Chapter 3, section 3.4.

Bone and meat fractions of MBM samples were determined by the flotation method described by Khajarern and Khajarern (1999). Meat and bone meal samples (10g) were weighed in a beaker and mixed well with carbon tetrachloride (90 ml) to dissolve the fat. The samples were then allowed to settle, and the floating meat and submerged bone fractions were separated on separate filter papers (Whatman no.4). Both fractions were dried at 110 ºC for 10 minutes, allowed to cool and weighed to calculate the proportions of bone and meat fractions.

Table 5.1. Ingredient composition and analysis (g/kg as-fed basis) of meat and bone meal (MBM) based diets

	MBM-1				MBM-2				MBM-3			
	20	40	60	80	20	40	60	80	20	40	60	80
Dextrose	717.2	701.6	686.08	670.48	717.2	701.6	686.08	670.48	717.2	701.6	686.08	670.48
Maize starch	179.3	175.4	171.52	167.62	179.3	175.4	171.52	167.62	179.3	175.4	171.52	167.62
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Meat and bone meal	20.0	40.0	60.0	80.0	20.0	40.0	60.0	80.0	20.0	40.0	60.0	80.0
Soybean oil	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Sodium chloride	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Potassium chloride	3.1	2.6	2.0	1.5	3.1	2.6	2.0	1.5	3.1	2.6	2.0	1.5
Titanium dioxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Trace mineral-vitamin premix[1]	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4
Calculated analysis												
Metabolisable energy, (MJ/kg)	15.17	15.04	14.91	14.78	15.17	15.04	14.91	14.78	15.17	15.04	14.91	14.78
Crude protein	10.72	21.45	32.17	42.89	9.75	19.5	29.25	39.00	9.48	18.97	28.45	37.94
Calcium[2]	1.43	2.86	4.30	5.73	2.36	4.72	7.09	9.45	2.29	4.58	6.88	9.17
Total phosphorus[2]	0.75	1.50	2.25	3.00	1.20	2.41	3.61	4.81	1.20	2.39	3.59	4.78
Non-phytate phosphorus	0.75	1.50	2.25	3.00	1.20	2.41	3.61	4.81	1.20	2.39	3.59	4.78
Analysed values (as-fed basis)												
Dry matter	912	915	915	915	916	917	916	917	915	916	916	919
Calcium	1.40	2.36	4.05	5.72	2.19	4.58	6.95	8.53	1.91	4.04	5.89	8.73
Phosphorus	1.11	1.68	2.36	3.59	1.35	2.75	4.22	5.15	1.42	2.32	3.78	4.85

[1]Supplied per kilogram of diet: vitamin A, 18,000 IU; cholecalciferol, 6,000 IU; thiamine, 4.5 mg; riboflavin, 13.5 mg; pyridoxine, 15 mg; folic acid, 4.5 mg; biotin, 0.38 mg; cyanocobalamin, 0.03 mg; dl-α-tochopherol acetate, 120 mg; niacin, 90 mg; Ca-D pentothenate, 22.5 mg; menadione, 6 mg; choline chloride, 900 mg; Co, 0.37 mg; I, 2.20 mg; Mo, 0.37 mg; Se, 0.38 mg; Mn, 147 mg; Cu, 15 mg; Zn, 117 mg; Fe, 88 mg; antioxidant, 147 mg.

[2] Calculated from the analysed values of MBM samples.

5.3.5. Calculations

The true ileal digestibility coefficient of Ca was calculated according to the procedure outlined by Dilger and Adeola (2006a) for the estimation of P digestibility. The apparent ileal digestibility coefficient (AIDC) of Ca of the test diets (at each concentration of inclusion) was calculated using the following equation.

$$AIDC = 1 - [(Ti_I/Ti_O) \times (Ca_O / Ca_I)] \quad [1]$$

where AIDC is apparent ileal digestibility coefficient of Ca, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta, and Ca_I is the Ca concentration in the diet. All analysed values were expressed as gram per kilogram of DM.

Total output of Ca in the ileal digesta, expressed as g/kg dry matter intake (DMI), was calculated by the following equation.

$$Ca_{O\text{-}DMI}\,(g/kg) = Ca_{O\text{-}DMO} \times (Ti_I/Ti_O) \quad [2]$$

where $Ca_{O\text{-}DMI}$ and $Ca_{O\text{-}DMO}$ represent Ca output concentrations on a DMI and DM output basis, respectively, and Ti_I and Ti_O represent the titanium concentration in the diet and digesta, respectively.

To generate the linear regression, digesta Ca outputs were regressed against dietary Ca concentrations by using the following statistical model:

$$Ca_{O\text{-}DMI}\,(g/kg) = (TCaI \times Ca_I) + IECaL \quad [3]$$

where $Ca_{O\text{-}DMI}$ represents the Ca concentration in digesta on DMI basis (dependent variable), Ca_I represents Ca concentration in diet on DM basis (independent variable), TCaI represents true Ca indigestibility, and IECaL represents the mean ileal endogenous Ca estimates on DM basis. In this equation, TCaI and ECaL are the slope and intercept, respectively, of the simple linear regression of $Ca_{O\text{-}DMI}$ on Ca_I.

True Ca indigestibility in an indirect measure of the inefficiency at which dietary Ca is extracted by the birds. The true ileal digestibility Ca digestibility was calculated by using the following equation.

TIDC = 1- TCaI [4]

where TIDC and TCaI represent the true ileal digestibility and true ileal indigestibility coefficients of Ca, respectively.

5.3.6. Statistical analysis

All data were analysed using the GLM procedure of SAS (2004). Cages served as the experimental unit and an alpha level of 0.05 was used. The regression model included MBM sample (2df) and MBM inclusion level (3df). Orthogonal polynomial contrasts were used to determine the effect of graded Ca intake from each MBM sample on weight gain, feed intake, Ca intake, digesta Ca output and apparent ileal digestibility of Ca. The mean true Ca utilisation coefficient and endogenous Ca losses (g/kg DMI) were estimated by regressing dietary Ca intake (g/kg DM) against digesta Ca output (g/kg DMI). Thus, standard errors for these regression coefficients were based on total of 16 observations for each MBM sample. Regression coefficients (slopes) between MBM samples were compared using a Student's t-test.

5.4. Results

Analysed Ca and P concentrations of the experimental diets are presented in Table 5.1. Dietary Ca and P concentrations increased with increasing dietary inclusion levels of each MBM sample in the diets. Analysed dietary Ca concentrations were 0.01 to 0.99 g/kg lower than the calculated values. Analysed dietary P concentrations were 0.07 to 0.61 g/kg higher than the calculated values. The nutrient composition of the three MBM samples is shown in Table 5.2. Analysed Ca and P concentrations of MBM-1, MBM-2 and MBM-3 were 71, 118 and 114, and 37, 60 and 59 g/kg, respectively.

Particle size distribution of the three MBM samples is presented in Figure 5.1. In the current study, particle size distribution was classified as fine (< 0.5 mm), medium (0.5-1.0 mm) and coarse (> 1.0 mm). According to this classification, proportions of fine, medium and coarse particles in MBM-1, MBM-2 and MBM-3 were 5, 41 and 5, and 63, 34 and 60, and 32, 25 and 35%, respectively. Geometric mean diameter (GMD) of MBM-1, MBM-2 and MBM-3, was determined to be 0.866, 0.622 and 0.875 mm, respectively. The corresponding geometric standard deviations were 1.52, 1.95 and 1.51, respectively.

Table 5.2. Analysed nutrient composition of the three meat and bone meal (MBM) samples (g/kg, as-fed basis)[1]

	Dry matter	Crude protein	Ash	Crude fat	Calcium	Phosphorus	Ca:P ratio
MBM-1	925	536	237	114	71	37	1.91
MBM-2	934	488	357	93	118	60	1.96
MBM-3	956	474	362	88	114	59	1.92

[1] Samples were analysed in duplicates

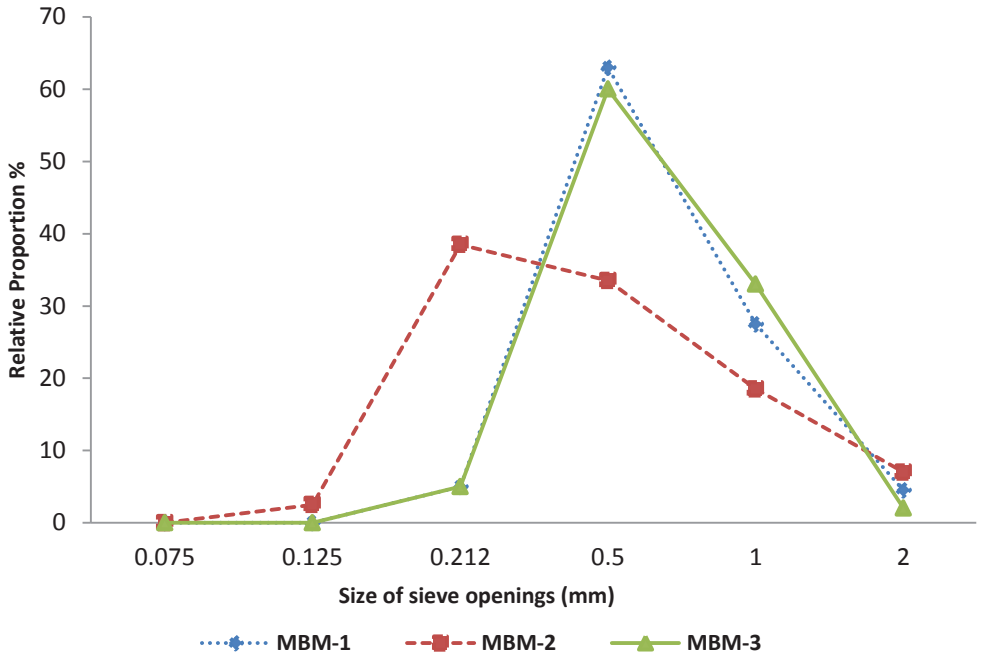

Figure 5.1. Particle size distribution of the three meat and bone meal samples

Bone and meat (soft tissue) fractions of the MBM samples are presented in Table 5.3. Bone and soft tissue fractions of MBM-2 and MBM-3 were similar, but MBM-1 contained lower bone and higher soft tissue fractions compared to MBM-2 and MBM-3.

Table 5.3. Percentage composition of bone and soft tissue fractions and bone to soft tissue ratio of three meat and bone meal (MBM) samples[1]

	Bone	Soft tissue	Bone : Soft tissue
MBM-1	40.2	59.8	1:1.49
MBM-2	50.5	49.5	1:0.98
MBM-3	52.1	47.9	1:0.92

[1] Samples were analysed in duplicates

Body weight gain and feed intake of birds fed the experimental diets during the three-day experimental period are summarized in Table 5.4. Feeding all MBM diets resulted in body weight loss during the 3-day trial period. Graded increases in MBM inclusion had no effect ($P > 0.05$) on feed intake for birds fed MBM-1 and MBM-2 diets, while, a linear ($P < 0.05$) increase in feed intake was observed with increasing inclusion level for MBM-3 diets.

Dietary Ca intake (g/kg DM), ileal digesta Ca output (g/kg DMI) and apparent Ca digestibility of the three MBM samples are also presented in Table 5.4. In all three MBM samples, increasing dietary Ca concentrations had no influence ($P > 0.05$) on the apparent ileal Ca digestibility coefficient. The average apparent ileal digestibility coefficients of Ca for MBM-1, MBM-2 and MBM-3, were calculated to be 0.50, 0.44 and 0.45, respectively.

The influence of digesta Ca output regressed against dietary Ca concentration (g/kg DM) for the three MBM samples is shown in Figure 5.2. The results indicated a strong linear relationship between digesta Ca outputs and dietary Ca intakes for all MBM, which is a prerequisite for the application of regression method.

Ileal endogenous Ca losses and true Ca digestibility coefficients for three MBM samples are presented in Table 5.5. Ileal endogenous Ca losses were determined to be 292, 123 and 174 mg/kg DMI in birds fed diets with MBM-1, MBM-2 and MBM-3, respectively. True Ca digestibility coefficients of MBM-1, MBM-2 and MBM-3 were 0.60, 0.46 and 0.50, respectively. True Ca digestibility of MBM-1 was significantly higher ($P < 0.05$) than MBM-2 but was similar ($P > 0.05$) to MBM-3. No difference ($P > 0.05$) was observed between true ileal Ca digestibility of MBM-2 and MBM-3.

Table 5.4. Weight gain (g/bird/day), feed intake (g/bird/day), calcium intake (g/kg DM), digesta calcium output (g/kg DMI), and apparent ileal calcium digestibility coefficients of broilers fed graded concentrations of calcium from three meat and bone meal (MBM) samples[1]

		Inclusion level of MBM (g/kg)				SEM[2]	*P*-value	
		20	40	60	80		Linear	Quadratic
MBM-1	Weight gain	-48	-45	-45	-42	3.241	0.328	0.958
	Feed intake	77	73	78	79	4.960	0.647	0.629
	Calcium intake	1.54	2.58	4.43	6.25	-	-	-
	Digesta Ca output	0.78	1.55	1.95	2.81	0.179	<0.001	0.791
	Apparent ileal Ca digestibility coefficient	0.49	0.40	0.56	0.55	0.049	0.154	0.414
MBM-2	Weight gain	-45	-47	-43	-40	2.627	0.152	0.362
	Feed intake	80	81	76	85	2.350	0.393	0.094
	Calcium intake	2.39	5.00	7.59	9.30	-	-	-
	Digesta Ca output	1.47	2.62	4.40	5.04	0.198	<0.001	0.229
	Apparent ileal Ca digestibility coefficient	0.38	0.48	0.43	0.46	0.035	0.270	0.406
MBM-3	Weight gain	-52	-44	-39	-36	3.392	0.006	0.488
	Feed intake	74	78	81	87	3.284	0.016	0.781
	Calcium intake	2.09	4.41	6.43	9.50	-	-	-
	Digesta Ca output	1.27	2.35	3.37	4.98	0.185	<0.001	0.179
	Apparent ileal Ca digestibility coefficient	0.40	0.47	0.48	0.48	0.028	0.071	0.218

Diets were fed from days 29 to 32 of age.

[1]Each value represents the mean of four replicates (six birds per replicate).

[2] Pooled standard error of the mean.

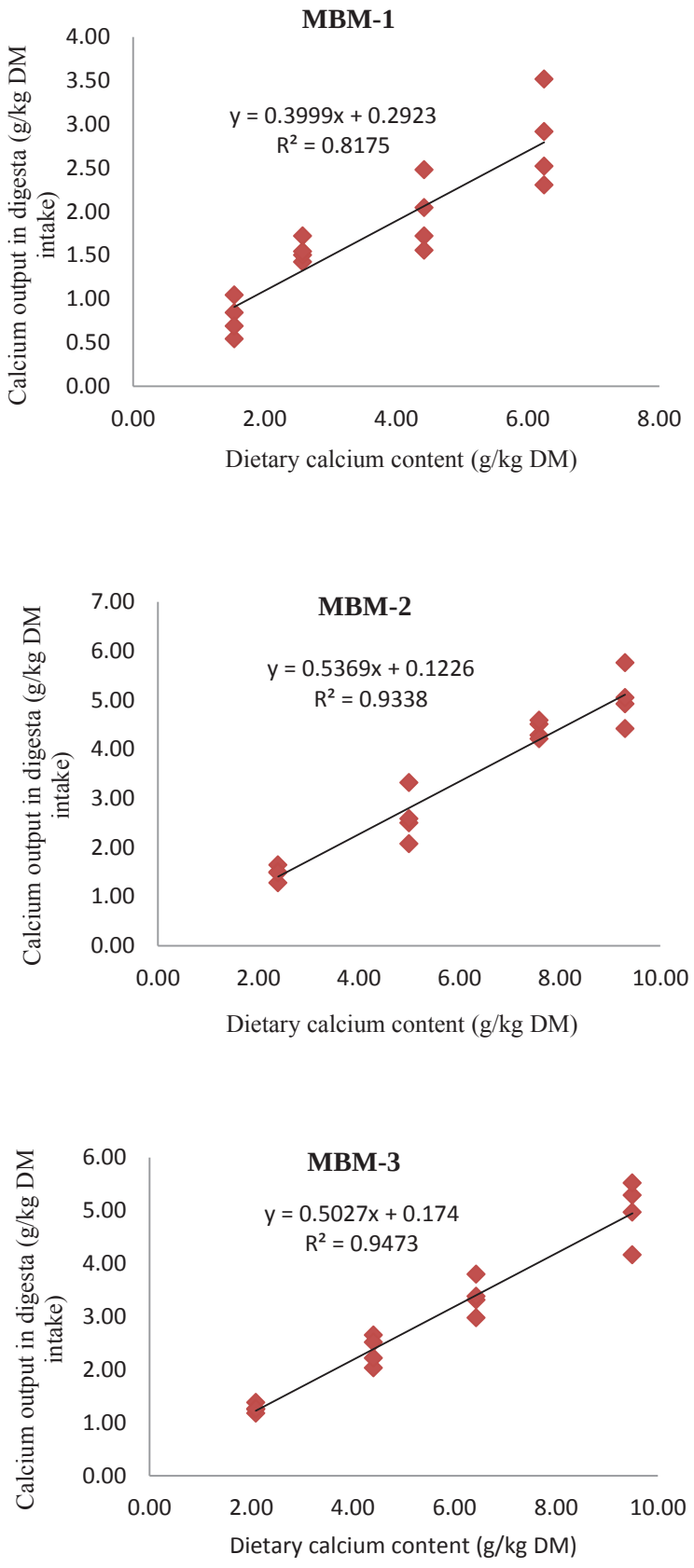

Figure 5.2. Digesta calcium outputs (g/kg of DMI) regressed against dietary calcium concentration (g/kg DM) in the three meat and bone meal (MBM) samples

Table 5.5. Linear relationship between digesta calcium outputs (g/kg of DMI) and dietary calcium concentrations (g/kg DM) of the three meat and bone meal samples

Meat and bone meal sample	Regression equation[1]	SE of the slope[2]	SE of the intercept[2]	r^2	Endogenous Ca losses (g/kg DMI)	Digestibility coefficient[3]
MBM-1	Y=0.3999X+0.2923	0.05	0.21	0.82	0.292	0.60[a]
MBM-2	Y=0.5369X+0.1226	0.04	0.25	0.93	0.123	0.46[b]
MBM-3	Y=0.5027X + 0.174	0.03	0.20	0.95	0.174	0.50[ab]

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).
[1] Regression of digesta Ca output (g/kg DMI) against dietary Ca concentration (g/kg DM) as determined by feeding chickens with graded levels of meat and bone meal. The slope represents the true Ca indigestibility and the intercept provides an estimate of endogenous Ca loss (g/kg DMI).
[2] Standard errors of regression components of the slope and intercept.
[3] Calculated as (1 − true Ca indigestibility coefficient), as described in Section 5.3.5 (Equation 4).

5.5. Discussion

According to the definition of Association of American Feed Control Officials (AAFCO, 2000), MBM is a rendered product of mammalian tissue and bones, exclusive of any added hair, horn, hoof and blood. It should contain a minimum of 40 g/kg P, a maximum of 550 g/kg CP, and the Ca concentration should not be more than 2.2 times the P concentration. If the P concentration is less than 40 g/kg and CP is more than 550 g/kg then the meal is considered meat meal. The CP, Ca and P concentrations of three samples used in this study were within the range to be considered as MBM. The Ca concentration of MBM from different sources has been reported to vary from 5.09 to 12.67 g/kg (Waldroup, 1999; Sulabo and Stein, 2013). This variability is attributed to differences in raw materials and the ratio between bone and soft tissues. Meat and bone meal of porcine origin has been reported to contain low ash, Ca and P concentrations compared to MBM of bovine origin (Traylor *et al*., 2005).

Ash and Ca concentrations of MBM-1, MBM-2 and MBM-3 were 237, 357 and 362 g/kg, and 72, 118 and 114 g/kg, respectively. It was observed that the Ca concentration of MBM samples were higher when there was a high ash concentration and vice versa. A similar trend has been reported previously by Sulabo and Stein (2013). In the current study, bone fractions of MBM-1, MBM-2 and MBM-3 were observed to be 40.2, 50.5 and 52.1%, respectively. Ash concentrations have been reported to be higher in MBM

samples with high bone fraction (Dale, 1997; Mendez and Dale, 1998) which is in agreement with the present data.

In the current study, analysed dietary Ca concentrations were lower, while P concentrations were higher than the expected calculated values. The interpretation of these findings is difficult because dietary values were calculated on the basis of analysed Ca and P concentrations of MBM samples, and MBM was the only source of both minerals in all experimental diets. Crude protein concentrations of all experimental diets were very low (9.5 to 43 g/kg) when compared to the recommended requirement (200 g/kg) in broiler finisher diets (Ross, 2007). The low dietary protein concentration is likely to be responsible for the observed weight losses.

The apparent ileal digestibility coefficient of Ca in the current study was determined to be 0.50, 0.44 and 0.45 for MBM-1, MBM-2 and MBM-3, respectively. Recent studies with pigs have shown that the apparent total tract digestibility coefficients of Ca varies from 0.53 to 0.81 (Sulabo and Stein, 2013).

A strong linear relationship ($P < 0.001$) was observed between dietary Ca intake and digesta Ca output, which justifies the use of the regression method. In poultry feed formulations, the availability of Ca in MBM is generally assumed to be 100%, but the current results show that this is not the case. True ileal digestibility coefficients of Ca were determined to be 0.60, 0.46 and 0.50 for MBM-1, MBM-2 and MBM-3 respectively. The factors responsible for the observed variability between MBM samples are not clear. However, nutrient composition (Traylor *et al*., 2005; Sulabo and Stein, 2013) and particle size distribution (Burnell *et al*., 1989) of MBM have been reported to influence the mineral utilisation in pigs. The apparent digestibility coefficient of Ca from different MBM samples for pigs has been reported to be negatively correlated to its bone to soft tissue ratio, and ash, Ca and P concentrations (Sulabo and Stein, 2013). In the current study, the bone to soft tissue ratio of MBM-1, MBM-2 and MBM-3 was determined to be 1:1.49, 1:0.98 and 1:0.92, respectively. The lower bone to soft tissue ratio and low ash, Ca and P concentrations of MBM-1 may partly explain its higher digestibility compared to MBM-2, but not its similar digestibility compared to MBM-3. In contrast, it has been reported that P in bones is more available to pigs than P in soft tissues (Traylor *et al*., 2005).

Average particle size (GMD) of MBM-1, MBM-2 and MBM-3 was determined to be 0.866, 0.622 and 0.875 mm, respectively. No data are available on the effect of

particle size of MBM on Ca utilisation in poultry. In pigs, P from large bone particles has been reported to be absorbed less efficiently than P from finely ground bones (Burnell *et al*., 1989). In present study, differences in the Ca digestibility coefficients of the three MBM samples cannot be explained on the basis of variations in the particle size, as average particle size of MBM-2 was lower than MBM-1 and MBM-3, while its digestibility coefficient was less than MBM-1 but was similar to MBM-3. However, there was no difference in average particle size and digestibility coefficients between MBM-1 and MBM-3.

Ileal endogenous Ca losses in broiler chickens, estimated as the intercept of the regression equations, were 292, 123 and 174 mg/kg DMI for the MBM-1, MBM-2 and MBM-3 based diets, respectively. Ileal endogenous Ca losses observed in this study were not different for different MBM samples. No previous published data are available on the ileal endogenous Ca losses in broiler chickens. In an earlier study (Chapter 4), ileal endogenous Ca losses in birds fed a Ca-P free diet were estimated to be 125 mg/kg DMI.

5.6. Conclusions

In the present study, the regression method was used to determine the true Ca digestibility of MBM. A strong linear relationship between dietary Ca concentrations and digesta Ca output indicated that the regression method can be successfully used for the estimation of true Ca digestibility of MBM. The findings also showed that Ca digestibility of MBM for broilers is not 100%, and the variations in the true ileal Ca digestibility coefficients of MBM samples may be partly explained by the differences in ash and Ca concentrations and bone to soft tissue ratio.

CHAPTER 6

Measurement of true ileal calcium digestibility in meat and bone meal for broiler chickens using the direct method

6.1. Abstract

The objective of this study was to determine the true ileal calcium (Ca) digestibility in meat and bone meal (MBM) for broiler chickens using the direct method. Four MBM samples (coded as MBM-1, MBM-2, MBM-3 and MBM-4) were obtained and analysed for nutrient composition, particle size distribution and bone to soft tissue ratio. The Ca concentrations of MBM-1, MBM-2, MBM-3 and MBM-4 were determined to be 71, 118, 114 and 81 g/kg, respectively. The corresponding geometric mean particle diameters and bone to soft tissue ratios were 0.866, 0.622, 0.875 and 0.781 mm, and 1:1.49, 1:0.98, 1:0.92 and 1:1.35, respectively. Five experimental diets, including four diets with similar Ca concentrations (8.3 g/kg) from each MBM sample and a Ca and phosphorus-free diet were developed. Meat and bone meal served as the sole source of Ca in the MBM diets. Titanium dioxide (3 g/kg) was incorporated in all diets as an indigestible marker. Each experimental diet was then randomly allotted to six replicate cages (eight birds per cage) and offered to broiler chickens from day 20 to 23 post-hatch. Apparent ileal Ca digestibility was calculated by the indicator method and corrected for ileal endogenous Ca losses to determine the true ileal Ca digestibility. Ileal endogenous Ca losses were determined to be 88 mg/kg dry matter intake. True ileal Ca digestibility coefficients of MBM-1, MBM-2, MBM-3 and MBM-4 were determined to be 0.56, 0.45, 0.52 and 0.41, respectively. True Ca digestibility of MBM-1 was higher ($P < 0.05$) than MBM-2 and MBM-4 but similar ($P > 0.05$) to that of MBM-3. True Ca digestibility of MBM-2 was similar ($P > 0.05$) to MBM-3 and MBM-4, while that of MBM-3 was higher ($P < 0.05$) than MBM-4. These results demonstrated that the direct method can be successfully used for the determination of true Ca digestibility in MBM, and that Ca in MBM is not highly available as is often assumed. The variability in true Ca digestibility of MBM samples could not be attributed to Ca content, percentage bones or particle size.

6.2. Introduction

Meat and bone meal (MBM) is an important organic calcium (Ca) source in poultry diets and contains 103 g/kg Ca on average (NRC, 1994). However, wide variations have been reported in the Ca contents of MBM from different sources depending on its origin and the proportion of meat and bones it contains. The Ca content of MBM is reported to range from 40 to 150 g (Drewyor and Waldroup, 2000; Sulabo and Stein, 2013). These variations in Ca contents may in turn cause variations in digestible Ca contents of MBM. A recent study by Sulabo and Stein (2013) has reported that total tract apparent Ca digestibility for pigs was reduced with increasing Ca contents of MBM.

Currently there is no established method available for the determination of Ca digestibility in poultry. However, three different methods, namely direct, difference and regression, are used for the determination of amino acid digestibility in feed ingredients (Ravindran and Bryden, 1999; Lemme *et al*., 2004). Historically Ca availability has been described in terms of bioavailability relative to calcium carbonate and it is generally assumed that Ca availability from Ca sources is 100%.

In our previous study (Chapter 5), the regression method was used for the determination of true ileal Ca digestibility in MBM for broiler chickens and it was found that the digestibility coefficients varied from 0.46 to 0.60. These values were lower than the apparent total tract digestibility of Ca (0.53 to 0.81) in MBM for pigs recently determined by the direct method by Sulabo and Stein (2013). The possible reasons for the observed discrepancy may include differences in animal species, source of MBM and the methodology used.

Apparent digestibility coefficients determined by the direct method must be corrected for endogenous Ca losses to determine the true Ca digestibility coefficients. No published data are available on endogenous Ca losses in broiler chickens. The purpose of this study was to determine the true ileal Ca digestibility of four MBM samples in broiler chickens using the direct method. Ileal endogenous Ca losses were also determined following feeding of a Ca-free diet.

6.3. Materials and methods

The experiment was conducted according to the New Zealand Revised Code of Ethical Conduct for the use of live animals for research, testing and teaching and approved by the Massey University Animal Ethic Committee.

6.3.1. Diets

Meat and bone meal samples from four commercial sources (coded as MBM-1, MBM-2, MBM-3 and MBM-4) were obtained and representative samples were analysed in triplicate for dry matter (DM), crude protein (CP), crude fat, ash, Ca, phosphorus (P), particle size distribution and, soft and bone tissue fractions. A total of five experimental diets were developed, one Ca- and P-free diet and four diets with similar dietary Ca concentrations containing the four MBM sources (Table 6.1). Meat and bone meal served as the sole source of Ca in the assay diets. The inclusion level of each MBM sample was set to obtain 8.3 g/kg of dietary Ca concentration, which was below the recommended dietary Ca requirement of broiler growers (Ross, 2007). Titanium oxide (3g/kg) was added in all diets as an indigestible marker.

6.3.2. Birds

Day-old male broilers (Ross 308) were obtained from a local hatchery and were raised according to the procedure described in Chapter 3, section 3.1. On day 20, 240 birds were individually weighed and allocated to 30 cages (eight birds per cage) on a weight basis so that the average bird weight per cage was similar. The five experimental diets were then randomly allotted to six replicate cages each. The diets, in mash form, were offered *ad libitum* and the birds had free access to water. Group body weights and feed intakes were recorded on days 20 and 23.

6.3.3. Digesta collection and processing

On day 23, all birds were euthanised by intravenous injection (1ml per 2 kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand) and digesta samples were collected, and processed as described in Chapter 3, section 3.2.

Table 6.1. Ingredient composition and analysis (g/kg as-fed basis) of experimental diets

	MBM diets				Ca and P-free diet
	MBM-1	MBM-2	MBM-3	MBM-4	
Maize starch	349.35	370.85	369.85	355.6	451.45
Dextrose	349.35	370.85	369.85	355.6	451.45
Dried egg albumen	100	100	100	100	-
Meat and bone meal	115	70	72	102	-
Cellulose	50	50	50	50	50
Soybean oil	20	20	20	20	20
Potassium bicarbonate	8	10	10	8.5	14.8
Sodium bicarbonate	3	3	3	3	3
Sodium chloride	-	-	-	-	4
Titanium dioxide	3	3	3	3	3
Trace mineral-vitamin premix[1]	2.3	2.3	2.3	2.3	2.3
Calculated analysis					
Metabolisable energy, (MJ/kg)	14.67	14.96	14.95	14.76	15.60
Crude protein	144.7	117.1	117.1	132.2	
Calcium[2]	8.30	8.30	8.30	8.30	-
Total phosphorus[2]	4.44	4.34	4.43	4.33	-
Non-phytate phosphorus	4.44	4.34	4.43	4.33	-
Ca: Non-phytate phosphorus	1.86	1.92	1.87	1.91	
Analysed values					
Dry matter	906	907	906	907	903
Calcium	9.89	8.03	9.71	8.25	0.18
Total phosphorus	4.34	3.54	4.45	3.64	0.27

[1]Supplied per kilogram of diet: vitamin A, 12,000 IU; cholecalciferol, 4,000 IU; thiamine, 3 mg; riboflavin, 9 mg; pyridoxine, 10 mg; folic acid, 3 mg; biotin, 0.25 mg; cyanocobalamin, 0.02 mg; dl-α-tocopherol acetate, 80 mg; niacin, 60 mg; Ca-D pantothenate, 15 mg; menadione, 4 mg; choline chloride, 600 mg; Co, 0.25 mg; I, 1.5 mg; Mo, 0.25 mg; Se, 0.26 mg; Mn, 100 mg; Cu, 10 mg; Zn, 80 mg; Fe, 60 mg; antioxidant, 100 mg.

[2]Calculated based on analysed values of MBM samples.

6.3.4. Chemical analysis

Representative samples of test diets and digesta were analysed for DM, Ca and titanium as described in Chapter 3, section 3.3. MBM samples were analysed for CP, crude fat, ash, Ca, and P as described in Chapter 3, section 3.3. The particle size distribution of MBM samples was determined as described in Chapter 3, section 3.4. Meat and bone fractions of the four MBM samples were determined as described in Chapter 5, section 5.3.4.

6.3.5. Calculations

Apparent digestibility coefficients of Ca in the test diets were calculated using the titanium ratio in the diets and digesta.

$$AIDC = 1 - [(Ti_I/Ti_O) \times (Ca_O / Ca_I)] \quad [1]$$

where AIDC is apparent ileal digestibility coefficient of Ca, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta, and Ca_I is the Ca concentration in the diet. All analysed values were expressed as gram per kilogram of DM.

Ileal endogenous Ca losses were calculated by the following formula.

$$IECaL = Ca_O \times (Ti_I/Ti_O) \quad [2]$$

where IECaL is ileal endogenous Ca losses, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta.

True ileal digestibility coefficients of Ca of the test diets were then calculated as follows:

$$TIDC = AIDC + [IECaL\ (g/kg\ of\ DMI)/Ca_I\ (g/kg\ of\ DM)] \quad [3]$$

where TIDC and AIDC represent the true ileal digestibility and apparent ileal digestibility coefficients of Ca, respectively, while IECaL represents the ileal endogenous Ca losses (g/kg of DMI) and Ca_I is the Ca concentration in the diet (g/kg of DM).

6.3.6. Statistical analysis

The data were analysed as a one-way ANOVA using the General Liner Model of SAS (2004). Cage means served as the experimental unit. Differences were considered significant at $P < 0.05$ and significant differences between means were separated by the Least Significant Difference test.

6.4. Results

Analysed Ca and P concentrations of the experimental diets are presented in Table 6.1. Analysed Ca concentrations of MBM-1 and MBM-3 diets were 1.59 and 1.41 g/kg higher while those of MBM-2 and MBM-4 diets were close to calculated values. The nutrient composition of the four MBM samples is shown in Table 6.2. Analysed Ca and P concentrations ranged between 71 and 118, and 37 and 59 g/kg, respectively.

Table 6.2. Analysed nutrient composition of the four meat and bone meal (MBM) samples (g/kg, as fed basis)[1]

	Dry matter	Crude protein	Crude fat	Ash	Calcium	Phosphorus	Ca:P ratio
MBM-1	925	536	114	237	71	37	1.91
MBM-2	934	488	93	357	118	60	1.96
MBM-3	956	474	88	362	114	59	1.92
MBM-4	953	482	128	251	81	41	1.97

[1] Samples were analysed in duplicates.

Particle size distribution of the four MBM samples is presented in Figure 6.1. In the current study, particle size distribution was classified as fine (< 0.5 mm), medium (0.5-1.0 mm) and coarse (> 1.0 mm). According to this classification, the proportions of fine, medium and coarse particles in MBM-1, MBM-2, MBM-3 and MBM-4 were 5, 41, 5 and 14, and 63, 34, 60 and 57, and 32, 25, 35 and 29%, respectively. Geometric mean diameters (GMD) of MBM-1, MBM-2 and MBM-3 were determined to be 0.866, 0.622, 0.875 and 0.781 mm, respectively. The corresponding geometric standard deviations were 1.52, 1.95, 1.51 and 1.61, respectively.

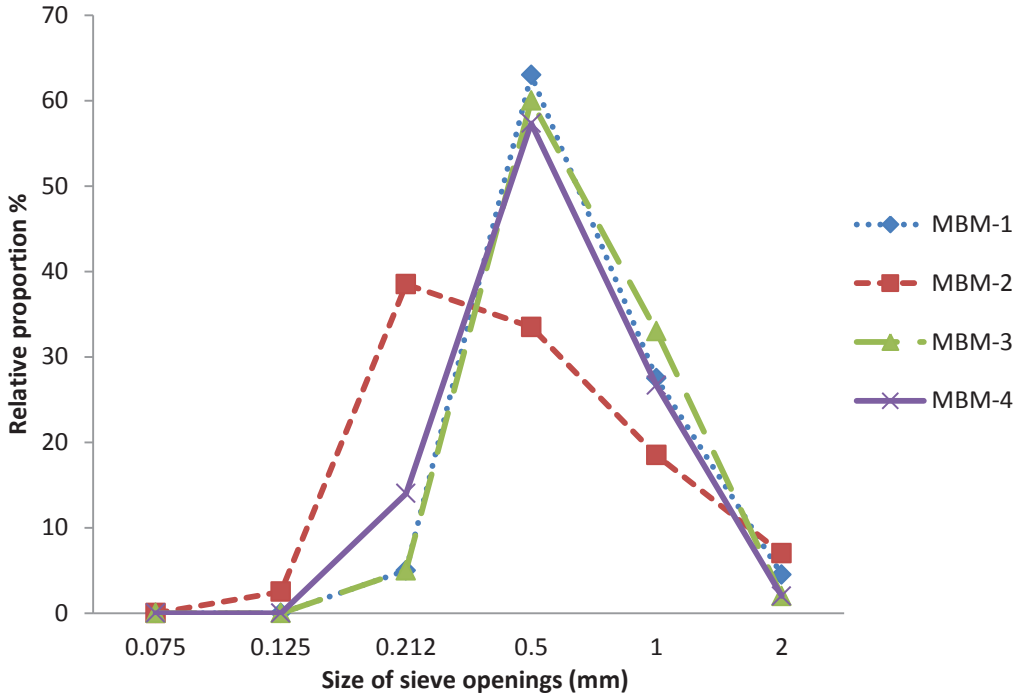

Figure 6.1. The particle size distribution of the four meat and bone meal samples

Bone and meat (soft tissue) proportions of the MBM samples are presented in Table 6.3. The bone percentages in MBM-1, MBM-2, MBM-3 and MBM-4 were observed to be 40.2, 50.5, 52.1 and 42.6%, respectively. Bone and soft tissue fractions of MBM-2 and MBM-3 were similar ($P > 0.05$) and their bone percentage was higher than MBM-1 and MBM-4. The lowest bone to soft tissue ratio was observed for MBM-1 while the highest was for MBM-3.

Table 6.3. Percentage composition of bone and soft tissue fractions and the bone to soft tissue ratio of four meat and bone meal (MBM) samples[1]

	Bone	Soft tissue	Bone : Soft tissue
MBM-1	40.2	59.8	1:1.49
MBM-2	50.5	49.5	1:0.98
MBM-3	52.1	47.9	1:0.92
MBM-4	42.6	57.4	1:1.35

[1] Samples were analysed in duplicate.

Body weight gain and feed intake of birds fed the experimental diets during the three-day experimental period are summarised in Table 6.4. Daily gain of birds fed the MBM-1 diet was higher ($P < 0.05$) than those of other MBM diets. There was no difference in the weight gain of birds fed MBM-3 and MBM-4 diets, while of that of birds fed the MBM-2 diet was lower ($P < 0.05$) than those of other diets and birds fed the MBM-1 diet had a higher ($P < 0.05$) weight gain than those of the other diets. Feed intake of birds was lower ($P < 0.05$) on MBM-2 diets compared to those of other diets.

Table 6.4. Body weight gain (g/bird/day) and feed intake (g/bird/day) of birds fed the experimental diets (20-23 days of age)[1]

	Feed intake	Weight gain
MBM-1	107[a]	46[a]
MBM-2	97[b]	28[c]
MBM-3	105[a]	35[b]
MBM-4	106[a]	37[b]
SEM[2]	1.68	1.64
Probability, *P*≤	0.001	0.001

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Pooled standard error of mean.

The apparent ileal digestibility coefficients of Ca for MBM-1, MBM-2, MBM-3 and MBM-4 were determined to be 0.55, 0.44, 0.51 and 0.40, respectively (Table 6.5). Ileal endogenous Ca losses were determined to be of 88 ± 21 mg/kg of DM intake and this value was used to calculate the true Ca digestibility coefficients.

True ileal Ca digestibility coefficients of MBM-1, MBM-2, MBM-3 and MBM-4 were 0.56, 0.45, 0.52 and 0.41, respectively (Table 6.5). The true Ca digestibility coefficient of MBM-1 was higher ($P < 0.05$) than those of MBM-2 and MBM-4, but similar ($P > 0.05$) to that of MBM-3. The true Ca digestibility of MBM-2 was similar ($P > 0.05$) to those of MBM-3 and MBM-4. The true Ca digestibility of MBM-3 was higher ($P < 0.05$) than that of MBM-4.

Table 6.5. Apparent and true ileal calcium digestibility coefficients of the four meat and bone meal (MBM) samples[1]

	Apparent ileal digestibility	True ileal digestibility
MBM-1	0.55[a]	0.56[a]
MBM-2	0.44[bc]	0.45[bc]
MBM-3	0.51[ab]	0.52[ab]
MBM-4	0.40[c]	0.41[c]
SEM[2]	0.033	0.033
Probability, *P*≤	0.03	0.03

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Pooled standard error of mean.

6.5. Discussion

As discussed in Chapter 5, section 5.5, all four samples used in current study can be categorised as MBM according to the definition of Association of American Food Control Officials (AAFCO, 2000). The Ca concentration of the four MBM samples used in the current study varied from 71 to 118 g/kg, which is within the range reported in previous studies (Waldroup 1999; Sulabo and Stein, 2013).

Ash and Ca concentrations of MBM-1, MBM-2, MBM-3 and MBM-4 were 237, 357, 362 and 251 g/kg, and 72, 118, 114 and 81 g/kg, respectively. Similar to the trend reported by Sulabo and Stein (2013), the Ca concentrations of the MBM samples were observed to be directly related to their ash content. In the present study, bone fractions of MBM-1, MBM-2 and MBM-3 were determined to be 40.2, 50.5, 52.1 and 42.6%, respectively. A direct relationship between the ash concentration and the bone fraction of the MBM samples has been described previously (Dale, 1997; Mendez and Dale, 1998).

The analysed dietary Ca concentrations of two of the four diets differed from the calculated values. These findings are difficult to interpret because the calculated dietary values were based on analysed Ca concentration of MBM samples and MBM was the only source of Ca in the experimental diets. However, the observed differences may be reflective of the difficulty in obtaining representative samples due to the particle size of Ca-containing components.

In the present study, the apparent ileal digestibility coefficient of Ca in the MBM samples ranged from 0.40 to 0.55 and the corresponding range for the true ileal digestibility coefficients were 0.41 to 0.56. A recent study with pigs has also reported wide variations in the apparent total tract digestibility of Ca in MBM, with coefficients ranging from 0.53 and 0.81 (Sulabo and Stein, 2013). Overall, these data do not support the general assumption that Ca in MBM is highly available.

In our previous study (Chapter 5), the regression method was used to determine the true Ca digestibility coefficients of three MBM samples. The digestibility estimates determined by the regression method are automatically corrected for endogenous losses and represent the true digestibility values, while the digestibility values determined by the direct method are apparent values and need to be corrected for endogenous losses. However, the direct method is less laborious, cheap and simple compared to the regression method as fewer birds are required for the direct method. True Ca digestibility coefficients of MBM samples determined by direct and regression method are presented in Table 6.6. The data from these two studies compared by two factor factorial demonstrate that there is no major difference in the true Ca digestibility of MBM determined by the two methods.

Table 6.6. True ileal calcium digestibility coefficients of meat and bone meal (MBM) samples as influenced by methodology

	True ileal digestibility	
	Direct method	Regression method
MBM-1	0.56[a]	0.60[a]
MBM-2	0.45[b]	0.46[b]
MBM-3	0.52[ab]	0.50[ab]
Probability, *P*≤	0.003	0.05

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

The factors responsible for the observed variability between MBM samples are not clear. However, ash and Ca concentration (Traylor *et al*., 2005; Sulabo and Stein, 2013) and particle size distribution (Burnell *et al*., 1989), have been reported to influence the Ca and P utilisation in pigs. The apparent digestibility coefficient of Ca in MBM for pigs has

been reported to be negatively correlated to its bone to soft tissue ratio and, concentrations of ash, Ca and P (Sulabo and Stein, 2013). In contrast, the bone to soft tissue ratio and nutrient profile of MBM-1 and MBM-4 in the present study were similar but their digestibility coefficients differed widely.

Average particle size (GMD) of MBM-1, MBM-2, MBM-3 and MBM-4 was determined to be 0.866, 0.622, 0.875 and 0.781 mm, respectively. No published data are available on the effect of particle size of the MBM on Ca utilisation in poultry. However, in pigs, P from large bone particles has been reported to be absorbed less efficiently than that from finely ground bones (Burnell *et al*., 1989). In the present study, the observed differences in the Ca digestibility of four MBM samples cannot be explained on the basis of variations in particle size, as the average particle size of MBM-2 was lower than the other three samples used, while its Ca digestibility coefficient was less than those of MBM-1 and MBM-4 but similar to that of MBM-3.

In the current study, ileal endogenous Ca losses were determined to be 88 mg/kg of DMI in birds fed a Ca and P-free diet. No previous published data are available on the ileal endogenous Ca losses in broiler chickens. In an earlier study (Chapter 4), ileal endogenous Ca losses were determined to be 125 mg/kg in broilers fed a similar Ca and P-free diet. It must be noted, however, that these endogenous flow estimates are not large in the context of undigested Ca in the ileal digesta, resulting in differences of less than 0.01 between apparent and true digestibility coefficients. A relevant question in practice, therefore, is whether the correction for endogenous Ca flow is necessary and whether the apparent value can be considered as an acceptable estimate of Ca digestibility in ingredients for poultry. The recommendation is to use apparent values as there is not much difference, however further studies are warranted to confirm it.

6.6. Conclusions

In conclusion, the present study indicates that the direct method can be successfully used for the estimation of true Ca digestibility of MBM. In the four MBM samples under test, the true ileal Ca digestibility coefficients ranged between 0.41 and 0.56, indicating that the Ca in MBM is not highly available. The observed variability in Ca digestibility in the MBM samples cannot be attributed to ash, Ca and bone content or mean particle size.

CHAPTER 7

Measurement of true ileal calcium digestibility in limestone for broiler chickens

7.1. Abstract

The objective of this study was to determine the true calcium (Ca) digestibility of limestone in broiler chickens as influenced by limestone source and dietary phosphorus (P) concentration. Limestone from three commercial sources (coded as LM-1, LM-2 and LM-3) were obtained, ground to pass through a 0.2 mm sieve and analysed for mineral composition and *in vitro* solubility. Analysed Ca concentrations and *in vitro* solubility coefficients of LM-1, LM-2 and LM-3 were 410, 390 and 420 g/kg, and 0.28, 0.29 and 0.27, respectively. Two experimental diets, containing 9 g/kg Ca, were developed from each limestone source with 0 and 4.5 g/kg dietary P. Titanium dioxide (3 g/kg) was incorporated as an indigestible marker. Each experimental diet was then randomly allotted to six replicate cages (eight birds per cage) and fed from day 21 to 24 post-hatch. The apparent ileal digestibility of Ca was calculated using the indicator method and corrected for endogenous Ca losses to determine the true Ca digestibility. Ileal endogenous Ca losses of 125 mg/kg of dry matter intake determined by feeding a Ca and P-free diet in a previous study (Chapter 4) were used. The true Ca digestibility coefficients of LM-1, LM-2 and LM-3 were determined to be 0.60, 0.62 and 0.56, respectively. True Ca digestibility of LM-3 was lower ($P < 0.05$) than those of LM-1 and LM-2. There was no difference ($P > 0.05$) between the true Ca digestibility of LM-1 and LM-2. Increasing the dietary P concentration from 0 to 4.5 g/kg in assay diets increased ($P < 0.05$) the true ileal Ca digestibility.

7.2. Introduction

Limestone is the major inorganic calcium (Ca) source used in poultry diets and contains 380 g/kg Ca (NRC, 1994). Determination of Ca digestibility has not been considered in the past due to the cheap availability and surplus global reserves of limestone. A recent initiative towards the use of digestible phosphorus (P) values in feed formulations (WPSA, 2013) has attracted interest in the measurement of the digestible Ca contents of

feedstuffs, because of the adverse effects of high dietary Ca levels on the availability of P (Ballam *et al*., 1984; Tamim and Angel, 2003; Plumstead *et al*., 2008) and other biologically important minerals such as zinc, magnesium and iron (Shafey *et al*., 1991). On the other hand, lower dietary P concentrations have also been reported to reduce Ca availability (Sebastian *et al*., 1996b; Viveros *et al*., 2002). The ratio between dietary Ca to P is critical for the absorption and post-absorptive utilisation of both minerals. Calcium in plasma is tightly regulated, and Ca or P deficient diets can influence the Ca homeostasis mechanism and can affect the estimation of digestible Ca contents of test ingredients (Proszkowiec-Weglarz and Angel, 2013). A Ca to non-phytate P ratio of 2:1 is generally accepted in poultry feed formulations (NRC, 1994), but it is probable that the move towards digestible values may change this ratio.

Historically Ca availability has been reported in terms of bioavailability relative to calcium carbonate. Although it is generally assumed that Ca from different Ca sources is highly available (Blair *et al*, 1965; Peeler, 1972; Reid and Weber, 1976), our previous studies (Chapters 5 and 6) have demonstrated that the true ileal Ca digestibility of meat and bone meal (MBM) for broilers only ranged from 0.41 and 0.60. A similar scenario may exist with limestone.

In vitro solubility has been used by the feed industry as an indicator of Ca availability in limestone (Cheng and Coon, 1990a). However, a number of techniques have been employed to determine the *in vitro* solubility of limestone and these include measurement of percentage weight loss, pH change, proton consumption, percentage hydrogen ion disappearance and pH plateau time (Cheng and Coon, 1990b). Of these, percentage weight loss is the most commonly used method. An inverse relationship between *in vitro* and *in vivo* solubilities of limestone has been observed (Zhang and Coon, 1997a; de Witt *et al*., 2006), with limestones of low *in vitro* solubility staying longer in the gizzard and, thus showing increased *in vivo* solubility and Ca availability in layer hens.

Currently there is no established method available for the determination of Ca digestibility in poultry. In our previous studies (Chapters 5 and 6), using regression and direct methods were used to determine the true Ca digestibility in MBM for broiler chickens and it was observed that both methods yielded comparable true Ca digestibility coefficients. The regression method is laborious, costly and time consuming because

each ingredient needs to be tested at least three inclusion levels to develop a regression line. On the other hand, the direct method is simple, but determines the apparent digestibility and a correction for endogenous Ca losses is required to calculate the true Ca digestibility. Recently, direct method has been used to determine the Ca digestibility of MBM in pigs (Sulabo and Stein, 2013). In the present study, the influence of the source of limestone, with and without dietary P concentrations on the true Ca digestibility of limestone for broiler chickens was determined using the direct method.

7.3. Materials and methods

The experiment was conducted according to the New Zealand Revised Code of Ethical Conduct for the use of live animals for research, testing and teaching, and approved by the Massey University Animal Ethics Committee.

7.3.1. Diets and experimental design

Limestone from three commercial sources (coded as LM-1, LM-2 and LM-3) were obtained, ground to pass through 0.2 mm sieve and analysed for mineral composition and *in vitro* solubility. Two experimental diets were developed from each limestone with 0 and 4.5 g/kg dietary P (Table 7.1). The limestone served as the sole source of dietary Ca in the experimental diets, and the inclusion level of limestone was set to maintain the recommended dietary Ca concentration (9 g/kg) for broiler growers (Ross, 2007). In diets supplemented with P, the Ca:non-phytate P ratio was maintained at 2:1. Titanium dioxide (3 g/kg) was incorporated in all diets as an indigestible marker.

7.3.2. Birds

Day-old male broilers (Ross 308) were obtained from a local hatchery and raised as described in Chapter 3, section 3.1. On day 14, birds were moved to colony cages to acclimatise them. Between days 14 and 20, the crumble starter diet was gradually changed to a mash-based broiler starter diet as the experimental diets were in mash form. On day 21, the birds were individually weighed and allocated to 36 cages (eight birds per cage) on weight basis so that the average bird weight per cage was similar. The six experimental diets were then randomly allotted to six replicate cages each. The diets, in mash form, were offered *ad libitum* from day 21 to day 24 post-hatch and the birds had free access to water. Group body weights and feed intake were recorded on days 21 and 24.

Table 7.1. Ingredient composition and analysis (g/kg as fed basis) of experimental diets

	Limestone source (with 0 g/kg P)			Limestone source (with 4.5 g/kg P)		
	1	2	3	1	2	3
Maize starch	391.95	391.95	391.95	387.4	387.4	387.4
Dextrose	391.95	391.95	391.95	387.4	387.4	387.4
Dried egg albumen	100	100	100	100	100	100
Cellulose	50	50	50	50	50	50
Limestone	23.6	23.6	23.6	23.6	23.6	23.6
Soybean oil	20	20	20	20	20	20
Monosodium phosphate	-	-	-	20	20	20
Potassium bicarbonate	9.2	9.2	9.2	2.3	2.3	2.3
Sodium bicarbonate	6	6	6	-	-	-
Sodium chloride	2	2	2	-	-	-
Potassium chloride	-	-	-	4	4	4
Titanium dioxide	3	3	3	3	3	3
Trace mineral-vitamin premix[1]	2.3	2.3	2.3	2.3	2.3	2.3
Calculated Analysis						
Metabolisable energy, (MJ/kg)	15.02	15.02	15.02	14.87	14.87	14.87
Crude protein	83.00	83.00	83.00	83.00	83.00	83.00
Calcium[2]	9.01	9.01	9.01	9.01	9.01	9.01
Total phosphorus[2]	0.12	0.12	0.12	4.48	4.48	4.48
Non-phytate phosphorus	0.12	0.12	0.12	4.48	4.48	4.48
Calcium:non-phytate phosphorus	-	-	-	2:1	2:1	2:1
Analysed values (as fed basis)						
Dry matter	906	914	902	915	904	917
Calcium	8.52	8.69	9.16	8.76	8.65	8.69

[1]Supplied per kilogram of diet: vitamin A, 12,000 IU; cholecalciferol, 4,000 IU; thiamine, 3 mg; riboflavin, 9 mg; pyridoxine, 10 mg; folic acid, 3 mg; biotin, 0.25 mg; cyanocobalamin, 0.02 mg; dl-α-tocopherol acetate, 80 mg; niacin, 60 mg; Ca-D pantothenate, 15 mg; menadione, 4 mg; choline chloride, 600 mg; Co, 0.25 mg; I, 1.5 mg; Mo, 0.25 mg; Se, 0.26 mg; Mn, 100 mg; Cu, 10 mg; Zn, 80 mg; Fe, 60 mg; antioxidant, 100 mg.

[2]Calculated based on NRC (1994) values.

7.3.3. Digesta collection and processing

On day 24, all birds were euthanised by intravenous injection (1ml per 2 kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand) and, ileal digesta were collected and processed as described in Chapter 3, section 3.2.

7.3.4. Chemical analysis

Representative samples of diets and ileal digesta were analysed for DM, Ca and titanium as described in Chapter 3, section 3.3. For mineral analysis, the limestone samples were wet acid digested with nitric and perchloric acid mixture, and concentrations of Ca, P, potassium, magnesium, sodium, iron, aluminium, arsenic, cadmium, lead and mercury were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) using a Thermo Jarrell Ash IRIS instrument (Thermo Jarrell Ash Corporation, Franklin, MA). The concentrations of copper, manganese and zinc were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) using a Perkin Elmer Elan 6000 instrument (Perkin Elmer, Melbourne, Victoria, Australia).

In vitro solubility of the limestone samples was determined by two weight loss methods described by Cheng and Coon (1990b) and Zhang and Coon (1997b), respectively. In the first method, hydrochloric acid (0.1 N; 100 ml) was heated for 15 minutes at 42 $^{\circ}$C in a water bath oscillating at 60 hertz and the limestone sample (2g) was added. After 10 minutes, the contents were filtered through Whatman 42 filter paper, dried at 70 $^{\circ}$C for 10 hours, cooled and weighed to determine the percent weight loss. In the second method, 0.2 N; 200ml hydrochloric acid was used instead of the 0.1 N 100ml hydrochloric acid.

7.3.5. Calculations

Apparent digestibility coefficients of Ca of limestone were calculated using the indigestible marker ratios.

$$AIDC = 1 - [(Ti_I/Ti_O) \times (Ca_O / Ca_I)]$$

where AIDC is apparent ileal digestibility coefficient of Ca, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta, and Ca_I is the Ca concentration in the diet. All analysed values were expressed as gram per kilogram of DM.

True digestibility coefficients of Ca were calculated by the following formula.

TIDC = AIDC + [IECaL (g/kg of DMI)/Ca_I (g/kg of DM)]

where TIDC is true ileal digestibility coefficient of Ca, AIDC is apparent ileal digestibility coefficient of Ca, IECaL is ileal endogenous Ca losses (g/kg of DMI) and Ca_I is the Ca concentration in the diet (g/kg of DM). Ileal endogenous calcium loss estimate of 125 mg/kg DM intake determined in Chapter 4 using a Ca and P-free diet was used.

7.3.6. Statistical analysis

The data were analysed as a 2×3 factorial arrangement of treatments using the general linear model procedure of SAS (2004). Cages served as the experimental unit and differences were considered to be significant at $P < 0.05$. Significant differences between means were separated by Least Significant Difference test.

7.4. Results

The analysed dietary Ca concentrations of five out of six experimental diets were 0.25 to 0.49 g/kg lower than the calculated values (Table 7.1). In one diet, the analysed value was 0.15 g/kg higher than calculated. The analysed values were used to determine the digestibility coefficients of the three limestone samples.

The mineral composition of the three limestone samples is presented in Table 7.2. Analysed Ca concentrations of LM-1, LM-2 and LM-3 were 410, 390 and 420 g/kg, respectively. Magnesium, iron and aluminium concentrations of LM-2 were higher than LM-1 and LM-3.

In vitro solubility coefficients of LM-1, LM-2 and LM3 were comparable and determined to be 0.28, 0.29 and 0.27, respectively. The corresponding values with 0.2 N, 200 ml hydrochloric acid were 0.60, 0.57 and 0.53, respectively.

Daily weight gain and feed intake of birds fed the experimental diets during the 3-day trial period are presented in Table 7.3. Body weight gain was not influenced ($P > 0.05$) by the limestone source, but there was a tendency ($P=0.07$) for dietary P concentration to affect weight gain. Birds fed diets with no P lost weight, whereas those fed diets with P tended to maintain body weight. Feed intake was not affected ($P > 0.05$) by limestone source or dietary P concentration. No interaction ($P > 0.05$) was observed between the limestone source and dietary P concentration.

The apparent ileal Ca digestibility coefficients of LM-1, LM-2 and LM-3 were 0.58, 0.61 and 0.54, respectively (Table 7.4). True ileal Ca digestibility coefficients were determined to be 0.60, 0.62 and 0.56 for LM-1, LM-2 and LM-3 respectively (Table 7.4). Apparent and true Ca digestibility of LM-3 was lower ($P < 0.05$) than those of LM-1 and LM-2, while no difference ($P > 0.05$) was observed between the digestibility of LM-1 and LM-2. The increase in dietary P concentration increased ($P < 0.05$) the ileal Ca digestibility. There was no interaction ($P > 0.05$) between the limestone source and dietary P concentration.

Table 7.2. Mineral analysis of limestone samples (as received basis)[1]

	Limestone-1	Limestone-2	Limestone-3
Macro-minerals (g/kg)			
Calcium	410	390	420
Magnesium	2.10	3.40	1.89
Potassium	<0.40	<0.40	<0.40
Sodium	0.70	<0.50	<0.50
Phosphorus	0.49	0.32	<0.20
Micro-minerals (mg/kg)			
Manganese	42	114	44
Zinc	13	19	<10
Copper	0.7	2.0	<0.5
Iron	1460	5000	420
Aluminium	360	1440	71
Cadmium	0.10	0.58	0.53
Lead	5.3	2.8	0.31
Arsenic	<1.0	10.3	<1.0
Mercury	<0.10	<0.10	<0.10

[1] Samples were analysed in duplicates.

Table 7.3. Body weight gain (g/bird/day) and feed intake (g/bird/day) of broilers as influenced by limestone source and dietary phosphorus concentration (21-24 days of age)[1]

	Phosphorus inclusion (g/kg)	Weight gain	Feed intake
Limestone-1	0	-2.8	69
	4.5	4.4	70
Limestone-2	0	-5.3	63
	4.5	0.3	61
Limestone-3	0	-6.5	60
	4.5	1.7	62
SEM[2]		4.49	6.89
Main Effects			
Limestone source			
Limestone-1		0.8	69
Limestone-2		-2.5	62
Limestone-3		-2.4	61
SEM[2]		3.17	4.87
Dietary phosphorus (g/kg)			
0		-4.9	64
4.5		2.1	64
SEM[2]		2.59	3.98
Probabilities, *P* ≤			
Limestone source		0.71	0.44
Dietary phosphorus		0.07	0.95
Limestone source x Dietary phosphorus		0.96	0.96

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Pooled standard error of mean.

Table 7.4. The effect of limestone source and dietary phosphorus concentration on apparent and true calcium digestibility in broiler chickens[1]

	Dietary phosphorus concentration (g/kg)	Apparent ileal calcium digestibility coefficient	True ileal calcium digestibility coefficient
Limestone-1	0	0.55	0.56
	4.5	0.62	0.63
Limestone-2	0	0.61	0.63
	4.5	0.60	0.62
Limestone-3	0	0.51	0.53
	4.5	0.57	0.59
SEM[2]		0.02	0.02
Main Effects			
Limestone source			
Limestone-1		0.58[a]	0.60[a]
Limestone-2		0.61[a]	0.62[a]
Limestone-3		0.54[b]	0.56[b]
SEM[2]		0.01	0.01
Dietary phosphorus (g/kg)			
0		0.56[a]	0.57[a]
4.5		0.60[b]	0.61[b]
SEM[2]		0.01	0.01
Probabilities, $P \leq$			
Limestone source		0.006	0.003
Dietary phosphorus		0.02	0.02
Limestone source x Dietary phosphorus		0.16	0.16

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Pooled standard error of mean.

7.5. Discussion

According to NRC (1994), the average Ca concentration in limestone is 380 g/kg. The analysed Ca concentration of three limestone samples was 10-30 g/kg higher than the NRC values. Variation in the Ca concentration of limestone has been previously reported (Reid and Weber, 1976; Browning and Cowieson, 2013; Wilkinson *et al*., 2013a). In these studies, Ca concentrations of limestone were reported to vary from 360

to 415 g/kg. The concentration of zinc, manganese and iron were reported to vary from 6 to 32, 40 to 414 and 131 to 1137 mg/kg, respectively. Similar variations in the mineral composition of limestone samples were observed in the current study.

In vitro solubility of the three limestone samples varied from 0.27 to 0.29. These values are comparable to those reported by Cheng and Coon (1990a), where *in vitro* solubility of nine limestone samples, determined by the same methodology, varied from 0.25 to 0.28. It must be noted that the *in vitro* solubility of limestone differs depending on the methodology used (Cheng and Coon, 1990a) and, higher solubility has been reported with increased normality and volume of hydrochloric acid (Zhang and Coon, 1997b). Similar results have been observed in the current study where *in vitro* solubility coefficients of the three limestone samples varied from 0.53 to 0.60 with higher normality (0.2) and volume (200ml) of hydrochloric acid.

In current study, the apparent ileal Ca digestibility coefficients were determined to be 0.58, 0.61 and 0.54 for LM-1, LM-2 and LM-3, respectively. Using the direct method, Proszkowiec-Weglarz *et al*., (2013) determined the apparent digestibility of limestone at different time intervals after feeding and found that the apparent digestibility of Ca decreased from 0.64 to 0.37 (24-32 hours after feeding) and then reduced to 0.48 (72-96 hours after feeding).

True Ca digestibility coefficients were determined to be 0.60, 0.62 and 0.56 for LM-1, LM-2 and LM-3, respectively. Currently there is no comparable published data on the true Ca digestibility of limestone for broiler chickens. Calcium availability has been historically described in terms of relative bioavailability (Blair *et al*, 1965; Peeler, 1972; Reid and Weber, 1976). Blair *et al*. (1965) reported a 102% relative bioavailability of Ca in limestone as compared to calcium carbonate. Relative bioavailability of Ca has also been reported to vary from 73 to 109% in broiler chickens for five limestone samples (Reid and Weber, 1976). The present data demonstrate that the true Ca digestibility in limestone is lower than the relative bioavailability estimates.

It is difficult to explain the reasons for the observed variability in Ca digestibility between the three limestone samples. Larger limestone particles have been reported to stay longer in the gizzard which increases their *in vivo* solubility and Ca availability (Zhang and Coon, 1997a; de Witt *et al*., 2006). There were no differences in the particle size of limestone samples in the current study since all three samples were ground to pass through 0.2 mm sieve, so the difference in Ca digestibility cannot be attributed to particle size. *In vitro* solubility of limestone has been reported to be inversely related to

its *in vivo* solubility (Zhang and Coon, 1997a; de Witt *et al*., 2006). In the current work, however, *in vitro* solubilities of limestone samples were similar and thus this does not explain the differences in Ca digestibility.

Addition of 4.5 g/kg dietary P increased the true ileal Ca digestibility as compared to diets without P. The average true Ca digestibility coefficients, without and with P, were 0.57 and 0.61, respectively. These findings are in agreement with previous studies where low dietary P concentrations have been reported to reduce Ca availability in poultry (Sebastian *et al*., 1996b; Viveros *et al*., 2002). Owing to the close relationship between Ca and P, the practical implication is that assay diets used in Ca digestibility studies with limestone should contain supplemental P.

7.6. Conclusions

In conclusion, the present data suggests that the true Ca digestibility of limestone for broiler chickens is not as high as previously assumed. The true Ca digestibility of the three limestone samples varied between 0.56 and 0.62, and the observed variability cannot be explained on the basis of *in vitro* solubility. The addition of P to assay diets increased the true Ca digestibility estimate.

CHAPTER 8

Effect of particle size and calcium to non-phytate phosphorus ratio on true calcium digestibility of limestone for broiler chickens

8.1. Abstract

The purpose of this study was to determine the effect of particle size and calcium (Ca) to non-phytate phosphorus (P) ratio on the true Ca digestibility of limestone for broiler chickens. A limestone sample was obtained from a commercial source, ground and then passed through a set of sieves to obtain fine (<0.5 mm) and coarse (1-2 mm) particles. The analysed Ca concentration of both particle sizes was similar (420 g/kg). Six experimental diets were developed using the two particle sizes with Ca:non-phytate P ratios of 1.5:1, 2.0:1 and 2.5:1, with ratios being adjusted by manipulating the dietary Ca concentrations. A Ca and P-free diet was also developed to determine the ileal endogenous Ca losses. Titanium dioxide (3 g/kg) was incorporated in all diets as an indigestible marker. Each experimental diet was then randomly allotted to six replicate cages (eight birds per cage) and fed from day 21 to 24 post-hatch. Apparent ileal digestibility of Ca was calculated using the indicator method and corrected for endogenous Ca losses to determine the true Ca digestibility. Ileal endogenous Ca losses were determined to be 127 mg/kg of dry matter intake. Increasing Ca:non-phytate P ratios reduced ($P < 0.05$) the true Ca digestibility of limestone. The true Ca digestibility coefficients of limestone with Ca:non-phytate P ratios of 1.5:1, 2.0:1 and 2.5:1 were 0.65, 0.57 and 0.49, respectively. The particle size of limestone influenced ($P < 0.05$) the Ca digestibility, with the digestibility being higher in coarse particles (0.71 vs. 0.43).

8.2. Introduction

Limestone is a major inorganic calcium (Ca) source and is extensively used in poultry diets throughout the world. Calcium from different Ca sources is generally assumed to be highly available (Blair *et al*, 1965; Peeler, 1972; Reid and Weber, 1976), but our previous studies (Chapters 5, 6 and 7) have demonstrated that the true Ca digestibility of meat and bone meal and limestone is not high, varying from 0.45 to 0.60 and 0.56 to

0.62, respectively. In the past, Ca availability in poultry has been described on the basis of egg shell characteristics (weight and thickness), Ca retention and bone mineralisation. Egg shell weight and thickness, and bone breaking force have been reported to increase with increasing particle size of limestone in layer diets (Cheng and Coon, 1990a). Larger particles have been reported to stay longer in the gizzard, increasing their *in vivo* solubility and utilisation (Zhang and Coon, 1997a). The effects of particle size of limestone on true ileal Ca digestibility in broiler chickens are yet to be explored.

In vitro solubility of limestone is influenced by its source and particle size (Cheng and Coon, 1990b; Zhang and Coon, 1997a; de Witt *et al*., 2006; Manangi and Coon, 2007). It has been reported that the *in vitro* solubility of limestone is negatively related to the *in vivo* solubility and availability of Ca in laying hens (Cheng and Coon, 1990a; Zhang and Coon, 1997a).

Dietary Ca and phosphorus (P) concentrations are critical for the absorption and post-absorptive utilisation of both minerals. Diets deficient in Ca or P reduce their plasma concentrations (Proszkowiec-Weglarz and Angel, 2013). Low plasma Ca or P concentration increase the production of parathyroid hormone (PTH) and production of 1,25-dihyroxy cholecalciferol (1, 25$(OH)_2D_3$) enhancing the intestinal Ca absorption (Adedokun and Adeola, 2013; Proszkowiec-Weglarz and Angel, 2013). Low dietary P also increases the production of Ca binding protein and increases the intestinal Ca absorption (Bar and Wasserman, 1973). High dietary Ca concentrations, on the other hand, have been reported to reduce the apparent ileal digestibility (Plumstead *et al*., 2008) and retention of Ca in broiler chickens (Sebastian *et al*., 1996a; Pintar *et al*., 2005). Both dietary Ca concentrations and Ca:non-phytate P ratios are critical for the absorption and utilisation of these two minerals. A Ca:non-phytate P ratio of 2:1 is considered optimal in poultry feed formulations (NRC, 1994), but it can be expected that the move towards digestible values may result in an attraction in digestible Ca to digestible P ratio.

The objective of the present study was to determine the effect of the particle size of limestone and Ca:non-phytate P ratio on the true Ca digestibility of limestone for broiler chickens.

8.3. Materials and methods

The experiment was conducted according to the New Zealand Revised Code of Ethical Conduct for the use of live animals for research, testing and teaching, and approved by the Massey University Animal Ethics Committee.

8.3.1. Diets and experimental design

A limestone sample was obtained from a commercial source, ground and passed through a set of sieves to obtain two particle sizes, namely fine (<0.5 mm) and coarse (1-2 mm) particles. Representative samples were analysed for mineral composition and *in vitro* solubility. Six experimental diets, with two particle sizes and three Ca:non-phytate P ratios (1.5:1, 2.0:1 and 2.5:1) were developed (Table 8.1). Limestone served as the sole source of dietary Ca in the experimental diets and the Ca to P ratio was adjusted by changing the inclusion level of limestone. A Ca and P-free diet was also developed to determine the ileal endogenous Ca losses. Titanium dioxide (3 g/kg) was incorporated in all diets as an indigestible marker.

8.3.2. Birds

Day-old male broilers (Ross 308) were obtained from a local hatchery and, raised on the floor and fed a commercial starter crumble as described in Chapter 3, section 3.1. On day 14, birds were moved to grower cages to acclimatise them. Between days 14 and 20, the crumble starter diet was gradually changed to mash as the experimental diets were in mash form. On day 21, the birds were individually weighed and allocated to 42 cages (eight birds per cage) on a weight basis so that the average bird weight per cage was similar. The seven experimental diets were then randomly allotted to six replicate cages each. The diets, in mash form, were offered *ad libitum* from day 21 to day 24 post-hatch and the birds had free access to water. Group body weights and feed intake were recorded on days 21 and 24.

8.3.3. Sample collection and processing

On day 24, all birds were euthanised by intravenous injection (1ml per 2 kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand), and ileal digesta were collected and processed as described in Chapter 3, section 3.2.

8.3.4. Chemical analysis

Representative samples of diets and ileal digesta were analysed for DM, Ca and titanium as described in Chapter 3, section 3.3. Mineral analysis of limestone was carried out as described in Chapter 7, section 7.3.4.

In vitro solubility of limestone samples was determined by the weight loss method, as described by Zhang and Coon (1997b). Hydrochloric acid (0.2 N; 200 ml) was heated for 15 minutes at 42 °C in a water bath oscillating at 60 Hertz and the limestone sample (2g) was added. After 10 minutes, the contents were filtered through a Whatman 42 filter paper, dried at 70 °C for 10 hours, cooled and weighed to determine the percentage weight loss.

8.3.5. Calculations

Apparent ileal digestibility coefficients of Ca were calculated using titanium ratio in the diets and digesta.

$$AIDC = 1 - [(Ti_I/Ti_O) \times (Ca_O / Ca_I)]$$

where AIDC is apparent ileal digestibility coefficient of Ca, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta, and Ca_I is the Ca concentration in the diet. All analysed values were expressed as gram per kilogram of DM.

Ileal endogenous Ca losses (g/kg DM intake) were calculated by the following formula.

$$IECaL = Ca_O \times (Ti_I/Ti_O)$$

where IECaL is ileal endogenous Ca losses, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta and Ca_O is the Ca concentration in the ileal digesta.

Table 8.1. Ingredient composition and analysis (g/kg as fed basis) of experimental diets

	Ca:non-phytate P, 1.5:1		Ca:non-phytate P, 2:1		Ca:non-phytate P, 2.5:1		Ca and P-free
	<0.5 mm	1.0-2.0 mm	<0.5 mm	1.0-2.0 mm	<0.50 mm	1.0-2.0 mm	diet
Maize starch	393.86	393.86	391.18	391.18	388.50	388.50	405.05
Dextrose	393.86	393.86	391.18	391.18	388.50	388.50	405.05
Dried egg albumen	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Soybean oil	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Limestone	15.98	15.98	21.33	21.33	26.69	26.69	-
Potassium bicarbonate	-	-	-	-	-	-	8.5
Sodium bicarbonate	-	-	-	-	-	-	5.0
Potassium chloride	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Mono-potassium phosphate	11.9	11.9	11.9	11.9	11.9	11.9	-
Mono-sodium phosphate	8.0	8.0	8.0	8.0	8.0	8.0	-
Titanium dioxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Trace mineral-vitamin premix[1]	2.3	2.3	2.3	2.3	2.3	2.3	2.3
Calculated Analysis							
Metabolisable energy (MJ/kg)	15.05	15.05	14.96	14.96	14.86	14.86	15.44
Crude protein	83.00	83.00	83.00	83.00	83.00	83.00	83.00
Calcium[2]	6.75	6.75	9.00	9.00	11.25	11.25	0.05
Total phosphorus	4.51	4.51	4.51	4.51	4.51	4.51	0.18
Non-phytate phosphorus	4.51	4.51	4.51	4.51	4.51	4.51	-
Ca: non-phytate P ratio	1.50	1.50	2.00	2.00	2.50	2.50	-
Analysed values (as fed basis)							
Dry matter	911	912	909	914	914	916	912
Calcium	6.44	6.90	8.22	9.17	11.00	11.12	0.15

[1]Supplied per kilogram of diet: vitamin A, 12,000 IU; cholecalciferol, 4,000 IU; thiamine, 3 mg; riboflavin, 9 mg; pyridoxine, 10 mg; folic acid, 3 mg; biotin, 0.25 mg; cyanocobalamin, 0.02 mg; dl-α-tocopherol acetate, 80 mg; niacin, 60 mg; Ca-D pantothenate, 15 mg; menadione, 4 mg; choline chloride, 600 mg; Co, 0.25 mg; I, 1.5 mg; Mo, 0.25 mg; Se, 0.26 mg; Mn, 100 mg; Cu, 10 mg; Zn, 80 mg; Fe, 60 mg; antioxidant, 100 mg.

[2] Calculated based on analysed values of limestone samples.

True ileal digestibility Ca coefficient was then calculated as follows:

$$TIDC = AIDC + [IECaL\ (g/kg\ of\ DMI)/Ca_I\ (g/kg\ of\ DM)]$$

where TIDC and AIDC represent the true ileal digestibility and apparent ileal digestibility coefficients of Ca, respectively, while IECaL represents the ileal endogenous Ca losses (g/kg of DMI) and Ca_I represents the Ca concentration in the diet (g/kg of DM).

8.3.6. Statistical analysis

The data were analysed as a 2×3 factorial arrangement of treatments using the general linear model procedure of SAS (2004). Cages served as the experimental unit and differences were considered to be significant at $P < 0.05$. Significant differences between means were separated by Least Significant Difference test.

8.4. Results

Analysed Ca concentrations of the experimental diets are presented in Table 8.1. The analysed dietary Ca concentrations of the experimental diets were close to calculated values in five of the six experimental diets. In one diet, the analysed Ca concentration was 0.78 g/kg lower than the calculated value. Analysed values were used to determine the digestibility coefficients of the three limestone samples.

The mineral composition of the two limestone samples are presented in Table 8.2. The Ca concentrations of fine and coarse limestone particle sizes (<0.5 and 1-2 mm) were similar and determined to be 420 g/kg. There was no major difference in the concentrations of other minerals in the two particle sizes. *In vitro* solubility coefficients of fine and coarse limestone particle were determined to be 0.60 and 0.33, respectively.

Daily weight gain and feed intake of birds fed the experimental diets during the 3-day trial period are presented in Table 8.3. Body weight gain and feed intake were not influenced ($P > 0.05$) by the limestone particle size or Ca:non-phytate P ratio. There was no interaction ($P > 0.05$) between the limestone particle size and Ca:non-phytate P ratio for weight gain and feed intake.

Table 8.2. Mineral analysis of limestone samples (as received basis)[1]

	Particle size, <0.5mm	Particle size, 1-2 mm
Dry matter (g/kg)	998	998
Macro minerals (g/kg)		
Calcium	420	420
Magnesium	2.10	2.20
Potassium	<0.40	<0.40
Sodium	1.21	1.13
Phosphorus	0.23	0.23
Micro minerals (mg/kg)		
Manganese	28	27
Zinc	<10	<10
Copper	<0.5	<0.5
Iron	780	782
Aluminium	151	187
Cadmium	0.09	0.08
Lead	1.48	1.42
Arsenic	<1.0	10.3
Mercury	<0.10	<0.10

[1]Samples were analysed in duplicate.

The influence of dietary Ca:non-phytate P ratio and particle size of limestone on apparent and true Ca digestibility are shown in Table 8.4. Significant main effects ($P < 0.05$) of Ca:non-phytate P ratio and particle size of limestone were observed for both the apparent and true Ca digestibility. Widening the dietary Ca:non-phytate P ratio from 1.5 to 2.5 decreased the apparent and true Ca digestibility of limestone, whilst increasing the particle size from <0.5 to 1-2 mm increased the digestibility. No interaction ($P > 0.5$) was observed between Ca:non-phytate P ratio and particle size for apparent and true Ca digestibility estimates. Apparent and true ileal Ca digestibility coefficients of limestone at Ca:non-phytate P ratios of 1.5:1, 2.0:1 and 2.5:1 were 0.63, 0.56 and 0.48, and 0.65, 0.57 and 0.49, respectively. The apparent and true Ca digestibility coefficients for fine and coarse particles were 0.42 and 0.70, and 0.43 and 0.71, respectively.

Ileal endogenous Ca losses, following the feeding of Ca- and P-free diet, were determined to be (mean ± SE) 127 ± 12 mg/kg of dry matter intake.

Table 8.3. Weight gain (g/bird/day) and feed intake (g/bird/day) of broilers as influenced by calcium to non-phytate phosphorus ratio and particle size of limestone (21-24 days of age)[1, 2]

Ca:non-phytate P ratio	Particle size (mm)	Weight gain	Feed intake
1.5	<0.5	18	98
	1-2	17	97
2.0	<0.5	17	98
	1-2	15	97
2.5	<0.5	15	95
	1-2	15	96
SEM[3]		2.12	2.92
Main Effects			
Ca:non-phytate P ratio			
1.5		18	98
2.0		15	97
2.5		16	96
SEM[3]		1.50	2.07
Particle size (mm)			
<0.5		17	97
1-2		16	97
SEM[3]		1.23	1.69
Probabilities, *P* ≤			
Ca:non-phytate P ratio		0.51	0.77
Particle size		0.52	0.81
Ca:non-phytate P ratio x Particle size		0.82	0.93

[1] Each treatment value represents the mean of six replicates (eight birds per replicate).

[2] Body weight gain and feed intake of birds the on Ca and P-free diet were 16 and 96 g/bird/day, respectively.

[3] Pooled standard error of mean.

Table 8.4. Effect of calcium to non-phytate phosphorus ratio and particle size of limestone on apparent and true calcium digestibility in broiler chickens[1]

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

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

[1] Each treatment value represents the mean of six replicates (eight birds per replicate).

[2] Pooled standard error of mean.

8.5. Discussion

The analysed Ca concentration of limestone with particle sizes of <0.5 and 1-2 mm was similar, but 40 g/kg higher than the NRC (1994) value of 380 g/kg. The present results are consistent with published data, wherein Ca concentrations of limestone have been reported to vary between 360 and 415 g/kg (Reid and Weber, 1976; Browning and Cowieson, 2013; Wilkinson *et al*., 2013a).

The present data demonstrated that increasing dietary Ca concentrations and/or widening Ca:non-phytate P ratios have a negative influence on Ca digestibility. In the current study, the different Ca:non-phytate P ratios were achieved by manipulating dietary Ca concentrations (6.75, 9.0 and 11.25 g/kg diet), while keeping the dietary P concentration constant (4.5 g/kg). A decrease in Ca retention with increasing dietary Ca concentrations has been reported previously. Sebastian *et al*. (1996a) observed a reduction in Ca retention in broilers with increasing dietary Ca concentrations. Calcium retention coefficients were 0.65, 0.49 and 0.43 on diets with 6.0, 10.0 and 12.5 g/kg Ca, respectively. Pintar *et al*. (2005) also found that Ca retention in broilers was decreased when the dietary Ca concentration increased from 6.0 to 10 g/kg of diet. These findings are also consistent with the findings of Plumstead *et al*. (2008), where the apparent ileal digestibility of Ca decreased from 0.47 to 0.37 when the dietary Ca concentration increased from 4.7 to 11.6 g/kg in broiler diets.

Two explanations may be provided for the observed effects of dietary Ca. First, the Ca homeostasis mechanism is important for Ca absorption and utilisation. Feeding of Ca deficient diets can lower the plasma Ca concentration, which increase the release of PTH and production of 1,25 $(OH)_2D_3$ in the kidneys resulting in increased Ca absorption from the small intestine and reabsorption of Ca from kidneys (Adedokun and Adeola, 2013; Proszkowiec-Weglarz and Angel, 2013). On the other hand, high dietary Ca can cause hypercalcaemia, which can cause a reduction in the secretion of PTH and production of 1,25 $(OH)_2D_3$, and lower the plasma Ca concentrations by decreasing its reabsorption from the kidney and absorption from the intestine (de Matos, 2008). The higher Ca digestibility (0.65 vs 0.57) on the diet with a low Ca concentration (6.75 g/kg), as compared to normal dietary Ca concentrations (9.0 g/kg) for broiler grower, may be attributed to these mechanisms. Second, Ca from the intestine is absorbed by active and passive pathways (Broner *et al*., 1987). Dietary intake of Ca plays an important role in Ca absorption through both of these mechanisms. Increasing dietary Ca concentrations increase the absorption of Ca through the passive pathway, while reducing its absorption through the active pathway because of the down regulation of Ca binding proteins (Morrissey and Waserman, 1971; Broner *et al*., 1987). Low dietary Ca intakes, on the other hand, enhance the Ca uptake and Ca extrusion activities by the cells through plasma membrane Ca ATPase or sodium/calcium ion exchange (Centeno *et al*., 2004). Although a high Ca intake increases the total amount of Ca absorbed by the passive pathway, the overall effect is a reduction in the percentage of Ca absorption

(Hurwitz and Bar, 1969), providing supporting evidence for the observed differences in Ca digestibility (0.65 vs 0.49) of limestone between the extreme dietary Ca concentrations tested (6.75 vs 11.25 g).

The absorption and utilisation of Ca is influenced not only by the dietary Ca concentration, but also by the ratio of dietary Ca to non-phytate P. In the current study, true Ca digestibility of limestone decreased from 0.65 to 0.49 when the dietary Ca:non-phytate P ratio was increased from 1.5 to 2.5. Sebastian *et al*. (1996b) reported that widening the Ca:total P ratio from 2:1 to 2.6:1 reduced the Ca retention coefficient from 0.41 to 0.32. Similar results were observed in a subsequent study, where Ca retention was reduced from 0.66 to 0.43 when Ca:total P ratio was increased from 1:1 to 2.22:1, respectively (Sebastian *et al*., 1996a). A similar reduction in the retention of Ca from 0.58 to 0.43 was observed when the dietary Ca:total P ratio was increased from 1.1:1 to 2:1 in broiler diets in a study by Qian *et al*. (1997).

The *in vitro* solubility coefficient of limestone with fine particles (<0.5 mm) was determined to be higher (0.60 vs 0.33) than coarse particles (1-2 mm), which was in agreement with previous studies (Cheng and Coon, 1990b; Zhang and Coon, 1997a; de Witt *et al*., 2006; Manangi and Coon, 2007). Calcium absorption from the less soluble source is reported to be higher due to its slower release when compared to a Ca source with high solubility (Zhang and Coon, 1997a). In the current study, true Ca digestibility coefficients of coarse and fine limestones were observed to be 0.71 and 0.43, respectively. Similarly, Manangi and Coon (2007) reported that the apparent Ca digestibility coefficient of coarse limestone (1.3 mm) in a maize-soy diet was higher (0.70 vs. 0.62) than that of fine particle size (0.4 mm). *In vitro* solubility of limestone has been reported to inversely related with its *in vivo* solubility (Zhang and Coon, 1997a; de Witt *et al*., 2006) and larger limestone particles have been reported to stay longer in the gizzard increasing the *in vivo* solubility and Ca availability (Rao and Roland, 1989; Zhang and Coon, 1997a; de Witt *et al*., 2006). These data, along with the findings of the current study, suggest that coarser limestone particles are more digestible than finer particles.

8.6. Conclusions

In conclusion, the present data demonstrate that increasing dietary Ca concentrations and wide Ca:non phytate P ratios lower the true Ca digestibility of limestone in broiler

chickens. The true Ca digestibility of coarse limestone particles was higher and the i*n vitro* solubility was lower than those determined for fine particles, indicating an inverse relationship between these two parameters.

CHAPTER 9

Effect of calcium source and particle size on true ileal digestibility and total tract retention of calcium in broiler chickens

9.1. Abstract

The influence of calcium (Ca) source and particle size on true ileal Ca digestibility and total tract Ca retention in broilers was assessed. Four experimental diets containing limestone and oyster shell, each provided as fine (<0.5 mm) and coarse (1-2 mm) particles, were fed to broiler chickens from 21 to 24 days of age. A Ca- and phosphorus-free diet was used to determine the ileal endogenous Ca losses. Limestone and oyster shell were obtained from commercial sources and ground to pass through a set of sieves to obtain the fine (<0.5 mm) and coarse (1-2 mm) particles. Titanium dioxide was incorporated in all diets as an indigestible marker. Each experimental diet was randomly allotted to six replicate cages (eight birds per cage). Apparent ileal digestibility and total tract retention of Ca were calculated using the indicator method and the true ileal digestibility values were calculated by correcting for endogenous Ca losses. Ileal endogenous Ca losses were determined to be 115 mg/kg of dry matter intake. The main effect of Ca source and the interaction between Ca source and particle size were not significant ($P > 0.05$) for the true ileal Ca digestibility and total tract Ca retention. Both these parameters were influenced ($P < 0.05$) by particle size, with coarser particles increasing the digestibility and retention values. Increased particle size increased the true Ca digestibility of limestone from 0.38 to 0.62 and that of oyster shell from 0.33 to 0.56. The corresponding increases in Ca retention were from 0.44 to 0.66 in limestone and from 0.40 to 0.60 in oyster shell. The Ca concentration of gizzard contents (mg/g of gizzard contents) of birds was higher ($P < 0.05$) for limestone as compared to oyster shell. Calcium concentration of gizzard contents was increased with increasing particle size and, this was strongly correlated with the true ileal Ca digestibility (r=0.81; $P < 0.01$) and total tract Ca retention (r=0.82; $P < 0.01$). A strong correlation (r=0.80, $P < 0.01$) was also observed between true ileal Ca digestibility and, total tract Ca retention.

9.2. Introduction

Limestone and oyster shell are major sources of calcium (Ca) used in poultry diets, and contain 380 g/kg Ca (NRC, 1994). In both sources, Ca is present in the form of calcium carbonate, but limestone is an inorganic Ca source of calcitic origin, while oyster shell is an organic Ca source of marine origin. Calcium bioavailability of limestone and oyster shell for broiler chickens has been reported to be high, varying between 73-109% and 87-108%, respectively (Reid and Weber, 1976). However, data from our previous study (Chapter 7) indicate that the true ileal Ca digestibility coefficient of limestone was not high, ranging from 0.52 to 0.60. A similar scenario may exist for oyster shell. Currently no published data are available for the true Ca digestibility of oyster shell for poultry.

Source and particle size of limestone and oyster shell have been reported to influence the calcium availability, measured in terms of growth and tibia ash content, in broiler chickens (McNaughton *et al*., 1974; Reid and Weber, 1976). In layers, shell weight, shell thickness and bone breaking force have been found to increase with increasing particle size of limestone (Cheng and Coon, 1990a). Large Ca particle size has been speculated to stay longer in the gizzard, thus increasing *in vivo* Ca solubility and utilisation in laying hens (Zhang and Coon, 1997a). The effects of particle size on true ileal Ca digestibility are yet to be explored.

In a previous study (Chapter 8), true ileal Ca digestibility coefficient of limestone with larger particles (1-2 mm) was observed to be markedly higher (0.71 vs. 0.43) compared to fine particles (<0.5 mm). These findings might be due to differences in the retention in the gizzard of different sized particles, and the possible slow Ca release and increased absorption. The objective of the present work was to determine the effect of particle size of limestone and oyster shell on true ileal Ca digestibility, total tract Ca retention and Ca retention in the gizzard of broiler chickens.

9.3. Materials and methods

The experiment was conducted according to the New Zealand Revised Code of Ethical Conduct for the use of live animals for research, testing and teaching, and approved by the Massey University Animal Ethics Committee.

9.3.1. Diets and experimental design

Limestone and oyster shell were obtained from commercial sources and ground to pass through a set of sieves to obtain fine (<0.5 mm) and coarse (1-2 mm) particles. Representative samples were analysed for mineral composition and *in vitro* solubility. Two experimental diets, with fine (<0.5 mm) and coarse particle size (1-2 mm) were developed using each Ca source, which served as the sole source of dietary Ca (Table 9.1). Inclusion levels of limestone and oyster shell were set to maintain the recommended dietary Ca concentration (9 g/kg) for broiler growers (Ross, 2007), with a Ca to non-phytate P ratio of 2:1. A Ca- and P-free diet was also developed to determine the ileal endogenous Ca losses. Titanium dioxide (3 g/kg) was incorporated in all diets as an indigestible marker.

9.3.2. Birds

Day-old male broilers (Ross 308) were obtained from a local hatchery and raised on the floor and fed a commercial starter crumble as described in Chapter 3, section 3.1. On day 14, birds were moved to grower cages to acclimatise them. Between days 14 and 20, the starter crumble was gradually changed to mash as the experimental diets were in mash form. On day 21, the birds were individually weighed and allocated to 30 cages (eight birds per cage) on a weight basis so that the average bird weight per cage was similar. The five experimental diets were then randomly allotted to six replicate cages each. The diets were offered *ad libitum* from day 21 to day 24 post-hatch and the birds had free access to water. Group body weights and feed intake were recorded on days 21 and 24.

9.3.3. Sample collection and processing

On day 23, excreta collection trays were introduced and grab samples of fresh excreta were collected for the last 24 hours, pooled within a cage and subsequently lyophilised. The lyophilised samples were then ground to pass through 0.5 mm sieve and store in air tight containers at 4 $^{\circ}$C untill chemical analysis. On day 24, all birds were euthanised by intravenous injection (1ml per 2 kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand) and, digesta samples from the lower ileum were collected and processed as described in Chapter 3, section 3.2.

Table 9.1. Ingredient composition and analysis (g/kg as fed basis) of experimental diets

	Limestone		Oyster shell		Ca- and P-free diet
	<0.5 mm	1.0-2.0 mm	<0.5 mm	1.0-2.0 mm	
Maize starch	391.18	391.18	389.74	389.74	406.55
Dextrose	391.18	391.18	389.74	389.74	406.55
Dried egg albumen	100.0	100.0	100.0	100.0	100.0
Cellulose	50.0	50.0	50.0	50.0	50.0
Limestone	21.34	21.34	-	-	-
Oyster shell	-	-	24.22	24.22	-
Soybean oil	20.0	20.0	20.0	20.0	20.0
Monopotassium phosphate	11.9	11.9	11.9	11.9	-
Potassium chloride	1.1	1.1	1.1	1.1	1.1
Monosodium phosphate	8.0	8.0	8.0	8.0	-
Potassium bicarbonate	-	-	-	-	8.5
Sodium bicarbonate	-	-	-	-	5.0
Titanium dioxide	3.0	3.0	3.0	3.0	3.0
Trace mineral-vitamin premix[1]	2.3	2.3	2.3	2.3	2.3
Calculated analysis					
Metabolisable energy (MJ/kg)	14.96	14.96	14.92	14.92	15.49
Crude protein	83.00	83.00	83.00	83.00	83.00
Calcium[2]	9.00	9.00	9.00	9.00	0.04
Total phosphorus	4.51	4.51	4.51	4.51	0.12
Non-phytate phosphorus	4.44	4.34	4.43	4.33	0.12
Ca: Non-phytate phosphorus	2.00	2.00	2.00	2.00	-
Analysed values					
Dry matter	915	917	917	914	911
Calcium	9.12	9.31	9.68	9.80	0.08

[1]Supplied per kilogram of diet: vitamin A, 12,000 IU; cholecalciferol, 4,000 IU; thiamine, 3 mg; riboflavin, 9 mg; pyridoxine, 10 mg; folic acid, 3 mg; biotin, 0.25 mg; cyanocobalamin, 0.02 mg; dl-α-tocopherol acetate, 80 mg; niacin, 60 mg; Ca-D pantothenate, 15 mg; menadione, 4 mg; choline chloride, 600 mg; Co, 0.25 mg; I, 1.5 mg; Mo, 0.25 mg; Se, 0.26 mg; Mn, 100 mg; Cu, 10 mg; Zn, 80 mg; Fe, 60 mg; antioxidant, 100 mg.

[2]Calculated based on analysed values of limestone and oyster shell samples.

9.3.4. Chemical analysis

Representative samples of diets, ileal digesta and excreta were analysed for dry matter (DM), Ca and titanium as described in Chapter 3, section 3.3. Mineral analysis of limestone and oyster shell was carried out as described in Chapter 7, section 7.3.4.

The *in vitro* solubility of limestone and oyster shell samples was determined by the weight loss method, as described by Zhang and Coon (1997b). Hydrochloric acid (0.2 N; 200 ml) was heated for 15 minutes at 42 °C in a water bath oscillating at 60 hertz and the limestone sample (2g) was added. After 10 minutes, the contents were filtered gravimetrically through Whatman 42 filter paper, dried at 70 °C for 10 hours, cooled and weighed to determine the percent weight loss.

9.3.5. Calculations

Apparent ileal digestibility coefficients of Ca were calculated using titanium ratio in the diets and digesta.

$$AIDC = 1 - [(Ti_I/Ti_O) \times (Ca_O / Ca_I)]$$

where AIDC is apparent ileal digestibility coefficient of Ca, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta, and Ca_I is the Ca concentration in the diet. All analysed values were expressed as gram per kilogram of DM.

Ileal endogenous Ca losses (g/kg DM intake) were calculated by the following formula.

$$IECaL = Ca_O \times (Ti_I/Ti_O)$$

where IECaL is ileal endogenous Ca losses, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta.

True ileal digestibility Ca coefficient was then calculated as follows:

$$TIDC = AIDC + [IECaL\ (g/kg\ of\ DMI)/Ca_I\ (g/kg\ of\ DM)]$$

where TIDC and AIDC represent the true ileal digestibility and apparent ileal digestibility coefficients of Ca, respectively, while IECaL represents the ileal endogenous Ca losses (g/kg of DMI) and Ca_I represents the Ca concentration in diet (g/kg of DM).

Apparent total tract Ca retention coefficients were calculated using titanium ratio in the diets and excreta.

Apparent total tract Ca retention coefficient = $1 - [(Ti_I/Ti_E) \times (Ca_E / Ca_I)]$

Where, Ti_I is the titanium concentration in the diet, Ti_E is the titanium concentration in the excreta, Ca_E is the Ca concentration in the excreta, and Ca_I is the Ca concentration in the diet. All analysed values were expressed as gram per kilogram of DM.

9.3.6. Statistical analysis

The data were analysed as a 2×2 factorial arrangement of treatments using the general linear model procedure of SAS (2004). Cages served as the experimental unit and differences were considered to be significant at $P < 0.05$. Significant differences between means were separated by Least Significant Difference test. Correlations between Ca retention in the gizzard contents and digestibility parameters were analysed by Pearson Correlation.

9.4. Results

The calculated and analysed dietary Ca concentrations of the experimental diets are presented in Table 9.1. Although the calculated values were based on the analysed Ca concentration of limestone and oyster shell samples, the analysed dietary Ca concentration of the diets was 0.12 to 0.80 g/kg higher than the calculated values. The analysed values were used to determine the digestibility coefficients of limestone and oyster shell.

The mineral composition of oyster shell and limestone is presented in Table 9.2. The Ca content of limestone and oyster shell differed and was determined to be 420 and 370 g/kg, respectively. In both Ca sources, particle size had no influence on Ca concentration. The concentration of other macro-minerals was somewhat similar in the

two Ca sources. However, the concentration of manganese, iron and aluminium was lower in oyster shell compared to limestone.

Table 9.2. Mineral analysis of limestone samples (as received basis)[1]

	Limestone		Oyster shell	
	<0.5mm	1-2 mm	<0.5mm	1-2 mm
Dry matter	998	998	998	998
Macro-minerals (g/kg)				
Calcium	420	420	370	370
Magnesium	2.10	2.20	1.70	1.40
Potassium	<0.40	<0.40	<0.40	<0.40
Sodium	1.21	1.13	4.0	4.1
Phosphorus	0.23	0.23	0.34	0.30
Micro-minerals (mg/kg)				
Manganese	28	27	4.6	4.4
Zinc	<10	<10	<10	<10
Copper	<0.5	<0.5	<0.5	<0.5
Iron	780	782	66	41
Aluminium	151	187	47	27
Cadmium	0.09	0.08	0.05	0.03
Lead	1.48	1.42	0.15	0.10
Arsenic	1.0	10.3	0.14	0.11
Mercury	<0.10	<0.10	<0.10	<0.10

[1]Samples were analysed in duplicate.

In both Ca sources, *in vitro* solubility was influenced by particle size with fine particles having higher solubility than coarse particles. *In vitro* solubility coefficients of fine and coarse particles of limestone were determined to be 0.60 and 0.33, respectively. The corresponding solubility coefficients for oyster shell were 0.60 and 0.37, respectively.

Daily weight gain and feed intake of birds fed the experimental diets during the 3-day trial period are presented in Table 9.3. Weight gain and feed intake were not influenced ($P > 0.05$) by the Ca source and particle size. No interaction ($P > 0.05$) was observed between the Ca source and particle size for weight gain or feed intake.

No differences ($P > 0.05$) were observed between the digestibility parameters of limestone and oyster shell (Table 9.4). An increase in particle size increased ($P < 0.05$) the apparent and true ileal Ca digestibility. There was no interaction ($P > 0.05$) between Ca source and particle size distribution for the digestibility parameters. Ileal endogenous Ca losses, following the feeding of the Ca- and P-free diet, were determined to be (mean ± SE) 115 ± 8 mg/kg of DM intake. The influence of the treatments on the total tract Ca retention followed the same pattern as digestibility parameters.

Table 9.3. Weight gain (g/bird/day) and feed intake (g/bird/day) of broilers fed different calcium sources and particle sizes (21-24 days of age)[1, 2]

	Particle size (mm)	Weight gain (g/bird/day)	Feed intake (g/bird/day)
Limestone	<0.5	30.1	79
	1.0-2.0	29.6	78
Oyster shell	<0.5	23.7	80
	1.0-2.0	30.1	85
SEM[3]		1.86	2.05
Main Effects			
Calcium source			
Limestone		29.9	79
Oyster shell		27.0	83
SEM[3]		1.32	1.45
Particle size (mm)			
< 0.5		26.9	80
1.0-2.0		29.9	82
SEM[3]		1.32	1.45
Probabilities, $P \leq$			
Calcium source		0.13	0.06
Particle size		0.12	0.37
Calcium source x Particle size		0.08	0.13

[1] Each treatment value represents the mean of six replicates (eight birds per replicate).

[2] Weight gain and feed intake of birds on Ca- and P-free diet were 30 and 87 g/bird/day, respectively.

[3]Pooled standard error of mean.

Table 9.4. Effect of limestone source particle size on the apparent and true ileal calcium digestibility and apparent total tract calcium retention in broiler chickens[1]

	Particle size (mm)	Apparent ileal calcium digestibility coefficient	True ileal calcium digestibility coefficient	Apparent calcium retention coefficient
Limestone	<0.5	0.37	0.38	0.44
	1.0-2.0	0.61	0.62	0.66
Oyster shell	<0.5	0.32	0.33	0.40
	1.0-2.0	0.55	0.56	0.60
SEM[2]		0.04	0.04	0.04
Main Effects				
Calcium source				
Limestone		0.49	0.50	0.55
Oyster shell		0.43	0.44	0.50
SEM[2]		0.03	0.03	0.03
Particle size (mm)				
< 0.5		0.35[b]	0.36[b]	0.42[b]
1.0-2.0		0.58[a]	0.59[a]	0.63[a]
SEM[2]		0.03	0.03	0.03
Probabilities, *P* ≤				
Calcium source		0.15	0.14	0.23
Particle size		0.001	0.001	0.001
Calcium source x Particle size		0.83	0.82	0.90

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Pooled standard error of mean.

Calcium concentration of gizzard contents was higher ($P < 0.05$) in birds fed limestone compared to oyster shell (Table 9.5). In both Ca sources, the increase in particle size increased ($P < 0.05$) the Ca concentration of gizzard contents. However, relative weight of gizzard digesta was not affected ($P > 0.05$) by Ca source or particle size. There was no interaction ($P > 0.05$) between Ca source and particle size for these parameters.

Table 9.5. Calcium concentration of gizzard contents (mg/g) and weight of gizzard digesta (mg/kg of body weight) in broilers as influenced by calcium source and particles size[1]

	Particle size (mm)	Calcium concentration of gizzard contents (mg/g)	Gizzard contents (mg/ kg of body weight)
Limestone	<0.5	150	159
	1.0-2.0	275	161
Oyster shell	<0.5	86	228
	1.0-2.0	213	181
SEM[2]		20	22
Main Effects			
Calcium source			
Limestone		212[a]	160
Oyster shell		149[b]	204
SEM[2]		14	16
Particle size (mm)			
< 0.5		118[a]	193
1.0-2.0		244[b]	171
SEM[2]		14	16
Probabilities, *P* ≤			
Calcium source		0.01	0.07
Particle size		0.001	0.34
Calcium source x Particle size		0.96	0.30

Diets were fed from day 21 to 24 of age.

[1]Calcium concentration in the gizzard contents (mg/g of gizzard contents) and gizzard contents (mg/kg of body weight) were calculated on dry matter basis.

[2]Pooled standard error of mean.

There were strong correlations between the Ca concentration of gizzard contents and, true ileal Ca digestibility (r=0.81; $P < 0.01$) and total tract Ca retention (r=0.82; $P < 0.01$). A strong correlation was also observed between true ileal Ca digestibility and total tract Ca retention (r=0.80; $P < 0.01$).

9.5. Discussion

According to NRC (1994), the Ca concentration of limestone and oyster shell is similar (380 g/kg). The Ca concentration of oyster shell evaluated in the current work was comparable to that reported by NRC (1994). The Ca concentration of the limestone sample, however, was 40 g/kg higher than the NRC (1994) value, but within the range of 360-428 g/kg reported in the literature (Reid and Weber, 1976; Ajakaiye *et al.,* 2003a; Browning and Cowieson, 2013; Wilkinson *et al*., 2013a).

In the present study, the *in vitro* solubility coefficient of both limestone and oyster shell increased with decreasing particle size. These findings are in agreement with previous studies where *in vitro* solubility of fine limestone particles were observed to be higher than that of coarse particles (Cheng and Coon, 1990b; Zhang and Coon, 1997a; de Witt *et al.,* 2006; Manangi and Coon, 2007; Saunders-Blades *et al.,* 2009; Chapter 8). It has been suggested that the flat long surface of large oyster shell particles (>2 mm) provide more surface area for acid reaction and resulting in higher solubility in oyster shell as compared to similar size of limestone (Saunders-Blades *et al.,* 2009). Similar results were observed in current study where the *in vitro* solubility of coarse particles of oyster shell was numerically higher (0.37 vs 0.33) than coarse limestone. *In vitro* solubility of limestone has been shown to be inversely related to its *in vivo* solubility (Zhang and Coon, 1997a; de Witt *et al.,* 2006) and *in vivo* availability in layers. Higher digestibility of coarse limestone and oyster shell with low *in vitro* solubility indicates a similar scenario in broiler chickens.

In the present study, the apparent ileal Ca digestibility coefficient of limestone and oyster shell were determined to be similar, with values ranging between 0.32 and 0.61 depending on the particle size. No published data are available on the Ca digestibility of oyster shell for poultry. In the past, Ca availability from different Ca sources has been reported in terms of bioavailability using $CaCO_3$ as a standard Ca source and bone ash and growth as response criteria. The Ca bioavailability in limestone and oyster shell has been reported to be very high (Table 9.6), however, the data reported in Chapter 7 indicated that apparent ileal Ca digestibility of three limestone samples varied from 0.51 to 0.59. A recent study by Proszkowiec-Weglarz *et al*. (2013) reported apparent Ca digestibility values from 0.37 to 0.64 in limestone for broiler chickens, which were dependent upon the adaptation period to assay diets of 32 and 24 hours, respectively.

True Ca digestibility coefficients of limestone and oyster shell samples determined in this study were similar and varied from 0.33 to 0.62 depending on the particle size. In our previous study (chapter 7), a digestibility range of 0.52 to 0.60 was determined for three limestone samples.

Table 9.6. Bioavailability of calcium from limestone and oyster shell for young chicks[1]

	Response criteria	Relative bioavailability	Reference
Limestone	Bone ash	102	Blair *et al*. (1965)[2]
	Growth	75-96	Reid and Weber (1976)
	Bone ash	73-109	Reid and Weber (1976)
	Bone ash	77	Augspurger and Baker (2004)
Oyster shell	Growth	87	Reid and Weber (1976)
	Bone Ash	108	Reid and Weber (1976)
	Bone Ash	95	Augspurger and Baker (2004)

[1] Considering calcium carbonate as the standard source with 100% availability.

[2] Adapted from Peeler (1972).

In the current study, large particle size increased the true Ca digestibility of both Ca sources. Similar results has been reported in our previous study (Chapter 8), where true Ca digestibility of limestone with a fine particle size was observed to be lower than that of limestone with a larger particle size (0.43 vs. 0.71). Larger particles of limestone shown increased its retention time in the gizzard which increases their *in vivo* solubility and availability (Zhang and Coon, 1997a; de Witt *et al*., 2006). In present work, Ca concentration in the gizzard contents increased with increasing particle size. A strong positive correlation between the Ca concentration of gizzard contents and the true Ca digestibility coefficient lends support for the slow release and subsequent higher Ca digestibility of coarse particles as compared to fine particles. Higher apparent Ca digestibility (0.70 vs. 0.62) has also been reported for coarse (1.3 mm) and fine (0.4 mm) limestone particles in a maize-soybean meal based diet (Manangi and Coon, 2007).

In current study, the apparent total tract Ca retention of fine and coarse particles of limestone and oyster shell was 0.44 and 0.66, and 0.40 and 0.60, respectively. Oso *et al*. (2011) also reported similar Ca retention values for limestone and oyster shell when

offered as fine particles (<0.5 mm) in a maize-soybean meal diet. In contrast to that observed effects of particle size on Ca digestibility, published data on retention have been contradictory. Guinotte *et al.* (1991) reported higher Ca retention of oyster shell (0.57 vs. 0.38) for fine (<0.075 mm) and coarse (>1.8 mm) particles in a maize-soybean meal diet. For limestone, however, similar particle sizes had no effect on Ca retention (0.40 vs. 0.41). In a subsequent study, Guinotte *et al.* (1995) reported higher Ca retention for limestone (0.48 vs 0.22) in broiler chickens fed fine particle (<0.5mm) as compared to coarse (>1.2 mm) particles in maize, wheat and soybean meal based diets. In these two studies it is difficult to explain the low Ca digestibility of limestone and oyster shell with coarse particles as compared to fine particles as it is evident from the previous studies (Cheng and Coon, 1990b; Zhang and Coon, 1997a; de Witt *et al.,* 2006; Manangi and Coon, 2007; Saunders-Blades *et al.,* 2009; Chapter 8), that coarse particles have low *in vitro* solubility which is inversely related to *in vivo* solubility and availability to the birds (Zhang and Coon, 1997a; de Witt *et al*., 2006).

9.6. Conclusions

In conclusion, the present study demonstrated that an increase in particle size of two different Ca sources increased the Ca concentration of gizzard contents, ileal Ca digestibility and Ca retention. Strong positive correlations were observed between Ca concentration of gizzard contents and, Ca digestibility and retention. No difference was observed between the true ileal Ca digestibility of limestone and oyster shell.

CHAPTER 10

True ileal calcium digestibility of some common feed ingredients for broiler chickens using the direct method

10.1. Abstract

Results from two studies are reported in this chapter. The first study was conducted to determine the true ileal calcium (Ca) digestibility of dicalcium phosphate (DCP), monocalcium phosphate (MCP), poultry by-product meal (PBPM), fish meal (FM) and canola meal (CM) for broiler chickens. Five experimental diets with DCP, MCP, PBPM and FM with dietary Ca concentrations of 9 g/kg and CM with a dietary Ca concentration of 5.71 g/kg were developed. A Ca- and phosphorus (P)-free diet was used to determine the basal ileal endogenous Ca losses. Titanium dioxide (3 g/kg) was incorporated in all diets as an indigestible marker. Each experimental diet was randomly allotted to six replicate cages (eight birds per cage) and fed from day 21 to 24 post-hatch. Apparent ileal digestibility was calculated using the indicator method and corrected for endogenous Ca losses to determine the true ileal Ca digestibility. The true Ca digestibility of DCP, MCP, PBPM, FM and CM were determined to be 0.28, 0.33, 0.29, 0.24 and 0.31, respectively. The true Ca digestibility of MCP and CM was higher ($P < 0.05$) than FM but similar ($P > 0.05$) to that of DCP and PBPM. It was speculated that the low Ca digestibility may be due, partly, to the length of diet adaptation. To examine this possibility, a second study was conducted to determine the effect of dietary adaptation length on the true ileal Ca digestibility of DCP and MCP. The experimental diets with DCP and MCP were similar to those used in Experiment 1 and a Ca- and P-free diet was also developed. Each diet was then randomly allotted to four replicate cages (15 birds per cage) and fed from day 21 to 24 post-hatch. Digesta samples of five birds from each replicate were collected after 24, 48 and 72 hours of feeding. Ileal endogenous Ca losses after 24, 48 and 72 hours of adaptation period were determined to be 84, 113 and 124 mg/kg of dry matter intake and were not influenced ($P > 0.05$) by adaption length. An interaction ($P < 0.05$) was observed between Ca source and dietary adaptation length. Dietary adaptation length did not affect the true Ca digestibility of DCP and MCP in this study, except a higher digestibility coefficient of DCP was

reduced after 24 hours. The true Ca digestibility of DCP and MCP after 24, 48 and 72 hours of adaptation period were 0.45, 0.36, and 0.35, and 0.30, 0.32 and 0.34, respectively.

10.2. Introduction

Dicalcium phosphate (DCP) and monocalcium phosphate (MCP) are the most commonly used inorganic phosphorus (P) sources, but also contribute a significant amount of dietary Ca in feed formulations. The Ca concentration of DCP and MCP is 220 and 160 g/kg, respectively (NRC, 1994). In addition to these inorganic phosphates sources, there are some feed ingredients which also contain relatively high Ca concentrations and their contribution towards total dietary Ca cannot be overlooked. Such ingredients include poultry by-product meal (PBPM), fish meal (FM) and canola meal (CM) which contains 30, 12-73 and 6.8 g/kg Ca, respectively (NRC, 1994). Currently there is no published data available on true Ca digestibility of these ingredients for poultry. It is widely assumed that Ca availability of these sources is high. However, in our previous studies, true Ca digestibility coefficients of meat and bone meal and limestone were determined to be 0.41 to 0.60 and 0.52 to 0.60, respectively (Chapter 5, 6, 7).

Plasma Ca and P concentrations are controlled within a narrow physiological range by feedback mechanisms involving parathyroid hormone, activated vitamin D_3, and calcitriol, and their respective receptors in the small intestine, bone and kidney. High or low plasma Ca and/or P can trigger the Ca homeostasis mechanism and influence the intestinal absorption of these minerals (Veum, 2010). Recent work has raised questions regarding the appropriate time for this response and the relevance of the length of feeding of experimental diets with imbalanced dietary Ca and P concentrations for the measurement of Ca and P digestibility (Proszkowiec-Weglarz *et al*., 2013). In our previous studies with meat and bone meal and limestone, dietary Ca and P concentrations were adjusted according to the recommended requirements of the bird (Ross, 2007). In the case of ingredients that inherently contain low concentrations of Ca relative to P, however, this will not be possible.

The purpose of Experiment 1 was to determine the true Ca digestibility of some common feed ingredients (DCP, MCP, PBPM, FM, and CM). A follow-up study was

conducted to determine the effect of dietary adaptation length on the true Ca digestibility of DCP and MCP.

10.3. Materials and method

The studies were conducted according to the New Zealand Revised Code of Ethical Conduct for the use of live animals for research, testing and teaching, and approved by the Massey University Animal Ethics Committee.

10.3.1. Experiment 1

10.3.1.1. Diets and experimental design

The ingredients (DCP, MCP, PBPM, FM and CM) were obtained from commercial sources. Representative samples of DCP and MCP were analysed for dry matter (DM), ash, Ca and P, while PBPM, FM and CM were analysed for DM, crude protein, fat, ash, Ca and P. Five experimental diets were developed using each of the above ingredients as the sole source of Ca. In diets with DCP, MCP, PBPM, and FM, the dietary Ca concentration was adjusted to 9 g/kg. In the case of CM, a dietary Ca concentration of only 5.71 g/kg was obtained at the maximum inclusion level of CM. A Ca- and P-free diet was developed to determine the ileal endogenous Ca losses (Table 10.1). Titanium dioxide (3 g/kg) was incorporated in the diets as an indigestible marker.

10.3.1.1. Birds

Day-old male broilers (Ross 308) were obtained from a local hatchery and, raised on the floor and fed a commercial starter crumble as described in Chapter 3, section 3.1. On day 14, birds were moved to grower cages. Between days 14 and 20, the crumbles were gradually changed to mash as the experimental diets were in mash form. On day 21, the birds were individually weighed and allocated to 36 cages (eight birds per cage) on a body weight basis so that the average bird weight per cage was similar. The six experimental diets were then randomly allotted to six replicate cages each. The diets, in mash form, were offered *ad libitum* from days 21 to 24 post-hatch and the birds had free access to water. Group body weights and feed intake were recorded on days 21 and 24.

10.3.1.2. Sample collection and processing

On day 24, all birds were euthanised by intravenous injection (1ml per 2 kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand) and, ileal digesta were collected and processed as described in Chapter 3, section 3.2.

Table 10.1. Ingredient composition and analysis (g/kg, as fed basis) of experimental diets

Ingredients	DCP[3]	MCP[3]	PBPM	FM	CM	Ca- and P-free
Maize starch	383.85	378	344.1	388.6	-	406.85
Dextrose	383.85	378	344.1	388.6	-	406.85
Dried egg albumen	100	100	-	-	-	100
Cellulose	50	50	50	50	-	50
Soybean oil	20	20	20	20	20	20
Dicalcium phosphate	46	-	-	-	-	-
Monocalcium phosphate	-	57.7	-	-	-	-
Poultry by-product meal	-	-	226	-	-	-
Fish meal	-	-	-	135.5	-	-
Canola meal	-	-	-	-	968.4	-
Potassium bicarbonate	8	8	7	8	-	8
Sodium chloride	-	-	1.5	2	3.3	-
Sodium bicarbonate	3	3	2	2	3	3
Titanium dioxide	3	3	3	3	3	3
Trace mineral-vitamin premix[1]	2.3	2.3	2.3	2.3	2.3	2.3
Calculated Analysis						
Apparent metabolisable energy (MJ/kg)	14.75	14.56	14.68	14.93	8.83	15.50
Crude protein	83.00	83.0	151.42	74.66	367.99	83.00
Calcium[2]	9.01	9.00	9.02	9.01	5.71	0.04
Total phosphorus[2]	8.31	12.88	6.01	6.31	6.31	0.12
Non-phytate phosphorus	8.31	12.88	6.01	6.31	2.91	0.12
Ca: Non-phytate phosphorus	1.08	0.70	1.50	1.43	1.97	0.33
Analysed values (as fed basis)						
Dry matter	912	913	903	897	899	911
Calcium	9.48	9.72	10.31	11.12	6.46	0.05
Total phosphorus	8.54	12.22	5.74	6.39	6.99	0.15
Ca: Total phosphorus	1.11	0.79	1.80	1.74	0.65	0.33

[1] Supplied per kilogram of diet: vitamin A, 12,000 IU; cholecalciferol, 4,000 IU; thiamine, 3 mg; riboflavin, 9 mg; pyridoxine, 10 mg; folic acid, 3 mg; biotin, 0.25 mg; cyanocobalamin, 0.02 mg; dl-α-tocopherol acetate, 80 mg; niacin, 60 mg; Ca-D pantothenate, 15 mg; menadione, 4 mg; choline chloride, 600 mg; Co, 0.25 mg; I, 1.5 mg; Mo, 0.25 mg; Se, 0.26 mg; Mn, 100 mg; Cu, 10 mg; Zn, 80 mg; Fe, 60 mg; antioxidant, 100 mg.

[2] Calculated based on analysed values of dicalcium phosphate, monocaclcium phosphate, poultry by-product meal, fish meal and canola meal.

[3] DCP sample was obtained from China, MCP sample was obained from Innophos, 259 Prospect Plains Road, Building A, Cranbury, USA.

10.3.2. Experiment 2

To examine the possibility that the low Ca digestibility values observed in Experiment 1 may have been due, partly, to the length of diet adaptation, a second study was conducted to determine the effect of dietary adaptation length on the true ileal Ca digestibility of DCP and MCP.

10.3.2.1. Diets and birds

The conduct of the experiment and the diet formulations with DCP and MCP were similar to those used in Experiment 1. A Ca- and P-free diet was also developed. Each diet was randomly allotted to four replicate cages (15 birds per cage) and fed from day 21 to 24 post-hatch.

10.3.2.2. Sample collection and processing

Five birds from each replicate were euthanised after 24, 48 and 72 hours of diet introduction on day 21, by intravenous injection (1ml per 2 kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand) and, the ileal digesta were collected and processed as described in Chapter 3, section 3.2.

10.3.3. Chemical analysis

Representative samples of diets and ileal digesta were analysed for DM, Ca and titanium as described in Chapter 3, section 3.3. Representative samples of DCP and MCP were analysed for DM, Ca and P and, of PBPM, FM and CM for DM, crude protein, crude fat, ash, Ca, and P as described in Chapter 3, section 3.3.

10.3.4. Calculations

Apparent ileal digestibility coefficients of Ca were calculated using titanium ratio in the diets and digesta.

$$AIDC = 1 - [(Ti_I/Ti_O) \times (Ca_O / Ca_I)]$$

where AIDC is apparent ileal digestibility coefficient of Ca, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta, and Ca_I is the Ca concentration in the diet. All analysed values were expressed as gram per kilogram of DM.

Ileal endogenous Ca losses (g/kg DM intake) were calculated by the following formula.

$$IECaL = Ca_O \times (Ti_I/Ti_O)$$

where IECaL is ileal endogenous Ca losses, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta.

True ileal Ca digestibility coefficient was then calculated as follows:

$$TIDC = AIDC + [IECaL\ (g/kg\ of\ DMI)/Ca_I\ (g/kg\ of\ DM)]$$

where TIDC and AIDC represent the true ileal digestibility and apparent ileal digestibility coefficients of Ca, respectively, while IECaL represents the ileal endogenous Ca losses (g/kg of DMI) and Ca_I represents the Ca concentration in diet (g/kg of DM).

10.3.5. Statistical analysis

In Experiment 1, the data were analysed by a one-way ANOVA using the General Linear Model of SAS (2004) in a completely randomised design. In Experiment 2, the data were analysed as a 2×3 factorial arrangement of treatments using the General Linear Model procedure (SAS, 2004) to determine the effect of Ca source, dietary adaptation length and their interaction. In both studies, cage served as the experimental unit. Differences were considered significant at $P < 0.05$ and significant differences between means were separated by the Least Significant Difference test.

10.4. Results

10.4.1. Experiment 1

Analysed Ca concentrations of the experimental diets with DCP, MCP, PBPM, FM and CM were 9.48, 9.72, 10.21 and 11.12 and 6.46 g/kg, respectively (Table 10.1).

The analysed composition of DCP, MCP, PBPM, FM and CM are presented in Table 10.2. Analysed Ca and P concentrations of DCP, MCP, PBPM, FM and CM were determined to be 195, 155, 40, 67 and 6, and, 178, 221, 27, 47 and 10 g/kg, respectively.

Table 10.2. Analysed composition of dicalcium phosphate, monocalcium phosphate, poultry by-product meal, fish meal and canola meal (g/kg, as fed basis)[1]

	Dry matter	Ash	Protein	Fat	Calcium	Phosphorus
Dicalcium phosphate	951	825	-	-	195	178
Monocalcium phosphate	947	794	-	-	155	221
Poultry by-product meal	959	151	670	119	40	27
Fish meal	941	293	551	75	67	47
Canola meal	901	69	379	31	6	10

[1]Samples were analysed in duplicate.

Body weight gain and feed intake of birds fed the experimental diets during the 3-day experimental period are summarised in Table 10.3. Daily gains of birds fed the CM and PBPM diets were higher ($P < 0.05$) than those fed the other diets. The lowest body weight gain was observed for diets containing MCP. The feed intakes of birds were lowest on the diet containing MCP ($P < 0.05$), while the highest ($P < 0.05$) feed intake was observed for diets containing CM and PBPM.

Table 10.3. Body weight gain and feed intake (g/bird/day) of birds fed the experimental diets (21-24 days of age)[1]

	Weight gain (g/bird/day)	Feed intake (g/bird/day)
Ca- and P-free	30^{b}	87^{bc}
Dicalcium phosphate	32^{b}	88^{b}
Monocalcium phosphate	2^{d}	56^{d}
Poultry by-product meal	56^{a}	91^{ab}
Fish meal	19^{c}	81^{c}
Canola meal	60^{a}	96^{a}
SEM[2]	2.63	2.14
Probability, *P*≤	0.001	0.001

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Pooled standard error of mean.

The apparent and true ileal digestibility coefficients of Ca for DCP, MCP, PBPM, FM and CM are presented in Table 10.4. Ileal endogenous Ca losses were determined to be 115 ± 8 mg/kg of DM intake and used to calculate the true Ca digestibility coefficients of the diets. The apparent and true Ca digestibility of MCP and CM was higher ($P < 0.05$) than FM but similar ($P > 0.05$) to that of DCP and PBPM.

Table 10.4. Apparent and true ileal calcium digestibility coefficients of feed ingredients for broiler chickens[1]

	Apparent ileal calcium digestibility	True ileal calcium digestibility
Dicalcium phosphate	0.27[ab]	0.28[ab]
Monocalcium phosphate	0.32[a]	0.33[a]
Poultry by-product meal	0.28[ab]	0.29[ab]
Fish meal	0.23[b]	0.24[b]
Canola meal	0.29[a]	0.31[a]
SEM[2]	0.02	0.02
Probability, *P*≤	0.04	0.04

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Pooled standard error of mean.

10.4.2. Experiment 2

Ileal endogenous Ca losses were determined to be of 84 ± 13, 113 ± 12 and 124 ± 16 mg/kg of DM intake following 24, 48 and 72 hours of dietary adaptation and were used to calculate the respective true Ca digestibility coefficients at each time period. No effect ($P > 0.05$) of dietary adaptation length was observed on ileal endogenous Ca losses. The Ca digestibility coefficient of DCP was higher after 24 hours as compared to 48 and 72 hours of dietary adaptation length. No differences were observed for MCP, resulting in a significant interaction ($P < 0.05$) between Ca source and dietary adaptation length (Table 10.5).

Table 10.5. Effect of calcium source and dietary adaptation length on apparent and true ileal calcium digestibility in broiler chickens[1]

Calcium source	Dietary adaptation length (hours)	Apparent ileal calcium digestibility coefficient	True ileal calcium digestibility coefficient
Dicalcium phosphate	24	0.44[a]	0.45[a]
	48	0.34[b]	0.36[b]
	72	0.33[b]	0.35[b]
Monocalcium phosphate	24	0.29[b]	0.30[b]
	48	0.30[b]	0.32[b]
	72	0.33[b]	0.34[b]
SEM[2]		0.02	0.02
Main Effects			
Calcium source			
Dicalcium phosphate		0.37	0.38
Monocalcium phosphate		0.31	0.32
SEM[2]		0.01	0.01
Dietary adaptation length			
24		0.37	0.38
48		0.32	0.34
72		0.33	0.35
SEM[2]		0.02	0.02
Probabilities, *P*≤			
Calcium source		0.008	0.007
Dietary adaptation length		0.251	0.304
Calcium source x Dietary adaptation length		0.033	0.036

[a, b, c] Values with a different superscript within a column differ significantly ($P < 0.05$).

[1] Each value represents the mean of four replicates (five birds per replicate).

[2] Pooled standard error of mean.

10.5. Discussion

In the current study, the analysed Ca concentrations in DCP, MCP, PBPM, FM and CM were determined to be 195, 155, 40, 67 and 6 g/kg, respectively. Calcium concentrations of MCP, PBPM, FM and CM in this study were comparable to the NRC (1994) values. However, the Ca concentration of DCP was lower than the NRC (1994) value of 220 g/kg and the range of 261-297 g/kg reported by Browning and Cowieson

(2013). It would therefore appear that the DCP used in the present work was a mixture of DCP and MCP, rather than 100% DCP.

A comparison of the Ca concentration of the ingredients used in current study with available literature is presented in Table 10.6.

Table 10.6. Comparison of analysed calcium concentration of dicalcium phosphate, monocalcium phosphate, poultry by-product meal, fish meal and canola meal samples with NRC (1994) and Browning and Cowieson (2013) values (g/kg, as fed basis)

	Current Study[1]	NRC (1994)	Browning and Cowieson (2013) Mean (Range)
Dicalcium phosphate	195	220	276 (261-294)
Monocalcium phosphate	155	160	164 (152-184)
Poultry by-product meal	40	30	32 (22-40)
Fish meal	67	12-73	-
Canola Meal	6	6.8	6 (3.7-7.1)

[1]Samples were analysed in duplicate.

10.5.1. Experiment 1

The analysed dietary Ca concentration of the experimental diets was 0.15 to 2.11 g/kg higher than the calculated values. Such differences between the analysed and calculated dietary Ca concentrations have also been observed in our previous studies (Chapters 5, 6, 7 and 8). The analysed values were used to determine the true Ca digestibility of the ingredients.

The true Ca digestibility of DCP, MCP, PBPM, FM and CM were determined to be low and, ranged between 0.24 and 0.33. Currently there is no published data available on the Ca digestibility of these ingredients for broilers. Proszkowiec-Weglarz *et al.* (2013) reported that the apparent Ca digestibility of MCP varied according to dietary adaptation length. The apparent Ca digestibility of MCP determined after 72 hours of adaptation in the current study was 0.32 and comparable to the value of 0.35 after 96 hours in their study.

The Ca digestibility of ingredients determined in the current study was much lower than the values determined for meat and bone meal (0.45-0.60) and limestone (0.52-0.60) in our previous studies (Chapters 5, 6 and 7). Dietary Ca and P concentrations are critical for their absorption and post-absorptive utilisation, and imbalance between these two minerals can affect the Ca digestibility (Proszkowiec-Weglarz and Angel, 2013). NRC (1994) recommends a Ca:non-phytate phosphorus ratio of about 2:1 as favourable for broilers. Widening or narrowing of this ratio is known to affect Ca absorption and retention (Hurwitz and Bar, 1969; Sebastian *et al*., 1996a, b; Pintar *et al*., 2005; Plumstead *et al*., 2008). In the present study, the Ca:non-phytate P ratio of all experimental diets was narrower than 2:1, which was inevitable given the Ca and P imbalance in the test ingredients. The ratios in diets with DCP, MCP, PBPM and FM were calculated to be 1.08:1, 0.70:1, 1.50:1 and 1.43:1, respectively. Alternative methodologies (regression or substitution) should be tested to determine the true C digestibility of such feed ingredients.

In current study, dietary P concentrations of all experimental diets were higher than recommended dietary P concentration (4.5 g/kg) for broiler growers (Ross, 2007). High dietary P intake can cause hyperphosphataemia which suppresses the renal 1- α hydroxylase to lower calcitriol production, which in turn reduces the intestinal absorption of Ca (Breves and Schroder, 1991). The high P concentrations in the experimental diets in current study may, at least in part, explain the lower Ca digestibility observed in the DCP, MCP, PBPM and FM. In the diet with CM, although the ratio between Ca and non-phytate P was close to 2:1, a lower Ca digestibility (0.31) was still observed. A possible reason for this may be the high phytate P (3 g/kg) concentration in CM (NRC, 1994). Phytate is well known to form a complex with Ca and reduces its availability (Angel *et al*., 2002; Selle *et al.,* 2009). Reduction in Ca retention has been reported previously with increase in dietary phytate P concentrations in broiler chickens (Viveros *et al*., 2002; Plumstead *et al*., 2008).

Studies with pigs suggest a higher standardised Ca digestibility value of DCP and MCP compared to the present data (Gonzalez-Vega *et al.,* 2015a, b). The standardised total tract Ca digestibility of DCP and MCP was determined to be 0.78 and 0.86, respectively (Gonzalez-Vega *et al.,* 2015a). The reasons for the observed discrepancies between the studies are not clear, but may be related to species differences and the experimental diets used. Maize-based diets were used in the pig study compared

to maize starch and dextrose in the present study. In a follow-up study, Gonzalez-Vega *et al.* (2015b) determined the standardised Ca digestibility of fish meal in pigs to be 0.46 in maize starch based diets, and 0.89 in maize-based diets which is suggestive of higher Ca digestibility in practical diets rather than experimental-type diets. The true Ca digestibility of fish meal for broiler chickens in the current study with maize starch and dextrose based diet was only 0.24.

10.5.2. Experiment 2

Calcium homeostasis is maintained by feedback mechanisms regulated by plasma Ca and/or P concentrations which trigger the release of hormones that affect intestinal Ca absorption (Veum, 2010). The response time for this mechanism is therefore important for determination of Ca digestibility in diets with an imbalance of Ca and/or P. In a study with MCP, the apparent ileal digestibility of Ca was found to be reduced from 0.70 to 0.35 at dietary adaptation lengths of 16 and 96 hours, respectively, in broiler chickens (Proszkowiec-Weglarz and Angel, 2013). In the current study, the true Ca digestibility of DCP and MCP was not affect by dietary adaptation length, although a higher digestibility coefficient was observed for DCP after 24 hours compared to 48 and 72. Though not conclusive, these findings suggest that an adaptation length up to 72 hours may not influence the Ca digestibility.

10.6. Conclusions

In conclusion, the measurement of true Ca digestibility of DCP, MCP, PBPM, FM and CM, using the direct method, yielded low values, ranging from 0.24 to 0.33. These low values are likely due to the high dietary P and/or narrow Ca:non-phytate P ratio in the test diets. Low Ca digestibility in CM may also be due partly to the presence of high concentrations of phytate P in CM. No clear evidence was observed for the influence of dietary adaptation length on Ca digestibility of DCP and MCP. Further research is required to determine the true Ca digestibility of these feed ingredients using alternate methodologies.

CHAPTER 11

Comparison of methodologies to determine the true ileal calcium digestibility of dicalcium phosphate for broiler chickens

11.1. Abstract

A study was conducted to determine the effect of methodology on true calcium (Ca) digestibility of dicalcium phosphate (DCP) for broiler chickens. Three different methodologies, namely direct, difference and regression methods, were used to determine the true Ca digestibility of DCP. In total, eight experimental diets were formulated. Diets 1, 2 and 3 were developed to determine the true Ca digestibility of DCP by the regression method; Diet 3 was also used to determine the true Ca digestibility of DCP by the direct method. Diets 4, 5, 6 and 7 were developed to determine the true Ca digestibility of DCP by the difference method at Ca:non-phytate phosphorus (P) ratios of 2:1 and 1.16:1. Dietary Ca concentrations were maintained by using limestone and DCP as Ca sources in these four diets. A Ca- and P-free diet was used to determine the basal ileal endogenous Ca losses. Titanium dioxide was incorporated in all diets as an indigestible marker. Each experimental diet was randomly allotted to six replicate cages (eight birds per cage) and fed from 21-24 days post-hatch. Apparent and true ileal digestibility of the experimental diets was calculated using the indicator method. Basal ileal endogenous Ca losses were determined to be 86 mg/kg of dry matter intake and this value was used to determine the true Ca digestibility by direct and difference methods. Negative endogenous losses were observed in the regression method. The true Ca digestibility coefficients of DCP determined by direct, difference and regression method with an intrinsic Ca:non-phytate P ratio of 1.16:1 were 0.34, 0.21 and 0.13, respectively. The true Ca digestibility of DCP determined by the direct method was higher ($P < 0.05$) than those determined by difference and regression methods. Calcium to non-phytate P ratio had no affect ($P > 0.05$) on the true Ca digestibility of DCP determined by the difference method. The true Ca digestibility coefficients of DCP with Ca:non-phytate P ratios of 2:1 and 1.16:1 were 0.25 and 0.21, respectively.

11.2. Introduction

Currently there is no established method for the determination of the true calcium (Ca) digestibility of feed ingredients for broiler chickens. However, three different methods, namely direct, difference and regression, are used for the determination of amino acid digestibility in feed ingredients (Ravindran and Bryden, 1999; Lemme *et al*., 2004). In our previous studies, regression (Chapter 5) and direct (Chapter 6) methods were used to determine the Ca digestibility of meat and bone meal for broiler chickens and it was observed that both methods yielded comparable Ca digestibility coefficients. As the direct method is less laborious and more cost effective, this method was used in subsequent studies to determine the true Ca digestibility of limestone and oyster shell (Chapters 7 to 9). When the direct method was used to evaluate monocalcium phosphate (MCP), dicalcium phosphate (DCP), poultry by-product meal (PBPM), fish meal (FM) and canola meal (CM), however, low true Ca digestibility coefficients were determined for these Ca sources (Chapter 10). It was speculated that the direct method may not be appropriate for these Ca sources and, therefore, a comparison with regression and difference methods is warranted.

The Ca to phosphorus (P) ratio in the diet is critical for the absorption and post-absorptive utilisation of these two minerals. It is well known that dietary Ca and P concentrations are critical as plasma Ca concentrations are tightly regulated and imbalance can affect the estimation of digestible Ca contents of the test ingredient (Proszkowiec-Weglarz and Angel, 2013). A Ca to non-phytate P ratio of 2:1 is recommended in poultry diets (NRC, 1994). In our previous studies (Chapters 5, 6 and 7) with meat and bone meal and limestone, dietary Ca and P concentrations were maintained or adjusted to meet this ratio (Ross, 2007). In Ca sources, where the P concentration is higher or closer to Ca concentration, the ratios will be narrower. Such inherent imbalance may be another contributory factor for the observed low Ca digestibility.

This study aimed to compare different methodologies to determine the true Ca digestibility of DCP for broiler chickens. An additional aim was to examine whether the true Ca digestibility measurement is influenced by Ca:non-phytate P ratios.

11.3. Materials and methods

The experiment was conducted according to the New Zealand Revised Code of Ethical Conduct for the use of live animals for research, testing and teaching, and approved by the Massey University Animal Ethics Committee.

11.3.1. Diets and experimental design

Dicalcium phosphate was obtained from a commercial source. Eight experimental diets were developed for this study. Diets 1, 2 and 3 were formulated to contain 13, 26 and 39 g/kg of DCP to determine the true Ca digestibility by the regression method. Diet 3 with 39 g/kg of DCP was also used to determine the Ca digestibility by the direct method. Diets 4, 5, 6 and 7 were developed to determine the true Ca digestibility of DCP using the difference method at different Ca:non-phytate P ratios (2:1 and 1.16:1). Diets 4 and 6 were developed with Ca:non-phytate P ratios of 2:1 and 1.16:1 by using limestone as a Ca source. Diet 5 was formulated by substituting a portion of the limestone in diet 4 with DCP to achieve 8.63 g/kg Ca and 4.32 g/kg P with a Ca:non-phytate P ratio of 2:1, and diet 7 by substituting a portion of limestone in diet 6 with DCP to achieve 8.63 g/kg Ca and 7.42 g/kg dietary P with a Ca:non-phytate P ratio of 1.16:1. Maximum dietary Ca concentration in all diets was set at 8.63 g/kg Ca, marginally below the recommended dietary Ca concentration of 9 g/kg for broiler growers (Ross, 2007). A Ca- and P-free diet was developed to determine the ileal endogenous Ca losses. Titanium dioxide (3 g/kg) was incorporated in the diets as an indigestible marker.

11.3.2. Birds

Day-old male broilers (Ross 308) were obtained from a local hatchery and, raised on the floor and fed a commercial starter crumble as described in Chapter 3, section 3.1. On day 14, birds were moved to grower cages. Between days 14 and 20, the starter crumble was gradually changed to mash as the experimental diets were in mash form. On day 21, the birds were individually weighed and allocated to 48 cages (eight birds per cage) on a body weight basis so that the average bird weight per cage was similar. The eight experimental diets were then randomly allotted to six replicate cages each. The diets, in mash form, were offered *ad libitum* from day 21 to day 24 post-hatch and the birds had free access to water. Group body weights and feed intake were recorded on days 21 and 24.

Table 11.1. Ingredient composition and analysis (g/kg, as fed basis) of experimental diets for experiment

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Ca-free diet
	1.3% DCP	2.6% DCP	3.9% DCP					
Maize starch	399.6	393.1	386.6	386.85	389.7	379.67	384.01	398.15
Dextrose	399.6	393.1	386.6	386.85	389.7	379.67	384.01	398.15
Dried egg albumen	100	100	100	100	100	100	100	100
Cellulose	50	50	50	50	50	50	50	50
Soybean oil	20	20	20	20	20	20	20	20
Limestone	-	-	-	22.6	11.3	22.6	11.3	-
Dicalcium phosphate[1]	13	26	39	0	19.5	0	19.5	-
Potassium bicarbonate	8	8	8	8	8	8	8	8
Sodium bicarbonate	3.5	3.5	3.5	-	3	-	-	-
Sodium chloride	1	1	1	1	1	1	1	1
Monosodium phosphate	-	-	-	19.4	2.5	33.76	16.88	19.4
Titanium dioxide	3	3	3	3	3	3	3	3
Trace mineral-vitamin premix[2]	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
Calculated Analysis								
Apparent metabolisable energy (MJ/kg)	15.26	15.05	14.84	14.85	14.94	14.62	14.76	15.22
Crude protein	83.00	83.00	83.00	83.00	83.00	83.00	83.00	83.00
Calcium[3]	2.90	5.76	8.63	8.63	8.63	8.63	8.63	0.04
Total phosphorus[3]	2.56	4.99	7.42	4.32	4.31	7.42	7.42	4.32
Non-phytate phosphorus[3]	2.56	4.99	7.42	4.32	4.31	7.42	7.42	4.32
Ca:non-phytate phosphorus[3]	1.14	1.16	1.16	2.00	2.00	1.16	1.16	0.01
Analysed values (as fed basis)								
Dry matter	897	900	905	897	906	895	904	919
Calcium	3.91	6.86	10.56	8.84	10.31	8.23	11.21	0.34
Total phosphorus	2.64	4.05	8.37	5.09	4.56	9.04	8.83	5.11

[1]Chinese origin; manufacturer unknown, [2]Supplied per kilogram of diet: vitamin A, 12,000 IU; cholecalciferol, 4,000 IU; thiamine, 3 mg; riboflavin, 9 mg; pyridoxine, 10 mg; folic acid, 3 mg; biotin, 0.25 mg; cyanocobalamin, 0.02 mg; dl-α-tocopherol acetate, 80 mg; niacin, 60 mg; Ca-D pantothenate, 15 mg; menadione, 4 mg; choline chloride, 600 mg; Co, 0.25 mg; I, 1.5 mg; Mo, 0.25 mg; Se, 0.26 mg; Mn, 100 mg; Cu, 10 mg; Zn, 80 mg; Fe, 60 mg; antioxidant, 100 mg. [3] Calculated based on NRC (1994) values.

11.3.3. Sample collection and processing

On day 24, all birds were euthanised by intravenous injection (1ml per 2 kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand) and, ileal digesta were collected and processed as described in Chapter 3, section 3.2.

11.3.4. Chemical analysis

Representative samples of DCP, limestone and monosodium phosphate were analysed for DM, Ca and P as described in Chapter 3, section 3.3. Representative samples of diets and ileal digesta were analysed for DM, Ca and titanium as described in Chapter 3, section 3.3.

11.3.5. Calculations

a) Regression method

The true ileal digestibility coefficient of Ca was calculated according to the procedure outlined by Dilger and Adeola (2006a) for the estimation of P digestibility. The apparent ileal digestibility coefficient of Ca of the test diets (at each inclusion concentration) was calculated using the following equation.

$$AIDC = 1 - [(Ti_I/Ti_O) \times (Ca_O / Ca_I)]$$

where AIDC is apparent ileal digestibility coefficient of Ca, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta, and Ca_I is the Ca concentration in the diet. All analysed values were expressed as gram per kilogram of DM.

Total output of Ca in the ileal digesta, expressed as g/kg dry matter intake (DMI), was calculated by the following equation.

$$Ca_{O\text{-}DMI}\,(g/kg) = Ca_{O\text{-}DMO} \times (Ti_I/Ti_O)$$

where $Ca_{O\text{-}DMI}$ and $Ca_{O\text{-}DMO}$ represent Ca output concentrations on DMI and DM output basis, respectively, and Ti_I and Ti_O represent the titanium concentration in diet and digesta, respectively.

To generate the linear regression, digesta Ca outputs were regressed against dietary Ca concentrations by using the following statistical model:

$Ca_{O\text{-}DMI}$ (g/kg) = (TCaI × Ca_I) + IECaL

where $Ca_{O\text{-}DMI}$ represents the Ca concentration in digesta on DMI basis (dependent variable), Ca_I represents Ca concentration in diet on DM basis (independent variable), TCaI represents true Ca indigestibility, and IECaL represents the mean ileal endogenous Ca estimates on DM basis. In this equation, TCaI and ECaL are the slope and intercept, respectively, of the simple linear regression of $Ca_{O\text{-}DMI}$ on Ca_I.

True Ca indigestibility is an indirect measure of the inefficiency at which dietary Ca is extracted by the birds. The true ileal digestibility Ca digestibility was calculated by using the following equation.

TIDC = 1- TCaI

where TIDC and TCaI represent the true ileal digestibility and true ileal indigestibility coefficients of Ca, respectively.

b) Direct method

Apparent ileal digestibility coefficients of Ca were calculated using titanium ratio in the diets and digesta.

AIDC = 1 – [(Ti_I/Ti_O) × (Ca_O / Ca_I)]

where AIDC is the apparent ileal digestibility coefficient of Ca, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta, and Ca_I is the Ca concentration in the diet. All analysed values were expressed as gram per kilogram of DM.

Ileal endogenous Ca losses (g/kg DM intake) were calculated by the following formula.

IECaL = Ca_O × (Ti_I/Ti_O)

where IECaL is the ileal endogenous Ca losses, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, Ca_O is the Ca concentration in the ileal digesta.

True ileal digestibility coefficient was then calculated as follows:

$$TIDC = AIDC + [IECaL\ (g/kg\ of\ DMI)/Ca_I\ (g/kg\ of\ DM)]$$

where TIDC and AIDC represent the true ileal digestibility and apparent ileal digestibility coefficients of Ca, respectively, while IECaL represents the ileal endogenous Ca losses (g/kg of DMI) and Ca_I represents the Ca concentration in diet (g/kg of DM).

c) Difference method

In difference method, true Ca digestibility was calculated according to the procedure describe by Adeola (2001) for the estimation of the nutrient digestibility of a single ingredient. Apparent and true Ca digestibility of the basal and test diets was determined as described above. The following equation was then used to determine the Ca digestibility of the test ingredient.

$$A = [(T \times Tp) - (B \times Bp)/Ap]$$

A = Digestibility coefficient of Ca in the test ingredient

Ap = Proportion of the component of Ca in assay diet contributed by test feed ingredient

B = Digestibility coefficient of Ca in the basal feed ingredient

Bp = Proportion of the component of Ca in assay diet contributed by basal feed ingredient.

T = Digestibility coefficient of Ca in test diet

Tp = Ap+Bp

11.3.6. Statistical analysis

For direct and difference methods, the data were analysed by a one-way ANOVA using the General Linear Model of SAS (2004). Cages served as the experimental unit and an alpha level of 0.05 was used for all analysis. In the regression method, mean true Ca

digestibility coefficients and endogenous Ca losses (g/kg DMI) were estimated by regressing dietary Ca intake (g/kg DMI) against digesta Ca output (g/kg DMI). Thus, standard errors for these regression coefficients were based on total of 18 observations. The significance of differences between the true Ca digestibility coefficients of DCP determined by the direct, difference and regression methods were compared by using means and confidence intervals.

11.4. Results

Analysed Ca and P concentrations of the experimental diets are presented in Table 11.1. The analysed dietary Ca concentration of diet 3 was 0.40 g/kg of diet lower, while those in the other diets were 0.21 to 3.14 g/kg higher than the calculated values. The analysed dietary P concentration of diet 6 was 0.94 g/kg lower than the calculated values while concentrations in the other diets was 0.25 to 1.62 g/kg higher than calculated values. Analysed values were used to determine the digestibility coefficients of DCP.

Analysed Ca and P concentrations of the DCP, limestone and monosodium phosphate samples are presented in Table 11.2. The Ca concentrations of DCP, limestone and monosodium phosphate were 322, 352 and 0.20 g/kg, respectively.

Table 11.2. Comparison of calcium and phosphorus concentrations of calcium sources used in this study with NRC (1994) values[1]

	Current study (g/kg)		NRC (1994)	
	Calcium[1]	Phosphorus[1]	Calcium	Phosphorus
Dicalcium phosphate	322	176	220	187
Limestone	352	0.18	380	-
Monosodium phosphate	0.20	248	-	218

[1]Samples were analysed in duplicate.

The body weight gain and feed intake of birds during the 3-day trial period are presented in Table 11.3. Body weight gain and feed intake were influenced ($P < 0.05$) by dietary treatments. The highest ($P < 0.05$) and lowest ($P < 0.05$) weight gains were observed in birds fed diets 4 and 6, respectively. Feed intake was highest ($P < 0.05$) on diet 5 and, lowest ($P < 0.05$) on diet 6 and the Ca- and P-free diet.

Table 11.3. Body weight gain and feed intake (g/bird/day) of birds fed the experimental diets (21-24 days of age)[1]

Diet[2]	Weight gain (g/bird/day)	Feed intake (g/bird/day)
1	20[b]	76[ab]
2	18[bc]	75[b]
3	20[b]	77[ab]
4	24[a]	74[b]
5	18[bc]	79[a]
6	13[d]	70[c]
7	18[bc]	75[b]
Ca- and P-free diet	16[c]	70[c]
SEM[3]	1.0	1.4
Probability, *P*≤	0.001	0.001

[a, b, c] Values with a different superscript within a column differ significantly (P < 0.05).

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Diets 1, 2 and 3 were used to determine the calcium digestibility of DCP by the regression method, diet 3 was used for the direct method and diets 4, 5, 6 and 7 for the difference method with Ca:non-phytate P ratios of 2:1 and 1.16:1.

[3] Pooled standard error of mean.

The apparent and true ileal Ca digestibility coefficients of the experimental diets are presented in Table 11.4. Apparent ileal Ca digestibility coefficients of the diets varied from 0.33 to 0.67. Ileal endogenous Ca losses (mean ± SE), following the feeding of Ca- and P-free diet, were determined to be 86 ± 11 mg/kg of DMI and were used to calculate the true Ca digestibility coefficient. The true Ca digestibility of the experimental diets varied from 0.34 to 0.68. Significant differences ($P < 0.05$) were observed between the true Ca digestibility of the experimental diets, being highest on diet 1 and lowest on diets 3 and 7.

Diets 1, 2 and 3 were used to determine the true Ca digestibility of DCP by the regression method. Figure 11.1 represents the effect of digesta Ca output (g/kg of DMI) regressed against dietary Ca concentration (g/kg DM). The results indicated a strong linear relationship ($R^2 = 0.98$) between digesta Ca outputs and dietary Ca intakes, which is a prerequisite for the application of regression method. The true Ca digestibility coefficient of DCP determined by the regression method was 0.13. Ileal endogenous Ca losses determined as the intercept of the regression line was -2.29 g/kg of DMI. The true Ca digestibility coefficient of DCP using the direct method was determined to be 0.34.

Table 11.4. Apparent and true ileal calcium digestibility coefficients of experimental diets for broiler[1]

Diet[2]	Apparent ileal calcium digestibility coefficient	True ileal calcium digestibility coefficient
1	0.67[a]	0.68[a]
2	0.42[d]	0.43[d]
3	0.33[e]	0.34[e]
4	0.60[b]	0.61[b]
5	0.42[d]	0.43[d]
6	0.52[c]	0.53[c]
7	0.37[de]	0.38[de]
SEM[3]	0.02	0.02
Probability, *P*≤	0.001	0.001

[a, b, c] Values with a different superscript within a column differ significantly (P < 0.05).

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Diets 1, 2 and 3 were used to determine the calcium digestibility of DCP by the regression method, diet 3 was used for the direct method and diets 4, 5, 6 and 7 for the difference method with Ca:non-phytate P ratios of 2:1 and 1.16:1.

[3] Pooled standard error of mean.

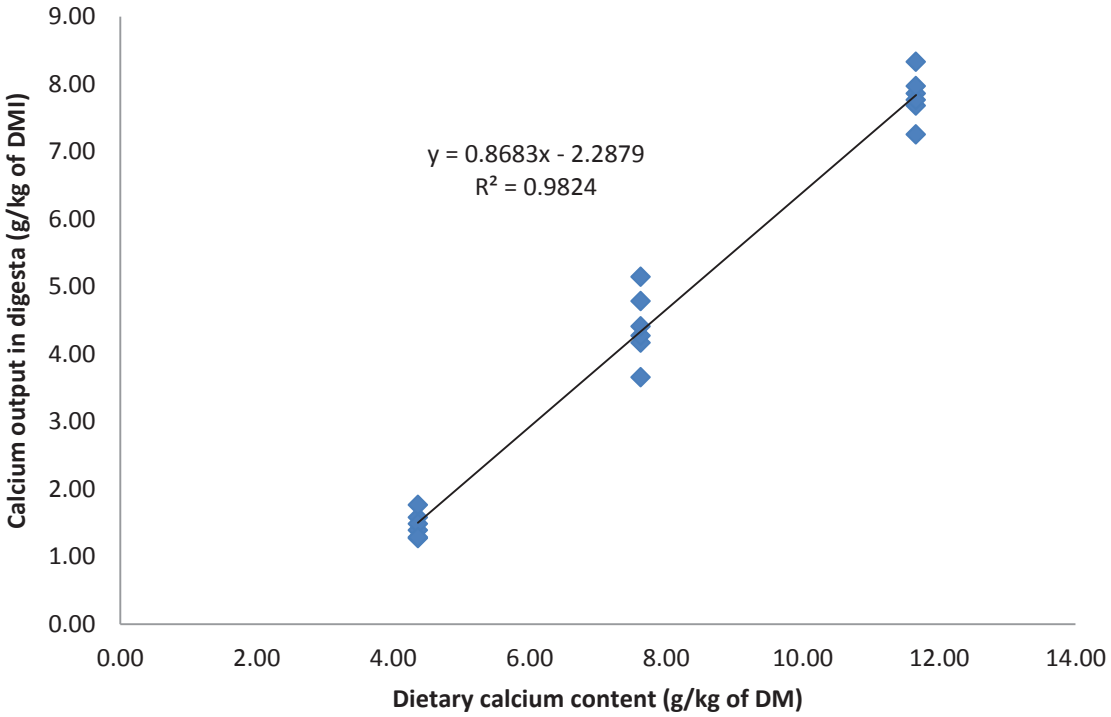

Figure 11.1. Digesta calcium output (g/kg of DMI) regressed against dietary calcium concentration (g/kg DM) in diets 1, 2 and 3

Table 11.5. Linear relationship between digesta calcium outputs (g/kg of DMI) and dietary calcium concentrations (g/kg DM) of dicalcium phosphate

Regression equation[1]	SE of the slope[2]	SE of the intercept[2]	r^2	Endogenous Ca losses (g/kg DMI)	Digestibility coefficient[3]
Y=0.868X – 2.289	0.029	0.245	0.98	-2.289	0.13

[1] Regression of digesta Ca output (g/kg DMI) against dietary Ca concentration (g/kg DM) as determined by feeding chickens with graded levels of dicalcium phosphate. The slope represents the true Ca indigestibility and the intercept provides an estimate of endogenous Ca loss (g/kg DMI).
[2] Standard errors of regression components of the slope and intercept.
[3] Calculated as (1 − true Ca indigestibility coefficient), as described in Section 11.3.5 (a).

The effect of the Ca:non-phytate P ratio on the true Ca digestibility of DCP determined by the difference method is presented in Table 11.6. There was no effect of the Ca:non-phytate P ratio ($P > 0.05$) on the true Ca digestibility of DCP for broiler chickens. The true Ca digestibility of DCP at Ca:non-phytate P ratios of 2:1 and 1.16:1 was 0.25 and 0.21, respectively.

Table 11.6. True ileal calcium digestibility coefficient of dicalcium phosphate with different calcium to non-phytate phosphorus ratios determined by difference method[1]

	Ca:non-phytate P	True ileal calcium digestibility
Difference method	2:1	0.25
	1.16:1	0.21
SEM[2]		0.03
Probability, *P*=		0.42

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Pooled standard error of mean.

Difference in the Ca digestibility coefficients of DCP using various methodologies was determined by comparing the means and confidence interval are represented in Figure 11.2. For the difference method, the true Ca digestibility coefficient of 0.21 at Ca:non-phytate P ratio of 1.16:1 was used in this comparison. The true Ca digestibility of DCP with the direct method was higher ($P < 0.05$) compared to

the regression and difference methods. No difference ($P > 0.05$) was observed between the true Ca digestibility of DCP determined by regression and difference methods.

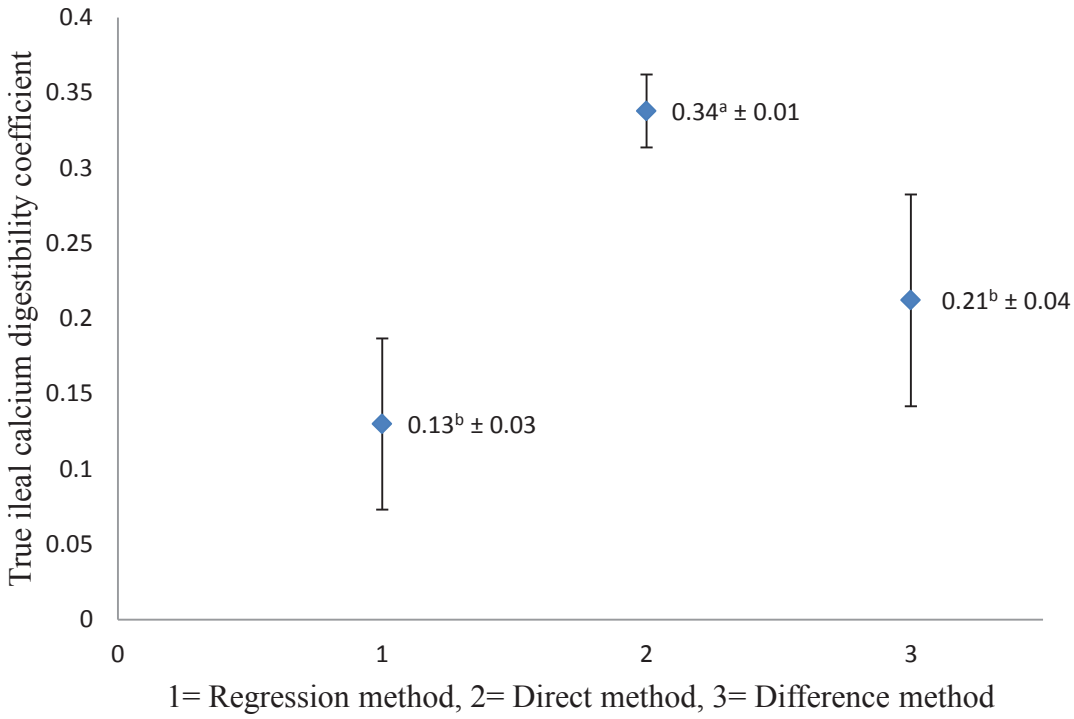

Figure 11.2. Comparison of true calcium digestibility coefficients (mean ± SE) of dicalcium phosphate as determined using the direct, difference and regression methods

[a, b, c] Values with a different superscript differ significantly ($P < 0.05$).

11.5. Discussion

According to NRC (1994), the average Ca concentration of limestone and DCP are 380 and 220 g/kg, respectively. In the current study, the analysed Ca concentration of limestone was comparable to the NRC (1994) value, whereas it was 102 g/kg higher in the DCP (Table 11.6). The DCP sample used in the present work was obtained from the same company as that used in Chapter 10 but was from a different batch. Differences in the Ca concentration of DCP samples from different batches highlight the need for mineral analysis of each batch before using in feed formulation. Higher Ca concentrations in DCP samples (261-296 g/kg) have also been reported previously (Browning and Cowieson, 2013).

The analysed dietary Ca concentration of the experimental diets containing limestone as the sole source of Ca was close to the calculated values (Table 11.1). However, the analysed dietary Ca concentration of experimental diets containing DCP was 1.1 to 2.58 g/kg higher than the calculated values, as the NRC (1994) Ca concentration of DCP was used for the diet formulations.

The true Ca digestibility coefficients of 0.13, 0.34 and 0.21 were determined by regression, direct and difference methods, respectively. Thus, contrary to expectation, the use of regression and difference methods did not resolve the issue of low digestibility estimates and, in fact, yielded estimates lower than that determined by the direct method. This finding is in contrast to our previous studies (Chapters 5 and 6) where no difference was observed between the direct and regression methods in assessing the true Ca digestibility of meat and bone meal. Currently there are no published data available on true Ca digestibility of DCP in poultry. The Ca digestibility of DCP in this study was very low as compared to the values determined for meat and bone meal and limestone in previous studies (Chapter 5, 6 and 7). However, the Ca digestibility of DCP determined by the direct method in the current study was comparable to the value of 0.28 for DCP determined in our previous study (Chapter 10).

In the regression method, the intercept represents endogenous losses. In this study, negative ileal endogenous Ca losses were observed using the regression method. Negative endogenous Ca losses are not physiologically possible, reflecting a possible limitation of the regression method here. In a previous study (chapter 5), positive endogenous Ca losses were observed with the regression method for meat and bone meal. However, negative endogenous P losses have been reported in several studies using the regression method (Liu *et al*., 2013; Mutucumarana *et al*., 2014a,b; 2015b). The negative endogenous losses determined in this study may account, in part, for the low true Ca digestibility of DCP measured with the regression method as compared to the direct and difference methods. Lower P digestibility of feed ingredients has been reported to be associated with negative endogenous P losses (Mutucumarana *et al*., 2014a,b; 2015b).

A prerequisite of the regression method is that the apparent digestibility coefficient is constant at all levels of Ca intake. This precondition was not satisfied, and in the current study, increasing dietary Ca concentrations (diets 1, 2 and 3) reduced the

apparent Ca digestibility coefficient of DCP which indicate that regression method is not suitable method for this study.

In current study, the highest Ca digestibility coefficient of 0.34 was observed with the direct method. However, this value is considerably lower than the Ca digestibility coefficient of 0.78 reported in pigs with the direct method (Gonzalez-Vega *et al*., 2015a). The possible reasons for this discrepancy may be explained by the differences in species and methodology used. In the pig study, maize-based basal diets were used compared to the present study in which purified diets based on maize starch and dextrose were used. Maize-based diets have been reported to produce higher Ca digestibility (0.46 vs. 0.89) of FM when compared to maize starch-based diets (Gonzalez-Vega *et al*., 2015b). Maize-based diets have been used frequently in pig studies to determine the true Ca digestibility of different feed ingredients (Sulabo and Stein, 2013; Gonzalez-Vega *et al*., 2015a, b; Merriman *et al*., 2016).

An additional aim of the present study was to investigate whether the Ca digestibility estimates could be improved by adjusting the Ca: non-phytate P ratios in assay diets, but no difference was observed between the true Ca digestibility of DCP with ratios of 2:1 and 1.16:1. Given the close relationship between these two minerals, this finding was unexpected.

11.6. Conclusions

In conclusion, the issue of low Ca digestibility in DCP, a Ca source with a narrow Ca: non-phytate P ratio was not resolved by the use of regression and difference methods or by manipulating the Ca: non-phytate ratio in assay diets.

CHAPTER 12

General discussion

12.1. Introduction

Calcium (Ca) and phosphorus (P) are vital minerals required for the skeletal development in animals. Almost 99% of the body Ca is present in the skeleton and the remaining 1% is involved in a wide array of metabolic and physiological functions in the body. Calcium plays important roles *inter alia* in blood clotting, muscle contraction, transmission of nerve impulses, hormone secretion, and regulation of heart function (Suttle, 2010). A deficiency in Ca can cause poor growth performance, rickets and tibial dyschondroplasia in broiler chickens. On the other hand, high dietary Ca concentrations can cause reduction in the availability of several important and costly nutrients, including P, protein and lipids (Ballam *et al*., 1984; Tamim and Angel, 2003; Mutucumarana *et al*., 2014c).

Measurement of Ca digestibility in poultry has received relatively little attention in the past due to the cheap availability of limestone, the major source of Ca in poultry diets. However, the recent interest in the measurement of digestible P in feed ingredients (WPSA, 2013) due to increasing price of inorganic phosphates and environmental P pollution necessitates a closer look at digestible Ca. If the poultry industry shifts towards a digestible P system, the close relationship between Ca and P during absorption and post-absorptive utilisation processes requires the development of a digestible Ca system to ensure that the Ca and P requirements of birds are precisely met. It is generally assumed that Ca in feed ingredients is highly available, but this assumption has never been tested and confirmed. At the time of the commencement of this thesis research, no published data were available on the digestibility of Ca in feed ingredients for poultry. Therefore, the major objectives of the studies reported herein were to develop a methodology for the determination of true Ca digestibility of major Ca sources for broiler chickens and to examine some factors influencing Ca digestibility.

12.2. Development of methodology for the measurement of true ileal calcium digestibility

When this project was initiated, no established method was available for the determination of true ileal Ca digestibility for poultry. However, several studies have been conducted during the recent past where the regression method (Dilger and Adeola, 2006a; Liu *et al*., 2013; Mutucumarana *et al*., 2014a,b; 2015a) and direct method (Mutucumarana and Ravindran, 2016) were used to determine the true P digestibility of feedstuffs for broiler chickens. In the regression method, diets with graded concentrations of the specific nutrient from the specific assay ingredient are formulated. Theoretically, the digestibility estimates determined by the regression method are automatically corrected for total endogenous losses and represent true digestibility values. In the direct method, test ingredients serve as the sole source of the nutrient in the diet. The digestibility values determined using the direct method are apparent values. Measurement of the endogenous loss of Ca is necessary for the estimation of true/standardised digestibility of Ca using the direct method. In several University of Illinois studies, basal endogenous Ca losses in pigs have been quantified following the feeding of Ca-free diets with P and wide variations have been reported, with values ranging from 123 to 670 mg/kg dry matter intake (Gonzalez-Vega and Stein, 2016) which were used to calculate the standardised Ca digestibility. In studies described in this thesis, ileal endogenous Ca losses were determined several times and also the uses of different techniques were compared.

12.2.1. Ileal endogenous calcium losses

The first study (Chapter 4) was conducted to measure the basal ileal endogenous losses using various methodologies, namely Ca- and P-free, egg albumen and maize gluten based diets. Ileal endogenous Ca losses were determined to be 125, 77 and 43 mg/kg of dry matter intake, respectively.

In this thesis research, ileal endogenous Ca losses were determined using a Ca- and P-free diet in eight separate assays, with an average (± SE) of 108±6 mg/kg dry matter intake (range, 84-127 mg/kg). It must be noted, however, that this estimate is very low when compared to the amount of undigested Ca in the ileum and its use for true ileal Ca digestibility corrections essentially made very little difference.

12.2.2. True ileal calcium digestibility in broilers using various methodologies

The second study (Chapter 5) was planned to determine the true Ca digestibility of three meat and bone meal (MBM) samples using the regression method. The strong linear relationship observed between dietary Ca concentrations and digesta Ca output indicated that the regression method could be used for the estimation of Ca digestibility of MBM. The true Ca digestibility coefficients of the three MBM samples ranged between 0.46 and 0.60. The exact reasons for the observed variability in Ca digestibility among MBM samples are unclear; however, this variation could not be attributed to the contents of ash, Ca and bone content or particle size.

As the regression method is laborious, costly and time consuming, the next study (Chapter 6) was planned to determine the true Ca digestibility of four MBM samples using the direct method. The true ileal Ca digestibility coefficient of the four samples varied from 0.41 to 0.56. Three of the MBM samples evaluated in these two studies (Chapters 5 and 6) were the same, thus enabling direct comparison between the two methods. The comparison of true Ca digestibility coefficients of MBM samples determined by the direct and regression methods showed that the ranking and trend in variation in digestibility among samples within each method were similar.

The direct method is simple, less laborious and cost effective, and yields comparable results to that of the regression method. For this reason, the direct method was employed in subsequent studies (Chapters 7, 8, 9 and 10), to determine the true Ca digestibility of limestone, oyster shell, dicalcium phosphate (DCP), monocalcium phosphate (MCP), poultry by-product meal (PBPM), fish meal (FM) and canola meal (CM).

Limestone is the major Ca source used in poultry feed formulations throughout the world. Therefore, the next study (Chapter 7) was conducted to determine the true ileal Ca digestibility of three limestone samples with and without 4.5 g/kg of dietary non-phytate P using the direct method. True ileal Ca digestibility coefficients of limestone samples varied from 0.56 to 0.62. Inclusion of dietary P increased the average ileal Ca digestibility of limestone from 0.57 to 0.61. These findings implied that the true Ca digestibility of limestone for broiler chickens is not as high as widely assumed.

Particle size and dietary Ca:non-phytate P ratios play important roles in Ca absorption (Cheng and Coon, 1990a; Zhang and Coon, 1997a; Adedokun and Adeola,

2013; Proszkowiec-Weglarz and Angel. 2013). The next study (Chapter 8) determined the effect of particle size and Ca:non-phytate P ratio on the true ileal Ca digestibility of limestone. Three Ca:non-phytate P ratios (1.5, 2.0 and 2.5) and two particle sizes (fine, <0.5 mm and coarse, 1-2 mm) were evaluated in this study. The Ca:non-phytate P ratio was adjusted by manipulating the dietary Ca concentration with a fixed amount (4.5 g/kg) of non-phytate P. Increasing Ca:non-phytate P ratios reduced the true Ca digestibility. The true Ca digestibility coefficients of limestone with Ca:non-phytate P ratios of 1.5, 2.0 and 2.5 were 0.65, 0.57 and 0.49, respectively. The true Ca digestibility of coarse particles was markedly greater compared with fine particles (0.71 vs. 0.43). These data demonstrated that narrow Ca:non phytate P ratios and coarse particle size improved the Ca digestibility of limestone for broiler chickens.

Oyster shell is another Ca source, used in poultry feed formulations. The next study (Chapter 9) was conducted to determine the effect of Ca source (limestone vs. oyster shell) and particle size (<0.5 and 1-2 mm) on true Ca digestibility. The true ileal Ca digestibility coefficients of limestone and oyster shell were found to be similar. Increasing the particle size from <0.5 mm to 1-2mm increased the Ca digestibility of both Ca sources. The true Ca digestibility coefficients of fine and coarse particles of limestone and oyster shell were 0.38 and 0.62, and 0.33 and 0.56, respectively. This study confirmed the positive impact of coarse particle size on the true Ca digestibility.

The next study (Chapter 10) was conducted to determine the true Ca digestibility of DCP, MCP, PBPM, FM and CM using the direct method. In this study, Ca digestibility coefficients of these ingredients were determined to range from 0.24 to 0.33. These Ca digestibility estimates were low, compared to those for limestone, oyster shell and MBM determined in previous studies. All ingredients evaluated in this study had an inherent imbalance in the Ca:non-phytate P ratio, which could have lowered Ca absorption. The Ca:non-phytate P ratios in DCP, MCP, PBPM and FM were 1.08:1, 0.70:1, 1.50:1 and 1.43:1, respectively.

Another possible reason for the low digestibility estimates may be the length of adaptation to assay diets. A study by Proszkowiec-Weglarz and Angel (2013) has raised questions regarding the effect of the length of dietary adaptation on the measurement of Ca and P digestibility of diets with an imbalance of dietary Ca and P concentrations. In the current study, a dietary adaption length of 72 hours was used before digesta

collection. Since animals have a very tight homeostatic regulation of Ca, a time course of changes in Ca digestibility must be established. Angel *et al.* (2013), using the direct method, determined the apparent Ca digestibility of four ingredients from 8 to 96 hours after the introduction of assay diets and reported that, based on changes in Ca digestibility, the best time to determine digestibility was around 32-40 hours. To further examine this possibility, a second study (Chapter 10) was conducted to evaluate the effect of dietary adaptation length (24, 48 and 72 hours) on the true ileal Ca digestibility of DCP and MCP. Overall, the results showed that the Ca digestibility was unaffected by an adaption length of 24 and 72 hours.

The tested dietary adaption lengths did not resolve the issue of the low Ca digestibility of DCP and MCP, and the digestibility coefficients varied between 0.35-0.45 and 0.30-0.34, respectively (Chapter 10). These values were much lower than the corresponding Ca digestibility coefficients of 0.78 and 0.86 of the same ingredients in pigs, respectively (Gonzalez-Vega *et al.,* 2015a). These differences may be related, at least in part, to methodology, as the difference method was used in the pig study compared to the direct method in our broiler study (Chapter 10). In the study reported in Chapter 11, therefore, the effect of methodology (direct, difference and regression method) on the true Ca digestibility of DCP was compared. It was found that the use of the other methods did not overcome the issue of the low digestibility estimate for DCP because the highest digestibility coefficient of 0.34 was observed with direct method, while difference and regression methods produced much lower values.

12.3. Comparison of calcium digestibility in broilers and pigs using empirical work

Currently there is no comparable data on the true Ca digestibility of feedstuffs for broiler chickens. However, several studies have been reported on the Ca digestibility of Ca sources in pigs. A comparison of the true ileal Ca digestibility coefficients of the Ca sources tested in the current study with the pig data showed that the Ca digestibility of limestone and MBM was comparable in broilers and pigs. However, for DCP, MCP, PBPM, FM and CM the Ca digestibility coefficients in broilers were much lower than those determined for pigs. It is difficult to explain this discrepancy. However, one possible reason may be the difference in the composition of basal diet. Maize based diets was used in pig studies, whereas purified diets based on maize starch and dextrose were used in our studies.

Gonzalez-Vega *et al*. (2015b) observed that the standardised total tract Ca digestibility of FM increased from 0.46 to 0.89 when dietary maize starch was replaced with maize in the diets. It was suggested that the increase in Ca digestibility may be due to the reduction in the pH in the digestive tract due to the higher soluble fibre content of the maize-based diets.

In the case of CM, phytic acid may be a factor contributing to the lower Ca digestibility in our studies as compared to pig study reported by Gonzalez-Vega *et al*. (2013b). In this pig study, CM was used at a maximum inclusion level of 500 g/kg as compared to 970 g/kg in our study (Chapter 10) which would have contributed much more phytate P in the assay diet, influencing the Ca digestibility.

In our studies, the true ileal Ca digestibility of limestone and oyster shell increased with increasing particle size (Chapter 8 and 9). In contrast, no differences were observed in the Ca digestibility of limestone in pigs where a range of four particle sizes 0.2, 0.5, 0.7 and 1.125 mm were tested (Merriman and Stein, 2016). Possible reasons may be the differences in species, especially the presence of gizzard in broilers, and smaller coarse particles (1.125 mm) in the pig study as compared to 1-2 mm in our broiler studies. Calcium digestibility in pigs were reported as total tract digestibility as compared to our data which was based on ileal digestibility and presence of larger hindgut in pigs might be another contributing factor for differences in digestibility values in chicken and pigs.

Table 12.1. Comparison of true ileal calcium digestibility coefficients of different feed ingredient determined in this study with calcium digestibility data from pigs

Ca source	Broiler data (current study)		Pig data	
	Chapter	True ileal Ca digestibility coefficient	Reference	Calcium digestibility coefficient
Meat and bone meal	Chapters 5 and 6	0.41-0.60	Sulabo and Stein (2013)	0.53-0.81[1]
Calcium carbonate	Chapter 7	0.61-0.70	Gonzalez-Vega *et al.* (2014)	0.53[2]
Calcium carbonate	Chapter 7	0.61-0.70	Gonzalez-Vega *et al.* (2014)	0.61[3]
Calcium carbonate	Chapter 7	0.61-0.70	Gonzalez-Vega *et al.* (2015a)	0.64[3]
Dicalcium phosphate	Chapters 10 and 11	0.13-0.45	Gonzalez-Vega *et al.* (2015a)	0.77[3]
Monocalcium phosphate	Chapter 10	0.30-0.34	Gonzalez-Vega *et al.* (2015a)	0.73[3]
Poultry by-product meal	Chapter 10	0.29	Merriman *et al.* (2016)	0.88[3]
Fish meal	Chapter 10	0.24	Gonzalez-Vega *et al.* (2015b)	0.46-0.89[3]
Canola meal	Chapter 10	0.31	Maison *et al.* (2015)	0.61[1]
Canola meal	Chapter 10	0.31	Gonzalez-Vega *et al.* (2013b)	0.47[3]

[1] Apparent total tract digestibility.

[2] Standardised ileal digestibility.

[3] Standardised total tract digestibility.

12.4. Comparison of the calcium digestibility of practical diets with predicted calcium digestibility using calcium digestibility coefficients determined in this thesis research

With the exception of those determined in our studies, no published data are available on the true Ca digestibility of feed ingredients for broiler chickens. However, several studies have reported the apparent ileal Ca digestibility of practical-type diets for broiler chickens (Ravindran *et al*., 2006; Walk *et al*., 2012b; Amerah *et al*., 2014; Tancharoenrat and Ravindran, 2014; Bradbury *et al*., 2016). In practical diets, 80-90% dietary Ca comes from the major Ca sources, limestone and inorganic phosphates (DCP and MCP). A comparison of the apparent ileal digestibility coefficient of Ca in practical diets with the Ca digestibility coefficients of those diets predicted using the Ca digestibility coefficient of major Ca sources (limestone, DCP and MCP) determined in the current thesis is presented in Table 12.2. The apparent ileal Ca digestibility coefficients of diets had a range from 0.26 to 0.62, while predicted true digestibility coefficient ranged from 0.37 to 0.48. While these two sets of values are not exactly comparable due to various confounding factors, the comparison appears to indicate that values generated in the current thesis are acceptable in general.

Table 12.2. A comparison of the calcium digestibility coefficients of experimental diets from previous studies with predicted calcium digestibility of diets using true ileal calcium digestibility determined in studies described in this thesis [1, 2,3]

Reference	Major calcium source	Total Dietary calcium (g/kg)	Calcium from limestone + DCP/MCP (g/kg)	Dietary available phosphorus (g/kg)	Apparent ileal calcium digestibility coefficient of diets	Predicted calcium digestibility coefficient of diets [4]
Ravindran *et al*. (2006)	Limestone + DCP	7.5	6.5	2.5	0.26	0.39
Ravindran *et al*. (2008)	Limestone + DCP	7.8	5.4	3.0	0.28	0.45
Walk *et al*. (2012b)	Limestone + MCP	9.0	7.7	4.5	0.56	0.46
Tancharoenrat and Ravindran (2014)	Limestone + DCP	7.0	5.9	5.0	0.53	0.37
Tancharoenrat and Ravindran (2014)	Limestone + DCP	10.0	8.9	5.0	0.46	0.43
Amerah *et al*. (2014)	Limestone + DCP	4.0	3.3	2.8	0.54	0.39
Amerah *et al*. (2014)	Limestone + DCP	6.0	5.3	2.8	0.50	0.44
Amerah *et al*. (2014)	Limestone + DCP	8.0	7.3	2.8	0.56	0.47
Amerah *et al*. (2014)	Limestone + DCP	10.0	9.3	2.8	0.62	0.48
Bradbury *et al*. (2016)	Limestone + DCP	4.0	2.8	2.5	0.55	0.40
Bradbury *et al*. (2016)	Limestone + DCP	5.7	4.5	2.5	0.47	0.45

[1] To determine the true calcium digestibility of diets, the limestone digestibility coefficient of 0.53 (average from chapter 7, 8 and 9) with a fine particle size and dietary calcium and non-phytate phosphorus concentration of 9 and 4.5 g/kg, respectively was used.

[2] For dicalcium phosphate (DCP) and monocalcium phosphate (MCP) the digestibility coefficients of 0.32 and 0.34 (average from Chapters 10 and 11) were used, respectively.

[3] Calcium contributions from limestone, dicalcium phosphate and monocalcium phosphate were determined using NRC (1994) values of 380, 220 and 160 g/kg, respectively.

[4] True calcium digestibility coefficients of diets were determined by using the digestibility coefficients of 0.53, 0.32 and 0.34 for limestone, DCP and MCP in the diets.

12.5. Problems faced during the study

12.5.1. Analysed calcium concentration of ingredients used

Differences in the analysed Ca concentration of different feed ingredients used in this study were observed as compared to standard NRC (1994) values. A comparison of the analysed Ca concentrations of feed ingredients in the current work with the values of NRC (1994) and Browning and Cowieson (2013) is presented in Table 12.3. For MBM, the analysed Ca concentration of different samples varied from 71-118 g/kg of diet on an as-fed basis. These variations can be related to the origin of the sample used. A wide variation in Ca concentration (165-322 g/kg) of different batches of DCP samples was observed in Chapters 10 and 11, although the DCP samples were from same supplier in China. It is therefore recommended to analyse the Ca concentration of each batch of Ca source before using it for feed formulations. For limestone samples, the analysed Ca concentration varied from 352-420 g/kg; but values higher than 380 g/kg are not possible scientifically, according to the formula of calcium carbonate ($CaCO_3$), which suggests possible analytical errors.

Table 12.3. A comparison of the analysed calcium concentrations of calcium sources used in the studies herein with NRC (1994) and Browning and Cowieson (2013) values (g/kg, as fed basis)

Calcium source	Current Study Mean (Range)	NRC (1994)	Browning and Cowieson (2013) Mean (Range)
Meat and bone meal	96(71-118)	103	109(51-148)
Limestone	393(352-420)	380	393(376-415)
Oyster shell	370	380	-
Dicalcium phosphate	258(195-322)	220	276 (261-294)
Monocalcium phosphate	155	160	164 (152-184)
Poultry by-product meal	40	30	32 (22-40)
Fish meal	67	12-73	-
Canola Meal	6	6.8	6 (3.7-7.1)

12.5.2. Calculated and analysed calcium concentration of experimental diets

A major problem faced in the current series of studies was the notable differences between analysed and calculated dietary Ca concentrations. Given that over 85% of

dietary Ca is supplied by inorganic Ca sources, analysing them prior to formulation should resolve this problem, but our experience shows that is not always the case. In the current study, the differences between analysed and calculated dietary Ca concentrations ranged from -0.78 to +2.58 g/kg diet. It is difficult to explain this variation as in most of the studies; the calculated values were based on the analysed Ca concentration of Ca sources used in those studies. One possible reason for the observed differences may be sampling errors. However these differences are in agreement with previous studies where similar variations have been reported (Walk *et al*., 2012b; Amerah *et al*., 2014; Kiarie *et al*., 2014; Bradbury *et al*., 2016).

12.6. Suggestions for the future work

Some issues that need to be resolved in future studies are briefly discussed below. Several of these issues have been highlighted previously (Adedokun and Adeola; 2013; Proszkowiec-Weglarz and Angel, 2013).

1. A 2:1 Ca:non-phytate P ratio was maintained in the assay diets used for limestone and MBM. But maintaining a 2:1 ratio is not possible in assays for inorganic phosphates (MCP and DCP) and common protein meals with the high P concentrations, relative to Ca. This resulted in much narrower ratios and possibly contributed to the observed low Ca digestibility estimates. Future studies are needed to explore the appropriate methodology for determination of true Ca digestibility of these ingredients.

2. Our data, presented in this thesis, show that almost half the Ca in limestone and MBM are unabsorbed and excreted. In this context, future research should explore the reasons for this poor absorption and how the absorption can be improved (Dudley-Cash, 2016).

3. Microbial phytase is now routinely used in poultry diets nowadays and, generation of data on ileal Ca and P digestibility of Ca sources with phytase supplementation are needed to be relevant in practical situations. This consideration is particularly critical given the differential effects of phytase on the release of Ca and P (Walk, 2016).

4. In the studies reported in this thesis (except Chapter 5), the true Ca digestibility of different Ca sources was determined for broiler chickens at day 24 post-hatch. Nutrient digestibility values determined with 3-5 week-old broilers are commonly used in feed formulations for broilers of different ages and for broilers and laying hens. Comparative values between bird classes are limited and the assumption of similar digestibility has never been validated. Some evidence indicates that Ca digestibility is reduced with age of broilers (Angel *et al.,* 2013). Since Ca metabolism is influenced by physiological needs (egg production, growth rate and skeletal growth) and has a very tight homeostatic control, such a validation is urgently needed.

5. The effect of vitamin D_3 (1, 25 dihydroxy D_3) on the absorption of Ca from the intestine and its homeostasis in the body is well known (Veum, 2010). In our assay diets, vitamin D_3 was supplied as 4,000 International Units cholecalciferol per kg diet. Although this supplementation level is reported to be sufficient to support live performance and bone mineralisation (Fritts and Waldroup, 2003), adequacy of this inclusion level for maximum Ca absorption has never been tested.

6. The physical form of Ca supplements is important to allow for maximum utilisation, and this has been demonstrated in our studies (Chapters 8 and 9) with limestone and oyster shell. However, because broiler diets are pelleted, the particle size of limestone may not be directly relevant to broiler feeding. A practical solution may be to feed these Ca sources as large particles separate from the rest of the broiler feed (Wilkinson *et al.,* 2013b; Abdollahi *et al.,* 2016).

7. The use of mash diets in the digestibility assay may also be criticised. The use of mash diets is standard practice in determining the ileal digestibility of nutrients in feed ingredients whether of amino acids, P or Ca. It is recognised however, that since practical broiler diets are fed as pellets, should be investigated the effect of pellet vs. mash diets on ileal Ca and P digestibility in future studies as published data on the effect of feed form on the digestibility of these minerals are contradictory (Table 12.4). The results generated using purified or semi-purified diets may not be applicable to practical-type diets. There are number of complex and interacting factors in practical diets that influence Ca digestibility,

the most important of which is phytate content. It noteworthy however, that semi-purified diets have been commonly used to determine the amino acid digestibility and the use of these values in practical feed formulations have been a great success (Dudley-Cash, 2016).

Table 12.4. Effect of feed form on apparent ileal calcium digestibility in broiler chickens

Reference	Dietary calcium (%)	Dietary phosphorus (%)	Apparent ileal calcium digestibility coefficient	
			Mash	Pellet
Abdollahi *et al.* (2013)[1]	1.0	0.52	0.42	0.50
Abdollahi *et al.* (2013)[2]	1.0	0.52	0.37	0.29
Abdollahi *et al.* (2014)[3]	1.0	0.53	0.49	0.36
Naderinejad *et al.* (2016)[1]	1.0	0.52	0.53-0.57	0.33-0.45

[1] Maize-based diets.

[2] Wheat-based diets.

[3] Sorghum-based diets.

12.7. Summary and main conclusions

The major aims of this thesis were to develop a methodology for the determination of true ileal Ca digestibility of Ca sources and to determine the effect of selected factors on the Ca digestibility in broilers. Overall, this research on Ca digestibility resulted in more questions than answers and number of challenges were encountered. In total, 9 studies were conducted to determine the true Ca digestibility of MBM, limestone, oyster shell, DCP, MCP, FM, PBPM and CM using different methodologies. Overall, the data suggest that the true Ca digestibility of Ca sources is not as high as was assumed in the past.

The regression and direct methods produced comparable Ca digestibility values for MBM samples. The negative endogenous Ca losses determined by using the regression method indicate another drawback of regression method as compared to the direct method. The low Ca digestibility estimates by direct, difference and regression methods for ingredients with inherent imbalance between Ca and P raises major challenges regarding the applicability of appropriate methodology.

Variations in the true Ca digestibility of limestone were observed with different particles size, dietary non-phytate P and Ca:non-phytate P ratios. The Ca digestibility coefficients of limestone and oyster shell were higher with coarser particles as compared to fine particles. Ileal endogenous Ca losses determined by feeding Ca- and P-free diets were low relative to Ca output in the digesta and therefore had almost no impact on correction for true ileal Ca digestibility.

It is clear that further studies are warranted to answer the questions raised in this thesis research. The ultimate goal is to develop appropriate methodology/methodologies for the determination of true ileal Ca digestibility of Ca sources and to develop a digestible Ca to digestible P ratio for optimum growth and P utilisation to minimise feed costs and environmental pollution.

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APPENDIX

Statement of contribution to doctoral thesis containing publications

Statement of contribution to doctoral thesis containing publications from Chapters 5, 6, 7, 8 and 9 are attached.

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(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Muhammad Naveed Anwar

Name/Title of Principal Supervisor: Professor Velmurugu Ravindran

Name of Published Research Output and full reference:

Anwar, M. N., Ravindran, V., Morel, P. C. H., Ravindran, G. and Cowieson, A. J. (2015). Measurement of true ileal calcium digestibility in meat and bone meal for broiler chickens. Animal Feed Science and Technology, 206:100-107.

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Anwar, M. N., Ravindran, V., Morel, P. C. H., Ravindran, G. and Cowieson, A. J. (2016). Measurement of true ileal calcium digestibility in meat and bone meal for broiler chickens using direct method. Poultry Science, 95:70-76.

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Anwar, M. N., Ravindran, V., Morel, P. C. H., Ravindran, G. and Cowieson, A. J. (2016). Apparent ileal digestibility of calcium in limestone for broiler chickens. Animal Feed Science and Technology, 213:142-147.

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

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