An investigation into the possible health-promoting modes of action of regular- and super- doses of phytase in the broiler chicken

By

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Abstract

The overall objective of this thesis was to study the effects of regular and high (super-) doses of phytase in the gut of broilers, with the aim of documenting the mechanism of their action leading to improvements in animal health.

Phytase is often supplemented to commercial broiler diets to facilitate the hydrolysis of plant phytate and release of phosphorus for utilisation. Although not the original intention of its addition, phytase supplementation leads to improvements in growth performance parameters and enhanced nutrient utilisation. Further benefits have also been observed following the addition of super-doses of phytase which are not explained by an increase in phosphorus release, and thus have been termed 'extra-phosphoric effects'. Using diets formulated to be adequate or marginally deficient in available phosphorus (aP; forming the negative control, NC), phytase was supplemented at 1,500 and 3,000 FTU/kg phytase in the first study (both super-doses) and the partitioning of nutrients within the body was investigated. It appeared that there were some metabolic changes between 1,500 and 3,000 FTU/kg, switching between protein and fat accretion, potentially as a consequence of nutrient availability, although these changes were not reflected by changes in growth performance parameters. However, the loss of the NC treatment without phytase on day 12 limits the comparison of the phytase within the NC treatment, but does allow for comparison of each dose at adequate or low dietary aP levels. As expected, a greater degree of phytate hydrolysis was achieved with 3,000 than with 1,500 FTU/kg phytase, but changes in carcass accretion characteristics were greater with 1,500 than 3,000 FTU/kg. Using these findings and the observation that there were no further changes in the parameters measured by increasing phytase from 1,500 to 3,000 FTU/kg (aside from phytate hydrolysis), 1,500 FTU/kg phytase was selected as the super-dose to be used in subsequent studies.

The next study considered the influence of regular (500 FTU/kg) and super doses (1,500 FTU/kg) of phytase from within the gut. Overall, it was observed that changes were occurring to the gut environment, which ultimately would influence the absorptive capacity and conditions for further phytate hydrolysis. Dietary treatment influenced gut conditions such as pH, intestinal morphology and bacterial populations which can subsequently influence nutrient utilisation

and potential for growth. The subsequent study was designed to investigate the effects within the gut in more detail. The release of nutrients from phytate hydrolysis and their bioavailability within the digesta can influence conditions within intestine, facilitating enhanced absorption. One of the parameters investigated was the expression of genes involved in the transport of nutrients in the intestine. Overall, there were few significant dietary treatment influences on gene expression in the intestine, however there was a dose-dependent response of phytase on the expression of the jejunual divalent mineral transporter. This indicates a change in divalent mineral bioavailability in the intestine, with correlations with inositol phosphate esters (IPs) being identified. This is likely explained by the IPs produced by phytase hydrolysis and accumulating in the digesta, differing between regular and high doses of phytase.

It became apparent that interactions between the products of phytate hydrolysis (IP3, IP4) and minerals in the digesta had the potential to influence the gut environment and subsequent nutrient bioavailability and overall phytase action. The final study was designed to increase the content of the IPs, and investigate the influence of phytase under these conditions. As the complete hydrolysis of phytate to myo-inositol has been reported to be beneficial due to its proposed insulin mimetic effects, myo-inositol was also supplemented to one of the diets to see if any further benefits would be observed when supplemented alongside super-doses of phytase. Neither increased concentrations of the higher IP esters (IP6, IP5 and IP4) nor myo-inositol (myo-) had any effect on broiler growth performance, however there were still apparent beneficial influences of phytase supplementation. The results suggest considerable and important interactions between minerals and IP esters within the digesta, which ultimately have the potential to influence gut conditions and thus nutrient utilisation and growth performance. Reduced concentrations of blood glucose in the high IP ester diet with additional phytase supplementation suggest some insulin-like effects of myo- production. Additionally, the lack of effect of myo- supplementation on blood glucose and insulin concentrations suggests a difference between the structure of phytase-produced myo- and supplemented myo-.

Although there were no improvements in growth performance by increasing phytase from 500 to 1,500 FTU/kg, there were changes occurring at the level of the gut and expression of genes in the intestine, influencing nutrient utilisation and the partitioning of nutrients within the body. There are many factors to be considered when supplementing phytase, with dietary nutrient content and nutrient release and IP production during phytate hydrolysis having an influence on phytase action, nutrient absorption and conditions within the gut. Super-doses of phytase may be beneficial for maintaining optimal gut conditions, clearing IP esters from the digesta, reducing their potential to form complexes with minerals and other nutrients, ultimately influencing the efficiency of production.

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Publications

Part of the work reported in this thesis has been communicated at different conference meetings or in scientific journals:

Abstracts:

L. Beeson, C. Walk, P. Hastie, K. Ajuwon, M. Bedford, and O. Olukosi (2016). Manipulation of dietary phytic acid, myo-inositol and exogenous phytase levels influenced the blood insulin to glucose ratio but not their overall concentration. (PSA, July 2016, New Orleans)

L. Beeson, C. Walk, M. Bedford, O. Olukosi (2016). Manipulation of the phytic acid and myo-inositol content of broiler diets on growth performance, gut pH and expression of genes in the jejunum, with and without additional supplementation of phytase. (WPC, September 2016, China)

L. Beeson, C. Walk, P. Hastie, M. Bedford, and O. Olukosi (2016). Gene expression of nutrient transporters and markers of liver anti-oxidants, gut level immune response and cellular integrity in broilers fed diets containing regular or high doses of phytase. (WPSA, April 2016, Chester, Oral)

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Abbreviations

4PL	Four parameter logistic curve fit
AA	Amino acid
AME	Apparent metabolisable energy
aP	Available phosphorus
ATP	Adenosine Tri-Phosphate
AW	Apical width
BW	Basal width
BWG	Body weight gain
C	Control diet
Ca	Calcium
Ca:P	Calcium-Phosphorus ratio
Ca_2PO_4	Calcium phosphate
$CaCO_3$	Calcium carbonate
CD	Crypt depth
Co	Cobalt
CP	Crude protein
Cu	Copper
d	Day
DM	Dry matter
DMD	Dry matter digestibility
DMT1	Divalent mineral transporter
ECP	E.Coli phytase
ER	Energy retention
FABP2	Fatty acid transporter
FBW	Final body weight
FCR	Feed conversion ratio
Fe	Iron
FI	Feed intake
FTU	Phytase unit, FTU/kg
FP1	Aspergillus ficuum, 3-phytase
FP2	Peniophora lycii, 6-phyase
GALT	Gastro-intestinal associated lymphoid tissue
GE	Gross energy

GIT	Gastro-intestinal tract
GLUT2/4	Glucose transporter 2/4
GPX4	Glutathione peroxidase
HCl	Hydrochloric acid
HKG	House-keeping gene
HP	Heat production
IBW	Initial body weight
IDE	Ileal digestible energy
IFNG	Interferon gamma
IGF	Insulin-like growth factor
IP	Inositol phosphate esters
IP6	Phytate
K	Potassium
LHS	Left hand side
LSD	Least significant differences
ME	Metabolisable energy
Mg	Magnesium
ML	Mucosa length
Mn	Manganese
MUC2	Mucin
MW	Molecular weight
MYO	Myo-inositol
N	Nitrogen
Na	Sodium
$NaHCO_3$	Sodium bicarbonate
NaPi-IIb	Sodium-phosphate co-transporter
NC	Negative control
Ni	Nickel
NPP	Non-Phytate P
P	Phosphorus
P5	Position 5 of the phytate ring
PAMPS	Pathogen associated molecular pattern sequences
PC	Positive control
PepT1	Peptide transporter
PHYT	Phytic acid salt

Pi	Inorganic P
pKa	Acid dissociation constant
PRRs	Pattern recognition receptors
RT PCR	Real-time Polymerase Chain Reaction
S	Sulphur
SA	Apparent villi surface area
SGLT1	Na-glucose co-transporter
SLC7A1	Protein transporter
SLC34A2	Phosphorus transporter
TD	Tibial dyschondroplasia
Ti	Titanium
TJ	Tight junctions
TLR2	Toll-like receptor 2
TLRs	Toll-like receptors
Tm	Melting temperature
tP	Total P
TRPV6	Calcium channel
Trt	Treatment form
TSAA	Total sulphur-containing amino acids
TTR	Total tract retention
VH	Villi height
Zn	Zinc
ZO	Zona occludins

Chapter 1: Review of literature

1.1 General Introduction

As with all other dietary nutrients and minerals, animals have a dietary requirement for phosphorus (P) for essential physiological roles. Although these minerals may be present in the feed, they may not be in a form that is readily available to the animal. In the case of P, there are usually adequate concentrations in the plant material making up the feed, but as a result of P being bound to phytate, it is largely unavailable for utilisation by animals.

Phytate is the plant storage form of P, where it also acts as a mineral reserve, especially during seed germination (Lott *et al.,* 2000). The structure of phytate encourages interaction with divalent cations, making the P essentially unavailable to interact with and be hydrolysed by endogenous phytase for the release of P. It is often incorrectly suggested that non-ruminant (mono-gastric) animals, such as poultry and pigs, do not have the endogenous phytase enzyme required to release the bound P from phytate (Clarke and Wiseman, 2000; Cowieson *et al.,* 2006b; Mitchell *et al.,* 1997) however this is not entirely correct. These animals do have intestinal phytase, however, it is not at sufficient concentrations to efficiently bind with and hydrolyse the phytate due to its interaction with other nutrients, resulting in poor P utilisation from plant based feed ingredients (Sebastian *et al.,* 1998). Therefore, if the animals' dietary P requirement is not met through the supplementation of inorganic P (Pi), a P deficiency may be observed as a reduction in animal performance and poor bone mineralisation. Deficiency symptoms may also be related to the unavailability of other dietary nutrients which are bound to phytate, thus making phytate an anti-nutrient. As well as diminishing animal performance, there are economical and environmental implications to animals being unable to utilise the phytate in feeds and subsequent requirement for supplementation of inorganic P.

As the majority of phytate in feed is not hydrolysed in the gut, a large proportion is excreted, which when applied in high concentrations to arable farm land as manure, the soil (or the surface layers of the soil) have a high P concentration. Surface run-off may occur, with P leaching into nearby

watercourses, where this can have a detrimental effect. Eutrophication is a common problem, whereby algal blooming becomes excessive and reduces the oxygen content of the water, killing many of the aquatic organisms. In some regions, this problem is so severe that legislation and testing of P concentrations of manure has been enforced in order to set the upper limit of P content in soils such as in Maryland USA and the Netherlands (Angel *et al.,* 2002; Biehl *et al.*, 1995; DeLaune *et al.,* 2004; Simons *et al.,* 1990).

The addition of Pi to diets has traditionally been used to meet P requirement in animal feeds. This is a costly practice and so the use of exogenous phytase in animal feeds is increasing. Not only can this have the beneficial effect of reducing environmental pollution and feed costs, but provide further enhancement of animal growth characteristics through its suggested 'extra-phosphoric effects'.

These 'extra-phosphoric effects' are associated with benefits in animal performance beyond those reported as purely through an increase in P release and utilisation. The focus of current research is into the use of phytase, the effects of super-dosing, and understanding these extra-phosphoric effects using growth performance, nutrient digestibility and parameters of gut-health and function. Although there is currently little published research on this topic, it is predicted that super-doses will not only increase the bioavailability of many nutrients in the feed, but also act to improve gut function and thereby immune status (Cowieson *et al.,* 2009; Onyango *et al.,* 2009; Wyatt *et al.*, 2008). Together these effects can benefit animal health and performance, having not only welfare implications, but also improving feed conversion ratio (FCR) and reducing feeding costs through enhanced nutrient utilisation.

1.2 Phosphorus

1.2.1 Physiological requirements

The characteristic symptoms of P deficiency reflect the physiological functions it is involved in. Phosphorus is involved in growth regulation and bone mineralisation via interaction with calcium (Arouca *et al.,* 2012), but is also involved in processes at a cellular level, being involved in metabolism, including:

maintenance of the electrolyte balance (Berner *et al.,* 1976), compensation against metabolic acidosis (Stauber *et al.,* 2005), formation of membrane phospholipids, regulation of carbohydrate and fat metabolism, nucleic acid synthesis (Singh, 2008), as well as being the hydrolysable component of the energy currency (Adenosine Tri-Phosphate) ATP (Suttle, 2010). Changes in bone mineralisation and growth and development may be indicative of the availability of P for cellular processes, generally being the measurement of choice in studies due to the relative ease with which differences can be detected.

1.2.2 Phosphorus deficiency

Inadequate dietary concentration, inefficient utilisation due to being bound to phytate, and location and/or form (i.e. available or unavailable) within the plant can result in P deficiency. As P requirements are influenced by species, age, sex, genotype and environment (amongst many other factors), it is important to ensure animals are fed at the level of requirement in order to reduce the risk of sub-optimal or deficient P levels. There is a requirement for P in gluconeogenesis, protein synthesis and fatty acid transport, primarily through the the involvement of P in ATP (Suttle, 2010), which indicates some of the essential function which may be in jeopardy if dietary P supply is limiting, reducing the potential for overall growth.

One of the implications associated with P deficiency is a reduction in feed intake (Berner *et al.,* 1976), which will consequently lead to a reduction in weight gain. This can quickly become a welfare and economic issue, with the current criteria of selection being for rapidly growing animals for high productivity. A high mortality rate has also been reported with P deficiency (Simons *et al.,* 1990).

Within the bone, P is important for cellular growth and differentiation, as well as for the mineralisation of the matrix component, which is also dependent on calcium (Ca) availability, with 80% of P in the body being in the form of calcium phosphate in the bone (Suttle, 2010). The phosphorus requirements for bone mineralisation are greater than those for growth, occurring as a secondary process only if supplies are adequate (Francesch and Geraert, 2009). During

periods of inadequate P supply, P is mobilised from the bone (Crenshaw *et al.*, 1981).

1.2.3 Absorption

Phosphorus transporters are found throughout the body, such as in the intestine and kidney (Suttle, 2010). In the intestine, the primary mechanism of P absorption is through the action of Na^{2+}-dependant ATPase transporters at the epithelial cell membrane (Berner *et al.,* 1976), of which the sodium-phosphate co-transporter NaPi-IIb is of significance. Phosphorus uptake is primarily in the proximal intestine (Katai *et al.*, 1999). Absorption can be via Na-dependent or Na-independent mechanisms, with Na-dependent absorption being the primary mechanism in the ileum, but Na-independent absorption being the primary mechanism in the duodenum and jejunum (Radanovic *et al.*, 2005).

Intestinal NaPi-IIb transporters can become saturated (Berner *et al.,* 1976), resulting in an increase in unabsorbed P being present in the excreta. As well as being regulated by nutritional and hormonal factors, P transporter activity may be pH dependant (Berner *et al.*, 1976); therefore other nutrients may indirectly influence P absorption. In addition to being regulated by sodium (Na) and P concentrations, the NaPi-IIb transporter is influenced by numerous other factors (Katai *et al.,* 1999) such as hormones, including estrogen (Xu *et al.,* 2003), epidermal growth factor (Xu *et al.,* 2001) and vitamin D (Edwards Jr, 1993; Katai *et al.,* 1999), and other nutrients such as Ca (Wasserman and Taylor, 1973). Phosphorus concentrations are also an important influential factor on NaPi-IIb expression and abundance. During periods of low dietary P, there is an upregulation of NaPi-IIb transporter abundance but no change in mRNA transcript abundance, suggesting that there is an increase in expression to facilitate P absorption (Hattenhauer *et al.,* 1999; Katai *et al.,* 1999; Radanovic *et al.,* 2005; Stauber *et al.,* 2005).

1.3 Phytate

1.3.1 Phytate in plant feedstuffs

Phytate consists of an inositol ring with up to six bound phosphate molecules; the prefix *myo-* refers to the position of the hydroxyl groups on the inositol ring. Of the 12 possible proton dissociation sites, 6 are strongly acidic, 3 weakly acidic and the remaining 3 strongly alkaline (Fontaine *et al.,* 1946). This characteristic determines the ease of displacement of the protons by minerals, resulting in the formation of an insoluble phytate-mineral complex (Fontaine *et al.,* 1946). The pKa (acid dissociation constant), which exists over a range of 1.5 to more than 10, will influence the dissociation at various pH (Maenz, 2001), as might be present through the GIT. Phosphate is released from the phytate molecule following the hydrolytic action of the phytase (Cowieson *et al.,* 2004; Simons *et al.,* 1990).

Phytate is the storage form of P in plants, where it is stored as globoid phytin crystals. Phytate, the salt of phytic acid, binds divalent minerals such as Ca^{2+}, Mg^{2+} and K^{+} and is also known as *myo*-inositol hexaphosphate (IP6). It is estimated that two thirds of the phosphorus in plant material is in the form of phytate (Camden *et al.,* 2001; El-Sherbiny *et al.,* 2010; O'Dell *et al.,* 1972; Simons *et al.,* 1990), however other studies estimate it to be closer to 30% (Angel *et al.,* 2002; Singh, 2008). The phytic acid content of the plant is dependent on the plant species, as well as environmental influences such as the soil and climate. Phytic acid is an important protein and energy store for the plant, as well as having anti-oxidant activity. Alongside this, the intermediate lower inositol-phosphate esters (IPs) released following phytate hydrolysis are involved in a variety of signalling cascades (Bohn *et al.,* 2008). The phosphorus concentration of phytic acid is estimated to be 282 g/kg (Singh, 2008). Adeola and Cowieson (2011) report that in vegetable-based feeds, there is between 5 and 25 g/kg of phytate, providing between 1.4 to 7 g/kg phytate-P. The concentration of phytate can vary between plant species; generally oilseed meals tend to have a greater concentration than cereal grains and cereal by-products. There is also a large degree of variation between these subgroups, for example in the case of cereal grains and by-products, wheat bran > oats > wheat/ barley and > sorghum; and for the oilseed meals: sunflower meal > cottonseed meal > rapeseed meal > soybean meal (Bedford and Partridge, 2001;

Maenz, 2001). There are also differences between the storage sites of phytate between different plant species. As a whole, the phytate-containing globoids are stored in protein-rich tissues of the plant, including the germ and aleurone layer (Kies *et al.,* 2006; O'Dell *et al.*, 1972). The aleurone layer is a protective barrier which surrounds seeds, protecting their contents against digestion and degradation, and is the phytate storage site in cereal plants (Lott *et al.,* 2000; Ogawa *et al.,* 1979) particularly wheat and rice (Maenz, 2001). However, in plants such as maize, phytate is stored within the endosperm. The endosperm is located within the seed and provides nutrition to the developing embryo. The location of phytate storage within the plant influences its susceptibility to digestion, and ease to which the phytase enzyme can interact with it. The procedure of milling and feed processing may influence its availability further by making the phytate more accessible for enzymatic digestion (Maenz, 2001).

1.3.2 Phytate bioavailability

Following ingestion, phytate hydrolysis is facilitated by intestinal phytases as well as by phytase activity of the microflora present in the intestine (Taylor, 1965).

The intestinal phytase activity can be specific or non-specific. Maenz and Classen (1998) proposed that mucosal phytase activity is highest in the duodenum and the total brush border phytase is highest in the proximal gut, with differences seen between laying and broiler hens. It was also identified that there are no differences in activity of endogenous phytases between 4 week old broiler chicks and mature laying hens; however the total brush border phytase activity was greater in the larger birds. It was suggested that this is a consequence of a larger body size, with a more mature and developed GIT and a larger intestinal surface area. Singh (2008) suggested that intestinal phytase levels increase with age.

The brush border intestinal phytases are non-specific alkaline phosphatases (Bitar and Reinhold, 1972; Maenz and Classen, 1998; Onyango *et al.,* 2006) which work at an optimum pH greater than 10. This is very different to the optimal pH of specific exogenous phytases, and allows a greater range of activity as

intestinal conditions change. Generally, the optimal pH for activity of exogenous (E. Coli) phytase is regarded to be around 3.5 to 4.5 (Golovan *et al.*, 1999; Rodriguez *et al.*, 1999; Wyss *et al.*, 1999). However, enzyme activity may vary according to the analytical procedure used, complicating the biological significance of these estimates.

Dietary Pi content has been suggested to affect the availability of phytate-P, with availability diminishing with increasing Pi concentrations. It is proposed that there is a potential adaptation mechanism of intestinal alkaline phosphatase to Pi concentrations, however there is a degree of conflict between studies on this mechanism (Leske and Coon, 1999; Moore and Veum, 1983). Onyango *et al*. (2006) observed an increase in intestinal phytase activity with low dietary P or supplemental vitamin D. Moore and Veum (1983) investigated the potential adaptation of intestinal alkaline phosphatase or phytase. They found that marginal or adequate dietary P had no effect on the amount of duodenal phytase or alkaline phosphatase, but observed a general increase in P absorption in the intestine in P deficient states. There were no significant increases in the synthesis of these intestinal enzymes with P deficiency, at least in this reported study with rats. The authors concluded that rats are able to increase phytate digestibility when fed low dietary P, however the mechanism is not thought to be from the increased action of these phosphatase enzymes. It is possible that adaption may have occurred at the level of intestinal microflora, however, the microbial hydrolysis of phytate in faeces was not considered during the investigation, so may have led to an over-estimation of phytate absorption in the intestine. Some studies in sterile (microbe-free) rats have indicated that some phytate hydrolysis was still occurring and suggested that intestinal microbes had no major role in the release of P from phytate (Applegate *et al.*, 2003a). Additionally, no influence on phytase activity was seen in high phosphate diets, which does not agree with the suggestion of adaption of phytase activity in response to high P (Mohammed *et al.*, 1991).

Nutritional interactions (including the formation of insoluble phytate complexes with dietary components such as minerals, protein and starch), can reduce the availability of P. These complexes prevent the interaction between phytate and phytase for hydrolysis and Pi release. Various studies have demonstrated dietary

interactions and the consequential reduction in P availability. High dietary levels of Ca and magnesium (Mg) have the effect of reducing phytate digestibility and utilisation due to the formation of insoluble complexes (Applegate *et al.,* 2003a). Calcium has been reported to increase phytase activity by 9% when dietary levels were decreased from 9 to 4 g/kg Ca, but this may be detrimental to growth and bone mineralisation (Applegate *et al.,* 2003a).

1.3.3 Phytate anti-nutritional effects

There are many phytate-nutrient complexes which can form, either within the raw material (plants), during processing, or following ingestion. Formation may vary according to intestinal and digesta pH and viscosity, nutrient availability and the presence of digestible enzymes within the gastro-intestinal tract (GIT). Reduced nutrient availability due to binding with phytate can have a detrimental impact on animal performance, with decreased growth and bone mineralisation occurring with poor nutrient utilisation, accretion and feed conversion efficiency (Liu *et al.,* 2008b; Sebastian *et al.,* 1997). Reduction in the abundance of these complexes within the digesta may lead to the observation of extra-phosphoric effects.

Phytase has been noted to improve the ileal digestibility of starch and crude protein, with greatest improvements seen in the jejunum, then the upper and lower ileum respectively (Selle *et al.,* 2012). In rats, phytate appears to inhibit the total tract digestibility of lipids, where phytate may trigger a compensatory increase in lipase output, contributing to endogenous amino acid flow and reduced nutrient utilisation (Selle *et al.,* 2012).

During the first 3 weeks of life, chicks are capable of absorbing small quantities of IP6 but the intestinal microflora are not sufficient to hydrolyse phytate (Adeola and Cowieson, 2011), therefore the saturated form of phytic acid, IP6, forms the highest proportion of unhydrolysed phytate found in excreta (Cowieson *et al.,* 2004); this molecule has a high affinity for chelating with other molecules such as protein (Selle *et al.,* 2006). As dietary phytate increases, there is a reduction in animal performance and digestive efficiency and an increase in nutrient excretion.

1.3.3.1 Influence on proteins

Complex formation

Dietary phytate can bind with protein, thereby reducing protein solubility and the ability of protein to interact with digestive enzymes for its digestion. Thus the digesta pH is reduced, increasing mucin and sodium bicarbonate (buffer) secretion and subsequently reducing Na-K-ATPase dependent nutrient transport and increasing nutrients being excreted. The nutrients which are affected the most through increased excretion include, P, amino acids (AA), Na, iron (Fe), sulphur (S), Ca and (TSAA) sulphur-containing amino acids (Fontaine *et al.*, 1946). These are the nutrients which interact with phytate to form insoluble complexes and as a consequence these nutrient-complexes are not digested and instead pass through the digestive system, being excreted in faeces.

Vaintraub and Bulmaga (1991) and Liu *et al*. (2008) described the potential inhibitory action of phytate on the action of proteases such as pepsin and trypsin resulting in reducing the overall digestibility of dietary proteins. Phytate may bind proteins which are closely associated with starch granules or form covalent bonds between starch polymers, reducing the digestibility of starch. Formation of complexes may occur during the process of seed germination (Selle *et al.*, 2006; Singh, 2008), feed processing and also within the GIT. The structure and the composition of protein and phytic acid may be of importance, as well as the phytic acid content of the diet in determining the degree of phytate-protein complex formation.

Phytate-mineral complexes are charged molecules which allow interaction with protein, allowing the formation of phytate-protein-mineral complexes (Maenz, 2001). These complexes reduce the solubility and hence availability of these bound nutrients (Ravindran *et al.,* 2001). The type of bond which forms between nutrients varies according to pH (Fontaine *et al.*, 1946). At low pH, electrostatic interactions occur between phytate and the free amino groups of arginine and lysine at the terminal amino group (Adeola and Sands, 2003). However, at high pH, salt bridges are more likely to form (Angel *et al.,* 2002). The amino acid composition of the protein therefore can influence the properties/ charge/ interactions with phytate in the formation of phytate-mineral complexes

(Ravindran *et al.,* 1999), with solubility varying at different pH. Basic AAs have been identified as the most effective at binding with phytate (Kies *et al.,* 2006).

Phytate-agglutinin complexes in soybean meal are an example of phytate-protein complexes which can subsequently form complexes with minerals such as Fe (Honig and Wolf, 1991). These phyate-agglutinin-mineral complexes are unavailable to bind with phytase, which explains why protein utilisation of soybean meal doesn't appear to be improved following the supplementation of phytase (Peter and Baker, 2001). However, Kies *et al.* (2006) reported a low presence of phytate-protein complexes in soybean meal, suggesting that the formation of phytate-protein complexes prior to feeding can be considered as almost irrelevant. It is therefore assumed that the majority of complex formation occurs in the GIT.

Endogenous protein loss – influence of phytic acid

Phytic acid is an anti-nutrient and increases endogenous protein losses (Cowieson and Ravindran, 2007). Endogenous protein loss includes the sloughing of intestinal cells, digestive secretions such as enzymes (e.g. pepsin) and mucus excretion and microflora (Hodgkinson *et al.,* 2000; Cowieson and Ravindran, 2007). Protein complexes are hypothesised to cause hypersecretion of mucin, sodium and bicarbonate and it is suggested that this may occur through an indirect mechanism (Cowieson *et al.,* 2008).

Influence of pH

There are two types of phytate-protein complexes which can form (Figure 1.1), depending on the digesta pH. At a lower pH, binary phytate-protein complexes form from direct binding between the protein and phytate molecules. Above pH 7, ternary phytate-protein complexes are more likely to form, which is the binding of a protein to a phytate-mineral complex (Kies *et al.,* 2006). A binary protein complex occurs when protein binds directly with phytate and a ternary complex is formed when calcium (or other mineral) is an intermediate between the binding of phytate and phosphorus (Kies et al., 2006). As the pH of the pig stomach and poultry crop, proventriculus and gizzard are around 1 to 6, binary complexes form at a higher proportion than ternary complexes (Kies *et al.,* 2006).

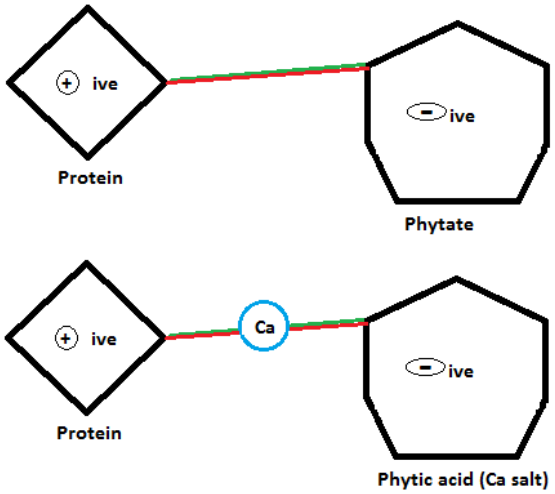


Figure 1.1 – An example of a binary phytate-protein complex (top) and ternary phytate-protein-mineral complex where calcium is the ternary mineral (bottom).

Protein-Phytate precipitation

At a low pH of around 2.5, the phytic acid molecule has a strong negative charge (Cowieson *et al.*, 2009) and is able to interact with a positive protein molecule (Singh, 2008). Increased phytate-protein complex dissociation occurs at low pH (Fontaine *et al.*, 1946). If the pH is above 4, the protein and phytic acid molecules have a net negative charge, with few complexes expected to form (Kies *et al.*, 2006). Dissociation and subsequently precipitation ensues once the isoelectric point has been reached as neutralisation of the charge occurs and phytic acid becomes soluble releasing the previously-bound protein (Ravindran *et al.*, 1999). The isoelectric point of proteins varies between plants and protein composition. Cereals such as wheat, maize and sorghum have a higher isoelectric point than oilseed meals such as cottonseed, soybean and rapeseed meals (Selle *et al.*, 2012) and so precipitation would occur under different conditions of the GIT. Microflora in the hindgut may also be involved in phytate-protein complex degradation (Mroz *et al.*, 1994). Although this has little effect on protein utilisation for the animal as there is little nutrient absorption occurring in the hindgut, there is an increase in substrate availability for use by the microflora.

1.3.1.3.2 Minerals

Phytate is able to interact with minerals to form insoluble complexes, which at neutral pH are resistant to degradation (Maenz *et al.,* 1999; Maenz, 2001), thus reducing mineral bioavailability (Lei and Porres, 2003). The phytate-mineral complexes also reduce the capacity of phytate-phytase interaction for hydrolysis and Pi release (Maenz, 2001). Mineral excretion has been noted to escalate with increasing phytate concentrations (Cowieson *et al.,* 2006a). Cowieson *et al.* (2006a) reported an increase in Ca, Mg, manganese (Mn) and Na excretion of 187, 39, 87 and 174% respectively following the ingestion of 1g phytate.

The formation of phytate-mineral complexes can have other indirect effects on nutrient utilisation, such as the formation of phytate-mineral starch complexes (Cowieson, 2005) and the formation of soaps in the GIT, which have the effect of reducing lipid utilisation (Ravindran *et al.,* 2001).

Phytic acid binds with K, Mg or Ca to form phytin, which is less soluble than the unbound phytic acid (Angel *et al.,* 2002; Maenz, 2001). This is because phytate has a high affinity to binding divalent cations (Biehl *et al.,* 1995; Kies *et al.,* 2006). The potency with which phytate binds varies according to the chemical properties of the mineral it is bound to. With increasing potency and stability of binding, interactions include: Fe < Ca < Mn < cobalt (Co) < nickel (Ni) < copper (Cu) < zinc (Zn) (Singh, 2008). The solubility of these complexes depends on the period of incubation, pH, presence and proportion of cations and the mineral: phytate ratio (Biehl *et al.,* 1995; Singh, 2008). For example, at neutral pH, a high mineral: phytate ratio may allow the formation of an insoluble complex between Zn and position 5 (P5) of the two phytate molecules, in effect forming a 'bridge'. As Cu tends to associate with P4 and P6 positions of the phytate molecule, the complex is likely to be more soluble (Maenz, 2001). Generally, the pH of the digesta increases as it passes distally through the GIT, which reduces solubility as mineral interaction and subsequent complex formation occurs (Angel *et al.,* 2002). As the complexes tend to be soluble at pH < 3.5 and insoluble at pH 4 to 7, the majority of complexes are insoluble in the duodenum, increasing mineral presence in excreta (Angel *et al.,* 2002).

Some of the most important minerals involved in phytate susceptibility to hydrolysis are identified and described in the following text. Others may have significant influences with phytate, but for the purpose of this thesis, Ca, Na and micro-minerals (Zn, Cu, Fe) will be the main focus of subsequent sections.

Calcium

Although not the most potent mineral to bind with phytate, Ca has the greatest effect on phytate availability through the formation of phytate-Ca complexes (Singh, 2008; Walk *et al.,* 2012a). This is because Ca is more abundant in diets than other minerals, and through the law of mass action, is the most common mineral to form phytate complexes (Angel *et al.,* 2002; Singh, 2008). Calcium is often provided in the diet as $CaCO_3$ (calcium carbonate), its solubilisation in the duodenum is directly related to the gastric acid secretions of the proventriculus and gizzard (Guinotte *et al.,* 1995). Dietary Ca is able to influence the pH of the GIT, which may have the effect of reducing IP6 solubility and therefore phytate hydrolysis (Angel *et al.,* 2002). Most Ca and P absorption occurs in the small intestine, where the relationship between the two is speculated to be through a chemical association in the intestinal lumen, rather than at the site of absorption (Hurwitz and Bar, 1971).

The absolute levels of dietary Ca are important when considering associations with phytate (Driver *et al.,* 2005) and it is important to ensure interactions are accounted for when calculating requirements. In order to do this, the following equation has been suggested: 0.6% phytate-P + 1.1 (Singh, 2008). This indicates the increasing Ca requirements as phytate level increases, due to the phytate-Ca binding. This relationship is in agreement with a study reported by Qian *et al.* (1997), where maximum weight gain was seen when the dietary calcium-phosphorus ratio (Ca:P) was 1.1:1, with supplemental phytase and vitamin D. With high levels of Ca, there is increased formation of Ca_2PO_4 (calcium phosphate) in the gut, which reduces P availability further (Driver *et al.,* 2005). This may help explain the antagonistic relationship between Ca and P in the gut (Driver *et al.,* 2005). In a P deficient, high Ca diet, Driver *et al.* (2005) identified Ca × P interaction for body weight gain but not for feed intake. However, Hurwitz and Bar (1971) and Sebastian *et al.* (1997) did not observe any

interactions for growth performance, but a reduction in feed intake led to a decrease in body weight gain.

Alongside direct P × Ca interaction, Ca is also able to bind with inositol phosphates (phytate) with a high affinity (Luttrell, 1993). Therefore, the Ca: phytate-P ratio can be considered to be of importance, with a high ratio increasing the formation of insoluble phytate-Ca complexes, particularly with the higher phytate esters (Selle *et al.*, 2012). These phytate-Ca complexes are able to chelate with fatty acids in the GIT, which has the effect of forming a soap-like mixture, and reducing fat digestibility (Singh, 2008). Therefore, by reducing the Ca level (whilst ensuring requirements are met), nutritional efficiency can be improved, as well as reducing the phytate-P content of excreta (Bedford and Schulze, 1998; Taylor, 1965), thereby helping to reduce environmental pollution. The Ca:P ratio is an important influencing factor, often considered to be more important than the absolute Ca level (Qian *et al.*, 1997). The benefits of using a narrow Ca:P ratio alongside phytase supplementation are reported in many studies (Edwards and Veltmann, 1983; Selle *et al.*, 2006)

Deficiency diseases such as rickets and tibial dyschondroplasia (TD) have a high incidence when P is high and Ca is low, but P retention is greatest when the Ca:P ratio is narrow, indicating the complex relationship and importance of Ca:P ratio (Edwards and Veltmann, 1983). Maximum bone ash in broilers was recorded when dietary P was at 1.12% and Ca at 2.15%, however there is no correlation identified between TD and bone ash % (Edwards and Veltmann, 1983). Calcium: aP ratios between 1.4:1 and 4.1:1 have been reported to have a detrimental influence on growth performance parameters (Shafey *et al.*, 1990).

As Ca is an important enzymatic cofactor, phytate-Ca complex formation reduces the Ca available for this role and may lead to a reduction in function, which can have many different consequences depending on the physiological process involved. Due to the wide range of possible enzymes potentially influenced by phytate-Ca binding, only a couple will be mentioned, giving particular reference to nutritional implications, whereby following phytase supplementation, ‘extra-phosphoric effects’ may be seen. Intestinal amylase is involved in starch digestion and due to Ca being a cofactor for its activity, an

increase in Ca and subsequently Ca-phytate binding and diminished amylase activity would be seen (Selle *et al.,* 2012). This has the effect of reducing the release of glucose from starch hydrolysis, which may have detrimental effects on growth performance (Cowieson, 2005; Liu *et al.,* 2008b). Similarly, the activation of trypsin from trypsinogen by enteropeptidase also requires the presence of Ca (Caldwell, 1992). Trypsinogen contains two Ca binding sites, and one trypsin binding site (Singh and Krikorian, 1982). Here Ca has two effects on carbohydrate digestion, the first being the activation of trypsin by trypsinogen binding, and the second being the inhibition of trypsin degradation by binding. In addition to this, a ternary complex between Ca-bound trypsin and phytate may form, rendering the enzyme dysfunctional.

Sodium

The importance of phytate on processes requiring Na is a result of the properties of phytic acid, with an increase in Na excretion occurring as a result of an increase in phytate levels (Cowieson *et al.,* 2004, 2009; Liu *et al.,* 2008b). Interactions between phytate, phytase and Na have been noted in studies on rats by (Liu *et al.,* 2008b). Increased Na excretion is observed following phytate supplementation in poultry, reducing the Na available for Na-dependant transport mechanisms (Cowieson *et al.,* 2009). The buffering effect of phytate may result in the movement of Na into the gut lumen and may cause Na to become limiting for other processes such as for the transport of glucose (Liu *et al.,* 2008b). Additionally, as sodium is important for glucose and amino acid transport in a dependant manner, the interaction of phytate is significant in the absorption of these substances and thus their bioavailability.

It is reasoned that the movement of Na as bicarbonate and bile salts (Cowieson *et al.,* 2006a, 2008) into the intestinal lumen restores the acid-base balance and acts as a buffer to phytic acid, which otherwise irritates the gut (Selle and Ravindran, 2007). Sodium is also important for the activity of many intestinal transporters (Gal-Garber *et al.,* 2003; Liu *et al.,* 2008b; Sklan and Noy, 2000) and for nutrient absorption the intestine through an ATPase generated chemical gradient (Liu *et al.,* 2008b). Therefore, reduced Na availability by phytate-Na complexes can have a number of effects in the body.

Micro-minerals

It is well documented that phytate is able to reduce the bioavailability of various micro-minerals, such as Cu, Fe, Mn and Zn (Davies and Nightingale, 1975; Tamim and Angel, 2003). The formation of phytate-mineral complexes reduces bioavailability of the minerals (Davies and Nightingale, 1975).

There are many complexes between Zn and phytate (Biehl *et al.*, 1995; O'Dell *et al.*, 1972) which can form in the small intestine at pH 6 (Kornegay, 2001), both binary and ternary. Binary complexes involve the direct connection between phytate and Zn, while the ternary complexes include the formation of phytate-Zn-Ca (Kornegay, 2001) and phytate-Zn-protein complexes (Lei and Porres, 2003; O'Dell and De Boland, 1976). Martinez *et al*. (2004) reported an increase in plasma Zn concentrations with increasing dietary Zn and phytase supplementation. The author also reported Zn × phytase interaction on renal Cu and liver Zn and P concentrations. It is suggested that physiological and morphological changes occur within the gut as Zn concentrations increase, which may lead to some of the 'extra-phosphoric effects' of phytase described later, and may also lead to an understanding of the health-promoting effects of super-dosing of phytase, as is the purpose of this investigation.

Other mineral complexes deemed of significance in this thesis are between phytate and Fe. Due to the divalency of Fe, complexes can form with phytate, reducing Fe bioavailability (Brigide and Canniatti-Brazaca, 2006; Nolan *et al.*, 1987; Tako *et al.*, 2014). If Fe is bound to phytate and consequently not absorbed, this has the potential to influence caecal bacterial populations (Deriu *et al.*, 2013; Kot *et al.*, 1995; Yeung *et al.*, 2005).

1.4 Phytase sources

There are exogenous and endogenous source of phytase for poultry. Endogenous sources include intestinal phytase occurring naturally within the animal, whereas exogenous are purified sources, often from bacteria.

The beneficial effects of phytase are the reduction in the anti-nutritional effects associated with dietary phytate (Cowieson *et al.*, 2004). Phytase allows

increased nutrient accretion, including P, minerals, proteins and other dietary components, potentially giving a greater benefit above supplying P at a rate which would meet requirements. These are often referred to as 'extra-phosphoric effects'. Despite current estimations of the P release from phytate without phytase supplementation being < 35% (Powell *et al.,* 2011), the use of phytase in the animal feeding industry is increasing. This may be due to the ease and practicality of use, the fact that it can be added to many different feeds, and also the extensive economical and environmental benefits (Angel *et al.,* 2002; Cowieson *et al.,* 2006b; Selle *et al.,* 2012).

1.4.1 Properties of phytase

Phytase; myo-inositol hexaphosphate phosphohydrolase, Enzyme Classification 3.1.3.8/ 3.1.3.26 (Mahajan and Dua, 1997; Onyango *et al.,* 2004), is an exogenous enzyme used to increase phytate hydrolysis into inositol X-phosphate and Pi. Phytase activity is influenced by intestinal viscosity and nutrient concentration (availability of nutrients to complex with phytate; Cowieson, 2005). A greater incubation period of enzyme and substrate within the intestine increases the potential for nutrient release (Bedford and Schulze, 1998), intensifying the degree of phytate hydrolysis, nutrient utilisation and efficiency of phytase (Selle *et al.,* 2006). Digesta pH is also an important factor influencing the action of phytase. The greater the pH, the lower the phytic acid solubility (Simons *et al.,* 1990), and the less likely phytate would be able to bind with phytase. The greatest exogenous phytase activity in pigs occurs in the stomach which has a pH around 2.5, but phytase activity becomes negligible by the time it reaches the distal ileum even at super doses (Applegate *et al.,* 2003b). However, work by Morgan *et al.* (2015) demonstrated that phytate hydrolysis was a result of both endogenous and exogenous phytase, detecting 45 U/kg endogenous phytase activity in the ileum of 14 day-old broilers. Feed ingredients can also influence phytase efficiency depending on the solubility at intestinal pH, for example, soybean meal is more soluble than sesame meal and can therefore more readily be hydrolysed by phytase (Singh, 2008). The supplementation of 500 FTU/kg of an *E.Coli* derived phytase can increase Pi release more in a soybean meal diet than a maize-based diet. This may be due

to a higher non-phytate-P (NPP) content or the location of phytate; there is a limited phytate availability in the maize (Adeola *et al.*, 2004).

The hydrolysis of phytate occurs in a step-wise manner with each step releasing a lower IP ester and phosphate, which can then be absorbed and utilised by the animal, until finally just the (myo-) inositol ring remains (Lei and Porres, 2003) as shown in Figure 1.2.

Figure 1.2 – The step-wise hydrolysis of phytate, with each step producing a lower inositol phosphate ester and releasing phosphate for absorption, with inositol being the final product of phytate hydrolysis.

The efficacy of phytase is determined by the end point ester of phytate hydrolysis, e.g. IP5 vs. IP2 (Cho *et al.*, 2006), however, it is considered to be of more importance to hydrolyse IP6 to IP5, than to hydrolyse completely to IP1 (Adeola and Cowieson, 2011). This is because the nutrient binding affinity of IP6 is greater than the lower esters, removal of which would increase the nutrient availability and improve animal performance.

1.4.2 Factors affecting phytase efficacy

1.4.2.1 pH

There are many factors which affect phytase efficacy such as temperature, substrate availability and pH (Lucas, 1983). These factors can be manipulated during feed manufacture, in processes such as heat treatment, body temperature, dietary composition and presence of phytate complexes; which may abate phytate-phytase interaction and subsequent phytate hydrolysis. Gastric and dietary pH can be affected by the substrate availability of the diets and the temperature at which they were processed at, subsequently influencing nutrient digestibility and phytase efficacy (Dono *et al.*, 2014; Olukosi and Dono,

2014; Ptak *et al.,* 2015). A lack of substrate at the intestinal lumen (i.e. during a period of feed deprivation) gives a low intestinal pH (Lucas, 1983), which may influence nutrient absorption, potentially leading to inflammation and damage to intestinal epithelia.

A change in molecular charge causes a subsequent change in pKa (acid dissociation constant) and optimal pH for enzymatic action and is referred to as the enzyme pH activity model. The charge can be altered through a variety of changes to its outer structure, influencing its potential for interaction. These include the replacement of ionic groups which are able to interact and form complexes, the replacement or removal of residues which form hydrogen bonds or salt bridges, as well as manipulation of the surface charge to alter associations (Leenhardt *et al.,* 2005). Intensifying digesta negativity may result in an increase in pKa and optimal pH. These manipulations may be beneficial for increasing phytase activity, where the pKa regulates complex precipitation (Leenhardt *et al.,* 2005). A decrease in gastric acid pH may increase the efficacy of phytase by making conditions more favourable for its action, and thereby benefit animal performance through accentuation of the 'extra-phosphoric effects'. Benefits may occur through increased nutrient bioavailability following their release from phytate hydrolysis and conditions being more favourable for their precipitation and absorption.

The interaction of phytate with nutrients (Taylor, 1965) and phytase (Leenhardt *et al.,* 2005; Taylor, 1965) is influenced by digesta pH. A suitable pH is also required for the digestion, absorption and utilisation of nutrients (Dono *et al.,* 2014; Ptak *et al.,* 2015), influencing (and influenced by) enzyme activity and the microbial profile (Dono *et al.,* 2014; Ewing and Cole, 1994; Fuller, 1984; Lan *et al.,* 2005; Ptak *et al.,* 2015). Feed enzymes, such as phytase, have been reported to influence digesta pH (Steiner, 2006; Walk *et al.,* 2012b). Walk *et al.* (2012b) reported an increase in GIT pH following the supplementation of super doses of phytase (2,500 FTU/kg), suggesting the response was due to reduced phytate concentrations and reduced phytate-calcium complexes, as Ca and the Ca:aP ratio can have a significant influence on digesta pH (Shafey, 1993).

1.4.2.2 Calcium

High levels of Ca present in the digesta can have a negative influence on the activity of phytase (Akter *et al.,* 2016; Applegate *et al.,* 2003a; Tamim *et al.,* 2004). The formation of phytate-Ca complexes, Ca-phytase binding and precipitation as calcium-phosphate may be responsible for the negative effects associated with high levels of Ca (Tamim *et al.,* 2004). Applegate *et al.* (2003a) suggested that "typical" dietary Ca concentrations were at such a level that there was a decrease in intestinal phytase activity, with activity being greater in diets containing 0.4% Ca than 0.9% Ca, which is closer to recommendations (NRC, 1994; Ross 308 Specifications, 2007).

Walk *et al.* (2012b) reported Ca × phytase interaction, where high dietary Ca levels increased gizzard and ileal pH, and appeared to interfere with P and crude protein digestibility. The author suggested that the use of super-doses of phytase (such as the 5,000 FTU/kg used in the reported study) would allow for a reduction in dietary Ca concentrations, whilst maintaining growth performance parameters. Similarly, Olukosi and Fru-Nji (2014a) observed that the best response to lower levels of phytase (1,000 FYT/kg) occurred when a nutrient matrix was applied with the supplementation of phytase and the Ca: total P (tP) ratio was narrow. Akter *et al.* (2016) also highlights the benefits of an enzyme matrix which considers the release of Ca and non-phytate phosphorus following phytate hydrolysis. The specifications of an enzyme matrix are specific to the enzyme supplemented and dose used. The matrix takes consideration of the release of nutrients (primarily minerals) which would be released during phytate hydrolysis. This allows them to be removed from the dietary formulations, enhancing their utilisation when released into the intestine, but also allowing a cost saving to the feed formulation. When higher doses of phytase were used (2,000 FYT/kg) in nutritionally adequate diets (without an enzyme matrix applied), the best response was observed with a wide Ca:tP ratio, and may have been a result of the restoration of an adequate Ca: available P (aP) balance as an extra-phosphoric influence of the super doses of phytase. A reduction in Ca:P ratio has the effect of improving growth performance, bone ash, and P digestibility when phytase is applied (Dilger and Adeola, 2006; Walk *et al.,* 2012b), which suggests that an optimal Ca:P ratio may exist for phytase activity. It is known that Ca and phytate may interact in the intestine, forming

complexes. In addition to this, a high dietary Ca concentration can have the effect of reducing the pH of the GIT, which may consequently reduce phytate hydrolysis. It is possible that phytase is able to reduce the negative effects of high dietary Ca levels (Walk *et al.*, 2012b).

1.4.3 Types of phytase

1.4.3.1 Endogenous phytase

Endogenous phytases are thought to be evolved from a family of histidine acid phosphatases, many of which are non-specific, including multiple inositol polyphosphate phosphatase, histidine acidic and alkaline phytases (Cho *et al.*, 2006; Lei and Porres, 2003). The acidic phytases hydrolyse metal-phytate, with a broad range of specificity, to produce myo-inositol mono-phosphate. The main product from specific alkaline phytase hydrolysis of phytate-Ca complexes is myo-inositol tri-phosphate. Alkaline phytases may be more prominent in the GIT of mono-gastrics due to their stable characteristics (Oh *et al.*, 2004). Other endogenous phytases include intestinal phytases which tend to have a higher affinity for lower esters, meaning exogenous phytases are required to hydrolyse the phytate and higher IP esters (Cowieson *et al.*, 2008).

The efficacy of endogenous phytase activity can vary between and within species. For example, phytase activity may be up to 60% greater in laying hens than broilers, but negligible in pigs (Bedford and Schulze, 1998). This may be due to differences in GIT and the rate of passage of digesta. The dry matter (DM) content of feeds may also be important, giving an indication of the concentration and solubility of nutrients and phytate within the feed, particularly as phytase supplementation has been reported to increase DM digestibility (Mroz *et al.*, 1994). It can be assumed that the higher rate of passage and concentrated solutes in the chick GIT may explain these species differences, with pigs having a larger volume of water and pancreatic juices in the intestine (Bedford and Schulze, 1998), diluting the endogenous phytase concentrations.

1.4.3.2 Exogenous phytase

Modern exogenous phytases have the ability to hydrolyse phytate in a more efficient manner than endogenous phytases, due to their relative resistance to GIT denaturisation (Olukosi and Adeola, 2008). Phytases have been extracted and purified from plant, microbial and yeast sources, with each having its own characteristics and effects. There is also a degree of phytase activity in plants which varies between species as follows: rye > wheat > barley > oats and maize (Taylor, 1965; Viveros *et al.*, 2000). However the process of feed manufacture and heat treatment renders most plant phytases inactive (Angel *et al.*, 2002; Kornegay, 2001). The use of more than one source of phytase in combination has not been found to have any additional beneficial effects (Lei and Porres, 2003); each phytase source will be discussed briefly in the following section.

1.4.3.3 Plant phytase

The low activity of plant phytase was noted as early as 1945 (Spitzer and Phillips, 1945), due to its poor stability, solubility and inactivation during feed processing (Bohn *et al.*, 2008). The effectiveness of phytase is greatly dependant on phytate bioavailability within the plant material, which may be influenced by phytic acid, mineral and fibre content (Bohn *et al.*, 2008). A positive correlation has been identified between total P and phytate-P, as well as between phytase/ acid phytase activity and phytate-P content (Viveros *et al.*, 2000).

There is a great degree of variability in phytase activity both between and within plant species (Bedford, 2000). An example of the degree of variability between plant phytases may be described for differences in optimal conditions, such as temperature and pH. Optimum conditions for peanut phytase are pH 4.8 to 6.0 and 25^{o}C, with Pi bioavailability increasing in peanut meal when digesta pH is above pH 1.7 due to conditions being optimal for the activity of plant phytase. In cottonseed, maximal P solubility (85%) occurs at pH 0.50 and 6.5 at 25^{o}C. Phosphorus is less soluble in cottonseed than peanut meal which may be due to a greater Mg/Ca: K ratio increasing the likelihood of phytate complex formation. There is also twice the P content in cottonseed meal than peanut meal which may also increase the formation of phytate-protein complexes (Fontaine *et al.*, 1946).

Plant phytases are more effective at hydrolysing lower esters, such as IP3 and IP4 (Lei and Porres, 2003), but are more rapidly degraded than microbial phytases during gastric digestion (Cowieson and Adeola, 2005) with an optimal pH of around 5 (Angel *et al.*, 2002).

1.4.3.4 Microbial phytase

With an optimum pH range of 2 to 6 (Angel *et al.*, 2002), phytate hydrolysis by microbial phytases are generally specific to IP6 (Lei and Porres, 2003). This confirms the proposal that microbial phytase is most suitable for commercial use due to its optimal pH and thermostability properties (Bohn *et al.*, 2008). Using pigs in the weaning-finishing stage fed maize-soybean meal diets; microbial phytase (*A. ficuum*) was found to give greater improvements in animal performance than cereal phytase (Han *et al.*, 1997).

There are two main classes of phytase; 3-phytase and 6-phytase, however a single 5-phytase has been identified from lily pollen (Bohn *et al.*, 2008). The 3-phytase has the characteristic of removing the Pi at position 3 of the phytate molecule, and 6-phytase from the 6th position (Cowieson *et al.*, 2008), as shown in Figure 1.3.

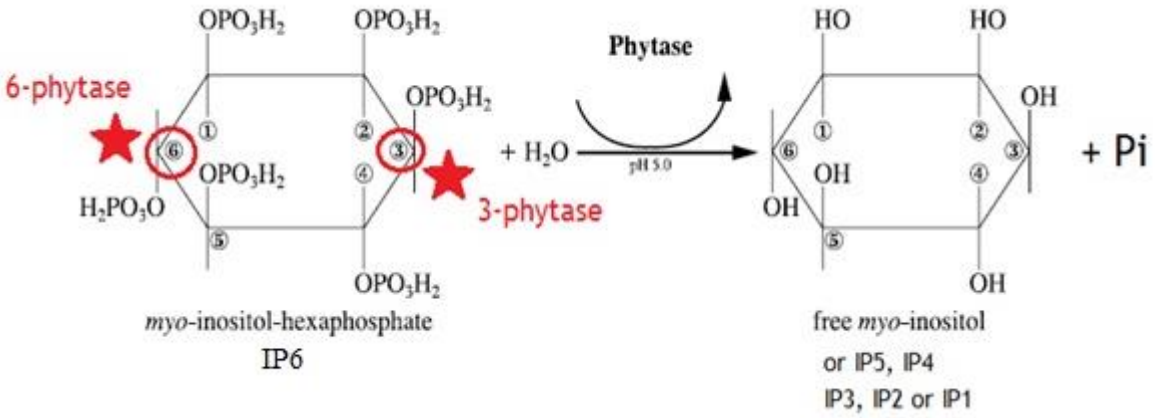


Figure 1.3 – The hydrolysis of phytate by phytase to produce lower IP esters and phosphate (Pi) and the site of action by 3-phytase and 6-phytase, adapted from (Liu *et al.*, 1998).

Examples of 3-phytase include *Aspergillus ficuum* and *Aspergillus niger* (Onyango *et al.,* 2004). *Aspergillus niger* is the most active source of phytase, having maximal activity at pH 2.5 and 5.5 at 60^{o}C (Adeola and Sands, 2003). *Aspergillus ficcum* is the most thermostable microbe producing the highest concentrations of phytase (Singh, 2008) and so is a beneficial source of exogenous phytase for supplementation. The 3-phytase may not have the ability to complete dephosphorylation, whereas due to high specificity to IP6, more complete dephosphorylation can occur with a 6-phytase, however full hydrolysis is unlikely to be achieved in the GIT (Angel *et al.,* 2002). Some examples of 6-phytases, which are occasionally referred to as 4-phytases, include *Peniophoria lycii* and *E.Coli* (Onyango *et al.,* 2004).

In a study with pigs, an *E.Coli* derived 6-phytase (ECP), purified from yeast and isolated from the pig intestine was seen to have a greater beneficial effect on pig performance than a 3-phytase isolated from *Aspergillus ficuum* (FP1) or a 6-phytase isolated from *Peniophora lycii* (FP2) (Augspurger and Baker, 2004). A greater increase in weight gain, feed conversion efficiency and tibia ash weight was found in chicks with ECP supplementation compared to 500 or 1,000 FTU/kg of FP1, however there was significant interaction between phytase source and level of activity at the higher levels of supplementation. All sources had a greater degree of activity when added at higher doses however increasing levels of FP1 and/or FP2 reach the same levels of activity as ECP, the differences are thought to be a result of the differing optimum pH. At 10,000 FTU/kg the tibia ash weight was increased to a greater proportion than 0.20% Pi supplementation which shows that phytase supplementation is more effective than Pi supplementation for improving bone mineralisation characteristics (Augspurger and Baker, 2004).

Onyango *et al*. (2004) reported different efficacies of different preparations of E. coli phytases on animal performance and bone strength. Applegate *et al*. (2003b) also studied the differences in activity and efficacy of phytase from different sources, observing that different sources may have different resistance characteristics to digestive enzymes such as pepsin and trypsin. The less resistant the phytase enzyme is to protease enzymes, the greater the potential for phytase activity and release of nutrients for utilisation. Enzyme sources with

a greater efficacy can be provided at a smaller concentration. Microbial phytase may be more effective than plant phytase due to the optimal conditions for its activity being more suited in the GIT of poultry and pigs, making it a suitable additive to animal feed in order to enhance animal performance.

1.4.4 Benefits of phytase supplementation

The efficacy of the phytase is often determined through the degree of ileal phytate degradation (Camden *et al.,* 2001), and can be influenced by factors such as dietary composition, animal species, age and genotype (Singh, 2008); for example, phytase activity is more efficient when dietary P is low (Akinmusire and Adeola, 2009). The benefits of exogenous phytase may occur primarily through depleting proportions of phytate in digesta, rather than through a reduction in phytate-Ca complexes (Walk *et al.,* 2012a). In addition to increasing P utilisation following phytate hydrolysis, the increased bioavailability of other nutrients enhances the effect on animal performance further, described as 'extra-phosphoric effects'.

1.4.4.1 Animal growth and performance

The hydrolysis of phytate by phytase releases lower inositol phosphate esters and Pi for utilisation by the animal, however, complete hydrolysis to myo-inositol is not regularly achieved. Despite the improvement in P availability, other nutrients may become limiting to growth, for instance those that form complexes with phytate, such as Ca (Singh, 2008). Enhanced animal growth, and subsequently performance, with exogenous phytase is considered to be a consequence of: phytate degradation, P release, reduced complex formation therefore increased bioavailability of other nutrients, as well as increased feed intake and improved feed conversion efficiency (Camden *et al.,* 2001; Liu *et al.,* 2008b). The absorption of inositol and/or soluble phytate into tissues prevents complex formation and enzyme inhibition (Bedford and Schulze, 1998). Decreased inhibition of enzymes such as pepsin and trypsin increases the availability of their products for utilisation by the animal, possibly explaining the 'extra-phosphoric effects'.

There are many studies which report the benefits of phytase on growth performance parameters. As well as leading to improvements in BWG (Adeola *et al.,* 2004; Biehl *et al.,* 1995; Camden *et al.,* 2001; Cowieson *et al.,* 2009; Lei *et al.,* 1993b; Liu *et al.,* 2008b; Nelson *et al.,* 1971; Qian *et al.,* 1997; Sebastian *et al.,* 1997), phytase has also been reported to decrease the incidence of mortality (Camden *et al.,* 2001; Cowieson *et al.,* 2009; Liu et al., 2008b; Qian *et al.,* 1997).

A phytase induced decrease in mortality may be a consequence of improved nutrient bioavailability, either through the reduction in phytate or greater feed intake (FI). An increase in feed intake will give a subsequent weight gain (BWG), as is generally reported in the literature (Adeola *et al.,* 2004; Camden *et al.,* 2001; Cowieson *et al.,* 2009; Liu *et al.,* 2008b; Powell *et al.,* 2011; Qian *et al.,* 1997; Sebastian *et al.,* 1997). There are some contradictory results in the general literature and the reduced BWG, as noted by Cowieson and Bedford (2009), may be a result of a reduction in FI. In this case, it was suggested that the deficiency of dietary P throughout the trial may have reduced the appetite enough that bodily reserves were mobilised to meet requirements, leading to the observed drop in body weight. As increases in both BWG and FI tend to occur following phytase supplementation, there are often no improvements in FCR (Powell *et al.,* 2011; Sebastian *et al.,* 1997). Powell *et al.* (2011) reported an increase in BWG and FI with the supplementation of 500 FTU/kg phytase, which consequently meant there were no improvements in feed conversion efficiency.

Pigs fed a low NPP diet, with supplementation of an *E.coli* derived phytase showed an increase in both FI and subsequently BWG (Adeola *et al.,* 2004). In this study it was found that the phytase had a greater influence on P availability and digestibility in pigs of a larger body weight, suggesting that the larger (and older) the pig, the more developed the GIT will be, with potentially more active endogenous phytase in the intestine to hydrolyse phytate and release P for absorption. Similarly, according to Sohail and Roland (1999), when the dietary NPP content was 0.225%, raising phytase from 0 to 300 FTU/kg increased the bone mineral content by 28.4% ($P < 0.01$). Supplementing phytase at 300 FTU/kg and increasing NPP from 0.225% to 0.325% gave a further increase in bone mineral content of 5.1%. It was found that 300 FTU/kg of phytase reversed the

negative effect of 0.225% NPP and 0.75% Ca on bone strength, preventing the onset of P deficiency symptoms; however, increasing phytase to 600 FTU/kg had no further effect. These results show that phytase is capable of improving P availability for bone mineralisation, but the degree to which this can occur is influenced by the proportion of dietary-P which is in a soluble form, NPP. Similar to these findings, Applegate *et al.* (2003b) observed a significant increase in tibia and toe ash content with phytase supplementation. Biehl *et al.* (1995) reported an in increase in tibia Zn and Mn content when phytase was fed alongside a vitamin D supplement and Singh (2008) observed a linear increase in bone ash following phytase supplementation. Supplementation of 10,000 FTU/kg phytase to nursery and grower/ finisher pigs increased the bone ash by 15% compared to the negative control and 5% relative to the positive control. This confirms the assertion that phytase has a greatest effect when P is deficient and that the phytase is able to improve animal performance measurements above an adequate P diet when provided to deficient diets. Overall, phytase is reported to lead to an increase in bone mineralisation (Camden *et al.,* 2001; Cowieson *et al.,* 2009; Liu *et al.,* 2008b; Nelson *et al.,* 1971; Powell *et al.,* 2011; Qian *et al.,* 1997), likely as a result of increase P and Ca bioavailability.

1.4.4.2 Nutrient utilisation

Improving nutrient utilisation will enhance animal performance through an increase in nutrient accretion and availability for the processes involved in growth and bone mineralisation. Following phytase supplementation, there is often an increase in nutritional utilisation and availability from increased ileal digestibility and total tract retention, however there is a degree of discrepancy between studies. Improvements have been recorded for the apparent ileal digestibility of P, Ca, Cu, nitrogen (N), Zn, starch, fat and energy.

Phytase has been reported to increase the ileal utilisation of P (Camden *et al.,* 2001; Mroz *et al.,* 1994; Singh, 2008), Ca (Mroz *et al.,* 1994; Singh, 2008), Cu and Zn (Singh, 2008). Starch, fat (Camden *et al.,* 2001; Singh, 2008), and energy (Bedford, 2003; Camden *et al.,* 2001; Singh, 2008) ileal utilisation can similarly be increased through phytase supplementation.

Total tract retention (TTR) is also increased with phytase, maximising nutrient bioavailability for utilisation. Many studies have reported an increase in P retention with phytase (Adedokun *et al.,* 2004; Adeola *et al.,* 2004; Cromwell *et al.,* 1995; Kies *et al.,* 2006; Lei *et al.,* 1993b; Singh, 2008). Additionally, increases in the TTR of Ca, Cu, Fe, K, Mg, Na and Zn have also been reported (Adeola and Cowieson, 2011; Kies *et al.,* 2006; Lei *et al.,* 1993a; Mroz *et al.,* 1994). Increasing phytase from 0 to 1,000 FTU, in a low NPP diet fed to pigs, increased Ca and P digestibility (Adeola *et al.*, 2004). Adeola *et al.* (2004) also identified an additional increase in P digestibility in 19 kg pigs compared to 13 kg pigs. It is thought that physiological, GIT pH and rate of passage differences may lead to this variation. In common with poultry, when increasing phytase supplementation in pig diets, total P digestibility and P digested increased and total P excreted reduced. Although similar patterns are seen between the two species, as would be expected, the degree to which phytase has an effect differs. Korneygay (2001) also reported a greater increase in total P digestibility in pigs (+26.8%) than poultry (+13.9%).

Mroz *et al.* (1994) reported an increase in N, Ca and P TTR and suggested that this is a result of the action of phytase. The potential mechanisms of action include: removal of inhibitory phytic acid, reduced phytate-protein complex formation, activation of microbial phytase by cleavage and reduced inhibition of trypsin and pepsin. For example, an increased availability of Ca may reduce the bioavailability of other minerals. Increased minerals may also influence the absorption of other minerals (Maenz and Classen, 1998; Maenz *et al.,* 1999), and potentially increase urine production to remove excess, which not only wastes the dietary supply, but also increases energy expenditure, reducing the energy available for growth (Kies *et al.,* 2006).

1.4.4.2.1 Protein/ AA discrepancies

Some studies report improvements in protein utilisation following the supplementation of phytase (Adeola and Cowieson, 2011; Bedford, 2003; Camden *et al.,* 2001; Kies *et al.,* 2006; Mroz *et al.,* 1994; Singh, 2008; Walk *et al.,* 2012b). However, other studies report no change or improvement in protein or AA digestibility with phytase (Adedokun *et al.,* 2004; Ravindran *et al.,* 1999;

Thompson and Serraino, 1986) or AA digestibility following the supplementation of phytase. Adeola and Sands (2003) suggest that the differences in the effect of phytase on apparent ileal AA/ protein digestibility and utilisation may be due to problems in trying to assign an 'AA response factor' or 'AA equivalency value', resulting in an inadequate nutrient supply.

Phytase supplementation increased serine, leucine, tyrosine, phenylalanine, histidine and glutamic acid utilisation, with significant interaction being identified between phytic acid × phytase interaction for glutamic acid, glycine and histidine being identified (Cowieson and Ravindran, 2008). Cowieson *et al.* (2008) reported a 20 to 30% reduction in endogenous AA flow following the supplementation of 5,000 FTU/kg phytase. Rutherfurd *et al.* (2004) observed that phytase supplementation improved AA digestibility, and showed the general doubling in phytate hydrolysis with phytase supplementation. The overall apparent ileal AA digestibility mean with 500 FTU/kg phytase was 84.6%; 3.6% higher than without phytase supplementation. It was concluded from this investigation that 500 FTU would be adequate to improve the AA digestibility of a low P diet. Ravindran *et al.* (2001) reported an improvement in BWG and feed conversion efficiency. It was suggested the release of lysine from phytate-protein complexes was responsible for the observed improvements in lysine. The author also observed an increase in the mean AA digestibility values with the addition of 500 FTU/kg (Ravindran *et al.*, 2001). Ravindran *et al.* (2001) also suggested that the AA response to phytase is associated with energy utilisation and content of the diet. Rutherfurd *et al.* (2002) reported an improvement in AA digestibility by 5 to 20% in wheat, 7 to 16% in rapeseed meal and 5 to 7% in maize, with the supplementation of phytase. This may be similar for other AAs, but threonine has been determined as the most responsive to phytase (Selle *et al.*, 2012). This is likely to be because threonine is an abundant AA in mucin (Lien *et al.*, 1997), therefore its bioavailability would be expected to increase following phytase supplementation. Phytate has been reported to lead to an increase in mucin gene expression (Onyango *et al.*, 2008) due to the phytate-initiated irritation in the gut (Onyango *et al.*, 2009) and subsequent reduction with the supplementation of phytase.

Interaction between phytase and protein source was identified by Peter *et al.* (2000), however, no interaction between specific AAs and phytase were observed. Zhang *et al.* (1999) reported the effects of phytase supplementation on 3 week old male broiler chicks fed diets containing reduced dietary lysine, TSAA and ME (metabolisable energy; Zhang *et al.*, 1999). Between weeks 3 and 7, no improvements in BWG, FI and FCR were seen with supplementation of phytase, relative to nutritionally adequate diets; these results are likely a consequence of the low dietary levels of lysine, TSAA and ME, which are important for animal growth and performance. However, a linear increase in BWG with increasing phytase (0, 250, 500, 750 FTU/kg) was seen in 3 week old chicks. Sebastian *et al.* (1997) also investigated the differences between male and female chickens for protein and AA digestibilities with phytase supplementation. In male chickens, no change in ileal digestibility of protein or AA was detected, except for methionine and phenylalanine which decreased. Digestibility of methionine and phenylalanine AA also decreased in female chickens, but the ileal digestibility of essential AA increased. Interaction between the Ca/ P level and phytase was observed for apparent ileal digestibilities of cystine, histidine, lysine, methionine and threonine in males, but no interaction was seen in females. The differences between sexes may be due to the medullary layer of bone in female birds, which acts as a Ca reserve. A reduction in Ca:P ratio has the effect of improving growth performance, bone ash, and P digestibility when phytase is applied (Dilger and Adeola, 2006; Walk *et al.*, 2012a), which suggests that an optimal Ca:P ratio may exist for phytase activity. It is known that Ca and phytate may interact in the intestine, forming complexes. In addition to this, a high dietary Ca concentration can have the effect of reducing the pH of the GIT, which may consequently reduce phytate hydrolysis. It is possible that phytase is able to reduce the negative effects of high Ca (Walk *et al.*, 2012a).

Rutherfurd *et al.* (2004) observed that phytase supplementation improved AA digestibility, and showed the general doubling in phytate hydrolysis. The overall apparent ileal AA digestibility mean with 500 FTU/kg phytase was 84.6%; 3.6% higher than without phytase supplementation. It was concluded from this investigation that 500 FTU would be adequate to improve the AA digestibility of a low P diet. Cowieson *et al.* (2008) reported a 20 to 30% reduction in

endogenous AA flow following the supplementation of 5,000 FTU/kg phytase. Ravindran *et al.* (2001) also suggested that the AA response to phytase is associated with energy utilisation and content of the diet.

A study by Selle *et al.* (2006) demonstrated that the increased amino acid digestibility of wheat was improved to a greater degree following phytase supplementation compared to maize. A consequence of reduced protein solubility through the formation of these complexes is the reduced absorption of Na, Ca and amino acids, as well as an excess of secretion of HCl, mucin, pepsin, bile and $NaHCO_3$, which may influence the capability and efficiency of Na-dependant transport as well as the intestinal lumen electrolyte balance (Adeola and Cowieson, 2011).

1.4.4.3 Phosphorus retention and excretion

A large proportion of dietary phytate passes through the body undigested and is present in excreta (Biehl et al., 1995). The majority of studies report a reduction in P excretion as a result of increased retention following phytase supplementation (Adedokun *et al.,* 2004; Adeola *et al.,* 2004; Cromwell *et al.,* 1995). Not only is this beneficial to the animal, reducing P deficiency symptoms, but also may have a significant impact on reducing environmental P pollution (Angel *et al.,* 2005; Costa *et al.,* 2000; El-Sherbiny *et al.,* 2010). Korneygay (2001) reported a decrease in total P excretion of -0.101% in pigs and –0.067% in poultry following phytase supplementation. A linear relationship between digested P and dietary P and reduction in P excretion and increasing phytase levels was observed by Akinmusire and Adeola (2009). This is similar to the conclusions reached by Nelson *et al.* (1971), who observed a proportional reduction in excreta P with phytase supplementation.

Despite a general consistency in observations of a reducing faecal P content with phytase supplementation, improvements are not always observed. Both Cromwell *et al.* (1995) and Cowieson *et al.* (2004) did not observe a significant reduction in excreta total P. Cromwell *et al.,* (1995) reported reductions of 82%, 79% and 73% of excreta P content with 250, 500 and 1,000 FTU/kg phytase supplementation, respectively. Although Cowieson *et al.* (2004) reported a

decrease in the proportion of phytate-P present in excreta, it was suggested that the hydrolysed Pi was not utilised. Both authors suggest that this was because the P levels in the diet were sufficient, so the Pi released was above requirements, and hence excreted. This indicates the importance and relevance of providing phytase to P deficient diets.

1.4.5 Phytase doses

The composition of feeds given to animals on a commercial basis is highly influenced by market price and may reflect the requirement for phytase supplementation (Bedford and Schulze, 1998). Even if phytases are present, if the phytate has formed a complex, then the presence of phytase is irrelevant, as without further intervention it will not be able to exert its effect and hydrolyse phytate (Angel *et al.*, 2002). Therefore it is important to consider the substrate available to phytase when formulating it into diets. In addition to the improvement in P utilisation, as was the initial aim of implementing phytase supplementation, recent evidence suggests that super-doses of phytase may result in further benefits to animal performance through an improvement in GIT health. The effects of super-dosing and possible influences on gut health and function are discussed in following sections.

1.4.5.1 Regular- doses

FTU, PU and U are the commonly used units to express amounts of phytase (Kornegay, 2001), with FTU being most predominant throughout the literature, hence being the unit of choice used in this thesis. Phytase activity is measured by the degree of Pi released from phytic acid and is given the connotation of 'Fytase Unit: FTU' (Singh, 2008), for which there are various definitions, one of which is defined as "the quantity of enzyme which releases 1 µmol of inorganic phosphorus per min from 0.00015 mol/L sodium phytate at pH 5.5 at 37^{o}C", (Camden *et al.*, 2001). It is important to ensure phytase is supplied at a sufficient dosage to ensure an optimum response (Bedford, 2003). Edwards (1993) reported a significant increase in phytate-P retention with 600 FTU/kg phytase. In general, the standard dose of phytase is between 500 to 1,000

FTU/kg, which has the potential to release 0.05 to 0.15% bioavailable P from feeds (Adeola and Cowieson, 2011; Augspurger and Baker, 2004).

1.4.5.2 Super- doses

Super-doses of phytase are generally reported to be above 2,500 FTU/kg (Adeola and Cowieson, 2011). However, the application of 3x the standard regular dose (500 FTU/kg), ≥ 1,500 FTU/kg depending on the phytase product, is suggested to be a super-dose and able to achieve the 'extra-phosphoric' benefits (Schlegel *et al.*, 2016; Wilcock and Walk, 2016).

It is generally perceived that there are no detrimental effects on animal health or performance following super-doses of phytase, as reported by Brana *et al.* (2006), in which 10,000 FTU/kg phytase was supplemented without any adverse effects. However, not all studies are in agreement of any extra beneficial effect of super-doses of phytase. Camden *et al.* (2001) and Liu *et al.* (2008a) reported no further improvements in animal performance at regular doses of phytase, increasing from 500 to 1,000 FTU/kg. Similarly, there were no additional benefits from the use of super-doses of phytase in a study reported by Biehl *et al.* (1995), where the greatest response to phytase occurred with an increase from 0 to 1,200 FTU/kg, compared to an increase from 1,200 to 1,800 FTU/kg. Pillai *et al.* (2006) observed the maximal release of P (95%) to occur at 1,000 FTU/kg of an *E.Coli* derived phytase, and Walk *et al.* (2012b) found N digestibility to increase then plateau at 500 FTU/kg. Despite these findings, there is evidence that increasing phytase doses beyond those regularly used in the commercial industries can have a further beneficial effect on animal performance. Some of these effects include: increase P liberation, reduction in the anti-nutritional influence of phytate, restoration of the Ca:P ratio, and the production of myo-inositol, which is deemed to have anti-oxidant properties (Adeola and Cowieson, 2011). Shirley and Edwards, (2003) observed an increase in P retention and utilisation, with a log-dose response relationship with phytase. Below 3,000 FTU/kg, there was little effect seen on Ca retention, but above 3,000 FTU/kg, Ca retention also increased with rising levels of phytase. Despite these changes, P levels remained higher than those of Ca, with P levels thought to be retained to a greater degree. The authors suggested that the

higher levels of phytase allowed restoration of the homeostatic balance between Ca and P, with the Ca:P ratio reducing from 1.72:1 to 1.28:1 as phytase increased from 300 to 10,000 FTU/kg.

Brana *et al.* (2006) reported an increase in the growth rate of finisher pigs with 10,000 FTU/kg phytase, suggesting this was a consequence of the 'extra-phosphoric effects' of phytase and the release of other nutrient inaddition to P. At 12,000 FTU, Shirley and Edwards (2003) reported a 33% increase in N retention, a 200 kcal improvement in ME/kg, with maximal P release (95%) occurring at this level of supplementation to a P-deficient diet. Further to this, an increase in body weight, feed intake and FCR equivalent to the control diet occurred with 120,000 FTU/kg. With increasing phytase levels, the Ca:P ratio decreased, alongside the mortality rate. Super-doses of phytase may give a bimodal effect (Cowieson *et al.*, 2011), primarily releasing digestible P from phytate and removal of phytate, and secondly, improving digestible nutrient intake (Adeola and Cowieson, 2011).

1.4.5.3 Extra-phosphoric effects of phytase

Benefits of phytase supplementation may extend to that beyond increased phosphorus availability, described as 'extra-phosphoric effects'. The three main potential mechanisms which have been identified in the literature include: increased P availability and restoration of the Ca:P balance; the proportional reduction in phytate with increasing lower IP esters; and production of myo-inositol (Adeola and Cowieson, 2011; Cowieson *et al.*, 2011). Other explanations include the reduction in phytate-nutrient complexes and increased nutrient utilisation (Adeola and Cowieson, 2011), reduced inhibition and enhanced efficacy of other digestive enzymes and improved gut health alongside reduced risk of hind-gut microflora colonisation. Research into the effect of phytase on improving gut health is evolving, and is a major point of consideration for this thesis.

1.4.5.3.1 Phytate hydrolysis

Since exogenous phytase exerts a greater degree of hydrolysis on IP6 than the

lower esters, the decrease in IP6 frees other nutrients such as Ca for utilisation. As IP6 is a stronger chelator in the formation of phytate-nutrient complexes than lower esters such as IP3 (Cowieson *et al.,* 2011), its removal has a significant effect. The lower IP esters are also more soluble in the intestine. Greater absorption increases the substrate available for conversion to inositol (Adeola and Cowieson, 2011), which will be described in more detail in subsequent sections.

1.4.5.3.2 Production of myo-inositol

Inositol is the final product of phytate hydrolysis, which following absorption, can be converted to myo-inositol in the mucosa, liver and blood (Adeola and Cowieson, 2011). Myo-inositols (MYO) are thought to have vitamin-like/ lipotropic effects, which have the overall effect of promoting growth. High doses of phytase are required to increase the intestinal myo-inositol content. Myo-inositol may drive some of the indirect protective effects of high phytase doses, anti-oxidant activity and increasing absorption, transport and utilisation of fat and fat-soluble nutrients such as vitamins A, D and E.

Many tissues are sensitive to MYO levels, being involved in processes such as myelination and mitochondrogenesis as part of the immature immune system (Burton *et al.,* 1976), glucose transport, gluconeogenesis and protein deposition, leading to inositol being described as an insulin-mimetic (Cowieson *et al.,* 2015). Additionally, MYO may have an influence on carcass accretion characteristics, due to its ability to influence protein deposition (Cowieson *et al.,* 2015) and fat accumulation (Chu and Geyer, 1983). The supplementation of phytase increases the production of inositol, and consequently blood inositol concentrations (Cowieson *et al.,* 2015; Schmeisser *et al.,* 2016).

Glucose uptake in mammals is facilitated by the transporter GLUT4, however, this is not present in birds (Sweazea and Braun, 2006; Tokushima *et al.,* 2005), suggesting that glucose is regulated by other insulin-sensitive mechanisms (Tokushima *et al.,* 2005). Phytase has been reported to increase blood glucose concentrations (Cowieson *et al.,* 2013; Johnson and Karunajeewa, 1985; Liu *et al.,* 2008b). High glucose concentrations are required to induce insulin secretion

in birds due to the naturally high circulating glucose levels (Rideau, 1988). Dietary MYO supplementation has been observed to increase blood insulin and glucagon concentrations (Cowieson *et al.,* 2013), again suggesting an insulin-like role. However, Cowieson *et al*. (2013) suggests that there is competition between MYO and glucose for Na-dependent transport, and that high blood glucose levels may have the negative effect of reducing MYO absorption. The author also suggested that the benefits of MYO and phytase occur through different mechanisms, as in their study the insulin concentration was not directly relatable to the glucose concentration. Phytase appeared to indirectly influence glucose concentrations via interactions with Na, with some mechanisms influencing glucose concentration being competitive between the action of MYO and phytase.

1.4.5.3.3 Reduced phytate concentrations

Phytase supplementation has the overall effect of reducing phytate concentrations. Due to the anti-nutrient nature of phytate and its binding to nutrients (such as starch, glucose, protein, AA and fat), reductions in the concentration of phytate reduces the potential for binding with these nutrients and thus increases their potential for absorption (Ravindran *et al.,* 2001). Increased mineral availability in the digesta, such as Ca and Zn, can have the effect of enhancing enzyme activity due to the role of these minerals as cofactors in the enzyme structure (Singh and Krikorian, 1982). There are many enzymes which have been reported to be inhibited by phytate, either by direct binding, or through binding of the cofactors (e.g. Ca) required for their activity. These include: trypsin (Maenz, 2001; Mroz *et al*., 1994; Sebastian *et al.,* 1997), α-amylase (Selle *et al.,* 2012), protease (Maenz and Classen, 1998; Singh, 2008), lipase (Maenz and Classen, 1998; Singh, 2008), maltase (Liu *et al.,* 2008b), tyrosinase (Mroz *et al.,* 1994; Sebastian *et al.,* 1997) and pepsin (Mroz *et al.,* 1994; Sebastian *et al.*, 1997).

Sufficient Ca is required for the activation of trypsin from trypsinogen (Caldwell, 1992) and its inhibition may lead to pancreatic hypertrophy (Selle *et al.,* 2003). Phytase increases the bioavailability of Ca by hydrolysing phytate, reducing phytate-Ca complexes and thus reducing Ca-induced inhibition of trypsin (Selle

et al., 2003). The inhibition of pepsin has a further influence other than a possible reduction in protein digestion. Pepsin is important in the release of enteric hormones, such as gastrin and cholecystokinin (Selle *et al.,* 2003), indicating the range of mechanisms to which phytate can influence. Increased protein availability through a reduction in phytate-protein complexes requires adequate pepsin available for its utilisation (Kies *et al.,* 2006). Pepsin is secreted in a compensatory manner, alongside hydrochloric acid (HCl), mucin and sodium bicarbonate ($NaHCO_3$), due to the presence of phytate-protein complexes in the GIT (Cowieson *et al.,* 2006a).

Phytase also has the potential to improve the efficacy of other enzymes (Maenz and Classen, 1998; Singh, 2008), such as xylanase (Olukosi and Adeola, 2008; Ravindran *et al.,* 1999; Selle *et al.,* 2003), for which phytate presence is a limiting factor (Francesch and Geraert, 2009). This interaction between phytase and other enzymes may improve nutrient utilisation, enhancing animal performance. Some of these effects include an increase in carbohydrate and soluble fibre digestion, which are improved when phytase is used alongside other enzymes (Campbell and Bedford, 1992; Officer and Batterham, 1992). Although phytase is able to improve the utilisation of other exogenous enzymes, they are able to have similar effects of the efficacy of phytase, highlighting synergism. Enzymes such as xylanase, ß-glucan and proteases act to degrade the endosperm and aleurone layer, which can increase the phytate substrate available for phytase hydrolysis, as well as increasing starch and protein digestion (Bedford and Schulze, 1998; Olukosi and Adeola, 2008; Ravindran *et al.,* 1999). Xylanases, phytases and cellulases are commonly supplemented together (Bedford, 2003; Cowieson, 2010). The sub-additive, full-additive or synergistic interactions between phytase and other exogenous enzymes (Cowieson and Bedford, 2009) is observed to improve digestive efficiency (Camden *et al.,* 2001; Liu *et al.,* 2008b; Officer and Batterham, 1992; Selle *et al.,* 2006).

1.4.5.3.4 Gut health and function

Gut health and function may be improved in the presence of phytase, either through reductions in phytic acid concentrations, improvement in nutrient availability or reduced quantity of digesta reaching the hind-gut. Removal of

phytate-nutrient complexes reduces the proportion of nutrients reaching the hindgut, diminishing the risk of microflora colonisation and disease (Bedford and Schulze, 1998). In addition, phytic acid acts as an irritant to the gut mucosa, stimulating mucin synthesis and excretion. Phytase may help to reduce endogenous AA/protein/Na flow, prevent gut irritation and stimulation of an immunological response, as well as improving the health and efficiency of physiological homeostasis.

The exact mechanisms of action of super-doses of phytase are not fully known, but alongside those described above, there is a potential for improvements in gut-health. The aim of this thesis is to identify the potential mechanisms of phytase super-doses and observe any beneficial effects on health.

1.4.6 Gut parameters

Various mechanisms have been identified which may be correlated with phytase supplementation and improvements in gut health and function. Using the chicken as an example, promotion of health will be discussed with indication to the potential mechanisms of action from within the gut.

Improvements in gut health generally lead to enhanced nutrient absorption and utilisation, reducing the proportion of nutrients reaching the hind-gut. Enhanced nutrient absorption in the gut reduces the substrate available for bacterial fermentation in the hind gut, reducing the risk of bacterial colonisation and disease. Additionally, changes in gut pH can influence nutrient solubility, as well as determining which bacterial species are able to survive and exert dominance through competitive exclusion. Many studies show the benefits of exogenous enzyme supplements on gut health (Bedford and Cowieson, 2011; Jia *et al.*, 2009; Kiarie *et al.,* 2013). A change in concentration, availability or activity of these exogenous enzymes may result in changes to the GIT, influencing mucin secretion, water loss in excreta and nutrient bioavailability (Cowieson *et al.*, 2004).

1.4.6.1 Gut integrity

Maintenance of gut integrity is essential for its function, namely as absorptive medium and acting as chemical and physical barrier (De Lange *et al.,* 2010). Overall, gut integrity is dependant on its degree of physical development, immune competence, gut enzyme activity, mucin production, gut flora, and epithelial damage (Williams, 2005). A selective permeable barrier allows nutrients to be absorbed and homeostatic fluid exchange to occur, but not other unwanted components such as bacteria (Anderson *et al.,* 1993; Derikx *et al.*, 2010; Tsukita *et al.*, 2008; Turner, 2009).

The physical development of the gut includes intestinal growth, development of the crypts and villus growth (Williams, 2005). The small intestine is the major site for nutrient absorption, containing a large number of villi, which are also the sites of enzymatic activity. This allows for efficient absorption of the products released by enzymatic hydrolysis during digestion, reducing nutrient loss and excretion. Many endogenous enzymes are found on the intestinal brush border membrane, including: sucrase, maltase, phytase and alkaline phosphatase (Uni *et al.,* 1998); the activity of these mucosal enzymes is correlated with the enterocyte density of the villus (Williams, 2005). As with the rest of the digestive system, gut health is critical for optimal absorption. The inner luminal wall consists of tight junctions between the enterocytes, villi and crypts (Dibner and Richards, 2004). Nutrients that are not absorbed enter the caeca, where they may remain for a relatively long period (compared to the small intestine), potentially being used as substrates for fermentation by the bacterial microflora. This may have a considerable influence on gut health, increasing the risk of pathogenic bacterial colonisation. However, the observation of adaptational changes of the caeca to nutrition indicates the importance of nutrition to gastro-intestinal health. For example, it has been shown that the caeca is smaller in domestic bird species, such as the chicken, compared to birds in the wild (Clench and Mathias, 1995). Additionally, the microflora populations are also different to wild birds, the loss of commensal microflora being a result of changes in feed and environment conditions (Clench and Mathias, 1995).

Intestinal development and maturation is important for the selective absorption of nutrients (Yegani and Korver, 2008). Dietary composition can have a major influence on intestinal characteristics, including: villus height, mucus production, goblet cell number and volume, and the capability of enterocyte migration. Deterioration of the gut structure is associated with a decrease in villus height, decrease in total brush border enzyme activity and an increase in paracellular permeability (measured through determination of the trans-epithelial resistance) indicating the effectiveness of the intestinal barrier function (Montagne *et al.,* 2007). Micro-dissection of the villi and crypts from intestinal histomorphology samples allow microscopic examination of the villus and crypt length and width. Disturbed structural integrity of the enterocyte may lead to epithelial mucosa cell death, such as following separation of epithelial cells from the basement membrane of the villi. This is indicated by the presence of an oedematous space during histomorphological examination (Olkowski *et al.*, 2008).

Villus height is important for efficient nutrient absorption and influenced by mucus production. If the mucus layer is too thick, then nutrient absorption is less efficient. Not only will this increase endogenous N loss (from the mucin), but may also increase the nutrients entering the colon, providing substrates for bacterial fermentation. Another important factor is the maintenance of tight junctions, which has the regulatory influence of nutrient absorption from the intestinal lumen (Madara, 1989, 2011).

Tight junctions, or zonula occludens (ZO), are important for intestinal barrier function, being permeable to small molecules they act to regulate nutrient absorption (Madara, 1989; Shen *et al.,* 2011; Turner, 2009). They are found between enterocytes in the intestinal lumen, and maintain the passage of molecules through interaction between proteins, including occludins and claudins (Derikx *et al.,* 2010).

Variation in resistance to permeability can occur according to the role of the surrounding epithelium (Madara, 1989), with protein-protein interactions regulating the action of the tight junctions, (Shen, 2012). Alteration to the physical characteristics of the pores and the probability of these channels being

in an open state can modify nutrient absorption (Madara, 1989). Occludins and claudins are members of a family of major sealing proteins, which interact with ZO-specific cytoplasmic proteins, preventing the passage of antigens, microbiota and toxins etc. into the circulation (Derikx *et al.,* 2010; Furuse *et al.,* 1993; Mitic and Anderson, 1998).

As Na absorption through tight junctions is essential; the Na^{2+}-K^{+}-ATPase pump may influence the fluid composition and subsequent absorption of ions (Shen, 2012). If a Na^{+} selective pore is able to form, permeability is able to increase, influencing nutrient uptake (Shen, 2012). This may be through an increase in osmolality resulting from a change in the ion charge gradient, causing solvent drag to occur (Madara, 1989). ZO-specific proteins include ZO-1 and angulin (Madara, 1989), which have an important regulatory function. ZO-1 may regulate the interaction of trans-membrane proteins in the tight junctions, influencing permeability (Shen, 2012). Leaky tight junctions are associated with diseases, potentially identified by the presence of ZO-specific proteins in excreta. The main effect of phytase on the improvement and maintenance of gut integrity is the dose-dependent reduction in the irritative action of phytate on the gut mucosa (Onyango *et al.,* 2009; Wyatt *et al.,* 2008). Additionally this allows an increase in villus height and crypt depth, also influenced by a reduction in mucin production, to improve nutrient utilisation

1.4.6.2 Nutrient absorption

Through the hydrolysis of phytate, there is likely to be an increase in the concentration of nutrients in the digesta. Without an adaptational increase in the presence of nutrient transporters, these additional nutrients will pass through the GIT unabsorbed and be excreted.

Intestinal absorption of amino acids and glucose are dependant on Na (and K). The Na-glucose co-transporter (SGLT1) is located in the brush border of the intestine, where it transports AA and glucose, requiring two Na molecules for each glucose molecule (Sklan and Noy, 2000). The expression of SGLT1 is influenced by nutritional status, observed to increase in food deprived and starved and re-fed birds (Gal-Garber *et al.,* 2000). Similarly, the primary

intestinal P transporter NaPi-IIb is regulated by digesta P, with the expression of the NaPi-IIb transporter increasing during periods of low P (Hattenhauer *et al.*, 1999).

1.4.6.3 Mucin production

Mucins, produced by goblet cells, consist of high molecular weight glycoproteins of which > 90% is a carbohydrate structure (Barcelo *et al.*, 2000; Lien *et al.*, 1997; Walk *et al.*, 2012a). The density of goblet cells accumulates proportionally with villi size and increase distally from the duodenum to the ileum (Horn *et al.*, 2009). Although the jejunum is the primary site of nutrient absorption, there is a greater goblet cell density reported in the ileum, which is likely to be due to the greater length of the ileum.

Mucus is constitutively secreted (Lien *et al.*, 1996), as a lubricant, for the protection of the gut. The mucus layer provides a barrier against physical or chemical damage, by digesta or microbes, influencing nutrient absorption, adhesion and epithelial cells signalling and differentiation (Gabriel and Mallet, 2006; Tanabe *et al.*, 2005). Due to the net negative charge of the mucin molecule, binding with positive cations, such as Ca^{2+}, may facilitate the intestinal absorption of these ions (Uni *et al.*, 2003). Acid mucins also have an important role in immune function during early life before the acquired immune system is fully developed (Uni *et al.*, 2003). Secretion may be up-regulated by proteolytic degradation, physical abrasion, or dietary components, such as the presence of phytate (Barcelo *et al.*, 2000; Lien *et al.*, 1996). Secretion has been identified as being of gastric or intestinal origin (Tanabe *et al.*, 2005), positively correlated with feed intake (Lien *et al.*, 1996, 1997). Degradation generally occurs in the large intestine in response to intestinal microflora, physical damage, proteolytic digestion or high concentrations of gastric acid and bile (Lien *et al.*, 1997).

There are two layers of intestinal mucus, which are often referred to as the 'unstirred water layer' (Tanabe *et al.*, 2005). The first layer is a thin, viscous, water-insoluble layer, which adheres to the epithelial surface through interaction with membrane bound mucins, the enterocyte glycocalyx, and acts

to limit the proteolytic degradation of the enterocyte. The trapped mucus makes up the second layer, protecting the intestinal lumen surface and facilitating the absorption of small nutrients. Importantly, this layer also contains bound enzymes and immunoglobulins (Lien *et al.,* 1996). This arrangement is essential for the protective mechanism of mucins, for example, inhibiting microbial adhesion prevents colonisation and toxin production, which may be detrimental to enterocyte cell health and nutrient absorption (Uni *et al.,* 2003). However, the carbohydrate component of mucin may actually allow interaction with bacteria following migration through the mucus layer, the bacteria may associate with the mucin binding protein receptors and release toxins to damage epithelial cells; the sloughing of epithelial cells also allows mucus to trap bacteria for excretion.

The MUC2 gene is the gene responsible for mucin production (Mattar *et al.,* 2002). Mucin is important for inhibition of bacteria through the intestinal barrier, but as it can be catabolised by bacteria in the hind-gut, a balance between synthesis and degradation is important to prevent bacterial colonisation (Koutsos and Arias, 2006; Mattar *et al.,* 2002). Probiotics have been identified as being able to stimulate MUC2 gene expression, which may have the benefit of reducing bacterial translocation (Mattar *et al.,* 2002). Consequently, an increase in mucin synthesis reduces nutrient retention and decreases growth if at excessive levels (Koutsos and Arias, 2006). It is well known that mucin has an important barrier function role, but the optimum thickness of the mucin layer for nutrient retention and bacterial excretion is unknown. The reduction in mucin production following phytase supplementation allows a decrease in energy expenditure and reducing endogenous AA and N reaching the hind gut (Cowieson *et al.,* 2009; Onyango *et al.,* 2009; Selle *et al.,* 2012; Wyatt *et al.,* 2008).

1.4.6.4 Microbial ecosystems

Diet is an important factor that helps to regulate intestinal bacterial populations for the benefit of the host (Brisbin *et al.,* 2008a; Gabriel and Mallet, 2006). Establishment of microflora can occur following adherence of the microbe to the enterocytes in the intestinal epithelium or a damaged intestinal epithelial barrier, however not all bacterial species are pathogenic (Bedford, 2000).

Bacterial populations are constantly changing, with variations occurring in individuals over time, consequently, there is only around 50% similarity in bacterial populations between birds reared in the same environment (Guarner and Malagelada, 2003). In a healthy animal, commensal (indigenous) intestinal bacteria will be of the highest proportion (Ogawa *et al.,* 2000). One species may dominate the intestinal environment through competitive exclusion. The dominant species are able to produce antimicrobial metabolites, bacteriocins, which inhibit the growth of other bacteria (Apajalahti *et al.,* 2004; Brisbin *et al.,* 2008a; Gabriel and Mallet, 2006; Guarner and Malagelada, 2003).

Generally, large populations of commensal bacteria exist in the crop, distal ileum, caeca and colon; populations are lower in the proventriculus and gizzard due to the low pH and high rate of passage (Gabriel and Mallet, 2006). If digesta pH is around 6 then conditions are beneficial for lactic acid bacteria colonisation. These bacteria only have a small amount of endogenous phytase activity, but as a result of their metabolism produce lactic acid, which also reduces the pH (Leenhardt *et al.,* 2005). There is an important dietary influence on properties of bacteria populations throughout the gut. For example, Cu has previously been used as a growth promoter in pig diets which is toxic to micro-organisms (Brisbin *et al.,* 2008b). Additionally, the use of antibiotics have been used to alter microflora populations, reducing colonic colonisation, although the use of antibiotics for growth promotion has been banned in the EU due to concerns of antibiotic resistance in animals and man (Brisbin *et al.,* 2008b), with increasing pressure to extend the ban to therapeutic antibiotics.

Many commensal bacteria may have a protective function, potentially being able to reduce carcinogens, increase resistance to infection, as well as enhance nutrient absorption (Guarner and Malagelada, 2003). Peristalsis of the small intestine reduces bacterial colonisation by preventing adherence of pioneer bacteria to the epithelial lumen (Guarner and Malagelada, 2003). Ileal conditions are most suitable for facultative anaerobes such as lactobacilli, enterococci and coliforms (Brisbin *et al.,* 2008a). It is possible that these established bacteria are able to alter gene expression, such as of receptors (Gabriel and Mallet, 2006), to manipulate environmental conditions to make them more optimal for their survival (Guarner and Malagelada, 2003).

Intestinal microflora populations may have a significant influence on physiological processes and homeostasis, through interaction with the GALT (gastro-intestinal associated lymphoid tissue), which regulates intestinal health (Cebra, 1999; Guarner and Malagelada, 2003). The innate immune response may be initiated by the presence of bacteria (Gabriel and Mallet, 2006; Guarner and Malagelada, 2003), having the potential to influence host gene expression (Kelly and Conway, 2001). Commensal bacteria also have an essential function in immune function (MacDonald and Pettersson, 2000), particularly of the acquired immune system (Brisbin *et al.,* 2008a). This indicates the importance of immuno-regulation, ensuring an immune response is only initiated against pathogenic bacteria (Brisbin *et al.,* 2008a). Increasing the potential for nutrient absorption reduces the nutrients available for fermentation in the large intestine, preventing the establishment of bacteria. Lower bacterial colonisation lessens the competition for nutrient resources and detrimental effects as a result of toxin production (Gabriel and Mallet, 2006). Consequently the capacity for nutrient absorption increases, improving animal growth and performance.

1.4.6.5 Immunological response

The immunological response to pathogens and antigens is influenced by interaction between mucins, the diet and bacterial ecosystems (Williams, 2005). The GALT is responsible for the immune regulation of the GIT in both innate and adaptive responses.

The detection of PAMPS (Pathogen Associated Molecular Pattern Sequences) by PRRs (Pattern Recognition Receptors) stimulate innate immunity (Akira *et al.,* 2006; Boehme and Compton, 2004). Numerous PRRs exist, with TLR (toll-like receptors) being of primary importance (Mitchell *et al.,* 2007). There are 12 TLR molecule types; TLR 3, 7, 8 and 9 act intra-cellularly, whilst TLR 1, 2, 4, 5 and 6 act extra-cellularly (Akira *et al.,* 2006). The expression of the markers occurs in response to cytokines and environmental stress.

Not all antigens are capable of stimulating an immune response (hence interaction between beneficial and pathogenic microflora). Antigens which are absorbed are referred to as tolerogenic, but those which penetrate the cell are

immunogenic and stimulate an immune response. Neutralisation of bacteria more commonly occurs (rather than elimination) due to the cross-reactivity of antigenic components between the beneficial and pathogenic bacteria (Yegani and Korver, 2008). The diet can influence the bacterial populations of the intestine; therefore effecting the production of lymphoid follicles and the subsequent immune response (Sydora *et al.,* 2003). These influences of bacteria on immune inflammation and health of the intestinal epithelium show the complexities of interactions within the gut (Shulzhenko *et al.,* 2011).

The initiation of an immune response reduces an animals potential for growth due to the requirement for energy (Yegani and Korver, 2008), therefore low nutrient absorption may result in immunosuppression (Williams, 2005). Additionally, immunologic stress results in a reduction in feed intake, increasing nutrient deficiency further. Additionally, proinflammatory cytokines can lead to a breakdown of the intestinal barrier (Sydora *et al.,* 2003), reducing integrity and conditions for nutrient absorption. Liu *et al.* (2008a) reported a role of phytase on GIT health and immune competence, with benefits being seen with 500 FTU/kg phytase, although no further improvements were seen when phytase was increased to 1,000 FTU/kg.

1.4.6.6 Carcass characteristics

Dietary composition will influence carcass composition due to the quantity and quality of nutrients available for maintenance and production processes. Jackson (1982) reported that dietary protein and energy can influence carcass composition. The authors reported that a dietary increase in protein decreased the fat and increased the protein compositions of the carcass. Differences in the carcass composition were partially explained by interaction between dietary protein and energy for the efficiency of protein and energy utilisation within the carcass (Jackson *et al.,* 1982), which ultimately influences the resources available for carcass accretion. Carcass protein accretion is more energetically demanding than fat accretion, therefore if there is not sufficient energy for protein accretion then fat accretion will occur instead.

In addition to the obvious nutritional influences (protein, energy), inositol (the final product of phytate hydrolysis) has been reported to influence carcass protein deposition (Cowieson *et al.,* 2015). Schmeisser *et al.* (2016) reported an upregulation of the expression of genes involved in breast muscle development following the supplementation of 1,000 U/kg phytase. The breast meat weight increased above the NC by 4.5g ($P < 0.05$) in the starter period and by 51.3g between days 22 and 36 ($P < 0.05$). The author suggested that the benefit of phytase was more than just an increase in Ca and P concentrations, and that the reported improvements in muscle growth with phytase were probably not through the same mechanism of muscle growth in the nutritionally adequate diets. The enhanced muscle production in the phytase-supplemented diets was likely through the production of myo-inositol, and subsequent influences through IGF (insulin-like growth factor), highlighting the possible extra-phosphoric effects. Additionally, nutrient release following phytate hydrolysis may change the absolute nutrient levels within the digesta, and the relative proportions of each. Phytase has been reported to improve the efficiency of producing a lean carcass through an increase in carcass protein and reduction in carcass fat (Olukosi and Adeola, 2008). The authors reported an increase in 2g/d DM ($P < 0.05$), 1.25 g/d crude protein (CP; $P < 0.05$) and 0.76 g/d fat ($P < 0.05$) relative to the negative control diet (NC), which was marginally deficient in nPP by 0.8 g/kg. However, these changes during phytase supplementation were on a g/d basis, there were no overall effects observed on the overall DM, CP or fat % compositions.

1.5 Aims and objectives of the thesis

The potential mechanisms of action of super-doses of phytase on animal health have been mentioned, however, there is little information about this in the literature. Therefore, the aim of the thesis was to document the effects of regular- and super- doses of phytase within the gut and to suggest the mechanism of action.

- Documentation of the effects of super-doses of phytase on animal performance through measurement of growth performance, nutrient utilisation, and the characteristics of phytate and degree of its hydrolysis in the different regions of the gastro-intestinal tract.
- Analysis of the responses to regular- and super- doses of phytase supplementation in the gut, on gut inflammation and intestinal microbial populations
- Investigation of the effects of regular and super-doses of phytase on the morphology and microbial profile of the gastro-intestinal tract and the influence on expression of markers of gut cell integrity and inflammation.
- Investigation of the possible effects of the inositol phosphate esters produced following phytate hydrolysis.
- Investigation of the influence of phytase on gut integrity and its subsequent ability to resist pathogenic colonisation, through the examination of gross morphology and expression of MUC2, which is a molecular marker for mucin production. As previously mentioned, mucin production is increased with high phytate levels. It was therefore proposed that the addition of phytase supplementation would reduce the expression and secretion of MUC2.
- Examination of the expression of markers of gut integrity, including occludin, were also examined. Combined, this information helps form an understanding of the mechanism by which the effects of super-doses of phytase are able to improve an animal's health and growth performance.

Chapter 2.

Super- doses of phytase on growth performance, nutrient utilisation and nutrient partitioning of the broiler chicken.

2.1 Introduction

It is well documented that non-ruminant species, particularly poultry and pigs, are unable to sufficiently utilise P from plant-based feed ingredients due to it binding with phytate, and the animals' inability to produce sufficient endogenous phytase. Exogenous phytase has been supplemented to commercial poultry and pig diets for many years, with the aim of improving growth performance in P deficient diets (Biehl *et al.,* 1995; DeLaune *et al.,* 2004). In addition, phytase has the benefit of reducing environmental P pollution (Angel *et al.,* 2002) since phytase has been reported to reduce the excreta P content by as much as 53% (Cowieson *et al.,* 2004). Regular doses of phytase have been reported to improve body weight gain, efficiency of feed conversion, nutrient utilisation and bone mineralisation in broilers fed diets containing low available P (Leske and Coon, 1999; Olukosi and Adeola, 2008; Selle *et al.,* 2012). Super-dosing is the addition of phytase at levels around or above 2,500 FTU/kg (Adeola and Cowieson, 2011) with the aim of improving growth performance and feed conversion ratio (Walk *et al.,* 2014). Improvements in growth performance observed are likely to be partly a result of an increase in availability of the nutrients which had previously been bound with phytate, including protein, and minerals such as Ca, Zn and Na (Adeola and Cowieson, 2011; Applegate *et al.,* 2003b; Maenz, 2001; Ravindran *et al.,* 2001). The 'extra-phosphoric effects' of super-doses of phytase are benefits resulting from more than just an increase in P availability. Increased phytate hydrolysis may improve overall performance and efficiency of production and the production of a carcass with a greater proportion of protein and less fat through manipulation of nutrient partitioning, thereby increasing the efficiency of production for poultry farmers. Therefore, the aim of the experiment reported here was to test the hypothesis that super-dosing of phytase will improve broiler growth performance, bone mineralisation, nutrient utilisation and enhance whole body nutrient accretion, through enhanced phytate hydrolysis and the production of lower inositol phosphate esters and myo-inositol, when supplemented to diets marginally deficient in aP%. The positive control (PC) diet consisted of a nutritionally adequate diet,

whilst the negative control (NC) was marginally deficient in aP relative to the PC, in order to distinguish differences between phytase-induced release of P from phytate, and extra-phosphoric effects. Two super-doses of phytase 1,500 and 3,000 FTU/kg were selected.

2.2 Materials and Methods

2.2.1 Experimental Design

A total of 344, one-day-old Ross 308 broilers were used for the study. All experimental procedures were approved by the SRUC Animal Experiment Committee in accordance with the Animals (Scientific Procedures) Act 1986 (Appendix 2.6.1). On the day of arrival (day 0), 8 birds formed an initial slaughter group and were euthanased without feeding to allow comparison of whole body nutrient accretion characteristics using the comparative slaughter technique. The remaining 336 birds were randomly allocated to one of the 6 dietary treatments (with 7 birds per replication and 8 replications per diet) in modified raised-floor metabolism cages, with feed and water provided *ad libitum* throughout the 21 day study. Dietary treatments were randomly allocated within blocks and subsequently blocks were spatially randomised within the house in a Randomised Complete Block Design. The treatments were initially based on a 2 x 3 factorial arrangement with two levels of dietary aP (0.45% and 0.30%) and three levels of phytase supplementation (0, 1,500 and 3,000 FTU/kg). The PC diet was formulated to meet Ross 308 energy and nutrient requirements (Ross 308 Specifications, 2007), whilst the NC diet was formulated to be marginally deficient in aP, relative to the PC. Titanium dioxide was used as an indigestible marker. The diets were as follows: (1) PC, aP at 0.45% and Ca at 0.99%; (2) PC + 1,500 FTU/kg phytase; (3) PC + 3,000 FTU/kg phytase; (4) NC, aP at 0.30% and Ca at 1.01%; (5) NC + 1,500 FTU/kg phytase; (6) NC + 3,000 FTU/kg phytase. Table 2.1 shows the ingredient composition of the dietary treatments.

Table 2.1 – The ingredient composition of the dietary treatments fed to broilers from 0 to 21 days post-hatch

Description of diets:	PC[1]	PC1500	PC3000	NC[2]	NC1500	NC3000
Phytase, FTU/kg:	0	1,500	3,000	0	1,500	3,000
Ingredients, g/kg						
Maize	523	523	523	525	525	525
Soybean meal	335	335	335	335	335	335
Soybean oil	50	50	50	50	50	50
DCP[3]	18.7	18.7	18.7	10.5	10.5	10.5
Limestone[4]	11.0	11.0	11.0	17.0	17.0	17.0
TiO_2	5	5	5	5	5	5
Enzyme premix[5]	0.0	10.0	20.0	0.0	10.0	20.0
Maize gluten meal	40.0	30.0	20.0	40.0	30.0	20.0
Vitamin-mineral premix[6]	5.0	5.0	5.0	5.0	5.0	5.0
Methionine	2.1	2.1	2.1	2.1	2.1	2.1
Lysine	5.2	5.2	5.2	5.2	5.2	5.2
Threonine	0.9	0.9	0.9	0.9	0.9	0.9
Salt, NaCl	4.0	4.0	4.0	4.0	4.0	4.0

Notes: [1]PC: Positive Control; [2]NC: Negative control; [3]DCP: 25.7% Ca, 17.5% P; [4]Limestone: 38.9% Ca; [5]Enzyme premix is mixed with maize gluten meal and has an activity of 150 FTU/g; [6]Premix supplies the following per kg diet: Vit. A, 5484 IU; Vit. D3, 2643 ICU; Vit E,11 IU; Menadione sodium bisulfite,4.38 mg; Riboflavin, 5.49 mg; d-pantothenic acid, 11 mg; Niacin, 44.1 mg; Choline chloride, 771 mg; Vit B12, 13.2 ug; Biotin, 55.2 ug; Thiamine mononitrate, 2.2 mg; Folic acid, 990 ug; Pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug. Also contains per g of premix: Vit. A, 1828 IU; Vit. D3, 881 ICU; Vit E,3.67 IU; Menadione sodium bisulfite,1.46 mg; Riboflavin, 1.83 mg; d-pantothenic acid, 3.67 mg; Niacin, 14.69 mg; Choline chloride, 257 mg; Vit B12, 4.4 ug; Biotin, 18.4 ug; Thiamine mononitrate, 735 ug; Folic acid, 330 ug; Pyridoxine hydrochloride, 1.1 mg; I, 370 ug; Mn, 22.02 mg; Cu, 1.48 mg; Fe, 14.69 mg; Zn, 14.69 mg; Se, 100 ug.

2.2.2 Sample Collection and Analyses

2.2.2.1 Growth performance

Data on body and feed weights for each cage were collected on days 0, 12 and 21 and was corrected for mortality.

2.2.2.2. Bone mineralisation

For bone mineral determination the left tibia was collected from two randomly selected birds from each cage on day 21, and surrounding skin, muscle, tendons and cartilage were removed. Samples were defatted by ether extraction and dried again before being placed in a Muffle furnace for 24 hours at 600^{o}C, using AOAC procedures. Bone ash was analysed for P and Ca content using inductively coupled plasma atomic emission spectroscopy procedures (S1015 UKAS accredited procedure, Sciantec Analytical Services Ltd, Cawood, UK).

2.2.2.3 Digesta collections

On day 21, birds were euthanased by cervical dislocation, and gizzard, jejunum and ileum digesta samples were collected from each bird and pooled per cage. The diet and digesta samples were ground through a 0.5 mm sieve and analysed for dry matter (DM), nitrogen (N), minerals, non-phytate P (NPP), and gross energy (GE) using AOAC procedures. Titanium (Ti) concentration was determined using the method described by Short *et al*. (1996).

For determination of inositol phosphate ester (IP) degradation, samples of digesta, feed and excreta were dried in a forced draught oven at 80^{o}C, for a minimum of 48 hours or until a constant dry weight was reached, and ground before being analysed by high-performance ion chromatography-based techniques (Blaabjerg *et al.*, 2010).

2.2.2.4 Comparative slaughter technique

Whole body nutrient and energy accretion for the 21-day period was determined using the comparative slaughter technique as described by Olukosi *et al*. (2008). In brief, 8 birds formed an initial slaughter group on arrival and were euthanased

by overdose of barbiturate without feeding. On day 21, one of the seven birds in each cage was selected for the final slaughter group, consisting of 40 birds providing 8 replicates for each of the 5 diets (excludes the NC without phytase treatment due to its termination on day 12). Feed was removed 2 hours prior to euthanasia to allow gut emptying. The whole birds (including feathers and guts) were frozen prior to preparation for analysis, which involved the chopping and coarse-grinding of individual chickens prior to freeze-drying and passing through a 0.5 mm sieve. Dry matter of the freeze-dried, minced-birds was determined in two stages: two fresh subsamples of the minced birds were taken from each cage and then weighed after freeze drying (constituting the first DM determination) with a second DM determination done on freeze-dried samples using AOAC procedures. The carcass samples were then analysed for DM, GE, ether extractable fat, and N content.

2.2.3 Calculations and statistical analyses

2.2.3.1 Nutrient utilisation

Nutrient digestibility was calculated using the following equation:

$$\text{Nutrient digestiblity (\%)} = \left[1 - \left(\frac{\text{Ti in diet}}{\text{Ti in sample}}\right) \times \left(\frac{\text{Nutrient in sample}}{\text{Nutrient in diet}}\right)\right] \times 100$$

Dry matter digestibility was calculated by:

$$\text{Dry matter digestibility (\%)} = 1 - \left(\frac{\text{Ti in diet}}{\text{Ti in excreta}}\right)$$

Mineral flow:

$$Mineral\ concentration\ per\ g\ DM \times \frac{Ti\ in\ diet}{Ti\ in\ ileum}$$

2.2.3.2 Comparative slaughter technique

The proportion of fat and protein accretion was determined and the whole body nutrient accretion rate was calculated using the following equations; where ‘whole body nutrient concentration’ is the analysed nutrient content of the

dried, minced samples, and the number of days is the age of the bird when the final slaughter group was euthanased (21 in this study).

(1) Whole body initial nutrient quantity (g)
= Whole body initial nutrient concentration (%)
×Initial weight of chick (g)

(2) Whole body final nutrient quantity (g)
= Whole body final nutrient concentration (%)
×Final weight of chick (g)

(3) Whole body nutrient accretion (g) = (2) − (1)

(4)Whole body nutrient accretion $(g/d) = \frac{Whole\ body\ nutrient\ accretion}{Number\ of\ days}$

Heat production:

Heat production corrected for BW (MJ) $= \frac{\text{Heat production (MJ)}}{\text{Final carcass wt DM(g)}^{0.6}}$

Heat production (MJ) = AME intake (MJ) − Energy retention (MJ)

AME intake (MJ) = AME (MJ/g)×DM intake (g)

Energy retention (MJ) = Final carcass GE (MJ) − Inital carcass GE (MJ)

2.2.3.3 Inositol phosphate esters

Conversion of inositol phosphate esters (used to assess the degree of phytate hydrolysis) from nmol per g DM to mg per g DM was calculated over a series of 3 equations, as follows:

(1): ng per g DM = MW×nmol per g DM

(2): µg per g DM $= \frac{\text{ng per g DM}}{1000}$

(3): mg/g DM $= \frac{\text{µg per g DM}}{1000}$

2.2.3.4 Statistics

Statistical analyses were performed using the General Analysis of variance function of Genstat (14th edition, VSN International Ltd.). Means (per pen) were pooled for each of the diets to allow comparison of the treatment effects. Data was checked for normality and suitably transformed when required, details are provided under the tables. Growth performance data from d 0 to d 12 were analysed as a 2 × 3 factorial and the model included aP, phytase and the aP x phytase interaction. Results obtained from d 0 to d 21 were analysed as a 2 × 2 + 1 factorial due to the removal of the NC treatment at day 12. The model included the treatment form (Trt; PC or phytase supplemented) nested within the aP, phytase and aP × phytase interaction. When effects were statistically significant, means were separated using Tukey's test. Significance was accepted at $P < 0.05$. Where correlations are presented (identified using Genstat), all r^2 are significant to at least $P < 0.05$ unless otherwise stated.

2.3 Results

The NC treatment (diet 4) was terminated on day 12 due to poor growth of the birds leading to welfare concerns should the treatment have been continued. The body weight of the birds was close to reaching < 65% of the breed specifications, which was the previously-agreed cut-off in the Home Office licence at the time, details are provided in Appendix 2.6.3. Statistical analysis was required to be done on the basis of a 2 x 2 + 1 factorial arrangement.

The calculated and analysed nutrient values of the dietary treatments are in Table 2.2. All 6 diets were formulated to be similar in all nutrients except for aP, which was deficient in the NC treatment. The analysed Ca content of the diets was higher than formulated, and as analysed P level was similar to formulated, the Ca:P level was consequently higher than formulated. In the PC treatments, the Ca:tP ratio was consistent at 1.7:1, however the Ca:aP ratio varied between 2.7:1 and 3.0:1. In the NC treatments the Ca:aP ratio was higher, ranging from 2.2:1 to 2.6:1. In the NC without phytase treatment the ratio was 4.6:1; 4.5:1 in the NC with 1,500 FTU/kg phytase and 4.2:1 in the NC with 3,000 FTU/kg. The high Ca:aP ratio of 4.6:1 in the NC treatment may have exacerbated the aP deficiency of the diet further explaining the poor

performance of the birds, which ultimately resulted in the termination of this treatment. All other analysed nutrients were close to formulated values.

2.3.1 Phytate hydrolysis

Phytate, inositol phosphate (IPx; x refers to number of bound phosphate molecules) esters and inositol content (mg/g) of the gizzard and ileum were analysed. In the gizzard (Table 2.3), there was no aP × phytase interaction on IP ester content, however inositol increased with increasing phytase supplementation ($P < 0.001$). The content of IP4 and inositol in the gizzard was influenced by the dietary aP levels; the IP4 ($P < 0.05$) and inositol ($P < 0.001$) content was lowest in the PC compared to birds fed the PC (0.45% aP) or NC (0.30% aP) supplemented with phytase. Phytase supplementation had the overall effect of reducing IP6 ($P < 0.001$) and IP5 content ($P < 0.001$) and increasing inositol ($P < 0.001$) in the gizzard compared to the PC.

In the ileum (Table 2.4), aP × phytase interaction was significant for IP5 ($P < 0.05$), IP4 ($P < 0.01$) and tended to influence IP3 ($P = 0.051$). The ileal content of IP5 was lowest in the treatments containing 3,000 FTU/kg and comparable between 0.30 and 0.45% aP at both doses. However, IP5 content in the ileum with the addition of 1,500 FTU/kg was comparable to the PC. The addition of 3,000 FTU/kg phytase to the adequate aP treatment increased levels of IP4 above the PC and this was achieved with 1,500 FTU/kg when aP was reduced to 0.30% and there were no further increases in IP4 with the addition of 3,000 FTU/kg. The supplementation of phytase reduced IP6 ($P < 0.001$) and tended to increase inositol content ($P = 0.054$), with reductions in IP6 being more dramatic at the higher phytase levels, but comparable between doses for the effect on inositol, when compared to the PC. There were no major effects of aP content on the IP ester contents in the ileum. The phytase-supplemented treatments were significantly lower in IP6 and inositol than the PC treatment without phytase ($P < 0.001$). Although there were no statistically significant interactive or phytase influences (except for phytase dose response of inositol) on the IP esters in the gizzard, some of the values appear to be numerically different.

Table 2.2 - Calculated and analysed nutrient and energy content of dietary treatments fed to broilers from 0 to 21 days post-hatch

Description of diets:	PC[1]	PC1,500	PC3,000	NC[2]	NC1,500	NC3,000
Phytase, FTU/kg:	0	1,500	3,000	0	1,500	3,000
Calculated nutrients and energy:						
ME, MJ/kg	12.9	12.9	12.9	12.9	12.9	12.9
Lysine, g/kg	15.3	15.3	15.3	15.3	15.3	15.3
Calcium, g/kg	9.9	9.9	9.9	10.1	10.1	10.1
Total phosphorus,	6.8	6.8	6.8	5.3	5.3	5.3
aP, g/kg	4.5	4.5	4.5	3.1	3.1	3.1
Ca: tP	1.5	1.5	1.5	1.9	1.9	1.9
Ca : aP	2.2	2.2	2.2	3.3	3.3	3.3
Phytase, FTU/kg	50	1,500	3,000	50	1,500	3,000
Analysed nutrient content:						
DM, %	89.0	88.7	88.7	89.3	89.2	90.0
Protein, g/kg	258	255	239	254	246	245
Calcium, g/kg	12.8	13.2	12.5	12.5	16.8	14.6
Total phosphorus,	7.6	7.8	7.4	5.8	6.5	6.1
Phytate P, g/kg	3.4	2.6	3.0	3.1	2.8	3.3
aP[3], g/kg	4.2	4.9	4.4	2.7	3.7	2.8
Ca: tP	1.7:1	1.7:1	1.7:1	2.2:1	2.6:1	2.4:1
Ca : aP	3.0:1	2.7:1	2.8:1	4.6:1	4.5:1	5.2:1
Phytase[4], FTU/kg	< 50	1995	4045	< 50	2,270	4,420

Notes: [1]PC: Positive Control; [2]NC: Negative control; [3]Available phosphorus (non-phytate phosphorus) = total phosphorus – phytate-P; [4]Quantification of phytase enzyme activity determined by AB Vista, Marlborough, UK

Table 2.3 – Phytate and myo-inositol phosphate ester content (mg/g DM) of the gizzard of broilers fed diets adequate or deficient in available phosphorus, with 1,500 or 3,000 FTU/kg phytase for 21 days[1,2]

Diet description	Phytase, FTU/kg	Gizzard IP6	IP5	IP4	IP3	Inositol
Positive control, aP 0.45%[3]		3.18	0.47	1.50	0.33	0.13
0.30%	1,500	0.18	-0.001	1.37	0.34	0.29
0.30%	3,000	0.12	0.002	1.47	0.37	0.42
0.45 %	1,500	0.17	0.000	1.60	0.40	0.26
0.45%	3,000	0.11	0.005	1.54	0.37	0.36
	SEM	0.099	0.014	0.069	0.022	0.021
Treatment [Trt]						
	PC	3.18[a]	0.47[a]	1.50	0.33	0.13[b]
	Enzyme	0.14[b]	0.002[b]	1.49	0.37	0.33[a]
	SEM	0.049	0.007	0.035	0.011	0.011
[Trt] aP						
PC, 0.45% aP (0 FTU)		3.18	0.47	1.50[ab]	0.33	0.13[b]
NC, 0.30% aP (phytase)		0.15	0.001	1.42[b]	0.35	0.36[a]
PC, 0.45% aP (phytase)		0.14	0.002	1.57[a]	0.39	0.31[a]
	SEM	0.070	0.010	0.049	0.015	0.015
[Trt] Phytase, FTU/kg						
	PC (0)	3.18	0.47	1.50	0.33	0.13[c]
	1,500	0.18	0.000	1.48	0.37	0.28[b]
	3,000	0.11	0.003	1.50	0.37	0.39[a]
	SEM	0.070	0.010	0.049	0.015	0.015
P values						
Trt[3]		<0.001	<0.001	0.904	0.109	<0.001
[Trt] aP%		0.862	0.895	0.039	0.136	0.034
[Trt] Phytase		0.542	0.792	0.780	0.867	<0.001
[Trt] aP x phytase		0.988	0.933	0.235	0.124	0.347

Notes: [1]Means were obtained from 7 birds per pen with 8 replicate pens per diet; [2]Birds from the NC were removed from the experiment at d 12 therefore, the data has been analysed as a 2 x 2 + 1 factorial, with the PC with 0 FTU/kg phytase nested within Treatment (PC vs. Enzyme), phytase dose and the interaction, using the ANOVA function of Genstat; [3]PC without phytase compared against the mean of all phytase-supplemented treatments; [a-b] Means within columns with different superscripts are significantly different for each factor (P < 0.05).

Table 2.4 - Phytate and myo-inositol phosphate ester content (mg/g DM) of the ileum of broilers fed diets adequate or deficient in available phosphorus, with 1,500 or 3,000 FTU/kg phytase for 21 days[1,]

Diet description	Phytase, FTU/kg	Ileum IP6[4]	IP5[4]	IP4	IP3	Inositol
Positive control, aP 0.45% (PC)[1]		3.72	0.33[a]	0.33[b]	0.28	0.30
0.30%	1,500	0.84	0.19[b]	0.62[ab]	0.26	0.60
0.30%	3,000	0.38	0.09[c]	0.80[a]	0.33	0.70
0.45 %	1,500	1.06	0.28[ab]	0.87[a]	0.34	0.56
0.45%	3,000	0.32	0.08[c]	0.65[a]	0.31	0.64
	SEM	0.287	0.024	0.071	0.018	0.043
Treatment [Trt]						
	PC	3.72[a]	0.33	0.33	0.28	0.30[b]
	Phytase	0.65[b]	0.16	0.74	0.31	0.63[a]
	SEM	0.144	0.012	0.035	0.012	0.022
[Trt] aP						
PC, 0.45% aP (0 FTU)		3.72	0.33	0.33	0.28	0.30
NC, 0.30% aP (phytase)		0.61	0.14	0.71	0.30	0.65
PC, 0.45% aP (phytase)		0.69	0.18	0.76	0.32	0.60
	SEM	0.203	0.017	0.050	0.018	0.031
[Trt] Phytase, FTU/kg						
	PC (0)	3.72[a]	0.33	0.33	0.28	0.30[b]
	1,500	0.95[b]	0.24	0.75	0.30	0.58[a]
	3,000	0.35[c]	0.08	0.72	0.32	0.67[a]
	SEM	0.203	0.017	0.050	0.018	0.031
P values						
Trt[3]		<0.001	<0.001	<0.001	0.348	<0.001
[Trt] aP%		0.916	0.750	0.485	0.255	0.239
[Trt] Phytase		<0.001	<0.001	0.741	0.431	0.054
[Trt] aP x phytase		0.334	0.019	0.007	0.051	0.852

Notes: [1]Means were obtained from 7 birds per pen with 8 replicate pens per diet; [2]Birds from the NC were removed from the experiment at d 12 therefore, the data has been analysed as a 2 x 2 + 1 factorial, with the PC with 0 FTU/kg phytase nested within Treatment (PC vs. Enzyme), phytase dose and the interaction, using the ANOVA function of Genstat; [3]PC without phytase compared against the mean of all phytase-supplemented treatments; [4]Log transformed prior to analysis; [a-b] Means within columns with different superscripts are significantly different for each factor (P < 0.05).

This may be explained by the observed influence of a significant influence of Trt (PC vs. average of phytase-supplemented treatments) resulting from the loss of the NC treatment. There were no diets deficient in aP or Ca for comparison for the effects of phytase, where we would expect to see a response.

2.3.2 Nutrient utilisation

Nutrient and energy digestibility at the level of the ileum is shown in Table 2.5, with the minerals in Table 2.6. Flow of the micro-minerals through the ileum are shown in Table 2.7. Interaction between aP and phytase was significant for the ileal digestibility of energy (IDE, $P < 0.05$), DM ($P \leq 0.01$), N ($P < 0.05$), Ca ($P < 0.01$) and Zn ($P < 0.01$), and tended to be significant for P ($P = 0.063$) digestibility.

The IDE was comparable to the PC with both doses of phytase to the 0.30% aP diet, but decreased relative to the PC when phytase was added to the 0.45% aP diets. The PC diet was comparable to all phytase treatments for ileal DM digestibility, however there was a phytase dose response in the 0.30% diets, decreasing as phytase increased from 1,500 to 3,000 FTU/kg.

Phytase supplementation increased Zn digestibility above the PC, tending to increase digestibility in the 0.45% treatments and decrease digestibility in the 0.30% treatments as phytase increased from 1,500 to 3,000 FTU/kg. The dietary aP level was important for the digestibility of P ($P < 0.05$), being greater when phytase was added to the NC diet for P, compared to the PC with or without phytase.

Magnesium flow through the ileum was influenced by aP × phytase interaction ($P < 0.01$). Although Mg flow did not differ from the control in any of the treatments, or with an increase in dose within the 0.30 and 0.45% aP diets, flow was greater in the 0.45% diet with 1,500 FTU/kg than when the same dose was supplemented to the 0.30% diet. There was a tendency for Na flow to decrease with phytase supplementation ($P = 0.063$), but no other treatment effects on K and Fe.

Table 2.5 – Ileal nutrient digestibility (coefficient of) of broilers fed diets adequate or deficient in available phosphorus, with 1,500 or 3,000 FTU/kg phytase for 21 days[1,2]

Diet description	Phytase FTU/kg	IDE[4] kcal/kg	DM	N	Phytate-P
Positive control, aP, 0.45%	0	3051[a]	0.70[ab]	0.75[a]	0.71
0.30%	1,500	3052[a]	0.73[a]	0.74[ab]	0.89
0.30%	3,000	2911[ab]	0.69[b]	0.71[b]	0.91
0.45 %	1,500	2845[b]	0.69[b]	0.72[ab]	0.82
0.45%	3,000	2892[b]	0.70[ab]	0.72[ab]	0.91
	SEM	34.43	0.008	0.009	0.031
Treatment [Trt]					
	PC	3051.5	0.70	0.75	0.71[b]
	Phytase	2925.7	0.70	0.72	0.88[a]
	SEM	17.21	0.004	0.004	0.015
[Trt] aP					
PC, 0.45% aP (0 FTU)		3051.5	0.70	0.75	0.71
NC, 0.30% aP (phytase)		2982.0	0.71	0.73	0.90
PC, 0.45% aP (phytase)		2869.3	0.70	0.72	0.86
	SEM	24.34	0.006	0.006	0.022
[Trt] Phytase FTU/kg	PC (0)	3051.5	0.70	0.75	0.71
	1,500	2949.1	0.71	0.73	0.85
	3,000	2902.2	0.69	0.72	0.91
	SEM	24.3	0.006	0.006	0.022
P values					
Trt[3]		0.003	0.991	0.012	<0.001
[Trt] aP%		0.003	0.095	0.368	0.281
[Trt] Phytase		0.184	0.052	0.118	0.086
[Trt] aP x phytase		0.011	0.001	0.021	0.222

Notes: [1]Means were obtained from 7 birds per pen with 8 replicate pens per diet; [2]Data analysed as a 2 x 2 + 1 factorial with the PC with 0 FTU/kg phytase nested within Treatment (PC vs. Enzyme), phytase dose and the interaction, using the ANOVA function of Genstat; [3]PC without phytase compared against the mean of all phytase-supplemented treatments; [4]Ileal digestible energy; [a-b]Means within columns with different superscripts are significantly different for each factor (P < 0.05).

Table 2.6 – Ileal P, Ca, Zn and Na mineral digestibility (coefficient of) of broilers fed diets adequate or deficient in available phosphorus, with 1,500 or 3,000 FTU/kg phytase for 21 days[1,2]

Diet description	Phytase FTU/kg	P	Ca	Zn
Positive control, aP, 0.45% (PC)[3]	0	0.43	0.37[b]	0.10[c]
0.30%	1,500	0.55	0.57[a]	0.40[a]
0.30%	3,000	0.49	0.40[b]	0.20[b]
0.45 %	1,500	0.46	0.36[b]	0.20[b]
0.45%	3,000	0.48	0.33[b]	0.30[ab]
	SEM	0.022	0.023	0.03
Treatment [Trt]				
	PC	0.43[b]	0.37	0.10[b]
	Phytase	0.50[a]	0.41	0.30[a]
	SEM	0.011	0.012	0.01
[Trt] aP				
PC, 0.45% aP (0 FTU)		0.43[b]	0.37[b]	0.10[b]
NC, 0.30% aP (phytase)		0.52[a]	0.48[a]	0.30[a]
PC, 0.45% aP (phytase)		0.47[ab]	0.34[b]	0.20[a]
	SEM	0.016	0.017	0.02
[Trt] Phytase, FTU/kg	PC (0)	0.43	0.37[b]	0.10
	1,500	0.51	0.46[a]	0.30
	3,000	0.48	0.36[b]	0.20
	SEM	0.016	0.017	0.02
P values				
Trt[3]		0.015	0.098	<0.001
[Trt] aP%		0.044	<0.001	0.032
[Trt] Phytase		0.310	<0.001	0.107
[Trt] aP x phytase		0.063	0.004	0.002

Notes: [1]Means were obtained from 7 birds per pen with 8 replicate pens per diet; [2]Data analysed as a 2 x 2 + 1 factorial with the PC with 0 FTU/kg phytase nested within Treatment (PC vs. Enzyme), phytase dose and the interaction, using the ANOVA function of Genstat; [3]PC without phytase compared against the mean of all phytase-supplemented treatments; [a-b]Means within columns with different superscripts are significantly different for each factor (P < 0.05)

Table 2.7 – Ileal Mg, K and Fe mineral flow of broilers fed diets adequate or deficient in available phosphorus, with 1,500 or 3,000 FTU/kg phytase for 21 days[1,2]

Diet description	Phytase FTU/kg	Mg, %	K, %	Fe, mg/kg	Na, %
Positive control, aP, 0.45%	0	0.102[ab]	0.187	96.8	0.190
0.30%	1,500	0.089[b]	0.183	83.7	0.157
0.30%	3,000	0.097[ab]	0.195	95.1	0.175
0.45 %	1,500	0.108[a]	0.209	94.7	0.178
0.45%	3,000	0.095[ab]	0.191	93.9	0.182
	SEM	0.0037	0.0098	3.76	0.0076
Treatment [Trt]					
	PC	0.102	0.187	96.8	0.190
	Phytase	0.097	0.195	91.9	0.173
	SEM	0.0019	0.0049	1.88	0.0038
[Trt] aP					
PC, 0.45% aP (0 FTU)		0.102	0.187	96.8	0.190
NC, 0.30% aP (phytase)		0.093	0.189	89.4	0.166
PC, 0.45% aP (phytase)		0.102	0.200	94.3	0.180
	SEM	0.0026	0.0070	2.66	0.0053
[Trt] Phytase, FTU/kg	PC (0)	0.102	0.187	96.8	0.190
	1,500	0.098	0.196	89.2	0.168
	3,000	0.096	0.193	94.5	0.179
	SEM	0.026	0.0070	2.66	0.0053
P values					
Trt[3]		0.263	0.502	0.247	0.063
[Trt] aP%		0.033	0.277	0.205	0.084
[Trt] Phytase		0.597	0.738	0.169	0.165
[Trt] aP x phytase		0.009	0.149	0.116	0.342

Notes: [1]Means were obtained from 7 birds per pen with 8 replicate pens per diet; [2]Data analysed as a 2 x 2 + 1 factorial with the PC with 0 FTU/kg phytase nested within Treatment (PC vs. Enzyme), phytase dose and the interaction, using the ANOVA function of Genstat; [3]PC without phytase compared against the mean of all phytase-supplemented treatments; [a-b]Means within columns with different superscripts are significantly different for each factor (P < 0.05).

There were no phytase dose influences within the 0.30% or 0.45% aP diets, however, N digestibility decreased relative to the PC in the 0.30% aP diet with 3,000 FTU/kg phytase. The addition of 1,500 FTU/kg phytase to the 0.30% aP diet increased Ca digestibility above all other treatments, however there were no other treatment differences.

2.3.3 Growth performance

Growth performance was also recorded for the initial 12 day period, Table 2.8. Due to significant aP × phytase interaction on initial body weight (IBW), this was included as a covariate for the analyses of BWG, FI, FCR and mortality. The BWG and FI were significantly lower in the 0.30% aP treatment without phytase, with phytase improving BWG and FI in the NC only ($P \leq 0.01$). There were no treatment effects on FCR or mortality.

As a consequence of poor growth performance, the NC treatment was terminated on d12 in order to stay within the predetermined severity limits allowed under the animal experiment committee guidelines. Table 2.9 shows the growth performance of the remaining treatments at day 21. There was a significant treatment (PC vs. phytase-supplemented) influence on IBW, with the IBW of the birds being 1.2g greater in the PC than the average of all of the phytase supplemented diets. Therefore IBW was included as a covariate in the statistical analyses. During the overall 21 day experimental period, there were no treatment effects on growth performance parameters or mortality. The overall 21 day BWG of the PC diet was 817g and 808g in the phytase supplemented treatments. There was a tendency for phytase to increase FI ($P = 0.077$), with birds fed the 1,500 FTU/kg level of phytase, eating 1157g each over the 21 days and 1194g when fed 3,000 FTU/kg phytase, compared to the birds fed the PC diet, which ate 1140g. However, the FCR was comparable between the PC and phytase supplemented diets, being 1.41 and 1.46 respectively. Mortality remained low at under 3%.

Table 2.8 -Growth performance of broilers fed diets adequate or deficient in available phosphorus, with 0, 1,500 or 3,000 FTU/kg phytase from 0 to 12 days post-hatch[1,2]

aP, %	Phytase, FTU/kg	IBW, g	BW gain, g	Feed intake, g	FCR[3]	Mortality[4], %
0.30	0	43.9	179.6[b]	303.5[b]	1.77	3.2
	1,500	44.5	245.5[a]	385.5[a]	1.58	3.8
	3,000	44.3	269.0[a]	372.4[a]	1.48	1.9
0.45	0	45.3	254.1[a]	370.2[a]	1.47	0.4
	1,500	43.8	251.4[a]	365.1[a]	1.55	-0.2
	3,000	43.9	265.7[a]	377.0[a]	1.47	1.7
	SEM	0.42	12.27	7.74	0.13	1.80
0.30		44.2	231.4	353.8	1.31	2.9
0.45		44.3	257.1	370.8	1.49	0.6
	SEM	0.24	6.91	4.35	0.07	1.01
	0	44.6	216.9	336.9	1.62	1.8
	1500	44.1	248.4	375.3	1.56	1.8
	3000	44.1	267.4	374.7	1.48	1.8
	SEM	0.30	8.56	5.44	0.09	1.3
P values						
aP%		0.733	0.014	0.010	0.291	0.113
Phytase		0.420	<0.001	<0.001	0.500	1.000
aP × phytase		0.033	0.010	<0.001	0.515	0.554
Covariate (IBW)			0.393	0.016	0.998	0.420

Notes: IBW: Initial body weight; BWG: Body weight gain; FI: Feed Intake; FCR: Feed conversion ratio; [1]Means were obtained from 7 birds per pen with 8 replicate pens per diet; [2]IBW was included as a covariate; [3]Log transformed; [4]Statistical analyses of mortality % was performed following the application of 1/(sqrtX+0.1) to allow for analysis of zero mortality; [a-b]Means within columns with different superscripts are significantly different for each factor (P < 0.05).

Table 2.9 - Growth performance of broilers fed diets adequate or deficient in available phosphorus, with 0, 1,500 or 3,000 FTU/kg phytase from 0 to 21 days post-hatch[1,2]

Diet description, aP%	Phytase, FTU/kg	IBW, g	BWG, g	FI, g	FCR[5]	Mortality[6] %
Treatment [Trt][3]						
	PC	45.3[a]	817	11404	1.409	0.2
	Phytase	44.1[b]	808	11756	1.460	2.2
	SEM	0.31	11.9	11.2	0.03	0.94
[Trt] aP						
PC, 0.45% aP (0 FTU)		45.3	817.3	1140.4	1.409	0.2
NC, 0.30% aP (phytase)		44.4	798.5	1182.7	1.492	2.9
PC, 0.45% aP (phytase)		43.8	818.7	1168.5	1.428	1.7
	SEM	0.22	16.3	15.3	0.04	1.29
[Trt] Phytase, FTU/kg	PC (0)	45.3	817.3	1140.4	1.409	0.2
	1,500	44.1	797.1	1157.0	1.453	1.8
	3,000	44.1	820.1	1194.2	1.467	2.6
	SEM	0.31	16.1	15.1	0.03	1.27
P values						
Trt[3]		0.020	0.637	0.162	0.174	0.301
[Trt] aP%		0.224	0.390	0.485	0.262	0.593
[Trt] Phytase		0.888	0.299	0.077	0.829	0.608
[Trt] aP x phytase		0.702	0.109	0.408	0.277	0.126
Covariate (IBW)[4]			0.444	0.024	0.573	0.809

Notes: IBW: Initial body weight; BWG: Body weight gain; FI: Feed Intake; FCR: Feed conversion ratio; [1]Means were obtained from 7 birds per pen with 8 replicate pens per diet; [2]Data analysed as a 2 x 2 + 1 factorial with the PC with 0 FTU/kg phytase nested within Treatment (PC vs. Enzyme), phytase dose and the interaction, using the ANOVA function of Genstat; [3]PC without phytase compared against the mean of all phytase-supplemented treatments; [4]IBW was included as a covariate; [5]Log transformed; [6]Statistical analyses of mortality % was performed following the application of 1/(sqrtX+0.1) to allow for analysis of zero mortality; [a-b]Means within columns with different superscripts are significantly different for each factor (P < 0.05).

2.3.4 Bone mineralisation

The bone ash and mineral content are shown in Table 2.10. There were no significant aP × phytase interactions on the ash or mineral contents, or main effects of phytase. However, there was a significant increase in bone Zn ($P < 0.05$) in the phytase-supplemented diets compared to the PC without phytase, but no difference in Zn content between the NC and PC supplemented with phytase. The Mg content of the bone was increased ($P < 0.001$) when phytase was added to the PC diet above both the phytase-supplemented NC diet and PC diet without phytase, which were comparable to each other. The overall addition of phytase increased the Zn ($P < 0.001$) and Mn ($P < 0.05$) content above the PC, and tended to increase the content of ash ($P = 0.059$) and Mg ($P = 0.055$). There were no treatment effects on the bone Ca and P contents.

2.3.5 Whole body nutrient accretion

The accretion of DM, CP and fat into the carcass was not influenced by aP × phytase interactions, as shown in Table 2.11. Phytase at 1,500 FTU/kg increased carcass fat accretion ($P < 0.01$) above the PC and 3,000 FTU/kg, which did not differ from the PC. The dietary aP level was more important for the accretion of DM and CP, being greater when phytase was supplemented to the adequate aP than the low aP diets, whilst not differing significantly from the PC without phytase ($P < 0.05$). Overall, there were no differences between the PC diet and phytase-supplemented diets for carcass DM, CP and fat accretion.

Nutrient accretion of the carcass may be partly explained by the characteristics of energy retention (ER) and its subsequent partitioning between different processes, as shown in Table 2.12. There was no aP × phytase interaction on overall carcass ER. The ER as fat ($P < 0.05$) was greatest with 1,500 FTU/kg, being greater than both the PC and 3,000 FTU/kg which were similar, corresponding with the effects seen on carcass fat accretion. Similarly, overall ER and ER as protein were greater when phytase was added to the adequate aP diets than when phytase was added to the low aP diets ($P < 0.05$). Heat production (HP) was greater with phytase in the 0.30% aP diet than the 0.45%, however neither significantly differed from the PC without phytase ($P < 0.01$).

Table 2.10 - Bone ash and mineral content of broilers fed diets adequate or deficient in available phosphorus, with 1,500 or 3,000 FTU/kg phytase for 21 days[1,2]

Diet description/ aP, %	Phytase FTU/kg	Ash, %	Ca, %	P, %	Zn, mg/kg	Mg, %	Mn, mg/kg
Treatment [Trt]							
	PC	51.6	19.2	9.1	197.6[b]	0.35	4.5[b]
	Phytase	52.2	19.7	9.3	222.2[a]	0.37	5.2[a]
	SEM	0.15	0.14	0.06	2.32	0.003	0.12
[Trt] aP							
PC, 0.45% aP (0 FTU)		51.6	19.2	9.1	197.6[b]	0.35[b]	4.5
NC, 0.30% aP (phytase)		52.2	19.9	9.3	227.2[a]	0.35[b]	5.3
PC, 0.45% aP (phytase)		52.2	19.6	9.3	217.2[a]	0.38[a]	5.1
	SEM	0.21	0.19	0.09	3.29	0.004	0.18
[Trt] Phytase, FTU/kg	PC (0)	51.6	19.2	9.1	197.6	0.35	4.5
	1,500	52.0	19.6	9.2	223.9	0.37	5.3
	3,000	52.4	19.8	9.3	220.6	0.37	5.1
	SEM	0.21	0.19	0.09	3.29	0.004	0.18
P values							
Trt[3]		0.059	0.109	0.291	<0.001	0.055	0.020
[Trt] aP%		0.835	0.281	0.934	0.041	<0.001	0.323
[Trt] Phytase		0.153	0.516	0.416	0.482	0.905	0.323
[Trt] aP x phytase		0.432	0.920	0.758	0.419	0.285	0.619

Notes: [1]Means were obtained from 7 birds per pen with 8 replicate pens per diet; [2]Data analysed as a 2 x 2 + 1 factorial with the PC with 0 FTU/kg phytase nested within Treatment (PC vs. Enzyme), phytase dose and the interaction, using the ANOVA function of Genstat; [3]PC without phytase compared against the mean of all phytase-supplemented treatments; [a-b]Means within columns with different superscripts are significantly different between factors (P < 0.05).

Table 2.11 – Whole body nutrient accretion of broilers fed diets adequate or deficient in available phosphorus, with 1,500 or 3,000 FTU/kg phytase for 21 days[1,2]

Diet description/ aP, %	Phytase, FTU/kg		DM, g	CP, g	Fat, g
Treatment (Trt)					
	PC		230.8	133.4	58.4
	Phytase		233.6	131.4	63.6
		SEM	4.34	2.72	1.88
[Trt] aP					
PC, 0.45% aP (0 FTU)			230.8[a]	133.4[ab]	58.4
NC, 0.30% aP (phytase)			223.4[a]	124.4[b]	62.6
PC, 0.45% aP (phytase)			243.7[a]	138.4[a]	64.7
		SEM	6.13	3.84	2.66
[Trt] Phytase, FTU/kg	PC (0)		230.8	133.4	58.4[b]
	1,500		235.1	129.0	68.5[a]
	3,000		232.0	133.8	58.8[b]
		SEM	6.13	3.84	2.66
P values					
Trt[3]			0.778	0.745	0.225
[Trt] aP%			0.026	0.016	0.582
[Trt] Phytase			0.724	0.376	0.015
[Trt] aP x phytase			0.105	0.176	0.190

Notes: [1]Means were obtained from 1 bird per pen of average BW with 8 replicate pens per diet; [2]Data analysed as a 2 x 2 + 1 factorial with the PC with 0 FTU/kg phytase nested within Treatment (PC vs. Enzyme), phytase dose and the interaction, using the ANOVA function of Genstat; [3]PC without phytase compared against the mean of all phytase-supplemented treatments; [a-b]Means within columns with different superscripts are significantly different between factors (P < 0.05).

Table 2.12 – Whole body energy retention (ER; MJ) and heat production (HP; MJ) of broilers fed diets adequate or deficient in available phosphorus, with 1,500 or 3,000 FTU/kg phytase for 21 days[1, 2]

Diet description, aP, %	Phytase, FTU/kg	ER	ER as protein	ER as fat	HP / $BW^{0.6}$
Treatment [Trt]					
	PC	5.52	3.15	2.23	0.32
	Phytase	5.62	3.11	2.43	0.31
	SEM	0.11	0.06	0.072	0.007
[Trt] aP					
PC, 0.45% aP (0 FTU)		5.52[a]	3.15[ab]	2.23	0.32[ab]
NC, 0.30% aP (phytase)		5.39[a]	2.94[b]	2.39	0.34[a]
PC, 0.45% aP (phytase)		5.84[a]	3.27[a]	2.47	0.28[b]
	SEM	0.16	0.09	0.102	0.010
[Trt] Phytase, FTU/kg	PC (0)	5.52	3.15	2.23[b]	0.32
	1,500	5.63	3.05	2.62[a]	0.31
	3,000	5.60	3.16	2.24[b]	0.30
	SEM	0.16	0.09	0.102	0.01
P values					
Trt[3]		0.682	0.745	0.225	0.515
[Trt] aP%		0.049	0.016	0.582	<0.001
[Trt] Phytase		0.904	0.376	0.015	0.818
[Trt] aP x phytase		0.109	0.176	0.190	0.116

Notes: [1]Means were obtained from 1 bird per pen of average BW with 8 replicate pens per diet; [2]Data analysed as a 2 x 2 + 1 factorial with the PC with 0 FTU/kg phytase nested within Treatment (PC vs. Enzyme), phytase dose and the interaction, using the ANOVA function of Genstat; [3]PC without phytase compared against the mean of all phytase-supplemented treatments; [a-b]Means within columns with different superscripts are significantly different for each factor (P < 0.05).

There were no overall differences between the phytase-supplemented diets and the PC. Table 2.13 shows the efficiency of ER and the proportion of energy required for maintenance. There were no interactive effects, although the efficiency of ER as fat was greater with 1,500 than 3,000 FTU/kg phytase ($P < 0.01$), but not greater than the PC without phytase. There was also a tendency for phytase to reduce ER as protein ($P = 0.052$) and increase the proportion of energy required for maintenance ($P = 0.052$) with 1,500 FTU/kg, however there were no differences between the PC and all phytase-supplemented treatments. Reducing the dietary aP level from 0.45 to 0.30% (in the presence of phytase) reduced the overall efficiency of ER ($P \leq 0.001$) and increased the energy required for maintenance processes ($P < 0.05$).

2.3.6 Correlations

Correlations between all the variables are presented in Table 2.14 The proportion of energy used for maintenance was negatively correated with carcass CP and DM accretion, efficiency of energy retention, overall energy retention and energy retained as protein (r^2 = -0.91, -0.75, -0.73, -0.87, -0.91, respectively; $P < 0.001$). Meanwhile, whole body CP accretion was negatively correlated with the coefficient of energy used for heat production (r^2 = -0.75; $P < 0.001$) but positively correlated with the efficiency of energy retention (r^2 = +0.75; $P < 0.001$). The coefficient of energy used for heat production was negatively correlated with the efficiency of energy retention (r^2 = 1.00; $P < 0.001$).

There were correlations between nutrient digestibility and other variables. Ileal Ca digestibility was positively correlated with ileal Mn digestibility (r^2 = +0.86; $P < 0.001$), whilst ileal Na flow was negatively correlated with the efficiency of energy use for whole body protein accretion (r^2 = -0.65; $P < 0.001$).

Inositol phosphate esters also appeared to be correlated with ileal nutrient digestibilities. The content of ileal IP3 was negatively correlated with ileal digestible energy, K, N and Na digestibilities/ flow (r^2 = -0.87, -0.60, -0.76, -0.72, respectively; $P < 0.001$). Ileal inositol content was also negatively correlated with ileal N digestibility (r^2 = -0.62; $P < 0.001$).

Table 2.13 – Efficiency of energy retention (ER) overall, for protein and fat deposition, and proportion of energy retained for maintenance of broilers fed diets adequate or deficient in available phosphorus, with 1,500 or 3,000 FTU/kg phytase for 21 days[1, 2]

Diet description, aP, %	Phytase FTU/kg	Efficiency of ER overall	Efficiency of ER as protein	Efficiency of ER as fat	Proportion of energy for maintenance, %
Treatment [Trt]					
	PC	0.40	0.57	0.41	15.78
	Phytase	0.41	0.55	0.44	16.25
	SEM	0.008	0.005	0.012	0.471
[Trt] aP					
PC, 0.45% aP (0 FTU)		0.40^{ab}	0.57	0.41	15.78^{ab}
NC, 0.30% aP (phytase)		0.38^{b}	0.55	0.45	17.42^{a}
PC, 0.45% aP (phytase)		0.44^{a}	0.56	0.41	15.03^{b}
	SEM	0.011	0.007	0.016	0.666
[Trt] Phytase,	PC (0)	0.40	0.57	0.41^{ab}	15.78
	1,500	0.41	0.54	0.47^{a}	17.21
	3,000	0.41	0.57	0.40^{b}	15.29
	SEM	0.011	0.007	0.016	0.666
P values					
Trt[3]		0.469	0.112	0.326	0.662
[Trt] aP%		0.001	0.113	0.217	0.015
[Trt] Phytase		0.975	0.052	0.005	0.052
[Trt] aP x phytase		0.107	0.729	0.998	0.172

Notes: [1]Means were obtained from 1 bird per pen of average BW with 8 replicate pens per diet; [2]*Data analysed as a 2 x 2 + 1 factorial with the PC with 0 FTU/kg phytase nested within Treatment (PC vs. Enzyme), phytase dose and the interaction, using the ANOVA function of Genstat;* [3]*PC without phytase compared against the mean of all phytase-supplemented treatments;* [a-b]*Means within columns with different superscripts are significantly different (P < 0.05).*

Table 2.14 - Correlations of interest identified within all study data

Variable 1	Variable 2	r^2	P value
Proportion of energy used for maintenance, %	Carcass CP accretion, g	-0.91	< 0.001
Proportion of energy used for maintenance, %	Carcass DM accretion, g	-0.75	< 0.001
Proportion of energy used for maintenance, %	Efficiency of energy retention	-0.73	< 0.001
Proportion of energy used for maintenance, %	Energy retention, MJ	-0.87	< 0.001
Proportion of energy used for maintenance, %	Energy retained as protein, MJ	-0.91	< 0.001
Carcass CP accretion, g	Coefficient of energy used for heat production	-0.75	< 0.001
Carcass CP accretion, g	Efficiency of energy retention	+0.75	< 0.001
Carcass CP accretion, g	Heat production/$BW^{0.6}$	-0.71	< 0.001
Coefficient of energy used for heat production	Efficiency of energy retention	-1.00	< 0.001
Ileal Ca digestibility, %	Ileal Mn, mg/kg	+0.86	< 0.001
Efficiency of energy use for protein accretion	Ileal Na flow, %	-0.65	< 0.001
IDE, kcal/kg	Ileal IP3, mg/g DM	-0.87	< 0.001
Ileal inositol, mg/g DM	Ileal N digestibility, %	-0.62	< 0.001
Ileal IP3, mg/g DM	Ileal K flow, %	-0.60	< 0.001
Ileal IP3, mg/g DM	Ileal N digestibility, %	-0.76	< 0.001
Ileal IP3, mg/g DM	Ileal Na flow, %	-0.72	< 0.001

2.4 Discussion

2.4.1 Phytate hydrolysis

Phytase hydrolyses phytate to release lower inositol phosphate esters and phosphate molecules in a step-wise manner, with each series of hydrolysis producing a lower ester from IP6 to IP1 and finally inositol (Lei and Porres, 2003). The phosphate molecules then have the potential to be absorbed in the intestine and utilised by the animal. The majority of phytase activity has been observed prior to the small intestine, primarily in the crop, gizzard and proventriculus due to the suitability of the pH for phytate solubility and enzyme activity (Maenz and Classen, 1998; Yi and Kornegay, 1996).

As expected, the results show significant phytate and IP5 hydrolysis in the gizzard and ileum, with hydrolysis increasing with phytase dose, indicating that the phytase used was efficacious in this intestinal region for full hydrolysis of the phytate molecule to produce inositol. At least for phytate and IP5, the release of inorganic P did not appear to negatively feedback and restrict further phytate hydrolysis. However, the effect of aP level on IP4 hydrolysis in the ileum and the gizzard may have been a result of inorganic-P feedback as indicated by the significant aP × phytase interaction. However, IP3 has been reported to enhance P retention (Zyla *et al.,* 2004) although high IP4 concentrations may inhibit the activity of intestinal phosphatases (Hu *et al.,* 1996).

In the current study, the production of inositol in both the gizzard and ileum was greater with higher phytase doses indicating that regardless of phytase level, full hydrolysis of phytate occurred. However, Wyss *et al.* (1999) reported that hydrolysis often terminates at IP1, whilst Rapp *et al.* (2001) suggested that once hydrolysis was initiated, 90-100% of the products of phytate degradation would be hydrolysed to at least IP2. Additionally, the dietary Ca level has an important influence on phytase activity and the degree of phytate hydrolysis achievable. High Ca reduces the efficacy of phytase (and intestinal phytase activity), through changes in pH, phytate-Ca complex formation, phytase-Ca binding and overall nutrient solubility (Applegate *et al.,* 2003a; Konietzny and Greiner, 2002; Tamim *et al.,* 2004). The phytase used in the current study may be more suited for IP6 and IP5 hydrolysis and due to the high affinity of IP6 binding with nutrients this may have resulted in the significant improvements in nutrient availability,

however further hydrolysis of the lower esters may have been achieved if dietary Ca levels were lower.

2.4.2 Nutrient utilisation

Phytate hydrolysis reduces the complexes with nutrients such as Ca, other minerals, energy and protein (Cabahug *et al.*, 1999; Fontaine *et al.*, 1946; Mahajan and Dua, 1997) which may then be absorbed and consequently lead to the improved digestibility, as seen in this study. For example, 1,500 FTU/kg phytase improved nutrient digestibility compared to the PC diet without phytase. However, in most cases, supplementing 3,000 FTU/kg phytase did not result in additional benefits beyond 1,500 FTU/kg for nutrient utilisation. Additionally, the highest nutrient digestibility was obtained in birds fed the NC with 1,500 FTU/kg of phytase. This may be because at 1,500 FTU/kg, P and Ca absorption met the nutritional requirements and at 3,000 FTU/kg the intestinal absorption may have become saturated (Wasserman and Taylor, 1973) preventing further absorption and resulting in the excess being excreted.

The release of previously-bound nutrients, primarily Ca, may also influence the efficacy of phytase and its ability to interact with the phytate molecule. The high dietary Ca levels and subsequent Ca:aP ratio experienced in this study may hinder the ability of phytase to hydrolyse phytate and utilise nutrients in the digesta. Calcium can form complexes with phytate (Hamdi *et al.*, 2015), which reduces its ability to interact with phytase (Selle *et al.*, 2009), or results in the formation of metallic soaps in the gut (Papakonstantinou *et al.*, 2003), reducing the ability of the animal to utilise associated nutrients and dietary fats, altering pH and nutrient absorption capabilities (Applegate *et al.*, 2003b; Tamim and Angel, 2003).

The overall phytase effect observed in this study was similar to other published literature in response. For example, Ravindran *et al.* (2000) used diets where different levels of phytase were added to diets containing different levels of aP and reported that phytase improved the ileal digestibility of AME (apparent metabolisable energy), P, N and AAs, as well as the retention of DM, P and N. The response of phytase was greater in diets with lower aP, except for AME,

which was greater in the diets with higher aP, which are similar to the finding presented here.

It is generally suggested that interaction between minerals and lower inositol esters are lower than the interaction between phytate and higher esters (Frolich *et al.,* 1986). However the current study suggests that interaction with the lower esters does occur, as seen with IP3 and ileal K, N and Na flow. There was an increase in ileal energy digestibility identified to be correlated with a decrease in the ileal IP3 content (r^2 = -0.87). Similarly, ileal digestibility and mineral flow of K (r^2 = -0.60), N (r^2 = -0.76) and Na (r^2 = -0.72) appear to increase as ileal IP3 content decreases, highlighting a negative influence of IP3 on mineral digestibility. A reduction in Na digestibility in the PC diets may be a response to the buffering nature of the secretion of $NaHCO_3$, suggesting that IP3 may lower the intestinal pH and may subsequently decrease the solubility, or increase the binding of phytate and other nutrients. Additionally, high Ca and P levels in the digesta (increased as a result of phytase hydrolysis) can contribute to deficiencies in other minerals, such as Na (Atia *et al.,* 2000). In the current study, as the mineral flow of Na increased, the efficiency of energy use for carcass protein accretion decreased (r^2 = -0.65). As the PC diets were nutritionally adequate, the additional release of nutrients and minerals from phytate hydrolysis may alter the dynamics of absorption and excretion. The digestibility of N is negatively correlated with the ileal inositol content (r^2 = -0.62), with N digestibility decreasing as the ileal inositol content increases. This observation may be a response of increased N release from phytate, as full hydrolysis occurs to produce inositol and preferential absorption of inositol over N. Perhaps the associated negative effects of the correlation between IP3 content and mineral digestibility would be minimised through full hydrolysis of phytate and its lower esters, preventing these esters from accumulating and forming complexes with minerals in the digesta.

2.4.3 Growth performance

The observed improvements seen in ileal nutrient digestibility were not reflected by improvements in growth performance. However, the results show the ability of phytase to allow birds fed diets marginally deficient in aP to match

the performance of birds fed nutritionally adequate diets and not appear to be deficient in P. Other published studies have indicated that reducing the dietary aP content has the effect of reducing growth performance in broilers (Perney *et al.,* 1993; Sebastian *et al.,* 1997; Watson *et al.,* 2006), and that phytase can compensate for low dietary aP (Gordon and Roland, 1997; Sebastian *et al.,* 1996; Waldroup *et al.,* 2000). The particularly poor performance of the NC diet without phytase in this study was not expected, but may have been due to the high analysed dietary Ca, which then resulted in a Ca:aP ratio of 4:1, which is very high and likely to have had detrimental effects. A number of studies have showed the ability of phytase to restore the performance of diets low in aP. Taheri *et al.* (2015) demonstated the ability of 1,000 FTU/kg phytase to restore growth performance of diets containing 0.23% aP and 4,000 FTU/kg in diets containingly 0.14% aP. Similarly, Cabahug *et al.* (1999) conducted a similar study to the one presented here, using 3 levels of phytase supplemented to either a low (0.23%) or adequate (0.45%) aP diet and observed growth performance. The authors found that BWG and FI were improved with increasing aP levels ($P < 0.05$, $P < 0.001$, respectively) and phytase supplementation ($P < 0.001$), with the greatest response seen following phytase addition to the lower aP level. As phytase increased FI and feed efficiency, it was suggested by the author that these improvements were a result of an increase in utilisation of nutrients in addition to P. Although no improvements in BWG were observed in the NC with phytase, the performance was comparable to the PC, suggesting that P was sufficient in all treatments. As the NC + phytase diets were compared to aP-adequate PC diets only, the lack of improvement above the PC or treatment differences is not unexpected.

2.4.4 Bone mineralisation

High dietary Ca has been reported to be implicated in the development of bone problems, such as rickets, however, effects are likely to only ever be subclinical unless there is a substantial increase in growth rate (Long *et al.,* 1984). The lack of effect on growth performance parameters in this study may explain why no adverse effects on bone mineralisation were observed despite the higher than formulated dietary Ca content. In a study by Sohail and Roland (1999), an increase in bone mineral content with 300 FTU/kg phytase supplemented to

diets with a low aP content of 0.225% ($P < 0.01$) was observed, with a further increase in bone mineral content of 5% when non-phytate P was increased to 0.325%. Similar results were reported by Brana *et al.* (2006), where 10,000 FTU/kg phytase increased bone ash by 15% relative to NC diet and 5% relative to PC diet, compared to when 500 FTU/kg was supplemented, suggesting an increase in available P.

In the current study, the absence of treatment effects on the bone mineral content of P and Ca of the NC diets is likely to be a result of the absence of the NC without phytase for comparison. As the mineral contents were similar between the PC and NC phytase supplemented diets, it is apparent that the phytase-supplemented NC diets did not lead to Ca and P deficiency during bone mineralisation, which would likely have occurred in the NC diet if phytase was absent. Although there were treatment influences on the bone mineral content, there were no significant effects on the Ca and P content, likely as a consequence of the dietary Ca levels, but also subsequent influences on other minerals.

Smith and Kabaja (1985) report an interaction between Ca and Mn, with a high Ca: P ratio leading to secondary Mn deficiency and subsequently leg problems. Although correlation between ileal digestibility of Ca and Mn were identified in the current study, there were positive correlations between the digestibility (r^2 = +0.86; Table 2.14) but no correlations with bone mineral content. These results suggest an interaction between Ca and Mn, but that the Ca levels were not severe enough for influences to be seen at the level of the bone, as there were no treatment effects on the bone Ca content, but the phytase supplemented treatments had a greater bone Mn content than the PC treatments without phytase. In the current study, phytase and aP level influenced bone Zn and Mg content, being greater in the PC phytase-supplemented treatments than the PC without phytase. Phytase supplementation increased bone Zn and tended to increase Mg, which is similar to a study by Qian *et al.* (1996), who reported a tendency for an increase in bone Mg with 800 U/kg phytase, although they didn't observe any treatment effect on Zn content, which may have been a result of the lower phytase dose used.

2.4.5 Implications on whole body nutrient partitioning

It is apparent that the addition of phytase alters the partitioning of energy and nutrients within the body, potentially via enhanced efficiency of production, although these effects were not reflected in growth performance. This may be because phytase was able to influence the partitioning of nutrients but the nutrient ratios may not have been sufficient to increase the overall nutrients available to be able to seen by improvements in growth performance. This may be particularly hindered by the massive imbalance between dietary Ca and P. It is also possible that the high dietary Ca levels experienced in this study may have contributed a reduction in the fat content of the PC diet, reducing the apparent effect of phytase supplementation against the non-supplemented diets. In a study with rats, Papakonstantinou *et al.* (2003) demonstrated that a high level of dietary Ca led to a decrease in BW and fat content, explained by a reduction in digestible energy intake, but did not see any differences in body temperature or energy expenditure.

The efficiency of utilisation of nutrients for whole body DM, CP accretion and energy retained as protein was more efficient in the PC than NC diet, even when phytase was supplemented, but still being comparable to the PC without phytase, with overall efficiency improved through reduced heat production and a lower energy requirement for maintenance. As the NC diet in the current study was formulated to be deficient in aP, this deficiency must be reversed before the additional nutrients released from phytate hydrolysis can be utilised for carcass accretion. The PC diet already met the nutritional breed-specific requirements for the broilers, so the released nutrients from phytase supplementation have the potential to be immediately utilised for production rather than for maintenance, as would be the case in the NC treatment. The greater DM and/or CP accretion in the PC diet with phytase may be an indication of extra-phosphoric effects of the super-dosing of phytase. As the PC diet is nutritionally adequate, the phytase-mediated release of P and other nutrients benefited carcass accretion. As all nutrients are in adequate supply or in excess, deficiency will not be limiting during growth or carcass accretion, but the relative levels and ratios between the different nutrients may become more beneficial following the additional nutrient release from phytate hydrolysis, facilitating more efficient production and a lower metabolic demand. Olukosi

and Adeola (2008) reported that the provision of phytase to a diet marginally deficient in P and ME gave a numerical increase in carcass protein and reduction in carcass fat, suggesting enhanced efficiency in producing a lean carcass with the addition of phytase. The authors observed that protein carcass accretion was twice that of fat accretion in the phytase supplemented diets compared to the NC, indicating that sufficient ME was also released for protein accretion.

The proportion of energy required for maintenance is negatively correlated (Table 2.14) with the accretion of carcass CP (r^2 = -0.91) and DM (r^2 = -0.75) and energy retention characteristics, such as the amount (r^2 = -0.87) and efficiency of overall ER, and ER as protein (r^2 = -0.73, r^2 = -0.91, respectively). As the accretion of CP increases, the energy used for heat production decreases (r^2 = -0.75), suggesting that this energy is partitioned towards CP accretion and growth, rather than heat production, also coinciding with an improvement in efficiency of energy retention (r^2 = 0.75) and reduction in heat production (r^2 = -0.71) as carcass CP increases. The efficiency of energy retention and the coefficient of energy used for heat production are negatively correlated (r^2 = -1.00).

Published studies also suggest that the extra-nutrients released and utilised following super-doses of phytase lead to an increased nutrient availability for whole body nutrient accretion (Olukosi and Adeola, 2008; Olukosi *et al.,* 2008). There may be a genetic limitation to the metabolic capability for the accretion of protein, resulting in excess energy being accreted as fat (Summers and Leeson, 1979). Similarly, Agbisit *et al.* (2013) suggested that the increase in fat accretion was from the storage of excess energy and nutrients released from phytate during hydrolysis by phytase. It is possible that the supplementation of 1,500 FTU/kg phytase in the current study led to nutrient excess, resulting in the deposition of carcass fat (Agbisit *et al.*, 2013). The extra availability of these nutrients and AME from the hydrolysis of phytate by super doses of phytase may result in their excess, particularly if a nutrient matrix is not applied, resulting in the deposition of fat (Lin, 1981). When supplying phytase to a NC diet formulated to be deficient in Ca, P and ME, Agbisit *et al.* (2013) also reported an increase in abdominal fat weight compared to the NC without phytase ($P < 0.05$), but matched levels of the PC diet. It was also apparent in a study reported by

Shelton *et al*. (2003) that increasing dietary energy was more effective at increasing carcass protein or fat deposition and energy retained, than phytase.

In the current study, the increase in fat accretion with 1,500 FTU/kg phytase in adequate aP diets was overcome by the addition of 3,000 FTU/kg, and may be a result of additional nutrient release (which may have been limiting for protein accretion) following phytate hydrolysis, as seen by the further decrease in IP6 content of the ileum as phytase increased from 1,500 to 3,000 FTU/kg. The accretion of carcass fat is less energetically expensive than the accretion of protein or DM as the energy requirement for the deposition of fat is lower than for the deposition of protein. The results suggest that the addition of 1,500 FTU/kg to an adequate aP diet may result in the diet being nutritionally adequate and higher than requirements for maintenance, but that some nutrients (or energy) may have been limiting for further protein deposition and thus fat was deposited. Additionally, the birds fed the NC diets may have had a higher metabolic demand with increased catabolism occurring to supply molecules for processes involved in cellular maintenance as a result of aP deficiency, thereby also increasing heat production. Heat production is energetically expensive and reduces production efficiency and may have been a result of a high metabolic demand following a deficiency in aP, potentially due to an inability to deposit muscle due to a lack in improvement in bone structure, resulting in energy instead being deposited as fat. The lower heat production in the PC with phytase than NC with phytase suggests an extra-phosphoric influence, with more efficient protein utilisation and accretion, and subsequently less deamination for fat synthesis.

A shift in nutrient partitioning may have occurred, changing the ratio between nutrients, such as Ca:P and protein: energy. The protein: energy ratio is important for the accretion of carcass protein and fat (Mcleod, 1982). It is possible that the additional release of nutrients following the supplementation of 3,000 FTU/kg results in a shift in the ratio, becoming more optimal for processes other than the accretion of fat, corresponding with the reduction in energy retention as fat with 3,000 FTU/kg compared to 1,500 FTU/kg.

2.5 Conclusion

- The high dietary Ca:aP levels experienced in this study may have prevented the phytase from reaching its full potential as phytase would exacerbate the amount of free Ca in the GIT through the hydrolysis of phytate, particularly in the reduced aP diets, with effects being more a consequence of this ratio than a reduction in aP.
- Phytase reduced ileal phytate concentrations, with the greatest reduction achieved with 3,000 FTU/kg phytase in the NC treatments.
- Inositol content increased with phytase, regardless of dose, however the mid-esters (IP5-4) were influenced by dietary aP and Ca levels. There were no significant treatment effects on ileal IP3 content.
- Improvements in nutrient utilisation or changes in whole body nutrient partitioning were not reflected by improvements in growth performance.
- The lack of treatment effect on bone Ca and P suggests that in all treatments neither Ca or P were limiting, so further release from phytase addition had no benefit. The influence on bone Zn, Mg and Mn by dietary aP and phytase supplementation suggests that in some cases these minerals were limiting.
- Where significant treatment effects were observed, nutrient utilisation was generally higher with 1,500 FTU/kg in the reduced aP diets (except for Mg, which decreased) and is likely a result of nutrient balance within the digesta for absorption and phytase action.
- Treatment effects on ileal nutrient utilisation or the partitioning of nutrients in the carcass and subsequent influences on the accretion of protein and fat were not substantially influential on overall growth performance and BWG.
- It appears that metabolic changes may be occurring when phytase is increased from 1,500 to 3,000 FTU/kg, switching between protein and fat accretion, potentially as a consequence of nutrient availability as maintenance requirements must be met before the nutrients can be utilised for growth.
- The reduction in BWG in the NC treatment without phytase at day 12 compared to all others indicates that a dietary imbalance was present, with performance following the supplementation of super-doses of

phytase matching that of the birds fed the nutritionally adequate diet. This initial data at day 12 reflects that the NC treatment had the expected response, so any improvements in the PC treatment with phytase supplementation can be explained by the extra-phosphoric effects of phytase.

- As there were no benefits of increasing phytase supplementation from 1,500 to 3,000 FTU/kg on growth performance, nutrient utilisation or carcass characteristics, to maintain commercial and economical applicability, 1,500 FTU/kg was the dose selected for the super-dose in following studies.

2.6 Appendix

2.6.1 Animal husbandry

Birds were monitored at least twice daily, ensuring good health and that feed and water supplies were clean and adequate, and were wing-tagged at day 8 for individual identification. The house temperature was as detailed in the Ross broiler manual (Ross 308 Specifications, 2007), where ambient temperature (measured at chick height) was 30^{o}C, litter temperature 28-30^{o}C and humidity at 60-70%. At day 3, the temperature was decreased at a rate of 1^{o}C per day, so that on day 21, the temperature was 22^{o}C. For the first 7 days, the lighting regime was set to 23:1 light: dark hours, with 30-40 lux intensity. All procedures were approved by the SRUC Animal Experiment Committee prior to commencement.

2.6.2 Enzyme information

The enzyme used was Quantum Blue, provided by AB Vista, with an initial activity level of 5,000 FTU/kg. The premix was made to 150 FTU/kg and added at the rate of 10 g/kg or 20 g/kg to give activity levels of 1,500 or 3,000 FTU/kg, respectively.

2.6.3 Termination of the NC treatment

The negative control treatment had to be terminated on day 12 of the study due to the poor growth of the birds on this treatment. It was pre-determined prior to the start of the study, that as per the home office requirements, if the average bird weight reached < 65% of the breed-specific recommended weight for any particular time period, then this treatment should be terminated in order to maintain animal welfare standards. This treatment was ended before birds reached the < 65% level, as it was anticipated that they would continue to defer from guidelines and in consideration of the birds welfare this treatment was ended on day 12.

2.6.4 Sample size calculations

2.6.4.1 Dietary treatment sample size (excludes birds for carcass samples)

1. Equation on page 41 of 'Design and Analysis of Experiments', 5th edition, Douglas C. Montgomery, Arizona state university

$$d = |\mu_1 - \mu_2| / 2\sigma$$

$\alpha = 0.05$ $\beta = 0.05$

2. Using a study by Lu *et al*. (2009) which uses a positive and negative control with levels of available P in broiler diets similar to that of this trial, 'd' can be calculated (for weight gain, g/d).

μ_1 = (PC, 0.45% available P) = 31.1; μ_2 = (NC, 0.30% available P) = 28.9;

σ = SD= pooled SEM × $\sqrt{n}$; SEM = 0.35 n = 2000; σ = 15.65

$d = (31.1 - 28.9) / (2 \times 15.65)$ $d = 0.07$

3. Using the operating characteristic curve (page 41), where the probability of accepting $H_0 = 0.8$, n^* is estimated at 15.

4. Using the equation on page 42, sample size can be calculated

$$n = (n^* + 1) / 2$$

$$n = (15 + 1) / 2$$

$$n = 8$$

5. Therefore number of replicates to be used is 8

6. 6 dietary treatments, 6 birds per pen, 8 replicates = 288 birds (48 pens required)

2.6.4.2 Comparative slaughter sample size

1. As above

2. Using a study by Olukosi *et al*. (2008) which involves carcass sampling, 'd' can be calculated for whole body protein composition (%).

μ_1 = (PC) = 0.617; μ_2 = (NC) = 0.607; σ = SD = 0.459;

d = (0.617-0.607) / (2 × 0.459) d = 0.0109; d = 0.01

3. Using the operating characteristic curve (page 41), where the probability of accepting H_0 = 0.8, n^* is estimated at 100

4. Using the equation on page 42, sample size can be calculated

$$n = (n^* + 1) / 2$$

$$n = (100 + 1) / 2$$

$$n = 50.5$$

5. Therefore number of replicates to be used is 51

5. 6 dietary treatments, 48 pens. For ease of collection, one bird to be taken from each pen on day 21 for carcass sampling, therefore final slaughter group = 48 birds. To ensure the minimum number of birds were used, with 48 pens being suitable to achieve adequate statistical power for the other parts of the study, this number was deemed to be acceptable for the carcass sampling, being close to 51 (rather than requiring 51 pens and an additional 18 birds).

Chapter 3.

The effect of regular- and super- doses of phytase on growth performance, nutrient utilisation, phytate hydrolysis and gut parameters

3.1 Introduction

Chapter 2 reported the ability of super-doses of phytase supplementation to allow birds fed a diet deficient in aP to achieve a similar level of growth performance and nutrient utilisation to those fed nutritionally adequate. There were no benefits of increasing phytase supplementation from 1,500 to 3,000 FTU/kg on growth performance, nutrient utilisation or carcass characteristics reported in Chapter 2. Therefore, to maintain commercial applicability, 1,500 FTU/kg was the dose selected for the super-dose in following studies. The aim of the subsequent study reported here was to investigate the potential influence of super-doses of phytase on gut health.

Super doses of phytase may create gut conditions more favourable to nutrient absorption, gut function and proliferation of beneficial bacterial populations, either as a direct influence of phytase, or via a reduction in the concentrations of phytate, which irritates the gut (Onyango *et al.*, 2009). A healthy gut environment is associated with good overall animal health and performance, playing a major role in immune function and health status (Choct, 2009). Dietary factors influence not only the physical stimulation of the gut but also the establishment of microbial populations, and are considered one of the most influential factors affecting gut health (Choct, 2009; Ewing and Cole, 1994; Shirkey *et al.,* 2006). The presence of beneficial bacterial species can be associated with increased villi length along with deeper crypts, increased turnover of epithelial cells and an increased rate of cell migration, particularly in the upper regions of the small intestine, which may beneficially influence nutrient absorption and characteristics of overall performance (Ewing and Cole, 1994). High doses of phytase may create gut conditions more favourable to nutrient absorption and proliferation of beneficial bacterial populations, either as a direct influence of phytase, or via a reduction in phytate concentrations, which irritates the gut (Onyango *et al.,* 2009). A healthy gut environment is associated with good overall animal health and performance, with the gut playing a major role in immune function and health status (Choct, 2009).

Dietary factors, not only influence physical stimulation of the gut, but also the establishment of microbial populations and are considered one of the most influential factors affecting gut health (Choct, 2009; Ewing and Cole, 1994; Shirkey *et al.,* 2006). The presence of beneficial bacterial species can be associated with increased villi length along with deeper crypts, increased turnover of epithelial cells and an increased rate of cell migration, particularly in the upper regions of the small intestine, which may beneficially influence nutrient absorption and characteristics of overall performance (Ewing and Cole, 1994).

The pH of the intestinal contents may also be influenced by the microbial species inhabiting the intestinal lumen and their fermentation products (Akyurek *et al.*, 2011; Dono *et al.*, 2014; Leenhardt *et al.*, 2005). The presence of beneficial bacteria, such as those which secrete antibiotics and organic acids, are able to lower digesta pH which may improve the microbial balance and enhance animal health and performance, creating conditions which are unsuitable for the survival of pathogenic micro-organisms (Steiner, 2006). Stable microbial populations within the gut are important for health, with the dominant population being maintained via competitive exclusion (Choct, 2009). The commensal bacterial populations have a significant influence on the potential colonisation of pathogenic populations (Tannock and Savage, 1974), through competitive exclusion, interaction with mucin secretion and the maintenance of tight junctions (Srikanth and McCormick, 2008). When phytase is provided in super doses, we expect a reduction in intestinal pH (Leenhardt *et al.*, 2005), making conditions favourable for establishment of beneficial bacteria. Decreasing pH may improve nutrient absorption by increasing nutrient solubility (Ravindran *et al.*, 1999, 2001), thereby increasing the nutrients available for utilisation by the animal and reducing substrates for bacterial fermentation. In addition to enhancing the capacity for nutrient absorption, super-doses of phytase may beneficially influence gut morphology, increasing villus height and thus surface area for absorption.

The objectives of the study were to document the effect of regular- and super-dosage of phytase on gut health parameters, including pH, morphology and

microbial profile. It is expected that super-doses of phytase will have an overall beneficial influence on broiler gut health.

3.2 Materials and Methods

3.2.1 Animals, Diets and Housing

A total of 384, one-day-old Ross 308 broilers were used for the study and housed in metabolism cages with water and experimental diets provided on an *ad libitum* basis, for 21 days. There were 48 cages each containing 8 birds. Birds and feed were weighed on days 0 and 21 for determination of growth performance.

Birds were monitored at least twice daily, ensuring good health and that feed and water supplies were clean and adequate, and were wing-tagged at day 8 for individual identification. The house temperature was as detailed in the Ross broiler manual. All experimental procedures were approved by the SRUC Animal Experiment Committee in accordance with the Animals (Scientific Procedures) Act 1986 (Appendix 3.6.1).

On arrival birds were randomly allocated to 6 treatments with 8 replicates each in a Randomised Complete Block Design and a 2 x 3 factorial arrangement. The factors included two levels of dietary available P (aP; 0.50% and 0.35%) and three levels of phytase supplementation (0, 500 and 1,500 FTU/kg; information provided in the Appendix 3.6.2). The PC diet was formulated to meet Ross 308 energy and nutrient requirements (Ross 308 Specifications, 2007). The NC diet was formulated to be deficient from the PC by 0.15% aP and 0.16% Ca, in accordance to the enzymes nutritional matrix specifications, applied relative to the PC diet. Therefore, the dietary treatments were: (1) Positive control, PC; (2) PC + 500 FTU/kg phytase; (3) PC + 1,500 FTU/kg phytase; (4) Negative control, NC; (5) NC + 500 FTU/kg phytase; (6) NC + 1,500 FTU/kg phytase. Titanium dioxide was used an indigestible marker for digestibility calculations. Table 3.1 shows the nutritional composition of the dietary treatments.

Table 3.1 – Ingredient composition of the experimental diets

Description of diets	PC	PC 500	PC 1,500	NC	NC 500	NC 1,500
Phytase (FTU/kg)	0	500	1,500	0	500	1,500
Ingredients, g/kg						
Maize	346.5	346.5	346.5	382.9	382.9	382.9
Wheat	200	200	200	200	200	200
Soybean meal	302	302	302	295	295	295
Soybean oil	50	50	50	28	28	28
DCP[1]	21.0	21.0	21.0	12.6	12.6	12.6
Limestone[2]	9.7	9.7	9.7	11.3	11.3	11.3
Titanium-dioxide premix[3]	25.0	25.0	25.0	25.0	25.0	25.0
MGM[4] for enzyme	30.0	20.0	0.0	30.0	20.0	0.0
Enzyme premix[5]	0.0	10.0	30.0	0.0	10.0	30.0
Vitamin-mineral premix[6]	5.0	5.0	5.0	5.0	5.0	5.0
Methionine	1.9	1.9	1.9	1.9	1.9	1.9
Lysine	3.6	3.6	3.6	3.8	3.8	3.8
Threonine	0.7	0.7	0.7	0.4	0.4	0.4
Salt NaCl	3.1	3.1	3.1	3.1	3.1	3.1
$NaHCO_3$	1.5	1.5	1.5	1.0	1.0	1.0
Total	1000	1000	1000	1000	1000	1000

Note: PC: Positive Control; NC: Negative control; [1]Di-calcium phosphate, 25.7% Ca, 17.5% P; [2]Limestone, 38.9% Ca; [3]3.5kg TiO_2 mixed with 13.8kg maize gluten meal; [4]Maize gluten meal forms the basis of the enzyme premix and is used as a filler in the place of phytase; [5]Phytase premix is mixed with maize and has an activity of 150 FTU/g; [6]Premix supplies the following per kg diet: Vit. A, 5484 IU; Vit. D3, 2643 ICU; Vit E,11 IU; Menadione sodium bisulfite,4.38 mg; Riboflavin, 5.49 mg; d-pantothenic acid, 11 mg; Niacin, 44.1 mg; Choline chloride, 771 mg; Vit B12, 13.2 ug; Biotin, 55.2 ug; Thiamine mononitrate, 2.2 mg; Folic acid, 990 ug; Pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug. Also contains per g of premix: Vit. A, 1828 IU; Vit. D3, 881 ICU; Vit E,3.67 IU; Menadione sodium bisulfite,1.46 mg; Riboflavin, 1.83 mg; d-pantothenic acid, 3.67 mg; Niacin, 14.69 mg; Choline chloride, 257 mg; Vit B12, 4.4 ug; Biotin, 18.4 ug; Thiamine mononitrate, 735 ug; Folic acid, 330 ug; Pyridoxine hydrochloride, 1.1 mg; I, 370 ug; Mn, 22.02 mg; Cu, 1.48 mg; Fe, 14.69 mg; Zn, 14.69 mg; Se, 100 ug.

3.2.2 Sample collection

Birds and feed were weighed on days 0 and 21 for determination of growth performance. On day 21, six birds per pen were euthanased by cervical dislocation and used for collection of ileal digesta.

Excreta were collected from each cage on days 19 and 20 to determine total tract nutrient retention. Ileal digesta were collected for determination of ileal nutrient digestibility.

Ileal digesta and gizzard contents were analysed for phytate P, inositol esters of phytate and phytase activity.

On day 22, the remaining two birds per pen were euthanised by injection of pentobarbital and used for the gut pH and morphology and microbial profiling study.

Immediately following euthanasia, two replicate pH readings were taken from the middle section of the duodenum, jejunum, ileum and caeca using a probe pH meter. The averages of the readings were taken for analysis. The birds selected for pH readings were then used for the gut morphology and microbial profiling studies. Using one of the two birds selected for pH measurements, approximately 3cm sections of duodenum, jejunum and ileum were cut and flushed with a saline phosphate buffer of pH 7.4 to allow for histological examination. The other bird was used for the purposes of sequencing the gut microbial profile. The ileum and caeca were isolated, the digesta removed and microbial populations determined using 16S rDNA-based techniques through MiSeq Illumina Next Generation Sequencing. This analysis was carried out at the Polyomics centre at the University of Glasgow.

3.2.3 Processing of samples

3.2.3.1 Titanium digestion

Determination of Ti concentration in diet, excreta and digesta samples were performed as described by Short *et al*. (1996).

3.2.3.2 Next generation sequencing

For the sequencing of the microbial populations, digesta from the ileum and caeca were collected into epindorf tubes and were snap-frozen in liquid nitrogen. Samples were stored at -80^{o}C until processing.

DNA extraction was carried out using the Qiagen stool DNA extraction technique (QIAamp DNA stool handbook, Sample and assay technologies, 2nd Edition, April 2010, Qiagen). Briefly, this involved the lysis and protein digestion of the sample using proteinase K, followed by a series of incubations and wash steps using ethanol and was finally eluted. 16S library preparation and sequencing were performed by Glasgow Polyomics, using an adapted protocol based on the protocol provided by Illumina (San Diego, CA, USA). In brief, purified DNA samples, containing bacterial DNA of interest, were used as template to produce an amplicon library of the V3/V4 regions of the bacterial 16S ribosomal subunit. This amplicon library was then further amplified with Illumina Nextera XT adapters and indexes (San Diego, CA, USA) to allow sample clustering and multiplexing. All samples were run simultaneously on an Illumina MiSeq (San Diego, CA, USA), in the presence of 10% PhiX, utilising a 600 cycle (2x 300 cycle) kit. Bioinformatic analysis was carried out by Graham Hamilton (Glasgow polyomics).

Due to pipetting error during the DNA extraction, the concentration of DNA was too dilute for the ileal digesta samples (Appendix 4.6.1). In order to facilitate the use of these samples, DNA concentration was performed using the ethanol precipitation method. This involved the addition of sodium acetate (3 molar, pH 5.2) and ethanol (100%), and incubation at -20^{o}C for a minimum of 24 hours. The samples were then centrifuged and the fluid removed, before washing in 70% ethanol and re-suspension in water.

3.2.3.3 Tissue preparation for histomorphology

Tissue sections were fixed with a 10% buffer formalin solution before being processed by the veterinary pathology laboratory at the University of Glasgow, where they were first dehydrated by transfer through a series of ethyl alcohols, of 70, 80 and 100%. Subsequently the samples were cleared using xylene before being embedded in wax and 2 µm transverse sections being cut and placed on glass slides. Samples were then stained with haematoxylin and eosin. Using Cell^D software and an Olympus BX51 DP71 camera attachment, photographs of each of the three sections for each sample were taken. Subsequently, the transverse section images were analysed using Image J software (Image J 1.49 c, Java 1.6.0_05, 32 bit). There were three sections of tissue from each sample on each slide, analysed as A, B and C, which were then pooled to give the mean data value for each sample. Where possible, three villi and crypt readings (villi height, apical width and basal width) were taken from each section at x4 magnification and the average taken to give the overall section value. At x1.25 magnification, total counts of villi and crypts were taken, as well as measurement of the mucosal length. Counts of the left hand side (LHS) longitudinal edge (except where damage to the LHS section made analysis of the RHS section more appropriate) were taken and multiplied by 2 to estimate the total villi number; only villi attached at the base to the submucosa were included in the total count. The apparent villus surface area were calculated using the following equation (Iji *et al.*, 2001):

$$\text{Villi surface area} = ((\text{Basal width} + \text{Apical width}))/(2\times\text{Villus height})$$

See the appendix (4.6.2) for illustrated diagram of measurements mentioned here. Using the image J software, measurements were calibrated to the superimposed scale on the image, produced by the Cell^D software and calibrated to the magnification used.

3.2.4 Calculations and Statistical analyses

Feed conversion ratio was calculated from the body weight gain and feed intake data. Feed intake was corrected for mortality through calculation of bird-days.

3.2.4.1 Digestibility

Nutrient digestibility was calculated using the following equations:

$$\text{Dry matter digestibility (\%)} = 1 - \left(\frac{Ti\ in\ diet}{Ti\ in\ excreta}\right)$$

$$\text{Nutrient digestibility (\%)} = \left[1 - \left(\frac{Ti\ in\ diet}{Ti\ in\ sample}\right) \times \left(\frac{Nutrient\ in\ sample}{Nutrient\ in\ diet}\right)\right] \times 100$$

Mineral flow:

$$Mineral\ concentration\ per\ g\ DM \times \frac{Ti\ in\ diet}{Ti\ in\ ileum}$$

Means (for each pen) were pooled for each of the diets to allow comparison of the treatment effects. Statistical analyses of the data were done using the General ANOVA function of Genstat (14th Edition, VSN International Ltd.). Data was checked for normality and suitably transformed when required and analysed as a 2 × 3 factorial where the model included aP level, phytase and the interaction between the two. When differences were significant, means were separated using Tukey's test. Significance between treatments was determined using orthogonal polynomial contrasts. Where correlations are presented (identified in Genstat), all r^2 are significant to $P < 0.0001$ unless otherwise stated.

3.3 Results

3.3.1 Chemical analyses of the diets

The analysed chemical compositions of the diets are presented in Table 3.2. Analysed Ca of the diets was higher than formulated, and consequently this raised the Ca:P ratio above anticipated (formulated 1.4:1 in PC and 1.5:1 in NC, analysed as 1.5 – 1.7: 1 in PC and 1.8:1 in NC). The phytase activity of the diets were similar to the expected levels, being <50, 715 to 744 and 1,880 to 1,960 when formulated as 0, 500 and 1,500, respectively.

3.3.2 Growth performance

Day 21 growth performance data is presented in Table 3.3. There were no treatment effects of IBW therefore this was not required to be added as a co-factor in the statistical model. There was significant aP x phytase interaction ($P < 0.01$) on BWG, with phytase leading to a quadratric increase in the NC ($P < 0.001$) but a linear increase in the PC ($P < 0.05$). Feed conversion ratio improved ($P < 0.001$) with the addition of phytase (quadratic, $P < 0.01$) but was comparable between the 500 and 1,500 FTU/kg treatments. There were no treatment effects on FI or mortality.

3.3.3 Nutrient utilisation

3.3.3.1 Ileal nutrient utilisation

There was significant aP x phytase interaction on ileal DM, N and P ($P < 0.05$) digestibility (Table 3.4). The digestibility of DM, N and P were increased with the addition of phytase in the NC diets (quadratic, $P < 0.01$), but not the PC. The digestibility of Ca increased as the dietary aP and Ca content were reduced ($P < 0.001$) but decreased with the addition of phytase (linear, $P < 0.001$).

There were no aP × phytase interactive effects on ileal mineral flow (Table 3.5). There was an increase ($P < 0.05$) in Cu flow as phytase dose increased (linear, $P < 0.05$), and a tendency for an increase in Na ($P = 0.058$) and Fe ($P = 0.051$; linear, $P < 0.05$) flow.

Table 3.2 – Calculated and analysed nutrient and energy content of the six formulated dietary treatments

Description of diets	PC	PC500	PC1,500	NC	NC500	NC1,500
Phytase, FTU/kg	0	500	1,500	0	500	1,500
Calculated nutrients and energy:						
Protein, g/kg	230.5	230.5	230.5	230.3	230.3	230.3
ME, MJ/kg	12.6	12.6	12.6	12.3	12.3	12.3
Lysine, g/kg	14.0	14.0	14.0	14.1	14.1	14.1
Ca, g/kg	10.0	10.0	10.0	8.4	8.4	8.4
P, g/kg	6.9	6.9	6.9	5.5	5.5	5.5
Available P, g/kg	5.0	5.0	5.0	3.5	3.5	3.5
Ca:P	1.4	1.4	1.4	1.5	1.5	1.5
Na	1.8	1.8	1.8	1.7	1.7	1.7
K	7.9	7.9	7.9	7.9	7.9	7.9
Cl	2.2	2.2	2.2	2.2	2.2	2.2
Mg	1.5	1.5	1.5	1.5	1.5	1.5
Total amino acids, g/kg						
Lys	14.0	14.0	14.0	14.1	14.1	14.1
Met	5.0	5.0	5.0	5.0	5.0	5.0
Cys	4.0	4.0	4.0	4.0	4.0	4.0
Phe+Tyr	17.5	17.5	17.5	17.5	17.5	17.5
Analysed nutrient content:						
Ca (g/kg)	12.2	11.1	10.7	10.8	10.4	10.2
P (g/kg)	7.5	6.7	7.1	6.1	5.8	5.7
aP (g/kg)[1]	5.8	4.3	4.9	4.4	3.8	3.9
Ca:P	1.6	1.6	1.5	1.8	1.8	1.8
Na	1.8	1.7	1.5	1.7	1.6	1.6
K	9.0	8.5	9.7	9.0	8.9	8.5
Mg	1.4	1.3	1.5	1.4	1.4	1.3
Phytate-P (g/kg)	1.7	2.4	2.2	1.7	2.0	1.8
Phytase (FTU/kg)	<50	715	1880	<50	744	1960

Note: PC: Positive Control; NC: Negative control; Dietary phytase quantification determined using ELISA method by AB Vista; Enzyme premix quantified at 56 FTU/kg using Quantum method by AB Vista; [1]aP calculated as total P – phytate P.

Table 3.3 - Mortality, initial (IBW, d0) and final (FBW, d21) body weights, feed intake (FI) and feed conversion ration (FCR) of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Diet		IBW, g	BWG, g	FI, g	FCR	Mortality [2] %
NC		41.1	729	1184	1.624	3.1
NC + 500 FTU/kg		40.9	852	1215	1.428	1.6
NC + 1,500 FTU/kg		41.2	849	1192	1.407	1.6
PC		41.8	840	1242	1.487	4.7
PC + 500 FTU/kg		41.4	874	1201	1.377	1.6
PC + 1,500 FTU/kg		41.3	882	1256	1.426	3.1
	SEM	0.350	14.159	31.87	0.040	2.10
aP, %	0.35	41.1	810	1197	1.486	2.1
	0.50	41.5	865	1233	1.430	3.1
	SEM	0.202	8.175	18.40	0.023	1.21
Phytase[1]	0	41.4	784	1213	1.555	3.9
	500	41.1	863	1208	1.402	1.6
	1,500	41.3	865	1224	1.417	2.3
	SEM	0.247	10.012	22.54	0.028	1.49
P values						
aP, %		0.153	<0.001	0.174	*0.095*	0.702
Phytase		0.749	<0.001	0.879	<0.001	0.673
	Linear				0.006	
	Quadratic				0.005	
aP × phytase		0.725	0.007	0.391	0.167	0.899
Contrasts						
	PC linear		0.043			
	PC quadratic		0.459			
	NC linear		<0.001			
	NC quadratic		<0.001			

Note: [1]FTU/kg phytase; [2]Statistical analyses of mortality % was performed following the application of 1/(sqrtX+0.1) to allow for analysis of zero mortality; 8 replications per diet.

Table 3.4 - Ileal nutrient digestibility of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

		Ileal digestibility coefficients			
Diet		DM	N	P	Ca
NC		0.54	0.51	0.42	0.47
NC + 500 FTU/kg		0.63	0.62	0.52	0.46
NC + 1,500 FTU/kg		0.59	0.59	0.41	0.30
PC		0.58	0.59	0.43	0.40
PC + 500 FTU/kg		0.57	0.57	0.37	0.29
PC + 1,500 FTU/kg		0.57	0.56	0.42	0.24
	SEM	0.012	0.018	0.032	0.033
aP, %	0.35	0.58	0.57	0.45	0.41
	0.50	0.57	0.57	0.41	0.31
	SEM	0.007	0.01	0.019	0.019
Phytase[1]	0	0.56	0.55	0.42	0.43
	500	0.60	0.60	0.45	0.38
	1,500	0.58	0.57	0.41	0.27
	SEM	0.009	0.013	0.023	0.033
P values					
aP, %		0.328	0.956	0.107	<0.001
Phytase		0.016	0.058	0.560	<0.001
	Linear				<0.001
	Quadratic				0.966
aP x phytase		<0.001	0.003	0.022	0.131
Contrasts					
	PC linear	0.521	0.299	0.774	
	PC quadratic	0.578	0.787	0.190	
	NC linear	0.004	0.007	0.878	
	NC quadratic	<0.001	0.002	0.008	

Notes: [1]FTU/kg phytase; 8 replications per diet; DM: Dry matter; N: Nitrogen; P: Phosphorus; Ca: Calcium.

Table 3.5 - Ileal mineral flow of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

		Ileal mineral flow (mg/100g DM intake)						
		Zn	Mg (g)	Mn	K (g)	Cu	Na (g)	Fe
NC		261.0	1.89	320.5	5.73	35.7	7.76	180.1
NC + 500 FTU/kg		281.4	2.03	342.1	5.48	41.9	7.50	178.0
NC + 1,500 FTU/kg		294.0	2.26	313.6	6.54	46.1	8.41	202.8
PC		262.7	2.05	317.2	5.24	40.0	7.82	173.8
PC + 500 FTU/kg		270.2	2.07	345.0	5.53	42.0	7.19	197.5
PC + 1,500 FTU/kg		253.3	2.05	365.2	5.48	42.9	8.48	203.1
	SEM	18.36	0.093	19.83	0.302	2.47	0.44	10.22
aP, %	0.35	278.8	2.06	342.5	5.92	41.2	7.89	187.0
	0.50	262.1	2.06	325.4	5.41	41.7	7.83	191.5
	SEM	10.60	0.054	11.45	0.174	1.42	0.255	5.90
Phytase[1]	0	261.9	1.97	318.9	5.49	37.8	7.79	177.0
	500	275.8	2.05	343.6	5.50	42.0	7.35	187.7
	1,500	273.7	2.15	339.4	6.01	44.5	8.44	202.9
	SEM	12.98	0.066	144.0	0.214	1.74	0.313	7.23
P values								
aP, %		0.273	0.985	0.299	0.050	0.830	0.866	0.596
Phytase		0.717	0.159	0.421	0.161	0.034	0.058	0.051
	Linear					0.014	0.077	0.016
	Quadratic					0.382	0.099	0.817
aP x phytase		0.504	0.148	0.329	0.200	0.328	0.890	0.431

Notes: [1]FTU/kg phytase; 8 replications per diet; Zn: Zinc; Mg: Magnesium; Mn: Manganese; K: Potassium; Cu: Copper; Na: Sodium; Fe: Iron.

The flow of K through the ileum was greater in the NC than PC diets ($P \leq 0.05$).

3.3.3.2 Total tract nutrient retention

The total tract nutrient retention (TTR) data are presented in Table 3.6. There was significant aP x phytase interaction on the TTR of DM, N and P ($P < 0.05$). The TTR of DM and N was significantly reduced in the NC, compared to the PC ($P < 0.01$, $P < 0.001$, respectively). There were no improvements in retention in the PC treatments, however DM and N retention increased (linear, $P < 0.001$) and had the tendency to increase (quadratic, $P = 0.059$) when phytase was added to the NC treatments. The TTR of Ca was greater in the NC than PC diets ($P < 0.01$).

There was no significant aP × phytase interaction on mineral total tract flow, Table 3.7, except for that of Na ($P < 0.05$). Na flow was significantly lower in the NC than the PC treatment ($P < 0.001$), and increased with increasing phytase dose in the NC (linear, $P < 0.001$), but not in the PC. There was a tendency ($P = 0.053$) for an increase in K (linear, $P < 0.05$) flow, but there were no treatment effects observed on the flow of the other minerals investigated.

3.3.4 Phytate hydrolysis

The treatment effects on the IP content of the gizzard are shown in Table 3.8. IP3 and IP2 were not detected in the gizzard. There was an aP × phytase interaction on the gizzard content of IP4 ($P < 0.01$). The addition of phytase to the PC had no effect on IP4 content, however, there was a quadratic increase in IP4 content when phytase was added to the NC ($P < 0.001$). There was a quadratic decrease ($P < 0.001$) in IP6 and IP5 content as phytase dose increased, whereas there was a quadratic increase ($P < 0.05$) in inositol content with phytase supplementation. The inositol content of the gizzard was greater in the NC than PC diets ($P < 0.05$).

Table 3.6 - Total tract nutrient retention of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

		Total tract retention coefficients			
Diet		DM	N	P	Ca
NC		0.70	0.63	0.62	0.51
NC + 500 FTU/kg		0.73	0.68	0.70	0.57
NC + 1,500 FTU/kg		0.74	0.70	0.68	0.54
PC		0.73	0.70	0.58	0.51
PC + 500 FTU/kg		0.72	0.68	0.53	0.46
PC + 1,500 FTU/kg		0.72	0.67	0.57	0.45
	SEM	0.007	0.011	0.023	0.029
aP, %	0.35	0.72	0.67	0.67	0.54
	0.50	0.72	0.68	0.56	0.47
	SEM	0.004	0.006	0.013	0.017
Phytase[1]	0	0.72	0.66	0.60	0.51
	500	0.73	0.68	0.62	0.51
	1,500	0.73	0.69	0.62	0.49
	SEM	0.005	0.008	0.016	0.020
P values					
aP, %		0.554	0.092	<0.001	0.008
Phytase		0.181	0.114	0.558	0.704
aP x phytase		0.002	<0.001	0.025	0.125
Contrasts					
	PC linear	0.260	0.112	0.693	
	PC quadratic	0.423	0.527	0.160	
	NC linear	<0.001	<0.001	0.073	
	NC quadratic	0.101	0.222	0.059	

Notes: [1]FTU/kg phytase; 8 replications per diet; DM: Dry matter; N: Nitrogen; P: Phosphorus; Ca: Calcium.

Table 3.7 -Total tract mineral flow of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days

		Total tract (mg/100g DM intake)						
		Zn	Mg (g)	Mn	K (g)	Cu	Na (g)	Fe
NC		714.5	4.93	767.0	27.86	73.7	2.03	348.3
NC + 500 FTU/kg		712.3	4.87	750.8	27.51	75.5	3.10	364.7
NC + 1,500 FTU/kg		742.7	5.45	849.7	31.64	83.2	3.42	399.5
PC		695.4	4.79	789.7	27.94	75.9	3.24	368.2
PC + 500 FTU/kg		691.8	4.87	790.4	27.48	78.8	3.51	377.3
PC + 1,500 FTU/kg		666.3	4.87	814.4	28.40	77.9	3.58	368.8
	SEM	31.83	0.201	46.93	1.074	3.37	0.178	16.57
aP, %	0.35	723.2	5.08	789.2	29.00	77.5	2.85	370.8
	0.50	684.5	4.85	798.2	27.94	77.6	3.44	371.5
	SEM	18.38	0.116	27.09	0.620	1.95	0.103	9.57
Phytase[1]	0	704.9	4.86	778.3	27.90	74.8	2.64	358.3
	500	702.1	4.87	770.6	27.49	77.2	3.30	371.0
	1,500	704.5	5.16	832.1	30.02	80.6	3.50	384.2
	SEM	22.50	0.142	33.18	0.760	2.38	0.126	11.72
P values								
aP, %		0.146	0.154	0.816	0.234	0.983	<0.001	0.964
Phytase		0.995	0.254	0.372	0.053	0.241	<0.001	0.307
	Linear				0.032			
	Quadratic				0.247			
aP x phytase		0.594	0.343	0.707	0.229	0.391	0.016	0.269
Contrasts								
	PC linear						0.188	
	NC linear						<0.001	
	NC quadratic						0.096	

Notes: [1]FTU/kg phytase; 8 replications per diet; Zn: Zinc; Mg: Magnesium; Mn: Manganese; K: Potassium; Cu: Copper; Na: Sodium; Fe: Iron.

Table 3.8 - Inositol phosphate ester content of the gizzard of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase.

Gizzard		mg/g DM[2]			
		IP6[1]	IP5[1]	IP4	Inositol
NC		3.58	0.50	0.55	0.09
NC + 500 FTU/kg		0.59	0.11	0.94	0.12
NC + 1,500 FTU/kg		0.52	0.07	0.67	0.14
PC		3.15	0.47	0.72	0.08
PC + 500 FTU/kg		0.65	0.12	0.83	0.11
PC + 1,500 FTU/kg		0.42	0.04	0.78	0.11
	SEM	0.11	0.02	0.04	0.008
aP, %	0.35	1.56	0.22	0.72	0.12
	0.50	1.41	0.21	0.78	0.10
	SEM	0.06	0.01	0.03	0.005
Phytase[3]	0	3.37	0.48	0.63	0.09
	500	0.62	0.11	0.89	0.12
	1,500	0.47	0.05	0.73	0.13
	SEM	0.08	0.01	0.03	0.006
P values					
aP, %		0.474	0.243	0.107	0.018
Phytase		<0.001	<0.001	<0.001	<0.001
	Linear	<0.001	<0.001	0.272	<0.001
	Quadratic	<0.001	<0.001	<0.001	0.047
aP x phytase		0.606	0.129	0.005	0.315
Contrasts					
	PC linear	<0.001	<0.001	0.328	0.016
	PC quadratic	0.002	0.254	0.148	0.117
	NC linear	<0.001	<0.001	0.041	<0.001
	NC quadratic	<0.001	0.002	<0.001	0.939

Notes: [1]Log transformation of IP6 and IP5 prior to analysis; [2]IP2 and IP3 not detected; [3]FTU/kg; 8 replications per diet; IP6-4: Inositol phosphate ester with 6- 4 bound phosphate molecules.

In the ileum (Table 3.9), there was no significant aP × phytase interaction on the IP content, however both the aP and phytase levels had a significant main effect. There was a decrease in IP6 and IP5 (quadratic, $P < 0.01$) and increase in inositol (quadratic, $P < 0.05$) content in the ileum as phytase dose increased. IP6 ($P < 0.01$), IP5 and IP4 ($P < 0.05$) were lower, and inositol ($P < 0.001$) higher, in the NC than the PC.

There was significant aP × phytase interaction on excreta IP content (Table 3.10), except for IP6. The excreta IP5 content was significantly reduced in the PC diet with 1,500 FTU/kg phytase (quadratic, $P < 0.001$) and was comparable to the NC diet with 1,500 FTU/kg. In the NC diets, 500 FTU/kg had the tendency to increase excreta IP5, but decrease with 1,500 FTU/kg, relative to the NC without phytase, but these differences were not significantly different. The excreta IP4, IP3 and inositol content increased ($P < 0.05$) with the addition of phytase in both the PC and NC diets. The content of IP5 decreased in both the PC and NC in a quadratic manner as phytase dose increased ($P < 0.001$), whilst IP4 increased with the addition of phytase in the PC (linear, $P < 0.001$) and NC (quadratic, $P < 0.05$), as did IP3 in both the PC (quadratic, $P < 0.001$) and NC (linear, $P < 0.001$). The inositol content was higher in the NC than PC, and did not increase significantly in the NC with phytase, however, in the PC the increase was linear ($P < 0.001$). The IP6 content was influenced by phytase ($P < 0.01$), decreasing as the level of supplementation increased (quadratic, $P < 0.05$).

3.3.5 Gut characteristics

3.3.5.1 Gut pH

In the jejunum, there was a significant aP and phytase interaction ($P < 0.01$), as shown in Table 3.11, whereas the addition of phytase increased the jejunal pH in both the PC and NC treatments. However, the response to phytase dose was quadratic ($P < 0.001$) in birds fed the PC and linear ($P < 0.01$) in birds fed the NC. There was a tendency for a decrease in duodenal pH as the dietary aP decreased ($P = 0.054$), and a tendency for an increase in caecal pH as phytase dose increased ($P = 0.059$). There were no treatment influences on ileal pH.

Table 3.9 - Inositol phosphate ester content of the ileum of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Ileum		mg/g DM				
		IP6[1]	IP5[1]	IP4	IP3	Inositol
NC		8.4	1.89	1.61	0.62	1.72
NC + 500 FTU/kg		3.6	0.55	1.04	0.67	2.26
NC + 1,500 FTU/kg		2.4	0.41	1.31	0.49	2.86
PC		14.9	2.29	1.69	0.44	1.23
PC + 500 FTU/kg		5.9	0.98	1.72	0.58	1.90
PC + 1,500 FTU/kg		2.5	0.45	1.48	0.63	2.07
	SEM	0.930	0.16	0.178	0.121	0.135
aP, %	0.35	4.79	0.95	1.32	0.59	2.28
	0.50	7.79	1.24	1.63	0.55	1.73
	SEM	0.537	0.094	0.126	0.070	0.078
Phytase[2]	0	11.67	2.09	1.65	0.53	1.47
	500	4.77	0.77	1.38	0.62	2.08
	1,500	2.44	0.43	1.39	0.56	2.47
	SEM	0.658	0.12	0.126	0.086	0.095
P values						
aP, %		0.008	0.035	0.041	0.670	<0.001
Phytase		<0.001	<0.001	0.257	0.734	<0.001
	Linear	<0.001	<0.001			<0.001
	Quadratic	0.004	<0.001			0.027
aP x phytase		0.105	0.106	0.199	0.410	0.276
Contrasts						
	PC linear	<0.001	<0.001	0.413	0.283	<0.001
	PC quadratic	0.724	0.777	0.518	0.750	0.149
	NC linear	<0.001	<0.001	0.246	0.444	<0.001
	NC quadratic	0.111	0.002	0.062	0.458	0.870

Notes: [1]Log transformation of IP5 and IP6 prior to analysis; [2]FTU/kg; [3]No IP2 detected; 8 replications per diet; IP6-3: Inositol phosphate ester with 6- 3 bound phosphate molecules.

Table 3.10 - Inositol phosphate ester content of the excreta of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Total tract		mg/g DM				
		IP6[1]	IP5	IP4[1]	IP3	Inositol
NC		35.6	3.60	1.83	0.17	1.21
NC + 500 FTU/kg		20.4	4.14	4.18	0.22	1.41
NC + 1,500 FTU/kg		12.9	3.14	7.05	0.24	1.46
PC		39.5	4.70	3.04	0.24	0.37
PC + 500 FTU/kg		20.7	4.76	4.93	0.18	0.89
PC + 1,500 FTU/kg		8.8	2.73	7.30	0.26	1.32
	SEM	1.687	0.173	0.389	0.010	0.130
aP, %	0.35	22.92	3.63	4.35	0.21	1.36
	0.50	23.01	4.07	5.09	0.23	0.86
	SEM	0.974	0.100	0.225	0.006	0.075
Phytase[2]	0	37.55	4.15	2.43	0.21	0.79
	500	20.55	4.45	4.55	0.20	1.15
	1,500	10.81	2.94	7.17	0.25	1.39
	SEM	1.193	0.12	0.275	0.007	0.092
P values						
aP, %		0.715	0.004	<0.001	0.033	<0.001
Phytase		<0.001	<0.001	<0.001	<0.001	<0.001
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.045	<0.001	<0.001	0.018	0.165
aP x phytase		0.162	<0.001	0.018	<0.001	0.034
Contrasts						
	PC linear	<0.001	<0.001	<0.001	0.415	<0.001
	PC quadratic	0.296	<0.001	0.691	<0.001	0.752
	NC linear	<0.001	0.069	<0.001	<0.001	0.192
	NC quadratic	0.682	<0.001	0.046	0.431	0.642

Notes: [1]Log transformation of IP4 and IP6 prior to analysis; [2]FTU/kg; 8 replications per diet; IP6-3: Inositol phosphate ester with 6- 3 bound phosphate molecules.

Table 3.11 - Digesta pH of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Diet		Duodenum	Jejunum	Ileum	Caecum
NC		6.43	6.25	6.55	6.63
NC + 500 FTU/kg		6.40	6.33	6.78	6.82
NC + 1,500 FTU/kg		6.49	6.40	6.99	6.81
PC		6.37	6.26	6.80	6.61
PC + 500 FTU/kg		6.41	6.38	7.00	6.84
PC + 1,500 FTU/kg		6.37	6.22	6.89	6.80
	SEM	0.037	0.032	0.142	0.092
aP, %	0.35	6.38	6.29	6.90	6.75
	0.50	6.44	6.33	6.77	6.75
	SEM	0.021	0.019	0.082	0.053
Phytase	0	6.40	6.26	6.67	6.62
	500	6.41	6.35	6.89	6.83
	1,500	6.43	6.31	6.94	6.80
	SEM	0.026	0.023	0.101	0.065
P values					
aP, %		0.054	0.138	0.307	0.998
Phytase		0.736	0.018	0.150	0.059
aP x phytase		0.201	0.002	0.419	0.968
Contrasts					
	PC linear		0.402		
	PC quadratic		0.001		
	NC linear		0.003		
	NC quadratic		0.954		

Note: [1]FTU/kg phytase; 8 replications per diet.

3.3.5.2 Gut morphology

In the duodenum, there was significant aP x phytase interaction on the apparent villi surface area (SA) in the duodenum ($P < 0.05$), Table 3.12. Following the addition of phytase, there was a quadratic increase in SA in the NC ($P < 0.05$), but no change in the PC. There was also a tendency for aP × phytase interaction on the basal width (BW; $P = 0.051$), whereas the BW increased in the NC diets (quadratic, $P < 0.01$), but there was no effect of phytase on BW in birds fed the PC diets. There were no other treatment effects on duodenal morphology (Table 3.13).

There was significant aP × phytase interaction on jejunal apical width (AW; $P < 0.05$; Table 3.14), with a linear increase in AW as phytase increased in the NC diet ($P < 0.01$) but no effect of phytase on apical width in birds fed the PC diet. There was a tendency for an aP × phytase interaction on the crypt depth (CD; $P = 0.09$; Table 3.15), where depth tended to increase with phytase supplementation in the NC (quadratic, $P = 0.094$) but not in the PC.

Tables 3.16 and 3.17 show the ileal histomorphology characteristics. Interaction between aP and phytase was significant ($P < 0.05$) for villi number and CD. There was a quadratic increase in villi number as phytase dose increased in the PC ($P < 0.05$), but there was no phytase effect in the NC treatments. Similarly, there was a linear decrease in CD as phytase dose increased in the PC ($P < 0.05$), with no effects observed in the NC.

The ileal BW also had the tendency to be influenced by aP x phytase interaction ($P = 0.063$), with a quadratic decrease in basal width as phytase supplementation increased in the NC ($P < 0.01$) but not the PC. There was a reduction in the crypt number in birds fed the NC diets compared with birds fed the PC diets ($P < 0.05$).

Table 3.12 – Duodenal villi histomorphology characteristics of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Duodenum: Diet		Villi n[1]	VH, µm	AW, µm	BW, µm	SA, mm [2,3]
NC		52	1697	146	216	310
NC + 500 FTU/kg		49	1741	144	254	350
NC + 1,500 FTU/kg		52	1610	126	182	250
PC		48	1548	144	209	270
PC + 500 FTU/kg		49	1621	139	232	300
PC + 1,500 FTU/kg		50	1734	135	229	320
	SEM	1.9	80.1	10.8	14.2	24.0
aP, %	0.35	51	1683	139	218	300
	0.50	49	1634	139	223	300
	SEM	1.1	46.3	6.2	8.2	13.8
Phytase	0	50	1623	145	212	290
	500	49	1681	141	243	320
	1,500	51	1672	130	206	280
	SEM	1.4	56.7	7.6	10.0	17.0
P values						
aP, %		0.176	0.465	0.965	0.624	0.740
Phytase		0.437	0.737	0.379	0.026	0.209
	Linear				0.327	
	Quadratic				0.012	
aP x	phytase	0.642	0.188	0.778	*0.051*	0.048
Contrasts						
PC linear					0.317	0.270
PC quadratic					0.436	0.870
NC linear					0.101	*0.076*
NC quadratic					0.003	0.028

Notes: [1]Villi (n) counted on half of section and ×2 to determine density of each transverse section [2]Calculated by: (basal width + apical width)/2 × villus height; [3]Data log transformed prior to analysis; VH: villi height; AW: apical width; BW: basal width; SA: apparent villi surface area.

Table 3.13 – Duodenal histomorphology characteristics of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Duodenum: Diet		Crypt n[1]	ML, µm	CD, µm	VH: CD
NC		285	179.8	93	20.02
NC + 500 FTU/kg		277	192.5	91	20.82
NC + 1,500 FTU/kg		257	199.7	93	18.07
PC		279	195.5	104	15.56
PC + 500 FTU/kg		277	184.2	85	22.16
PC + 1,500 FTU/kg		260	192.7	85	22.35
	SEM	12.7	9.48	7.4	2.29
aP, %	0.35	273	190.7	92	19.63
	0.50	272	190.8	91	20.02
	SEM	7.3	5.48	4.3	1.32
Phytase	0	282	187.6	99	17.79
	500	277	188.3	88	21.49
	1,500	259	196.2	89	20.21
	SEM	9.0	6.71	5.2	1.62
P values					
aP, %		0.932	0.989	0.893	0.836
Phytase		0.174	0.611	0.289	0.273
aP x phytase		0.932	0.372	0.352	0.167

Notes: [1]Crypt numbers (n) counted on half of section and ×2 to determine density of each transverse section; ML: mucosal length; CD: crypt depth; VH:CD: villi height : crypt depth.

Table 3.14 – Jejunal villi histomorphology characteristics of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Jejunum: Diet		Villi n[1]	VH, µm	AW, µm	BW, µm	SA, mm [2,3]
NC		57	950	157	195	170
NC + 500 FTU/kg		57	933	166	196	170
NC + 1,500 FTU/kg		57	955	211	202	190
PC		56	1045	178	209	200
PC + 500 FTU/kg		62	951	184	228	210
PC + 1,500 FTU/kg		54	1046	169	190	190
	SEM	2.6	60.5	13.6	15.2	19.6
aP, %	0.35	57	946	178	198	180
	0.50	58	1014	177	209	200
	SEM	1.5	34.9	7.9	8.8	11.3
Phytase	0	57	997	167	202	190
	500	59	942	175	212	190
	1,500	56	1000	190	196	190
	SEM	1.8	42.8	9.6	10.7	13.9
P values						
aP, %		0.815	0.176	0.933	0.349	0.240
Phytase		0.331	0.557	0.262	0.561	0.888
aP x phytase		0.305	0.769	0.047	0.360	0.447
Contrasts						
PC linear				0.656		
PC quadratic				0.524		
NC linear				0.009		
NC quadratic				0.275		

Notes: [1]Villi numbers counted on half of section and x2 to determine density of each transverse section; [2]Calculated by: (basal width + apical width)/(2 × villus height); [3]Data log transformed prior to analysis; VH: villi height; AW: apical width; BW: basal width; SA: apparent villi surface area.

Table 3.15 – Jejunal histomorphology characteristics of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Jejunum: Diet		Crypt n[1]	ML, µm	CD, µm	VH:CD
NC		282	163.8	71	13.6
NC + 500 FTU/kg		269	183.9	82	12.1
NC + 1,500 FTU/kg		268	183.0	75	13.3
PC		261	175.6	75	14.2
PC + 500 FTU/kg		269	172.6	67	14.7
PC + 1,500 FTU/kg		296	185.6	72	14.7
	SEM	11.6	9.01	4.3	1.12
aP, %	0.35	273	176.9	76	13.0
	0.50	276	177.9	71	14.5
	SEM	6.7	5.20	2.5	0.65
Phytase	0	272	169.7	73	13.9
	500	269	178.3	74	13.4
	1,500	282	184.3	73	14.0
	SEM	8.2	6.37	3.0	0.79
P values					
aP, %		0.802	0.890	0.236	0.108
Phytase		0.490	0.281	0.938	0.855
aP x phytase		0.132	0.446	*0.090*	0.686
Contrasts					
	PC linear			0.528	
	PC quadratic			0.232	
	NC linear			0.516	
	NC quadratic			0.094	

Notes: [1]Crypt numbers (n) counted on half of section and ×2 to determine density of each transverse section; ML: mucosal length; CD: crypt depth; VH:CD: villi height : crypt depth.

Table 3.16 – Ileal villi histomorphology characteristics of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Ileum: Diet		Villi n[1]	VH, µm	AW, µm	BW, µm	SA, mm[2,3]
NC		59	557	124	145	76
NC + 500 FTU/kg		54	615	156	185	100
NC + 1,500 FTU/kg		58	623	145	164	96
PC		57	594	123	152	91
PC + 500 FTU/kg		63	579	135	149	85
PC + 1,500 FTU/kg		54	609	138	152	95
	SEM	2.4	44.2	11.4	9.0	10.0
aP, %	0.35	57	598	142	165	92.2
	0.50	58	594	132	151	90.2
	SEM	1.4	25.5	6.6	5.2	5.8
Phytase	0	58	575	124	149	83.6
	500	58	597	146	167	94.7
	1,500	56	616	142	158	95.3
	SEM	1.7	31.2	8.0	6.4	7.1
P values						
aP, %		0.553	0.910	0.311	0.069	0.451
Phytase		0.551	0.663	0.134	0.144	0.516
aP x phytase		0.037	0.702	0.672	0.063	0.173
Contrasts						
PC linear		0.367			0.952	
PC quadratic		0.028			0.761	
NC linear		0.690			0.150	
NC quadratic		0.150			0.008	

Notes: [1]Villi and crypt numbers counted on half of section and ×2 to determine density of each transverse section; [2]Calculated by: (basal width + apical width)/(2 × villus height); [3]Data log transformed prior to analysis; VH: villi height; AW: apical width; BW: basal width; SA: apparent villi surface area.

Table 3.17 – Ileal histomorphology characteristics of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Ileum: Diet		Crypt n[1]	ML, µm	CD, µm	VH: CD
NC		210	176.8	56	10.0
NC + 500 FTU/kg		216	184.2	63	9.7
NC + 1,500 FTU/kg		207	177.4	61	10.3
PC		236	166.4	65	9.4
PC + 500 FTU/kg		225	183.4	63	9.5
PC + 1,500 FTU/kg		223	165.9	57	10.9
	SEM	8.8	8.63	2.4	0.79
aP, %	0.35	211	179.4	60	10.0
	0.50	228	171.9	61	9.9
	SEM	5.1	4.98	1.4	0.45
Phytase	0	223	171.6	61	9.7
	500	221	183.8	63	9.6
	1,500	215	171.7	59	10.6
	SEM	6.2	6.10	1.7	0.56
P values					
aP, %		0.024	0.293	0.591	0.923
Phytase		0.660	0.280	0.240	0.408
aP x phytase		0.619	0.793	0.032	0.752
Contrasts					
	PC linear			0.022	
	PC quadratic			0.500	
	NC linear			0.169	
	NC quadratic			0.132	

Notes: [1]Crypt numbers counted on half of section and ×2 to determine density of each transverse section; ML: mucosal length; CD; crypt depth; VH:CD: villi height : crypt depth.

3.3.5.3 Microbial profiling

After consideration of the grouping of samples within a dendrogram of the kingdom (not presented), there is a clear separation of the ileal and caecal samples, except for ileal sample 42, which is grouped with the caecal samples. Therefore, ileal sample 42 was removed from all analyses.

Many species were detected in both the ileum and the caecum. Although there were a similar number of genera identified in the ileum and caeca as shown in Table 3.18, there was greater species diversity in the ileum, as indicated by the Shannon Species diversity values. Table 3.19 gives further indication of the spread and diversity of the bacterial species detected. The principle co-ordinate analysis outputs of the genera and species are presented in Figures 3.1 and 3.2. There is closer grouping of the genera of the caeca samples than those from the ileum (Figure 3.1). The greater variation in the sequencing of the ileal samples may be due to the greater environmental fluidity with dietary fluidity, and possible contamination from the less-digested feed components. Similar patterns can be seen in the grouping of the species (Figure 3.2).

Due to the large volume of data, 7 genera were selected for statistical analysis based on their relevance and on current published literature. The genera selected are: *Bacteroides*, *Bifidobacterium*, *Campylobacter*, *Clostridium*, *Escherichia*, *Lactobacillus* and *Salmonella*. All were identified except for *Salmonella*. Proportionally the highest 10 genera counts identified in each of the ileum and caecum are shown in Table 3.20. Of the 10 genera of interest, *Lactobacillus, Clostridium* and *Escherichia* appeared to be predominant in both samples, but all 6 will still be considered for further discussion.

The genera of the ileum and caeca are presented in Figures 3.3 and 3.4, respectively. *Lactobacillus* were the predominant genera in the ileum, being over 50% of the genus populations in the ileum, with the remaining genera of interest being 11.9% of the bacteria identified.

Table 3.18 - Representation of the species and genera identified in the ileal and caecal contents of broilers.

N	Ileum	Caeca
Samples	36 of 48	46 of 48
Genera	158	168
Species	1404 (191 above 100 counts)	666 (161 above 100 counts)

Table 3.19 – Counts and diversification of bacterial species identified in the ileum and caecum of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

		Number of Reads	% Reads Classified to Genus	Shannon Species Diversity[1]	Number of Species Identified
Ileum	Min	64503	55.52	1.40	163
	Max	185856	97.16	2.25	468
	Range	121353	41.64	0.85	305
	Mean	113463	88.42	1.81	310
Caeca	Min	43694	75.21	1.03	184
	Max	188365	87.31	2.06	291
	Range	144671	12.10	1.03	107
	Mean	118732	80.13	1.44	233

Notes: [1]Shannon species diversity: quantitative measure of the number of different species and how the individual samples are distributed among the species.

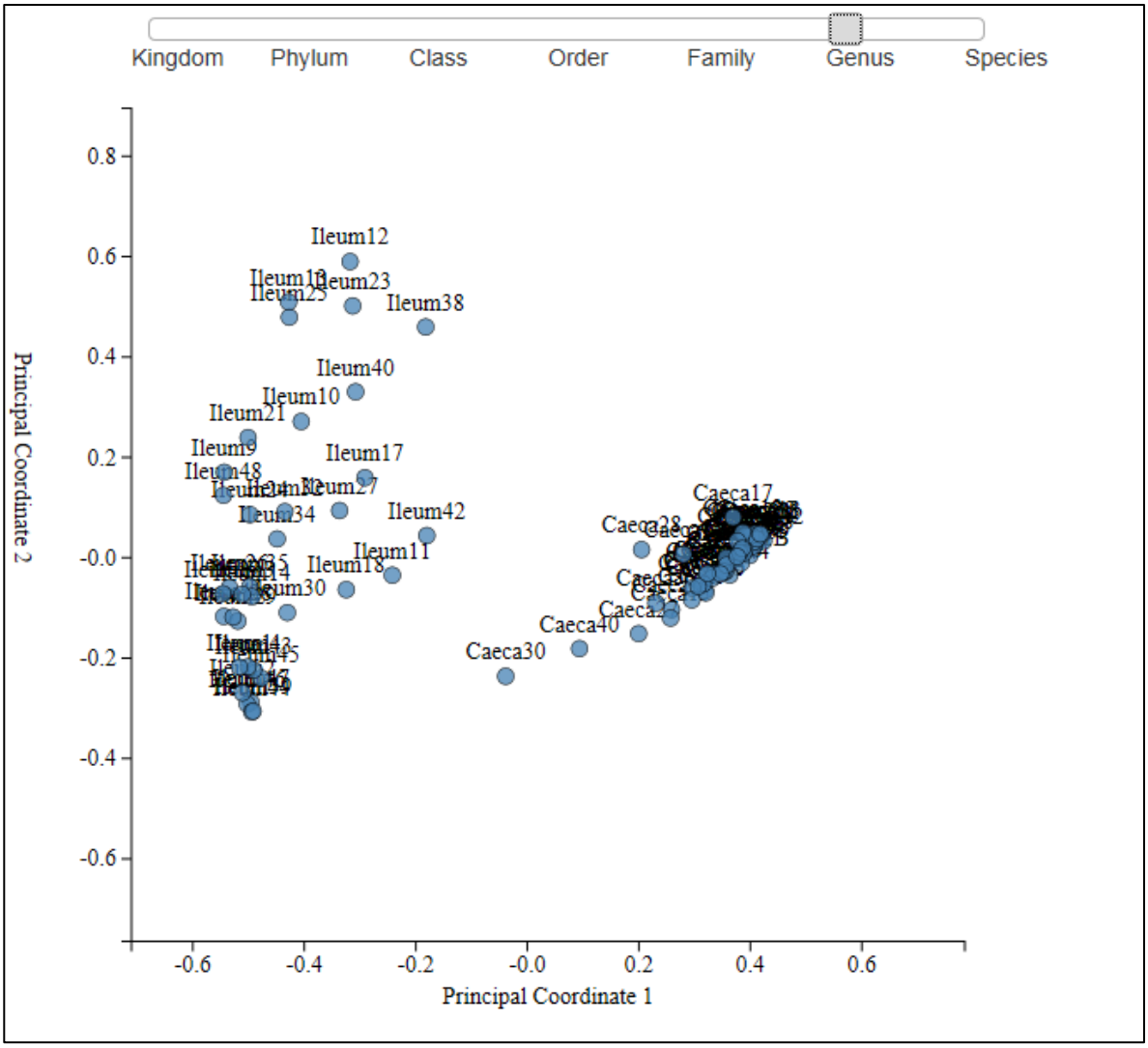


Figure 3.1 – Principle co-ordinate analysis of the spread of genera in the ileum and caeca of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

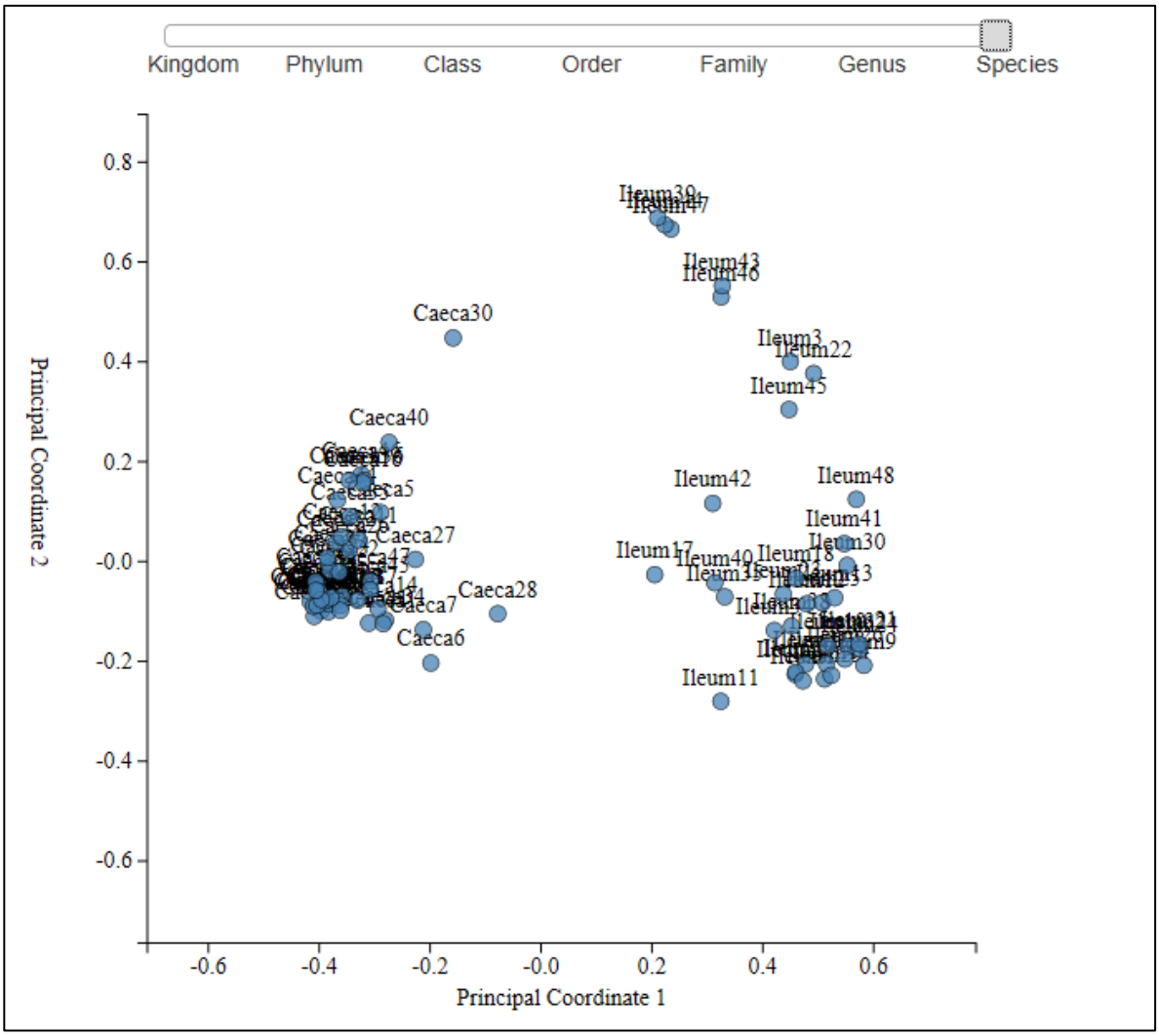


Figure 3.2 – Principle co-ordinate analysis of the spread of species in the ileum and caeca of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Table 3.20– The top 10 genera counts in the ileum and caeca of broilers.

Top 10 genera counts	
Ileum	Caeca
Lactobacillus	Flavobacterium
Calothrix	Oscillospira
Clostridium	Blautia
Escherichia	Faecalibacterium
Alkaliphilus	**Clostridium**
Flavobacterium	Ruminococcus
Blautia	Alkaliphilus
Faecalibacterium	**Lactobacillus**
Oscillospira	Anaerofilum
Rickettsia	**Escherichia**

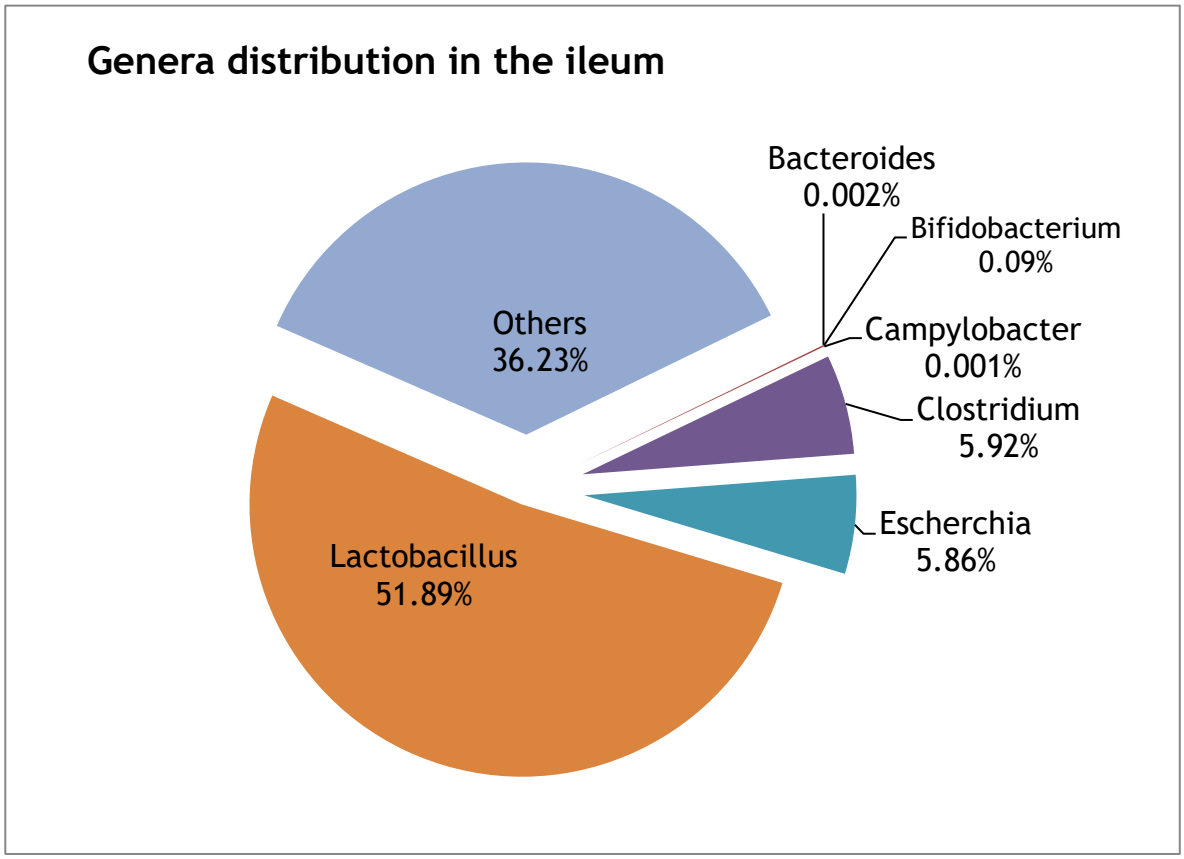


Figure 3.3 – The proportional distribution of genera in the ileum of broilers.

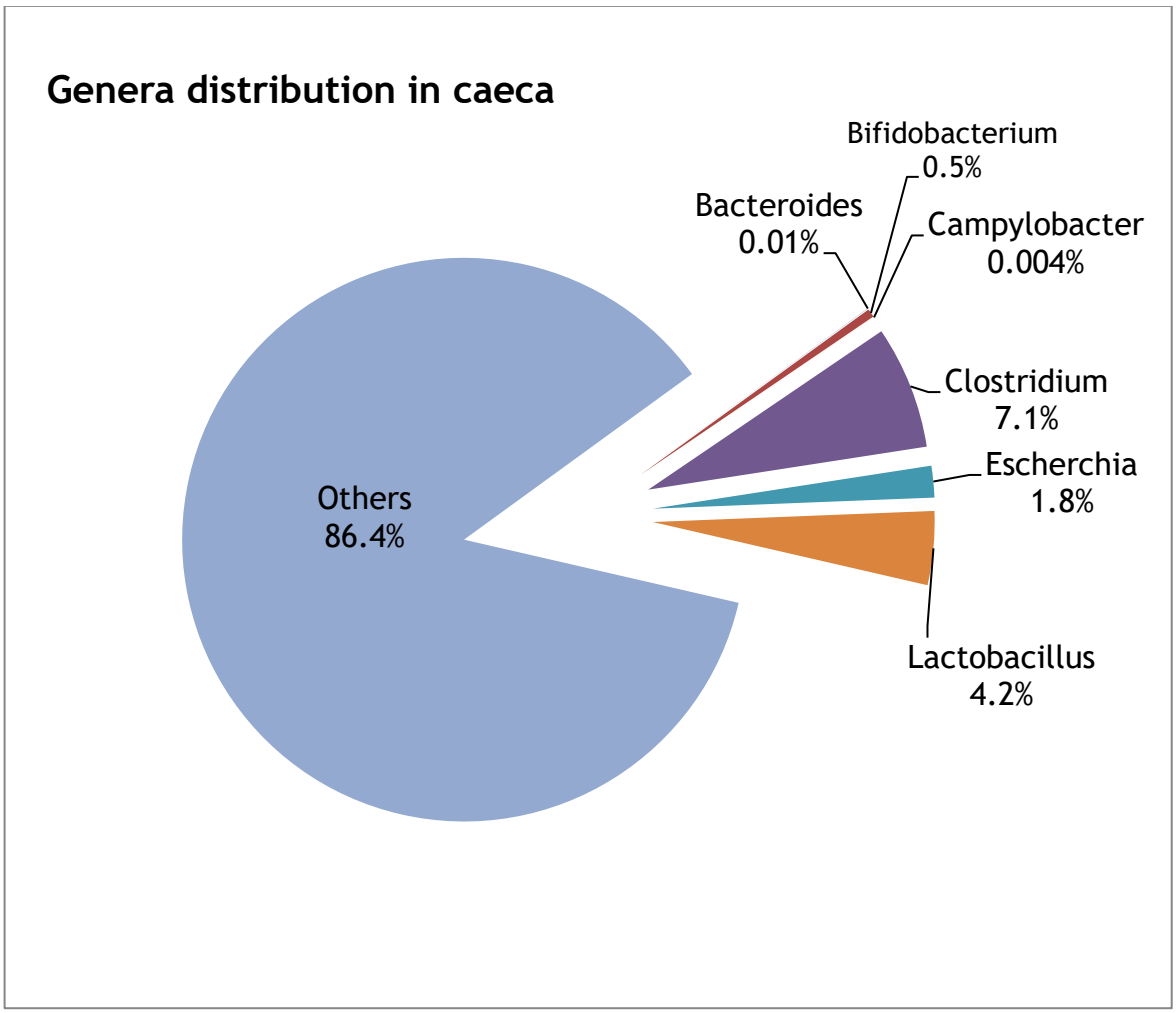


Figure 3.4 – The proportional distribution of genera in the caeca of broilers.

The remaining 36.2% consisted of the other 622 genera sequenced. Compared to the ileal sequences, there were proportionally fewer genera of interest in the caecum. The other 446 genera sequenced in the caeca form 86% of the overall genera present. The proportions of *Bacteroides, Bifidobacterium, Campylobacter* and *Clostridium* were similar between the ileum and caeca. There appear to be a greater presence of *Escherichia* in the ileum (5.9%) than the caeca (1.8%); however these differences may be inconsequential to the health status of the bird. The proportion of *Lactobacillus* was considerably lower in the caeca (4%) than the ileum (52%), as would be expected.

Where the predominant 5 genera in the ileum and caeca did not consist of the 7 genera of interest, these were included for statistical analyses. In the ileum there were *Calothrix* and *Alkaliphilus*, and in the caeca were *Flavobacterium, Oscillospira, Blautia* and *Faecalibacterium*. Ileal results are presented in Table 4.11. There was significant aP x phytase ($P < 0.05$) interaction on the ileal *Lactobacillus* populations. The Lactobacillus populations decreased quadratically in the PC ($P < 0.01$) and tended to show a linear deacrease in the NC ($P = 0.055$) treatments. There was a tendency for an interactive influence on ileal *Clostridium* populations ($P = 0.095$). There were no treatment effects on ileal populations of *Calothrix* and *Alkaliphilus*.

The prominent five bacterial genera and additional genera of interest in the caecum are presented in Table 3.22. There were no significant aP × phytase interactions on the populations of the predominant bacteria in the caeca. The only significant treatment effect was that *Flavobacterium* populations (the most predominant bacteria in the caecum) were lower in NC treatments ($P < 0.05$) compared with the PC. There was a tendency for a phytase effect on populations of *Oscillospira* and *Clostridium*. *Oscillospira* populations tended to decrease as phytase addition increased ($P = 0.097$), whilst *Clostridium* populations tended to increase with 500 FTU/kg but decrease with 1,500 FTU/kg phytase ($P = 0.063$).

Table 3.21 – Genera counts in the ileum of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Ileum: Diet		Lactobacillus	Calothrix[1]	Clostridium[1]	Escherichia[1]	Alkaliphilus[1]
NC		66782	9393	6546	567	1368
NC + 500 FTU/kg		59867	18283	3083	264	1357
NC + 1,500 FTU/kg		42360	21952	3881	323	2234
PC		49163	21838	4134	249	2779
PC + 500 FTU/kg		23169	30602	9775	533	2394
PC + 1,500 FTU/kg		57972	16914	2718	643	1816
	SEM	8510.8	5196.9	1699.3	219.5	1094.5
aP, %	0.35	56337	16543	4503	385	1653
	0.50	43435	23118	5543	475	2330
	SEM	4913.7	3000.4	981.1	126.8	631.9
Phytase	0	57973	15615	5340	408	2074
	500	41518	24442	6429	399	1875
	1,500	50166	19433	3300	483	2025
	SEM	6018.0	3674.7	1201.6	155.2	773.9
P values						
aP		*0.077*	0.427	0.651	0.211	0.924
Phytase		0.178	0.250	0.517	0.958	0.483
aP × phytase		0.018[2]	0.151	*0.095*	0.933	0.450

Notes: [1]Log transformed prior to analyses, raw data of means presented; [2]Contrasts: NC linear P = 0.055, PC quadratic P = 0.008.

Table 3.22 – Genera counts in the caeca of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Diet:		Flavobacterium	Oscillospira	Blautia	Faecalibacterium[1]	Clostridium	Escherichia[1]	Lactobacillus[1]
NC		11886	15725	11911	9349	6675	262	3705
NC + 500 FTU/kg		23157	15388	15061	5967	7690	424	3043
NC + 1,500 FTU/kg		13897	12629	11594	4942	4877	676	5446
PC		22392	16655	15205	6045	5444	390	3077
PC + 500 FTU/kg		23800	16260	14662	8789	6906	384	2906
PC + 1,500 FTU/kg		24381	11905	14210	4897	5922	228	3313
	SEM	4012.6	1936.8	1656.6	1509.3	784.4	152.1	977.5
aP, %	0.35	16313	14581	12856	6753	6414	454	4065
	0.50	23524	14940	14693	6577	6090	334	3099
	SEM	2316.7	1118.2	956.4	871.4	452.9	87.8	564.4
Phytase	0	17139	16190	13558	7697	6059	326	3391
	500	23478	15824	14862	7378	7298	404	2974
	1,500	19139	12267	12902	4920	5400	452	4380
	SEM	2837.3	1369.6	1171.4	1067.2	554.6	107.6	691.2
P values								
aP		0.035	0.822	0.184	0.964	0.617	0.909	0.192
Phytase		0.285	*0.097*	0.492	0.144	*0.063*	0.730	0.632
aP x phytase		0.377	0.890	0.502	0.631	0.320	0.297	0.951

Notes: [1]Log transformed prior to analyses, raw data of means presented.

Total counts of the species sequenced of the genera of interest in the ileum and caecum were considered (excluding those with counts below 10 overall, in each region) for further analysis. In the ileum, 191 species were sequenced; however *Lactobacillus*, *Clostridium* and *Calothrix* were considerably the predominant genera present. Therefore, these 3 genera were selected for analysis of treatment effects on species, with only the predominant species being selected for analyses, presented in Table 3.23, 3.24 and 3.25.

Only *Calothrix parietina* was detected, which was influenced by aP x phytase interaction ($P < 0.01$), with populations linearly decreasing with increasing phytase dose in the NC ($P < 0.05$) and linearly increasing in the PC ($P < 0.05$). Of the 5 *Clostridiu*m species selected for analysis, only *C. frigor*is was influenced by dietary aP ($P < 0.01$), being greater in the NC than PC. There were no other treatment effects on the populations of the other *Clostridium* species, except for a tendency for aP × phytase interaction ($P = 0.069$) on the *C. frigoris*, with a tendency to linearly decrease in the NC and no effect in the PC with the addition of phytase.

There were 6 *Lactobacillus* species analysed in the ileum, with only 3 being influenced by the dietary treatments. There were aP x phytase interactions on *L. johnsonii* and *L. taiwanensis* ($P < 0.05$). Populations of *L. johnsonii* and L. taiwanesis decreased in the NC (quadratic, $P < 0.01$) with increasing phytase.

In the PC diets, *L. johnsonii* appeared to increase with 500 and decrease with 1,500 FTU/kg; *L. taiwanensis* populations were comparable at both doses of phytase compared to when it was absent, with populations being greater in the PC than the NC treatments with the addition of 500 FTU/kg. Populations *of L. salivarius* decreased ($P < 0.05$) when 1,500 FTU/kg phytase were supplemented, but was comparable between 0 and 500 FTU/kg doses.

Table 3.23 - Calothrix counts in the ileum of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Diet:		Calothrix parietina
NC		29217
NC + 500 FTU/kg		27141
NC + 1,500 FTU/kg		15453
PC		10937
PC + 500 FTU/kg		10277
PC + 1,500 FTU/kg		19963
	SEM	4888.8
aP, %	0.35	23937
	0.50	13726
	SEM	2822.6
Phytase	0	20077
	500	18709
	1,500	17708
	SEM	3456.9
P values		
aP		0.005
Phytase		0.981
aP x phytase		0.006[1]

Notes: Data log transformed prior to analysis; [1]PC linear, P = 0.035, NC linear, P = 0.021.

Table 3.24 - Clostridium counts in the ileum of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

Notes: All data log transformed prior to analysis; [1]NC linear, P = 0.046; PC linear, P = 0.386.

		Ileal Clostridium				
Diet:		C. alkalicellulosi	C. frigoris	C. histolyticum	C. taeniosporum	C. thermosuccinogenes
NC		60.3	411	27.0	41.0	27.8
NC + 500 FTU/kg		21.8	356	22.0	27.7	23.6
NC + 1,500 FTU/kg		33.4	347	27.4	36.9	35.1
PC		29.9	143	24.3	24.1	38.8
PC + 500 FTU/kg		26.9	217	36.4	43.3	53.8
PC + 1,500 FTU/kg		37.0	176	34.7	29.3	74.0
	SEM	17.54	127.1	9.53	12.41	19.77
aP, %	0.35	38.5	371	25.5	35.2	28.8
	0.50	31.3	179	31.8	32.2	55.5
	SEM	10.13	73.4	5.50	7.17	11.42
Phytase	0	45.1	277	25.6	32.5	33.3
	500	24.4	286	29.2	35.5	38.7
	1,500	35.2	262	31.0	33.1	54.5
	SEM	12.41	89.9	6.74	8.78	13.98
P values						
aP		0.977	0.008	0.412	0.635	0.478
Phytase		0.574	0.624	0.923	0.714	0.254
aP x phytase		0.542	*0.069[1]*	0.737	0.309	0.938

Table 3.25 - Lactobacilli counts in the ileum of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days.

			Ileal Lactobacilli					
Diet:			acidophilus	crispatus	johnsonii	salivarius	taiwanensis	ultunensis
NC			1431	2640	16466	1871	1620	3512
NC + 500 FTU/kg			2784	4688	2747	1916	374	7202
NC + 1,500 FTU/kg			3693	6172	42455	604	4098	9751
PC			3007	5246	28401	1148	2714	7429
PC + 500 FTU/kg			2151	3902	33162	13556	3135	5423
PC + 1,500 FTU/kg			2140	3211	26914	1467	2528	5902
		SEM	832.5	1357.6	6108.3	468.1	555.3	2221.4
aP, %	0.35		2636	4500	20556	1463	2031	6822
	0.50		2432	4120	29492	1324	2792	6251
		SEM	480.7	783.8	3526.7	270.2	320.6	1282.5
Phytase	0		2219	3943	22433	1510[a]	2167	5470
	500		2468	4295	17955	1636[a]	1755	6312
	1,500		2916	4691	34685	1036[b]	3313	7826
		SEM	588.7	960	4319.3	331	392.7	1570.8
P values								
aP			0.847	0.802	0.531	0.129	0.140	0.970
Phytase			0.887	0.962	0.236	0.018[1]	*0.052*	0.899
aP x phytase			0.312	0.358	0.049[2]	0.139	0.012[3]	0.326

Notes: All data log transformed prior to analysis; [1]Phytase, linear P < 0.01; [2]NC linear, P = 0.449; NC quadratic, P = 0.047; PC linear, P = 0.114; PC quadratic, P = 0.142; [3]NC linear, P = 0.077; NC quadratic, P = 0.003; PC linear, P = 0.217; PC quadratic, P = 0.193.

In the caeca, the predominant genera were *Flavobacterium*, *Oscillospira* and *Clostridium*. Due to the number of species sequenced, the predominant species of *Clostridium* and *Lactobacillus* were selected for analysis of treatment effects. There were 50 *Clostridium* species sequenced, but the predominant 9 were selected for analysis, Table 3.26. There was a significant aP × phytase interaction on the caecal populations of *C. taeniosporum* ($P < 0.05$), decreasing (linear, $P < 0.05$) in the PC and tending to quadratically decrease in the NC ($P = 0.074$). There was a phytase ($P < 0.05$) influence on *C. termitidis*, increasing as phytase dose increased (linear, $P < 0.01$), and a tendency for *C. thermosuccinogenes* ($P = 0.057$) to increase as phytase increased (linear, $P < 0.05$). Dietary aP had no influence on the *Clostridium* species populations investigated.

The populations of 6 *Lactobacillus* species in the caeca were analysed, Table 3.27. There was no aP x phytase interaction, or main effects of phytase, on the predominant *Lactobacillus* species. Lowering the dietary aP significantly reduced the populations of *L. acidophilus* ($P < 0.05$), *L. crispatus* ($P < 0.05$), *L. johnsonii* ($P < 0.05$), *L. taiwanensis* ($P < 0.01$) and *L. ultunensis* ($P < 0.01$).

3.3.6 Correlations

Correlations between the data are presented in Table 3.28. *Escherichia* populations in the ileum were negatively correlated with day 21 BWG ($r^2 = -0.69$; $P < 0.001$) and ileal villi SA and BW ($r^2 = -0.77$, -0.79, respectively, $P < 0.001$), and positively correlated with ileal IP3 content ($r^2 = +0.72$; $P < 0.001$). The *Escherichia* populations in the caecal content were positively correlated with jejunal VH, AW and SA ($r^2 = +0.75$, $+0.66$, $+0.79$, respectively; $P < 0.001$). Duodenal villi BW was negatively correlated with ileal *Bifidobacterium* populations ($r^2 = -0.76$; $P < 0.001$). Ileal *Bifidobacterium* populations were also positively correlated with ileal *Campylobacter* populations ($r^2 = +0.69$; $P < 0.001$). The caecal *Bacteroides* populations were positively correlated with day 21 BWG ($r^2 = +0.66$; $P < 0.001$). Correlations were also identified between ileal Ca digestibility and ileal P digestibility and excreta IP4 content ($r^2 = +0.68$, -0.66, respectively; $P < 0.001$). Excreta IP4 and IP6 contents were also negatively correlated ($r^2 = -0.82$; $P < 0.001$).

Table 3.26 - The predominant Clostridium species in the caecum of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days

Notes: All data log transformed prior to analysis, raw data means presented; [1]Phytase; linear, P = 0.007; quadratic, P = 0.199; Phytase;

Diet:		alkalicellulosi	cadaveris	caenicola	frigoris	histolyticum	taeniosporum	termitidis	thermos-succinogenes	vincentii
NC		568	50.2	33.2	44.3	446	134	53	352	68.0
NC + 500 FTU/kg		729	43.2	32.0	49.6	548	91	87	429	49.6
NC + 1,500 FTU/kg		622	55.5	36.4	46.0	499	142	232	753	89.5
PC		837	47.0	35.4	58.9	582	203	93	377	70.8
PC + 500 FTU/kg		660	63.9	38.5	64.2	518	194	152	842	52.9
PC + 1,500 FTU/kg		528	43.2	43.1	48.2	647	110	131	944	83.0
	SEM	96.5	8.24	7.07	8.22	107.1	28.0	35.5	201	16.19
aP, %	0.35	640	49.7	33.9	46.6	498	122	124	511	69.0
	0.50	675	51.4	39.0	57.1	583	169	125	721	68.9
	SEM	55.7	4.76	4.08	4.75	61.8	16.2	20.5	116.1	9.35
Phytase	0	703	48.6	34.3	51.6	514	169	73	365	69.4
	500	695	53.6	35.3	56.9	533	142	119	636	51.3
	1,500	575	49.4	39.7	47.1	573	126	182	849	86.3
	SEM	68.2	5.83	5.00	5.81	75.7	19.8	25.1	142.2	11.45
P values										
aP		0.282	0.859	0.499	0.120	0.338	0.044	0.705	0.337	0.804
Phytase		0.624	0.782	0.610	0.725	0.854	0.210	0.012[1]	*0.057*[2]	0.121
aP x phytase		0.839	0.357	0.913	0.659	0.652	0.034[3]	0.111	0.619	0.967

linear, P = 0.021; quadratic, P = 0.533; [3]PC linear, P = 0.021; PC quadratic, P = 0.233; NC linear, P = 0.961; NC quadratic, P = 0.074.

*T*able 3.27- Lactobacilli counts in the caecum of broilers fed diets adequate or deficient in aP and Ca, with 0, 500 or 1,500 FTU/kg phytase, for 21 days

		Caecal Lactobacillus					
Diet:		acidophilus	crispatus	johnsonii	salivarius	taiwanensis	ultunensis
NC		235	401	216	100	134	792
NC + 500 FTU/kg		65	102	210	244	98	327
NC + 1,500 FTU/kg		182	273	321	121	200	632
PC		320	502	453	100	221	979
PC + 500 FTU/kg		271	439	503	98	175	959
PC + 1,500 FTU/kg		326	539	507	133	278	1090
	SEM	73.8	118.5	141.1	38.1	69.0	249.0
aP, %	0.35	161	258	249	155	144	584
	0.50	306	493	488	110	225	1009
	SEM	42.6	68.4	81.5	22	39.8	143.8
Phytase	0	277	451	334	100	178	886
	500	168	270	357	171	137	643
	1,500	254	406	414	127	239	861
	SEM	52.2	83.8	99.8	27	48.8	176.1
P values							
aP		0.020	0.017	0.021	0.502	0.007	0.004
Phytase		0.162	0.231	0.900	0.580	0.287	0.104
aP x phytase		0.239	0.243	0.724	0.157	0.881	0.321

Notes: All data log transformed prior to analysis, raw data means presented.

Table 3.28 - Correlations of interest.

Variable 1	Variable 2	r^2	P value
Ileal Ca digestibility	Ileal P digestibility	+0.68	< 0.001
Excreta IP6 content	Excreta IP4 content	-0.82	< 0.001
Excreta IP4 content	Ileal Ca digestibility	-0.66	< 0.001
Caecal Bacteroides populations	Day 21 BWG	+0.66	< 0.001
Ileal Escherichia populations	Day 21 BWG	-0.69	< 0.001
Caecal Escherichia	Jejunal apical width	+0.66	< 0.001
Caecal Escherichia populations	Jejunal villi surface area	+0.75	< 0.001
Caecal Escherichia populations	Jejunal villi height	+0.79	< 0.001
Ileal Campylobacter	Ileal Bifidobacterium	+0.69	< 0.001
Ileal Escherichia populations	Ileal basal width	-0.79	< 0.001
Ileal Escherichia populations	Ileal villi surface area	-0.77	< 0.001
Ileal Escherichia populations	Ileal IP3 content	+0.72	< 0.001
Ileal Bifidobacterium populations	Duodenal basal width	-0.76	< 0.001

3.4 Discussion

The results from Chapter 2 suggest that there were changes occurring within the body with the different doses of phytase, possibly because of the characteristics of phytate hydrolysis, consequently influencing intestinal conditions. The aim of the current study was to observe the influence of both regular and super doses of phytase on the gut environment to try and explain the effects observed at the different levels of phytase supplementation.

3.4.1 Growth performance

There are many reports that the addition of phytase has the benefit of improving growth performance, in particular BWG and FCR, with the greatest improvements seen in phosphorus deficient diets (Akyurek *et al.,* 2011; Cowieson *et al.,* 2011; Liu *et al.,* 2008a; Sebastian *et al.,* 1996). This study shows that phytase supplementation in diets low in available P is able to prevent the P deficiency symptoms and associated depression in growth performance, allowing the birds to match the growth performance of those birds fed nutritionally adequate diets. Overall, the specific benefits of phytase supplementation to NC diets may vary between studies, according to the level of aP (and other nutrient) deficiency, ultimately giving different responses to phytase and the dose effects.

For BWG in the current study, the dietary level of aP had an important influence on the efficacy of phytase, with phytase effects only observed in the NC. The additional release of nutrients following phytate hydrolysis may lead to further interactions between nutrients and phytate within the digesta, and reduce the efficacy of phytase and potential for improvements in growth performance. In the NC diets where aP was limiting, subsequent Ca release from phytate hydrolysis can have detrimental effects on performance (Shafey, 1993) and may influence the Ca:aP ratio, which is important for phytase efficacy and performance (Cowieson *et al.,* 2011; Rao *et al.,* 2006; Shafey *et al.,* 1990). The Ca:aP ratio is deemed more critical than absolute Ca and aP levels, having a detrimental effect of phytase at the higher doses (Cowieson *et al.,* 2011; Shafey *et al.,* 1990). Qian *et al.* (1997) reported that a greater Ca: aP ratio resulted in a

reduction in growth performance, with the negative influence of a wide ratio being more apparent at the lower levels of phytase supplementation. Olukosi and Fru (2014b) reported that a wide Ca:aP ratio had a muting effect on the efficacy of phytase on performance parameters, with a wide ratio being more detrimental in reduced Ca/ aP diets. In the current study, the analysed dietary Ca content was higher than formulated, which following the release of Ca from phytate hydrolysis, may have had the effect of further widening the Ca:aP ratio, leading to the observed difference in phytase effects between the PC and NC diets.

3.4.2 Nutrient utilisation

Ravindran *et al.* (2006) reported a phytase mediated increase in Ca digestibility, regardless of the dietary phytate content. In the current study, ileal Ca digestibility was also influenced by phytase dose and not dietary aP/ Ca level. Calcium digestibility decreased with 1,500 FTU/kg phytase, and this effect was likely a consequence of increased Ca released into the digesta following phytate hydrolysis, with absorption already being saturated due to the higher than anticipated dietary Ca levels. High Ca concentrations following release from phytate hydrolysis and thus a widening of the Ca:aP ratio can reduce the availability of other nutrients, primarily divalent minerals, in the digesta for absorption and increase their excretion (Shafey, 1993). The ratio between aP and Ca is important for nutrient utilisation. Shafey *et al.* (1990) reported that the retention of P was not influenced over a wide range of dietary aP concentrations, provided adequate Ca was also provided to maintain the balance. Additionally, Ca has been reported to form insoluble Ca-phosphate complexes, which subsequently reduces their absorption (Shafey *et al.*, 1990; Underwood, 1999). Nutrient release following phytate hydrolysis can result in antagonism between the minerals in the digesta, reducing or preventing their absorption (Sebastian *et al.*, 1996).

Wilkinson *et al.* (2011) reported an inverse relationship between dietary Ca levels and P, Mg and K digestibility. In the current study positive relationship was observed between Ca and P digestibility (r^2 = +0.68), however Ca

digestibility was only influenced by phytase supplementation and not the dietary aP and Ca levels. Perhaps if a more severe deficiency of aP and Ca had been implemented in the current study, then similar observations to Wilkinson *et al*. (2011) may have been observed. Similarly, Sebastian *et al*. (1996) observed an increase in TTR of P and Ca when phytase was added to low P diets. Olukosi and Fru (2014a) also reported an increase in Ca and P TTR with the addition of phytase to diets with a 2:1 Ca:aP ratio, but not when the ratio was increased to 2.5:1, again highlighting the importance of the Ca:aP ratio. The authors also concluded that the negative influence of a wide Ca:aP ratio was more pronounced in low aP and Ca diets. In this study, there were no treatment effects on Ca TTR, whilst P TTR was comparable in the NC treatments, with and without phytase, however retention was lower in the PC than the NC at each dose. The lack of observed effect in this study may have been due to the high dietary Ca content.

Higher doses of phytase were required to improve the digestibility of protein and P at lower phytate levels, as reported by Ravindran *et al*. (2006). Although in the current study the overall dietary phytate was comparable between treatments, by the time the digesta reached the ileum, there were treatment differences on the IP6 content. Consequently, this may have then influenced the digesta environment, ileal nutrient utilisation and TTR. Phytase increased N digestibility in the NC diets but not the PC, with digestibility being greater than the NC when 500 FTU/kg phytase was added, but not with 1,500 FTU. This difference in phytase dose response compared to a study by Ravindran *et al*. (2006) may be due to the additional release of Ca and Pi with 1,500 FTU/kg, thus changing the gut conditions for phytase action and nutrient absorption.

The overall lack of correlation between growth performance parameters and nutrient utilisation suggests that the observed improvements in growth performance following phytase supplementation are a result of extra-phosphoric effects, and may be explained by the characteristics of phytate hydrolysis throughout the gastro-intestinal tract.

3.4.3 Phytate hydrolysis

As digesta flowed from the ileum through the remainder of the digestive tract, it appears that dietary aP had more of an influence on the activity of phytase than it had in the gizzard and ileum. However, Li *et al*. (2016) reported that the proventriculus and gizzard are the most active sites of phytate hydrolysis, and may be an influence of the solubility of IP6, which is important for its interaction with phytase, which was also suggested by Zeller *et al*. (2015a). Inositol phosphate esters must be soluble to interact with phytase, therefore the high IP6 content of the ileum may be due to its high solubility and passage through the digestive tract (Li *et al.*, 2016). The IP6 content of the excreta was numerically greater than in the ileum and the initial dietary content which suggests that there may be some re-phosphorylation of the inositol phosphate ester molecules as they pass through the caeca, aided perhaps by the caecal microflora, or the increased concentration of P in the digesta following initial phytate hydrolysis. Despite this, the higher doses of phytase had a greater effect of reducing IP6 in the excreta. Between the ileum and caeca, only super doses of phytase were effective at reducing the IP5 content of the PC diets which suggests that the higher aP level may have hindered the hydrolysis of IP5 through this section of the gut.

It appears that the majority of the dietary IP6 may be hydrolysed (step-wise) through to IP4 and IP3, with the IP5 and IP4 esters produced by phytase hydrolysis being more susceptible to hydrolysis than those occurring in the feed. Li *et al*. (2016) reported the accumulation of IP6 in the distal ileum regardless of dietary factors, suggesting that in the absence of phytase, there are little dietary influences on the disappearance of IP6. When the author supplemented phytase, there were no observed differences in the ability of phytase to hydrolyse phytate at different levels of dietary Ca, aP and phytate-P. However, in a study reported by Li *et al*. (2016), the low dietary Ca levels used were generally higher than the 'high dietary Ca' treatments in many other studies. Therefore, the chelation sites for IP6 with Ca may have already been full at the 'low' Ca level, as a result of which no differences would have been observed by increasing the Ca levels further, either through dietary manipulation or following phytate hydrolysis. As there were treatment effects in the current study on the

excreta content of IP5, IP4 and IP3, but not in the ileum (with numerically greater concentrations in the excreta than the ileum, even in the treatments where phytase was absent), some IP6 hydrolysis appears to have occurred in the caecum, and may be a result of bacterial action, as the phytase enzyme would be expected to be completely degraded by this point (Zeller *et al.*, 2015a). Perhaps the greater IP4 content of the phytase supplemented diets may reflect the inability of the caecal bacteria to hydrolyse phytase-produced IP4, which may have a different structure to the IP4 present in the feed (Bedford, 2015, personal communication). Zeller *et al.* (2015a) also reported different enantiomers of the IPs produced during phytate hydrolysis.

Additional observation of the ratios between IP6 and IP4 contents of the ileal digesta and excreta suggests this may be the case. In the ileum and excreta, the ratio of IP6:IP4 decreases as phytase increases, in both the PC and NC diets, reaching 1:1 in the excreta when high doses of phytase had been used. In the absence of phytase in the ileum, the IP6 content was greater than the IP4. As the phytase dose increased, the ratio decreased as IP6 was hydrolysed and IP4 content marginally increased. Differences were more apparent in the excreta, where the ratio decreased as phytase dose increased. The increased IP6 hydrolysis led to increased IP4 content and became relatively proportional when 1,500 FTU/kg phytase is used, resulting in the ratio of 1:1 of IP6:IP4; strong negative correlations between excreta IP4 and IP6 content were identified (r^2 = -0.82; $P < 0.0001$). These results suggest that IP4 is more susceptible to hydrolysis post-ileum in the control diets than the phytase supplemented diets. The interactive effect of dietary aP appeared to become irrelevant for the inositol content in the excreta. As expected, inositol content increased with increasing doses of phytase, indicating that high levels of phytase are more effective at increasing inositol concentrations than regular levels. Inositol content of the excreta is numerically lower than the content in the ileum, which suggests potential uptake of inositol in the terminal region of the ileum, or by bacteria residing in the caeca.

3.4.4 Gut function parameters

In addition to improved growth performance and nutrient utilisation of aP-deficient diets, super-doses of phytase have the potential to enhance gut function. A healthy gut environment is associated with good overall animal health and performance, playing a major role in immune function and health status. Macro- and micro- properties of gut integrity, microfloral balance and immune status, all have a major influence on gut health and overall animal health. Dietary factors, such as nutritional components and composition, influence not only the physical stimulation of the gut but also the establishment of microbial populations, and are considered one of the most influential factors affecting gut health (Choct, 2009; Shirkey *et al.*, 2006). The interaction between the host physiology, diet and intestinal microflora are strongly influenced by environmental factors and stress (Steiner, 2006; Tannock and Savage, 1974). The relative balance of pathogenic and beneficial bacterial species within the gut is important for animal health, and intestinal pH may be an important proxy for gut health. Similarly, reducing the irritating influence of phytate in the gut may lead to beneficial alterations to gut histomorphology, such as the development of intestinal villi. This hypothesis of the influence of phytase on the gut can be tested through measurement of the pH, bacterial profiles and gut morphology.

3.4.5 pH

Phytase efficacy is determined by pH (Leenhardt *et al.*, 2005), but may differ between animal species and phytase sources. Gastric conditions, such as the crop in poultry and stomach in pigs, are optimal for phytase activity as the phytate is likely to be more soluble and able to interact with phytase (Campbell and Bedford, 1992; Kornegay *et al.*, 1996). The pH at which phytate-phytase interaction is optimal is dependent on the precipitation characteristics of the nutrients or cations it has formed a complex with (Taylor, 1965). A lower pH is considered beneficial, as this may create conditions which are beyond the optimal for the survival of pathogenic bacteria, as well as potentially increasing activity of digestive enzymes, such as pepsin (Ewing and Cole, 1994; Fuller, 1984). A change in pH may also influence nutrient absorption, increasing the nutrients available for utilisation by the animal and reducing substrates for

bacterial fermentation, influencing the microbial profile, and their activity (Dono *et al.*, 2014; Ptak *et al.*, 2015).

In a study by Walk *et al.* (2012b) super-doses of phytase (5,000 FTU/kg) increased the pH of the gastro-intestinal tract. The increased pH was likely a result of a reduced acidogenic effect of phytate, as well as from reduced concentrations of phytate-Ca complexes. In the current study, it is possible that the observed changes in jejunal pH with phytase supplementation may influence the solubility of some of the nutrients in the digesta, enhancing their absorption once they enter the ileum. As no further phytate hydrolysis is likely to occur in the ileum due to its proteolytic degradation, the reduction in IP6 detected in the ileum (Chapter 3) is likely to be reflective of hydrolysis in the previous regions. The optimal pH for phytate hydrolysis is between 2 and 6 (Maenz and Classen, 1998; Wodzinski and Ullah, 1996), with phytate-mineral complexes being most soluble when the pH is low (Shafey *et al.*, 1991). Therefore, the increases in pH with phytase addition to the PC diet and 500 FTU/kg to the NC diet may actually be having a detrimental effect on the hydrolysis of phytate in the jejunum. The lowest IP6 content, suggestive of the greatest phytate hydrolysis, was seen in the PC diet with 1,500 FTU/kg and the NC with both doses of phytase. However, the pH of the jejunum was considerably higher with the PC with 1,500 FTU/kg than the NC with 1,500 FTU/kg (6.40 vs. 6.23). Therefore, it is difficult to link jejunal pH with IP content of the ileum as a result of altered pH-mediated phytase activity specifically in the jejunum.

As there were no treatment influences on the pH of the duodenum, the effects on ileal nutrient utilisation with phytase supplementation may be a consequence of more favourable conditions for absorption as digesta passes through the jejunum. Assuming the optimal pH for the phytase used in this study is close to 6.0, these results suggest that optimal phytate hydrolysis in the jejunum would occur when 1,500 FTU/kg phytase is provided to diets which are marginally deficient in aP. However, when the super doses of 1,500 FTU/kg are provided in diets which have adequate aP and Ca (through the use of inorganic supplements), this may detrimentally influence conditions for phytase efficacy. This may be a consequence of an abundance of minerals in the digesta, namely

aP and Ca, and may be aggravated by the presence of inorganic P and Ca and their influence on pH.

The Ca:aP ratio can influence digesta pH, consequently influencing nutrient solubility and thus absorption (Shafey, 1993). Ptak *et al.* (2015) reported a decrease in caecal pH as dietary Ca and digestible P were decreased, suggesting that the higher Ca content of the PC resulted in higher pH. This was not seen in this study, although there was a tendency for phytase supplementation to influence caecal pH. Differences in response between this and the study reported by Ptak *et al.* (2015) may be explained by the dietary aP, Ca and phytase levels. In the PC diet fed by Ptak *et al.* (2015), the Ca and digestible P levels were lower than in the current study, being closer to our NC treatments than the PC. The tendency for a phytase influence on caecal pH with phytase in the current study may be a direct influence of Ca and aP release from phytate hydrolysis, rather than directly from the supplementation of phytase. This is because of the lack of effect of 5,000 FTU/kg phytase reported by Ptak *et al.* (2015), whilst a tendency for 500 or 1,500 FTU/kg phytase to have an influence in this study. These differences in study design likely also explain the differences in response observed on ileal pH. Ptak *et al.* (2015) reported an interaction between the Ca and aP content of the diets and phytase supplementation, with phytase increasing pH in the NC diets, but not the PC. No ileal pH effects were observed in the current study, with differences explained by the differences in dietary Ca and aP between the treatments.

Phytase, being an enzyme and therefore a protein, is thought to be degraded by the time it reaches the ileum, but some activity has been measured in the jejunum, but this may be from intestinal phytases (Morgan *et al.*, 2015; Onyango *et al.*, 2005; Yu *et al.*, 2004). Perhaps the improvements in nutrient digestibility detected in the ileum are sequential to desirable conditions in the jejunum for nutrient digestion and absorption, through reduced phytate-mineral, phytate-protein and phytate-nutrient interactions due to reduced phytate concentrations (Oberleas, 1973). However, the increase in jejunal pH of the PC diets with 1,500 FTU/kg, compared to when it was absent, and consideration of potential influences on nutrient bioavailability for nutrients entering the ileum does not

correspond with increases in ileal nutrient digestibility. In the PC diets, N and digestibility improved with phytase but there were no influences on Ca and P. Although 1,500 FTU/kg significantly increased jejunual pH, it was not different from the addition of 500 FTU/kg, which did not itself change in relation to the PC. The application of reduced Ca and aP in the NC diets resulted in the reduction in jejunal pH with 1,500 FTU/kg compared to when 500 or 0 FTU/kg phytase were used. The ileal digestibility in these NC treatments does not differ between either dose of phytase. These results suggest that the changes in jejunal pH with the addition of phytase to NC and PC diets do not beneficially influence the bioavailability of nutrients within the digesta for absorption in the ileum.

3.4.6 Gut morphological characteristics

The important morphological and physiological factors which have an influence on gut health and nutrient utilisation include villus height, crypt depth and the number and density of goblet cells. Feed enzymes may modify gut physiology and morphology altering digesta pH, nutrient bioavailability, solubility and absorption (Olukosi and Dono, 2014; Steiner, 2006). However, this maintenance of the gut structure and physiology has a high protein and energy requirement, which is greater than the maintenance requirements of many other organs (Choct, 2009). Short villi and deep crypts are associated with the presence of toxins and reduce the surface area available for nutrient absorption (Choct, 2009). The presence of large crypts suggests rapid cellular turnover, which has a high energy demand (Choct, 2009; Shakouri *et al.*, 2009), partitioning energy away from production. Therefore, the villi height: crypt depth ratio is an indicator of gut health and also a measure of functional efficiency of the intestine and gut (Chen *et al.*, 2015; Wu *et al.*, 2015). Wu *et al.* (2004) observed that 500 FTU/kg phytase increased the duodenal villus height and reduced goblet cell numbers in the jejunum of broilers fed adequate-P wheat-soy diets.

In the current study, treatment effects on morphological characteristics were more prominent in the ileum. The addition of phytase to the PC diets quadratically decreased the villi numbers and linearly increased crypt depth.

Chen *et al*. (2015) suggested that an increase in villi height and decrease in crypt depth is indicative of good gut health and function. Good gut function is important for facilitating nutrient absorption and improving the efficiency of nutrient use for immune function and growth. Additionally, phytase supplementation in the PC increased villi number and decreased crypt depth, suggesting improved gut function, and may subsequently lead to improved nutrient absorption and utilisation by the animal. It is interesting to observe that overall there was a greater phytase effect on intestinal morphology in the PC than the NC diets.

Emami *et al*. (2013) observed a phytase-mediated increase in duodenal, jejunal and ileal villus height, and duodenal villus height: crypt depth ratio, of NC diets (of similar aP levels to the current study), increasing to comparable conditions of the PC diet. The author also reported a reduction in villi height and villi height: crypt depth ratio, and increase in crypt depth, in the negative control treatments. Although the specifics in the morphological changes were different to those seen in our study (i.e. villi height vs. villi width or apparent surface area), the overall effect of phytase in preventing the detrimental influences of a NC diet is common. The minor disparity in observations between the studies may be related to differences in study design, such as the dietary composition, degree of deficiency in the NC diets, and the management and pathogenic challenge of the birds, as Emami *et al*. (2013) suggested that the observed changes in morphology may be a result of changes in the gut microbial populations.

Despite the negative association with microbes and gut morphology, the presence of beneficial bacterial species can be associated with increased villi length, increased turnover of epithelial cells, deeper crypts and an increased rate of cell migration, particularly in the upper regions of the small intestine, which may have a beneficial influence on nutrient absorption efficiency and overall performance characteristics (Ewing and Cole, 1994).

3.4.7 Microbial profiling

Using 16S next generation sequencing, a relative new sequencing tool, it was possible to take a snapshot of the bacterial populations in the ileum and caecum. The differences in sequencing procedure make direct comparisons to other sequenced populations in the literature complex, however, once this procedure becomes more developed and commonly used, it will make a good basis for having an idea of the bacterial populations in the gut at a given time. However, it must be remembered that bacterial sequencing just gives a "snapshot" of a "moment in time", and that populations are likely to be fluid, and vary between birds (species, age), environment and dietary composition (Torok *et al.*, 2008), making it difficult to determine how well the results are representing the changes which might actually be happening in the gut (Rudi *et al.*, 2007).

Numerous bacterial species have been identified in the gastrointestinal tract of poultry, including: *Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Fusobacterium*, and *Streptococcus* (Ewing and Cole, 1994; Richards *et al.*, 2005; Rinttila and Apajalahti, 2013), all of which were identified in the current study. Generally, facultative anaerobes are found from the crop to the terminal ileum, with strict anaerobes being most abundant in the caeca (Fuller, 1984). The concentrations of microflora increase distally along the small intestine towards the large intestine due to the larger volume of digesta, slower rate of passage and more favourable intestinal conditions (Barnes, 1972; Ewing and Cole, 1994; Rinttila and Apajalahti, 2013). Bacteria present in the gastrointestinal tract can have a substantial influence on the host's metabolism and physiology, therefore achieving optimal conditions is important for animal health (Kiarie *et al.,* 2013; Richards *et al.*, 2005; Yang *et al.*, 2009). Bacteria such as *Lactobacilli* may increase nutrient absorption through increased enzyme activity at the brush border, influencing gut morphology, digesta pH with additional immune-stimulatory anti-microbial and anti-tumour effects (Ewing and Cole, 1994; Lan *et al.*, 2005).

Stable microbial populations within the gut are important for health, with the dominant populations being maintained via antagonistic activity (Choct, 2009).

The commensal bacterial populations have a significant influence on the potential colonisation of pathogenic populations in the gut (Tannock and Savage, 1974), through competitive exclusion, interaction with mucin secretion and the maintenance of tight junctions (Srikanth and McCormick, 2008). Microbial populations are influenced by many factors such as diet, stress (Kiarie *et al.*, 2013; Tannock and Savage, 1974) and the environment, with diet likely being the most important (Choct, 2009; Lan *et al.*, 2005). Ingested feed passes through the gut, and depending on the degree of digestion and absorption, can provide a substrate for bacterial fermentation (Choct, 2009), with feed enzymes reducing the amount of undigested dietary substrates available (Kiarie *et al.*, 2013). The dietary Ca and aP levels are thought to have an important influence on the phytase effect on microbial populations throughout the gut and may lead to some of the observed benefits on growth performance following phytase supplementation (Ptak *et al.*, 2015). Similarly, Zeller (2015a) suggested a reduction in the activity of microbial and mucosa phytases from end product Pi inhibition, particularly when phytase was supplemented alongside inorganic P supplements such as mono-calcium phosphate. The author also suggested an influence on endogenous phytase, through down regulation of endogenous phytase expression or from a change in bacterial populations away from those which are able to produce phytase, as well as inhibition of the supplemented phytase by phytate-Ca complexes. Aydin *et al.*, (2010) suggested that a reduction in pathogenic bacteria populations with phytase may ultimately improve gut heatlh, more specifically reporting a reduction in total anaerobic bacteria, coliforms and *E. Coli* in the ileum following the supplementation of 750 FTU/kg phytase.

Our findings were similar in that *Lactobacilli* were the predominant bacteria in the ileum, but *Flavobacteria* were predominant in the caeca. *Lactobacillus johnsonii* and *L. taiwanesensis* observed to decrease with 500 FTU/kg in the NC, with *L. taiwanesensis* populations being greatest in the PC + 500 FTU/kg treatment. The tendency for an increase in *Clostridium* populations, particularly of *C. Frigoris*, with the addition of 500 FTU/kg phytase in the PC may be leading to a decrease in *Lactobacillus* populations through competitive exclusion. Munyaka (2015) identified *Lactobacilli* as the prominent bacteria in the ileum

and anaerobic bacteria such as *Lachnospiracea* and *Clostridium* as the predominant bacteria in the caeca. Amit-Romach *et al*. (2004) reported that 50% of the bacteria in the caeca at day 25 were *Lactobacilli* and *Bifidobacterium* species, and another 30% consisting of *Escherichia* and *Clostridium*. Differences can likely be explained by differences in dietary composition and/or bird age.

Metlzer-Zebeli *et al*. (2010) suggested that changes in ileal bacterial populations are a result of a phytase-mediated increase in Ca and P concentrations. The author did not detect a phytase response on ileal *Lactobacilli* populations, but observed that populations were influenced by dietary Ca. Ptak *et al*. (2015) reported a decrease in total bacterial counts as the dietary Ca and aP were reduced, but an increase following the supplementation of phytase. The author also reported a decrease in ileal *Clostridium perfringens* and *Enterobacteriacae* counts in reduced Ca and aP NC treatments, but no direct influence of phytase addition. However, they did observe a phytase-induced increase in lactic acid producing bacteria (*Lactobacilli*) and a dietary Ca, aP and phytase interaction on the populations of *Clostridium, Streptococcus* and *Lactococcus*. In the current study, phytase increased *Clostridium leptum* populations in the NC, but not the PC, and decreased *Streptococcus* and *Lactococcus* populations in the PC but not the NC. *Bifidobacterium* populations were increased (by the main effects) in the NC treatment and decreased following the supplementation of phytase. There were no treatment effects on the *Bacteroides* counts.

In the current study, the dietary Ca was higher than desired in the PC + 500 FTU/kg treatment and may explain some of the effects seen. Calcium has been shown to be involved in preventing the establishment of pathogenic bacteria (Metzler-Zebeli *et al.*, 2010) such as *Salmonella* (Ten Bruggencate *et al.*, 2004) and *Escherichia. coli* (Larsen *et al.*, 2007), through acting as a buffer in a calcium-phosphate complex in the gut (Ten Bruggencate *et al.*, 2004) and facilitating the binding of *Lactobacilli* in the gut, preventing *E. coli* binding (Larsen *et al.*, 2007). The tendency for a linear decrease in ileal *Lactobacilli* populations in the NC treatment may be an influence of the observed linear increase in jejunual pH. The high Ca levels, increased by the release following

phytate hydrolysis, may increase the digesta pH (Ptak *et al.*, 2015), as seen in the jejunum, creating gut conditions which were not optimal for *Lactobacilli* colonisation. In the current study, although there were no differences in the ileal nutrient utilisation of the PC diets between 500 and 1,500 FTU/kg, there were tendencies for the main effects of 1,500 FTU/kg to increase ileal Na and Fe flow above 500 FTU/kg. Considering the IP content of the ileum, there was significantly less IP6 and more inositol present in the PC diet containing 1,500 than 500 FTU/kg phytase. At the level of the excreta, there was greater clearance of IP5, IP4 and IP3 in the PC diets with 1,500 FTU/kg compared to when 500 FTU/kg was supplemented. These changes in the content of the higher IP esters and ileal Na and Fe may make conditions less suited for the colonisation of *Lactobacilli*, as suggested above, however no direct significant correlations were identified in the data.

3.4.8 Correlations

Considering the correlations identified within the data (Table 3.28), it appears that the bacterial populations are influenced by nutrient utilisation and the degree of phytate hydrolysis and the production of the lower IP esters (e.g. excreta IP4 and ileal Ca, r^2 = -0.66). These two factors are also interlinked, with nutrient bioavailability being influenced by the characterisation of phytate hydrolysis and interactions with nutrients in the digesta. Bacterial populations can also influence the gut morphological characteristics (and thus indirectly nutrient absorption), with correlations identified between ileal bacteria profiles and changes in ileal morphology (e.g. ileal *Escherichia* and ileal basal width and ileal villi surface area, r^2 = -0.79, -0.77, respectively; and ileal *Bifidobacterium* and duodenal basal width, r^2 = -0.76), and changes in the jejunal morphology being associated with changes in the caecal bacterial profiles (e.g. caecal *Escherichia* and jejunal apical width, villi surface area and villi height, r^2 = +0.66, +0.75 and +0.79, respectively), likely through a change in the nutrient profile entering the caeca. Baurhoo *et al.* (2007) suggested that bacteria such as *Lactobacilli* and *Bifidobacterium* can have an influence on gut morphological characteristics, such as villi height. Ileal *Escherichia* and caecal *Bacteroides* populations were identified to have a negative (r^2 = -0.69) and positive (r^2 =

+0.66), respectively, influence on BWG, which shows how all the aforementioned effects and interactions may lead to the observed changes in growth performance.

3.5 Conclusion

- Phytase supplementation was able to prevent the depressions in growth performance observed in the NC diet without phytase, being comparable to the PC, with or without phytase. No further improvements were seen with the high doses of phytase, likely explained by an increase in nutrients in the digesta following phytate hydrolysis, resulting in a nutrient imbalance in the digesta.
- An imbalance between Ca:aP is of particular importance, and may have led to the aP × phytase interaction observed on ileal nutrient digestibility.
- The high dietary Ca content may have also led to changes in the gut environment, such as pH.
- Despite this, there was no interaction on phytate hydrolysis in the ileum, with phytase decreasing IP6 and increasing inositol contents. However, the observed aP × phytase interaction on the content of IP3 to IP5 in the excreta may suggest changes in the gut environment, such as the microbial profile.
- Nutrient imbalance may be mediated by nutrient release from phytate hydrolysis and have further downstream effects on hydrolysis of the inositol phosphate esters and gut characteristics.
- The overall influence of phytase on broiler growth performance is the result of a multi-factorial influence, potentially initiating within the gut.
- Increased nutrient bioavailability following the supplementation of phytase and hydrolysis of phytate likely lead to changes in the environment of the gut.
- Changes in pH, intestinal morphology and bacterial populations, through a variety of interacting mechanisms, can lead to an overall influence of the production potential of the bird.
- Super-doses of phytase may be more suited to maintaining a good gut environment for these benefits to occur.

- The results suggest that phytate hydrolysis may ‘become stuck’ at IP4 when regular doses are used, potentially having a negative effect.
- Accumulation of IP4 may reduce mineral bioavailability, altering the nutrient profile of the digesta and gut health status, potentially resulting in the increased maintenance requirements observed in the first study.

3.6 Appendix

3.6.1 Animal housing

Birds were monitored at least twice daily, ensuring good health and that feed and water supplies were clean and adequate. Birds were wing-tagged at day 8 for individual identification. The house temperature was as detailed in the Ross broiler manual (Ross 308 Specifications, 2007), where ambient temperature (measured at chick height) was 30^{o}C, litter temperature 28-30^{o}C and humidity at 60-70%. At day 3, the temperature was decreased at a rate of 1^{o}C per day, so that on day 21, the temperature was 22^{o}C. For the first 7 days, the lighting regime was set to 23:1 light: dark hours, with 30-40 lux intensity. All procedures were approved by the SRUC Animal Experiment Committee prior to commencement.

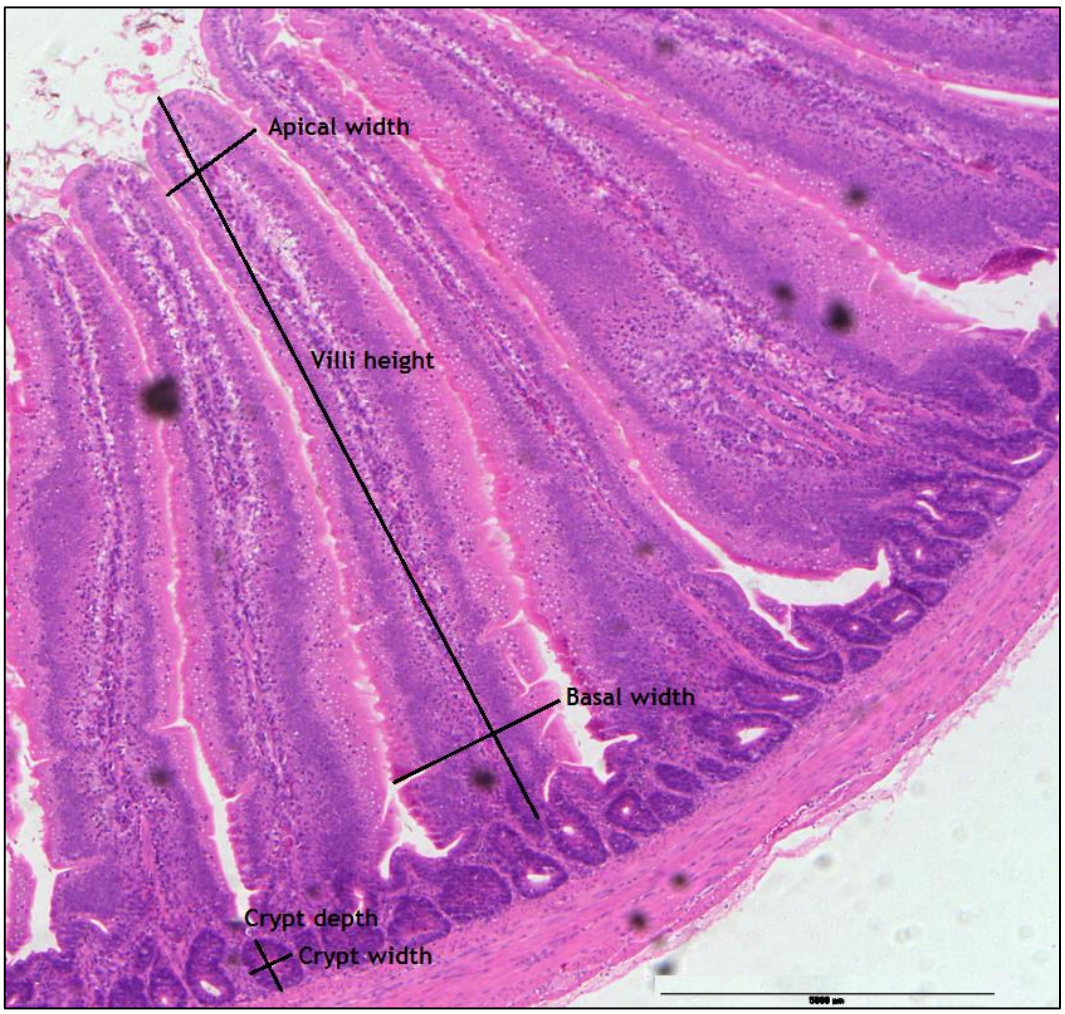
3.6.2 Enzyme information

The enzyme used was Quantum Blue, provided by AB Vista, with an initial activity level of 5,000 FTU/kg. The premix was made to 500 FTU/kg and added at the rate of 10 g/kg or 30 g/kg to give activity levels of 500 or 1,500 FTU/kg, respectively.

3.6.3 DNA concentration

1. Determine volume of current DNA sample
2. Add 1/10 of the sample volume of sodium acetate (3 molar, pH 5.2)
   e.g. if DNA sample is 100µl then add 10µl sodium acetate
3. Add double the volume of sample + sodium acetate of 100% ethanol
   e.g. 100µl DNA sample + 10µl sodium acetate, then add 220µl ethanol
4. Shake. Incubate at -20^{O}C overnight
5. Place in centrifuge for 15 minutes at max speed (at cool temperature)
6. Remove supernatant
7. Wash in 200-300µl ethanol (70%). Spin and remove supernatant.
8. Re-suspend in water (1/2 original sample volume)

3.6.4 Morphological measurements



Chapter 4.
Super-doses of phytase reduce the accumulation of intermediate inositol phosphate esters and thus reduce subsequent binding with minerals in the digesta

4.1 Introduction

The initial study looked at the effects of phytase on the broiler at the whole body level, whilst the second considered the effects within the gut, and the intestinal environment, and how this may be used to describe the influence observed in production parameters. Phytase and dietary aP influenced the environment of the gut (pH, morphology, bacterial profiles), with phytase appearing to have greater beneficial effects in diets deficient in aP. Influences on the environment of the gut may have subsequent effects on the efficacy of phytase and digestive enzymes, nutrient bioavailability and absorption, and ultimately animal health and the potential for production performance.

Additional benefits associated with extra-phosphoric effects of super-doses of phytase may be explained by changes occurring within the gut. Improvements in gut health and integrity may allow for suitable conditions for enhanced nutrient absorption, through increased expression of intestinal nutrient transporters (Vigors *et al.*, 2014). Phytate is an irritant to the gut, causing inflammation (Onyango *et al.*, 2009). Reducing concentrations of phytate can improve the health status of the gut by reducing inflammation and the nutrients and energy required to provide immune protection, maintaining gut integrity and facilitating nutrient absorption (Chen *et al.*, 2015; Liu *et al.*, 2008b; Vigors *et al.*, 2014).

Gut integrity is important for protection against pathogens and regulation of the absorption of ingested materials, influencing the nutrient status and overall health of the animal. Occludin is an important protein in the formation of tight junctions, which regulate mucosal permeability (Derikx *et al.*, 2010; Tsukita *et al.*, 2008; Turner, 2009). Toll-like receptor 2 (TLR2) is an immune molecule involved in regulation of the gut barrier integrity, which is upregulated to help restore barrier function when gut integrity is compromised (Derikx *et al.*, 2010;

Turner, 2009). The presence of glutathione peroxidase (GPX4) in the liver was selected as a response parameter due to its protection against anti-oxidant damage (Michiels *et al.*, 1994). Similarly, the expression of intestinal transporters involved in the absorption of nutrients released during phytate hydrolysis (divalent mineral, phosphate, calcium channels) are expected to increase following phytase supplementation (Vigors *et al.*, 2014).

The aim of this study was to investigate the influence of phytase on the environment of the gut, through the documentation of the effects of phytase on gut pH and intestinal gene expression.

4.2 Materials and Methods

4.2.1 Animals, diets and housing

On arrival birds were allocated to one of 6 treatments with 6 replicates per treatment in a Randomised Complete Block Design. The dietary treatments were randomly allocated within blocks and subsequently blocks were spatially randomised within the house. The dietary treatments were designed to have a 2 x 3 factorial arrangement where the factors were two levels of dietary available P (0.45% and 0.30%) and three levels of phytase supplementation (0, 500 and 1,500 FTU/kg). Enzyme information is provided in the Appendix 5.6.2. The positive control diet (0.45% aP) was formulated to meet Ross 308 energy and nutrient requirements. The negative control diet was formulated to be deficient from the PC by 0.15% aP and 0.16% Ca, with a matrix applied relative to the PC. The dietary treatments are as follows: (1) Positive Control, PC, aP at 0.45%; (2) PC + 500 FTU/kg phytase; (3) PC + 1,500 FTU/kg phytase; (4) Negative Control, NC, aP at 0.30%; (5) NC + 500 FTU/kg and (6) NC + 1,500 FTU/kg. Titanium was used as an indigestible marker. The treatment ingredient and nutrient contents are shown in Tables 4.1.

A total of 360, one-day-old Ross 308 broilers were used for the study. On arrival, all birds were housed on deep litter. There were 36 pens, each containing 10 birds, with 6 replications per treatment. Water and experimental diets were provided *ad libitum* basis from d0 to d21.

Table 4.1 – Ingredient and nutrient composition of the experimental diets

Description of diets	PC[1]	PC500	PC1500	NC[2]	NC500	NC1500
Phytase (FTU/kg)	0	500	1,500	0	500	1,500
Ingredients, g/kg						
Maize	423	423	423	435	435	435
Wheat	200	200	200	200	200	200
Soybean meal	259	259	259	259	259	259
Soybean oil	15	15	15	11	11	11
DCP[3]	18.2	18.2	18.2	10	10	10
Limestone[4]	9.9	9.9	9.9	11	11	11
Titanium dioxide	25	25	25	25	25	25
Maize gluten meal for	30	20	0	30	20	0
Enzyme premix[5]	0	10	30	0	10	30
Vitamin-mineral	5	5	5	5	5	5
Methionine	2	2	2	2	2	2
Lysine	5	5	5	5	5	5
Threonine	1.5	1.5	1.5	1.5	1.5	1.5
Salt NaCl	1.3	1.3	1.3	1.4	1.4	1.4
$NaHCO_3$	4.8	4.8	4.8	4.8	4.8	4.8
Total, g	1000	1000	1000	1000	1000	1000
Formulated nutrients						
Calcium %	1.00	1.00	1.00	0.84	0.84	0.84
Phosphorus %	0.69	0.69	0.69	0.54	0.54	0.54
aP, %	0.45	0.45	0.45	0.30	0.30	0.30
Crude protein %	23.0	23.0	23.0	23.1	23.1	23.1
ME MJ/kg	12.6	12.6	12.6	12.6	12.6	12.6
Lysine, g/kg	14.4	14.4	14.4	14.4	14.4	14.4
DM, %	87.2	87.2	87.2	87.2	87.2	87.2

Notes: [1]PC: Positive Control; [2]NC: Negative control; [3]Di-Calcium Phosphate: 25.7% Ca, 17.5% P; [4]Limestone: 38.9% Ca; [5]Enzyme premix is mixed with maize gluten meal and has an activity of 150 FTU/g; [6]Premix supplies the following per kg diet: Vit. A, 5484 IU; Vit. D3, 2643 ICU; Vit E,11 IU; Menadione sodium bisulfite,4.38 mg; Riboflavin, 5.49 mg; d-pantothenic acid, 11 mg; Niacin, 44.1 mg; Choline chloride, 771 mg; Vit B12, 13.2 ug; Biotin, 55.2 ug; Thiamine mononitrate, 2.2 mg; Folic acid, 990 ug; Pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug. Also contains per g of premix: Vit. A, 1828 IU; Vit. D3, 881 ICU; Vit E,3.67 IU; Menadione sodium bisulfite,1.46 mg; Riboflavin, 1.83 mg; d-pantothenic acid, 3.67 mg; Niacin, 14.69 mg; Choline chloride, 257 mg; Vit B12, 4.4 ug; Biotin, 18.4 ug; Thiamine mononitrate, 735 ug; Folic acid, 330 ug; Pyridoxine hydrochloride, 1.1 mg; I, 370 ug; Mn, 22.02 mg; Cu, 1.48 mg; Fe, 14.69 mg; Zn, 14.69 mg; Se, 100 ug.

All experimental procedures were approved by the SRUC Animal Experiment Committee (Appendix 4.6.1), in accordance with the Animals (Scientific Procedures) Act 1986. Sample size calculations are presented in Appendix 4.6.3.

4.2.2 Sample collection

4.2.2.1 Euthanasia

Two birds per pen, with body weights close to the pen average, were euthanased by overdose of barbiturate and were later used for gene expression studies. The remaining birds were euthanased by stunning and exsanguination and used for the ileal digesta collections. All birds were used for growth performance measurements.

4.2.2.2 Growth performance

All birds and feed were weighed on day of arrival and prior to euthanasia on day 21 for calculation of BWG, FI and FCR.

4.2.2.3 Nutrient utilisation

Ileal digesta were collected from 8 birds on day 21. Diet and digesta samples were ground through a 0.5 mm sieve and analysed for DM, N, minerals and nPP using AOAC procedures. Titanium concentration was determined using the method described by Short *et al*. (1996).

For determination of IP degradation, samples of digesta, feed and excreta were dried in a forced draught oven at 80^{o}C, for a minimum of 48 hours or until a constant dry weight was reached, and ground before being analysed by high-performance ion chromatography-based techniques (Blaabjerg *et al.*, 2010).

4.2.2.4 Gene expression studies

Jejunal and liver tissue samples were collected post-mortem from the 2 birds for the gene expression studies. Samples were placed into RNAlater® (Sigma-

Aldrich) prior to PCR analysis. Genes investigated include those responsible for nutrient transport, and markers of gut cell integrity and are shown in Table 4.2.

Selection of house-keeping genes

Initially, GAPDH was selected as the house-keeping gene (HKG) of choice, as it is regularly cited in the literature involving similar studies (Kono *et al.*, 2005; Ruhnke *et al.*, 2015; Wils-Plotz and Dilger, 2013), however, other studies suggest that GAPDH is not always the most suitable internal reference gene (Bohannon-Stewart, 2014; De Boever *et al.*, 2008; Kuchipudi *et al.*, 2012; Li *et al.*, 2011). Therefore, a number of HKG (TBP, PMM1, YWHAZ, 28S and GAPDH) were selected for testing and the two most suitable genes used for the calculation of the geomean, which is reported to be the most suitable function for the use of more than one HKG (Vandesompele *et al.*, 2002). Initially these genes were tested in the liver tissue and two suitable genes selected, YWHAZ and 28S and subsequently tested in the jejunum tissue to ensure they would be suitable for their use in both tissues. Primers, Table 4.3, were supplied by Eurofins genomics (Anzingerstraße, Ebersberg). Information regarding the primer design and experimental procedures can be found in the Appendix.

4.2.3 Statistical analyses

4.2.3.1 Growth performance

Overall BWG, FI and FCR were calculated from bird weight and feed intake data, corrected for mortality. The data were analysed using the general ANOVA function of Genstat (14th Edition, VSN International Ltd), with significance determined where $P \leq 0.05$. Where statistical significance was observed, means were separated using orthogonal contrasts.

Table 4.2 – Details of the genes selected for gene expression and their roles

Gene expression (level of) parameters investigated:		
Nutrient transporters:		
	SGLT1	Sodium-glucose co-transporter
	PepT1	Di-/tri-peptide transporter
	DMT1	Divalent metal transporter
	NaPi-IIb	Sodium-phosphate co-transporter
	TRPV6	Intestinal calcium channel
Gut integrity:		
	Occludin	Protein involved in tight junction formation
Immune response:		
	TLR2	Involved in pathogen recognition and innate
Liver anti-oxidant activity:		
	GPX4	Glutathione peroxidase level

Table 4.3 – Information on the primers selected for investigation, including melting temperature (Tm, °C) and molecular weight (MW, g/mol)

Oligo name	Forward (F) and Reverse (R) primers (length, bp)	Tm [°C]	MW, g/mol
TBP	F: GGCCCAGCAGTTTTTCGGGT (20)	61.4	6140
	R: GCCACTATTTGATCTAACTCCTGTTTAATCCAG (33)	65.8	10013
PMM1	F: CCGGGAGAAGTTTGTGGCAGC (21)	63.7	6527
	R: CGAAGAAGTGGATGGTGTCAAACCTC (26)	64.8	8044
YWHAZ	F: GAGTCGTCTCAAGTATCGAACAAAAGACG (29)	65.3	8944
	R: TTCTGCTTGCGAAGCATTGGG (21)	59.8	6468
28S	F: TTATTCCTGATAAACTGAAAAGGCAGAGGTTTATA (35)	63.6	10817
	R: AAGGACGAGTCACGTCTGCTTCATTC (26)	64.8	7946
GAPDH	F: TCTCCTGTGACTTCAATGGTGACAGC (26)	64.8	7937
	R: CTCCTTGGATGCCATGTGGACC (22)	64.0	6702
GPX4	F: TGAGGGCCAAGTGGTGAAAAGGTA (24)	62.7	7531
	R: TTCAAGGCAGGCCGTCATGG (20)	61.4	6158
Claudin-2	F: TGCCCTCGGTCCCGCAC (17)	62.4	5083
	R: CGTTTACCTTTTCATGGCCCTGCT (24)	62.7	7237
Occludin	F: ACAGCCCCTTGTTGGCCATGT (21)	61.8	6373
	R: GCGCGTCTTCTGGGCGAAG (19)	63.1	5861
IL-6	F: TGACGAAGCTCTCCAGCGGC (20)	63.5	6103
	R: GACCACTTCATCGGGATTTATCACCAT (27)	63.4	8194
IFNG	F: ACTTACAACTTGTTTGTTCTGTCTGTCATCATG (33)	64.5	10050
	R: TTCTCTACAATAATAGGTCCACCGTCAGCTAC (32)	66.9	9703
TLR2-2	F: AATTTGCACGGCCTTGGCTG (20)	59.4	6124
	R: TATGGGCCAGGTTTAACACCGTGATT (26)	63.2	8001
MUC2	F: GAGCACCACGCCTGTTACCAGC (22)	65.8	6665
	R: TCGATCCAATCAGTCCAGATGCAGT (25)	63.0	7626
NaPi-IIb	F: CAATCGCTACTAATGACGAATCAGAAA (28)	62.2	8559
	R: CATGGTCCACGTCACATTTCCTTCT (25)	63.0	7519
SLC15A1	F: CCTGCGGTGGGATGACAACTTT (22)	62.1	6766
	R: TGACTGCCTGCCCAATTGTATAGACA (26)	63.2	7930
TRPV6	F: GGGTACCTTTATTTGGTGATAGCAAACCC (29)	65.3	8908
	R: CTTGGCAGCTTGAAGGAGTGGG (22)	64.0	6871
SLC11A1[1]	F: CCCCGGATCATCCTGTGGC (19)	63.1	5741
	R: TGTCCAGGAAGAGGAAGACGAAGGT (25)	64.6	7829
SLC5A1[2]	F: TGACAATGCCGGAGTATCTGAGGAAG (26)	64.8	8084
	R: CAAATACAGATTCAGCCCCATGGC (24)	62.7	7291

*Note: sequence 5' → 3'; Purification by HPSF; *Gene of interest tested in jejunal tissue, except for GPX4 which was tested in the liver; [1]SLC11A1 is a variant/ alternate gene name of DMT1; [2]SLC5A1 is a variant/ alternate gene name of SGLT1*

4.2.3.2 Nutrient utilisation

Nutrient digestibility was calculated using the index method using the following equations:

$$Nutrient\ digestiblity\ (\%) = [1 - (\frac{Ti\ in\ diet}{Ti\ in\ sample}) \times (\frac{Nutrient\ in\ sample}{Nutrient\ in\ diet})] \times 100$$

Dry matter digestibility (DMD) was calculated by:

$$Dry\ matter\ digestibility\ (\%) = 1 - (\frac{Ti\ in\ diet}{Ti\ in\ excreta})$$

4.2.3.3 Inositol phosphate esters

Conversion of inositol phosphate esters from nmol per g DM to mg per g DM was calculated over a series of 3 equations, as follows:

$$(1): \text{ng per g DM} = \text{MW} \times \text{nmol per g DM}$$

$$(2): \mu\text{g per g DM} = \frac{\text{ng per g DM}}{1000}$$

$$(3): \text{mg per g DM} = \frac{\mu\text{g per g DM}}{1000}$$

IP flow (mg/g DM) was calculated by:

$$\text{mg per g DM} \times \frac{\text{Ti in diet}}{\text{Ti in ileum}}$$

4.2.3.4. Gene expression

The output generated by the RT machine (MxPro QPC software, Mx3000Pv4.10. Build 389, Schema 95 2007), was exported into excel, to allow calculations using the cT values. The threshold value was set at 1,000. The dissociation curve was used to consider the appropriateness of the individual samples. Where the dissociation curve of the individual sample did not conform with the majority of samples and overall dissociation curve output, it was considered as a failure and not used in data analysis. Data were analysed using the ΔΔcT Pfaffl technique (Pfaffl, 2004). First, the average of the replicates (A and B) were taken for each of the genes. The ΔcT for each gene was calculated by

subtracting the measured cT value of each gene from the average cT of that gene, before being used in the $2^{\Delta cT}$ equation. The geomean of the $2^{\Delta cT}$ values for YWHAZ and 28S were used in the calculation of the relative expression of each gene of interest. A log-2 transformation was applied to each of the relative expression outputs (except for that of NaPi-IIb which was already normally distributed) for normalisation prior to statistical analyses. Outliers in the HKG were considered following the $2^{\Delta cT}$ calculation, prior to determination of the geomean.

4.3 Results

The analysed nutrient content of the diets (Table 4.4) was similar to the formulated nutrient composition for all nutrients, except aP. The analysed aP content was lower than formulated in the PC treatmetnts, with some differences also occurring within the PC and NC treatments. The average analysed aP content of the PC diets was 0.36% compared to the formulated 0.45%, but 0.29% compared to 0.30% in the NC. Phytase levels were also similar to formulated.

4.3.1 Growth performance

The broiler growth performance data up to day 21 are presented in Table 4.5. Mortality remained low throughout the study (< 5%) and was not influenced by treatment ($P > 0.05$). It is apparent that the IBW of the birds differed significantly between the two levels of dietary aP. Growth performance data were reanalysed using IBW as a covariate. There was a significant aP × phytase interaction on BWG ($P < 0.05$). Phytase had no influence in the PC treatment but there was an increase in BWG of the birds fed the NC ($P < 0.001$) as phytase increased. The greatest BWG was in the NC + 1,500 FTU/kg treatment. There were no treatment effects on FI, however FCR improved with phytase supplementation ($P < 0.05$), decreasing linearly ($P < 0.05$) as phytase dose increased. At 1,500 FTU/kg, FCR was significantly improved compared to when phytase was absent, but comparable to when 500 FTU/kg was supplemented.

Table 4.4 – The calculated and analysed (%) nutrient composition of the six dietary treatments based in a positive and negative control diet, with the addition of 0, 500 or 1,500 FTU/kg phytase

Diets:	PC	PC500	PC1,500	NC	NC500	NC1,500
Calculated nutrients, %						
ME, kcal/kg	3000	3000	3000	3000	3000	3000
Crude protein	23.00	23.00	23.00	23.08	23.08	23.08
DM	87.17	87.17	87.17	87.04	87.04	87.04
Calcium	1.00	1.00	1.00	0.84	0.84	0.84
Phosphorus	0.69	0.69	0.69	0.54	0.54	0.54
Ca:P	1.4	1.4	1.4	1.6	1.6	1.6
aP	0.45	0.45	0.45	0.30	0.30	0.30
Phytate P	0.24	0.24	0.24	0.24	0.24	0.24
Phytase, FTU/kg	0	500	1,500	0	500	1,500
Analysed nutrients						
DM,	86.1	86.2	86.2	86.3	86.2	86.5
Calcium	0.97	0.99	0.92	0.90	0.84	0.88
Phosphorus	0.62	0.62	0.59	0.53	0.46	0.51
Ca:P	1.6	1.6	1.6	1.7	1.8	1.7
aP	0.37	0.38	0.34	0.33	0.25	0.30
Phytate P	0.25	0.24	0.25	0.20	0.21	0.21
Phytase, FTU/kg	<50	625	1600	<50	607	1510

Notes: DM: Dry matter; Ca:P, calcium: phosphorus ratio; aP: non-phytate phosphorus, calculated by: total phosphorus – phytate P; analysed aP calculated using analysed values of total P and phytate P.

Table 4.5 – Growth performance data of broilers fed diets either nutritionally adequate (PC), or marginally deficient (NC) in available phosphorus, with 0, 500 or 1,500 FTU/kg phytase supplementation, for 21 days, analysed using IBW as a covariate

aP, %	Phytase, FTU/kg	IBW (g)	BWG (g)	FI[1] (g/b)	FCR	Mortality[2], %
0.30	0	40.6	675[c]	1030	1.527	0
	500	40.7	718[bc]	1040	1.452	1.4
	1,500	40.9	777[a]	1088	1.400	1.7
0.45	0	41.0	742[ab]	1070	1.442	1.7
	500	41.3	770[ab]	1074	1.397	3.8
	1,500	41.3	772[ab]	1088	1.413	8.8
	SEM	0.182	13.13	26.34	0.0267	2.43
0.30		40.7[b]	723.6	1053.2	1.460	0.8
0.45		41.2[a]	761.6	1078.0	1.417	4.7
	SEM	0.105	8.50	17.05	0.017	1.57
	0	40.8	708.7	1050.8	1.485[a]	0.6
	500	41.0	744.5	1057.6	1.424[ab]	2.6
	1,500	41.0	774.6	1088.4	1.407[b]	5.2
	SEM	0.129	9.08	18.22	0.018	1.68
P values						
aP		0.005	0.023	0.407	0.207	0.225
Phytase		0.275	<0.001	0.318	0.018[3]	0.352
aP × phytase		0.839	0.020	0.703	0.167	0.815
	Covariate		*0.057*	0.890	*0.077*	0.774

Note: [a-b] Means within columns with different superscripts are significantly different for each variable, (P < 0.05); polynomial contrasts run on treatment means. BWG: Body weight gain; FI: Feed intake; FCR: Feed conversion ratio. [1]Feed intake was corrected for mortality through calculation of bird-days. [2]Statistical analyses of mortality % was performed following the application of 1/(sqrtX+0.1) to allow for analysis of zero mortality; [3]Phytase, linear P = 0.013, quadratic P = 0.139.

4.3.2 Nutrient utilisation

The ileal coefficients of digestibility of DM, Ca, Fe, Na, Zn, Mn, P, and N are shown in Tables 4.6 and 4.7. Pen 1 was excluded from analyses due to a number of outliers, including Ti/To (Ti in diet/ Ti in sample, i.e. ileal content or excreta), which was used to calculate the other digestibility values and may increase the error of these points.

There was significant aP × phytase interaction on the ileal coefficient of digestibility of Ca, Fe, Na, P and Zn ($P < 0.05$).

Sodium digestibility increased with 1,500 FTU/kg phytase in the NC, but phytase had no effect at either dose in the PC treatments ($P < 0.01$).

There were no changes in P digestibility following phytase supplementation in the NC or PC treatments, however, digestibility was significantly greater in the NC + 1,500 FTU/kg phytase treatment than the PC ($P < 0.05$).

Calcium digestibility decreased in the NC treatment at both levels of phytase, whilst there were no changes compared to the PC when phytase was added, although digestibility was greater in the PC + 500 FTU/kg than the PC + 1,500 FTU/kg treatment ($P \leq 0.001$).

The addition of 1,500 FTU/kg decreased Fe digestibility in the NC treatments, but 500 FTU/kg had no effect ($P \leq 0.001$). In the PC treatments, 500 FTU/kg increased whilst 1,500 FTU/kg decreased Fe digestibility. With 1,500 FTU/kg supplementation, Fe digestibility was comparable between the NC and PC treatments. Supplementation of 1,500 FTU/kg phytase to the NC treatment increased Zn digestibility above the NC without phytase, whereas in the PC treatment, 500 FTU/kg phytase was required to increase digestibility above the PC, which was not achieved with 1,500 FTU/kg.

Table 4.6 – Coefficients of ileal nutrient digestibility of broilers fed either 0.45% or 0.30% aP with 0, 500 or 1,500 FTU/kg phytase for 21 days.

aP, %	Phytase, FTU/kg	Ca	DM	N	P
0.30	0	0.63[a]	0.71	0.82	0.59[ab]
	500	0.52[b]	0.70	0.81	0.56[abc]
	1,500	0.51[b]	0.75	0.80	0.63[a]
0.45	0	0.35[cd]	0.73	0.82	0.49[c]
	500	0.44[bc]	0.76	0.82	0.56[abc]
	1,500	0.33[d]	0.76	0.83	0.54[bc]
	SEM	0.025	0.009	0.008	0.020
0.30		0.55	0.74[b]	0.81	0.60
0.45		0.37	0.75[a]	0.82	0.53
	SEM	0.014	0.005	0.005	0.011
	0	0.49	0.72[a]	0.82	0.54
	500	0.48	0.75[b]	0.82	0.56
	1,500	0.42	0.76[b]	0.82	0.58
	SEM	0.018	0.006	0.006	0.014
P values					
aP		<0.001	0.048	*0.095*	<0.001
Phytase		0.022	0.001[1]	0.940	0.111
aP × phytase		0.001	0.713	0.137	0.026

Notes: [a-b] Means within columns with different superscripts are significantly different for each variable, (P < 0.05); [1]Phytase, linear P = 0.001, quadratic P = 0.045; Ca: Calcium; DM: Dry matter; N: Nitrogen; P: Phosphorus.

Table 4.7 – Coefficients of ileal mineral digestibility of broilers fed either 0.45% or 0.30% aP with 0, 500 or 1,500 FTU/kg phytase for 21 days.

aP, %	Phytase, FTU/kg	Fe	Mn	Na	Zn
0.30	0	0.21^{d}	-0.15	-0.37^{c}	-0.08^{c}
	500	0.14^{d}	-0.10	-0.22^{bc}	0.02^{bc}
	1,500	0.45^{c}	-0.05	0.20^{a}	0.20^{b}
0.45	0	0.70^{b}	-0.20	-0.03^{ab}	0.03^{bc}
	500	0.80^{a}	-0.04	0.08^{ab}	0.40^{a}
	1,500	0.45^{c}	-0.19	0.01^{ab}	0.09^{bc}
	SEM	0.020	0.059	0.070	0.045
0.30		0.27	-0.10	-0.13	0.05
0.45		0.65	-0.15	0.02	0.17
	SEM	0.011	0.034	0.041	0.026
	0	0.46	-0.18	-0.20	-0.02
	500	0.47	-0.07	-0.07	0.21
	1,500	0.45	-0.12	0.10	0.14
	SEM	0.014	0.042	0.050	0.032
P values					
aP		<0.001	0.392	*0.017*	0.002
Phytase		0.440	0.223	<0.001	<0.001
aP × phytase		<0.001	0.251	0.002	<0.001

Notes: $^{a-b}$ Means within columns with different superscripts are significantly different for each variable, (P < 0.05); Fe: Iron; Mn: Manganese; Na: Sodium; Zn: Zinc.

Phytase increased DMD (P < 0.05), but was comparable between 500 and 1,500 FTU/kg. Reducing the dietary aP% had the effect of reducing DMD (P < 0.05). There were no treatment effects on the ileal digestibility of Mn or N.

4.3.3 Phytate hydrolysis

Table 4.8 shows the data on the content of the different IP esters in the ileal digesta of the broilers. There were no aP × phytase interactions on the content of the different IP esters in the ileum. The ileal content of IP6, IP5 and IP4 were lower (P < 0.05), with IP3 also tending to be lower (P = 0.08), and inositol being greater (P < 0.01) in the NC compared to the PC. Phytase supplementation linearly (P < 0.01) reduced IP6 and IP5 and linearly increased (P < 0.05) IP3 and inositol.

The data on flow of inositol phosphate esters in the ileal digesta are shown in Table 4.9; there were no aP × phytase interactions. The ileal flow of IP6 (P < 0.01) and IP5 were, and the IP4 flow tended to be (P = 0.07), lower in the NC compared with PC. There was also an increase in flow of inositol (P < 0.001) in the low aP treatments. Phytase linearly decreased IP6 flow and both doses (P < 0.001), but required 1,500 FTU/kg to decrease IP5 compared to when phytase was absent (P < 0.01). Also, 1,500 FTU/kg phytase tended to increase inositol flow (P = 0.082).

4.3.4 Gene expression

4.3.4.1 Selection of house-keeping genes

Five genes were selected to be tested for their suitability as housekeeping genes in the liver tissue: TBP, PMM1, YWHAZ, 28S and GAPDH. The dissociation curves of the PCR product of these genes in the liver tissue are shown in Figure 4.1, A-E. As indicated by the red arrows against curves for PMM1 and GAPDH (B-C), there was more than one peak identified on the dissociation curve, therefore these genes were excluded from consideration for use as a HKG.

Table 4.8 – The content of the different inositol phosphate (IP) esters and inositol in the ileum, mg/g DM.

Ileum		mg/g DM					
aP, %	Phytase	IP6	IP5	IP4	IP3	IP2	Inositol
0.30	0	7.3	1.47	0.35	0.18	0.59	1.20
	500	2.8	0.75	0.60	0.22	0.60	1.31
	1,500	1.8	0.65	0.81	0.25	0.59	1.42
0.45	0	9.9	1.62	0.30	0.14	0.55	0.40
	500	6.8	1.59	1.20	0.33	0.59	0.54
	1,500	2.0	0.93	1.92	0.50	0.62	0.75
	SEM	0.98	0.23	0.29	0.06	0.05	0.10
0.30		3.97[b]	0.95[b]	0.59[b]	0.22	0.60	1.31[a]
0.45		6.25[a]	1.38[a]	1.14[a]	0.32	0.58	0.56[b]
	SEM	0.56	0.14	0.17	0.04	0.03	0.06
	0	8.61[a]	1.54[a]	0.33	0.16[b]	0.57	0.80[b]
	500	4.83[b]	0.17[b]	0.90	0.27[ab]	0.60	0.93[ab]
	1,500	1.89[b]	0.79[ab]	1.37	0.38[a]	0.61	1.09[a]
	SEM	0.69	0.17	0.21	0.04	0.04	0.07
P values							
	aP	0.004	0.005	0.049	*0.084*	0.589	<0.001
	Phytase	<0.001	0.008	0.371	0.046	0.828	0.037
	aP × phytase	0.395	0.404	0.240	0.154	0.822	0.797
Phytase	Linear	<0.001	0.003		0.025		0.012
	Quadratic	0.293	0.286		0.271		0.749

Notes: Data log transformed for statistical analyses (except inositol); [a-b] Means within columns with different superscripts are significantly different for each variable, (P < 0.05); IP6: inositol phosphate 6, number refers to number of phosphate molecules bound; Inositol is the final product of phytate hydrolysis and has no bound phosphate molecules.

Table 4.9 – The flow of the different inositol phosphate (IP) esters and inositol through the ileum, mg/g DM.

		Flow: mg/g DM					
aP, %	Phytase	IP6	IP5	IP4	IP3	IP2	Inositol
0.30	0	1.89	0.38	0.10	0.05	0.17	0.36
	500	0.69	0.18	0.14	0.05	0.15	0.34
	1,500	0.42	0.15	0.19	0.06	0.15	0.35
0.45	0	2.51	0.42	0.08	0.04	0.14	0.11
	500	1.57	0.36	0.27	0.08	0.14	0.14
	1,500	0.48	0.22	0.44	0.12	0.15	0.18
	SEM	0.25	0.06	0.07	0.015	0.01	0.03
0.30		1.00[b]	0.24[b]	0.14	0.06	0.16	0.35[a]
0.45		1.52[a]	0.33[a]	0.26	0.08	0.14	0.14[b]
	SEM	0.14	0.03	0.04	0.009	0.008	0.02
	0	2.20[a]	0.40[a]	0.09	0.05	0.16	0.24
	500	1.13[b]	0.27[ab]	0.21	0.07	0.15	0.24
	1,500	0.45[b]	0.18[b]	0.31	0.09	0.15	0.27
	SEM	0.17	0.04	0.05	0.01	0.009	0.02
P values							
	aP	0.006	0.008	0.067	0.152	0.241	<0.001
	Phytase	<0.001	0.004	0.533	0.155	0.598	0.082
	aP × phytase	0.417	0.390	0.221	0.143	0.637	0.095
Phytase	Linear	<0.001	0.002				0.028
	Quadratic	0.205	0.214				0.802

Notes: Data log transformed for statistical analyses; [a-b] Means within columns with different superscripts are significantly different for each variable, (P < 0.05); IP6: inositol phosphate 6, number refers to number of phosphate molecules bound; Inositol is the final product of phytate hydrolysis and has no bound phosphate molecules.

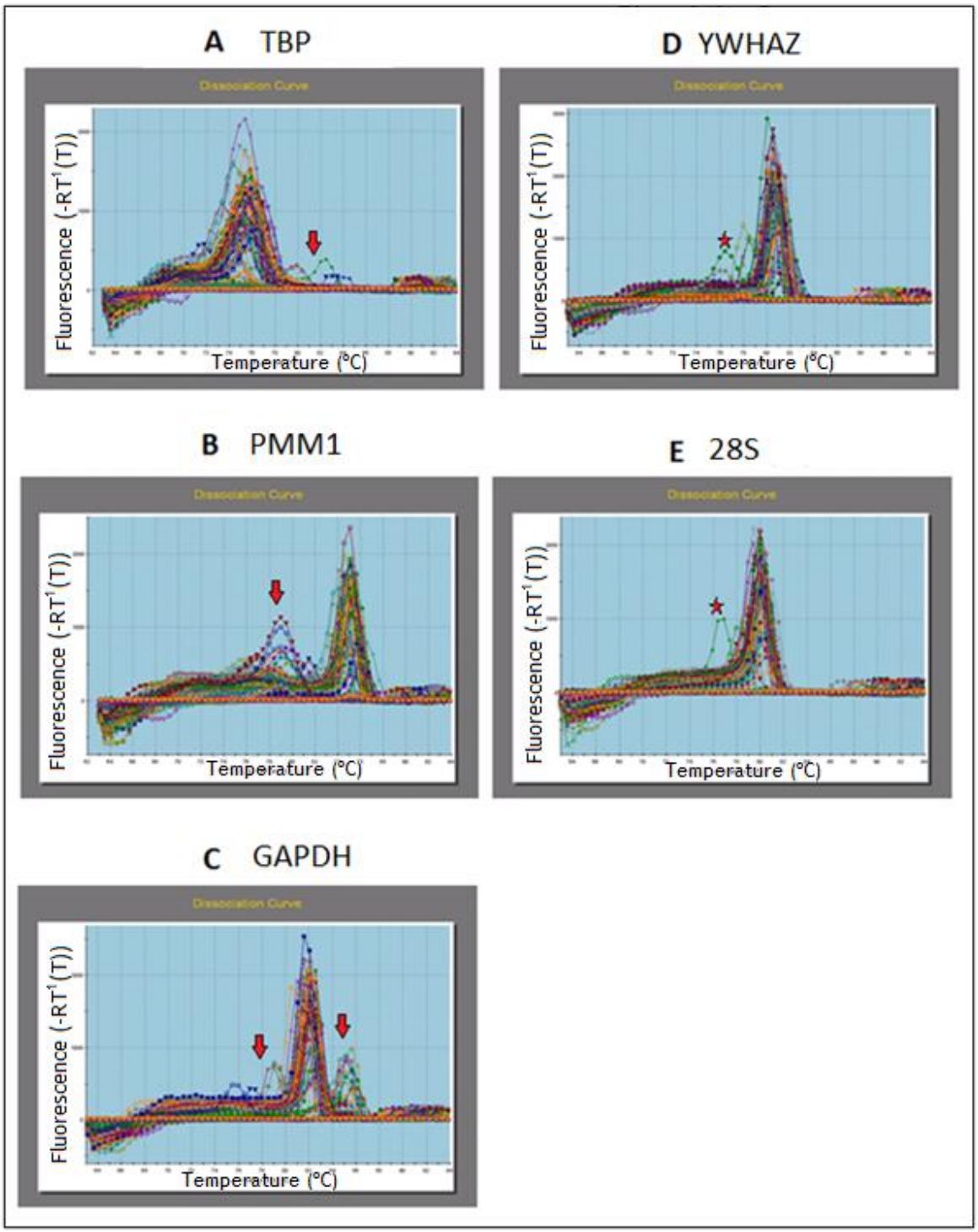


Figure 4.1- Testing of suitable candidates for use as housekeeping genes for gene expression studies in liver tissue. Dissociation curve outputs from RT PCR of TBP (A), PMM1 (B), GAPDH (C), YWHAZ (D) and 28S (E) in the liver tissue.

For the dissociation curve of TBP (A), although there is just one peak, there is also a larger spread of curves around the peak, therefore this gene was also excluded for use as a HKG. Aside from one or two outlying curves, identified by the stars against points on the curves for YWHAZ and 28S (D and E), there appeared to be a single, tight-fitting peak for the dissociation curve, therefore these genes were considered as suitable candidates for their use as a HKG in the liver.

Using this information, YWHAZ and 28S were subsequently tested in the jejunal tissue to ensure that they would be suitable for HKG in this tissue too. GAPDH was also tested in order to test the suitability of this gene in broiler gene expression studies, as this gene is commonly used as a reference gene. The dissociation curves of the PCR product of these genes tested in the jejunal tissue are shown in Figure 4.2.

Further information regarding gene expression, such as replicate size and primer success rates, can be found in the Appendix.

4.3.4.2 Liver gene expression

There were no significant aP × phytase interactive or main effects on the expression of GPX4 in the liver (Table 4.10), although the phytase supplementation was close to having a tendency for an effect ($P = 0.131$), with expression increasing by 2.2 fold with 1,500 FTU/kg phytase compared to when it was absent.

4.3.4.3 Jejunal gene expression

The data on expression of various genes involved in gut cellular integrity and nutrient transport are presented in Table 4.11. There were no significant aP × phytase interactions on the expression of any of the genes investigated. Phytase decreased the expression of the divalent mineral transporter DMT1 (quadratic, $P \leq 0.001$), decreasing with 500 FTU/kg, but being comparable between 0 and 1,500 FTU/kg phytase.

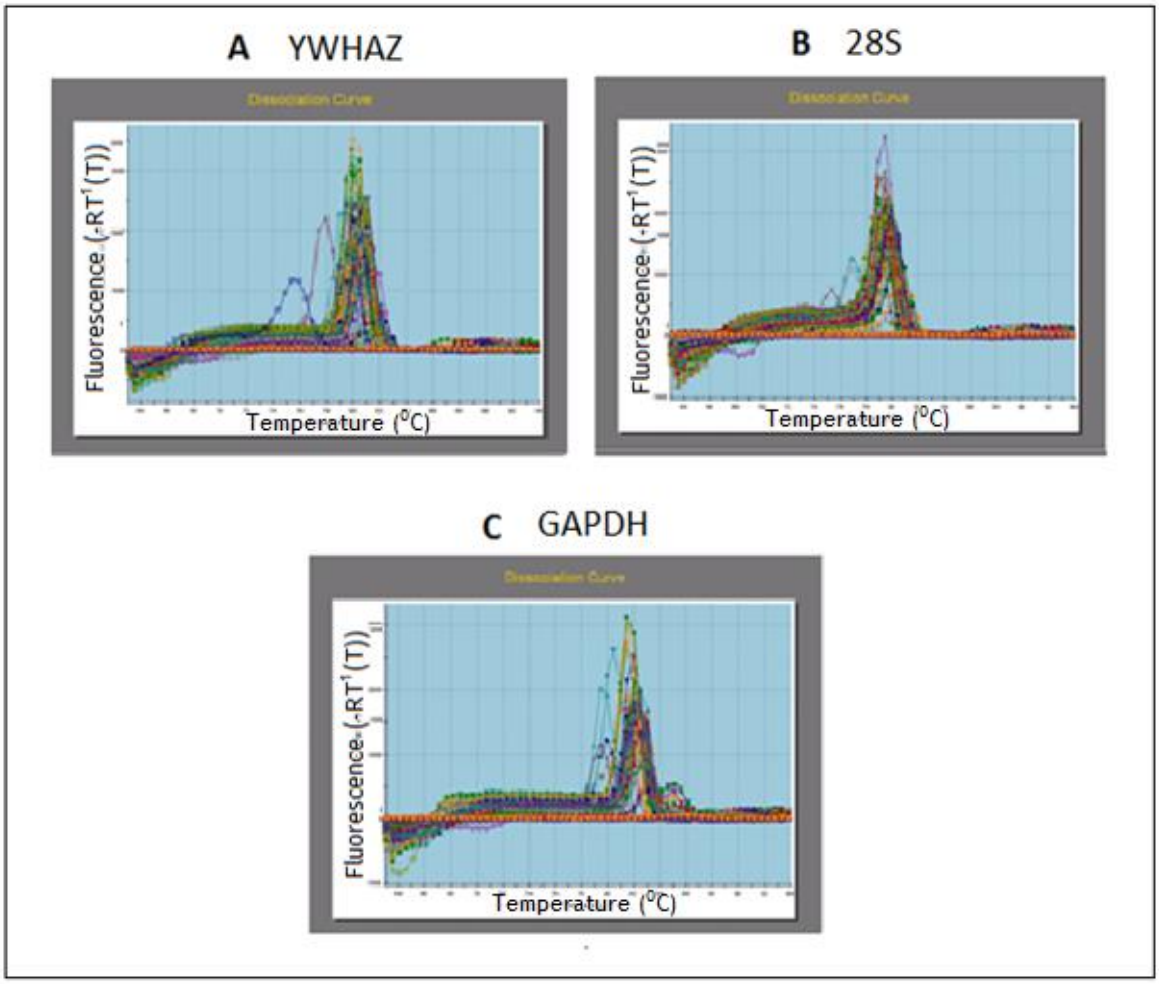


Figure 4.2- Testing of suitable candidates for use as housekeeping genes for gene expression studies in jejunal tissue. Dissociation curve outputs from RT PCR of YWHAZ (A), 28S (B) and GAPDH (C) in the liver tissue.

Table 4.10 – The expression of glutathione peroxidase (GPX4) relative to 2 housekeeping genes (28S and YWHAZ) in the liver of broilers fed diets containing 0.45% or 0.30% aP and either 0, 500 or 1,500 FTU/kg phytase for 21 days

aP, %	Phytase	GPX4
0.30	0	1.69
	500	3.63
	1,500	3.27
0.45	0	2.39
	500	1.28
	1,500	5.50
	SEM	1.47
0.30		2.86
0.45		3.06
	SEM	0.85
	0	2.04
	500	2.45
	1,500	4.38
	SEM	1.04
P values		
	aP	0.614
	Phytase	0.131
	aP × phytase	0.697

Notes: Values presented are ΔCt values normalised against the two reference genes (28S and YWHAZ) using the Pfaffl technique; Data was log-2 transformed prior to analysis.

Table 4.11 – The expression of genes involved in gut integrity and nutrient transport in the jejunum, relative to 2 housekeeping genes (28S and YWHAZ), of broilers fed diets containing 0.45% or 0.30% aP and either 0, 500 or 1,500 FTU/kg phytase for 21 days.

aP, %	Phytase	Occludin	TLR2	NaPi-IIb	PepT1	TRPV6	DMT1	SGLT1
0.30	0	1.75	0.77	0.59	1.52	0.51	1.38	1.56
	500	0.85	2.09	0.46	2.11	0.78	0.43	2.37
	1,500	1.01	1.25	0.49	3.01	0.64	1.10	2.52
0.45	0	2.70	1.71	0.47	2.09	0.54	1.37	2.11
	500	1.15	1.03	0.55	1.07	1.73	0.58	1.42
	1,500	1.44	0.53	0.44	2.58	0.73	1.21	2.78
	SEM	0.50	0.63	0.11	0.72	0.31	0.31	0.90
0.30		1.20	1.37	0.51	2.21	0.64	0.97	2.15
0.45		1.76	1.09	0.49	1.92	1.00	1.05	2.11
	SEM	0.29	0.37	0.06	0.42	0.18	0.18	0.52
	0	2.22	1.24	0.53	1.81	0.52	1.38	1.84
	500	1.00	1.56	0.51	1.59	1.26	0.50	1.89
	1,500	1.22	0.89	0.46	2.80	0.68	1.16	2.65
	SEM	0.35	0.45	0.08	0.51	0.22	0.22	0.64
P values								
aP		0.104	0.497	0.772	0.188	0.126	0.628	0.127
Phytase		0.203	0.330	0.810	*0.089*	0.119	0.001	0.157
aP × phytase		0.926	0.329	0.610	0.982	0.744	0.844	0.962
Phytase	Linear				*0.328*		0.981	
	Quadratic				*0.048*		<0.001	

Notes: *Values presented are ΔCt values normalised against the two reference genes (28S and YWHAZ) using the Pfaffl technique; Data was log-2 transformed prior to analysis (except NaPi-IIb).*

There was also a tendency (P = 0.089) for phytase to decrease (quadratic, P < 0.05) the expression of PepT1 (peptide transporter), and a trend for occludin expression to decrease in the low aP diets (P = 0.104). There were no treatment effects on the expression of NaPi-IIb, TRPV6 (calcium channel) or SGLT1 (sodium-glucose co-transporter), or the immune protein TLR2.

4.3.5 Correlations

A number of correlations within the data were identified (Table 4.12), including nutrient transporters, as well as with IP esters. The expression of DMT1 was correlated with the expression of SGLT1 (r^2 = + 0.67; P < 0.001) and TRPV6 with occludin (r^2 = -0.71; P < 0.01). The ileal IP2 content was also correlated with DMT2 expression (r^2= + 0.67; P < 0.01), ileal IP4 was correlated with GPX4 expression (r^2 = +0.75; P < 0.01) and IP5 with N digestibility (r^2 = +0.66; P < 0.01).

Table 4.12 - Significant correlations identified between the parameters measured throughout the study

Variable 1	Variable 2	r^2	P value
SGLT1	DMT1	+ 0.67	< 0.001
IP2	DMT1	+ 0.67	< 0.004
IP4	GPX4	+ 0.75	< 0.007
IP5	N digestibility	+ 0.66	< 0.010
TRPV6	Occludin	- 0.71	< 0.011

4.4 Discussion

Super-doses of phytase may indirectly lead to improvements in growth performance and nutrient utilisation through extra-phosphoric effects. These effects may occur through the reduction in phytate concentrations, reducing the anti-nutrient and inflammatory effects of phytate within the gut. Improved environmental conditions in the gut may increase the potential for nutrient absorption, possibly through increased gene expression of nutrient transporters, increasing nutrient bioavailability and leading to the observed improvements in production parameters.

In Chapter 2 of this thesis, phytase was demonstrated to influence the partitioning of nutrients within the carcass and proportion of energy required for maintenance, suggesting that there were changes occurring at a metabolic level. In this chapter, the intention was to discuss the changes which may be occurring on nutrient transport and thus utilisation and production potential. This discussion is split to focus on the expression of genes involved in the different aspects of health and nutrient absorption parameters within the gut.

4.4.1 Growth performance

Phytase supplementation to diets low in aP prevented P-deficiency-associated depressions in growth performance, with the birds having comparable growth performance to those receiving nutritionally-adequate diets. As there were improvements in ileal nutrient utilisation following the dietary addition of phytase to both the PC and NC in the absence of performance benefits in the PC, this may suggest there are alternative uses of nutrients within the body aside from production.

4.4.2 Gene expression

Reducing the concentrations of phytate in the gut may lead to the observed improvements through a reduction in inflammation, therefore reduced immune stimulation, allowing improved gut integrity and potential for enhanced nutrient absorption. Increased nutrient bioavailability may lead to changes in expression of genes involved in nutrient transport and nutrients available for growth

processes. Full phytate hydrolysis and increased inositol concentrations may lead to increased protection against anti-oxidant damage, as measured by the expression of GPX4 in the liver in this study. The following discussion on gene expression will be split into sections according to the overall function. Gut integrity will cover tight junctions, markers of immune response and liver anti-oxidant activity. Nutrient utilisation and transport will be discussed together.

4.4.3 Gut integrity

Gut integrity is an important factor which affects gut health, nutrient transport and immune function. The gut is a barrier to the rest of the body from ingested pathogens, but also facilitates the uptake and utilisation of nutrients. As investigated in Chapter 3, gut integrity can be defined by morphological characteristics such as those of the villi length and crypt depth, but also extends to the characteristics of the tight junctions (TJ) throughout the intestine (Turner, 2009), as investigated in the current study. The passage of digesta through the gut exposes the intestinal lumen epithelial cells to bacteria and potential antigens.

Occludin, an intercellular barrier-forming protein, is an important functional component of tight junctions and cell adhesion which interacts with TJ-associated proteins (Derikx *et al.,* 2010; Furuse *et al.,* 1993; Mitic and Anderson, 1998). Occludin is bound to ZO-1 and ZO-2 on the cytoplasmic junction (Anderson and Itallie, 1995; Tsukita *et al.*, 2008), which are important proteins required for the formation of tight junctions (Shen *et al.*, 2011; Tsukita *et al.*, 2008). Permeability of the tight junctions can be influenced by physiological and pathophysiological stimuli, such as nutrients, cytokines and bacteria (Capaldo and Nusrat, 2009; Turner, 2009), kinases, Ca^{2+}, phospholipases and calmodulin, amongst others (Mitic and Anderson, 1998), through cytoskeletal modifications and altered binding between ZO-1 and occludin (Capaldo and Nusrat, 2009; Shen *et al.*, 2011).

The activation of TLR2 may help restore barrier function (Derikx *et al.,* 2010; Turner, 2009), and hence TLR2 was selected as a marker of immune function due to its involvement in gut barrier integrity. Inefficient regulation of gut integrity

and barrier function can lead to poor health and subsequent disease. Should pathogens enter the body through the intestine then this can lead to cellular damage. An increase in TLR2 expression is associated with an increase in the detection of pathogens in the gut (Sansonetti, 2004). Pathogenic bacteria such a C*lostridium difficile* and *Bacteroides fragilis* adhere to the gut cells and secrete toxins, which influence the transcription of pro-inflammatory genes, whereas commensal bacteria are not expected to initiate this response (Sansonetti, 2004). The lack of treatment effect on TLR2 expression suggests there were no negative influences on the intestinal bacteria following phytase supplementation (which fits with the bacterial profiling results in Chapter 3). Low level TLR2 expression is expected in a normal intestine with low bacterial counts (Sansonetti, 2004).

In the current study, the lack of treatment influence on TLR2 and GPX4 (which was selected as a marker for protection against anti-oxidant damage in the liver (Michiels *et al.*, 1994)) did not help to explain the increased maintenance requirement observed with 1,500 FTU/kg in Chapter 2. This suggests that the increased maintenance requirements were not a result of gut inflammation (which would be expected to increase TLR2 expression) or oxidative damage (which would be expected to increase GPX4 expression), but could result from altered metabolism or processes such as muscle maintenance. However it would be beneficial to investigate a more comprehensive list of genes to reach this conclusion with confidence. Similarly, the lack of treatment effect on the expression of occludin as a measure for gut integrity did not indicate any damage to the gut which would increase maintenance requirements.

One reason for the lack of effect on gene expression throughout the study, particularly on immune response and liver anti-oxidant activity, may be that the birds were healthy and not pathogenically or immunologically challenged. An increase in GPX4 would be expected following an increased pathogenic or immunologic threat or in response to oxidative damage. As there were no treatment effects on the expression of GPX4, this confirms that, as suspected, the birds were healthy. There was a positive correlation between the ileal IP4 content and GPX4 expression (r^2 = +0.75, $P < 0.001$; Table 5.13). If we consider the latter points that at regular doses exogenous phytase activity ceases at IP4,

then the accumulation of IP4 in the gut may lead to irritation, oxidative stress and the initiation of an immune response, consequently increasing the expression of GPX4. If the dietary IP4 levels were higher, then perhaps a significant increase in GPX4 may have been observed in the current study.

4.4.4 Nutrient transport

If intestinal environmental conditions allow, then an effective way of increasing the capacity for nutrient absorption would be to increase the number of nutrient transporters present in the intestine. Of particular importance as far as phytase is concerned, due to the known affinity for binding to divalent minerals and potential influence on protein and glucose utilisation, are those transporters which require Na for their function or those involved in the transport of minerals or peptides.

A study by Vigors *et al*. (2014) involved the addition of phytase to diets with low P levels and explored the influence on gene expression in the jejunum and ileum. The authors found that there was a tendency for a treatment influence on the expression of the protein transporter SLC7A1 and mineral transporters TRPV6 and SLC34A2 in the jejunum. No treatment effects on the TRPV6 expression were observed in the current study. Vigors *et al*. (2014) also observed the effects on ileal nutrient transporters, and found that there were phytase induced increases in the ileal expression of the fatty acid transporter FABP2, PepT1, TRPV6 and phosphorus-transporter SLC34A2, when 1,020 FTU/kg phytase was supplemented to pig diets with low dietary P. Perhaps the differences in the negative control treatments and species between the studies explains the differences in results, but suggests that under certain dietary conditions, there may be some phytase influences on the expression of jejunal, and more prominently ileal, nutrient transporters.

4.4.4.1 Phosphate

One of the most relevant transporters investigated, considering its role in the absorption of Pi and the role of phytase in releasing Pi and Na from phytate, is the sodium-phosphate co-transporter NaPi-IIb. The NaPi-IIb co-transporter is

present in many tissues, including the small intestine, where it is found at the brush border membrane of enterocytes for the absorption of inorganic phosphate (Hilfiker *et al.*, 1998). The NaPi-IIb co-transporter is important to regulate the uptake and balance of Pi, and is influenced by pH (Hilfiker *et al.*, 1998) and dietary Pi content (Hattenhauer *et al.*, 1999). When dietary Pi is low, the abundance of the co-transporters at the brush border membrane is increased to enhance intestinal Pi absorption (Hattenhauer *et al.*, 1999). Liu *et al.* (2016) reported that NaPi-IIb was upregulated when NPP was decreased to 0.18%. Therefore, the supplementation of phytase and subsequent release of Pi would be expected to influence the expression of NaPi-IIb to facilitate uptake of the increased concentrations of Pi. The lack of treatment effect in the current study was not as expected, as not only was there an increase in Na and P digestibility which would suggest an increase in NaPi-IIb transporters, but in phytase studies NaPi-IIb is commonly observed to be influenced by phytase supplementation. Differences in treatment influences between this study and that of Hattenhauer *et al.* (1999) may be due to the dietary composition, method of analysis (PCR vs. blotting) but also that they measured transport at the brush border membrane, whereas sections of jejunual tissue were analysed in this study. Vigors *et al.* (2014) observed that there was correlation between changes in expression of genes involved in nutrient transport and changes in nutrient digestibility, but little statistical significance in the data to back it up.

The lack of treatment effect, particularly of phytase supplementation, on the expression of NaPi-IIb is surprising, due to the primary role of phytase in releasing P from phytate. Many studies have highlighted the effect of diets low in P on increasing NaPi-IIb expression (Hattenhauer *et al.*, 1999; Li *et al.*, 2012) and phytase-mediated adaptations to P transporters (Huber *et al.*, 2015). The content of Ca and Ca:P ratio in the digesta can also have an important influence on the expression of NaPi-IIb (Li *et al.*, 2012), so perhaps in the current study, the environmental conditions were not optimal (or detrimental) enough to lead to changes in NaPi-IIb expression. However, Hattenhauer *et al.* (1999) suggested that not all changes in NaPi-IIb transporters were induced by a change in gene expression, but an increase in the number of transporters in the intestine. These results suggest changes in the post-transcriptional regulation, and that the

dietary levels and time exposed to these levels can influence the regulation of nutrient transporters (Hattenhauer *et al.*, 1999; Saddoris, 2007).

4.4.4.2 Protein

For protein absorption, intestinal PepT1 (Fei *et al.*, 1994; Gilbert *et al.*, 2007; Leibach and Ganapathy, 1996) expression was measured, with expression expected to increase following phytase supplementation (Vigors *et al.*, 2014). Phytate hydrolysis would prevent any potential complexes forming between protein and phytate, increasing protein concentrations in the digesta. The expression of the PepT1 transporter has been reported to be influenced by dietary protein concentrations, being upregulated during periods of high or low dietary protein, through effects on gene expression and mRNA stability (Boudry *et al.*, 2010; Shiraga *et al.*, 1999). The influence of phytase on protein utilisation is still a controversial issue, with many alternate views highlighted in the literature (Cowieson *et al.*, 2004; Cowieson and Ravindran, 2007; Peter and Baker, 2001; Selle *et al.*, 2000). However, the tendency for a dose dependent phytase effect on PepT1 expression in the current study suggests a phytase induced influence on protein bioavailability and capacity for absorption. Although not statistically significant, there was numerically greater expression in the PepT1 transporter with 1,500 FTU/kg phytase, which suggests that at super-doses, phytase sufficiently hydrolyses phytate and reduces its binding with protein, increasing its concentration and bioavailability in the digesta. Additionally, the positive correlation between ileal IP5 content and ileal N digestibility (r^2 = +0.66, $P < 0.001$; Table 5.13), suggests that there may be some influence of phytase on protein utilisation under certain conditions.

4.4.4.3 Glucose

The sodium-glucose co-transporter SGLT1 was selected to indicate the potential for glucose absorption (Moran *et al.*, 2010), due to its expression being influenced by dietary P (Vigors *et al.*, 2014) and glucose (Margolskee *et al.*, 2007) concentrations. Jejunal SGLT1 expression has been reported to increase following supplementation of 500 FTU/kg phytase (Woyengo *et al.*, 2011), which may be a result of increased glucose availability, which has been observed to

induce SGLT1 gene expression (Margolskee *et al.*, 2007). Kellett (2001) suggested that the expression of SGLT1 could be increased by reducing the phytate-mediated effect on the Na electrochemical gradient and Na-dependant transport, following the supplementation of phytase. As an increase in Na digestibility when phytase was added to the NC diets was observed, it could be assumed that increased Na bioavailability would increase the expression of nutrient transporters, such as the Na-dependent SGLT1 transporter, but particularly that of NaPi-IIb, to maintain an adequate P balance within the intestinal lumen.

As there were no treatment effects on the expression of the sodium-glucose co-transporter SGLT1, it can be assumed, that although there was an increase in Na bioavailability, there were not sufficient increases in glucose to warrant an increase in expression of the SGLT1 transporter. Gal-Garber *et al.* (2000) reported an increase in SGLT1 expression in food-deprived and also in starved then re-fed chickens, showing the influence dietary substrate on its expression. Miyamoto *et al.* (1993) also showed an increase in the expression of SGLT1 and GLUT2 glucose transporters in mice as dietary glucose increased. This suggests that if an increase in SGLT1 or GLUT2 expression is observed, then this is likely to be the result of an increase in glucose in the intestinal lumen. In a study using pigs, Woyengo *et al.* (2011) observed a phytase-induced increase in jejunal SGLT1 expression, but a reduction in expression in the duodenum and ileum. The authors suggested that the observed increases in jejunal SGLT1 expression were a result of decreased phytate concentrations, alleviating its negative effect on the SGLT1 transporter and/or glucose bioavailability. In a pig study, Vigors *et al.* (2014) reported an increase in glucose bioavailability following phytate hydrolysis, which was reflected by an increase in expression of SGLT1 and GLUT2. Therefore, considering these reported studies, we would have expected to see similar effects in the current study.

4.4.4.4 Minerals

Divalent mineral transport was measured through observation of the expression of the divalent mineral transporter DMT1, which regulates and is regulated by dietary mineral content (Arredondo *et al.*, 2003; Bai *et al.*, 2013; Trinder *et al.*,

2000; Yeh *et al.*, 2000). There may be an influence of Na concentrations in the digesta, or a requirement for Na or energy, on the action of the DMT1 transporter, as shown by correlation between SGLT1 and DMT1 (r^2 = +0.67, P < 0.001; Table 5.13). Bindslev *et al.* (1997) also reported correlation between high dietary Na content and SGLT1 expression, with increased dietary Na increasing the expression of SGLT1 in the colon of hens. However, the lack of treatment effect on SGLT1 but linear increase in Na digestibility (thereby assumed absorption) as phytase dose increased in the NC in the current study, suggests that although there was a treatment influence on Na utilisation, glucose may have been limiting for increased expression of SGLT1, and although SGLT1 and DMT1 functions and pattern of expression may be related, it is unlikely that one is dependent on the other.

4.4.4.5 Calcium

As Ca is important considering phytase supplementation (Huber *et al.*, 2015; Sebastian *et al.*, 1996; Selle *et al.*, 2009; Vigors *et al.*, 2014), the expression of TRPV6, a Ca channel (van Abel *et al.*, 2005), was also selected for measurement in the current study. There were no significant treatment effects, however aP (P = 0.126) and phytase (P = 0.119) were close to having a tendency to influence on the expression of TRPV6. The expression of TRPV6 was 1.6 times greater in the PC than the NC. The addition of 500 FTU/kg also increased TRPV6 expression 2.5 x greater than when phytase was absent, and 1.9 x greater when 1,500 FTU/kg phytase was supplemented. As there was an interactive effect of aP × phytase on Ca digestibility, and an absolute lack of an interactive effect (P = 0.744) on the expression of TRPV6, perhaps the fluctuations in digesta Ca (dietary and Ca released from phytate) were not severe enough to lead to changes in gene expression.

4.4.5 Phytate hydrolysis

The inositol phosphate esters produced by phytate hydrolysis may influence intestinal nutrient transporter gene expression through the accumulation of the lower inositol phosphate esters in digesta. As discussed in Chapter 3 of this thesis, due to the conformation of these inositol isomers, a difference in

structure compared to endogenously produced isomers of the same degree, may mean that these exogenously produced IP esters are not able to be hydrolysed by endogenous phytases or bacteria (Bedford, 2015, personal communication).

Phytate hydrolysis in the duodenum may also have an indirect effect on nutrient solubility and bioavailability of the digesta passing into the jejunum. For example, phytate has been observed to negatively influence carbohydrase enzyme activity, protein digestion and glucose uptake in the jejunum (Liu *et al.*, 2008b). Therefore reduced phytate concentrations in the jejunal digesta, through the supplementation of phytase, would be expected to increase nutrient bioavailability through increased digestive enzyme activity in the jejunum (Liu *et al.*, 2008b). The majority of nutrient transporters investigated in this study were not influenced by treatment. However, the decrease in the expression of the divalent mineral transporter DMT1 with 500 FTU/kg phytase, but restoration to levels similar to the PC with 1,500 FTU/kg, suggests that there may be different effects in the gut between 500 and 1,500 FTU/kg. Changes in gene expression of nutrient transporters are likely to be in response to changes in nutrient concentrations in the digesta, the bacterial populations and adequate provision of nutrients and energy. Bacteria may directly interact or, through the production of toxins and other such products, indirectly influence gut barrier integrity and permeability (Sansonetti, 2004).

If not cleared through the supplementation of 1,500 FTU/kg phytase, it is possible that the lower IP esters (IP4 is of particular interest, with phytase having no significant influence on its content in the ileum) may accumulate in the digesta. The dietary P influence on IP4 (IP4 content greater when aP is higher) suggests there may be product (Pi) feedback, thereby free P and endogenous phytase activity may ultimately regulate the clearance of IP4 from the ileum. A reduction in the expression of the DMT1 divalent mineral transporter with 500 FTU/kg, but restoration to control levels with 1,500 FTU/kg, suggests that there is a reduction in divalent mineral bioavailability with 500 FTU/kg (which have high affinity binding to phytate esters) for absorption in the jejunum. There was positive correlation between the ileal IP2 content and DMT1 expression (r^2 = +0.67, $P < 0.001$; Table 5.13). As there were no treatment effects on the ileal IP2 content, but a linear increase in IP3

content as phytase dose increased, the addition of phytase may lead to accumulation of IP3 which is unable to be further hydrolysed to IP2 at regular doses, so is instead available for binding to divalent minerals in the digesta. This increased binding then reduces the bioavailability of the mineral for absorption, and consequently influence the expression of the transporters involved in their uptake. Increased hydrolysis from IP3 to IP2, facilitated by the super-dosing of phytase, is likely to increase the bioavailability and utilisation of these minerals. The results showed a linear increase in the ileal IP3 content as phytase dose increased, but decrease in DMT1 expression with 500 FTU/kg. That the ileal IP2 content was not influenced by treatment suggests that the rate of IP2 production and hydrolysis was similar, and that greater hydrolysis occurred, as seen by the linear increase in inositol production with increasing dose of phytase. There was aP × phytase interaction on the utilisation of the divalent minerals Fe^{2+} and Zn^{2+}, with a quadratic increase when phytase was added to the PC for both, but quadratic decrease in the NC for Fe and linear increase for Zn. Perhaps the ratio between these different divalent minerals has an important influence on the intestinal expression of DMT1, however, the dietary levels of each of these must be considered. If Fe and Zn are already sufficient in the diet, increased concentrations following phytase supplementation may increase their levels above requirement and thus limit their absorption.

There is a lot of complexity between responses which occur in the gut, within the cells and the body as a whole, and that effects seen following phytase supplementation, or any other dietary manipulation, are often interlinked. An example of this is the correlation identified between TRPV6 and occludin expression (r^2 = -0.71, $P < 0.01$; Table 4.12). Again, there were no significant treatment effects observed in the current study therefore described correlations remain speculative. As suggested by the correlation, occludin expression may decrease as TRPV6 expression increases (or vice versa, as is more likely due to the release of Ca on phytate hydrolysis), which suggests that low dietary Ca (known to increase TRPV6 expression (Bodding and Flockerzi, 2004; Woudenberg-Vrenken *et al.*, 2012) has a detrimental influence on tight junction barrier function of integrity. Calcium has an important function in the formation and maintenance of tight junctions (Mitic and Anderson, 1998; Turner, 2009). This suggests that in cases of substantial Ca concentrations, such as when phytate

hydrolysis releases Ca into the digesta, there is increased Ca transport via para-cellular mechanisms rather than via TRPV6 (Bodding and Flockerzi, 2004), but overall, the increase in Ca bioavailability increases the potential for initiation of occuldin expression.

4.5 Conclusion

- Although phytase improved broiler growth performance and nutrient utilisation, particularly in diets low in aP, there were few significant treatment effects on the expression of genes in the gut.
- This may be explained by the birds being healthy and productive, even in the negative control treatment.
- However, there was a dose-dependent response to phytase on the divalent mineral transporter, suggesting that mineral release from phytate has an influence within the gut.
- The next study will investigate the influence of different concentrations of inositol phosphate esters and inositol on growth, nutrient utilisation (with focus on minerals), and expression of nutrient transporters.

4.6 Appendix:

4.6.1 Animal housing

Birds were monitored at least twice daily, ensuring good health and that feed and water supplies were clean and adequate, and were wing-tagged at day 8 for individual identification. The house temperature was as detailed in the Ross broiler manual, where ambient temperature (measured at chick height) was 30^{o}C, litter temperature 28-30^{o}C and humidity at 60-70%. At day 3, the temperature was decreased at a rate of 1^{o}C per day, so that on day 21, the temperature was 22^{o}C. For the first 7 days, the lighting regime was set to 23:1 light: dark hours, with 30-40 lux intensity. All procedures were approved by the SRUC Animal Experiment Committee prior to commencement and were in accordance with the Animals (Scientific procedures) Act 1986.

4.6.2 Enzyme information

The enzyme used was Quantum Blue, provided by AB Vista, with an initial activity level of 5,000 FTU/kg. The premix was made to 150 FTU/kg and added at the rate of 10 g/kg or 20 g/kg to give activity levels of 1,500 or 3,000 FTU/kg, respectively.

4.6.3 Sample size calculations

1. Equation on page 41 of 'Design and Analysis of Experiments', 5th edition, Douglas C. Montgomery, Arizona state university

$$d = |\mu 1 - \mu 2| \; / \; 2\sigma$$

$\alpha = 0.05$ $\beta = 0.05$

2. Using a study by Liu *et al*. (2008) whereby the author provided 0, 500 or 1000 FTU/kg phytase to diets containing 2.2 or 4.4g/kg phytate, and measured gene expression and enzyme activities of digestive enzymes; 'd' can be calculated (for mRNA expression).

$\mu 1$ = (PC, 4.4 g/kg phytate) = 1.37; $\mu 2$ = (NC, 2.2 g/kg phytate) = 1.53;

σ = SD= pooled SEM*$\sqrt{n}$; SEM=0.04 n=36; σ= 0.24

d = (1.37-1.53) / (2*0.24) d = 0.67

3. Using the operating characteristic curve (page 41), where the probability of accepting H0 = 0.8, n* is estimated at 7.

4. Using the equation on page 42, sample size can be calculated

$$n = (n^* + 1) \; / \; 2$$

$$n = (7 + 1) \; / \; 2$$

$$n = 4$$

5. Therefore the minimum number of replicates determined was 4

6. 6 dietary treatments, 6 birds per pen, **4 replicates = 144 birds**

(24 pens required)

To increase the power of the experiment for growth performance measurements (as based on previous experience), 6 replicates per treatment were used, but this indicates 4 replicates would be adequate for mRNA expression studies.

Overall bird numbers:

Using the suitability of the previous study, the approximate sample size was determined, whilst maintaining the standards for the 3R's (reduction, replication, refinement).

Previously 6 birds were used for nutrient utilisation, which was adequate for the chemical analyses required, but in order to have enough sample for all analyses, it would not have been preferable to use fewer birds.

With 6 replicates to maintain high power, 10 birds per pen and 6 diets, a total of 360 birds will be required.

4.6.4 Primer design

The gene of interest was searched for in the unigene database of pubmed (http://www.ncbi.nlm.nih.gov/pubmed) and the 'model' or 'mRNA sequence' selected. The gene bank entry was selected and the FASTA sequence accessed. The FASTA sequence was then copied into the Primer express (ABI PRISM Primer express, version 2.0.0, Applied Biosystems) software. The parameters used for primer design are shown in Figure 4.3.

Temperature,	*°C*
Max	65
Min	64
Optimum	65
Amplicon requirements,	*bp*
Max length	200
Min length	160

Figure 4.3 – The parameters used for the design of primers via pubmed to be used in the current study

The FASTA sequence was also searched in the ncbi.blast web programme (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastnandPAGE_TYPE=BlastSearchandLINK_LOC=blasthome). Using the map viewer, the region of interest was expanded and primers were matched up with the primer options identified with the primer express software whilst searching for the greatest intron length (>1000bp), and using the information provided about the primer length, start and finish positions, were selected for use. Primer information is in Table 5.3 and were supplied by Eurofins genomics (Anzingerstraße, Ebersberg).

Other genes of interest were HPRT, SFRS4 and IgA, however, using the above described procedure, no results were found which corresponded with the chicken genome.

4.6.5 RNA extraction and cDNA synthesis

Working under the fume hood, with 6 samples at a time:

Step 1: Extraction 1

1. Isolate 100mg of each sample from RNAse-later solution
2. Place into Rybolyzer tubes and add 1ml Trizol
3. Place tubes into Rybolyzer and spin for 2 x 20 seconds at 6.5 speed
4. Add 300µl chloroform and shake, solution will turn cloudy/ milky
5. Leave at room temperature for 10 minutes
6. Centrifuge at room temperature for 10 minutes and max speed (13K rpm)
7. Transfer supernatant to labelled 1.5ml tubes (take care not to touch protein middle layer)
8. Measure volume using a pipette and add the same volume of isopropanol (2-propanol)
9. Freeze samples overnight at -20^{o}C

Step 2: Extraction 2

1. Centrifuge samples at 4^{o}C at 14000 rpm for 10 minutes
2. Discard supernatant into waste bottle
3. Wash pellet with 0.6ml 70% ethanol
4. Vortex and centrifuge at 4^{o}C at 14000 rpm for 5 minutes

5. Dry off pellet (pulse spin in centrifuge)
6. Remove any remaining fluid
7. Re-suspend with RNA-free water (~200µl)
8. Transfer to 0.5ml tube
9. Freeze in liquid nitrogen

Step 3: Reverse transcription

1. Prepare tubes and heat waterbath to 42°C
2. Prepare mastermix

Mastermix (for 40 tubes)	µl per tube	µl for 40 tubes
Buffer x5 concentration	2	80
DTT 10MM	1	40
Random hexamers (100pmol/µ)	0.2	8.0
DNTP	0.2	8.0
RNAse block	0.26	10.40
Reverse transcriptase (SSIII)*	0.26	10.40
RNA-free water	2.08	83.8
Total:	6	240

**Superscript III reverse transcriptase, 10,000U (200 U/µl) invitrogen)*

3. To each tube, add 6µl mastermix and 4µl RNA, at opposite sides of the tube to avoid mixing
4. Pulse in centrifuge
5. Incubate at room temperature for 3 minutes
6. Place in the waterbath at 42°C and immediately increase the temperature to 50°C, incubate for 1 hour
7. Pulse in centrifuge
8. Freeze at -20°C

.

4.6.6 Real time PCR

Step 1: Preparation of PCR aliquots

To make up 3 aliquots, double volumes to make 6

1. Set up 3 racks of 0.5ml for each sample
2. Thaw cDNA samples
3. Pipette 35µl LOTE into one tube
4. Add 1µl cDNA to LOTE in corresponding tube
5. Mix and dispense 12µl each into the other 2 tubes for each sample
6. Freeze at -20^{o}C

Step 2: Primer preparation

1. Add required volume of water as indicated by information provided with primers to prepare all primers to the same concentration
2. To a 0.5ml tube add 20µl forward primer, 20µl reverse primer and 60µl water
3. Mix and split into two 0.5ml tubes of 50µl each
4. Free at -20^{o}C

Step 3: RT PCR

1. Turn on the heat/sealing machine, thaw aliquots and pulse centrifuge, turn on RT machine to warm lamp
2. Preapre syber mix (syber green brilliant II QPCR mastermix, Aligent technologies) in 1.5ml tube (403µl syber II + 17µl primer mix)
3. Pipette 4.8µl syber mix into each well
4. Pipette 4.8µl aliquot into relevant well
5. Pipette 4.8µl LOTE into negative control (NTC) wes
6. Seal with plastic film (shiny side up)
7. Centrifuge at 1000rpm for 1 min at 20^{o}C
8. Place in RT machine (Mx3000P Stratagene, OS v6.22, control v5.20, firmware v110.61), set up plate alignment and thermal profile. Run. Takes around 1hour 15minutes

PCR thermal profile conditions

Segment	Temperature, °C	Time, minutes: seconds	Cycles
1	95	07:30	1
2	95	00:25	40
	63	00:25	
	72	00:25	
3	95	01:00	1
	63	00:30	
	95	00:30	

Samples were run in duplicate, and labelled as A and B. Two wells contained the syber-primer mix, but LOTE rather than a sample, to act as a negative control (NTC). A single aliquot was used for the liver tissue samples as there was only a single gene to be analysed, with RT results generated for 2 HKG and the gene of interest. For the jejunal tissue analysis, 3 aliquots were made up, allowing 2 HKG to be tested within each aliquot. Results for the expression of the genes of interest were corrected to the HKG output within each aliquot.

4.6.7 Further details on gene expression

Table 4.13 shows the number of replicates of each gene used for data analysis. Overall the success rate (proportion of viable results for n samples run through PCR procedure) was high at > 70%, except for a 60% success rate for measurement of the expression of SLC11A1 and a poor success rate of 21% for interferon gamma (IFNG). Due to the poor replication size of the IFNG expression results this data is excluded from analysis. There are no gene expression results for claudin-2, IL-6 and MUC2, as there were no distinct peaks on the dissociation curve, which may have been a result of primer dimerisation or binding of regions of DNA additional to the desired region of interest. The success rate of the expression of the different samples for the different genes of interest was high at > 76%. For samples 15B and 22B, only 50% of the results for the genes tested displayed dissociation curves as expected as per the combined sample output. Samples 1B and 25A had a success rate of 70%, which is acceptable. The raw data points were considered prior to analyses and values which were ± 3 standard deviations were removed as outliers, although overall there were only 4 additional values removed from the entire data set (not counting the initial identification of outliers from the dissociation curve).

Table 4.13- The number of replicates and allocation to aliquots, of each gene run through the RT PCR procedure

Tissue	Aliquot	Gene	n	n successful	success, %
Liver	1	YWHAZ	35	24	71.1
		28S	38	28	73.7
		GPX4	38	32	84.4
Jejunum	2	YWHAZ	38	31	81.6
		28S	38	27	71.1
		Claudin-2	38	-	-
		Occludin	38	28	73.7
		IL-6	38	-	-
		IFNG	33	3	21.1
	3	YWHAZ	38	31	81.6
		28S	38	31	81.6
		TLR2-2	38	32	84.2
		MUC2	38	-	-
		NaPi-IIb	38	36	94.7
		SLC15A1	38	36	94.7
	4	YWHAZ	38	31	81.6
		28S	38	38	100
		TRPV6	31	29	94.7
		SLC11A1	35	20	60.5
		SLC5A1	34	31	92.1

Notes: n = number of samples run through PCR, includes 2x NTC (negative control); n successful = number of samples run through PCR with meaningful results (as indicated by individual consideration of the dissociation peak).

Chapter 5.
Inositol and inositol phosphate ester effects in the gut

5.1 Introduction

The previous chapters have highlighted the beneficial influences of the addition of phytase on growth performance and nutrient utilisation. Correlations were also identified between the degree of phytate hydrolysis and subsequently the IP esters present in the digesta, with the utilisation of minerals (such as Ca) as well as the expression of intestinal nutrient transporters involved in the absorption of minerals and other nutrients. Consideration of these correlations and potential influences is a novel idea and leads to the design of the current study.

The complete dephosphorylation of phytate to myo-inositol has been suggested to result in the extra-phosphoric benefits associated with super-dosing of phytase. Myo-inositol is the cyclical product remaining following the complete de-phosphorylation of phytate by phytase hydrolysis (Cowieson *et al.*, 2015; Stephens and Irvine, 1990). Due to its structure and its similarity to the structure of vitamins, myo-inositol has been found to have some vitamin-like effects, such as being involved in fat-soluble nutrient transport and anti-oxidant protection (Adeola and Cowieson, 2011; Cowieson *et al.*, 2011; Zyla *et al.*, 2012). Increased inositol concentrations through the supplementation of super-doses of phytase (Cowieson *et al.*, 2011, 2015) have been suggested to give further health benefits, and is likely to be involved in the additional extra-phosphoric effects. Inositol is also suggested to be an insulin mimetic and therefore involved in insulin and glucose homeostatic regulation (Cowieson *et al.*, 2013, 2015; Lerner and Smagula, 1979). Increasing dietary myo-inositol concentrations, with and without the additional supplementation of phytase, allows for observation of the effects of high myo-inositol concentrations on gut characteristics, the expression of genes involved in nutrient transport, and the content of glucose, insulin and myo-inositol in the blood, considering its suggested influence on glucose-insulin regulation.

The dietary inositol phosphate ester and myo-inositol concentrations in the current study were manipulated with the intention of explaining some of the extra-phosphoric effects of super-doses of phytase within the gut, and how these

may differ from when regular-doses are used. The influence on the gut environment was reported through the measurement of pH and gene expression of intestinal nutrient transporters, as well as the influence of myo-inositol concentrations on blood constituents, to determine any potential effects on insulin and glucose homeostatic mechanisms.

5.2 Materials and Methods

5.2.1 Animals, Diets and Housing

A total of 384 Ross 708 broilers were allocated to 6 dietary treatments, with 8 replicates per diet type. All experimental procedures were approved by the SRUC Animal Experiment Committee in accordance with the Animals (Scientific Procedures) Act 1986 and the Purdue Animal Care and Use Committee (Appendix 5.6.1). Sample size calculations are provided in the Appendix 5.6.3.

On arrival birds were allocated to one of 6 dietary treatments in a Randomised Complete Block Design. The dietary treatments were randomly allocated within blocks and subsequently blocks were spatially randomised within the house. The birds were housed in a battery cage system with 8 birds per cage for a total of 21 days.

The dietary treatments were arranged in a 2 x 3 factorial where the factors were two levels of phytase supplementation (0 and 1,500 FTU/kg; enzyme information is provided in Appendix 5.6.2) and three types of control diets with differing inositol phosphate ester profiles. The dietary treatments were based on a standard, nutritionally adequate diet, formulated to meet Ross 708 energy and nutrient requirements (Ross 708 Specifications, 2007), with either myo-inositol (MYO, >90%, I5125 Sigma®) or phytic acid salt to increase IP esters IP4 to IP6 (PHYT, phytic acid sodium salt hydrate, P8810 Sigma®), added at a 0.10% and 1.04% inclusion rate, respectively. The dietary treatments (Table 5.1) were: (1) A nutritionally adequate Control diet (C); (2) C + phytic acid salt, increasing dietary phytate to 1.5% (PHYT); (3) C + myo-inositol (MYO); (4) C + 1,500 FTU/kg phytase; (5) PHYT + 1,500 FTU/kg phytase; (6) MYO + 1,500 FTU/kg phytase.

Table 5.1 – Ingredient and calculated nutrient composition of the diets

g/kg	C	MYO	PHYT	C	MYO	PHYT
Phytase, FTU/kg	0	0	0	1,500	1,500	1,500
Wheat	200	200	200	200	200	200
Maize	367.7	337.2	331.4	336.0	305.5	300.6
Soybean meal	330	330	333	330	330	332.2
Soya oil	27.8	28.3	32.7	29.5	30.0	34.3
Salt	1.7	1.7	1.6	1.7	1.7	1.6
Limestone[1]	13.7	13.7	13.7	13.7	13.7	13.7
Mono-cal Phos, 22%P	14.3	14.3	14.3	14.3	14.3	14.3
Sodium Bicarbonate	5.5	5.5	4.0	5.5	5.5	4.0
L-Lysine HCl	4.3	4.3	4.3	4.3	4.3	4.3
DL-Methionine	3	3	3	3	3	3
L-Threonine	2	2	2	2	2	2
Vitamin-Mineral[2] premix[2]	5	5	5	5	5	5
Phytase premix[3]	0	0	0	30	30	30
TiO_2 Marker[4]	25	25	25	25	25	25
PHYT premix[5]	0	0	30	0	0	30
MYO premix[6]	0	30	0	0	30	0
Total:	1000	1000	1000	1000	1000	1000
Calculated nutrients:						
Crude protein, %	23.07	23.06	23.08	23.03	23.02	23.01
ME, MJ/kg	12.6	12.6	12.6	12.6	12.6	12.6
Lysine, g/kg	15.3	15.3	15.4	15.3	15.3	15.3
Ca, %	1.01	1.01	1.01	1.01	1.01	1.01
P, %	0.70	0.70	0.88	0.70	0.70	0.88
aP[7], %	0.45	0.45	0.46	0.45	0.45	0.46
Phytate-P, %	0.25	0.25	0.[42]	0.25	0.25	0.42

Notes: C: Control; PHYT: C + phytic acid salt; MYO: C + myo-inositiol; [1]Limestone: 38.9% Ca; [2]Premix supplies the following per kg diet: Vit. A, 5484 IU; Vit. D3, 2643 ICU; Vit E,11 IU; Menadione sodium bisulfite,4.38 mg; Riboflavin, 5.49 mg; d-pantothenic acid, 11 mg; Niacin, 44.1 mg; Choline chloride, 771 mg; Vit B12, 13.2 ug; Biotin, 55.2 ug; Thiamine mononitrate, 2.2 mg; Folic acid, 990 ug; Pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug. Also contains per g of premix: Vit. A, 1828 IU; Vit. D3, 881 ICU; Vit E,3.67 IU; Menadione sodium bisulfite,1.46 mg; Riboflavin, 1.83 mg; d-pantothenic acid, 3.67 mg; Niacin, 14.69 mg; Choline chloride, 257 mg; Vit B12, 4.4 ug; Biotin, 18.4 ug; Thiamine mononitrate, 735 ug; Folic acid, 330 ug; Pyridoxine hydrochloride, 1.1 mg; I, 370 ug; Mn, 22.02 mg; Cu, 1.48 mg; Fe, 14.69 mg; Zn, 14.69 mg; Se, 100 ug; ; [3]Phytase premix is mixed with maize and has an activity of 150 FTU/g; [4]4.19kg TiO_2 to 16.78kg maize; [5]2 step dilution, 2274g phytic acid salt to 4776g maize; [6]2 step dilution, 250g MYO to 7250g maize; [7]aP = total P – phytate P.

Titanium dioxide was used as an indigestible marker. Feed and water were provided *ad libitum* throughout the 21 days.

5.2.2 Euthanasia

On conclusion of the experiment, the birds were confined to a 5 gallon plastic bucket. CO_2 was administered at 3 psi for 30 seconds, held for 2 minutes, after which the pressure increased to 5 psi, at which point the regulator was turned off and the bucket closed for an additional 3 to 5 minutes until all birds were dead.

5.2.3 Sample collection

5.2.3.1 Growth performance

Bird and feed weights were taken at the start (d0) and end (d21) of the trial for determination of BWG, FI and FCR and corrected for mortality.

5.2.3.2 Excreta and digesta collections

Excreta were collected on days 19 and 20, pooled for each cage and analysed for inositol phosphate esters. Digesta were collected from the gizzard and ileum from 6 birds per cage on day 21.

5.2.3.3 Blood collection

On day 21, blood was collected post-mortem from 2 birds per cage by cardiac puncture.

5.2.3.4 Gut pH

On day 21, the pH of the duodenum, jejunum, ileum and caecum were measured using a probe pH meter in the 2 birds used for blood collection. Two replicate pH readings were taken from the middle section of each region and the averages of the readings were used for analysis.

5.2.3.5 Jejunal tissue collection

A small section of jejunal tissue was collected (and placed directly into trizol solution) for gene expression studies from the 2 birds used for blood collection on day 21.

5.2.4 Processing of samples

5.2.4.1 Diets, digesta and excreta

Diets, digesta and excreta were freeze dried over the period of a week, before being ground and chemically analysed. All samples were analysed by inositol phosphate esters by using high-performance ion chromatography-based techniques (Blaabjerg *et al.*, 2010). Ileal samples were also analysed for DM, minerals and phytate-P using AOAC procedures for characterisation of nutrient utilisation. Titanium concentration was determined using the method described by Short *et al.* (1996).

5.2.4.2 Gut pH

From 2 birds used for blood collection, the pH of the duodenum, jejunum, ileum and caecum were measured on day 21.

5.2.4.3 Jejunal tissue collection

A small section of jejunal tissue was collected mid-region, placed directly into trizol solution and used for gene expression studies by PCR. The genes investigated were: calbindin, GLUT2 (glucose transporter), NaPi-IIb (sodium-phosphate co-transporter), SGLT1 (sodium-glucose transporter), TRPV6 (calcium channel), MUC2 (mucin), PepT1 (peptide transporter) and Ferroportin. Primer and house-keeping gene (HKG) information is in Appendix 5.6.4.

5.2.4.4 Blood collection

Following serum collection, samples were stored at -20^{o}C. Two hours prior to analysis samples were thawed and brought to room temperature. Reagents

provided in the kits for glucose, insulin and myo-inositol detection were brought to room temperature for 30 minutes prior to use.

5.2.4.5 Insulin analyses

Insulin analyses were performed using the chicken insulin ELISA kit from MyBioSource (mybiosource.com; supplied by Cusa Bio, catalogue number: MBS701713). In brief, 96-well plates were used, each containing 2 blank wells and 50 µl of 5 standards (3.12, 6.25, 12.5, 25 and 50 µlU/ml) in duplicate. Samples were also added (also 50 µl) in duplicate, with samples 1 to 40 in the first plate and 41 to 48 in the second. To each well (except the blank), 50 µl conjugate was added and the plate incubated at 37°C for 60 minutes. Incubation at 37°C was achieved through the use of a covered water bath, which allowed the samples to be kept in a dark environment. Following incubation, the wells were aspirated and washed 3 times using a wash buffer (1x), before 50 µl HRP-avidin was added to each well (except the blank) and placed for further incubation at 37°C for 30 minutes. The wells were aspirated and washed 3 times, and 50 µl substrate A and 50 µl substrate B added to each well. The plate was incubated at 37°C for 15 minutes, ensuring protection from light. Following this, 50 µl stop solution was added to each well and the optical density of each well read within 10 minutes using a microplate reader set at 450nm (endpoint fluorescence by VERSAmax tunable absorbance microplate reader from the Molecular Devices Corporation) using the soft max pro 4.3 software (life sciences addition, 2002 molecular devices, serial number BN02352).

Data duplicates were averaged and the mean values normalised against the absorbance reading (nm) of the blank well. The predicted insulin concentrations were calculated by generating a four parameter logistic (4PL) curve-fit from www.elisaanalysis.com (Elisakit.com Pty Ltd, 201). Values were verified by plotting a standard curve ($y = 8.0489x^{-1.063}$, $r^2 = 0.999$, Figure 5.1) and a log-linear standard curved ($y = -1.0633x + 2.0855$, $r^2 = 0.999$, Figure 5.2). The values given by www.elisaanalysis.com were identical to those given using the standard curve and < 2.7% different to the log-linear curve. Therefore, it can be assumed that the www.elisaanalysis.com programme used the equation $y = 8.0489x^{-1.063}$ and that the data is suitably reliable for analysis.

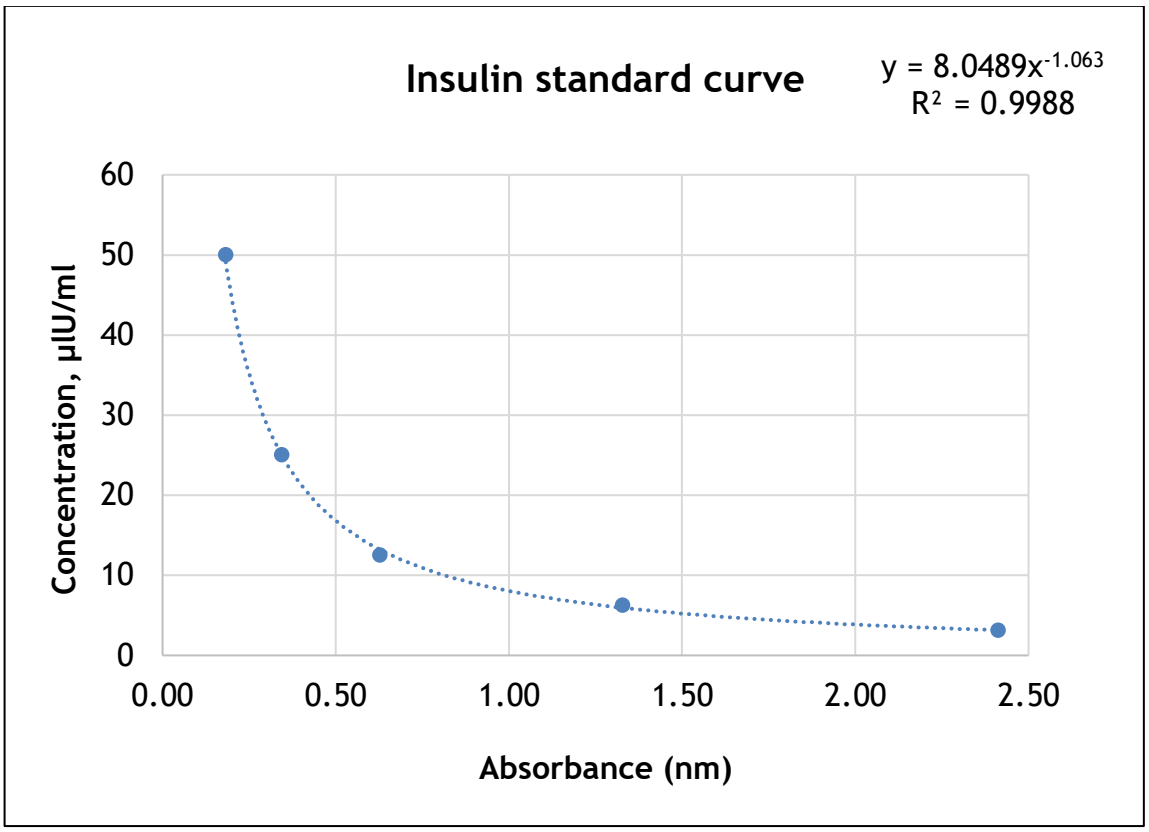


Figure 5.1 – Standard curve for insulin analyses based on the predicted standard curve equation using the absorbance of the standards provided in the ELISA kit.

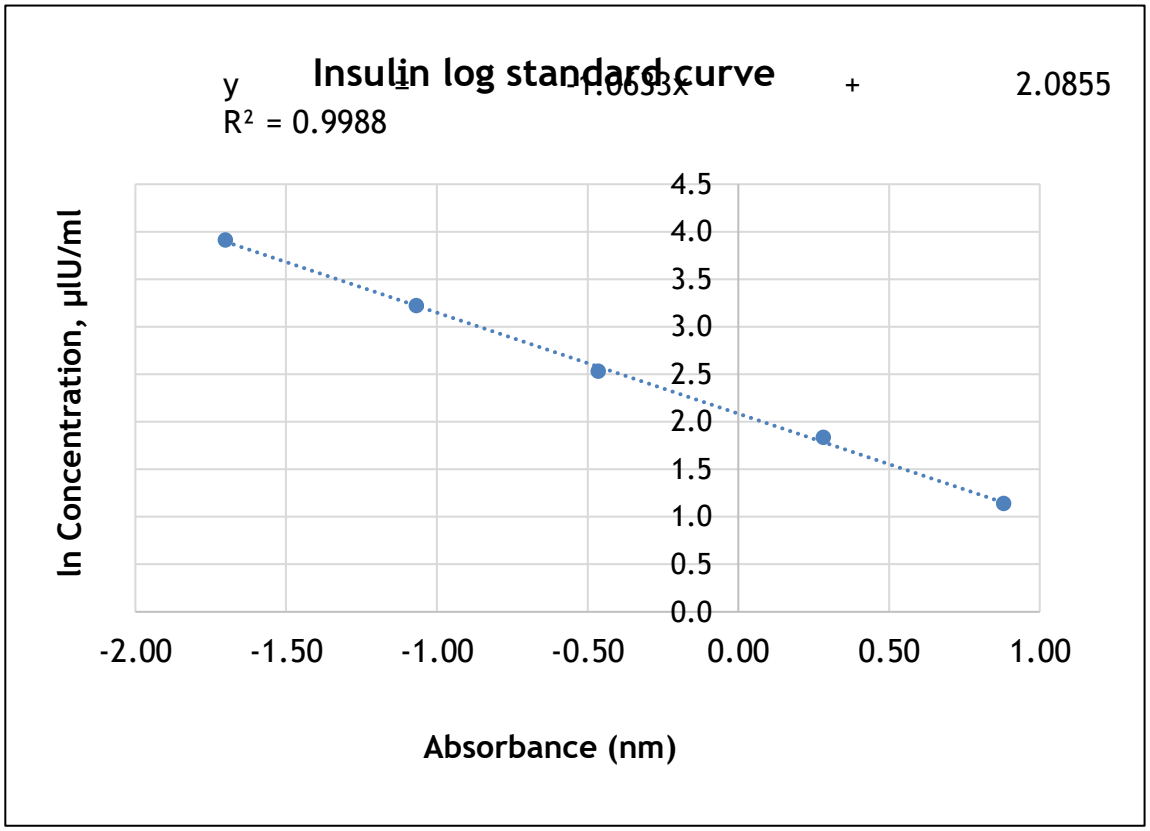


Figure 5.2 – Log-linear standard curve for insulin analysis based on the predicted log-linear standard curve equation using the absorbance of the standards provided in the ELISA kit.

Due to the greater precision of the 4PL curve, the values generated from www.elisaanalysis.com will be used for statistical analyses, following conversion to ng/mg, using the equation:

$$ng/mg = \mu lU/ml \times 0.0347$$

where 0.0347 is the conversion factor for (human) insulin (WHO/BS/10.2143, working document QAS/10.381;(WHO Expert Committee on Biological Standardization, 1990).

5.2.4.6 Glucose analyses

Glucose concentration was determined using a modified protocol of the Wako autokit glucose microtiter procedure (Wako Pure Chemical Industries, catalogue number 439-90901). Using a 96-well plate, 3 µl of the standard (200 mg/dL) and each sample were pipetted in duplicate, leaving four wells blank. To each well (except two of the blanks) 300 µl of the reagent solution was added, before the well was incubated at 37°C for 10 minutes. Incubation at 37°C was achieved through the use of a covered water bath, which allowed the samples to be kept in a dark environment. The optical density of each well was read within 10 minutes using a microplate reader set at 505 nm (endpoint fluorescence by VERSAmax tunable absorbance microplate reader from the Molecular Devices Corporation) using the soft max pro 4.3 software (life sciences addition, 2002 molecular devices, serial number BN02352).

Prior to calculation of the glucose concentration of each of the test samples, the average of each of the duplicate samples was calculated, and the average absorbance of the wells containing the reagent solution only subtracted from each of the average glucose concentrations.

Glucose concentration (mg/L) was calculated using the following equation:

$$\frac{\left(\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard} \times Concentration\ of\ standard\right)}{10}$$

where the unit of absorbance is nm, and the concentration of the standard is mg/dL.

5.2.4.7 Myo-inositol analyses

Serum myo-inositol concentration was analysed using a modified Megazyme myo-inositol protocol (K-INOSL 02/14, Megazyme International Ireland 2014). Prior to use, all serum samples were diluted on a 1:1 ratio using distilled water to decrease the colour of the sample. Using a 96-well plate, 40 µl distilled water, 10 µl pH 7.5 buffer, 10 µl sample, 10 µl ATP and 2 µl hexokinase were added and mixed, before being incubated at room temperature for 15 minutes. In the first column of the plate, there was a blank well, containing all reagents but without a sample, and 5 standards, all in duplicate, as indicated in the Appendix 5.6.5. All samples were run in duplicate.

Following incubation, 100 µl pH 9.5 buffer, 50 µl NAD/IND and 2 µl diaphorase were added and mixed. After 3 minutes, the absorbance of the solutions (A_1) were read at 492 nm, and the reaction started by the addition of 2 µl IDH. A second absorbance reading (A_2) was taken at the end of the reaction after 10 minutes.

Prior to calculation of the myo-inositol concentration, the absorbance differences for the blank and samples were determined and mean values of the duplicates for each sample calculated. $\Delta A_{Inositol}$ was calculated by determining the absorbance difference for the blank and samples (A_2 – A_1) and subtracting the absorbance difference of the blank from each of the samples. Using these values, the concentration of inositol (mg/L) was then calculated using the following equation:

$$c = \left(\frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{\text{Inositol}} \times DF\right) \times 1000$$

where:

V = final volume (mL) = 0.226 mL

MW = molecular weight of myo-inositol (g/mol) = 180.16

ε = extinction coefficient of INT-formazan at 492 nm = 19900 (L x mol^{-1} x cm^{-1})
d = light path (cm) = 0.6; corrected for use of 96 well plate (Lampinen *et al.*, 2012)
v = sample volume (mL) = 0.01 mL
DF = Dilution factor = 2

5.2.4.8 Nutrient utilisation

Nutrient digestibility was calculated using the following equations:

$$\text{Dry matter digestibility (\%)} = 1 - \left(\frac{Ti\ in\ diet}{Ti\ in\ excreta}\right)$$

$$\text{Nutrient digestibility (\%)} = \left[1 - \left(\frac{Ti\ in\ diet}{Ti\ in\ sample}\right) \times \left(\frac{Nutrient\ in\ sample}{Nutrient\ in\ diet}\right)\right] \times 100$$

5.2.4.9 Inositol phosphate esters

Conversion of inositol phosphate esters from nmol per g dry matter (DM), to mg per g DM was calculated over a series of 3 equations, as follows:

$$(1)\ \text{ng per g DM} = \text{MW} \times \text{nmol per g DM}$$

$$(2)\ \mu\text{g per g DM} = \frac{\text{ng per g DM}}{1000}$$

$$(3)\ \text{mg per g DM} = \frac{\mu\text{g per g DM}}{1000}$$

5.2.5 Statistical analyses

Data was checked for normality and suitably transformed when required and analysed as a 2 x 3 factorial where the model included diet type, phytase and the interaction between the two. Means (per pen) were pooled for each of the diets to allow comparison of the diet type effects. Statistical analyses of the data were done using the general ANOVA function of Genstat (14th Edition, VSN

International Ltd.). Data were checked for normality and transformed when required; details are provided under the tables. When differences were significant, means were separated using Tukey's test. Where the P value was significant but no differences were identified using Tukey's test, treatment means were separated by Fisher's least significant differences (LSD), with protected LSDs ($P < 0.05$). Where correlations are presented (identified using Genstat), all r^2 are significant to at least $P < 0.05$ unless otherwise stated.

5.3 Results

The analysed nutrient content of the diets are presented in Table 5.2. Analysed nutrient levels were similar to the expected formulated levels. IP2 was not detected in any of the treatments.

5.3.1 Growth performance

There were no interactive influences between the diet type and addition of phytase on the day 21 growth performance, Table 5.3. The supplementation of 1,500 FTU/kg phytase significantly increased the BWG ($P < 0.05$). There were no influences of the addition of PHYT or MYO on the FI or FCR.

5.3.2 Nutrient utilisation

Diet form × phytase interaction was significant for the ileal utilisation of DM, Na, N and Mg (Table 5.4). Phytase supplementation significantly ($P < 0.01$) increased DM, N, and Mg digestibility in birds fed the control and phytate diet, but there was no effect of phytase in birds fed MYO.

Table 5.2 – Analysed inositol ester, inositol and nutrient composition of the diets

Diet form:	Control	Control	MYO	MYO	PHYT	PHYT
Phytase[1]	0	1,500	0	1,500	0	1,500
Analysed nutrient composition, %						
DM	87.1	87	86.8	85.3	86.7	87.3
N	3.37	3.52	3.45	3.49	3.48	3.47
Ca	1.30	0.96	1.06	1.09	0.91	1.14
Fe	0.03	0.03	0.03	0.03	0.02	0.03
K	1.08	1.06	1.02	1.01	1.06	1.06
Na	0.17	0.15	0.17	0.17	0.17	0.19
P	0.69	0.66	0.68	0.67	0.72	0.88
Zn	0.02	0.02	0.02	0.02	0.02	0.02
Phytase[1]	<50	1620	<50	1600	<50	1700
Analysed IP ester and inositol content, mg/g DM						
IP6	16.968		8.559		25.546	
IP5	1.706		0.965		4.604	
IP4	0.141		0.126		0.653	
IP3	0.401		0.181		0.356	
IP2	nd		nd		nd	
Sum IP6 to IP4	19.216		9.831		31.159	
Inositol	0.167		0.887		0.172	

Notes: [1]Phytase, FTU/kg; nd, not detected.

Table 5.3 – Day 21 growth performance of broilers fed diets consisting of either a standard nutritionally adequate diet or diets with addition MYO or phytic acid salt, with either 0 or 1,500 FTU/kg phytase supplemented.

Phytase, FTU/kg	Diet type	IBW[1], g	BWG[2], g	FI[3], g	FCR[4]	Mortality %[5]
0	Control	42	843	1088	1.292	1.56
	MYO	42	852	1094	1.285	0
	PHYT	42	844	1105	1.284	0
1,500	Control	42	881	1113	1.265	1.56
	MYO	42	878	1121	1.279	0
	PHYT	42	855	1089	1.276	1.56
	SEM	0.077	14.2	4.7	0.012	1.051
	Control	42	862	1101	1.279	0.78
	MYO	42	865	1108	1.282	0
	PHYT	42	849	1097	1.280	1.56
	SEM	0.055	10.0	10.8	0.008	1.051
0		42	846[b]	1096	1.287	0.52
1,500		42	871[a]	1108	1.273	1.04
	SEM	0.045	8.2	8.8	0.007	0.607
P values						
Phytase		0.301	0.040	0.350	0.164	0.642
Diet type		0.934	0.526	0.777	0.958	0.548
Diet type x phytase		0.934	0.638	0.300	0.632	0.694

Note: [1]Initial body weight; [2]Body weight gain; [3]Feed intake; [4]Feed conversion ratio; [5]Statistical analyses of mortality % was performed following the application of 1/(sqrtX+0.1) to allow for analysis of zero mortality; 8 replications per diet; [a-b] Means within columns with different superscripts are significantly different for each response (P < 0.05).

Table 5.4 – Digestibility coefficients for ileal nutrient utilisation at day 21 of broilers fed diets consisting of either a standard nutritionally adequate diet or diets with addition MYO or phytic acid salt, with either 0 or 1,500 FTU/kg phytase supplemented.

Notes: DMD: Dry matter digestibility; N: Nitrogen; Ca: Calcium; Fe: Iron; K: Potassium; Mg: Magnesium; P: Phosphorus; Zn: Zinc; [a-b]

Phytase, FTU/kg	Diet form	DMD	N (%)	Ca (%)	Fe (mg/g)	K (%)	Mg (mg/g)	P (%)	Zn (%)
0	Control	0.655[c]	0.782[b]	0.584	0.19	0.885	0.11[bc]	0.552	0.203
	MYO	0.709[ab]	0.818[a]	0.480	0.06	0.892	0.15[ab]	0.638	0.024
	PHYT	0.668[bc]	0.783[b]	0.451	0.11	0.878	0.00[c]	0.619	0.092
1500	Control	0.731[a]	0.833[a]	0.576	0.20	0.908	0.23[a]	0.619	0.469
	MYO	0.707[abc]	0.809[ab]	0.533	0.19	0.900	0.17[ab]	0.671	0.167
	PHYT	0.724[a]	0.837[a]	0.573	0.26	0.900	0.19[ab]	0.644	0.188
	SEM	0.0124	0.0072	0.0275	0.040	0.0046	0.025	0.0381	0.0483
Diet form	Control	0.693	0.808	0.580[a]	0.19	0.897	0.17	0.586	0.336[a]
	MYO	0.708	0.814	0.506[b]	0.12	0.896	0.16	0.654	0.095[b]
	PHYT	0.696	0.810	0.512[b]	0.18	0.889	0.10	0.631	0.140[b]
	SEM	0.0088	0.0051	0.0195	0.028	0.0033	0.018	0.0269	0.0341
Phytase	0	0.677	0.794	0.505[b]	0.12[b]	0.885[b]	0.09	0.603	0.106[b]
	1500	0.721	0.827	0.560[a]	0.21[a]	0.903[a]	0.20	0.645	0.275[a]
	SEM	0.0072	0.0041	0.0159	0.023	0.0027	0.015	0.0220	0.0279
P values									
Diet form		0.442	0.688	0.019	0.197	N.169	0.013	0.200	<0.001
Phytase		<0.001	<0.001	0.019	0.005	<0.001	<0.001	0.186	<0.001
Diet form x phytase		0.009	<0.001	0.075	0.177	0.207	0.008	0.844	0.208

Means within columns with different superscripts are significantly different for each response (P < 0.05).

The control diet had the greatest ileal utilisation of Ca ($P < 0.05$) and Zn ($P < 0.001$) compared to the MYO and PHYT treatments, which were comparable to each other. The addition of phytase increased the ileal utilisation of Ca ($P < 0.05$), Fe ($P < 0.01$), K ($P < 0.001$) and Zn ($P < 0.001$). There were no significant treatment effects on P utilisation.

There was significant diet form × phytase interaction for the TTR of all of the nutrients, except for P, for which there were no significant treatment effects (Table 5.5). Phytase increased the DM retention of the control and MYO treatments, but not the PHYT ($P < 0.001$). Phytase increased N retention in the C and the PHYT diet, but there was no effect of phytase on N retention in birds fed the MYO diet

Calcium TTR was comparable between the non- and the phytase supplemented treatments in the C and MYO diets and phytase significantly increased Ca retention in the diets supplemented with PHYT ($P < 0.001$). Phytase-supplementation had no effect on birds fed the control diet, however, when phytase was absent Fe retention was significantly lower in the MYO and PHYT treatment ($P < 0.01$). Phytase supplementation increased the TTR of K and Mg in birds fed the C and PHYT, but significantly decreased TTR of K and Mg in birds fed MYO. Zinc TTR was comparable between treatments when phytase was absent and was increased in all treatments when phytase was supplemented, but greatest in the control treatment ($P < 0.001$).

6.3.3 Phytate hydrolysis

There were no significant diet type × phytase interactions on the IP content of the gizzard digesta (Table 5.6). The inositol content was comparable between the control and PHYT treatments, but increased in the MYO diet type ($P < 0.001$). The addition of phytase decreased IP6 and IP5 ($P < 0.001$) and increased IP4, IP3 and inositol content in the gizzard ($P < 0.05$).

Table 5.5 – Digestibility coefficients for total tract nutrient retention at day 21 of broilers fed diets consisting of either a standard nutritionally adequate diet or diets with addition MYO or phytic acid salt, with either 0 or 1,500 FTU/kg phytase supplemented.

Phytase, FTU/kg	Diet form	DM	N (%)	Ca (%)	Fe (mg/g)	K (%)	Mg (mg/g)	P (%)	Zn (%)
0	Control	0.704^{de}	0.638^{c}	0.515^{a}	0.24^{ab}	0.250^{d}	0.22^{b}	0.479	0.101^{cd}
	MYO	0.738^{b}	0.685^{b}	0.405^{b}	-0.04^{c}	0.317^{b}	0.23^{b}	0.505	0.038^{d}
	PHYT	0.690^{bc}	0.626^{c}	0.388^{b}	0.10^{bc}	0.165^{e}	0.08^{d}	0.491	0.115^{cd}
1500	Control	0.771^{a}	0.729^{a}	0.467^{ab}	0.29^{a}	0.394^{a}	0.31^{a}	0.511	0.471^{a}
	MYO	0.717^{cd}	0.666^{b}	0.514^{ab}	0.26^{a}	0.266^{cd}	0.17^{c}	0.556	0.177^{bc}
	PHYT	0.734^{bc}	0.688^{b}	0.520^{a}	0.27^{a}	0.304^{bc}	0.20^{bc}	0.498	0.201^{b}
	SEM	0.0044	0.0059	0.0186	0.035	0.0105	0.011	0.0342	0.0187
Diet form	Control	0.738	0.684	0.491	0.26	0.322	0.26	0.495	0.2862
	MYO	0.727	0.676	0.46	0.11	0.292	0.20	0.531	0.1073
	PHYT	0.712	0.657	0.454	0.19	0.234	0.14	0.495	0.1584
	SEM	0.0031	0.0034	0.0131	0.025	0.0074	0.008	0.0242	0.0132
Phytase	0	0.711	0.65	0.436	0.10	0.244	0.18	0.492	0.0848
	1500	0.741	0.695	0.500	0.28	0.321	0.23	0.522	0.2831
	SEM	0.0025	0.0034	0.0107	0.02	0.0061	0.006	0.0197	0.011
P values									
Diet form		<0.001	<0.001	0.116	<0.001	<0.001	<0.001	0.486	<0.001
Phytase		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.289	<0.001
Diet form x phytase		<0.001	<0.001	<0.001	0.005	<0.001	<0.001	0.814	<0.001

Notes: DM: Dry matter; N: Nitrogen; Ca: Calcium; Fe: Iron; K: Potassium; Mg: Magnesium; P: Phosphorus; Zn: Zinc;$^{a-b}$ Means within columns with different superscripts are significantly different for each response (P < 0.05).

Table 5.6 – Gizzard IP content (mg/g DM) of broilers fed diets consisting of either a standard nutritionally adequate diet or diets with addition of MYO or phytic acid salt, and supplemented with 0 or 1,500 FTU/kg phytase, for 21 days.

Gizzard IP content, mg/g DM						
Phytase[2]	Diet type	IP6[1]	IP5[1]	IP4[1]	IP3	Inositol
0	Control	3.78	0.567	0.156	0.159	0.067
	MYO	3.38	0.599	0.229	0.171	0.283
	PHYT	4.89	0.923	0.246	0.154	0.104
1,500	Control	0.32	0.206	0.317	0.202	0.237
	MYO	0.42	0.219	0.343	0.194	0.467
	PHYT	0.64	0.267	0.435	0.197	0.315
	SEM	0.188	0.060	0.072	0.017	0.028
Phytase	0	4.02[a]	0.696[a]	0.210[b]	0.161[b]	0.151[b]
	1,500	0.46[b]	0.231[b]	0.365[a]	0.198[a]	0.340[a]
	SEM	0.109	0.034	0.042	0.010	0.016
Diet type	Control	2.05	0.386	0.236	0.180	0.152[b]
	MYO	1.90	0.409	0.286	0.182	0.375[a]
	PHYT	2.77	0.595	0.340	0.176	0.209[b]
	SEM	0.133	0.042	0.051	0.012	0.020
P values						
Phytase		<0.001	<0.001	0.024	0.013	0.001
Diet type		0.255	0.098	0.212	0.920	<0.001
Diet type × phytase		0.647	0.794	0.765	0.801	0.772

Notes: [1]Log transformed prior to analysis; [2]Phytase, FTU/kg; [a-b] Means within columns with different superscripts are significantly different for each response (P < 0.05).

There was significant diet type × phytase interaction on the ileal IP3 content (Table 5.7). When phytase was absent, the IP3 content was comparable between the different treatments, however, when 1,500 FTU/kg phytase was supplemented, the IP3 content increased in the control, MYO and PHYT treatments, and was greater in the PHYT with phytase than the MYO with phytase ($P < 0.05$). The diet type had a significant influence on IP6, IP5, IP4 and inositol in the ileum. IP6, IP5 and IP4 were greater ($P < 0.001$) in the PHYT treatment than both the control and MYO, which had similar levels of these IP esters.

The inositol content was greater in the MYO compared to the control and PHYT treatments, but the addition of MYO and PHYT both increased inositol content to greater levels than the control ($P < 0.001$). The addition of phytase to the treatments had the overall effect of decreasing IP6 ($P < 0.001$) and increasing IP4, IP3 and inositol contents ($P < 0.001$).

There were significant diet type × phytase interactions on the IP3 and inositol content of the excreta ($P < 0.05$; Table 5.8). When phytase was absent, the IP3 content of both the MYO and PHYT treatment were comparable to the control, but higher in the PHYT than MYO diet type. Following phytase addition, IP3 was greater in all diet types compared to when it was absent, but greater in the PHYT treatment than both the MYO and control. The inositol content of the PHYT treatment without phytase was comparable to both the MYO and control treatments, but greater in the MYO than control, all without phytase. When phytase was added, the inositol content was greater for each of the diet types, than when it was absent.Both the MYO and PHYT treatments with phytase had greater inositol contents than the control with phytase, with the greatest inositol content being in the MYO with phytase treatment.

The diet type had a significant effect on the IP content of the excreta ($P < 0.001$). IP6, IP5, IP4 and IP3 levels were greatest in the PHYT than the MYO and control treatments, which were comparable. The addition of 1,500 FTU/kg phytase decreased IP6 and IP5 and increased the IP4, IP3 and inositol content of the excreta ($P < 0.01$).

Table 5.7 – Ileal IP content of broilers fed diets consisting of either a standard nutritionally adequate diet or diets with addition MYO or phytic acid salt, with either 0 or 1,500 FTU/kg phytase supplemented, for 21 days.

		Ileal IP content, mg/g DM				
Phytase[2]	Diet type	IP6[1]	IP5[1]	IP4[1]	IP3	Inositol
0	Control	31.55	2.260	0.271	0.192^{c}	0.485
	MYO	30.85	2.273	0.335	0.216^{c}	1.957
	PHYT	49.11	4.900	0.665	0.232^{c}	1.394
1,500	Control	9.06	2.245	3.549	0.892^{ab}	1.931
	MYO	8.24	1.878	2.689	0.738^{b}	3.840
	PHYT	16.69	4.542	5.391	1.265^{a}	3.441
	SEM	1.4893	0.2622	0.3499	0.0936	0.1697
Phytase	0	37.17^{a}	3.144	0.424^{b}	0.214	1.279^{b}
	1,500	11.33^{b}	2.888	3.876^{a}	0.965	3.071^{a}
	SEM	0.8598	0.1514	0.2020	0.0541	0.0980
Diet type	Control	20.30^{b}	2.252^{b}	1.910^{b}	0.542	1.208^{c}
	MYO	19.54^{b}	2.075^{b}	1.512^{b}	0.477	2.898^{a}
	PHYT	32.90^{a}	4.721^{a}	3.028^{a}	0.749	2.418^{b}
	SEM	1.0531	0.1854	0.2474	0.0662	0.1200
P values						
Phytase		<0.001	0.154	<0.001	<0.001	<0.001
Diet type		<0.001	<0.001	<0.001	0.018	<0.001
Diet type × phytase		0.366	0.505	0.118	0.032	0.205

Notes: [1]Log transformed prior to analysis; [2]Phytase, FTU/kg; [a-b] Means within columns with different superscripts are significantly different (P < 0.05).

Table 5.8 – Excreta IP content of broilers fed diets consisting of either a standard nutritionally adequate diet or diets with addition MYO or phytic acid salt, with either 0 or 1,500 FTU/kg phytase supplemented, for 21 days.

		Excreta IP content, mg/g DM				
Phytase[3]	Diet type	IP6[2]	IP5	IP4[1]	IP3[1]	Inositol
0	Control	28.35	1.870	0.407	0.535^{cd}	0.439^{d}
	MYO	30.54	1.970	0.351	0.507^{d}	1.180^{bc}
	PHYT	46.92	4.742	0.684	0.631^{c}	0.817^{cd}
1,500	Control	8.59	1.741	2.519	1.366^{b}	1.075^{c}
	MYO	8.09	1.569	2.483	1.355^{b}	2.460^{a}
	PHYT	16.58	3.852	5.566	2.082^{a}	1.581^{b}
	SEM	0.7952	0.1981	0.2416	0.0588	0.1057
0		35.27^{a}	2.861^{a}	0.481^{b}	0.588	0.812
1,500		11.09^{b}	2.388^{b}	3.523^{a}	1.601	1.705
	SEM	0.4591	0.1144	0.1395	0.0339	0.0610
	Control	18.47^{b}	1.806^{b}	1.463^{b}	0.950	0.757
	MYO	19.32^{b}	1.770^{b}	1.417^{b}	0.931	1.820
	PHYT	31.75^{a}	4.297^{a}	3.125^{a}	1.356	1.199
	SEM	0.5623	0.1401	0.1708	0.0416	0.0747
P values						
Phytase		<0.001	0.006	<0.001	<0.001	<0.001
Diet type		<0.001	<0.001	<0.001	<0.001	<0.001
Diet type × phytase		0.091	0.166	0.623	0.027	0.011

Notes: [1]Log transformed prior to analysis; [2]Square root transformed prior to analysis; [3]Phytase, FTU/kg; [a-b] Means within columns with different superscripts are significantly different for each response (P < 0.05).

5.3.4 Gut pH

There were no diet type × phytase interactive influences on the digesta pH of the duodenum, jejunum, ileum or caeca, Table 5.9. When phytase was supplemented, there was an increase in ileal ($P < 0.001$) and caecal pH ($P < 0.01$). The diet type also influenced the pH of the caeca ($P < 0.001$). The PHYT treatment decreased ($P < 0.001$) the pH of the caeca below that of the control and MYO treatments.

5.3.5 Jejunal gene expression

Information regarding the primer optimisation process and selection of HKGs is presented in the Appendix 5.6.6.

Considering all the evidence and experience from the previous study, the normalisation and analyses of the RT PCR data was done using GAPDH due to its wide use in similar studies. Prior to statistical analyses, GLUT2, MUC2 and ferroportin were log transformed.

There was an interactive effect between the diet type and phytase on the expression of NaPi-IIb (Table 5.10) in the jejunum of broilers fed the control, MYO and PHYT diets with 0 or 1,500 FTU/kg phytase. The PHYT treatment had greater expression of NaPi-IIb than the control ($P < 0.01$), but did not differ to any other diet type with phytase addition.

The expression of NaPi-IIb was similar to the control diet, with or without phytase, in all other diet types. There was a tendency for diet type × phytase interaction on SGLT1 expression ($P = 0.083$) and for 1,500 FTU/kg phytase to increase the expression of GLUT2 ($P = 0.052$).

There was an interactive effect between the diet type and phytase on the expression of ferroportin (Table 5.11) in the jejunum of broilers fed the control, MYO and PHYT diets with 0 or 1,500 FTU/kg phytase.

Table 5.9 - The pH of the small intestine and caeca of broilers fed diets consisting of either a standard nutritionally adequate diet or diets with addition MYO or phytic acid salt, with either 0 or 1,500 FTU/kg phytase supplemented, for 21 days.

Diet type	Phytase, FTU/kg	Duodenum	Jejunum	Ileum	Caeca
Control	0	6.10	5.89	6.21	6.06
MYO	0	6.18	5.92	6.21	6.16
PHYT	0	6.13	5.94	5.90	5.74
Control	1,500	6.07	5.97	6.54	6.35
MYO	1,500	6.12	6.00	6.35	6.37
PHYT	1,500	6.09	5.95	6.46	5.92
	SEM	0.045	0.046	0.107	0.097
Diet type	Control	6.08	5.93	6.37	6.20[a]
	MYO	6.15	5.96	6.28	6.27[a]
	PHYT	6.11	5.95	6.18	5.83[b]
	SEM	0.032	0.033	0.076	0.068
Phytase	0	6.13	5.92	6.11[b]	5.99[b]
	1,500	6.09	5.97	6.45[a]	6.21[a]
	SEM	0.026	0.027	0.062	0.056
P values					
Phytase		0.258	0.129	<0.001	0.007
Diet type		0.335	0.823	0.219	<0.001
Diet type x phytase		0.962	0.692	0.161	0.825

Notes: [a-b] Means within columns with different superscripts are significantly different for each response (P < 0.05).

Table 5.10 – The expression of genes involved in nutrient transport of Ca, Na and glucose, relative to GAPDH, of broilers fed diets based on a control, PHYT or MYO, with 0 or 1,500 FTU/kg phytase supplementation, for 21 days

Diet type	Phytase, FTU/kg	Calbindin	GLUT2[1]	NaPi-IIb	SGLT1	TRPV6
Control	0	1.102	1.133	0.780[b]	0.727	1.041
MYO	0	1.120	0.853	0.982[ab]	1.293	0.909
PHYT	0	1.633	1.193	1.904[a]	1.322	0.828
Control	1,500	1.126	1.204	1.616[ab]	1.524	0.894
MYO	1,500	0.914	1.907	0.837[b]	1.150	1.502
PHYT	1,500	1.192	1.045	1.265[ab]	1.333	1.129
	SEM	0.205	0.335	0.229	0.217	0.206
Diet type	Control	1.114	0.949	1.198	1.125	0.967
	MYO	1.017	1.550	0.909	1.221	1.206
	PHYT	1.412	1.168	1.584	1.328	0.978
	SEM	0.145	0.237	0.162	0.154	0.146
Phytase	0	1.285	1.059	1.222	1.114	0.926
FTU/kg	1,500	1.077	1.385	1.239	1.336	1.175
	SEM	0.118	0.193	0.132	0.126	0.119
P values						
Phytase		0.223	0.052	0.927	0.220	0.148
Diet type		0.147	0.125	0.020	0.652	0.435
Diet type x phytase		0.530	0.622	0.009	0.083	0.210

Notes: [1]Data log transformed prior to statistical analyses, raw diet type means presented; [a-b] Means within columns with different superscripts are significantly different for each response (P < 0.05); Calbindin: calcium binding protein; GLUT2: glucose transporter; NaPi-IIb: sodium-phosphate co-transporter; SGLT1: sodium-glucose co-transporter; TRPV6: calcium transporter.

Table 5.11 – The expression of genes involved in protein and divalent mineral transport and gut integrity in the jejunum, relative to GAPDH, of broilers fed diets based on a control, PHYT or MYO, with 0 or 1,500 FTU/kg phytase supplementation, for 21 days

Diet type	Phytase, FTU/kg	MUC2[1]	PepT1	Ferroportin[1]
Control	0	1.118	1.289	1.358[ab]
MYO	0	0.899	1.193	0.724[b]
PHYT	0	0.877	1.102	0.954[b]
Control	1,500	1.813	1.802	1.910[a]
MYO	1,500	1.274	1.434	0.847[b]
PHYT	1,500	1.505	0.928	0.866[b]
	SEM	0.243	0.207	0.192
Diet type	Control	1.466	1.545[a]	1.634
	MYO	1.086	1.313[ab]	0.786
	PHYT	1.190	1.015[b]	0.910
	SEM	0.172	0.146	0.136
Phytase	0	0.965[b]	1.194	1.012
FTU/kg	1,500	1.531[a]	1.388	1.208
	SEM	0.14	0.119	0.111
P values				
Phytase		0.020	0.260	0.237
Diet type		0.621	0.048	<0.001
Diet type x phytase		0.936	0.261	0.049

Notes: [1]Data log transformed prior to statistical analyses, raw diet type means presented; [a-b] Means within columns with different superscripts are significantly different for each response (P < 0.05); MUC2: mucin, marker of gut integrity; PepT1: peptide transporter; Ferroportin: divalent mineral transporter.

The expression of ferroportin was comparable between the control diet and the MYO and PHYT diets, with and without phytase ($P < 0.05$), however expression increased when 1,500 FTU/kg was added to the control only, but this was not significantly different to the control without phytase.

The diet type had a significant effect on the expression of PepT1 ($P < 0.05$). The expression of PepT1 was comparable between the MYO and PHYT treatments, but with the PHYT treatment having lower expression than the control. Supplementation of 1,500 FTU/kg phytase increased the expression of MUC2 ($P < 0.05$)

5.3.6 Blood constituent analyses

Insulin, glucose and myo-inositol concentrations are shown in Table 5.12. There were significant diet type × phytase interactive effects on glucose concentrations ($P < 0.05$) and the insulin-glucose ratio ($P < 0.01$). When phytase was absent, the glucose concentration was comparable between diet types, however when phytase was added, the glucose content of the PHYT treatment was significantly greater ($P < 0.05$) than the control, but both were comparable to the MYO treatment. The glucose content was greater in the PHYT without phytase than when it was added, but had no effect within the other diet types. The insulin-glucose ratio was comparable between diet types in the absence of phytase, however, when phytase was added, the ratio was greater ($P < 0.01$) in the PHYT than the MYO and control treatments, which were comparable to each other.

5.3.7 Correlations

A large number of correlations between the response variables measured were identified and presented in Table 5.13. Ileal Fe utilisation was positively correlated with BWG and Ca TTR ($r^2 = +0.82, +0.78$, respectively; $P < 0.001$) and Fe TTR was correlated with Zn TTR ($r^2 = +0.65$), ileal IP6 content ($r^2 = -0.68$; $P < 0.001$). Ileal Zn utilisation was positively ($r^2 = +0.62$) and negatively ($r^2 = -0.62$) correlated with MUC2 expression and ileal IP6 content, respectively ($P < 0.001$).

Table 5.12 – The serum glucose (mg/L), insulin (mg/L) and myo-inositol (mg/L) concentrations, and insulin-glucose ratio (mg/L) of broilers fed diets consisting of either a standard nutritionally adequate diet or diets with addition MYO or PHYT, with either 0 or 1,500 FTU/kg phytase supplemented, for 21 days.

Phytase, FTU/kg	Diet type	Glucose, mg/L	Insulin concentration mg/L	Insulin-Glucose ratio (mg/L)	Myo-inositol, mg/L
0	Control	24.69^{ab}	396.18	16.30^{ab}	19.98
	MYO	23.13^{ab}	370.22	16.39^{ab}	20.70
	PHYT	26.43^{a}	386.21	14.91^{b}	21.44
1,500	Control	25.62^{a}	367.59	14.60^{b}	19.22
	MYO	24.35^{ab}	348.43	14.59^{b}	22.39
	PHYT	21.40^{b}	379.41	18.02^{a}	31.38
	SEM	1.195	15.282	0.835	4.387
Diet type	Control	25.15	381.89	15.45	19.60
	MYO	23.74	359.33	15.49	21.55
	PHYT	23.91	382.81	16.46	26.41
	SEM	0.845	10.806	0.591	3.102
Phytase	0	24.75	384.20	15.86	20.71
	1,500	23.79	365.14	15.74	24.33
	SEM	0.690	8.823	0.482	2.533
P values					
Phytase		0.445	0.235	0.403	0.296
Diet type		0.333	0.136	0.852	0.322
Diet type x phytase		0.012	0.768	0.009	0.453

Notes: $^{a-b}$ Means within columns with different superscripts are significantly different for each response ($P < 0.05$) according to Fisher's LSD test.

Table 5.13 – Correlations of interest between the various response parameters investigated

Variable 1	Variable 2	r^2
Ileal Fe utilisation	BWG	+ 0.82
Ileal Fe utilisation	TTR of Ca	+ 0.78
TTR of Fe	Calbindin expression	+ 0.71
Ileal IP6 content	TTR of Fe	- 0.68
Ileal Mg utilisation	Calbindin expression	+ 0.71
Ileal Zn utilisation	MUC2	+ 0.62
Ileal Zn utilisation	Ileal IP6 content	- 0.62
TTR of Zn	TTR of Fe	+ 0.65
Excreta IP3 content	Ileal digesta pH	+ 0.62
Blood glucose concentrations	Insulin-Glucose ratio	- 0.64
IBW	Blood insulin concentrations	+ 0.62
IBW	Blood myo-inositol concentrations	+ 0.61
Ileal IP3 content	Ileal digesta pH	+ 0.62
Ileal IP4 content	Ileal IP6 content	- 0.65
Ileal IP4 content	Ileal digesta pH	+ 0.70
Ileal IP4 content	Jejunal digesta pH	+ 0.63
PepT1 expression	SGLT1 expression	+ 0.63

Calbindin expression was correlated with ileal Fe and Mg utilisation (r^2 = +0.71; P < 0.001). Correlations were also identified beween PepT1 and SGLT1 expression (r^2 = +0.63; P < 0.001).

A number of correlations were identified between IP content and ileal digesta pH. The ileal IP3 and IP4 content were positively correlated with the ileal digesta pH (r^2 = +0.62, +0.70, respectively; P < 0.001), whilst ileal digesta pH and excreta IP3 content were also correlated (r^2 = +0.62; P < 0.001). The ileal IP4 content was positively correlated with jejunal digesta pH (r^2 = 0.63; P < 0.001) and negatively correlated with ileal IP6 content (r^2 = -0.65; P < 0.001).

The intial body weight of the chick appeared to be correlated with the blood insulin and myo-inositol concentrations (r^2 = 0.62, +0.61, respectively; P < 0.001) with the insulin-glucose ratio being correlated with blood glucose concentrations as would be expected (r^2 = -0.64; P < 0.001).

There were no changes in the insulin-glucose ratio when phytase was added to each of the diet types, but increased when phytase was added to the PHYT treatment. There were no significant treatment effects on the insulin or myo-inositol concentrations.

5.4 Discussion

The results from the previous studies allow it to be hypothesised that the products of endogenous and exogenous phytase may have different structures, with the structures of the inositol phosphate esters produced by exogenous phytase being incompatible to bind with and be hydrolysed by endogenous phytase in the gut. If regular doses of phytase are used, phytate hydrolysis may occur as far as IP4, as seen by the accumulation of IP4 in the ileal digesta relative to the lower esters, suggesting that hydrolysis is unable to continue past this point with regular- doses, which may not be at substantial quantities to facilitate further hydrolysis of IP4 to the lower esters. It is likey that there are not enough 'free binding sites' for further hydrolysis, or that the exogenous phytase is degraded before it can remove the remaining phosphate molecules from IP4. Therefore, if the endogenous phytases in the intestine are unable to bind and hydrolyse the exogenously produced IP4 (due to possibly having a different structure which doesn't allow binding with the endogenous phytae), then the IP4 is likely to accumulate in the digesta and have the potential to bind with other nutrients and minerals present. Therefore, the addition of super-dose of phytase would be expected to reduce the possible accumulation of IP4 by providing a greater number of binding sites, increasing the degree of hydrolysis before the enzyme is degraded in the intestine, as seen by increased concentrations of the lower esters following the addition of super-doses of phytase.

The hydrolysis of phytate releases complexed nutrients, in particular phosphate and divalent minerals, into the digesta (Cowieson *et al.*, 2011; Maenz *et al.*, 1999, 2001; Selle and Ravindran, 2007). The hydrolysis of phytate therefore changes the overall characteristics of the digesta, influencing the relative abundance and ratio of nutrients released from phytate and those already present in the digesta. As a result, nutrient complexes may form with other nutrients or phytase, reducing the bioavailability of the complexed nutrients, altering the expression of genes involved in their transport (Kellett, 2001; Li *et al.*, 2012; Vigors *et al.*, 2014) and possibly also preventing phytase action. Gut pH is influenced by the contents of the digesta (Angel *et al.*, 2002; Lucas, 1983) and so it may be altered by increased release of these nutrients following phytate hydrolysis. A change in digesta pH has the potential to alter the

conditions away from (or towards) optimal for nutrient absorption, nutrient complex precipitation and phytase efficacy.

The hypothesised accumulation of IP4 and nutrients in the digesta with the use of regular doses of phytase increases the potential for their binding, reducing nutrient bioavailability and potential for utilisation for growth processes, but may also prevent phytase from binding and further hydrolysing IP4. When super-doses of phytase are provided, this may facilitate full hydrolysis of phytate, rather than halting at IP4, thereby preventing the possible association with the nutrients in the digesta, allowing an increase in nutrient absorption and possible influence on the expression of the genes involved in their transport.

5.4.1 Growth performance

The addition of MYO and PHYT had no influence on the growth performance parameters of the birds, however there was an expected overall increase in BWG with the supplementation of phytase. It was hypothesised that the PHYT would decrease, and the MYO increase growth performance. Cowieson *et al*. (2013) reported improvements in growth performance of phytase similar to those achieved with MYO supplementation, irrespective of the dietary aP. The author also suggested a partial additive effect of phytase and MYO on BWG and FCR, which was not observed in the current study. The lack of MYO effect on growth performance in the current study may be due to the difference in MYO content between the diets of the two studies. Cowieson *et al*. (2013) used levels of 500 FTU/kg phytase, and an inclusion rate of 0.15% MYO, whereas in this study, 1,500 FTU/kg phytase was supplemented and MYO included at a 0.10%. It would appear that any additional production of inositol from 1,500 FTU/kg in the 0.10% MYO treatment of the current study did not reach the same inositol levels of 500 FTU/kg and 0.15% MYO, therefore did not lead to the benefits observed by Cowieson *et al*. (2013). In the current study, the MYO treatments were analysed to contain 0.887 g/kg inositol, which is slightly lower than the 1.5 g/kg provision by Cowieson *et al*. (2013), assuming the analysed dietary composition was as formulated. The ileal digesta inositol content in the current study of the MYO treatment was 2.90 g/kg, however this was not considered as part of Cowieson *et al*. (2013)'s study. In the case of the lack of effect of PHYT on performance,

perhaps the dietary phytate content of the control was at such a level that there were no further negative effects of the addition of PHYT, and the improvements with phytase were a response of reduced phytate concentrations.

5.4.2 Nutrient utilisation

Main effects of phytase supplementation and diet form were observed in the ileum and interactive effects were observed in the excreta as the digesta passed from the ileum and through the caeca. This is likely to be a consequence of the environment in the caeca and influence on complex precipitation releasing the nutrients into the digesta and/or microbial populations. Both the diet type and phytase supplementation influenced the pH of the caecal content, which may have subsequently altered the conditions for nutrient precipitation and nutrient availability in the digesta for (or be a consequence of) microbial fermentation.

The discussion will focus on the treatment effects on the utilisation of the minerals hypothesised to be significantly influenced by the intermediate products of phytate hydrolysis.

Phytase supplementation increases P utilisation and TTR when added to diets low in dietary aP (Chung *et al.*, 2013; Olukosi *et al.*, 2013; Shang *et al.*, 2015). The lack of treatment effect in the current study on P utilisation is not surprising as P requirements were met by the dietary treatments in the absence of phytase, so the additional release of P from phytate hydrolysis would be unlikely to be absorbed. However, there were also no treatment effects on the TTR of P. If there was no additional utilisation of P at the level of the ileum, then it may be expected that there may be an increase in free P detected in the excreta, which was not observed. The decrease in Ca and Zn utilisation in the PHYT and MYO treatments compared to the control suggests that there was increased binding (Chapter 4) between the increased concentrations of the intermediate IP esters and these minerals in the digesta, released from phytate hydrolysis (Maenz *et al.*, 1999, 2001). These effects were as previously hypothesised and confirmed by the increase in utilisation following the supplementation of phytase, concluding that there is a negative effect of phytate on mineral utilisation.

5.4.3 Inositol phosphates

In the gizzard, there was a greater inositol content in the MYO treatment, as expected, but there were no higher concentrations of IP6, IP5 and IP4 in the PHYT treatment, as per the dietary content. The lack of higher IP content in the PHYT treatment may be explained by the high solubility of these higher esters and thus greater rate of flow through the gizzard (Li *et al.*, 2016). Li *et al.* (2016) reported that the gizzard is a site of a high rate of phytate hydrolysis and thus a degree of phytate hydrolysis of the PHYT treatment may have already occurred at the time of measurement. This hypothesis is confirmed by the tendency for the PHYT treatment to have a greater IP5 content. The phytase effect on reducing IP6 and IP5, and increasing IP4, IP3 and inositol is as expected, and confirms the activity of phytase within the gizzard.

By the time the digesta reached the ileum, interactive effects between the diet type and phytase supplementation were seen for the IP3 content. It appears that the addition of phytase altered the gut environment as such to facilitate the PHYT treatment to have a higher IP3 content than the MYO. The PHYT treatment had higher dietary IP6, IP5 and IP4 contents, therefore these esters were at a higher concentration and thus more bioavailable for rapid hydrolysis than the MYO. As the IP3 content of the PHYT and MYO treatments were not different to the control (all without phytase), but that the PHYT content was higher than the MYO, suggests that there may have been a degree of product (inositol) inhibition (Zeller *et al.*, 2015a) in the MYO treatment. As expected, the inositol content was greatest in the MYO treatment, however, the inositol content of the PHYT treatment was greater than the control. The dietary inositol content was comparable between the PHYT and control, which suggests that the increased inositol in the PHYT treatment was a result of endogenous phosphatase activity. There may have been a greater bioavailability or solubility of the lower IP esters in the PHYT treatment, allowing hydrolysis by the intestinal phosphatases.

The excreta inositol and IP3 contents were influenced by interaction between diet type and phytase supplementation, which suggests that their production and/or hydrolysis is influenced by their presence in the digesta. The greater inositol concentration in the MYO treatment with and without phytase is as expected with the addition of MYO to the diet formulation. The inositol content

of the PHYT with phytase was greater than the control with phytase, and is likely a result of a more abundant availability of IP esters for hydrolysis. The excreta IP3 was greater in the PHYT than the MYO and is likely a result of greater IP4 concentrations in the digesta of PHYT treatment available for hydrolysis. Even after passage through the entire digestive tract, there were still greater IP6, IP5 and IP4 concentrations in the excreta of the PHYT treatment.

5.4.4 Digesta pH

There were no treatment effects on the pH of the duodenal and jejunal digesta, indicating that the treatment effects occurred after or during nutrient digestion and absorption. Phytase increased the ileal digesta pH, suggesting that phytate hydrolysis released previously-bound nutrients into the digesta for absorption in the jejunum, influencing the characteristics of the digesta entering the ileum. Correlations were identified (Table 5.11) between the ileal digesta pH and the IP4 and IP3 content of the ileum (r^2 = +0.70, +0.62, respectively) and excreta (r^2 = +0.62). These correlations suggest that increased phytate hydrolysis by phytase to produce IP4 and IP3 may lead to the observed increase in pH. Digesta pH is important for the solubility of nutrients in the digesta for absorption, and also for the action of digestive enzymes, influencing the nutritional components and digesta characteristics entering the caeca for phytate hydrolysis (Dono *et al.*, 2014; Olukosi and Dono, 2014; Ptak *et al.*, 2015; Steiner, 2006). The decrease in caecal pH of the PHYT treatment below the control and MYO diets, and increase following phytase supplementation, is as expected according to the characteristics of IP6 and nutrient content of the digesta. The high concentrations of IP6, IP5 and IP4 of the PHYT diet may increase nutrient-complex formation thereby increasing the abundance of nutrients entering the caeca. This increase in phytate-mineral complexes entering the caeca (as would be expected in the PHYT treatments) may alter the microbial populations of the caeca and thus the products of their fermentation and subsequent pH of the caecal contents. Hydrolysis of phytate and the subsequent release of the bound minerals may explain the increase in pH following phytase supplementation, with the previously-bound minerals being cations.

5.4.5 Jejunal gene expression

5.4.5.1 NaPi-IIb

The diet type × phytase interaction on the expression of NaPi-IIb was greater in the PHYT than the control, in the absence of phytase, however, following the supplementation of phytase, the PHYT and C treatments were comparable. There were no obvious differences in the mineral content between the diets; Na content was similar between treatments, although P appeared to be slightly higher in the MYO + 1,500 FTU/kg phytase and MYO treatments compared to the others. Therefore, these effects cannot be explained as a result of differences in the dietary mineral content, particularly Na, between the control and PHYT due to the addition of the phytic acid salt. Sodium was not detected in the majority of the ileal digesta samples so cannot be considered at the level of the ileum to explain the observed effects on NaPi-IIb gene expression.

As phytase supplementation releases P through phytate hydrolysis, an increase in P bioavailability would be expected to maintain or reduce NaPi-IIb expression, as was observed in the current study following the addition of phytase. Diets low in P often lead to an increase in NaPi-IIb expression to facilitate maximal P absorption (Hattenhauer *et al.*, 1999; Li *et al.*, 2012; Saddoris, 2007). The greater expression of NaPi-IIb in the PHYT treatment suggests a lower bioavailability of P (as expected due to the characteristic binding of phytate and P). As the addition of phytase appeared to diminish these differences, it can be suggested that the release of other minerals and other nutrients through phytate hydrolysis restores the balance and bioavailability within the digesta, primarily of Na and P, relative to the control.

5.4.5.2 Mineral transport

The previous studies presented in this thesis suggest a link between IP esters and binding with minerals (DMT expression in Chapter 4). In the current study, there were no obvious differences in the dietary Ca concentrations between the treatments and there were no treatment effects on the Ca transporters calbindin and TRPV6. Although there were no significant treatment effects on jejunal calbindin expression, correlations between calbindin expression and Fe TTR were identified (r^2 = +0.71; Table 5.13) which may help explain some of the

absence of effects. As correlations were also identified between ileal Fe utilisation and Ca TTR (r^2 = +0.78), this suggests that the observed increase in Fe ileal utilisation with phytase supplementation may have had a consequence of reducing Ca bioavailability and thus prevented an adaptational increase in calbindin expression.

Despite the increase in digesta Ca concentration following the supplementation of phytase and the hydrolysis of phytate-Ca complexes, the diets were already sufficient in Ca (and other released minerals) so requirements were already being met, limiting the capacity for an increase in absorption through increased expression of Ca transporters. High levels of Ca have a detrimental influence on nutrient absorption and phytase activity (Applegate *et al.*, 2003a; Konietzny and Greiner, 2002; Tamim *et al.*, 2004). The release of Ca by phytase may make gut conditions less favourable for Ca absorption and phytase activity. However, the extra-phosphoric influence of the super-doses of phytase might ameliorate this negative effect through release of other nutrients (primarily P) to maintain nutrient balances for optimal Ca/ nutrient absorption, such as between Ca and P, and through the production of lower IP esters and inositol, reducing phytate-nutrient complexes in the digesta.

In Chapter 3 the potential for IP3 to bind with divalent minerals, such as Fe^{2+}, reducing their bioavailability was discussed. If these minerals are already present in the diet in sufficient quantities as is the case in the current study, then their absorption may be limited due to saturation of the nutrient transporters, increasing the likelihood of forming complexes with the increasing concentrations of the lower esters (IP3) with phytase supplementation. In the current study, there was a significant reduction in ileal IP3 content in each of the diet types with the addition of phytase, although the PHYT + 1,500 and MYO + 1,500 treatments were comparable to the Control + 1,500 diet type. If the previously suggested hypothesis regarding binding between IP3 and minerals is correct, then reduced IP3 may lead to increased minerals, i.e. Fe^{2+} in the digesta, which will either be excreted, or will initiate an increase in the gene expression of their transporters for removal from the digesta. Ferroportin (an iron-regulated transporter) expression would be expected to increase during periods of low iron (Ganz, 2005), therefore in the current study, assuming a

release of Fe following phytase supplementation, thereby further exceeding requirements, either a relative decrease, or stability in expression in the phytase supplemented diets would be expected. The decrease in the expression of the ferroportin transporter with the addition of phytase to the PHYT and MYO treatments, compared to the control with phytase suggests that there was an increase in iron bioavailability in these phytase-supplemented treatments, which was confirmed by an increase in Fe TTR in these treatments.

5.4.5.3 Peptide transport

PepT1 is the transporter responsible for peptide absorption, with high and low dietary protein concentrations resulting in an increase in PepT1 expression (Boudry *et al.*, 2010; Shiraga *et al.*, 1999). Many studies have been reported to increase protein utilisation following the supplementation of phytase (Bedford, 2003;Kies *et al.*, 2006;Walk *et al.*, 2012b) and phytase has also been reported to increase PepT1 expression through increased protein bioavailability from reduced phytate-protein complexes (Vigors *et al.*, 2014). In the previous study (Chapter 4), although no significant diet type effects on N utilisation were observed, there was a tendency for a phytase influence on PepT1 expression. In Chapter 4, a numerically greater level of PepT1 expression following the supplementation of 1,500 FTU/kg compared to 0 and 500 FTU/kg (but no effect of supplementing 1,500 FTU/kg in the current study) was reported. These effects were suggested to be a result of reduced protein-phytate complexes following phytase supplementation.

In the current study, there were no significant phytase effects on PepT1 expression, but this is not unexpected due to the birds' protein requirements being met in each of the treatments. The observed influence of diet type, with decreased PepT1 expression in the PHYT treatment compared to the control, confirms the suggestion that there is substantial protein-phytate complex formation within the digesta, reducing the protein bioavailability and thus substrate for PepT1 transport. This may link to the phytase-induced increase in ileal N utilisation in the control and PHYT treatments but not the MYO.

5.4.5.4 MUC2

Mucin is constitutively secreted in the gut to provide a protective layer against abrasion and microbial colonisation (Caballero-Franco *et al.,* 2007; Gabriel and Mallet, 2006; Lien *et al.*, 1996; Tanabe *et al.*, 2005). As phytate causes irritation in the gut (Onyango *et al.*, 2009), secretion is increased to provide lubrication and protection to the gut when dietary phytate concentrations are high (Barcelo *et al.*, 2000; Lien *et al.*, 1996), leading to a reduction in nutrient retention and subsequent reduction in growth if mucin secretion is excessive (Koutsos and Arias, 2006). However, enzymes and probiotics have been reported to increase MUC2 expression (Mattar *et al.*, 2002), as also observed in the current study by the increase in MUC2 expression with the supplementation of 1,500 FTU/kg phytase. Reducing phytate concentrations will reduce gut irritation and allow an improvement in gut morphology, integrity and health (Steiner, 2006). As gut barrier failure is associated with a reduction in the expression of occludin and MUC2 (Chen *et al.*, 2015), the observed increase in MUC2 following phytase supplementation in the current study is as expected and suggests an improvement in gut barrier integrity (Chen *et al.*, 2015). As constitutive expression of mucin is important for the protection of the gut (Lien *et al.*, 1996), studies involving the supplementation of phytase are not always observed to influence MUC2 gene expression (Olukosi *et al.*, 2013) as mucin will always be expected to be present and there may already be considerable mucin secretion, with phytase not appearing to have any futher effect.

5.4.6 Blood constituents

The diet type × phytase interaction on the concentration of glucose in the blood may coincide with the interactive effects seen on IP3 in the ileum, however there were no correlations (Table 5.11) between any of the inositol esters and blood constituent levels. As glucose levels were comparable between diet types in the absence of phytase, the decrease in the PHYT with phytase compared to the control suggests these effects on glucose levels are a response to increased hydrolysis of the higher IP esters as supplied by the PHYT diet type. The decrease in glucose in the PHYT with phytase treatment could be due to increased removal from the blood for other processes, although any processes this may be used for was not reflected in the growth performance responses.

Alternatively, the PHYT with phytase treatment could be more energetically demanding and require more glucose utilisation, and thus reduce its concentration in the blood. The tendency for an increase in GLUT2 expression with phytase suggests that there was an increase in glucose in the digesta for absorption. Birds do not have the GLUT4 transporter which is responsible for glucose uptake in mammals (Sweazea and Braun, 2006; Tokushima *et al.*, 2005), therefore glucose concentrations must be regulated by other insulin-sensitive transport mechanisms (Tokushima *et al.*, 2005). Inositol has been described as an insulin mimetic, having an insulin-like effect on glucose transport and gluconeogenesis (Cowieson *et al.*, 2015). Although only a tendency for increased GLUT2 expression was observed even at high phytase doses in the current study, chickens generally have very high blood glucose levels and therefore require high concentrations of insulin to regulate glucose levels (Rideau, 1988; Sweazea and Braun, 2006). Perhaps in this study, the dietary treatments did not induce a great enough increase in inositol and/ or glucose levels to induce an increase in GLUT2 expression for glucose uptake. Similarly, as phytate has been reported to increase SGLT1 expression in the duodenum (Liu *et al.*, 2008b) it could be suggested that an increase in plasma glucose concentrations would be seen in the PHYT treatment, however, in the current study the glucose concentrations of the PHYT treatments were not significantly different from the control diets.

In other studies, phytate was reported to decrease serum glucose concentrations, but increase following the addition of phytase (Liu *et al.*, 2008b). Phytase-induced increases in glucose concentration have been observed to be greater in diets with reduced Ca and aP (Cowieson *et al.*, 2013; Johnson and Karunajeewa, 1985), however Cowieson *et al.* (2015) reported the absence of an effect of dietary P or Ca and 1,000 or 2,000 FYT/kg phytase supplementation on glucose concentrations. Cowieson *et al.* (2013) reported an increase in glucose concentration with the supplementation of MYO and phytase and suggested that the benefits of MYO and phytase were through different mechanisms, as insulin concentrations were not directly relatable to the circulating glucose concentrations.

High glucose levels are required to induce insulin secretion in birds (Rideau, 1988). Therefore, as none of the treatments differed from the control in glucose

concentrations, the lack of treatment effect on insulin concentrations is not surprising. The point of time of blood sampling is not expected to have any effect on glucose concentrations as feed was available up to the point of euthanasia and blood was immediately withdrawn. Similarly, Twiest and Smith (1970) observed that glucose concentrations were significantly different between periods of light and dark, but did not significantly differ during the light period. Therefore, the lack of observed effect on glucose concentration in the current study reflects the inability of the treatments to influence glucose concentrations. However, MYO supplementation increased insulin and glucagon concentrations in a study by Cowieson *et al.* (2013) with differences between the observed responses between the treatments likely being explained by the differences in the level of dietary MYO supplementation.

The change in insulin-glucose ratio in the PHYT treatment with and without phytase is due to the increase in glucose concentrations of the PHYT without phytase compared to the PHYT with 1,500 FTU/kg phytase, as there were no significant diet type effects on insulin concentrations.

It is difficult to conclude that the effects on glucose concentrations in the PHYT with phytase diet type in the current study are a response of increased inositol production from increased substrate for hydrolysis, as there were no changes in blood concentration of the MYO diet type with or without phytase, compared to the control. Given the proposed effects of inositol on glucose homeostasis, an influence on glucose or insulin concentrations would be expected. There were also no treatment effects on inositol concentrations in the blood, as would be expected, particularly in the MYO treatment. It could be that the rate of removal of inositol from the blood is at the same rate as its uptake, or that the increased inositol is not effectively utilised and absorbed, as suggested by the highest excreta inositol content of the MYO treatment with and without phytase. However, super doses of phytase have been reported to increase plasma inositol concentrations (Cowieson *et al.*, 2015; Olukosi *et al.*, 2014; Schmeisser *et al.*, 2016), with the increase reported to be greater in NC than PC diets as a result of increased phytate-P digestibility following the removal of dietary Ca and aP (Cowieson *et al.*, 2015). The differences observed between the study reported by Cowieson *et al.* (2015) and the current study may be a consequence of

different blood inositol concentrations in the PC diets. Birds fed the PC diet in the study reported by Cowieson *et al*. (2015) had a similar concentration of glucose to the current study, however the blood inositol concentrations were substantially greater (39.3 mg/L, vs. 19.98 mg/L). Cowieson *et al*. (2013) suggested that some competition between myo-inositol and glucose occurred for Na-dependent transport, with high glucose levels being likely to reduce MYO absorption. However, this is unlikely to be the case in the current study as the glucose concentrations of the MYO diet were not different from those of the control diets, with and without phytase. Olukosi *et al*. (2014) suggested that an increase in plasma inositol following the supplementation of 1,000 and 2,000 FYT/kg was an extra-phosphoric effect, most likely related to utilisation of dietary phytate-P than total P.

5.5 Conclusion

- The addition of MYO or PHYT to the diets of broilers had no influence on growth performance, however BWG increased following phytase supplementation.
- The greater solubility of the higher IP esters (IP6, IP5, IP4) and thus greater rate of passage explains why no differences between the content of these esters in the gizzard were observed.
- Mixing and increased retention time through the gastrointestinal tract may explain why differences in the content of the higher IPs were not apparent until the ileum.
- The high IP content of the PHYT treatment was responsible for a decrease in caecal content pH, irrespective of the level of phytase, but suggests that the reductions in pH with phytase supplementation were through a reduction in phytate concentrations.
- Overall, the gene expression results suggest that minerals in the diet and/or digesta are important due to their interactions with the products of phytate hydrolysis and possible influence on the gut environment, and are likely to change as a result of dietary composition.

- There were reduced concentrations of blood glucose in the PHYT + phytase treatment and it is possible that these effects are an insulin-like response of myo-inositol production.
- Perhaps there is a difference in myo-inositol between the inositol produced from phytase in the PHYT diet type and the supplemented MYO, suggesting that the supplementation of super doses of phytase are more beneficial than exogenous MYO.

5.6 Appendix

5.6.1 Animal husbandry

Birds were monitored at least twice daily, ensuring good health and that feed and water supplies were clean and adequate. The birds were wing-tagged at day 8 for individual identification. The house temperature was as detailed in the Ross broiler manual (Ross 708 Specifications, 2007), where ambient temperature (measured at chick height) was 30^{o}C, litter temperature 28-30^{o}C and humidity at 60-70%. At day 3, the house temperature was decreased at a rate of 1^{o}C per day until the temperature reached 22^{o}C. For the first 7 days, the lighting regime was set to 23:1 light-dark hours, with 30-40 lux intensity. All procedures were approved by the SRUC Animal Experiment Committee prior to commencement and the Purdue Animal Care and Use Committee.

5.6.2 Enzyme information

The phytase used was Quantum Blue, provided by AB Vista, with an initial activity level of 5,000 FTU/kg. The premix was made to 50 FTU/kg and added at the rate of 10 g/kg or 30 g/kg to give activity levels of 500 or 1,500 FTU/kg, respectively.

5.6.3 Sample size calculations

1. Equation on page 41 of 'Design and Analysis of Experiments', 5th edition, Douglas C. Montgomery, Arizona state university

$$d = |\mu 1 - \mu 2| / 2\sigma$$

$\alpha = 0.05$ $\beta = 0.05$

2. Using a study by Liu *et al*. (2008b) whereby the author provided 0, 500 or 1,000 FTU/kg phytase to diets containing 2.2 or 4.4 g/kg phytate, and measured gene expression and activities of digestive enzymes; 'd' can be calculated (for mRNA expression).

$\mu 1$ = (PC, 4.4 g/kg phytate) = 1.37; $\mu 2$ = (NC, 2.2 g/kg phytate) = 1.53;

σ = SD= pooled SEM $\times \sqrt{n}$; SEM=0.04 n=36; σ= 0.24

$d = (1.37-1.53) / (2 \times 0.24)$ $d = 0.67$

3. Using the operating characteristic curve ('Design and Analysis of Experiments', page 41), where the probability of accepting H0 = 0.8, n* is estimated at 7.

4. Using the equation on page 42, sample size can be calculated

$$n = (n^* + 1) / 2$$

$$n = (7 + 1) / 2$$

$$n = 4$$

Therefore the minimum number of replicates to be used is 4

5. 6 dietary treatments, 6 birds per pen, **4 replicates = 144 birds**

(24 pens required)

To increase the power of the experiment for growth performance measurements (as based on previous experience), 8 replicates per diet type will be used, but this indicates 4 replicates would be adequate for mRNA expression studies.

Overall bird numbers: With consideration of the previous studies which make up part of this thesis, the approximate sample size can be determined, whilst

maintaining the standards for the 3R's (reduction, replication, refinement). Previously 6 birds were used for nutrient utilisation, which was adequate for the chemical analyses required, but in order to have enough sample for all analyses it would not have been preferable to use fewer birds. Additionally, using each bird for more than one sample collection (i.e. pH and tissue collection from the same bird) allows overall bird numbers to be reduced.

5.6.4 Primers and house-keeping genes

Following the optimisation process, the optimal temperature and efficiencies of each of the primers were determined, presented in the Table 5.14. All primers were used at the same concentration.

It is beneficial to use more than one reference gene to minimise error (Hamlet *et al.*, 2015). In the previous study 5 housekeeping genes (TBP, PMM1, YWHAZ, 28S and GAPDH) were tested prior to running the RT PCR to determine the best genes to use. The above were selected based on the findings of others in the laboratory, gained through experience. From this selection and consideration of factors such as the peak melt curve, 28S and GAPDH were selected as being most suitable for the reference genes. The expression of each of these was compared against GAPDH (Table 5.15), which is regularly the housekeeping gene of choice, however it has been suggested that this gene may not be the best selection for use as a reference gene (Bohannon-Stewart, 2014; Li *et al.*, 2011). It is apparent that there is an increase in the expression of 28S and YWHAZ, relative to GAPDH, with the addition of 1,500 FTU/kg phytase.

The selection of a reference gene is based on the assumption that there is no change in expression between treatments for that particular gene. In order to further validate the choice of reference genes used in the study, the expression of 28S against YWHAZ and GAPDH against YWHAZ was considered. There were no differences in relative expression between 28S and YWHAZ, or when GAPDH was normalised to YWHAZ. However, the phytase influence on the expression of both 28S and YWHAZ relative to GAPDH is of concern, considering that there is still a tendency for a phytase effect when the geomean of 28S and YWHAZ expression

were considered relative to GAPDH. Nevertheless, this doesn't discount the possible inappropriateness of the use of GAPDH, as this has not been tested against another reference gene, which may in some cases be disproved as suitable for use as a housekeeping gene.

Table 5.14 – The expression of 28S, YWHAZ and the geomean of both, relative to GAPDH

Diet type	Phytase, FTU/kg		28S	YWHAZ	Geomean
Control	0		0.943	1.073	0.987
MYO	0		0.942	0.974	0.900
PHYT	0		0.922	0.759	0.967
Control	1,500		1.239	1.425	1.284
MYO	1,500		1.216	0.996	1.279
PHYT	1,500		1.067	1.59	1.065
		SEM	0.116	0.213	0.156
Diet type	Control		1.091	1.249	1.136
	MYO		1.079	0.985	0.987
	PHYT		0.994	1.175	1.123
		SEM	0.082	0.151	0.110
Phytase	0		0.935^{b}	0.935^{b}	0.954
	1,500		1.174^{a}	1.337^{a}	1.209
		SEM	0.067	0.123	0.090
P values					
Diet type			0.67	0.45	0.58
Phytase			0.016	0.027	0.053
Diet type x phytase			0.78	0.18	0.86

Notes: $^{a-b}$ *Means within columns with different superscripts are significantly different for each response (P < 0.05).*

Table 5.15 – The relative expression of 28S and GAPDH against YWHAZ

Phytase, FTU/kg		28S vs YWHAZ	GAPDH
0		1.036	0.987
0		1.063	1.008
0		0.900	0.997
1,500		0.975	1.02
1,500		1.464	0.988
1,500		0.830	1.006
	SEM	0.336	0.016
Control		1.005	1.004
MYO		1.264	0.998
PHYT		0.865	1.001
	SEM	0.238	0.011
0		1.000	0.997
1,500		1.009	1.004
SEM		0.194	0.009
P values			
Diet type		0.14	0.94
Phytase		0.58	0.59
Diet type x phytase		0.40	0.25

5.6.5 Myo-inositol standards

The standards used for the myo-inositol protocol for calculation of myo-inositol concentrations, and location in the 96 well plate.

Location in 96 well plate	Myo-inositol standard solution*, µl	Distilled water, µl
A1, A2	0	10
B1, B2	0.63	9.38
C1, C2	1.25	8.75
D1, D2	2.5	7.5
E1, E2	5	5
F1, F2	10	0

*Notes: *Myo-inositol standard solution, 0.25 mg/mL, in 0.02% (w/v) sodium azide*

5.6.6 Primer optimal temperature and efficiency determination through primer optimisation.

Primer	Optimal temperature, ºC	Efficiency, %
YWHAZ	60	1.95
Calbindin	60	2
SLC11A1	64	1.64
TRPV6	50	2
PEPT1	51	2
GLUT2	50	2
SLC32A2	56	1.96
28S	53	2
SGLT1	56	2
NaPi-IIb	56	2
Ferroportin	53	2

Chapter 6.

Overall discussion

Due to the inability of non-ruminants (which includes chickens, the model used in this series of experiments) to sufficiently hydrolyse phytate into its lower IP esters, and the large quantities of phytate-P entering the environment, exogenous phytase has been supplemented to their diets for a number of years. Following the observation of the beneficial influences of phytase on growth performance and nutrient utilisation, it became apparent there were additional benefits to phytase supplementation above an increase in P availability (Pirgozliev *et al.*, 2008; Selle *et al.*, 1999; Shirley and Edwards, 2003). As a result, there is increasing interest in the use of high or so called super-doses of phytase, which are typically at or above 1,500 FTU/kg (assuming 500 FTU/kg is the regular, commercial dose), particularly in diets with low P (Manobhaven *et al.*, 2015). Additional benefits have been suggested to be a result of extra-phosphoric effects, with the proposed mechanisms of action being: (1) a reduction in the concentration of the anti-nutrient phytate; (2) restoration of the Ca: available P (aP) balance and (3) the production of myo-inositol (MYO). With these suggestions in mind, the studies in this thesis were designed to provide evidence of how phytase may be working, at regular- and super-doses, within the gut and how this may promote animal health.

The purpose of this thesis was to investigate the health-promoting effects of phytase supplementation in the broiler (meat) chicken. It is anticipated that the findings can be used to improve knowledge of the action of super-doses of phytase, to help implement an optimal feeding strategy to improve bird health and welfare, alongside performance parameters to benefit the producer.

6.1 Growth *performance*

Growth performance results in Chapters 2, 3 and 5 indicated the potential for phytase, at both regular and super-doses and regardless of the dietary IP ester content, to improve the performance of birds fed diets marginally deficient in aP and Ca, and to match the growth performance of those birds fed nutritionally-adequate diets (except for Chapter 2, where improvements to low aP diets can only be suggested due to the lack of the low aP diets without

phytase for comparison). There were no changes in any of the growth performance parameters when the birds were fed diets containing different proportions of the IP esters and MYO.

Improvements in growth performance following phytase supplementation are likely a result of an increase in nutrient bioavailability from phytate hydrolysis, as shown in Chapters 2, 3 and 5, also suggested by Shirley and Edwards (2003), amongst others. Additionally, interaction between aP and phytase was reported for nutrient utilisation in Chapters 2, 3 and 5, however there was no significant interaction for growth performance parameters. This suggests that overall nutrients were sufficient to meet requirements for maintenance, but they were not increased enough with phytase supplementation to result in further significant improvements in BWG, but with phytase improving FCR in some cases. However there may be some changes occurring in regards to the partitioning of nutrients within the carcass and efficiency of utilisation of those nutrients as indicated by numeric improvements in feed conversion ratio by 2 to 5 points in birds fed the low aP diets (Chapters 3 and 5) and this has been previously reported by Walk *et al*. (2013, 2014).

6.2 Nutrient partitioning

Myo-inositol has also been reported to have influences on carcass nutrient partitioning, although this was not directly measured in this series of experiments but may explain some of the effects of phytase. Chu and Geyer (1983) suggested that MYO concentrations might influence fat accumulation, with a high carbohydrate or fat diet diverting glucose-6-phosphate (the substrate for myo-inositol synthesis) towards lipogenesis rather than myo-inositol synthesis, particularly if there is already sufficient dietary MYO. Cowieson *et al*. (2015) also reported an influence of inositol on protein deposition.

In Chapter 2, both levels of super-doses of phytase (1,500 and 3,000 FTU/kg) led to differences in whole body nutrient accretion indicating there were some phytase-mediated influences. Metabolic changes may have occurred as phytase supplementation was increased from 1,500 to 3,000 FTU/kg, switching between protein and fat accretion, although this effect may have been partly muted by

the high Ca: aP in the analysed diets. The greater fat accretion in the 1,500 FTU/kg supplemented diets than the 3,000 FTU/kg may be the result of the dietary energy bioavailability. Protein accretion requires more energy than fat accretion, so with 1,500 FTU/kg digesta energy bioavailability may have increased from the phytase supplementation, but was not sufficient for protein accretion so was instead laid down as fat. The greater protein accretion in the low-aP and Ca diets with phytase when compared to the nutritionally adequate diets with phytase highlights the influence of dietary aP and Ca on protein accretion. Similar effects have been reported by Olukosi *et al.* (2008) and Olukosi and Adeola (2008). There were no differences between the different levels of phytase used on protein accretion, but fat accretion was lower with the supplementation of 3,000 FTU/kg than 1,500 FTU/kg. This suggests that some change in the nutrient concentrations of the digesta occurred (Ca, aP, energy substrates) leading to metabolic changes. Perhaps if the dietary aP and Ca levels were further reduced in the phytase nutritional matrix, then an enhancement of protein accretion might occur alongside a reduction in fat accretion.

6.3 Phytate as an anti-nutrient

Phytate is considered as an anti-nutrient due to its affinity for binding to other nutrients (e.g. minerals, protein, starch) in the digesta (Shirley and Edwards, 2003). The hydrolysis of phytate releases Pi and these other phytate-bound nutrients for absorption. Dietary aP can have an important influence on phytase-induced improvements in nutrient utilisation (Chapters 2, 3 and 4). Increased release of P and Ca during phytate hydrolysis may further alter intestinal conditions and influence nutrient absorption. Changing the concentration and relative proportions of nutrients in the digesta following phytate hydrolysis can influence digesta characteristics such as pH (Chapter 3), as well as the environment for interaction with digestive enzymes and subsequent absorption (Dono *et al.*, 2014; Ptak *et al.*, 2015; Selle *et al.*, 2012).

6.4 Calcium-phosphorus balance

The release of Ca from phytate is important in the restoration of the digesta Ca: aP ratio. The Ca: aP ratio is considered more important than the absolute levels

of each of the minerals, influencing many processes such as enzyme activity and nutrient absorption (Angel *et al.*, 2002; Qian *et al.*, 1997; Selle *et al.*, 2012). Due to the potentially large quantities of Ca bound to phytate, its hydrolysis by phytase may substantially increase the digesta Ca content, which alongside the release of Pi may make the digesta environment more suitable for nutrient digestion and absorption. Chapter 2 demonstrated the detrimental effect of high dietary Ca and a wide Ca: aP ratio. The Ca: aP ratio was greater than desired in the study presented in Chapter 2 so may have prevented phytase from reaching its full potential. In Chapters 2, 3 and 5, there was significant interaction between the dietary aP and Ca and phytase levels on nutrient digestibility and growth performance in Chapters 3 and 5, indicating the importance of these nutrients to be considered when supplementing phytase.

6.5 Nutrient absorption

Many of the nutrient transporters in the jejunum responsible for absorption are dependant on specific nutrients (many nutrients are co-transported with Na) and may be limited or inhibited by the presence or absence of nutrients in the digesta. Release of nutrients from phytate hydrolysis will increase their concentrations in the digesta and in order to facilitate their absorption there may be some changes in the expression of genes responsible for production of these transporters. In Chapter 4, the expression of the divalent mineral transporter DMT1 was decreased with the addition of 500 FTU/kg phytase compared to 0 or 1,500 FTU/kg, suggesting significant release of minerals from phytate at 500 FTU/kg, subsequently had an influence within the gut, which was also reported for the expression of DMT1 (Bai *et al.*, 2013). Similarly, this was also observed in Chapter 4 by the quadratic decrease in DMT1 expression with 500 FTU/kg but subsequent increase with 1,500 FTU/kg to levels similar to when phytase was absent.

Positive correlations were identified between the expression of the sodium-glucose co-transporter SGLT1 and DMT1, which suggests there may be some common factor, such as Na concentration, or the change in digesta content from one transporter has a beneficial influence on the expression of the other. As there were no significant treatment effects on the expression of SGLT1, it could

be that a nutrient could be limiting (such as Na, which is required for SGLT1 function), or that sufficient minerals are required to be removed from the digesta by DMT1 for the action of SGLT1. Conversely, as a decrease in expression was observed only with 500 FTU/kg and not 1,500 FTU/kg, hydrolysis of phytate to the lower esters and mineral release may have altered the dynamics of the complexes formed in the digesta. Accumulation of lower IP esters (namely IP4 and IP3) in the digesta with regular doses of phytase may increase the formation of phytate-mineral complexes in the digesta, thus reducing mineral bioavailability and leading to the observed reduction in DMT1 expression. Supplementation of super doses of phytase may be more effective at hydrolysing these lower esters and clearing them from the digesta, returning the mineral status of the digesta to more regular levels. The results in Chapters 2, 3 and 5 suggest that regular doses of phytase may be unable to hydrolyse IP esters below IP4 and/ or IP3, thereby allowing these esters to interact with minerals (Fe in particular) in the digesta and reduce their overall bioavailability. With this in mind, the study described in Chapter 5 was designed to further explore this hypothesis. The PHYT treatment was intended to increase the dietary abundance of IP4 esters relative to the other treatments, and to observe the effects of super doses of phytase on clearing the different IP esters and influences on mineral bioavailability. The addition of 1,500 FTU/kg phytase increased the IP4 and IP3 content of the ileal digesta, which was not consistent with results in Chapters 2 and 3 and could possibly be an effect of the dietary aP (or Ca) level in Chapter 5, which was adequate in all diets. Additionally, in Chapter 5 the higher concentration of the higher IP esters gave a higher substrate concentration for phytate hydrolysis, and as phytase wlll bind to the higher esters with greater affinity than the lower esters, then the benefits of super-dosing may be seen. Intestinal phytase activity is often increased when dietary P is low (Mohammed *et al.*, 1991; Onyango *et al.*, 2006), but inhibited by the products of lower IP products of phytate hydrolysis (Greiner *et al.*, 1993; Kornegay *et al.*, 1996). The relative balance between Ca and P is also important for phytase efficacy (Qian *et al.*, 1996).

6.6 The gut environment

The gut is the site of nutrient digestion and absorption and thus has a critical influence on processes such as growth. A well developed, healthy gut allows optimal nutrient absorption and thus greater nutrient bioavailability for various physiological processes within the body. The provision of sufficient nutrients in the correct proportion means that the maintenance requirements are met and remaining nutrients and energy can be used for growth with any excess being excreted. The proportion at which the different nutrients are provided influences the partitioning of nutrients within the carcass. If sufficient energy is available, increased muscle production through increased protein accretion can occur which will be beneficial to the producer by producing a good lean carcass, otherwise fat accretion is more likely to occur.

As everything that is ingested passes through the gut, the gut has a critical role to play in protection and prevention against illness and disease. Many factors can influence the gut environment and thus its health and the animals overall health. Gut integrity is important for nutrient absorption and protection against bacterial invasion. The digesta and microbial profile can influence the gut environment through the pH and presence of toxins and other irritants, such as phytate. Therefore, the supplementation of phytase, at both regular- and super-doses, is likely to have a beneficial influence on the gut environment by reducing the concentrations of phytate, leading to overall indirect improvements in gut health.

6.7 Microbial profile

There will always be bacteria present in the gut, however, dependant on the species present, this can either facilitate nutrient absorption and maintenance of a healthy gut or can lead to pathogen colonisation and disease. Phytase hydrolysis of phytate will release nutrients for absorption, reducing the nutrients entering the terminal gut for bacterial fermentation (provided sufficient absorption occurs). This may reduce the overall bacterial colonisation of the hind gut. The microbial data in Chapter 3 shows the importance of both aP and phytase at influencing bacterial populations, with interaction being of significance for ileal *Lactobacillus* populations. The decrease in *Lactobacillus*

populations in the nutritionally adequate diet with 500 FTU/kg phytase compared to the diet marginally deficient in aP and Ca without phytase, suggests that this treatment had a negative influence on the bacterial profile, since *Lactobacillus* are beneficial bacteria and often used in probiotic feed additives. In Chapter 3, there were no direct influences of aP or Ca on *Lactobacillus* populations, however previous studies have indicated an increase in *Lactobacillus* with high dietary Ca and P (Mann *et al.*, 2014) and CaP precipitation (Bovee-Oudenhoven *et al.*, 1999).

Altering the nutrient profile of the digesta passing through the gut can change the pH, which can also influence the bacteria present. Different bacteria require different conditions for their establishment and survival. This is shown by the aP × phytase interaction on jejunal digesta pH (likely a consequence of the aP influence on nutrient digestion in the duodenum) and the tendency for phytase to influence caecal pH in Chapter 3. The linear increase in the NC and quadratic increase in the PC of the jejunal pH was reflected by the linear decrease in the NC and quadratic decrease in the PC of the ileal *Lactobacillus* populations (Chapter 3). It is likely that this response was a result of the change in digesta composition influencing the digesta pH and nutrients available for bacterial fermentation in the subsequent GIT regions (ileum and caeca).

The bacteria present in the gut, particularly in the jejunum (and ileum) where nutrient absorption occurs, can also influence the morphological characteristics of the gut and potential for nutrient absorption. Beneficial bacterial species are associated with morphological changes and may be associated with improved nutrient absorption through an increased villi length (Ewing and Cole, 1994). In Chapter 3, correlations between the presence of *Escherichia* in the caeca and morphological changes in the jejunum were identified, suggesting that perhaps the greatest changes occur from the presence of pathogenic bacteria. The secretion of toxins and the products of fermentation by *Escherichia* may damage the villi. However, as there were no treatment effects on *Escherichia* populations, the changes in morphology cannot be directly inferred from the results. Similarly, the changes in *Lactobacilli* populations in the ileum however do not appear to reflect the morphological changes in the jejunum in pattern of

effects, suggesting the observed changes were not a result of the microbial profile (Chapter 3).

6.8 Digesta pH

Digesta pH is influenced by the nutrient content, nutrient-complexes and products of digestive enzyme activity and microbial fermentation. Phytase supplementation can influence the nutrient composition (through phytate hydrolysis and subsequent nutrient release) and potentially the formation of nutrient-complexes. In Chapters 3 and 5, it was observed that the formation of IP4 and IP3 esters by phytase, along with nutrient release from phytate hydrolysis, may lead to an increase the formation of phytate-mineral (in particular Fe) complexes in the digesta, which may subsequently influence digesta pH. Although there were no correlations identified between minerals and digesta pH, in Chapter 5 correlations were observed between the ileal digesta pH and the ileal IP3 and IP4 content, and also jejunal digesta pH and ileal IP4 content. A change in digesta pH according to the charge associated with the IP esters and minerals present in the digesta may subsequently lead to conditions more suitable for the formation of IP-mineral complexes to form.

Enzymes, both endogenous digestive enzymes and supplemented exogenous enzymes such as phytase, require an optimal pH for their activity. If the pH is not optimal, the enzyme will not be fully efficacious, or its substrate will not be soluble in the digesta and thus will not be able to interact with the enzyme. Bacterial (*E. Coli*) phytase requires an optimal pH of around 3.5 to 4.5 (Golovan *et al.*, 1999; Greiner *et al.*, 1993; Menezes-Blackburn *et al.*, 2015). If the pH is altered through the nutrient content of the digesta (indicated by the aP × phytase interaction in Chapters 2 to 5) then phytase activity may not be optimal. The release of nutrients from phytate hydrolysis can influence the digesta pH and this may subsequently influence the efficacy of the phytase enzyme and thus the subsequent hydrolysis of the lower IP esters. The supplementation of the super doses of phytase may more fully hydrolyse phytate, preventing the accumulation of IP4 and IP3 in the digesta (as shown in Chapters 3 and 5) and thus the binding between the IP esters and minerals in the digesta and potential for change in pH as a result of a change in the nutrient profile of the digesta.

6.9 Gut integrity

Gut integrity is consideration of the optimal conditions for the desired function of regulating the passage of substances across the intestinal brush border. This includes morphological parameters such as villi length/ width/ surface area and crypt depth and numbers, as well as the tight junctions within the villi. Cellular turnover and gut integrity may be reflected by the expression of genes involved in the formation and maintenance of tight junctions, such as occludin. Good gut integrity provides a good environment for nutrient absorption and protection against the invasion of bacteria across the gut wall and protects against oxidative damage, inflammation and the risk of microbial disease.

The observation of treatment effects observed in Chapter 3 suggests that changes are occurring to the morphological characteristics of the gut, likely due to changes in nutrient concentration in the digesta following phytate hydrolysis. The high variation in the gene expression data presented in Chapter 4 may have also dampened the possibility of significant treatment effects. There was no evidence for an improvement in gut integrity in the study reported in Chapter 4 due to the lack of treatment effect on the expression of occludin, a protein involved in the production of tight junctions. Despite this lack of effect, this does not mean that there were no treatment effects on gut integrity, only that the birds were already healthy and that integrity was not significantly compromised. Additionally, occludin is not be the only gene worth investigating as a measure of gut integrity, and some effects may have been observed on the expression of other genes involved in gut integrity. The lack of treatment effect on the expression of GPX4 as a marker for anti-oxidant protection in Chapter 4 also suggests that bird health was not compromised. Additionally, this suggests that gut integrity was not compromised to allow bacteria to enter the blood and eventually the liver, where GPX4 was measured.

6.10 Inositol production

Inositol has been suggested to have beneficial effects due to its cyclic structure and role of myo-inositol in the body once it has been absorbed. The study in Chapter 5 was designed to specifically observe the effects on myo-inositol in the gut. The lack of MYO-induced improvement in BWG, but positive influence of

phytase, suggests that perhaps a reduction in phytate concentration was more important than any benefits MYO might have. As all diets were nutritionally adequate, there would likely be other limiting factors (such as overall nutrient supply) preventing any further improvements in growth performance. Additionally, there may be differences in the structure or bioavailability of MYO produced by phytase hydrolysis and that of the form supplemented in the diet.

Many of the reported benefits of MYO suggest that improvements are due to insulin-like effects, with MYO being an insulin-mimetic. However, in Chapter 5, there were no significant differences in the concentrations of insulin or myo-inositol in the blood reported. Despite this, there were treatment differences in glucose concentrations and the insulin: glucose ratio in the blood, with significant diet × phytase interactions with effects suggested being a response to the difference in IP esters in the PHYT treatment. The reported increases in plasma inositol concentrations with high levels of phytase supplementation in the literature (Cowieson *et al.*, 2015; Olukosi *et al.*, 2014; Schmeisser *et al.*, 2016) may have been repeated in Chapter 5 if the dietary Ca and aP levels were decreased.

It is difficult to conclude from the results in Chapter 5 that extra-phosphoric effects are a result of increase inositol production and its proposed insulin-mimetic effects. Perhaps these effects would be more apparent if a nutrition matrix was applied to account for the additional release of nutrients from phytase supplementation to provide a more optimal nutrient and mineral balance within the digesta.

6.11 Accumulation of IP4 and IP3 in the digesta

The studies presented in Chapters 3 to 6 have all hinted to a significant influence of IP4 and IP3 in the digesta and the formation of mineral complexes following the release of both from phytate hydrolysis. High IP4 concentrations have also been reported to inhibit intestinal phosphatase activity (Hu *et al.*, 1996), further increasing accumulation in the digesta and potential for nutrient or mineral complex formation. When super doses of phytase were supplemented in Chapter 2, there was full hydrolysis to increase inositol concentration of the digesta and thus IP4 or IP3 accumulation. Interaction with minerals in the

digesta was not observed to be a problem with the high levels of phytase, in fact, IP3 concentrations were not influenced by the dietary treatments. Despite IP3 and IP4 accumulation not being considered a problem with super-doses of phytase in Chapter 3, there were correlations in the data between IP3 and the ileal digestibility of K and N suggesting that negative effects may occur.

The greater excreta IP4 content of the phytase-supplemented treatments and excreta IP6:IP4 ratio in Chapter 3 suggested that the structure of the exogenous-phytase-produced IP4 may differ from that produced by endogenous-phytase, preventing interaction with intestinal phosphatases or fermentation by caecal bacteria, resulting in its accumulation in the excreta. Zeller *et al*. (2015b) reported the contribution of endogenous and microbial phytases to IP6 hydrolysis, and differences in accumulation of IP4 and IP3 with different sources of exogenous phytase, although the author was unable to distinguish any differences in the structure of the hydrolysis products. Correlations reported in Chapter 3 suggest that IP4-Ca complex formation may alter the gut environment, subsequently influencing which bacterial species are able to colonise and ultimately lead to changes in gut morphology and thus the potential for nutrient absorption.

In Chapter 4 it was shown that the accumulation of IP4 may occur as a result of product inhibition of the Pi produced by endogenous phytase. The correlation identified between ileal IP2 content and DMT1 expression suggests that should IP4 and IP3 be hydrolysed to IP2, the release of minerals from these esters may be sufficient for an adaptational increase in the expression of the mineral transporter DMT1. However, it was apparent that the IP2 content was not influenced by treatment but IP3 increased linearly with increasing doses of phytase, suggesting that super-doses of phytase would be required to prevent the accumulation of lower IP esters in the digesta, with IP4 likely to accumulate with the use of regular-doses of phytase. However, as suggested in Chapter 5, it is possible that there may be a degree of phytase-inhibition by increasing concentrations of inositol, but this is unlikely to be a problem with the use of super-doses of phytase due to the reported increases in plasma inositol with phytase (Cowieson *et al.*, 2015; Olukosi *et al.*, 2014).

Many significant interactions between diet (i.e. phytate, IP ester and MYO proportions) and phytase supplementation on mineral utilisation and total tract retention were reported in Chapter 5 and this confirms the importance of mineral release from phytase hydrolysis and subsequent interactions within the gut. Correlations of Fe and Zn ileal utilisation with IP ester concentrations in the ileum were identified in Chapter 5, in addition to those reported in Chapters 2 and 3.

Chapter 7.
Overview

- Although there were changes in the whole body nutrient accretion characteristics between 1,500 and 3,000 FTU/kg phytase in Chapter 2, there were no additional benefits on growth performance by increasing the level of supplementation from 1,500 to 3,000 FTU/kg.
- Changes in nutrient partitioning of the bird were not assessed on an economical basis, however as the cost of implementation of the use of super-doses of phytase has to be considered for the study to have commercial implications, 1,500 FTU/kg phytase was considered suitable as a super-dose for continuation of the study and investigation of the mechanism of action.
- The presence of growth performance differences between NC and PC diets in Chapter 2 at day 12 indicates that the NC diet may have been deficient in aP, and thus allows suggestion, that as there were no detrimental effects at day 21 following the supplementation of phytase to the NC diets, that phytase may have been able to release sufficient P and prevent deficiency.
- It appears that exogenous phytase hydrolysis halts at IP4 with regular doses of phytase, thereby increasing IP ester-mineral complex formation and preventing further hydrolysis and leading to changes in the gut environment.
- This may have an influence on digesta pH and change the substrates available for bacterial fermentation (as well as overall nutrient bioavailability to the animal), ultimately influencing gut morphology and potential for nutrient absorption.
- The application of a nutrient matrix would be beneficial alongside phytase supplementation to maintain a more optimal nutrient balance, particularly with aP, Ca and the Ca:aP ratio having a significant influence on the gut environment and phytase activity.
- Future experiments could be designed to implement a greater enzyme matrix, including additional minerals, such as Na and Fe, to investigate their effect on phytase efficacy and interaction with the lower IP esters in the digesta.

- Similarly, the investigated phytase effects on parameters such as gut morphology, integrity and immune protection may have been more apparent if the birds were immunologically challenged.
- Throughout the series of experiments (excluding the birds culled for poor performance on the aP deficient diet in Chapter 2), all birds were of good health, with minimal nutrient deficiencies implemented, particularly in Chapters 3 to 6.
- Super-dosing is not a simple fix, but under the right dietary conditions, can lead to substantially greater phytate and IP hydrolysis, allowing for reductions in feed costs, improvements in growth performance, and potentially improvements in the gut environment to facilitate improved gut health and function.
- Reductions in phytate concentrations and abundance of nutrients entering the hind gut for bacterial fermentation with phytase supplementation decreases the risk of pathogenic bacterial colonisation, reduces inflammation and increases the overall nutrient bioavailability to the animal.
- Gut integrity (morphology) and beneficial bacterial populations are integral to gut and overall animal health. Good gut health can lead to more efficient nutrient absorption, allowing more nutrients to be available for growth processes.

Recommendations:

- The use of super-doses of phytase (along with a full enzyme matrix to allow reduced concentrations of aP, Ca and Na) for maximal removal of IP esters from the digesta, an improved gut environment and greater potential for enhanced growth performance.
- The producer may expect to benefit through reduced use of dietary additives such as inorganic phosphorus and improved overall animal health, and thus the associated costs of these, such as reduced therapeutic animal care and feed costs.

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