Harper Adams University

Understanding the importance of *myo*-inositol for poultry production.

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Abstract

Recently, free myo-inositol (Ins) availability in the gastrointestinal tract (GIT) of growing poultry has increased due to the dietary inclusion of phytase enzymes enhancing the digestibility of organic phosphorus (P). Increasing phytase dosage, known as super dosing, has ‘extra-phosphoric effects’, including improved feeding efficiency and increasing concentrations of Ins and inositol phosphates. It is therefore hypothesised Ins may improve bird performance, acting as a growth promoter, explaining the beneficial effects of super dosing phytase. Ins is a cyclohexitol sugar alcohol, involved in many biological pathways. In humans, the requirement for Ins is met from de novo synthesis, GIT uptake of Ins or the recycling of Ins containing compounds. It is unclear if this is the case in poultry. The objective of this thesis was to evaluate the efficacy and explanatory mechanisms of Ins relating to bird performance, Ins homeostasis and health of broiler chickens. Results indicated that 4.5 g/kg Ins improved bird growth performance, although greater dosages of Ins (30 g/kg) were not beneficial. Ins in tissues could be manipulated (except brain) from either phytase supplementation or direct free Ins, with increases in kidney, muscle tissue and blood plasma observed. Further results indicated that despite clear increases in Ins in the jejunum and kidney tissue, the relative gene expression did not demonstrate the expected manipulation of Ins homeostasis. Additionally, negative effects of feeding Ins at 3.5 and 7.5 g/kg Ins were observed with significant decreases in bird growth performance. The studies also demonstrated no Ins related antioxidant activity in growing broiler chickens and marginal effects on chicken health despite increases in blood plasma and free Ins in the GIT. In conclusion, there are no clear growth or health benefits of feeding growing broiler chickens with free Ins suggesting alternative mechanisms are associated with the extra-phosphoric effects associated with super dosing phytase.

Declaration and Acknowledgments

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I would like to dedicate this thesis to my Gran (Jean Charlotte Tomasina Gershom), who sadly passed away before its completion.

I declare that this thesis has been composed entirely by the author. No part of this report has previously been submitted for any qualification at this or any other university. Where I have consulted published work of others I have clearly attributed and stated sources given. Parts of this thesis has been published in conference abstracts:

Arthur, C., Rose, S., Mansbridge, S.C., Kühn, I. and Pirgozliev. V. 2022. The modulation of *myo*-inositol (Ins) transporter proteins in the jejunum and kidney of broiler chickens supplemented with dietary phytase, Ins and glucose. 26th World's Poultry Congress, Paris. Abstract number: 2274.

Scott-Cook, H., **Arthur, C.,** Rose, S., Mansbridge, S.C., Boffey, R., Kühn, I. and Pirgozliev. V. 2022. The effects of *myo*inositol and phytase supplementation on the tibia bone characteristics of 35 day old broiler chickens. Proceedings of the World's Poultry Science Association (UK Branch), 13-14th April 2022, Nottingham UK.

Hinch, L., **Arthur, C.,** Rose, S., Mansbridge, S.C., Karim, F., Boffey, R., Kühn, I. and Pirgozliev. V. 2022. The effect of dietary phytase and *myo*-inositol on the breast fillet meat quality of broilers chickens reared to 35 days of age. Proceedings of the World's Poultry Science Association (UK Branch), 13-14th April 2022, Nottingham UK.

Arthur, C., Rose, S., Mansbridge, S.C., Brearley, C., Kühn, I. and Pirgozliev. V. 2021. The correlation between *myo*inositol (Ins) concentration in the jejunum digesta of broiler chickens and the Ins concentrations of key tissues associated with the uptake, circulation and catabolism of Ins. Proceedings of the World's Poultry Science Association (UK Branch), 15-16th April 2021, Virtual conference, UK. Abstract number: 2.

Arthur, C., Rose, S., Mansbridge, S.C., Brearley, C., Whitfield, H., Kühn, I. and Pirgozliev. V. 2021. *myo*-Inositol concentration in chicken tissues is influenced by dietary *myo*-inositol

and phytase. Proceedings of the World's Poultry Science Association (UK Branch), 15-16th April 2021, Virtual conference, UK. Abstract number: 23.

Arthur, C., Rose, S., Mansbridge, S.C., Brearley, C., Kühn, I. and Pirgozliev. V. 2020. The effect of graded levels of *myo*-inositol on apparent ileal mineral digestibility and plasma mineral concentration of broiler chickens at day 21 of age. Proceedings of the World's Poultry Science Association (UK Branch), meeting cancelled, UK. p.3-4.

Arthur, C., Rose, S., Mansbridge, S.C., Kühn, I. and Pirgozliev. V. 2020. Antioxidant status of broiler chickens in response to graded levels of dietary levels of *myo*-Inositol. 26th World's Poultry Congress, Paris. Abstract number: 1176. (accepted in 2020, published 2022, conference postponed)

Arthur, C., Rose, S., Mansbridge, S.C., Kühn, I. and Pirgozliev. V. 2020. The effects of *myo*-inositol and phytase supplementation on the body weight of broiler chickens reared to 21 days and pre-caecal dry mater and nitrogen digestibility. 26th World's Poultry Congress, Paris. Abstract number: 1178. (accepted in 2020, published 2022, conference postponed)

Arthur, C., Rose, S., Mansbridge, S.C., Brearley, C., Kühn, I., Bedford, M.R. and Pirgozliev. V. 2019. Effect of graded levels of dietary *myo*-inositol and phytase supplementation on growth performance and plasma *myo*-inositol concentrations in broilers. Proceedings of the World's Poultry Science Association (UK Branch), 10-11th April 2019, Edinburgh, UK. p. 12.

Charlotte Arthur

Undergraduate Honours Research Projects

The following undergraduate research projects have been supported through the final experiment (Chapter 6) of this thesis and some data is reproduced or re-analysed in/from these publications:

Clark, L. 2022. Understanding the importance of dietary phytase and *myo*-inositol, alone or in a combination on the development and red blood cell counts of broiler chickens: *being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree.* Newport: Harper Adams University.

Bedford, D. 2022. The effects of *myo*-inositol and phytase supplementation on the growth performance, plasma mineral concentration, mineral digestibility and of growing broiler chickens: *being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree.* Newport: Harper Adams University.

Hinch, L. 2022. The effect of dietary phytase and *myo*-inositol on the breast fillet meat quality of broilers chickens reared to 35 days of age: *being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree.* Newport: Harper Adams University.

Scott-Cook, H. 2022. The effects of *myo*-inositol and phytase supplementation on the tibia bone characteristics of 35 day old broiler chickens: *being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree.* Newport: Harper Adams University.

List of Common Abbreviations

ADFI Average Daily Feed Intake

ADWG Average Daily Weight Gain

AME Apparent Metabolisable Energy

AMEn Nitrogen corrected apparent metabolisable energy

ANOVA Analysis of Variance

APL Alkaline Phosphatase

BW Body weight

Ca Calcium

Cu Copper

CV% Coefficient of Variation

DM Dry Matter

FCR Feed Conversion Ratio

Fe Iron

FR Fat retention

FTU Phytase Units

GE Gross energy

GIT Gastrointestinal tract

HCL Hydrochloric acid

HPLC High Performance Liquid Chromatography

ICP-MS Inductively Coupled Plasma - Mass Spectrometer

Ins *myo*-inositol

IP_1 *myo*-inositol monophosphate

IP_2 *myo*-inositol bisphosphate

IP_3 *myo*-inositol trisphosphate

IP_4 *myo*-inositol tetrakisphosphate

IP_5 *myo*-Inositol pentakisphosphate

IP_6 *myo*-inositol hexakisphosphate

IPs Inositol phosphates

KEGG Kyoto Encyclopaedia of Genes and Genomes

MCH Mean Corpuscular Haemoglobin

MCV Mean Corpuscular Volume

ME Metabolisable energy

Mg Magnesium

Mn Manganese

Mo Molybdenum

Na Sodium

NC Negative Control

NH_4 Nitric acid

NIR Near Inferred

NR Nitrogen retention

P Phosphorus

PC Positive Control

RBC Red Blood Cells

RBW% Red Blood Cell Distribution Width

RT-qPCR Reverse Transcription -quantitative Polymerase Chain Reaction

SEM Standard Error of the Mean

Zn Zinc

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Chapter one: General Introduction

Global consumption of meat proteins is set to increase by 14% over the next decade, this is mainly driven by increased population and wealth. Protein from poultry is predicted to increase by 17.8% by 2030, this is due to its affordability in lower income countries and in richer countries preferences are towards white meats with perceived health benefits (OECD/FAO, 2021). Poultry is cheap and easy to produce, whether that be on a small-scale back yard system or large-scale intensive systems.

The first chicken was domesticated around 8000 years ago from red junglefowl (Lawal et al., 2020). Genetic improvements of broiler chickens since the 1950s has resulted in a highly efficient and fast-growing birds with performance objectives for feed conversion ratios (FCRs) of 1.399 for production (Aviagen, 2022). The nutrition of poultry is critical in producing a healthy and profitable bird. The cost of feed is the most expensive part of producing a broiler chicken for meat, making up 70% of the total cost. With increased raw feed costs and uncertainty in the market due to conflict, finite resources and changes in the law, understanding the nutrition of broiler chickens is of the upmost importance to produce an affordable and healthy bird. In 2021, 1.116 billion broilers, million boiling fowl, 12 million turkeys and 10 million ducks and geese were slaughtered in the UK (Figure 1.1).

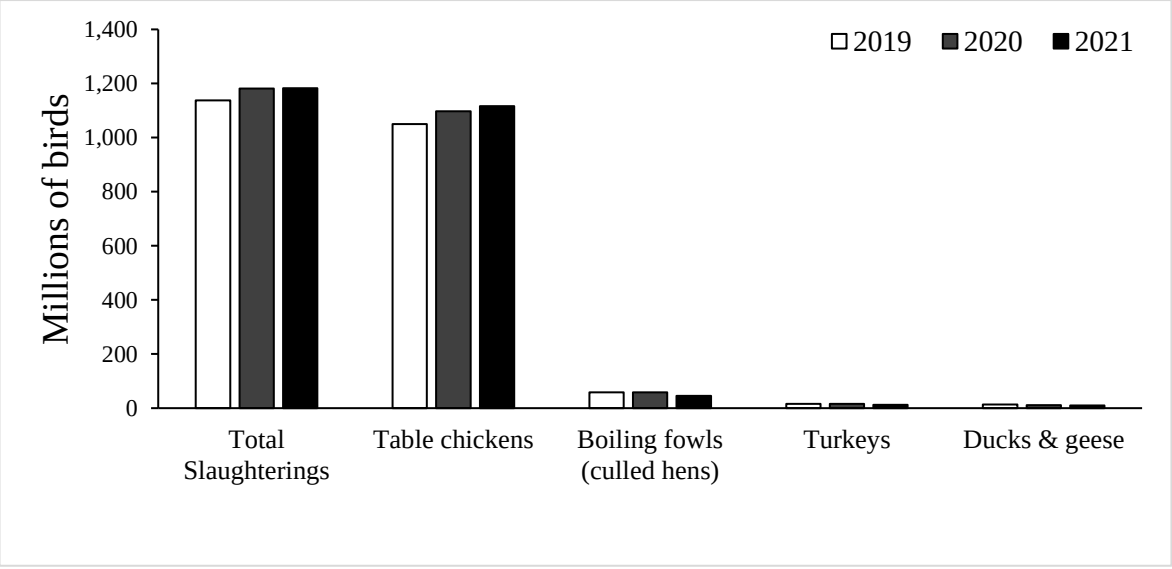


Figure 1.1: Yearly slaughtering numbers of poultry in the UK, data obtained from DEFRA, 2022.

Feed enzymes are supplemented into poultry diets, which can increase the digestibility of feed or produce beneficial products which aid in the performance and health of the bird. The global enzyme market reached $12.46 billion in 2022 and is expected to grow to $20.5 billion by 2030 (Precedence Research, 2021). With increases in demand due to the growth of animal feed, food and biofuel markets (Kumar, 2014).

Broiler chickens have a dietary requirement for available phosphorus in diets, with starter diets requiring 0.48 % available phosphorus, grower requiring 0.435 % and finisher 0.395 % (Aviagen, 2014). Phosphorus is supplied in two main forms, as inorganic mined phosphorus or as organic phosphorus. However, organic phosphorus may not be in a form which can easily be utilised by the animal. Typically, there is adequate concentrations of organic phosphorus found in the plant material making up the feed, however the phosphorus is bound in the molecule called phytate making it largely unavailable to the animal. Phytases are often supplemented in poultry diets as it can hydrolyse phytate, releasing bound phosphorus. Monogastric animals including chickens do have endogenous phytase enzymes which can release the bound phosphorus from phytate but not in effective quantities. Phytase mode of action involves the dephosphorylation of phytate by hydrolysing the phosphate groups in a stepwise manner, causing the release of phosphate groups and forming lower inositol phosphates (IPs). The last step of IP_1 to Ins is mediated by the bird's own alkaline phosphatase. The complete dephosphorylation of phytate results in the formation of a sugar called *myo*-inositol (Ins) with bioactive properties (Bedford and Partridge, 2010).

Recent studies have demonstrated supplementing third generation phytases (with higher affinities to the lower inositol phosphates such as IP_3) at high or super doses improves FCR and increases the concentrations of lower IPs and Ins in the excreta and digesta of chickens and pigs (Zeller et al., 2015[a,b]; Kuehn et al., 2016; Beeson et al., 2017; Walk et al., 2014; Gautier et al., 2017; Farhadi et al., 2017). This improvement is partly due to the releasing of nutrients such as protein and minerals which was previously bound to phytate. The 'extra-phosphoric effects' of super-doses of phytase can be defined as the extra benefits resulting from more than just an increase in P availability. Furthermore, phytase has other beneficial effects including removing the anti-nutritional properties of phytate, by reducing the formation of cation ions with minerals and reducing the negative environmental impacts of high phosphorus contents in manures. Since the positive impacts of phytase are well understood, greater attention is now being given to the concept of super-dosing phytase. However, few have assessed the role Ins may play in the improved production performance seen when formulating diets with phytases. Therefore, it is a logical assumption that Ins may play a role in improved performance and contribute to the beneficial extra-phosphoric effects of super dosing phytase as Ins is being released into the GIT of chickens at levels which would not normally be seen with diets not supplemented with phytase. However, the link to Ins and improved performance is not fully understood (Lee and Bedford, 2016). Ins is a cyclohexitol sugar alcohol and due to the "vitamin like" properties of Ins, it may be a factor in why there is an increase in the performance of broiler chickens. Ins is an important biological molecule and is

involved in many different pathways as its self or used as a precursor for more complex Ins containing molecules. Ins can be formed from endogenous *de novo* synthesis, or from the complete dephosphorylation of phytate or inositol phosphates by phytase and APL in the GIT. However, little is known about Ins in poultry production and is of interest due to the possible benefits it may give to growing birds.

The object of this thesis is to try and understand the importance of Ins for poultry production. Phytase supplementation is routinely used in poultry diets to primarily to increase the availability of organic P. Super dosing phytase has many 'extra-phosphoric effects' including improving feeding efficiency, mineral digestibility, amino acid digestibility, reducing environmental P pollution and increasing the concentration of Ins produced in the GIT. Therefore, it is a logical assumption that Ins may play a role in improved performance and contribute to the beneficial extra-phosphoric effects of super dosing phytase. However, few have assessed the role Ins may play in poultry production. The objective of this thesis was therefore to evaluate the efficacy and possible explanatory mechanisms of Ins involvement in the performance and health of broiler chickens.

1. Supplementing broiler chicken diets with free Ins may aid in bird performance.

2. Increasing free Ins to broiler chickens will manipulate the regulation, concentrations, synthesis of Ins and may aid in bird health.

A schematic diagram of the studies conducted in this thesis is summarised in Figure 1.2.

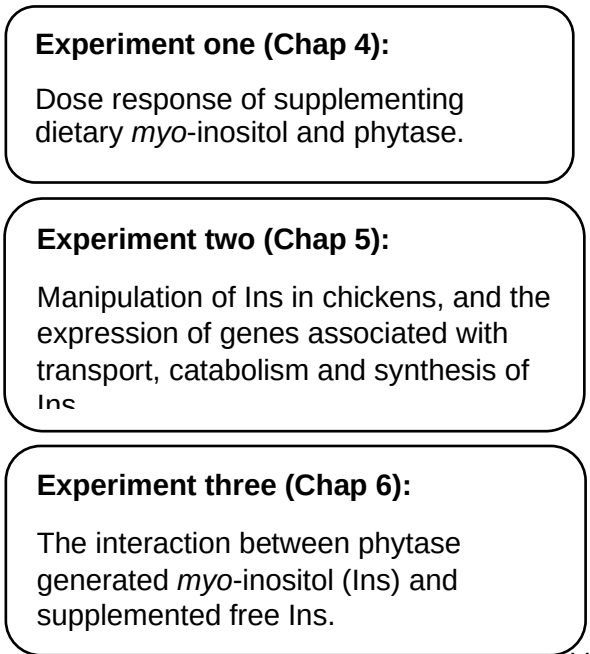


Figure 1.2: Schematic overview of the experiments conducted in this thesis.

Chapter two: Literature review

The following chapter will review current literature and thinking on Ins and its importance in broiler and animal production. The review will establish current gaps in knowledge on poultry production and Ins, Ins supplementation and associated biochemical pathways.

2.1 myo-Inositol

myo-Inositol: is a cyclohexitol sugar alcohol and is one of the nine possible stereoisomers of inositol (cyclohexane-1,2,3,4,5,6-hexol) (figure 2.1). Depending on the spatial orientation of its six hydroxyl groups determines the other possible isomers (Mullaney et al., 2007). The other eight isomers configurations can be seen in figure 2.2. *myo*-Inositol as a versatile chemical building block and is found in many different compounds for example phosphatidylinositol and phytate.

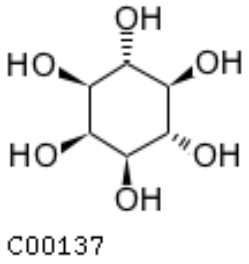


Figure 2.2: structure of *myo*-inositol with the atomic mass of 180.0634 and formula $C_6H_{12}O_6$ (KEGG, 2018).

In literature *myo*-inositol (or cis-1,2,3,5-trans-4,6-cyclohexanehexol) is inconsistently abbreviated for example a study by Loewus and Murthy, (2000) shortened it to MI, Lee and Bedford, (2016) used inositol and Yorek et al., (1993) did not abbreviate. Within the British Poultry journal there are also different abbreviations used in the literature INS, and MYO.

In this literature review *myo*-inositol will be abbreviated to Ins as per the suggestions made by the Nomenclature Committee of the International Union of Biochemistry (NC-IUB) (NC-IUB, 1989). It is also important to note that inositol is an approved synonym for *myo*-inositol (CSID:10239179.). However, without naming which stereoisomer is being discussed can lead to ambiguity, it is therefore important to clarify these terms as each stereoisomer has different biological properties. In this review inositol will be used as a broad term to describe all possible isomers.

2.1.1 History of Ins

Inositol was first discovered by Scherer in 1850, isolating it from meat juice. It was called “inosite” due to its sweet taste (Scherer, 1850). A series of classic papers then followed,

by Maquenne in 1887, describing the purification of inosite and some chemical properties. These first observations included its inert characteristic (compared to glucose) and molecular weight of its acetyl and benzoyl esters (Maquenne,1887[a], Maquenne,1887[b] and Maquenne,1887[c]). Unlike Scherer, Maquenne used plant leaves to extract the inositol, but later used large quantities of horse urine which required large amounts of boiling. This process apparently led to complaints from his neighbours (Irvine, 2016). In 1942 its configuration was finally elucidated by Dangschat and Fischer, (1942) and Posternak, (1942). Furthermore Posternak, established the configuration of the principle inositol, which underpins the 9 possible isomers of inositol naming (Posternak, 1942). Ins was once regarded as a B vitamin however it is no longer classified as an essential nutrient as human and monogastric animals can synthesise sufficient amounts from D-glucose (Regidor and Schindle, 2016). More recently it has been suggested that Ins could be classed as a semi-essential nutrient in poultry nutrition in a review by Gonzalez-Uarquin et al., 2020[c].

2.1.2 Importance in human health

Ins and their derivatives, inositol phosphates and phosphatidylinositol (Ins lipids) are all important biological molecules and commonly found in plant and mammalian cells. The broad spectrum of their activities has been shown to be related to; decrease insulin resistance, increase insulin sensitivity, energy homeostasis, antioxidant and anti-inflammatory activities, role as neurotransmitters, metabolic disorders including polycystic ovary syndrome, gestational diabetes mellitus, infertility, and thyroid disorders (Larner, 2002; Asplin et al., 1993; Heimark et al., 2014; Chatree et al., 2020; Palmieri et al., 2017; Jiang et al., 2009; Wu et al., 2015; Colodny and Hoffman, 1998; Concerto et al., 2023; Bizzarri and Carlomagno, 2014; Benvenga et al., 2016).

The dietary requirements of Ins in humans can either be met from synthesis from glucose or from ingestion and subsequent uptake from the GIT. In a typical human diet of 1800 kcal will contain 225 to 1500 mg of Ins (Clements and Darnell, 1980). Ins is half the sweetness of sucrose (Awuchi et al., 2019). There are no guidelines set by the World Health Organisation on how much Ins we should have from our diets or indeed artificial supplementation as healthy humans can meet the requirements from synthesis. The content of Ins found in food items varies for example a slice of bread made from stone ground flour contains 278.5 mg, in comparison a whole egg contains just 4.5 mg. Citrus fruits contain exceptionally high quantities of Ins, 120 mL of grapefruit juice contains 470 mg of Ins. Poultry products contain a relatively low amount of Ins, typically a portion size of cooked chicken breast would contain 9 mg of Ins, 4-0.6% of the typical diet uptake (Clements and Darnell, 1980). In diets Ins often forms parts of other molecules and is not normally in its free form.

Ins deficiency may occur through a plethora of different mechanisms, which could include reduced dietary intake, decreased synthesis, increased catabolism or inhibition of intestinal and cellular uptake (Dinicola et al., 2017). In the treatment of PSOS in women, a review paper by Facchinetti et al., (2020) compiled dosages in a meta-analysis given to patients which varied from 100 mg to 4 g of Ins. The study concluded that 4 g of inositol (Ins and D-*chiro*-inositol in a molar ratio of 40:1) improved insulin sensitivity and ovarian physiology.

2.2 The "other" inositol isomers

Ins is the most abundant isomer found in nature, of the 9 isomers 6 are naturally occurring (*myo*-, L-*chiro*-, D-*chiro*-, *scyllo*-, *muco*- and *neo*-). The "other" (excluding Ins) inositol isomers are involved in many biological processes and have bioactive properties. The other inositol's are often overlooked due to their rarity, complexity, not being able to quantify them and scientific focus on *myo*-inositol. A brief overview of each of the other inositol isomers can be seen below:

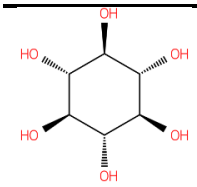
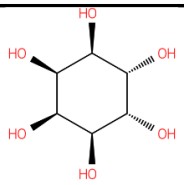
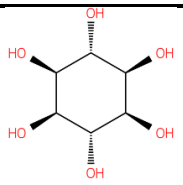
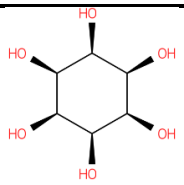
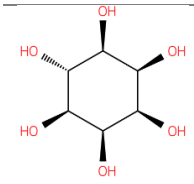
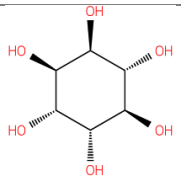
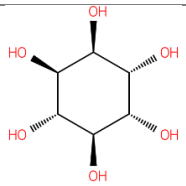
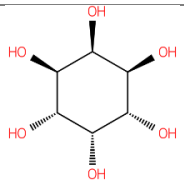
			
***scyllo*-inositol**	*allo*-inositol	*muco*-inositol	*cis*-inositol
			
***epi*-inositol**	L-*chiro*-inositol	D-*chiro*-inositol	*neo*-inositol

Figure 2.2: The other 8 possible isomers of inositol, for *myo*-inositol see figure 1 (ChemSpider, 2018).

scyllo-Inositol: discovered in mammal urine in the 1950s (Helleu, 1957). Studies have shown (both in rats and humans) that small quantities of *scyllo*-inositol are converted from Ins (Sherman et al.,1968; Groenen et al., 2003). *Scyllo*-inositol has been identified as a potential drug for the treatment of Alzheimer's disease. Alzheimer's disease can cause amyloid plaques in the brain (distributing connections between neurones), *scyllo*-Inositol can bind to the proteins involved and prevent the formation of amyloid plaques (Ma et al., 2012). Current research is looking at ways of enzymatically producing *scyllo*-Inositol (Michon et al, 2020, Yoshida; Ramp et al., 2021) *scyllo*-Inositol metabolism (Wu et al., 2022) and qualifying concentrations in various sample types (Monnard et al., 2020).

L-*chiro*-Inositol: the “mirror image” of D-*chiro*-inositol and is referred to as chiral. L-*chiro*-inositol and D-*chiro*-inositol are a pair of enantiomers. As a result, they possess very different biological activities. There is very limited research carried out the biological role of L-*chiro*-inositol, it can be synthesised enzymatically from D-xylose (Kornienko et al., 1997) or from the microbial oxidation of bromobenzene (Brammer et al., 1998).

D-*chiro*-Inositol: can be absorbed from the diet only, it cannot be synthesized endogenously nor produced from Ins (Lin et al., 2009). This contradicts earlier work by Y Pak et al., (1992) suggesting that Ins can be converted into D-*chiro*-inositol by the specific epimerase in the blood, muscle and liver respectively. Some bone related diseases are associated with D-*chiro*-inositol, as it can inhibit the formation of osteoclasts (type of bone cell) which could provide a possible candidate to treat inflammatory bone related diseases (Yu et al., 2012[a]). Furthermore, D-*chiro*-inositol is widely used in clinical practice to treat polycystic ovary syndrome in women by inducing ovulation. D-*chiro*-inositol is involved in two different mechanisms, improving insulin signalling therefore improving insulin levels and can down regulate the expression of steroidogenic enzyme aromatase (conversion of androgens to estrogens) at a cellular level (Gambioli et al., 2021).

allo-Inositol: can be synthesised from carbohydrate-derived dialdehydes (Stockton et al., 2014) or *p*-benzoquinone (Podeschwa et al., 2003). *allo*-Inositol can be found in different plant species, a study by Ratiu et al., (2019) analysed 40 different plant species for novel sources of cyclitols. Blueberries fruits contained the highest levels of *allo*-inositol at 10.84 mg/g DM followed by elder fruits at 3.49 mg/g DM. There are very limited studies on the role *allo*-inositol in animal production, however it was measured as a metabolite in Caecal Chyme by Wu et al., (2021). The study compared control diet with a mix of a probiotic Xylan oligosaccharides (XOS) and bacteria (*Pediococcus acidilactic*). There was a 25-fold increase from the control diet when compared to the mix diet (P=0.044) of *allo*-inositol. The mix diet also improved FCR when compared to the control (P=0.028).

muco-Inositol: First synthesised in the 1930s (Dangschat and Fischer, 1939). More recently, it can be chemoenzymatically synthesised starting from bromobenzene (Brammer and Hudlicky, 1998). There is very limited research carried out on the biological role of *muco*-inositol. A study by Yap et al., (2007) demonstrated that *muco*-inositol could induce glucose uptake by inducing translocation of glucose transporter 4 in rat L6 myotubes in vitro. However, if this happens in vivo is still not fully understood.

cis-Inositol: as *cis*-inositol is not known to naturally occur in nature, the literature possibly describing its biological role is therefore lacking. It can be synthesised from a one-step hydrogenation of tetrahydroxyquinone, however this process requires complex

chromatography (Angyal et al., 1995). Alternatively, is can be synthesised from *epi*-inositol in a seven-step process (Angyal and Hickman, 1971).

epi-Inositol: can be synthesised from carbohydrate-derived dialdehydes (Stockton et al., 2014). The influence by *epi*-inositol of cholinergic and serotonergic induced seizures on lithium treated rats was examined by Williams et al., (1995). *epi*-Inositol was able to block seizures; however, the underlying mechanisms are not understood. *epi*-Inositol can reduce the formation of amyloid plaques in the brain associated with Alzheimer's disease, however this cannot be maintained overtime. Furthermore, *epi*-inositol can inhibit the expression on the gene coding inositol-1-phosphate synthase (INO1), which can reduce the conversion of glucose-6-phosphate into D-*myo*-inositol 3-phosphate (Shaldubina et al., 2002).

neo-Inosiol: synthesised from Ins in 5 steps with good yields (Riley et al., 1998). Interestingly, *neo*-inositol is the least water-soluble of the inositol isomers due to its stable crystal structure (Angyal and Craig, 1994) In mammalian biology, *neo*-inositol has been found in bovine brains and formed by a specific epimerase (Hipps et al., 1997).

2.3 Inositol lipids

Inositol lipids are a diverse family of compounds and are now known to be central to many aspects of cell biology. This is a huge topic area because of the diversity of inositol lipids and their derivatives/cousins and the inositol phosphates impinge on a substantial proportion of cell biology (Irvine, 2016). A few of the common phosphoinositides can be seen in figure 2.3. Regulating lipid transfer and generating and maintaining lipid gradients in the membrane structure of cells is of importance to allow the normal functioning of the cell. It is believed that inositol lipids, also known as phosphoinositides (PI) play an important role in the cellular membrane. The roles of inositol lipids in eukaryotic cells include transmembrane signalling, ion channel regulation, lipid composition, lipid homeostasis and cellular trafficking (Kim et al., 2013). PI is found in the phospholipid component of cell membranes of eukaryotic cells, although the inositol-containing phospholipids are widespread they only make up a minor part of the complex membrane structure. They are synthesised in the endoplasmic reticulum by combining Ins and cytidine diphosphate-diacylglycerol (CDP-DAG) and transported to the cell membrane by either vesicles or cytosolic transfer proteins. The synthesis of PIs is catalysed by phosphatidylinositol synthase which has a high affinity for Ins (K_m = 1.5- 2.5 mM) suggesting that the regulation of Ins is important in the cell (Croze and Soulage, 2013). PIs contain large amounts of stearic acid in the sn-1 positions and arachidonic acid in the sn-2 position of the glycerol backbone (Holub, 1986). The inositol head group of PI can be phosphorylated in different locations, which can produce a range of different

phosphoinositides with different roles in cell signalling. Within the cell these phosphoinositides are present in different organelles; phosphatidylinositol- PI(4)P can be found in the Golgi apparatus, PI(3)P in the endosome and PI(4,5)P_2 in the plasma membrane (Holub, 1986). Cellular phosphatidylinositol phosphate lipids (PIP) are a product of phosphoinositide 3-kinases (PI 3-kinases) and acting on PIs. PI 3-kinases are a family of enzymes involved in a number of cellular functions including cell growth, proliferation, differentiation and survival. Inositol phosphates (IPs) are also hydrolysed by phospholipase C from phosphatidylinositol phosphates and can also be transformed or synthesised by many kinases and phosphatases (Croze and Soulage, 2013; Abel et al., 2001). Although little work has been carried out on the role of inositol lipids in chicken tissues and cells it is believed that many of the cellular process are highly conserved between species.

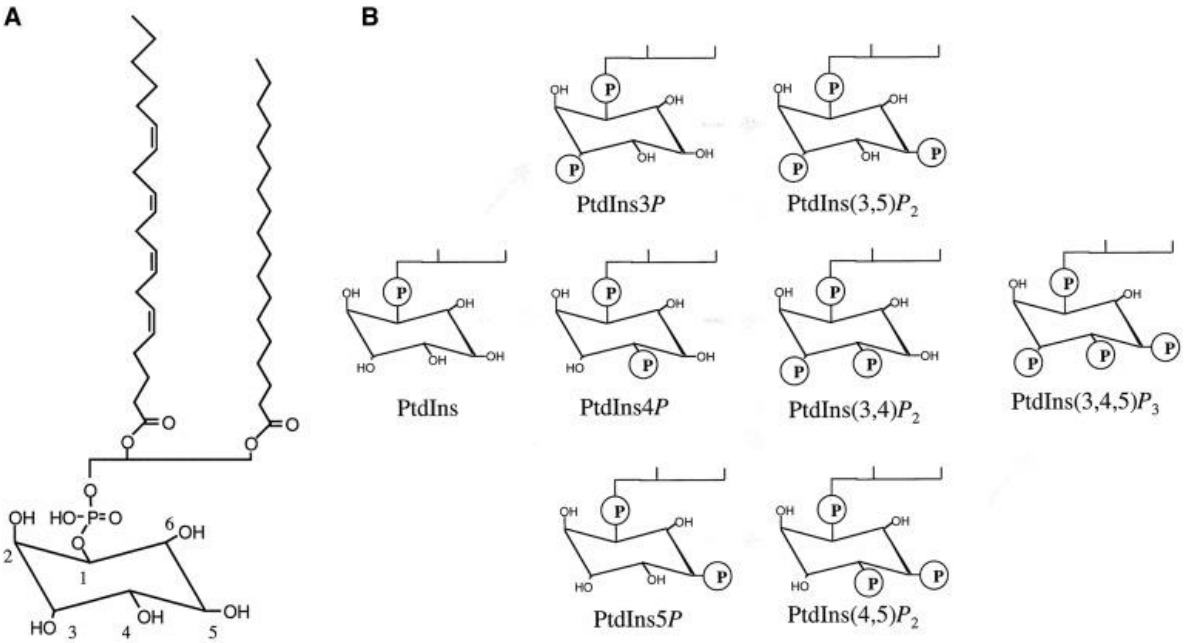


Figure 2.3: The polyphosphoinositol lipids (PI) (A) and a schematic depiction of PI and the seven polyphosphoinositol lipids (B). Extracted from Irvine (2016).

2.4 Inositol phosphates

Ins can be phosphorylated to form inositol phosphates, which typically contain between one and six phosphorus groups (Table 2.1). Due to the properties of inositol and which of the carbons in the carbon ring are phosphorylated there are many different arrangements of IPs. Posternak was also responsible for the identification of phytate as being *myo*-inositol hexakisphosphate in the first decade of the 20th century and the structure of phytate (Posternak 1919). Seeds use phytate as a phosphorus store resulting in plant tissues typically having a greater concentration of phytate than animal tissues. This is because phytate is important in the germination and growing of seeds (Khattak *et al.*, 2007). Phytate is considered to have anti-nutritional properties, chelating minerals into

indigestible complexes and reducing phosphorus digestibility. As a result phytase supplementation in poultry and pig diets is routine, and this area has been given considerable attention.

Table 2.1: The *myo*-inositol phosphates and accepted abbreviations.

Full Name	Number of phosphate groups	IUPAC abbreviation	Common abbreviations
myo-Inositol	0	Ins	Ins
myo-Inositol monophosphate	1	Ins*P*$_1$	IP$_1$
myo-Inositol bisphosphate	2	Ins*P*$_2$	IP$_2$
myo-Inositol trisphosphate	3	Ins*P*$_3$	IP$_3$
myo-Inositol tetrakisphosphate	4	Ins*P*$_4$	IP$_4$
myo-Inositol pentakisphosphate	5	Ins*P*$_5$	IP$_5$
myo-Inositol hexakisphosphate	6	Ins*P*$_6$	IP$_6$

2.4.1 Inositol phosphates in feed stuffs

Plant seeds use phytate as the primary store of P and as a result due to the relance of seeds and their processed products (soyabean meal and rapeseed meal) in poultry diets, makes phytate an important source of organic P. Poultry diets typically contain 0.2-0.3% of Phytate-P depending on processing, feed stuff and genotypes. Figure 2.4, shows the phytate-P, P and inositol phosphate esters of common feed stuffs, with barley containing the most phytate-P in this study. When looking at the study by Rodehutscord et al., (2016) (Figure 2.4), it is important to note the variation within one feed stuff, this variation is likely due to the different genotypes, soil type and fertiliser usage. Inositol pentakisphosphates and other inositol esters can be found in common feed stuffs but only at marginal concentrations. Processing grains to produce oilseed meals can shift the inositol phosphate profile by increasing the IP$_5$ concentration (Rodehutscord et al., 2017).

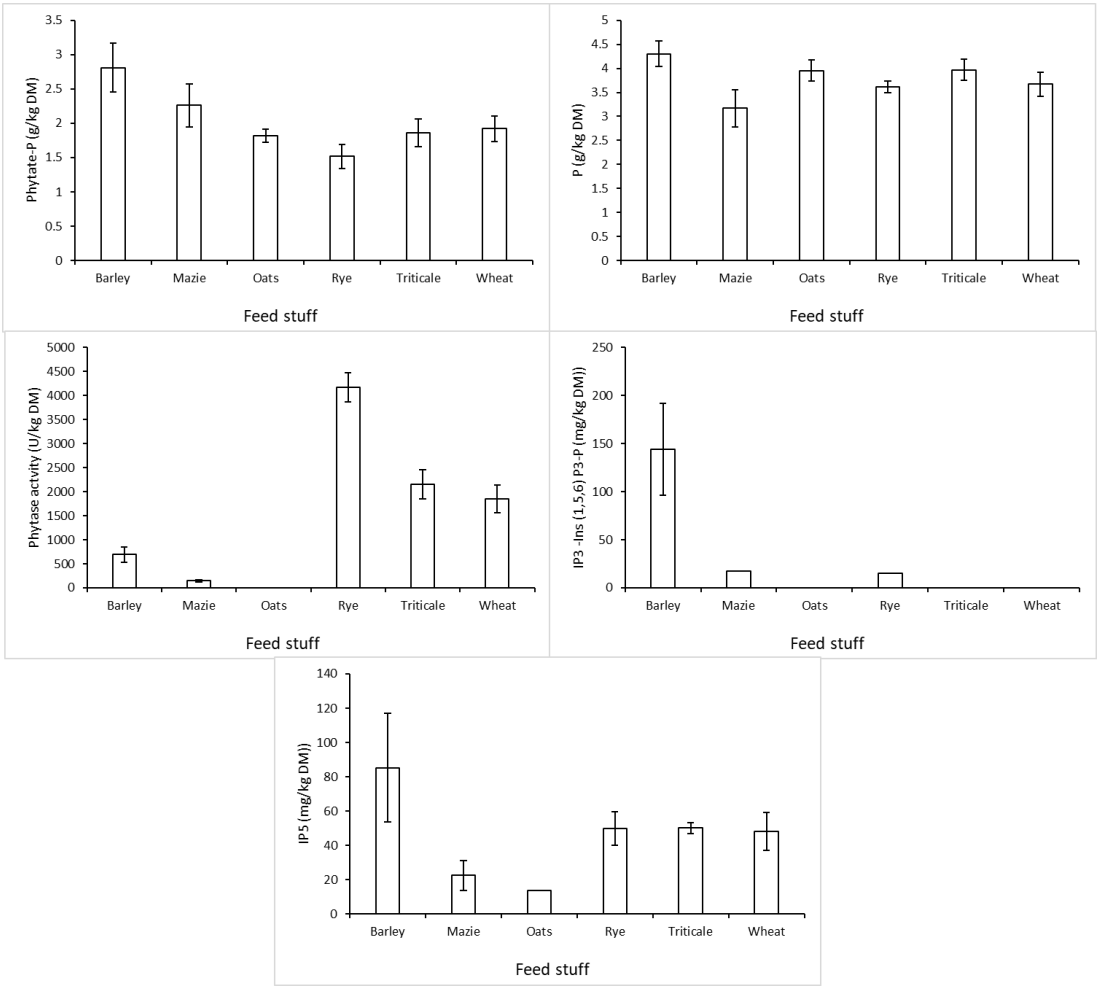


Figure 2.4: The phytate-P, P, phytase activity, IP3 (Ins (1,5,6) P3-P) and IP5 (Ins (1,2,3,4,6) P5-P, s (1,2,3,4,5) P5-P and Ins (1,2,4,5,6) P5-P) contents of common feed stuffs used in poultry diets, data extracted from Rodehutscord et al. (2016). Data is based on winter barley (n = 21), maize (n = 27), oats (n = 14), winter rye (n = 22), winter triticale (n = 21) and winter wheat (n = 29).

2.5 Phytases

Phytates (phosphoric monoester hydrolases (EC 3.1.30)) are a diverse group of phosphatase enzymes which have a specific substrate of phytate. The mode of action includes dephosphorylating a phosphate group off the phytate molecule, releasing water and phosphate (see figure 2.5). Phytases can be grouped based on their catalytic mechanism and include histidine acid phytases (HAPhy), β-propeller phytases (BPPhy), cysteine phytases (CPhy) and purple acid phytases (PAPhy). Phytases can be classified further based on their optimum pH conditions, acid and alkaline phytases. Acid phytases are of most interest in monogastric nutrition as they are able to remain viable in the acidic conditions of animals stomachs. Finally, phytases can be characterised based on which carbon of the inositol ring is dephosphorylated first, 3-phytases (E.C. 3.1.3.8) remove the phosphate group on the D-3 position first, 6-phytases (E.C. 3.1.3.26) remove the

phosphate group on the L-6 (D4) position and 5-phytases remove the phosphate group on the D-5 position (E.C. 3.1.3.72) (Bedford and Partridge, 2010). These enzymes are able to break the ester bond between the phosphate groups and Ins ring producing intermediary products including the mono-, di-, tri-, tetra- and penta-phosphate forms of inositol (See Table 1) (Turner *et al.*, 2007). Phytases are routinely added to poultry and pig diets as they are able to release phosphorus into the upper parts of the digestive track and reduce the anti-nutritional factors of phytate. Modern 3rd generation phytases used in animal nutrition are 6-phytases with increased thermostability and have higher affinity for the lower IPs which result in a greater proportion of phytate being completely dephosphorylated and increased generation of Ins (Bedford and Partridge, 2010). Furthermore, phytase has the added benefit of reducing environmental P pollution and protecting water systems, as phytase has been reported to reduce the excreta P content by as much as 53% (Cowieson et al., 2004).

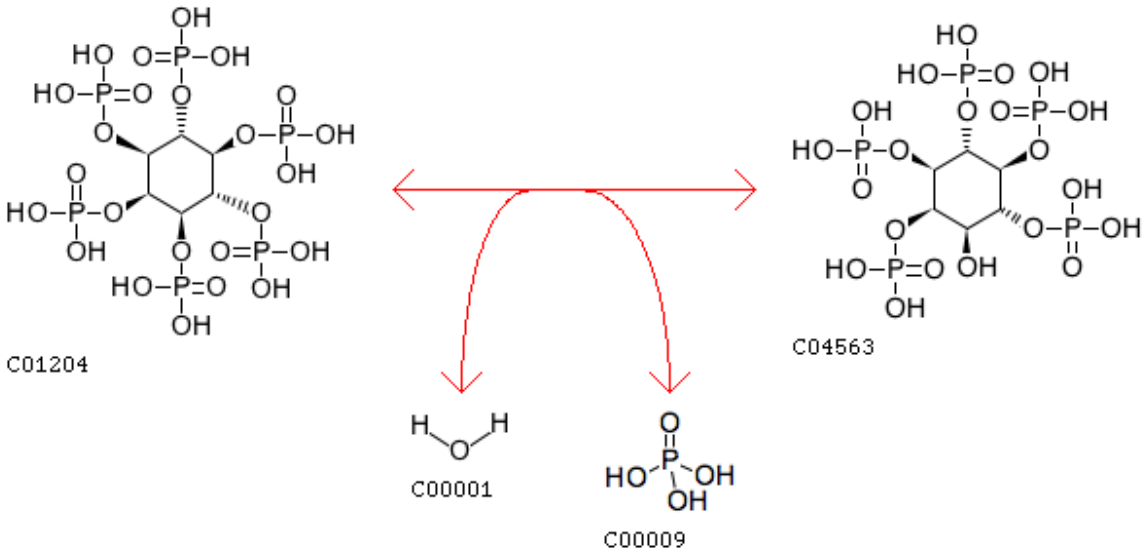


Figure 2.5: Hydrolysis of phytate by phytase enzymes (KEGG, 2019).

2.6 *De novo* biosynthesis of *myo*-inositol

Ins can be synthesised endogenously from D-glucose in three steps and this process can occur in the testis, brain, kidney and liver of rats (Hauser and Finelli, 1963). Firstly glucose is phosphorylated by hexokinase (EC:2.7.1), next radical cyclization of D-glucose 6-phosphate to 1 L-*myo*-inositol 1-phosphate by 1L-*myo*-Inositol-1-phosphate synthase (EC:5.5.1.4) and finally 1 L-myo-inositol 1-phosphate is dephosphorylated by inositol 1-monophosphatase (EC: 3.1.3.25) to make the final product of Ins (Figure 2.6). The second step in the process is rate-limiting in most animals (Croze and Soulage, 2013).

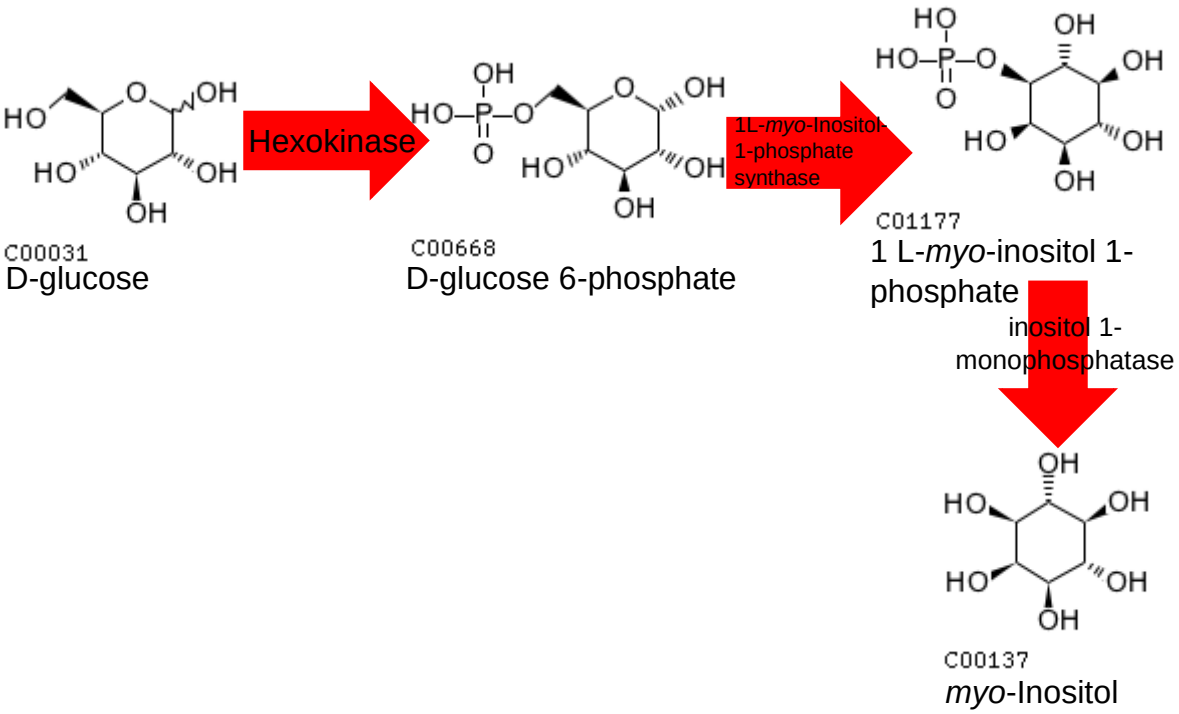


Figure 2.6: *De novo* biosynthesis of *myo*-inositol from D-glucose pathway (KEGG, 2019).

In humans and other animals endogenous biosynthesis of Ins is critical, it appears that all cells contain varying concentrations of Ins. Tissues containing higher levels of Ins such as the brain, kidney, liver and testis have been shown to be able to transform Ins from D-glucose. The kidney can produce 2 grams of Ins and the brain can produce 4 grams a day, thus demonstrating its importance as the typical human intake is considerably lower than the biosynthesis of the brain and kidney alone (Clements and Diethelm, 1979). Spector and Lorenzo, (1975) demonstrated that one half of the unbound Ins in a brain of a rabbit was derived from endogenous biosynthesis from D-glucose with the remainder of Ins being transported into the brain from the blood.

2.7 Digestion and absorption

With very small quantities of free Ins in feed, most is consumed as phytate, found in plant foodstuffs. Monogastric animals can produce phytase in the intestinal mucosa, forming intermediate esters of inositol (IP_{5-1}) and free Ins. Despite routine use of dietary phytase in poultry feeds, poultry do have sufficient quantities of phytase in the small intestinal mucosa (Maenz and Classen, 1998). However due to low phytate solubility in the lower part of the intestine can affect the efficacy of the endogenous phytase (Lee and Bedford, 2016). The last step to release free Ins is typically carried out by the hydrolysis of IP_1 by endogenous alkaline phosphatase. Furthermore, Ins can also be consumed as part of phosphatidylinositol (PI) (lipid bound), hydrolysed in the intestinal lumen by pancreatic phospholipase A to form lyso-phosphatidylinositol (lysoPI). lysoPI is then reacylated via acyltransferase upon entering the intestine cell and further hydrolysed to glycerylphosphorylinositol (Schlemmer et al., 2009; Holub, 1986).

Free Ins and phosphatidylinositol is rapidly absorbed with high efficacy from the small intestine (Kohlmeier, 2003). In humans almost all free Ins is absorbed from the gastrointestinal track (99.8%) and in healthy humans Ins transported in the blood at 30 µM with an approx. half-life of 20 mins (Holub, 1986). Ins is also present in serum lipoproteins as PI and as phytate with a range of 0.1-0.4 µM (Croze and Soulage, 2013). Ins uptake in the intestine is mediated by an active transport system, a study by Caspary and Crane, (1970) showed that Ins was rapidly uptaken against a concentration gradient. Thus, proving that active transport is needed for Ins to be uptaken from cells. Furthermore this process is temperature dependent, pH sensitive, stereospecific and the binding and absorption is dependent on Na^+ (Takenawa *et al.*, 1977). Lewin *et al.*, (1976[a]) tracked the dispersal of radioactive Ins in male rats following a intraperitoneal injection. After a few hours the radioactive Ins was located in the spleen, liver, pituitary gland, prostate, kidney and thyroid glands of the rats. However, muscle tissues (the heart and diaphragm) and brain, concentrated little of the radioactive Ins. Most of the Ins found in the liver was contained in the lipid fraction.

2.8 Cellular uptake

Cells can obtain Ins from a number of sources; from the *de novo* biosynthesis of D-glucose, breakdown of PIs or via specialist Ins transporter proteins actively transporting Ins from the extracellular fluid (Croze and Soulage, 2013). Currently three transporter systems have been identified, two sodium (Na^+) dependent (SMIT1 and SMIT2) and one proton (H^+) dependent (HWIT). SMIT 1 and 2, transport Ins by co-transporting 2 sodium ions along the concentration gradient to generate the energy required to transport one molecule of Ins across the membrane and into the cell. HWIT also generates a concentration gradient to transport Ins but with H^+ rather than Na^+ (Fu *et al.*, 2012). The gene encoding for SMIT1 is *SLC5A3* and is part of the Solute Carrier Family 5 (SLC5A) (Berry et al., 1995; Aouameur et al., 2007). The respective gene for SMIT2 is *SLC5A11* and on an amino acid level shows 43 % sequence identity to SMIT1 (Schneider, 2015). Both proteins are located on the plasma membranes but are found in abundancies in different organs. SMIT1 is expressed in the kidney, brain, placenta, heart, skeletal muscle and lung, were as SMIT2 is expressed in the kidney, placenta, heart, skeletal muscle, liver and poorly expressed in the brain (Roll et al., 2002; Berry et al., 1995). The proton coupled Ins transporter, HMIT is expressed by the gene *SLC2A13* and part of the Major Facilitator superfamily. SMIT1 preferred substrate is Ins (K_m = 55 µM), SMIT2 also accepts D-*chiro*-inositol (K_m = 130 µM) in addition to Ins (K_m = 120 µM), both transporters have a low affinity for glucose with a K_m of 50 mM for SMIT1 and 30 mM for SMIT2 respectively (Schneider, 2015). High concentrations of L-fucose can act as a competitor inhibitor to Ins in the SMIT1 transporter system. D-*Chiro*-inositol can compete with Ins in SMIT2

mediated transport, therefore it is possible to distinguish between SMIT1 and SMIT2 activity *in vivo* by the transported substrates (Schneider, 2015; Croze and Soulage, 2013). Lerner and Smagula, (1979) investigated Ins transporter systems in the small intestines of chickens. It was observed that Ins transport could be inhibited by phlorizin, sulfhydryl reagents, removal of sodium and high levels of glucose. Moreover, the addition of 16.6 mM of glucose (a concentration typically used to simulate the intestinal milieu) could suppress the transport of Ins concentrations ranging from 1-20 mM. This study suggests a semi-synthetic diet for broiler chickens containing high amounts of glucose could influence Ins uptake. Furthermore, this has greater impacts with starch rich diets, whereby rapid generation of glucose and maltose in the brush border of the intestine could again compete with the transporter proteins due to a higher affinity to glucose than Ins (Lee and Bedford, 2016).

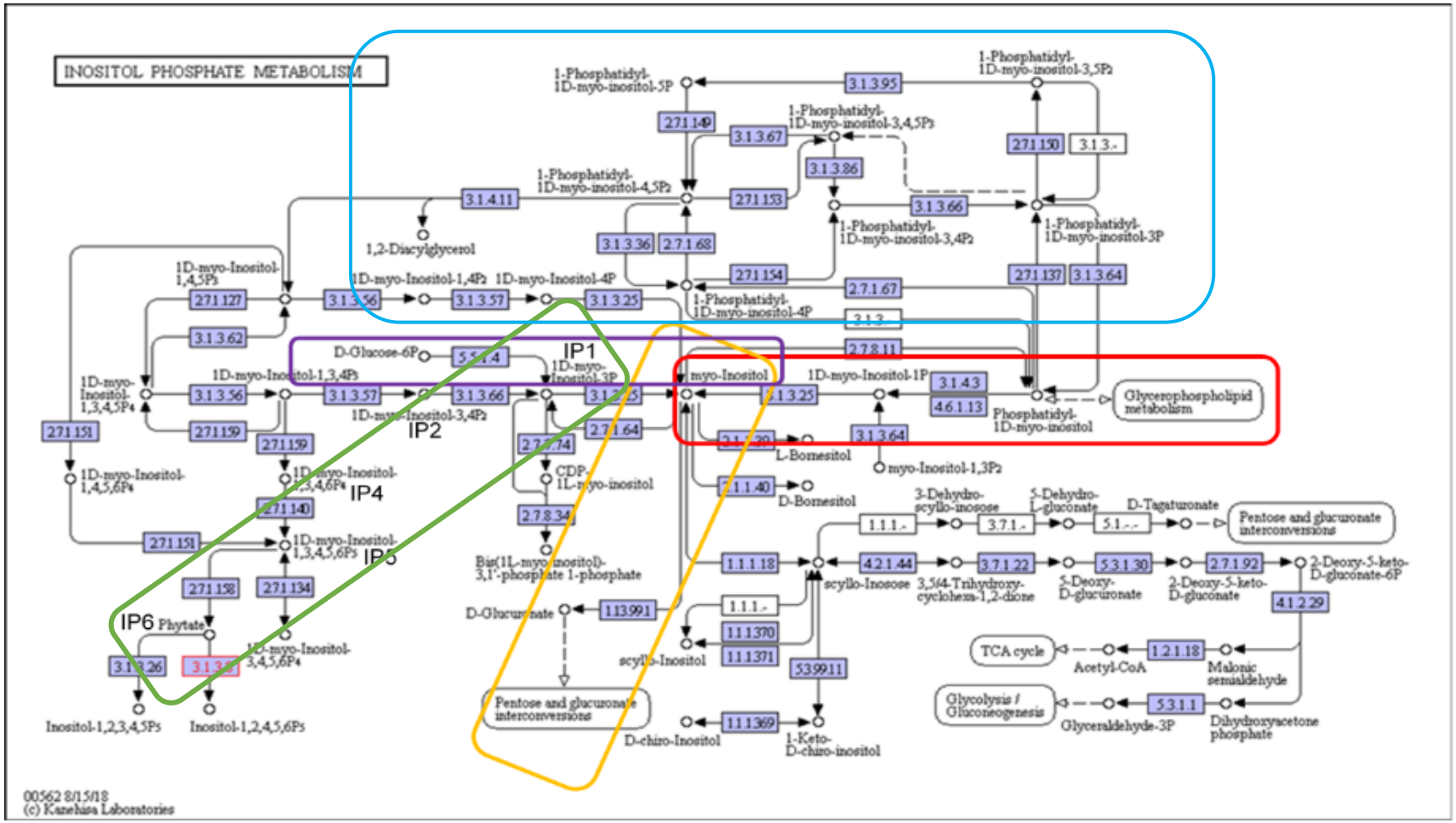


Figure 2.7: *Myo*-inositol associated pathways, pathways of interest are highlighted: Green- destruction of phytate to form Ins, Purple- biosynthesis of Ins from D-glucose, Yellow- catabolism of Ins by pentose and glucuronate pathway, Blue- Phosphoinositide metabolism and Red- phosphatidylinositol metabolism/catabolism. Extracted from KEGG, (2019).

2.9 Catabolism of *myo*-inositol

The only significant organ involved in the catabolism of Ins is the kidney, since radioactively labelled Ins was not degraded to respiratory ^{14}carbon dioxide in rats that had there kidneys removed (Howard and Anderson, 1967). Ins is catabolised to D-glucuronic acid by *myo*-inositol oxygenase (MIOX) (EC:1.13.99.1), whereby it is transported to the liver and is metabolised to D-xylulose 5-phosphate, and subsequently enters the pentose phosphate pathway (Figure 2.8). Furthermore, Ins can also be oxidised to carbon dioxide and only a small fraction of the disposal of Ins was accounted by urinary excretion in rats and humans (Lewin et al., 1976; Hankes et al., 1970; Moscatelli and Larner, 1959; Charalampous and Lyras, 1957). The kidney is the site of regulation of Ins, with poor kidney function associated with increased Ins in blood (Holub, 1986). Therefore the kidney plays a key role in controlling the plasma concentration of Ins and movement of Ins around the body.

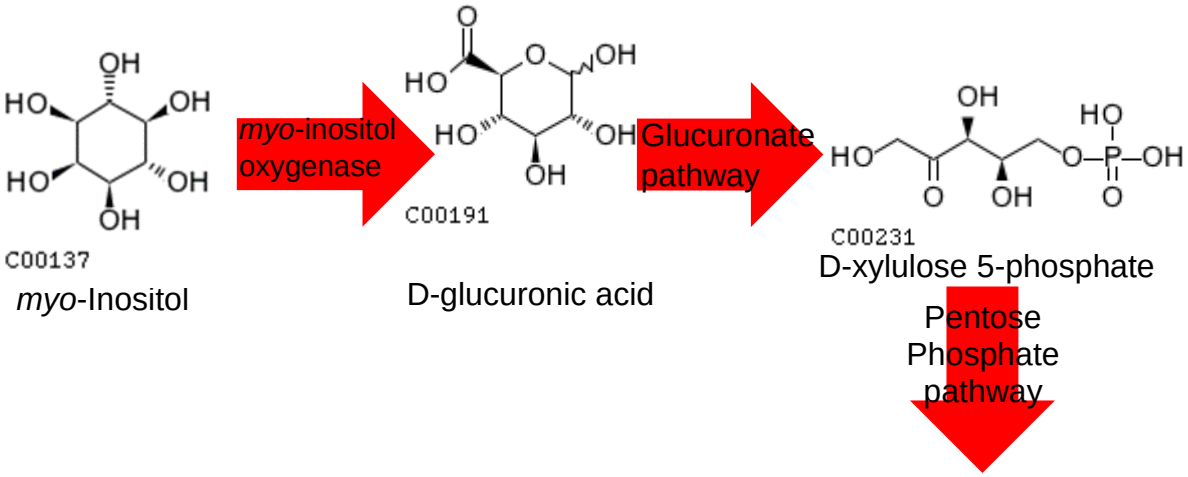


Figure 2.8: Catabolism of *myo*-inositol via Glucuronate and Pentose Phosphate pathways (KEGG, 2019).

2.10 Current understanding of *myo*-inositol in animal production

2.10.1 myo-Inositol in broiler chicken production

Much of our current understanding of *myo*-inositol has come from experiments looking at improving broiler chicken performance as a result of phytase supplementation rather than the direct supplementation of Ins into diets. Phytase can dephosphorylate of phytate to IP_1 and the animals own alkaline phosphatase can complete the dephosphorylation to produce Ins. The recommended dosages of phytase is 250-2500 FTU/kg in feed for fattening chickens. Feeding exogenous phytases can change the inositol phosphate profiles in the broiler and releasing phosphorus which was once bound to phytate. Figure 2.9, clearly shows the increase in Ins content and lower inositol phosphates ($IP_{5\text{-}1}$) in the ileal digesta by

phytase. Improving the phosphorus digestibility and improves growth performance of the chicken (Gautier *et al.*, 2018).

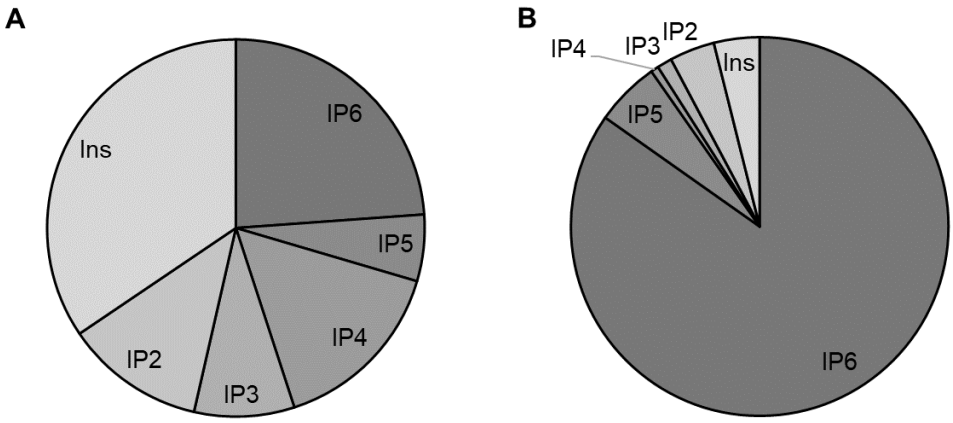


Figure 2.9: The effect of phytase supplementation (1500 FTU/kg) (A) and un-supplemented phytase (B) diets on inositol phosphate profiles in the ileal digesta of broiler chickens. Data extracted from *(*Gautier et al., 2018*)*.

More recently the concept of “super-dosing” phytase has demonstrated improved weight gain and performance compared to a standard enzyme dose. However, the concept of a super-dose was first reported by Nelson et al., (1971) and demonstrated improvements in phytate destruction from 38.9% with 950 FTU/kg to 99.4% with 7600 FTU/kg (Figure 9). There was also linear response between phytase supplementation dosage and weight gain and bone ash, with the 7600 FTU/kg improving weight gain by 131% compared to the un-supplemented diet.

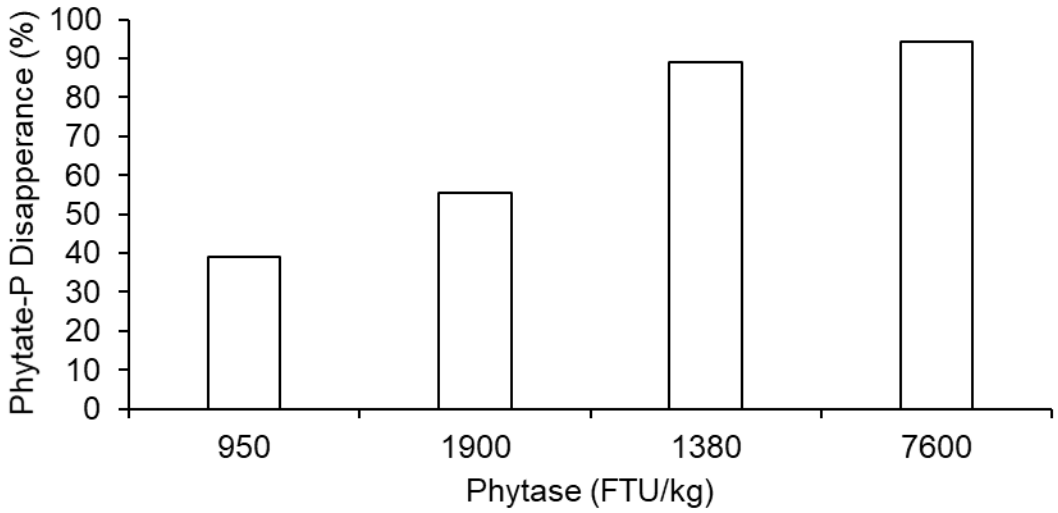


Figure 2.10 Effect of graded levels phytase on the disappearance of phytate in broiler chicks. Data extracted from *(*Nelson et al., 1971*)*.

Since the initial research by Nelson and commercialisation of phytase more attention has been given to the idea of super-dosing phytase. Shirley and Edwards, (2003) supplemented corn-based diets with a range of phytase dosages (See table 2.2) and reported a quadratic increase in phytate disappearance, body weight gain and feed intake.

Table 2.2: Effect of graded levels of phytase on broiler chicks 0-16 days on growth performance and phytate disappearance. Data extracted from (Shirley and Edwards, 2003)**.**

Phytase (U/kg)	Body weight gain (g)	Feed intake (g)	FCR	Phytate disappearance (%)
0	287	381	1.33	40.3
94	354	441	1.25	42.3
188	358	441	1.23	51.9
375	399	490	1.23	49.5
750	424	505	1.19	58.4
1500	459	548	1.19	65.2
3000	481	558	1.16	73.5
6000	494	580	1.17	84.9
12000	515	595	1.15	94.8
P-vaule	P<0.001	P<0.001	P<0.001	P<0.001

Further studies Pirgozliev *et al.*, (2007), Augspurger and Baker, (2004) and Simons et al., (1990) have also shown an improvement with growth performance and increasing levels of phytase supplementation above the conventional levels. Although these reports clearly state the benefits of super-dosing there is no clear understanding on the underpinning mechanisms involved. There are three main explanations on why a super-dose of phytase may elicit a beneficial effect on the broiler. Firstly, a reduction in residual phytate and inositol phosphates resulting in the removal of the anti-nutritional effects that these compounds

have. Secondly, an increased and more rapid phosphorus digestion. Finally, the increased generation of Ins with potential beneficial activities.

There are many studies looking at the effects of phytase generated Ins rather than direct supplementation, however it is difficult to get a good understand of Ins due to the lower inositol phosphates and the removal of the anti-nutritional effects of phytate. Studies on the effect of free dietary Ins supplementation on performance, bird health and other variables can be found summarised in Table 2.3. There are limited studies assessing the effects of supplementing broiler diets with Ins, the main outcomes include bird performance, nutrient and energy digestibility and blood chemistry. As summarised in table 2.3, many studies indicate that Ins has no effect on the study variables which is surprising given the bioactive nature of Ins. This may be due to other factors including phosphorus and calcium levels in diets or more likely that the broiler chicken is able to produce enough Ins from *de novo* biosynthesis and the lack of understand of Ins in broiler chickens.

Overall, when comparing the studies, the effects of Ins are inconsistent and demonstrate that more understanding is required to understand the effects and pathways involved with Ins in chickens. Furthermore, Ins supplementation didn't show any clear response regarding performance benefits/reductions due to what stage of growth it was supplemented at. Due to the lack of literature it is hard to make recommendations on how much Ins or phytase should be added to diets to meet the Ins requirements of the growth broiler and at what stage of development it is required at.

Table 2.3: The use of free dietary Ins in poultry experiments.

Reference	Ins dosages used	Duration	Poultry Type	Variables measured	Outcomes
Hegsted et al., 1941	0.1% (1 g/kg)	0-28 days	Broiler chickens	BW gain	Ins increased BW gain by 18 to 52 g
Dam, 1944	1.5% (15 g/kg)	From day 0	White leghorn chickens	Exudative diathesis and encephalomalacia (Vitamin E deficiencies)	Ins reduced the symptoms of encephalomalacia and exudative diathesis
Pearce, 1975	0.25% (2.5 g/kg)	1-8 weeks	Ross broiler chickens	BW gain Mortality from fatty liver and kidney syndrome (FLKS) Liver weight, lipid content, DM, and lipogenic enzyme activities	Ins reduced BW gain by 72 g and Increased liver weight (as percentage of BW) Ins did not affect the incidence of FLKS or liver lipid metabolism
Zyla et al., 2004	0.1% (1 g/kg)	0-21 days	Ross broiler chickens	Performance Toe ash, P and Ca retention Liver weight	Ins increased BW gain and reduced FCR Ins reduced P retention by 6% Ins increased liver weight
Józefiak, 2012	0.15% (1.5 g/kg)	0-42 days	Broiler chickens	Performance	Experiment one: Ins significantly increased FCR for 1-10 days but reduced FCR for 21-42 days and overall FI was reduced during 21-42 day period and overall Experiment two: Ins significantly increased BW during 21-42 and 0-42 days FI was increased by Ins during 21-42 days FCR was significantly reduced with Ins supplementation during 21-42 and 0-42 days
Zyla et al., 2012	0.1% (1 g/kg)	50-62 weeks	Bovans Brown laying hens	FI and layer performance Eggshell quality, yolk cholesterol and fatty acid deposition	Ins reduced FI, egg production and deposition of eicosanoid fatty acids in yolks

Zyla et al., 2013a	0.1% (1 g/kg)	0-42 days (in two phases 0-21 and 22-42 days)	Ross broiler chickens	Performance Toe ash Concentration of triglycerides and HDL cholesterol in blood serum.	Ins improved FI, BW gain and FCR (compared to control)
Zyla et al., 2013b	0.1% (1 g/kg)	50-62 weeks	Bovans Brown laying Hens	Haematology (Haemoglobin, haematocrit, erythrocytes, leucocytes, lymphocytes, monocytes, heterophils, eosinophils and Basophils)	Ins reduced basophils percent in the white blood cells compared to the control
Cowieson et al., 2013	1.5% (15 g/kg)	1-42 days (experiment one and two)	Ross broiler chickens	Experiment one: Performance Experiment two: Performance Blood parameters (glucagon, insulin, glucose, total cholesterol, HDL cholesterol, and triglycerides)	Experiment one: Starter period (1-10 days) Ins increased FCR Finisher period (21-42 days) Ins reduced FI and improved FCR Overall (0-42 days) Ins reduced FI and improved FCR Experiment two: Finisher period (21-42 days) Ins increased BW gain, FI and improved FCR Overall, (0-42 days) Ins increased BW gain improved FCR Ins increased glucagon insulin, glucose concentrations and reduced HDL cholesterol
Beeson, 2017	3% (30 g/kg)	0-21 days	Ross 708 broilers	Performance Digestibility Inositol phosphate esters Gut pH Gene expression of genes involved in nutrient transport of Ca, Na and glucose Blood serum chemistry	No significant difference in performance Ins increased DM, N and Mg ileal digestibility Ins reduced Ca and Zn ileal digestibility Ins increased total DM, N and K digestibility Ins reduced total Ca, Fe and Zn digestibility Ins increased pH in caeca

					Ins was increased in the excreta, gizzard and ileal digesta with birds supplemented with Ins IP3 was reduced in the excreta and ileal digesta with birds supplemented with Ins Ins caused the down regulation of Ferroportin in the jejunum
Lee et al., 2017	0.3% (3 g/kg)	0-42 days	Cobb 500 broiler chickens	Performance Bone parameters	No significant of Ins on performance Ins reduced bone strength but increased P % in bone ash
Pirgozliev et al., 2017	0, 0.25, 0.5 and 0.7% (0, 2.5, 5 and 7 g/kg)	7-17 days	Ross broiler chickens	Performance Dietary energy, sialic acid excretion and digestibility of DM, N, Fat and P	Quadratic response seen in FI, ADWG, AMEn intake and DM digestibility (with 2.5 g/kg improving variables) Increasing Ins reduced secretion of sialic acid in excreta
Farhadi et al., 2017	0.15 % (1.5 g/kg)	0-42 days	Ross Broiler chickens	Performance Serum metabolites Gait, incidence of foot pad lesions and hock burn scores	Ins increased total protein in serum Ins reduced ileum P content Ins reduced litter moisture on day 42
Sommerfeld et al., 2018	0.38% (3.8 g/kg) starter (1-11 days) 0.35% (3.5 g/kg) grower (11-22 days)	0-22 days	Ross Broiler chickens	Performance Inositol phosphate esters Concentrations of Ins in the digestive tract and blood plasma Bone parameters Prececal digestibility of amino Acids.	Ins significantly improved G:F ratio for each phase and overall Ins increased the concentration of IP_5 (Ins(1,2,3,4,5)P5) and Ins in terminal ileum Ins increased the concentration of IP_4 (Ins(1,2,5,6)P4), Ins and IP_3 in the crop Ins increased the concentration of Ins in blood plasma Ins reduced the digestibility of arginine, isoleucine, leucine, phenylananine, asparagine, glutamic acid, proline and tyrosine.
Farhadi et al., 2019	0.15% (1.5 g/kg)	1-23 days	Ross broiler chickens	pH of digesta and diets subjected to in vitro digestion in gizzard and jejunum.	Ins supplementation decreased the pH of during in vitro digestion for gastric and gastric-small intestine

				Ca, P, Zn, and Fe solubility of digesta and diets subjected to in vitro digestion in gizzard and jejunum.	
Pirgozliev et al., 2019	0.3 and 3% (3 and 30 g/kg)	7-21 days	Ross broiler chickens	Performance and liver weights AME, fat digestibility, DM and nitrogen retention Ca, P, fat and N digestibility and retention Sialic acid excreta concentration Plasma Ins, APL and Vitamin E	30 g/kg Ins increased Ins plasma and ALP concentrations Ins increased liver weights and hepatic N retention in a dose dependent manner
Greene et al., 2019	0.3% (3 g/kg)	0-56 days	Cobb 500 broiler chickens	Performance and carcass confirmation Plasma metabolite and Ins levels in muscle WB incidence Gene expression of pathways associated with muscle hypoxia	Ins improved FCR compared to control but no significant difference in carcass confirmation, plasma metabolites or muscle minerals Ins supplementation increased Ins in muscle compared to the control HBBR mRNA (oxygen-sensing gene) was reduced by Ins compared to control HBM, HBA1 and ENGL1 mRNA (oxygen-sensing genes) was increased by Ins compared to control
Herwig et al., 2019	0.16% (1.6 g/kg)	19-59 weeks	Lohmann LSL lite hens	Behaviour observations and novel object tests Heterophil-to-lymphocyte (H/L) ratios, end of trial feather coverage, skin lesions and mortality	Ins reduced the tendency to peck at the novel object tests by 300 sec Ins reduced vent feather cover by 12% and tended to increase mortality by 13% Ins reduced fearfulness in laying hens
Gonzalez-Uarquin et al., 2020	1-11 days 0.38% (3.8 g/kg) 11-22 days 0.35% (3.5 g/kg)	1-22 days	Ross broiler chickens	Plasma Ins Plasma metabolome profile	Ins supplementation significantly increased plasma concentrations of Ins, dopamine and serotonin

Cowieson and Zhai, 2021	1, 2, 3, 4 and 5% (10, 20, 30, 40 and 50 g/kg)	8-18 days	Cobb 500 broiler chickens	Performance Bone parameters DM, GE, CP, Ca, P and IDE Ileal digestibility DM, GE, AMEn, AME, P and Ca retention	Positive linear increase with increasing Ins for final BW, WG and CP retention Negative linear increase with increasing Ins for FCR, DM, GE, Ca and IDE Ileal digestibility and AMEn
Whitfield et al., 2022	0.3% (3 g/kg)	0-56 days	Cobb 500 broiler chickens	Plasma and liver Ins concentrations	Samples obtained from Greene et al., 2019 Ins increased Ins connections in plasma day 18 and liver day 18

2.10.2 myo-Inositol in aqua culture

It is also important to assess *myo*-inositol supplementation in other monogastric animals including its importance and benefits in aquaculture. Shiau and Su, (2005) reported that *myo*-inositol was required for maximum growth of juvenile tilapia (*oreochromis niloticus*× *oreochromis aureus*), in dose response designed experiment it was observed that there was a liner response between Ins supplementation and hepatic Ins concentration. This study concluded that to grow tilapia with maximum growth, 400 mg/kg of Ins in diets is required. Interestingly, the addition of an antibiotic to the basal diet did not affect hepatic Ins or growth performance suggesting the gut bacterial microbiome synthesis was not a significant source of Ins. Moreover, Shiau and Su, (2004) reported a statistical improvement in juvenile grass shrimp (*penaeus monodon*) weight gain ($P<0.05$), protein efficiency and Ins in the midgut gland with 3943 mg/kg Ins in diet compared to 2036 mg/kg Ins in diet. Regression analysis indicated that the requirement to grow shrimp was 3400 mg/kg diet. Other species used in aquaculture have ins dietary requirements of Ins for maximum performance, parrot fish (*oplegnathus fasciatus*) require 100 mg/kg of Ins in diet (Khosravi et al., 2015), grass carp (*ctrnopharyngodon idella*) require 166-214 mg/kg of ins in diet (Wen et al., 2007), Olive flounder (*paralichthys olivaceus*) needs 617 mg/kg of dietary Ins in feed (Lee et al., 2008) and juvenile gibel carp (*carassius auratus gibelio*) also has a Ins requirement of 165 mg per a kg of diet (Gong et al., 2014). These studies provide information on the importance of Ins supplementation in animal production. Most importantly not all species are able to meet the requirements of Ins for maximum growth by *de novo* synthesis alone and prove that Ins is required to maximise performance.

However, a study by Burtle and Lovell, (1989), observed that there was no statistically difference in Ins tissue content (liver brain and kidney) in channel catfish (*ictalurus punctatus*) with diets formulated with 400 mg/kg or without Ins and the *de novo* biosynthesis was sufficient to meet the tissue requirement of the catfish. The only affect Ins supplementation had on the fish was to reduce the lipid content of the brain. The two enzymes involved in the regulation of Ins (*myo*-inositol-1-phosphate synthase and inositol-1-phosphatase) was increased in the liver when there was no Ins supplementation, thus suggesting the fish was able to regulate Ins to sufficient levels. Furthermore, a study using juvenile sunshine bass (*morone chrysops* ♀× *morone saxatilis* ♂) showed that the fish could produce sufficient amounts of Ins by *de novo* synthesis and there was no difference between brain and liver content of Ins by treatment groups (graded levels) (Deng et al., 2002). Further studies on Abalone (*haliotis discus hannai ino*), have also found that these species of fish are able to synthesize sufficient quantities of Ins (Mai et al., 2001).

There are many aquaculture trials suggesting a benefit of supplementing Ins in diets. However, it is important to note that a number of species are able to synthesise the Ins

requirements of the animal. There are no guidelines stating the Ins requirement of Ross 308 broiler chickens. It may be the case that boiler chickens may not require Ins supplementation for maximum growth performance. Research using more than one dose of Ins in treatment diets is very limited in boiler chickens' publications and do not report consistent findings. Pirgozliev et al. (2007) suggested that 2.5 g/kg of supplemented Ins in diets maybe required for maximum growth for 7-17 days of age, however further work is required to quantify varying levels of Ins in diets and the resulting growth performance in chickens.

Although these studies were not on broiler chickens, it is still important to understand that there is variation in the dietary requirements of different aquaculture species. Much of this initial research in aquaculture on Ins was due to Ins being once classified as a vitamin, rather than its link with super-dosing phytase and the subsequent increase generation of Ins.

More recently focus has changed in aquaculture research to focus on the link between antioxidant properties of dietary Ins supplementation. Antioxidants are a group of molecules that inhibit or stop free radical reactions, preventing or delaying cellular damage. Some compounds are able act as *in vivo* antioxidants increasing the level of endogenous antioxidant defences including increasing the expression of enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Nimse and Pal, 2015). Interestingly no dose response studies using Ins in broiler production has been carried out and subsequent antioxidative status of the broiler chicken. Several studies in aquaculture have been carried out on the potential antioxidant properties of Ins. In a study looking at juvenile Jian carp (*Cyprinus carpio var.* Jian), fish were fed graded levels of Ins (163.5, 232.7, 384.2, 535.8, 687.3, 838.8 and 990.3 mg/kg diet) for 60 days. This resulted in an improvement in enzymatic antioxidant capacity of SOD, CAT, glutathione-S-transferase (GST), GPx and glutathione reducase (GR) when Ins was supplemented into the diets ($P<0.05$), thus demonstrating Ins have some antioxidant characteristics and the and antioxidative status was improved (Jiang, J. *et al.*, 2010). Further studies on juvenile Jian carp have also concluded that Ins supplementation inhibits oxygen radical species and prevents oxidative damage (Jiang, W. *et al.*, 2009). This may account for some of the performance benefits seen in previous studies of broiler chickens, however the mechanisms underpinning this are not fully understood.

2.10.3 myo-Inositol in swine production

Like discussed in the poultry model there is increased interest in super dosing phytase in pig diets and the same theory applies that the extra phosphoric effects of super dosing phytase may be due to the increased formation of Ins. There is even less published

literature on direct supplementation of Ins into pig diets, with most literature focusing on the effect of super-dosing of phytase in the diets of pigs rather than direct use of Ins. One of the first studies looking at the role of Ins in swine production was by Lindley and Cunha (1946), concluding that it was required to improve feed efficiency. More recently a study by Moran *et al.*, (2019) used three levels of Ins (0, 1.5 and 3 g/kg diet) and two levels of phytase (0 and 2,500 FTU/kg) were combined into a 2 x 3 factorial arrangement, measuring growth performance and Ins in blood plasma. The study used weaned pigs at approx. 22 days of age for 42 days. Ins supplementation improved average daily gain and average daily feed intake in a quadratic manner ($P<0.10$), however Ins had no significant effect on body weight gain and gain to feed ratio. Increasing levels of Ins in diets significantly increased the Ins in the blood plasma in a linear correlation ($P<0.001$). A study by Lu et al., (2019) conducted an experiment looking at the role Ins plays in the extra phosphoric effects of super dosing phytase on growth performance. Supplementing pig diets with 2 g/kg Ins and 2 dosages of phytase at 1,000 FTU/kg and 3,000 FTU/kg. The study concluded that the beneficial effects of super dosing phytase at 3,000 FTU/kg improved feed efficiency and may be due to the formation of Ins and the release of P and may not be solely due to Ins release by this dosage of phytase. An additional study by Lu et al., (2019) also assessed the role of phytase generated Ins on plasma inositol concentrations and glucose transporter type 4 abundance in muscle membranes in weanling pigs. Ins plasma concentrations was increased by super dosing phytase at 2,000 FTU/kg. Phytase generated Ins could contribute to the increased expression of GLUT4 in muscle plasma membranes. The study concluded that further work is required to understand if this increase of GLUT4 is associated with enhanced cellular glucose uptake and utilisation. A study by Ogunribido et al., (2022) observed no effect of Ins supplementation on performance at 2 g/kg for pigs feed from weening to 4 weeks post weening. Due to the lack of pig performance by Ins additional *in vitro* study was conducted to determine the direct effect of Ins on the intestinal epithelium that might not be reflected in growth performance. The results indicate that Ins may directly regulate gut barrier integrity by enhancing integrity in the IPEC-J2 monolayer. The papers discussed here assessing free Ins supplementation of pig show similar trends that Ins doesn't consistently deliver clear performance benefits.

In terms of the biochemical role of Ins in pigs little information is available using the pig model as much of the research is basis towards humans and rats. A study by in pigs by Lu et al., (2020) assed the expression of the Ins transporter proteins SMIT1 and HMIT in the duodenal loop, jejunum and ileum tissue. Pigs were fed four different diets: PC with adequate Ca and P, NC with reduced Ca and P, NC+1500 FTU/kg or NC+3000 FTU/kg for 28 days from weaning. High levels of phytase supplementation increased the

performance of the nursery pigs used in this study. There was no significant effect on the relative expression of SMIT1 in the duodenum (P=0.81), jejunum (P=0.23) and ileum (P=0.26). There was no significant effect on the relative expression of HMIT in the jejunum (P=0.08) and ileum (P=0.12). However, the presence of phytase in the NC reduced the expression in duodenum (P=0.01). Further study is required to understand in the importance of Ins for pigs. Based on the literature discussed is not enough information to conclude if Ins is required to maximise performance in swine production.

2.11 Knowledge gap and Conclusions

Dietary phytase supplementation is routine in the poultry industry to prevent the anti-nutritional effects of phytate and release the organic P stored in phytate. Super-dosing of phytases increases the Ins being formed in the GIT of chickens. Therefore, the assumption is that Ins is a major mediator of the extra phosphoric effect of phytase in poultry. There is limited research examining the potential benefits of direct supplementation of Ins in poultry diets, as a potential feed additive to improve growth performance and bird health. In addition, there is limited information on Ins in chickens as there are very limited studies assessing the biochemical role Ins has in chickens. The published studies do not show a clear benefit of Ins supplementation, with studies having both negative and positive impacts on bird health and performance. Therefore, more work is required to understand the role of Ins in the chicken model and how the effects of Ins generated by phytase, or direct free dietary Ins has on the production of poultry.

Chapter Three: General materials and methods.

3.0 Introduction

The following chapter is made up of common materials and methods used in the experimental chapters (chapters 4-6). For specific methods exclusive to a chapter, can be found in the materials and methods section of that chapter.

3.1 Animals, Husbandry and Diets

Ethical approval for all experiments was obtained from Harper Adams University Research Ethics Committee before the start of each experiment and reported in accordance with the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020). The principles of the 3Rs (Replacement, Reduction and Refinement) were taken into careful consideration when designing all experiments.

The chickens (*Gallus gallus domesticus*) used in this thesis were male Ross 308 (Aviagen, UK) broilers. These chickens are representative of common practice in commercial broiler farming in the UK, accounting for 70-80% of the market (Competition and Markets Authority, 2018). Chicks were obtained from a commercial hatchery, Cyril Bason Ltd (Craven Arms, UK), as sexed day-old chicks. Chicks were vaccinated against Infectious Bronchitis, Bursal disease and Marek's disease at the hatchery. Experiments were conducted at the National Institute of Poultry Husbandry, Harper Adams University, Shropshire, UK.

Birds were housed in raised floor pens (60 X 60 cm with 0.36 m^2 total area) with a solid floor that could be replaced for short periods of time with a wire mesh to allow for excreta collection. Pens were equipped with individual feeders and nipple drinkers and were adjusted according to the birds' height. Birds were reared according to the recommendations of the Aviagen Ross 308 broiler management handbook (2018), which briefly comprised of a lighting regime of 23:1 (light: dark) for the first week, darkness was then increased an hour each day until an 18:6 (light: dark) pattern was established and maintained for the remaining life of the birds. Temperature of the shed was maintained at 30 ˚C for the first three days and was reduced to 28 ˚C for the next three days. On day six, the temperature was reduced to 27 ˚C for the next three days, after which the temperature was reduced by 1 ˚C every three days until day 27, when 21 ˚C was then maintained throughout the duration of the study. Heat, light and ventilation were controlled using a Climatec control system (Climatec, Old Wharf Industrial Estate, Dymock Rd, Ledbury, UK) Birds were checked a minimum of twice a day which consisted of temperature and humidity recording, health of the birds, feeding, watering and mortality recording. Ventilation and humidity were adjusted to the birds' age and behaviour. Cardboard

(corrugated cardboard chick paper (Dalton Engineering Ltd)) was used as litter and was regularly changed.

All diets were formulated to meet the nutritional requirements for Ross 308 broilers (Aviagen, UK) and fed in a phase regime. Feed was produced at Target Feeds Ltd (Market Drayton, Shropshire, UK). The addition of diogenous earth (Multi-Mite®) was used as an insoluble digestibility marker at the inclusion rate of 20 g per kg (AIA recovery rate of 48.8%). The mineral and vitamin premix used in all diets contained vitamin A, 10,000 IU; vitamin D3, 2,500 IU; vitamin E, 25 IU; vitamin E, 50 mg; vitamin K3, 1.5 mg; vitamin B1, 2 mg; vitamin B2, 7.5 mg; vitamin B6, 3.5 mg; vitamin B12, 20 µg; niacin, 35 mg; pantothenic acid, 12 mg; choline chloride, 460 mg; folic acid, 1.0 mg; biotin, 0.2 mg; Fe as iron sulphate, 265 mg; Cu as copper sulphate, 48 mg; Mn as manganese oxide, 140 mg; Zn as zinc sulphate, 165 mg; I as potassium iodide, 1.2 mg; and Se as sodium selenite. Phytase (Quantum® Blue P40, AB Vista, UK) and *myo*-inositol (98 ± % *myo*-inositol, Thermo Fisher Scientific, experiments one and two; 98 ± % *myo*-inositol, Carbosynth Ltd, experiment three) were added on top of basal diets to specified levels (see respective experimental chapters). Products were individually mixed in a food processor for 2 mins with a small quantity of feed before being incorporated into a larger feed ribbon mixer for 10 mins, to ensure even distribution. Diets in experimental chapters two and three were cold pelleted (without steam) according to Azhar et al. (2019) using a KAHL pelleter (Amandus Kahl GmbH & Co. KG, Reinbek, Germany). The diameter and size of the pellet was 3 mm and 4-7 mm respectively. Pellets were produced at 60 °C.

3.2 Performance variables

The birds and feed were weighed at pre-determined timepoints to calculate, Feed Intake (FI), Average Daily Feed Intake (ADFI), Weight Gain (WG), Average Daily Weight Gain (ADWG) and Feed Conversion Ratio (FCR), corrected for mortality. Performance was calculated on a pen basis. The weight and date of any dead or culled birds was recorded. Performance objective equations used:

Feed Intake (g):

- $\mathrm{FI\ (g)} = \text{feed start weight (g)} - \text{feed end weight (g)}$

Bird Days (d), every 24-hour period for one bird is equal to one bird day:

- $\text{Bird Days} = \text{number of birds X number of days of study period}$

Average Daily Feed Intake (ADFI) (g/b/d):

- $\mathrm{ADFI\ (g/b/d)} = \mathrm{FI(g)/bird\ days\ (d)}$

Weight Gain (WG) (g):

- $\mathrm{WG\ (g)} = \big((\text{final weight of birds in a pen (g)} - \text{start weight of birds in a pen (g)}\big) + \text{weight gain of dead or culled birds in pen (g)})$

Average Daily Weight Gain (ADWG) (g/b/d):

- $\mathrm{ADWG\ (g/b/d)\ } = \mathrm{WG} * \mathrm{(g)} \,/\, \text{bird days (d)}$

Feed Conversion Ratio (FCR) (g/g):

- $\mathrm{FCR\ (g/g)} = \mathrm{ADFI\ (g)/ADWG(g)}$

3.3 Nutritional Analysis

3.3.1 Collection of Excreta

Excreta was collected for the last 48 hours of each feeding phase by removing the solid floor and placing the birds on a wire mesh with a tray underneath to collect the excreta. Excreta was collected after the first 24 hours and stored at –20 °C, then defrosted at room temp and the remaining 24-hour collection mixed in and weighed. This was then sub-sampled and dried at 60 °C in a forced draft oven. Excreta was then milled through a 0.75 mm screen.

3.3.2 Dry Matter (DM)

Excreta and feed were dried for four days or until a constant weight was obtained at 60 °C in a forced draft oven, after which it was allowed to cool for 24 hours at room temperature. Feed and excreta were then milled (see above) and further dried in a forced draft oven at 105 °C to a constant weight (AOAC 2000; method 934.01). DM was calculated using the equation below:

- $\text{Dry Matter \% (DM)} = ((\text{Start weight (g)} - \text{final weight (g)})/\text{start weight (g)})$

3.3.3 Gross Energy (GE)

Dried and milled excreta samples were analysed for GE, by using an adiabatic bomb calorimeter (Parr 6200, Moline, IL, USA) with Benzoic acid as the standard (Hill and Anderson, 1958).

3.3.4 Nitrogen (N)

Ileal digesta, feed and excreta (dried and milled) were analysed for crude protein (6.25 X N) by the combustion method (AOAC, 2000; method 990.03) using a Leco (FP-528 N, Leco Corp., St. Joseph, MI) with Na_2EDTA as the standard. In brief, 100 mg of sample was weighed into a foil cone and dropped into a furnace at 950 ˚C with a helium and oxygen atmosphere. As the sample is burnt the nitrogen released is then measure against the standard.

3.3.5 Digestibility marker (acid insoluble ash)

The method by Van Keulen and Young (1977) was used to determine the acid insoluble ash (AIA) in feed, ileal digesta and excreta as the indigestible digestibility marker. This briefly involved weighing out dried milled sample in duplicate into a crucible and placing into a muffle furnace for four hours at 550 ˚C and weighing the remaining ash. The following equations were used to determine the ash content of the samples:

- $\text{Weight of ash (g)} = \text{weight of crucible and sample (g)} - \text{weight of crucible (g)}$
- $\text{Ash in sample (g/kg)} = (\text{weight of ash (g)}/\text{sample weight (g)})\ \text{X}\ 1000$

The remaining ash was then boiled in 100 mL of 2 M Hydrochloric acid at 175 ˚C for 10 mins in Kjeldal tubes. The acidic digest was washed from the tubes using hot water and the contents captured in filter paper. The filter paper and digested sample was then folded and placed back into its crucible in the furnace for a further four hours at 550 ˚C. The remaining sample was weighed, and the weight of the crucible deducted to calculate the ashed acid sample weight (g). AIA was calculated using the following equation:

- $\text{AIA (g/kg)} = (\text{ashed acid sample (g)}/\text{start sample weight (g)})\ \text{X}\ 1000$

Furthermore, in Figure 3.1, initial sample weights were tested for AIA recovery using a pooled sample of excreta samples and subsequent variation. Samples over 6 g gave more consistent results with less variation. As a result, 6-8 g of sample was used to determine AIA.

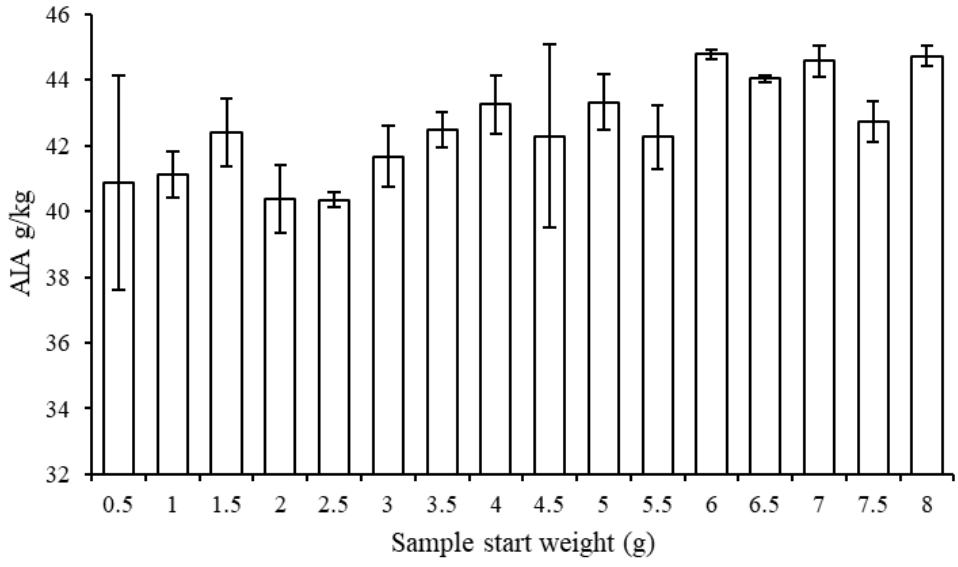


Figure 3.1: The recovery rate of AIA (g/kg) with different sample weights (pooled excreta), error bars represent SD with three replicates per each sample.

3.3.6 Apparent Metabolisable Energy (AME)

To calculate AME, first Dry Matter Retention (DMR) was calculated using the following equation:

- Dry Matter Retention (DMR) = (AIA in excreta (g/kg) − AIA in feed (g/kg))/ (AIA in excreta (g/kg))

To calculate AME the following equation was used:

- Apparent Metabolisable Energy (AME) = GE in feed (MJ/kg) − ((1 − DMR) X GE in excreta (MJ/kg)

To calculate the ratio of AME to GE, the following equation was used.

- AME: GE = AME / GE (MJ/kg) in feed

3.3.7 Nitrogen-corrected Apparent Metabolisable Energy (AMEn)

To calculate Nitrogen Retention (NR) the following equation was used:

- Nitrogen Retained (NR) = N in feed (g/kg) − ((N in excreta (g/kg) X AIA in feed (g/ kg))/ AIA in excreta (g/kg))

To calculate AMEn the following equation was used:

- Nitrogen corrected Apparent Metabolisable Energy (AMEn) = GE in feed − ((GE in feed X AIA in excreta)/AIA in excreta) − ((34.39 X NR)/1000)

To calculate the ratio of AMEn to GE, the following equation was used.

- AMEn: GE = AME / GE (MJ/kg) in feed

3.3.8 Near Infrared Spectrometry

Phytate-P levels in feed were predicted using NIR (FOSS, Cheshire, UK), based on AUNIR calibration standards (designated by ESC as method: SAM120).

3.3.9 Total phyate-P

Total phytate-P was analysed by Enzyme Services & Consultancy (ESC) (Caerphilly, UK) using a modified Megazyme method K-PHYT 08/14 (Megazyme International Ireland). Modifications can be found in Mansbridge (2017).

3.3.10 Phytase activity

Phytase activity in diets were analysed by Enzyme Services & Consultancy (ESC) (Caerphilly, UK) using a modified ELISA test with Quantiplate Kits for Quantum Blue phytase (Envirologix, USA), based on Envirologix method AP181, Rev. 12-28-11. Modifications can be found in Mansbridge (2017).

3.4 *myo*-Inositol and Inositol phosphate analysis

myo-Inositol and inositol phosphates were extracted at Harper Adams University before being sent to the School of Biological Sciences, University of East Anglia for high-performance liquid chromatography pulsed amperometry (HPLC-PAD) on a on a Dionex DX-600 HPLC System to determine Ins in plasma and tissues and IP_{3-6} concentrations in digesta samples. IP_{3-6} in tissue was extracted and analysed at UEA. *myo*-Inositol was extracted according to sample type:

For blood plasma, blood was collected into lithium heparin vacutainers and subject to centrifugation at 1,500 x g for 15 mins at 4 ˚C. An aliquot of the supernatant was taken and mixed with two volumes of ice cold 1N-perchloric acid and put on ice for 15 mins to allow the protein to be precipitated. Samples were subject to further centrifugation at 16,000 x *g* for 10 mins at 4 ˚C and diluted 1:50 in 18.2 MOhm.cm water.

For analysis of Ins in chicken tissue samples (e.g. brain, kidney or jejunum), 2-3 g of the tissue was immediately frozen following dissection and stored at –80 ˚C. 100 mg of the frozen tissue was then weighed into tubes and was homogenized in 1 mL of ice-cold 5 % w/v (0.83 N) perchloric acid, 20 mM EDTA,Na_2. The homogenate was then put on ice for 15 mins before being subject to centrifugation for 10 mins at 15,000 x *g* at 4 ˚C. The supernatant was diluted 50-fold in 18.2 mOhm cm water.

For analysis of Ins and inositol phosphates (IP_6, IP_5, IP_4, IP_3) in feed and digesta samples, samples were dried and milled and 100 mg weighed into tubes and were extracted in 5 mL of 20 mM EDTA, 100 mM NaF (pH 10), on a rotary shaker for 15 mins followed by sonication in an ice bath sonicator for 15 mins. The samples were held at 4 ˚C for two h before centrifugation at 14,000 x *g* for 15 mins. The supernatant was filtered through a 13 mm x 45 µm pore PTFE filter before analysis (20 µL injection) by HPLC-PAD.

Twenty micro litres of the sample were injected onto a 4 mm × 50 mm CarboPac PA1 column (Dionex, UK) arranged in series with a 4 mm × 250 mm CarboPac MA1 column with 4 mm × 50 mm guard column of the same material. The column was eluted with 150 mM NaOH with the flow rate of 0.4 mL/min. The Ins was eluted from the CarboPac PA1 column onto the CarboPac MA1 column and the flow through the CarboPac PA1 column was modified at 1.5 mins to 750 mM NaOH, at the same flow rate of 0.4 mL/min. Ins was detected from the CarboPac MA1 column eluent using an ED50 electrochemical detector (Dionex) with a gold electrode. Ins was eluted at approximately 10.5 min. Calibration curves used to determine Ins concentration were made from inositol standards (0.01–0.2 nM in 20 µl).

For analysis of inositol phosphates (IP_6, IP_5, IP_4, IP_3) in tissue, tissue samples were prepared using the same method as described above. All steps were carried out at 4 ˚C to prevent inositol phosphate degradation. The method used can be found in Sprigg et al., (2022), and in brief 5 mg of Titansphere1TiO_2 beads in 50 µL of 1 M perchloric acid was added to the lysate and vortexed briefly before being mixed for 30 mins on a rocker. Samples were then subject to centrifugation at 3500 x *g* for five mins to pellet the TiO_2 beads and the perchloric acid supernatant discarded. To release the bound inositol phosphates, the TiO_2 beads were resuspended in 200 µL of 3 % ammonium hydroxide solution (pH 10), vortexed and incubated on a rocker for five mins. Samples were then

subject to centrifugation at 3500 x *g* for one min and supernatant containing the inositol phosphates were transferred to a clean 1.5 mL tube. This process was then repeated to ensure all inositol phosphates were eluted and pooled. Samples were then vacuum evaporated until dry and resuspended in 100 µL of 18.2 MOhm.cm water. Samples were then analysed using HPLC and UV detection at 290 nm after post column reaction with ferric ion, on a 250 x 3 mm Dionex CarboPac PA200 column (Thermo Scientific) with a corresponding 3 x 50 mm guard column of the same material. The column was eluted at a flow rate of 0.4 mL/min with an increasing gradient of methanesulfonic acid, derived from buffer reservoirs containing (A) water and (B) 0.6 M methanesulfonic acid, by mixing according to the following schedule: time (minutes), % B; 0, 0; 25, 100; 38, 100. Fe[NO3)3 in 2 % HClO4 was used as the post-column reagent added at a flow rate of 0.2 mL/min. The elution order of InsPs was established using acid hydrolysed $InsP_6$ standards. Concentration of InsPs was established by reference to UV detector response to injection of $InsP_6$ (Merck, UK).

3.5 Mineral Concentrations

Inorganic elements (Ca, P, Mg, Fe, Na, Co, Mn, Cu, Zn and Mo) were quantified in the ileal digesta and feed using inductively coupled plasma mass spectrometry (ICP-MS). Digesta and feed were prepared in duplicate according to Cope et al. (2009). 0.5 g of milled and dried digesta or feed was weighed into 50 mL plastic DigiTubes. A combination of 1 mL of concentrated analytical grade hydrochloric acid (Fisher Scientific), 6 mL of concentrated analytical grade nitric acid (Fisher Scientific) and heat was used to digest samples using a DigiPREP heating block with the following cycle (Table 3.1). A reagent blank was also prepared at the same time using the combined acid and heating cycle digestion (Table 3.1).

Table 3.1: DigiPREP heating programme for the acid digest of feed and digesta samples for mineral quantification by ICP-MS.

Program	Time (mins)	Set point temperature
To temp	30	45˚C
At temp	1	
To temp	25	65˚C
At temp	5	
To temp	15	100˚C
At temp	45	

Once the samples and reagent blanks were digested, they were made up to 50 mL volume with Purite water. The acidic diluent was composed of an analytical grade mixture

of 0.5 % Nitric acid, 0.05 % Trition-X and 2 % methanol. Gallium was used as an internal standard (50 µL of 1000 ppb gallium was diluted in 50 mL of the acidic diluent). The sample diluting acid was made up of 10.204 g of the diluted gallium and made up to 1 L using the acidic diluent. Samples were diluted 1:50 and were mixed using a vortex mixer in a 5 mL auto sampler tube.

Blood plasma samples were analysed for Ca, P, Mg, Fe, Na, Cu and Zn by ICP-MS as described by Cope et al. (2009). Briefly, plasma was defrosted and diluted 1:50 in a 5 mL auto sampler tubes with the diluent described above. Plasma samples were analysed in duplicate. Plasma minerals were validated against ClinCheck certified lyophilised plasma control sample 2 (Product no. 8885 RECIPE; Chemicals and Instruments GmbH, Munich, Germany), to ensure precision and accuracy of the minerals tested.

All analyses were carried out on a NexION 2000 ICP-MS using the following parameters: the wash and carrier solution were the same as the acidic diluent, the spray chamber (glass cyclonic) was kept at 2 ˚C, RF power 1600 W and Nebuliser. NexION setup solution (PerkinElmer Inc.) (10 µg/L: Be, Ce, Fe, In, Li, Mg, Pb, U) was used to calibrate the ICP-MS to ensure operation within normal parameters. Samples were analysed by three sweeps of three replicates, resulting in each sample being read nine times.

Calibration curves were used to determine element concentrations. 1000 ppm standards were diluted in the acidic diluent to make up a final concentration of 10, 50, 100, 200 and 400 ppb of the major elements and 1, 5, 10, 20 and 40 ppb of the minor elements. Each run of analysis was validated against European Commission certified reference material (Hay powder - BSR® 129) for feed and faecal samples. Mineral digestibility was calculated using the equation below:

- Nutrient digestibility coefficients: $1 - [\frac{\text{AIA in feed (g/kg) X Mineral in waste (g/kg)}}{\text{AIA in waste (g/kg) X Mineral in feed (g/kg)}}]$

Waste denotes digesta (ileum) or excreta.

3.6 Haematology

At the end of the trial period one bird per pen was killed by electrical stunning followed by exsanguination. At this time, blood was collected into 6 mL lithium heparin vacutainers tubes. Whole blood was analysed for red blood cell counts using a ProCyte Dx Haematology Analyser (Grange House, Wetherby, UK). Blood was analysed within 24 hours of collection and stored a 4 °C. Blood was inverted using a roller before analysis to ensure blood was mixed. The analysis of a control blood sample (WD 1154) (ABX Diagnostics, Bedfordshire, UK) was used prior to experimental sample analysis to ensure instrument validation.

3.7 Antioxidants

Total antioxidant status (TAS) was determined in blood plasma (fresh) using a Cobas Miras plus auto-analyser (ABX Diagnostics, Bedfordshire, UK). Activity of TAS was determined using the kit NX2332 (Randox Laboratories, Antrim, UK). Colour change was measured spectrophotometrically at 600 nm and antioxidants present in the plasma caused the suppression of the colour production which was proportional to the concentration. The manufactures recommendations for instrument settings were followed without any modifications. Randox total antioxidant control (NX2331) was prepared according to kit recommendations and analysed within the expected range, before experimental samples were tested, to ensure validation.

Lysed whole blood Superoxidase Dismutase (SOD) activity was determined using kit SD125 (Randox Laboratories, Antrim, UK) which has been designed for use on the Cobas Miras Plus auto-analyser. Colour change was measured spectrophotometrically at 500 nm. The manufactures recommendations for instrument settings were followed without any modifications. The method was modified to use whole blood: samples of 2 mL of whole blood were defrosted and vortex mixed to ensure a lysed and homogenised sample. Samples were then diluted 1:4 with purite water and then further diluted with sample diluent (SD124) 1:50. Samples were then analysed according to kit method. Ransod Control (SD126) was prepared according to kit recommendations and analysed within expected range, before experimental samples to validate analysis.

Glutathione Peroxidase (GPx) was determined in whole blood (fresh) using kit RS504 (Randox Laboratories, Antrim, UK) which has been designed for use on the Cobas Miras Plus auto-analyser. The manufactures recommendations were followed. GPx activity is measured at 340 nm. Ransel Control (SC692) was prepared according to kit recommendations and analysed within expected range, before experimental samples to validate analysis.

3.8 Relative development of gastrointestinal tract and organs

The relative development of the gastrointestinal tract (GIT) and organs were expressed as a percentage of bird body weight. Feed was not removed before birds were killed. One bird per pen was killed and dissected. A detailed diagram of a broiler chicken GIT and sampled organs can be found in Figure 3.2. Sections of GIT were emptied by careful palpation to remove digesta. Weights of spleen, liver, pancreas, duodenum, jejunum, ileum, caeca (left and right), proventriculus and ventriculus (weighed together) were recorded. The relative organs were then calculated with the following equation:

- $\text{Relative weight of organ } (\%) = \text{weight of organ (g)} / \text{ weight of bird (g)}$

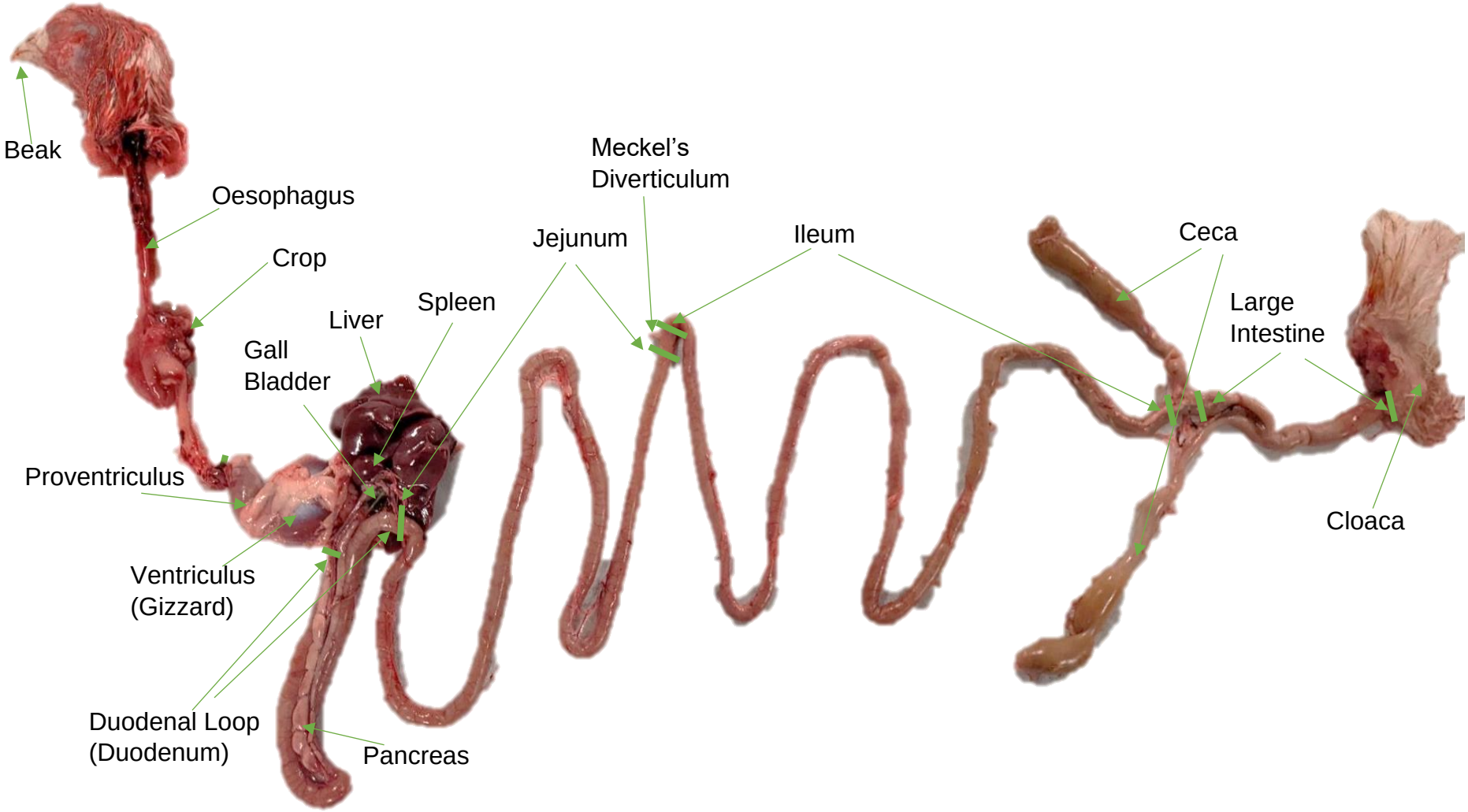


Figure 3.2: The gastrointestinal tract of a male broiler chicken at 28 days of age (authors own image).

3.9 Statistical analysis

Data handling and calculations were carried out in Excel 2018 (Microsoft Corporation). Data were analysed using analysis of variance (ANOVA) in GenStat® (21st edition, Rothamstead, Hertfordshire, UK), differences were reported as significant at $P<0.05$. Post hoc testing using protected Fisher Least Significant Difference was used to differentiate between treatment means. Values greater than or smaller than 3 standard deviations away from the numerical mean were removed from the analysis. Graphics were either produced in R (R Core Team, 2021) or Excel (Microsoft Corporation), please refer to each experimental chapter for details.

Chapter Four: Effects of supplementing dietary *myo*-inositol and phytase on broiler chickens from 0-21 days of age.

4.0 Introduction

Poultry species are known to not be able to efficiently utilise organic phosphates in feed, due to these being stored in the mixed metal salt form of phytic acid in plant-based ingredients such as grains due to insufficient production of endogenous phytase enzymes in the GIT (Dersjant-Li et al., 2015; Maenz and Classen, 1998). Poultry diets are therefore typically formulated to contain exogenous phytase enzymes, to give the chicken sufficient phytases to degrade phytate and reduce the reliance on inorganic phosphorus. Phytase addition improves P digestibility which in turn improves production performance as there is increased available P to the growing bird (Bedford, 2000).

The primary role of phytate in seeds and grains is to act as an essential P store for the germinating seed and providing the necessary nutrients for an emerging seedling. However, in animal production, phytate is classified as an anti-nutritional compound, by reducing the available phosphorus and chelating to various minerals and proteins forming indigestible complexes (Cowieson et al., 2004; Pirgozliev et al., 2007, 2008). Phytase mode of action involves the dephosphorylation of phytate by hydrolysing the phosphate groups in a stepwise manner, causing the release of phosphate groups and forming lower IPs. The last step of IP_1 to Ins is mediated by the bird's own ALP. The complete dephosphorylation of phytate results in the formation of a sugar called *myo*-inositol (Ins) with bioactive properties (Bedford and Partridge, 2010).

Many studies have demonstrated the improvement in growth performance and bird health that phytase supplementation provides in monogastric diets, by improving body weight gain, feed efficiency, nutrient utilisation and bone parameters (Simons et al., 1990; Shirley and Edwards, 2003; Pirgoziev and Bedford, 2013; Gautier et al., 2018; Moita et al., 2021). Super-dosing phytase is the addition of phytase above conventional levels and is around or above 2500 FTU/kg of diet (Adeola and Cowieson, 2011; Mansbridge, 2017). Recent studies have demonstrated supplementing third generation phytases (with higher affinities to the lower inositol phosphates such as IP_3) at high or super doses improves FCR and increases the concentrations of lower IPs and Ins in the excreta and digesta of chickens and pigs (Zeller et al., 2015[a,b]; Kuehn et al., 2016; Beeson et al., 2017; Walk et al., 2014; Gautier et al., 2017; Farhadi et al., 2017). This improvement is partly due to the releasing of nutrients such as protein and minerals which was previously bound to phytate. The 'extra-phosphoric effects' of super-doses of phytase can be defined as the extra benefits resulting from more than just an increase in P availability. However, few have assessed

the role Ins may play in the improved production performance seen when formulating diets with phytases. Therefore, it is a logical assumption that Ins may play a role in improved performance and contribute to the beneficial extra-phosphoric effects of super dosing phytase as Ins is being released into the GIT of chickens at levels which would not normally be seen with diets not supplemented with phytase. However, the link to Ins and improved performance is not fully understood (Lee and Bedford, 2016). Studies assessing the performance benefits and energy utilisation of Ins have shown an inconsistent picture, with no difference shown by Pirgozliev et al. (2019), reduction by Cowieson et al. (2013) and improvement by Cowieson and Zhai (2021). Optimal dosage is therefore still not understood due to the inconsistency in the results and the lack of understanding of the biological role Ins has in growing chickens.

There are increasing number of studies assessing the role dietary Ins may play in poultry production. Studies by Somerfield et al. (2018) and Pirgozliev et al. (2019) have shown that supplemented dietary Ins was associated with increases of Ins in intestinal chymus and blood plasma. However, there is very limited information on Ins distribution across different tissues in chickens and if the quantities of Ins found in these tissues can be influenced by diet (phytase or direct Ins supplementation), due to the chicken model being poorly studied.

The effects of feeding phytase on mineral availability and digestibility are well understood. It is well known that including phytase increases mineral availability as the salt, phytic acid, forms complexes with cations in the following descending order of strength: Cu^{2+}> Zn^{2+}> Co^{2+}> Mn^{2+}> Fe^{3+}> Ca^{2+} (Nolan et al., 1987). The interaction between Ins and lower IPs may play on mineral digestibility has not been studied.

4.1 Objectives

The aim of the study was to quantify the response to feeding three levels of *myo*-inositol (1.5 g, 3 g or 30 g per a kg of diet), two levels of phytase (1,500 or 4,500 FTU/kg) and a basal diet (Control) to broiler chickens from 0-21 days of age. Measurable outcomes studied were growth performance (Average BW, ADFI, ADWG and FCR), dietary energy (AME and AME:GE), IP_{3-6} and Ins concentrations in ileum digesta, apparent mineral ileal nutrient digestibility (Ca, P, Mg, Fe, Na, Co, Mn, Cu, Zn, Mo and N), plasma mineral concentrations (Ca, P, Mg, Fe, Na, Cu and Zn), plasma Ins concentrations, IP_{3-6} kidney tissue concentrations and Ins tissue concentrations (brain, kidney, breast and leg muscle).

Hypotheses:

1. Increasing available Ins in diets (either from the enzymatic action of supplemented phytase or direct supplementation of free Ins) will significantly improve broiler chicken production performance, above the control basal diet (0 g/kg Ins).
2. Increasing available Ins in diets will not significantly affect AME or AME:GE compared to the control diet.
3. Supplementing diets with phytase will alter the IP_{3-6} ileal profiles compared to basal and diets supplemented with Ins.
4. Increasing available Ins in diets will significantly modify mineral digestibility compared to the control diet.
5. Increasing available Ins in diets will not significantly influence the plasma mineral concentrations.
6. Increasing available Ins in diets will significantly increase the plasma and ileal Ins concentrations compared to the control.
7. Increasing available Ins in diets will significantly increase Ins concentrations in chicken tissues compared to the control diet.
8. Increasing available Ins in diets will not affect the IP_{3-6} concentrations in kidney tissues.

4.2 Materials and Methods

The experiment was conducted at the National Institute of Poultry Husbandry (Newport, Shropshire, UK) and was approved by Harper Adams University Research Ethics Committee (code: 0398-201809-PGMPHD) before the initiation of the experiment. The study is reported here in accordance with the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020).

For general Materials and Methods relating to this section please see Chapter three.

4.2.1 Animals

Five hundred male Ross 308 chicks were obtained from a commercial hatchery (Cyril Bason Ltd, Craven Arms, UK). Four hundred and eighty were randomly allocated into sixty raised floor pens and weighed (eight birds per pen) and fed one of the five experimental diets from day 0. The remaining chicks were kept in two pens fed on the control diet and used to replace birds which were withdrawn or died during the first 7 days of the experiment. Growth performance was determined from 0-21 days of age.

4.2.2 Sample collection

During the last three days of the study, the solid floor of the pens was removed and replaced with a wire mesh to allow for excreta collection, which was then dried at 60˚C until a constant weight and then milled. At the end of the study, one bird per pen was electrically stunned and killed by exsanguination. At this time blood was collected into

lithium heparin BD Vacutainers ®. A further three birds per pen were despatched by cervical dislocation. Ileum digesta was collected from the four birds killed from each pen, digesta was removed from the Meckel's diverticulum to the caecal junction and pooled before freeze drying to a constant weight. Digestibility marker (AIA) was determined in ileal digesta and feed. Feed, plasma and ileal digesta were analysed for mineral content using ICP-MS. Gross energy was determined on feed and excreta.

Tissue samples were taken from the bird that was killed by electrical stunning followed by exsanguination. Sections of tissue (2- 3 g) were sampled from the left breast (*pectoralis major*), left leg (taken above the femur (*iliotibialis cranialis* and *iliotibialis lateralis*)), left kidney and brain (left hemisphere). Tissue samples were frozen at -80°C until further analysis. Feed, ileal digesta and tissue samples were analysed for IPs, please refer to the general materials and methods (Chapter 3) for full details.

4.2.3 Treatment diets

A maize-soybean meal was produced at Target Feeds Ltd. (Whitchurch, Shropshire, UK) and a digestibility marker of diatomaceous earth was included at 20 g per kg of feed. The basal diet was formulated to meet breeder's nutrient recommendations for Ross 308 broiler meat chickens (Aviagen Ltd., Edinburgh, UK). Composition of the basal diet can be seen in Table 4.1; vitamin premix formulation can be found in the general materials and methods.

Table 4.1: Ingredient composition of basal diet (g/kg).

Ingredient	g/kg
Maize	580
Soybean meal (48 % CP)	300
Corn gluten meal	40
Vegetable oil	20
Salt	3.6
DL Methionine (M)	4.2
Lysine HCl (L)	3.0
Limestone	6.7
Dicalcium Phosphate	18.5
Vitamin Mineral premix	4.0
Diatomaceous Earth	20.0
Calculated values	
Dry Matter (DM), g/kg	0.90
Crude protein (N x 6.25), g/kg	250
Metabolisable Energy, MJ/kg	16
Calcium (Ca), g/kg	9.545
Available Phosphorus (P), g/kg	4.856
L	3
M+ Cystine	11.23
Determined values	
DM, g/kg	0.903
Gross Energy, MJ/kg	16.387
Crude protein (N x 6.25), g/kg	250
Total Ca, g/kg	0.95
Total P, g/kg	6.8

The basal diet was then split into six parts, two of the parts were supplemented with one of two levels of the zootechnical additive phytase (6-phytase EC 3.1.3.26) produced by a genetically modified strain of *Trichoderma reesei* (Quantum^{TM} Blue P40, AB Vista, UK) at the inclusion rate of 1,500 or 4,500 FTU/kg. Three additional parts were supplemented with *myo*-inositol (98±% *myo*-inositol, Alfa Aesar, Thermo Fisher Scientific) at either 1.5 g, 3 g or 30 g per kg of basal diet. The Ins and the phytase were mixed on top of the diets in powder form and incorporated together in a ribbon mixer for approximately 5 minutes. The remaining part was left un-supplemented as a control (C). The experiment consisted of a randomised design with ten pen replicates per dietary treatment. All diets were fed as mash. Phytate-P content of samples was determined by wet chemistry method as high levels of Ins in diets effected NIR results.

4.2.4 Statistical analysis

Data were statistically analysed using one-way analysis of variance (ANOVA), without blocking, in GenStat® (19th edition), with a protected Fisher's least significant difference test to separate means. Differences were reported as significant at $P<0.05$ and tendencies considered at $P>0.05$ - $P<0.10$. Graphics and correlations (Pearsons) were produced in

"ggpubr" package version 0.1.5. in R version 3.5.1 (Feather Spray) (R Core Team, 2018). Each pen was considered as an experimental unit in this study.

4.3 Results

4.3.1 Analysis of feed.

Analysed phytase activity (Table 4.2) confirmed that it was close to the formulated activity of 1500 FTU/kg and 4500 FTU/kg with the recovered activity of 1740 and 4310 FTU/kg, respectively. Ins recovery was close to expected dosages of 1.5, 3, and 30 g/kg with determined values being 1.2, 4.4 and 34.1 g/kg, respectively. Additionally, phytate-phosphorus and IP_{3-6} were also consistent across diets, which was expected.

Table 4.2: Recovered phytase activity, phosphorus bound phytate contents, Ins recovery and IP_{3-6} profiles of experimental diets.

Treatment Diet	phytase activity FTU/kg[1]	phytate-P content (g/100g)	Ins recovery (g/kg)	IP_3 (nmol/g)	IP_4 (nmol/g)	IP_5 (nmol/g)	IP_6 (µmol/g)
Ins (g/kg)							
0	<50	0.199	<0.18	15	142	1647	14.0
1.5 g	<50	0.253	1.2	22	177	1827	14.2
3 g	<50	0.181	4.4	34	559	1841	15.4
30 g	<50	0.207	34.1	26	178	1858	15.3
Phytase (FTU/kg)							
1500	1740	0.201	<0.18	17	152	1876	15.6
4500	4310	0.204`	<0.18	31	195	1630	14.0

[1]One FTU is defined as the amount of enzyme required to release 1 mmol of inorganic P per minute from sodium phytate at 37°C and pH 5.5.

Following ICP-MS analysis of the feed, the selected minerals of the six diets can be seen in Table 4.3. Results for the EU certified reference hay powder (BCR-129) were shown to be in range or marginally higher or lower than the expected range for all mineral analysed, except for Cu and Zn. Mineral levels are broadly similar across all diets, which is to be expected as they were derived from the same basal diet before supplementation of phytase and Ins.

Table 4.3: Analysed selected mineral content of experimental diets by ICP-MS.

Diet	Ins (g/kg)				Phytase (FTU/kg)	
	0	1.5	3	30	1500	4500
P (g/kg)	6.8	7.4	7.1	7.4	7.3	7.3
Ca (g/kg)	0.95	1.1	1.1	1.1	1.1	1.1
Mg (g/kg)	1.6	1.7	1.6	1.6	1.7	1.7
Fe (g/kg)	0.68	0.73	0.72	0.73	0.74	0.69
Na (g/kg)	1.3	1.6	1.5	1.5	1.6	1.4
Co (mg/kg)	0.42	0.38	0.39	0.34	0.42	0.35
Mn (mg/kg)	0.16	0.16	0.14	1.1	0.17	0.13
Cu (mg/kg)	19	18	20	18	23	16
Zn (g/kg)	0.95	0.83	1.2	0.97	1.0	0.85
Mo (mg/kg)	2.2	2.2	1.9	2.1	2.3	2.0
N (g/kg)	40	39	37	37	39	39

Ins, added dietary *myo*-inositol. Three samples of treatment diets were analysed on a dry matter basis.

4.3.2 Mortality

The birds remined healthy throughout the study and the mortality was overall low with no difference at high dosages of Ins. The overall mortality was 3.5%, which is below the 5% tolerance level recommended by the Red Tractor Assurance scheme (2018).

Table 4.4: Mortality rates expressed as a percentage of individual broiler chickens fed on each experimental diet during the study period, 0-21 days.

Treatment Diet	Mortality (total number of birds removed, culled or deceased)
Ins (g/kg)	
0	3.8% (3)
1.5 g	7.5% (6)
3 g	2.5% (2)
30 g	2.5% (2)
Phytase (FTU/kg)	
1500	2.5% (2)
4500	2.5% (2)

There were 80 individuals per treatment diet.

4.3.3 Performance and Energy Utilisation of Broilers

Chick weights were around 10% below the breed standard of 43 g (Aviagen, 2019), however, there was no significant difference in chick weight allocation across treatment diets (Table 4.5). There was an overall significant difference (P=0.001) between the treatment factors for average BW at day 21. Birds fed 3 or 30 g/kg Ins had a reduced body weight at day 21 compared to the birds fed on the basal diet (0 g/kg Ins). The treatment diet containing 30 g/kg Ins significantly reduced body weight at day 21 compared to all other diets except 3 g/kg Ins ($P<0.05$). Phytase improved ADWG compared to the basal diet, increasing weight gain by 3 g and 0.7 g per day (1500 FTU/kg and 4500 FTU/kg respectively). Ins supplementation had a negative effect on ADWG, whereby all three levels reduced the weight gain compared to the basal and phytase supplemented diets.

FCR was increased in the 1.5 and 30 g/kg Ins diets compared to the basal diet, however, the FCR was improved compared to the control by 3 g/kg Ins and the two levels of phytase. Co-efficient of variation (CV%) on growth parameters was relatively low, ranging from 4.2 % for FCR and 7.2 % for ADWG.

AME and AME:GE was not affected by any of the supplementation in diets (P>0.05) which can be seen in table 4.6. Inclusions of 30 g/kg of Ins in diets significantly differed from the addition of 3 g/kg Ins, reducing overall ileum dry matter digestibility (IDMD) by 1.6 % compared to the basal diet. Ins included at 3 g/kg improved IDMD compared to the control and there was an overall significant effect of treatments on IDMD (P>0.05).

Table 4.5: The effect of supplementary *myo*-inositol and phytase on growth performance (Average BW, ADFI, ADWG and FCR) of broiler chickens from 0 to 21 days of age.

Treatment factor	Average BW (g)		ADFI (g/b/d)	ADWG (g/b/d)	FCR
	Day 0	Day 21			
Ins (g/kg)					
0	39	753^{b}	48^{b}	33^{b}	1.44^{bc}
1.5 g	39	759^{b}	48^{b}	33^{bc}	1.46^{ab}
3 g	38	732^{bc}	47^{b}	32^{bc}	1.43^{bc}
30 g	39	699^{c}	47^{b}	31^{c}	1.50^{a}
Phytase (FTU/kg)					
1500	39	758^{b}	48^{b}	34^{b}	1.41^{bc}
4500	39	815^{a}	51^{a}	36^{a}	1.40^{c}
SEM	0.4	17	0.7	0.8	0.019
P-value	0.830	0.001	0.002	<0.001	0.005
CV%	3.1	7.1	4.7	7.2	4.2

FCR, feed conversion ratio; Ins, added dietary *myo*-inositol; SEM, pooled standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

Table 4.6: The effect of supplementary *myo*-inositol and phytase on the apparent metabolisable energy (AME) and apparent metabolisable: gross energy ratio (AME:GE). Ileum dry matter digestibility (IDMD) was determined at day 21 of age.

Treatment factor	AME[1] (MJ/kg DM)	AME: GE[1]	IDMD %[2]
Ins (g/kg)			
0	12.8	0.78	55.4[ab]
1.5 g	12.5	0.76	57.4[ab]
3 g	12.4	0.76	58.6[a]
30 g	12.7	0.78	53.9[b]
Phytase (FTU/kg)			
1500	12.7	0.77	54.1[ab]
4500	12.5	0.76	55.9[ab]
SEM	0.202	0.0123	1.1
P-value	0.734	0.734	0.027
CV%	5.1	5.1	6.2

Ins, added dietary *myo*-inositol; SEM, pooled standard error of means; CV% coefficient of variation; AME, apparent metabolisable energy; GE, gross energy.
[1] based off a four-day collection (19-21 days) of excreta.
[2]based on day 21 ileal collection from 4 birds per an observation.
There were 10 observations per treatment factor.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

4.3.4 Inositol Phosphates in Ileal Digesta

The concentration of Ins in the ileum digesta was affected by the treatment diets (P<0.001) (Table 4.7). The treatment diet of 30 g/kg Ins significantly increased the Ins content in the digesta compared to the other diets (P<0.05), although the two phytase treatment groups did not significantly differ from one another or to the un-supplemented diet (P>0.05). The content of phytate (IP_6) in the ileum digesta was affected by the diets (P<0.01), there was clear separation of treatment means as 0 g/kg Ins, 30 g/kg Ins, 1500 FTU/kg and 4500 FTU/kg all differed from one another (P<0.05). IP_5 content of digesta was influenced by diets (P<0.001). The 1500 FTU/kg diet increased the concentration of IP_5 compared to the Ins treatment groups (P<0.05), however, the 4500 FTU/kg diet reduced the concentration of IP_5 compared to the Ins treatment groups (P<0.05). The quantity of IP_4 found in the digesta was affected by the diets (P<0.001), with the two treatment groups containing phytase increasing the concentration present compared to the Ins diets (P<0.05). Finally, IP_3 was also affected by treatment groups (P<0.01) with Ins and phytase treatment groups differing from one another (P<0.05).

Table 4.7: The effect of supplementary *myo*-inositol and phytase on IP_{6-3} and *myo*-inositol concentrations in ileum digesta µmol/g DM of broilers at 21 days of age.

Treatment	IP_3	IP_4	IP_5	IP_6	Ins
Ins (g/kg)					
0	0.071^{b}	0.936^{c}	4.196^{b}	66.81^{a}	1.55^{c}
1.5	0.078^{b}	0.930^{c}	4.022^{b}	64.65^{ab}	15.29^{bc}
3	0.094^{b}	0.995^{c}	4.601^{b}	66.88^{a}	35.59^{b}
30	0.260^{b}	1.618^{c}	4.040^{b}	56.05^{b}	398.57^{a}
Phytase (FTU/kg)					
1500	4.089^{a}	14.164^{a}	6.202^{a}	18.88^{c}	4.68^{bc}
4500	4.221^{a}	9.757^{b}	0.841^{c}	3.05^{d}	22.83^{bc}
SEM	0.284	0.794	0.255	3.364	11.39
P-value	<0.001	<0.001	<0.001	<0.001	<0.001
CV%	61.3	53.1	20.3	23.1	45.2

Ins, added dietary *myo*-inositol; IP_6, *myo*-inositol hexakisphosphate; IP_5, *myo*-Inositol pentakisphosphate; IP_4, *myo*-inositol tetrakisphosphate; IP_3, *myo*-inositol trisphosphate; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, based on a pooled ileum digesta from 4 birds per an observation.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

4.3.5 Mineral Digestibility, Blood Plasma Minerals and Ins plasma concentrations.

Mineral concentrations in ileal digesta were determined by IPC-MS, and digestibility calculated using the internal digestibility marker (AIA). The treatment diets had a significant effect on mineral digestibility on all minerals in Table 4 (P<0.05) except for Ca (P=0.175). Feeding 4500 FTU/kg of phytase in diets reduced the digestibility of Co, Mn, Cu and Zn compared to the un-supplemented diet (P<0.05) but improved P digestibility (P<0.05). The CV% of digestibility data varied from 3.5 % to 133.2 % (Table 4.8).

There was no significant effect on the concentration of the plasma minerals due to the experimental diets (P>0.05) (Table 4.9). The CV% of data varied from 4.9 % to 74.9%. In addition, there was a significant difference (P<0.001) in Ins concentrations in blood plasma, whereby, 30 g/kg differed from the other treatments (P<0.05).

Table 4.8: The effect of supplementary *myo*-inositol and phytase on apparent mineral ileal nutrient digestibility (%) of broilers at 21 days of age.

Treatment factor	Ca %	P %	Mg %	Fe %	Na %	Co %	Mn %	Cu %	Zn %	Mo %	N %
Ins (g/kg)											
0	53.2	61.7^{b}	31.5^{ab}	28.7^{ab}	-37.5^{c}	48.6^{a}	54.5^{a}	34.0^{a}	36.7^{a}	72.9^{a}	32.8^{ab}
1.5 g	59.1	64.2^{b}	34.4^{a}	32.0^{a}	-7.2^{ab}	40.7^{a}	50.5^{ab}	30.8^{a}	24.6^{bc}	69.7^{ab}	31.1^{c}
3 g	57.1	62.3^{b}	28.1^{ab}	28.0^{ab}	-7.9^{ab}	26.7^{c}	19.9^{e}	23.0^{a}	20.2^{b}	63.5^{c}	32.3^{b}
30 g	55.0	65.3^{b}	34.4^{a}	28.1^{ab}	4.6^{a}	33.6^{c}	27.1^{de}	30.9^{a}	31.0^{ab}	66.8^{bc}	32.7^{b}
Phytase (FTU/kg)											
1500	52.7	65.7^{b}	23.8^{b}	20.0^{c}	-19.6^{bc}	33.4^{b}	42.1^{bc}	26.9^{a}	16.0^{c}	67.5^{abc}	32.5^{b}
4500	53.8	71.8^{a}	27.6^{ab}	22.6^{bc}	-20.7^{bc}	30.5^{b}	35.6^{cd}	0.1^{b}	15.2^{c}	68.3^{abc}	33.8^{a}
SEM	2.1	1.6	2.8	2.4	6.5	2.2	2.8	6.0	4.0	1.9	0.4
P-value	0.175	<0.001	0.007	0.046	<0.001	<0.001	<0.001	0.001	<0.001	0.002	<0.001
CV%	11.4	7.3	27.7	26.8	133.2	18.6	21.6	74.5	49.4	7.0	3.5

Ins, added dietary *myo*-inositol; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, based on a pooled ileum digesta from 4 birds per an observation.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05)

Table 4.9: The effect of supplementary *myo*-inositol and phytase of plasma mineral concentration and plasma *myo*-inositol broilers at 21 days of age.

Treatment factor	Ins[2] (nmol/mL)	Ca (mmol/L)	P (mmol/L)	Mg (mmol/L)	Fe (µmol/L)	Na (mmol/L)	Cu (µmol/L)	Zn (µmol/L)
Ins[1] (g/kg)								
0	219[c]	2.903	5.550	0.987	19.30	149.53	1.71	22.95
1.5 g	435[b]	2.919	5.401	0.979	19.32	148.73	1.68	25.53
3 g	491[b]	2.911	5.476	0.982	22.02	149.76	2.03	23.50
30 g	1933[a]	2.924	5.320	0.978	18.72	148.27	2.76	22.24
Phytase (FTU/kg)								
1500	341[bc]	2.777	5.242	0.911	18.36	142.81	2.18	21.25
4500	491[b]	2.899	5.778	1.006	20.22	148.66	1.67	22.62
SEM	71.3	0.0641	0.2086	0.0373	1.201	2.311	0.475	1.011
P-value	<0.001	0.591	0.535	0.588	0.319	0.299	0.550	0.088
CV%	34.6	7.0	12.1	12.1	19.3	4.9	74.9	13.9

Ins[1], added dietary *myo*-inositol; Ins[2] plasma *myo*-inositol; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, based on one bird per an observation.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

4.3.6 myo-Inositol in Tissues

Feeding either Ins or phytase increased Ins found in the kidney, leg and breast muscle. Feeding 30 g/kg Ins significantly increased Ins concentration in all tissues except the brain compared to the control (P<0.05). There was no significant effect on the concentration of Ins found in the brain due to treatment diets (P>0.05) (Table 4.10).

Table 4.10: The effect of supplementary *myo*-inositol and phytase on *myo*-inositol found in tissues (mmol/kg FWT) of broiler chickens at 21 days of age.

Treatment factor	Breast (mmol/kg FWT)	Leg (mmol/kg FWT)	Kidney (mmol/kg FWT)	Brain (mmol/kg FWT)
Ins (g/kg)				
0	0.452^{a}	1.62^{a}	4.39^{a}	12.3
1.5	0.756^{ab}	1.48^{a}	5.90^{ab}	17.9
3	0.638^{ab}	1.49^{a}	5.17^{ab}	21.5
30	1.31^{c}	2.71^{b}	10.2^{c}	19.3
Phytase (FTU/kg)				
1500	0.624^{ab}	1.60^{a}	7.65^{bc}	14.7
4500	0.925^{bc}	1.65^{a}	7.03^{ab}	18.4
SEM (df=23)	0.1368	0.2407	0.984	3.03
P-value	0.006	0.015	0.007	0.354
CV%	34.9	27.4	29.2	34.9

Ins, added dietary *myo*-inositol; FWT, fresh weight; SEM, standard error of means; CV% coefficient of variation.
There were 4 observations per treatment factor.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

4.3.7 The interaction between Ins in plasma and tissues.

Several correlations were identified between Ins concentrations in the blood plasma and in kidney, breast and leg muscle (Figure 4.1). The strongest positive correlation between variables assessed was between breast and leg muscle Ins concentrations (R=0.76, P<0.001), followed by kidney and leg muscle (R=0.55, P<0.001) and kidney and breast muscle (R=0.53, P<0.05).

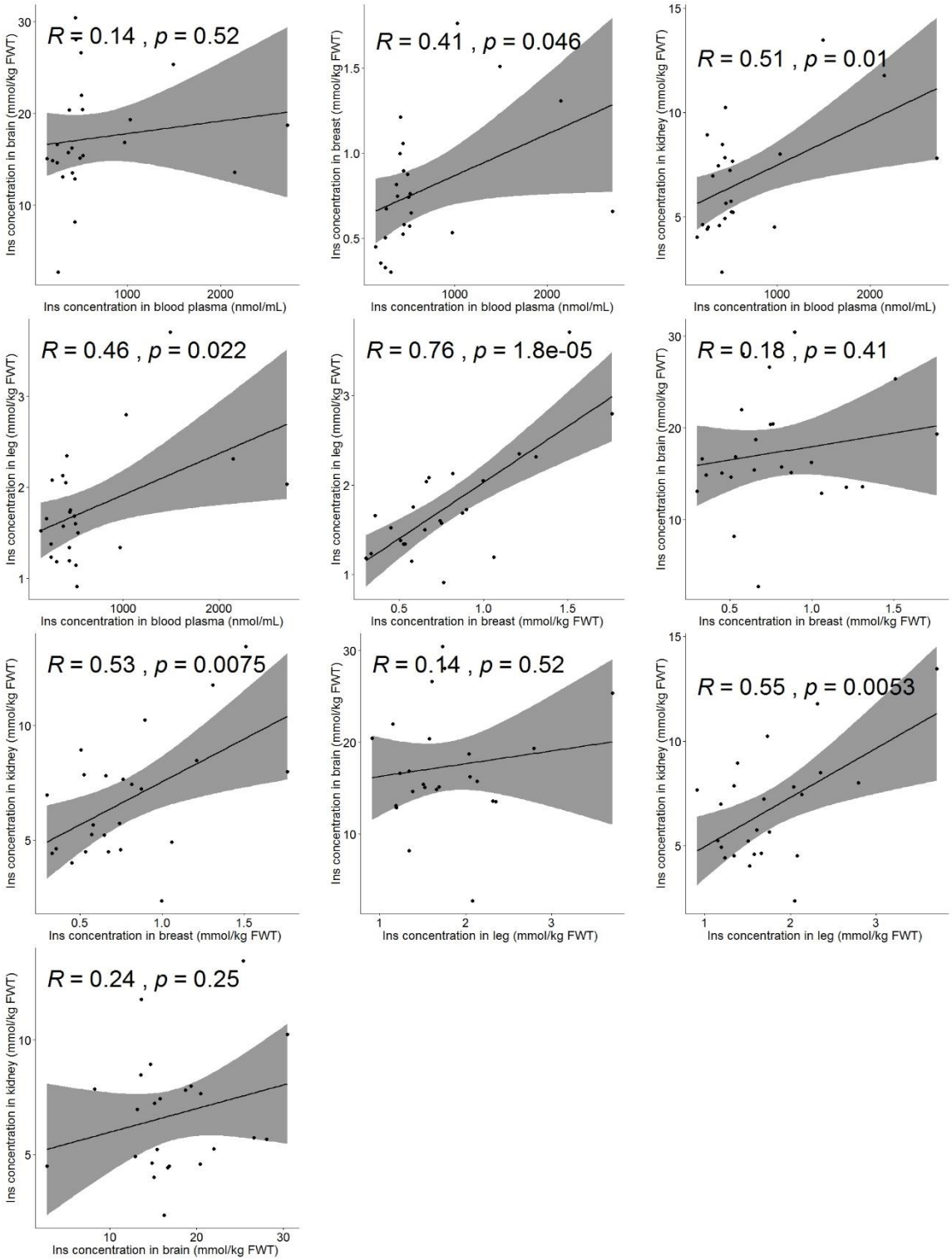


Figure 4.1: The Correlation of *myo*-inositol found in tissues and blood plasma. N= 24.

4.3.8 Inositol phosphates (IP_{3-6}) in kidney tissues.

The total IPs ($_{3-6}$) found in kidney tissue was not affected by treatment factors (P=0.675) and ranged from 296- 414 nmol/g FWT. IP_3, IP_4, IP_5 and IP_6 concentrations were also not affected by treatment diet (P>0.05).

Table 4.11: The effect of supplementary *myo*-inositol and phytase on Inositol phosphates (IP_{3-6}) in kidney tissue (nmol/g FWT) of broiler chickens at 21 days of age.

Treatment factor	Total IP_{3-6}	IP_3	IP_4	IP_5	IP_6
Ins (g/kg)					
0	314	56.9	78.3	135	43.8
1.5	414	64.6	96.3	217	36.3
3	296	57.9	76.5	134	27.1
30	334	50.0	69.9	173	40.7
Phytase (FTU/kg)					
1500	360	52.9	98.7	178	30.2
4500	345	60.4	96.4	157	30.7
SEM	51.6	10.06	20.33	27.0	4.69
P-value	0.675	0.930	0.860	0.272	0.102
CV%	45.1	52.9	70.9	48.8	40.4

Ins, added dietary *myo*-inositol; FWT, fresh weight; SEM, standard error of means; CV% coefficient of variation.
There were 9 observations per treatment factor.

4.4 Discussion

4.4.1 Dosage

The optimal dosage of Ins for broiler chickens is still not very well understood. Predictions on what dosage may be beneficial to broiler chickens has been based on the amount that maybe liberated by different dosages of phytase in the GIT and the subsequent improvement in performance observed with increasing dosages of phytase. The lowest level of supplemented Ins at 1.5 g/kg, was designed to replicate the quantity of Ins produced by 1500 FTU/kg of phytase. Furthermore, the 3 g/kg of Ins in diets was formulated to equate to the quantity of Ins produced by a super-dose of phytase (4500 FTU/kg). The highest level of added Ins, at 30 g/kg was selected to maximise any effect of Ins has on the study variables; approximately 10 times the quantity of Ins produced with a super-dose of phytase. Previous studies investigating Ins supplementation in broiler chicken diets have formulated diets to contain a range of dosages from 0.1 to 7.5 g/kg (Sommerfeld et al., 2018; Gonzalez-Uarquin[a] et al., 2020; Pirgozliev et al., 2007). The treatment of 30 g/kg Ins did not increase mortality compared to the control diet, this confirms that high levels of Ins are safe for broiler chickens. As chickens are not a model organism little information is available on the biological requirement of Ins or if it is required at all in healthy birds. Ins deficiency may occur through a plethora of different mechanisms, which could include reduced dietary intake, decreased synthesis, increased catabolism or inhibition of intestinal and cellular uptake (Dinicola et al., 2017). In the treatment of PSOS in women, a review paper by Facchinetti et al. (2020) compiled dosages in a meta-analysis given to patients which varied from 100 mg to 4 g of Ins. The study concluded that 4 g of inositol (Ins and D-*chiro*-inositol in a molar ratio of 40:1) improved insulin sensitivity and ovarian physiology. Other animals are also known to have

deficiency due to diet are gerbils (Chu and Hegsted, 1980). Ins is required in chickens either in its free form or used in other compounds to regulate and maintain essential biological pathways. However, the balance of *de novo* synthesis and dietary intake is not understood. In addition, the fast-growing nature of modern broiler chickens may result in deficiency due to the improvements seen when super-dosing phytase. Therefore, this study has provided valuable information on refining the dosage and knowledge on what dosage of Ins supplementation might be beneficial to chickens.

4.4.2 Growth Performance

In this and other studies testing different levels of Ins into broiler chicken diets it is important to note that removing the anti-nutritional compound phytate in itself can aid in improved performance and mask the effects of Ins. There is an increasing number of studies looking at Ins supplementation in poultry diets, however there is no clear link that Ins is a growth promoter. Super-dosing phytase increases the formation of Ins and lower IPs in the upper parts of the gastrointestinal tract in chickens and turkeys (Beeson et al., 2017; Pirgozliev et al., 2019). However, supplemented Ins used in the study would have been available to the bird to absorb straight away and would not have to wait for phytase and the birds own APL to produce the Ins for it to be available for uptake. Therefore, comparing the free Ins dosages and phytase liberated Ins is not necessarily a fair comparison as there is a greater availability and chance for uptake from free supplementation. Due to increased formation of Ins due to super-dosing phytase it is a logical assumption that the increased formation of Ins is a factor in increasing growth performance (Lee and Bedford, 2016). This therefore complicates understanding the importance of Ins in poultry diets and its links to phytate destruction and bird performance. The growth performance of the broiler chickens in this study was typically lower than the breed standard, however, this can be accounted for by the feeding of mash diets. Pirgozliev et al. (2016) observed approx. 20% reduction performance in feeding mash diets compared to the same diet pelleted. Feed intake is the major component in determining body weight gain and feed efficiency in broiler chicken poultry diets, but FI can easily be influenced by several factors (Classen, 2017; Ferket and Gernat, 2016). In this study, FI was significantly improved ($P=0.02$) by a dosage of 4500 FTU/kg phytase by 3 g/b/d when compared to the control diet. Feeding phytase may change feed intake of broiler chickens. A study by Ptak et al. (2015) also observed an increased in FI during the 0-14 day period when feeding a super dose of phytase at 5000 FTU/kg ($P<0.001$) but no difference between 14-21 day period ($P=0.361$) when compared to the control. Similarly, Pirgozliev et al. (2007), observed a step wise increase in FI with increasing dosage during 0-28 day period ($P<0.001$). However, Zanu et al. (2020), observed the opposite with a reduction in FI between 500 and 1500 FTU/kg phytase ($P=0.01$) for 0-14 days of age and

no difference in dosages 0-21 days (P= 0.787). Ins supplementation in this study did not affect FI, which agrees with the work of Cowieson and Zhai (2021) who assessed graded levels of Ins at 0, 1, 2, 3, 4 and 5 % in diets from 8 to 18 days of age (P=0.131) and Sommerfeild et al. (2018), compared three dosages of phytase, control and with 3.8 g/kg Ins with no effect on FI from 1-22 days of age (P=0.504).

A comparable study by Gonzalez-Uarquin et al. (2020[a]) assessed three inclusion levels of phytase at 500, 1500 and 3000 FTU/kg, one level of Ins at 3.5 g/kg and a control diet. There was no difference in body weight on day 22 (P>0.05) with all birds performing well compared to the breeders recommendations and ranging from 1084-1188 g. This study partially agrees with the findings of Gonzalez-Uarquin et al. (2020[a]) that the final body weights of the control, 1.5, 3 g/kg and 1500 FTU/kg were comparable. However, the high dosage of Ins at 30 g/kg reduced final BW at day 21 by 54 g when compared to the control diet and 4500 FTU/kg improving BW by 62 g again compared to the control diet (P=0.001). Pirgozliev et al. (2019) also did not see any differences in day 21 body weights with birds feed comparably high dosage of Ins at 30 g/kg Ins (P>0.05). Based off this experiment it is possible to suggest that 30 g/kg of Ins may be above the optimal dosage.

The overall FCR for the study from 0-21 days of age was increased by the highest level of Ins at 30 g/kg, and there was no significant difference between 0, 1.5, 3 g/kg or 1500 FTU/kg of phytase. In the published literature there is not a consistent picture of Ins dosages improving FCR. Pirgozliev et al. (2019) did not observe any differences in FCR due to the Ins dosages of 0, 3 and 30 g/kg Ins. However, Cowieson and Zhai (2021) did observe a positive linear response with increasing Ins for improving FCR (P<0.001) ranging from 0-5 % Ins in diets. It is also important to note that available P may affect the overall FCR, in this study available P was determined at 4.8 g/kg, which meant the birds were not limited by available P. A study produced by Zyła et al. (2013) demonstrated that supplementation of 1 g/kg Ins in diets containing 1.5 g/kg of available phosphorus could improve growth of broilers from 0-21 days. Furthermore, in a dose designed experiment by Pirgozliev et al., (2007), maximal growth was reported at 2.5 g/kg Ins with 2.5 g/kg available P. Which suggests that Ins may be more beneficial when added to diets containing lower available P.

4.4.3 AME and IDMD

There is limited information on how Ins supplementation or phytase liberated Ins effects energy utilisation by the bird. The results from this chapter for AME and AME:GE were not affected by treatment diets (P=0.734 and P=0.734 respectively). Cowieson and Zhai (2021) observed no significant effects of Ins supplementation on AME (no linear (P=0.143), or quadratic response (P=0.427)). This agrees with the chapter findings, with

Ins having no effect on AME. A study by Pirgozliev et al. (2019) also agrees with the chapter results whereby nutrient retention (AME and AME:GE) were not affected (P>0.05) by dietary Ins content of 0, 3 or 30 g/kg Ins. Ileum dry matter digestibility (IDMD) however was affected by treatment diet (P=0.027), with 30 g/kg Ins significantly reducing IDMD to 53.9% compared to 58.6% in table 4.6. IDMD was affected by Ins supplementation, with a significant linear decrease observed with increasing Ins dosage (P<0.01) (Cowieson and Zhai, 2021). This work suggests that higher dosages of Ins may reduce IDMD.

4.4.4 Plasma Ins

The uptake of Ins from the gastrointestinal tract in healthy humans is a highly efficient process with 99.8% of Ins being absorbed via three transporter proteins (SMIT1, SMIT2 and HMIT) (Croze and Soulage, 2013). The values reported here (Table 4.9) for Ins in blood plasma varied according to the treatment factors with 219, 436, 491, 1933, 341 and 491 nmol/mL observed for 0, 1.5, 3, 30 g/kg Ins, 1500 and 4500 FTU/kg respectively (P<0.001). There are an increasing number of papers reporting Ins in chicken blood plasma with birds being fed a range of Ins dosages and phytase dosages (Cowieson et al., 2015; Sommerfeld et al., 2018; Lee et al., 2018; Pirgozliev et al., 2019; Whitfield et al., 2022; Gonzalez-Uarquin[a] et al., 2020; Gonzalez-Uarquin et al., 2020[b]). Based on the papers listed, control diet or diets not containing Ins or phytase, Ins in blood plasma varied from 140-584 nmol/mL with the average value being 282 nmol/mL. Base levels of Ins in blood plasma do appear to vary in the published literature and warrants further investigation. It is important to note that available P and Ca, age of birds and environmental conditions may affect the results published and therefore more data is required to further refine normal ranges under specific conditions. The data reported in this chapter agrees with this average control value and suggests that the chicken will maintain Ins homeostasis around 282 nmol/mL without phytase or direct Ins supplementation. When phytase or Ins is included into diets there is typically an increase seen in Ins levels in blood plasma above the control values. For example, Pirgozliev et al. (2019) observed an increase of Ins in blood plasma in broilers supplemented with Ins, 584 (no Ins supplementation), 663 (3 g/kg) and 982 nmol/mL (30 g/kg). The data in this chapter agrees with the published literature that Ins in blood plasma can be increased and manipulated by dietary Ins, either supplied in its free form or produced by the complete dephosphorylation of phytate. This leads to the conclusion that Ins, regardless of origin can be used by the bird for metabolic purposes and is of equal value.

4.4.5 Tissue Ins and Inositol Phosphate esters

myo-Inositol concentrations in different tissues varies (Table 4.10), this is due to different requirements and roles these tissues have. The only significant organ involved in the catabolism of Ins is the kidney, since radioactively labelled Ins was not degraded to

respiratory ^{14}carbon dioxide in nephrectomised rats (Howard and Anderson, 1967). Although there is no reported range of Ins in chicken kidneys, expected ranges in other animals are 2.51- 8.21 mmol/kg FWT (Frieler et al., 2009). The kidney plays an important role in controlling blood plasma and movement of Ins which could explain why there is a significant increase of Ins in the kidney when birds are fed high dose of Ins (30 g/kg) and this effect is also seen in the blood plasma (Holub, 1986). Concentrations of Ins in the brain is tightly controlled and is less dependent on dietary supply, only 2 % of Ins is absorbed from the blood thus Ins in the brain originates from the central nervous system in rats (Godfrey et al., 1982; Barkai, 1981). This could account for why no differences were observed in Ins concentrations in the brain of chickens despite increases seen in the other tissues. In addition, Ins levels in the brain are typically much higher than other tissues and blood plasma (Frieler et al., 2009), again these effects were also observed. Reported values of Ins in muscle tissue ranges from 0.33 to 2.4 mmol/kg FWT in different species of animals (Sun et al., 2002; Reddi et al., 1990; Battaglia et al., 1961), the values shown here fit within this range. In addition, Greene et al. (2019) also found increased Ins levels in muscle tissues in broilers fed Ins at 3 g/kg or phytase beyond a level of 1000 FTU/kg feed. It is important to note that the Ins produced by the bird cannot be distinguished from the complete dephosphorylation of phytate or direct Ins supplementation. Due to the limited research in this area, it is not possible to conclude if the broiler chickens can meet the tissue requirements of Ins by *de novo* biosynthesis alone. This study demonstrates that Ins can be manipulated in tissues (except the brain) by diet. Further work is required to understand the pathways effected by changes in Ins concentrations in the chicken.

There is very limited information on Inositol phosphates (IP_{3-6}) in kidney tissue of broiler chickens and if this ester profile can be manipulated by phytase or Ins supplementation. In this study there was no significant effect on IP_{3-6} or total IPs when birds were fed either Ins or phytase in the feed. Sprigg et al. (2022) is the only study to date to have measured IPs in kidney tissue with birds being fed one of three diets, control, control + 500 FTU/kg and control + 6000 FTU/kg. The study reported a significant difference in Control vs 6000 FTU/kg for IP_4, P=0.023. There was also a significant difference in Control vs 6000 FTU/kg (P=0.006) and 300 vs 6000 FTU/kg (P=0.013) for IP_5. Furthermore, there was a significant difference in Control vs 6000 FTU/kg for phytate concentrations (P=0.003). The dominant IP isomers found in the kidney are not the direct result of absorption of the IP isomers being produced by the 6-phytase used in this study. The results in this chapter did not find any significant differences by the treatment diets which disagrees with the findings of Sprigg et al. (2022). However, this is the first time the effects of direct supplementation of Ins has been assessed to see if it could manipulate the IP in tissues,

which could have shown changes in IP pathways due to Ins, further work is required in this area.

4.4.6 Inositol Phosphates (IP_{3-6}) in ileal digesta

Inositol phosphates (IP_{3-6}) esters and Ins was determined in the ileal digesta. As might be expected, Ins supplementation (either 1.5, 3 or 30 g/kg Ins) increased Ins concentration in the ileal digesta and had no impact on phytate (IP_6) degradation or concentrations of IP_{5-3}, this observation was also seen by Sommerfeld et al. (2018). There was a stepwise reduction of IP_6 in the ileal digesta with increasing phytase dosage, which is again expected given pervious research by Sprigg et al. (2022), Sommerfeld et al. (2018) and Gautier et al. (2018). Furthermore, the higher dosage of phytase at 4500 FTU/kg was able to degrade IP_6 more effectively than the lower dosage of 1500 FTU/kg, this pattern was also seen in IP_5 and IP_4 concentrations. The concentrations of IP_3 were comparable to one another, however there was a significant increase in the concentration of Ins by 4500 FTU/kg suggesting that more of the phytate had been completely dephosphorylated to Ins.

4.4.7 Plasma Minerals

The use of plasma mineral concentrations is often used as an indicator of bird health (Stout et al., 2010). In this study there was no significant effect on plasma mineral concentrations ($P>0.05$), by treatment diets. Minerals are tightly regulated by the bird to ensure homeostasis and it is known that phytate can lower the bioavailability of minerals by forming cations. Uptake of minerals out if the GIT is tightly regulated along with the detoxification of minerals, which could explain why no differences were seen in the blood plasma of birds at 21 days of age. Ins did not affect the mineral concentrations in the blood plasma, despite the concentrations of Ins increasing by direct Ins or phytase supplementation, this suggests that Ins doesn't bind or interact with Ins in the blood plasma.

4.4.8 Mineral digestibility

The formation of these phytate mineral complexes can be influenced by several factors these include pH of the digesta, diet type and concentrations of other minerals (Champagne, 1988; Hill and Link 2009). Mineral absorption is typically reliant on specific active transporter proteins and associated cotransporter mechanisms and require the availability of other minerals and nutrients (e.g. Na-P co-transporter system). Minerals in ileal digesta were determined by IPC-MS, and digestibility calculated using the internal digestibility marker (AIA). The treatment diets had a significant effect on mineral digestibility on all minerals in Table 4.8 ($P<0.05$) except for Ca ($P=0.175$). Feeding 4500 FTU/kg of phytase in diets reduced the digestibility of Co, Mn, Cu and Zn compared to the

un-supplemented diet ($P<0.05$) but improved P digestibility ($P<0.05$). The nature of mineral digestibility is complex and in this study many minerals did not form recognisable patterns or trends.

Phytate forms strong divalent mineral cations with the following descending order of strength: Cu^{2+}> Zn^{2+}>Co^{2+}>Mn^{2+}>Fe^{3+} >Ca^{2+} (Nolan et al., 1987). In cereal grains it is suggested that phytate forms strong agonist between Mn and Zn but not Cu. Research evaluating Cu in pigs found that phytase supplementation without added Cu did not improve Cu status of the pigs (Bikker et al., 2012). In this chapter Cu digestibility was significantly decreased by 4500 FTU/kg, which doesn't agree with these findings and warrants further investigation as it is believed that Cu is not bound to phytate in grains (Walk et al., 2016). Diet can alter iron absorption, in humans iron absorption can be improved by eating wheat without bran compared to those who ate higher bran level (Brune et al., 1989). Furthermore, iron status can also be improved by eating genetically modified low phytate varieties of wheat in humans and rat models compared to conventional varieties (Kruger et al., 2013 and Mendoza et al., 1998). This suggests that phytate can bind Fe^{2+} and reduce availability of the mineral. However, in the chicken model, a study by Biehl et al. (1997) fed phytase in a soybean meal-based diet without added iron and found no difference in the blood haemoglobin level. In this chapter the addition of phytase typically reduced iron digestibility, despite phytate being degraded by dietary phytase. Although, blood plasma concentrations were not affected. This requires further investigation and any implications this may have on the haematology status of the chicken. There is very limited information on the effects of manganese availability due to phytase supplementation, a study by Mohanna and Nys (1999) found that a dosage of 1200 FTU/kg of phytase improved Mg digestibility. In this study there is not a clear trend if phytase can improve digestibility and therefore availability. Zinc plays many important roles in protein formation and repair, zinc bioavailability from feed stuffs can be improved by genetically modified low-phytate cultivars and low phytate content varieties in rats, pigs and chickens (Walk et al., 2016). In this study digestibility of Zn was reduced by the addition of phytase but Zn plasma levels were not affected. Studies by Jondreville et al. (2007) and Schlegel et al. (2010) both found that the Zn status was improved by the addition of phytase into broiler chicken diets which disagrees with the results in this chapter.

Transport of Ins via SMIT2 relies on an active sodium dependent process, at a ratio of two sodium ions to one molecule of Ins in the brush border (Huber, 2016). A study by Walk et al. (2018) observed an increase in Na digestibility and the up-regulation of SMIT2 (SLC5A11) in the jejunum of broiler chickens at day 21 with increasing phytase supplementation. Here, (Table 4.8) also observed that phytase increased Na digestibility.

More interestingly the inclusion rate of 30 g/kg differed from the basal diet (P<0.05), thus it is possible to suggest that SMIT2 was up-regulated or was more active due to the shift in Na digestibility (Huber, 2016; Walk et al., 2018). However, the expression of SLC5A11 is needed to confirm this.

Removing phytate can increase the digestibility of these minerals however, lower IPs (IP_{3-4}) also have anti-nutritive characteristics which can interfere with mineral digestibility (Bedford and Walk, 2016). Further work is required by *in vivo* studies to see how these lower IPs interact and form cations with the minerals discussed in this chapter. However, pure lower IPs isomers are very expensive, but this is very much warranted due to increased reliance of phytase to supply the P requirements of a growing bird due to the increased cost of inorganic P and other mined minerals. This may account for why some mineral digestibility was reduced by phytase compared to the basal diet.

4.5 Conclusions

In conclusion, this experiment indicated there is an upper limit of inclusion for dietary Ins, whereby high doses (30 g/kg) may have a negative impact on growth performance of broiler chickens in the first 21 days of production. However, P and Ca levels were not limited in this study, which suggests that the benefits of Ins may be more apparent under more limiting diet formulations. There is a rapid uptake of Ins from the ileum into the blood plasma and tissues (excluding the brain). *myo*-Inositol or phytase supplementation in diets have been shown in this study to manipulate the concentration of Ins found in the kidney and muscle. Further investigation is needed to understand the biological pathways that are affected by phytase and Ins supplementation to obtain if the broiler chicken can rely on *de novo* synthesis alone for maximum productivity.

4.6 Outcomes:

1. Feeding high levels of Ins at 30 g/kg significantly reduced the FCR and performance of growing broiler chickens (P<0.05). This suggests an upper feeding limit for Ins of below 30 g/kg Ins.
2. Increasing available Ins (from phytase or direct supplementation) in diets did not significantly affect AME or AME:GE compared to the control diet (P>0.05).
3. Diets containing Ins, increased Ins in the ileum digesta, diets containing phytase reduced the concentration of IP_6, increased the concentration of IP_4, IP_5 and Ins compared to the control diet.
4. The significant effects of the treatment diets had on the mineral ileal digestibility resulted in complex relationships and patterns being observed which did not follow recognisable patterns trends except for P.

5. Increasing available Ins in diets did not significantly influence the plasma mineral concentrations despite differences observed in the ileal mineral digestibility.
6. Increasing available Ins in diets either from direct supplementation or from phytase significantly increased the plasma and ileal Ins concentrations compared to the control.
7. Increasing available Ins in diets did significantly increase Ins concentrations in chicken tissues compared to the control diet except from the brain due to Ins not being transported across the blood brain barrier.
8. Increasing available Ins in diets did not affect the IP_{3-6} concentrations in kidney tissues, suggesting that available lower IPs formed by phytase were not absorbed.

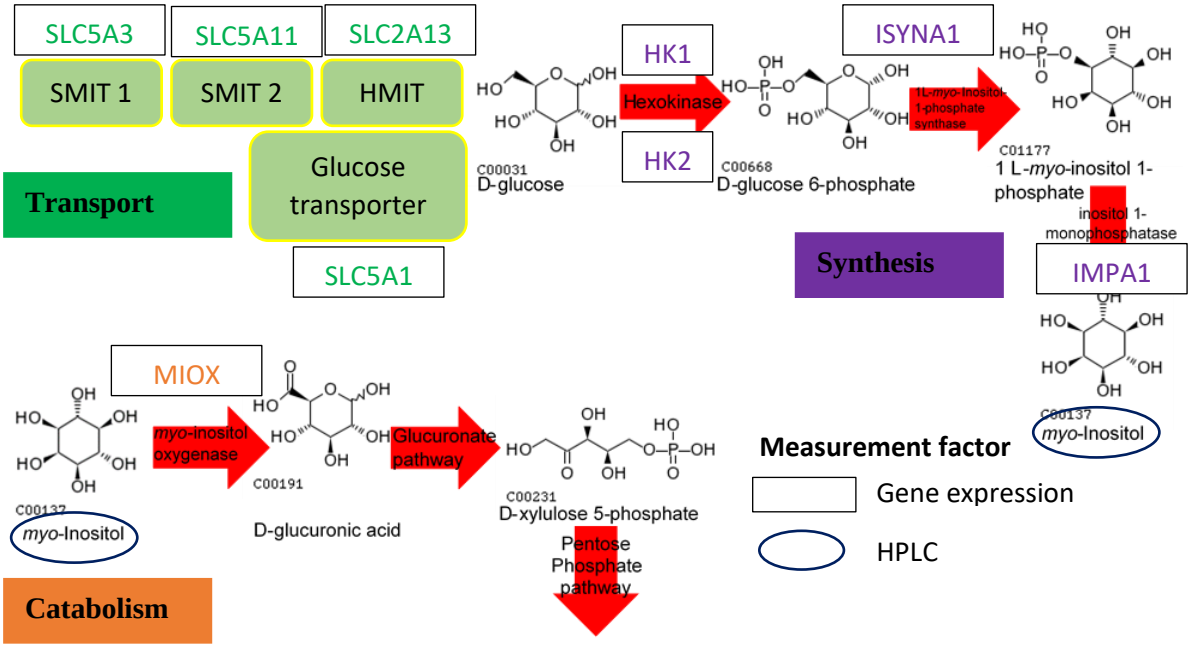
Chapter Five: The effect of *myo*-inositol (Ins), phytase and glucose supplementation on bird performance, manipulation of Ins, and the expression of genes associated with transport, catabolism and synthesis of Ins in 21-day old broilers.

5.0 Introduction

The first study (Chapter 4) examined the effects of using different levels of supplemented Ins and phytase in poultry diets. Super-dosing phytase at 4500 FTU/kg led to improvements in feed efficiency and high dosages of Ins at 30 g/kg reduced performance. The study also looked at the nutritional value and interaction Ins has in poultry diets assessing mineral digestibility and AME. Improvements in performance are often associated with the increased dephosphorylation of phytate (IP_6) by phytase and the formation of lower inositol phosphates (IP_5, IP_4, IP_3) and *myo*-inositol (Ins). Rapid uptake of free Ins was observed by increased Ins found in blood plasma and tissues but not IP_{3-6} in the kidney. However, the mechanisms underpinning the uptake, catabolism and synthesis of Ins in chickens is poorly understood and how dietary supply interacts with these mechanisms. The aim of the subsequent study reported here is to understand pathways associated with Ins homoeostasis in broiler chickens.

Free Ins can be actively transported from GIT via 3 transporter proteins, two of which depend on sodium (Na^+) (SMIT1 or SLC5A3 and SMIT2 or SLC5A11) and one proton (H^+) dependent (HMIT or SLC2A13). Cells can also obtain free Ins from the *de novo* biosynthesis of D-glucose, in three steps. Firstly D-glucose is phosphorylated by hexokinase (HK1 or HK2) producing D-glucose 6-phosphate, it is then synthesised by 1L-*myo*-inositol-1-phosphate synthase (ISYNA1) into 1 L-*myo*-inositol 1-phosphate, finally, it is dephosphorylated by inositol 1-monophosphatase (IMPA1) into Ins. The only significant organ involved in the catabolism of Ins appears to be the kidney, whereby it is catabolised to D-glucuronic acid by *myo*-inositol oxygenase (MIOX) and subsequently enters the (glucuronate-xylulose pathway) pentose phosphate pathway (Figure 5.1) (Croze and Soulage, 2013).

Figure 5.1: Genes of interest (in bold) associated with the active transport (green), synthesis (purple), and catabolism (orange) of *myo*-inositol (KEGG 2021).



myo-Inositol is a cyclohexo sugar and depending on the arrangement of the hydroxy groups there are nine possible stereoisomers (Mullaney et al., 2007). Although Ins is the most abundant form found in nature and is seen in various biological compounds as itself or forming parts of lipids or IPs (Croze and Soulage, 2013). Ins plays an important role in several important biological pathways including cell signalling, cell survival, osmolarity and reproduction (Eagle et al., 1957; Unfer et al., 2012). A recent review by Gonzalez-Uarquin et al. (2020[c]), suggests that Ins could be classified as a semi essential nutrient for poultry. Healthy humans are able to produce sufficient quantities of Ins from D-glucose in a 3-step process (endogenous synthesis), accounting for approximately 80 % of the Ins required. The remaining 20 % is absorbed from the diet (Croze and Soulage, 2013). However, it is unclear what dosage of Ins is required in broiler chicken diets or if requirements can be met by endogenous synthesis alone.

There are an increasing number of studies assessing the role dietary Ins may play in poultry production. Studies by Somerfield et al. (2018) and Pirgozliev et al. (2019) have shown that supplemented dietary Ins was associated with increases of Ins in intestinal chymus and blood plasma which was also seen in the previous chapter. Ins is rapidly absorbed from the GIT via 3 transporter proteins using active transport (SMIT 1, SMIT2 and HMIT). However, increasing plasma Ins does not provide clear information on the resulting health and nutrient availability to the bird, as there a several factors dictating this including the birds age, diet formulation and environmental stress. There is very limited information on Ins distribution across different tissues in chickens and if the quantities of

Ins found in these tissues can be influenced by diet (phytase or direct Ins supplementation) due to the chicken model being poorly studied.

5.1 Objectives

The aim of the study was to quantify the response to feeding two levels of *myo*-inositol (4.5 g and 13.5 per kg of diet), one dosage of phytase (4,500 FTU/kg), one level of glucose (4.5 g/kg) and basal diet (Control) to broiler chickens from 0-21 days of age. With the measurable outcomes being assessed as growth performance (Average BW, ADFI, ADWG and FCR), dietary energy (AME, AME_n, AME:GE, AME_n:GE, NR and DMR), IP_{6-3} and Ins concentrations in jejunum digesta, blood plasma, kidney and jejunum Ins concentrations. Gene expression of Ins transporter proteins; SMIT1 (SLC5A11), SMIT2 (SLC5A11) and HMIT (SLC2A13), glucose transporter (SLC5A1), enzymes involved in Ins synthesis; Hexokinase 1 (HK1), Hexokinase 2 (HK2), 1L-*myo*-inositol-1-phosphate synthase (ISYNA1) and inositol-1-monophosphatase (IMPA1) and the enzyme involved in Ins metabolism *myo*-inositol oxygenase (MIOX) by qPCR in the kidney and jejunum tissue.

Hypotheses:

1. Increasing available Ins in diets (either from the enzymatic action of supplemented phytase or direct supplementation of free Ins) will significantly improve broiler chicken performance, above control diet and glucose diets.
2. Increasing available Ins in diets will not significantly affect AME, AME_n, AME:GE, AME_n:GE, NR or DMR compared to the control diet.
3. Supplementing diets with phytase will alter the IP_{3-6} jejunum profiles compared to control and diets supplemented with Ins and glucose.
4. Increasing available Ins in diets will significantly increase Ins concentrations in blood plasma, kidney and jejunum tissues compared to the control diet.
5. Increasing available Ins in diets will manipulate the expression of genes associated with Ins in kidney and Jejunum tissues.

5.2 Materials and Methods

The experiment was conducted at the National Institute of Poultry Husbandry (Newport, Shropshire, UK) and was approved by Harper Adams University Research Ethics Committee (code: 0283-202002-PGMPHD-CO1) before the initiation of the experiment. The study is reported here in accordance with the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020).

For general Materials and Methods relating to this section please see chapter three.

5.2.1 Animals

One hundred and fifty male Ross 308, day old chicks were obtained from a local hatchery (Cyril Bason Ltd, Craven Arms, UK) and were raised in one communal pen and fed on a commercial starter diet. On day seven, the smallest and largest birds were removed from the flock and the remaining one hundred birds were randomly allocated into fifty raised floor pens (two birds per pen). Animal well-being was checked twice daily, and records used in the assessment of the humane endpoint (any suffering above the commercial baseline). Growth performance was determined from 7-21 days of age on pen basis.

5.2.2 Sample collection

On day 19 of the study, the solid floor of the pens was removed and replaced with a wire mesh to allow for excreta collection, which was then dried at 60˚C until a constant rate and then milled. The digestibility marker (AIA) was determined in the excreta and feed along with GE. At the end of the study, one bird per pen was electrically stunned and killed by exsanguination. At this time blood was collected into lithium heparin BD Vacutainers ®. The bird was then dissected, tissue samples of jejunum and kidney were removed and the contents of the jejunum, extracted by careful pupation of the intestine from the end of the duodenal loop Meckel's diverticulum was collected. The samples were immediately frozen at -80 °C for later analysis. Further samples of jejunum and kidney were taken and stored in RNAlater® (Sigma-Aldrich, USA) at -80 °C prior to analysis of the relative expression of selected genes. The jejunum digesta was then freeze dried and along with the feed samples Ins and IPs were determined using HPLC. The Ins concentrations in blood plasma, jejunum and kidney tissue was also determined using HPLC.

5.2.3 Treatment diets

A maize-soy-based diet was produced at Target Feeds Ltd (Whitchurch, Shropshire, UK), which was formulated to meet breeder's recommendations (Aviagen, 2014), with the addition of a digestibility marker of acid insoluble ash (AIA) (Table 5.1). The basal diet was then split into five parts and supplemented with either Ins (98 ± % *myo*-inositol, Thermo Fisher Scientific) at 4.5 g/kg or 13.5 g/kg, glucose at 4.5 g/kg (Thermo Fisher Scientific), phytase (QuantumTM Blue P40, ABVista, UK) at the inclusion rate of 4,500 FTU/kg and the final part was left un-supplemented as a control. Diets were then pelleted according to Azhar et al. (2019). Each diet was fed to ten pens to give a total of five treatments in a randomised design (allocation by random number generator). Diets were fed from 7 to 21 days and was available *ad libitum*.

Table 5.1: Ingredient composition of basal diet (g/kg).

Ingredient	g/kg
Mazie	580
Soybean meal (48 % CP)	300
Corn gluten meal	40
Vegetable oil	20
Salt	3.6
DL Methionine (M)	4.2
Lysine HCl (L)	3.0
Limestone	6.7
Dicalcium Phosphate	18.5
Vitamin Mineral premix	4.0
Diatomaceous Earth	20.0
Calculated values	
Dry Matter %	90.0
Crude protein (N x 6.25), g/kg	211.2
Metabolisable Energy, MJ/kg	12.58
Calcium (Ca), g/kg	9.545
Available Phosphorus (P), g/kg	4.856
Lysine	1.3364
M+ Cysteine	1.123
Determined values	
DM %	88.3
Gross Energy, MJ/kg	17.36
Crude protein (N x 6.25), g/kg	212
Total Ca, g/kg	0.95
Total P, g/kg	6.8

5.2.4 Gene expression analysis by qPCR

The relative expression of genes of interest in the kidney and jejunum were performed by qStandard (Middlesex, UK) using their standard pipeline. A cross section of jejunum stored in RNAlater® was sampled for RNA extraction and slices of left kidney were taken from the kidney stored in RNAlater® for RNA extraction. All equipment, plasticware and solutions used were nuclease-free.

5.2.4.1 Tissue homogenisation

Tissue was removed from RNAlater®, and 10-3 0 mg of tissue was excised using a clean scalpel and immediately placed into a 2 mL round-bottomed tube containing two 5 mm sterile stainless-steel beads. 600 µL of RLT buffer were added to each tube and tubes were placed into a Qiagen Tissulyser LT (Qiagen, UK) and homogenised at 30 Hz for 10 min. The lysate was centrifuged at top speed for 1 minute to pellet cellular debris and 600 µL of supernatant were transferred to a 1.5 mL tube to which an equal volume of 70 % ethanol was added with immediate mixing by pipetting.

5.2.4.2 RNA extraction.

RNA was extracted using a Qiagen RNeasy kit (Qiagen, UK) following the manufacturer's protocol. Briefly, half of the lysate was transferred to an RNAeasy spin column and

centrifuged at 8000 x *g* for 30 sec to bind RNA, and the flow-through was discarded. This was repeated for the second half of the lysate.

Seven hundred micro litres of RW1 buffer were added to the column, the column centrifuged as above, and the flow through discarded. This was repeated twice with 500 µL RPE buffer. The column was then transferred to a fresh 2 mL collection tube and centrifuged at 13,000 x *g* for 2 min to dry the membrane. The column was placed into a 1.5 mL tube and 30 µL of nuclease-free water were pipetted onto the membrane and the column was incubated at room temperature for 5 min before being centrifuged at 8000 x *g* for 1 min to elute the RNA.

5.2.4.3 Total RNA quantification, purity and normalisation

All samples were quantified using a Nanodrop spectrophotometer to provide an estimate of RNA purity and concentration. All samples exhibited a peak absorbance at 260 nm and A260/A280 ratio of > 2 and A260/230 > 1. Samples were normalised to 100 ng/µL with tRNA 0.5 µg/µL.

5.2.4.4 RNA Integrity

All samples were assessed for RNA integrity using an Agilent Bioanalyzer and an RNA nano 6000 kit (Agilent, UK). The Bioanalyzer electrode pins were cleaned with RNAse Zap and rinsed with water before use. Reagents were brought to room temperature for at least 30 min before use. The gel matrix was prepared according to the manufacturer's instructions: 1 µL of dye was added to 65 µL of gel, mixed by vortexing and centrifuged for 10 min at 13,000 x *g*. A chip was placed on the priming station and 9 µL of gel-dye mix were pipetted into the third well down in the 4th column (marked G). The plunger was positioned at 1 mL, the priming station closed, and the plunger pushed until retained by the clip. Once exactly 30 sec had elapsed the clip was released and five seconds later the plunger was returned to the 1 mL position. Nine micro litres of gel-dye mix was then pipetted into the two remaining wells marked G. Five microlitres of RNA marker was pipetted into all remaining empty wells, and 1 µL of RNA ladder was pipetted into the well-marked with a ladder symbol. Finally, 1 µL of sample was pipetted into each of the 12 sample well, 1 sample per well. The chip was placed into an IKA vortexer set at 2400 rpm for 1 minute. The chip was placed into the Bioanalyzer and the RNA Nano programme was run for eukaryotic total RNA. Data, including electropherograms and RIN were saved as a PDF file.

5.2.4.5 Reverse Transcription

Ten microlitres (1 µg) of normalised RNA were reverse transcribed to cDNA using a Quantitect Reverse Transcription kit (Qiagen, UK). First, gDNA was removed by adding 2 µL of gDNA wipeout buffer to 10 µL RNA and 2 µL water in a 200 µL PCR tube followed

by incubation for 4 min at 4 ˚C in an RGQ qPCR machine (Qiagen). A master mix comprising 1 µL Quantiscript reverse transcriptase, 4 µL RT buffer and 1 µL RT primer mix (random/oligo dT primers) per sample was prepared and 6 mL of master mix were added to each sample followed by incubation at 42 ˚C for 15 min and 95 ˚C for 3 min in the RGQ machine. Ten samples were reverse transcribed in duplicate to provide a measure of RT repeatability. Completed reactions were diluted 10-fold with yeast tRNA 500 ng mL in water (Roche, UK).

5.2.4.6 Primers

Primers were designed and wet-lab validated for specificity, sensitivity, linearity over 7 log, and efficiency >95% by qStandard (UK). For each target, a standard of 107 copies was prepared from purified PCR products and serially diluted 10-fold to 10 copies. Primer sequences are reported in Table 5.2.

Table 5.2: GenBank accession number, sequences of forward and reverse primers and fragments sizes used for quantitative real-time PCR.

Target	Accession number	Primer sequence	Size (bp)
HK1	NM_204101.1	F: 5'- TTCACAGATTGAGAGCGACAGG -3' R: 5'- CATAACTGAGCTGCTCGCTTTGA -3'	141
HK2	NM_204212.1	F: 5'- TCATCGTCAAGGAGGTGTGC -3' R: 5'- ATGATGGCCGAGAAGTGTGG -3'	175
IMPA	XM_004939935.2	F: 5'- ACAGGTTTCCATTTGTGGCAGTT -3' R: 5'- TCTGGCAGTGTACATCTTGTCTTCAA -3'	148
ISYNA1	XM_015300143.1	F: 5'- GTCTTTGATGGGTGGGACATCTC -3' R: 5'- CGATGAACTCGGGGATGTAAATGGA -3'	148
MIOX	XM_015281706.3	F: 5'- TCCATGATTCCACCTTCCACGA -3' R: 5'- ATCACTCTGTACATGTACTCGTCGT-3'	139
SLC2A13	XM_001232939.6	F: 5'-GTTTTCTTTGCACCTGGGATGG-3' R: 5'- GCAGAAAGGTCAGTGACACCAG- 3'	142
SLC5A1	NM_001293240.1	F: 5'-ATCAAAGCTGGGGTGGTGACA-3' R: 5'- GCCAACTGTATAAACACAGCTCCAG -3'	155
SLC5A3	NM_204474.1	F: 5'- GGCGTTCTATGACCTGGGTAGC-3' R: 5'-TCCAGATCCTGCGAGCCCAATG -3'	89
SLC5A11	XM_414862.5	F: 5'-AGATGGTGTGGTGGCCTGTTG-3' R: 5'- ATTCCACTCATAGGCTGTTGCAG -3'	122
ACTB	NM_205518.1	F: 5'- GACAATGGCTCCGGTATGTG-3' R: 5'- CAACCATCACACCCTGATGTC -3'	107
B2M	NM_001001750.1	F: 5'-GTACTCCGACATGTCCTTCAAG -3' R: 5'- CACAGCTCAGAACTCGGGAT -3'	157
GAPDH	NM_204305.1	F: 5'- TGTGACTTCAATGGTGACAGC -3' R: 5'- CCAAACTCATTGTCATACCAGGA -3'	107
PPIA	NM_001166326.1	F: 5'- TGACAAGGTGCCCATAACAG -3' R: 5'- CGTAAAGTCACCACCCTGAC -3'	127
YWHAZ	NM_001031343.1	F: 5'- TCTTGATCCCCAATGCTTCG -3' R: 5'- TGCTCCACAATCCCTTTCTT -3'	122

5.2.4.7 qPCR

Two microlitres of DNA were amplified in an RGQ qPCR machine (Qiagen, UK) in a 10 µL total reaction volume using Brilliant III SYBR Green master mix (Agilent, UK) with each primer at a final concentration of 500 nmol/L. The no-template control reactions (NTC) contained 2 µL of tRNA (0.5 µg/mL). DNA standards (107-101 copies reaction-1) for each target were included in each run. Reactions were pipetted robotically using a Qiagility (Qiagen, UK).

Cycling parameters were: 95 ˚C for 5 min followed by 40 cycles of 95 ˚C for 5 sec and 60 ˚C for 1 sec. At the end of 40 cycles the temperature was raised from 60 ˚C to 95 ˚C rising by 1 ˚C each step to provide melt curve data.

Melt curves were checked for product specificity (single peak) and the presence of primer-dimers. Sample amplification curves were checked to ensure they were parallel with amplification curves for standards to identify qPCR inhibition. Copy numbers/reaction were derived from the standard curves using the Rotor-Gene software.

5.2.4.8 Data normalisation and analysis

Reference gene copy numbers were uploaded into geNorm software to determine the number and identity of optimal reference genes for normalisation. The normalisation factor was calculated by the software for five stable reference genes, which were ACTB, B2M, GAPDH, PPIA and YWHAZ. For each gene of interest, the copy number in a sample was divided by its respective normalisation factor to derive normalised copy numbers per reaction. RT means were first calculated for the 10 samples reverse transcriped in duplicated before group means were calculated and compared.

5.2.5 Statistical analysis

All data were checked for normality and homogeneity of residuals prior to ANOVA. Data was analysed by one-way ANOVA in Genstat® (21st edition, Rothamstead, Hertfordshire, UK), with differences reported as significant at $P<0.05$ and tendencies considered at $P>0.05$ - $P<0.10$. Treatment means were separated by post hoc protected Fisher Least Significant Difference test. Values greater than or smaller than 3 standard deviations from the arithmetic mean were removed as outliers. Graphics were produced in Excel 2018 (Microsoft). Each pen is considered was an experimental unit in this study. Normalised mRNA copy number was transformed using $\log_{10}$ prior to ANOVA.

5.3 Results

5.3.1 Analysis of feed

Feed was analysed by Enzyme Services and Consultancy, UK for phytate-P and phytase recovery by NIR (Table 5.3), Ins recovery and IP_{3-6} was determined using HPLC. Analysed

phytase activity confirmed that it was close to the formulated activity of 4500 FTU/kg with the recovered activity of 3780 FTU/kg. Ins recovery was close to expected dosages of 4.5 and 13.5 g/kg with determined values being 4.12 and 15.43 g/kg respectively. Additionally, phytate-phosphorus and IP_{3-6} was also consistent across diets which was expected.

Table 5.3: Recovered phytase activity, phosphorus bound phytate contents, Ins recovery and IP_{3-6} profiles of experimental diets.

Treatment Diet	phytase activity FTU/kg[1]	phytate-P content (g/100g)	Ins recovery (g/kg)	IP_3 (nmol/g)	IP_4 (nmol/g)	IP_5 (nmol/g)	IP_6 (µmol/g)
Control	<50	0.26	<0.18	<10	185	2449	16.3
4500 FTU/kg	3780	0.26	<0.18	<10	412	1709	10.9
4.5 g/kg Ins	<50	0.28	4.12	<10	246	2684	18.1
13.5 g/kg Ins	<50	0.31	15.43	<10	225	2533	16.5
4.5 g/kg glucose	<50	0.27	<0.18	<10	193	2761	18.8

[1]One FTU is defined as the amount of enzyme required to release 1 mmol of inorganic P per minute from sodium phytate at 37°C and pH 5.5.

5.3.2 Mortality

During the experiment only one bird was culled during 0-7 days of age in the communal pen. No birds died or were culled during the 7-21 day period of the experiment, with all birds remaining healthy.

5.3.3 Performance and Energy Utilisation of Broilers

To determine if growth performance was influenced by either Ins, phytase or glucose supplementation to the basal diet, average BW, ADFI, ADWG and FCR was measured for the trial period of 7-21 days (Table 5.4). Chick weights on day 7 were around 20% below the breed standard of 213 g (Aviagen, 2019), there was no significant difference in chick weight allocation across treatment diets. FI and average BW was only influenced by 4.5 g/kg glucose treatment group, with birds consuming less food ($P<0.001$) and gaining less weight ($P=0.002$). Overall FCR was influenced by treatment diets ($P=0.003$), Ins supplementation at 4.5 g/kg improved FCR by 0.06 compared to the control. The FCR of the birds fed 4.5 g/kg Ins was comparable to 4500 FTU/kg and 4.5 g/kg glucose treatment groups. Coefficients of variation (CV%) of FI, WG, and FCR were 4.3 %, 5.1 %, and 2.6 % respectively.

A three-day collection was used to determine AME, AMEn, AME:GE, AMEn:GE, NR and DMR in the excreta of the study chickens, collected from 19-21 days (Table 5.5). The treatment diets significantly affected AME ($P=0.044$), AME:GE ($P=0.044$), NR ($P=0.022$), DMR ($P=0.002$) with a tendency seen in AME_n ($P=0.089$). However, there was no effect on AME_n by treatment diets ($P=0.138$). Supplementing diets with 4.5 g/kg of Ins significantly improved AME by 0.31 MJ compared to the control ($P<0.05$). NR was improved by 4.5 g/kg Ins, compared to the control and was reduced by 13.5 g/kg Ins

compared to the control. DMR was also improved by 4500 FTU/kg and 4.5 g/kg compared to the other treatment groups.

Table 5.4: The effect of supplementary Ins, phytase and glucose on growth performance of broiler chickens fed a pelleted feed from 7 to 21 days of age.

Treatment factor	Average BW (g)		ADFI (g/b/d)	ADWG (g/b/d)	FCR
	Day 7	Day 21			
Control	170	930^{b}	84^{b}	63^{b}	1.32^{c}
4500 FTU/kg	167	956^{b}	84^{b}	66^{b}	1.28^{ab}
4.5 g/kg Ins	169	952^{b}	82^{b}	65^{b}	1.26^{a}
13.5 g/kg Ins	170	928^{b}	82^{b}	63^{b}	1.30^{bc}
4.5 g/kg glucose	168	889^{a}	77^{a}	60^{a}	1.28^{ab}
SEM	1.2	12.5	1.1	1.0	0.01
P-value	0.34	0.004	<0.001	0.003	0.003
CV%	2.2	4.2	4.3	5.1	2.6

FCR, feed conversion ratio; Ins, added dietary *myo*-inositol; SEM, pooled standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor.
Means within a column with data not sharing a common superscript letter significantly differ.

Table 5.5: The effect of supplementary Ins, phytase and glucose on AME, AME_n, AME:GE, AME_n:GE, NR and DMR based on a total excreta collection from 19 to 21 days of age.

Treatment factor	AME	AME_n	AME:GE	AME_n:GE	NR	DMR
Control	13.30^{a}	12.43	0.767^{a}	0.721	0.679^{ab}	0.722^{a}
4500 FTU/kg	13.59^{bc}	12.68	0.783^{bc}	0.731	0.699^{bc}	0.741^{b}
4.5 g/kg Ins	13.61^{c}	12.69	0.785^{c}	0.731	0.706^{c}	0.741^{b}
13.5 g/kg Ins	13.37^{ab}	12.53	0.771^{ab}	0.722	0.668^{a}	0.724^{a}
4.5 g/kg glucose	13.43^{abc}	12.53	0.774^{abc}	0.722	0.687^{abc}	0.729^{a}
SEM	0.08	0.07	0.0047	0.0036	0.008	0.004
P-value	0.044	0.089	0.044	0.138	0.022	0.002
CV%	1.9	1.9	1.9	1.6	3.9	1.7

AME, Apparent metabolisable energy (MJ/kg DM); AME_n, Nitrogen-corrected apparent metabolisable energy (MJ/kg DM); GE, Gross energy (MJ/kg DM); NR, Nitrogen retention coefficient, DMR, Dry mater retention coefficient; Ins, added dietary *myo*-inositol; SEM, pooled standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor.
Means within a column with data not sharing a common superscript letter significantly differ.

5.3.4 Inositol Phosphates and myo-inositol concentrations

myo-Inositol concentrations in tissues (kidney and jejunum), blood plasma and jejunum digesta was affected by treatment diets (P<0.001). Concentrations of Ins in the blood plasma was significantly increased by 13.5 g/kg Ins to 671 nmol/mL compared to the other diets (P<0.05). The addition of phytase at 4500 FTU/kg did not significantly increase plasma Ins compared to the control or 4.5 g/kg glucose (P>0.05). In the kidney, Ins concentration was also significantly increased by 13.5 g/kg Ins to 5.35 mmol/kg (fresh

weight), compared to the other diets (P<0.05). The addition of phytase into diets did significantly increase Ins concentrations when compared to 4.5 g/kg glucose diet (P<0.05). Ins concentrations in the jejunum tissue was increased the most by 13.5 g/kg Ins to 7.3 mmol/kg with increases also seen in 4.5 g/kg Ins and 4500 FTU/kg treatment diets when compared to 4.5 g/kg glucose. The Ins concentrations in the dry jejunum digesta was increased by 4500 FTU/kg, 4.5 and 13.5 g/kg Ins compared to the control and 4.5 g/kg glucose diet. The CV% varied from 13 to 45 %. The concentrations of inositol phosphate esters (IP_{3-6}) in the jejunum digesta were significantly affected by treatment diets (P<0.001). Phytase supplementation significantly reduced IP_6 and IP_5 concentrations (P<0.001), and increased IP_4 and IP_3 concentrations (P<0.001).

Table 5.6: The effect of supplementary Ins, phytase and glucose on Ins concentrations in blood plasma, kidney tissue, jejunum tissue and jejunum digesta at 21 days of age.

Treatment factor	Plasma (nmol/mL)	Kidney tissue (mmol/kg FWT)	Jejunum tissue (mmol/kg FWT)	Jejunum digesta (µmol/g DM)
Control	47^{a}	3.61^{ab}	2.92^{ab}	8^{a}
4500 FTU/kg	147^{a}	4.01^{bc}	3.90^{b}	98^{b}
4.5 g/kg Ins[1]	336^{b}	4.37^{c}	5.66^{c}	280^{c}
13.5 g/kg Ins[1]	671^{c}	5.35^{d}	7.30^{d}	1030^{d}
4.5 g/kg glucose	67^{a}	3.40^{a}	2.76^{a}	8^{a}
SEM	36	0.17	0.36	23
P-value	<0.001	<0.001	<0.001	<0.001
CV%	45	13	25	25

[1]Ins, added dietary *myo*-inositol; SEM, pooled standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor.
Means within a column with data not sharing a common superscript letter significantly differ.

Table 5.7: The effect of supplementary Ins, phytase and glucose on inositol phosphate esters (IP_{3-6}) in jejunum digesta at 21 days of age.

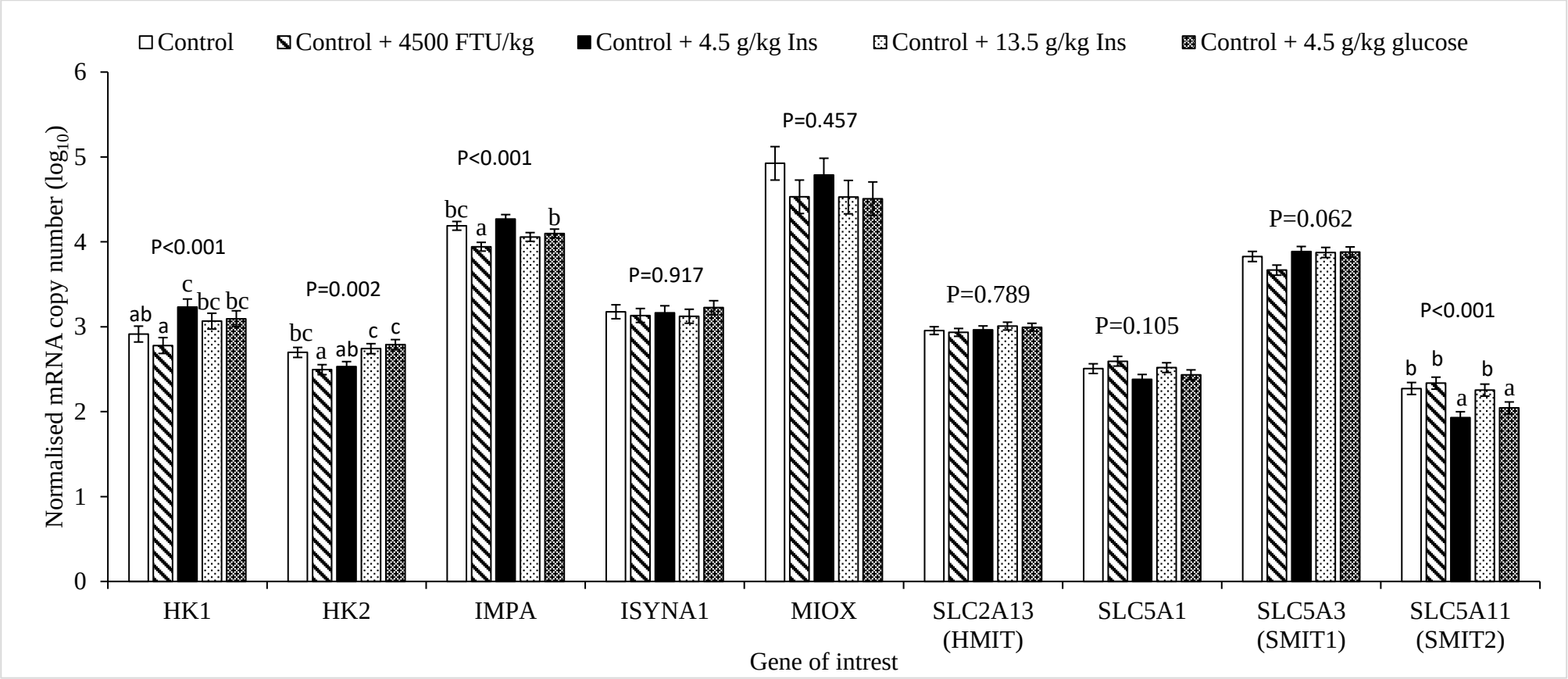
Treatment factor	IP_3 (µmol/g DM)	IP_4 (µmol/g DM)	IP_5 (µmol/g DM)	IP_6 (µmol/g DM)
Control	0.18^{a}	0.94^{a}	3.9^{b}	36^{b}
4500 FTU/kg	2.4^{b}	4.6^{b}	0.68^{a}	2.9^{a}
4.5 g/kg Ins	0.15^{a}	1.0^{a}	3.9^{b}	37^{b}
13.5 g/kg Ins	0.078^{a}	1.0^{a}	3.8^{b}	37^{b}
4.5 g/kg glucose	0.17^{a}	1.1^{a}	4.1^{b}	37^{b}
SEM	0.18	0.39	0.26	2.0
P-value	<0.001	<0.001	<0.001	<0.001
CV%	95	71	25	22

Ins, added dietary *myo*-inositol; SEM, pooled standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor.
Means within a column with data not sharing a common superscript letter significantly differ.

5.3.5 qPCR results of the genes of interest in the kidney.

There was a significant difference between the expression of the gene HK1 in the kidney of broiler chickens fed different treatment groups ($P<0.001$). The addition of sugars (Ins or glucose) increased relative expression compared to 4500 FTU/kg phytase treatment group. HK2 relative mRNA expression was affected by treatment diet in the kidney ($P=0.02$). In the kidney relative expression was decreased by 4500 FTU/kg diet compared to control diet, 13.5 g/kg and 4.5 g/kg glucose ($P<0.05$). Experimental diets did not affect the expression levels of ISYNA1 in the kidney ($P=0.917$). Treatment diets significantly affected the expression level of IMPA in kidney tissue ($P<0.001$), with 4500 FTU/kg treatment diet reducing copy numbers of IMPA compared to the control, 4.5 g/kg Ins and 4.5 g/kg glucose. In addition, birds fed 4.5 g/kg Ins in diets, increased expression of IMPA when compared to 4500 FTU/kg, 13.5 g/kg Ins and 4.5 g/kg glucose treatment groups. Expression of MIOX was not affected by treatment diet in the kidney ($P=0.457$). Expression of SLC2A13 encoding for the Ins transporter HMIT was not affected by treatment diet ($P=0.789$) in the kidney. The expression SMIT2 (SLC5A11) was affected by treatment diets in the kidney tissue of broiler chickens ($P<0.001$). The control, 4500 FTU/kg and 13.5 g/kg Ins diets increased expression compared to the other diets of 4.5 g/kg Ins and 4.5 g/kg glucose. Expression of SLC5A3 encoding for the Ins transporter SMIT1 was not significantly affected by treatment diet ($P>0.05$) but showed a tendency ($P=0.062$). The phytase treatment group of 4500 FTU/kg reduced expression compared to the other diets. Expression of SLC5A1 which encodes for a glucose transporter was not significantly changed by treatment diet in the kidney of the chickens used in this study ($P=0.105$), but there was a tenancy whereby 4500 FTU/kg diet increased expression compared to 4.5 g/kg Ins.

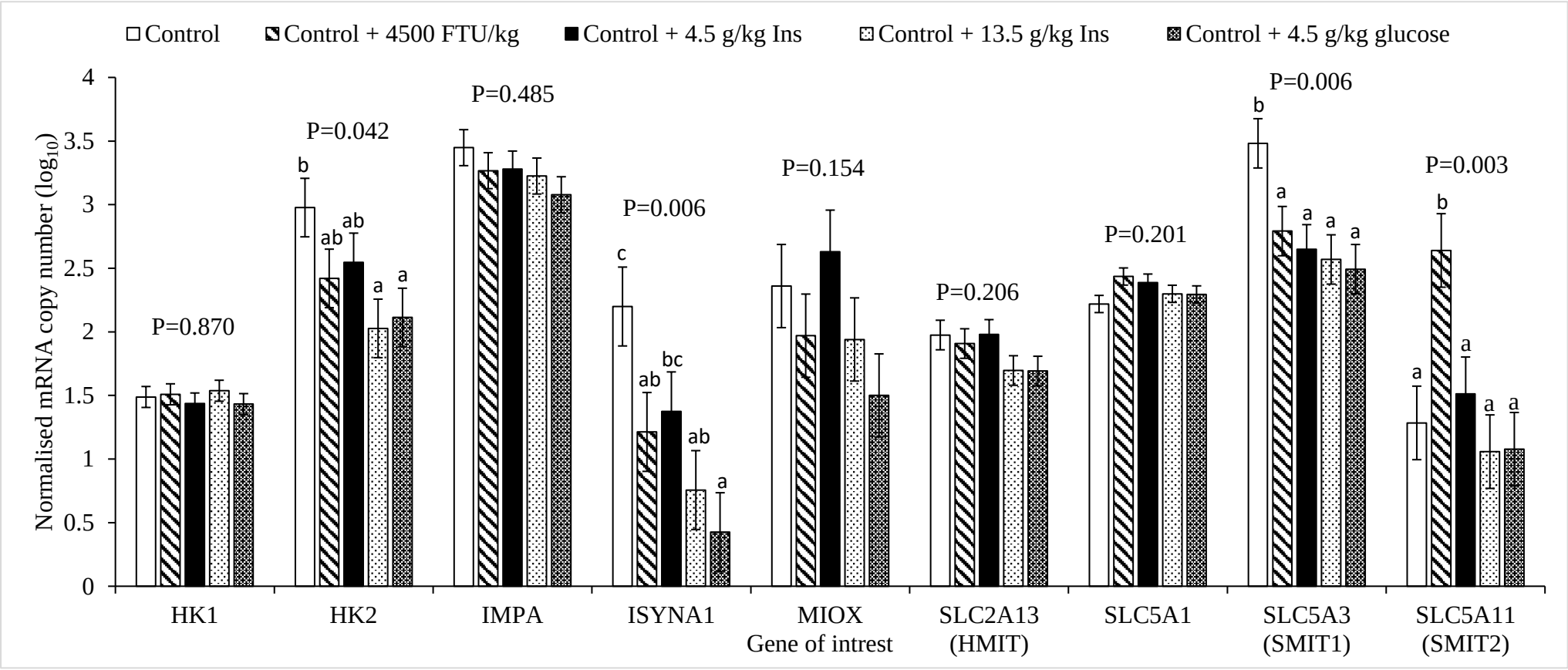
Figure 5.2: The effect of *myo*-inositol (Ins), phytase (4500 FTU/kg) and glucose supplementation on the normalised mRNA copy number (per reaction) (Log_{10}) of the genes associated with Ins pathways in the kidney of broiler chickens at 21 days of age. Error bars represent pooled SEM.



5.3.6 qPCR results of the genes of interest in the jejunum tissue.

There was no significant difference was observed for the expression of HK1 in the jejunum tissue of broiler chickens (P=0.870). HK2 relative mRNA expression was affected by treatment diet in jejunum tissue (P=0.042). Copy numbers were increased in the control diet compared to 13.5 g/kg and 4.5 g/kg glucose (P<0.05). The expression of ISYNA1 in the jejunum tissue of broiler chickens at 21 days of age was not affected by diet (P=0.006). Expression of IMPA was not affected by treatment diets in the jejunum tissue of chickens (P=0.485). MIOX expression was not significantly affected by diet in the jejunum tissue of the broiler chickens used in this study (P=0.154). The transporter protein HMIT which is encoded by SLC2A13 was not affected by treatment diet (P=0.206) in the jejunum. The Ins transporter SMIT2 (SLC5A11) expression was affected by treatment diets (P=0.003) in the jejunum tissue. The treatment diet containing 4500 FTU/kg phytase increased expression compared to the other diets. Expression of SLC5A3 encoding for the Ins transporter SMIT1 was significantly affected by the different treatment diets (P=0.006), with the control diet causing greater expression compared to the other diets. The glucose transporter (SLC5A1) was not significantly affected by the treatment diets (P=0.201) in jejunum tissue.

Figure 5.3: The effect of *myo*-inositol (Ins), phytase (4500 FTU/kg) and glucose supplementation on the normalised mRNA copy number (per reaction) of the genes associated with Ins pathways in the jejunum tissue of broiler chickens at 21 days of age. Error bars represent pooled SEM.



5.4 Discussion

5.4.1 Growth performance and energy utilisation

One of the aims of this study was to investigate the effects of phytase, Ins and glucose supplementation on the growth performance and energy unitisation of growing birds. There are a growing number of reports assessing the addition of free Ins into the diets of broiler chickens. The growth performance of the broiler chickens in this study was slightly lower than the breed recommendations. FCR was significantly improved by phytase dosage of 4500 FTU/kg and 4.5 g/kg compared to the control diet ($P=0.003$). The higher addition of 13.5 g/kg was comparable to the control diet ($P<0.05$) but did numerically improve FCR by 0.02. This once again raises the question of what the optimal dosage of Ins is for improved performance. In this study it appears that 4.5 g/kg of Ins was optimal for improving FCR. Studies by Zyła et al. (2013) and Pirgozliev et al. (2007) found that the optimal dosage was 1 g/kg and 2.5 g/kg respectively, however available P was limited. The Ca and available P was not limited, and diets were fed in pellet form in this study, this may explain why phytase diet was comparable to 4.5 g/kg Ins because the antinutritional effects of phytate was not observed and the positive impacts of Ins were observed. This study provides evidence that Ins could aid in increased broiler performance. The previous chapter found no difference between the FCR of birds fed 0, 1.5, 3 g/kg Ins or 1500 FTU/kg, but the diet was fed in mash form.

There are only a few studies published on the effects of Ins on energy utilisation in broiler chickens, with all studies looking at growing birds of around 18 to 21 days of age. The direct caloric value of Ins is estimated to be approximately 2,800 kcal/kg and for glucose 3,700 kcal/kg (Potter, 1979). When comparing diets containing 4.5 g/kg Ins and 4.5 g/kg glucose, there was no significant difference ($P<0.05$) for AME, AME_n or FCR. Despite there being a 25% reduction in the energy density of glucose. Cowieson and Zhai (2021), assessed graded levels of Ins at 0, 1, 2, 3, 4, 5 % in diets by sequential displacement of increments of 1% dextrose with Ins from 8 to 18 days of age. Excreta was collected from day 15 to 17 and AME, AME_n and DMR was determined. The inclusion of graded levels of Ins had no linear ($P=0.143$), or quadratic response ($P=0.427$) for AME, measured as MJ/kg. However, there was a significant positive linear increase ($P<0.05$) for AME_n with increasing dosage, but no quadratic response ($P=0.256$). DMD ranged from 0.713 to 0.732, with a significant negative linear response with increasing Ins dosage ($P<0.001$), with no quadratic response ($P=0.455$). Ileal digestible energy (IDE) in this study was also measured, with a significant decrease observed with increasing Ins dosage ($P<0.01$) measured as MJ/kg. However, FCR was shown to be improved with increasing Ins dosage in this study. The disappearance of GE in the small intestine does not necessarily reflect the energy status of the growing bird as heat production, energy partitioning and

maintenance energy are not accounted for in these types of digestibility studies. Furthermore, birds in this study were only fed the treatment diets for 7 days before excreta collection started which may not have been long enough to get a true reflection of the effects of Ins on energy utilisation.

The effects of Ins supplementation on nutrient retention were reported by Pirgozliev et al. (2019), whereby nutrient retention and digestibility coefficients (AME, AME:GE, DMR, NR and FD) were not affected (P>0.05) by dietary Ins content of 0, 3 or 30 g/kg Ins. AME ranged from 14.41 to 14.57 MJ/kg DM, AME:GE ratio ranged from 0.856 to 0.865, DMR ranged from 0.746 to 0.756 and NR ranged from 0.634 to 0.641. These values reported in this study have a similar range the those shown in table 5.5. There was no effect on AME_n by treatment diets (P=0.138) in this chapter which agrees to the findings by Pirgozliev et al. (2019). However, supplementing diets with 4.5 g/kg of Ins significantly improved AME by 0.31 MJ and NR compared to the control (P<0.05). DMR was also improved by 4500 FTU/kg and 4.5 g/kg Ins compared to the other treatment groups.

A previous study by Pirgozliev et al. (2007) also observed no significant effects of Ins on nutrient retention and digestibility coefficients (AME_n, AME_n:GE, DMD, ND and FD) (P>0.05). Diets were supplemented with increasing levels of Ins at 0, 2.5, 5 and 7.5 g/kg Ins, there was no significant linear or quadratic response to AME_n, AME_n:GE, DMD, ND or FD (P>0.05), with excreta being collected from 13-17 days. AME_n ranged from 13.92 to 14.21, AMEn:GE ranged from 0.830-0.848, DMD ranged from 0.670-0.686 and ND ranged from 0.596 to 0.615. Again, these results in this chapter fit with the ranges published by Pirgozliev et al. (2007). However significant effects of the treatment diets were observed for AME, DMR and NR. More work is required to understand the effects Ins has on energy utilisation of older birds as to date there is no published literature on it.

5.5.2 myo-Inositol and IP_{3-6} esters

Like other studies that have examined the effects of Ins in diets (either from direct supplementation or produced from phytase), Ins was increased in blood plasma and tissues (jejunum and kidney). Concentrations of Ins in the blood plasma was significantly increased by 13.5 g/kg Ins to 671 nmol/mL compared to the other diets (P<0.05). The addition of phytase at 4500 FTU/kg did not significantly increase plasma Ins compared to the control or 4.5 g/kg glucose (P>0.05). The concentration of Ins found in the control diet of 47 nmol/mL is much lower than published values available, with a range of 140-584 nmol/mL with the average value being 282 nmol/mL (Cowieson et al., 2015; Sommerfeld et al., 2018; Lee et al., 2018; Pirgozliev et al., 2019; Whitfield et al., 2022; Gonzalez-Uarquin et al., 2020[a]; Gonzalez-Uarquin et al., 2020[b]). A study by Gonzalez-Uarquin et al. (2020[a]) assessed the effects of three inclusions of phytase at 500, 1500 and 3000

FTU/kg, one level of Ins at 3.5 g/kg and a control diet on plasma Ins concentrations of broiler chickens. The dosages of 500 and 1500 FTU/kg significantly increased plasma Ins compared to the control (P=0.012), but not the highest dosage of 3000 FTU/kg. This result of high dosages of dietary phytase not increasing plasma Ins was also seen in this chapter despite Ins in the jejunum digesta increasing. This suggests that the homeostasis and regulation of Ins may have been altered by the phytase dosage due to no significant difference in plasma Ins compared to the control diet. There was no significant effect of diet on the Ins transporter proteins in the jejunum except for an increase of SMIT2 for 4500 FTU/kg compared to the control. Furthermore, the concentration of jejunum was not increased by 4500 FTU/kg despite increases in expression of SMIT2 compared to the control.

Novotny et al., (2022) compared the breakdown of phytate and subsequent generation of Ins in the GIT of broiler chickens and turkeys in a 2 by 2 by 2 factorial arrangement (2 species, 2 levels of phytase and 2 levels of Ca and P). When comparing the Ins generated in the crop and gizzard of turkeys and broiler chickens, it was found that more Ins was found in the digesta of the crop (P=0.001) and gizzard (P<0.001) of turkeys than broilers. However more Ins was found in the ileum digesta (P<0.001) and a tendency for blood plasma in broiler chickens compared to turkeys (P=0.055). In addition, there was a positive linear relationship between Ins in blood plasma and ileal digesta for boilers (R^2=0.69) and turkeys (R^2=0.53), however the regression slope was greater in turkeys than broilers. It was speculated that this increased uptake of Ins in the turkey was caused by changes in Ins transporters, different turnover rate in the enterocytes or different Ins requirement in organs. Concluding that broiler chickens have poorer uptake of Ins compared to turkeys. Despite supplemented Ins being freely available for absorption from the moment of ingestion, rather than Ins originating from phytate. In this experimental chapter it was observed that blood plasma Ins concentration was only doubled (approximately) between 4.5 g/kg Ins and 13.5 g/kg, jejunum tissue increasing by 1.64 nmol/kg and kidney concentration only 0.98 nmol/kg despite the dosages triplicating. Which supports the hypotheses of Novotny et al. (2022) that broiler chickens are not efficiently absorbing Ins. A study by Beeson (2017), found that Ins in the excreta was increased by birds fed 3% Ins into diets. This suggests that all the available free Ins is not absorbed.

The $IP_{3\text{-}6}$ esters seen in Table 5.7, demonstrated that there is a reduction of phytate (IP_6) and the increased presence of Ins in the digesta when diets are supplemented with phytase. This is consistent with the findings by (Walk et al., 2014; Zeller et al., 2015[a]; Zeller et al., 2015[b]). A super dosage of phytase at 4500 FTU/kg was able to significantly

increase Ins the jejunum digesta compared to the control (P<0.05) which demonstrates that phytase on its own can manipulate Ins in broiler chickens.

5.4.3 Gene expression (qPCR)

There has been limited studies carried out on the expression of genes associated with Ins in broiler chickens.

5.4.3.1 HK1

Relative expression of the gene HK1 was not influenced by treatment diet in jejunum tissue (P=0.870) but was influenced in kidney tissue (P<0.001). HK1 is involved in the first step of *de novo* synthesis of Ins, whereby D-glucose is phosphorylated into D-glucose 6-phosphate which is ATP-dependent. This enzymatic reaction is also the first step in glycolysis and other glucose metabolism pathways (Litwack, 2022). HK1 can also phosphorylate several other hexoses including D-glucosamine, D-fructose, D-mannose and 2-deoxy-D-glucose (Magnani et al., 1992; Wang et al., 2014; Wolf et al., 2016). HK1 is expressed in most tissues, however higher levels of expression can be found in brain, kidney, and heart (Seki et al., 2005[a]). This gene encodes a ubiquitous form of hexokinase which localises to the outer membrane of mitochondria and play an important role in maintaining the integrity and preventing apoptosis of the cell (Nakamura et al., 2008). A study by Seki et al. (2005[a]) found that chicken HK1 was 87.4 % the same as human HK1 by comparing full-length cDNA sequence and deduced amino acid sequences. Chickens typically have a blood glucose level that is twice as high than in most mammals (Hazelwood and Lorenz, 1959). There is limited information of HK1 in chickens as most research focuses on human biology and disease. Seki et al., (2005[a]) also observed if birds were fed normally (ie no feed withdrawal) mRNA expression in skeletal muscle was lower than in birds which were fasted for 48 hours for HK1 and 2. Birds which were then re-fed, mRNA expression was lowered compared to the fasting birds but still higher than the normally fed birds. The paper concludes that the regulatory mechanisms of chicken skeletal muscles are different to mammals and HKI and 2 have a unique role in the glucose homeostasis. Interestingly, an early study looking at gluconeogenic, glycolytic, and pentose shunt enzymes in chicken kidney tissue by Shen and Mistry (1978) looked at activities of hexokinase. The study measured activities of hexokinase pre and post hatch and found that 4 days pre hatch activity was around 0.25 µM/min/g of kidney tissue, post hatch the activity had increased to around 0.5 on day 5. Activity then decreased until day 30 where it then levelled off until day 45. In acute hypoglycaemia of chickens induced with tolbutamide, caused plasma glucose concentration to fall from 8.6 mM to 3.7 mM. mRNA expression of HK1 after 4 hours was significantly increased (P<0.05) compared to the control. This suggests that HK1 regulates in the acute hypoglycaemic state only in chickens and not in persistent hypoglycaemia, induced by sequential administration of

tolbutamide as expression of HK1 was unchanged (Seki et al., 2005[b]). In Figure 5.2, there was an increased level of expression of HK1 for the Ins and glucose treatment groups compared to the control in the kidney. This may be due to increased need to regulate glucose and sugars in the kidney to maintain glucose homeostasis, as HK1 is required in the first step of glycolysis. This also may explain why no difference in expression HK1 was found in the jejunum tissue as its role is not to regulate glucose in the bird. Furthermore, expression of HK1 is higher in kidney than HK2 this is because it is typically expressed in a greater amount in the kidney than HK2, this was also observed by Seki et al. (2005[a]).

5.4.3.2 HK2

HK2 relative mRNA expression was affected by treatment diet in the kidney (P=0.02) and in jejunum tissue (P=0.042). Like HK1, HK2 also plays a key role in maintaining the integrity of the outer mitochondrial membrane by preventing the release of apoptogenic molecules from the intermembrane space and subsequent apoptosis (Nakamura et al., 2008). A study by Seki et al. (2005[a]) found that chicken HK2 was 85.9 % the same as human HK2 by comparing full-length cDNA sequence and deduced amino acid sequences. In addition, the study observed that chicken HK2 mRNA was highly expressed in skeletal muscle and heart, and lower expression was seen in the liver, brain and kidney which is also seen in humans. This suggests that HK2 is more regulated as is expressed in fewer tissues than HK2. Furthermore, the study also tracked mRNA levels of HK2 in birds that were fed, starved for 48 hours and birds which were re-fed after starvation. Starvation for 48 hours or refeeding for 24 hours significantly increased expression compared to normal feeding (P<0.05). Like HK1, HK2 is also thought to have a unique role in the glucose homeostasis. In persistent hypoglycaemia and acute hypoglycaemia induced by tolbutamide HK2 mRNA levels remained unchanged in chickens (Seki et al., 2005[b]). HK2 can also be simulated by an injection of insulin in 3-week-old chickens, HK2 mRNA expression were significantly increased (P<0.01) at 3 h after insulin injection in skeletal muscle but HK1 expression was not significantly affected. In Figures 1 and 2 HK2 was affected by treatment diets but there is very limited information to explain the results in the kidney and jejunum (Seki et al., 2005[b]). A study by Xu et al. (2017), assessed effects of selenium on cadmium-induced apoptosis in chicken spleens, they found that mRNA expression of HK2 was lower in birds injected with a lethal dose of cadmium than the control and selenium birds. The study concluded that cadmium treatment promoted a mitochondrial dynamic imbalance and reduced energy metabolism. Further work in humans have concluded that HK2 can facilitates autophagy in response to glucose deprivation to protect cardiomyocytes. HK2 can function as a molecular switch from glycolysis to autophagy to ensure cellular energy homeostasis under starvation conditions. In this study the birds were not starved or re-fed after starvation, but 4.5 g/kg

glucose diet reduced feed intake compared to the other diets but had the highest expression level in the kidney. It may be possible to speculate that lower feed intakes caused the higher level of expression due to the birds not eating the same as the other diets. As HK2 is involved in many pathways it is hard to understand the role it plays in chickens as there is very little information available. Furthermore, a grater copy number does not necessarily result in a greater enzymatic activity of HK2 (Zhao et al., 2011).

5.4.3.3 ISYNA1

ISYNA1 gene encodes for 1L-*myo*-inositol-1-phosphate synthase enzyme. The enzyme catalyses the conversion of glucose 6-phosphate to 1L-*myo*-inositol-1-phosphate which is the rate limiting step in the synthases of Ins and requires nicotinamide adenine dinucleotide (NAD+) as a co-enzyme (Croze and Soulage, 2013). Experimental diets affected the expression of ISYNA1 in the jejunum tissue of broiler chickens at 21 days of age (P=0.006) but did not affect the expression in the kidney (P=0.917). Birds fed the control diet caused the expression of ISYNA1 gene in jejunum tissue to increase compared to the other diets. It is possible to suggest that less *de novo* synthesis is required with diets directly supplemented with Ins or phytase as there is an increase in the concentrations of Ins in the jejunum digesta and tissue. As the cells in the jejunum tissue can use the free Ins which is present. Therefore, expression of ISYNA1 was increased as it is the rate limiting step in *de novo* synthesis as more Ins synthesis was required. In humans, ISYNA1 is expressed in different amounts in different organs and tissues. Human testis, ovary, heart, placenta, and pancreas express relatively high level of ISYNA1 and low expression levels can be found in blood leukocyte, thymus, skeletal muscle, brain, kidney, lung and colon (Chauvin and Griswold, 2004; Guan et al., 2003). In this study expression levels of ISYNA1 in the kidney were typically lower than IMPA, MIOX and SLC5A3, and in the jejunum ISYNA1 expression was the lowest compared to the other genes of interest. In mammals, the mammalian ISYNA1 transcription is not controlled by extracellular inositol levels in cell culture using HepG2 cells (Guan et al., 2003). However, the study found that mammalian ISYNA1 mRNA levels increased 2 to 4 fold in the presence of glucose (Guan et al., 2003). In this study the glucose treatment diet of 4.5 g/kg caused the reduction of ISYNA1 mRNA levels in the jejunum compared to the control diet. However, glucose regulation is different in chickens compared to humans which may explain the results seen here. Furthermore, ISYNA1 expression is also controlled by the p53 oncogene (also known as a tumour suppressor) which plays an important role in DNA repair and apoptosis (Su et al., 2022). Overall, there is limited information available about the regulation of ISYNA1 in chickens as it is not a well-studied species. However, a recent study by Greene et al. (2020) used 28-day old male broiler chickens to assess the response of concentrations of Ins and expression of ISYNA by real

time qPCR in feathers and blood plasma when birds were fed a commercially available phytase for 30 hours. Blood plasma and feather Ins levels were significantly increased by the supplemented phytase at 6 to 8 hours post introduction. ISYNA expression in whole blood were significantly down regulated at all periods compared to the baseline levels and no significant change in expression was observed in the feathers.

5.4.3.4 IMPA1

The gene IMPA encodes the enzyme inositol-1-monophosphatase, which dephosphorylates *myo*-inositol monophosphate (IP_1) to generate free Ins and in chickens it is located on chromosome 2. The free Ins can then be used by the cell in its free form or as a precursor of phosphatidylinositol and other inositol containing compounds (Croze and Soulage, 2013). In mammal cells there are two known inositol monophosphatase homologs, IMPA1 and IMPA2. In human health IMPA is best known for its involvement with bipolar disorder. Lithium is commonly used to treat the condition and works by blocking the IMPA1 activity which then increases the IP_1 level and decreases the ratio of Ins to IP_1 in human cells (Harwood, 2005). Inositol monophosphates are sensitive to lithium and can inhibited in at least two ways (Lopez et al., 1999; Sherman et al., 1985). Firstly, Inositol monophosphates relays on magnesium as a co-factor but lithium is a non-competitive inhibitor of the enzyme in vitro (Hallcher and Sherman, 1980). Secondly, in yeast and human cells lithium also suppresses IMPA gene expression (Murray and Greenberg, 2000; Seelan et al., 2004). The depletion of Ins for phosphatidylinositol synthesis may explain the anti-manic and anti-depressive effects of lithium administered to treat bipolar disorder (Seelan et al., 2004). There are no studies to date assessing the role lithium plays in Ins production in chickens. Furthermore, in yeasts expression of IMPA is activated by Ins but repressed by alternative carbon sources, but these observations have not been discovered in higher eukaryotes (Murray and Greenberg, 2000). In this study there was no significant difference in the expression levels of IMPA in the jejunum tissue despite there being an increased concentration of Ins in the tissue and digesta. It is possible to suggest that chicken jejunum cells do not behave like the yeast cells described by Murray and Greenberg, 2000 as there was no significant change in expression levels. In the kidney cells IMPA expression was affected by diet, with the control diet typically having much higher expression than the treatment groups. It is possible to speculate that as there was increased free Ins in the kidney tissue from dietary sources less expression of IMPA was required as the dietary sources were able to meet the requirements for Ins, however further work is required to explore this hypothesis. The only study to date looking at the activity of IMPA in chickens is Gonzalez-Uarquin et al. (2020[b]) who assessed the activity of inositol monophosphates in the livers of chickens fed one of four diets; the

control birds were fed a basal diet adequate in Ca and P, the negative control birds were fed diets with reduced Ca and P, the negative control diet was supplemented with two dosages of phytase at 1500 and 3000 FTU/kg to make up the other two diets. The livers were sampled at day 22 and activity measured, there was no significant difference in the activity of inositol monophosphates (P=0.34) with activity ranging from 1.20 to 1.36 pg/mg of total protein. In addition, they also measured the Ins concentration in the liver but diets did not significantly affect the concentration (P=0.38). As there was no difference in the Ins concentration in the liver the diets may not have generated enough free Ins to manipulate the expression and therefore activity of inositol monophosphates in this study, which may account for the changes in expression seen in the kidney tissue (Figure 5.2).

5.4.3.5 MIOX

In mammalian cells, the only pathway for Ins catabolism involves *myo*-inositol oxygenase which catalyses dioxygen-dependent cleavage between C1 and C6 of the inositol ring to form D-glucuronate. It is then transported into the liver where it is metabolised into D-xylulose 5-phosphate, and subsequently enters the glucuronate-xylulose pathway (pentose phosphate pathway) (Thorsell et al., 2008). In figure 5.2, MIOX expressed most in the kidney compared to the other genes which is logical when the kidney is the site of catabolism. In addition, in humans MIOX is expressed the most in the kidney, when compared variety of different tissue and organ types (Fagerberg et al., 2014). Ins catabolism is required to maintain Ins homeostasis, and poor kidney function associated with increased Ins in blood (Holub, 1986). In human medicine, MIOX maybe a target for future cure for diabetic complications as inhibiting *myo*-inositol oxygenase could reverse complications caused by inositol depletion (Thorsell et al., 2008). In this study expression of MIOX was not affected by treatment diet in the kidney (P=0.457) or jejunum (P=0.154). There is very little information of MIOX in chickens despite the increased research in Ins and Inositol phosphate chemistry and nutrition. A study by Gonzalez-Uarquin et al. (2020[b]) carried out a study in chickens assessing *myo*-inositol oxygenase activity in the kidney. The study (as described above) found no significant differences in kidney *myo*-inositol oxygenase activity (P=0.90), with values ranging from 1.17 to 1.39 pg/mg of total protein. However, kidney Ins concentrations was increased by the lower dosage of phytase at 1500 FTU/kg compared to the negative control. Phytase supplementation can change the Ins concentration in kidney tissue but the increased concentration does not mean increased activity of *myo*-inositol oxygenase which is key to maintaining Ins homeostasis in this study. In figures 5.2 and 5.3 there was no significant difference in expression levels which was surprising when high levels of Ins at 13.5 g/kg was added and there were significant increases in Ins found in the kidney and jejunum tissues. More research is necessary to understand and elucidate the underlying causes and mechanisms. To better

understand the Ins homeostasis in chickens, measurements of the metabolite D-xylulose 5-phosphate would be required, to see if concentrations increase due to increased levels of Ins present in the kidney, which would demonstrate Ins regulation.

5.4.3.6 SLC2A13 (HMIT)

HMIT is encoded by the gene *SLC2A13* and part of the Major Facilitator superfamily. The transporter relies on active transport to generate a concentration gradient by using H^+(Fu *et al.*, 2012). In humans it is found in most tissue types, with highest levels of expression found in the brain, medium expression found in the small intestine and kidney and low levels of expression found in the liver, heart and testis (Fagerberg et al., 2014). A study by Ajuwon et al. (2019) assessed the expression of the Ins transporter proteins in the duodenal-jejunal bush border and the distal ileum bush border of broiler chickens at 22 days of age. Birds were fed one of 4 diets: positive control with adequate in Ca and P, negative control with reduced Ca and P, negative control with 1500 FTU/kg and negative control with 3000 FTU/kg. The expression of HMIT was not affected by the treatment diets in either the duodenal-jejunal bush border (P=0.24) or distal ileum bush border (P=0.88). Despite the Ins concentration in blood plasma being increased by 1500 FTU/kg compared to the other diets (P<0.001). In this study there was no differences seen in the expression of HMIT in the kidney (P=0.789) or jejunum tissue (P=0.206). These results agree with the findings of Ajuwon et al. (2019), whereby the addition of phytase did not significantly change the expression of HMIT transporter. In addition, a study by Walk et al. (2018) examined the expression of SLC2A13 in liver, kidney, jejunum and ileum in 21-day old broiler chickens. Birds were fed a control diet, which was formulated to contain a 0.16% reduction in Ca and 0.15% reduction in available P. The control diet was then supplemented with 0, 500, 1500 and 4500 FTU/kg to create four experimental diets. Expression of SLC2A13 in the jejunum was not significantly affected by diet (P=0.09), however there was a tendency with highest expression found in birds fed on 1500 FTU/kg and lowest expression found in birds fed 500 FTU/kg. There was no linear (P=0.314) or quadratic response (0.112) with increasing phytase dosage. These findings are also in agreement with this study and Ajuwon et al. (2019). In addition, Walk et al. (2018) also measured the gene expression of HMIT in the ileum and observed a tendency (P=0.055) with highest expression in the highest phytase dosage of 4500 FTU/kg and with a linear response (P=0.07) and no quadratic response (P=0.965). These studies all demonstrate that HMIT is present in the GIT of broiler chickens which is also observed in the human model but is not manipulated by Ins (generated by phytase or free supplemented Ins). Expression of HMIT in the kidney was not affected by phytase dosage (P=0.174), however a negative linear response (P=0.044) was observed with increasing phytase dosage and no quadratic response (P=0.565) (Walk et al., 2018). Walk et al. (2018) also assessed

liver HMIT expression and found no significant difference (P=0.379), no linear response (P=0.481) or quadratic (P=0.443). In figure 5.3, the expression of SLC2A13 was not affected by treatment diet (P=0.789), which agrees with Walk et al. (2018). The role of HMIT plays in Ins homeostasis is poorly understood in the chicken model but also in humans and remains a controversial topic.

5.4.3.7 SLC5A11 (SMIT2)

The respective gene for SMIT2 is SLC5A11 and belongs to the Solute Carrier Family 5 (SLC5A). SMIT2 requires two sodium ions to transport one molecule of Ins across the plasma membrane and on an amino acid level shows 43 % sequence identity to SMIT1 (Schneider, 2015). SMIT2 is expressed in the kidney, placenta, heart, skeletal muscle and liver but is poorly expressed in the brain (Roll et al., 2002; Berry et al., 1995). Walk et al. (2018), (as previously described above) assessed SMIT2 expression in the GIT of 21-day old broiler chickens. In the jejunum expression was affected by diet (P=0.002) and had a positive linear (P=0.003) and quadratic response (P=0.017) with increasing levels of phytase. However, Ajuwon et al. (2019) did not observe any significant changes in expression in the duodenal-jejunal bush border when birds were also fed different dosages of phytase (P=0.40). In this study (figure 5.3), there was a significant effect of Ins or phytase supplementation manipulating expression of SMIT2 (P=0.003). Which disagrees with the findings of Ajuwon et al., (2019) but is consistent with the findings of Walk et al., (2018). These studies all show that SMIT2 is expressed in the GIT of broiler chickens which suggests that SMIT2 could be involved in the uptake of free Ins out of the digesta and into the blood plasma of chickens. Ins in the jejunum tissue was increased by phytase and Ins supplementation compared to the control and glucose diets. Both Walk et al. (2018) and Ajuwon et al. (2019) also measured expression of SMIT2 in the ileum with no significant differences reported due to phytase dosage (P>0.05). The stereoisomer of Ins, D-*Chiro*-inositol can compete with Ins for SMIT2 mediated transport and can be used to identify which SMIT is active *in vivo* (Schneider, 2015; Croze and Soulage, 2013). An early study by Lerner and Smagula (1979), investigated Ins transporter systems in the small intestines of chickens. They discovered that Ins transport could be inhibited by phlorizin, sulfhydryl reagents, removal of sodium and high levels of glucose. Furthermore, the addition of 16.6 mM of glucose (a concentration typically used to simulate the intestinal milieu) could suppress the transport of Ins concentrations ranging from 1-20 mM. This study suggests that poultry diets high in sugars could compete with the Ins transporter proteins due to glucose having a higher affinity to the transporter proteins (Lee and Bedford, 2016). However, this study assessed the effects of 4.5 g/kg glucose in diets and found no difference in the concentration of Ins in jejunum and kidney tissue, blood plasma or jejunum digesta compared to the control diet. Further work would be required to

assess the interaction glucose and Ins uptake has on the subsequent concentration of Ins in tissues and digestas. Expression of SMIT2 in the kidney of broiler chickens in this study was affected (P<0.001), with 4500 FTU/kg diet increased expression compared to the other diets. Walk et al. (2018) also did not observe a change in SMIT2 expression due to phytase supplementation (P=0.410), with no linear (P=0.17) or quadratic responses (P=0.921). In addition, no significant differences were observed in the liver (P=0.247), with no linear response (P=0.846) and a tendency towards a quadratic response (P=0.069). The study agrees with the with Walk et al. (2018) that SMIT2 can be found in the kidney of broiler chickens.

5.4.3.8 SLC5A3 (SMIT1)

Relative mRNA expression of SLC5A3 encoding for the Ins transporter SMIT1 in the kidney was not affected by treatment but showed a tendency (P=0.062) with the phytase treatment group of 4500 FTU/kg reducing expression compared to the other diets. There is limited information on the expression of SMIT1 in the kidney of broiler chickens. Walk et al. (2018) did not observe any differences in the reported values for expression of SMIT1 in the kidney of 21-day old broiler chickens (P=0.234). Birds were fed diets supplemented with 0, 500, 1500 and 4500 FTU/kg. There was also no linear (P=0.390) or quadratic (P=0.516) response seen in the graded levels of phytase fed to the birds for expression of SMIT1. In addition, no significant affect or response was seen in the liver (P>0.05). This study agrees with the results of this chapter, that SMIT1 is not affected by Ins (either liberated from phytate or as a purified supplement) despite increases of Ins seen in the kidney tissue and blood plasma.

In the jejunum tissue expression of SMIT1 was significantly affected by the different treatment diets (P=0.006), with the control diet causing greater expression compared to the other diets in jejunum tissue, meaning that increasing Ins in the jejunum digesta potentially caused downregulation of SMIT. A study by in pigs by Lu et al. (2020) assessed the expression of SMIT1 in the duodenal loop, jejunum and ileum. Pigs were fed four different diets: PC with adequate Ca and P, NC with reduced Ca and P, NC+1500 FTU/kg or NC+3000 FTU/kg for 28 days from weaning. There was no significant effect on the relative expression of SMIT1 in the duodenum (P=0.81), jejunum (P=0.23) and ileum (P=0.26). In broiler chickens both Walk et al. (2018) and Ajuwon et al. (2019) did not see any significant change in expression in the GIT (P>0.05). It is important to note that active transport is required to transport Ins across plasma membranes and into cells, so despite the increases in Ins concentrations in tissues and plasma expression levels were not influenced by this additional Ins transported initially from the GIT and distributed across different tissues via the blood plasma.

5.4.3.9 SLC5A1 (SGLT1)

Glucose is absorbed by enterocytes lining the microvilli of the small intestine, by sodium dependent glucose cotransporters (SGLT) mediating the uptake of glucose and galactose (Byers et al., 2017). Expression of SLC5A1 also known as sodium glucose transporter one (SGLT1) was not significantly changed by treatment diet in the kidney (P=0.105) or jejunum (P=0.201) of the chickens used in this study. A study by Li et al. (2008) profiled the expression of 162 SLC genes belonging to 41 SLC families, post and pre hatch in chickens. The study found that expression was low during embryogenesis but rose continuously to day 14 when the study ended. In addition, SLC5A1 was expressed in all three segments of the GIT, with greater expression seen in the jejunum than the duodenum or ileum. In this chapter we can confirm with this study that SLC5A1 was also found and expressed in the jejunum. It was proposed by Lee and Bedford, (2016) that due to high sugars in poultry diets, Ins uptake could be reduced because of a higher affinity to glucose by Ins transporters. This chapter assessed the effects of 4.5 g/kg glucose in diets and found no difference in expression of SLC5A1. This suggests that SLC5A1 was not affected by the addition of glucose to the diet and expression was not altered despite the increase in available glucose in the diet. Further work is required to understand what role Ins has on glucose regulation and uptake in chickens.

5.5 Conclusions

In conclusion, the addition of phytase at the dosage of 4500 FTU/kg and 4.5 g/kg improved FCR compared to the control diet (P=0.003) if fed from 7-21 days of age. The highest dosage of Ins used in this chapter at 13.5 g/kg was comparable to the control diet for FCR, suggesting that high levels of Ins do not have any negative affect on performance of growing chickens. The energy utilisation of the growing broiler chickens was affected by diet for AME, DMR and NR, however further work is required to understand what effects Ins has on the energy metabolism of chickens. The relative mRNA expression of the genes associated with Ins uptake and regulation showed that the genes of interest were expressed in jejunum and kidney tissue. This is the first study to date, to assess the genes associated with the synthesis, transport and catabolism of Ins in broiler chickens. Overall the expression of the genes of interest did not follow recognisable patterns and warrants further investigation of whether increasing Ins causes the up or down regulation of the genes of interest.

5.6 Outcomes

1. FCR of the growing broilers from 7-21 days of age was significantly improved by phytase at the dosage of 4500 FTU/kg and 4.5 g/kg compared to the control diet.

The highest dosage of Ins used in this chapter at 13.5 g/kg was comparable to the control diet.

2. The energy utilisation of the growing broiler chickens was affected by diet for AME, AME:GE, DMR and NR but not AME_n or AME_n:GE.
3. The addition of phytase into diets reduced phytate content by 92 % compared to the other diets and reduced IP_5 content 83 % compared to the other diets in jejunum digesta. The concentration of IP_3 and IP_4 was increased by phytase in jejunum digesta.
4. Increasing available Ins in diets by direct Ins supplementation (4.5 g/kg and 13.5 g/kg) increased Ins in in the blood plasma, kidney tissue, jejunum tissue and jejunum tissue ($P<0.001$) when compared to the control diet. However, the addition of phytase at 4500 FTU/kg did not significantly increase blood plasma, kidney tissue or jejunum tissue when compared to the control diet ($P>0.05$).
5. The relative expression of genes associated with Ins catabolism, synthesis and transport in kidney tissue was affected by diet for HK1, HK2, IMPA and SMIT2, but not ISYNA1, MIOX, HMIT, SLC5A1 and SMIT1.
6. The relative expression of genes associated with Ins catabolism, synthesis and transport in jejunum tissue was affected by diet for HK2, ISYNA1, SMIT1 and SMIT2 but not and HK1, IMPA, MIOX, HMIT and SLC5A1.

Chapter Six: The interaction between phytase generated *myo*-inositol (Ins) and supplemented free Ins on bird performance and health.

6.0 Introduction

Dietary phytase supplementation is routine in the poultry industry to prevent the anti-nutritional effects of phytate that originates naturally in plant-based feeds. Free *myo*-inositol and inositol phosphates are subsequently generated in the gastrointestinal tract of the bird, which was observed in the previous two chapters. Studies by Pirgozliev et al. (2007), Augspurger and Baker (2004) and Simons et al. (1990) have shown an improvement with growth performance by increasing levels of phytase supplementation above the conventional levels. Although these reports clearly state the benefits of super-dosing there is no clear understanding on the underpinning mechanisms involved. As a result, it is thought that Ins may aid performance. A dosage of 4.5 g/kg Ins in the previous chapter was able to significantly improve the FCR between 7 and 21 days of age. In addition to this the first experiment showed that 30 g/kg of Ins reduced performance suggesting a very high dosage has a negative impact on performance. Furthermore, 1.5 and 3 g/kg Ins was not significantly different to phytase supplemented diets, suggesting that Ins may play an important role in improved growth. This suggests that a dosage of around 3 to 4.5 g/kg maybe optimum and warrants further investigation. A dosage of 3.8 g/kg improved FCR compared to the control (Sommerfeld et al., 2018). Based on these results it is possible to hypothesis that a dosage of 3.5 g/kg maybe be beneficial to a growing broiler chicken. For phytase to work in the GIT of a chicken, phytate (the substrate) must be present in sufficient quantities. Therefore, if there was an increase of phytate into diets, it is assumed that there would be an increase in Ins and lower IPs. Therefore, the following study was formulated to contain 8% sunflower meal which is known to have a high phytate content. It has been suggested by Lee and Bedford (2016) that Ins may account for up to a third of the performance gain when feeding super dosages of phytases, therefore if we can generate more Ins in the GIT of a chicken by feeding bird diets with higher phytate content, improved performance may also be observed. In addition, there are limited studies assessing the performance of Ins supplementation past 21 days of age. The following study will observe the performance of broilers from 0-35 days which will be fed in two phases 0-21 and 22-35 days.

Bird health combines many aspects of poultry production. Antioxidants are a group of molecules that inhibit or stop free radical reactions, preventing or delaying cellular damage. Some compounds can act as *in vivo* antioxidants increasing the level of endogenous antioxidant defences including increasing the expression of enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Nimse and Pal, 2015). More recently, in aquaculture research, the link between

antioxidant properties of dietary Ins supplementation has been explored (Jiang, J. et al., 2010; Jiang, W. et al., 2009). There are currently no studies assessing the potential role Ins has at improving the antioxidant status of chickens which could potentially understand the importance of Ins as a bioactive compound in poultry.

The development of the GIT and organs are very important in the development of broiler chickens. Based on the previous chapter assessing the relative expression of genes associated with Ins synthesis, catabolism, and uptake, which found that expression of some of these genes can be altered either by Ins, phytase or glucose supplementation. Therefore, it is important to establish if there are any subsequent physical developmental changes in the GIT and organs of growing broiler chickens. In addition, based on the results seen in the first experimental chapter (chapter 3) the mineral digestibility data showed that the minerals assessed formed complex interactions and did not follow a recognisable pattern except for P, therefore this area warrants further investigation.

Meat quality is of the upmost importance in chicken meat production. Woody breast (WB) is a myopathy that can affect the tactile characteristics of meat, making it firmer upon palpation compared to normal breast fillets and in extreme cases cause a prominent ridgelike bulge on the caudal area of the fillet. WB and other myopathies are associated with the fast-growing rates of modern broiler chicken lines. The exact cause of these myopathies is unknown (undefined), however there is evidence to suggest that WB is caused by necrosis, fibrosis and lipidosis of muscle tissue and connective tissues. A recent study by Green et al. (2019) indicated that WB related to systemic and local breast muscle hypoxia. Dysregulated oxygen homeostasis causes hypoxic conditions, which limits the regenerative capacity of muscle. This causes degenerated muscle fibres to be replaced with lipid and fibrotic tissue causing the change in myopathy. A study by Whitfield et al. (2022), assessed the effects of Ins (liberated by supplementary phytase) on Ins(1,3,4,5,6)P5, (IP_5) production in chicken erythrocytes and the possible benefits to enhance oxygen availability and help alleviate WB myopathy. The study showed a positive correlation between blood Ins and IP_5, suggesting that phytase liberated Ins could improve haemoglobin function as phytase supplementation has been shown to reverse hypoxic responses of blood and tissues in animals showing WB phenotypes (Green et al., 2019). However further work is required to understand the links between Ins, erythrocyte IP_5, haematology and meat quality.

6.1 Objectives

The aim of the study was to quantify the response of 6 treatment diets in a 2 by 3 factorial arrangement. The factors being phosphorus source, standard P (recommend levels) with no phytase supplementation and a reduction of 1.5 g/kg of available P with the addition of

phytase at 4500 FTU/kg. The other three factors being different dosages of Ins at 0 g/kg, 3.5 g/kg and 7 g/kg. The diets were fed in two phases 0-21 days and 22-35 days. With the measurable outcomes being assessed were growth performance (0-21, 22-35 and 0-35), energy utilisation (day 21 and day 35), GIT and organ development (day 21 and day 35), haematology (day 21 and day 35), antioxidant status (day 21 and day 35), ileal inositol phosphate esters (IP_{2-6}) and Ins concentrations (day 21 and day 35), plasma Ins concentrations (day 21 and day 35) and plasma mineral concentrations (day 21 and day 35).

Hypotheses:

1. Changing available Ins or P source in diets will significantly affect growth performance of broiler chickens from 0-35 days (0-21, 22-35 and 0-35) of age and there will be no interaction between Ins and P source.
2. Changing available Ins or P source in diets will not significantly affect AME, AME_n, AME:GE, AME_n:GE, NR or DMR (measured at 19-21 days and 33-35 days) and there will be no interaction between Ins and P source.
3. Supplementing diets with phytase will alter the IP_{2-6} ileum ester profiles but supplementing diets with Ins will not alter the IP_{2-6} ileum ester profiles and there will be no interaction between Ins and P source at either day 21 or day 35.
4. Increasing available Ins in diets (either from the enzymatic action of supplemented phytase or direct supplementation of free Ins) will significantly increase the plasma and ileum Ins concentrations either on day 21 or day 35 and there will be no interaction between Ins and P source.
5. Changing available Ins or P source in diets will not significantly influence the plasma mineral concentrations and there will be no interaction between Ins and P source on either day 21 or day 35.
6. Changing available Ins or P source in diets will significantly influence the antioxidant status and there will be no interaction between Ins and P source on either day 21 or day 35.
7. Changing available Ins or P source in diets will not significantly influence the haematology profile of the broiler chickens either on day 21 or day 35 and there will be no interaction between Ins and P source.
8. Changing available Ins or P source in diets will not significantly influence the development of the GIT and organs either on day 21 or day 35 and there will be no interaction between Ins and P source.

6.2 Materials and methods

The experiment was conducted at the National Institute of Poultry Husbandry (Newport, Shropshire, UK) and in accordance with the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020). The study reported here, was approved by Harper Adams University Research Ethics Committee (code: 0283-202002-PGMPHD-CO2) before the initiation of the experiment.

For general Materials and Methods relating to this section please see Chapter three.

6.2.1 Animals

A total of 400 Ross 308 male day-old chicks were obtained from a local hatchery (Cyril Bason Ltd, Craven Arms, UK). Upon arrival, the chicks were checked, and any small, weak or deformed chicks were humanely killed. Three hundred and sixty chicks were then randomly allocated into sixty raised floor pens (with each pen holding six chicks). Chicks were replaced during the first 7 days if withdrawn or dead. Animal well-being was checked twice daily, and records used in the assessment of the humane endpoint (any suffering above the commercial baseline). Feed and water were offered *ab libitum* throughout the study. Growth performance was determined from 0- 21, 22- 35 and 0- 35 days of age on pen basis.

6.2.2 Sample collection

On day 19 of the study, the solid floor of the pens was removed and replaced with a wire mesh to allow for excreta collection and the solid floor was reinstated on day 21. The excreta samples were then dried at 60 ˚C until a constant weight and then milled. On day 21, one bird per pen was randomly selected and killed by electrical stunning followed by exsanguination. At this time blood was collected into lithium heparin BD Vacutainers ®. The bird was then dissected to remove the different sections of the GIT and organs, sections of GIT were emptied by careful palpitation and the ileum digesta collected. The GIT sections and organs were then weighed and expressed as a percentage of the body weight of the bird. The remaining birds were then transferred to the next feeding phase. On day 33 of the study, the solid floor of the pens was removed and replaced with a wire mesh to allow for excreta collection and the same sample collection was repeated for day 35 (the end of the study).

6.2.3 Treatment diets

Diets were fed in a 2 by 3 factorial designed experiment, whereby P availability, either from formulated recommended P or phytase supplementation with 1.5 g/kg less formulated P will be compared with three inclusion levels of Ins at 0 g/kg, 3.5 g/kg and 7 g/kg in two phases (Table 6.1).

Table 6.1: Diet design for a 2 by 3 factorial experiment assessing *myo*-inositol (Ins) supplementation, fed in two feeding phases (0-21 and 22-35 days).

0-21 days Feeding phase one	0 g/kg Ins	3.5 g/kg Ins	7 g/kg Ins	22-35 days Feeding phase two	0 g/kg Ins	3.5 g/kg Ins	7 g/kg Ins
Phytase (4500 FTU/kg) 1.5 g/kg less available P	Diet 1	Diet 2	Diet 3	Phytase (4500 FTU/kg) 1.5 g/kg less available P	Diet 7	Diet 8	Diet 9
No phytase Recommended available P	Diet 4	Diet 5	Diet 6	No phytase Recommended available P	Diet 10	Diet 11	Diet 12

Diets were produced at Target Feeds Ltd (Whitchurch, Shropshire, UK), with the addition of a digestibility marker of acid insoluble ash (AIA) at 20 g per kg of feed (Diatomaceous Earth, MULTI MITE, Wiltshire, UK). Four maize-soya basal diets were designed to contain 8% sunflower meal, to increase the phytate levels in the diet. Two of the four basal diets were designed to meet the recommended available P for each of the feeding phases and the remaining two basal diets to contain 1.5 g/kg less available P for each of the feeding phases by the removal of inorganic phosphorus (dicalcium phosphate) (for formulations please see Table 6.2). Diets containing less available P were supplemented with phytase (Quantum™ Blue P40, AB Vista, UK) at the inclusion rate of 4,500 FTU/kg. The four basal diets will then be supplemented with Ins (98± % *myo*-inositol, Carbosynth Ltd) at 0, 3.5 and 7 g/kg, which will create 12 diets in total (Table 6.1).

Table 6.2: Ingredient composition of the four basal diets (g/kg).

Ingredient	0-21 days % (diet 4)	0-21 days % (diet 1)	22-35 days % (diet 10)	22-35 days % (diet 7)
Mazie	51.055	51.355	53.7750	54.0750
Soya (48% CP)	30	30	26	26
Sunflower meal (27% CP)	8	8	8	8
Soya oil	5	5	6.5	6.5
Salt	0.3	0.3	0.3	0.3
DL Methionine	0.37	0.37	0.39	0.39
Lysine HCl	0.3	0.3	0.16	0.16
Limestone	0.7	1.25	0.7	1.23
Dicalcium Phosphate	1.8	0.95	1.7	0.87
Sodium bicarbonate	0.075	0.075	0.075	0.075
Vitamin Mineral premix	0.4	0.4	0.4	0.4
Diatomaceous Earth (AIA)	2	2	2	2
Calculated values				
DM %	90	90	90	90
Crude protein (N x 6.25), g/kg	206.732	20.6942	18.8225	18.8435
Metabolisable Energy, MJ/kg	12.6857	12.7271	13.1687	13.2101
Calcium (Ca), g/kg	9.504	9.525	9.165	9.158
Av Phosphorus (P), g/kg	4.771	3.244	4.539	3.049
Lysine	1.3674	1.3681	1.1377	1.1384
M+ Cysteine	1.0450	1.0459	1.0169	1.0178
Determined values				
Dry Matter %	90.3	90.5	89.9	90.0
GE, MJ/kg	16.292	16.7835	16.9325	16.7385
Crude protein (N x 6.25), g/kg	215	213	195	204
Total Ca, g/kg	9.504	9.525	9.165	9.158
Total P, g/kg	7.196	5.674	6.844	5.358

6.2.4 Chemical analysis

Gross energy was determined on both sets of excreta collected at the end of each feeding phase. Ileal digesta was freeze dried and Ins and IP_{2-6} determined. The haematological profile of erythrocytes was carried out on fresh blood within 6 hours of collection. Ins in blood plasma and plasma minerals (Ca, P, Na, Cu, Mo, Mn, Fe, Mg and Co) was determined using HPLC and ICP-MS. The antioxidant status was determined in blood by measuring SOD, GSH-Px and TAS.

6.2.5 Statistical analysis

Data was statistically analysed using analysis of variance (ANOVA), without blocking, in GenStat® (22nd edition), with a protected Fisher's least significant difference test to separate means. Differences were reported as significant at $P<0.05$ and tendencies

considered at P>0.05 - P<0.01. Each pen is considered was an experimental unit in this study.

6.3 Results

6.3.1 Analysis of feed.

Analysed phytase activity confirmed that it was close to the formulated activity of 4500 FTU/kg with the recovered activity of 5210 FTU/kg for the first feeding phase (0-21 days) and 5130 FTU/kg for the second feeding phase (22-35 days). Ins recovery was slightly lower than expected. Additionally, phytate-phosphorus and IP_{3-6} was also consistent across basal diets which was expected.

Table 6.3: Recovered phytase activity, Ins recovery and IP_{2-6} profiles of experimental diets.

Treatment Diet	phytase activity FTU/kg[1]	Ins recovery (g/kg)	IP_3 (nmol/g)	IP_4 (nmol/g)	IP_5 (nmol/g)	IP_6 (µmol/g)
Phase: 0-21 days						
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	5210	<0.18	49.6	804	2136	20.5
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	5210	2.30	<10	794	1978	19.8
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	5210	5.20	30.2	743	1976	19.5
No Phytase (standard P) + 0 g/kg Ins	<50	<0.18	<10	483	1998	20.1
No Phytase (standard P) + 3.5 g/kg Ins	<50	2.29	29.3	770	1958	19.9
No Phytase (standard P) + 7 g/kg Ins	<50	5.02	29.6	751	1808	19.4
Phase: 22-35 days						
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	5130	<0.18	44.0	720	1729	18.6
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	5130	2.23	56.9	659	1747	19.5
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	5130	4.7	65.4	748	1876	18.6
No Phytase (standard P) + 0 g/kg Ins	<50	<0.18	28.3	605	1721	18.9
No Phytase (standard P) + 3.5 g/kg Ins	<50	1.99	29.9	672	1893	18.3
No Phytase (standard P) + 7 g/kg Ins	<50	5.48	38.5	765	1936	18.8

[1]One FTU is defined as the amount of enzyme required to release 1 mmol of inorganic P per minute from sodium phytate at 37°C and pH 5.5.

6.3.2 Mortality

Birds remained healthy throughout the study. The overall mortality was 4.17%, which is below the 5% tolerance level recommended by the Red Tractor scheme 2018. Mortality rates for individual diets can be seen in Table 6.4.

Table 6.4: Mortality rates expressed as a percentage of individual birds fed on each experimental diet of broiler chickens during study, 0-35 days.

Treatment	Mortality (total number of birds removed, culled or deceased)
Diet	
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	5 % (3)
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	3.3 % (2)
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	1.67 % (1)
No Phytase (standard P) + 0 g/kg Ins	1.67 % (1)
No Phytase (standard P) + 3.5 g/kg Ins	5 % (3)
No Phytase (standard P) + 7 g/kg Ins	8.3 % (5)

Ins, added dietary *myo*-inositol; P, phosphorus; Phytase dosage was at 4500 FTU/kg. There were 60 observations per treatment diet.

6.3.3 Growth performance

To determine the effects P source and Ins had on performance, weekly body weight, ADFI, ADWG and FCR was calculated for each phase and overall (Table 6.5 and 6.6). Chick weights were around 10 % below the breed standard of 44g (Aviagen, 2021), there was no significant difference in chick weight allocation across treatment diets. After seven days, treatment diets and P source had affected body weight ($P<0.001$), with phytase supplemented diets improving body weight when compared to standard P. Ins dosage did not affect body weight at day 7 ($P=0.169$), and there was no interaction between Ins and P source. Chick weights were still below the breed objectives; however, this is likely a carry-over effect from small start weights, however the highest Ins dosage of 7g/kg, further reduced body weight when compared to the objectives.

Average body weight at day 14 was affected by diet ($P<0.001$), P source ($P<0.001$) and Ins dosage ($P<0.01$) with no significant interaction ($P>0.05$). The treatment diet containing phytase and 0 g/kg Ins had the highest average body weight at day 14, with reductions in body weight observed with increasing Ins dosage across phytase containing diets, this trend was also seen with standard P diets supplemented with Ins. When comparing P source, diets supplemented with phytase improved body weight by 60 g compared to standard P. The Ins dosage of 7 g/kg significantly reduced average body weight compared 0 g/kg Ins ($P<0.05$).

At the end of the first feeding phase (day 21), average bird weights were affected by diet ($P<0.001$), P source ($P<0.001$) and Ins dosage ($P<0.001$) with no significant interaction ($P=0.243$). Diets containing phytase and 0 g/kg Ins had met the breed recommendations

for average body weight for day 21. Diets with less available P supplemented with phytase, improved body weight compared to standard P. Diets supplemented with 3.5 and 7 g/kg Ins reduced body weigh compared to diets not supplemented with Ins ($P<0.05$).

On day 28, average body weight was affected by treatment diets ($P<0.001$) with standard P supplemented with 3.5 or 7 g/kg Ins significantly reducing body weight compared to the other diets. Phytase supplemented diets improved body weight compared to standard P ($P<0.001$), increasing Ins dosage reduced average body weight ($P<0.001$). There was no significant interaction between P source but there was a tendency ($P=0.072$).

Final body weights on day 35 were just below the breed performance objectives ranging from 96.6 to 87.2 % of the target weights. Body weights were affected by diet ($P=0.012$) and Ins dosage ($P=0.008$) but was not affected by P source ($P=0.072$). Ins dosage was again reduced by 3.5 and 7 g/kg Ins compared to 0 g/kg Ins, with 7 g/kg reducing final body weight by 174 g compared to no Ins in diets. There was no significant interaction between P source and Ins dosage.

Overall, diets formulated with phytase improved body weight when compared to standard P (day 7, 14, 21, 28 and 35) and diets supplemented with Ins at 3.5 g/kg or 7 g/kg reduced body weights (day 14, 21 and 35). CV% was relatively low and comparable to other studies.

ADFI, ADWG and FCR were all affected by treatment diets and treatment factors (P source and Ins dosage) for the first feeding phase (0-21 days of age) ($P<0.001$), except for P source FCR, whereby a tendency ($P=0.073$) was observed. The addition of phytase to the diet containing 1.5 g/kg less available P still significantly increased ADFI, ADWG and reduced FCR compared to the standard P diet ($P<0.05$). The addition of 7 g/kg Ins reduced ADFI compared to 0 g/kg Ins ($P<0.05$). The dosages containing Ins (3.5 and 7 g/kg) significantly reduced ADWG and increased FCR ($P<0.05$).

Measuring performance for the second feeding period (22-35 days of age), resulted in no significant differences in treatment diets and treatment factors (P source and Ins dosage) for ADFI ($P>0.05$). However, FCR was affected by treatment diets ($P=0.041$) and there was a tendency for treatment diets to affect ADWG ($P=0.054$). No significant differences in P source were observed for FCR or ADWG ($P=0.167$ and $P=0.835$ respectively). Ins dosage did significantly affect FCR and ADWG whereby the addition of Ins (3.5 or 7 g/kg) significantly reduced ADWG and increased FCR ($P<0.05$).

Overall performance (0-35 days), ADFI, ADWG and FCR were all significantly affected by treatment diets and treatment factors (P source and Ins dosage) except for no differences were seen for P source FCR ($P=0.879$). The addition of Ins (3.5 or 7 g/kg) significantly

reduced ADWG and increased FCR ($P<0.05$) compared to 0 g/kg Ins and the addition of 7 g/kg Ins also reduced ADFI ($P<0.05$). In addition, there was no significant interactions ($P>0.05$) between the factors of P source and Ins dosage for all of the performance parameter found in table 6.6.

Table 6.5: Average body weight of male broiler chickens at day 0, 7, 14, 21, 28 and 35, numbers in brackets () represent the percentage of the 2021 Ross 308 male broiler chicken objectives reached (Avaigen, 2021). Observations based on total pen weights.

Treatment	Average weight day 0 (g)	Average weight day 7 (g)	Average weight day 14 (g)	Average weight day 21 (g)	Average weight day 28 (g)	Average weight day 35 (g)
Diet						
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	39.4 (89.5)	185^{d} (91.8)	511^{e} (98.6)	1014^{c} (100.0)	1627^{b} (97.8)	2330^{c} (96.6)
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	40.0 (91.0)	182^{cd} (90.6)	496de (95.8)	959^{b} (94.6)	1597^{b} (96.0)	2281^{bc} (94.6)
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	39.3 (89.2)	173^{bc} (86.3)	475^{cd} (91.8)	961^{b} (94.8)	1627^{b} (97.8)	2190^{abc} (90.8)
No Phytase (standard P) + 0 g/kg Ins	39.5 (89.8)	167^{ab} (83.0)	459^{bc} (88.6)	942^{b} (92.9)	1601^{b} (96.2)	2311^{c} (95.9)
No Phytase (standard P) + 3.5 g/kg Ins	39.4 (89.4)	165^{ab} (81.8)	433^{ab} (83.6)	871^{a} (85.9)	1481^{a} (89.0)	2131^{ab} (88.4)
No Phytase (standard P) + 7 g/kg Ins	39.8 (90.4)	161^{a} (80.3)	410^{a} (79.1)	841^{a} (82.9)	1427^{a} (85.8)	2102^{a} (87.2)
SEM	0.673	3.73	9.34	14.3	34.7	53.5
CV	5.4	6.9	6.4	4.8	7.0	7.6
P-value	0.967	<0.001	<0.001	<0.001	<0.001	0.012
P source						
Phytase (-1.5 g/kg av P)	39.6 (89.9)	180 (89.6)	494 (95.4)	978 (96.4)	1617 (97.2)	2267 (94.0)
No Phytase (standard P)	39.5 (89.9)	164 (81.7)	434 (83.8)	884 (87.2)	1503 (90.3)	2181 (90.5)
SEM	0.378	2.19	6.14	10.3	21.7	33.1
CV	5.2	7.0	7.2	6.0	7.6	8.1
P-value	0.992	<0.001	<0.001	<0.001	<0.001	0.072
Ins dosage						
0 g/kg Ins	39.4 (89.6)	176 (87.4)	485^{b} (93.6)	978^{b} (96.4)	1614 (97.0)	2320^{b} (96.2)
3.5 g/kg Ins	39.7 (90.2)	173 (86.2)	465^{ab} (89.7)	915^{a} (90.2)	1539 (92.5)	2204^{a} (91.5)
7 g/kg Ins	39.5 (89.8)	167 (83.3)	443^{a} (85.5)	901^{a} (88.9)	1527 (91.8)	2146^{a} (89.0)
SEM	0.467	3.15	9.45	14.7	28.4	38.6
CV	5.3	8.2	9.1	7.1	8.1	7.8
P-value	0.932	0.169	0.01	0.001	0.072	0.008
Interaction						
P source*Ins						
SEM	0.673	3.73	9.34	14.3	34.7	53.5
CV	5.4	6.9	6.4	4.8	7.0	7.6
P-value	0.675	0.690	0.736	0.243	0.052	0.478

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

Table 6.6: Growth performance parameters (ADFI, ADWG and FCR) of broiler chickens for first (0-21) and second feeding (22-35) phases and overall (0-35). Observations based on total pen weights and feed intakes.

Treatment	ADFI (g/b/d) 0-21 days	ADWG (g/b/d) 0-21 days	FCR (g/g) 0-21 days	ADFI (g/b/d) 22-35 days	ADWG (g/b/d) 22-35 days	FCR (g/g) 22-35 days	ADFI (g/b/d) 0-35 days	ADWG (g/b/d) 0-35 days	FCR (g/g) 0-35 days
Diet									
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	59.7^{c}	46.4^{c}	1.29^{a}	144.9	93.08	1.561^{ab}	89.61^{b}	62.82^{d}	1.427^{a}
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	59.2^{c}	43.8^{b}	1.36^{cd}	143.3	90.62	1.584^{b}	88.82^{b}	60.28^{cd}	1.475^{b}
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	57.8^{bc}	43.6^{b}	1.33^{bc}	142.5	88.61	1.613^{b}	87.60^{b}	59.40^{bc}	1.476^{b}
No Phytase (standard P) + 0 g/kg Ins	56.3^{b}	42.9^{b}	1.31^{ab}	146.0	96.89	1.510^{a}	87.84^{b}	61.89^{cd}	1.420^{a}
No Phytase (standard P) + 3.5 g/kg Ins	53.3^{a}	39.4^{a}	1.36^{cd}	141.6	89.05	1.595^{b}	83.66^{a}	56.44^{ab}	1.485^{b}
No Phytase (standard P) + 7 g/kg Ins	52.1^{a}	38.1^{a}	1.37^{d}	137.5	87.60	1.572^{ab}	81.60^{a}	55.21^{a}	1.479^{b}
SEM	0.746	0.674	0.0126	2.62	2.268	0.02246	1.326	1.083	0.01353
CV	4.2	5.0	3.0	5.8	7.9	4.5	4.8	5.8	2.9
P-value	<0.001	<0.001	<0.001	0.275	0.054	0.041	<0.001	<0.001	0.002
P source									
Phytase (-1.5 g/kg av P)	58.9	44.58	1.32	143.6	90.77	1.5858	88.68	60.83	1.4593
No Phytase (standard P)	53.9	40.13	1.35	141.7	91.18	1.5588	84.37	57.85	1.4612
SEM	0.489	0.492	0.0086	1.54	1.393	0.01366	0.821	0.739	0.00897
CV		6.4	3.5	5.9	8.4	4.8	5.2	6.8	3.4
P-value	<0.001	<0.001	0.073	0.390	0.835	0.167	<0.001	0.006	0.879
Ins dosage									
0 g/kg Ins	58.0^{b}	44.7^{b}	1.30^{a}	145.5	94.98^{b}	1.5354^{a}	88.73^{b}	62.35^{b}	1.424^{a}
3.5 g/kg Ins	56.3^{ab}	41.6^{a}	1.36^{b}	142.4	89.94^{a}	1.5892^{b}	86.24^{ab}	58.36^{a}	1.480^{b}
7 g/kg Ins	54.9^{a}	40.8^{a}	1.35^{b}	140.0	88.11^{a}	1.5923^{b}	84.60^{a}	57.30^{a}	1.477^{b}
SEM	0.781	0.696	0.0093	1.84	1.586	0.01608	1.059	0.837	0.00935
CV	8.2	7.3	3.1	5.8	7.8	4.6	5.5	6.3	2.9
P-value	0.025	<0.001	<0.001	0.117	0.009	0.025	0.027	<0.001	<0.001
Interaction									
P source*Ins									
SEM	0.746	0.674	0.013	2.62	2.268	0.02246	1.326	1.083	0.01353
CV	4.2	5.0	3.0	5.8	7.9	4.5	4.8	5.8	2.9
P-value	0.197	0.35	0.265	0.508	0.432	0.334	0.248	0.263	0.804

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

6.3.4 Energy Utilisation of Broilers Chickens

A three-day collection was used to determine AME, AMEn, AME:GE, AMEn:GE, NR and DMR in the excreta of the study chickens, collected from 19-21 days for the first feeding phase. The treatment diets and treatment factor, P source, significantly affected AME, AMEn, AME:GE, AMEn:GE, NR and DMR ($P<0.001$). The addition of phytase to the diet containing 1.5 g/kg less available P still significantly reduced AMEn, AME:GE, AMEn:GE, NR, DMR and increased AME compared to the standard P diet ($P<0.05$). There was no significant difference observed for the treatment factor, Ins dosage for AME, AMEn, AME:GE, AMEn:GE, NR and DMR ($P>0.05$). There was an significant interaction between P source and Ins dosage for DMR ($P=0.002$) and tendencies seen for AME ($P=0.073$) and AME:GE ($P=0.070$).

A three-day excreta collection was also used to determine AME, AMEn, AME:GE, AMEn:GE, NR and DMR, collected from 33-35 days for the second feeding phase. AME ($P<0.001$), AMEn:GE ($P=0.009$), NR ($P<0.001$) and DMR ($P<0.003$) were all significantly affected by treatment diets and a tendency was seen for AME:GE ($P<0.08$). Except for AME_n where no significant difference was observed for treatment diets ($P=0.183$). The factor of P source significantly affected AME ($P<0.001$), AMEn ($P=0.012$), AMEn:GE ($P<0.001$), NR ($P<0.001$) and DMR ($P<0.001$), with only a tenancy seen for AME:GE ($P=0.077$). The addition of phytase to the diet containing 1.5 g/kg less available P still significantly reduced AME, AME:GE, NR and DMR and increased AMEn and AMEn:GE compared to the standard P diet ($P<0.05$). Ins dosage of 3.5 or 7 g/kg significantly reduced AME compared to the 0 g/kg diets ($P<0.05$) and 3.5 g/kg reduced AME:GE compared to 0 g/kg Ins ($P<0.05$). Ins dosage did not significantly affect AMEn, AME:GE NR or DMR ($P>0.05$). In addition, there was no significant interactions ($P>0.05$) between the factors of P source and Ins dosage for all of the energy utilisation parameters found in table 6.8.

Table 6.7: The effect of supplementary Ins and P source on AME, AMEn, AME:GE, AMEn:GE, NR and DMR based on a total excreta collection from 19 to 21 days.

Treatment	AME	AME_n	AME:GE	AME_n:GE	NR	DMR
Diet						
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	12.39^{c}	11.45^{b}	0.7382^{b}	0.6825^{b}	0.8652^{b}	0.6780^{b}
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	12.34^{c}	11.54^{b}	0.7352^{b}	0.6875^{b}	0.8406^{b}	0.6672^{b}
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	12.24^{c}	11.51^{b}	0.7290^{b}	0.6860^{b}	0.8441^{b}	0.6702^{b}
No Phytase (standard P) + 0 g/kg Ins	11.32^{a}	10.08^{a}	0.6950^{a}	0.6185^{a}	0.6690^{a}	0.6398^{a}
No Phytase (standard P) + 3.5 g/kg Ins	11.82^{b}	10.16^{a}	0.7258^{b}	0.6235^{a}	0.6869^{a}	0.6673^{b}
No Phytase (standard P) + 7 g/kg Ins	11.43^{a}	10.17^{a}	0.7016^{a}	0.6241^{a}	0.6593^{a}	0.6474^{a}
SEM	0.1176	0.0716	0.00715	0.00431	0.01035	0.00522
CV	3.1	2.1	3.1	2.1	4.3	2.5
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
P source						
Phytase (-1.5 g/kg av P)	12.32	11.501	0.7341	0.6853	0.8503	0.6719
No Phytase (standard P)	11.52	10.135	0.7072	0.6221	0.6719	0.6528
SEM	0.0715	0.0404	0.00436	0.00244	0.00613	0.00331
CV	3.3	2.0	3.3	2.0	4.4	2.7
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Ins dosage						
0 g/kg Ins	11.88	10.80	0.7177	0.6522	0.778	0.6622
3.5 g/kg Ins	12.08	10.85	0.7305	0.6555	0.760	0.6672
7 g/kg Ins	11.83	10.84	0.7153	0.6551	0.752	0.6588
SEM	0.1250	0.1646	0.00602	0.00785	0.0217	0.00458
CV	4.7	6.8	3.7	5.4	12.7	3.1
P-value	0.339	0.977	0.170	0.948	0.682	0.430
Interaction						
P source*Ins						
SEM	0.1176	0.0716	0.00715	0.00431	0.01035	0.00522
CV	3.1	2.1	3.1	2.1	4.3	2.5
P-value	0.073	0.966	0.070	0.963	0.115	0.002

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

Table 6.8: The effect of supplementary Ins and P source on AME, AMEn, AME:GE, AMEn:GE, NR and DMR based on a total excreta collection from 33 to 35 days.

Treatment	AME	AME_n	AME:GE	AME_n:GE	NR	DMR
Diet						
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	12.168^{bc}	10.936	0.7269	0.6533^{b}	0.6002^{a}	0.6624^{a}
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	11.892^{a}	10.886	0.7105	0.6504^{b}	0.5799^{a}	0.6574^{a}
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	11.969^{ab}	10.778	0.7150	0.6439^{ab}	0.5959^{a}	0.6619^{a}
No Phytase (standard P) + 0 g/kg Ins	12.422^{d}	10.657	0.7336	0.6294^{a}	0.7000^{b}	0.6836^{c}
No Phytase (standard P) + 3.5 g/kg Ins	12.246^{cd}	10.681	0.7177	0.6308^{a}	0.6746^{b}	0.6672^{ab}
No Phytase (standard P) + 7 g/kg Ins	12.233^{cd}	10.604	0.7297	0.6262^{a}	0.6978^{b}	0.6822^{bc}
SEM	0.0876	0.1061	0.00633	0.00630	0.01138	0.00544
CV	2.3	3.1	2.8	3.1	5.6	2.6
P-value	<0.001	0.183	0.080	0.009	<0.001	0.003
P source						
Phytase (-1.5 g/kg av P)	12.009	10.867	0.7175	0.6492	0.5920	0.6606
No Phytase (standard P)	12.305	10.647	0.7270	0.6288	0.6908	0.6777
SEM	0.0524	0.0599	0.00375	0.00356	0.00662	0.00320
CV	2.4	3.0	2.8	3.0	5.7	2.6
P-value	<0.001	0.012	0.077	<0.001	<0.001	<0.001
Ins dosage						
0 g/kg Ins	12.30^{b}	10.796	0.7303^{b}	0.6413	0.6501	0.6730
3.5 g/kg Ins	12.06^{a}	10.784	0.7141^{a}	0.6406	0.6273	0.6623
7 g/kg Ins	12.09^{a}	10.691	0.7224^{ab}	0.6351	0.6468	0.6721
SEM	0.0690	0.0774	0.00451	0.00494	0.01378	0.00427
CV	2.5	3.2	2.8	3.5	9.6	2.9
P-value	0.041	0.577	0.047	0.619	0.452	0.154
Interaction						
P source*Ins						
SEM	0.0876	0.1061	0.00633	0.00630	0.01138	0.00544
CV	2.3	3.1	2.8	3.1	5.6	2.6
P-value	0.822	0.879	0.780	0.877	0.950	0.509

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.050).

6.3.5 GIT Development

Day 21

Diets and factors (P source and Ins dosage) did not significantly affect the relative weights of proventriculus and ventriculus, spleen or caeca expressed as a percentage of body weights (P>0.05) (Table 6.9). There was no interaction between P source and Ins dosage across all organs and sections of GIT. CV% was with expected values, but spleen and caeca had higher CV%. Livers (expressed as a percentage of body weight) were affected by treatment diets (P<0.05), P source (P<0.05) and Ins dosage (P<0.05). The treatment diet containing phytase and 0 g/kg Ins had the lowest percentage liver content, and a numerical trend of increases in liver % observed with increasing Ins dosage across phytase supplemented diets, this trend was also seen with standard P diets supplemented with Ins. Standard P diets increased relative liver weights compared to diets containing phytase (P<0.05). Pancreas relative weights were affected by treatment diet (P<0.05) and P source (P<0.001) but not Ins dosage (P=0.243). Percentages ranged from 0.3734% to 0.2876%. Standard P increased relative weights compared to diets containing phytase. Duodenum %, was significantly affected by treatment diets (P<0.001), P source (P<0.001) and Ins dosage (P<0.05). Relative weights increased with increasing Ins dosage from 1.106 to 1.272 % (0 g/kg to 7 g/kg Ins). Relative weights of jejunum were significantly affected by diets (P<0.001), P source (P<0.001) and Ins dosage (P<0.05). The treatment diet containing phytase and 0 g/kg Ins had the lowest percentage jejunum weight, a numerical trend of with increases in jejunum % observed with increasing Ins dosage, this trend was also seen with standard P diets supplemented with Ins. This pattern was also observed with the relative percentage of livers. Standard P diets increased relative weights compared to diets supplemented with phytase. Ins dosages of 3.5 and 7 g/kg significantly increased weights compared to 0 g/kg Ins. Ileum relative weights, were only affected by P source (P<0.05) and not treatment diets (P>0.05) or Ins dosage (P>0.05). Standard P increased relative weights from 1.27% to 1.37% when compared to phytase diets. Overall small intestine relative weights were affected by diet (P<0.001), P source (P<0.001) and Ins dosage (P<0.001). Treatment diets followed the same numerical pattern as liver and jejunum relative weights, whereby phytase or standard P diets supplemented with 0, 3.5 and 7 g/kg Ins increased relative weights with Ins dosage. When comparing P source, standard P increased relative weights compared to phytase diets. Furthermore, with increasing Ins dosage relative weights also increased from 4.295% to 4.953% (0 to 7 g/kg Ins).

Day 35

At the end of the study relative GIT and organ weights were calculated on day 35. Treatment diets, factors (P source and Ins dosage) and interaction (P source x Ins dosage) did not significantly affect the relative weights of proventriculus and ventriculus, spleen, pancreas, ileum or caeca expressed as a percentage of body weights ($P>0.05$) (Table 6.10). Although, there was a tenancy for Ins dosage to affect relative caeca weights ($P=0.075$). Relative weights of the Liver were not significantly affected by Treatment diets or factors (P source and Ins dosage), but there was a significant interaction between P source and Ins dosage ($P=0.036$). Duodenum %, was significantly affected by treatment diets ($P=0.015$) and P source ($P<0.001$), with no phytase (standard P) diets increasing relative weights, but not Ins dosage ($P=0.600$). Jejunum %, was also significantly affected by treatment diets ($P<0.001$) and P source ($P<0.001$), with no phytase (standard P) diet increasing relative weights, but not Ins dosage ($P=0.891$). Small intestine relative weight also followed the same pattern as jejunum and duodenum weights whereby treatment diets and P source had a significant effect on relative weights ($P=0.001$ and $P<0.001$ respectively) and Ins dosage had no significant affect ($P=0.640$).

Table 6.9: The effect of supplementary Ins and P source on the development of the GIT and organs, expressed as a percentage of the body weight of the chicken at 21 days of age.

Treatment	Proventriculus and Ventriculus %	Spleen %	Liver %	Duodenum %	Pancreas %	Jejunum %	Ileum %	Caeca %	SI %
Diet									
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	2.068	0.1008	2.90^{a}	1.021^{a}	0.3159^{ab}	1.758^{a}	1.212	0.450	3.991^{a}
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	1.975	0.0883	2.89^{a}	1.100^{ab}	0.2876^{a}	1.957^{ab}	1.283	0.426	4.341^{ab}
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	2.078	0.1004	3.061^{ab}	1.121^{ab}	0.3178^{ab}	2.02^{b}	1.313	0.497	4.455^{ab}
No Phytase (standard P) + 0 g/kg Ins	1.946	0.1027	2.986^{ab}	1.192^{b}	0.3540^{bc}	2.087^{b}	1.320	0.422	4.599^{b}
No Phytase (standard P) + 3.5 g/kg Ins	2.050	0.0864	3.076^{ab}	1.372^{c}	0.3453^{bc}	2.402^{c}	1.346	0.537	5.12^{c}
No Phytase (standard P) + 7 g/kg Ins	1.959	0.0810	3.185^{b}	1.423^{c}	0.3734^{c}	2.572^{c}	1.456	0.548	5.451^{c}
SEM	0.09	0.00606	0.0712	0.0531	0.01561	0.0844	0.0627	0.0454	0.1683
CV	14.1	20.6	7.5	13.9	14.9	12.5	15.0	29.9	11.4
P-value	0.824	0.059	0.039	<0.001	0.004	<0.001	0.167	0.210	<0.001
P source									
Phytase (-1.5 g/kg av P)	2.040	0.0965	2.950	1.081	0.3071	1.912	1.270	0.458	4.262
No Phytase (standard P)	1.985	0.09	3.082	1.329	0.3576	2.353	1.374	0.502	5.057
SEM	0.051	0.00367	0.0423	0.0328	0.00901	0.0559	0.0362	0.0267	0.1076
CV	13.8	21.5	7.7	14.9	14.9	14.4	15.0	30.4	12.6
P-value	0.445	0.218	0.032	<0.001	<0.001	<0.001	0.046	0.240	<0.001
Ins dosage									
0 g/kg Ins	2.007	0.1018	2.943^{a}	1.106^{a}	0.335	1.922^{a}	1.266	0.436	4.295^{a}
3.5 g/kg Ins	2.013	0.0873	2.983^{ab}	1.236^{ab}	0.3164	2.179^{b}	1.315	0.482	4.730^{b}
7 g/kg Ins	2.018	0.0907	3.123^{b}	1.272^{b}	0.3456	2.296^{b}	1.384	0.522	4.953^{b}
SEM	0.0632	0.00437	0.0515	0.0468	0.01224	0.0778	0.0449	0.0323	0.1485
CV	14.0	20.9	7.6	17.4	16.5	16.3	15.2	30.1	14.3
P-value	0.992	0.058	0.042	0.038	0.243	0.004	0.183	0.18	0.009
Interaction									
P source*Ins									
SEM	0.09	0.00606	0.0712	0.0531	0.01591	0.0844	0.0627	0.0454	0.1683
CV	14.1	20.6	7.5	13.9	14.9	12.5	15	29.9	11.4
P-value	0.459	0.183	0.781	0.446	0.791	0.422	0.818	0.313	0.517

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

Table 6.10: The effect of supplementary Ins and P source on the development of the GIT and organs, expressed as a percentage of the body weight of the chicken at 35 days of age.

Treatment	Proventriculus and ventriculus %	Spleen %	Liver %	Duodenum %	Pancreas %	Jejunum %	Ileum %	Caeca %	SI %
Diet									
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	1.298	0.1386	2.711	0.7451^{ab}	0.1974	1.386^{a}	1.050	0.3331	3.181^{a}
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	1.128	0.1258	3.009	0.7073^{a}	0.2149	1.312^{a}	0.925	0.3672	2.945^{a}
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	1.168	0.1440	2.946	0.7220^{a}	0.2047	1.398^{a}	1.061	0.4013	3.180^{a}
No Phytase (standard P) + 0 g/kg Ins	1.145	0.1423	2.893	0.8950^{c}	0.2238	1.629^{b}	1.143	0.3347	3.668^{b}
No Phytase (standard P) + 3.5 g/kg Ins	1.175	0.1031	2.702	0.8552^{bc}	0.2050	1.711^{b}	1.043	0.3603	3.609^{b}
No Phytase (standard P) + 7 g/kg Ins	1.193	0.1338	2.826	0.8277^{abc}	0.2268	1.688^{b}	1.067	0.3589	3.583^{b}
SEM	0.0788	0.01195	0.0930	0.0440	0.01016	0.0641	0.0525	0.02029	0.1387
CV	21.0	28.8	10.3	17.6	15.1	13.3	15.8	17.9	13.1
P-value	0.715	0.167	0.126	0.015	0.266	<0.001	0.130	0.200	0.001
P source									
Phytase (-1.5 g/kg av P)	1.198	0.1361	2.889	0.725	0.2056	1.365	1.012	0.3672	3.102
No Phytase (standard P)	1.171	0.1264	2.807	0.859	0.2186	1.676	1.084	0.3513	3.620
SEM	0.0450	0.00708	0.0555	0.0249	0.00588	0.0641	0.0308	0.01198	0.0788
CV	20.8	29.6	10.7	17.2	15.2	13.3	16.1	18.3	12.8
P-value	0.673	0.337	0.303	<0.001	0.125	<0.001	0.103	0.353	<0.001
Ins dosage									
0 g/kg Ins	1.222	0.1405	2.802	0.820	0.2106	1.508	1.097	0.3339	3.424
3.5 g/kg Ins	1.151	0.1145	2.856	0.781	0.2099	1.511	0.984	0.3637	3.277
7 g/kg Ins	1.180	0.1389	2.886	0.775	0.2157	1.543	1.064	0.3801	3.382
SEM	0.0553	0.00839	0.0687	0.0341	0.00739	0.0572	0.0374	0.01425	0.1131
CV	20.9	28.6	10.8	19.2	15.6	16.8	16.0	17.7	15.1
P-value	0.666	0.057	0.683	0.600	0.831	0.891	0.101	0.075	0.640
Interaction									
P source*Ins									
SEM	0.0788	0.01195	0.0930	0.0440	0.01016	0.0641	0.0525	0.02029	0.1387
CV	21.0	28.8	10.3	17.6	15.1	13.3	15.8	17.9	13.1
P-value	0.390	0.548	0.036	0.852	0.158	0.469	0.541	0.519	0.632

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

6.3.6 Haematology

Whole blood was analysed for haematology at the end of each feeding phase, day 21 and day 35. Blood was analysed for RBC count (x10^12/L), haematocrit (L/L), haemoglobin (g/L), MCV (fL), MCH (pg), MCHC (g/L) and RDW (%). For chickens sampled on day 21, treatment diets significantly affected haemoglobin (g/L) (P=0.028), MCV (fL) (P=0.001), MCH (pg) (P<0.001), MCHC (g/L) (P=0.01) and RDW (%) (P<0.001) with a tendency seen for haematocrit (L/L) (P=0.077). No significant difference was observed for RBC count (x10^12/L) (P=0.530). The factor of P source significantly affected Haemoglobin (g/L) (P=0.025), MCV (fL) (P<0.001), MCH (pg) (P<0.001), MCHC (g/L) (P=0.017) and RDW (%) (P=0.025) with a tendency seen for haematocrit (L/L) (P=0.055).The addition of phytase to the diet containing 1.5 g/kg less available P significantly increased haemoglobin, MCV, MCH, MCHC and reduced RDW compared to the standard P diet (P<0.05). The addition of the three levels of Ins, did significantly affect RDW% only (P=0.01), with no significant effects seen on the haematology parameters. There were significant interactions between P source and Ins, haematocrit (P=0.045), haemoglobin (P=0.026), MCH (P=0.002), and RDW (P=0.038) and with tendencies seen in MCV (P=0.065), MCHC (P=0.054).

For birds sampled on day 35, there was no significant difference for treatment diets in the following haematology parameters; haematocrit (L/L), haemoglobin (g/L), MCV (fL), MCH (pg), MCHC (g/L) or RDW (%) (P>0.05). There was however a significant difference in RDW % for treatment diets (P=0.006). In addition, there was also no significant difference in Ins dosage for the following haematology parameters; RBC count (x10^12/L), haematocrit (L/L), haemoglobin (g/L), MCV (fL), MCH (pg) or MCHC (g/L) (P>0.05), however a tendency was seen in RDW% (P=0.061). The addition of phytase to the diet containing 1.5 g/kg less available P significantly increased RBC count, haematocrit, haemoglobin, MCHC and RDW compared to the standard P diet (P<0.05). There were no significant interactions between P source and Ins dosage for haematology parameters seen in table 6.12, apart from a tendency was seen in RDW% (P=0.061).

Table 6.11: The effect of supplementary Ins and P source on haematology parameters of chickens sampled at 21 days of age.

Treatment	Red Blood Cell count x10^12/L	Haematocrit L/L	Haemoglobin g/L	MCV fL	MCH pg	MCHC g/L	RDW %
Diet							
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	2.071	0.2733	68.30^{abc}	132.1^{bc}	32.99^{b}	249.9^{b}	10.64^{ab}
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	2.214	0.2944	74.00^{c}	133.5^{c}	33.40^{b}	250.4^{b}	11.00^{ab}
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	2.135	0.2853	71.80^{bc}	133.7^{c}	33.57^{b}	251.5^{b}	11.19^{ab}
No Phytase (standard P) + 0 g/kg Ins	2.161	0.2819	71.10^{bc}	130.6^{bc}	32.91^{b}	251.9^{b}	10.58^{a}
No Phytase (standard P) + 3.5 g/kg Ins	2.055	0.2619	63.40^{a}	128.0^{ab}	30.90^{a}	241.4^{a}	13.16^{c}
No Phytase (standard P) + 7 g/kg Ins	2.194	0.2697	65.30^{ab}	123.3^{a}	29.85^{a}	241.8^{a}	11.82^{b}
SEM	0.0706	0.00805	2.46	1.854	0.498	2.64	0.431
CV	10.4	9.2	11.3	4.5	4.9	3.4	12.0
P-value	0.530	0.077	0.028	0.001	<0.001	0.010	<0.001
P source							
Phytase (-1.5 g/kg av P)	2.140	0.2843	71.4	133.08	33.32	250.6	10.94
No Phytase (standard P)	2.137	0.2712	66.6	127.27	31.22	245.0	11.85
SEM	0.0408	0.00475	1.47	1.110	0.325	1.61	0.279
CV	10.5	9.4	11.7	4.7	5.5	3.6	13.4
P-value	0.954	0.055	0.025	<0.001	<0.001	0.017	0.025
Ins dosage							
0 g/kg Ins	2.116	0.2776	69.7	131.34	32.95	250.9	10.61^{a}
3.5 g/kg Ins	2.135	0.2782	68.7	130.72	32.15	245.9	12.08^{b}
7 g/kg Ins	2.164	0.2775	68.5	128.47	31.71	246.7	11.50^{ab}
SEM	0.0502	0.00606	1.89	1.499	0.453	2.02	0.332
CV	10.5	9.8	12.3	5.1	6.3	3.7	13.0
P-value	0.789	0.997	0.897	0.370	0.156	0.179	0.010
Interaction							
P source*Ins							
SEM	0.0706	0.00805	2.46	1.854	0.498	2.64	0.431
CV	10.4	9.2	11.3	4.5	4.9	3.4	12.0
P-value	0.167	0.045	0.026	0.065	0.002	0.054	0.038

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

Table 6.12: The effect of supplementary Ins and P source on haematology parameters of chickens sampled at 35 days of age.

Treatment	Red Blood Cell count x10^12/L	Haematocrit L/L	Haemoglobin g/L	MCV fL	MCH pg	MCHC g/L	RDW %
Diet							
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	2.582	0.3131	85.6	121.09	33.09	273.5	9.267[a]
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	2.570	0.3159	87.4	120.73	33.35	276.1	9.520[ab]
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	2.603	0.3289	88.1	122.33	33.82	276.3	9.180[a]
No Phytase (standard P) + 0 g/kg Ins	2.417	0.2942	80.3	121.86	33.22	272.7	9.130[a]
No Phytase (standard P) + 3.5 g/kg Ins	2.458	0.2936	79.6	120.08	32.61	271.4	10.033[b]
No Phytase (standard P) + 7 g/kg Ins	2.528	0.2988	80.9	121.39	32.88	270.9	10.190[b]
SEM	0.0720	0.01003	3.00	1.051	0.414	2.49	0.2365
CV	9.0	10.4	11.3	2.7	3.9	2.9	7.8
P-value	0.391	0.274	0.173	0.711	0.425	0.523	0.006
P source							
Phytase (-1.5 g/kg av P)	2.586	0.3160	87.0	121.35	33.42	275.3	9.78
No Phytase (standard P)	2.466	0.2957	80.3	121.14	32.91	271.69	9.32
SEM	0.0405	0.00560	1.68	0.599	0.236	1.399	0.147
CV	8.8	10.4	11.0	2.7	3.9	2.8	8.4
P-value	0.041	0.013	0.006	0.808	0.135	0.073	0.034
Ins dosage							
0 g/kg Ins	2.499	0.3036	83.0	121.47	33.16	273.1	9.19
3.5 g/kg Ins	2.517	0.3060	83.9	120.42	33.00	273.9	9.76
7 g/kg Ins	2.567	0.3088	84.5	121.84	33.35	273.6	9.69
SEM	0.0515	0.00728	2.21	0.728	0.296	1.78	0.180
CV	9.1	10.6	11.8	2.7	4.0	2.9	8.4
P-value	0.629	0.880	0.881	0.367	0.705	0.950	0.061
Interaction							
P source*Ins							
SEM	0.0720	0.01003	3.00	1.051	0.414	2.49	0.2365
CV	9.0	10.4	11.3	2.7	3.9	2.9	7.8
P-value	0.822	0.986	0.911	0.685	0.396	0.614	0.061

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

6.3.7 Antioxidant Status

The total antioxidant status was measured in fresh plasma, with birds being sampled at day 21 and day 35. TAS (mmol/L) was not affected by Ins dosage (P=0.797) but was significantly affected by treatment diet (P=0.011) and P source (P=0.003) on day 21. There was a tendency towards an interaction between P source and Ins dosage (P=0.061). Birds sampled on day 21 saw no significant difference or interaction for SOD activity (u/mL), except for P source where a tendency was observed (P=0.079). GPx (u/L) activity was significantly affected by treatment diet (P=0.004) and P source (P<0.001) on day 21. However, there was no significant effect of Ins dosage on GPx activity but there was a tendency for an interaction between P source and Ins dosage. Antioxidant status (TAS, SOD and GPx) was not affected by Ins dosage (P>0.05) and did not have any significant interaction between treatment factors (P>0.05) when birds were sampled at day 35. Furthermore, P source did either significantly affect or had a tenancy to affect antioxidant parameters, with diets supplemented with no phytase and containing standard P reducing parameters compared to diets containing phytase and 1.5 g/kg less available P (TAS (P=0.004), SOD (P=0.099) and GPx (P=0.071)).

Table 6.13: The effect of supplementary Ins and P source on Total Antioxidant Status (TAS) (fresh plasma), Superoxidase Dismutase (SOD) (lysed whole blood) and Glutathione Peroxidase (GPx) (fresh whole blood) of broiler chickens sampled at day 21 and 35.

Treatment	TAS mmol/L (day 21)	SOD u/mL (day 21)	GPx u/L (day 21)	TAS mmol/L (day 35)	SOD u/mL (day 35)	GPx u/L (day 35)
Diet						
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	1.187^{ab}	90.5	419.1^{bc}	0.8420^{b}	94.6	462
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	1.305^{b}	95.8	476.0^{c}	0.8770^{b}	89.0	471
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	1.291^{b}	93.2	440.6^{c}	0.8710^{b}	90.8	431
No Phytase (standard P) + 0 g/kg Ins	1.195^{ab}	87.3	404.1^{abc}	0.7740^{ab}	87.3	461
No Phytase (standard P) + 3.5 g/kg Ins	1.139^{a}	87.5	329.9^{a}	0.8080^{ab}	90.1	389
No Phytase (standard P) + 7 g/kg Ins	1.101^{a}	89.0	339.7^{ab}	0.7140^{a}	76.2	375
SEM	0.0445	3.67	28.7	0.0403	5.03	30.5
CV	11.7	12.8	22.6	15.6	18.1	22.3
P-value	0.011	0.525	0.004	0.047	0.187	0.127
P source						
Phytase (-1.5 g/kg av P)	1.261	93.2	445	0.863	91.5	455
No Phytase (standard P)	1.145	87.9	358	0.765	84.5	408
SEM	0.0262	2.06	16.9	0.0231	2.93	17.8
CV	12.0	12.5	23.0	15.5	18.2	22.6
P-value	0.003	0.079	<0.001	0.004	0.099	0.071
Ins dosage						
0 g/kg Ins	1.191	88.9	412	0.808	90.9	462
3.5 g/kg Ins	1.222	91.7	403	0.843	89.5	430
7 g/kg Ins	1.196	91.1	390	0.792	83.5	403
SEM	0.0349	2.61	23.0	0.0303	3.63	22.0
CV	13.0	12.9	25.7	16.6	18.4	22.8
P-value	0.797	0.731	0.804	0.494	0.311	0.173
Interaction						
P source*Ins						
SEM	0.0445	3.67	28.7	0.0403	5.03	30.5
CV	11.7	12.8	22.6	15.6	18.1	22.3
P-value	0.061	0.764	0.077	0.453	0.301	0.411

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

6.3.8 Plasma Ins, Ins and inositol phosphate esters (IP2-6) concentrations in ileum digesta

myo-Inositol concentrations in blood plasma and ileum digesta was affected by treatment diets, P source and Ins dosage at day 21 and 35 ($P<0.05$) (Table 6.14 and 6.15). In addition, there was a significant interaction between P source and Ins dosage for Ins digesta (day 21 and 35) and day 35 Ins plasma ($P<0.001$). Phytate (IP_6), IP_5 and IP_3 was affected by treatment diets and P source at day 21 and 35 ($P<0.05$), but not by Ins dosage ($P>0.05$) in ileum digesta. Treatment diets effected IP_4 concentrations at day 21 and 35 ($P<0.05$), however P source had no significant effect on day 35 ($P=0.311$) but did have an effect at day 21 ($P<0.001$). Furthermore, IP_4 concentrations were not affected by Ins dosage. P source had a clear significant effect of increasing IP_2 concentrations with the addition of phytase on day 21 and day 35 ($P<0.05$).

Table 6.14: The effects of supplementary Ins and P source on plasma Ins concentration, Ins digesta concentration and inositol phosphate esters (IP_{2-6}) in ileum digesta at 21 days of age.

Treatment	Ins plasma nmol/mL	Ins digesta µmol/g	IP_2 nmol/g	IP_3 µmol/g	IP_4 µmol/g	IP_5 µmol/g	IP_6 µmol/g
Diet							
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	232.0^{b}	11.4^{b}	1543^{c}	2.3^{b}	5.3^{c}	1.0^{a}	4.8^{a}
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	357.0^{c}	51.3^{d}	540^{ab}	1.4^{b}	4.1^{bc}	1.8^{b}	11.0^{b}
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	459.5^{d}	89.9^{e}	1342^{bc}	2.3^{b}	5.1^{c}	1.5^{ab}	8.5^{ab}
No Phytase (standard P) + 0 g/kg Ins	130.7^{a}	1.73^{a}	0^{a}	0.14^{a}	1.7^{a}	4.7^{c}	61.2^{c}
No Phytase (standard P) + 3.5 g/kg Ins	283.8^{b}	25.3^{c}	0^{a}	0.1^{a}	1.8^{ab}	5.0^{c}	60.2^{c}
No Phytase (standard P) + 7 g/kg Ins	418.9^{cd}	57.5^{d}	0^{a}	0.082^{a}	1.6^{a}	4.2^{c}	58.8^{c}
SEM	23.4	2.78	288.3	0.44	0.81	2.62	1.84
CV	23.6	22.2	159.8	131.8	79.0	27.3	17.1
P-value	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001
P source							
Phytase (-1.5 g/kg av P)	349.5	50.85	1141	2.0	4.8	1.5	8.1
No Phytase (standard P)	277.8	28.16	0	0.11	1.7	4.6	60.1
SEM	23.6	5.40	170.4	0.25	0.459	0.157	1.09
CV	41.3	74.8	163.6	130.6	77.2	28.3	17.5
P-value	0.036	0.004	<0.001	<0.001	<0.001	<0.001	<0.001
Ins dosage							
0 g/kg Ins	181.4^{a}	65.6^{a}	771	1.23	3.5	2.9	33.0
3.5 g/kg Ins	320.4^{b}	38.3^{b}	270	0.74	3.0	3.4	35.6
7 g/kg Ins	439.2^{c}	73.7^{c}	671	1.19	3.3	2.9	33.7
SEM	18.3	3.41	242.9	0.38	0.67	4.10	6.11
CV	26.1	38.6	190.3	0.16	92.0	60.3	80.2
P-value	<0.001	<0.001	0.311	0.601	0.839	0.609	0.952
Interaction							
P source*Ins							
SEM	23.4	27.7	288.3	0.44	0.81	0.26	1.85
CV	23.6	2.22	159.8	131.8	79.0	27.3	17.1
P-value	0.437	<0.001	0.194	0.493	0.648	0.188	0.119

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

Table 6.15: The effects of supplementary Ins and P source on plasma Ins concentration, Ins digesta concentration and inositol phosphate esters (IP_{2-6}) in ileum digesta at 35 days of age.

Treatment	Ins plasma nmol/mL	Ins digesta µmol/g	IP_2 nmol/g	IP_3 µmol/g	IP_4 µmol/g	IP_5 µmol/g	IP_6 µmol/g
Diet							
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	309.7^{b}	7.98^{a}	141	0.29^{c}	1.0^{a}	0.78^{a}	6.6^{a}
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	300.9^{b}	45.84^{c}	90	0.18^{abc}	0.83^{a}	1.1^{ab}	9.8^{a}
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	423.4^{c}	83.88^{e}	42	0.29^{bc}	1.1^{a}	1.4^{b}	11.6^{a}
No Phytase (standard P) + 0 g/kg Ins	108.1^{a}	0.32^{a}	61	0.20^{abc}	1.7^{b}	4.3^{d}	56.9^{b}
No Phytase (standard P) + 3.5 g/kg Ins	317.2^{b}	22.61^{b}	0	0.078^{a}	0.81^{a}	3.7^{c}	58.1^{b}
No Phytase (standard P) + 7 g/kg Ins	406.2^{c}	57.12^{d}	36	0.14^{ab}	0.95^{a}	3.5^{c}	53.5^{b}
SEM	20.9	3.64	64.4	0.044	0.21	0.17	2.2
CV	21.3	31.8	330.2	73.8	62.6	22.0	20.9
P-value	<0.001	<0.001	0.715	0.020	0.050	<0.001	<0.001
P source							
Phytase (-1.5 g/kg av P)	344.7	45.9	91	0.24	0.96	1.1	9.3
No Phytase (standard P)	277.2	7.76	32	0.14	1.14	3.9	56.2
SEM	21.4	5.49	36.4	0.026	0.127	0.111	126.2
CV	37.7	82.8	323.4	76.1	66.2	24.6	21.1
P-value	0.030	0.016	0.0255	0.009	0.311	<0.001	<0.001
Ins dosage							
0 g/kg Ins	208.9^{a}	4.15^{a}	101	0.24	1.3	2.6	31.7
3.5 g/kg Ins	309.0^{b}	34.22^{b}	45	0.13	0.82	2.4	34.0
7 g/kg Ins	414.8^{c}	70.50^{c}	39	0.21	1.0	2.4	32.6
SEM	19.7	3.47	45.0	0.032	0.15	0.34	5.6
CV	28.3	42.8	326.6	77.3	64.0	62.6	76.4
P-value	<0.001	<0.001	0.565	0.059	0.052	0.940	0.961
Interaction							
P source*Ins							
SEM	20.9	3.64	64.4	0.044	0.21	0.17	2.2
CV	21.3	31.8	330.2	73.8	62.6	22.0	20.9
P-value	<0.001	0.026	0.778	0.943	0.126	<0.001	0.140

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

6.3.9 Plasma mineral concentrations

Blood plasma mineral concentrations was determined using ICP-MS for birds sampled at day 21 and 35. There was no significant effect of diet on Ca, Mg, Cu or Mn ($P>0.05$) concentrations, however P ($P=0.046$), Na ($P=0.025$), Fe ($P=0.002$), Mo ($P<0.001$) were affected by treatment diets on day 21. Diets containing standard P with no phytase significantly decreased the concentrations of P, Na, Cu and Fe ($P<0.05$) compared to phytase (with 1.5 g/kg less available P) except for Mo whereby standard P significantly increased Mo concentration. There was no significant difference between the three phytase and standard P diets for Ca, Mg and Mn for plasma concentrations. The factor of P source significantly affected P ($P=0.005$), Na ($P<0.001$), Cu ($P=0.025$) Fe ($P<0.001$) and Mo ($P<0.001$), but not Ca ($P=0.243$), Mg ($P=0.189$) and Mn ($P=0.230$) plasma concentrations on day 21. Ins dosage did not significantly affect Ca, P, Mg, Na, Cu, Fe or Mn ($P>0.05$), but Mo was increased with increasing Ins dosage ($P=0.019$). There were no significant interactions between P source and Ins dosage for day 21 plasma minerals except for Mo ($P=0.019$).

On day 35, plasma mineral concentrations (Ca, P, Mg, Na, Fe and Mn) were not significantly affected by treatment diet ($P>0.05$), but concentrations of Cu and Mo were significantly affected $P=0.013$ and $P<0.001$ respectively. Ins dosage had no significant effect on Ca, P, Mg, Na, Cu, Fe and Mn plasma concentrations ($P>0.05$). However, the plasma concentration of Mo was significantly affected by Ins dosage ($P=0.008$). Mg, Cu and Mo were significantly affected by P source ($P<0.05$) but not Ca, P, Na, Fe and Mn ($P>0.05$). There were no significant interactions between P source and Ins dosage for day 35 plasma minerals except for Mo ($P<0.001$).

Table 6.16: The effects of supplementary Ins and P source on plasma mineral concentration at 21 days of age.

Treatment	Ca mmol/L	P mmol/L	Mg mmol/L	Na mmol/L	Cu µmol/L	Fe µmol/L	Mo µmol/L	Mn µmol/L
Diet								
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	2.995	6.189^{ab}	1.123	176.0^{c}	2.329	24.27^{b}	2.320^{a}	0.330
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	3.052	6.466^{b}	1.124	175.4^{bc}	2.328	24.02^{b}	2.013^{a}	0.217
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	2.933	6.135^{ab}	1.079	175.7^{bc}	2.500	24.47^{b}	2.479^{a}	0.292
No Phytase (standard P) + 0 g/kg Ins	3.000	5.902^{a}	1.089	169.3^{a}	2.280	19.00^{a}	2.452^{a}	0.222
No Phytase (standard P) + 3.5 g/kg Ins	2.985	5.842^{a}	1.072	172.1^{abc}	2.234	17.25^{a}	5.179^{b}	0.235
No Phytase (standard P) + 7 g/kg Ins	2.781	5.919^{a}	1.050	171.2^{ab}	2.195	18.93^{a}	6.364^{b}	0.217
SEM	0.0725	0.1509	0.0363	1.683	0.0830	1.578	0.536	0.0568
CV	7.8	7.9	10.5	3.1	11.4	23.4	48.9	71.2
P-value	0.151	0.046	0.663	0.025	0.168	0.002	<0.001	0.622
P source								
Phytase (-1.5 g/kg av P)	2.993	6.256	1.109	175.71	2.392	24.25	2.27	0.280
No Phytase (standard P)	2.922	5.888	1.070	170.86	2.237	18.38	4.67	0.225
SEM	0.0430	0.0862	0.0205	0.950	0.0475	0.885	0.369	0.032
CV	8.0	7.8	10.3	3.0	11.3	22.8	58.3	69.9
P-value	0.243	0.004	0.189	<0.001	0.025	<0.001	<0.001	0.230
Ins dosage								
0 g/kg Ins	2.997	6.045	1.106	172.50	2.303	21.63	2.386^{a}	0.273
3.5 g/kg Ins	3.018	6.137	1.098	173.77	2.281	20.63	3.596^{ab}	0.226
7 g/kg Ins	2.857	6.027	1.065	173.43	2.356	21.85	4.422^{b}	0.253
SEM	0.0511	0.114	0.0254	1.294	0.0610	1.279	0.497	0.0400
CV	7.7	8.4	10.4	3.3	11.8	26.8	64.0	71.4
P-value	0.061	0.766	0.485	0.773	0.676	0.775	0.019	0.707
Interaction								
P source*Ins								
SEM	0.0725	0.1509	0.0363	1.683	0.0830	1.578	0.536	0.0568
CV	7.8	7.9	10.5	3.1	11.4	23.4	48.9	71.2
P-value	0.558	0.359	0.0363	0.587	0.268	0.881	0.002	0.522

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05).

Table 6.17: The effects of supplementary Ins and P source on plasma mineral concentration at 35 days of age.

Treatment	Ca mmol/L	P mmol/L	Mg mmol/L	Na mmol/L	Cu µmol/L	Fe µmol/L	Mo µmol/L	Mn µmol/L
Diet								
Phytase (-1.5 g/kg av P) + 0 g/kg Ins	3.183	6.343	1.0007	195.5	3.247^{b}	20.08	2.382^{a}	0.2833
Phytase (-1.5 g/kg av P) + 3.5 g/kg Ins	3.298	6.516	0.9838	188.0	3.320^{b}	17.95	1.855^{a}	0.2365
Phytase (-1.5 g/kg av P) + 7 g/kg Ins	3.415	6.125	0.9742	211.9	3.082^{b}	19.62	2.274^{a}	0.2684
No Phytase (standard P) + 0 g/kg Ins	3.466	6.404	0.9766	193.6	2.879^{ab}	17.05	1.951^{a}	0.2119
No Phytase (standard P) + 3.5 g/kg Ins	3.290	6.282	0.9315	190.0	2.463^{a}	21.27	3.888^{b}	0.2231
No Phytase (standard P) + 7 g/kg Ins	3.391	6.604	0.9206	175.2	2.410^{a}	17.72	4.558^{b}	0.2724
SEM	0.1928	0.2098	0.02328	11.34	0.2158	2.413	0.2374	0.02071
CV	18.3	10.4	7.6	18.6	23.5	40.3	26.6	26.3
P-value	0.919	0.665	0.125	0.366	0.013	0.807	<0.001	0.094
P source								
Phytase (-1.5 g/kg av P)	3.303	6.328	0.9861	186.3	3.222	18.68	2.16	0.2632
No Phytase (standard P)	3.382	6.424	0.9437	198.6	2.582	19.23	3.41	0.2362
SEM	0.1084	0.1197	0.01341	6.54	0.1235	1.368	0.199	0.01228
CV	17.8	10.3	7.6	18.6	23.3	39.5	39.1	26.9
P-value	0.606	0.571	0.029	0.188	<0.001	0.776	<0.001	0.127
Ins dosage								
0 g/kg Ins	3.332	6.375	0.9880	194.5	3.063	18.40	2.155^{a}	0.2436
3.5 g/kg Ins	3.294	6.393	0.9576	189.0	2.917	19.79	2.818^{ab}	0.2294
7 g/kg Ins	3.404	6.365	0.9474	193.5	2.709	18.62	3.356^{b}	0.2704
SEM	0.1339	0.1484	0.01682	8.18	0.1662	1.687	0.262	0.01499
CV	17.9	10.4	7.8	19.0	25.7	39.8	42.1	27.0
P-value	0.841	0.991	0.216	0.880	0.325	0.821	0.008	0.156
Interaction								
P source*Ins								
SEM	0.1928	0.2098	0.02328	11.34	0.2158	2.413	0.2374	0.02071
CV	18.3	10.4	7.6	18.6	23.5	40.3	26.6	26.3
P-value	0.671	0.242	0.774	0.180	0.524	0.379	<0.001	0.173

Ins, added dietary *myo*-inositol; P, phosphorus; SEM, standard error of means; CV% coefficient of variation.
There were 10 observations per treatment factor, Phytase dosage was at 4500 FTU/kg.
Means within a column with data not sharing a common superscript letter significantly differ (P<0.05

6.4 Discussion

6.4.1 Performance and energy utilisation

The aim of this study was to assess the interaction between P source and Ins on the growth performance of broiler chickens. It is predicted that phytase supplementation can release approximately 1.5 g/kg of bound P from phytate in the GIT of broiler chickens (Walk et al., 2018). Based on the meta-analysis by Bougouin et al. (2014), super dosing phytase can increase available P from organic sources (IPs and phytate) which can aid in improved performance. In this chapter, in order to balance the P availability in the diets for each feeding phase, two diets were formulated to contain standard P availability and 1.5 g/kg less available P supplemented with 4500 FTU/kg of phytase (by the reduction of inorganic P). In the hope to offer the same available P to the birds but from different sources, as phytase would increase the availability of organic P in the reduced P diets. Sunflower meal was included in all diets at 8% of the ration, to increase the phytate concentrations as this provides plenty of substrate but not reduce the bird performance (Araújo et al., 2014). As previously discussed, the optimal dosage of Ins is still not fully understood, it was predicted that with the increases in the phytate concentration in diets and a super dosage of phytase that 3.5 g/kg of Ins could be produced. Based on the previous chapters a dosage around 3-4.5 g/kg was beneficial. Therefore, the aim was trying to generate a beneficial dosage of Ins by phytase in the GIT by increasing the phytate and phytase into the diets. In order to assess the interaction, a 2 by 3 factorial was designed. Ins dosages were chosen to either match the predicted Ins produced by phytase at 3.5 g/kg or a stepwise increase to 7 g/kg Ins. However, there was a careful consideration between increasing phytate and increasing other anti-nutritional factors and while wanting to maintain a diet which could produce birds that meet the Ross 308 performance objectives.

The growth performance was calculated for each feeding phase and overall (Table 6.6) and weekly average BWs (Table 6.5). Surprisingly, when comparing Ins dosages on weekly BW there was a clear reduction in the average BW by Ins, with 7 g/kg Ins significantly reducing average BW on day 14, 21 and 35. Furthermore, Ins during the first feeding phase significantly reduced ADFI, ADWG and increased FCR. This was also seen in the second feeding phase whereby Ins significantly reduced ADWG and increased FCR. Overall performance (0-35 days) results followed a similar effect, whereby reductions in ADWG and ADFI and increases in FCR was seen due to Ins supplementation. This is the first study to clearly demonstrate the negative feeding effects

of Ins on performance during the different feeding phases of broiler chickens. Studies showing reductions in performance due to Ins include; Pearce, 1975 used a 2.5 g/kg inclusion and saw a reduction of 72 g in final body weight, Józefiak, 2012 used a inclusion of 1.5 g/kg Ins and saw a significant increase in FCR for the starter period (1-10 days) and Zyla et al., 2012 in laying hens saw a reduction in feed intake and egg production with the addition of 1 g/kg Ins into diets. The performance results in this chapter clearly show the stepwise reduction in performance due to Ins which has not been seen before in published literature, as the literature described above only assessed one inclusion level of Ins into diets. This again raises the question on what the optimum dosage of Ins in growing broiler chickens is and in what stage of production supplementary Ins would be beneficial or at all. Furthermore, these results show that Ins generation may not contribute to the beneficial extra phosphoric effects often associated with Ins generated by phytase in the GIT of broiler chickens.

There are limited studies published on the effects of Ins on energy utilisation in broiler chickens, with all studies looking at growing birds, collecting excreta from 18 to 21 days of age. As a result, energy utilisation was determined at the end of each feeding phase using excreta collected from days 19-21 and 33-35. Energy utilisation and nutrient digestibility was not affected by Ins dosages on excreta collected during the first feeding phase, however, AME and AME:GE were affected by Ins dosage during the second feeding phase, whereby 3 g/kg Ins reduced AME and AME:GE compared to 0 g/kg Ins ($P<0.05$). As previously mentioned, the direct caloric value of Ins is estimated to be approximately 2,800 kcal/kg (Potter, 1979). The results in this chapter fit with the ranges published by Pirgozliev et al. (2007) and the previous chapters. In addition, Pirgozliev et al. (2007) observed no significant effects of graded levels of Ins on energy utilisation and digestibility coefficients, which was also seen in a later work whereby nutrient retention and digestibility coefficients (AME, AME:GE, DMR, NR and FD) were not affected ($P>0.05$) by dietary Ins content of 0, 3 or 30 g/kg Ins (Pirgozliev et al., 2019). However, Cowieson and Zhai (2021), observed a positive linear increase ($P<0.05$) for AME_n with increasing dosage of Ins fed to growing broiler chickens. The results in this chapter clearly show that the basal diets used from 0-21 days had more effect on the energy utilisation, as seen by clear separations and groupings by the post hoc test results when comparing all the treatment diets. In addition, when comparing P source during the first feeding phase, phytase with lower available P increased the study variables in table 6.8. This may have masked any effects of Ins during this phase. This is the first study to date to measure the

effects of energy utilisation on older broiler chickens, therefore the literature on effects of Ins on energy usage limited for birds aged over 21 days. Further work is required to understand the effects Ins has on manipulating the pathways associated with energy utilisation in growing chickens.

6.4.2 GIT and organ development

The development of organs and GIT was determined at day 21 and day 35 and expressed as a percentage of the body weight, which can be seen in tables 6.9 and 6.10. Ins increased the relative liver weights (P=0.042) on day 21, but not on day 35. There are a number of studies reporting that direct Ins supplementation can increase liver weights which include; Pearce (1975), supplementing diets with 2.5 g/kg Ins, Zyla et al. (2004), supplementing diets with 2.5 g/kg Ins and Pirgozliev et al. (2019), supplementing diets with 3 and 30 g/kg. Furthermore, Pirgozliev et al. (2019) observed an increase in hepatic N retention in a dose dependent manner due to Ins supplementation. However, the physiological changes of why Ins can increase relative liver weights remain unclear.

The avian spleen is a lymphoid organ which can produce and store lymphocytes (white blood cells) and aid in blood filtration, removing aged RBCs and antigens (Nagy et al., 2016; Jeurissen, 1991). A study by Zyla et al. (2013[b]) in laying hens observed a reduction of basophils percent in the white blood cells due to the addition of 1 g/kg Ins into diets. The relative weights of the spleen were not affected by treatment diets (P>0.05) in this chapter, despite changes in the haematology of red blood cells (see tables 6.11 and 6.12). The avian pancreas in brief, is classified into 2 sections; the exocrine pancreas which supplies digestive juice through ducts to aid in small intestinal digestion and the endocrine pancreas which regulates glucose concentrations (Pilny, 2008). The relative weights of the pancreas were not affected by Ins dosages; however, the weights were affected by treatment diet and P source on day 21, whereby standard P diets increased relative weights. The implications of the function of the pancreas were not studied in this chapter and warrants further investigation. A study by Cowieson et al. (2013), observed an increase in glucagon insulin, glucose concentrations and reduction HDL cholesterol by a dosage of 1.5 g/kg included into diets. However, the link between the hormones and blood chemistry being affected by Ins is still not fully understood in chcikens.

The development of a functional GIT is critical in providing the nutrients needed to aid the rapid growth of a broiler chicken. This is the first study assessing the physiological effects of Ins supplementation on the development of the GIT. The relative weights of the proventriculus and ventriculus were not affected by the treatment diets at either day 21 or

day 35. However other parts of the GIT were, and followed some similar trends; the first being diets fed without phytase (standard P) saw a significant increase in relative weights of duodenum on day 21 and 35, jejunum on day 21 and 35, ileum day 35 and the overall weight of the small intestine on day 21 and day 35. The second trend being that Ins supplementation increased the relative weights of duodenum on day 21, jejunum on day 21 and overall weight of the small intestine on day 21. Although the diets were designed to provide a comparable amount of available P to the birds either from organic P liberated by phytase or inorganic P, feeding diets with different levels of available minerals may trigger the alterations in the microbial activity and composition. To overcome the short fall in any nutritional requirements or by the alteration of the physiologic and chemical environment in the gut lumen the small intestine may alter in relative size (Tilocca et al., 2016). The trend of phytase supplemented diets reducing GIT segments was also observed by Wu et al. (2004), whereby phytase (500 FTU/kg) with basal diet reduced the relative weights of duodenum, jejunum, ileum and small intestine ($P<0.05$). Moreover, it is important to note the value of free Ins and IPs to the microbial colony present in the GIT of a broiler chicken, as there is no information of the effects of free Ins on the microbiome of broiler chickens. The microbiota present in the GIT of poultry can utilise 10-25% of dietary phytate. Phytase supplementation can have an indirect effect of the microbiome through changes in the buffering capacity of the digesta and release of IP isomers as well as Ins (Jozefiak et al., 2016). A study by Okazaki et al. (2018) in rats assessed diets containing high sucrose diets or high starch diets with or without phytate to determine if there were any modulation to the gut microbiota. In addition, they supplemented high sucrose diets with 0.2% Ins. The study showed that Ins increased the percentage of *Lactobacillus spp*. expressed as a percentage of total bacteria and the concentration of n-Butyrate in the digesta. The addition of phytate also saw an increase in the percentage of *Lactobacillus spp*. in the digesta. Concluding, that the changes in gut microbiota may be due to the ring of Ins and may be responsible for the effects seen with diets supplemented with phytate. The underlying mechanisms of the importance of Ins for the GIT microbiome needs to be further investigated and elucidated in broiler chickens.

6.4.3 Haematology

Inositol is involved in many biological roles, including acting as a precursor for IP_5 which is needed in the formation of red blood cells of avian species including chickens. Unlike mammals that use 2,3-diphosphoglycerate, adult birds use IP_5 to control oxygen affinity in the red blood cells (Lutz, 1980). A study by Isaacks et al. (1982) used C^{14} inositol

(radioactively labelled) to incubate 5-day old chicks to determine how free Ins is incorporated into IP_5 and what effects this then had on the plasma Ins concentrations. The study showed that increasing the incubation time increased the uptake of Ins by the erythrocytes and the subsequent Ins being phosphorylated to IP_5. This also resulted in the blood plasma Ins concentrations falling from 14.6 to 10.7 µg/mL from 0 to 6 hours. This demonstrates the importance of Ins in erythrocyte formation in chickens and one of the many pathways that Ins is used in the chicken. A more recent study by Whitefeild et al. (2022) observed a positive correlation with increasing chick erythrocyte IP_5 and Ins concentrations (with parent stock being fed increasing dosages of phytase). This also demonstrates that liberated free Ins from phytase is used in IP_5 formation by erythrocytes. In the present study, one of the aims was to see if there were any physical changes or changes in the concentrations of red blood cells by Ins liberated phytase or free dietary Ins. When comparing the Ins dosages, the only significant affect that was observed was on day 21 whereby RDW% was increased to 12.08 % by Ins dosage of 3.5 g/kg compared to the 0 g/kg Ins of 10.61 % (P=0.01). An increased RDW can mean that there is more difference between the sizes of the red blood cells and is often associated with anaemia. This suggests that Ins can increase the variation of red blood cells in growing broiler chickens, however the interactions this then plays on the oxygen capacity was not measured in this study. Moreover, IP_5 in erythrocytes was also not measured which could have possibly shown than some of the Ins was used in the formation of IP_5. When comparing the effects of P source on the haematology parameters measured on day 21 and 35. Diets containing phytase with reduced available P, saw an increase in haemoglobin, MCV, MCH and MCHC on day 21 and an increase in red blood cell count, haematocrit, haemoglobin on day 35. This suggests that the release of organic P into the GIT by phytase and subsequent uptake was increased by diets with phytase as there was an increase in P concentrations in the blood plasma (Tables 6.16 and 6.17) The increase in available P may be more important in red blood cell formation than free Ins which can easily be synthesised *de novo* as blood plasma P concentrations was increased by phytase containing diets. Zanu et al. (2020) assessed the effects of phytase (high and low dosages of 500 and 5,000 FTU/kg) with the same levels of available P in diets and did not see any significant effects of phytase for red blood cells, haemoglobin, PCV, MCV, MCH or MCHC. However available P was not limited to or formulated to balance the extra available P produced by phytase. A study by Fijabio et al. (2020) observed no significant difference in haematology parameters with increasing dosages of phytase, again this study did not balance the P availability. Aureli et al. (2011) assessed the efficacy of a

novel phytase and observed that MCH was decreased by phytase supplementation, but no other effects on the other haematology parameters. Therefore, it is possible to conclude that increasing the available P in the blood is more important than increasing plasma Ins to increase haematology parameters, as Ins requires rephosphorylation to from IP_5. The link between Ins, IP_5 and WB is yet to be fully understood. A study by Greeen et al. (2019) demonstrated that birds with WB had lower haemoglobin levels than normal birds, indicating that WB is associated with muscle hypoxia and this could be improved by phytase supplementation and reverse hypoxic responses of blood and tissues. More work is required to understand the mechanisms that P availability and Ins has on the haematology and the subsequent meat quality of broiler chickens.

6.4.4 Antioxidant status

The antioxidant potential of Ins in growing broiler chickens is unknown. In this experimental chapter, antioxidant status (SOD, TAS and GPx) was not affected by Ins dosage at either day 21 or day 35 of broiler chickens. Despite recent findings in a number of different aquaculture species including juvenile Jian carp (*Cyprinus carpio var.* Jian), juvenile hybrid grouper (♀ *Epinephelus fuscoguttatus* × ♂ *E. lanceolatu*) and Chinese mitten crab (*Eriocheir sinensis*) which have all shown that Ins can improve antioxidant status (Jiang et al., 2009; Bu et al., 2022; Pan et al., 2022). This suggests that Ins does not act as an *in vivo* antioxidant in broiler chickens and does not affect the antioxidant defences of key enzymes or total antioxidant capacity in this chapter. Furthermore, this chapter also shows the rapid intake of Ins into blood, but despite its increased presence doesn't reduce the antioxidant status and therefore pose any harm to the bird's response to oxidative stress.

The antioxidant status of the chickens used in this experiment was affected by P source, with the diets containing 1.5 g/kg less available P supplemented with 4500 FTU/kg of phytase significantly or had a tendency to increase TAS ($P=0.03$), SOD ($P=0.079$) and GPx ($P<0.001$) at day 21 and TAS ($P=0.004$), SOD ($P=0.099$) and GPx ($P=0.071$) at day 35 compared to the standard P diets. The first line of defence for protecting against oxygen radicals is the enzyme SOD which catalyses the dismutation endogenous HO_2 to H_2O_2 or can form different metal-coordinated forms of SOD which is able to catalyse the superoxide radical H_2O_2 or O_2 (Hayyan et al., 2016). Hydrogen peroxide is further detoxified into H_2O and O_2 by catalyse and GPx (Chelikani et al., 2004). Oxidative damage can be caused by Ca and P deficiencies in fast growing broiler chickens and

ducks (Zhang et al., 2020; Karadas et al., 2010; Liu et al., 2020). Blood plasma concentrations of Ca and P was measured on day 21 and 35 and showed plasma concentrations of P on day 21 was significantly reduced by diets containing standard P compared to diets supplemented phytase with 1.5 g/kg less available P. This result was surprising since it was hoped that the recommend P and Ca used in the basal diet would be sufficient. A study by Zhang et al., (2020) demonstrated that broiler chickens fed low Ca and P diets reduced antioxidant capacity, by significantly reducing the concentrations of TAS, catalyse and GPx present in the serum ($P<0.05$) but did not significantly affect the levels of SOD ($P= 0.567$). In this study, phytase supplementation (with lower available P) increased antioxidant defences of key enzymes studies and total antioxidant capacity. Despite the increased antioxidant stress placed on the birds fed standard P in diets, Ins was not able to overcome these stresses as the post hoc test did not differentiate diets with standard P from one another.

6.4.5 Ileal inositol phosphate esters (IP_{2-6}) and Ins concentrations

The inositol phosphate esters IP_{2-6} was determined in the ileal digesta on day 21 and day 35 (Tables 6.14 and 6.15). The results showed that the super dose of phytase at 4500 FTU/kg was effective in removing the majority of the phytate (IP_6) in the ileum at day 21 and day 35 ($P<0.001$), which agrees with (Walk et al., 2014; Zeller et al., 2015[a]; Zeller et al., 2015[b]). Furthermore, this study showed that phytase is effective at removing increased phytate levels, by the inclusion of 8% sunflower meal. Ins supplementation did not significantly affect the concentrations of IP_{2-6} in the digesta on either day 21 or day 35 which agrees with the previous chapters and Sommerfeld et al. (2018). Furthermore, phytase supplementation increased the concentrations of IP_3 and IP_4 and reduced IP_5. This study also shows the increase of IP_2 in the ileal digesta by phytase, which provides a closer link between Ins generation by phytase in the GIT of chickens. Finally, as expected free Ins supplementation increased the concentrations of Ins in the digesta, which also suggests that Ins is not fully absorbed out of the GIT by the levels used in this study.

The Ins concentration in blood plasma was affected by treatment diets, P source and Ins dosage ($P<0.05$) on day 21 and day 35. Supplementing diets with either phytase or Ins increased the concentration of Ins found in the blood plasma. The origin of Ins in the blood plasma above the basal concentrations is likely to be from the increased Ins in the GIT (however radioactive labelled Ins is needed to confirm this). In addition, the concentrations of Ins were broadly similar between day 21 and day 35, suggesting the addition of phytase and or Ins can maintain these levels of Ins. There are increasing number of studies

publishing Ins concentrations in blood plasma of broiler chickens, with a range of 140-584 nmol/mL for control diets with the average value being 282 nmol/mL (Cowieson et al., 2015; Sommerfeld et al., 2018; Lee et al., 2018; Pirgozliev et al., 2019; Whitfield et al., 2022; Gonzalez-Uarquin et al., 2020[a]; Gonzalez-Uarquin et al., 2020[b]). The values shown in table 6.14 and 6.15 fit within the published literature and agree with their findings that supplementary Ins and exogenous phytase can increase the concentration of Ins in blood plasma.

6.4.6 Plasma mineral concentrations

The mineral content of blood plasma was determined at day 21 and 35, and unlike the first experimental study (Chap 4) significant differences were observed for some minerals. Expected ranges of the minerals analysed (Ca, P, Mg, Na, Cu, Fe, Mo and Mn) vary considerably in the published literature (Atteh and Leeson 1982; Sebastian et al., 1996; Hernández et al., 2006; Mondal et al., 2007; Nourmohamm et al., 2010; Chamorro et al., 2013; Vanderhasselt et al., 2013; Akbarian et al 2014; Akter et al., 2017; Abbas et al., 2018; Mohammadigheisar et al., 2020; Mohammed et al., 2021; Straková et al., 2021; Groff-Urayama et al., 2022). However, the concentrations of the blood plasma minerals represented in this chapter fit within the published values. Ins supplementation appears to not affect the mineral content of the blood plasma except for Mo, where by a significant step wise increase of Mo was seen in the blood on day 21 and day 35. The biological reason of why Mo and Ins may interact with one another is not understood and requires further investigation. There are limited studies assessing the effects of phytase on the minor plasma minerals for broiler chickens, with studies typically reporting Ca and P concentrations only. Phytase is known to improve the availability of minerals, however the increased availability of these minerals does not necessary result in increased concentrations in the blood plasma, as seen in chapter four. A study using broiler chickens supplemented with and without 500 FTU/kg phytase observed a significant reduction of Ca and Mg in the blood plasma with phytase addition and an increase in P with phytase. No significant difference was seen in Zn levels due to phytase addition (Viveros et al., 2002). Birds sampled on day 21 in this chapter, diets containing phytase significantly increased the concentrations of P, Na, Cu and Fe ($P<0.05$) compared to standard P diets, except for Mo whereby standard P significantly increased Mo concentration. Similar results were also seen on day 35, whereby diets containing phytase increased the concentrations of Mg, Cu and Mo in the blood plasma when compared to standard P diets. The result here of phytase increasing Mg contradicts the findings of

Viveros et al. (2002). There are several studies in pigs measuring the effects of phytase supplementation on plasma mineral concentrations. A study by Zeng et al. (2015), measured the response of graded levels of phytase into growing pig diets and observed no significant effect on the concentrations of Mg, Cu and Fe but saw significant increases in Ca and P. A further study in growing pigs by Czech et al. (2022) saw an increase in concentrations of P, Ca, Cu and Zn with diets supplemented with graded levels of phytase but no difference seen in Fe and Mg concentrations. To conclude it appears that Ins does not interact with the plasma minerals except for Mo, phytase supplementation is well known to manipulate the mineral digestibility and therefore can affect the uptake of minerals by changing the bioavailability of them.

6.5 Conclusions

The growth performance results in this chapter clearly show that supplementing diets with Ins had a negative impact on growth performance and feeding efficiency, which contradicts previous chapters. These results indicate that Ins generation may not contribute to the beneficial extra phosphoric effects often associated with Ins generated by phytase in the GIT of broiler chickens. It also raises the question of not only what dosage would be beneficial but also in what situation (ie Ca and P availability). Ins supplementation had no significant effect on the antioxidant status of broiler chickens, despite improvements seen other in species. Furthermore, Ins increased liver weights and relative weights of the small intestine on day 21, increased red cell width on day 21 and increased plasma Mo. The reasons why changes in the physiology were seen warrants further investigation. Finally, like previous chapters supplementing free Ins into diets results in increased Ins in blood plasma and digesta, however this doesn't provide clear information on whether this increase is beneficial to growing broiler chickens. Finally, this study clearly showed that Ins has negative feeding effects, the reasons why Ins supplementation into diets reduced performance needs to be investigated further.

6.6 Outcomes

1. Ins supplementation reduced bird performance from 0-21, 22-35 and 0-35 days. Phytase improved performance despite reductions in available P in diets, which suggests that Ins in this chapter did not contribute to the extra phosphoric effects.
2. Treatment diets and P source in diets did significantly affect AME, AME_n, AME:GE, AME_n:GE, NR or DMR, but Ins had no significant effect (collected from 19-21 days). Treatment diets and P source in diets did significantly affect AME, AME_n,

AME:GE, AME_n:GE, NR or DMR, but Ins had no significant effect (collected from 33-35 days).

3. Supplementing diets with phytase did alter the IP_{2-6} ileum ester profiles but supplementing diets with Ins did not alter the IP_{2-6} ileum ester profiles.
4. Increasing available Ins in diets (either from the enzymatic action of supplemented phytase or direct supplementation of free Ins) did significantly increase the plasma and ileum Ins concentrations either on day 21 or day 35.
5. Changing available Ins or P source in diets did significantly influence the plasma mineral concentrations on day 21 or day 35.
6. Dietary Ins supplementation did not significantly affect antioxidant defences of key enzymes studied or total antioxidant capacity, but standard P diets reduced antioxidant status compared to diets supplemented with phytase with less available P.
7. Supplementing diets with Ins only significantly affected RDW% on day 21, P source in diets did significantly influence the haematology profile with phytase supplementation increasing in haemoglobin, MCV, MCH and MCHC on day 21 and increasing in RBC, haematocrit, haemoglobin on day 35.
8. Increasing available Ins from direct supplementation increased the relative liver weights, increased the relative weights of duodenum, jejunum and overall weight of the small intestine on day 21. The effects of P source resulted in phytase (standard P) diets increasing relative weights of duodenum on day 21 and 35, jejunum on day 21 and 35, ileum day 35 and the overall weight of the small intestine on day 21 and day 35.

Chapter Seven: General discussion and conclusions on the effects of supplementary *myo*-inositol and phytase liberated *myo*-inositol on broiler chickens.

7.0 Introduction

The object of this thesis was to understand the importance of Ins for poultry production. The first study (Chapter 3) explored the effects of phytase and Ins supplementation on growth performance, energy utilisation, Ins in tissues and the concentrations of IPs in digesta and tissue. The second study explored homeostasis of Ins in chickens, measuring the relative expression of the genes associated with the synthesis, transport and catabolism of Ins in jejunum and kidney tissues. The final experimental chapter (Chap 6) explored the interaction between P source and Ins dosage in a 2 by 3 factorial experiment, on bird health and performance. Ins can be formed from endogenous *de novo* synthesis, recycling of Ins containing compounds or from the complete dephosphorylation of phytate by phytase and APL in the GIT. Phytase supplementation is routinely used in poultry diets to primarily to increase the availability of organic P. Super dosing phytase has many 'extra-phosphoric effects' including improving feeding efficiency, mineral digestibility, amino acid digestibility, reducing environmental P pollution (Refs). Furthermore, super-dosing phytase increases the concentration of Ins and lower inositol phosphates in the GIT. In brief Ins is an important biological compound used in many pathways and processes as its self or used as a precursor in more complex compounds. Due to the biological importance of Ins in biology, it is a logical assumption that Ins may play a role in improved performance and contribute to the beneficial extra-phosphoric effects of super dosing phytase. However, few have assessed the role Ins may play in poultry production. The objective of this thesis was therefore to evaluate the efficacy and possible explanatory mechanisms of Ins involvement in performance of broiler chickens.

7.1 Bird growth performance

Formulating diets that are nutritionally adequate and balanced is only one part of optimising broiler performance. Birds in this thesis were grown under experimental conditions which aim to replicate the commercial conditions used in the UK. The optimal dosage of Ins for broiler chickens is not well understood and the results in the literature appear to be inconsistent (see Table 2.3). Predictions on what dosage may be beneficial to broiler chickens has been based on the amount that maybe liberated by phytase in the GIT and the subsequent improvement in performance observed with super-dosing phytase. Ins dosages used in this thesis were Chap 4: 3 and 30 g/kg Ins; Chap 5: 4.5 and

13.5 g/kg and Chap 6: 3.5 and 7 g/kg Ins. These dosages were either chosen to replicate the levels that were predicted to be generated by phytase or above the levels to maximise any effects and explore the upper limit of Ins. A summary of the growth performance variables measured in this thesis can be seen in figure 7.1. Based on this thesis results, the benefits and negatives of feeding Ins to broiler chickens is still not fully understood. There was no clear explanation of why chap 6 saw reduced growth performance when comparable dosages of 3 g/kg used in chap 4 saw improvements. It could be suggested that the feeding of mash form diets in chap 4, may have caused this phenomenon, however in chap 5 used pelted feed produced in the same manner as chap 6.

Comparing supplementary dietary Ins and phytase liberated Ins is not necessary a fair comparison as the free Ins is available straight away, rather than having to wait for phytate to be dephosphorylated. Although studies by Novotny et al., (2022) and Sommerfeld et al., (2017) have shown than phytase can liberate Ins in the upper parts of the GIT. Finally, commercially the use of direct supplementation of Ins would not be financially viable due to expensive production costs. Therefore, to produce sufficient quantities of Ins would have to be produced by phytase.

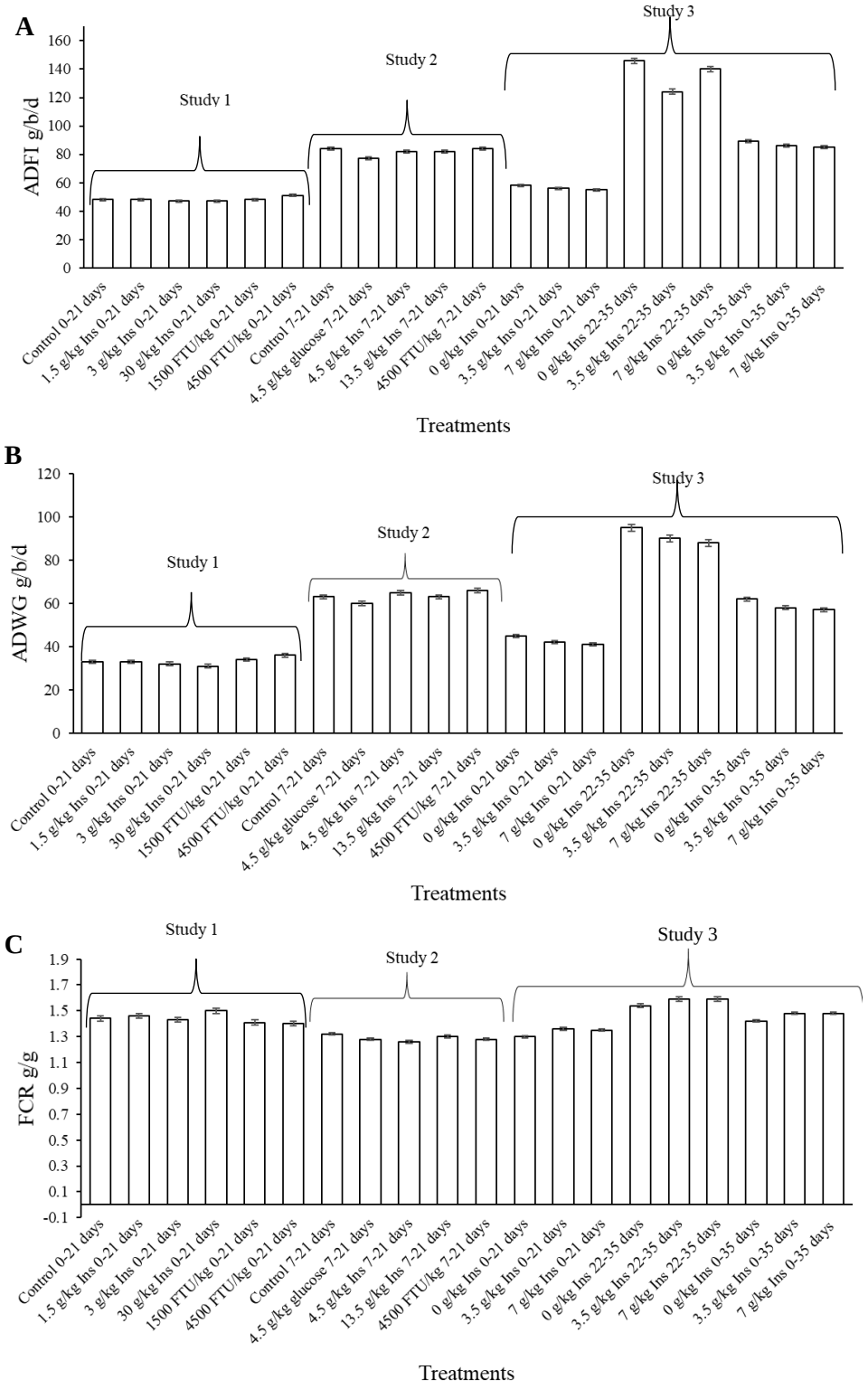


Figure 7.1: A summary of the bird performance obtained in this thesis; (A) average daily feed intake, (B) average daily weight gain, (C) feed conversion ratio for each experimental chapter, Ins, added dietary *myo*-inositol. For diet information please refer to the experimental chapters.

7.2 The role of Ins in broiler chickens

The biological role of Ins in broiler chickens is not fully understood. This thesis aimed to try and explore some of the regulatory pathways and the other roles Ins may play in broiler chickens.

7.2.1 Ins homeostasis

In order to understand the fait and implications of increasing available Ins by free Ins supplementation or phytate liberated Ins on the homeostasis of Ins, the expression of key genes associated with Ins were determined. The relative mRNA expression of Ins transporter proteins; SMIT1 (SLC5A11), SMIT2 (SLC5A11) and HMIT (SLC2A13), glucose transporter (SLC5A1), enzymes involved in Ins synthesis; Hexokinase 1 (HK1), Hexokinase 2 (HK2), 1L-*myo*-inositol-1-phosphate synthase (ISYNA1) and inositol-1-monophosphatase (IMPA1) and the enzyme involved in Ins metabolism *myo*-inositol oxygenase (MIOX) was determined using qPCR in the kidney and jejunum tissue of 21 day old broiler chickens (Chap 5). This is the first study to date, to assess the genes associated with the synthesis, transport and catabolism of Ins in broiler chickens. In the kidney tissue the Ins transporter proteins, SMIT1 and HMIT were not significantly affected by treatment diet but SMIT2 was ($P<0.001$). With 4.5 g/kg glucose and 4.5 g/kg Ins reducing expression compared to the other diets. Ins in the kidney tissue was significantly increased by the inclusion either 4.5 g/kg Ins or 13.5 g/kg into the diets, compared to the control. Despite the changes in relative expression seen with SMIT2, there doesn't seem to be an overall shift in the expression of these transporters with, in increasing the concentration of Ins in the blood plasma or kidney tissue. This suggests that the transporters can mediate additional transportation of Ins across plasma membranes without the need for up-regulation. The relative expression of the transporter proteins SMIT1 ($P=0.006$) and SMIT2 ($P=0.003$) in the jejunum were affected by diet except from HMIT. It appears that Ins is actively transported from the jejunum digesta and into the adjacent tissue via these transporters as the concentration of Ins was increased by all diets containing Ins or phytase compared to the control. However, the expression of the transporter proteins did not follow a stepwise increase despite there being an increase of Ins present in the digesta, with the control diet increasing relative expression of SMIT1 and phytase dosage of 4500 FTU/kg increasing the expression of SMIT2, which disagrees with the findings of Walk et al. (2018).

The gene associated with the first step in Ins catabolism is MIOX which was not affected by treatment diet in the jejunum or kidney tissue. The blood plasma Ins concentration was

only doubled (approximately) between 4.5 g/kg Ins and 13.5 g/kg, jejunum tissue increasing by 1.64 nmol/kg and kidney concentration only 0.98 nmol/kg despite the dosages triplicating. It is possible to suggest that either that the chicken is not actively absorbing all the Ins out of the GIT therefore limiting the uptake (ie the transporter proteins are not effectively absorbing Ins) or the action of *myo*-inositol oxygenase encoded in the gene MIOX was able to somewhat regulate the concentration of Ins despite no changes in the expression seen. More work is required to further understand the regulation of Ins by measuring the metabolite D-xylulose 5-phosphate which is produced in catabolism of Ins.

The genes involved in the first step of Ins synthesis are HK1 and HK2, which were affected by treatment diet in the jejunum and kidney ($P<0.05$) except for HK1 in the jejunum. The next step evolves ISYNA1, which was affected by treatment diets in the jejunum ($P=0.006$) but not in the kidney. The final step in Ins synthesis is reliant on the expression of IMPA, which was affected by diets in the jejunum ($P=0.006$) but not the kidney. The genes associated with the synthesis of Ins do not follow an overall pattern i.e. increasing available dietary Ins would cause the down regulation expression of these genes, which would help regulate Ins in cells. This could suggest that expression of these genes is tightly regulated and increasing Ins in broiler chickens is someway beneficial, or at least to a point. It is important to note that synthesised Ins and Ins obtained from the GIT cannot be distinguished in this study and biologically they are the same compound.

7.2.2 Manipulation of Ins in tissues and plasma

The uptake of Ins from the gastrointestinal tract in healthy humans is a highly efficient process with 99.8% of Ins being absorbed via three transporter proteins (SMIT1, SMIT2 and HMIT) (Croze and Soulage, 2013). However, in chickens the process doesn't appear to be so efficient. Chapter four used dosages of 0, 1.5, 3 and 30 g/kg which resulted in Ins blood plasma concentrations of 219, 436, 491 and 1933 nmol/mL respectively on day 21. Chapter five used dosages of 0, 4.5 and 13.5 g/kg Ins which resulted in Ins blood plasma concentrations of 47, 147 and 336 nmol/mL respectively on day 21. Chapter six used dosages of 0, 3.5 and 7 g/kg Ins which resulted in Ins blood plasma concentrations of 181, 320 and 439 nmol/mL respectively on day 21 and on day 35 resulted in Ins blood plasma concentrations of 209, 309 and 415 nmol/mL. When comparing diets containing Ins in this thesis and the subsequent increase in blood plasma there appears to be some common theme of inefficient uptake due to the non linear increase, ie doubling free Ins into diets doesn't result in doubled plasma concentrations. Therefore, it appears that there is a point

at which the GIT becomes too saturated with Ins to allow for the complete uptake. Furthermore, this inefficient uptake may be a deliberate response of high levels of Ins in the GIT to help regulate the Ins present in the chicken, although as described above the regulation of Ins transporters do not follow a noticeable trend. These findings also agree with Sommerfeld et al., (2017), Beeson (2017), Cowieson et al., (2013), Greene et al., (2019), Spigg et al., (2022), Lee et al., (2017) and Pirgozliev et al., (2019) that Ins supplementation or phytase supplementation can increase the concentration of Ins in the blood plasma.

The origin of Ins in the blood plasma above the basal concentrations is likely to be from the increased Ins in the GIT (however radioactive labelled Ins is needed to confirm this). In addition, in chapter six, the concentrations of Ins were broadly similar between day 21 and day 35, suggesting the addition of phytase and or Ins can maintain these levels of Ins throughout the growth of a broiler chicken. Furthermore, the correlation of Ins in the digesta and blood plasma from the studies used in this thesis can be seen in figure 7.2. All studies and sample points show the positive linear increase of increasing free Ins in the digesta of the GIT and the subsequent increase in blood plasma. Novotny et al., (2022) compared the breakdown of phytate and subsequent generation of Ins in the GIT of broiler chickens and turkeys. The study showed there was a positive linear relationship between Ins in blood plasma and ileal digesta for boilers (R^2=0.69) and turkeys (R^2=0.53), however the regression slope was greater in turkeys than broilers. In figure 7.2, shows that the greatest positive linear relationship between digesta Ins and blood plasma was for study one (Chapter 4). However, the reasons why some birds responded better than others still remain unclear.

In addition, Ins is also manipulated in tissue, chapter four showed that feeding high levels of Ins at 30 g/kg significantly increased Ins concentration in all tissues (breast, leg and kidney) except the brain. These results were also echoed in chapter five, whereby concentration of Ins found in the kidney and jejunum tissue were increased by 13.5 g/kg Ins. Again, the implications on the biochemical pathways altered remain unclear.

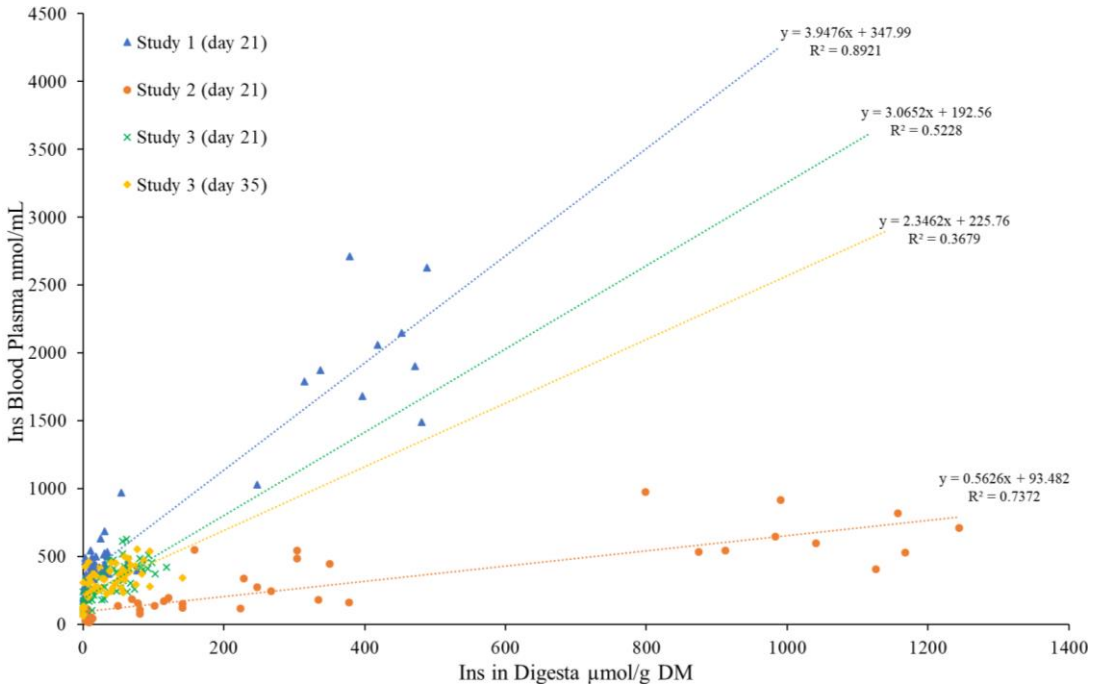


Figure 7.2: Linear regression of *myo*-inositol (Ins) concentrations in digesta and blood plasma of broiler chickens fed experiment diets used in this thesis. Digesta source, study one and three ileum and study two jejunum. Study 1 (Chap 4), birds sampled on day 21 and ileal digesta analysed. Study 2 (Chap 5), birds sampled on day 21 and jejunum digesta analysed. Study 3 (Chap 6) birds sampled on day 21 and day 35 and ileal digesta analysed. For diet information please refer to the experimental chapters.

7.2.3 Health status

Ins is an important biological compound therefore increasing Ins above basal levels may have implications on the health of the bird. Despite positive and negative impacts of the growth performance of Ins. This thesis demonstrated that Ins doesn't act as an antioxidant or effect the haematology of chickens.

7.3 General conclusion

The thesis evaluated the effects of supplementing poultry diets with free Ins and Ins liberated from phytate. Whether free dietary Ins supplementation may be beneficial to broiler chickens in terms of improving performance is still not fully understood. The results in this thesis did not give a clear and consistent indication of the benefits Ins can provide above diets not supplemented with phytase or free Ins. It was hypothesised that a proportion of the beneficial extra phosphoric effects of super dosing phytase could be associated with the increase of available Ins. The thesis demonstrated that Ins in blood plasma could be manipulated by either free supplementary Ins or phytase liberated Ins. However, this thesis did not provide repeatable positive effects of Ins in the experimental chapters, therefore, it is difficult to associate the extra Ins generated by super dosing phytase beneficial, suggesting alternative mechanisms are at play.

7.4 Areas for further research

Several areas have been identified for further research in this thesis and include:

- Ins is a biologically valuable compound, not just to chickens but also to the microbiome that lives within the GIT of a chicken. This raises the question of, is Ins beneficial to the microbial colony found in the GIT or is the increased P availability in the GIT produced by dietary phytase more important than the formation of increased Ins.
- The positive feeding effects of Ins on growth performance still appear to be inconsistent. Further work is required not only to better understand dosage but also at what point in production to supplement diets with extra Ins or at all. The quantities of Ins produced can be manipulated by supplementary phytase and this may help in controlling the quantity of Ins present in the GIT of a bird as Ins would be too expensive to use on a commercial scale.
- The homeostasis of Ins in chickens is still poorly understood. The results indicate that the free Ins in the GIT (either from direct supplementation or phytase generated) is absorbed due to the subsequent increases in blood plasma and tissue. However, with isotope labelling of Ins could provide clear results and the balance of how much Ins is synthesised and how much is absorbed in broiler chickens. Furthermore, this would also provide additional information of the fait and use of Ins in the bird by tracking the labelled Ins as it enters various pathways.

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