STUDIES ON THE MEASUREMENT OF CALCIUM DIGESTIBILITY IN RAW MATERIALS FOR POULTRY AND OF DIGESTIBLE CALCIUM REQUIREMENT OF BROILER STARTERS

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ABSTRACT

The measurement of calcium (Ca) digestibility in feed ingredients for poultry is receiving increasing attention because of recent interest in formulating diets based on digestible phosphorous (P) and the close relationship between these two minerals in their absorption and post absorptive utilisation. Data on the ileal Ca digestibility of Ca sources and factors influencing Ca digestibility in poultry are scant. The current thesis presents follow-up studies of the previous work conducted at Massey University. The Ca digestibility values of Ca sources determined in the thesis, along with previous published data, were then used to develop dietary treatments to assess the digestible Ca requirement of broiler starters.

The first study reported in Chapter 3 was conducted to examine the effect of basal diet composition on true ileal Ca digestibility of four Ca sources namely, limestone, meat and bone meal (MBM), monocalcium phosphate (MCP) and dicalcium phosphate (DCP) in broiler chickens. Two basal diets, namely a maize-based diet and a maize-starch-based purified diet, with each Ca source were tested. The results showed that the average true ileal Ca digestibility was higher in the maize-based diet (0.46) than that in the purified diet (0.37). True ileal Ca digestibility of limestone, MBM, MCP and DCP were determined to be 0.51, 0.41, 0.43 and 0.32, respectively.

In the study reported in Chapter 4, the effect of dietary indicator-type and dietary adaptation length on the apparent ileal Ca digestibility of limestone were evaluated. In Experiment 1, the use of two indicators namely, titanium dioxide and acid insoluble ash (Celite) were compared and the findings showed that the ileal Ca digestibility was unaffected by dietary indicator. In Experiment 2, four dietary adaptation lengths namely, 24, 72, 120 and 168 hours were examined and it was found that the Ca digestibility was unchanged between 72 and 120 hours but decreased at 168 hours of adaptation length.

The third study, presented in Chapter 5, was conducted to measure the influence of age (7, 14, 21, 28, 35 and 42 days post-hatch) on the Ca digestibility of limestone for broiler chickens. The findings revealed that the apparent ileal Ca digestibility coefficients were linearly decreased from day 7 to day 42. The ileal Ca digestibility coefficients were determined to be 0.51, 0.53, 0.36, 0.34, 0.41 and 0.27 at days 7, 14, 21, 28, 35 and 42, respectively. A secondary objective of this study was to examine the influence of dietary crude protein concentration (79 and 153 g/kg) on the apparent ileal Ca digestibility at 21 days of age. It was found the apparent ileal Ca digestibility was not influenced by dietary protein concentrations.

The studies reported in Chapter 6 were conducted to measure the influence of phytase doses (0, 500 and 2000 FTU/kg) on the Ca and P digestibility of soybean meal (SBM) and canola meal (CM) in broiler starters (Experiment 1) and finishers (Experiment 2). True ileal Ca digestibility coefficients of SBM and CM, with no supplemental phytase, were determined to be 0.51 and 0.53, respectively, for broiler starters and 0.33 and 0.22, respectively, for broiler finishers. True ileal P digestibility coefficients of maize-SBM diet and maize-CM diet, with no phytase were determined to be 0.89 and 0.66, respectively, for broiler starters and 0.82 and 0.57, respectively, for broiler finishers. Microbial phytase increased the true ileal digestibility of Ca and P in maize-SBM diet and maize-CM diet, but the effect was more pronounced for the maize-CM diet. Superdosing of phytase (2000 FTU/kg) increased the Ca digestibility in CM and SBM by two-fold compared to the normal phytase dose (500 FTU/kg).

The studies reported in Chapter 7 were conducted to determine the ileal Ca digestibility coefficients of two limestone sources in broilers and layers. The results showed that, in both sources, the apparent ileal Ca digestibility of limestone was found to be higher in laying hens (0.62 and 0.70) compared to broilers (0.50 and 0.43). The

findings indicated that laying hens absorb Ca more efficiently than broilers which may be attributed to their high demand of Ca for eggshell formation.

Using Ca digestibility values of Ca sources measured in this thesis work and previous published data, a growth study (Chapter 8) was conducted to estimate the standardised ileal digestible (SID) Ca requirement for 1 to 10 day-old broilers fed different dietary concentrations of both SID Ca (3.3, 3.9, 4.4, 5.0 and 5.5 g/kg) and SID P (4, 5 and 6 g/kg). Based on response surface models, the growth performance, bone mineralisation and mineral utilisation of broiler starters were found to be optimised at 5 g/kg SID P concentration. The concentrations of SID Ca that maximised body weight gain, tibia ash, tibia Ca, tibia P and toe ash were estimated to be 3.32, 4.51, 4.72, 4.36 and 4.78 g/kg, respectively, which corresponds to SID Ca to SID P ratios of 0.66, 0.90, 0.94, 0.87 and 0.96, respectively. Bone mineralisation required more SID Ca than for growth.

Most of the findings reported in this thesis are novel and contribute to the advancement of current knowledge on the measurement of ileal Ca digestibility in poultry and the factors influencing Ca digestibility. The array of factors examined were hitherto unexplored and included the effects of basal diet type, dietary adaptation length, broiler age, phytase dose, Ca source and bird type (broilers vs. layers). Another notable contribution was to establish the requirements of digestible Ca, digestible P and the ratio of digestible Ca to digestible P for broiler starters (1-10-day old).

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PUBLICATIONS

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree centigrade
µm	Micrometre
µmol	Micromole
ABC	Acid binding capacity
AIDC	Apparent ileal digestibility coefficient
AME	Apparent metabolisable energy
AOAC	Association of Official Analytical Chemists
ATTRC	Apparent total tract retention coefficient
C	Celsius
Ca	Calcium
Cu	Copper
DAL	Dietary adaptation length
DCP	Dicalcium phosphate
DCI	Digestible calcium intake
DFP	Defluorinated phosphate
DM	Dry matter
DMI	Dry matter intake
EO	Excreta output
Fe	Iron
FI	Feed intake
FTU	Phytase unit
g	Gram
GLM	General linear model
HCl	Hydrochloric acid
IEL	Ileal endogenous losses
Ind	Indicator

K	Potassium
kg	Kilogram
MBM	Meat and bone meal
MCP	Monocalcium phosphate
Mg	Magnesium
mg	Milligram
MJ	Mega joule
mm	Millimetre
Mn	Manganese
MP-AES	Microwave Plasma Atomic Emission Spectrometry
N	Nitrogen
Na	Sodium
NRC	National Research Council
P	Phosphorous
P	Probability
PTH	Parathyroid hormone
RCI	Retainable calcium intake
SAS	Statistical analysis software
SD	Standard deviation
SE	Standard error
SEM	Pooled standard error of mean
SID	Standardised ileal digestible
SIDC	Standardised ileal digestibility coefficient
TCP	Tricalcium phosphate
Ti	Titanium dioxide
TIDC	True ileal digestibility coefficient
UV	Ultraviolet
WPSA	World’s Poultry Science Association
Zn	Zinc

CHAPTER 1

GENERAL INTRODUCTION

Calcium (Ca) is a macro-mineral that is essential for the growth and health of animals. Calcium not only plays a major role in building up the skeletal system, but also contributes to an array of physiological functions (Crenshaw, 2001). The metabolism of Ca is closely associated with that of phosphorous (P). In poultry diets, Ca is supplied by means of inorganic Ca sources as well as feed ingredients. However, the Ca content of feed ingredients of plant origin are very low (NRC, 1994) compared to inorganic Ca sources and, therefore, inorganic Ca supplements are included to meet the Ca requirements. Limestone, monocalcium phosphate (MCP) and dicalcium phosphate (DCP) are the commonly used Ca sources, but limestone, with 380 g/kg Ca, is the major supplement.

Calcium metabolism in poultry is closely regulated by parathyroid hormone (PTH), vitamin D_3 and, calcitonin and receptors in the small intestine, bone, liver and kidneys. Most of the Ca is reported to be absorbed in the duodenum and jejunum of poultry (Hurwitz and Bar, 1970, 1971; van der Klis *et al*., 1990; Mutucumarana *et al*., 2014a). The absorption takes place by passive or active transport depending on the dietary Ca concentration. Passive absorption is facilitated when the Ca intake is adequate or high, whereas the active absorption is activated when the Ca intake is low. Active transport is associated with upper duodenum and depends on vitamin D_3 (Bronner, 1999).

In the past, the measurement of Ca digestibility in poultry has received no attention because limestone is cheap and not economically important. Historically, Ca and P requirements of poultry have been reported on a total basis (NRC, 1950). Over time, the P requirement was changed to available or non-phytate P (NRC, 1994), but the basis of Ca requirement remained unchanged as total Ca. In recent years, digestible P has been suggested as preferred term to express P availability in feed ingredients (WPSA, 2013) and the poultry

industry is currently moving towards a digestible P system. Calcium and P are closely associated with each other in terms of digestion and post-absorptive metabolism and a total Ca to available P ratio of 2:1 is maintained in broiler diets (Angel, 2013). The shift to digestible P system may result in the oversupply of Ca (Walk, 2016). Thus there is an urgent need to determine digestible Ca values for feed ingredients in order to develop an appropriate digestible Ca to digestible P ratio for poultry.

The digestibility of nutrients can be expressed as apparent or true digestibility. True digestibility is obtained by correcting the apparent digestibility for endogenous losses. The apparent and true Ca digestibility for a number of feed ingredients for pigs have been reported from the University of Illinois (González-Vega *et al*., 2013, 2015a,b; Merriman *et al*., 2016) and for poultry from Massey University (Anwar *et al*. 2015; 2016a, b, c; 2017; 2018). It was found that the true ileal Ca digestibility of limestone and meat and bone meal (MBM) in broilers was comparable with those of pigs. However, the Ca digestibility for DCP, MCP, poultry by-product meal, fish meal and canola meal in poultry were much lower than those determined for pigs. There may be several possible reasons for this discrepancy. First, the differences in the composition of basal diet used in the assays. Maize-based basal diets were used in the pig studies, whereas purified diets based on maize starch and dextrose were used in the broiler studies. Second, there are differences in the way digestibility is calculated. Total excreta collection was used in pig studies, whereas titanium indicator ratios in the diet and digesta were used in broiler studies. It may be speculated that the lower Ca digestibility values determined in broilers may be due to, at least in part, to interaction between titanium and Ca. It is known that different minerals interact with each other in their absorption and metabolism (Suttle, 2010). Third, the length of adaptation to dietary treatments may influence Ca digestibility measurements. The dietary adaptation period employed in poultry studies was three days, whereas five days were used in pig studies. Anwar *et al*. (2018) observed that the Ca

digestibility of DCP was higher at 24 hours of dietary adaptation length compared to those of 48 and 72 hours. Further research is warranted to determine the effects of basal diet composition, indicator effect and dietary adaptation length on Ca digestibility measurements.

Ca digestibility in poultry is influenced by dietary, animal and physiological factors. The dietary factors include Ca: P ratio, phytate, phytase, diet composition, Ca source, form of Ca, dietary Ca concentration and Ca status of birds. Microbial phytase, an important addition to feed formulations in recent years, is an enzyme that hydrolyses the phytate-bound P present in plant-based ingredients and improves the bioavailability of P. It is now well documented that phytase not only improves the digestibility of P, but also of Ca and other minerals (Selle *et al*., 2009a; Walk *et al*., 2012b). In addition, phytase doses above the recommendation (super dose) are known to hydrolyse the phytate as well as the lower phytate esters (IP5 to IP1) and thereby reducing their anti-nutritive effect in poultry (Cowieson *et al*., 2011). However, high dietary Ca concentration in poultry diets has been shown to inhibit the efficacy of phytases (Paiva *et al*., 2013). Therefore, establishing the digestible Ca of diets with a background of phytase is crucial in order to meet the birds' requirement.

Protein is a critical nutrient that is necessary for growth performance and its dietary concentration may affect Ca digestibility in broilers. Evidence from human studies indicates that protein influences Ca digestion (Kerstetter *et al*., 2003). However, the effect of dietary protein concentration on Ca digestibility in poultry has not been previously investigated. The previous Ca digestibility studies conducted by our laboratory (Anwar *et al.*, 2015; 2016a,b,c; 2017; 2018) have used a lower dietary protein concentrations (around 80 g/kg) than the standard protein requirement of broiler growers (215 g/kg; Ross, 2019) and growers (215 g/kg) as the practical protein feed ingredients cannot be used in these studies in order to eliminate the Ca contribution from these supplements.

Age, gender and genotype may also influence the Ca digestibility, but their effects on Ca digestibility are not well understood. The digestibility of major nutrients (protein, lipids and starch) generally increases with the age of birds as the younger birds have underdeveloped digestive system (Batal and Parsons, 2002; Morgan *et al*., 2015). However, studies on age effects on the mineral digestibility in broilers are scant. In addition, the Ca digestibility data determined with different age group of birds are inconsistent (Fonolla *et al*., 1981; Thomas and Ravindran, 2010; Morgan *et al*., 2015) and there are no reports on age effect for the entire growth period of broilers.

Calcium metabolism is unique in laying hens. In general, Ca utilisation in chickens depends on the dietary Ca solubilisation in the digestive tract. In broilers, the solubility of Ca in the gastrointestinal tract is negatively correlated to pH, with Ca being solubilised more at lower pH (Guinotte *et al.*, 1995). However, despite having a higher pH, the Ca solubility in the intestine of laying hens is greater during the time of eggshell formation (Mongin, 1976). No studies are available on the ileal Ca digestibility of Ca sources in laying hens and most of the Ca availability values in laying hens are based on retention. Studies comparing the ileal Ca digestibility between broilers and layers are warranted.

The end point of digestibility estimates is to determine the ileal digestible Ca requirements for different classes of poultry. In the final study, the digestible Ca and digestible P requirements for broiler starters were generated based on response surface models.

The overall aim of this thesis research was to refine the protocol for ileal Ca digestibility measurements, establish a dataset of the Ca digestibility of feed ingredients for poultry, and examine some key factors that influence Ca digestibility. This thesis consists of nine chapters. The first two chapters address the framework for the experimental research with Chapter 1 discussing the rationale for the focus of the research. Chapter 2 reviews the different

terminologies related to Ca digestibility and factors affecting the Ca digestibility and the research gaps. Chapter 3 to 8 present the experimental work in this thesis where each chapter includes an abstract, introduction, materials and methods, results and discussion and conclusions. Chapter 9 is explaining the overall view of the thesis as general discussion.

The specific objects of the experiments were:

1. To examine the effects of basal diet composition, indicator type and dietary adaptation length on ileal Ca digestibility in broilers.
2. To evaluate some factors influencing the Ca digestibility, including age of broilers, dietary crude protein, phytase and bird-type (broilers vs layers) in poultry.
3. To establish requirements of digestible Ca, digestible P and the ratio of digestible Ca to digestible P for use in broiler starter diets.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The concentration of minerals in animal body is only 20-50 g/kg, depending on the species, and the largest amount is located in the skeletal system (Gillespie, 1987). Calcium (Ca) is the most abundant macro-mineral in the animal body followed by phosphorous (Kellems and Church, 1998). Calcium is important to build up skeletal health and a wide range of functions in the body such as blood clotting, muscle contraction, nerve impulse transmission, egg production, enzyme activation, metabolic reactions, protein synthesis, maintenance of osmotic and acid-base balances and, components in membranes (Crenshaw, 2001). Calcium is required at high concentrations in poultry compared to other minerals and a balance needs to be maintained between Ca and phosphorus (P) because of their close interaction which influences the absorption and utilisation of both minerals (Crenshaw, 2001). Excess or deficiency in one of the minerals can lead to reduced utilisation of the other (Shafey *et al*., 1991).

Measurement of Ca digestibility in poultry has not received any attention in the past. The primary focus has been on P digestibility, because environment problems like eutrophication due to excess P excretion in poultry excreta and increasing price of inorganic phosphates. Studies have shown that increasing concentration of one mineral (Ca or P) causes a decrease in absorption of the other (Mutucumarana *et al*., 2014a; Wilkinson *et al*., 2014). Furthermore, the poultry sector is moving towards a digestible nutrient system rather than a total or available nutrient system. Thus, there is a need to develop a digestible calcium system in order to meet the requirement of animals with accurate digestible Ca to digestible P ratio.

2.2. Calcium metabolism

Several factors are involved in Ca metabolism and these include parathyroid hormone (PTH), vitamin D_3 and, calcitonin and receptors in the small intestine, bone and kidneys. Hurwitz and

Bar (1970) reported that the Ca absorption takes place in the duodenum and jejunum. However, recent findings suggest that the jejunum and ileum are the major segments of Ca absorption in poultry and that the Ca digestibility was highly negative in the duodenum (Mutucumarana, 2014a).

In the body, 99% of the Ca is present in the skeleton, with the remaining 1% is either present in intracellular or extracellular spaces. Extracellular Ca (0.1%) is present in the forms of ionised Ca, protein-bound Ca and anion-bound Ca where the ionised Ca is the physiologically active form. Two mechanisms, namely active (saturable) and passive (unsaturable), are involved in intestinal Ca absorption. Passive absorption is characterised by Ca ion movement from the intestinal lumen to blood circulation along the chemical gradient through spaces between cells (Bronner, 1998). When Ca intake is either high or at adequate concentrations, passive transport of Ca predominates. Active absorption involves three steps namely entry across the cell wall, diffusion through the cytoplasm and exit at the basolateral cell membrane. Active transport is associated with upper duodenum, depends on vitamin D_3 and takes place at low dietary Ca concentrations (Bronner, 1999).

Vitamin D_3 is bound with protein and stored in fat or transported to the liver. This inactive form of vitamin D_3 undergoes a double hydroxylation reaction by means of 24-hydroxylase and 1α-hydroxylase and resulting the formation of 25-hydroxy D_3 ($25(OH)D_3$) and $1,25(OH)_2D_3$) or active form of vitamin D_3, respectively (Jones *et al*., 1998). The hormones responsible for this double hydroxylation reaction are activated by PTH. Parathyroid hormone is released when the blood Ca concentration is low. Consequently, the active vitamin D_3 increases the intestinal Ca absorption by means of stimulating Ca transporter named calbindin. In addition, PTH influences an increase Ca reabsorption from renal tubes (Proszkowiec-Weglarz and Angel, 2013). Calbindin is a Ca binding protein which helps in transporting Ca from the brush border membrane to the basolateral membrane of duodenal cell. Calbindin D9k

and D28k are responsible for transcellular diffusion of Ca in the intestinal and renal tissues, respectively (Proszkowiec-Weglarz and Angel, 2013).

Calcium metabolism is unique in laying hens. In general, Ca utilisation in chickens depends on the dietary Ca solubilisation in the digestive tract. In broilers, the solubility of Ca in the gastrointestinal tract is negatively correlated to pH, with Ca being solubilised more at lower pH (Guinotte *et al.*, 1995). However, despite having high pH, the Ca solubility in the intestine of laying hens is greater during the time of eggshell formation (Mongin, 1976). The enhancement of soluble Ca in laying hens is achieved by the increase hydrochloric acid secretion at the onset of dark period (Mongin, 1976). The solubility and retention of Ca in the gut is increased prior to oviposition due to increased Ca demand (Mongin, 1976; Rao and Roland, 1990). Medullary bone plays a major role in layers to supply Ca when the dietary Ca is insufficient for egg production (Etches, 1987). Medullary bone is the Ca reservoir which differs in its Ca mobilisation from that of cortical bone. In laying hens, the medullary bone is distributed in the marrow cavity of some parts of the skeleton including tibia, femur, sternum, ribs, toes etc. with more prominent in the shaft of the femur (Simkiss, 1961). On normal Ca diets, the medullary reserves are adequate to buffer the cyclic changes in Ca demand caused by egg formation (Dacke *et al*., 1993). However, it has been reported that on Ca-deficient diet, the medullary bone starts to reduce its volume after 16 days. During prolonged Ca-deficiency, the hens respond by increasing the size of the medullary reservoir at the expense of the less labile cortical bone (Dacke *et al*., 1993). As a result, the percentage of medullary bone in the skeleton increases during egg-laying on a Ca-deficient diet. Comar and Driggers (1949) found that 25-40% of the eggshell Ca comes from the skeleton and the remainder from ingested Ca. Similarly, Dacke *et al*. (1993) indicated that the medullary bone supplies around 40% of the Ca for eggshell formation.

It is well known that increasing dietary Ca concentration negatively affects the utilisation of other minerals and nutrients in poultry (Shafey *et al*., 1991; Selle *et al*., 2009a; Mutucumarana *et al*., 2014a). High Ca is known to reduce the Ca retention and bone Ca in broilers (Atteh and Leeson, 1984). A reduced Ca retention with increasing Ca concentrations was also reported in layers (Cheng and Coon,1990b). Kebreab *et al*. (2009) reported that Ca retained in body and eggs in laying hens were 62.5, 51.4 and 50.5% at dietary Ca concentration of 25 (low), 35 (medium) and 45 (high), respectively. Furthermore, high dietary Ca concentrations have been shown to reduce the availability of other minerals (P, iron [Fe], magnesium [Mg], manganese [Mn] and zinc [Zn]) and protein (Shafey *et al*., 1991; Mutucumarana *et al*., 2014a). This may be because of increased intestinal pH, by the addition of limestone, reduces the soluble fraction of mineral complexes (Shafey *et al*., 1991). High dietary Ca also adversely affects the utilisation of fat (Edwards *et al*., 1960; Mutucumarana *et al*., 2014a) and metabolisable energy (Atteh and Leeson, 1984), with the eventual outcome of reduced growth performance and feed efficiency (Davis, 1959; Shafey and McDonald, 1991; Tancharoenrat and Ravindran, 2014) and, increased leg abnormalities in broilers (Ogura, 1981). According to Mutucumarana *et al*. (2014a), the site of digestion of P and nitrogen also shifts depending on dietary Ca concentrations. In egg-type pullets, some metabolic disorders like hypercalcemia and hypophosphatemia have been reported as a result of high dietary Ca (Guo *et al*., 2008). Ensuring adequate amounts of Ca in poultry diets is important especially when other minerals are marginal in the diets.

2.3. Calcium sources

Over 80% of Ca in broiler diets is supplied by inorganic calcium sources such as limestone, dicalcium phosphate (DCP), monocalcium phosphate (MCP), mono-dicalcium phosphate (MDCP) and oyster shell. The value of lesser-known Ca sources has also been evaluated. Calcium citrate and Ca citrate-malate are reported to have similar relative bioavailability to

that of limestone (Hendry and Pesti, 2002; Augspurger and Baker, 2004). Hurwitz and Rand (1965) indicated that the Ca availability in gypsum (Ca sulphate) is similar to that in limestone and replacing 11 g/kg Ca from limestone by Ca sulphate in a diet containing 30g/kg Ca had no detrimental effect on layers. Although other inorganic sources like agricultural grade phosphates and raw rock phosphates are cheaper than DCP, these contain high concentrations of heavy metals and can be toxic to animals (Fernandes *et al*., 1999). Animal-based feed ingredients (meat and bone meal [MBM], bone meal, poultry by-product meal) and some plant-based ingredients (canola meal, soybean meal) can also contribute reasonable amount calcium to poultry diets. On the other hand, Ca contents of most plant-based ingredients (maize, wheat, rice, sorghum, barley) are very low (NRC, 1994). The Ca contents of common Ca sources are given in table 2.1.

Table 2.1. Common calcium (Ca) sources and their Ca contents

Ca sources	Ca content (g/kg)
Inorganic sources	
Limestone	380
Oyster shell	380
Dicalcium phosphate	220
Monocalcium phosphate	160
Animal-based sources	
Meat and bone meal	103
Fish meal	22.9 - 51.1
Poultry by-product meal	30
Plant-based sources	
Canola meal	6.8
Corn gluten feed (with bran)	4.0
Soybean meal	2.7-2.9
Sunflower meal	2.1

Source: NRC (1994)

Limestone is the common and abundant Ca source used in poultry feed formulations. The primary component of limestone is calcite, but it may also contain the minerals aragonite and dolomite ($CaMg(CO_3)_2$). Calcite and aragonite have different crystal arrangements of the calcium carbonate ($CaCO_3$). Different types of limestone are formed through a variety of

processes such as precipitation from water, secretion by marine organisms, shells of dead-sea creatures and cementation of sand or mud by calcite. Limestone can be divided into three categories based on its depositional environment such as platform, basin and geosynclinals (Sloss, 1947). There are many different names used for limestone based upon how the rock is formed, its appearance or its composition, and other factors. Different colours of limestone (tan, grey etc.) are due to impurities like sand, clay, iron oxides and organic materials. The Ca content of limestone may vary from 360 to 415 g/kg (Reid and Weber, 1976; Browning and Cowieson, 2014).

Oyster shell is the second most widely used Ca source for poultry. The Ca content of oyster shell is 380 g/kg (NRC, 1994). According to Reid and Weber (1976), the Ca content of oyster shell varies from 344 to 390 g/kg. The relative bioavailability of Ca from oyster shell is similar to limestone (Augspurger and Baker, 2004). Scott *et al*. (1971) reported that feeding Ca carbonate in the form of oyster shells is more effective than feeding the same amount of finely ground limestone, because of the larger particles that are solubilised slowly resulting in higher Ca absorption. Overall, however, most studies report that feeding oyster shell has similar benefits as that of feeding limestone (Roland, 1986).

Dicalcium phosphate ($CaHPO_4$) is the Ca phosphate with its dihydrate, an odourless white coloured powder. Dicalcium phosphate contains about 220 g/kg Ca and 190 g/kg P (NRC, 1994). There are three forms namely dihydrate ($CaHPO_4.2H_2O$, the mineral brushite), hemihydrate ($CaHPO_4.0.5H_2O$) and anhydrous (CaHPO4, the mineral monetite). Depending on the form of DCP, the P and Ca contents may vary (Viljoen, 2001). Dicalcium phosphate is produced by the neutralisation of Ca hydroxide with phosphoric acid, which precipitates the dihydrate as a solid or by reacting phosphoric acid with limestone. According to Lima *et al.* (1995), DCP is a mixture of DCP, MCP, phosphoric acid, Ca carbonate and impurities depending on the origin of the raw material and procedures employed in its industrial

production. Dicalcium phosphate could also be produced through precipitation from bones and it is a co-product from the gelatine manufacture (Sullivan *et al*., 1994). Figure 2.1 illustrates the production process of DCP from bones. The relative biological value of bone-precipitated dicalcium phosphate was reported to be higher than commercial feed phosphates (Sullivan *et al*., 1994).

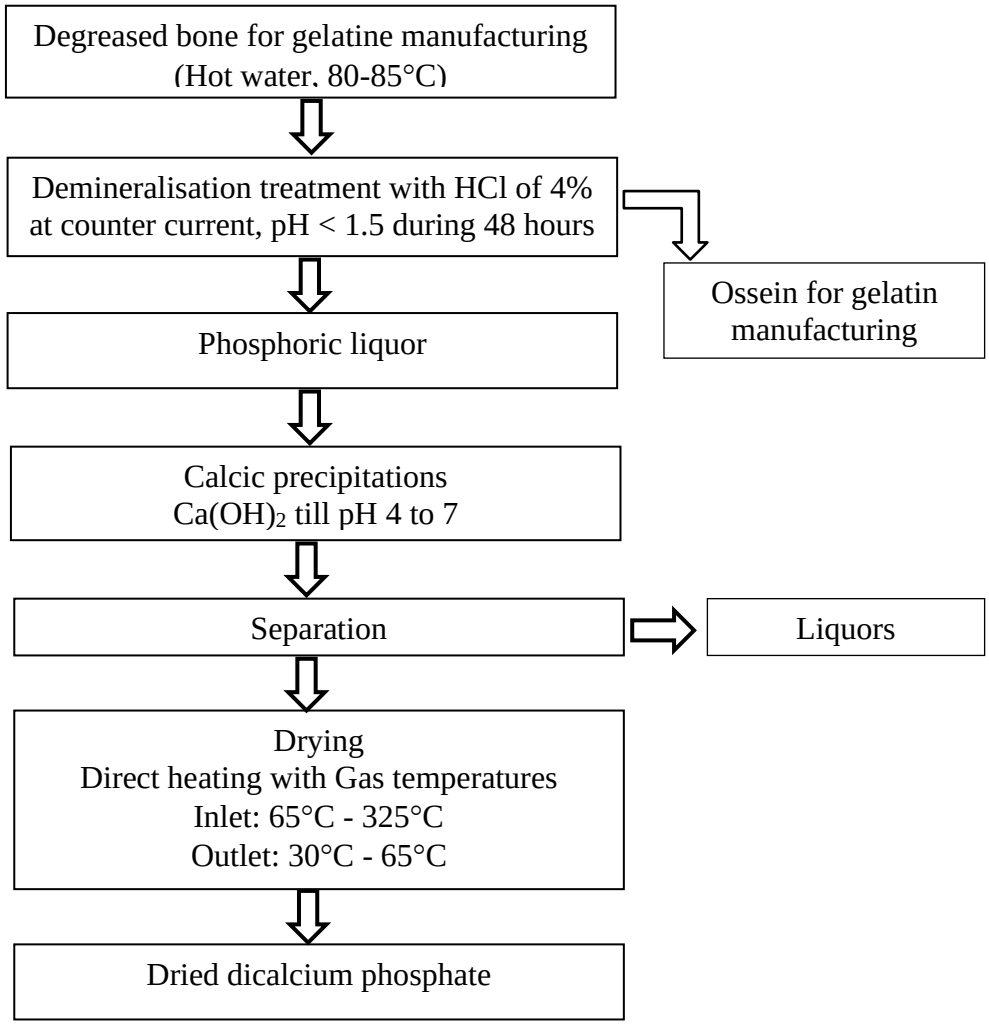

Figure 2.1. Production process of DCP (Scientific steering committee, 2003)

Monocalcium phosphate is an inorganic compound ($Ca(H_2PO_4)_2$) and is commonly found as the monohydrate ($Ca(H_2PO_4)2.H_2O$). Monocalcium phosphate is produced by treating Ca hydroxide with phosphoric acid which contains around 160 g/kg Ca and 220 g/kg P (NRC,

1994). Even though MCP is a mixture of MCP and DCP, more than 80% of P should be derived from the MCP fraction, to be classified as a MCP, while MDCP may contain from lower than 50% to 80 % P from MCP (Viljoen, 2001).

Tricalcium phosphate ($Ca_3(PO4)_2$) is a calcium salt of phosphoric acid which is a white solid of low solubility. It exists as three crystalline polymorphs such as α, α' and β. The α and α' states are stable at high temperatures (Carrodeguas and De Aza, 2011). Tricalcium phosphate (TCP) is produced commercially by treating hydroxyapatite with phosphoric acid and slaked lime. Tricalcium phosphate is also produced by heating a mixture of Ca pyrophosphate ($Ca_2P_2O_7$) and Ca carbonate ($CaCO_3$). Tricalcium phosphate occurs naturally in several forms such as rock, skeletons and teeth of vertebrate animals and milk. In some countries, TCP is used as inorganic phosphate supplement in poultry diets (Ravindran, 2013b). The production process of TCP from bones involves the degreasing of the bones in counter-flow with hot water (bone chips less than 14 mm). Then it undergoes continuous cooking with steam at 145°C during 30 minutes at 4 bars and the separation of the protein broth from the hydroxyapatite (TCP) by centrifugation. The granulation of TCP is carried out after drying in a fluid bed with air at 200°C. This TCP is not pure and, on average, composed of 750 g/kg hydroxyapatite, 170 g/kg gelatine, 4 g/kg fat and 4 g/kg moisture (Scientific Steering Committee, 2003). Feed grade TCPs may be available in different trade names depending on the production process. For example, Hamdi *et al.* (2015) used a TCP called lipocal, which is a TCP powder that has been treated with lecithin to reduce its interactions with other minerals and feed ingredients, especially in aqueous media. Rao *et al.* (1995) used a TCP called Multifos, which is a deflourinated TCP derived from phosphate rock. Rao *et al.* (1995) reported that the *in vivo* solubility of TCP (Multifos) was lower than those of DCP (Dynafos) or MCP (Biofos). Kwon and Kim (2017) found that the Ca digestibility to be lower for TCP than those for DCP and

MCP. In contrast, Hamdi *et al.* (2017) indicated recently that the P availability of TCP is similar to that of MCP.

Defluorinated phosphate ($Ca_4Na(PO_4)_3$) is also used as a mineral supplement to poultry. Defluorinated feed phosphate (DFP) is produced by removing the fluorine from the phosphate rock, which is toxic to animals. Natural phosphate rocks vary in fluorine content from less than 10g/kg to above 40g/kg (Jacob *et al*., 1933). Phosphate rock and sand are ground, mixed in a definite ratio and fed as a slurry to a rotary kiln. The material is treated at temperatures of 2700° to 2900° F. Water vapor is introduced at the hot end of the kiln and quick cooling of the product is done. The discharged, compacted residue which is not fused, is ground and bagged. The product contains about 20% phosphorus pentoxide and 0.1% or less fluorine. The phosphate constituent is α-tricalcium phosphate (Whitney and Hollingsworth, 1949). Defluorinated feed phosphate is a source of highly available P. Defluorinated feed phosphate is non-hydroscopic and non-caking powder or granules and is light-brown to dark-brown in color, and odorless. Compared to other mineral P sources, the DFP supplies Ca, P and sodium to animals at minimum concentration of heavy metals and harmful components, promotes skeletal hardening in animals, increases shell quality of intensively egg-laying birds and is well soluble in the gut.

Other than the inorganic Ca sources, animal protein sources such MBM, fish meal, and poultry by-product meal also supply significant amounts of Ca in poultry diets. Meat and bone meal is a product of the rendering industry and an important organic Ca source which contains an average of 103 g/kg Ca (NRC, 1994). However, Ca concentration of MBM widely varies depending on different sources (Waldroup and Adams, 1994; Sulabo and Stein, 2013; Anwar *et al*., 2015; 2016b). This variation in Ca concentration could be due to the nature of raw materials as well as the processing methods. Meat and bone meal is made from animal offal

that is not suited for human consumption. Offal is cooked, defatted, sterilised and ground to obtain MBM. Depending on the proportions of bone and soft tissues used in the manufacture, the finished product is categorised as meat meal (containing > 55 g/100 g crude protein and < 4.4 g/100 g P) or meat and bone meal (containing < 55 g/100 g crude protein and > 4.4 g/100 g P). Similarly, fish meal is manufactured by cooking, pressing, drying and grinding the fish or fish waste that are not intended for human consumption. In the same way, poultry by-product meal is made by rendering process.

Plant-based feed ingredients are mostly used in poultry diets as an energy or protein supplement. They are however, contribute less Ca to the poultry diet. Among the plant-derived feed ingredients, a plant protein source, canola meal contributes comparatively higher Ca (6.8 g/kg; NRC, 1994) to the poultry diets. The main energy and protein sources are maize and soybean meal, respectively, and they supply 0.2 and 2.8 g/kg (NRC, 1994) Ca, respectively, to the poultry diets.

2.4. Total calcium

Historically, 'total Ca' was used for formulating poultry diets (NRC, 1950). Total Ca in a diet refers to the entire Ca content of the diet, regardless of the portion available for absorption and utilisation by the animal. Calcium and P are interrelated in terms of their metabolism, and their requirements were expressed as total Ca and total P during 1950s. The recommended requirements of total Ca and total P for broilers during this period were 10 and 6 g/kg, respectively (NRC, 1950). Although the expression of P requirement was changed to available P and non-phytate P in NRC (1984) and NRC (1994), respectively, the expression of Ca requirement remained unchanged because of lack of studies on available Ca. The total Ca to available P and total Ca to non-phytate P ratios of broilers used during these periods ranged from 2.22 to 2.28 (up to 8 weeks of age) and 2.22 to 2.67 (depending on the growth stage), respectively (Angel, 2013). Regardless of the P term (available or non-phytate), the total Ca to

P ratio is normally maintained at around 2:1 in poultry diets. The use of total Ca system, however, is not precise (Walk, 2016; Li *et al*., 2017a), because Ca is clearly oversupplied.

There are reports that highlighted an over Ca supply for broilers. It has been indicated that the total Ca concentrations of 6.5 and 6.0 g/kg were sufficient to meet the nutrient requirement of broilers from day 1 to 14 and from day 15 to 49, respectively (Li *et al*., 2017). Similarly, Sebastian *et al*. (1996) reported that optimum growth performance and mineral utilisation were achieved at the dietary Ca concentration of 6 g/kg with phytase supplement. Furthermore, Applegate *et al*. (2003) found that the recommended dietary Ca concentration of 9 g/kg reduced the intestinal phytase activity and apparent ileal phytate-P hydrolysis. A Ca concentration of 10 g/kg has been shown to reduce the ileal digestibility of P and protein in broilers (Akter *et al*., 2018). It is now well documented that excess Ca negatively affects not only the digestibility of P, but also of other minerals, metabolisable energy, lipids and protein as shown in Table 2.2. In the future, it is expected that the poultry sector will shift towards the digestible Ca system.

2.5. Calcium availability

Calcium availability is also known as bioavailability or biological availability of Ca. According to Peeler (1972), there are number of terms that have been used in literature to define the utilisation or availability of major minerals such as percent utilisation, percent apparent digestibility, percent true digestibility, percent absorption, percent net retention, percent apparent availability, percent true availability, biological availability etc., but meaning of these terms differ from each other. Biological availability of Ca is determined using slope ratio assays. The slope ratio procedure involves feeding graded levels of Ca from a test ingredient below the requirement to induce an experimental deficiency response and the response criterion is plotted against Ca intake of animals fed diets containing the test ingredient diet and a known standard that is presumed to be 100% available (Kiarie and Nyachoti, 2010). The response

criteria are growth response (weight gain and feed conversion) and bone measurements (tibia ash, bone breaking strength and bone density).

Table 2.2. Effects of excess calcium (Ca) on the digestibility of nutrients in poultry

Dietary Ca (g/kg)	Bird class	Effect	Reference
20.0	Broilers	↓ iron retention	Sell (1965)
16.0	Broilers	↓ retention of fat, Ca and Mg	Atteh and Leeson (1984)
15.3, 21.8, 22.6	Broilers	↑ insoluble form of Ca, Mg, Zn and Fe	Shafey *et al*. (1991)
12.5	Broilers	↓ Ca Retention	Sebastian *et al*. (1996)
12.0	Broilers	↓ P, N and fat digestibility	Mutucumarana *et al*. (2014a)
10.3, 13.3	Broilers	↓ ileal digestibility and retention of P	Abdollahi *et al*. (2016)
10.0	Broilers	↓ ileal P & protein digestibility	Akter *et al*. (2018)
16.0	Roosters	↓ digestibility of high-melting triglycerides and hydrogenated fats	Edwards *et al*. (1960)
12	Roosters	↓ lowered Mg in bone ash	Nugara and Edwards (1963)
45.0	Layers	↓ body, egg and bone Ca retention	Kebreab *et al*. (2009)

↓ : Reduce ↑ : Increase

In early research reports, the availability of Ca was determined to be high in Ca supplements. Dilworth *et al*. (1964) reported that the Ca availability of Ca carbonate, low fluorine rock phosphate, defluorinated phosphate and soft phosphate were 100, 90, 92-95 and 68%, respectively. Calcium carbonate was found to have 100% biological availability, while the values for limestone, bone meal, TCP and DCP were 102, 109, 115 and 113%, respectively. However, dolomitic limestone and soft rock phosphate have relatively low Ca availabilities of 65 and 70%, respectively (Suttle, 2010). According to Reid and Weber (1976), the Ca availability of ground limestone and oyster shell were 73-109 and 107.8%, respectively, in broilers. The corresponding values in laying hens were 82.4-98.4 and 100%, respectively. Available Ca concentrations are rarely used in feed formulations, partly because the Ca content in plant ingredients is low. In addition, the phytate found in plant materials reduces Ca availability by binding Ca in phytate-mineral complexes (Taylor, 1965).

Even though the terms available P and non-phytate P are different, they are normally used interchangeably. Mutucumarana (2014b) reported that the P evaluation system based on non-phytate P is not precise as the values for true digestible P were higher than those for non-phytate P in maize and canola meal. This observation suggests that a portion of phytate-bound P is utilised by broilers as reported by Tamim *et al*. (2004).

Compared with considerable research reported on the biological availability of P sources, only limited research studies have been carried out with Ca sources. Recent findings suggest that current commercial diets for both broilers and layers are formulated to contain excess Ca, and Ca could be reduced without affecting their production and welfare (Li *et al*., 2017a). Therefore, initiatives have been taken to address the need for digestible Ca values in feedstuffs (Anwar, 2015; 2016a,b,c; 2017; 2018).

2.6. Calcium digestibility

The current interest in changing the P system to digestible P has led to attention on digestible Ca system. Digestibility of nutrients is an estimate of availability if all the nutrients that disappear from the intestinal tract are absorbed and if all the absorbed nutrients are available for utilisation by the animal (Stein *et al*., 2007; Ammerman, 1995). Digestibility of nutrients can be expressed as apparent and true digestibility (Stein *et al*., 2007). True digestibility is measured by correcting the apparent digestibility for endogenous losses. Endogenous losses can be categorised into basal and specific endogenous losses based on dietary independence and dependence, respectively. Since basal endogenous losses are easier to measure than total endogenous losses, basal endogenous losses are used to calculate the true digestibility (Stein *et al*., 2007; Almeida and Stein, 2010). It is opined that the basal losses must be charged against the feedstuff and specific losses against the animal.

Calcium digestibility in poultry and pigs can be presented as diet digestibility and ingredient digestibility. In ingredient digestibility studies, the particular ingredient or Ca supplement serves as the sole source of Ca, whereas in diet digestibility the Ca assumes that Ca from individual ingredients in the diet is additive.

The methodology for Ca digestibility measurements can be direct, difference (substitution) and regression methods (Anwar *et al*., 2018). A test ingredient serves as the sole source of nutrient in the diet in the direct method. In the difference method, a basal and a test diet are formulated, and the test diet comprises of a mixture (usually 50:50) of the basal and test ingredient. The digestibility of particular nutrient in the test ingredient is then determined based on the difference in digestibility between the two assay diets and the concentration of the specific nutrient in the test diet (Lemme *et al*., 2004). Regression method involves establishing a linear relationship between nutrient output in ileal digesta and dietary nutrient input. For this, diets with graded concentrations of the specific nutrient from the assay ingredient are formulated. In this method, digestibility estimates are automatically corrected for endogenous losses and represent true digestibility values. However, Anwar *et al*. (2018) recently reported lower Ca digestibility coefficients in DCP with difference (0.21) and regression (0.13) methods when compared to direct (0.34) method.

2.6.1. Calcium retention

Retainable Ca is the proportion of dietary total Ca that is deposited in the body of an animal (Rodehutscord, 2013) and is measured as the 'total tract Ca digestibility'. Retainable Ca measurement is reflective of both digestive and post-absorptive utilisation of Ca. (Li *et al*., 2017a). Excess Ca which is not absorbed in an animal's body is known to be excreted in the urine (Rao and Roland, 1990). Thus, the measurement of retainable Ca requires the determination of Ca intake and output (faeces and urine). In poultry, faeces and urine are excreted together which enable the Ca retention measurement easy when compared to that in

other monogastric animals. The determination of Ca retention involves either total collection method or index method (Zhang and Adeola, 2017). In the total collection method, total amount of Ca consumed and total amount of Ca output in excreta must be determined to calculate the Ca digestibility, whereas in the index method, the amount of indigestible indicator in the diet and excreta is measured. Cheng and Coon (1990b) compared these two methodologies and reported that the use of acid insoluble ash as an indigestible indicator resulted in higher Ca retention when compared to the total collection method in laying hens fed a maize-soybean based diet. There are several reports on the Ca retention of diets for broilers (Atteh and Leeson, 1983; Leeson *et al*., 1987; Thomas and Ravindran, 2010; Oso *et al*., 2011; Shastak *et al*., 2012; Tancharoenrat and Ravindran, 2014; Cowieson *et al*., 2015; Akter *et al*., 2018). However, published data on the Ca retention in Ca sources are limited and are summarised in Table 2.3.

2.6.2. Ileal digestibility

Ileal digestibility is the proportion of dietary Ca that is not recovered in the digesta at the terminal ileum. It is currently accepted that the nutrient digestibility assays in poultry should be based on the analysis of ileal digesta rather than of excreta, because of the variable and modifying effects of hindgut microflora and possible urine contamination (Ravindran *et al*., 1999). Ileal digestibility determination involves the use of indigestible indicators such as chromic oxide, titanium dioxide and acid-insoluble ash, but the results may differ depending on the indicator (Scott and Boldaji, 1997). Many reports are available on the ileal Ca digestibility of diets (Tamim *et al.*, 2004; Cowieson and Ravindran, 2008; Shastak *et al.*, 2012; Walk *et al.*, 2012b; Amerah *et al.*, 2014; Tancharoenrat and Ravindran, 2014; Hamdi *et al.*, 2015; Bradbury *et al.*, 2017; Bradbury *et al*., 2018; Li *et al.*, 2018) and limited number of reports are available on the ileal Ca digestibility of Ca sources (Table 2.4) in broilers. The apparent and true ileal Ca digestibility of some common Ca sources in broilers have been

reported previously by Anwar *et al*. (2015; 2016a,b,c; 2017; 2018). However, Ca digestibility values determined in broilers are in disagreement with those reported in pigs in the literature.

2.7. Additivity of nutrients

When ingredients are combined in feed mixtures, it is assumed that the amount of digestible nutrients is equal to the sum of digestible nutrients supplied by each dietary ingredient (Ravindran, 2007). This concept is known as additivity. Number of studies have reported the additivity of different nutrients including amino acids (Angkanaporn *et al*., 1996; Kong and Adeola, 2013), metabolisable energy (Hong *et al*., 2001, 2002; Cilliers *et al*., 1998) and P (Fang *et al*., 2007) in mixed diets for poultry and pigs.

It has been shown that true P digestibility values, rather than apparent, are additive in soybean meal-based pig diets containing low levels of phytate P (Fang *et al*., 2007). Zhang and Adeola (2017) reported that true total tract digestibility of Ca in limestone and DCP were more additive than apparent digestibility in growing pigs fed semi-purified diets. However, there are no studies on the additivity of digestible Ca in poultry.

Several factors are known to influence the additivity of nutrients of ingredients in a mixed diet and these include protein content (Stein *et al*., 2005), amino acid content (Xue *et al*., 2014), endogenous losses and anti-nutritive factors (Kim *et al*., 2017a). The reason for the lack of additivity of apparent ileal digestibility values can be explained by the underestimation of the apparent ileal digestibility due to a greater contribution of endogenous losses in ileal digesta (Stein *et al*., 2005).

Table 2.3. Reported apparent total tract calcium (Ca) retention coefficients of Ca sources in broiler chickens

Ca source	Dietary Ca (g/kg)	Ca:aP/npP ratio	Diet[1] type	Age (day)	Ca retention coefficient	Reference
Seashells (<0.5mm)	10	2.2	W	18	0.48	Guinotte *et al*. (1995)
Seashells (>1.2 mm)	10	2.2	W	18	0.22	Guinotte *et al*. (1995)
Limestone	9	2.0	P	24	0.55	Anwar *et al*. (2017)
Limestone	3.3-5.3	0.9-1.0	SP	24-27	0.57-0.70	Zhang and Adeola (2018)
Oyster shell	9	2.0	P	24	0.50	Anwar *et al*. (2017)
Dicalcium phosphate	3.3-5.3	0.9-1.0	SP	24-27	0.58-0.64	Zhang and Adeola (2018)

[1]W: wheat-based, P: purified diet (maize starch-dextrose based), SP: semi purified diet (maize-maize starch based)
aP: available phosphorous, npP: non-phytate phosphorous

Table 2.4. Reported apparent (AIDC) and true (TIDC) ileal digestibility coefficients of calcium (Ca) for Ca sources in broiler chickens

Ca source	Dietary Ca (g/kg)	Ca: npP ratio	Age (day)	AIDC	TIDC	Methodology	Reference
Limestone	6.9	-	11	0.50	-	Direct	Angel *et al.* (2013)
Limestone	6.9	-	25	0.32	-	Direct	Angel *et al.* (2013)
Limestone	6.8	1.5	24	0.63	0.65	Direct	Anwar *et al*. (2016a)
Limestone	9.0	2.0	24	0.56	0.57	Direct	Anwar *et al*. (2016a)
Limestone	11.3	2.5	24	0.48	0.49	Direct	Anwar *et al*. (2016a)
Limestone	9.0	2.0	24	0.60	0.61	Direct	Anwar (2017) and Anwar *et al*. (2016c)
Limestone	9.0	2.0	24	0.49	0.50	Direct	Anwar *et al*. (2017)
Limestone	3.3	0.9	27	0.56	0.64	Regression	Zhang and Adeola (2018)
Limestone	4.3	1.0	27	0.60	0.64	Regression	Zhang and Adeola (2018)
Limestone	5.3	1.0	27	0.62	0.64	Regression	Zhang and Adeola (2018)
Oyster shell	9.0	2.0	24	0.43	0.44	Direct	Anwar *et al*. (2017)
Dicalcium phosphate	9.0	1.1	24	0.27	0.28	Direct	Anwar *et al*. (2018)
Dicalcium phosphate	3.3	0.9	27	0.61	0.67	Regression	Zhang and Adeola (2018)
Dicalcium phosphate	4.3	1.0	27	0.63	0.67	Regression	Zhang and Adeola (2018)
Dicalcium phosphate	5.3	1.0	27	0.65	0.67	Regression	Zhang and Adeola (2018)
Monocalcium phosphate	9.0	0.7	24	0.32	0.33	Direct	Anwar *et al*. (2018)
Monocalcium phosphate	7.3	-	11	0.77	-	Direct	Angel *et al.* (2013)
Monocalcium phosphate	7.3	-	25	0.56	-	Direct	Angel *et al.* (2013)
Meat and bone meal	8.0	1.9	31	0.38-0.56	0.46-0.60	Regression	Anwar *et al*. (2015)
Meat and bone meal				0.40-0.55	0.41-0.56	Direct	Anwar *et al*. (2016b)
Fish meal	9.0	1.4	24	0.23	0.24	Direct	Anwar *et al*. (2018)
Poultry by-product meal	9.0	1.5	24	0.28	0.29	Direct	Anwar *et al*. (2018)
Canola meal	6.0	2.0	24	0.29	0.31	Direct	Anwar *et al*. (2018)

npP: non-phytate phosphorous.

2.8. Factors influencing calcium digestibility

2.8.1. Animal species

2.8.1.1. Broilers and laying hens

It is conventional wisdom that Ca digestibility will differ among different species of animals. In addition, there can be differences in Ca digestibility values between different classes within a species, for example broilers and layers. Studies on the Ca digestibility in laying hens are limited compared to broilers. Recently, Mtei *et al.* (2019a,b) reported a higher apparent ileal Ca digestibility in laying hens when compared to pullets and broilers. In addition to skeletal development, Ca plays a major role in eggshell formation in laying hens. On average, an eggshell contains around 1.6-2.4 g Ca (Taylor, 1963). The recommended dietary Ca requirement of contemporary laying hens is in the range from 39.0 to 49.0 g/kg (ISA, 2014; Hy-Line Brown, 2018) whereas the requirement of broilers is in the range from 7.2 to 9.6 g/kg (Ross, 2019; Arbor Acres, 2019), depending on their growth and production stage. The broilers and laying hens have the ability to utilise Ca more efficiently at low dietary Ca concentrations. Rao and Roland (1990) reported that the laying hens in Ca-deficient status retained and solubilised more Ca *in vivo* than the birds at normal status. The laying hens that were maintained on normal Ca regimen (37.5 g/kg) one week prior to the experiment showed Ca retention values of 69, 65 and 51%, respectively, at dietary Ca concentrations of 15, 30 and 45 g/kg. The corresponding values for hens maintained on deficient Ca regimen (10 g/kg) one week prior to the experiment were 88, 72 and 62%, respectively (Rao and Roland, 1990). No published data are available on the Ca digestibility of Ca sources in laying hens. Table 2.5 and 2.6 summarise the reported coefficients of apparent total tract Ca retention and apparent ileal Ca digestibility of diets, respectively, in laying hens.

Table 2.5. Reported apparent total tract calcium (Ca) retention coefficients of diets in laying hens

Main Ca sources	Diet type[1]	Dietary Ca (g/kg)	aP/TP (g/kg)	Age (weeks)	Ca retention	Reference
Ca carbonate + soybean oil meal	G	26.4	5.0	-	0.60	Hurwitz and Griminger (1961)
Ca carbonate + soybean meal	MS	39.3	7.5*	~ 36	0.43	Hurwitz and Bar (1966)
Ca carbonate + soybean meal	MS	18.2	7.5*	~ 36	0.66	Hurwitz and Bar (1966)
Limestone + DCP + meat meal + fish meal	MS	26.8	-	~ 48	0.67	Tortuero and Centeno (1973)
Limestone + DCP + meat meal + fish meal	MS	38.6	-	~ 48	0.63	Tortuero and Centeno (1973)
Limestone + DCP + meat meal + fish meal	MS	41.8	-	~ 48	0.58	Tortuero and Centeno (1973)
Limestone + soybean meal	MS	15-60	-	36, 40	0.41-0.78	Rao and Roland (1990)
Limestone + DCP	MS	20-45	-	36	0.42-0.73	Cheng and Coon (1990b)
Limestone + MDCP	MS	36	2.5, 4.5	28	0.46, 0.55	Nahason *et al*. (1994)
Limestone + DCP	MS	38	4.0	25, 108	0.39-0.54	Scheideler (1998)
Limestone + MCP	MWS	41-45	5.2-5.7*	56	0.51-0.58	Lichovnikova (2007)
Limestone + Oyster shell + MCP	MWS	40	5.4*	56	0.52	Lichovnikova (2007)
Limestone + Eggshells + MCP	MWS	40	5.2*	56	0.58	Lichovnikova (2007)
Limestone + DCP	MS	39-41	3.8	~ 27	0.83-0.86	Araujo *et al*. (2011)
Limestone + MCP	MWS	37-42	3.9	45	0.55-0.57	Swiatkiewicz *et al*. (2015)
Limestone + MDCP	MS	35-40	4.0	98	0.48-0.51	Plaimast *et al*. (2015)
Oyster shell + DCP + fish meal	MG	20-43	4.0, 6.0*	70	0.49-0.77	Kalango and Ademosun (1973)
Seashells	MWS	35	-	30	0.51-0.54	Guinotte *et al*. (1995)

[1]MS: maize-soybean meal, MWS: maize-wheat-soybean meal, MG: maize-groundnut cake, G: glucose-based
aP: available phosphorous; *TP: total phosphorous
DCP: dicalcium phosphate; MCP: monocalcium phosphate, MDCP: monodicalcium phosphate

Table 2.6. Reported apparent ileal digestibility coefficients (AIDC) of calcium (Ca) in diets for laying hens

Ca source	Diet type[1]	Dietary Ca (g/kg)	npP/aP (g/kg)	Age (weeks)	AIDC	Reference
Limestone + DCP	MS	33	2.8	28	0.45	Liu *et al*. (2007)
Limestone + MCP	MS	33	1.3	24	0.36-0.42	Kozłowski and Jeroch (2011)
Limestone + MCP	MS	36	2.5	24	0.46	Kozłowski and Jeroch (2011)
Ca carbonate + MCP	MWS	36	3.5*	23	0.47-0.52	Hafeez *et al.* (2015)
Limestone + MCP	MWS	34	1.8	60	0.50	Englmaierová *et al*. (2017)
Limestone + MCP	MWS	35	1.6	27	0.44	Musilova *et al*. (2017)
Limestone + MCP	MWS	35	2.0	27	0.37	Musilova *et al*. (2017)
Ca carbonate + DCP	MSC	37	3.8	32-70	0.44-0.72	Bello and Korver (2019)

[1]MS: Maize-soybean meal, MWS: Maize-wheat-soybean meal, MSC: Maize-soybean meal-canola meal
npP: non-phytate phosphorous, aP: available phosphorous, DCP: dicalcium phosphate, MCP: monocalcium phosphate
* Total phosphorous

The time of feeding have an influence on the eggshell formation in layers. Evening feeding before shell formation is known to increase the shell quality. According to Lichovnikova (2007), the Ca retention was higher in midnight fed layers than that of the daytime fed layers regardless of the composition of diet. The shell gland is more active during the night when shell is being formed and the layers depend on Ca from medullary bones since they are not fed during night times (Scanes *et al*., 1987). Medullary bone serves as a temporary Ca reserve from the time of sexual maturity and release Ca when there is insufficient Ca supply from feed (Etches, 1987). The Ca is deposited on the eggshell at a rate of 100-150 mg/h (Taylor, 1963). It has been reported that dietary Ca is responsible for shell formation from morning to noon and from midnight to morning while the skeletal Ca is responsible from noon to midnight (Taylor, 1963).

2.8.1.2.Calcium digestibility studies in pigs

Number of studies have been carried out in pigs to determine the Ca digestibility in Ca sources and, the available data on both apparent and true total tract Ca digestibility coefficients are summarised in Table 2.7. Anwar *et al*. (2015; 2016a,b,c) found that the true ileal Ca digestibility of limestone and MBM in broilers was comparable with those of pigs. However, the Ca digestibility for DCP, MCP, poultry by-product meal, fish meal and canola meal in poultry were much lower than those determined for pigs.

There may be several possible reasons for this discrepancy. First, the composition of basal diet used in the assays differed. Maize-based basal diets were used in the pig studies, whereas purified diets based on maize starch and dextrose were used in the broiler studies. Second, there are differences in the way digestibility is calculated. Total excreta collection was used in pig studies, whereas titanium indicator ratios in the diet and digesta were used in broiler

studies. Third, the length of adaptation to dietary treatments could have influenced the digestibility measurements. The dietary adaptation period employed in poultry studies was three days, whereas five days were used in pig studies. Anwar *et al*. (2018) observed the Ca digestibility of DCP was higher at 24 hours of dietary adaptation length compared to those of 48 and 72 hours. Further research is warranted to determine the effects of basal diet composition, methodology and dietary adaptation length on Ca digestibility measurements.

Table 2.7. Reported total tract calcium (Ca) digestibility coefficients of Ca sources in pigs

Ca source	Apparent digestibility	True digestibility	Reference
Limestone[2]	0.67	0.70	Zhang and Adeola (2017)
Ca carbonate[1]	0.58	0.60	González-Vega *et al*. (2015a)
Ca carbonate[3]	0.61-0.71	-	Stein *et al*. (2011)
Ca carbonate[3]	0.70-0.74	0.74-0.78	Merriman and Stein (2016)
Dicalcium phosphate[2]	0.70	0.76	Zhang and Adeola (2017)
Dicalcium phosphate[1]	0.75	0.78	González-Vega *et al*. (2015a)
Monocalcium phosphate[1]	0.83	0.86	González-Vega *et al*. (2015a)
Canola meal[2]	0.34- 0.43	0.47	González-Vega *et al*. (2013)
Fish meal (maize-starch-based diet)[3]	0.40-0.57	0.46-0.62	González-Vega *et al*. (2015b)
Fish meal (maize-based diet)[3]	0.78-0.84	0.82-0.89	González-Vega *et al*. (2015b)
Meat and bone meal[3]	0.53-0.81	-	Sulabo and Stein (2013)
Soybean meal[1]	0.47	-	Bohlke *et al*. (2005)

[1]Difference method [2]Regression method [3]Direct method

As a result of the shift from total Ca to digestible Ca, recommendations for standardised digestible Ca to standardised digestible P requirements have been proposed for pigs in NRC (2012). For the maximum growth performance, the digestible Ca to digestible P ratios less than 1.40:1, 1.35:1, 1.25:1 and 1.10:1 were recommended for pigs having 11-25, 25-50, 50-85 and 100-130 kg body weights, respectively. Corresponding ratios recommended for maximum bone ash were 1.70:1, 1.80:1, 2.00:1 and 2.30:1, respectively (Lee *et al*., 2019). Similar changes

in the ratio of digestible Ca to digestible P are envisaged when poultry feed formulations move towards a digestible system.

2.8.2. Calcium: phosphorous ratio

Dietary Ca to P ratio is known to influence the absorption and utilisation of Ca (Adedokun and Adeola, 2013). Maintaining a proper Ca to P ratio in poultry diets is important to minimise the detrimental effects on the utilisation of both minerals. High Ca to P ratio will increase digesta pH because of the buffering action of limestone and lower the utilisation of several nutrients (Sebastian *et al*., 1996).

The terminology used to describe Ca:P ratio has undergone significant changes over the years. The ratio of total Ca to total P was considered in early feed formulations (NRC, 1950) and a ratio of 1.66 was recommended for broilers. This was changed to total Ca to inorganic P ratio in NRC (1954) recommendations. As researchers started to recognise the negative influence of phytate present in grains and other plant-based ingredients on P bioavailability, a change was made to total Ca to available P ratio. Although P requirements of broilers were stated as available P in NRC (1984), the Ca requirement remained unchanged. For broilers, from hatch to 8 weeks of age, the recommended Ca to available P ratio was 2.22 to 2.28. Subsequently, the term non-phytate P replaced available P (NRC, 1994) and the total Ca to non-phytate P ratio recommended for broilers ranged from 2.22 to 2.67 depending on growth stage (Angel, 2013). According to Wilkinson *et al*. (2014), the ratio of Ca to non-phytate P is more important than the absolute dietary concentrations of each macro-mineral. Imbalanced Ca to non-phytate P ratios negatively affected growth performance and resulted in lower utilisation of non-phytate P at high dietary Ca concentrations (Gautier *et al*., 2017).

During the past three decades, total Ca to P ratio was maintained at 2:1 in broiler diets regardless of the terms available or non-phytate P (Angel, 2013). Gautier *et al*. (2017) observed that maintaining a 2:1 Ca to non-phytate P ratio appears beneficial to broilers during the starter period and that individual dietary concentrations of Ca and non-phytate P are also important. However, other studies have reported differing Ca to P ratios for maximum performance. Driver *et al*. (2005a) reported that broiler starters (day-old to 16 day of age) need 1:1 total Ca to total P to maximise weight gain and feed to gain. A subsequent study reported ratios of 0.77, 0.99, 1.14 for weight gain, feed to gain and tibia ash, respectively for the broilers aged from day-old to 16 days (Driver *et al*., 2005b). Amerah *et al*. (2014) reported that the ileal Ca digestibility was higher at a Ca to available P ratio of 1.43 than a ratio of 2.14 (55 vs. 46%). Anwar *et al*. (2016a) similarly reported that widening the Ca to non-phytate P ratios, from 1.5 to 2.5, in broiler diets reduced the true ileal Ca digestibility coefficients (0.65 and 0.49, respectively) of limestone.

2.8.3. Phytate

Phosphorous in plant seeds is stored as phytic acid. Phytate is a salt of phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate, IP6) and occurs as a complex of Ca or Mg with myo-inositol (Cosgrove, 1980). Phytates are widely distributed in cereals, grain legumes and oilseed meals at concentrations ranging from 7-8 g/kg in maize to 50-60 g/kg in cereal by-products (Eeckhout and De Paepe, 1994). Phytates are mostly located in the outer membranes of grains, predominantly in the aleurone layer (wheat and barley) or in the embryo (maize). Phytates are therefore enriched in the cereal bran which also has a high content of dietary fibre (O'Dell *et al*., 1972). In dicotyledonous seeds, phytate is located in the cotyledons (Kies, 2005; Bohn *et al*., 2008).

Phytic acid contains six phosphoric acid groups that are removed in a step-wise manner by phytases (Heinzl, 1996). The term 'phytin' denotes a Ca-magnesium salt of phytic acid, whereas phytate refers to the mono to dodeca anion of phytic acid (Maga, 1982). Catabolites of phytic acid are called lower inositol penta- (IP5), tetra- (IP4) and tri (IP3) phosphates. Phytate has negatively charged phosphate groups that strongly bind to number of biologically important cations such as Ca, Mg, potassium [K], copper [Co], Zn and Fe, and make them insoluble and unavailable for absorption in monogastric animals (Angel *et al*., 2002).

The binding of Ca with phytic acid is pH dependent (Dendougui and Schwedt, 2004). According to Nelson *et al*. (1968), 3.6 g/kg of the Ca can be bound by 10 g/kg phytate. Phytate also binds with protein, starch and lipids (Selle *et al*., 2009a), negatively affecting their digestibility.

In general, P and inositol in the phytate are not bioavailable to monogastric animals as these animals lack endogenous phytases. The ruminants, on the other hand, are able to digest phytate as this enzyme is produced by rumen microorganisms. Poultry and pigs are mainly fed on grain-based diets and therefore, the P in phytate form in these diets is unavailable for absorption and passes through the gastrointestinal tract. Consequently, significant proportion of the P is excreted in the manure and leads to environmental problems such as eutrophication (Mallin, 2003).

Poultry are capable of utilising at least a portion of dietary phytate phosphorous (Angel *et al*., 2002). Phytate-P utilisation in the gastrointestinal tract of chickens is known to be influenced by the concentration of Ca, P, non-phytate P, vitamin D_3 and its metabolites, feed processing and particle size (Angel *et al*., 2002). Studies have shown that increased Ca content in broiler diets reduces the phytate-P hydrolysis and P digestibility (Tamim and Angel, 2003;

Tamim *et al*., 2004; Plumstead *et al*., 2008). This finding could be partly explained by the increased digesta pH due to high Ca concentrations which may lead to the formation of Ca-phytate complex (Selle *et al*., 2009a). According to Tamim *et al*. (2004), a Ca form that is not reactive with phytic acid molecule would be beneficial to increase the utilisation of Ca and phytate-P. The effective approach to increase the P digestibility of diets by means of phytate P hydrolysis is the use of microbial phytases.

2.8.4. Phytase

Phytase (myo-inositol hexaphosphate phosphohydrolase) is the phosphatase enzyme that catalyses the hydrolysis of phytic acid, an indigestible P form found in plant-based ingredients, and releases a usable form of inorganic P to increase the phytate-P utilisation in animals. Monogastric animals cannot utilise phytates as there is no endogenous phytase activity in their digestive tract. Therefore, the addition of exogenous phytases to poultry diets has now become routine.

Dephosphorylation of the six phosphate groups in the phytic acid by phytases takes place at different rates and in an orderly manner yielding products that become substrates for further hydrolysis. These dephosphorylated products from phytic acid are known as lower inositol phosphate esters (IP5 to IP1) which ultimately yield inositol and inorganic P (Selle *et al*., 2000). Most of the phytases are able to cleave five of the six phosphate groups from phytic acid (Konietzny and Greiner, 2002). Phytases have been classified based on the first phosphate position of hydrolysis onset, namely 3-phytases and 6-phtases (International Union of Biochemistry, 1979). The 3-phytase (EC 3.1.3.8) hydrolyses the ester bond of myo-inositol hexakisphosphate at third position to D-myo-inositol 1, 2, 4, 5, 6-pentakisphosphate and orthophosphate and the 6-phytase (EC 3.1.3.26) first hydrolyses the sixth position of myo-

inositol hexakisphosphate to 1-L-myo-inositol- 1, 2, 3, 4, 5-pentakisphosphate and orthophosphate. Most phytases produced by microorganisms (bacteria, fungi and yeasts) are 3-phytases (Maenz, 2001) except for few derived from *Basidiomycete* fungi and *Escherichia coli* bacteria which are 6-phytases (Greiner *et al*., 1993; Lassen *et al*., 2001). Most of the 6-phytases are isolated from higher plants (Cosgrove and Irving, 1980) except for soybean phytases which is 3-phytase (Sandberg and Andlid, 2002). High concentration of phytase has been reported in wheat, rye, triticale and barley (Eeckhout and De Paepe, 1994; Viveros *et al*., 2000). However, phytase in feed ingredients may be inactivated by several factors like feed processing temperature, low pH in the upper digestive tract of birds and action of pepsin on gastric secretions (Wodzinski and Ullah, 1996; Phillipy, 1999). Phytases have also been grouped as histidine acid phosphatases, β-propeller phytases, purple acid phosphatases and protein tyrosine phosphatase-like phytases (Puhl *et al*., 2007). Only few phytases belong to the protein tyrosine phosphatase-like phytase class and have been described as highly specific for phytic acid. These include phytases from bacterial species like *Bacillus*, *Aspergillus* and *Escherichia coli* (Konietzny and Greiner, 2002). The optimum pH for microbial and plant-based phytases are reported to be around 2-6 and 5, respectively (Wodzinski and Ullah, 1996). Other than the exogenous microorganisms and plant-based feedstuffs, very little quantity of phytase is found in monogasric animal's body (endogenous phytase) by means of digestive secretions and gut microbes (Ravindran, 1995; Sebastian *et al*., 1998). Endogenous phytase activity is reported to be efficient in poultry than in pigs (Rodehutscord and Rosenfelder., 2016).

Phytase enzyme activity is expressed in activity units (FTU) where 1 FTU is the amount of enzyme that liberates 1 µmol inorganic orthophosphate per minute from 0.0051 $molL^{-1}$ sodium phytate at pH 5.5 and a temperature of 37°C (Engelen *et al*. 1994). Other abbreviations,

including FYT, U and PU have also been used to denote phytase activity as per the literature (Selle and Ravindran, 2007). The doses of phytases tested in poultry and pig trials vary from 150 to 24,000 FTU per kg diet (Cowieson *et al*., 2006; Browning *et al*., 2012; Walk *et al*., 2012a,b; Paiva *et al*., 2013), but the recommended dosage of most commercial phytases range between 150 (for layers) to 1000 FTU/kg diet (for broilers and pigs). The doses higher than the recommendation are known as superdoses which are currently being used by the industry and reported to improve the growth performance and nutrient utilisation (Cowieson *et al*., 2011). Phytase has been known to hydrolyse not only the phytic acid but also the lower phytate esters (IP5 to IP1), especially at superdoses. Administration of these phytase superdoses have been found to prevent the build-up of lower phytate esters in the digestive tract of poultry and thereby reduces the anti-nutritive effect of phytate as well as lower phytate esters in poultry (Cowieson *et al*., 2011; Beeson *et al*., 2017; Bedford and Rousseau, 2017).

A large volume of published data is available on the influence of phytase addition in poultry and pig diets. It is well documented that the addition of exogenous phytases to poultry diets increases the phytate-phosphorous utilisation and the availability of minerals, energy, protein and amino acids (Selle *et al*., 2000, 2009b; Woyengo and Nyachoti, 2010). Even though, the digestibility of amino acids and energy are maximised at recommended doses of phytases, the mineral availability seems to non-linearly increase with incremental doses of phytase (Cowieson *et al*., 2006). Release of phytate P depends on several factors like added phytase dose, dietary phytate content, source of phytate, dietary non-phytate P content, dietary Ca content, dietary Ca: total P ratio and vitamin D (Selle and Ravindran, 2007). For example, several studies have shown that increasing phytase doses increase the ileal P digestibility in broilers (Cowieson *et al*., 2015; Walters *et al*., 2019). Also, the P and Ca digestibility responses

vary depending on the type of phytases. Tamim *et al*. (2004) reported that the inclusion of 3-phytase (from *Aspergillus ficuum)* improved ileal P absorption and phytate-P disappearance by 71 and 132%, respectively at 7 g/kg dietary Ca content while the improvement was 58 and 77%, respectively with 6-phytase (from *Peniophora lycii*). Furthermore, it has been shown that the increment percentage in ileal P digestibility (15%) of the diet containing high non-phytate P (4.5 g/kg) was lower than that of the low (65%) non-phytate P (2.3 g/kg) diet (Ravindran *et al*., 2000). This indicates that the amount of supplemental inorganic P can be reduced when phytase is added to the poultry diets. Consequently, the amount of P excretion in poultry is reduced which can positively affect the environment. It has been reported that approximately 1 g/kg of inorganic P can be replaced by approximately 800 FTU/kg phytase activity (Selle and Ravindran, 2007). For the purpose of feed formulation, the efficacy of an enzyme is expressed by its nutrient matrix value. The nutrient matrix value of phytase indicates the amount of a nutrient that would be released when phytase is added to the diet (Shelton *et al*., 2004). Knowledge of the correct matrix value of a nutrient would facilitate accurate diet formulation.

Phytases not only have influence on P digestibility but also affect Ca digestibility in poultry. Because, phytase hydrolyses the phytate-bound Ca to make it available for absorption in the broiler's digestive tract. Inclusion of phytases in the diets of poultry and pigs has been shown to increase the Ca digestibility (Kornegay *et al*., 1996; Tamim *et al*., 2004; Selle *et al*., 2009a; Walk *et al*., 2012b; González-Vega *et al*., 2015a; Blavi *et al*., 2017; Walters *et al*., 2019). Qian *et al*. (1996) reported that the apparent Ca retention was increased linearly in turkeys, with the increased phytase doses (0, 300, 600 and 900 FTU/kg). Similarly, Walters *et al*. (2019) reported that increasing phytase doses (250-3000 FTU/kg) increased the Ca digestibility in broilers. However, high concentration of dietary Ca content inhibits the efficacy

of both endogenous and exogenous phytases (Angel *et al*., 2002; Applegate *et al*., 2003; Paiva *et al*., 2013). Presumably, this could be a consequence of insoluble Ca-phytate complex formation. In an *in vitro* assay, Tamim and Angel (2003) reported that addition of 5g/kg Ca from limestone reduced phytate-P hydrolysis at both pH 2.5 and pH 6.5. According to Akter *et al*. (2016), dietary Ca of 10 g/kg diet negatively affects the phytase activity and phytate-P hydrolysis in broilers especially when the non-phytate P is low. Moreover, Qian *et al*. (1996) reported that narrowing the dietary Ca to total P ratio (from 2:1 to 1.2:1) increased the phytase efficacy by 16% in improving the performance, digestibility of Ca and P, bone measurements and serum Ca levels in weanling pigs. Apart from improving major mineral digestibility, several studies have shown that phytase also improved growth performance, digestibility of trace minerals and bone mineralisation in broilers (Powell *et al*., 2011; Moss *et al*., 2018; Walters *et al*., 2019).

Recent studies reported that the age of broilers has significant influence on phytase efficacy (Li *et al*., 2018; Babatunde *et al*., 2019). According to Babatunde *et al*. (2019), the age effect on phytase efficacy in terms of apparent digestibility of Ca and P was more evident at younger age (day 14) than at older age (day 22). Similar effect was reported by Li *et al*. (2018) at days 9 vs. 21 in broilers. Possible reason could be the inability of younger birds to utilise phytate P as they have an immature digestive tract (Ravindran, 2013a).

2.8.5. Dietary adaptation length

Calcium digestibility estimates in poultry could also be influenced by dietary adaptation length. Proszkowiec-Weglarz and Angel (2013) reported a reduced apparent ileal Ca digestibility in broilers with increased dietary adaptation length. Similarly, Anwar *et al*. (2018) found that the apparent and true Ca digestibility of DCP were higher at 24-hours of adaptation when

compared to those at 48 and 72 hours. The true Ca digestibility values reported for DCP at 24, 48 and 72 hours of adaptation length were 0.45, 0.36 and 0.35, respectively (Anwar *et al*., 2018). In the same study, however, the adaptation length had no influence on the Ca digestibility of MCP. Similarly, Perryman *et al*. (2016) conducted a study with broilers to test the effect of dietary adaptation length on P utilisation and reported a higher apparent ileal P digestibility at 24 hours of adaptation when compared to 0 and 72 hours of adaptation. However, there was no clear pattern to the effect of dietary adaptation length on P digestibility, since the results were inconsistent.

The dietary adaptation lengths used in the measurement of Ca digestibility are variable depending on the methodology and species. Anwar *et al*. (2015; 2016a,b,c; 2017; 2018) used a 3-day dietary adaptation period for broilers to measure the true ileal Ca digestibility. However, trials conducted to measure the Ca retention have employed different adaptation lengths and excreta collection periods in poultry. Nahason *et al*. (1994) practiced a 7-day adaptation period in layers followed by a 3-day excreta collection period. Cheng and Coon (1990b) used a 4-week adaptation followed by a 3-day excreta collection period in layers. In pig assays, a 5-day adaptation period was practiced followed by 6 days of faecal collection (González-Vega, *et al*., 2015a,b). Longer than 5-day adaptation was proposed by WPSA (2013) for P utilisation studies in broilers with semi-purified ingredients. Therefore, establishing a standard dietary adaptation length is necessary to reduce the variations in the measurement of Ca digestibility.

2.8.6. Age of birds

Age of the birds may influence the Ca digestibility in poultry. It is well recognised that the digestion of other nutrients is influenced by the age of poultry (Lima *et al*., 2012; Li *et al*.,

2015). In particular, the digestion of major nutrients (starch, fat and protein) and metabolisability of energy are known to be compromised in the newly hatched broiler chick and increase with advancing age (Zelenka, 1968; Noy and Sklan, 1995; Uni *et al*., 1995; Batal and Parsons, 2002; Thomas *et al*., 2008; Tancharoenrat *et al*., 2013). However, reports on the effect of age on mineral digestibility in poultry are limited. Some reports are available on Ca digestibility among different age groups of broilers, but most relate to estimates during the first three weeks of age. Several studies have reported a decreasing trend in Ca digestibility with advancing age of broilers (Fonolla *et al*., 1981; Shastak *et al*., 2012; Angel *et al*., 2013; Li *et al*., 2018). In contrast, Morgan *et al*. (2015) reported that the ileal Ca digestibility of a maize-soybean meal diet was higher at week 2 compared to that at week 1. Comparable results of decreasing trends in digestibility have also been reported for phosphorous (P) digestibility in broilers (Fonolla *et al*., 1981; Shastak *et al*., 2012; Angel *et al*., 2013). However, no studies to date have investigated the age effect on Ca digestibility over the entire growth phase of broilers.

2.8.7. Other factors

There are number of other factors that influence the Ca digestibility in poultry such as dietary, physiological and animal factors. Dietary factors include Ca source, diet composition, form and amount of Ca fed and Ca status of birds. Difference in Ca digestibility of different Ca sources is obvious and to be expected. For example, Angel (2013) indicated that the true Ca digestibility of MCP (67.9%) is higher than the limestone (34.1%). Hamdi *et al*. (2015) reported a higher Ca digestibility (73.7%) for calcium chloride than for limestone (67.1%) and TCP (66.8%). In addition, the Ca digestibility reported for TCP was lower than MCP.

Dietary composition may also affect the Ca digestibility in poultry. Crude protein content of the diet is one of the chief nutrients that is necessary for the growth of animals.

However, there are no studies available on the effect of dietary protein concentrations on the Ca digestibility in poultry. The dietary protein concentrations that have been maintained in the previous Ca digestibility studies in broilers were lower than the standard requirement of birds as the practical protein feed ingredients cannot be used in these studies in order to eliminate the Ca contribution from these supplements. Because the digestibility studies use a particular Ca source to be served as a sole source of Ca. Although purified protein sources without any Ca content can be used to increase the protein content of the diet in the digestibility studies, there is a certain usage limit of these ingredients in digestibility studies based on the recommendation of WPSA (2013). Dried egg albumen, maize gluten meal and potato protein extract are some of the purified protein sources that have been used to formulate diets in the digestibility studies for poultry and pigs.

Another dietary factor that influences Ca digestibility is the concentration of Ca in the diets. Feeding lower dietary Ca contents resulted an improvement in performance and mineral digestibility in poultry (Paiva *et al*., 2013; Rao and Roland, 1990; Selle *et al*., 2009a). However, particle size and Ca status could interact with dietary Ca concentration to influence the Ca retention (Rao and Roland, 1990). Particle size of Ca source has been shown to influence Ca digestibility. Coarse particles (1-2 mm) of limestone and oyster shell increase the true Ca digestibility and retention in broilers (Anwar *et al*., 2017). Similarly, the Ca retention in layers consuming large limestone particles was higher than those consuming small limestone particles (Rao and Roland, 1990). Rao and Roland (1990) reported that the hens in the Ca-deficient status solubilised and retained more Ca than that of the birds at normal status.

Anti-nutritive factors present in feed ingredients also could reduce the Ca digestibility in poultry. Mahmood *et al*. (2014) reported that the tannin content in sorghum at the level of 30 g/kg reduced the absorption of Ca, P and other minerals in layers.

Duration of steam conditioning of diets may also influence Ca digestibility. Attar *et al*. (2017) reported that duration of 2-minutes steam conditioning improved the Ca retention in broilers than 0- and 4-minutes duration. Addition of carbohydrase enzyme complex has shown to increase the Ca digestibility in broilers fed wheat-soybean based diets by lowering the digesta viscosity (Neto *et al*., 2015).

Physiological factors like vitamin D_3 level, plasma hormone level and gut health are other factors that may affect Ca digestibility. Gut abnormalities or inflammation may affect the Ca digestibility (Adedokun and Adeola, 2013). Furthermore, the passage rate determines the resident time of digesta inside the gut and thereby altering the Ca digestibility. Digesta viscosity may alter the passage rate and subsequently the digestibility. Length of gut is another factor that could affect Ca digestibility. A longer ileum enables the digesta to be spent longer and thereby increasing the absorption of Ca (Adedokun and Adeola, 2013).

Animal factors such as sex, body weight, production level, etc. could influence the Ca digestibility in poultry. Sex has been shown to influence the digestibility of other nutrients in poultry (Ten Doeschate *et al*., 2007). Body weight and growth rate of animals may alter the Ca digestibility in poultry (Kemme *et al*., 1997). Calcium digestibility was increased with increasing body weights in pigs housed in metabolic crates regardless of methodology (total collection and indicator method). However, no body weight effect was reported for pigs housed in pens (Kemme *et al*., 1997). Pigs kept in pens had higher Ca digestibility than those in metabolic cages, indicating that exercise is beneficial to improve Ca digestibility.

2.9. Research gaps

Anwar *et al*. (2015; 2016a,b,c; 2017) found that the true ileal Ca digestibility of limestone and MBM in broilers was comparable with those of pigs. However, the Ca digestibility for DCP, MCP, poultry by-product meal, fish meal and canola meal in poultry were much lower than those determined for pigs. There may be several possible reasons for this discrepancy. First, the differences in the composition of basal diet used in the assays. Maize-based basal diets were used in the pig studies, whereas purified diets based on maize starch and dextrose were used in the broiler studies. Second, there are differences in the way digestibility is calculated. Total excreta collection was used in pig studies, whereas titanium indicator ratios in the diet and digesta were used in broiler studies. It may be speculated that the lower Ca digestibility values determined in broilers may be due to, at least in part, to interaction between titanium and Ca. It is known that different minerals interact with each other in their absorption and metabolism (Suttle, 2010), but there are no published reports on any possible interference of titanium with Ca analysis or absorption. Third, the length of adaptation to dietary treatments may influence Ca digestibility measurements. The dietary adaptation period employed in poultry studies was three days, whereas five days were used in pig studies. Anwar *et al*. (2018) observed the Ca digestibility of DCP was higher at 24 hours of dietary adaptation length compared to those of 48 and 72 hours. Further research is warranted to determine the effects of basal diet composition, methodology and dietary adaptation length on Ca digestibility measurements.

Age of poultry has significant effect on nutrient digestibility in poultry (Lima *et al*., 2012; Li *et al*., 2015). However, reports on Ca digestibility between different age groups of birds are limited. Anwar *et al*. (2016a,c; 2017; 2018) reported the Ca digestibility values for

feed ingredients in broilers aged between days 21 and 24. Studies are warranted to estimate the Ca digestibility between different age groups of poultry.

It is well documented that the addition of exogenous phytases to poultry diets increases the phytate-P utilisation and the availability of minerals (Selle *et al*., 2000, 2009b; Woyengo and Nyachoti, 2010). The Ca digestibility values for some Ca sources with and without phytase have been reported recently in pigs (González-Vega *et al*., 2015a,b). However, none of the studies reported the Ca digestibility of Ca sources with phytase in poultry.

There can be differences in the Ca digestibility between different classes of poultry namely broilers and layers. Studies on the Ca digestibility in laying hens are limited and research is needed to determine the Ca digestibility values in layers.

As a result of the shift from total Ca to digestible Ca, recommendations for standardised digestible Ca to standardised digestible P requirements for pigs have been proposed recently (NRC, 2012). Digestible Ca to digestible P ratios between 1.1:1 and 1.40:1 are recommended for the growth performance of pigs having 11-130 kg body weight. Similar changes in the ratio of digestible Ca to digestible P will take place when poultry feed formulations move towards a digestible system.

CHAPTER 3

Effect of basal diet composition on the calcium digestibility in broiler chickens

3.1. Abstract

The objective of the study was to determine the effect of basal diet composition on true ileal calcium (Ca) digestibility of limestone, meat and bone meal (MBM), monocalcium phosphate (MCP) and dicalcium phosphate (DCP) in broiler chickens. Eight experimental diets were developed based on two basal diets (maize-based or maize starch-based) with each of the four Ca sources. Two Ca-free diets representing both basal diets were used to determine the endogenous Ca losses. Each diet was randomly allotted to six replicate cages (six birds per cage) and fed from 21 to 24-day post-hatch. Titanium dioxide was incorporated in all diets as an indigestible indicator. Apparent ileal Ca digestibility was calculated using the indicator method and the true ileal Ca digestibility was calculated by correcting for endogenous Ca losses. Ileal endogenous Ca losses were determined to be 253 and 131 mg/kg of dry matter intake in the birds fed maize-based diet and purified diet, respectively. Calcium digestibility of maize-based diet was higher ($P < 0.05$) than the maize starch-based purified diet. The average true Ca digestibility coefficients of limestone, MBM, MCP, and DCP were determined to be 0.51, 0.41, 0.43, and 0.32, respectively. These findings suggest that the Ca digestibility in broiler chickens is influenced by the composition of basal diet.

3.2. Introduction

The measurement of calcium (Ca) digestibility has received little attention in the past due to the abundance and low cost of limestone, the major source of Ca in poultry diets. However, the recent interest in digestible phosphorus (P) has necessitated a closer look at Ca digestibility. When feed formulations shift to a digestible P system, the relationship between these two

minerals during the absorption and utilisation requires the development of a digestible Ca database to ensure that Ca and P requirements of birds are precisely met. The Ca digestibility of Ca sources for growing pigs (González-Vega *et al*., 2013; 2015a,b; Merriman *et al*., 2016) and broiler chickens (Anwar *et al*., 2015; 2016a,b,c; 2017; 2018) has been reported in several recent studies. A comparison of digestibility estimates indicates that the true ileal Ca digestibility of some Ca sources (limestone, and meat and bone meal [MBM]) in broilers were comparable with those in pigs, whereas Ca digestibility of other Ca sources (dicalcium phosphate [DCP] and monocalcium phosphate [MCP]) were considerably lower (0.73 vs 0.28 and 0.78 vs 0.33 for DCP and MCP, respectively). Several reasons may be responsible for the observed differences between the two animal species. One of them might be the composition of basal diet used in the assays differed. Maize-based diets were used in the pig studies, whereas purified diets based on maize starch and dextrose were used in the broiler studies. The use of maize-based diets in pig assays was justified because of the negligible Ca concentration in maize (0.2 g/kg; NRC, 1994). The aim of this experiment was to determine the effect of basal diet composition on the Ca digestibility of four Ca sources.

3.3. 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.

3.3.1. Experimental diets

Limestone, MBM, MCP and DCP samples were obtained from commercial sources and analysed for minerals (methods 968.08D; AOAC 2005) and proximate (methods 930.15, 968.06, 991.36; AOAC, 2005) composition (Table 3.1). Limestone sample was analysed for

particle size by dry sieving method (Baker and Herrman, 2002) and for *in vitro* solubility by the weight loss method (Zhang and Coon, 1997b). Eight experimental diets were generated using two basal diet types (maize-based and maize starch-based purified diets) with each of the four Ca sources (limestone, MBM, MCP, and DCP) as the sole source of Ca. The contribution of Ca from maize in the maize-based diets was negligible (< 0.02 g/kg). A dietary Ca content of 9.0 g/kg was maintained in all diets (Table 3.2), which was above the recommended dietary Ca requirement (8.5 g/kg) for broiler finishers (Ross, 2014). Two Ca-free diets representing maize-based and purified diets were also developed to determine the ileal endogenous Ca losses. Titanium dioxide (TiO_2) was incorporated in all diets as an indigestible indicator. The 10 experimental diets were then randomly allocated to 6 replicate cages each (6 birds per cage).

3.3.2. Birds and housing

Day-old male broilers (Ross 308) were obtained from a commercial hatchery and raised on floor pens in an environmentally controlled room. Temperature was maintained at 31˚C on day 1 and gradually reduced to 22◦C by 21-day post-hatch. The birds were fed commercial broiler starter crumbles (230 g/kg crude protein, 10 g/kg Ca and 5.2 g/kg non-phytate P). On day 14, the birds were moved to grower cages for acclimatisation. Between days 14 and 20, the crumbles were gradually changed to mash as the experimental diets were in mash form. On day 21, birds were individually weighed and allocated to 60 cages (6 birds per cage) so that the average body weight per cage was similar. A lighting schedule of 20 hours light per day was provided. The experimental diets, in mash form, were offered *ad libitum* for three days from 21- to 24-day post-hatch and the birds had free access to water.

Table 3.1. Analysed mineral and nutrient composition of calcium sources (as received basis)[1]

	Limestone	Meat and bone meal	Monocalcium phosphate	Dicalcium phosphate
Dry matter (g/kg)	1000	978	941	969
Ash (g/kg)	996	265	792	848
Fat (g/kg)	-	88	-	-
Protein (g/kg)	-	596	-	-
Macro minerals (g/kg)				
Calcium	400	90	174	260
Phosphorous	0.56	45	260	190
Magnesium	2.2	1.96	1.06	8.70
Potassium	< 0.40	2.90	6.70	0.65
Sodium	< 0.50	4.90	< 0.50	0.71
Micro minerals (mg/kg)				
Iron	500	510	187	530
Copper	0.60	8.3	< 0.5	3.30
Zinc	<10	87	< 10	600
Manganese	61	24	10.2	2700
Chloride	< 100	2700	< 90	< 100
Aluminium	93	174	2900	780
Lead	0.36	4.90	< 0.10	0.98
Arsenic	<1.0	< 0.50	< 1.0	19
Cadmium	0.70	0.036	0.20	1.66

[1]Samples were analysed in duplicate

3.3.3. Collection and processing of ileal digesta

Group body weights and feed intake were recorded on days 21 and 24. All birds were euthanised on day 24 by intravenous injection (0.5 mL per kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand), and the ileal digesta were collected and processed as described by Ravindran *et al*. (2005). The ileum was defined as that portion of the small intestine extending from the Meckel's diverticulum to a point approximately 40 mm proximal to the ileo-cecal junction. The ileum was then divided into two halves, and the digesta were collected from the lower half towards the ileo-cecal junction. Digesta were gently flushed with deionised distilled water, immediately frozen and subsequently lyophilised. Lyophilised digesta samples were ground to pass through 0.5 mm sieve and stored in airtight containers at 4◦C till chemical analysis.

Table 3.2. Ingredient composition and analysis of experimental diets (g/kg, as fed basis)[1]

Ingredient	Maize-based diet					Purified diet				
	Limestone	MBM	MCP	DCP	Ca-free	Limestone	MBM	MCP	DCP	Ca-free
Maize	915	853	912	932	970	-	-	-	-	-
Maize starch	-	-	-	-	-	380	349	380	389	408
Dextrose	-	-	-	-	-	380	349	380	389	408
Dried egg albumen	-	-	-	-	-	100	100	100	100	100
Cellulose	-	-	-	-	-	50	50	50	50	50
Limestone	23.2	-	-	-	-	23.7	-	-	-	-
MBM	-	105	-	-	-	-	107	-	-	-
MCP[2]	-	-	58.8	-	-	-	-	60	-	-
DCP[2]	-	-	-	37.9	-	-	-	-	38.7	
Monosodium phosphate	32.7	12.7	-	0.6	1	36.2	15.5	-	3.5	4.6
Soybean oil	20	20	20	20	20	20	20	20	20	20
Sodium chloride	2	2	2	2	2	2	2	2	2	2
Titanium dioxide	5	5	5	5	5	5	5	5	5	5
Trace mineral-vitamin premix[3]	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
Calculated analysis										
Metabolisable energy (MJ/kg)	13.6	13.7	13.6	13.9	14.4	12.7	12.8	12.8	13.0	13.6
Crude protein	77.8	125	77.5	79.3	82.4	82.4	136	82.4	82.4	82.4
Calcium[4]	9.00	9.00	9.00	9.00	0.19	9.00	9.00	9.00	9.00	-
Total phosphorus[4]	9.69	9.59	15.90	9.73	2.93	7.89	7.90	13.62	7.91	1.00
Non-phytate phosphorous[5]	7.86	7.88	14.08	7.87	0.99	7.89	7.90	13.62	7.91	1.00
Ca: Non-phytate phosphorous	1.14	1.14	0.64	1.14	0.20	1.14	1.14	0.66	1.14	-

Analysed composition (as fed basis)										
Dry matter	921	934	925	929	933	933	935	933	949	929
Calcium	10.8	9.4	10.2	9.9	0.3	9.5	9.3	8.9	10.5	0.2

[1]MBM: meat and bone meal, MCP: monocalcium phosphate, DCP: dicalcium phosphate.

[2]DCP was of Chinese origin (Luteng Tianbao Phosphorous Chemical Co., LTD, Yun Nan, China). MCP was manufactured by Innophos, Cranbury, NJ.

[3]Supplied per kilogram of diet: vitamin A (trans-retinyl acetate), 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 IU; 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.

[4]Calculated based on analysed values for MBM, MCP and DCP and NRC (1994) for limestone.

[5]Calculated based on both NRC (1994) and analysed values where non-phytate phosphorous contents of Ca sources and maize were obtained from analysed values and NRC (1994), respectively.

3.3.4. Chemical analysis

Representative samples of the experimental diets and ileal digesta were analysed for dry matter (DM), Ca and TiO_2. Dry matter was determined using standard procedure (method 930.15; AOAC International, 2005). Titanium was determined on a UV spectrophotometer according to the method of Short *et al*. (1996). Calcium analysis was carried out according to the method of Karlsson *et al*. (2015). The samples were digested with concentrated nitric acid, followed by the addition of 2 M hydrochloric acid and strontium-caesium solution (25,000 mg/L). The Ca concentration was determined by Microwave Plasma Atomic Emission Spectrometry (Agilent Technologies, 4200 MP-AES, Santa Clara, CA) at 714.815 nm wavelength.

3.3.5. Calculations

Apparent ileal digestibility coefficients of Ca were calculated using the titanium ratio in the diets and digesta (Ravindran *et al*., 2005) as indicated below:

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

where AIDC is the apparent ileal digestibility coefficient, 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 concentrations were expressed as g/kg DM. Analysed Ca values were used to calculate the Ca digestibility.

Ileal endogenous Ca losses were determined in birds fed the Ca-free diet and the values (g/kg DM intake [DMI]) were calculated by the following formula (Anwar *et al*., 2018).

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

where IEL 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 concentration of Ca in the ileal digesta.

True ileal digestibility coefficients were then calculated as follows:

$$TIDC = AIDC + (IEL / Ca_I)$$

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

3.3.6. Statistical Analysis

The data were analysed as a 2 × 4 factorial arrangement of treatments using the General Linear Model procedure of SAS (2004) to determine the effect of basal diet type, Ca source, and their interaction. Differences were considered significant at $P < 0.05$ and significant differences between means were separated by the Least Significant Difference test. Cage served as the experimental unit.

3.4. Results

The geometric mean particle diameter of the limestone was determined to be 370 µm with a geometric standard deviation of 2.28 µm (Baker and Herrman, 2002). *In vitro* Ca solubility coefficient of limestone, determined by the weight loss method (Zhang and Coon, 1997b) was 0.47. Analysed Ca concentrations of limestone, MBM, MCP, and DCP were determined to be 400, 90, 174 and 260 g/kg, respectively (Table 3.1). Analysed Ca concentrations of the experimental diets ranged between 8.9 to 10.8 g/kg (Table 3.2).

The performance of birds during the 3-day experimental period (Table 3.3) is provided only as supporting data. However, of interest is the weight loss in birds fed diets containing MCP which was associated with lowest feed intake.

The influence of basal diet composition on apparent and true ileal Ca digestibility coefficients of the four Ca sources is presented in Table 3.4. Ileal endogenous Ca losses (mean ± SE; n = 6) were determined to be 253 ± 65 and 131 ± 25 mg/kg DM intake in birds fed maize-based and purified diets, respectively, and these values were used to calculate the true ileal Ca

digestibility. The results showed that the main effects of diet and Ca source were significant ($P < 0.05$) and that there was no interaction ($P > 0.05$).

Table 3.3. Influence of basal diet composition on growth performance of broiler chickens[1]

Diet	Feed intake (g/bird/day)	Body weight gain (g/bird/day)
Maize-based diet:		
Limestone	99.2^{ef}	19.6^{de}
Meat and bone meal	116.1^{cd}	40.9^{b}
Monocalcium phosphate	86.6^{g}	-8.0^{g}
Dicalcium phosphate	107.3^{de}	12.6^{e}
Ca-free	105.1^{e}	11.3^{ef}
Purified diet:		
Limestone	89.6^{g}	9.8^{ef}
Meat and bone meal	126.5^{b}	56.1^{a}
Monocalcium phosphate	94.0^{fg}	-0.4^{fg}
Dicalcium phosphate	124.9^{bc}	28.4^{cd}
Ca-free	135.9^{a}	35.5^{bc}
SEM[2]	3.31	4.17
Main effects		
Diet		
Maize-based	102.9	15.3
Purified	114.2	25.9
SEM	1.48	1.87
Ca source		
Limestone	94.4	14.7
Meat and bone meal	121.3	48.5
Monocalcium phosphate	90.3	-4.2
Dicalcium phosphate	116.1	20.5
Ca-free	120.5	23.4
SEM	2.34	2.95
Probabilities, $P \leq$		
Diet	0.001	0.001
Ca source	0.001	0.001
Diet × Ca source	0.001	0.003

[a-g] Means having different superscripts are significantly different ($P < 0.05$).
[1]Each value represents the mean of six replicates (six birds per replicate).
[2]Pooled standard error of mean.

Calcium digestibility in birds fed maize-based diets was higher ($P < 0.05$) than those fed maize starch-based purified diets. The Ca digestibility in limestone was higher ($P < 0.05$) than those

in the other three sources, whereas the Ca digestibility in DCP was lower ($P < 0.05$) than those in the other sources. The digestibility of Ca in MBM and MCP was similar ($P > 0.05$).

Table 3.4. Influence of basal diet composition on apparent and true ileal calcium (Ca) digestibility of limestone, meat and bone meal, monocalcium phosphate and dicalcium phosphate in broiler chickens[1,2]

Diet	Ca source	Apparent ileal digestibility	True ileal digestibility
Maize-based diet	Limestone	0.53	0.55
	Meat and bone meal	0.43	0.45
	Monocalcium phosphate	0.45	0.48
	Dicalcium phosphate	0.33	0.36
Purified diet	Limestone	0.45	0.47
	Meat and bone meal	0.35	0.36
	Monocalcium phosphate	0.37	0.39
	Dicalcium phosphate	0.27	0.28
SEM[3]		0.033	0.033
Main effects			
Diet			
Maize-based		0.44[a]	0.46[a]
Purified		0.36[b]	0.37[b]
SEM		0.016	0.016
Ca source			
Limestone		0.49[a]	0.51[a]
Meat and bone meal		0.39[b]	0.41[b]
Monocalcium phosphate		0.41[b]	0.43[b]
Dicalcium phosphate		0.30[c]	0.32[c]
SEM		0.023	0.023
Probabilities, $P \leq$			
Diet		0.003	0.001
Ca source		0.001	0.001
Diet × Ca source		0.994	0.994

[a-c] Means having different superscripts within the column are significantly different ($P < 0.05$).
[1]Each value represents the mean of six replicates (six birds per replicate).
[2]The endogenous Ca losses of maize-based and purified diets were determined to be 253 and 131 mg/kg, dry matter intake, respectively.
[3]Pooled standard error of mean.

3.5.Discussion

Calcium concentration of MBM in this study was lower than the NRC (1994) values of 103 g/kg, whereas the Ca concentrations of limestone, MCP and DCP were higher than the NRC (1994) values of 380, 160 and 220 g/kg, respectively. However, the Ca concentrations of limestone, MBM and MCP were within the reported ranges of 376 to 415, 51 to 148 and 152 to 184 g/kg, respectively (Browning and Cowieson, 2014). The Ca concentration of DCP was lower than the range of 261-294 g/kg reported by Browning and Cowieson (2014). Analysed Ca concentrations of experimental diets varied between 0.1 to 1.8 g/kg from the calculated values. Such differences between formulated and actual Ca concentrations have also been observed in previous studies (Anwar *et al*., 2015; 2018) and highlighted by Walk (2016).

The Ca digestibility determined in birds fed the maize-based diet was higher than those fed the maize starch-based purified diet. A possible explanation may lie in the coarser particle size of the maize-based diet, which may have influenced the rate of passage enabling more contact time of the Ca source with digestive secretions resulting in greater Ca solubility and digestibility (O'Dell *et al*., 1959; Rochell *et al*., 2012). According to O'Dell *et al*. (1959), the passage rates of purified and maize-based diets in the digestive tract of chickens were 135 and 165 min, respectively. To the best of our knowledge, there are no reports comparing the Ca digestibility determined using maize-based and purified diets in poultry. However, our findings are in agreement with those reported in pigs by González-Vega *et al*. (2015b), wherein the standardised total tract Ca digestibility in fish meal was greater in a maize-based diet than in a maize starch-based diet. Another possible reason for the observed difference between these two basal diets may be the differences in the content and composition of fibre as speculated by González-Vega *et al*. (2015b).

Ileal endogenous Ca losses observed in the current study for the birds fed the purified diet (131 mg/kg DM intake) is close to the upper range of losses reported in previous studies

(between 88 to 127 mg/kg DM intake; Anwar *et al*., 2015; 2016a,b,c; 2017; 2018). The ileal endogenous Ca loss was higher (253 mg/kg DM intake) in the birds fed the maize-based diet. Phytic acid contributed by the maize is possibly the major contributor to the increased endogenous losses (Cowieson and Ravindran, 2007). Similar findings were observed in pigs (González-Vega *et al*., 2015b) where a maize-based Ca-free diet induced a higher (396 mg/kg DM intake) endogenous Ca losses than a maize starch-based Ca-free diet (220 mg/kg DM intake).

In the current study, the true ileal digestibility coefficient of limestone (0.47) and MBM (0.36) in birds fed the purified diet were lower compared to the values reported by Anwar *et al*. (2015; 2016a,b). For the limestone, the observed discrepancy may be explained by differences in the *in vitro* solubility and particle size of samples used. In the study of Anwar *et al*. (2016a), the *in vitro* solubility coefficients of the same limestone having geometric mean particle diameters of < 0.5 and 1 to 2 mm were 0.60 and 0.33, respectively. The geometric mean particle diameter of limestone used in the current study was 0.37 mm and the *in vitro* solubility coefficient was 0.47. It is accepted that the *in vitro* solubility of coarser limestone particle is lower than that of fine particles (Cheng and Coon, 1990a; Manangi and Coon, 2007) which can increase the Ca digestibility by staying longer in the gizzard (Zhang and Coon, 1997a). True ileal Ca digestibility coefficient reported for the MBM (0.41) in the current study is close to the lower range of digestibility value reported in previous studies (Anwar *et al*., 2015; 2016b). Variations in the true ileal Ca digestibility coefficient from 0.41 to 0.60 among four MBM samples have been reported by Anwar *et al*. (2015; 2016b).

In the current study, the digestibility of Ca in MBM was similar to that of MCP, but greater than that of DCP. The low Ca digestibility of MCP (0.39) and DCP (0.28) in broilers fed the purified diet are in agreement with those of Anwar *et al*. (2018) who reported digestibility coefficients for MCP and DCP to be 0.33 and 0.28, respectively. It is difficult to

provide a definitive explanation for the observed discrepancy in Ca digestibility between MCP and DCP in poultry. Possible reasons may include differences in Ca to non-phytate P ratio, particle size, and Ca solubility. It is, however, worth noting that markedly higher Ca digestibility in MCP (0.77) and DCP (0.73) determined in pigs, and that there were no differences in Ca digestibility between these two inorganic phosphates (González-Vega *et al*., 2015a).

Overall, the present findings indicate that the composition of basal diet affected the Ca digestibility measurement in broilers and that the use of maize-based should be considered in future assays. The digestibility estimates determined with the maize-based diet, however, were still lower than the pig data (González-Vega *et al*., 2015a; Zhang and Adeola, 2017).

3.6. Conclusions

These results indicate that the measurement of Ca digestibility is influenced by the composition of basal diet. The Ca digestibility estimates for the Ca sources were higher in the maize-based diet compared to those in the purified diet, but the digestibility values are still lower than the pig data.

CHAPTER 4

Influence of indicator type and dietary adaptation length on calcium digestibility of limestone in broiler chickens

4.1. Abstract

Results from two experiments relating to the measurement of apparent ileal calcium (Ca) digestibility of limestone in broiler chickens are reported in this chapter. The first experiment was conducted to examine the effect of indicator type on the ileal Ca digestibility of limestone. Two experimental diets with either titanium dioxide or acid insoluble ash (Celite) were developed. Each diet was randomly allocated to six replicate cages (eight birds per cage) and fed from 21- to 24-day post-hatch. Total tract Ca retention was also measured using the indicator ratios. Indicator type had no influence ($P > 0.05$) on the digestibility measurements. Calcium retention determined using acid insoluble ash was higher ($P < 0.05$) compared to that determined using titanium dioxide. The second experiment was conducted to determine the effect of dietary adaptation length on apparent Ca digestibility of limestone. The experimental diet was offered from day 21 to six replicates (six birds per cage) each for 24, 72, 120 or 168 hours and the ileal digesta were collected. Calcium digestibility at 24-hour was higher ($P < 0.05$), and increasing the adaptation length from 72 to 120 hours had no effect ($P > 0.05$) on the digestibility. The coefficients of apparent ileal Ca digestibility of limestone after 24, 72, 120 and 168 hours of adaptation length were determined to be 0.65, 0.46, 0.44 and 0.36, respectively.

4.2. Introduction

Indigestible dietary indicators are routinely used in the measurement of nutrient digestibility, both at ileal and total tract levels. The indicator-based digestibility assays are not subject to the limitations which arise in the total collection method such as the adherence of droppings to plumage, contamination of excreta with scurf, feathers and regurgitated feed, changes in

chemical composition of excreta due to fermentation, excreta losses during removal and transfer, birds excreting away from the tray and variation in moisture content of feed during the assay (Sales and Janssens, 2003). Number of indicators have been used in poultry digestibility assays and the common ones include titanium dioxide (TiO_2), chromic oxide, ferric oxide and acid insoluble ash (AIA).

As previously noted, despite using similar assay methodology, Ca digestibility values reported for some Ca sources for broilers (Anwar *et al*., 2018) are markedly lower than those reported for pigs (González-Vega *et al*., 2015a). A difference between the broiler and pig studies was that TiO_2 indicator was used in the former and total excreta collection in the latter. Thus, it may be speculated that the lower Ca digestibility values determined in broilers may be due to, at least in part, to the interaction between titanium and Ca. It is well known that minerals interact with each other in their absorption and metabolism (Suttle, 2010), but there are no published reports on the possible interference of titanium with Ca analysis or absorption. Titanium dioxide has been widely used to determine the digestibility of different minerals, including phosphorus. For example, van Harn *et al*. (2017) reported high ileal phosphorus digestibility coefficients for monocalcium phosphate (0.88) and dicalcium phosphate (0.82) with the use of TiO_2 indicator in broiler assays.

It is also possible that differences in dietary adaptation length (DAL) may partly explain the lower Ca digestibility in poultry. Different DAL have been used in the measurement of Ca digestibility, depending on the methodology and species. Anwar *et al*. (2015; 2016a,b,c; 2017; 2018) used a three-day DAL in broiler assays. In pig assays, a five-day adaptation period was practiced followed by six days of faecal collection (González-Vega *et al*., 2015a,b). Longer than 5-day adaptation was proposed by WPSA (2013) for phosphorous utilisation studies in broilers fed semi-purified ingredients. Studies on the effect of different DAL on Ca digestibility

in poultry are limited (Proszkowiec-Weglarz and Angel, 2013; Anwar *et al*., 2018). Establishing a standard DAL may reduce the variations in the measurement of Ca digestibility.

Therefore, experiments 1 and 2 were conducted to compare the effects of indicator type and DAL, respectively, on the apparent ileal Ca digestibility of limestone.

4.3. Materials and Methods

The experiments 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.

In both experiments, day-old male broilers (Ross 308) were obtained from a local hatchery and raised on floor pens in an environmentally controlled room. Temperature and lighting schedule were maintained as described in Chapter 3, section 3.3.2. The birds were fed commercial broiler starter crumbles (230 g/kg crude protein, 10 g/kg Ca and 5.2 g/kg non-phytate phosphorous). On day 14, birds were moved to grower cages for acclimatisation. 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 weighed and allocated to 12 cages (eight birds each) in Experiment 1 or 24 cages (six birds each) in Experiment 2 so that the average body weight per cage was similar. The birds were fasted overnight before the introduction of experimental diets.

4.3.1. Experiment 1

This experiment was initiated to determine the effect of two dietary indicator types (TiO_2 and AIA) on the apparent ileal digestibility and total tract retention of Ca in limestone. Two experimental diets were formulated using either TiO_2 or AIA (Celite) as indicators (5 or 20 g/kg, respectively) in a maize-based diet (Table 4.1). Limestone served as the sole source of Ca in assay diets and the dietary Ca content was maintained as 6 g/kg in both diets. Each diet

was randomly assigned to six replicate cages (eight birds per cage). The experimental diets, in mash form, were offered *ad libitum* for three days from 21 to 24-day post-hatch and the birds had free access to water.

Table 4.1. Ingredient composition and analysis of experimental diets (g/kg as fed basis), Experiments 1 and 2

Ingredient	Experiment 1		Experiment 2
	Titanium dioxide diet	Acid insoluble ash diet	
Maize	945	930	930
Limestone	15.5	15.5	23.2
Soybean oil	20	20	20
Monosodium phosphate	10.5	10.5	17.2
Sodium chloride	2	2	2
Titanium dioxide	5	-	5
Celite[1]	-	20	-
Trace mineral-vitamin premix[2]	2.3	2.3	2.3
Calculated analysis[3]			
Metabolisable energy (MJ/kg)	14.07	13.86	13.87
Crude protein	80.30	79.02	79.08
Calcium	6.08	6.08	9.00
Total phosphorus	5.03	4.99	6.45
Non-phytate phosphorous	3.04	3.03	4.49
Ca: Non-phytate phosphorous	2.00	2.00	2.00
Analysed values (as fed basis)			
Dry matter	910	898	909
Calcium	6.90	5.80	9.70

[1]Source of acid-insoluble ash, ECP Ltd., Birkenhead, Auckland.

[2]Supplied per kilogram of diet: vitamin A (trans-retinyl acetate), 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 IU; 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.

4.3.2. Experiment 2

Experiment 2 was conducted to determine the effect of different DAL on the apparent ileal Ca digestibility of limestone. Four time periods, namely 24, 72, 120 and 168 hours of DAL were tested. An experimental diet was formulated using limestone as the sole Ca source in a maize-

based diet (Table 4.1) and offered from day 21, in six replicates (six birds per cage) for 24, 72, 120 or 168 hours corresponding to the treatment groups. Titanium dioxide was incorporated in the diet (5 g/kg) as an indigestible indicator.

4.3.3. Collection and processing of ileal digesta and excreta

In Experiments 1, all birds were euthanised on day 24 by intravenous injection (0.5 mL per kg body weight) of sodium pentobarbitone (Provet NZ Pty. Ltd., Auckland, New Zealand), and the ileal digesta were collected and processed as described by Ravindran *et al*. (2005). Total excreta samples were also collected for three consecutive days from 21 to 24 days, weighed and pooled within a cage. Pooled excreta were mixed well and representative samples were obtained and lyophilised. In Experiment 2, starting from day 21, the birds were euthanised after 24 or 72 or 120 or 168 hours of diet introduction for ileal digesta collection. Lyophilised samples (digesta and excreta) were processed as described in Chapter 3, section 3.3.3.

4.3.4. Chemical analysis

Representative samples of diets, ileal digesta and excreta were analysed for dry matter (DM), Ca and TiO_2 as described in Chapter 3, section 3.3.4. Acid insoluble ash was measured after ashing and treating the ash with boiling 4 *M* hydrochloric acid (Siriwan *et al*., 1993).

4.3.5. Calculations

Apparent ileal Ca digestibility coefficients were calculated using the indicator (TiO_2 or AIA) ratios in the diet and digesta (Ravindran *et al*., 2005) as shown below:

$$AIDC = 1 - [(Ind_I / Ind_O) \times (Ca_O / Ca_I)]$$

where AIDC is apparent ileal digestibility coefficient of Ca, Ind_I is the indicator concentration in the diet, Ind_O is the indicator 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 concentrations were expressed as g/kg DM. Analysed Ca values were used to calculate the Ca digestibility.

Total tract Ca retention was calculated using the indicator ratios in the diet and excreta as indicated below:

$$\text{Ca retention} = 1 - [(Ind_I / Ind_E) \times (Ca_E / Ca_I)]$$

where Ind_I is the indicator concentration in the diet, Ind_O is the indicator concentration in the excreta, Ca_E is the Ca concentration in the excreta, and Ca_I is the Ca concentration in the diet. Analysed Ca values were used to calculate the Ca retention.

4.3.6. Statistical Analysis

In experiment 1, the means were compared by Student's t-test using SAS (2004). In experiment 2, the data were analysed using orthogonal polynomials to determine the linear and quadratic responses to increasing DAL. Differences were considered significant at $P < 0.05$ and significant differences between means were separated by the Least Significant Difference test. In both experiments, cage served as the experimental unit.

4.4.Results

4.4.1. Experiment 1

Analysed Ca concentration of the titanium and AIA diets were 0.9 g/kg higher and 0.2 g/kg lower, respectively, than the calculated concentrations (Table 4.1).

Coefficients of apparent ileal digestibility and total tract Ca retention of limestone in birds fed the TiO_2 and AIA diets are presented in Table 4.2. The apparent ileal Ca digestibility was not influenced ($P > 0.05$) by the type of indicator. Calcium retention was influenced ($P < 0.05$) by the type of indicator used in the assay with AIA yielding higher ($P < 0.05$) estimates.

4.4.2. Experiment 2

The apparent ileal Ca digestibility in limestone was influenced when the DAL was increased from 24 to 168 hours (Table 4.3; linear effect, $P < 0.001$; quadratic effect, $P = 0.07$). The Ca

digestibility decreased linearly with increasing DAL, but there was a tendency to plateau beyond 72 hours.

Table 4.2. Influence of indicator type on the apparent ileal digestibility and total tract retention of calcium (Ca) in limestone for broilers, Experiment 1[1]

	Apparent ileal Ca digestibility	Ca retention[2,3]
Titanium dioxide	0.59	0.59[b]
Acid insoluble ash	0.59	0.65[a]
SEM[4]	0.023	0.013
Probability, $P \leq$	0.955	0.001

[a,b] Means having different superscripts within the column are significantly different ($P < 0.05$).
[1]Each value represents the mean of six replicates (eight birds per cage).
[2]Calcium retention, provided in the Table, was calculated using indicator ratios.
[3]Ca retention calculated by the total collection method was 0.61 and 0.57, respectively, in birds fed TiO_2 and AIA diets. Retention value calculated by total collection was similar ($P > 0.05$) to that calculated by TiO_2 index method, whereas retention value calculated by total collection was lower ($P < 0.05$) to that calculated by AIA index method.
[4]Pooled standard error of mean.

Table 4.3. Influence of dietary adaptation length on the apparent ileal calcium (Ca) digestibility of limestone for broiler chickens, Experiment 2[1]

Dietary adaptation length (hours)	Apparent ileal Ca digestibility
24	0.65
72	0.46
120	0.44
168	0.36
SEM[2]	0.032
Probability, $P \leq$	
Linear	0.001
Quadratic	0.071

[1]Each value represents the mean of six replicates (six birds per cage).
[2]Pooled standard error of mean.

4.5. Discussion

4.5.1. Experiment 1

The inclusion of indigestible dietary indicators is obligatory for the calculation of digestibility in the ileal digesta. In the present study, the type of indicator had no influence on the apparent ileal Ca digestibility. These findings confirm that there is no interaction between titanium and

Ca during the digestion and absorption of these two minerals and that both indicators (TiO_2 and AIA) can be used for the determination of ileal Ca digestibility in broilers. Similar results have been reported in pigs by Favero *et al*. (2014) where TiO_2 and AIA yielded similar apparent ileal Ca digestibility (0.77 and 0.73, respectively) in soybean meal diet. However, AIA resulted in a higher apparent ileal Ca digestibility for a canola meal diet when compared to TiO_2. The present findings suggest that the issue of lower ileal Ca digestibility values in poultry cannot be explained by the basis of indicator effect.

In contrast, total tract Ca retention was influenced by the indicator type, with AIA resulting in a higher Ca retention coefficient (0.65) than TiO_2 (0.59). It must be noted that the Ca retention coefficient determined based on total collection method (0.57) was lower than that calculated by AIA-based index method (0.65), whereas no differences between total collection and index method were observed for TiO_2 (0.61 vs. 0.59). Cheng and Coon (1990b) similarly reported that, in laying hens, the index method (with AIA) based Ca retention values were higher than that of the total collection method. The possible reasons for the higher Ca retention estimated with AIA may include partial loss of AIA (McCarthy *et al*., 1974) and analytical imprecision (Sales and Janssens, 2003) causing underestimation of AIA in the excreta, thus overestimating the retention.

4.5.2. Experiment 2

The apparent ileal Ca digestibility of limestone was higher at 24 hours of DAL and markedly declined at 72 hours. Increasing the DAL from 72 hours to 120 hours had little effect on Ca digestibility. Anwar *et al*. (2018) also reported that the ileal Ca digestibility of dicalcium phosphate in broilers at 24 hours (0.45) DAL was higher when compared to that of 48 (0.36) and 72 hours (0.35), and that there were no differences between 48 and 72 hours. In their study, Ca digestibility of monocalcium phosphate was not influenced by the DAL. The higher apparent Ca digestibility values obtained at 24 hours DAL may reflect, at least in part, the

lower endogenous Ca losses reported at 24 h DAL. Anwar *et al*. (2018) found that the ileal endogenous Ca losses at 24, 48, and 72 h DAL were 84, 113, and 124 mg/kg DM intake, respectively. Proszkowiec-Weglarz and Angel (2013) similarly reported that the Ca digestibility of MCP in broilers was reduced from 0.70 at 16 hours DAL to 0.35 at 96 hours DAL. Perryman *et al*. (2016) conducted a study to examine the effect of DAL on phosphorous utilisation in broilers and reported a higher apparent ileal phosphorous digestibility at 24 hours DAL when compared to 0 and 72 hours. In their study, there were inconsistencies in the results among diet types, and it was concluded that there is no clear evidence to demonstrate the effect of DAL. The current findings suggest that the DAL between 3- and 5-day does not influence the Ca digestibility of limestone in broilers. However, the Ca digestibility was reduced at 7-day DAL. In pig studies, a 5-day DAL has been used (González-Vega, *et al*., 2015a,b; Merriman *et al*., 2016). Based on present studies, it may be concluded that the differences in Ca digestibility cannot be explained on the basis of DAL.

Among the other possible reasons for the dissimilarities between broilers and pigs, differences in assay methodology may be of some relevance. The direct method was used in broiler assays (Anwar *et al*., 2018), whereas a modified difference method was used in pig studies (Gonzalez-Vega *et al*., 2015a). Zhang and Adeola (2018) recently reported, using the regression method, a higher Ca digestibility coefficient of 0.67 for DCP. However, it must be noted that Ca deficient diets, which is a requirement to obtain linearity, were used in this study. Digestible Ca values determined under deficient conditions will be influenced by homeostatic mechanisms and their applicability to commercial diets is questionable (Proszkowiec-Weglarz and Angel, 2013).

Calcium metabolism in avian and mammalian species is very similar (Proszkowiec-Weglarz and Angel, 2013) and unlikely to have contributed to the observed discrepancy. Differences in anatomical and physiological differences between these species, however,

require some reflection. The crop, gizzard and antiperistaltic intestinal refluxes are unique features in birds and could influence nutrient digestion. We therefore hypothesize that different limitations may exist between the 2 species in the capacity to digest Ca. Nutrient digestion is a 3-tier process involving enzymatic hydrolysis / solubilisation, absorption and transport across the enterocyte. No comparative data are available of these processes in broilers and pigs, and therefore it cannot be established in any general way that there are no differences between the two species. The evidence reported in P availability studies, however, strongly support the view that the digestive efficiency is influenced by species differences. For example, it is pertinent to note that the extent of phytic acid degradation and appearance of degradation products in digesta are distinctly different in poultry and pigs (Rodehutscord and Rosenfelder, 2016). It appears that the activities of endogenous mucosal and microbial phytases play a major role in broilers fed diets severely limited in P, resulting in considerably greater phytic acid disappearance than in pigs.

4.6.Conclusions

Apparent ileal Ca digestibility of limestone is unaffected by the type of dietary indicator. Dietary adaptation lengths from 72 to 120 hours had no effect on the apparent ileal Ca digestibility of limestone. These data suggest that the lower ileal Ca digestibility of inorganic phosphates (MCP and DCP) in poultry, compared to pigs, cannot be explained based on the type of indicator and DAL.

CHAPTER 5

Effect of age and dietary crude protein content on the apparent ileal calcium digestibility of limestone in broiler chickens

5.1. Abstract

The objective of the present study was to investigate the effects of age on the apparent ileal calcium (Ca) digestibility of limestone for broiler chickens. Six treatment groups of different ages, namely days 1-7, 8-14, 15-21, 22-28, 29-35 and 36-42, were utilised. A maize-based diet (crude protein, 79 g/kg) containing limestone as the sole Ca source and, supplying 9.0 g/kg Ca, was fed to six replicate cages of broilers during each of the six periods. The birds were fed a commercial broiler starter diet until the introduction of the assay diet, except the day 1-7 age group. Ileal digesta were collected on day 7 (twelve birds per cage), 14 (ten birds per cage), 21 (eight birds per cage), 28 (six birds per cage), 35 (six birds per cage) or 42 (six birds per cage). A secondary objective was to examine the influence of dietary protein content on Ca digestibility. An additional diet containing a crude protein content of 153 g/kg was developed and fed to six replicate cages (eight birds per cage) from day 15 to 21 post-hatch. The birds in the age group of 15-21 days and fed the 79 g/kg crude protein diet served as the control treatment. Titanium dioxide (5 g/kg) was included in all diets as an indigestible indicator for apparent ileal digestibility measurements. Total tract Ca retention, pH of gizzard digesta and relative weights of gizzard and gizzard digesta were also measured. Apparent ileal Ca digestibility coefficients declined linearly ($P < 0.001$) with advancing age, from 0.51 at day 7 to 0.27 at day 42. Calcium retention coefficients declined quadratically ($P < 0.01$) with advancing age, from 0.56 at day 7 to 0.30 at day 28 and then plateaued. Age quadratically ($P < 0.01$) affected the gizzard pH where the pH declined from day 7 to 21 and then increased. Relative weights of gizzard and gizzard digesta were quadratically decreased ($P < 0.001$) with advancing age. Increasing the dietary protein content had no effect ($P > 0.05$) on the apparent

ileal digestibility and retention of Ca. Increasing dietary protein had no effect ($P > 0.05$) on the relative weights of gizzard and gizzard digesta. In conclusion, the present results showed that the Ca digestibility of limestone in broilers, fed a maize-based diet with limestone supplying 9 g/kg Ca, declined with advancing age.

5.2. Introduction

It is well recognised that the digestion of nutrients is influenced by the age of poultry. In particular, the digestion of major nutrients (starch, fat and protein) and metabolisability of energy are compromised in the newly hatched broiler chick and increase with advancing age. Apparent metabolisable energy (Zelenka, 1968; Batal and Parsons, 2002; Thomas *et al*., 2008), ileal protein digestibility (Noy and Sklan, 1995; Uni *et al*., 1995) and total tract digestibility of fat (Tancharoenrat *et al*., 2013) have been shown to be lower during the first week of life and increase thereafter. The low digestibility of these nutrients in the hatchling is attributed to the poor development and maturation of the gastrointestinal tract. The digestive system of the newly hatched chick is juvenile and, its capacity to digest the feed and absorb nutrients appears to be limiting during early growth (Uni *et al*., 1995). The digestion and nutrient absorption during the initial growth period of broilers depend on a well-developed gastrointestinal tract with sufficient digestive enzyme secretion and developed intestinal morphology (Noy and Uni, 2010). Growth and development of the digestive tract of poultry is very rapid and exceeds that of body weight from 10 to14 days post-hatch (Nitsan *et al*., 1991; Sell *et al*., 1991; Obst and Diamond, 1992). In addition, the secretion and activities of lipase, amylase and proteases, which are responsible for the enzymatic digestion of major nutrients, are reported to increase during the first week of age (Nitsan *et al*., 1991; Nir *et al*., 1993). The villus height and crypt depth, which are the direct representation of the absorptive surface of small intestine, also increase between 4 and 10 days of age (Uni *et al*., 1995).

On the other hand, reports on the effect of age on mineral digestibility in poultry are limited. Some reports are available on Ca digestibility among different age groups of broilers, but most relate to estimates during the first three weeks of age. Several studies have reported a decreasing trend in Ca digestibility in older broilers (Fonolla *et al*., 1981; Shastak *et al*., 2012; Angel *et al*., 2013; Li *et al*., 2018). In contrast, Morgan *et al*. (2015) reported that the ileal Ca digestibility of maize-soybean meal diet was higher at week 2 compared to that at week 1. Comparable results of decreasing trends with advancing age have also been reported for phosphorous (P) digestibility in broilers (Fonolla *et al*., 1981; Shastak *et al*., 2012; Angel *et al*., 2013). However, no studies to date have investigated the age effect on Ca digestibility over the entire growth period of broilers. The current experiment was designed to test the null hypothesis that Ca digestibility of limestone is not different among different age groups (7, 14, 21, 28, 35 and 42 days) of broilers.

Protein is a critical nutrient that is necessary for growth and development, and its deficiency may affect digestibility of nutrients, including minerals. The dietary crude protein concentrations (78-136 g/kg) that were maintained in previous Ca digestibility assays (Anwar *et al*., 2016a, 2017; Chapters 3 and 4) were lower than the recommended requirement for broilers (Ross, 2019). Therefore, an additional objective of the current study was to examine the influence of dietary protein concentration on the Ca digestibility of limestone.

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. Experimental diets, birds and housing

Same limestone that has been used in the previous study (Chapter 3) was used in the current experimental diets. A maize-based diet, with a Ca concentration of 9.0 g/kg was formulated using limestone as the sole Ca source (Table 5.1). A total of 288, day-old male broilers (Ross 308) were obtained from a commercial hatchery and the assay diet was fed to six replicate cages of broilers during six periods, namely days 1-7, 8-14, 15-21, 22-28, 29-35 and 36-42. Ileal digesta were collected on day 7 (twelve birds per cage), 14 (ten birds per cage), 21 (eight birds per cage), 28 (six birds per cage), 35 (six birds per cage) or 42 (six birds per cage). An additional diet containing a crude protein content of 153 g/kg was developed to examine the effect of dietary protein on Ca digestibility on day 21 and fed to six replicate cages (eight birds per cage) from day 15 to 21 post-hatch. The high protein diet was formulated by the inclusion of 100 g/kg dried egg albumen in the low protein diet at the expense of maize (Table 5.1). Titanium dioxide (5 g/kg) was added to the experimental diet as an indigestible indicator. The birds were raised in floor pens and fed a commercial broiler starter crumble (230 g/kg crude protein, 10 g/kg Ca and 5.2 g/kg non-phytate P) until the introduction of assay diets, except in the day 7 age group. The diets were offered *ad libitum* and the birds had free access to water. Temperature and lighting schedule were maintained as described in Chapter 3, section 3.3.2.

5.3.2. Collection and processing of ileal digesta and excreta

At the end of respective experimental periods (days 7 or 14 or 21 or 28 or 35 or 42 for age study and day 21 for crude protein study), the birds were euthanised by intravenous injection (0.5 mL per kg body weight) of sodium pentobarbitone and the ileal digesta were collected and processed as described in Chapter 3, section 3.3.3. Grab excreta samples were also collected during the last three days of experimental period, pooled within a cage and processed as described in Chapter 4, section 4.3.3.

Table 5.1. Ingredient composition and analysis (g/kg, as fed basis) of assay diets

Ingredient	Age effect study	High protein diet – protein effect study
Maize	930	830
Limestone	23.2	23.3
Dried egg albumen	-	100
Soybean oil	20	20
Monosodium phosphate	17.2	17.6
Sodium chloride	2.0	2.0
Titanium dioxide	5.0	5.0
Trace mineral-vitamin premix[1, 2]	2.3	2.3
Calculated analysis[3]		
Metabolisable energy (MJ/kg)	13.87	14.02
Crude protein	79.1	152.9
Calcium	9.00	9.00
Total phosphorous	6.45	6.24
Non-phytate phosphorous	4.50	4.50
Ca: Non-phytate phosphorous	2.00	2.00
Analysed values (as fed basis)		
Dry matter	921	940
Calcium	9.90	8.90

[1]Supplied per kg diet: vitamin A (trans-retinyl acetate), 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 IU; 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]The trace mineral-vitamin premix contained no Ca.
[3]Calculated based on NRC (1994) values.

5.3.3. Measurement of gizzard parameters

Four birds in each replicate cage were used for gizzard pH measurements. The gizzard was carefully detached at the points of proventricular opening and duodenal opening and the digesta pH was measured with a calibrated digital pH meter (pH spear, Oakton Instruments, Vernon Hill, IL) by inserting the probe directly into two places in the gizzard (proximal and distal) for each bird as described by Morgan *et al*. (2014). Readings were recorded after stabilisation of the value and average of two readings was considered as final pH value. Full and empty gizzard weights were measured and, the weight of gizzard digesta was determined as the difference

between the full and empty gizzard weights. The relative weights of gizzard and gizzard digesta were calculated as a percentage of body weight.

5.3.4. 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 3.3.4.

5.3.5. Calculations

The coefficients of apparent ileal Ca digestibility were calculated using the titanium ratios in the diet and digesta (Ravindran *et al*., 2005) as indicated below:

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

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 g/kg DM.

Apparent total tract Ca retention coefficients (ATTRC) were calculated using titanium ratio in the diet and excreta as indicated below:

$$ATTRC = 1- [(Ti_I / Ti_E) \times (Ca_E/ Ca_I)]$$

where RC is the apparent total tract Ca retention coefficient, 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 g/kg DM.

Digestible and retainable Ca intakes were calculated as indicated below:

$$DCI = \text{Feed intake} \times Ca_I \times AIDC$$

$$RCI = \text{Feed intake} \times Ca_I \times ATTRC$$

where DCI is the digestible Ca intake (g/bird), Ca_I is the dietary Ca concentration (g/kg DM), and AIDC is apparent ileal Ca digestibility coefficient, RCI is the retainable Ca intake (g/bird), and ATTRC is the apparent total tract Ca retention coefficient.

5.3.6. Statistical Analysis

The data were analysed using the General Linear Model procedure of SAS (2004). Orthogonal polynomial contrasts were performed to determine the linear and quadratic effects of broiler age. The crude protein effect was analysed by the Student's t-test (SAS, 2004). Cage served as the experimental unit. The relationships between Ca digestibility and other measured parameters were analysed by Pearson Correlation.

5.4. Results

Analysed Ca concentrations of the experimental diets were close to calculated Ca concentrations (Table 5.1). Analysed values were used in the calculations of apparent ileal digestibility and retention of Ca.

All birds in the experiment remained healthy and there was no mortality. As could be expected, the body weight of birds and feed intake increased with age (Table 5.2). The average body weight at days 1, 8, 15, 22, 29, 36 and 42 were 39, 168, 502, 1025, 1844, 2719 and 3705 g/bird, respectively. The average feed intake during 1-7, 8-14, 15-21, 22-28, 29-35 and 36-42 days were 56, 167, 378, 559, 791 and 974 g/bird, respectively.

Table 5.3 summarises the effect of age on the ileal digestibility and retention of Ca for limestone in broiler chickens. Apparent ileal Ca digestibility coefficients declined linearly ($P < 0.001$) with advancing age, from 0.51 at day 7 to 0.27 at day 42 (Figure 5.1a). Calcium retention quadratically ($P < 0.01$) declined with age, from 0.56 at day 7 to 0.30 at day 28 and then plateaued (Figure 5.1b).

Table 5.2. Body weight and feed intake in broiler chickens as influenced by age[1]

Age (days)	Initial body weight (g/bird)	Final body weight (g/bird)	Feed intake (g/bird)
0-7	39	56	56
8-14	168	198	167
15-21	502	559	378
22-28	1025	1049	559
29-35	1844	1861	791
36-42	2719	2720	974
SEM[2]	13.8	108.6	19.2
Probabilities, $P \leq$			
Linear	0.001	0.001	0.001
Quadratic	0.001	0.001	0.017

[1]Each value represents the mean of six replicates (twelve, ten and eight birds per replicate for ages at 0-7, 8-14, 15-21, respectively, and six birds per replicate for ages at 22-28, 29-35 and 36-42, respectively).
[2]Pooled standard error of mean.

Table 5.3. Apparent ileal digestibility and retention of calcium (Ca) in limestone for broiler chickens as influenced by age[1]

Age (days)	Apparent ileal Ca digestibility	Ca retention
7	0.51	0.56
14	0.53	0.47
21	0.36	0.45
28	0.34	0.30
35	0.41	0.33
42	0.27	0.31
SEM[2]	0.028	0.017
Probabilities, $P \leq$		
Linear	0.001	0.001
Quadratic	0.529	0.004

[1]Each value represents the mean of six replicates (twelve, ten and eight birds per replicate for ages at 7, 14 and 21, respectively and six birds per replicate for ages at 28, 35 and 42, respectively).
[2]Pooled standard error of mean.

The effects of broiler age on the pH of gizzard digesta and, relative weights of gizzard and gizzard digesta are summarised in Table 5.4. Age quadratically ($P < 0.01$) affected the gizzard pH where the pH declined from day 7 to 21 and then increased to day 42 (Figure 5.1c).

Relative weights of gizzard and gizzard digesta were reduced with advancing age, but the decline was greater between days 7 and 21 resulting in a quadratic effect ($P < 0.001$).

Table 5.4. Gizzard digesta pH and relative weights of gizzard and gizzard digesta in broiler chickens as influenced by age[1]

Age (days)	Gizzard pH	Gizzard weight[2]	Digesta weight[2]
7	2.39	3.85	2.09
14	2.26	2.22	1.48
21	2.00	1.63	0.88
28	2.47	1.30	0.73
35	2.53	0.95	0.53
42	2.73	0.79	0.36
SEM[3]	0.095	0.033	0.029
Probabilities, $P \leq$			
Linear	0.001	0.001	0.001
Quadratic	0.002	0.001	0.001

[1]Each value represents the mean of six replicates (twelve, ten and eight birds per replicate for ages at 7, 14 and 21, respectively, and six birds per replicate for ages at 28, 35 and 42, respectively).
[2]Calculated as a % of body weight.
[3]Pooled standard error of mean.

Table 5.5 summarises the digestible and retainable Ca intakes in different age groups. Digestible and retainable Ca intakes were linearly increased ($P < 0.001$) with the advancing age of broilers.

Table 5.5. Intake (g/bird) of digestible and retainable calcium (Ca) in broiler chickens as influenced by age[1]

Age (days)	Digestible Ca intake	Retainable Ca intake
1-7	0.28	0.31
8-14	0.87	0.77
15-21	1.34	1.68
22-28	1.87	1.68
29-35	3.18	2.58
36-42	2.61	2.97
SEM[2]	0.208	0.101
Probabilities, $P \leq$		
Linear	0.001	0.001
Quadratic	0.210	0.673

[1]Each value represents the mean of six replicates (twelve, ten and eight birds per replicate for ages at 0-7, 8-14 and 15-21, respectively and six birds per replicate for ages at 22-28, 29-35 and 36-42, respectively).
[2]Pooled standard error of mean.

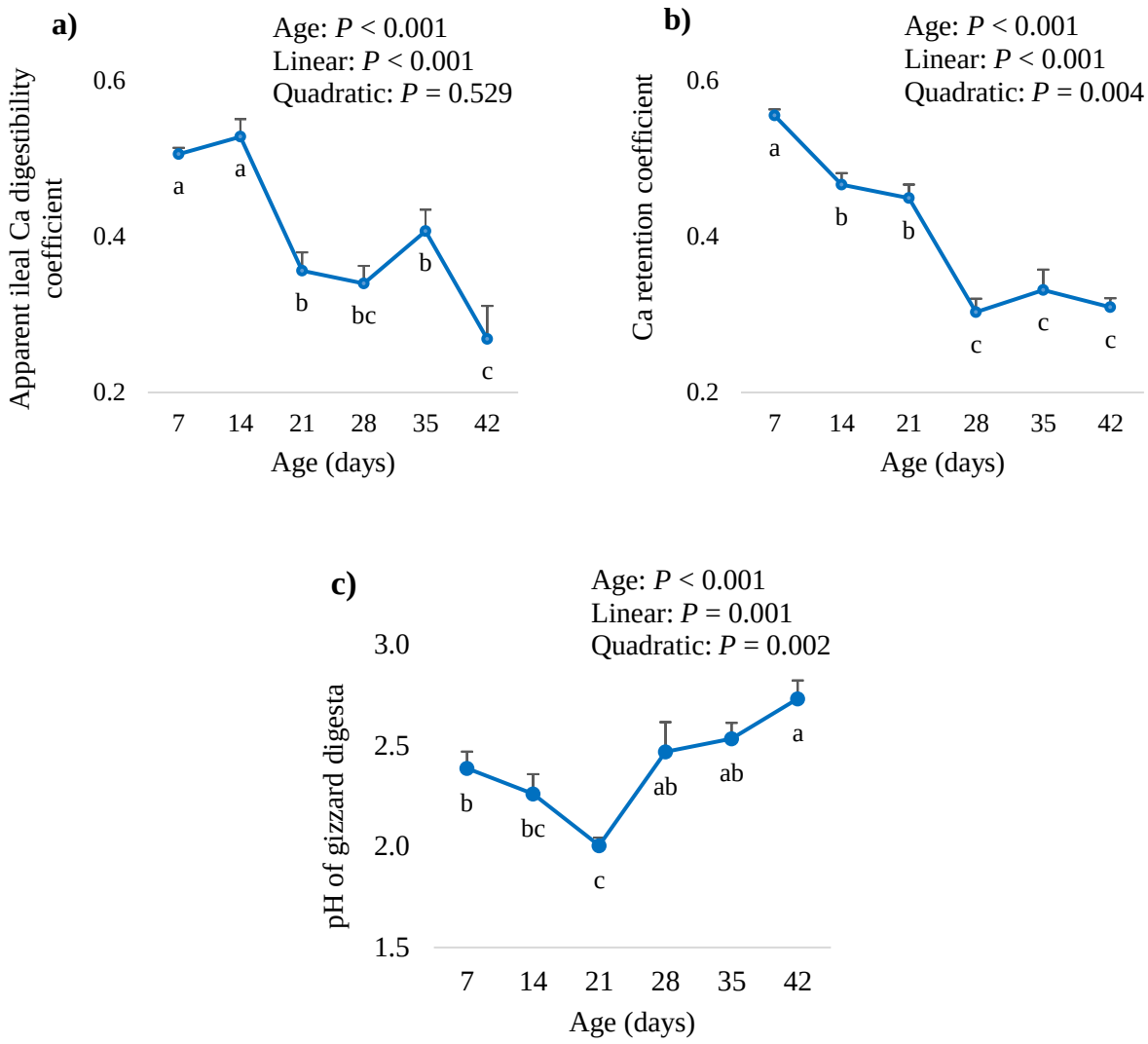

Figure 5.1. Effect of broiler age on apparent ileal Ca digestibility coefficient (a), total tract Ca retention coefficient (b) and gizzard pH, mean ± standard deviation.

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

Table 5.6 summarises the correlation coefficients between Ca digestibility and other measured parameters. Apparent ileal Ca digestibility and Ca retention measurements were positively correlated (r=0.58; $P < 0.001$) in the current study. Gizzard pH was positively correlated with digestible Ca (r=0.42; $P < 0.01$) and retainable Ca intakes (r=0.34; $P < 0.05$). There was a strong positive correlation (r=0.84; $P < 0.001$) between digestible Ca and retainable Ca intakes. However, apparent ileal Ca digestibility was negatively correlated with

retainable Ca intake (r=-0.66; $P < 0.001$). Calcium retention was negatively correlated with gizzard pH (r=-0.49; $P < 0.01$), digestible Ca intake (r=-0.76; $P < 0.001$) and retainable Ca intake (r=-0.71; $P < 0.001$).

Table 5.6. Pearson correlations (probability values in parentheses) between ileal calcium (Ca) digestibility and measured parameters

	AIDC	Ca retention	Gizzard pH	DCI	RCI
AIDC	1.000	0.580 (0.0002)	-0.215 (0.2079)	-0.294 (0.0815)	-0.659 (< 0.0001)
Ca retention	0.580 (0.0002)	1.000	-0.494 (0.0022)	-0.758 (< 0.0001)	-0.708 (< 0.0001)
Gizzard pH	-0.215 (0.2079)	-0.494 (0.0022)	1.000	0.424 (0.0099)	0.344 (0.0400)
DCI	-0.294 (0.0815)	-0.758 (< 0.0001)	0.424 (0.0099)	1.000	0.844 (< 0.0001)
RCI	-0.659 (< 0.0001)	-0.708 (< 0.0001)	0.344 (0.0400)	0.844 (< 0.0001)	1.000

AIDC: apparent ileal Ca digestibility coefficient, DCI: digestible Ca intake, RCI: retainable Ca intake

The effect of dietary protein content on the growth performance, gizzard parameters and Ca digestibility (apparent ileal Ca digestibility and Ca retention) of broilers is summarised in Table 5.7. Weight gain and feed intake were higher ($P < 0.001$) in broilers fed the high protein diet. High protein diet increased ($P < 0.05$) the gizzard pH. The relative weights of gizzard and gizzard digesta were not affected ($P > 0.05$) by the dietary protein content. Apparent ileal digestibility and retention of Ca were not influenced ($P > 0.05$) by the dietary protein content.

5.5.Discussion

All birds in the experiment remained healthy. As expected, the body weight and feed intake were increased with advancing age of broilers.

Table 5.7. Effect of dietary crude protein content on growth performance, gizzard pH, relative weights of gizzard and gizzard digesta, apparent ileal calcium (Ca) digestibility and total tract Ca retention in broiler chickens from day 15 to 21[1]

Parameters	Dietary crude protein (g/kg)		SEM[2]	Probabilities, $P \leq$
	79	153		
Body weight gain (g/bird/day)	8.08[b]	36.89[a]	1.079	0.001
Feed intake (g/bird/day)	54.00[b]	72.19[a]	1.645	0.001
Gizzard pH	2.00[b]	2.46[a]	0.125	0.004
Gizzard weight[3]	1.63	1.59	0.053	0.429
Digesta weight[3]	0.88	0.82	0.043	0.157
Apparent ileal Ca digestibility	0.36	0.32	0.029	0.214
Ca retention	0.45	0.49	0.031	0.177

[a,b] Means having different superscripts within the row are significantly different ($P < 0.05$).

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

[2]Pooled standard error of mean.

[3]Calculated as % of body weight.

In the current study, apparent ileal Ca digestibility linearly decreased with advancing broiler age and therefore the null hypothesis was rejected. The Ca digestibility was higher at days 7 and 14 compared to later ages. These results are contrary to the trends observed in the digestibility of major nutrients (starch, lipids and protein). It is well accepted that the digestibility of major nutrients is low in the newly hatched chick, but increases with age. Total tract digestibility of starch of maize-soybean meal diets in broilers have been shown to increase from hatch to 14 days of age (Uni *et al*., 1995; Batal and Parsons, 2002; Thomas *et al*., 2008). The total tract digestibility of soybean oil and tallow has been observed to increase from 0.59 and 0.37 on day 7 to 0.90 and 0.65, respectively on day 14 (Tancharoenrat *et al*., 2013). A significant age effect on the apparent amino acid digestibility has also been reported (Noy and Sklan, 1995; Uni *et al*., 1995; Batal and Parsons, 2002; Huang *et al*., 2005). According to Batal and Parsons (2002), total tract amino acid digestibility of a maize-soybean meal diet increased from 1 to 10 days of age. Huang *et al*. (2005) studied the digestibility of eight feed ingredients (canola meal, cotton seed meal, soybean meal, wheat, sorghum, maize, mill run and, meat and bone meal) at 14, 28 and 42 days of age in broilers and, based on the analysis of combined

results, reported an increased amino acid digestibility with increasing age. Noy and Sklan (1995) and Uni *et al*. (1995) found that the ileal protein digestibility of a maize-soybean diet in broilers was low at day 4 post-hatch and increased at days 14 or 21 post-hatch. Similarly, apparent metabolisable energy has been shown to be lower during 3-9 days post-hatch and then increase (Zelenka, 1968; Thomas *et al*., 2008). Batal and Parsons (2002) found a progressive increase in the apparent metabolisable energy of maize-soybean meal and maize-canola meal diets from 1 to 14 days of age. In general, available data demonstrate improvements in nutrient utilisation with age of broilers. In the current work, however, an exactly opposite trend occurred with regard to Ca digestibility. This unexpected finding may be explained partly by differences in their mode of digestion, with those of starch, fat and protein depending on enzymic action and, that of minerals largely on their solubility. The secretion and activity of digestive enzymes are lower at hatch and increases with age (Nitsan *et al*., 1991; Noy and Sklan, 1995). Nitsan *et al*. (1991) reported that the activities of enzymes measured in the pancreas and intestinal contents were increased with advancing age of broilers, with maximal values attained on days 4, 8, 11, 17 for intestinal lipase, pancreatic amylase and lipase, trypsin and intestinal amylase, respectively. Similarly, Noy and Sklan (1995) found that the duodenal secretion of amylase, trypsin, and lipase was low at 4 days and increased 100-, 50-, and 20-fold, respectively, by 21 days of age. On the other hand, Ca release and absorption depend largely on the solubilisation of Ca in the digestive tract (Rao and Roland, 1989; Zhang and Coon, 1997a).

Studies on the age effects on Ca digestibility in broilers are limited and none have examined over the entire growth phase. Angel *et al*. (2013) reported a higher ileal Ca digestibility values for ingredients (soybean meal, limestone and monocalcium phosphate) and diets (maize-starch-soybean meal-based) at day 11 than at day 25. Similarly, Fonolla *et al*. (1981) found that Ca (0.55-0.58 vs 0.46-0.47) retention at day 21 was higher than those at day 52. Shastak *et al*. (2012) observed that the ileal digestibility of Ca in a maize-soy diet at 21

days of age was higher than that at 35 days. Li *et al*. (2018) reported the apparent ileal Ca digestibility coefficients of a maize-soybean diet with Ca concentration of 6.5 g/kg in young (7-9 days of age) and older birds (19-21 days of age) to be 0.59 and 0.50, respectively. The corresponding values at a dietary Ca concentration of 8 g/kg were 0.53 and 0.48, respectively. However, the digestibility coefficients observed at 9.5 g/kg of dietary Ca concentration were similar between the two age groups (0.58 and 0.59, respectively). In contrast, Morgan *et al*. (2015) reported that the apparent ileal Ca digestibility of a maize-soybean diet (10 g/kg dietary Ca) was higher at day 14 (0.72) compared to those at days 4, 6, 8, 10 and 12 (range, 0.56-0.67). There were no differences in Ca digestibility among days 6, 8, 10 and 12, but the digestibility was lowest (0.56) at day 4. Overall, the available data are in general agreement with the decreasing Ca digestibility trend, similar to that observed in the current study.

Several reasons could be provided for the higher Ca digestibility in the newly hatched broiler chick. First, it would appear that Ca absorption is more efficient during the first two weeks due the greater demand on the intestines to absorb more Ca to meet the needs for rapid bone formation. According to Skinner and Waldroup (1995), the percentage increase in tibia Ca concentration in broilers was greater during the first week compared to those at other ages (up to eight weeks), highlighting a very high demand for Ca during the first week post-hatch. Second, the residual yolk sac is not only a rich source of Ca (Komazaki *et al*., 1993; Moran, Jr., 2007; Tong *et al*., 2008), but its presence may also exert a positive influence on Ca absorption. It has been speculated that the yolk sac has a beneficial effect on the utilisation of energy during the first few days after hatch (Zelenka, 1968; Thomas *et al*., 2008) and a similar positive effect may exist for Ca utilisation. The third possibility is endogenous Ca losses may have increased with age and may have partly contributed to the lower apparent Ca digestibility values in later ages. Even though endogenous Ca losses have been measured in some studies (Anwar *et al*., 2016a, b; 2017; 2018), there are no published studies examining the losses at

different ages of broilers. Endogenous Ca originates from bile, digestive secretions, mucins and desquamated cells from intestinal lining; of these, bile is the major source of endogenous Ca (Gleeson *et al*., 1990). It is known the secretions of bile and digestive enzymes increase with age (Nitsan *et al*., 1991).

It is worth highlighting two limitations in the experimental design employed. One was that, to enable valid comparisons, the same diet was offered in the current work to broilers of all ages. The consequence of this approach was that the dietary Ca concentration (9 g/kg) was adequate for early growth phases, but slightly in excess for later phases. However, the recommended dietary Ca concentration for broiler finishers is 7.9 g/kg (Ross *et al*., 2019), which is only 1.1 g/kg lower than the concentration used in our assay diet. Published data on the effects of high Ca concentrations on Ca digestibility are contradictory, with some studies reporting a reduction in Ca digestibility with increasing dietary Ca concentration (Swaminathan *et al*., 1978; Zhang and Coon, 1997a; Sebastian *et al*., 1996; Rao *et al*., 2003), some reporting increased digestibility (Tamim and Angel, 2003; Tamim *et al*., 2004), while others observed no effect (Paiva *et al*., 2013; Mutucumarana *et al*., 2014a). Such inconsistency in published reports is to be expected, since a multitude of factors including differences in dietary concentrations of Ca and P, Ca:P ratio and dietary phytate concentration could influence Ca absorption. Excess Ca has negative consequences on Ca utilisation only when P is deficient, which is not the case in the assay diet wherein adequate P was maintained.

Another limitation in the current design relates to the time given to adapt to the assay. An adaptation length of seven days was employed in our study. In one of our previous studies (Chapter 4), Ca digestibility was determined to be lower following a 7-day adaptation length compared to 3- and 5- days. On reflection, therefore, Ca digestibility should have been measured after three days of feeding assay diets instead of seven days, which would have enabled better comparisons with published data. In previous studies, which used three days of

adaptation, estimates ranging between 0.43 and 0.71 were reported for Ca digestibility in limestone at 21 days of age (Anwar *et al*., 2016a, 2017), as against the value of 0.36 measured in the current work. Interestingly, the apparent ileal Ca digestibility coefficient of limestone determined at day 21 (0.36) in the current study was similar to the results of the previous study following a 7-day dietary adaptation length. In the current study, the apparent ileal Ca digestibility coefficient of limestone at day 42 post-hatch was determined to be 0.27, which was half of the estimate at seven days.

Calcium retention followed somewhat a similar pattern as ileal Ca digestibility where the retention declined with age, from 0.56 at day 7 to 0.30 at day 28 and then plateaued. The current finding is in general agreement with those of Fonolla *et al*. (1981) who reported lower Ca retention coefficients in broilers at 52 days (0.46-0.47) than those at 21 days (0.55-0.58).

Gizzard digesta pH declined from day 7 to 21 and then increased beyond day 21. This decline in gizzard pH until day 21 may be explained by the changes in the gastric acid secretion with the advancing age of broilers. The digestive secretions have been known to increase during the first 14 days of age in broilers (Nitsan *et al*., 1991). Concurrent increase in hydrochloric acid secretions is to be expected and this would have increased the acidity of digesta during this period. A similar reduction in gizzard pH at the age of day 14 has been reported by Angel *et al*. (2010), based on the analysis of 15 published studies. Younger birds, due to the immaturity of the proventriculus, are more vulnerable to alterations in the gastrointestinal environment and unable to react to the greater Ca carbonate load by increasing proventricular hydrochloric acid secretion. Rynsburger *et al*. (2009) reported that the production and secretion of gastric acid in the proventriculus is limited at a very young age and increased from 2 to 15 days of age, resulting in decreased pH of proventriculus and gizzard. Gizzard pH values obtained in the current study at day 7 (2.39) was comparable with those of 2.37 and 2.42 reported by Angel *et al*. (2010) and Morgan *et al*. (2014), respectively, for broilers of similar

age. Gizzard pH values obtained in the current study ranged between 2.00 (day 21) and 2.73 (day 42) which lie within the range reported by Morgan *et al*. (2014) in 7-42 day-old broilers.

A possible explanation for the increased gizzard pH after day 21 could be higher feed intake, which may have outstripped acid secretion capacity thereby diluting the concentration of hydrochloric acid. The pH of practical poultry diets is around 6.0 (Ao *et al*., 2008) and an increase in gizzard pH is to be expected unless gastric juice secretion is able to increase in accordance with intake (Ravindran, 2013a; Svihus, 2014). The gastric juice secreted from the proventriculus of poultry has been reported to have a pH of around 2 (Duke, 1986) and the pH of gizzard contents in broilers range from 2.5 to 3.5 (Ravindran, 2013a). According to Svihus (2011), the pH of gizzard contents in broilers varies between 1.9 and 4.5, depending on the dietary Ca carbonate, retention time and feed form. In the present study, increased Ca carbonate intake, due to increased feed intake during this period, could also have resulted in an elevated pH as a result of an increase in the ratio of limestone (g): acid produced (g). Morgan *et al*. (2014) reported a higher gizzard pH in broilers fed the diet with high inclusion of limestone than that of a low-limestone diet. Calcium digestibility in poultry depends on the solubilisation of Ca source in the gizzard and continued solubility into the small intestine where it is absorbed (Mutucumarana *et al*., 2014a). Solubility of any Ca source is increased when the gizzard pH is lower. In the current study, the Ca retention was negatively correlated with gizzard pH. It may therefore be speculated that the lower Ca retention values obtained in broilers at later growth stages (from day 28 to 42) may be related with increased gizzard pH after day 21. Surprisingly, however, there was no correlation between ileal Ca digestibility and gizzard pH, even though the digestibility and retention were positively correlated. The lack of correlation between Ca digestibility and gizzard pH is a key finding and may be suggestive that widely used *in vitro* solubility measurement is not an ideal test for the prediction of Ca availability.

The effect of dietary protein concentration on Ca digestibility in poultry has not been previously investigated. However, evidence from human studies indicates that protein influences Ca digestion. According to Kerstetter *et al*. (2003), consumption of low protein diets reduced Ca absorption whereas high protein diets increased both Ca absorption and urinary Ca excretion in humans. In contrast, dietary protein intake had no influence on Ca retention in other human studies (Walker and Linkswiler, 1972; Kerstetter *et al*., 2005). In the present study, the Ca digestibility of limestone was unaffected by dietary protein content and therefore the null hypothesis is accepted. As expected, high dietary protein increased the weight gain and feed intake in broilers. The dietary protein content influenced the gizzard digesta pH with values of 2.00 and 2.46 for low and high protein diets, respectively. Despite this large difference in gizzard pH, Ca digestibility was similar between the low and high protein diets, further highlighting the limitation of *in vitro* solubility tests. The pH difference between the gizzard contents of birds fed high and low protein diets cannot be explained based on the relative gizzard size or the amount of gizzard digesta. However, as discussed earlier, it is possible that the higher feed intake of birds fed the high protein diet may have diluted the hydrochloric acid to maintain the acidity of digesta in the gizzard. Another possibility may be the buffering action of protein leading to the increased pH (Menna-Govela *et al*., 2019).

5.6. Conclusions

The current data demonstrate that the ileal digestibility of Ca in limestone is influenced by broiler age, with higher digestibility in the newly hatched chick compared to older birds. These findings suggest that the digestibility estimated with older birds may not be applicable to younger birds. Calcium digestibility was unaffected by dietary protein concentrations. It is noteworthy that Ca digestibility appears to be unrelated to gizzard pH, challenging the use of *in vitro* acid solubility assays to predict *in vivo* digestibility. Another key observation is the negative correlation between Ca digestibility, and digestible and retainable Ca intakes, which

points to the problems in using ileal digestibility of Ca alone as an indicator how well the diet will supply the needs of the bird for growth and bone development. This is because intake as opposed to digestibility per se is by far the greater determinant of digestible nutrient intake, which is particularly pertinent with Ca concentration since it influences intake as much if not more than the digestibility.

CHAPTER 6

True ileal calcium digestibility in soybean meal and canola meal, and true ileal phosphorous digestibility in maize-soybean meal and maize-canola meal diets, without and with microbial phytase, for broiler growers and finishers

6.1. Abstract

Published data on the ileal calcium (Ca) digestibility in soybean meal (SBM) and canola meal (CM), and the effect of microbial phytase on the Ca digestibility of these ingredients are limited. Therefore, two experiments were conducted, with the primary objective of determining the true ileal digestibility of Ca in SBM and CM, without and with microbial phytase, during broiler grower (Experiment 1) and finisher (Experiment 2) periods. A secondary objective was to investigate the influence of microbial phytase on the true ileal digestibility of phosphorus (P), apparent digestibility of nitrogen (N) and minerals, and phytate disappearance in maize-SBM and maize-CM diets. Six experimental diets based on SBM and CM, with three phytase doses (0, 500 and 2000 FTU/kg), were fed to broilers from day 18 to 21 (Experiment 1) or 39 to 42 (Experiment 2) post-hatch. A Ca- and P- free diet, with no added phytase, was also developed to determine the endogenous Ca and P losses. Titanium dioxide was incorporated in all diets as an indigestible indicator. Each experimental diet was randomly allocated to six replicate cages (eight birds per cage). Apparent ileal digestibility was calculated using the indicator method and the true ileal digestibility was calculated by correcting for endogenous losses. Apparent total tract retention coefficient (ATTRC) of Ca and P was also measured. Ileal endogenous losses of Ca and P were determined to be 236 and 310 mg/kg of dry matter intake (DMI), respectively, in broiler growers and 29 and 130 mg/kg of DMI, respectively, in broiler finishers. True ileal Ca digestibility coefficients of SBM and CM, without added phytase, were determined to be 0.51 and 0.53, respectively, in growers and 0.33 and 0.22, respectively, in finishers. Increasing phytase doses increased ($P < 0.05$) the true ileal Ca digestibility of CM in

both broiler growers and finishers, but Ca digestibility of SBM increased ($P < 0.05$) only at the superdose (2000 FTU/kg) in broiler finishers. The ATTRC of Ca ($P < 0.001$) in growers was higher in CM than in SBM and was increased in both ingredients by increasing phytase doses. In finishers, the ATTRC of Ca was increased ($P < 0.001$) by both phytase doses in CM, but only by the superdose in SBM, resulting in an ingredient × phytase interaction ($P < 0.001$). True ileal P digestibility coefficients of maize-SBM and maize-CM diets, without added phytase, were determined to be 0.89 and 0.66, respectively, in growers and 0.82 and 0.57, respectively, in finishers. Supplemental phytase increased ($P < 0.05$) the true ileal P digestibility of the maize-CM diet in both broiler growers and finishers. However, the P digestibility of the maize-SBM diet was increased ($P < 0.05$) in broiler finishers only at the superdose (2000 FTU/kg). The ATTRC of P was higher ($P < 0.001$) in the maize-SBM diet during both periods. The apparent ileal digestibility of N, Mg, K and Mn was higher ($P < 0.001$) in the maize-SBM diet for growers and finishers. Phytase addition had no effect ($P > 0.05$) on the apparent digestibility of N and minerals in growers and finishers. Increasing phytase doses increased IP6 disappearance in the maize-CM diet, but not in the maize-SBM diet, resulting in an ingredient × phytase interaction ($P < 0.05$) for growers and finishers. In conclusion, true ileal Ca digestibility coefficients of SBM and CM for broilers were determined in this study. The findings confirmed the influence of broiler age on Ca digestibility. Superdosing of phytase increased the digestibility and ATTRC of Ca in CM and SBM by two-fold compared to the normal phytase dose.

6.2. Introduction

Data on ileal digestibility of calcium (Ca) of major inorganic and animal-based Ca sources for broilers are now becoming available (Anwar *et al*., 2015; 2016a,b,c; 2017; 2018; Chapter 3). Soybean meal (SBM) and canola meal (CM) are two plant-based protein sources which contain reasonable amounts of Ca (2.9 and 6.8 g/kg, respectively; NRC, 1994). In a broiler diet with

350 g/kg SBM or 300 g/kg CM, SBM can contribute up to 15% of the dietary Ca and CM can contribute up to 25% of the dietary Ca. Currently no published values are available for the Ca digestibility in SBM and limited studies (Anwar *et al*., 2018; Moss *et al*., 2018) are available for the Ca digestibility in CM.

Microbial phytases are now routinely added in poultry diets to improve the bioavailability of phosphorous (P) bound to phytate (*myo*-inositol hexaphosphate; IP_6) and P digestibility. The effect of phytase addition on Ca digestibility, however, is contradictory (Tamim *et al*., 2004; Ravindran *et al*., 2008; Walk *et al*., 2012a). The dosage of phytase generally recommended for use in broiler diets is 500 FTU/kg. However, higher doses, typically over four times the recommended dose and referred to as superdoses, are currently being used by the industry and reported to improve the growth performance and nutrient digestibility (Cowieson *et al*., 2011). Furthermore, administration of phytase superdose has been found to prevent the build-up of lower phytate esters (IP5 to IP1) in the digestive tract and thereby reducing the anti-nutritive effects of IP6 as well as lower phytate esters in poultry (Cowieson *et al*., 2011; Beeson *et al*., 2017; Bedford and Rousseau, 2017).

The primary aim of the present study was to measure the true ileal digestibility of Ca in SBM and CM, without and with microbial phytase (0, 500 and 2000 FTU/kg), for broiler growers and finishers. The influence of microbial phytase on the true ileal digestibility of P, apparent digestibility of nitrogen (N) and minerals, and phytate disappearance in maize-SBM and maize-CM diets during grower and finisher periods was also investigated.

6.3. Materials and Methods

The experiments 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.

Two experiments were conducted to measure the digestibility of Ca in SBM and CM for broiler growers (Experiment 1) and broiler finishers (Experiment 2). The ingredients were purchased from a local commercial source. Six experimental diets were formulated using a 2 × 3 factorial arrangement of treatments with two ingredients (SBM and CM) and three doses of microbial phytase (0, 500 and 2000 FTU/kg; Quantum Blue, AB Vista, Marlborough, U.K.) as indicated in Table 6.1. The same batch of ingredients was used in diet formulations of both experiments. The experimental diets were maize-based (Chapter 3) and the contribution of Ca from maize (0.2 g/kg; NRC, 1994) was assumed to be negligible. Soybean meal and CM were included at 500 and 300 g/kg, respectively, in respective diets and served as the sole source of Ca. A Ca- and P-free diet was also generated, without phytase, to measure the endogenous Ca and P losses. Titanium dioxide (5 g/kg) was added to all diets as an indigestible indicator. Table 6.2 summarises the analysed nutrient composition of diets.

In both experiments, day-old male broilers (Ross 308) were obtained from a commercial hatchery, raised in floor pens and fed a commercial broiler starter diet (230 g/kg CP, 10 g/kg Ca, and 5.2 g/kg non-phytate P). Since birds used in Experiment 1 were euthanised for ileal digesta collection, a different set of birds have to be used for Experiment 2. Fresh wood shavings were used as the bedding material. Temperature and lighting schedule were maintained as described in Chapter 3, section 3.3.2.

6.3.1. Experiment 1

On day 14, the birds were moved to grower cages. Between days 14 and 18, the crumbles were gradually changed to mash as the experimental diets were in mash form. On day 18, the birds were individually weighed and allocated to 42 cages (eight birds per cage) so that the average bird weight per cage (mean ± SD, 0.72 ± 0.02 kg) was similar. Each diet was randomly assigned to six replicate cages. The birds were fasted overnight prior to the introduction of experimental

diets on day 18. The experimental diets were offered *ad libitum* for three days from day 18 to 21 post-hatch and the birds had free access to water.

Table 6.1. Ingredient composition of diets (g/kg as fed basis), Experiments 1 and 2[1]

Ingredient	Soybean meal			Canola meal			Ca and P free diet
	0[1]	500[1]	2000[1]	0[1]	500[1]	2000[1]	
Maize	472	472	472	672	672	672	-
Soybean meal	500	500	500	-	-	-	-
Canola meal	-	-	-	300	300	300	-
Soybean oil	20	20	20	20	20	20	20
Sodium chloride	2	2	2	2	2	2	2
Titanium dioxide	5	5	5	5	5	5	5
Trace mineral-vitamin premix[2,3]	1	1	1	1	1	1	1
Maize starch	-	-	-	-	-	-	411
Dextrose	-	-	-	-	-	-	411
Dried egg albumen	-	-	-	-	-	-	100
Cellulose	-	-	-	-	-	-	50
Phytase	-	0.1	0.4	-	0.1	0.4	-
Calculated analysis[4]							
Metabolisable energy (MJ/kg)	11.98	11.98	11.98	12.63	12.63	12.63	13.54
Crude protein	283	283	283	171	171	171	82.4
Calcium	1.44	1.44	1.44	2.17	2.17	2.17	-
Total phosphorous	4.42	4.42	4.42	5.39	5.39	5.39	-
Non-phytate phosphorous	1.48	1.48	1.48	1.44	1.44	1.44	-
Phytate phosphorous	2.94	2.94	2.94	3.95	3.95	3.95	-
Ca: Non-phytate phosphorous	0.98	0.98	0.98	1.51	1.51	1.51	-

[1]Phytase recovery percent of grower diets were 116, 108, 109 and 81%, respectively, for SBM with 500 and 2000 FTU/kg and CM with 500 and 2000 FTU/kg and of finisher diets were 87, 83, 133 and 89%, respectively.
[2]Supplied per kilogram of diet: vitamin A (trans-retinyl acetate), 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; 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]The trace mineral-vitamin premix was calcium-free.
[4]Calculated based on NRC (1994) values.

6.3.2 Experiment 2

On day 35, the birds were moved to grower cages. The birds were fed commercial broiler grower crumbles until day 21 and commercial broiler finisher pellets from day 22 to 34. Between days 35 and 39, the crumbles were gradually changed to mash as the experimental

diets would be in mash form. On day 39, the birds were individually weighed and allocated to 42 cages (8 birds per cage) so that the average bird weight per cage (mean ± SD, 2.66 ±0.09 kg) was similar. Each diet was randomly assigned to six replicate cages. The birds were fasted overnight prior to the introduction of experimental diets on day 39. The experimental diets, in mash form, were offered *ad libitum* for three days from day 39 to 42 and the birds had free access to water.

Table 6.2. Analysed composition of diets (g/kg, as fed basis), Experiments 1 and 2[1]

Ingredient	Soybean meal			Canola meal			Ca and P free diet
	0	500	2000	0	500	2000	
			Experiment 1				
Dry matter (DM)	927	916	924	919	918	918	923
Calcium (Ca)	1.71	1.77	1.74	1.98	2.00	1.69	0.26
Phosphorous (P)	5.00	5.00	5.00	5.40	5.60	5.50	0.30
IP5(µmol/g DM)	2.47	-	-	1.98	-	-	-
IP6 (µmol/g DM)	26.49	-	-	21.15	-	-	-
Nitrogen	44	-	-	27	-	-	-
Magnesium	2.1	-	-	2.2	-	-	-
Potassium	14.7	-	-	6.1	-	-	-
Sodium (mg/kg)	830	-	-	1070	-	-	-
Iron (mg/kg)	183	-	-	179	-	-	-
Copper (mg/kg)	38	-	-	30	-	-	-
Manganese (mg/kg)	180	-	-	161	-	-	-
Zinc (mg/kg)	125	-	-	132	-	-	-
			Experiment 2				
DM	920	920	923	917	921	926	923
Ca	1.74	1.78	1.74	1.91	1.96	1.92	0.18
P	4.6	4.2	4.5	5.1	4.9	5.1	0.1
Phytate-P (g/kg)	2.82	2.80	2.83	3.27	3.30	3.19	-
IP5(µmol/g DM)	3.05	-	-	3.46	-	-	-
IP6 (µmol/g DM)	22.14	-	-	24.99	-	-	-
Nitrogen	48	-	-	29	-	-	-
Magnesium	2.3	-	-	2.5	-	-	-
Potassium	16.1	-	-	6.8	-	-	-
Sodium (mg/kg)	804	-	-	1243	-	-	-
Iron (mg/kg)	129	-	-	123	-	-	-
Copper (mg/kg)	24	-	-	18	-	-	-
Manganese (mg/kg)	78	-	-	69	-	-	-
Zinc (mg/kg)	75	-	-	84	-	-	-

[1]Samples were analysed in triplicate

6.3.3. Collection and processing of digesta and excreta

On day 21 (Experiment 1) or 42 (Experiment 2), all birds were euthanised by an intravenous injection (0.5 mL per 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 3.3.3. Grab excreta samples were also collected during the last two days of the experimental period and processed as described in Chapter 4, section 4.3.3.

6.3.4. Measurement of gizzard parameters

Four birds in each replicate cages were used for gizzard pH measurement. The pH of the gizzard content was measured as described in Chapter 5, section 5.3.3. Full and empty gizzard weights were recorded and, the weight of gizzard digesta was calculated as the difference between the full and empty gizzard weights. The relative weights of gizzard and gizzard digesta were calculated as percentage of body weight.

6.3.5. Chemical analysis

Ingredient samples (maize, SBM and CM) were analysed for proximate composition (methods 930.15, 942.05, 968.06; AOAC, 2005), Ca (method 968.08D; AOAC 2005), P (method 968.08D; AOAC, 2005) and phytate (Caldwell, 1992). Diets, digesta and excreta were analysed for dry matter (DM), Ca, P and titanium as described in Chapter 3, section 3.3.4. Diets and digesta samples were also analysed for N (Method 968.06; AOAC, 2005) and minerals (Na, K, Mg, Fe, Cu, Mn and Zn). For the analysis of minerals, the samples were wet acid digested with nitric and perchloric acid mixture, and concentrations of K, Mg, Na and Fe were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) using a Thermo Jarrell Ash IRIS instrument. The concentrations of Cu, Mn and Zn were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) using a Perkin Elmer Elan 6000 instrument. Inositol phosphate esters (IP6 and IP5) were determined by high-performance ion chromatography-based techniques, as described by Blaabjerg *et al*. (2010).

6.3.6. Calculations

The coefficients of apparent ileal digestibility of minerals were calculated using the titanium ratio in diets and digesta (Ravindran *et al*., 1999) as indicated below:

$$AIDC = 1 - [(Ti_I / Ti_O) \times (M_O / M_I)]$$

where AIDC is the apparent ileal digestibility coefficient, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, M_O is the mineral concentration in the ileal digesta, and M_I is the mineral concentration in the diet. All concentrations were expressed as g/kg DM.

Ileal endogenous losses of Ca and P were determined following the feeding of a Ca- and P-free diet and the values (g/kg DM intake [DMI]) were calculated by the following formula (Anwar *et al*., 2018).

$$IEL = M_O \times (Ti_I/Ti_O)$$

where IEL is ileal endogenous losses of Ca or P, Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, M_O is the concentration of Ca or P in the ileal digesta.

True ileal digestibility coefficient (TIDC) was then calculated as follows:

$$TIDC = AIDC + (IEL / M_I)$$

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

Apparent total tract retention coefficients (ATTRC) of Ca and P were calculated using the titanium ratio in diets and digesta as indicated below:

$$ATTRC = 1- [(Ti_I / Ti_E) \times (M_E/ M_I)]$$

where Ti_I is the titanium concentration in the diet, Ti_E is the titanium concentration in the excreta, M_E is the concentration of Ca or P in the excreta, and M_I is the concentration of Ca or P in the diet.

The coefficients of ileal IP6 disappearance were calculated using the titanium ratio in diets and digesta (Zeller *et al*., 2015) as indicated below:

$$\text{Coefficient of ileal IP6 disappearance} = 1 - [(Ti_I / Ti_O) \times (IP_O / IP_I)]$$

where Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, IP_O is the IP6 concentration in the ileal digesta, and IP_I is the IP6 concentration in the diet. All concentrations were expressed as g/kg DM.

6.3.7. Statistical Analysis

In both experiments, the data were analysed as a 2 × 3 factorial arrangement of treatments to examine the main effects of ingredient and phytase dose and their interaction using the General Linear Model procedure of SAS (2019). Cage served as the experimental unit and differences were considered to be significant at $P < 0.05$. Significant differences between means were separated by the Least Significant Difference test.

6.4. Results

Analysed nutrient and mineral compositions of SBM, CM and maize are presented in Table 6.3. Calcium concentrations of SBM, CM and maize were analysed to be 2.9, 5.4 and 0.09 g/kg, respectively, whereas the analysed P concentrations were 6.3, 9.7 and 2.5 g/kg, respectively.

Table 6.4 summarises the phytase recovery in diets used for Experiments 1 (growers) and 2 (finishers). The phytase recovery ranged from 81-116% for grower diets and 87-133% for finisher diets.

Table 6.3. Analysed nutrient and mineral composition of soybean meal, canola meal and maize (g/kg, as received basis)[1]

Nutrient	Soybean meal	Canola meal	Maize
Dry matter	907	913	896
Ash	65	64	12
Protein	435	373	71
Fat	19	39	32
Neutral detergent fibre	79	212	60
Phytate	10	20	-
Phytate phosphorous[2]	2.8	5.6	-
Non-phytate phosphorous[3]	3.5	4.1	-
Macro-minerals (g/kg)			
Calcium	2.9	5.4	0.09
Phosphorous	6.3	9.7	2.5
Magnesium	3.2	4.8	0.87
Potassium	25	12.8	3.4
Sodium	0.11	1.12	< 0.05
Micro-minerals (mg/kg)			
Iron	109	131	33
Copper	15.8	4.8	1.52
Zinc	39	67	18.8
Manganese	49	60	9.1
Chloride	150	550	-
Iodine	0.012	0.028	-
Selenium	0.29	0.39	-

[1]Samples were analysed in duplicate.
[2]Values were calculated based on the assumption that a phytate molecule contains 28% of phytate phosphorous.
[3]Calculated as the difference between total phosphorous and phytate phosphorous.

Analysed Ca concentrations of SBM-based and CM-based grower diets ranged from 1.71-1.77 and 1.69-2.00 g/kg, respectively, and the analysed P concentrations were 5.0 g/kg and 5.4-5.6 g/kg, respectively (Table 6.2).

Analysed Ca concentrations of SBM-based and CM-based finisher diets ranged from 1.74-1.78 and 1.91-1.96 g/kg, respectively (Table 6.2). Analysed P concentrations of SBM-based and CM-based diets were 4.2-4.6 and 4.9-5.1 g/kg, respectively. Analysed Ca and P values were used for digestibility calculations.

Table 6.4. Calculated phytase recovery (%) of diets, Experiments 1 (growers) and 2 (finishers)

Diet	Phytase	Grower diets	Finisher diets
Soybean meal	500	116	87
	2000	108	83
Canola meal	500	109	133
	2000	81	89

6.4.1. Experiment 1: Broiler growers

The growth performance of broiler growers fed SBM and CM diets with different phytase doses, over the 3-day experimental period, are summarised in Table 6.5. The effects of ingredient, phytase dose and their interaction were not significant ($P > 0.05$) for the growth performance.

Table 6.6 summarises the true ileal digestibility and ATTRC of Ca in broiler growers fed SBM and CM with different phytase doses. Calcium digestibility coefficients in SBM and CM, without added phytase, were determined to be 0.51 and 0.53 respectively. Ileal endogenous Ca losses were determined to be 236 mg/kg of DMI. Calcium digestibility was increased by added phytase in CM, but not in SBM, resulting in an ingredient × phytase interaction ($P < 0.05$). The ATTRC of Ca was higher ($P < 0.001$) in CM than SBM. Phytase increased ($P < 0.001$) ATTRC of Ca in both ingredients.

It must be noted that the P digestibility and ATTRC data do not relate to the ingredients, but to maize and ingredient (SBM or CM) mixtures. Maize contained 2.5 g/kg total P and contributed 27 and 37% of the P in SBM and CM diets, respectively; hence the P utilisation data must be interpreted in this context.

Ileal P digestibility coefficients in maize-SBM and maize-CM diets, without added phytase, were determined to be 0.89 and 0.66, respectively (Table 6.7). Ileal endogenous P loss was determined to be 310 mg/kg of DMI. An interaction ($P < 0.001$) was observed between ingredient and phytase for the true ileal P digestibility. Phosphorus digestibility was increased

with increasing doses of phytase in both diets, but the increments were greater in the maize-CM diet. The ATTRC of P was markedly higher ($P < 0.001$) in the maize-SBM diet than the maize-CM diet. Although numerical improvements were observed, the effects of phytase addition on ATTRC of P were not statistically significant ($P = 0.122$).

Table 6.5. Effect of phytase doses on the growth performance of broiler growers fed soybean meal and canola meal diets (Experiment 1), measured from day 18 to 21 post-hatch[1]

Ingredient	Phytase (FTU/kg)	Body weight gain (g/bird/day)	Feed intake (g/bird/day)	Feed conversion ratio
Soybean meal	0	55	101	1.84
	500	58	104	1.78
	2000	62	107	1.73
Canola meal	0	56	105	1.90
	500	55	106	1.94
	2000	55	110	2.04
SEM[2]		2.4	3.2	0.095
Main Effects				
Ingredient				
Soybean meal		58	104	1.78[a]
Canola meal		55	107	1.96[b]
SEM		1.4	1.8	0.055
Phytase				
0		55	103	1.87
500		57	105	1.86
2000		59	109	1.89
SEM		1.7	2.3	0.067
Probability, $P \leq$				
Ingredient		0.129	0.257	0.029
Phytase		0.389	0.181	0.966
Ingredient × phytase		0.300	0.966	0.430

[1]Each value represents the mean of six replicates (eight birds per replicate).
[a,b] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]Pooled standard error of mean.

Table 6.8 summarises the effect of phytase on the apparent ileal digestibility of N and minerals in maize-SBM and maize-CM diets for broiler growers.

Table 6.6. Effect of phytase doses on true ileal digestibility and apparent total tract retention coefficients (ATTRC) of calcium (Ca) in soybean and canola meals in broiler growers (Experiment 1), measured on day 21[1,2]

Ingredient	Phytase (FTU/kg)	True ileal Ca digestibility[3]	ATTRC of Ca[3]
Soybean meal	0	0.51[c]	0.34
	500	0.54[c]	0.49
	2000	0.57[bc]	0.58
Canola meal	0	0.53[c]	0.52
	500	0.64[b]	0.69
	2000	0.75[a]	0.78
SEM[4]		0.027	0.033
Main Effects			
Ingredient			
Soybean meal		0.54	0.47[b]
Canola meal		0.64	0.66[a]
SEM		0.015	0.019
Phytase			
0		0.52	0.43[c]
500		0.59	0.59[b]
2000		0.66	0.68[a]
SEM		0.019	0.024
Probabilities, $P \leq$			
Ingredient		0.001	0.001
Phytase		0.001	0.001
Ingredient × phytase		0.025	0.903

[1]Each value represents the mean of six replicates (eight birds per replicate).
[a-c] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]The ileal endogenous Ca losses were 236 mg/kg.
[3]The Ca digestibility and ATTRC data relate to the ingredient (soybean meal or canola meal) as the Ca content in maize is negligible.
[4]Pooled standard error of mean.

The ileal digestibility of N and minerals, except of Na, Fe and Zn, was higher ($P < 0.01$ to 0.001) in maize-SBM diet than the maize-CM diet. Sodium digestibility was negative in all diets and it was greater in the maize-CM diet. The digestibility of Fe and Zn were unaffected ($P > 0.05$) by the ingredient. Phytase addition had no effect ($P > 0.05$) on the digestibility of N or minerals, except Na. Sodium digestibility tended ($P = 0.06$) to be increased by 2000 FTU/kg

phytase. There was no interaction ($P > 0.05$) between the ingredient and phytase for the digestibility of minerals, except Mn. The apparent ileal digestibility coefficient of Mn in maize-CM diets was affected ($P < 0.05$) by added phytase, but not in maize-SBM diets.

Table 6.7. Effect of phytase doses on true ileal digestibility and apparent total tract retention coefficients (ATTRC) of phosphorous (P) in soybean meal- and canola meal-based diets in broiler growers (Experiment 1), measured on day 21[1,2]

Ingredient	Phytase (FTU/kg)	True ileal P digestibility[3]	ATTRC of P[3]
Soybean meal	0	0.89[b]	0.50
	500	0.92[ab]	0.52
	2000	0.94[a]	0.60
Canola meal	0	0.66[d]	0.27
	500	0.75[c]	0.38
	2000	0.88[b]	0.37
SEM[4]		0.015	0.048
Main Effects			
Ingredient			
Soybean meal		0.92	0.54[a]
Canola meal		0.76	0.34[b]
SEM		0.009	0.028
Phytase			
0		0.77	0.39
500		0.83	0.45
2000		0.91	0.49
SEM		0.010	0.034
Probability, $P \leq$			
Ingredient		0.001	0.001
Phytase		0.001	0.122
Ingredient × phytase		0.001	0.546

[1]Each value represents the mean of six replicates (eight birds per replicate).
[a-d] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]The ileal endogenous P losses were 310 mg/kg.
[3]The P digestibility and ATTRC data relate to combination of maize and soybean meal or canola meal.
[4]Pooled standard error of mean.

Table 6.8. Effect of phytase doses on the apparent ileal digestibility coefficients of minerals in soybean meal and canola meal diets in broiler growers (Experiment 1), measured on day 21[1]

Ingredient	Phytase (FTU/kg)	Apparent ileal digestibility coefficients							
		N	Mg	K	Na	Fe	Cu	Mn	Zn
Soybean meal	0	0.83	0.19	0.90	-1.33	0.15	0.00	-0.06[a]	0.01
	500	0.83	0.17	0.89	-1.44	0.13	-0.03	-0.02[a]	-0.02
	2000	0.84	0.23	0.90	-1.09	0.17	0.07	0.01[a]	0.06
Canola meal	0	0.78	0.03	0.80	-0.87	0.04	-0.09	0.00[a]	-0.03
	500	0.77	0.04	0.80	-0.84	0.13	-0.09	-0.20[b]	-0.08
	2000	0.77	0.08	0.80	-0.59	0.11	-0.06	-0.23[b]	-0.03
SEM[2]		0.010	0.033	0.010	0.130	0.060	0.040	0.046	0.050
Main Effects									
Ingredient									
Soybean meal		0.83[a]	0.19[a]	0.90[a]	-1.28[b]	0.15	0.01[a]	-0.02	0.01
Canola meal		0.77[b]	0.05[b]	0.80[b]	-0.77[a]	0.09	-0.08[b]	-0.15	-0.05
SEM		0.006	0.019	0.006	0.075	0.035	0.023	0.027	0.029
Phytase									
0		0.80	0.11	0.85	-1.10	0.10	-0.04	-0.03	-0.01
500		0.80	0.10	0.85	-1.14	0.13	-0.06	-0.11	-0.05
2000		0.81	0.15	0.85	-0.84	0.14	0.01	-0.11	0.01
SEM		0.007	0.023	0.007	0.092	0.043	0.029	0.033	0.035
Probabilities, $P \leq$									
Ingredient		0.001	0.001	0.001	0.001	0.269	0.008	0.003	0.147
Phytase		0.836	0.233	0.871	0.055	0.790	0.234	0.123	0.471
Ingredient × phytase		0.820	0.907	0.875	0.847	0.650	0.636	0.007	0.856

[1]Each value represents the mean of six replicates (eight birds per replicate).
[a,b] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]Pooled standard error of mean.

Table 6.9 summarises the effect of different phytase doses on gizzard pH and relative weights of gizzard and gizzard digesta in broiler growers fed the maize-SBM and maize-CM diets. The acidity of gizzard contents differed ($P < 0.001$) between ingredients, with gizzard pH being higher in birds fed maize-SBM diets. Phytase addition had no effect ($P > 0.05$) on the gizzard pH. No correlation ($P < 0.05$) was observed between the gizzard pH and ileal Ca digestibility. The ATTRC of Ca was negatively correlated (r=-0.39, $P < 0.05$) with the gizzard pH. The relative weights of gizzard and gizzard contents were higher ($P < 0.01$) in birds fed maize-CM diet. Phytase addition influenced the relative weight of gizzard, with birds receiving the 2000 FTU/kg having a smaller ($P < 0.05$) gizzard.

The effect of different phytase doses on IP6 disappearance and concentrations of IP5 and IP6, in the terminal ileum of broiler growers fed maize-SBM and maize-CM diets is summarised in Table 6.15. The IP6 disappearance in unsupplemented maize-SBM and maize-CM diets was found to be 0.94 and 0.56, respectively. The IP6 disappearance coefficients was highly correlated (r=0.97; $P < 0.001$) with true ileal P digestibility coefficients. Coefficient of IP6 disappearance increased with increasing phytase doses in the maize-CM diet, but not in SBM, resulting in an ingredient x phytase interaction ($P < 0.001$).

The concentration of IP6 in the ileal digesta decreased with increasing doses of microbial phytase in the maize-CM diet, but not in the maize-SBM diet, resulting in an interaction ($P < 0.001$) between the ingredient and phytase. The concentration of IP5 differed ($P < 0.001$) between ingredients, with the concentration being higher in birds fed maize-CM diets. Concentration of IP5 was affected neither by the phytase nor by the interaction between ingredient and phytase.

Table 6.9. Effect of phytase doses on gizzard pH and relative weights (g/kg body weight) of gizzard and gizzard digesta in broiler growers fed soybean meal and canola meal diets (Experiment 1), measured on day 21[1]

Ingredient	Phytase (FTU/kg)	Gizzard pH	Gizzard weight[2]	Digesta weight[2]
Soybean meal	0	2.06	1.34	0.53
	500	2.26	1.29	0.41
	2000	2.29	1.24	0.42
Canola meal	0	1.68	1.38	0.75
	500	1.68	1.37	0.72
	2000	1.80	1.31	0.66
SEM[3]		0.097	0.028	0.049
Main Effects				
Ingredient				
Soybean meal		2.20[a]	1.29[b]	0.45[b]
Canola meal		1.72[b]	1.35[a]	0.71[a]
SEM		0.056	0.016	0.028
Phytase				
0		1.87	1.36[a]	0.64
500		1.97	1.33[a]	0.56
2000		2.04	1.28[b]	0.54
SEM		0.069		0.035
Probabilities, $P \leq$				
Ingredient		0.001	0.009	0.001
Phytase		0.210	0.023	0.104
Ingredient × phytase		0.623	0.784	0.639

[1]Each value represents the mean of six replicates (four birds per replicate).
[a,b] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]Calculated as a percentage of body weight.
[3]Pooled standard error of mean.

6.4.2. Experiment 2: Broiler finishers

Table 6.10 summarises the growth performance of broiler finishers fed maize-SBM and maize-CM diets with different phytase doses, over the 3-day experimental period. The effects of ingredient, phytase doses and their interaction were not significant ($P > 0.05$) for the growth performance.

Table 6.10. Effect of phytase doses on the growth performance of broiler finishers fed soybean meal and canola meal diets (Experiment 2), measured from day 39 to 42 post-hatch [1]

Ingredient	Phytase (FTU/kg)	Body weight (g/bird)	Feed intake (g/bird)	FCR
Soybean meal	0	103	228	2.21
	500	100	225	2.31
	2000	107	215	2.05
Canola meal	0	113	214	1.97
	500	109	230	2.16
	2000	110	233	2.14
SEM[2]		6.5	11.9	0.2
Main Effects				
Ingredient				
Soybean meal		103	223	2.19
Canola meal		111	226	2.09
SEM		3.8	6.9	0.1
Phytase				
0		108	221	2.09
500		105	228	2.24
2000		109	224	2.09
SEM		4.6	8.4	0.1
Probabilities, $P \leq$				
Ingredient		0.178	0.750	0.484
Phytase		0.803	0.867	0.634
Ingredient × phytase		0.855	0.395	0.628

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

Tables 6.11 and 6.12 summarise the true ileal digestibility and ATTRC of Ca and P, respectively, for SBM and CM with different phytase doses for broiler finishers. True ileal Ca digestibility coefficients in SBM and CM, without added phytase, were determined to be 0.33 and 0.22, respectively. Ileal endogenous Ca losses were determined to be 29 mg/kg of DMI.

True ileal Ca digestibility of CM was increased by both phytase doses (500 and 2000 FTU/kg) while that of SBM was increased only by the 2000 FTU/kg, resulting in an interaction ($P < 0.001$) between the ingredient and phytase.

The ATTRC of Ca was increased ($P < 0.001$) by both phytase doses in CM, with no difference between birds receiving 500 and 2000 FTU phytase/kg diet. However, the ATTRC was increased at 2000 FTU/kg phytase dose in SBM, resulting in an interaction ($P < 0.001$) between the ingredient and phytase.

Table 6.11. Effect of phytase doses on true ileal digestibility and apparent total tract retention coefficients (ATTRC) of calcium (Ca) in soybean and canola meals for broiler finishers (Experiment 2), measured on day 42[1,2]

Ingredient	Phytase (FTU/kg)	True ileal Ca digestibility[3]	ATTRC of Ca[3]
Soybean meal	0	0.33[c]	0.51[cd]
	500	0.39[bc]	0.55[c]
	2000	0.44[b]	0.62[b]
Canola meal	0	0.22[d]	0.50[d]
	500	0.46[b]	0.72[a]
	2000	0.65[a]	0.76[a]
SEM[4]		0.033	0.016
Main Effects			
Ingredient			
Soybean meal		0.39	0.56
Canola meal		0.44	0.66
SEM		0.019	0.010
Phytase			
0		0.28	0.51
500		0.42	0.64
2000		0.54	0.69
SEM		0.024	0.012
Probabilities, $P \leq$			
Ingredient		0.066	0.001
Phytase		0.001	0.001
Ingredient × phytase		0.001	0.001

[1]Each value represents the mean of six replicates (eight birds per replicate).
[a-d] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]The ileal endogenous Ca losses were 29 mg/kg.
[3]The Ca digestibility and ATTRC data relate to the ingredient (soybean meal or canola meal) as the Ca content in maize is negligible.
[4]Pooled standard error of mean.

Table 6.12. Effect of phytase doses on true ileal digestibility and apparent total tract retention (ATTR) coefficients of phosphorous (P) in soybean meal- and canola meal-based diets in broiler finishers (Experiment 2), measured on day 42[1,2]

Ingredient	Phytase (FTU/kg)	True ileal P digestibility[3]	ATTRC of P[3]
Soybean meal	0	0.82[b]	0.47[a]
	500	0.85[ab]	0.36[b]
	2000	0.91[a]	0.51[a]
Canola meal	0	0.57[d]	0.26[c]
	500	0.71[c]	0.28[c]
	2000	0.89[a]	0.31[bc]
SEM[4]		0.022	0.027
Main Effects			
Ingredient			
Soybean meal		0.86	0.45
Canola meal		0.72	0.28
SEM		0.013	0.016
Phytase			
0		0.69	0.36
500		0.78	0.32
2000		0.90	0.41
SEM		0.016	0.019
Probabilities, $P \leq$			
Ingredient		0.001	0.001
Phytase		0.001	0.009
Ingredient × phytase		0.001	0.046

[1]Each value represents the mean of six replicates (eight birds per replicate).
[a-d] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]The ileal endogenous P losses were 130 mg/kg.
[3]The P digestibility and ATTRC data relate to combination of maize and soybean meal or canola meal.
[4]Pooled standard error of mean.

An interaction ($P < 0.001$) was observed between the ingredient and phytase for the true ileal P digestibility. Phosphorous digestibility coefficients for maize-SBM and maize-CM diets, without added phytase, were determined to be 0.82 and 0.57, respectively. Ileal endogenous P losses were determined to be 130 mg/kg of DMI. True ileal P digestibility of the maize-CM diet was increased by both phytase doses (500 and 2000 FTU/kg) while the P digestibility of maize-SBM diet was increased only by the super phytase dose (2000 FTU/kg).

The ATTRC of P lowered by 500 FTU/kg phytase in the maize-SBM diet, whereas no phytase effect was seen in the maize-CM diet, resulting in an ingredient x phytase interaction ($P < 0.05$).

Table 6.13 summarises the effect of phytase on the apparent ileal digestibility of N and minerals in maize-SBM and maize-CM diets for broiler finishers. Ileal digestibility of N and minerals, except Fe, Cu and Zn, was higher ($P < 0.001$) in maize-SBM diets. Sodium digestibility was determined to be negative. The digestibility of Fe, Cu and Zn were unaffected ($P > 0.05$) by the ingredient. Phytase addition had no effect ($P > 0.05$) on the digestibility of N or minerals. There was no interaction ($P > 0.05$) between the ingredient and phytase for the digestibility of minerals.

Gizzard pH and relative weights of gizzard and gizzard digesta of broiler finishers fed maize-SBM and maize-CM diets with different phytase doses is summarised in Table 6.14. Supplemental phytase increased the gizzard pH for maize-CM diets, but not for maize-SBM diets, resulting in an interaction ($P < 0.05$) between the ingredient and phytase. The acidity of gizzard contents differed between ingredients, with gizzard pH being higher ($P < 0.05$) in birds fed maize-SBM diet. Relative gizzard weight was not influenced by the ingredient, phytase and their interaction. Relative gizzard contents were higher ($P < 0.001$) in birds fed maize-CM diet.

The effect of phytase doses on IP6 disappearance and concentrations of IP5 and IP6 in the terminal ileum of broiler growers and finishers fed maize-SBM and maize-CM diets is summarised in Table 6.15. The IP6 disappearance coefficient in unsupplemented maize-SBM and maize-CM diets was found to be 0.89 and 0.50, respectively. Similar to the grower phase, IP6 disappearance was highly correlated (r=0.95; $P < 0.001$) with the true ileal P digestibility. IP6 disappearance increased with increasing phytase doses in the maize-CM diet, but not in SBM, resulting in an ingredient x phytase interaction ($P < 0.001$).

Table 6.13. Effect of phytase doses on the apparent ileal digestibility coefficients of minerals in soybean meal and canola meal diets in broiler finishers (Experiment 2), measured on day 42[1]

Ingredient	Phytase[3] (FTU/kg)	Apparent ileal digestibility coefficients							
		N	Mg	K	Na	Fe	Cu	Mn	Zn
Soybean meal	0	0.81	0.19	0.82	-2.91	0.17	0.21	-0.06	0.04
	500	0.81	0.20	0.82	-2.79	0.17	0.18	-0.00	0.06
	2000	0.82	0.20	0.82	-2.56	0.15	0.13	0.01	0.09
Canola meal	0	0.77	0.06	0.61	-1.81	0.18	0.18	-0.24	0.06
	500	0.77	0.03	0.60	-1.77	0.13	0.17	-0.24	0.01
	2000	0.78	0.12	0.62	-1.48	0.15	0.16	-0.15	0.06
SEM[2]		0.011	0.037	0.018	0.221	0.031	0.041	0.060	0.041
Main Effects									
Ingredient									
Soybean meal		0.82[a]	0.19[a]	0.82[a]	-2.75[b]	0.17	0.17	-0.02[a]	0.06
Canola meal		0.77[b]	0.07[b]	0.61[b]	-1.68[a]	0.15	0.17	-0.21[b]	0.04
SEM		0.006	0.022	0.010	0.128	0.018	0.024	0.035	0.024
Phytase									
0		0.79	0.12	0.72	-2.36	0.18	0.19	-0.15	0.05
500		0.79	0.11	0.71	-2.28	0.15	0.17	-0.12	0.03
2000		0.80	0.16	0.72	-2.02	0.15	0.14	-0.07	0.08
SEM		0.008	0.027	0.013	0.157	0.022	0.029	0.042	0.029
Probabilities, $P \leq$									
Ingredient		0.001	0.001	0.001	0.001	0.595	0.951	0.001	0.562
Phytase		0.735	0.434	0.955	0.281	0.579	0.481	0.409	0.570
Ingredient × phytase		0.949	0.506	0.907	0.980	0.629	0.833	0.762	0.699

[1]Each value represents the mean of six replicates (eight birds per replicate).
[a,b] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]Pooled standard error of mean.

Table 6.14. Effect of phytase doses on gizzard pH and relative weights (g/kg body weight) of gizzard and gizzard digesta in broiler finishers fed soybean meal- and canola meal-base diets (Experiment 2), measured on day 42[1]

Ingredient	Phytase (FTU/kg)	Gizzard pH	Gizzard weight[2]	Digesta weight[2]
Soybean meal	0	2.70[a]	0.79	0.18
	500	2.65[ab]	0.79	0.18
	2000	2.54[ab]	0.80	0.15
Canola meal	0	2.12[c]	0.81	0.30
	500	2.40[b]	0.81	0.36
	2000	2.55[ab]	0.78	0.28
SEM[3]		0.094	0.020	0.033
Main Effects				
Ingredient				
Soybean meal		2.63	0.79	0.17[b]
Canola meal		2.36	0.80	0.31[a]
SEM		0.054	0.012	0.019
Phytase				
0		2.41	0.80	0.24
500		2.52	0.80	0.27
2000		2.55	0.79	0.21
SEM		0.067	0.014	0.023
Probability, $P \leq$				
Ingredient		0.002	0.696	0.001
Phytase		0.313	0.851	0.213
Ingredient × phytase		0.016	0.469	0.630

[1]Each value represents the mean of six replicates (four birds per replicate).
[a-c] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]Calculated as a percentage of body weight.
[3]Pooled standard error of mean.

The ileal concentration of IP6 decreased with increasing doses of microbial phytase in the maize-CM diet, but not in the maize-SBM diet, resulting in an interaction ($P < 0.001$) between the ingredient and phytase. Concentration of IP5 was reduced ($P < 0.05$) by phytase doses in both diets, but the magnitude of response was greater in the maize-SBM diet resulting in an ingredient x phytase interaction.

Table 6.15. Effect of phytase doses on concentration of IP5 and IP6 (nmol/ g DM) and IP6 disappearance coefficient in the terminal ileum of broiler growers and finishers fed soybean meal and canola meal diets, measured on days 21 and 42[1]

	Soybean meal			Canola meal			SEM[3]	Probabilities, $P \leq$		
	0[2]	500[2]	2000[2]	0	500	2000		Ingredient	Phytase	Interaction
Growers										
IP5	26[c]	57[c]	0.0[c]	872[ab]	988[a]	589[b]	120	0.001	0.170	0.344
IP6	4658[c]	3746[c]	2027[c]	30,115[a]	19,867[b]	6894[c]	1946	0.001	0.001	0.001
IP6 disappearance	0.94[a]	0.95[a]	0.98[a]	0.56[c]	0.72[b]	0.90[a]	0.034	0.001	0.001	0.001
Finishers										
IP5	685[b]	117[c]	133[c]	2870[a]	2116[a]	841[b]	309.3	0.001	0.001	0.047
IP6	7186[c]	3044[c]	2000[c]	40,830[a]	20,364[b]	6643[c]	2386.8	0.001	0.001	0.001
IP6 disappearance	0.89[a]	0.96[a]	0.97[a]	0.50[c]	0.74[b]	0.92[a]	0.034	0.001	0.001	0.001

[1]Each value represents the mean of six replicates (eight birds per replicate).
[a-c] Means having different superscripts within the row are significantly different ($P < 0.05$).
[2]Phytase doses (FTU/kg).
[3]Pooled standard error of mean.

Tables 6.16 summarises the Pearson Correlations between the digestibility measurements for Ca and P in broiler growers and finishers. Strong positive correlations were observed between all measured parameters for Ca and P.

Table 6.16. Pearson correlations between true ileal digestibility (TIDC) and apparent total tract retention (ATTRC) coefficients of calcium and phosphorous for growers (day 21) and finishers (day 42)

	$TIDC_{21}$	$TIDC_{42}$	$ATTRC_{21}$	$ATTRC_{42}$
Calcium				
$TIDC_{21}$	1.000	0.62*	0.70*	0.75*
$TIDC_{42}$	0.62*	1.000	0.60*	0.82*
$ATTRC_{21}$	0.70*	0.60*	1.000	0.74*
$ATTRC_{42}$	0.75*	0.82*	0.74*	1.000
Phosphorous				
$TIDC_{21}$	1.000	0.81*	0.56*	0.64*
$TIDC_{42}$	0.81*	1.000	0.56*	0.54*
$ATTRC_{21}$	0.56*	0.56*	1.000	0.58*
$ATTRC_{42}$	0.64*	0.54*	0.58*	1.000

* $P < 0.001$.

6.5. Discussion

In general, the concentration of proximate components, phytate, Ca, P and other minerals of SBM and CM were within the range reported in the literature (NRC, 1994; Browning and Cowieson, 2014; Mutucumarana *et al*., 2014b,c; 2015a).

6.5.1. Broiler growers

The ileal digestibility coefficients of Ca in SBM and CM, without added phytase, were determined to be 0.51 and 0.53, respectively. The current study was the first to report the ileal Ca digestibility of SBM in maize-based diet. For the CM, Anwar *et al*. (2018) has previously reported a much lower true ileal Ca digestibility coefficient of 0.31. An even lower apparent ileal Ca digestibility of 0.18 was reported for CM by Moss *et al*. (2018) for broiler growers. This discrepancy may be attributed, at least in part, to differences in assay diet composition.

Purified diets were used in these two CM evaluations, whereas maize-based assay diet was utilised in the current work. As reported in Chapter 3, lower Ca digestibility was determined for limestone when a purified assay diet was used compared to a maize-based diet.

Calcium digestibility was increased by added phytase in CM, but not in SBM. The ileal Ca digestibility coefficient of CM was increased by 21% (0.64) and 42% (0.75), respectively, at phytase doses of 500 and 2000FTU/kg. A possible reason for this difference could be the higher reactive phytate concentrations in the CM compared to SBM (Selle and Ravindran, 2007). According to Morgan (2014), percentage of reactive phytate in the digestive tract of broilers was higher for CM (46.8-56.2%) than SBM (43.6-53.3%).

The ATTRC of Ca, with no added phytase, was higher in CM (0.52) than in SBM (0.34), suggesting better utilisation of Ca from CM. An explanation for this difference in utilisation is difficult to provide. In contrast to the trends observed in Ca digestibility, phytase effects on the ATTRC of Ca were similar in SBM and CM.

True ileal P digestibility was increased by supplemental phytase in both assay diets, but the magnitude of responses was considerably greater in maize-CM diets at both phytase doses. True ileal P digestibility of maize-CM diet was increased by 14 and 34%, respectively, at phytase doses 500 and 2000 FTU/kg. The corresponding increases in the maize-SBM diet were only 3 and 7%, respectively. Phosphorous digestibility determined in the unsupplemented maize-SBM diet was remarkably high (0.89) and this incongruity could explain its lower responses to phytase addition. Such a high P digestibility in unsupplemented maize-SBM diet was unexpected and most likely due to the very low dietary Ca concentration. It is well established that P absorption increases at deficient dietary Ca concentrations (Abdollahi *et al*., 2015, 2016; Blahos *et al*., 1987; Tamim *et al*., 2004; Mutucumarana *et al*., 2014a).

The current finding on ileal endogenous Ca losses (236 mg/kg DMI) in broiler growers were substantially higher than the value (131 mg/kg DMI) reported in Chapter 3 and the range of 84-127 mg/kg DMI reported by Anwar *et al*. (2016a,c; 2017) for broilers of similar age following the feeding of Ca-free purified diets. In the present work, a Ca- and P- free purified diet was used to measure the endogenous losses. However, the endogenous Ca losses determined in the current study (236 mg/kg DMI) were similar to that obtained with maize-based Ca- free diet (253 mg/ kg DMI) in a previous study (Chapter 3). These discrepancies are difficult to comprehend, but may be explained by differences in dietary and nutritional composition among the assays.

Endogenous P losses in broiler growers was estimated to be 310 mg/kg DMI in the current study, which is closer to the value of 354 mg/kg DMI reported by Mutucumarana and Ravindran (2016). However, there are inconsistencies in published data on ileal endogenous P losses in broilers. A lower value of 235 mg/kg DMI has been reported by Dilger and Adeola (2006a) using the regression method. According to Mutucumarna *et al*. (2014b,c; 2015b), the ileal endogenous P losses, based on the regression method, ranged from -487 to 609 mg/kg DMI.

The apparent ileal digestibility coefficients of N, Mg, K and Cu were higher in the maize-SBM diet than in the maize-CM diet. The lower digestibility in maize-CM diet may be due to the negative effects of hulls and tannins present in the CM (Bell, 1993; Wickramasuriya *et al*., 2015). Among the minerals, higher digestibility was found for K and, negative digestibility for Na, Cu, Mn and Zn. Similar results have been reported by Selle *et al*. (2009b) and Mtei *et al*. (2019b). In the case of Na, the negative estimates are reflective of significant endogenous secretion of sodium bicarbonate into the digestive tract, in response to dietary

phytate (Ravindran *et al*., 2006). Supplemental phytase had no effect on mineral digestibility, a finding similar to that reported by Moss *et al*. (2018).

Phytase addition had no effect on the gizzard pH, in agreement with the findings of Walk *et al*. (2012a). Feeding maize-CM diets resulted in lower gizzard pH than maize-SBM diets. The higher pH in birds fed maize-SBM diets may be due, at least in part, to presence of limestone added as a flow agent during SBM processing (Ravindran *et al*., 2014). On the other hand, the lower pH in birds fed maize-CM diets may be attributed to the higher fibre content of CM which may have increased the gizzard size, prolonged the digesta retention time in the gizzard, and increased gizzard motility and hydrochloric acid secretion (Svihus, 2011; Mateos *et al*., 2012). Another contributing factor may be the buffering effect of lower protein content of the maize-CM diet, which could have reduced the gizzard pH compared to that of maize-SBM diet. The buffering capacity of low protein diets has been previously noted (Chapter 5). Moss *et al*. (2019) similarly reported that the gizzard pH was reduced from 2.80 to 2.35 when the dietary crude protein was decreased from 220 to 170 g/kg. According to Mejicanos *et al*. (2016), the concentration of neutral and basic amino acids like arginine, lysine, threonine and tryptophan were lower in CM than SBM which may also have reduced the gizzard pH.

Increasing phytase doses increased the ileal disappearance of IP6 in the maize-CM diet. The increments at at phytase doses 500 and 2000 FTU/kg, over the unsupplemented diet, were 29 and 61%, respectively. The increase in IP6 disappearance by added phytase in maize-CM diet was consistent with the trends in ileal P digestibility. In contrast, no phytase effect on IP6 disapperance was observed in maize-SBM diets. The lack of phytase response in maize-SBM diets could be attributed to the extremely high IP6 disappearance (0.94) determined in the unsupplemented maize-SBM diet. As noted earlier, the deficient dietary Ca level may have

contributed to the increased IP6 hydrolysis in broilers fed maize-SBM diets (Li *et al*., 2017b; Sommerfeld *et al*., 2018a).

The ileal concentration of IP6 was higher in maize-CM diets, consistent with the high phytate content in CM (Selle *et al*., 2000). As expected, supplemental phytase reduced the ileal IP6 concentration in the maize-CM diet. The concentration of IP5 was also lowered at the superdose, mirroring the step-wise degradation of IP6. In contrast, ileal concentrations of IP6 and IP5 were unaffected by supplemental phytase in maize-SBM diets. Virtually all the IP6 was hydrolysed in the unsupplemented control, explaining the lack of phytase effect. IP6 disappearance was highly correlated (r=0.97; $P < 0.001$) with true ileal P digestibility.

6.5.2. Broiler finishers

The true ileal Ca digestibility coefficients of SBM and CM, without supplemental phytase, were determined to be 0.33 and 0.22, respectively. No comparable data for these ingredients with 42-day-old broilers are available.

True ileal Ca digestibility was increased by both phytase doses in CM and only at the super phytase dose in SBM. The percentage increase in Ca digestibility coefficient of CM was 109 and 195%, respectively, at 500 and 2000 FTU/kg phytase when compared to 18 and 33%, respectively, in SBM. As discussed for broiler growers, the higher reactive phytate concentration of CM may explain this disparity (Ravindran *et al*., 2006).

The effect of supplemental phytase on the ATTRC of Ca followed a similar trend as that on the ileal Ca digestibility. The increment in ATTRC of Ca as a result of added phytase doses were 44-52% and 8-22% for CM and SBM, respectively.

Responses in true ileal P digestibility to added phytase followed a pattern similar to those in Ca digestibility. The increase in P digestibility by added phytase was greater in maize-

CM diet than in maize-SBM diet. As discussed above, the likely reason for the lack of phytase effect was not the phytase efficacy but the anomaly of high ileal P digestibility (0.82) determined for the unsupplemented maize-SBM diet.

The endogenous Ca losses measured in broiler finishers (29 mg/kg DMI) were markedly lower than the previously reported values (Chapter 3; Anwar *et al*., 2016a,c; 2017) and than those measured in broiler growers (236 mg/kg DMI) in the current work. No comparable data are available for endogenous Ca losses in 42-day-old broilers. Such a surprisingly low estimate for endogenous Ca flow is counter-intuitive and it is difficult to provide an explanation. The endogenous P losses measured in broiler finishers (130 mg/kg DMI) were also lower than those measured in broiler growers (310 mg/kg DMI), suggesting an age effect on endogenous nutrient losses.

The digestibility of N and minerals in the finisher phase followed a trend similar to that of the grower phase. As discussed earlier, the lower mineral digestibility in maize-CM diets could be due to the presence of hulls and tannins (Bell, 1993). Microbial phytase had no effect on mineral digestibility, in agreement with those observed for broiler growers in the present work.

Gizzard pH in birds fed the unsupplemented maize-CM diet (2.12) was lower than that in birds fed the unsupplemented maize-SBM diet (2.70). Supplemental phytase increased the pH in birds fed maize-CM diets, but not in those fed maize-SBM diets. Phytic acid is known to have an acidogenic influence in the gizzard of broilers (Walk *et al*., 2012a). Hydrolysis of higher reactive phytate concentration in the maize-CM diet (Morgan, 2014) may also have contributed to the increased gizzard pH in maize-CM diets.

The data on IP6 disappearance and concentrations of lower phytate esters followed a similar trend as observed in the grower phase. Increasing phytase doses increased the IP6

disappearance only in the maize-CM diet and the increments in IP6 disappearance at phytase doses 500 and 2000 FTU/kg were 48 and 84%, respectively.

6.5.3. Broiler growers vs. finishers

The current study was not designed to provide statistical comparisons between the two age groups. However, because same ingredients and formulation were used, the results have ramifications to the understanding of age effects on Ca and P utilisation. The results showed that the Ca digestibility determined for broiler finishers were lower than those of the broiler growers, regardless of the ingredient type. This observation confirms our previous findings (Chapter 5) that the Ca digestibility coefficients decrease with advancing age of broilers.

It is well accepted that the microbial phytase improves P absorption in poultry by increasing the bioavailability of phytate-P (Selle and Ravindran, 2007), but the effects on the enzyme on Ca digestibility remains contradictory. Some reports indicate that phytase addition improves Ca digestibility (Ravindran *et al*., 2006; Walk *et al*., 2012b; Kim *et al*., 2018), whereas other have found little or no benefit (Sebastian *et al*., 1996; Walk *et al*., 2012a). The current findings demonstrate that supplemental phytase is consequential for Ca absorption and retention of Ca. These benefits are likely to be linked to a reduction in the formation of insoluble Ca-phytate complexes, rather than release of Ca from phytate-complexes. The magnitude of phytase effect on Ca digestibility was greater in finishers than in growers, regardless of ingredient type and phytase doses. The better responsiveness may be attributed to lower intestinal phytase setting in older birds (Ravindran *et al*., 1995a) due to reduced need for Ca and P.

As noted earlier, the interpretation of data on the digestibility and ATTRC of P and gizzard pH is confounded by differences in ingredient contribution to P and phytate-P in assay diets. The ingredient effects are difficult to discern because of different maize inclusion levels

in assay diets. Studies indicate that the digestibility and ATTRC of P is increased by the superdose (Shirley and Edwards, 2003; Cowieson *et al*., 2011), in agreement with current findings. Similar to Ca digestibility, the phytase effect on true ileal P digestibility was greater in finishers than in growers, particularly for maize-CM diets, suggesting greater phytate hydrolysis during the finisher phase. It is also possible that more phytate remained in the control birds to be hydrolysed by the supplemental phytase.

Studies on the influence of phytase on Ca digestibility in CM are limited. Moss *et al*. (2018) reported a 70% increase in the apparent ileal Ca digestibility of CM with 1000 FTU/kg added phytase. Overall, the response of phytase on IP6 disappearance, P digestibility and Ca digestibility in the CM paralleled implying that the higher phytate content was the primary reason for the phytase effects.

6.6. Conclusions

There are several outcomes of note, which will be useful in feed formulations. First, the ileal Ca digestibility coefficients of SBM and CM for broilers were determined. Second, the effect of broiler age on Ca digestibility was demonstrated. Third, supplemental phytase is consequential for Ca absorption and retention.

CHAPTER 7

Comparison of the apparent ileal calcium digestibility of limestone in broilers and layers

7.1. Abstract

The apparent ileal calcium (Ca) digestibility coefficients of two limestone sources in growing broilers and layers were determined in two separate experiments. In each experiment, two maize-based diets were developed with one of the two limestone sources (A, experiment 1 and B, experiment 2) to contain either 8.0 g/kg Ca for broilers or 40 g/kg Ca for layers. The two sources differed *inter alia* in particle size, with limestone A being finer and limestone B being coarser. Titanium dioxide was included in all diets as an indigestible indicator. Each experimental diet was randomly allotted to six replicate cages (six birds per cage for broilers or five birds per cage for layers) and offered for three days from 19 to 21-day post-hatch to broilers and during 40 weeks of age to layers. The total tract Ca retention was also measured using the indicator ratios in the diet and excreta. In both experiments, apparent ileal Ca digestibility of limestone, gizzard pH and gizzard Ca concentration were higher ($P < 0.05$) in layers than in broilers. The apparent ileal digestibility coefficient of limestone A for broilers and layers were 0.50 and 0.62, respectively. The corresponding values for limestone B were 0.43 and 0.70, respectively. The apparent total tract retention of Ca was similar ($P > 0.05$) between broilers and layers in both experiments, and between the two sources. The present data showed that layers are more efficient in absorbing Ca than broilers.

7.2. Introduction

Calcium (Ca) plays a major role in the growth and development of poultry. Apart from skeletal growth, Ca is important for a wide range of functions in the body, for example, blood clotting, muscle contraction, nerve impulse transmission, enzyme activation, metabolic reactions, protein synthesis, maintenance of osmotic and acid-base balances and, components in

membranes (Crenshaw, 2001). In layers, Ca is also important for eggshell formation and shell quality. Eggshell of an average egg, weighing 60 g, contains around 2.0 g of Ca and only 0.12 g phosphorus (Taylor, 1963). The dietary Ca requirement of contemporary laying hens is in the range from 39.0 to 49.0 g/kg (ISA, 2014; Hy-Line Brown, 2018) whereas the requirement of broilers is in the range from 7.2 to 9.6 g/kg (Ross, 2019; Arbor Acres, 2019), depending on their growth and production stage. Laying birds have a high Ca requirement for egg production and bone maintenance. Calcium metabolism is unique in layers. In general, Ca utilisation in chickens depends on the dietary Ca solubilisation in the digestive tract. In broilers, the solubility of Ca in the gastrointestinal tract is negatively correlated to pH, with Ca being solubilised more at lower pH (Guinotte *et al*., 1995). However, despite having high pH, the Ca solubility in the intestine of laying hens is greater during the time of eggshell formation (Mongin, 1976). Furthermore, the bone biology differs between the broilers and layers. In laying hens, apart from cortical and trabecular bone tissues, another specialised bone (medullary bone) is found which acts as a labile source of Ca to support shell formation during the dark hours (Simkiss, 1961). Medullary bone Ca is mobilised and redeposited daily in response to Ca demand during the time of shell formation. Several reports on the ileal Ca digestibility of Ca sources for broilers are now available (Chapters 3 and 4; Anwar, 2017). Owing to the greater Ca demand for shell formation, it was hypothesised that layers will be more efficient than broilers in absorbing Ca but no studies to date have compared the ileal digestibility of Ca sources between these two bird types. Therefore, objective of the current work is to test the hypothesis that the ileal Ca digestibility of limestone will be higher in laying hens compared to broilers.

7.3. Materials and methods

The experiments 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.

7.3.1. Experimental diets

Two limestone samples namely, limestone A and B, were obtained from commercial sources. The samples were of different origins, as confirmed by mineral analysis (Table 7.1) and, also differed in particle size and *in vitro* solubility. The particle size of limestone A was finer with a geometric mean diameter of 462 µm, whereas that of limestone B was coarser with a geometric mean diameter of 1301 µm. The two limestones were evaluated in separate experiments and it was not intended to compare particle size effects because the origins differed.

Two experiments were conducted to determine the effect of bird type (broilers and layers) on the ileal Ca digestibility in two limestone sources, A (Experiment 1) and B (Experiment 2). In each experiment, two maize-based diets were generated for each limestone sample to have either 8.0 g/kg Ca for broilers or 40 g/kg Ca for layers (Table 7.2). These Ca concentrations in assay diets were based on breeder recommendations (High-line brown, 2018; Ross, 2019).

The direct method was used for the measurement of Ca digestibility in the present study. Previous studies have shown that, in addition to its simplicity, this method yields comparable Ca digestibility estimates to that of the regression method (Anwar *et al*. 2016b, 2018). The use of maize-based diets was justified because of the negligible Ca concentration in maize (0.2 g/kg) and there is only a vestigial Ca contribution to assay diets (Gonzalez-Vega *et al*., 2015a,b; Chapter 3).

Published data are available for the ileal endogenous Ca losses in broilers (Anwar, 2017; Chapters 3 and 6), but corresponding information for layers is non-existent. For this reason, a Ca-free diet was also developed to measure the endogenous Ca losses in layers. Titanium dioxide (5 g/kg) was added to all diets as an indigestible indicator to calculate the coefficients of apparent ileal digestibility and apparent total tract retention (ATTRC) of Ca.

7.3.2. Birds

Broilers: One-day-old male broiler (Ross 308) chicks were obtained from a commercial hatchery, housed in floor pens, and fed a commercial broiler starter diet until day 18.

Table 7.1. Analysed mineral composition, particle size and *in vitro* solubility of limestone samples (as received basis)[1]

	Limestone A	Limestone B
Macro minerals (g/kg)		
Calcium	409	430
Phosphorous	0.4	< 0.1
Magnesium	2.0	1.4
Potassium	0.2	< 0.1
Sodium	0.1	< 0.1
Micro minerals (mg/kg)		
Iron	1500	400
Copper	4.0	< 1.0
Zinc	9.0	4.0
Manganese	70	21
Chloride	< 100	< 100
Aluminium	852	106
Lead	0.6	0.3
Arsenic	0.9	0.2
Cadmium	< 1.0	< 1.0
Particle size		
Geometric mean diameter (µm)	462	1301
Geometric standard deviation (µm)	2.42	1.78
In vitro solubility, %	55.5	22.5

[1]Samples were analysed in duplicate.

Table 7.2. Ingredient composition and analysis of diets (g/kg, as fed basis), Experiments 1 and 2

Ingredient	Experiment 1 (Limestone A)		Experiment 2 (Limestone B)		Ca-free layer diet
	Broiler	Layer	Broiler	Layer	(Experiment 1)
Maize	935.5	847.2	935.5	847.2	-
Limestone A	20.5	104.8	-	-	-
Limestone B	-	-	20.5	104.8	-
Monosodium phosphate	15	19	15	19	22.3
Soybean oil	20	20	20	20	20
Sodium chloride	2	2	2	2	2
Titanium dioxide	5	5	5	5	5
Trace mineral-vitamin premix[1,2]	2	2	2	2	2
Maize starch	-	-	-	-	399.4
Dextrose	-	-	-	-	399.3
Dried egg albumen	-	-	-	-	100
Cellulose	-	-	-	-	50
Calculated analysis[3]					
Metabolisable energy (MJ/kg)	13.83	12.59	13.83	12.59	12.23
Crude protein	79.52	72.0	79.52	72.0	82.40
Calcium	8.0	40.0	8.0	40.0	-
Total phosphorus	5.89	6.51	5.89	6.51	4.86
Non-phytate phosphorous	4.02	4.82	4.02	4.82	4.86
Phytate P	1.87	1.69	1.87	1.69	-
Ca: Non-phytate phosphorous	2.0	8.3	2.0	8.3	-
Analysed values (g/kg, as fed basis)					
Dry matter	913	917	915	921	915
Calcium	8.4	40.8	9.5	41.2	0.4

[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 IU; Mo, 0.25 mg; Se, 0.26 mg; Mn, 100 mg; Cu, 10 mg; Zn, 80 mg; Fe, 60 mg; antioxidant, 100 mg.
[2]The trace mineral-vitamin premix was calcium-free.
[3]Calculated based on NRC (1994) values.

Layers: Hens (Hy-Line brown) were obtained from a commercial farm at 38 weeks of age, with the peak laying rate of over 95%. Upon arrival, the birds were housed in layer cages, fed a commercial layer diet and given a two-week acclimatisation period to cages.

At the introduction of experimental diets, the broilers were19 days of age (body weight, mean ± SD, 0.93 ± 0.02 kg) and the layers were 40-week old (body weight, mean ± SD, 2.16

± 0.07 kg). In each experiment, a total of 36 broilers and 30 layers were assigned to six replicate cages (six broilers per cage or five layers per cage), so that the average body weight per cage within the bird type were similar. In experiment 1, the Ca-free diet was fed to an additional six replicate cages of layers (five birds per cage) to determine the endogenous Ca losses. The two bird types were randomly assigned to cages in an environmentally controlled room. The feed was removed for 8 hours before the introduction of experimental diets. The experimental diets, in mash form, were offered *ad libitum* for consumption during the 3-day assay period and birds had free access to water.

7.3.3. Digesta and excreta collection and processing

After the three days of experimental period, all birds were euthanised between 10:00 and 12:00 hours by intravenous injection (0.5 mL per 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 3.3.3. Total excreta samples were also collected during the last three days of experimental period and processed as described in Chapter 4, section 4.3.3.

7.3.4. Measurement of gizzard parameters

Gizzard was collected from four birds per replicate cage. The pH of the gizzard digesta content was measured as described in Chapter 5, section 5.3.3. Gizzard Ca retention was also determined. Individual gizzard contents were gently flushed in the plastic containers, frozen immediately and subsequently lyophilised. Dried gizzard samples were weighed, pooled within a cage, ground to pass through 0.5 mm sieve and stored in air-tight containers at 4◦C until chemical analysis. The relative weight of dried gizzard digesta content was calculated as g per kg of body weight.

7.3.5. Sample analysis

In vitro Ca solubility of limestone was determined using the weight loss method as reported by Zhang and Coon (1997b). The particle size was determined by dry sieving using the method described by Baker and Herrman (2002). For the analysis of minerals, the samples were wet acid digested with a mixture of nitric and perchloric acid, and concentrations of Ca, P, K, Mg and Na were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) using a Thermo Jarrell Ash IRIS instrument (Thermo Jarrell Ash Corporation, Franklin, MA, USA). The concentrations of micro-minerals were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) using a Perkin Elmer Elan 6000 instrument (Melbourne, Victoria, Australia). Representative samples of diets, ileal digesta and excreta were analysed for dry matter (DM), Ca (AOAC, 2016) and titanium dioxide as described in Chapter 3, section 3.3.4.

7.3.6. Calculations

Apparent ileal Ca digestibility coefficients were calculated using the titanium ratio in the diets and digesta (Ravindran *et al*., 2005) as indicated below:

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

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 concentration of Ca in the ileal digesta, Ca_I is the concentration of Ca in the diet. All concentrations were expressed as g/kg DM.

Apparent total tract Ca retention coefficients were calculated using the indicator ratios in the diet and excreta as indicated below:

$$\text{Ca retention} = 1 - [(Ti_I / Ti_E) \times (Ca_E / Ca_I)]$$

where Ti_I is the titanium concentration in the diet, Ti_E is the titanium concentration in the excreta, Ca_E is the concentration of Ca in the excreta, Ca_I is the concentration of Ca in the diet.

7.3.7. Statistical Analysis

In both experiments, the mean values were compared by Student's t-test (SAS, 2019). Cage served as the experimental unit and differences were considered to be significant at $P < 0.05$.

7.4. Results

The geometric mean diameter of limestone A and coarse limestone B was determined to be 462 and 1301 µm, respectively, with the geometric standard deviation of 2.42 and 1.78 µm, respectively. The *In vitro* solubility of limestone A and B was 55.5 and 22.5%, respectively.

For both bird types, the ileal Ca digestibility values are reported on apparent basis. It was intended to correct for true Ca digestibility in layers using the endogenous Ca loss value measured in birds fed the Ca-free diet and in broilers using an average estimate previously reported from our laboratory. But true digestibility values in layers could not be calculated because of extreme variations determined in the endogenous Ca losses (620 to 26,250 mg/kg DM intake) among replicates. It was observed that some layers receiving the Ca-free diet consumed their eggshells ostensibly to satisfy the Ca needs for egg lay as evidenced by the high incidence of broken eggs in cages and the presence of eggshells in the ileal digesta.

7.4.1. Experiment 1

Table 7.3 summarises the influence of bird type on Ca digestibility and gizzard parameters for limestone A. The apparent ileal Ca digestibility, gizzard pH and gizzard Ca concentration were higher ($P < 0.05$) in layers than in broilers. The ATTRC of Ca was similar ($P > 0.05$) between broilers and layers. The relative weight of gizzard digesta was higher ($P < 0.001$) in broilers than in layers.

Table 7.3. Apparent ileal calcium (Ca) digestibility coefficients and gizzard parameters in broilers and layers fed limestone A (Experiment 1)[1,2]

	Broilers	Layers	SEM[3]	Probabilities, $P \leq$
Apparent ileal Ca digestibility coefficient	0.50[b]	0.62[a]	0.042	0.015
Apparent total tract Ca retention coefficient	0.57	0.59	0.026	0.554
Gizzard pH	2.15[b]	3.13[a]	0.197	0.001
Gizzard Ca concentration, g/kg	3.80[b]	75.9[a]	14.173	0.001
Relative weight of gizzard digesta, g/kg body weight	3.74[a]	1.33[b]	0.213	0.001

[1]Each value represents the mean of six replicates (six birds per replicate for broilers and five birds per replicate for layers).
[2]Geometric mean diameter, *in vitro* solubility and analysed Ca concentration of limestone A were 462 µm (2.42 µm, geometric standard deviation), 55.5% and 409 g/kg, respectively.
[a,b] Means having different superscripts within the row are significantly different ($P < 0.05$).
[3]Pooled standard error of mean.

7.4.2. Experiment 2

Table 7.4 summarises the influence of bird type on Ca digestibility and gizzard parameters for limestone B. Apparent ileal Ca digestibility, gizzard pH and gizzard Ca concentration were higher ($P < 0.001$) in layers than in broilers. The ATTRC of Ca was similar ($P > 0.05$) between broilers and layers. Relative weight of gizzard digesta was higher ($P < 0.05$) in broilers than in layers.

Table 7.4. Apparent ileal calcium (Ca) digestibility coefficients and gizzard parameters in broilers and layers fed limestone B (Experiment 2)[1,2]

	Broilers	Layers	SEM[3]	Probabilities, $P \leq$
Apparent ileal Ca digestibility coefficient	0.43[b]	0.70[a]	0.027	0.001
Apparent total tract Ca retention coefficient	0.56	0.58	0.035	0.565
Gizzard pH	2.22[b]	3.12[a]	0.164	0.001
Gizzard Ca concentration, g/kg	45.7[b]	224[a]	11.72	0.001
Relative weight of gizzard digesta, g/kg body weight	3.92[a]	2.54[b]	0.350	0.003

[1]Each value represents the mean of six replicates (six birds per replicate for broilers and five birds per replicate for layers).
[2]Geometric mean diameter, *in vitro* solubility and analysed Ca concentration of limestone B were 1301 µm (1.78 µm, geometric standard deviation), 22.5% and 430 g/kg, respectively.
[a,b] Means having different superscripts within the row are significantly different ($P < 0.05$).
[3]Pooled standard error of mean.

7.5. Discussion

It was originally planned to determine the ileal endogenous Ca losses in layers to calculate the true ileal Ca digestibility. The current study highlighted the difficulty in feeding a Ca-free diet to birds in lay which have a very high Ca requirement. It was noted that some layers tended to maintain Ca intake by consuming the shells of their own eggs. Laying hens are known to possess a specific appetite for Ca (Wood-Gush and Kare, 1966; Bradbury *et al*., 2014), when Ca-deprived and can self-regulate their Ca intake (Hughes and Wood-Gush, 1971). Wood-Gush and Kare (1966) found that Ca-deprived layers increased their preference for Ca-enriched diet when offered a choice between two diets, without and with Ca carbonate. This feeding behavior explains the consumption of their own eggs when in a Ca-deficient state. Because of the resultant variation in endogenous Ca loss estimates, apparent digestibility values were not corrected for true ileal digestibility in the current study.

The effect of bird type on ingredient Ca digestibility has not been previously investigated. To the best of our knowledge, this is the first report on the ileal Ca digestibility of limestone in layers. The apparent ileal Ca digestibility of practical diets in layers have been determined in some studies, with a range between 0.37 and 0.72 depending on various interacting factors (Liu *et al*. 2007; Englmaierová *et al*., 2017; Musilova *et al*., 2017; Bello and Korver, 2019). Hurwitz and Bar (1969) used yttrium-91, an unabsorbed reference substance, to measure the Ca absorption in colostomised laying hens and reported an absorption of 62% in birds fed diets with 39.4 g/kg dietary Ca (Hurwitz and Bar, 1969). On the other hand, published data on the ileal Ca digestibility of Ca sources for broilers are now available (Chapters 3, 4, 5 and 6; Angel *et al*., 2013; Anwar *et al*., 2015; 2016a,b,c; 2017; 2018; Zhang and Adeola, 2018). The ileal Ca digestibility coefficients recorded for broilers in the present study were 0.50 and 0.43 for limestone A and B, respectively, which are closer to the lower range (0.49-0.65) of previously reported values (Chapter 3 and 4; Anwar *et al*., 2016a,c; 2017).

The current work was designed to test the thesis that layers absorb Ca more efficiently than broilers and the present findings support this premise. The apparent Ca digestibility coefficients recorded in both limestones for layers (0.62 and 0.70) were greater than those of broilers (0.50 and 0.43). Laying hens require more Ca to meet their high metabolic needs for bone resorption and eggshell formation (Etches, 1987), and these needs drive the higher absorption.

The use of different Ca concentrations in assay diets for the broilers and layers, and the validity of digestibility data generated may be questioned. When measuring and comparing Ca digestibility in different bird types, appropriate recommended Ca concentrations must be used. A previous study from our laboratory found that feeding laying hens a diet containing 8.5 g/kg Ca (instead of recommended levels of 40.0 g/kg Ca) resulted in unusually high Ca digestibility coefficients of 0.81-0.90 (Mtei *et al*., 2019a,b). The high Ca digestibility was an expected outcome, because negative Ca balance in layers fed a Ca-deficient diet has been shown to increase Ca absorption (Hurwitz and Bar, 1969), probably through increasing Ca-binding proteins in intestinal epithelial cells (Bar and Wasserman, 1973).

It may be argued that the differences in Ca digestibility between broilers and layers may be reflective, at least in part, of age effects. Layers are mature birds with fully developed digestive tract and functions whereas the broilers are growing birds. However, the higher Ca digestibility in layers cannot be explained based on bird age. Recent evidence shows that, contrary to the trends observed for the digestibility of major nutrients (starch, lipids, and protein), Ca digestibility declines with advancing age paralleling the decreasing Ca demand (Chapter 5).

In both experiments, the ATTRC of Ca was not influenced by the bird type. The coefficient of ATTRC of Ca recorded for broilers in both experiments (0.57 and 0.56) confirm the value (0.59) obtained in the study reported in Chapter 4. In the case of layers fed limestone

B, despite having higher ileal digestibility (70%), only around 58% of Ca was retained, suggestive of some urinary excretion of Ca. It is difficult to explain the exact reason for this observation. It is possible that the higher plasma Ca concentration from absorbed Ca may have induced the excretion of some absorbed dietary Ca (Proszkowiec-Weglarz and Angel, 2013). However, the involvement of dietary P may be discounted since the dietary P concentration was maintained at recommended level for layers in the current study (Hy-Line brown, 2018). Another possible reason could be the time of sample collection. Ileal digesta samples were collected 4 hours after the oviposition when there is little or no shell formation. The length of the laying cycle is about 24-25 hours, with the shell being deposited during the last 21 hours (Johnson, 2015). At the time of sampling (12:00 hours), the hens have completed their oviposition and several hours into the next ovulation cycle. On the other hand, excreta samples were collected over three days, which would have included both non-shell formation and calcification phases. Because the removal of Ca from blood for eggshell formation is affected by the stage of eggshell calcification and changes continually (Taylor and Hertelendy, 1961; Parsons and Combs, 1980; Etches, 1987), it may be argued that such changes could influence the excreta Ca levels and contribute to differences noted between the absorption and retention. The continual changes in serum Ca in layers has been confirmed by Roland *et al.* (1972) who also observed that the pattern of Ca excretion was exactly the reverse of serum Ca and, that the highest serum Ca and the lowest excreta Ca were at oviposition.

A related issue is the unique mechanism involved in the eggshell calcification in laying hens. The Ca is provided via the blood following absorption from the intestine or resorption from the medullary bone. During the calcification of shell, intestinal absorption is insufficient to satisfy the high Ca needs and as much as 40% of the shell Ca may be derived from bone. The bone reservoir is subsequently replenished with Ca from intestinal absorption during periods of eggshell gland inactivity (Leeson and Summers, 2005; Nys and Guyot, 2011;

Korver, 2020). Thus, the relative contributions of absorbed Ca and bone Ca to plasms Ca levels vary depending on the time of day and it is unclear whether this may partly explain the observed anomaly in the ATTRC. Absence of any correlation between the ileal digestibility and ATTRC of Ca in layers in the current study may further indicate possible loss of Ca via the urine.

An unresolved question that arose during the present study is the influence of stage of ovulation cycle on the Ca absorption in laying hens. There is some evidence relating Ca absorption to shell deposition. Hurwitz and Bar (1969), using an yttrium-91 marker, demonstrated that the Ca absorption increased during shell calcification. Calcium absorption estimates of 40, 68 and 72% were determined during different calcification stages (none, early and late, respectively) in layers fed diets with 35.6 g/kg dietary Ca. Future ileal digestibility assays are warranted to further explore this aspect.

The gizzard pH was influenced by bird type, with the layers having a higher pH than broilers. This is to be expected due to the higher limestone content in the layer diet. The high buffering capacity of limestone is well documented (Morgan *et al*., 2014). The higher Ca concentration in gizzard digesta than in broilers further supports this finding. Gizzard pH was positively correlated (r=0.71; $P = 0.010$ in experiment 1 and r=0.85; $P < 0.001$ in experiment 2) with gizzard Ca concentration. Gizzard pH influences the solubilisation of Ca source and subsequent Ca absorption in poultry. Calcium sources are solubilised more when the gizzard pH is low (Mongin, 1976). However, regardless of the higher gizzard pH, Ca absorption was higher in layers than in broilers, which can be explained by the unique Ca metabolism in laying hens. Layers can increase their gastric acid secretion by means of crop dilation due to the increased feed consumption early in the photoperiod (Mongin, 1976). This increased gastric acid secretion increases the soluble Ca concentration and consequently the Ca absorption in layers. Regardless of the limestone source (Experiments 1 and 2), measured gizzard pH values

were similar within bird type (2.15 and 2.22 for broilers, and 3.13 and 3.12 for layers, respectively) in the current study. It is, however, worth noting that the gizzard Ca concentration was substantially greater (not statistically compared) in birds fed limestone B than A (45.7 vs. 3.8 g/kg for broilers and 224 vs. 75.9 g/kg for layers).

Calcium concentration of gizzard digesta was higher in layers, which reflects the high dietary Ca concentration of the layer diet. Although the current study was not designed to perform a statistical comparison between two limestone sources, there were marked numerical differences in the gizzard Ca concentration between the broilers and layers receiving limestone A and B, with those fed the coarser limestone B having higher gizzard Ca concentrations. The coarser particles are known to be retained longer in the gizzard (Scott *et al*., 1971; Rao and Roland, 1990; Zhang and Coon, 1997a), increasing digesta Ca concentration. Similarly, Anwar *et al*. (2017) reported a higher gizzard Ca concentration in broilers fed coarse particles (1000-2000 µm) of limestone or oyster shell than those fed fine particles (< 500 µm).

7.6. Conclusions

The current findings support the hypothesis that the laying hens, at 40 weeks of age, absorb Ca more efficiently than the broilers. The apparent ileal digestibility coefficient of limestone A (with finer particles) for broilers and layers were determined to be 0.50 and 0.62, respectively. The corresponding values for limestone B (with coarser particles) were 0.43 and 0.70 respectively. This is the first published report of the ileal Ca digestibility of limestone for laying hens.

CHAPTER 8

Requirement of digestible calcium at different dietary concentrations of digestible phosphorus for broiler chickens during day 1 to 10 post-hatch

8.1. Abstract

An experiment was conducted to determine the digestible calcium (Ca) and digestible phosphorous (P) requirements of 10-day-old broiler chickens. Fifteen maize-soybean meal-based diets containing 3.3, 3.9, 4.4, 5.0 and 5.5 g/kg standardised ileal digestible (SID) Ca and 4.0, 5.0 and 6.0 g/kg SID P were fed to broilers from days 1 to 10. Each experimental diet was randomly allocated to six replicate cages (12 birds per cage). Body weight and feed intake were recorded at the start and end of experiment and the feed conversion ratio was calculated. On day 10, birds were euthanised to collect ileal digesta, toes and tibia for the determination of digestible Ca and P, toe ash concentration and the concentrations of ash, Ca and P in tibia. Titanium dioxide (5 g/kg) was included in all diets as an indigestible indicator for apparent ileal digestibility measurements. Total excreta were collected from day 1 to 10 for the measurement of total tract retention of Ca and P. The growth performance, bone mineralisation and mineral utilisation of broiler starters were found to be optimised at 5 g/kg SID P concentration. Required SID Ca for maximum weight gain and bone mineralisation were determined to be 3.32 and 4.36-4.78 g/kg, respectively, at 5 g/kg SID P concentration, which correspond to SID Ca to SID P ratios of 0.66 and 0.87-0.96, respectively. The estimated SID Ca requirement for weight gain is lower than the current Ca recommendation (9.6 g/kg total Ca or 4.4 g/kg SID Ca) for broiler starters. However, bone mineralisation is maximised around the current total Ca recommendation at 8.9-9.8 g/kg (4.36-4.78 g/kg SID Ca) and indicates that bone mineralisation requires more Ca than growth performance.

8.2. Introduction

Calcium is important to build up skeletal health and a wide range of functions in the body such as blood clotting, muscle contraction, nerve impulse transmission, egg production, enzyme activation, metabolic reactions, protein synthesis, maintenance of osmotic and acid-base balances and, components in membranes (Crenshaw, 2001). Dietary P also plays a vital role in skeletal development and synthesis of other tissues in broilers (Bertram, 1995). A balance needs to be maintained between Ca and P because of their close interaction which influences the absorption and utilisation of both minerals (Crenshaw, 2001). In general, total Ca to available P ratio of 2:1 is being maintained in commercial broiler diets (Angel, 2013). Excess or deficiency in one of the minerals can lead to reduced utilisation of the other (Shafey *et al*., 1991).

In recent years, digestible P has been suggested as preferred term to express P availability in feed ingredients (WPSA, 2013). However, in poultry, the requirement of P is still being considered on available P basis (Ross, 2019). In pigs, however, recommendations for the requirement of digestible Ca and digestible P for growth performance, bone ash and Ca retention have now been established (González-Vega *et al*., 2016a,b; Merriman *et al*., 2017; Lagos *et al*., 2019a,b; Lee *et al*., 2019). It has been reported that pigs of 11-25, 25-50, 50-85 and 100-130 kg body weights should be fed the standardised total tract digestible (STTD) Ca concentration not more than 4.6 (Lagos *et al*., 2019b), 3.6 (González-Vega *et al*., 2016b), 3.4 (Lagos *et al*., 2019a) and 2.9 (Merriman *et al*., 2017) g/kg, respectively, for maximum growth performance, which corresponds to STTD Ca to STTD P ratios of less than 1.40:1, 1.35:1, 1.25:1 and 1.10:1, respectively. On the other hand, the reported dietary STTD Ca requirement for the pigs of 11-25 (Lagos *et al*., 2019b), 25-50 (González-Vega *et al*., 2016b) and 50-85 (Lagos *et al*., 2019a) kg body weights to maximise bone ash were 4.8, 5.6 and 5.5 g/kg, respectively, which corresponds to a STTD Ca to STTD P ratio of 1.70:1, 1.80:1 and 2.00:1,

respectively. However, similar comprehensive studies in poultry are non-existent and total Ca still being used in feed formulations. The shift to digestible P system may result in the oversupply of Ca. Unlike pigs, the nutrient digestibility measured at the terminal ileum (Lemme *et al*., 2004) is unquestionably accepted in poultry because of limitations in the total tract digestibility measurement (Ravindran *et al*., 1999). There is only one report available on digestible Ca requirement (Angel, 2018), but no experimental data were provided. The requirements of digestible Ca and digestible P for 1 to 10 day old broilers were 6.1 and 5.3 g/kg, respectively, which correspond to a SID Ca to SID P ratio of 1.15 (Angel, 2018; van Krimpen, 2016). Clearly research is warranted to investigate the requirements of digestible Ca and digestible P for broilers. Towards this end, the current study was designed to determine the requirements of standardised ileal digestible (SID) Ca and SID P for broiler starters (day 1-10 post-hatch) with different concentrations of digestible Ca and digestible P. Because ileal Ca digestibility coefficients have been reported recently for the main Ca sources in broilers (Anwar *et al*., 2015; 2016a,b,c; 2017; 2018; Chapters 3 to 7) and it is now possible to formulate diets based on the digestible Ca in feed ingredients. The objective of the current study was to determine the requirements for digestible Ca and digestible P in broiler chickens during day 1 to 10 post-hatch to maximise growth performance, bone mineralisation and Ca and P retention.

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. Experimental diets

The ingredients (maize, soybean meal, limestone, dicalcium phosphate and monosodium phosphate) were obtained from commercial sources and analysed for nutrient composition. The analysed Ca and P concentrations were used to formulate the assay diets.

The recommended requirements of total Ca and available P for Ross 308 broiler starters (1-10 day posthatch) are 9.6 and 4.8 g/kg, respectively (Ross, 2019). Based on published values of digestible Ca and P in feed ingredients (Tables 8.1 and 8.2), equivalent SID Ca and SID P values were 4.4 and 5.4 g/kg, respectively. Therefore, a range of digestible Ca (3.3 to 5.5 g/kg) and digestible P (4 to 6 g/kg) which are below and above these recommended values were considered in the development of treatments. Fifteen experimental starter diets based on maize-soybean meal were formulated in a 5 × 3 factorial arrangement with diets containing five concentrations of Ca and three concentrations of P (Table 8.3). Diets were formulated to contain 3.3, 3.9, 4.4, 5.0 and 5.5 g/kg SID Ca (corresponding to 7, 8, 9, 10 and 11 g/kg total Ca, respectively), and 4, 5 and 6 g/kg SID P (corresponding to 5.3, 6.8 and 8.3 g/kg total P, respectively) as indicated in Table 8.4. Concentrations of SID Ca ranged from 0.73-1.15 times the equivalent requirements for total Ca (Ross, 2019). All experimental diets were isoenergetic and isonitrogenous, and formulated by varying the inclusions of corn, soybean meal and soybean oil. Each diet was separately mixed and pelleted. The diets were steam-conditioned to 70°C for 30 s and pelleted using a pellet mill (Model Orbit 15; Richard Sizer Ltd., Kingston-upon-Hull, UK) capable of manufacturing 180 kg of feed/h and equipped with a die ring with 3 mm holes and 35 mm thickness.

Table 8.1. Total and standardised ileal digestible (SID) phosphorous (P) contents of feed ingredients

Ingredient	Total P (g/kg)[1]	SID P digestibility (%)	SID P (g/kg)
Maize	2.30	70[2]	1.61
Soybean meal	5.90	75[2]	4.43
Dicalcium phosphate	185	79[3]	146
Monosodium phosphate	225	67[4]	151

[1]Analysed values.
[2] NRC. (2015a).
[3]van Harn *et al*. (2017).
[4]Shastak *et al*. (2012).

8.3.2. Birds

A total of 1080, day-old male broilers (Ross 308) were obtained from a commercial hatchery, weighed, and allocated (mean ± SD, 45 ± 0.41 g) to 90 electrically heated battery brooders (12 per brooder cage). Each of the 15 diets was randomly assigned into six replicate cages. The experimental diets were offered *ad libitum* from day 1 to 10 post-hatch and water was available at all the times.

Table 8.2. Total and standardised ileal digestible (SID) calcium (Ca) content of feed ingredients

Ingredients	Total Ca (g/kg)[1]	SID Ca digestibility (%)	SID Ca (g/kg)
Maize	0.20	50[2]	0.10
Soybean meal	3.50	54[3]	1.89
Dicalcium phosphate	260	36[4]	93.6
Limestone	410	55[4,5]	226

[1] Analysed values.
[2] Assumed value.
[3] Chapter 6.
[4] Chapter 3.
[5] Anwar *et al*. (2016c).

8.3.3. Measurements

8.3.3.1. Growth performance

Body weights and feed intake were recorded on a cage basis at the start and end of the experimental period. Mortality was recorded daily. Feed conversion ratio (FCR) was corrected for the body weight of any bird that died during the experiment.

8.3.3.2. Ileal digestibility and apparent total tract retention of Ca and P

At the end of the experiment, eight birds per replicate were euthanised by cervical dislocation and the contents of the lower half of ileum were collected and processed as described in Chapter 3, section 3.3.3. Total excreta samples were also collected during all ten days, pooled within a cage and processed as described in Chapter 4, section 4.3.3.

Table 8.3. Ingredient composition of experimental diets (g/kg, as fed basis)

SID Ca	**3.3**			**3.9**			**4.4**			**5.0**			**5.5**		
SID P	**4.0**	**5.0**	**6.0**	**4.0**	**5.0**	**6.0**	**4.0**	**5.0**	**6.0**	**4.0**	**5.0**	**6.0**	**4.0**	**5.0**	**6.0**
Total calcium	7.0	7.0	7.0	8.0	8.0	8.0	9.0	9.0	9.0	10.0	10.0	10.0	11.0	11.0	11.0
SID Ca: SID P	0.84	0.67	0.56	0.97	0.78	0.65	1.11	0.89	0.74	1.25	1.00	0.83	1.39	1.11	0.92
Maize	568	565	553	563	560	548	558	555	543	553	550	538	548	545	533
Soybean meal	361	362	364	362	362	364	363	363	365	363	364	366	364	365	367
Di-calcium phosphate	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.2	10.2	10.2	10.2	10.2	10.2
Monosodium phosphate	0.00	6.7	13.4	0.00	6.7	13.4	0.00	6.7	13.4	0.00	6.7	13.4	0.00	6.7	13.4
Limestone	7.3	7.3	7.3	9.7	9.7	9.7	12.2	12.2	12.2	14.6	14.6	14.6	17.0	17.0	17.0
Sodium chloride	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Sodium bicarbonate	5.9	1.0	0.00	5.9	1.0	0.00	5.9	1.0	0.00	5.8	1.0	0.00	5.8	1.0	0.00
DL Methionine	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
Lysine HCl	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.7	4.8	4.8	4.7
L Threonine	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
L Valine	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Vitamin premix[1]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mineral premix[1]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Choline chloride 60%	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Titanium dioxide	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Soybean oil	26.4	27.7	31.7	28.1	29.4	33.4	29.8	31.1	35.1	31.5	32.8	36.8	33.2	34.5	38.5

[1]Supplied per kilogram of diet: vitamin A (trans-retinyl acetate), 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 IU; 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.
[1]Vitamin and mineral premix contained no calcium.
SID Ca: standardised ileal digestible calcium, SID P: Standardised ileal digestible phosphorous.

Table 8.4. Calculated and analysed nutrient composition of experimental diets (g/kg, as fed basis)

SID Ca	**3.3**			**3.9**			**4.4**			**5.0**			**5.5**		
SID P	**4.0**	**5.0**	**6.0**	**4.0**	**5.0**	**6.0**	**4.0**	**5.0**	**6.0**	**4.0**	**5.0**	**6.0**	**4.0**	**5.0**	**6.0**
SID Ca: SID P	0.84	0.67	0.56	0.97	0.78	0.65	1.11	0.89	0.74	1.25	1.00	0.83	1.39	1.11	0.92
Total Ca	7.0	7.0	7.0	8.0	8.0	8.0	9.0	9.0	9.0	10.0	10.0	10.0	11.0	11.0	11.0
Non-phytate P	3.37	4.87	6.38	3.37	4.87	6.39	3.38	4.88	6.39	3.38	4.88	6.40	3.39	4.89	6.40
Total Ca: Non-phytate P	2.08	1.44	1.10	2.37	1.64	1.25	2.66	1.85	1.41	2.96	2.05	1.56	3.25	2.25	1.72
Dry matter	883	882	878	882	881	878	880	879	876	879	878	875	878	877	873
AME (kcal/kg)	3000	3000	3000	3000	3000	3000	3000	3000	3000	3000	3000	3000	3000	3000	3000
Crude protein	220	220	220	220	220	220	220	220	220	220	220	220	220	220	220
Digestible protein	179	179	179	179	179	179	179	179	179	179	179	179	179	179	179
Starch	356	354	347	353	351	344	350	348	340	347	345	337	344	342	334
Crude fat	47.9	48.9	52.2	49.3	50.3	53.6	50.7	51.7	55.0	52.1	53.1	56.4	53.5	54.5	57.8
Crude fiber	28.3	28.2	28.1	28.2	28.2	28.0	28.2	28.1	27.9	28.1	28.0	27.9	28.0	28.0	27.8
Total Ca	7.0	7.0	7.0	8.0	8.0	8.0	9.0	9.0	9.0	10.0	10.0	10.0	11.0	11.0	11.0
SID Ca	3.33	3.33	3.33	3.88	3.88	3.88	4.43	4.43	4.43	4.98	4.98	4.98	5.53	5.53	5.53
Total P	5.30	6.80	8.30	5.30	6.80	8.30	5.30	6.80	8.30	5.30	6.80	8.30	5.30	6.80	8.30
Phytate P	1.93	1.93	1.92	1.93	1.93	1.91	1.92	1.92	1.91	1.92	1.92	1.90	1.91	1.91	1.90
SID P	4.0	5.0	6.0	4.0	5.0	6.0	4.0	5.0	6.0	4.0	5.0	6.0	4.0	5.0	6.0
Chloride	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
Sodium	2.3	2.3	3.3	2.3	2.3	3.3	2.3	2.3	3.3	2.3	2.3	3.3	2.3	2.3	3.3
Potassium	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
Choline (mg/kg)	1700	1700	1700	1700	1700	1700	1700	1700	1700	1700	1700	1700	1700	1700	1700
Dig. threonine	8.60	8.60	8.60	8.60	8.60	8.60	8.60	8.60	8.60	8.60	8.60	8.60	8.60	8.60	8.60
Dig. alanine	8.13	8.12	8.09	8.12	8.11	8.08	8.10	8.10	8.07	8.09	8.08	8.06	8.08	8.07	8.05
Dig. valine	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60
Dig. isoleucine	7.29	7.29	7.30	7.30	7.30	7.30	7.30	7.30	7.31	7.30	7.30	7.31	7.30	7.30	7.31
Dig. leucine	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	14.9	15.0	15.0	14.9	15.0	14.9	14.9
Dig. lysine	12.8	12.8	12.8	12.8	12.8	12.8	12.8	12.8	12.8	12.8	12.8	12.8	12.8	12.8	12.8
Dig. arginine	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6	12.6
Dig. cysteine	2.93	2.93	2.92	2.93	2.93	2.91	2.92	2.92	2.91	2.92	2.92	2.90	2.91	2.91	2.90
Dig. methionine	6.57	6.57	6.58	6.57	6.57	6.59	6.58	6.58	6.59	6.58	6.58	6.60	6.59	6.59	6.60
Dig. methionine + cysteine	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50

Analysed values (as fed basis)[1]																
Dry matter	883	882	878	882	881	878	880	879	876	879	878	875	878	877	873	
Total Ca	6.9	7.1	7.3	7.9	7.7	7.8	8.2	9.2	9.1	11.2	10.7	10.2	11.4	11.6	11.0	
Total P	5.5	7.5	8.7	5.6	7.1	8.6	6.9	7.4	9.0	6.8	7.5	8.7	5.9	7.6	8.9	

Ca: calcium, P: phosphorous, SID: standardised ileal digestible, AME: apparent metabolisable energy, Dig.: digestible.
[1]Samples were analysed in triplicate.

8.3.3.3. Bone mineralisation

Middle toe of the left feet and right tibia were removed from 8 birds per replicate (from the birds euthanised for ileal digesta collection) and immediately frozen at -20 °C. Tibiae were cleaned from all adherent tissues and were kept frozen in airtight plastic bags until the measurements. Tibiae were oven dried at 105 °C for 24 hours, de-fatted by refluxing petroleum ether in a Soxhlet apparatus for 16 hours, oven-dried at 105 °C overnight for dry defatted bone weight determination, and ashed in ceramic crucibles for 24 hours at 600 °C for fat-free ash weight determination. Tibia ash content was expressed as a percentage of dry bone weight. The toe samples were weighed and dry ashed at 550 °C for 24 hours for determination of toe ash (Ravindran *et al*., 1995b). Tibia Ca and P concentrations were determined and expressed as g/kg dried defatted bone.

8.3.3.4. Carcass retention of Ca and P

At the start of the trial (day 1), 10 additional chicks were killed by cervical dislocation. At the end of experiment (day 10), four birds per replicate were randomly selected, fasted overnight, weighed and killed by cervical dislocation with minimum blood loss. At both ages, feathers were removed, the carcass weight was recorded and defeathered carcasses were stored at -20°C. In this thesis, the term ‘carcass’ refers to the whole body without feathers. The frozen carcasses were cut into small pieces and minced twice to obtain homogenous sub-samples. Feathers were removed at both ages, freeze-dried, weighed, and analysed for Ca and P to study the Ca and P retention in feathers, but these were not included in carcass retention calculations. The concentration of Ca and P and the retention of Ca and P in the feathers at both ages are presented in Appendix C.4.

8.3.4. Chemical Analysis

Ingredients were analysed for dry matter (DM; method 930.15; AOAC, 2016), ash (942.05; AOAC, 2016), nitrogen (968.06; AOAC, 2016), fat (AOAC 2003.06), crude fiber (AOAC 2002.04), Ca and total P (method 968.08D; AOAC, 2016) and phytate P (Caldwell, 1992). The concentrations of ash (AOAC 942.05; AOAC 2016), Ca and P (968.06; AOAC, 2016) of tibia and the concentration of toe ash (AOAC 942.05; AOAC 2016) were determined using standard procedures. The diet, ileal digesta and excreta samples were analysed for DM (method 930.15; AOAC, 2016), Ca and total P (method 968.08D; AOAC, 2016) and titanium dioxide (Short *et al*., 1996). Subsamples of the minced carcass were analysed for DM (method 930.15; AOAC, 2016), Ca and P (method 968.08D; AOAC, 2016).

8.3.5. Calculations

The apparent ileal digestibility coefficients (AIDC) of Ca and P were calculated using titanium marker ratios in the diet and ileal digesta (Ravindran *et al*., 1999) as indicated below. Analysed values were used in digestibility and retention calculations.

$$\text{AIDC of Ca or P} = 1 - [(Ti_I / Ti_O) \times (M_O / M_I)]$$

where Ti_I is the titanium concentration in the diet, Ti_O is the titanium concentration in the ileal digesta, M_O is the concentration of Ca or P in the ileal digesta, and M_I is the concentration of Ca or P in the diet. All concentrations were expressed as g/kg DM.

Standardised ileal digestibility coefficients (SIDC) of Ca and P were then calculated, based on previously determined values for endogenous Ca (108 mg/kg DM intake, Anwar, 2017) and P (25 mg/kg DM intake, Mutucumarana and Ravindran, 2020) values, as follows:

$$SIDC = AIDC + (IEL/M_I)$$

where SIDC and AIDC represent the coefficients of standardised ileal digestibility and apparent ileal digestibility of Ca or P, respectively, IEL represents the ileal endogenous losses

(mg per kg DM intake) of Ca or P and M_I represents the concentration of Ca or P in the diet (g per kg DM).

The apparent total tract retention coefficient (ATTRC) of Ca and P (% intake) was calculated using following equation:

$$\text{ATTRC of Ca or P} = [(M_I \times FI) - (M_E \times EO) / M_I \times FI]$$

where M_I is the concentration of Ca or P in the diet (g/kg DM), FI is the feed intake of birds (g, DM basis), M_E is the concentration of Ca or P in the excreta (g/kg DM) and EO is the excreta output (g, DM basis).

The intake of SID Ca or P and the retained Ca or P (g/bird) were calculated using following equations:

$$\text{Intake of SID Ca or P} = (FI \times M_I \times SIDC)$$

$$\text{Retained Ca or P} = (FI \times M_I \times ATTRC)$$

where FI is the feed intake of birds (g/bird, DM basis), M_I is the concentration of Ca or P in the diet (g/kg DM).

The retained Ca or P (g/bird) in the carcass was calculated using following equation:

$$\text{Retained Ca or P} = [(Mc \times CW)_{D10} - (Mc \times CW)_{D1}]$$

where M_c is the concentration of Ca or P in the carcass (g/kg DM), CW is the carcass weight (g/bird) and D10 and D1 denote 10-day old and day-old birds, respectively.

8.3.6. Statistical Analysis

Data were analysed using the General Linear Model (GLM) procedure of SAS (2019), with cage serving as the experimental unit. Two sets of analyses were conducted. First, as a factorial arrangement of treatments examining the effects dietary concentrations of SID Ca and SID P and their interaction. The effects were considered significant at $P \leq 0.05$. Second, if the

interaction or main effects were significant, then the parameter estimates for the second-order response surface model were determined using GLM procedure of SAS (2019). All calculations started with the full model, but if needed, the model was reduced by removing parameter estimates that were not significant ($P > 0.05$) and the estimates were recalculated using the reduced model as described by González-Vega *et al*., 2016b). Linear and quadratic effects of both SID Ca and SID P and the interaction between SID Ca and SID P were included in the full model as follows:

$$Y = a + b \times \text{SID Ca} + c \times \text{SID Ca}^2 + d \times \text{SID P} + e \times \text{SID P}^2 + f \times \text{SID Ca} \times \text{SID P}$$

Where Y is the dependent variable, a is the intercept, b, c, d, e and f are the coefficients, and SID Ca and SID P are the concentrations (g/kg) of dietary SID Ca and SID P.

The concentrations of SID Ca at the maximum response values were calculated using the following equation:

$$\text{SID Ca}_{\text{max}}\,(\text{g/kg}) = [(-f \times \text{SID P}) - b)] / (2 \times c)$$

Where $\text{SID Ca}_{\text{max}}$ is the concentration of SID Ca at the maximum response and SID P is the concentration of SID P in the diet.

The maximum response values were, therefore, calculated using the respective model equations with the concentrations of SID Ca at the maximum response for each concentration of SID P.

The response surface plots were also generated for all measured parameters using R-programme, version 3.6.1. (2019) and are presented in Appendix C.1.

The relationship between measured parameters were analysed by Pearson Correlations (SAS, 2019).

8.4. Results

Analysed total Ca concentrations of the ingredients are presented in Table 8.5. Analysed Ca concentrations of the experimental diets differed by -0.84 to 1.18 g/kg from calculated concentrations. Analysed P concentrations were 0.20-1.60 g/kg higher than the calculated concentrations. Analysed Ca and P values were used to calculate the Ca and P digestibility of assay diets.

Table 8.5. Analysed nutrient and mineral composition of calcium and phosphorous (P) supplements (g/kg, as received basis)[1]

Nutrient	Maize	Soybean meal	Limestone	Dicalcium phosphate	Monosodium phosphate
Dry matter	897	906	1,000	969	974
Ash	12	64	996	848	828
Crude protein	75	456	-	-	-
Fat	37	25	-	-	1.0
Neutral detergent fibre	85	82	-	-	-
Calcium	0.2	3.5	400	260	-
Total P	2.3	5.9	0.56	190	225
Phytate	5.0	11	-	-	-
Phytate P	1.5	3.0	-	-	-
Non-phytate P[2]	0.8	2.9	-	-	-
Sodium	< 0.05	< 0.05	< 0.50	0.71	196

[1]Samples were analysed in duplicate.
[2]Calculated as the difference between total P and phytate P.

Determined concentrations of SID Ca and SID P of the 15 assay diets, in comparison with formulated values, are summarised in Table 8.6. The calculated concentrations of both SID Ca and SID P, based on published ingredient values (Tables 8.1 and 8.2) were remarkably closer to determined concentrations.

8.4.1. Growth performance

All birds remained healthy during the experiment. Eight birds out of 1080 died and the deaths were not related to specific dietary treatment.

Table 8.6. Comparison of calculated and determined[1] values of standardised ileal digestible calcium (SID Ca) and standardised ileal digestible phosphorous (SID P) of experimental diets (g/kg, as fed basis)

SID Ca	3.3			3.9			4.4			5.0			5.5		
SID P	4.0	5.0	6.0	4.0	5.0	6.0	4.0	5.0	6.0	4.0	5.0	6.0	4.0	5.0	6.0
SID Ca: SID P	0.84	0.67	0.56	0.97	0.78	0.65	1.11	0.89	0.74	1.25	1.00	0.83	1.39	1.11	0.92
Determined SID Ca	3.3	3.3	3.6	3.9	3.7	3.8	4.5	4.3	4.0	5.8	5.1	4.9	5.4	4.9	5.1
Determined SID P	3.4	4.4	6.0	3.3	4.4	5.8	3.3	4.4	5.5	3.2	4.1	5.5	2.9	3.8	5.2
Determined SID Ca: SID P	0.97	0.75	0.60	1.18	0.84	0.66	1.36	0.98	0.73	1.81	1.24	0.89	1.86	1.29	0.98
Difference (calculated minus determined)															
SID Ca	0.0	0.0	-0.3	0.0	0.2	0.1	-0.1	0.1	0.4	-0.8	-0.1	0.1	0.1	0.6	0.4
SID P	0.6	0.6	0.0	0.7	0.6	0.2	0.7	0.6	0.5	0.8	0.9	0.5	1.1	1.2	0.8

[1] Dietary calcium or phosphorous concentration x Determined SID Ca or SID P for the respective experimental diet.

The body weight gain, feed intake and FCR from 1 to 10 days of chicks fed diets containing different concentrations of SID Ca and SID P are summarised in Table 8.7 and the trends are illustrated in Figure 8.1. There were interactions ($P < 0.001$) between SID Ca and SID P for all growth parameters. For body weight gain and feed intake, the full model was used to predict the SID Ca at maximum response. However, a reduced model was used for FCR. At lower SID Ca concentrations (3.3 and 3.9 g/kg), the weight gain was higher at 5 g/kg SID P concentration than at 4 and 6 g/kg. However, the weight gain was higher at 6 g/kg SID P concentration if the dietary SID Ca was 5.5 g/kg and was lower at 4 g/kg SID P if the SID Ca concentrations were above 4.4 -5.5 g/kg. At 4 g/kg SID P concentration, the weight gain was observed to be depressed ($P < 0.001$) with increasing SID Ca concentrations. The predicted maximum body weight gains at SID P concentrations of 4, 5 and 6 g/kg were 248, 255 and 247 g/bird, at SID Ca concentrations of 2.02, 3.32 and 4.62 g/kg, respectively. These values correspond to SID Ca to SID P ratios of 0.51, 0.66 and 0.77, respectively.

Similarly, feed intake was higher ($P < 0.05$) at 5 g/kg SID P if the SID Ca concentration was below 4.4 g/kg and was lower at 4 g/kg SID P if the SID Ca concentrations were above 4.4 g/kg. The predicted maximum feed intake at SID P concentrations of 5 and 6 g/kg were 272 and 257 g/bird, at SID Ca concentrations of 2.33 and 3.87 g/kg, respectively. These values correspond to SID Ca to SID P ratios of 0.47 and 0.65, respectively. Maximum value was not calculated for feed intake at 4 g/kg due to the lack of response. Maximum values were not calculated for FCR due to the linear nature of the response.

8.4.2. Standardised ileal Ca and P digestibility coefficients, intake of both SID Ca and SID P and the ratio between SID Ca and SID P intakes

Data on standardised ileal digestibility coefficients of Ca and P, the intake of SID Ca and SID P and the ratio of SID Ca intake to SID P intake in 1 to 10-day old broilers are presented in Table 8.8 and Figure 8.2.

Table 8.7. Growth performance of broiler chickens fed diets containing different concentrations of standardised ileal digestible (SID) calcium (Ca) and SID phosphorous (P) from day 1 to 10[1]

SID Ca (g/kg)	SID P (g/kg)	Body weight gain (g/bird)	Feed intake (g/bird)	Feed conversion ratio
3.3	4	240^{ef}	258^{bcd}	1.08^{a}
	5	257^{a}	269^{a}	1.06^{abc}
	6	241^{def}	255^{cd}	1.06^{ab}
3.9	4	240^{ef}	254^{cde}	1.05^{abc}
	5	253^{ab}	268^{ab}	1.07^{a}
	6	242^{def}	256^{cd}	1.06^{abc}
4.4	4	224^{g}	240^{f}	1.07^{a}
	5	251^{abc}	260^{abc}	1.04^{bcd}
	6	248^{bcd}	258^{bcd}	1.04^{bcd}
5.0	4	213^{h}	225^{g}	1.07^{a}
	5	244^{cde}	253^{cde}	1.04^{cd}
	6	248^{bcd}	252^{cde}	1.02^{d}
5.5	4	204^{i}	220^{g}	1.08^{a}
	5	235^{f}	245^{ef}	1.06^{abc}
	6	244^{cde}	250^{def}	1.02^{d}
SEM[2]		2.8	3.6	0.009
Main Effects				
SID Ca				
3.3		246	261	1.07
3.9		245	259	1.06
4.4		241	253	1.05
5.0		235	243	1.04
5.5		228	238	1.05
SEM		1.6	2.1	0.005
SID P				
4		224	239	1.07
5		248	259	1.05
6		245	254	1.04
SEM		1.3	1.6	0.004
Probabilities, $P \leq$				
SID Ca		0.001	0.001	0.008
SID P		0.001	0.001	0.001
SID Ca × SID P		0.001	0.001	0.032

[1]Each value represents the mean of six replicates (twelve birds per replicate).
[a-i] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]Pooled standard error of mean.

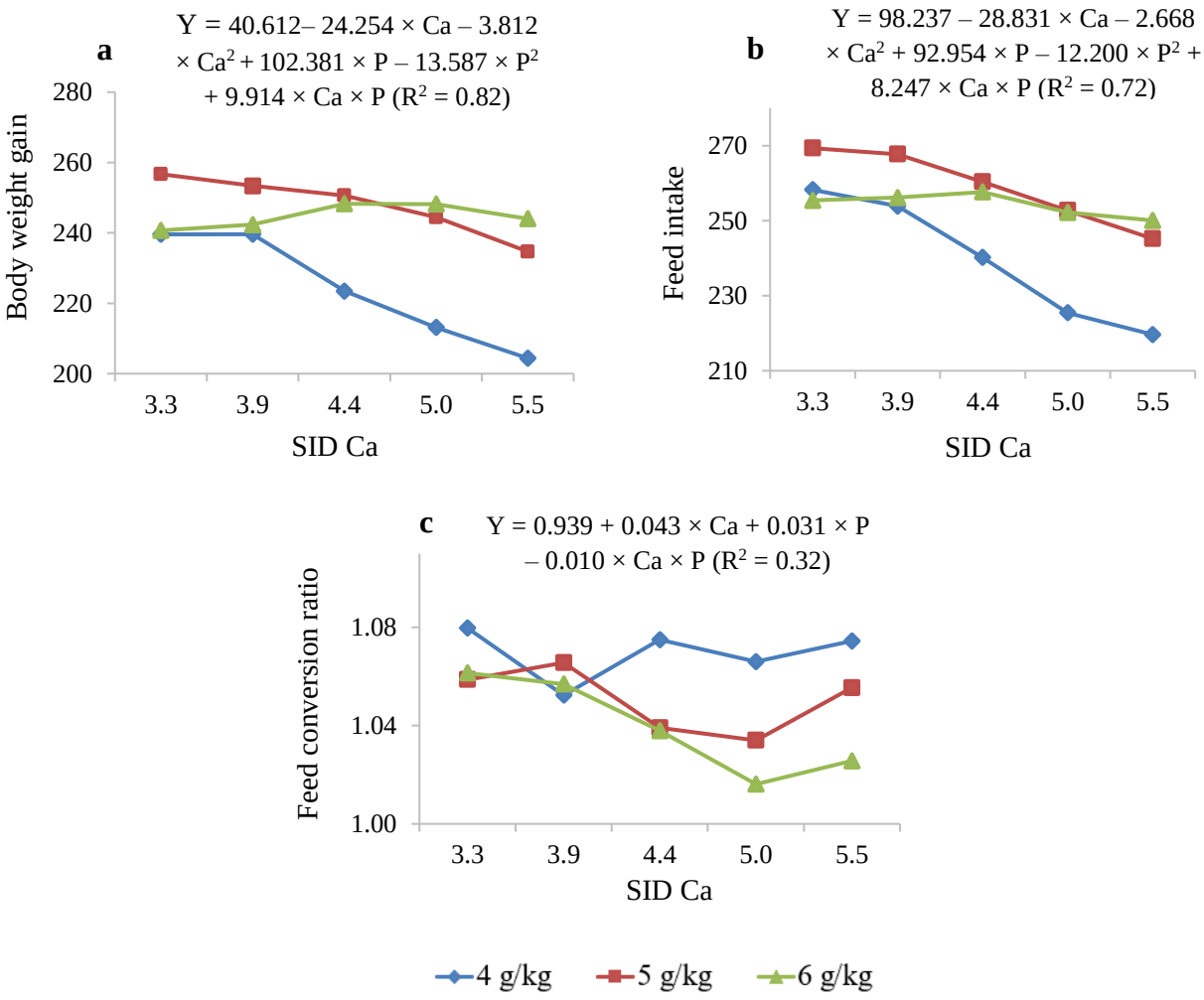

Figure 8.1. (a) Body weight gain (g/bird); (b) feed intake (g/bird) and (c) feed conversion ratio of broiler chickens fed different standardised ileal digestible (SID) calcium (Ca) and SID phosphorous (P) concentrations (4, 5 and 6 g/kg) from day 1 to 10

The reduced model was used to predict these parameters. For the standardised ileal Ca digestibility coefficient, the main effects and their interaction were not significant and therefore, the maximum values were not calculated. The digestible Ca intake was influenced by dietary SID Ca concentration where the intake was linearly increased ($P < 0.001$) as the SID Ca concentration increased. Therefore, the maximum values were not calculated for digestible Ca intake. Standardised ileal P digestibility linearly increased ($P < 0.001$) with increasing SID P and reducing SID Ca concentrations**.** The intake of digestible P was increased ($P < 0.001$) with increasing SID P concentration and with decreasing SID Ca concentrations.

Table 8.8. Standardised ileal digestibility coefficient (SIDC) of calcium (Ca) and phosphorous (P), intake (g/bird) of standardised ileal digestible (SID) Ca and SID P, and the ratio of SID Ca intake to SID P intake, in broiler chickens fed different concentrations (g/kg) of SID Ca and SID P from day 1 to 10[1]

SID Ca	SID P	SIDC of Ca	SID Ca intake	SIDC of P	SID P intake	SID Ca intake: SID P intake
3.3	4	0.47	0.85	0.56	0.87	0.98^{d}
	5	0.46	0.88	0.60	1.18	0.74^{fg}
	6	0.51	0.92	0.69	1.54	0.60^{h}
3.9	4	0.50	0.99	0.54	0.83	1.20^{c}
	5	0.47	0.98	0.60	1.17	0.84^{ef}
	6	0.48	0.97	0.68	1.49	0.65^{gh}
4.4	4	0.51	1.09	0.54	0.79	1.39^{b}
	5	0.49	1.13	0.60	1.15	0.97^{d}
	6	0.46	1.04	0.64	1.42	0.73^{fg}
5.0	4	0.54	1.31	0.52	0.71	1.83^{a}
	5	0.48	1.29	0.56	1.03	1.25^{c}
	6	0.46	1.24	0.63	1.39	0.89^{de}
5.5	4	0.48	1.19	0.48	0.64	1.85^{a}
	5	0.43	1.21	0.52	0.93	1.29^{bc}
	6	0.45	1.28	0.60	1.30	0.98^{d}
SEM[2]		0.024	0.058	0.015	0.031	0.043
Main effects						
SID Ca						
3.3		0.48	0.89^{d}	0.62^{a}	1.20^{a}	0.77
3.9		0.49	0.98^{c}	0.61^{a}	1.16^{ab}	0.90
4.4		0.49	1.08^{b}	0.59^{ab}	1.12^{b}	1.03
5.0		0.49	1.28^{a}	0.57^{b}	1.04^{c}	1.32
5.5		0.45	1.22^{a}	0.53^{c}	0.96^{d}	1.38
SEM		0.014	0.034	0.009	0.018	0.025
SID P						
4		0.50	1.09	0.53^{c}	0.77^{c}	1.45
5		0.47	1.10	0.57^{b}	1.09^{b}	1.02
6		0.47	1.09	0.65^{a}	1.43^{a}	0.77
SEM		0.011	0.026	0.007	0.014	0.019
Probabilities, $P \leq$						
SID Ca		0.301	0.001	0.001	0.001	0.001
SID P		0.071	0.938	0.001	0.001	0.001
SID Ca × SID P		0.391	0.878	0.664	0.848	0.001

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

[a-h] Means having different superscripts within the column are significantly different ($P < 0.05$).

[2]Pooled standard error of mean.

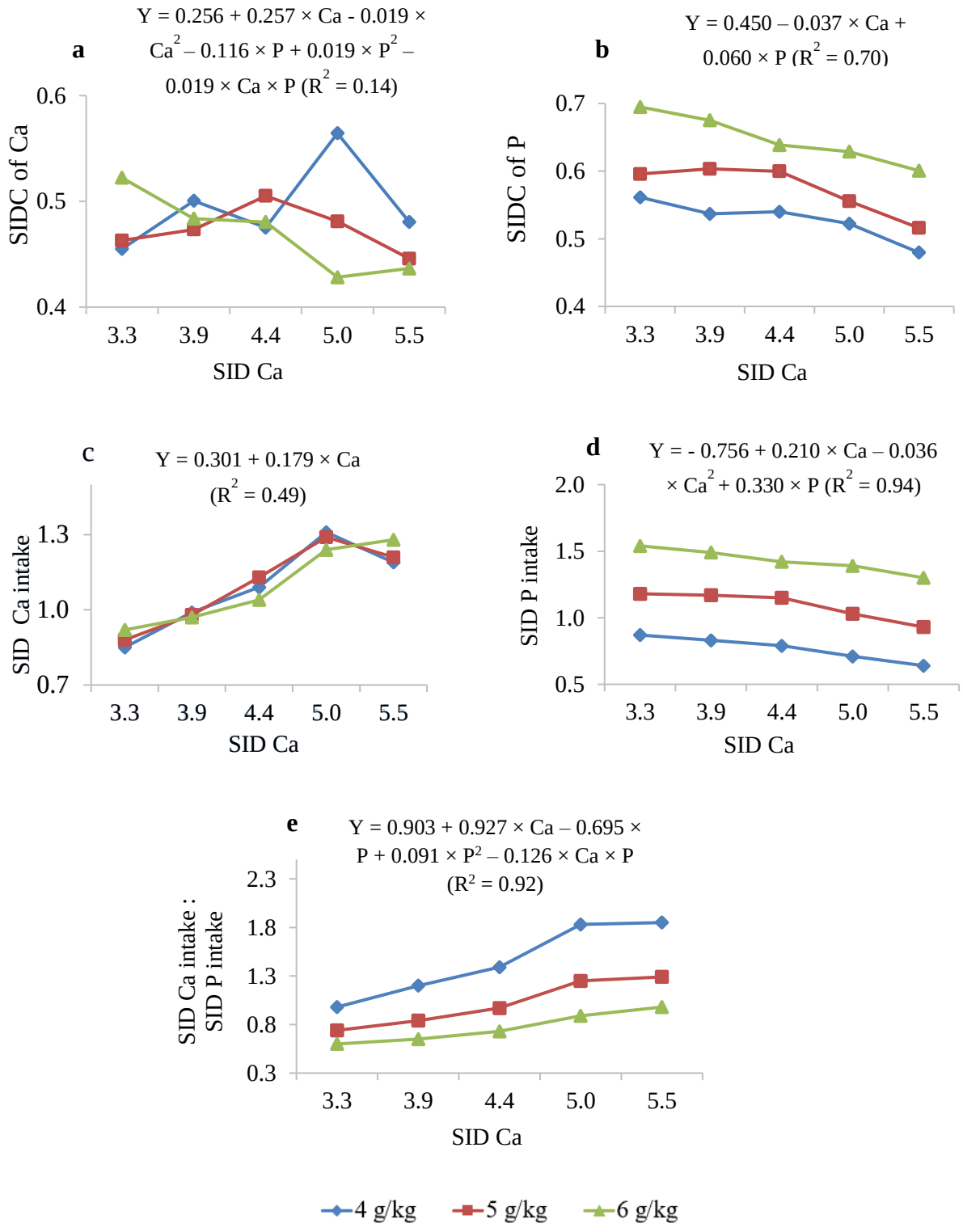

Figure 8.2. a) Standardised ileal digestibility coefficients (SIDC) of calcium (Ca) and b) phosphorous (P); intake (g/bird) of c) standardised ileal digestible (SID) Ca and d) SID P; and e) ratio of SID Ca intake: SID P intake, of broiler chickens fed different concentrations of SID Ca and SID P (4, 5 and 6 g/kg) from day 1 to 10

The predicted maximum intake of digestible P at SID P concentrations of 4, 5 and 6 g/kg were 0.80, 1.09 and 1.38 g/bird, at the SID Ca concentration of 2.91 g/kg. These values correspond to SID Ca to SID P ratios of 0.73, 0.58 and 0.49, respectively. The ratio of SID Ca intake to SID P intake was increased ($P < 0.001$) by increasing Ca concentrations and by decreasing SID P concentrations, resulting a SID Ca × SID P interaction ($P < 0.001$). The maximum values were not calculated for the SID P intake and the ratio of SID Ca intake: SID P intake due to the linear Ca effect.

8.4.3. Bone mineralisation

Table 8.9 and Figure 8.3 present the concentrations of ash, Ca and P of tibia and toe ash content of 10-day old broilers fed diets containing different SID Ca and SID P. The full model was used to predict the tibia parameters. The predicted maximum tibia ash content at SID P concentrations of 4, 5 and 6 g/kg was 352, 409 and 415 g/kg, at SID Ca concentrations of 3.47, 4.51 and 5.54 g/kg, respectively. These values correspond to SID Ca to SID P ratios of 0.87, 0.90 and 0.92, respectively. The predicted maximum tibia Ca content at SID P concentrations of 4, 5 and 6 g/kg was 115, 132 and 138 g/kg, at SID Ca concentrations of 3.27, 4.72 and 6.17 g/kg, respectively. These values correspond to SID Ca to SID P ratios of 0.82, 0.94 and 1.03, respectively. The predicted maximum tibia P content at SID P concentrations of 4, 5 and 6 g/kg was 55.5, 66.4 and 67.7 g/kg, at SID Ca concentrations of 3.22, 4.36 and 5.49 g/kg, respectively. These values correspond to SID Ca to SID P ratios of 0.80, 0.87 and 0.92, respectively. The toe ash was increased by increasing SID P concentrations at SID Ca concentrations of 3.3, 3.9 and 4.4 g/kg. The predicted maximum concentration of toe ash at SID P concentrations of 4, 5 and 6 g/kg was 34.4, 43.2 and 45.4 g/kg, at SID Ca concentrations of 3.96, 4.78 and 5.60 g/kg, respectively. These values correspond to SID Ca to SID P ratios of 0.99, 0.96 and 0.93, respectively.

Table 8.9. Concentration of ash, calcium (Ca) and phosphorous (P) in tibia (g/kg dried defatted matter) and toe ash concentration (g/kg, as received basis) in broiler chickens fed diets containing different concentrations (g/kg) of standardised ileal digestible (SID) Ca and SID P from day 1 to 10[1]

SID Ca	SID P	Tibia ash	Tibia Ca	Tibia P	Toe ash
3.3	4	354de	115de	56.0^{c}	34.8^{de}
	5	395^{bc}	126^{bc}	64.3^{ab}	40.3^{c}
	6	384^{c}	121^{cd}	62.5^{b}	40.8^{c}
3.9	4	357^{d}	116de	56.0^{c}	35.7^{d}
	5	401^{ab}	129^{ab}	65.1^{ab}	40.9^{c}
	6	401^{ab}	127^{ab}	65.8^{ab}	44.3^{a}
4.4	4	341^{ef}	109^{ef}	53.1^{cd}	33.1^{f}
	5	409 ^{a}	133^{ab}	66.3^{a}	42.5^{b}
	6	408^{ab}	132^{ab}	66.7^{a}	44.5^{a}
5.0	4	336^{fg}	110^{ef}	51.7^{de}	33.4^{ef}
	5	411^{a}	131^{ab}	66.3^{a}	44.9^{a}
	6	411^{a}	135^{a}	67.3^{a}	44.4^{a}
5.5	4	322^{g}	102^{f}	49.4^{e}	31.6^{g}
	5	410^{a}	134^{ab}	66.6^{a}	44.4^{a}
	6	411^{a}	135^{ab}	66.6^{a}	44.4^{a}
SEM[2]		5.0	3.1	1.24	0.54
Main Effects					
SID Ca					
3.3		377	121	60.9	38.6
3.9		387	124	62.3	40.3
4.4		386	125	62.0	40.0
5.0		386	125	61.8	40.9
5.5		381	124	60.9	40.1
SEM		2.9	1.8	0.71	0.31
SID P					
4		342	110	53.3	33.7
5		405	131	65.7	42.6
6		403	130	65.8	43.7
SEM		2.2	1.4	0.55	0.24
Probabilities, $P \leq$					
SID Ca		0.108	0.458	0.513	0.001
SID P		0.001	0.001	0.001	0.001
SID Ca × SID P		0.001	0.001	0.001	0.001

[1]Each value represents the mean of six replicates (eight birds per replicate).
[a-g] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]Pooled standard error of means.

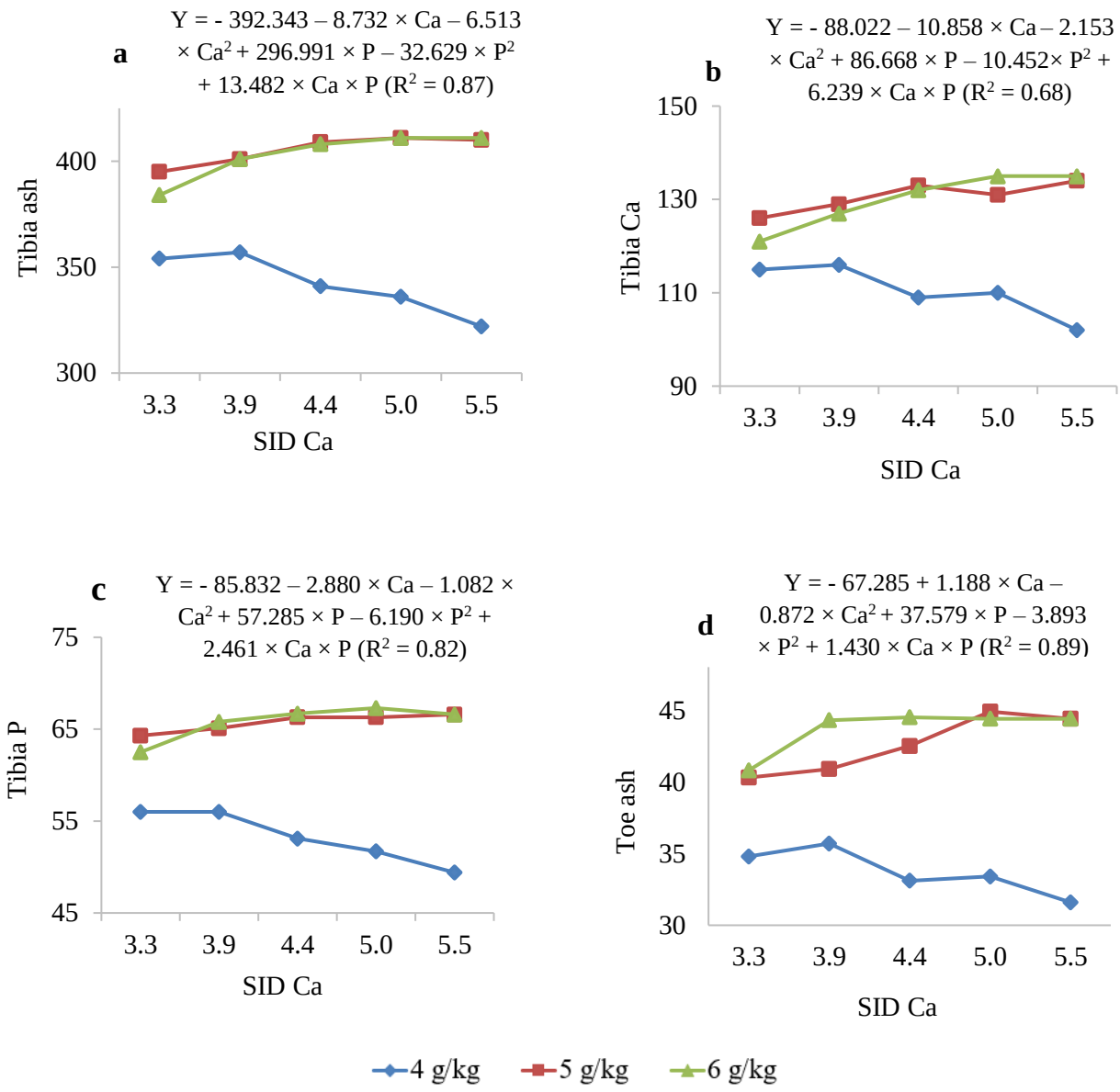

Figure 8.3. Concentrations (g/kg dried defatted matter) of a) ash, b) calcium (Ca) and c) phosphorous (P) of tibia and d) toe ash concentration (g/kg, as received basis) in broiler chickens fed different concentrations (g/kg) of standardised ileal digestible (SID) Ca and SID P (4, 5 and 6 g/kg) from day 1 to 10

8.4.4. Coefficients of apparent total tract retention and retained Ca and P

Table 8.10 summarises the total tract retained Ca and P in 10-day old birds fed diets containing different SID Ca and SID P. Apparent total tract retention coefficient (ATTRC) of Ca was lower at 4 g/kg SID P and increased with increasing SID P concentrations at all SID Ca concentrations. But the magnitude of increases was greater as the SID Ca concentrations increased, resulting in a SID Ca × SID P interaction ($P < 0.05$). A similar trend was observed for the total tract retained Ca (g/bird). The maximum values were not estimated for these parameters due to the linear Ca effect. At all SID Ca concentrations, the ATTRC of P was reduced at 6 g/kg SID P, but the magnitude of reductions differed in different SID Ca. This resulted in a SID Ca × SID P interaction ($P < 0.001$). Retained P (g/bird), on the other hand, increased at or above 5 g/kg SID P and the degree of increment varied at different SID Ca concentrations, resulting in a SID Ca × SID P interaction ($P < 0.001$). The predicted maximum retained P (g/bird) at SID P concentrations of 4, 5 and 6 g/kg was 1.00, 1.16 and 1.16 g/bird, at SID Ca concentrations of 2.62, 3.64 and 4.65 g/kg, respectively. These values correspond to SID Ca to SID P ratios of 0.66, 0.73 and 0.78, respectively. The ratio between retained Ca and retained P was higher ($P < 0.05$) at or above 5.0 g/kg SID Ca concentrations (Figure 8.4). The ratio was unaffected ($P > 0.05$) by SID P when the SID Ca was less than 4.4 g/kg and variable responses were noted at levels above 5.0 g/kg Ca, resulting in a SID Ca × SID P interaction ($P < 0.05$). Figure 8.4 illustrates the retention of Ca and P in 10-day old broiler chickens fed different SID Ca and SID P.

8.4.5. Carcass retention of Ca and P

Data on retention of Ca and P in the carcass of 10-day old birds fed diets containing different concentrations of SID Ca and SID P are presented in Table 8.11 and Figure 8.5. A reduced model was used for these parameters. An interaction ($P < 0.05$) was observed between SID Ca and SID P for carcass retention of Ca and P.

Table 8.10. Apparent total tract retention coefficients (ATTRC) of calcium (Ca) and phosphorous (P) and retained Ca and P in 10-day old broilers fed diets containing different concentrations of (g/kg) standardised ileal digestible (SID) Ca and SID phosphorous (P)[1]

SID Ca (g/kg)	SID P (g/kg)	ATTRC of Ca	Retained Ca (g/bird)	ATTRC of P	Retained P (g/bird)	Retained Ca: retained P ratio
3.3	4	0.51^{cd}	0.93^{gh}	0.65^{a}	1.02^{f}	0.92^{e}
	5	0.60^{a}	1.14^{de}	0.58^{bc}	1.15^{bcd}	0.99^{e}
	6	0.57^{ab}	1.03^{fg}	0.49^{e}	1.08^{def}	0.96^{e}
3.9	4	0.47^{e}	0.93^{gh}	0.60^{bc}	0.93^{g}	1.01^{e}
	5	0.55^{bc}	1.14^{de}	0.60^{bc}	1.17^{ab}	0.98^{e}
	6	0.57^{ab}	1.13^{de}	0.51^{e}	1.12^{bcde}	1.01^{e}
4.4	4	0.40^{f}	0.84^{h}	0.61^{b}	0.88^{g}	0.96^{e}
	5	0.55^{bc}	1.28^{bc}	0.60^{bc}	1.15^{bc}	1.11^{d}
	6	0.53^{bc}	1.21^{cd}	0.55^{d}	1.23^{a}	0.99 ^{e}
5.0	4	0.46^{e}	1.10^{ef}	0.59^{bc}	0.80^{h}	1.37^{ab}
	5	0.57^{ab}	1.53^{a}	0.58^{bc}	1.08^{cdef}	1.41^{a}
	6	0.54^{bc}	1.47^{a}	0.51^{e}	1.13^{bcde}	1.29^{bc}
5.5	4	0.40^{f}	1.01^{fg}	0.57^{cd}	0.77^{h}	1.32^{ab}
	5	0.53^{c}	1.46^{a}	0.59^{bc}	1.06^{ef}	1.38^{ab}
	6	0.48^{cd}	1.35^{b}	0.51^{e}	1.12^{bcde}	1.21^{c}
SEM[2]		0.014	0.036	0.011	0.026	0.033
Main Effects						
SID Ca						
3.3		0.56	1.03	0.57	1.08	0.96
3.9		0.53	1.07	0.57	1.07	1.00
4.4		0.49	1.11	0.59	1.09	1.02
5.0		0.52	1.36	0.56	1.00	1.36
5.5		0.47	1.27	0.56	0.98	1.30
SEM		0.008	0.021	0.006	0.015	0.019
SID P						
4		0.45	0.96	0.60	0.88	1.12
5		0.56	1.31	0.59	1.12	1.17
6		0.54	1.24	0.51	1.13	1.09
SEM		0.006	0.016	0.005	0.012	0.015
Probabilities, $P \leq$						
SID Ca		0.001	0.001	0.027	0.001	0.001
SID P		0.001	0.001	0.001	0.001	0.001
SID Ca × SID P		0.042	0.001	0.001	0.001	0.037

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

[a-h] Means having different superscripts within the column are significantly different ($P < 0.05$).

[2]Pooled standard error of mean.

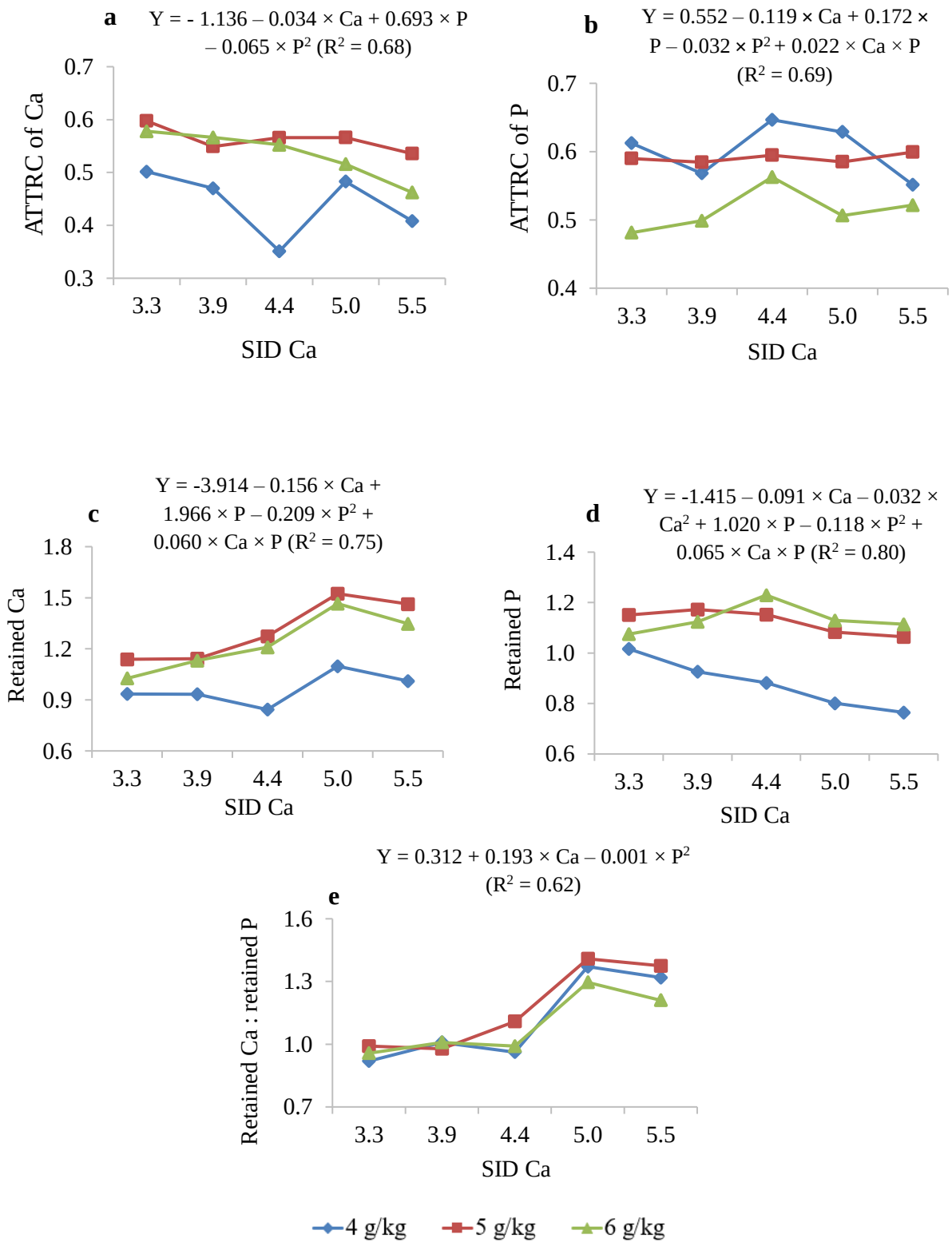

Figure 8.4. Apparent total tract retention coefficient (ATTRC) of a) Ca and b) phosphorous (P); retained (g/bird) c) Ca and d) P; e) ratio of retained Ca to retained P, of broiler chickens fed different concentrations (g/kg) of standardised ileal digestible (SID) Ca and SID P (4, 5 and 6 g/kg) from day 1 to 10

Table 8.11. Retention (g/bird) of calcium (Ca) and phosphorous (P) in the whole body (carcass) of 10-day old broilers fed diets containing different concentrations of (g/kg) standardised ileal digestible (SID) Ca and SID P[1,2,3]

SID Ca	SID P	Ca	P
3.3	4	0.94^{bc}	0.99
	5	1.07^{bc}	1.07
	6	1.07^{bc}	1.06
3.9	4	0.93^{bc}	0.94
	5	1.10^{b}	1.03
	6	1.34^{a}	1.21
4.4	4	1.01^{bc}	0.98
	5	1.33^{a}	1.17
	6	1.34^{a}	1.20
5.0	4	0.87^{c}	0.90
	5	1.38^{a}	1.19
	6	1.40^{a}	1.21
5.5	4	0.89^{bc}	0.90
	5	1.45^{a}	1.22
	6	1.31^{a}	1.17
SEM[4]		0.073	0.050
Main Effects			
SID Ca			
3.3		1.03	1.04
3.9		1.12	1.06
4.4		1.23	1.12
5.0		1.22	1.10
5.5		1.22	1.10
SEM		0.042	0.029
SID P			
4		0.93	0.94^{b}
5		1.27	1.13^{a}
6		1.29	1.17^{a}
SEM		0.033	0.022
Probabilities, $P \leq$			
SID Ca		0.003	0.271
SID P		0.001	0.001
SID Ca × SID P		0.019	0.062

[1]Each value represents the mean of six replicates (four birds per replicate). The term 'carcass' refers to the whole body without feathers.

[a-c] Means having different superscripts within the column are significantly different ($P < 0.05$).

[2]Ca and P in the carcass of day-old bird is 0.140 and 0.123 g/bird, respectively, and these values are deducted from the total retained Ca or P at day 10.

[3]Pooled standard error of mean.

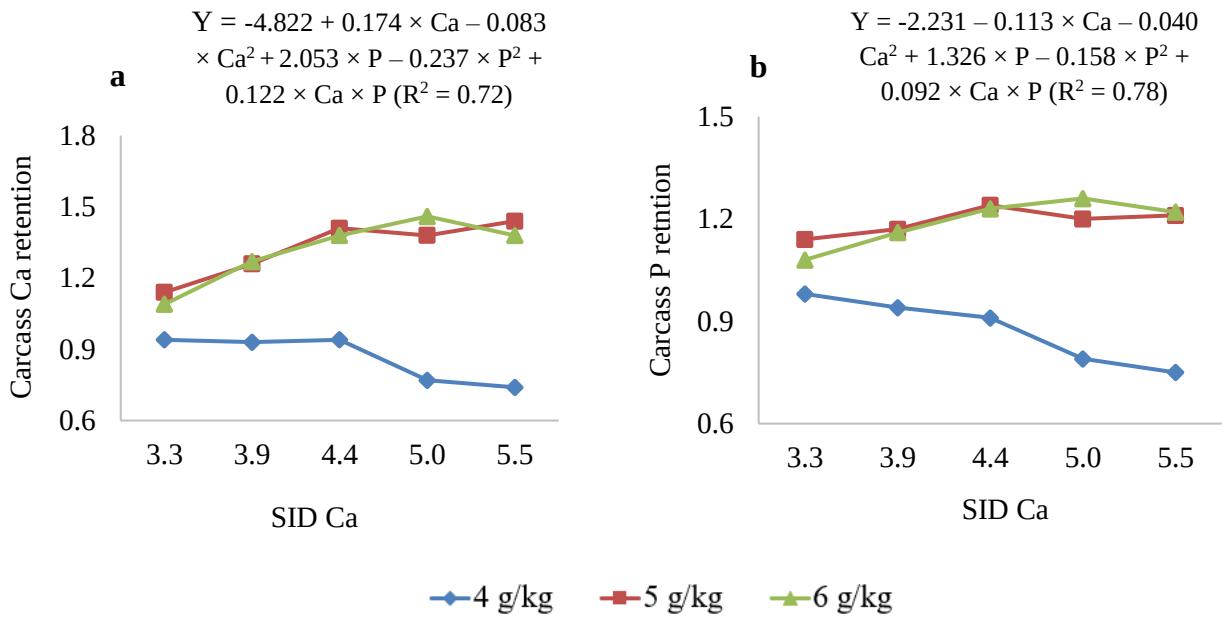

Figure 8.5. Carcass retention (g/bird) of a) calcium (Ca) and b) phosphorous (P) in 10-day old broiler chickens fed different concentrations (g/kg) of standardised ileal digestible (SID) Ca and SID P (4, 5 and 6 g/kg)

At all SID Ca concentrations, the carcass Ca retention was increased at 5 and 6 g/kg SID P, but the magnitude of increment was greater with increasing SID Ca. The predicted maximum carcass Ca retention at SID P concentrations of 4, 5 and 6 g/kg was 0.93, 1.38 and 1.46 g/bird, at SID Ca concentrations of 4.00, 4.74 and 5.47 g/kg, respectively. These values correspond to SID Ca to SID P ratios of 1.00, 0.95 and 0.91, respectively. The predicted maximum carcass P retention at SID P concentrations of 4, 5 and 6 g/kg was 0.96, 1.22 and 1.26 g/bird, at SID Ca concentrations of 3.19, 4.35 and 5.50 g/kg, respectively. These values correspond to SID Ca to SID P ratios of 0.80, 0.87 and 0.92, respectively.

8.5. Discussion

In general, analysed concentrations of proximate components, phytate, Ca and P of ingredients (maize, soybean meal, limestone, dicalcium phosphate and monosodium phosphate) were

within the range reported in the literature (NRC, 1994, 2012; Browning and Cowieson, 2014; Mutucumarana *et al*., 2014b,c; 2015).

A noteworthy finding of the current work was that the calculated SID Ca concentrations of the 15 dietary treatments, in general, were representative of the determined SID concentrations. The closeness between determined and formulated values provides confidence in the published values for the SID Ca of ingredients. The similarity also provides indirect evidence for the additivity of SID Ca in ingredients when combined in feed mixtures. It must be noted that the SID Ca coefficients used in the calculation of dietary SID concentrations were determined in our laboratory and that the same Ca sources (limestone and dicalcium phosphate) evaluated in Ca digestibility assays were used in the formulation of treatment diets. On the other hand, calculated and determined SID P concentrations differed by as much as 25%. The SID P coefficients used in our calculations were from literature (Table 8.1) and the differences likely to reflect the variations reported among laboratories as demonstrated in a ring test involving 17 research stations by Rodehutscord *et al.* (2017).

8.5.1. Requirements for SID P to maximise growth performance, bone mineralisation and utilisation of Ca and P

In the current study, three SID P concentrations were used to determine the SID Ca and SID P requirements in 1-10-day old broilers. As stated earlier, a range of digestible P (4 to 6 g/kg) which is below and above the recommended dietary P concentration (4.8 g/kg available P; Ross, 2019) were considered in the development of dietary treatments. Based on current data, the SID P concentration of 5 g/kg is recommended for broiler starters (1-10 day post-hatch). The results further indicate that any increase in dietary SID P requires increased dietary SID Ca to maximise both growth performance and bone mineralisation. The current finding is in

agreement with the SID P values (5.3 and 5.4 g/kg, respectively) reported by Angel (2018) and van Krimpen (2016) for 1-10 day old broilers.

8.5.2. Requirements for SID Ca to maximise growth performance

In the present study, increasing dietary Ca reduced the feed intake when the dietary SID P was lower (4 g/kg). Consequently, a decline in body weight gain was observed as dietary Ca increased at the dietary SID P of 4 g/kg, suggesting a detrimental effect of Ca on growth performance at low dietary P. Similarly, Walk *et al.* (2012b) reported a reduced feed intake and weight gain with increasing dietary Ca concentrations (4.5, 6.0, 7.5, 9.0 g/kg) in broilers. Similarly, Abdollahi *et al*. (2016) reported a reduced feed intake and weight gain with increasing dietary Ca concentrations (1.3, 4.3, 7.3, 10.3 and 13.3 g/kg) in broilers. However, the negative effect of Ca at 4 g/kg SID P, was ameliorated as the dietary P concentration was increased to 5 and 6 g/kg. The reason for the negative effect on growth performance with increasing dietary Ca concentration is likely the formation of Ca-P complexes in the digestive tract due to excess Ca and the resultant reduction in P digestibility (Mutucumarana *et al*., 2014a; Stein *et al*., 2011). Similar findings of negative Ca effects have been reported in pigs (González-Vega *et al*., 2016b; Merriman *et al*., 2017; Lagos *et al*., 2019a,b) and poultry (Hurwitz *et al*., 1995; Xie *et al*., 2009; Akter *et al*., 2016; Kim *et al*., 2017b).

The linear response of FCR to increasing dietary SID Ca observed in the current study prevented prediction of a maximum response and it was not possible to estimate an optimal concentration of SID Ca at each concentration of SID P for FCR. The FCR data also cannot be used as a reliable parameter to measure the mineral requirement for growth performance because of the wide variation in body weights of broilers in the current study.

For the reasons presented above, the SID P concentration recommended for maximum growth performance of 1-10 day-old broilers is 5 g/kg. The concentrations of SID Ca that

maximised body weight gain and feed intake at 5 g/kg SID P were 3.32 and 2.33 g/kg, respectively, which correspond to SID Ca to SID P ratios of 0.66 and 0.47, respectively. For feed intake, although the quadratic Ca effect was not significant in the full model, it was included in the model as there was no difference in the R^2 values between the models with and without quadratic Ca effect. This inclusion of non-significant quadratic Ca in the model may have lowered the predicted value of SID Ca (2.33 g/kg) at 5 g/kg for feed intake when compared to that for the weight gain (3.32 g/kg). In addition, it must be noted that a maximum value was not calculated for feed intake at 4 g/kg SID P due to the lack of response. Overall, these predicted values indicate that the growth of broiler starters is maximised below the current Ross (2019) Ca recommendation (9.6 g/kg total Ca or 4.4 g/kg SID Ca).

8.5.3. Requirements for SID Ca to maximise standardised ileal digestibility and intake of Ca and P

The quantity of Ca and P consumed and the ratio between them influence the absorption and post-absorptive utilisation of these minerals. However, in the current study, the standardised ileal Ca digestibility was not influenced by the dietary concentrations or ratios of SID Ca or SID P. Similarly, Mutucumarana *et al*. (2014a) found no effect of increasing dietary Ca concentrations (6, 9 and 12 g/kg) on the ileal Ca digestibility. However, SID Ca intake, a function of feed intake and SID Ca, was linearly increased as the dietary SID Ca concentration increased, which was expected. Similarly, Abdollahi *et al*. (2016) reported an increased total Ca intake with increasing dietary Ca concentrations (1.3, 4.3, 7.3, 10.3 and 13.3 g/kg) in 1-7-day old broilers. As noted earlier, reduced feed intake with increasing dietary SID Ca further suggests that the birds adjust their feed intake depending on dietary Ca concentration. As a result, it was not possible to estimate an optimal concentration of SID Ca at a given concentration of SID P for these parameters.

Dietary P plays a vital role in skeletal development and synthesis of other tissues in broilers. Absorption and retention of P in broilers is affected by multitude of interacting factors including dietary concentrations of Ca (Mutucumarana *et al*., 2014a) and P (Rodehutscord *et al*., 2012), phytic acid (Ravindran *et al*., 2006), phytase (Selle *et al*., 2009a; Chapter 6) and age of birds (Fonolla *et al*. 1981). In the current study, the coefficient of standardised ileal P digestibility was increased with decreasing dietary Ca and with increasing SID P concentrations, confirming the negative effect of excess Ca for ileal P absorption. Similarly, the ileal P digestibility has been shown to increase with decreasing dietary Ca concentration (Mutucumarana *et al*., 2014a) and increasing SID P concentrations (Rodehutscord *et al*., 2012) in broilers. The finding also demonstrates that the standardised ileal P digestibility reduces when the SID Ca to SID P ratio increases, confirming improvements in P digestibility with narrowing Ca:P ratios (Liu *et al*., 2013; Sebastian *et al*., 1996; Qian *et al*., 1996; 1997; Selle *et al*., 2009a). A similar trend was observed in the current study for the SID P intake where the intake was increased with the decreasing dietary Ca concentrations and with increasing SID P concentrations. Based on the response surface model, the concentration of SID Ca that maximised digestible P intake at different SID P concentrations was similar (2.91 g/kg) because there was no interaction between SID Ca and SID P. Corresponding SID Ca to SID P ratio at 5 g/kg SID P was 0.58 which is closer to the ratio (0.66) that maximised the body weight gain of broilers. It must be noted that the digestible P intake was positively correlated ($r=0.67$; $P < 0.001$) with the body weight gain in the current study. Dietary P is important for body protein deposition and consequent muscle growth in animals (Bertram, 1995).

8.5.4. Requirements for SID Ca to maximise bone mineralisation

Bone mineralisation is a process of deposition of minerals (Ca and P) on the organic bone matrix for the development of bones. Almost 99% of ingested Ca and 80% of P is deposited in the bones (Veum, 2010). Concentration of bone ash and bone Ca and P are two criteria that are

currently being used to measure the bone mineralisation in broilers. In the current study, the bone parameters were influenced by the interaction effect between SID Ca and SID P. As expected, the lowest concentrations of P (4 g/kg) and Ca (3.3 g/kg) reduced the concentrations of ash and, Ca and P of tibia. The combination of lowest SID P (4 g/kg) with higher SID Ca resulted in lower concentrations of ash, Ca and P of tibia compared to all other combinations of SID P and SID Ca. These findings are in agreement with those of Li *et al*. (2012) who reported lowest concentrations of ash, Ca and P of tibia in broilers fed a lower non-phytate P (2.7 vs 5.0 g/kg) and higher total Ca (11.0 vs 6.0 g/kg) when compared to those fed lower concentrations of both non-phytate P and total Ca. Similarly, Rousseau *et al*. (2013) reported a lower tibia ash in broilers fed a diet with lower non-phytate P of 3.0 g/kg and higher total Ca (10.0 g/kg) compared to those fed other combinations of non-phytate P (3.0 and 4.5 g/kg) and total Ca (6.0 and 10.0 g/kg). These data, along with the findings of the current study, highlight the negative effects of excess Ca on absorbable Ca and P through the formation of insoluble Ca-P complex, but the effect is much greater on P than Ca.

In the current study, concentrations of SID Ca that maximised the concentrations of ash, Ca and P of tibia and toe ash concentration at 5 g/kg SID P were 4.51, 4.72, 4.36 and 4.78 g/kg, respectively, which correspond to SID Ca to SID P ratios of 0.90, 0.94, 0.87 and 0.96, respectively. These values are well above the requirement of SID Ca and ratio of SID Ca to SID P for growth performance (2.33-3.32 g/kg and 0.47-0.66, respectively), demonstrating that the birds require Ca and P for bone tissue synthesis beyond the needs for body tissues (Gautier *et al*., 2017). Similar findings have been reported in pigs (González-Vega *et al*., 2016b; Lagos *et al*., 2019a,b). However, in general, a digestible Ca recommendation has been proposed for 1 to 10 day old broilers as 6.1 g/kg (Angel, 2018) which is higher than the values obtained in the current study.

Tibia ash is the most frequently used criterion for the determination of Ca and P requirements in poultry. Early studies have used tibia ash as a criterion to determine the biological value of inorganic phosphates (Nelson and Peeler, 1960; Nelson and Walker, 1964). Subsequently, toe ash was also used as an alternative to determine the bioavailability of P in various phosphate sources (Fritz *et al*., 1968; Potter, 1988; Potter *et al*., 1995; Ravindran *et al*., 1995b). However, some studies reported that the toe ash was not an appropriate criterion as the tibia ash (Huff, 1980; Scholey and Buron, 2017) while others reported that the toe ash was more sensitive than tibia ash (Ravindran *et al*., 1995b). In the current study, the responses of tibia ash and toe ash to different dietary Ca and P concentrations were similar which agrees with the finding of Potter (1988) and Jiménez-Moreno *et al*. (2013). The toe ash concentration was positively correlated with those of tibia ash (r=0.94; $P < 0.001$), tibia Ca (r=0.83; $P < 0.001$) and tibia P (r=0.91; $P < 0.001$), suggesting that toe ash can be used as an effective criterion to determine bone mineralisation in broilers.

8.5.5. Requirements for SID Ca to maximise total tract retention of Ca and P

Retention of dietary Ca is important to build up the skeletal system. Absorption and retention of Ca may be affected by dietary, physiological and animal factors. In the current study, the total tract Ca retention and retainable Ca contents were influenced by the interaction between Ca and P, suggesting the importance of formulating diets with proper Ca and P ratio. The linear nature of Ca retention with increasing dietary SID Ca prevented the prediction of maximum response and it was not possible to estimate an optimal concentration of SID Ca for Ca retention at different concentrations of SID P. Although Ca digestibility was unaffected by dietary treatments, both percentage (intake to output) and absolute (g/bird) Ca retentions were reduced at lower dietary SID P concentrations (4 g/kg), suggesting the need to maintain appropriate dietary P to maximise the Ca retention. In addition, the Ca retention was similar in birds fed SID P concentrations 5 and 6 g/kg, regardless of dietary Ca concentrations. Interestingly, the

retained Ca followed a trend similar to that of the bone Ca deposition in the current study where the bone Ca concentrations were similar at 5 and 6 g/kg SID P concentrations. In addition, at the lowest P concentration (4 g/kg), both Ca retention and bone Ca were higher at 3.3 g/kg SID Ca compared to higher Ca concentrations, highlighting the negative effect of excess Ca on Ca retention (Gautier *et al*., 2017). These findings further confirm the verity that almost all the Ca is stored in the skeletal tissue (Veum, 2010). It is also worth noting that Ca retention was positively correlated (r=0.61; $P < 0.001$) with tibia Ca in the current study. However, dietary Ca requirement studies on pigs (González-Vega *et al*., 2016a,b) found that the SID Ca to SID P ratio needed to maximise Ca retention was higher than the ratio needed to maximise the bone Ca.

In contrast to the trend in ileal P absorption, P retention was influenced by the interaction between SID Ca and SID P where the retention was reduced at the highest dietary P concentration (6 g/kg), indicating urinary excretion of absorbed P. When the dietary P is above the physiological threshold for maximum retention, additional P is known to be excreted through the kidney (Leske and Coon, 2002). At 6 g/kg SID P, the percentage P retention was lower than the percentage P absorption. However, the P retention values were higher than the P absorption values at 4 g/kg SID P. At 5 g/kg SID P, the percentage P retention was comparable to percentage P absorption, indicating that most of the absorbed P was retained at 5 g/kg dietary SID P. These findings further confirm that 5 g/kg SID P is the more appropriate requirement for 1-10 day-old broilers. At 5 g/kg SID P, the retained P (g/bird) was lowest at the 5.5 g/kg dietary SID Ca concentration, further confirming the negative effect of excess Ca on P absorption and retention (Mutucumarana *et al*., 2014a). Based on the response surface model, the linear nature of P retention to increasing dietary SID Ca observed in the current study prevented the prediction of a maximum response. A negative correlation (r=-0.31; $P < 0.01$) was observed between the P retention and tibia P results in the current study was

unexpected. Ostensibly most of the retained P was utilised for the growth of broilers as P utilisation is highly correlated with muscle protein synthesis (Bertram, 1995). The existence of strong, positive correlation (r=0.84; $P < 0.001$) between retained P (g/bird) and body weight gain further lends support to this thesis.

In the current study, the ratio between retained SID Ca and retained SID P was positively correlated (r=0.56; $P < 0.001$) with the ratio between SID Ca intake and SID P intake. Regardless of dietary treatments, the ratio between retained SID Ca and retained SID P ranged from 0.92 to 1.41, with the upper range being at SID Ca concentrations above 5.0. At 5 g/kg dietary SID P, the ratios ranged from 0.98 to 1.41 depending on the dietary SID Ca concentrations. However, the body weight gain was maximised at lower dietary SID Ca concentrations of 3.3 g/kg and the ratio of retained SID Ca to retained SID P at 3.3 g/kg SID Ca and 5 g/kg SID P was 0.99. Corresponding ratio of retained SID Ca to retained SID P for maximum bone mineralisation was 1.11 in the current study.

8.5.6. Requirements for SID Ca to maximise carcass retention of Ca and P

The Ca concentration of carcasses of day-old and 10-day old birds in the current study was 12.4 and 8.2-19.0 g/kg, respectively. Ostensibly, the use of different dietary Ca and P concentrations resulted in wide range of carcass Ca concentration in 10-day old broilers in the current study. Caldas *et al*. (2019) studied carcass composition of broilers at different points of growth period and, reported a carcass Ca concentration of 11.7 g/kg in day-old birds and a Ca concentration of 18.9 g/kg at 12-day of age in birds fed 9.0 g/kg dietary Ca which are comparable to values in the current work. The carcass P concentration of day-old and 10-day old birds in the current study were 10.8 and 10.0-15.6 g/kg, respectively. Similarly, Caldas *et al*. (2019) reported a carcass P concentration of 10.3 g/kg in day-old chicks and 15.3 g/kg in 12-day old broilers.

A notable observation was that the carcass Ca and P retention was representative of total tract retention values. The retained (g/bird) carcass Ca (r=0.66; $P < 0.001$) and P (r=0.60; $P < 0.001$) was positively correlated with the total tract Ca and P retention (g/bird), giving additional strength to the determined total tract retention estimates. It must be noted that the total excreta samples were collected during all 10 days of the experimental period. Interestingly, at 5 g/kg SID P, the carcass Ca retention and the tibia Ca were maximised at a SID Ca concentration of 4.72-4.74 whereas the carcass P retention and tibia P were maximised at a SID Ca concentration of 4.35-4.36, suggesting a close association between the tibia mineralisation and the carcass mineral retention. Such an association is to be expected since 990 g/kg of Ca in the body is in the bones.

8.6. Conclusions

Overall, the present data demonstrated that growth performance, bone mineralisation and Ca and P utilisation of broiler starters were optimised at 5 g/kg SID P concentration. The estimate of 5.0 g/kg SID P compares closely to the current Ross (2019) recommendation for available P (4.8 g/kg). Growth performance was negatively affected by dietary SID Ca concentrations above 5.0 g/kg at the SID P concentration of 5 g/kg or below. The SID Ca required for maximum weight gain and bone mineralisation is 3.32 and 4.36-4.78, respectively, at 5 g/kg SID P, which correspond to SID Ca to SID P ratios of 0.66 and 0.87-0.96, respectively. The current Ross (2019) Ca recommendation (9.6 g/kg total Ca or 4.4 g/kg SID Ca) for broiler starters is higher than the current estimate (3.32 g/kg SID Ca) for weight gain. However, the bone mineralisation is maximised around the current total Ca requirement at 8.9-9.8 g/kg (4.36-4.78 g/kg SID Ca). Bone mineralisation requires more Ca than growth performance demonstrating, as expected, that the birds use Ca exclusively for the synthesis of bone tissues.

CHAPTER 9

GENERAL DISCUSSION

9.1. Introduction

The measurement of digestible nutrients in feed ingredients has now become the cornerstone of contemporary poultry nutrition to formulate diets that closely match the nutrient requirements for optimal and sustainable production. Improvements in productivity could lead to lower feed costs and, increased profitability and sustainability. Historically, digestibility in poultry has been determined over the total digestive tract but this approach suffers from several limitations. Today, the superiority of nutrient digestibility measured at the terminal ileum is accepted unquestionably by the poultry industry (Lemme *et al*., 2004). The classic example is the move in the industry from using total amino acids and total tract digestible amino acids to ileal digestible amino acids in feed formulations and this shift had been an immense success. However, the requirement of phosphorous (P) is still being considered on available P basis and that of calcium (Ca) on total Ca basis. Due to the ever-increasing price of inorganic phosphates and the growing concern over environmental P pollution, the measurement of P digestibility in feed ingredients has received attention in the recent past and currently considered as the preferable method to measure P availability for poultry (WPSA, 2013). Concurrent measurement of Ca digestibility in feed ingredients is necessary because of the close relationship between Ca and P in the absorption and post-absorptive utilisation of these minerals. Applying the digestible nutrient principle to P and Ca will be a natural extension of the use of digestible amino acids and also aligns these two minerals with routine feed evaluation.

When the current PhD project was initiated, an assay based on regression for the determination of P digestibility for poultry has been advocated (WPSA, 2013) but there was no established method for that of Ca digestibility. In previous studies from our laboratory,

Anwar (2017), using direct, difference and regression methods, measured the Ca digestibility in major Ca sources. It was found that, regardless of the methodology, the ileal digestibility of Ca is around 50-60% for limestone and meat and bone meal and less than 40% for other Ca sources (dicalcium phosphate, monocalcium phosphate, poultry by-product meal, fish meal and canola meal). In contrast, using direct, regression and difference methods or modifications thereof, much higher Ca digestibility values reported in pig studies (Table 9.1) for these Ca sources necessitating the follow-up studies investigated in this thesis.

Table 9.1. Comparison of reported standardised calcium (Ca) digestibility coefficients between poultry and pigs

Ca source	Poultry	Pigs
Limestone	0.49-0.65[1,3]	0.70[2]
Oyster shell	0.44[1]	-
Dicalcium phosphate	0.28[1], 0.67[3]	0.76-0.78[2,4]
Monocalcium phosphate	0.33[1]	0.86
Meat and bone meal	0.41-0.60[1,5]	0.53-0.81[*6]
Fish meal	0.24[1]	0.46-0.89[7]
Poultry by-product meal	0.29[1]	-
Canola meal	0.31[1]	0.47[8]
Soybean meal	-	0.47[*9]

[*]Apparent calcium digestibility
[1]Anwar (2017) using direct method
[2]Zhang and Adeola (2017) using regression method
[3]Zhang and Adeola (2018) using regression method
[4]González-Vega *et al*. (2015a) using difference method
[5]Anwar (2017) using regression method
[6]Sulabo and Stein (2013) using direct method
[7]González-Vega *et al*. (2015b) using direct method
[8]González-Vega *et al*. (2013) using regression method
[9]Bohlke *et al*. (2005) using difference method

This apparent anomaly between poultry and pig data requires resolution, and possible contributing factors need to be examined. Available evidence indicates that the Ca metabolism in avian and mammalian species are not different and the same homeostatic mechanisms are involved (Proszkowiec-Weglarz and Angel, 2013). There are dissimilarities in the anatomy and digestive physiology between the two species and it may be argued that the higher Ca

digestibility in pigs is a reflection of species differences. For example, differences between monogastric (poultry vs. pigs) species (Dilger and Adeola, 2006a,b) and among poultry (broilers, turkeys, ducks, quails) species (Rodehutscord and Dieckmann, 2005; Ingelmann *et al*., 2019) in P utilisation are known and similar differences may exist for Ca. On the other hand, it may be that the differences in assay procedures have some relevance. The main differences between poultry and pig assays include the composition of basal diet, methodology (indicator or total collection) and dietary adaptation length. The influence of these factors was investigated in the studies reported in Chapters 3 and 4. These studies were followed by studies examining the influence of bird age, dietary protein, supplemental phytase and bird-type on Ca digestibility (Chapters 5, 6 and 7). In the study reported in the final chapter (Chapter 8), the Ca digestibility values of Ca sources determined in this thesis and published data were used to develop dietary treatments to study the digestible Ca requirement for broiler starters from day 1 to 10 post-hatch.

9.2. Resolving the issue of lower Ca digestibility estimates in poultry

One of the differences between poultry and pig assays is the composition of basal diet. Maize-based basal diets were used in the pig studies (González-Vega *et al*., 2013, 2015a,b; Merriman *et al*., 2016), whereas purified diets based on maize starch and dextrose were used in our previous broiler studies (Anwar, 2017). The Ca digestibility of fish meal was reported to be higher in the maize-based diet than in the maize-starch-based purified diet in pigs (González-Vega *et al*., 2015b). However, none of the studies have compared the influence of basal diet composition on the Ca digestibility of Ca sources in poultry. Other than the report of Zhang and Adeola (2018) who used a semi-purified diet (maize and maize starch-based) to measure the Ca digestibility of limestone and dicalcium phosphate in broilers, there were no reports on Ca digestibility of Ca sources in the maize-based diet as indicated in Table 9.2. It is worth noting that the contribution of Ca from maize in the maize-based diets is negligible (< 0.02

g/kg) and therefore the determined Ca digestibility values with maize-based diet relate to the Ca source. In this context, the study reported in Chapter 3 was conducted to examine the effect of basal diet composition (purified diet vs. maize-based diet) on the true ileal Ca digestibility of four Ca sources namely, limestone, meat and bone meal, monocalcium phosphate and dicalcium phosphate.

Table 9.2. Apparent ileal digestibility coefficient (AIDC) of calcium (Ca) in Ca sources determined with purified, semi-purified and maize-based diets in broilers[1]

Ca sources	AIDC	Basal diet-type[2]	Reference
Inorganic Ca sources			
Limestone	0.34-0.75	Purified	Anwar *et al*. (2016a)
Limestone	0.54-0.61	Purified	Anwar *et al*. (2016c)
Limestone	0.37-0.61	Purified	Anwar *et al*. (2017)
Limestone	0.32-0.50	Purified	Angel *et al*. (2013)
Limestone	0.56-0.62	Semi-purified	Zhang and Adeola (2018)
Limestone	0.45	Purified	Chapter 3
Limestone	0.53	Maize-based	Chapter 3
Limestone	0.36-0.65	Maize-based	Chapter 4
Limestone	0.27-0.53	Maize-based	Chapter 5
Limestone	0.43-0.50	Maize-based	Chapter 7
Oyster shell	0.32-0.55	Purified	Anwar *et al*. (2017)
Monocalcium phosphate	0.32	Purified	Anwar *et al*. (2018)
MCP	0.56-0.77	Purified	Angel *et al*. (2013)
MCP	0.37	Purified	Chapter 3
MCP	0.45	Maize-based	Chapter 3
Dicalcium phosphate	0.27	Purified	Anwar *et al*. (2018)
DCP	0.61-0.65	Semi-purified	Zhang and Adeola (2018)
DCP	0.27	Purified	Chapter 3
DCP	0.33	Maize-based	Chapter 3
Animal-based Ca sources			
Fish meal	0.23	Purified	Anwar *et al*. (2018)
Poultry by-product meal	0.28	Purified	Anwar *et al*. (2018)
Meat and bone meal	0.38-0.56	Purified	Anwar *et al*. (2015; 2016b)
Meat and bone meal	0.35	Purified	Chapter 3
Meat and bone meal	0.43	Maize-based	Chapter 3
Plant-based Ca sources			
Canola meal	0.29	Purified	Anwar *et al*. (2018)
Canola meal	0.22-0.53	Maize-based	Chapter 6
Soybean meal	0.33-0.51	Maize-based	Chapter 6

[1]The Ca digestibility determined with maize-based diet relates to the Ca source as the Ca content in maize is negligible.
[2]Purified: maize starch-dextrose-based; semi-purified: maize and maize starch-based

The results indicate that the basal diet influenced Ca digestibility measurement, with the maize-based diet (average, 0.46) having higher true ileal Ca digestibility coefficients than those of the purified diet (average, 0.37). This finding is in agreement with the results of pig study (González-Vega *et al*., 2015b). True ileal Ca digestibility coefficients of limestone, meat and bone meal, monocalcium phosphate and dicalcium phosphate were determined to be 0.51, 0.41, 0.43 and 0.32, respectively, in the current work. However, the poultry estimates were still noticeably lower than the pig data and do not fully explain the differences in Ca digestibility. Therefore, the composition of basal diet alone did not resolve the issue of lower Ca digestibility estimates in poultry.

The findings from Chapter 3 also indicate an influence of basal diet type on ileal endogenous Ca losses with the higher losses for the maize-based diet (253 mg/kg dry matter intake [DMI]) than the purified diet (131 mg/kg DMI). Phytic acid contributed by the maize is possibly the major contributor to the increased endogenous losses (Cowieson and Ravindran, 2007). Similar findings were observed in pigs (González-Vega *et al*., 2015b) where a maize-based Ca-free diet induced a higher (396 mg/kg DMI) endogenous Ca losses than a maize starch-based Ca-free diet (220 mg/kg DMI).

Methodology, which was the second difference between poultry and pig assays, was explored in the study reported in Chapter 4. Methodologies that have been used in pig and poultry assays were differed in the way the digestibility is calculated and in the length of dietary adaptation. First, the Ca digestibility was calculated by the total excreta collection (6 days) method in pig assays (González-Vega *et al*., 2015a, b) whereas titanium indicator ratios in the diet and digesta were used in broiler studies (Anwar, 2017). It may be speculated that the lower Ca digestibility values determined in broilers may be due, at least in part, to interaction between titanium and Ca. It is known that different minerals interact with each other in their absorption and metabolism (Suttle, 2010), but there are no published reports on any possible interference

of titanium with Ca analysis or absorption. Therefore, the effects of dietary indicator (titanium dioxide vs. acid insoluble ash) on the Ca digestibility of limestone was investigated in the first Experiment presented in Chapter 4. The results showed that the apparent ileal Ca digestibility was unaffected by the type of dietary indicator and therefore, the indicator used did not contribute to the issue of lower Ca digestibility estimates in poultry. It is clear from the current finding that titanium dioxide can be used as an effective indigestible dietary indicator for the measurement of Ca digestibility in poultry.

Second, a five-day adaptation period followed by six days of faecal collection (González-Vega *et al*., 2015a,b) was practiced in pig assays, whereas a three-day dietary adaptation length was used in our previous broiler studies (Anwar, 2017). Dietary adaptation length longer than 5 days was proposed by WPSA (2013) for P utilisation studies in broilers with semi-purified ingredients. It was speculated that the lower Ca digestibility values determined in broilers may be due to a shorter adaptation length (3 days) when compared to the adaptation length (5 days) used in pig assays. Therefore, the effect of different dietary adaptation lengths (24, 72, 120 and 168 hours) on the Ca digestibility of limestone was investigated for broilers in the second experiment presented in Chapter 4. The results showed that the ileal Ca digestibility was affected by the dietary adaptation length with a higher digestibility at 24 hours (0.65) adaptation length, steady digestibility values between 72 (0.46) and 120 (0.44) hours and a decrease at 168 hours (0.36). These findings demonstrate that in poultry, there is no difference in Ca digestibility between 3- (72 hours) and 5- (120 hours) day adaptation lengths and there is no increment in Ca digestibility beyond 5 days. Therefore, the issue of lower ileal Ca digestibility values in poultry cannot be explained on the basis of indicator type or adaptation length. Nonetheless, the current findings indicate that the methodology for Ca digestibility measurement in poultry could use a 3-day adaptation length as the standard practice.

9.3. Investigations into other possible factors influencing Ca digestibility in poultry

There are several other factors that may contribute significantly to the variability in Ca digestibility summarised in Table 9.3. Most of these factors are reported to have an influence on the Ca digestibility of diets. However, the current thesis focused on the effect of these factors on the ileal digestibility of Ca sources and the findings reported herein are novel.

Table 9.3. Factors affecting Ca digestibility in poultry

Factors	Reference
Age	Fonolla *et al*. (1981); Shastak *et al*. (2012); Angel *et al*. (2013); Li *et al*. (2018)
Phytate	Ravindran *et al*. (2006); Selle *et al*. (2009a)
Phytase	Ravindran *et al*. (2006); Selle *et al*. (2009a); Walk *et al*. (2012b); Kim *et al*. (2018); Bradbury *et al*. (2018)
Dietary P	Anwar *et al*. (2016c); Sebatian *et al.* (1996); Viveros *et al*. (2002)
Dietary Ca	Rao and Roland (1990); Plumstead *et al.* (2008); Gautier *et al*. (2017); Imari *et al*. (2020)
Ca:P ratio	Plumstead *et al.* (2008); van Krimpen *et al*. (2013); Amerah *et al*. (2014); Anwar *et al*. (2016a)
Species	Mtei *et al.* (2019a,b); Ingelmann *et al*. (2019)
Ca source	Angel (2013); Hamdi *et al.* (2015); Anwar *et al*. (2018)
Particle size of Ca source	Rao and Roland (1990); Anwar *et al*. (2017); Li *et al.* (2020); Majeed *et al*. (2020)
In vitro solubility of Ca source	Anwar *et al*. (2016c)
Dietary adaptation length	Angel *et al*. (2013); Anwar *et al*. (2018)
Ca status of the bird	Rao and Roland (1990)
Steam conditioning	Attar *et al*. (2017)
Other enzymes or combination of enzymes	Neto *et al*. (2015); Moss *et al.* (2018)
Dietary anti-nutritive factors	Mahmood *et al*. (2014)
Body weight and growth rate	Kemme *et al*. (1997)

Age of birds is one of the key factors that influences the major nutrient digestibility (Batal and Parsons, 2002; Lima *et al*., 2012; Li *et al*., 2015; Morgan *et al*., 2015). However, there are limited reports on the effect of broiler age on Ca digestibility (Table 9.4). Among these, only one study has reported the age effect on ingredient Ca digestibility (Angel *et al*., 2013). Furthermore, the Ca digestibility estimates reported in most of these studies are limited to the first three weeks and none has covered the entire growth period of broilers. Therefore,

the first experiment reported in Chapter 5 was conducted to measure the effect of age (7, 14, 21, 28, 35 and 42 days, post-hatch) on the Ca digestibility of limestone. The findings revealed that the apparent ileal Ca digestibility coefficients were linearly decreased from day 7 (0.51) to day 42 (0.27) in broilers. It is clear from the finding that the use of digestible Ca values of ingredients is not interchangeable among different age groups and age-dependant values must be considered when formulating diets for different age groups of broilers.

Table 9.4. Reported apparent ileal digestibility coefficients (AIDC) of calcium (Ca) in diets/Ca sources at different ages of broilers

Reference	Main Ca source/s	Age (days)	AIDC
Shastak *et al*. (2012)	soybean meal, limestone, dicalcium phosphate	20	0.51-0.63
		34	0.35-0.59
Angel *et al.* (2013)[1]	limestone	11	0.50
		25	0.32
	monocalcium phosphate	11	0.77
		25	0.56
	soybean meal	11	0.66
		25	0.54
Morgan *et al*. (2015)	soybean meal, limestone, dicalcium phosphate	4	0.56
		6	0.63
		8	0.61
		10	0.62
		12	0.67
		14	0.72
Li *et al*. (2018)	soybean meal, limestone, monocalcium phosphate	9	0.53-0.59
		21	0.48-0.59
Dersjant-Li *et al*. (2018)	soybean meal, canola meal, wheat bran, dicalcium phosphate, limestone	10	0.29
		27	0.41
		41	0.31
Majeed *et al*. (2020)	soybean meal, poultry by-product meal, limestone	14	0.56-0.65
		35	0.55-0.48

[1]Semi-purified diet was used and therefore, the values of AIDC relate to the ingredients.

Calcium digestibility in poultry may also be affected by the dietary composition. Protein content is a main nutrient that is necessary for animal growth. Evidence from human

studies indicates that protein influences Ca digestion (Kerstetter *et al.,* 2003). However, the effect of dietary crude protein concentrations on the Ca digestibility in poultry is unknown. The previous Ca digestibility studies conducted by our laboratory have used a lower dietary protein concentrations (around 80 g/kg) than the standard protein requirement (Ross, 2019) of broiler starters (230 g/kg) and growers (215 g/kg) as the practical protein feed ingredients cannot be used in these studies in order to eliminate the Ca contribution from these supplements. Hence, the second experiment reported in Chapter 5 was conducted to test the null hypothesis that the Ca digestibility in limestone is similar between the broilers fed two crude protein concentrations (79 vs 153 g/kg). The findings revealed that dietary protein concentration did not influence the Ca digestibility in broilers. However, both protein concentrations (79 and 153 g/kg) used in the current experiment are lower than the standard recommendation (Ross, 2019), which is a limitation of this study. The high protein diet (153 g/kg) used in this study was obtained by adding dried egg albumen at a usage limit of 100 g/kg as recommended by WPSA (2013) for P digestibility studies, which prevented the use of a protein concentration above 153 g/kg. Hence, this aspect may need to be explored in future studies.

It is well established that supplemental microbial phytase improves P absorption in poultry by increasing the bioavailability of phytate-P (Selle and Ravindran, 2007), but the effects on Ca digestibility remains contradictory. Some reports indicate that phytase addition improves Ca digestibility of diets (Ravindran *et al*., 2006; Walk *et al*., 2012b; Kim *et al*., 2018), whereas others have found little or no benefit (Sebastian *et al*., 1996; Walk *et al*., 2012a). On the other hand, phytase doses higher than the recommended doses, and referred to as superdoses, are known to hydrolyse the phytate (IP6) as well as the lower phytate esters (IP5 to IP1). Superdosing is currently used by the industry and reported to improve the growth performance and nutrient utilisation over the normal doses by removing the anti-nutritive effects of lower phytate esters (Cowieson *et al*., 2011). Table 9.5 summarises the reported

apparent ileal Ca digestibility in practical diets without and with supplemental phytase superdoses (≥ 1500 FTU/kg) for broilers.

Table 9.5. Reported apparent ileal calcium (Ca) digestibility coefficients (AIDC) without (-) and with (+) phytase superdoses in broilers

Main Ca sources[1]	Phytase dose	AIDC of Ca		Reference
		-	+	
Limestone, DCP, SBM, DDGS	5000	0.52-0.66	0.51-0.69	Walk *et al*. (2012a)
Limestone, MCP, SBM, HSC	2500	0.55	0.59	Walk *et al*. (2012b)
Limestone, MCP, SBM	2000	0.34	0.43	Kiarie *et al.* (2015)
Limestone, DCP, SBM, wheat	1500	0.40, 0.47	0.24, 0.30	Beeson *et al*. (2017)
Limestone, DCP, SBM	2000	0.52	0.57	Farhadi *et al*. (2017)
	3000	0.52	0.58	
	4000	0.52	0.58	
	5000	0.52	0.60	
	6000	0.52	0.60	
Limestone, MCP, wheat, extracted SBM	1500	0.45	0.53	Sommerfeld *et al*. (2018b)
	3000	0.45	0.52	
Limestone, DCP, SBM, wheat bran, RSM, SFM	2000	0.42	0.41	Walk and Olukosi (2019)
	4000	0.42	0.53	
Limestone, MCP, dehulled-SBM	2000	0.58-0.61	0.49-0.56	Walters *et al*. (2019)
	3000	0.58-0.61	0.49-0.55	
Limestone, MCP, SBM	1500	0.60	0.65	Ajuwon *et al*. (2020)
	3000	0.60	0.64	

[1]DCP: dicalcium phosphate; MCP: monocalcium phosphate; SBM: soybean meal; DDGS: distillers' dried grains with solubles; HSC: highly soluble Ca; RSM: rapeseed meal; SFM: sunflower meal

However, the influence of microbial phytase (including the superdose) on the Ca digestibility of Ca sources has not been previously reported. The studies reported in Chapter 6 were conducted to measure the influence of phytase doses (0, 500 and 2000 FTU/kg) on the Ca and P digestibility of soybean meal (SBM) and canola meal (CM) in broiler starters (Experiment 1) and finishers (Experiment 2). True ileal Ca digestibility coefficients of SBM and CM, with no phytase, were determined to be 0.51 and 0.53, respectively, for broiler starters and 0.33 and 0.22, respectively, for broiler finishers. These results also confirm (not statistically compared)

the finding that the Ca digestibility decreases with advancing age of broilers, as reported in Chapter 5.

True ileal P digestibility coefficients of maize-SBM diet and maize-CM diet, with no phytase, were determined to be 0.89 and 0.66, respectively, for starters and 0.82 and 0.57, respectively, for finishers. Extremely higher P digestibility estimated in maize-SBM at both broiler ages may be explained partly by the comparatively low dietary Ca concentration (Mutucumarana *et al*., 2014a), narrow Ca: total P ratio (Liu *et al*., 2013) and low dietary phytate concentration (Ravindran *et al.*, 2006). Microbial phytase increased the true ileal digestibility coefficients of Ca and P in maize-SBM diet and maize-CM diet, but the effect was more pronounced for the maize-CM diet. This finding indicates that comparatively more phytate-bound Ca and P were released by phytase in the maize-CM diet, which may be due to the relatively high phytate concentration of CM. Surprisingly, the superdosing of phytase (2000 FTU/kg) increased the Ca digestibility in CM and SBM by two-fold compared to the normal phytase dose (500 FTU/kg). These findings support the conventional wisdom that the use of phytase superdose will be more beneficial in diets based on high-phytate ingredients.

Ileal endogenous Ca losses used for true digestibility calculations for broiler growers and finishers in this study were determined to be 236 and 29 mg/kg of DMI, respectively. The endogenous Ca losses measured in broiler finishers (29 mg/kg DMI) were markedly lower than the previously reported (84-127 mg/kg DMI) values (Anwar *et al*. 2016a, 2016c, 2017) and than those measured in broiler growers in the current work. However, no comparable data are available for endogenous Ca losses in 42-day-old broilers. Ileal endogenous P losses in growers and finishers were determined to be 310 and 130 mg/kg of DMI, respectively. These findings clearly indicate an age effect on the endogenous mineral losses with higher losses in younger birds.

Bird type is another factor that may affect the Ca digestibility. Ileal Ca digestibility studies comparing the digestibility between broilers and layers are scant. In addition, no studies have been reported on ingredient Ca digestibility in laying hens. Therefore, studies reported in Chapter 7 were conducted to determine the apparent ileal Ca digestibility coefficients of two limestone sources in broilers and layers. Apparent ileal Ca digestibility of limestone was found to be higher in laying hens compared to broilers. The apparent ileal Ca digestibility coefficient of limestone A for broilers and layers were 0.50 and 0.62, respectively. The corresponding values for limestone B were 0.43 and 0.70, respectively. The findings indicate that laying hens digest Ca more efficiently than broilers which may be attributed to a high demand of Ca for eggshell formation in laying hens. Hence, the application of these bird type-specific digestible Ca values may need to be given major importance in feed formulations.

9.4. Digestible Ca to digestible P requirements of broiler starters

Using the digestible Ca content of Ca sources that have been measured in previous studies, a growth study (Chapter 8) was conducted to estimate the standardised ileal digestible (SID) Ca requirement for 1 to 10 day-old broilers fed different dietary concentrations of both SID Ca (3.3, 3.9, 4.4, 5.0 and 5.5 g/kg) and SID P (4, 5 and 6 g/kg). The findings indicate that growth performance, bone mineralisation and mineral utilisation of broiler starters were optimised at 5 g/kg SID P concentration, which is closer to the current Ross 308 (2019) recommendation for available P (4.8 g/kg). The concentrations of SID Ca that maximised body weight gain, tibia ash, tibia Ca, tibia P and toe ash were estimated to be 3.32, 4.51, 4.72, 4.36 and 4.78 g/kg, respectively which corresponds to SID Ca to SID P ratios of 0.66, 0.90, 0.94, 0.87 and 0.96. The SID Ca requirement for maximum growth performance (3.32 g/kg) of 10-day old broilers is equivalent to a total Ca concentration of 7 g/kg, which is less than the Ross (2019) recommendation of 9.6 g/kg total Ca. However, the bone mineralisation was maximised around the current recommendation (8.9-9.8 g/kg total Ca). These data showed that bone

mineralisation requires more Ca than growth performance which is in agreement with those from pig studies (González-Vega *et al*., 2016a,b; Merriman *et al*., 2017; Lagos *et al*., 2019 a,b).

9.5. Challenges and problems encountered during the study

9.5.1. Variations in the calculated and analysed dietary Ca concentrations

In the thesis studies, there were differences between calculated and analysed dietary Ca concentrations as summarised in Table 9.6. The differences (calculated minus analysed) ranged from -1.5 to 1.8 g/kg, on an as-fed basis. Most of the analysed values were higher than the calculated values and this could be due to errors in mixing, sampling and/or analytical procedures. These differences are an unresolved practical issue in Ca digestibility calculations.

Table 9.6. Comparison of calculated and analysed dietary calcium (Ca) concentrations (g/kg, as fed basis) in the current experiments

Chapter	Calculated	Analysed	Diet type	Difference[1]
Chapter 3	9.00	8.9-10.5	Purified	-0.1 to 1.5
Chapter 3	9.00	9.4-10.8	Maize-based	0.4 to 1.8
Chapter 4	6.08	5.80-6.90	Maize-based	-0.82 to 0.28
Chapter 4	9.00	9.70	Maize-based	-0.70
Chapter 5	9.00	8.90-9.90	Maize-based	-0.9 to 0.1
Chapter 6	1.44	1.71-1.78	Maize-based	-0.34 to -0.27
Chapter 6	2.17	1.69-2.00	Maize-based	0.17 to 0.48
Chapter 7	8.00	8.40-9.50	Maize-based	-1.5 to -0.4
Chapter 7	40.0	40.8-41.2	Maize-based	-1.2 to 0.8
Chapter 8	7.00	6.90-7.10	Maize-soybean meal	-0.1 to 0.1
Chapter 8	8.00	7.70-7.90	Maize-soybean meal	0.1 to 0.3
Chapter 8	9.00	8.20-9.20	Maize-soybean meal	-0.2 to 0.8
Chapter 8	10.0	10.2-11.2	Maize-soybean meal	-1.2 to 0.2
Chapter 8	11.0	11.0-11.6	Maize-soybean meal	-0.6

[1] Calculated dietary Ca concentration minus analysed dietary Ca concentration.

9.5.2. Differences in particle size and solubility of limestone samples

Particle size and solubility are two important factors that contribute to differences in Ca digestibility among limestone sources (Anwar *et al*., 2017; Zhang and Coon, 1997a). Therefore,

the digestible Ca determined for a particular limestone sample will not be applicable to other samples with different particle sizes and solubility. This is another challenge when an average value is used in feed formulations. Furthermore, the most widely used method for the measurement *in vitro* Ca solubility is that of Zhang and Coon (1997a) which is based on 0.2 N hydrochloric acid. This method involves a very acidic solution (pH 0.76) and a one-time point (10 minutes) solubility determination. Recently, however, a dynamic model (Kim *et al*., 2019) with more than one solubility time point (5, 15 and 30 minutes) and of a solution that closely represent gizzard conditions (pH 3 and buffered) has been reported to correlate better with *in vivo* Ca digestibility when compared to the digestibility results obtained at one time-point assay. Accordingly, these researchers have developed prediction models for ileal Ca digestibility which include the solubility at 15- and 30-minute time points as these time points were more relevant compared to that at a 5-minute time point. These models may be useful for the characterisation of limestone samples in future studies. Table 9.7 summarises the particle size and *in vitro* solubility of limestone samples that have been tested in previous (Anwar, 2017) and current studies.

Table 9.7. Geometric mean diameter and *in vitro* solubility of limestone samples that have been used in previous (Anwar, 2017) and current studies

Geometric mean diameter (mm)	*In vitro* solubility[1]	Reference
< 0.5	0.60	Anwar *et al.* (2016a)
1-2	0.33	Anwar *et al.* (2016a)
0.2	0.53-0.60	Anwar *et al.* (2016c)
0.37	0.47	Chapter 3, 4, 5 and 8
0.46	0.56	Chapter 7
1.30	0.23	Chapter 7

[1]Zhang and Coon (1997b) method (200 ml, 0.2 N hydrochloric acid)

9.5.3. Variations in estimated endogenous Ca losses

The presence of Ca in the ileal digesta, from non-dietary sources, is referred to as endogenous Ca. These endogenous Ca losses originate from various sources, including bile secretions, digestive enzymes and desquamated mucosa (Davies *et al*., 2004). Estimation of ileal endogenous Ca losses is needed to correct the apparent ileal Ca digestibility to true digestibility values. In previous studies, Anwar (2017) measured the ileal endogenous Ca losses in broilers by feeding a Ca- and P-free diet (Table 9.8). In the current thesis, ileal endogenous Ca losses were estimated in two studies (Chapters 3 and 6) as summarised in Table 9.8. However, there were variations in the endogenous Ca losses between the studies and these variations can be associated with different methodologies (diet-type, dietary adaptation length, age) as indicated by Anwar *et al*. (2018) and Anwar and Ravindran (2020). It must be noted, however, unlike for amino acids, the estimated endogenous Ca losses are low relative to unabsorbed dietary Ca in the ileal digesta and have almost no impact on correction with only an increase of 0.02-0.03 in true digestibility coefficients (Anwar, 2017; Chapters 3 and 6). It may be concluded that an average value is used instead of investing on the measurement of endogenous Ca losses in the future.

Table 9.8. Endogenous losses (mg/kg dry matter intake) of calcium (Ca) and phosphorous (P) measured in broilers

Reference	Endogenous losses		Age of birds	Ca- and/or P-free basal diet type
	Ca	P		
Anwar *et al.* (2020)	125	133	28	Dextrose-based
Anwar *et al.* (2020)	77	-	28	Maize gluten-based
Anwar *et al.* (2020)	46	110	28	Dried egg albumen-based
Anwar *et al.* (2016a)	127	-	24	Maize starch-based
Anwar *et al.* (2016b)	88	-	31	Maize starch-based
Anwar *et al.* (2017)	115	-	24	Maize starch-based
Anwar *et al.* (2018)	84-124	-	24	Maize starch-based
Chapter 3	131	-	24	Maize starch-based[1]
Chapter 3	253	-	24	Maize-based[1]
Chapter 6	236	310	21	Maize-based
Chapter 6	29	130	42	Maize-based

[1]Ca-free diet with P.

9.5.4. Dietary Ca:P ratio on Ca digestibility assays

Estimation of Ca digestibility in Ca sources is not simple and straightforward as it is influenced by multitude interacting factors including P concentration, phytate and Ca:P ratio. For the assays in the current work, a Ca:P ratio of 2.0 was maintained in experimental diets, except in studies reported in Chapters 3 and 6. In Chapter 3, the Ca:P ratios used for the experimental diets were lower than 2.0 (1.14 for limestone-, MBM- and DCP-diets and 0.64-0.66 for MCP-diets). Because of the greater P concentrations of these ingredients (except limestone), it was not possible to maintain a Ca: P ratio of 2.0. The 1.14 ratio in the limestone-diet was due to the inclusion of monosodium phosphate to supply P in the experimental diet. Higher P concentration, relative to Ca, in the MCP resulted in much lower Ca: P ratio of 0.64-0.66 in the MCP-diet. In the case of Ca digestibility assays in plant-based ingredients, maintaining a dietary Ca:P ratio of 2.0 was not possible because of the low Ca concentrations (Chapter 6). These limitations in maintaining the Ca:P ratio are unavoidable and would have influenced the digestibility estimates.

9.5.5. Appropriate model selection for Ca requirement study

Studies on digestible Ca requirement in poultry are non-existent. Although one report is available for digestible Ca requirements, no experimental details were provided (Angel, 2018). The study reported in Chapter 8 investigated the Ca requirement of 1-10-day old broilers. The response surface model was used to determine the digestible Ca and digestible P requirements. Response surface models are commonly used in research studies when there is more than one independent variable associated with a response variable. The full model equation, used in Chapter 8, comprised of linear and quadratic effects of both Ca and P and the interaction between linear Ca and linear P effects. The quadratic interactions between Ca and P were excluded in the models to avoid the complexity in data analysis. The predicted requirement values for response variables would have been different but only marginally if quadratic

interactions were included in the model. The model used in this thesis work has been employed previously for Ca requirement studies in pigs and considered appropriate based on the requirement data generated in this thesis.

9.6. Suggestions for future studies

Some questions that have been raised in the previous study (Anwar, 2017) and some issues that have been described in current thesis that need to be explored in future studies are outlined below.

9.6.1. Age-related digestibility studies

Ileal Ca digestibility of limestone at different broiler ages was evaluated in this thesis (Chapter 5). It was found that the digestibility decreased with advancing age. Future studies are needed to establish whether similar age effects exist in other major Ca sources (meat and bone meal, MCP and DCP).

9.6.2. Studies on the effect of dietary protein

The protein concentrations (79 and 153 g/kg) studied in Chapter 5 are lower than the standard recommendation for broilers (Ross, 2019). Investigation of the effect of standard protein concentration on Ca digestibility may be of interest and may be explored using a high protein ingredient such as dried egg albumen at levels above 100 g/kg.

9.6.3. Studies on microbial phytase

Data on ileal Ca digestibility of SBM and CM were generated without and with microbial phytase (at both normal and super doses) in broilers (Chapter 6). The current findings showed that supplemental phytase increases the Ca digestibility in SBM and CM, which is the first report on ingredient Ca digestibility with phytase. Similar studies to measure the ileal Ca digestibility of other plant feed ingredients with normal and superdoses of supplemental phytase will be of interest.

Phytase superdoses are known to hydrolyse the phytate as well as the lower phytate esters (IP5 to IP1) thereby reducing their anti-nutritive effects. Chapter 6 reported the effect of phytase doses on the concentration of IP5 and IP6 and the disappearance of IP6 in the terminal ileum of broilers fed maize-SBM and maize-CM diets. Studies are warranted to determine the concentrations and disappearance of lower phytate esters in the ileum of broilers fed other plant-based feed ingredients.

9.6.4. Studies with laying hens

To the best of our knowledge, the current thesis was the first to report the ileal Ca digestibility of limestone in laying hens (Chapter 7). Age of the layers that were used in this study was 40-weeks. Similar studies with limestone and other Ca sources are needed for different age groups and physiological stages (pullets, early lay, peak lay and late lay).

The influence of stage of ovulation on the Ca digestibility in laying hens is an unresolved question. Hurwitz and Bar (1969), using an yttrium-91 marker, demonstrated that the Ca absorption increased during shell calcification stage. It will be of interest to examine whether a diurnal variation exists and the digestibility is greater during the dark period.

9.6.5. Studies on acid binding capacity of limestone samples

Limestone, the major Ca source in poultry diets, has a high acid binding capacity (ABC; Lawlor *et al*., 2005; Gilani *et al*., 2013; Hamdi *et al*., 2015; Lu *et al*., 2016), which can increase the digesta pH in birds. An increase in digesta pH may influence solubility and digestibility of Ca sources. In addition to solubility and particle size, the ABC of limestone samples may influence Ca digestibility. Future studies are warranted to determine the ABC of different limestone samples and the influence of ABC on Ca digestibility of limestone.

9.6.6. Digestible Ca requirement studies

A comprehensive study on the digestible Ca and digestible P requirements of broiler starters (0-10 days) was reported in Chapter 8. There is clearly a need for studies to determine the requirements of digestible Ca and digestible P for other growth phases of broilers, namely growers (11-24 days) and finishers (25-42 days).

9.7. Conclusions

Research into the measurement of Ca digestibility for poultry is still in its infancy. Most of the current data on Ca digestibility procedures and digestibility estimates have been generated from Massey University (see review by Walk *et al*., 2021). The present thesis continued the exhaustive Ca digestibility research of Anwar (2017). The major aims were to resolve the issue of lower Ca digestibility estimates in poultry compared to pigs, to examine some key factors affecting Ca digestibility and, to establish the digestible Ca requirement for broiler starters. A total of eight studies was conducted to accomplish these aims. Most of the findings of this research were novel and not examined hitherto.

This thesis work unambiguously demonstrated that the reported differences in Ca digestibility between poultry and pigs are truly reflective of species effect and that the Ca digestibility in poultry is lower than in pigs. This contention was further confirmed by the notable similarity observed between calculated and determined SID Ca contents in the Ca requirement study (Chapter 8).

In the current research, the direct method wherein the ingredient serves as the sole source of Ca was used to determine the Ca digestibility. This method was favoured based on previous data from our laboratory by Anwar (2017) comparing different methodologies. In the case of amino acid digestibility measurements, the direct method suffers from some limitations (Lemme *et al*., 2004) but these do not apply to Ca digestibility. The dietary treatments tested

in Chapter 8 were formulated based on Ca digestibility values generated by the direct method and it was found that the determined SID Ca concentrations compared closely with those of calculated values, giving confidence in the direct method. Based on these findings, it is concluded that the direct method is useful for Ca digestibility assays. Accordingly, following assay procedures are suggested: (i) use of maize-based diets instead of maize starch-based purified diet, (ii) titanium dioxide as the indigestible indicator and (iii) a dietary adaptation of 3 days.

Another novel finding in this thesis was the age effects on Ca digestibility. The ileal Ca digestibility of limestone was highest during week 1 and declined with advancing age, in complete contrast to the general trends reported for major nutrients and energy.

True ileal Ca digestibility coefficient of main plant-based ingredients, SBM and CM, were determined for the first time. The data showed that supplemental phytase is consequential for Ca absorption in broilers. In particular, the use of phytase superdose (2000 FTU/kg) was found to release more phytate-bound Ca from CM, a high phytate-ingredient, increasing the Ca digestibility by two-fold the normal dose (500 FTU/kg). It seems that the, use of phytase superdose will be more beneficial in diets based on high-phytate ingredients.

Bird-type influences the Ca digestibility where the digestibility is higher in layers than in broilers. In laying hens, the measurement of ileal endogenous Ca losses was found to be a challenge because of the difficulty in feeding a Ca-free diet to birds in lay which have a very high Ca requirement. One option to resolve this issue, at least in part, is increasing the number of birds in replicate cages which may help to obtain adequate digesta samples for analysis. Also measures must be taken to avoid the consumption of own eggs by layers fed the Ca-free diet.

Considerable data are available now on the Ca digestibility of ingredients (Anwar, 2017; Chapters 3 to 7) to move towards the digestible Ca formulation system. As the first step,

requirements of digestible Ca, digestible P and the ratio of digestible Ca to digestible P were identified for broiler starters (0-10-day old) in this thesis. Accordingly, the digestible Ca requirement for growth performance of 0-10-day old broilers is lower than the current Ross (2019) recommendation of total Ca while the requirement for bone mineralisation is around the recommendation.

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APPENDICES

Appendix A. Determination of Ca

Calcium contents of diets, digesta and excreta samples were determined following the method of Karlsson *et al*. (2015) as indicated below. The samples were subjected to nitric acid digestion and the concentration of Ca was read using microwave plasma atomic emission spectroscopy (MP-AES).

Sample preparation:

1. Oven dried (at 65°C) ground sub sample of 0.1 g was weighed into a 25 mL-marked digestion tube using an accurate four decimal balance in triplicate. Accuracy of the measurements was assessed by analysing the samples in parallel with a certified standard reference material (NIST 1573a [National Institute of Standards and Technology; tomato leaves]) and a blank.
2. Each tube was added with 4 mL of concentrated nitric acid (69% v/v) in the fume cupboard, placed with small glass funnel on top and digested at 150°C for 3 to 4 hours until brown fuming stops and clear solution obtained.
3. The funnel tops were removed, and the temperature was increased gradually (≤ 200°C) to evaporate moisture and obtain dryness. Then the tubes were removed, added with 5 mL of 2M hydrochloric acid (HCl) and mixed using vortex mixture. The solution was mixed thoroughly three times in 15 minutes interval.
4. Each tube was added with strontium, caesium (Sr, Cs) solution (25,000 mg/L) of 1 mL and then diluted with deionised water up to the 25 mL mark. The solutions were mixed well using vortex mixer and the contents were transferred to labelled, 35 mL screw top plastic containers.
5. The samples having approximately higher Ca concentration such as inorganic Ca sources were diluted using 1000 ppm Sr, Cs solution, before reading them in MP-AES. Limestone

sample was diluted by 50 times whereas MBM, MCP, DCP and standard reference material were diluted by 10 times. Similarly, the diet and digesta samples were diluted by 10 times except those related to the Ca free diet. For that, 1 mL of sub sample was pipette out into volumetric flasks (50 mL or 10 mL) and diluted with 1000 ppm Sr, Cs solution up to 50 mL or 10 mL mark.

6. Then sub samples were transferred to 10 mL screw top plastic tubes. A series of calcium standard solutions (0, 20, 40, 60, 80 and 100 mg Ca/L) were also used to develop the calibration curve, and prepared from the calcium nitrate ($Ca(NO_3)_2$) stock standard solution (1000 mg Ca/L). The Ca concentration of samples was then determined by MP-AES (Agilent Technologies, 4200 MP-AES, Santa Clara, CA) at 714.815 nm wavelength.

Calculation

The Ca concentration of the samples were calculated by the following formula:

$$\% Ca = [(C \times DF) / (W \times 10000)]$$

Where,

C = MP-AES reading at 714.815 nm (ppm)

DF = dilution factor

W = Weight of the sample (g)

10000 = factor to convert mg/kg to %.

Appendix B. Determination of particle size

To determine the particle size of limestone sources, a set of sieves (Endocotts Ltd., London, UK) sized 2.0, 1.0, 0.5, 0.25, 0.125, and 0.063 mm and a Tyler To-Tap sieve shaker were used as described by Baker and Herrman (2002). The samples were passed through the sieve stack on shakers for 10 minutes. The weight 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. Three replicates per sample were analysed. The following equations were used to calculate the GMD and GSD.

$$di = (du \times do) \wedge 0.5$$

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

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

Where,

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

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

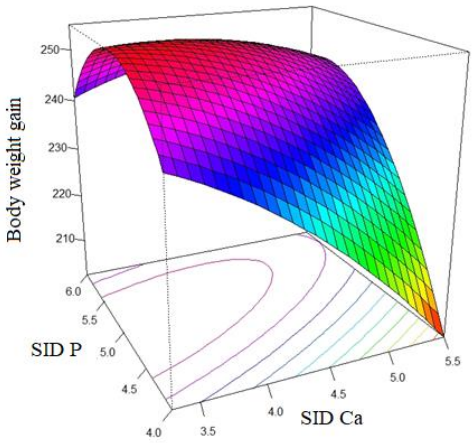
do = diameter opening through which particles were not passed (i^{th} sieve)

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

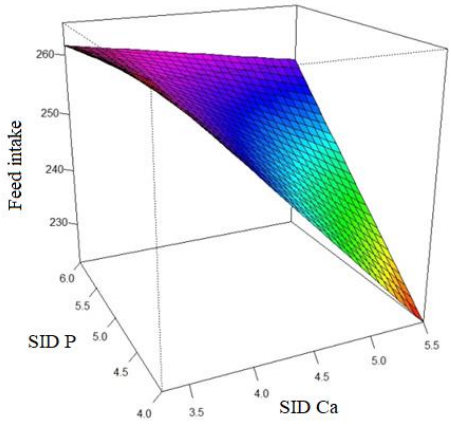
Appendix C. Chapter 8

C.1. Response surface models

a)



b)



c)

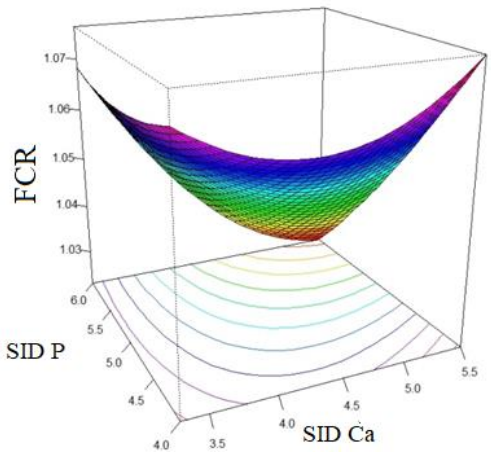


Figure 1. (a) Body weight gain (g/bird), (b) feed intake (g/bird) and (c) feed conversion ratio (FCR) of broiler chickens fed different concentrations (g/kg) of standardised ileal digestible (SID) calcium (Ca) and SID phosphorous (P) from day 1 to 10

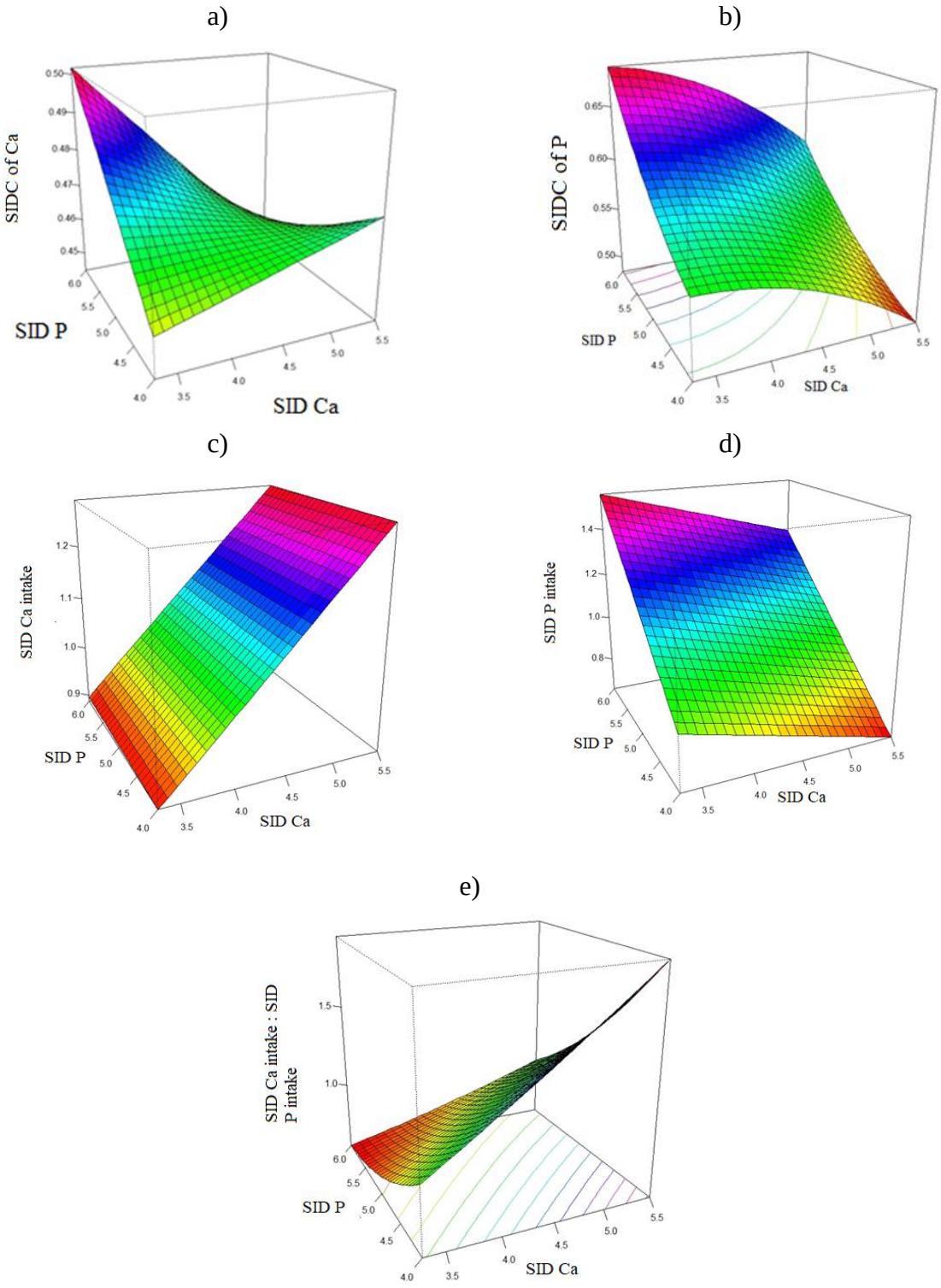


Figure 2. Standardised ileal digestibility coefficient (SIDC) of a) calcium (Ca) and b) phosphorous (P), Intakes (g/bird) of c) standardised ileal digestible (SID) Ca and b) SID P, and c) ratio of SID Ca intake to SID P intake, of broiler chickens fed different concentrations (g/kg) of SID Ca and SID P from day 1 to 10

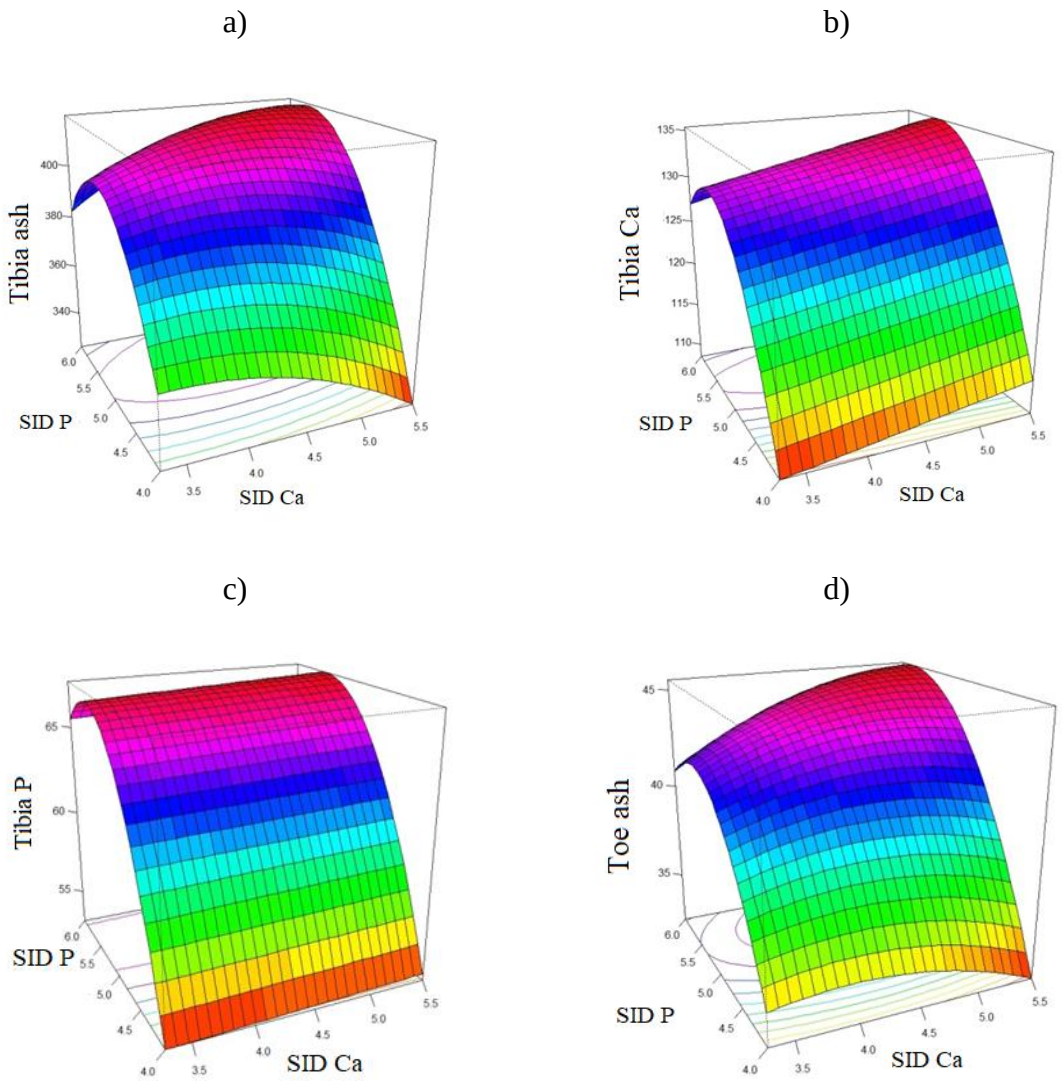


Figure 3. Concentrations (g/kg dried defatted matter) of a) ash, b) calcium (Ca) and c) phosphorous (P) of tibia and d) toe ash content (g/kg, as received basis) in broiler chickens fed different concentrations (g/kg) of standardised ileal digestible (SID) Ca and SID P from day 1 to 10

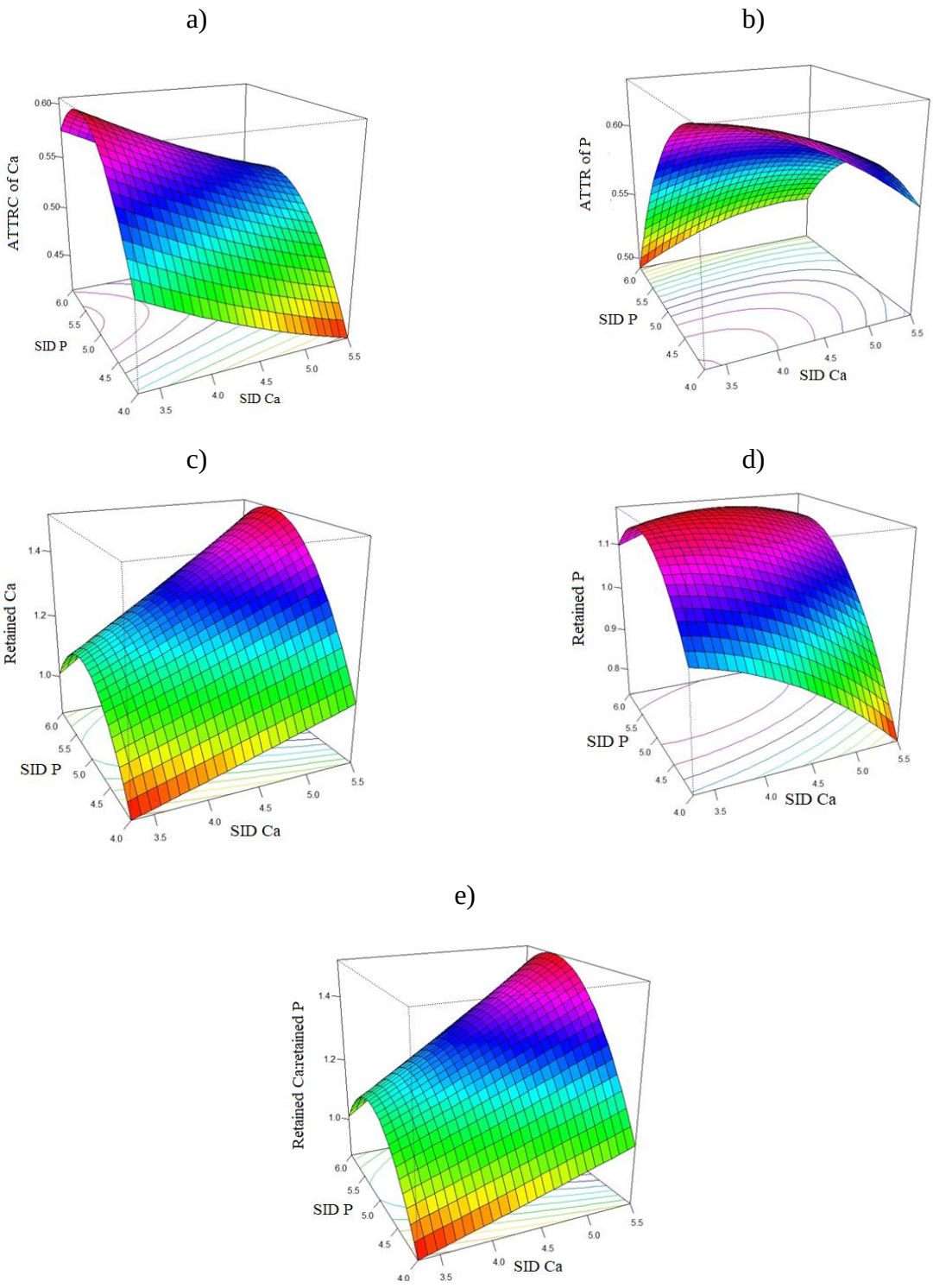


Figure 4. a) Apparent total tract retention coefficient (ATTRC) of calcium (Ca), b) ATTRC of phosphorous (P), c) total tract retained Ca (g/bird), d) total tract retained P (g/bird), e) ratio of retained Ca to retained P, of broiler chickens fed different concentrations (g/kg) of standardised ileal digestible (SID) Ca and SID P from day 1 to 10

a) b)

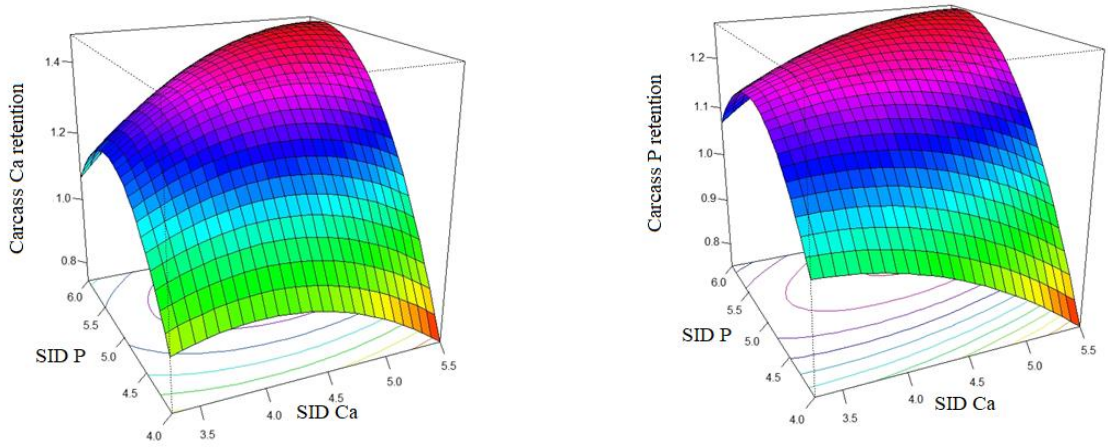


Figure 5. Carcass retention (g/bird) of a) calcium (Ca) and b) phosphorous (P) of broiler chickens fed different concentrations (g/kg) of standardised ileal digestible (SID) Ca and SID P from day 1 to 10

C.2. Digestive tract measurements

Table 1. Relative lengths (m/kg body weight) and weights (g/kg body weight) of digestive tract for 10-day old broilers fed the diets containing different concentrations (g/kg) of standardised ileal digestible (SID) calcium (Ca) and SID phosphorous (P)[1]

SID Ca	SID P	Relative length				Relative weight						
		Duodenum	Jejunum	Ileum	Caeca	Crop	Proventriculus	Gizzard	Duodenum	Jejunum	Ileum	Caeca
3.3	4	0.63	1.49^{cde}	1.29^{cdef}	0.29	3.38^{bcd}	4.43	16.52	5.70	8.29	6.13	2.10
	5	0.57	1.45^{de}	1.27^{def}	0.27	3.15^{cde}	4.62	16.79	5.23	7.50	5.63	1.88
	6	0.62	1.53^{bcd}	1.39^{bcd}	0.29	3.05^{de}	4.50	15.47	5.49	9.68	6.50	1.91
3.9	4	0.63	1.46^{de}	1.32^{bcde}	0.29	2.90^{e}	4.39	16.16	5.72	8.32	6.07	1.87
	5	0.62	1.49^{cde}	1.30^{cdef}	0.29	3.31^{bcd}	4.66	16.42	6.42	9.60	6.99	1.99
	6	0.62	1.50^{bcde}	1.35^{bcde}	0.28	3.34^{bcd}	4.78	16.24	6.35	9.27	6.32	1.95
4.4	4	0.65	1.60^{abc}	1.40^{bc}	0.31	3.50^{abc}	4.38	17.20	5.45	8.59	6.19	1.91
	5	0.60	1.47^{de}	1.28^{cdef}	0.29	3.17^{cde}	4.42	16.77	5.76	9.76	6.64	2.08
	6	0.61	1.44^{de}	1.23^{ef}	0.28	3.18^{cde}	5.09	16.83	6.06	9.02	6.47	2.23
5.0	4	0.71	1.62^{ab}	1.44^{ab}	0.26	3.51^{abc}	4.57	17.16	6.45	9.51	7.02	2.49
	5	0.62	1.56^{bcd}	1.36^{bcd}	0.26	3.25^{bcde}	4.33	16.18	5.25	8.92	6.75	1.99
	6	0.62	1.46^{de}	1.28^{cdef}	0.28	3.56^{ab}	4.42	16.07	5.43	8.86	6.36	1.86
5.5	4	0.73	1.71^{a}	1.54^{a}	0.35	3.86^{a}	4.16	16.89	6.52	9.30	6.29	2.17
	5	0.67	1.60^{abc}	1.37^{bcd}	0.31	3.45^{bc}	4.38	17.21	5.52	8.34	6.21	2.15
	6	0.60	1.40^{e}	1.19^{f}	0.29	3.26^{bcde}	5.16	16.79	5.76	7.90	5.89	2.10
SEM[2]		0.022	0.045	0.045	0.021	0.133	0.275	0.589	0.593	1.185	0.603	0.162
Main Effects												
SID Ca												
3.3		0.61^{d}	1.49	1.32	0.29	3.19	4.52	16.26	5.47	8.49	6.08	1.96
3.9		0.62^{bc}	1.48	1.32	0.29	3.19	4.61	16.27	6.16	9.06	6.46	1.94
4.4		0.62^{bc}	1.50	1.31	0.30	3.28	4.63	16.93	5.76	9.12	6.43	2.07
5.0		0.65^{ab}	1.55	1.36	0.27	3.44	4.44	16.47	5.71	9.10	6.71	2.11
5.5		0.67^{a}	1.57	1.37	0.31	3.52	4.56	16.96	5.93	8.51	6.13	2.14
SEM		0.012	0.026	0.026	0.012	0.077	0.159	0.340	0.342	0.684	0.348	0.09

SID P											
4	0.67[a]	1.58	1.40	0.30	3.43	4.39	16.79	5.97	8.80	6.34	2.11
5	0.62[b]	1.51	1.32	0.28	3.26	4.48	16.67	5.64	8.82	6.44	2.02
6	0.61[b]	1.47	1.29	0.29	3.28	4.79	16.28	5.82	8.95	6.31	2.01
SEM	0.010	0.020	0.020	0.009	0.059	0.123	0.264	0.265	0.530	0.270	0.07
Probabilities, $P \leq$											
SID Ca	0.008	0.083	0.373	0.076	0.006	0.916	0.398	0.687	0.924	0.701	0.448
SID P	0.001	0.001	0.001	0.492	0.087	0.060	0.367	0.672	0.979	0.933	0.572
SID Ca × SID P	0.096	0.004	0.001	0.864	0.007	0.516	0.892	0.772	0.877	0.912	0.231

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

[a-f] Means having different superscripts within the column are significantly different ($P < 0.05$).

[2]Pooled standard error of mean.

C.3. Weight and length of tibia

Table 2. Relative lengths (mm/kg body weight) and weights (g/kg body weight) of tibia for 10-day old broilers fed the diets containing different concentrations (g/kg) of standardised ileal digestible (SID) calcium (Ca) and SID phosphorous (P)[1]

SID Ca	SID P	Tibia weight		Tibia length
		Wet basis	Dry matter basis	
3.3	4	7.40	2.31^{g}	177^{defg}
	5	7.36	2.44^{ef}	170^{h}
	6	7.58	2.38^{fg}	179de
3.9	4	7.62	2.38^{fg}	178^{def}
	5	7.51	2.48^{de}	172^{gh}
	6	7.75	2.56^{bcd}	177^{defg}
4.4	4	7.26	2.32^{g}	185^{bc}
	5	7.29	2.52^{cde}	173^{fgh}
	6	7.63	2.56^{bcd}	176^{defg}
5.0	4	7.56	2.34^{fg}	190 ^{ab}
	5	7.41	2.59^{abc}	175^{efgh}
	6	7.61	2.53^{bcde}	174^{efgh}
5.5	4	7.48	2.34^{g}	195^{a}
	5	7.63	2.62^{ab}	181^{cd}
	6	7.92	2.66^{a}	175^{efgh}
SEM[2]		0.131	0.034	1.9
Main Effects				
SID Ca				
3.3		7.45	2.38	175
3.9		7.62	2.48	176
4.4		7.39	2.47	178
5.0		7.53	2.49	179
5.5		7.68	2.54	184
SEM		0.076	0.020	1.1
SID P				
4		7.46^{b}	2.34	185
5		7.44^{b}	2.53	174
6		7.70^{a}	2.54	176
SEM		0.059	0.015	0.9
Probabilities, $P \leq$				
SID Ca		0.054	0.001	0.001
SID P		0.004	0.001	0.001
SID Ca × SID P		0.900	0.015	0.001

[1]Each value represents the mean of six replicates (eight birds per replicate).
[a-h] Means having different superscripts within the column are significantly different ($P < 0.05$).
[2]Pooled standard error of mean.

C.4. Feathers

Table 3. The weight of feathers and the concentration of calcium (Ca) and phosphorous (P) in feathers of day-old and 10-day old broilers

	Day-old birds	10-day old birds
Carcass weight (g/bird)	46.0[1]	335 (314-359)[2]
Carcass DM (%)	24.8[1]	27.9[2]
Feather weight (g/bird)[3]	0.73	2.44
Feather DM (%)[3]	92.3	92.6
Feather percentage (% BW)[3]	1.73	0.55
Feather Ca content (g/bird)[3]	0.001	< 0.003
Feather P content (g/bird) [3]	< 0.001	0.006
Feather Ca retention at 10 days (g/bird)	-	0.002[4]
Feather P retention at 10 days (g/bird)	-	0.005[4]

[1]Value represents the mean of ten birds.
[2]Each value represents the mean of six replicates (four birds per replicate).
[3]Each value represents the mean of four birds.
[4]The retention of Ca and P in feathers at 10 days are negligible and represents only 0.17 and 0.46% of carcass Ca and P retention, respectively.

Appendix D. Statement of contribution to doctoral thesis containing publications

Statement of contribution to doctoral thesis containing publications from Chapters 3, 4, 5, 6, 7 and 8 are attached.

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We, the candidate and the candidate's Primary 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*.

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Name/title of Primary Supervisor:	Professor Velmurugu Ravindran
In which chapter is the manuscript /published work:	Chapter 3 and 4

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(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

David, L. S., Abdollahi, M. R., Ravindran, G., Walk, C. L. and Ravindran, V. (2019). Studies on the measurement of ileal calcium digestibility of calcium sources in broiler chickens. Poultry Science,98: 5582-5589.

( ) The manuscript is currently under review for publication – please indicate:

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- Please provide the full reference of the Research Output:

David, L. S., Abdollahi, M. R., Bedford, M. R. and Ravindran, V. (2020). Effect of age and dietary crude protein content on the apparent ileal calcium digestibility of limestone in broiler chickens. Animal Feed Science and Technology, 263:e114468.

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- Please provide the full reference of the Research Output:

David, L. S., Abdollahi, M. R., Bedford, M. R. and Ravindran, V. (2020). True ileal calcium digestibility in soybean meal and canola meal, and true ileal phosphorous digestibility in maize-soybean meal and maize-canola meal diets, without and with microbial phytase, for broiler growers and finishers. British Poultry Science. 62:293-303.

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In which chapter is the manuscript /published work:	Chapter 7

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- Please provide the full reference of the Research Output:

David, L. S., Abdollahi, M. R., Bedford, M. R. and Ravindran, V. (2021). Comparison of the apparent ileal calcium digestibility of limestone in broilers and layers. British Poultry Science. DOI:10.1080/00071668.2021.1943313.

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In which chapter is the manuscript /published work:	Chapter 8

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- Please provide the full reference of the Research Output:

David, L. S., Abdollahi, M. R., Bedford, M. R. and Ravindran, V. (2021). Requirement of digestible calcium at different dietary concentrations of digestible phosphorus for broiler chickens. 1. Broiler starters (d 1 to 10 post-hatch). Poultry Science. DOI: 10.1016/j.psj.2021.101439.

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