Maize feeds and influence of xylanase on broiler digestion of starch

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ABSTRACT

The primary cereal grain used in poultry feed worldwide is maize. An understanding of how and where broiler chickens digest native starches, this being the major energy component of maize, and the influence that exogenous xylanase has on this process, is essential when optimising nutrient utilisation of a diet. In this project, both *in vitro* and *in vivo* models were used to understand broiler digestion of starches in different maize-based diets: native maize starch, maize grit and maize-soy with or without xylanase (Econase XT 25 at 100 g/t; 18,100 BXU/kg).

An *in vitro* model to simulate the digestive tract of broilers using a ball milling technique to mimic the chicken gizzard was designed to investigate differences in the digestion of starches between the various diets. Reduced starch crystallinity and particle size of the diet was shown when samples were ball milled in HCl-pepsin, compared to roller mixed samples, leading to a greater glucose release when these samples were incubated with pancreatin. The maize-soy diet gave the greatest glucose release with pancreatin, with native maize starch giving the lowest, indicating that the processing of diets prior to digestion as well as the surrounding food matrix can impact on the digestion of starches in feed.

In all *in vivo* trials, starch fraction measurements and SEM imaging of digesta collected from the gizzard, upper and lower small intestine, revealed the progression of starch granule digestion through the tract. Despite birds being starved prior to feeding, broilers ingested little of the native maize starch diet. Nonetheless, predominantly intact granules were evident in the gizzard, with signs of amylase attack becoming apparent in the small intestine. Following starvation, starch granules were still observed throughout the tract of birds, suggesting the presence of resistant starches. Higher digesta pH values were observed when birds were fed a maize-soy diet which gave pH 3.56, 6.70, 7.70 for the gizzard, and upper and lower small intestine, respectively, compared to pH 2.47, 6.49, 7.34 for the maize grit diet, an effect that could potentially impact on digestive processes.

In feeding trials with broilers, inclusion of xylanase into the maize-soy diet and feeding on a single occasion revealed no apparent difference in the matrices surrounding the starch granules in digesta. This suggested that xylanase may be working indirectly *in vivo* through some physiological change in the digestion mechanism rather than direct action on the feed. To test this hypothesis, birds were fed xylanase for different lengths of time before slaughter. Peptide YY concentration in the blood was higher during the first few weeks of supplementation, with longer periods of supplementation nulling this effect, suggesting that xylanase may be acting through a prebiotic mechanism. RT-qPCR results revealed a trend towards an increase in glucose transporter (GLUT2 and SGLT1) expression at 2 and 3 weeks of xylanase supplementation, respectively. This also correlated to an increase in glucose concentration in the blood from 3 weeks of xylanase inclusion, suggesting a greater absorption capacity of birds. These results may indicate a potential mechanism of xylanase action in maize-based diets, although further work is required to confirm these findings.

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LIST OF ABREVIATIONS

ATR	Attenuated total reflectance
A/X	Arabinose/xylose
AXOS	Arabinoxylo-oligosaccharides
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
Con A	Concanavalin A
Ct	Cycle threshold
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
DP	Degree of polymerisation
dsDNA	Double stranded deoxyribonucleic acid
d.w.b	Dry weight basis
FCR	Feed conversion ratio
FTIR	Fourier transform infrared spectroscopy
GLUT2	Glucose transporter 2
GOPOD	Glucose oxidase/peroxidase reagent
NSP	Non-starch polysaccharides
PYY	Peptide YY
RDS	Rapidly digestible starch
RS	Resistant starch
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SDS	Slowly digestible starch
SEM	Scanning electron microscopy
SGLT1	Sodium-glucose cotransporter 1
VFA	Volatile fatty acids
WAXD	Wide angle x-ray diffraction

CHAPTER 1

INTRODUCTION

1.1 Project summary

Cereals predominate as the primary energy component of poultry feed, with maize currently being the mostly widely used. The nutritional value of maize has, in the past, been considered relatively constant with a high degree of digestibility. However, maize quality and composition have been shown to fluctuate due to variable agronomic factors and this can affect the nutritive value of the maize in animal feed and therefore animal performance. Moreover, the demand for such cereals in the biofuel and human food sector has led to elevated cereal prices. Consequently, growing pressure for lower feed costs and improved performance, in the animal feed industry, has given attention to the necessity for maximising nutrient utilisation.

Starches from cereals provide the greatest energy source in animal feed. Un-cooked starches are often fed to poultry in a mash diet, yet starch granules in their native form are known to be highly resistant to digestion. The accessibility and availability of the starches in a food matrix will also vary greatly depending on diet processing and feed ingredients. Therefore it is of interest for formulators to understand the effect that inclusion of different dietary ingredients might have on the digestibility of individual macronutrients.

Exogenous enzymes are routinely included into poultry feed world-wide to improve utilisation of the diet, moderate environmental pollution and reduce feed cost. Despite widespread acceptance and use, the mechanisms by which these enzymes function in the digestive tract of birds are still not fully understood. Inclusion of xylanases into maize-based diets is becoming more commonplace, with reports of improved bird performance. However, the mechanisms for achieving these results are still poorly defined.

In this work, the digestion of maize starch under *in vitro* and *in vivo* conditions was studied to better understand how and where native starches are digested in the tract and to determine what the limiting factors of digestion might be. Using techniques to study changes in starch content, composition and physical structure, an assessment on the digestion of starches was made. The mechanisms of exogenous xylanase action in a maize diet were also investigated to reveal any potential impact on starch digestion. These findings will contribute to the current and ongoing research into optimising nutrient utilisation of animal feed to ensure high animal performance as well as profitability.

1.2 Thesis outline

In this thesis, a literature review exploring background information on the use of maize in poultry feed is presented in chapter 2. Composition of the maize kernel is discussed with particular focus on the major storage glucan, starch. Native starch granule composition, structure and susceptibility to enzymatic hydrolysis are reviewed in this chapter. The digestive anatomy and physiology of the chicken is presented to highlight the current understanding of starch digestion in the bird. Inclusion of exogenous enzymes, such as xylanases, into poultry feed and the potential mechanisms of enzyme action are also discussed. Based on this review, a series of project aims and objectives were set and these are presented at the end of the chapter.

The materials and methodologies used in this work are defined in chapter 3. In this chapter, several techniques that were used to study starch content, composition and morphology are described. Moreover, *in vitro* and *in vivo* models used to examine the digestion of starches are presented.

Development of an *in vitro* model to simulate the digestive tract of birds is shown in chapter 4. Diets were characterised according to their starch content, composition, particle size, and

food matrix. The influence of gizzard action on starch digestion was assessed, including the extent of starch digestion among the different diets.

Broiler digestion of maize starch *in vivo* is explored in chapter 5. Starch granule hydrolysis through the tract of birds is discussed, along with the impact of a food matrix on starch digestion. Changes to digesta pH and blood glucose concentration in response to feed consumption are also considered.

The effect of supplementing a maize diet with xylanase on broiler digestion of starch is presented in chapter 6. Firstly, bird response to feeding xylanase on a single occasion was investigated to explore enzymatic action on the cell wall matrix and to determine how this may impact starch digestion as well as other digestive processes. Subsequently, the effect of supplementing maize diets with xylanase over different lengths of time is discussed. The direct mechanism of xylanase action on cell wall matrices was studied as previously described, as well as potential indirect mechanisms. These include modification of gut hormone secretion and intestinal glucose transporter expression. The impact of these changes on starch digestion and the potential influence on bird performance are discussed in this chapter.

In the final chapter, the importance of understanding digestive processes together with the effect of altering food matrices when studying starch digestion *in vitro* and *in vivo* are highlighted. A general conclusion is drawn on the use of maize in poultry feed and the process of starch digestion, including its limitations. From the findings presented in this thesis, a detailed view on the digestion of maize starch is revealed and the potential mechanisms by which xylanase may act in such a diet to elicit a beneficial response is presented.

CHAPTER 2

LITERATURE REVIEW

2.1 Broiler production

Poultry production began as small-scale, family-based units for local meat and egg consumption. After the Second World War, production rapidly developed with increasing knowledge of genetics, nutrition and disease control (Fletcher, 2004) to become the extensive industrial practice known today. Consumption of poultry products has coincidently increased alongside poultry production with continuing demand. Genetic selection has improved performance as well as product quality, with birds being selectively bred either for their meat (broilers) or eggs (layers). America is the greatest producer of chicken meat, accounting for 44 % of the world total production. World chicken meat production has increased by 64 % from 2000-2013, with approximately 96 million tonnes being produced in 2013 (Fig 2.1). This continual annual increase in broiler production may be explained by a number of factors. Poultry is a relatively low cost meat with desired nutritional qualities making poultry consumption generally universally accepted, while not being affected by any religious considerations. Also, a relatively short production cycle means that birds can reach market weights in less than 6 weeks. This rapid growth needs to be supported by the diet. However, feed costs represent an estimated 60-70 % of the total cost of broiler production (Agah and Norollahi, 2008), and therefore this could be considered one of the most important factors in controlling profitability.

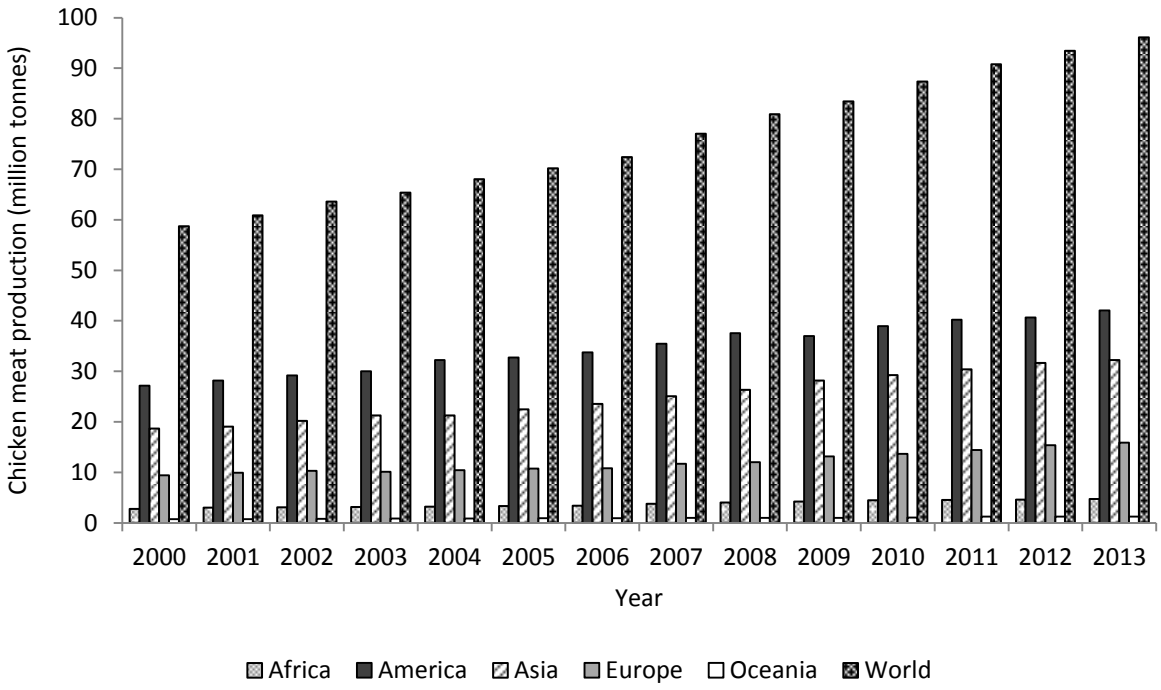


Figure 2.1 Chicken meat production (FAO, 2015)

2.2 Cereal production

Cereals are important for both human and animal diets. In developed countries, around 70 % of cereals are used as animal feed while in developing countries approximately 90 % of cereals produced are directly consumed by humans (Betschart, 1982). Wheat, barley, maize and rice represent 95 % of the world cereal production (Table 2.1), while rye, oats, millet and sorghum comprise only around 5 %. Altogether, these cereals provide 56 % and 50 % of the world's food energy and protein consumption, respectively (Stoskopf, 1985). With increasing cereal usage in animal feeds there is pressure to improve the grain quality, for example starch and protein quantity and composition, to effectively enhance the nutritional status of the cereal.

2.2.1 Cereals in poultry feed

Cereal grains such as wheat, maize and sorghum are commonly used in poultry feed as these provide the greatest source of available energy. Other cereals such as oats, barley and rye are generally less well digested and have a lower energy value in poultry feed (Classen, 1996). For optimal performance, poultry need a balanced supply of energy, protein, essential amino acids, minerals, vitamins and water. Poultry feed consists mainly of cereal grains which provide starch as the primary source of available energy. Other industrial by-products, such as soybean meal, are often included in feed to improve the nutritional value of the diet, by providing a supplementary source of protein. Fats and oils are also commonly included to improve palatability of the diet as well as energy intake (Poorghasemi *et al.*, 2013).

Table 2.1 Cereal produced (1000 million tonnes) in 2013 (FAO, 2015)

Country	Wheat	Barley	Maize	Rye	Oats	Rice	Millet	Sorghum
World	715,909	143,960	1,018,112	16,687	23,881	740,903	29,864	62,295
Africa	28,286	7,144	70,987	93	285	28,742	15,002	25,645
Americas	118,689	21,141	522,363	465	6,713	36,077	430	23,583
Asia	319,890	21,848	305,113	1,149	1,200	671,017	13,758	9,581
Europe	225,741	85,939	118,922	14,945	14,542	3,895	635	1,252
Oceania	23,303	7,888	726	35	1,141	1,172	40	2,235
United Kingdom	11,921	7,092	-	33	964	-	-	-

Although advances in poultry nutrition have led to an increased understanding of nutrient requirements, metabolism and availability, variations in the nutritional value of cereals need to be considered. Differences in the feeding value of cereals can be linked to cultivar,

environment, quantity and composition of starch, presence of non-starch polysaccharides (NSP) and anti-nutritional factors, as well as the processing methods used (Scott, 1996). Moreover, the digestive capacity of birds is an important factor in feed utilisation. This capacity is affected by bird age, sex, breed, environment, previous diet, endogenous enzyme production, health and microbial status. Therefore, all these factors need to be taken into account when formulating feed to achieve optimal broiler performance.

2.3 Maize

Maize (*Zea mays*) is the most cultivated crop in the world, with America accounting for half of the world's maize production in 2013 (Table 2.1). Approximately 70-80 % of maize produced worldwide is used in animal feed. Maize-based diets for broilers are favourable due to the relatively high starch content and low concentrations of anti-nutritional factors, such as non-starch polysaccharides, when compared to other cereals such as wheat and rye (Choct, 1997). However, maize has a relatively low crude protein content with a poor balance of amino acids, being deficient in a number of essential amino acids, particularly lysine and tryptophan. Therefore, poultry feed comprising maize must be supplemented with essential amino acids or a high-quality protein. Maize quality and composition can also vary noticeably with environmental factors, growth region, agronomic input and variety (Leeson and Summers, 1976; Leeson *et al.*, 1993; Masey O'Neill *et al.*, 2012). As a consequence, the nutritional value of the maize and subsequent animal performance has been shown to differ, making quality a key concern for formulators and producers.

2.3.1 Maize kernel structure

An appreciation of maize structure and composition is essential for an understanding of how this cereal is to be digested and utilised by the animal. The maize grain, commonly known as

the kernel, contributes around 42 % of the dry weight of the crop. The maize kernel is botanically classified as a caryopsis; a dry, indehiscent, single-seeded fruit. Figure 2.2 illustrates the three main elements that make up the kernel; the germ, endosperm and pericarp. The percentage weight of each element and the composition of these parts of the whole dent maize kernel are presented in Table 2.2.

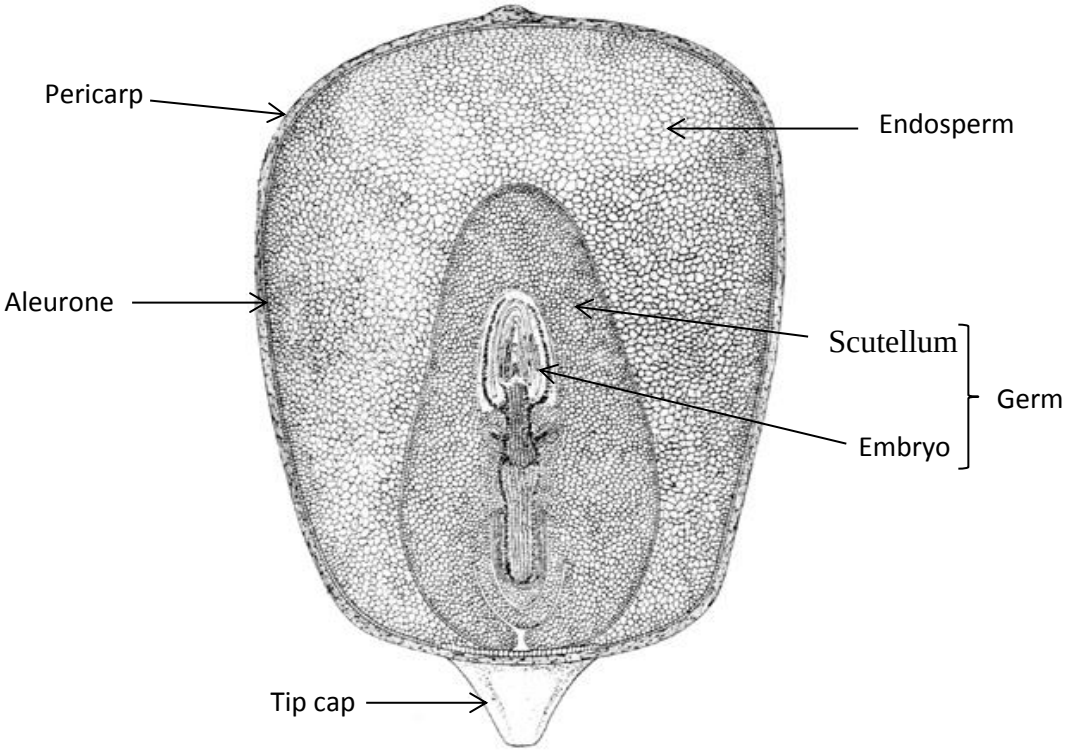


Figure 2.2 Mature dent maize kernel structure (image adapted from Coe (2001))

The germ comprises up to 10-13 % of the kernel dry weight and consists of the embryo and the scutellum. The scutellum absorbs nutrients from the endosperm during germination and transports it to the embryo. The germ contributes around 84 % of the total kernel oil mostly in the form of triacylglycerides (Tan and Morrison, 1979). The endosperm surrounds the germ and makes up approximately 80-84 % of the kernel dry weight and is composed of around 86

% starch by weight. Starches stored in the endosperm are held within a continuous protein matrix in which storage proteins, also known as protein bodies, are held. There are two types of starchy endosperm, namely, horny (hard) and floury (soft). The central core of the endosperm is where the floury endosperm resides with starch granules being held loosely in the protein matrix (Kikuchi *et al.*, 1982). Conversely, the harder horny endosperm, that surrounds the floury endosperm, holds the granules more tightly. In general, there is twice the amount of horny endosperm compared to floury endosperm (Watson, 1984), although proportions will vary depending on the type of corn, the general classes of which are flint corn, flour corn, dent corn, popcorn and sweet corn.

Table 2.2 Composition of dent corn kernel elements (Earle *et al.*, 1946)

Corn kernel element		Composition (% d.w.b)					
		Proportion of whole kernel	Starch	Sugar	Protein	Oil	Ash
Endosperm	Mean	82.3	86.82	0.61	8.63	0.86	0.31
	Range	80.3-83.5	83.9-88.9	0.47-0.82	6.7-11.1	0.7-1.1	0.22-0.46
Germ	Mean	11.5	8.26	11.00	18.53	34.35	10.27
	Range	10.2-13.1	5.1-10.0	10.00-12.51	17.3-20.0	31.1-38.9	9.38-11.31
Pericarp	Mean	5.3	7.31	0.34	3.54	0.98	0.68
	Range	4.4-6.2	3.5-10.4	0.19-0.52	2.9-3.9	0.7-1.2	0.29-1.01

The outer layer that surrounds the endosperm, the aleurone layer, is a single layer of cells that contain oil bodies as well as protein and mineral reserves (Becraft and Yi, 2011). It is important for the secretion of hydrolytic enzymes that digest the stored starch and proteins in the endosperm (Fath *et al.*, 2000), thereby producing free sugars and amino acids essential for embryo development. The pericarp, or hull, is situated at the periphery of the kernel and

constitutes 5-6 % of the kernel dry weight. This is the kernel's dense outer coating comprised of dead cells, underneath which is a spongy layer known the tube cell and cross cell layer which facilitate water absorption into the kernel (Watson, 1984). Below the spongy layer is a semi-permeable membrane, known as the seed coat or testa, which acts as a barrier against larger molecules entering the endosperm. The tip cap of the kernel is a fibrous structure which attaches the kernel to the cob and is also important for water adsorption.

2.3.2 Carbohydrates

2.3.2.1 Non-starch polysaccharides (NSP)

The fibre component of the kernel consists primarily of NSP, which can comprise up to 90 % of the plant cell wall structure (Bach Knudsen, 2001). The predominant cell wall's NSP include cellulose, hemicellulose and pectins. These are a heterogeneous group of polysaccharides whose main constituents include glucose, xylose, arabinose, galactose, mannose and uronic acids (Choct, 1997). The physiological impact of a particular NSP is dependent on the sugar component and the type of linkages between the sugars. Hemicelluloses include xylan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan, all of which provide a β-(1→4) linked backbone. Arabinoxylans are a linear polysaccharide β-(1→4) xylan substituted with arabinose residues (Fig 2.3), and comprise a substantial proportion of the NSP in the cell walls of maize kernels (Huisman *et al.*, 2000). Therefore it is not surprising that arabinoxylans have become one of the main focuses when investigating the effect of fibre on digestive processes.

Maize contains a relatively low concentration (l g/kg) of soluble NSP, with arabinoxylan making up the majority (Choct, 1997; Englyst *et al.*, 1989), compared to wheat (24 g/kg), barely (45 g/kg) and rye (46 g/kg). Soluble NSP have been demonstrated to have an anti-

nutritive effect on nutrient digestibility and absorption in poultry (Annison and Choct, 1991). These effects are due to the ability of soluble NSP to increase digesta viscosity thereby reducing digesta transit rate, nutrient digestibility, and increasing fermentation in the small intestine (Choct *et al.*, 1996). Insoluble NSP such as cellulose and hemicellulose are not hydrolysed by endogenous enzymes and thus can negatively affect feed efficiency. Broiler chickens do not synthesise endogenous enzymes that digest NSP and thus they rely on acid digestion in the proventriculus, grinding in the gizzard and microbial digestion in the hind gut to break down NSP. Exogenous NSP enzymes are often added to poultry diets to aid digestion.

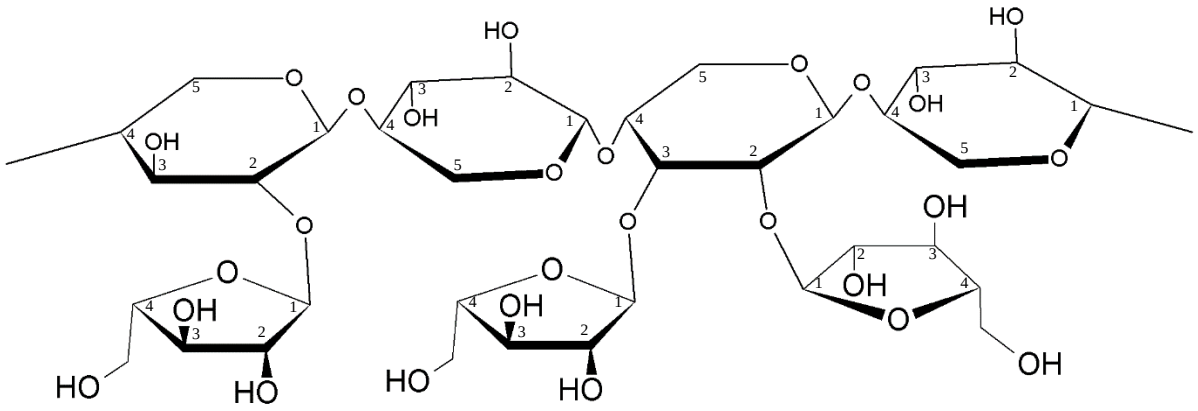


Figure 2.3 Schematic representation of arabinoxylan with a xylan backbone and arabinose residues

2.3.2.2 Starch

The main carbohydrate component of maize is starch, contributing around 72 % of the total kernel weight (FAO, 1992). Starch, the predominant storage glucan in cereals, is synthesised into a granular form within a cellular organelle known as an amyloplast. These starch granules can be isolated from seeds, roots, tubers, stems, leaves, fruits and pollen (Pérez and Bertoft, 2010), having variation in size, shape, and chemical composition depending on the botanical source.

2.3.2.2.1 Chemical composition of starch

Starch is made up of two polysaccharides, amylose and amylopectin (Fig 2.4), composed solely of glucose monomers. Amylose is an essentially linear α-(1→4) linked glucan having a low degree of branching with approximately < 1 % (1→6)-α- linkages (Delcour *et al.*, 2010). This polymer is the smaller out of the two polysaccharides in starch with a molecular weight range of approximately 10^5-10^6 (Buleon *et al.*, 1998) and a degree of polymerisation (DP) between 100-10,000. Conversely, amylopectin is a highly branched glucan consisting of around 95 % α-(1→4) and 5 % α-(1→6) linkages (Buleon *et al.*, 1998). On average, an amylopectin molecule has one branch point every 20-25 residues. It is a larger molecule than amylose with a molecular weight of approximately 10^7-10^9 and a DP between 10,000-100,000 (Keeling and Myers, 2010).

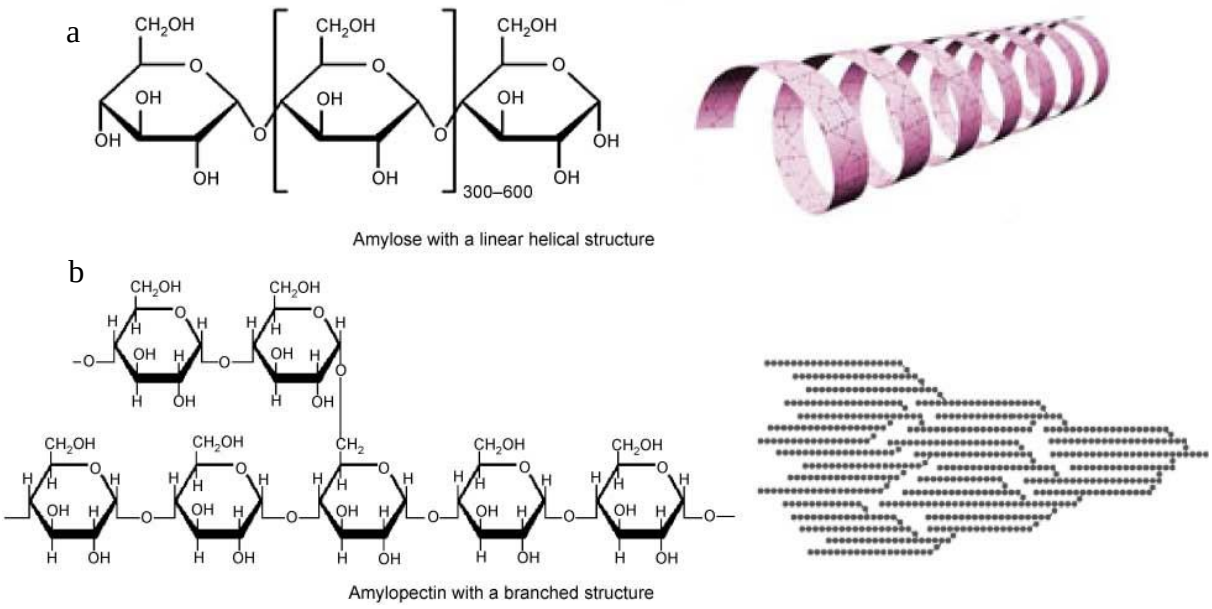


Figure 2.4 Chemical structure of amylose (a) and amylopectin (b) (Moulay, 2013)

Maize starch can be classified according to the amylose/amylopectin ratio. Native maize starch generally consists of 25-30 % amylose and 70-75 % amylopectin. Waxy starches contain near to 100 % amylopectin as a result of mutations in the genes encoding the granule bound starch synthase which is essential for amylose synthesis (Rahman *et al.*, 2000). High amylose starches, such as amylomaize, contain between 45-80 % amylose (Zobel, 1984). This ratio has a significant effect on the structure and physiochemical properties of the starch. Evidence suggests that increasing amylose content results in decreased digestibility of starches by enzymes.

2.3.2.2.2 Starch granule structure

Granule structure is complex and is highly dependent on the botanical origin. Starch granules differ in shape (spherical, oval, elongated, polygonal or lenticular) and in size (<1 μm to 100 μm diameter) depending on the botanical source. Maize starch granules have diameters ranging from 5-25 μm in the mature kernel (White and Johnson, 2003). These granules are generally spherical in shape, although becoming more polygonal as the granules expand and pack into the endosperm. The structure of the starch granule has been observed through light and electron microscopy showing a distinctive layered structure, known as "growth rings" (Fig 2.5).

The biosynthesis of growth rings initiates at the centre of the granule, known as the hilum, and continues by apposition to the periphery. The rings are alternating regions of low and high density; the disordered arrangement of amylose and amylopectin form the low-density amorphous layers while the alternating amorphous and crystalline lamellae generate the dense semi-crystalline layers (Pérez and Bertoft, 2010). The crystalline lamellae are primarily formed by amylopectin double helix side chains arranged into clusters, whereas the amorphous lamellae correspond to the branch points. Linear amylose polymers may be found

interspersed between amylopectin molecules in both crystalline and amorphous lamella (Oates, 1997), although the orientation will differ between botanical sources.

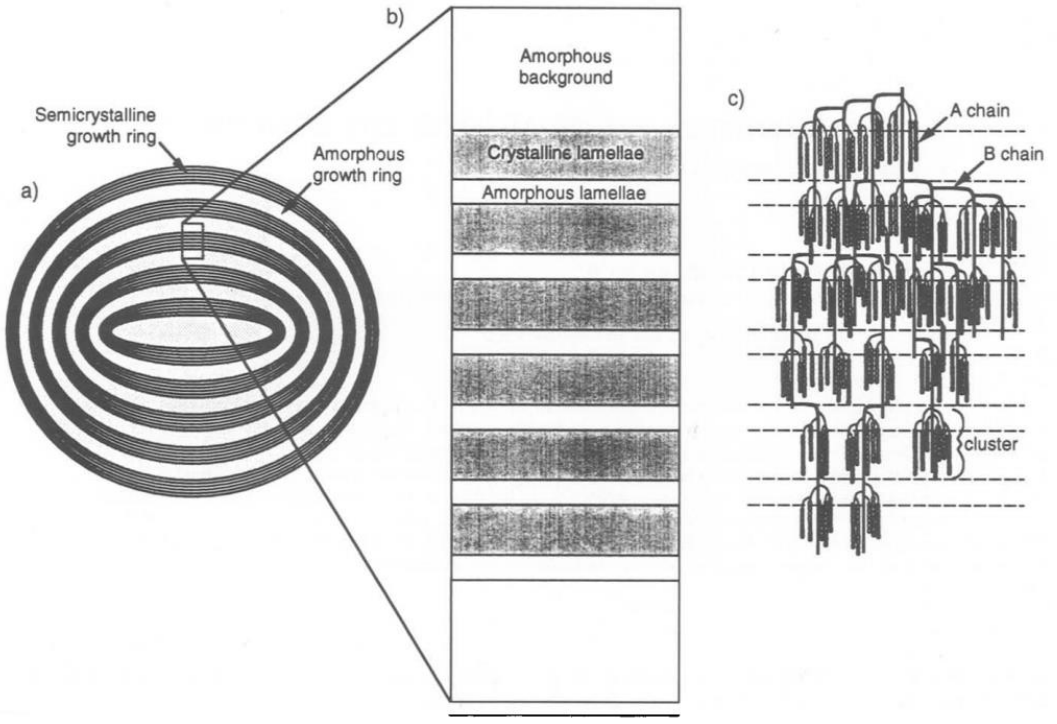


Figure 2.5 Overview of starch granule structure: (a) the starch granule, (b) the lamellae, and (c) the amylopectin cluster model (Jenkins and Donald, 1995)

The majority of starch granules demonstrate bifringence of polarised light, exhibiting a Maltese Cross due to the high degree of molecular order within the granule (Gallant *et al.*, 1997). X-ray diffraction techniques are often used to obtain information about the relative amounts of crystalline and amorphous regions. Native starch granules can differ in their crystallinity from 15 to 45 % (Zobel, 1988a) and show characteristic X-ray diffraction patterns of A, B and C type, as well as an additional V-type (Fig 2.6).

Cereal starches, such as maize, show an A-type pattern whereby amylopectin double helices are in close proximity to one another with water molecules between the structures.

Conversely, tuber starches and amylose-rich starches demonstrate a B-type pattern with a more open structure containing water molecules within the helical core. Legume and root starches as well as various fruit and stem starches may exhibit a C-type pattern, which is a combination of the A- and B-type patterns. Bogracheva *et al.* (1998) proposed that C-type starch granules are composed of B-type structures at the centre of the granule with A-type structures at the periphery. The V-type patterns are only found in amylose helical complex starches after starch gelatinization and complexing with suitable complexing agents. Generally, A-type starch has a higher crystallinity than that of B-type starch, although this depends greatly on the moisture content in the granule (Bogracheva *et al.*, 2002). This highly complex granule structure is ideal for energy storage in the plant, however in order to release this energy, enzymes must be able to access and hydrolyse the starch within the granule.

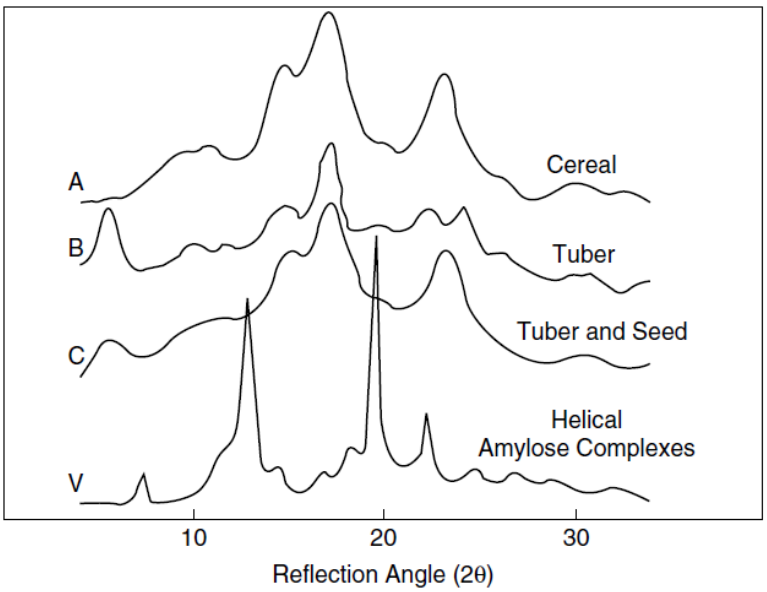


Figure 2.6 X-ray diffractograms of different starches. Tracings show A-type pattern from cereal starches (A), B-type from tuber starch (B), C-type from seed starches (C), and V-type from helical amylose complexes (V) (Zobel, 1988b)

2.4 Current understanding of starch digestion

2.4.1 Enzymatic hydrolysis

Starch hydrolysis is performed by enzymes, known as starch hydrolases (numbered EC 3.2.1), which act by cleaving the glucosidic linkage between two glucose molecules. These enzymes can be specific for α-(1→4) linkages, α-(1→6) linkages or both. For the purpose of the current study, only key starch hydrolases found in the chicken, namely α-amylase, maltase and isomaltase (Fig 2.7), and those used in digestion assays, specifically amyloglucosidase, will be discussed.

2.4.1.1 α-Amylase

α-Amylase (EC3.2.1.1) is an endo-enzyme widely distributed in plants, animals and microbes. This enzyme randomly cleaves α-(1→4) linkages in polysaccharides composed of three or more linear glucose residues (Richardson and Gorton, 2003) to produce a mixture of maltose, branched oligosaccharides (α-limit dextrins) and either maltotriose or glucose depending on the relative concentrations of enzyme and substrate (Manners, 1989). The tightly arranged intramolecular and intermolecular hydrogen bonds found in amylose increase the hydrophobicity of starch and consequently increase resistance to amylase digestion (Zhou *et al.,* 2010). Contrastingly, amylopectin's highly branched structure increases its surface area for amylase degradation and thus it is generally considered that starch digestibility increases with amylopectin content and decreases with amylose content (Gallant *et al.*, 1992).

2.4.1.2 Brush border enzymes

In the chicken, products of starch digestion by α-amylase are hydrolysed further by brush border enzymes. Maltase (EC 3.2.1.20), also known as α-glucosidase, is an exo-enzyme that hydrolyses α-(1→4) linkages from the non-reducing end primarily of maltose and maltotriose, but also oligosaccharides and polysaccharides at a slower rate, producing glucose

monomers (Richardson and Gorton, 2003). Another brush border enzyme is isomaltase (EC 3.2.1.10). This enzyme hydrolyses α-(1→6) linkages in α-limit dextrins, producing maltotriose, maltose and glucose. Once released, glucose monomers are absorbed into intestinal cells (enterocytes) to be used as energy by the bird.

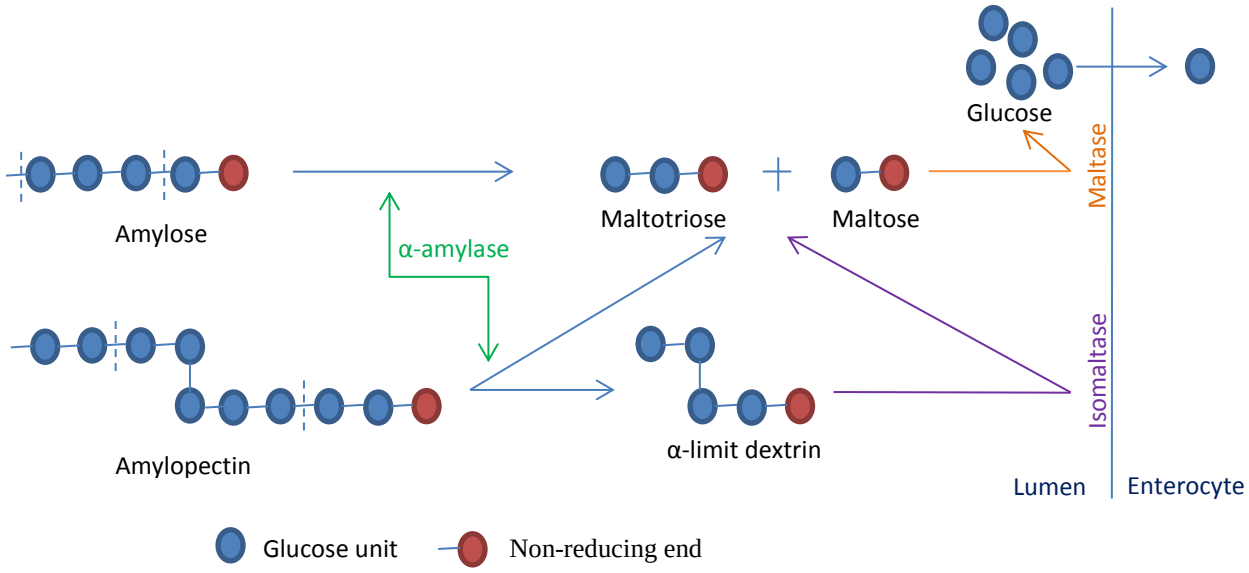


Figure 2.7 Hydrolysis of amylose and amylopectin to glucose by digestive tract enzymes. Pancreatic α-amylase hydrolyses α-(1→4) linkages of amylose and amylopectin to produce maltotriose and maltose, as well as α-limit dextrins from amylopectin. Dextrinases on the brush border hydrolyse α-(1→6) linkages of dextrin to produce maltotriose and maltose. These molecules are then hydrolysed to glucose monomers by glucoamylases on the brush border, and are subsequently actively taken up into the enterocyte for transport into the blood stream.

2.4.1.3 Amyloglucosidase

Amyloglucosidase (EC 3.2.1.3) is an exo-enzyme found primarily in moulds. This enzyme successively hydrolyses terminal α-(1→4) linked glucose residues from the non-reducing ends of malto-oligosaccharides composed of a minimum of two glucose residues, resulting in the release of glucose monomers (Richardson and Gorton, 2003). Furthermore,

amyloglucosidase can also hydrolyse α-(1→6) linkages when the subsequent linkage is α-(1→4). However, this results in a reduced rate of hydrolysis compared to the hydrolysis of linear polysaccharides. Moreover, this enzyme's rate of hydrolysis of polysaccharides is much greater than that of oligosaccharides, and it has been proposed that amyloglucosidase hydrolyses amylopectin twice as fast as maltotriose and 3-8 times faster than maltose (Matheson and McCleary, 1985).

2.4.2 Starch digestibility

Starch digestibility values are commonly used to specify the nutritional value of specific cereals, with a higher digestibility correlating to more available energy. Starch digestibility can be considered a function of granule surface area, starch structure and degree of crystallinity (Moran, 1982) as well as granule interactions with proteins and cell wall structures. It is also affected by extrinsic factors including, the processing of starch sources, gastrointestinal tract milieu, passage rate and influence of other feed material.

2.4.2.1 Susceptibility of native starch granules to enzymatic amylolysis

It is generally accepted that starches with an A-type X-ray diffraction pattern are more susceptible to enzymatic digestion than those with a B-type pattern. Shorter double helices and branched linkages within crystalline lamellae of A-type starches creates 'weak points' that make these starches more readily susceptible to digestion (Jane *et al.*, 1997). Morphological investigations into specific granule surface features have shown the presence of naturally occurring openings, known as pores, that are randomly distributed over the granule surface (Fannon *et al.*, 1992). The finding that porosity correlates with enzyme digestion patterns suggests that these openings are the 'weak points' that give enzymes direct access to the granule interior (hilum). Fannon *et al.* (1993), later reinforced this by observing that pores are openings to serpentine channels that penetrate to the granule hilum.

Enzyme adsorption onto the granule surface, forming an enzyme-substrate complex, is the initial step of enzyme action. Since amylase needs at least 3 glucose units in its active site for hydrolysis of the glycosidic linkage, the binding of amylase to the starch molecule would need to be in a direction parallel to the double helices in the internal channel (Zhang *et al.*, 2006a). The current starch model (Fig. 4.5) proposes the perpendicular alignment of crystalline regions to the granule surface. Consequently, hydrolysis from the sides of the lamellae would result in the enlargement of pores and channels and ultimately, fractionation of granules. This mechanism is known as side-by-side digestion (Zhang *et al.*, 2006a), and is concurrently followed by hydrolysis from the hilum towards the granule surface, in an inside-out digestion pattern (Wang *et al.*, 1995). It is suggested that the disordered amorphous rings are more susceptible to enzyme attack than the ordered crystalline regions (Gallant *et al.*, 1992) and therefore the amorphous regions are digested first.

A relationship between granule particle size and enzymatic susceptibility has been established (Kong *et al.*, 2003); with a smaller granule size, and thus a larger surface area, resulting in a higher digestibility. Differences have been observed in the mode of enzyme action on large and small granules. For large granules, pores were evident on the granule surface after amylase digestion, supporting previously discussed enzyme access to the granule. While conversely, no pores were seen on small granules, instead a rough surface was apparent, characterised by surface erosion and solubilisation (Franco *et al.*, 1992; MacGregor and Ballance, 1980). These results may suggest a difference in the physical structure of small and large granules. Therefore, a greater enzyme susceptibly of smaller granules may not solely be credited to surface area but also structural differences that can affect rate of digestion.

2.4.2.2 Classification

Dietary starch has been classified into rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (Englyst *et al.*, 1992). RDS is digested and absorbed

rapidly in the duodenum and proximal small intestine, resulting in elevated postprandial blood insulin and glucose levels (high-glycaemic index). SDS is slowly digested through the whole tract of the small intestine, allowing a slow, sustained glucose release (low glycaemic-index) and thus avoids these large glucose fluctuations. RS can be defined as "the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals" (Asp, 1992). Instead RS is fermented in the large intestine into short chain fatty acids which aid colonic health and also provides a low glyceamic-index. Moreover, RS can be further classified into four types (Englyst *et al.*, 1992); RS1-4. RS type 1 is defined as enzyme physically inaccessible starch, RS type 2 are starch granules that are resistant to digestion, RS type 3 is retrograded starch and RS type 4 is a chemically modified starch. The estimated rate and extent of digestion of these three main classes of starch can be determined *in vitro* using Englyst's method (Englyst *et al.*, 1992); RDS is the fraction of starch digested within the first 20 min of incubation, SDS is the fraction of starch digested between 20-120 min of incubation, and RS is the remaining fraction that cannot be further digested after 120 min. In human nutrition, it is well established that consuming SDS or RS rather than RDS is beneficial for health (Birt *et al.*, 2013; Lehmann and Robin, 2007). While conversely, RS in broiler nutrition imposes a problem of energy wastage. Current evidence also suggests that SDS has a positive effect on broiler performance, compared to RDS (del Alamo *et al.*, 2009; Weurding *et al.*, 2003)

Weurding *et al.* (2001) showed that site, rate and extent of starch digestion in the small intestine of broilers varies depending on the botanical starch source. Digestion rate can influence broiler performance through the regulation of insulin and protein deposition. It has been argued that a slow, continuous release of glucose from SDS improves broiler performance by having an amino acid sparing effect (Weurding *et al.*, 2003), thereby enhancing protein and energy utilisation in birds. Weurding *et al.* (2001) also concluded that

site of starch digestion is not an accurate indicator for starch digestion rate due to the influence that diet composition has on feed passage rate. Therefore, it is important to take into account not only the type of starch being fed, but also the other food components, as this will affect the rate and extent of starch digestion in broilers.

2.5 The anatomy and digestive physiology of the chicken

Intensive selection for growth in the modern-day broiler has led to huge pressures on the digestive tract of birds (Fig 2.8). An average adult broiler consumes approximately 10 % of its live weight per day, signifying that the digestive tract has to cope with the ingestion of around 7 g feed per hour (Svihus, 2014a). This high intake of feed must be met with a high digestibility and absorption capacity to prevent losses. The feed conversion ratio (FCR) is a measure of the amount of feed (kg) required to produce 1 kg poultry meat and is used to assess broiler performance. A lower FCR indicates an enhanced bird performance through improved utilisation of the diet. Functionality of the digestive tract will influence the response to a diet and therefore understanding digestive processes that occur in the bird is essential for diet selection and results interpretation.

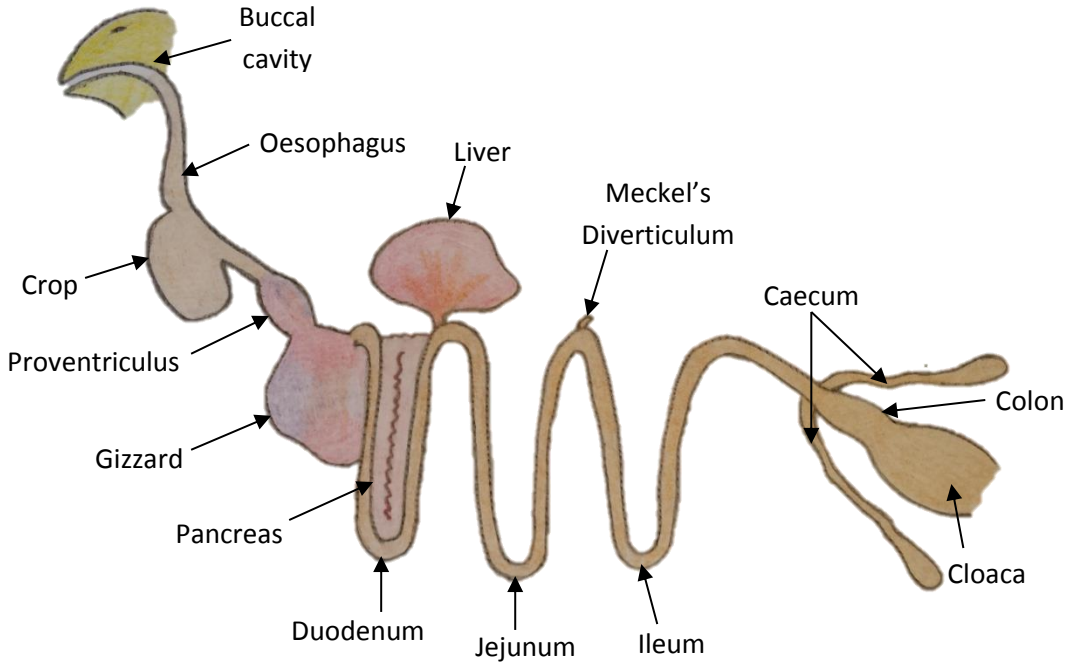


Figure 2.8 Schematic representation of the digestive tract of broilers

2.5.1 The buccal cavity

Poultry do not contain teeth and therefore there is no mastication of feed in the mouth, instead feed is consumed whole. However, the mouth does contain salivary glands to moisten the bolus of food aiding its passage into the oesophagus. Adult birds can produce 7-30 ml saliva per day depending on feeding environment (Larbier and Leclercq, 1994). Jerrett and Goodge (1973) reported negligible secretion of amylase from chicken salivary glands and thus it can be assumed that no starch digestion takes place in the mouth.

2.5.2 Oesophagus and crop

The oesophagus or gullet is a distensible tube that transports the food bolus from the mouth to the proventriculus via peristaltic movements. It can be divided into two parts; the upper tract is cervical and closely connected to the arterial system, while the lower tract is thoracic, being

positioned above the heart (Larbier and Leclercq, 1994). These two parts are separated by a dilatory lobe known as the crop. The crop is a pouch in the oesophagus that temporarily stores food thereby creating a reservoir that regulates release of food into the proventriculus. Therefore, if a bird is on restricted feeding or fed in meals, it can rapidly store large amounts of food in the crop and steadily release it throughout the day. Feed within the crop becomes moistened by mucus secretions from the mucosa of the crop wall. The pH of the crop is around 5 (Farner, 1942). Philips and Fuller (1983) reported amylase activity in the crop of chickens, indicating that some carbohydrate digestion may occur in this section. The presence of amylase may be due to intestinal reflux, plant or bacterial sources. Hydrolysed starches are either absorbed, transported down the digestive tract or converted into alcohol or lactic acid (Bolton, 1965). Fisher and Weiss (1956) reported that cropectomy of 1 week old chicks has no effect on food intake or weight gain if birds were fed *ad libitum*. Conversely, if feed was resisted during the day, growth was impaired (Feigenbaum *et al.*, 1962). Therefore, the main function of the crop is food storage; it is not essential for growth, food intake or digestion when feed is unrestricted. Emptying of the crop is dependent on crop capacity, gizzard fullness, and feed particle size and moistening.

2.5.3 Proventriculus and gizzard

Food leaves the crop via the lower oesophagus tract and enters the glandular stomach known as the proventriculus. The proventriculus is lined with a glandular mucus membrane that secretes hydrochloric acid (HCl) and pepsinogen, which is subsequently cleaved into pepsin, initiating food digestion. Basal gastric secretions have an average pH of 2.6 (Joyner and Kokas, 1971). Food remains in the proventriculus for a relatively short time, from minutes up to an hour, before it leaves via the isthmus, termed the *zona intermedia gastric*, to enter the gizzard (Denbow, 2015). The highly muscular gizzard (or ventriculus) is a major site of mechanical grinding that breaks feed down into smaller particles. The circular thin muscle

pair and thick muscle pair of the gizzard act in opposition (Denbow, 2015) to mechanically grind food particles. HCl and pepsin continue their function (gastric proteolysis) within the gizzard. Grinding of feed material is aided by insoluble grit that is retained in the gizzard. The inner surface of the gizzard is lined with a proteinaceous substance, known as koilin, which protects the muscles of the gizzard from the damaging effects of the gastric acid and mechanical action (Gussekloo, 2006). The green-brown colour of the koilin layer is due to bile reflux from duodenum (Denbow, 2015). Food chyme from the gizzard enters the duodenum via the pyloric region, which in the chicken is only 0.5 cm in length (Denbow, 2015), and thus prevents large particles from entering the duodenum.

2.5.4 Small intestine

In the adult chicken, the total length of the small intestine is around 120 cm and is divided into three parts: duodenum, jejunum, and ileum (Larbier and Leclercq, 1994). The duodenum is approximately 24 cm in length and is U-shaped, with the pancreas being enclosed within the "U". The pancreas has three ducts that, along with the two bile ducts from the liver, drain into the ascending loop of the duodenum (Denbow, 2015). Pancreatic secretions contain a cocktail of enzymes for the hydrolysis of protein, carbohydrate and lipid. It also comprises bicarbonate to increase the pH of the gastric chime to a level above pH 6, allowing optimal enzyme activity.

Studies have shown that 97 % maize starch is digested in the small intestine of broilers (Weurding *et al.*, 2001). Riesenfeld *et al.* (1980) reported digestion of around 65 % ingested starch by the end of the duodenum, 85 % up to the posterior jejunum and 97 % at the terminal ileum. The jejunum and ileum are distinguished by an appendage of the small intestine, known as Meckel's diverticulum, which was used in the embryo to join the intestine to the vitelline sac for the transfer nutrients. Amylase is secreted by both the pancreas and intestine

(Osman, 1982). Although amylase is active in all parts of the small intestine, approximately 80 % of amylase activity occurs in the jejunum. High enzymatic activity in this section is presumably due to pancreatic secretions entering near to the anterior jejunum. The optimum pH for intestinal and pancreatic amylase activity is pH 6.9 and 7.5, respectively (Denbow, 2015). Chicks are adapted to starch digestion very early on in development. Amylase, maltase and isomaltase activities are detectable in the embryo at day 18 of incubation, with pancreatic amylase reaching its maximum activity just 4 days after hatching (Moran, 1985). Products of starch digestion, namely maltose, maltotriose and α-dextrins are subsequently hydrolysed into glucose monomers by the major brush-border enzymes, maltase and isomaltase (Svihus, 2011). This allows active uptake of this monosaccharide into the enterocyte for transport into the blood stream.

Intestinal absorption of glucose is mediated by transporters localised on the brush border membranes of enterocytes. Glucose is transported across the enterocyte membrane via a sodium-glucose cotransporter (SGLT1). SGLT1 is a high affinity, low capacity transporter situated on the apical side of enterocytes (Braun and Sweazea, 2008). It actively transports glucose and galactose, along with two sodium ions down a concentration gradient from the intestinal lumen into the enterocyte (Kimmich and Randles, 1984). On the basolateral membrane of enterocytes, a glucose transporter (GLUT2) moves glucose out of the cell via facilitated diffusion. Absorption of carbohydrates is essential for maintaining energy supplies in birds for growth. This process is influenced by luminal digestion, brush border digestion and transport into the enterocyte. Quantifying expression of intestinal transporters provides valuable information regarding the control of nutrient uptake. SGLT1 and GLUT2 mRNA expression have been demonstrated to increase linearly with age from embryonic day 20 to day 14 post-hatch (Gilbert *et al.*, 2007), with expression being greatest in the jejunum (Gilbert *et al.*, 2008). Moreover, Gal-Garber *et al.* (2000) revealed that SGLT1 mRNA expression is

affected by nutritional status, with enhanced expression in starved and re-fed birds compared to fed birds. Conversely, GLUT2 expression in the jejunum is unaffected by either fasting or re-feeding (Duarte *et al.*, 2011).

2.5.5 Large intestine (caecum and colon)

In chickens, two caeca, approximately 20 cm in length, can be found at the beginning of the rectum (colon), separately connected to the lateral walls of the intestine. At the opening of the caeca, sphincter muscles have been suggested to be present along with well-developed villi to control chyme movement through the ileo-caeco-colonic junction (Denbow, 2015). The caeca of adult chickens are the sites of greatest micro-organism fermentation. The pH of the caeca is generally considered to be around pH 7, although this will alter depending on the diet (McNab, 1973), and consequently microbial content will also change accordingly. Fermentation in the caeca generates volatile fatty acids (VFA) such as acetate, propionate and butyrate, which are passively absorbed from the lumen into the epithelial cells lining the caeca (Denbow, 2015). Glucose absorption in the caecum has also been documented, with accumulation of sugar becoming more localised to the proximal region of the caeca during development (Planas *et al.*, 1986; Savory and Mitchell, 1991). Sugar transport appears to be through a sodium-dependant mechanism (Ferrer *et al.*, 1986) against a concentration gradient, similar to that of the small intestine. The rectum (or colon) is a relatively short tract connecting the ileum to the cloaca. The main role of this intestinal region is the absorption of water.

2.5.6 Cloaca

The cloaca provides as a common pathway for excretory, reproductive and digestive wastes. It can be divided into three sections; the coprodeum, urodeum and proctodeum (Denbow, 2015). The contents of the colon empty into and accumulate in the coprodeum. Regular

defecation is achieved by the rapid contraction of muscles within the coprodeum (Larbier and Leclercq, 1994). This section then leads onto the urodeum, into which the urinary and reproductive tracts empty their contents. Urine entering the urodeum may be transported to the caeca by antiperistalsis where electrolytes and water are absorbed. This results in concentrated urine in the form of insoluble urates which coat the excrement producing a white paste. The final section, the proctodeum, expels the contents from the body through the anus.

2.6 Carbohydrases in poultry feed

Exogenous enzymes have been used in animal feed for several years to reduce feed costs and pollution. Most commercial enzyme products currently consist of multiple enzymes to target various components of the feedstuff thereby increasing its digestibility. Current knowledge on the function of exogenous enzymes, in particular xylanase, in maize-based diets is not only limited, but also inconsistent. Multiple enzyme complexes (xylanase, protease, and amylase) have demonstrated improved performance of broilers fed maize-soy diets (Aftab, 2012; Cowieson and Ravindran, 2008a; Cowieson and Ravindran, 2008b; D'Alfonso, 2005). Zanella *et al.* (1999) reported a 3 % improvement in energy and amino acid digestibility of a maize-based diet for broilers supplemented with a xylanase, amylase and protease complex. While other studies did not find any significant effect of xylanase supplementation on performance in maize fed birds (Ritz *et al.*, 1995; Singh *et al.*, 2012). Consequently, part of this project focuses on the application of exogenous xylanase singly to a maize-soy diet, in order to better understand mechanisms of enzyme action.

2.6.1 Xylanase

Poultry do not produce endogenous enzymes that hydrolyse dietary NSP. Therefore, the NSP fraction remains relatively intact through the tract, resulting in a poor nutritive value of diets (Annison and Choct, 1991). This gives opportunity for supplementation of diets with exogenous enzymes that hydrolyse NSP, reducing intestinal viscosity and improving nutrient digestibility. Considerable attention has been given to improve nutrient utilisation of diets through the use of exogenous carbohydrases (Aftab, 2012; Masey O'Neill *et al.*, 2014b), including amylases, cellulases and xylanases, to release more energy from the diet. Xylanase enzymes hydrolyse arabinoxylans into xylose thereby cleaving hemicellulose, a major component of plant cell walls. Traditionally, exogenous xylanases have been added to viscous diets, such as wheat, barley, and rye, whereby significant levels of soluble arabinoxylans have been demonstrated to have anti-nutritive effects on nutrient digestibility and absorption in poultry (Annison and Choct, 1991; Bedford and Schulze, 1998; Choct, 2006). These effects are often accredited to the ability of soluble NSP to increase digesta viscosity thereby reducing digesta transit rate, nutrient digestibility, and increasing fermentation in the small intestine (Choct *et al.*, 1996). Maize contains a relatively low concentration of soluble NSP (Choct, 1997) compared to other cereals, such as wheat, suggesting a possible explanation for the lower magnitude of broiler response to xylanase supplementation in maize-based diets. However, there is evidence to suggest a beneficial effect of xylanase inclusion in maize-based diets through improved broiler performance (Cowieson, 2010; Zanella *et al.*, 1999), indicating that additional mechanisms other than reduction in viscosity are important. It is important to note however, that these studies focus on the use of multi-enzyme applications and therefore the mechanisms of xylanase action exclusively must also be considered.

2.6.1.1 Direct mechanism

It is still unclear how such enzymes improve digestibility in maize-based diets. One well documented explanation is cell wall dissolution, whereby xylanases hydrolyse the insoluble NSP fraction in maize (Meng and Slominski, 2005). This leads to the release of nutrients, including starch, protein and lipids, from the cell wall matrix thereby positively aiding nutrient digestibility (Bedford, 2002). However, the site of enzyme action in the gut combined with digesta transit time suggests that there is insufficient time for exogenous xylanases to significantly degrade cell wall material directly by the small intestine. Therefore, it has been suggested that xylanases may be working via an indirect mechanism (Singh *et al.*, 2012).

2.6.1.2 Indirect mechanism

Endo-xylanase hydrolysis of arabinoxylans leads to the production of beneficial arabinoxylo-oligosaccharides (AXOS). Courtin *et al.* (2008) reported a comparable improvement in FCR of birds fed a maize diet supplemented with either xylanase or wheat bran oligosaccharides derived from xylanase-treated wheat bran. This suggested that oligosaccharides produced from xylanase degradation of fibrous material are influential to broiler performance. An increase in movement of xylo-oligomers to the caeca has been linked to an increase in VFA concentration through fermentation in this section (Choct *et al.*, 1996; Choct *et al.*, 1999). The presence of VFA in the intestinal lumen is suggested to promote peptide YY (PYY) release from endocrine L-cells, located predominantly in the distal ileum and colon, into the blood (Cuche *et al.*, 2000). PYY is a neuropeptide that acts on the hypothalamus in the brain to delay gastric emptying, a process also known as ileal brake or gastroparesia. Feed is therefore retained in the proventriculus and gizzard for longer, resulting in a finer ‘grind’ and improved protein digestion. As the protein coating the starch granules is digested it allows for greater access of the amylase to the granules, thereby improving starch digestibility. AXOS

have also been described as having a prebiotic value through promoting the colonisation of beneficial microflora in the gut (Nian *et al.*, 2011). The VFA produced through AXOS fermentation, predominantly propionate, not only act to stimulate PYY secretion but also supress potential bacterial outbreaks, such as salmonella (Bedford, 1996; Hinton *et al.*, 1993). It is also possible that greater digestion in the more proximal regions of the digestive tract reduces the amount of undigested macromolecules in the ileum that can cause growth of detrimental bacteria that compete for nutrients.

2.7 Project aims and objectives

Maize is widely used as an animal feed ingredient, providing a high energy value. Although maize is generally considered to be highly digestible, variability in composition and quality can have detrimental effects on the nutritive value of feed and subsequent animal performance. Therefore, predicting quality is of great interest to formulators and producers. However, there is still a demand for an appropriate method for predicting quality accurately *in vitro*. There is a lack of integration between the vast number of techniques and expertise applied in the human food sector and that which is employed in the animal feed industry. Consequently, part of this project aimed to investigate better ways of characterising and describing maize, while creating a link to animal nutritional value.

Additionally, while supplementation of animal feed with exogenous enzymes is common practice world-wide, broiler response to xylanase supplementation of a maize-based diet still appears to be inconclusive. To better understand what response the bird has to feed additives, it is firstly important to understand the mechanisms by which these additives are acting. Therefore, another purpose of this study was to determine the mechanisms of xylanase action in a maize-based diet.

With potential industrial application, the specific objectives for the work performed and reported in this thesis were as follows:

- To develop an *in vitro* model to mimic the digestive tract of broilers
- To identify the role of the gizzard in the digestion of starch
- To understand where and how native starches are digested in the broiler
- To identify some of the limitations to starch digestion
- To investigate the effects that other feed components have on starch digestion
- To determine the relevance of exogenous xylanase supplementation for starch digestion

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

The general chemicals used in this study are listed in Table 3.1. All the chemicals were of analytical grade.

Table 3.1 Chemical list and respective suppliers

Chemicals and product code	**Suppliers**
Acetic acid glacial (CHE1012)	Scientific laboratory supplies
Amylase from *Aspergillus oryzae* (A8220)	Sigma Aldrich
Calcium chloride (C1016)	Sigma Aldrich
Dimethyl sulfoxide (DMSO) (9463)	Sigma Aldrich
Ethanol Absolute (10048291)	Fisher scientific
Hydrochloric acid (38282)	Sigma Aldrich
MOPS (M9027)	Sigma Aldrich
Pancreatin from porcine pancreas (P1750)	Sigma Aldrich
Pepsin from porcine gastric mucosa (P7000)	Sigma Aldrich
Sodium bicarbonate (217125000)	Acros Organics
Sodium hydroxide (S8045)	Sigma Aldrich
Xylanase (Econase® XT 25)	AB Vista

3.1.2 Digestive enzymes

3.1.2.1 α-amylase

An aqueous solution of α-amylase from *Aspergillus oryzae* was obtained from Sigma-Aldrich. Enzyme activity was ≥ 800 FAU/g, with one fungal amylase unit (FAU) being defined as the amount of enzyme which hydrolyses 5.26 g starch per hour at 37 °C, pH 4.7.

3.1.2.2 Pancreatin

Powdered pancreatin from porcine pancreas was acquired from Sigma-Aldrich, with an activity of 4 x United States Pharmacopeia (USP) unit. Pancreatin will hydrolyse ≥ 25 times its weight of potato starch in 5 min in water at 40 °C, will digest ≥ 25 times its weight of casein in 1 h at 40 °C, pH 7.5, and will liberate 2 ≥ microequivalents of acid per min, per mg pancreatin, from olive oil at 37 °C, pH 9.

3.1.2.3 Pepsin

Powdered pepsin from porcine gastric mucosa was obtained from, Sigma-Aldrich, with an activity of ≥ 250 U/mg. One unit will produce a ΔA_{280} of 0.001 per min at 37 °C, pH 2, measured as trichloroacetic acid (TCA)-soluble products using haemoglobin as a substrate.

3.1.2.4 Xylanase

A commercial xylanase, Econase XT 25, was supplied by AB Vista. Econase XT 25 is an intrinsically thermostable endo-1, 4-β-xylanase produced by a strain of *Trichoderma reesei*. Recovery of the enzyme in feed, using an ELISA method, confirmed an activity level of 18,100 BXU/kg feed. One xylanase unit (BXU) is the amount of endo-1, 4-β-xylanase that liberates 1 mole xylose from birch xylan per sec, at 50 °C, pH 5.3.

3.1.3 Diets

Unmodified regular maize starch was purchased from Sigma Aldrich. To improve palatability in feeding trials, maize starch was mixed in a ratio of 60:40 with water, producing a crumb. Maize grit (ground maize) and maize-soy diets were supplied by Target Feeds (Whitchurch, Shropshire, UK). Both diets were ground through a 5 mm screen by the supplier. Diet composition for the maize-soy mash is shown in Table 3.2. Nutritional information for the maize grit diet was not available, as this diet was intended for direct use in mash feed.

Table 3.2 Composition of raw materials and nutrient content of maize-soy diet

Ingredient	g/kg	Nutrient	g/kg
Maize	624.5	AME MJ/kg	12.9
Soya extract hipro	260.0	Crude protein	204.3
Full fat soybean extruded	50.0	Calcium	9.4
Llysine HCl	4.0	Available phosphorous	4.8
DL-methionine	4.0		
L-threonine	1.5		
Soya oil	20		
Limestone	12.5		
Monocalcium phosphate	15.0		
Sodium chloride	2.5		
Sodium bicarbonate	1.5		
Vitamin and Mineral premix	4.0		
Elancoban (anticoccidial)	0.5		

3.2 Methods

3.2.1 In vitro digestion

An *in vitro* model to simulate the digestive tract of chickens was designed and adapted from Bedford and Classen (1993) and Walk *et al.* (2012). Starch digestion in a maize grit diet was

explored according to the plan given in Figure 3.1, to understand the effect that gizzard conditions (HCl-pepsin and gizzard grinding) have on starch digestion. Subsequently, starch digestion in different maize diets (native maize starch, maize grit and maize-soy) was investigated (Fig 3.2) to better understand how diet composition influences the extent of starch digestion.

3.2.1.1 Gastric phase

In a method from Walk *et al.* (2012), 1 g maize diet was incubated with 0.1 M HCl (4.5 ml) containing 2000 U pepsin/ml to mimic the gastric phase. The pH of this mix was tested using an Inolab pH level 1 meter and gave a reading of pH 1.22. This pH was lower than *in vivo* gizzard content readings of pH 2-3, and therefore a modification of the method was made using a 0.01 M HCl solution (pH 2). When 0.01 M HCl (4.5 ml) containing 2000 U pepsin/ml was added to 1 g maize grit diet it gave a pH of 2.8, which was within the desired pH range and thus was used as a solution for *in vitro* digestions. Distilled water was also used as a solution for comparison, to determine the effect that HCl-pepsin has on maize starch digestion.

To mimic the grinding action of the gizzard, diets were ground using a ball mill (Fritsch, Pulverisette 5 ball mill). Once weighed, samples were poured into a 500 ml zirconium oxide (94.2 % ZrO_2) grinding bowl containing five grinding balls (20 mm). Samples were either dry milled or suspended in 4.5 ml solution (HCl-pepsin or water). A milling cycle consisted of a 10 min milling period at a rotational speed of 60 rpm, followed by a 5 min pause to avoid overheating, with 2 cycle repetitions, and a total milling time of 45 min. Following milling, samples were washed from the bowl using a wash bottle filled with distilled water and transferred into a 50 ml centrifuge tube. Additional water was added until the fill line was reached.

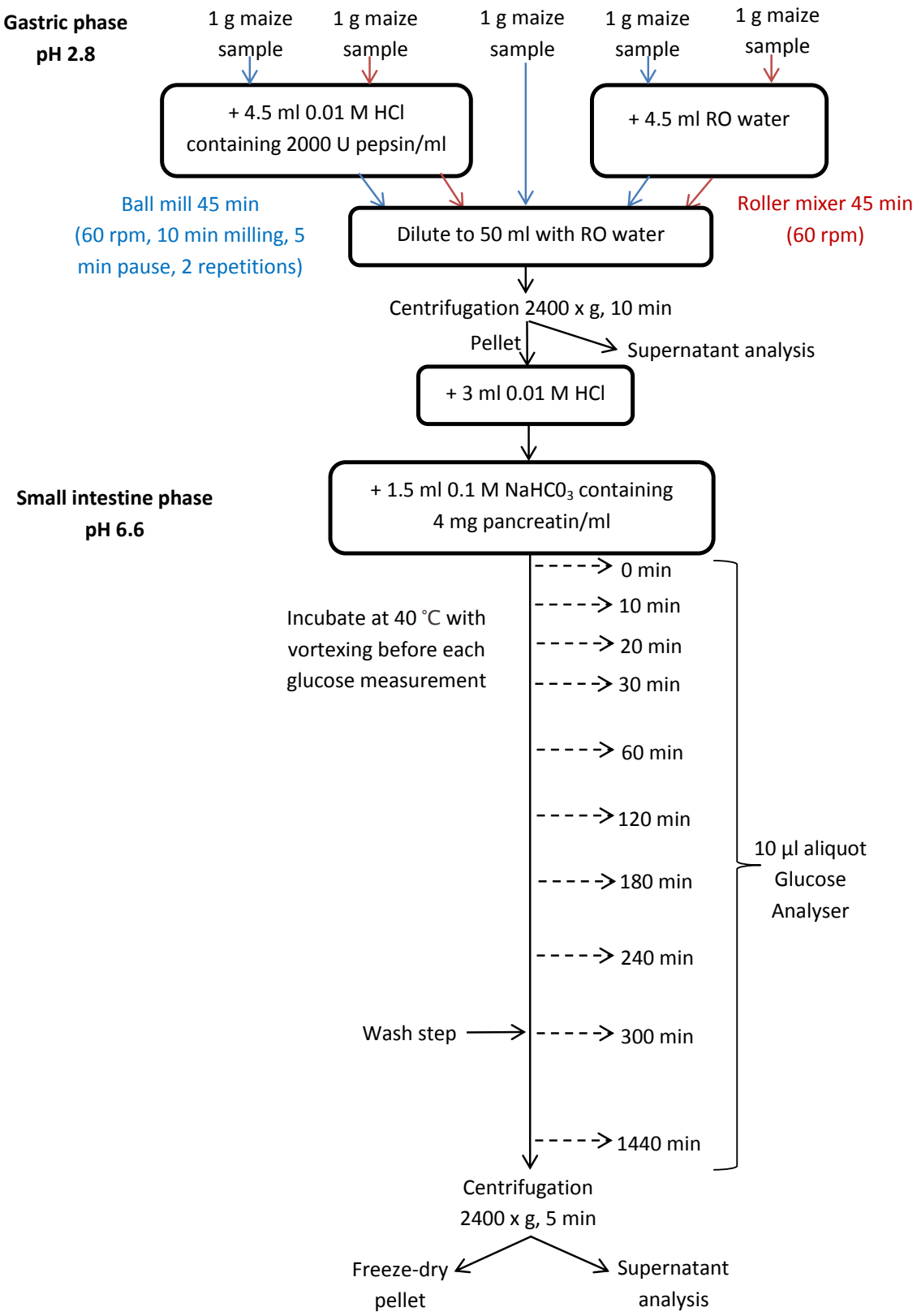


Figure 3.1 *In vitro* digestion procedure for determining the effect that gizzard conditions have on starch digestion in a maize grit diet.

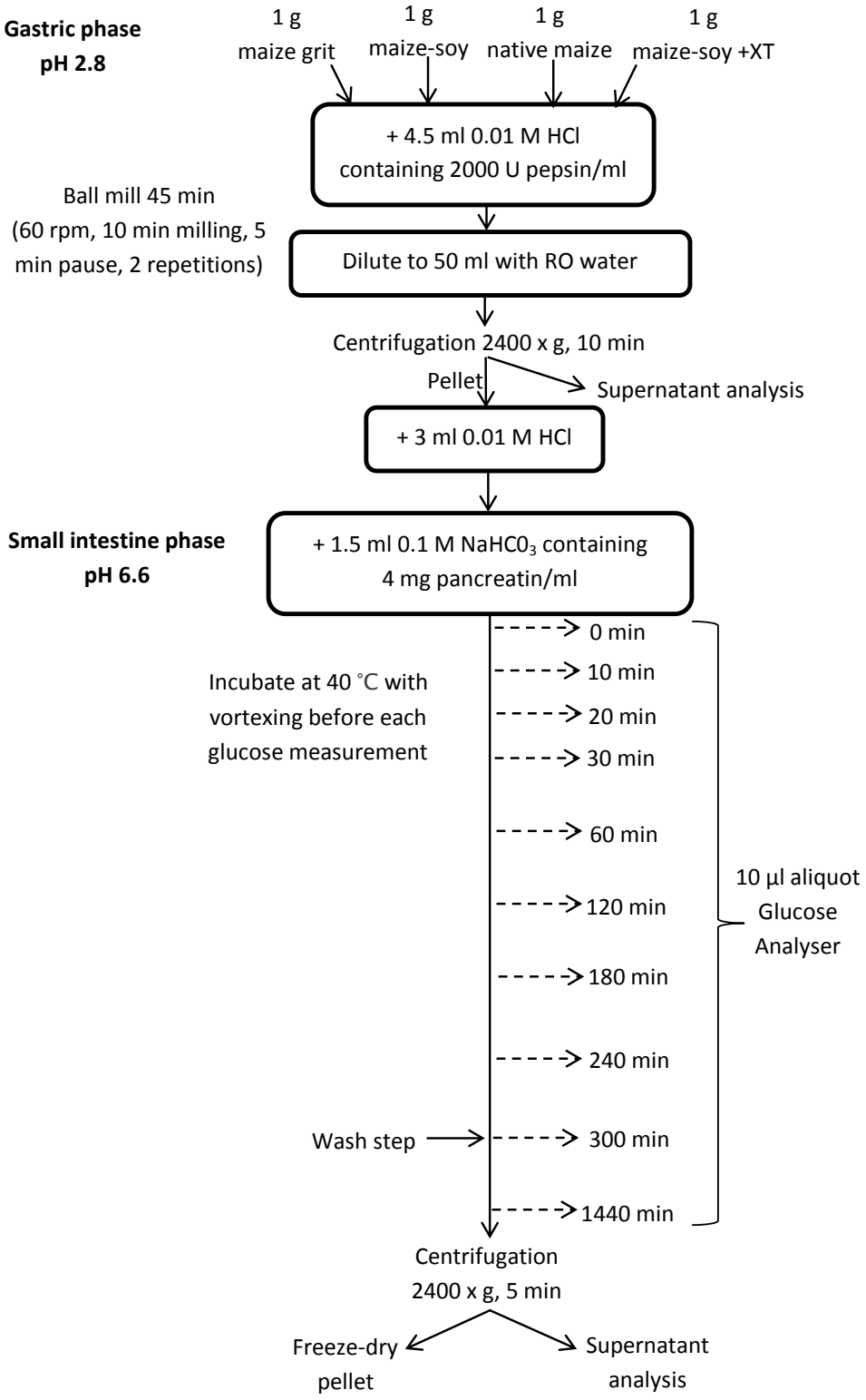


Figure 3.2 *In vitro* digestion procedure for determining the extent of starch digestion in different maize diets.

To compare the effect that ball milling has on the digestion of starch in a maize diet, samples were also simply mixed. Maize samples (1 g) were weighed into a 50 ml centrifuge tube and 4.5 ml solution added. Tubes were placed on a tube roller (Stuart, SRT9D) and rotated at 60 rpm for 45 min. Subsequently, samples were diluted with distilled water to the fill line. All samples were then centrifuged (Thermo Scientific Jouan CR3i multifunction centrifuge) at 2400 x *g* for 10 min, with the supernatant being collected in a 70 ml sterile container. At this point, pellets were either freeze-dried (Edwards Super Modulyo freeze dryer) or digested in the intestinal phase.

3.2.1.2 Intestinal phase

Following the gastric phase, 0.01 M HCl (3 ml) was added to the pellets to resemble gastric juices entering the duodenum. Immediately, the pH was increased to around pH 6.6 with addition of 0.1 M $NaHCO_3$ (1.5 ml) containing 4 mg pancreatin/ml. Samples were vortexed and glucose concentration measured using a glucose analyser (Analox GM9 Analyser), see section 3.2.4.5. Samples were then incubated in a water bath at 40 °C for 24 h, with samples being vortexed and glucose concentration measurements taken at 10, 20, 30, 60, 120, 180, 240, 300 and 1440 min.

The method was adapted during the experimental process to include a wash step at 300 min for the collection of soluble starch fractions produced during the digestion process. Repeat digestion of the maize-soy diet with and without xylanase supplementation consisted of washes every 60 min until 300 min. A wash step was performed after the glucose measurement was taken and involved dilution of the sample to 50 ml with distilled water. Subsequently, samples were centrifuged at 2400 x *g* for 5 min, with the supernatant being collected in a 70 ml sterile container. Pellets were re-suspended with 0.1 M $NaHCO_3$ (3 ml) and 0.1 M $NaHCO_3$ (1.5 ml) containing 4 mg pancreatin/ml and the sample re-incubated.

At the end of the digestion process, final glucose concentrations were measured and the sample diluted to 50 ml with distilled water. Samples were then centrifuged at 2400 x *g* for 5 min, with the supernatant being collected in a 70 ml sterile container. Pellets were frozen at -20 °C before freeze-drying. Supernatants were diluted to 50 ml with distilled water and the glucose concentration measured. Aliquots (200 µl) of supernatant were digested with 10 µl amyloglucosidase (Megazyme International Ireland Ltd, K-TSTSA, 33 U) at 50 °C for 1 h and the concentration of glucose measured.

The concentration of glucose released from starch was calculated by the following equations:

$$Glucose\ released\ (mg) = \frac{Glucose\ concentration\ \left(\frac{mg}{dl}\right) * 6}{100}$$

$$Glucose\ released\ \left(\frac{mg}{g}\ Starch\right) = \frac{Glucose\ released\ (mg)}{Starch\ content\ (g)}$$

3.2.2 In vivo digestion

3.2.2.1 Animals and housing

Day-old male Ross 308 broiler chicks were supplied from a commercial hatchery. Male broiler chicks weighing closest to 42 g at day 1 were selected and raised on a maize-soy mash diet. Diet changes are discussed in appropriate chapters. Water was provided *ad libitum* throughout the trials. Chicks were kept in an appropriate warm environment with a progressive decrease in temperature from 35 to 21°C. All birds were group housed until specified for a particular trial.

3.2.2.2 Sample collection

On the day of slaughter, birds were euthanised by an intravenous injection of pentobarbital with cervical dislocation to confirm death. Gastrointestinal tract contents (digesta) were flushed with 10 ml distilled water from the gizzard, upper (to) and lower (from Meckel's Diverticulum) small intestine, and collected in sterile containers. Caeca contents were also collected in the final trial. Digesta pH was measured using an Inolab pH level 1 meter. The brachial vein under the wing was cut using a scalpel blade and blood glucose measured using an Accu-chek mobile blood glucose monitor (Roche). Digesta samples were stored at -80 °C before being frozen in liquid nitrogen and freeze-dried.

3.2.2.3 Peptide YY analysis

Following cervical dislocation, blood was drawn from the aorta using a needle and syringe, and collected in a centrifuge tube at room temperature, to allow coagulation. Samples were then centrifuged at 1623 x *g* for 10 min to allow separation of plasma and serum. Serum was transferred into an Eppendorf® tube and stored at -20 °C. Serum samples were analysed using a chick specific PYY ELISA (Cusabio, China) as previously validated at Nottingham Trent University. Samples were analysed in duplicate using 25 µl serum, and repeated at 50 µl per well where samples were below detection limits. Sample PYY concentrations were calculated using a specific plate reader program (Multiskan Ascent) calibrated with the kit standards, which were run for each plate assayed. Two quality control samples were also measured with each plate to monitor inter assay coefficient of variation. Samples with coefficients of variation greater than 15 % were repeated.

3.2.3 Starch granule morphology

3.2.3.1 Optical microscopy

Surface properties and crystallinity of maize starches were examined using optical microscopy. The studies were performed using a Leitz Diaplan microscope attached with a PixeLINK PL-A662 camera for capturing still-images. Samples were viewed using unpolarised white light as well as polarised light to visualise birefringence of starch granules. Polarised light was produced using two polarising filters, known as the polariser and the analyser. The polariser is positioned in the light path beneath the sample stage and the analyser in the optical path above the objectives. When the polariser and analyser are 90 ° apart, the field of view will become dark, also known as extinction. However, when adjusted some light is able to pass through the filters at different angles. In an isotropic sample, light will simply pass through making it appear dark, while conversely in an anisotropic or birefringent sample, such as a starch granule, light will be reflected making it appear bright in contrast. Images were taken as various magnifications (x10, x25 and x40).

3.2.3.2 Scanning electron microscopy (SEM)

3.2.3.2.1 Principle

Starch granule morphology in maize diets and *in vivo* digesta was examined using a scanning electron microscope (SEM; Jeol JSM-6490LV), with an energy of 10-15 kV. The SEM (Fig 3.3a) uses a high energy electron beam, generated by a heated tungsten filament. The electrons are focused into a small beam by a series of electromagnetic lenses along the SEM column. Scanning coils near the end of the column direct and position the beams onto the sample surface. A high vacuum minimises scattering of the electron beam before it reaches the specimen. As the electron beam hits the sample surface it causes electrons to be emitted from the specimen due to elastic and inelastic scattering events.

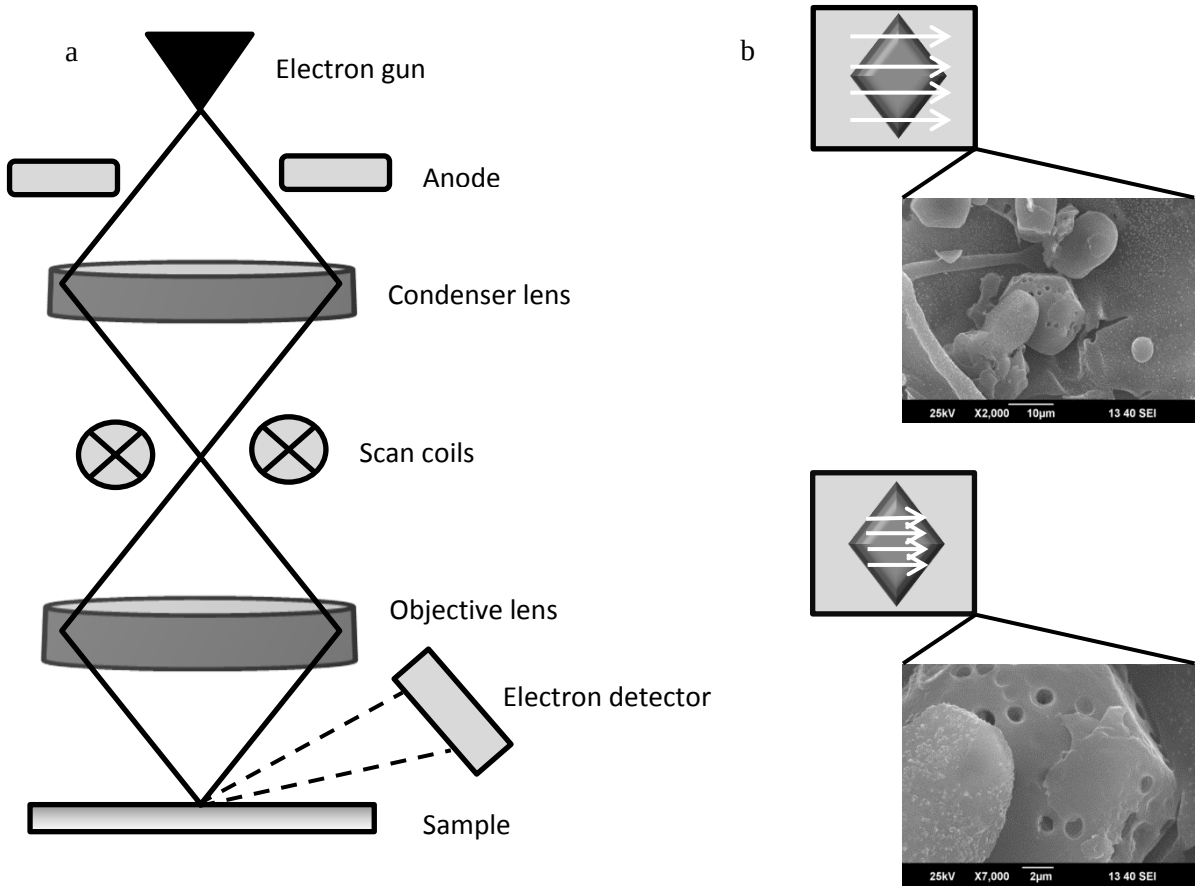


Figure 3.3 Schematic presentation of a scanning electron microscope (a) and the effect of scanning area on the magnification of samples (b).

Low energy (< 50 eV) secondary electrons are formed by the electron beam knocking into atoms and displacing electrons from the sample (inelastic event). These negatively charged electrons are attracted to a positive voltage detector producing a high resolution image of the surface morphology. The topography of surface features influences the number of secondary electrons that reach the detector. The variation in electron intensity over the sample creates image contrast that reveals surface morphology. High energy (> 50 eV) backscattered electrons are produced by the beam's electrons ejecting back out of the sample (elastic event). The intensity of backscattering is proportional to the mean atomic number of the sample material; a material with a higher atomic number appears brighter than a material with a

lower atomic number. Therefore, images produced by backscattering provide information on variations in sample composition. The beam is scanned in a rectangular scanning (raster) pattern; from left to right and top to bottom. The image is viewed on a monitor in a point-by-point translation of the rastering pattern of the specimen. Increasing magnification is achieved by decreasing the size of the scanning area (Fig 3.3b). In this way, high magnification and resolution images can be obtained.

3.2.3.2.2 Sample preparation

Freeze-dried samples were mounted onto a conductive carbon tape attached to an aluminium stub. A manual air blower was used to remove excess sample. Non-conductive samples are coated to reduce surface charging which can block secondary electron path and lead to a distorted image. Also, it provides a surface area that produces a higher secondary electron yield than a non-coated specimen, and therefore increases the signal to noise ratio. Samples were gold coated at 25 kV for 5 min using a vacuum sputter coater (Polaron Emitech SC7640 Sputter Coater). The sputter coater uses an electric field and argon gas. The sample is placed in a small chamber that is at a vacuum. Argon gas is leaked into the vacuum chamber to provide a medium for ionisation. Electrons emitted from the cathode collide with the argon gas molecules, producing positive ions. Argon ions become attracted to the negatively charged gold and knock atoms from the surface of the gold foil. These gold atoms fall and settle onto the surface of the sample producing a thin gold coating. Following coating, samples were viewed and photographed in the SEM unit at various magnifications (x250, x500 and x1000).

3.2.4 Starch characterisation and structure analysis

3.2.4.1 Particle sizing

Particle size distribution was measured mechanically by a nested column of sieves with decreasing mesh sizes (2 mm, 1 mm, 600 μm, 425 μm, 250 μm and 106 μm) with a receiver at the base to collect the smallest particles. Freeze-dried samples were weighed to 50 g and placed onto the largest sieve (2 mm) at the top of the column, with a lid added to prevent loss of sample. The sieves were set on a mechanical shaker (Retsch Vibro apparatus) at an amplitude of 3.00 mm/"g" for 2 min. Sample fractions retained on each sieve were weighed and the distribution given as a percentage of total weight.

3.2.4.2 Moisture content

The dry basis moisture content of samples was determined gravimetrically. This procedure involves drying a sample to a constant weight to allow determination of water content in the sample. The water content is expressed as a percentage of the dry sample weight.

Firstly, an aluminium container was dried in an oven (Sanyo convection oven MOV-112F) at 105 ^{0}C for 10 min and allowed to cool in a desiccator for a further 10 min. The weight of the aluminium container was recorded followed immediately by the sample weight (~2 g), known as the 'sample wet weight'. The wet sample was dried to a constant weight in the oven for 24 h. The dry sample was allowed to cool in a desiccator for 10 min before reweighing. The aluminium container weight was then subtracted from this weight to give the 'sample dry weight'. All samples were weighed and dried in duplicate.

The moisture content of samples was determined using the following calculation:

$$Moisture\ content\ (\%) = \left(\frac{Sample\ wet\ weight - Sample\ dry\ weight}{Sample\ dry\ weight}\right) x\ 100$$

3.2.4.3 Total starch assay

3.2.4.3.1 Principle

The total starch content of maize diets and digesta was determined using the Megazyme total starch assay kit (K-TSTSA; Megazyme International Ireland Ltd). This procedure (Fig 3.4) includes an ethanol wash to remove soluble glucose and maltodextrins, followed by a pre-dissolution step using dimethyl sulfoxide (DMSO) to solubilise starches, including any RS. Subsequent to starch gelatinisation in DMSO, a thermostable α-amylase hydrolyses starch into shorter, soluble chains of branched and unbranched maltodextrins. These maltodextrins are then further hydrolysed by amyloglucosidase into D-glucose monomers.

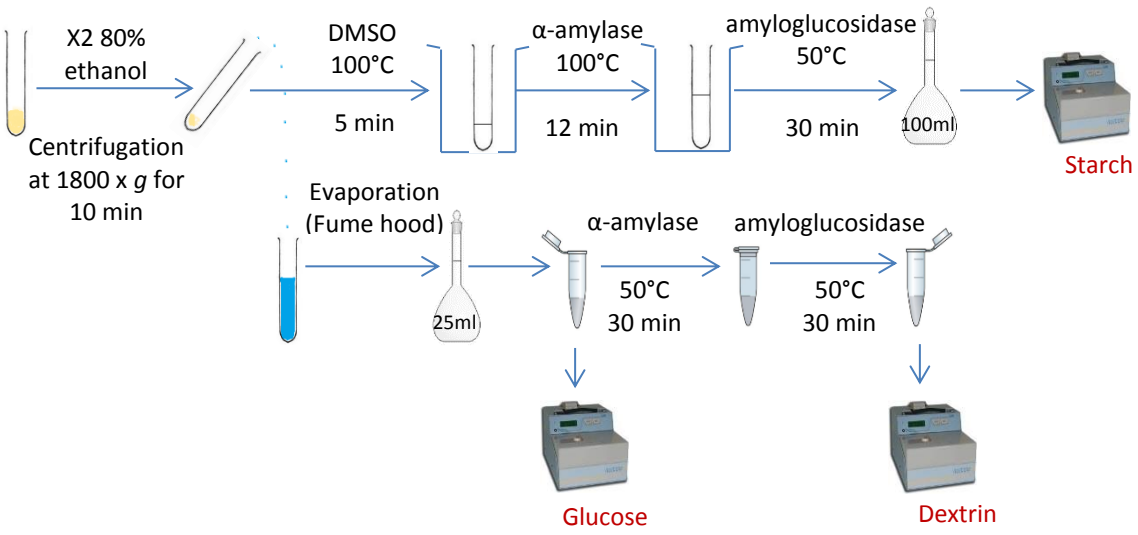


Figure 3.4 Schematic diagram of the total starch assay procedure

3.2.4.3.2 Procedure

Approximately 100 mg of sample was accurately weighed into a 15 ml centrifuge tube. Subsequently, 5 ml aqueous ethanol (80 % v/v) was added to the tube and the sample incubated at 80-85 ^{0}C for 5 min. The contents were then mixed on a vortex mixer and a

further 5 ml 80 % ethanol added. The tube was centrifuged at 1800 *x g* for 10 min and the supernatant collected in a 70 ml sterile container. The pellet was re-suspended in 10 ml 80 % ethanol and stirred on a vortex mixer, followed by centrifugation at 1800 x *g* for 10 min and collection of supernatant. Immediately, 2 ml DMSO was added to the pellet, and the tube mixed and placed in a vigorously boiling water bath for 5 min, with constant stirring. Immediately, 3 ml of thermostable α-amylase (300 U), diluted 1:30 in MOPS buffer (50 mM, pH 7.0) with calcium chloride (5 mM), was added and the tube incubated in a boiling water bath for 12 min with intermittent vortexing. The tube was then placed in a water bath at 50 ^{0}C with addition of 4 ml sodium acetate buffer (200 mM, pH 4.5), followed by 100 µl amyloglucosidase (330 U). The tube was mixed and incubated at 50 ^{0}C for 30 min. Subsequently, the tube's content was quantitatively transferred into a 100 ml volumetric flask using a funnel and wash bottle, and then adjusted to volume with distilled water. An aliquot of this solution was taken and the glucose concentration measured using the glucose analyser (see section 3.2.4.5).

Starch '*as is*' (w/w) was then calculated by the following calculation:

$$Starch\ 'as\ is' = \left(\frac{glucose\ concentration\ \left(\frac{mg}{dl}\right) x\ 100\ (dilution\ factor)}{sample\ weight\ (mg)} \right) x\ 0.9$$

The value of 0.9 was included for the adjustment from free-glucose to anhydro-glucose in starch. The total starch content (g/100g) of the sample was then calculated, taking into account the sample's moisture content to give values on a dry weight basis:

$$Total\ starch\ \left(\frac{g}{100g}\right) d.w.b = \frac{starch\ 'as\ is' x\ 100}{100 - moisture\ content}$$

The ethanol washes, assumed to contain soluble dextrin and glucose, were collected and the ethanol evaporated in a fume hood. Following ethanol evaporation, the remaining contents of the supernatant were quantitatively transferred into a 25 ml volumetric flask and adjusted to volume with distilled water. This was known as 'ethanol wash dilute'. Glucose concentration of this ethanol wash dilute was measured using the glucose analyser. Subsequently, 25 µl α-amylase (*A.oryzae;* Sigma, A8220, 24 FAU) was added to a 200 µl aliquot of ethanol wash dilute and incubated at 50 °C for 30 min. This was followed by the addition of 25 µl amyloglucosidase (Megazyme; 82.5 U) and incubation at 50 °C for a further 30 min. Glucose concentration after digestion was then measured using the glucose analyser. The difference in glucose concentration before and after digestion gave the amount of dextrin in the sample. From this method, the amount of starch (mg), dextrin (mg) and glucose (mg) in the total digesta sample collected was calculated using the following equations:

$$Total\ digesta\ starch\ (mg) = \left(\frac{total\ starch \left(\frac{g}{100g} \right)}{digesta\ sample\ weight\ (g)} \right) x\ 1000$$

$$Total\ digesta\ dextrin\ (mg)$$

$$= \left(dextrin\ \left(\frac{mg}{ml} \right)\ x\ 25\ ml \right)\ x\ (\frac{digesta\ sample\ weight\ (mg)}{total\ starch\ assay\ sample\ weight\ (mg)})$$

$$Total\ digesta\ glucose\ (mg)$$

$$= \left(glucose\ \left(\frac{mg}{ml} \right)\ x\ 25\ ml \right)\ x\ (\frac{digesta\ sample\ weight\ (mg)}{total\ starch\ assay\ sample\ weight\ (mg)})$$

3.2.4.4 Amylose-amylopectin ratio

3.2.4.4.1 Principle

The composition of starch was determined using the amylose: amylopectin ratio assay (K-AMYL; Megazyme International Ireland Ltd). In this method, starch is dispersed by heating in DMSO and then precipitated in ethanol to remove lipids. Following dissolution of the precipitated starch in DMSO and an acetate/salt solution, Concanavalin A (Con A) is added to precipitate amylopectin. Con A specifically complexes branched polysaccharides based on α-D-glucopyranosyl or α-D-mannopyranosyl units at numerous non-reducing end-groups, forming a precipitate. Therefore, Con A will complex with amylopectin, yet not with the predominantly linear amylose fraction. The remaining amylose component is then enzymatically hydrolysed to D-glucose, which is measured colourimetrically using a glucose oxidase/peroxidase reagent. Concurrently, the total starch in a separate aliquot of the acetate/salt solution is hydrolysed to D-glucose and measured colourimetrically. Amylopectin content is deducted by the difference between total starch and amylose content.

3.2.4.4.2 Procedure

Samples were accurately weighed (20-25 mg) into a test tube and then solubilised in 1 ml DMSO by stirring and then heating in a boiling water bath for 15 min with intermittent mixing. Tubes were allowed to stand at room temperature for 5 min, followed by addition of 2 ml 95 % (v/v) aqueous ethanol with continuous stirring. A further 4 ml 95 % ethanol was added and the tube sealed with parafilm before mixing by inversion. The tube was allowed to stand for at least 15 min for a starch precipitate to form. The tube was then centrifuged at 2000 x *g* for 5 min with the supernatant being discarded and the tube drained on tissue paper for 10 min to ensure complete removal of ethanol.

The starch pellet was then gently mixed with 2 ml DMSO and incubated in a boiling water bath for 15 min with intermittent stirring. Immediately, 4 ml Con A solvent (600 mM, pH 6.4 sodium acetate buffer) was added, the tube mixed and the content quantitatively transferred to a 25 ml volumetric flask and adjusted to volume with Con A solvent. This was known as solution A.

3.2.4.4.2.1 Determination of amylose

A 1 ml aliquot of solution A was transferred to a 2 ml Eppendorf microfuge tube. To this tube, 0.5 ml Con A solution (200 mg lectin Con A dissolved in 50 ml Con A solvent) was added and the tube gently mixed by inversion. The tube was allowed to stand for 1 h at room temperature before centrifugation at 14000 x *g* for 10 min in a microfuge (Thermo Scientific Heraeus, FRESCO21). Subsequently, 1 ml supernatant was transferred into a 15 ml centrifuge tube and mixed with 3 ml sodium acetate buffer (100 mM, pH 4.5). The tube was then placed in a boiling water bath for 5 min to denature the Con A and then the contents allowed to equilibrate in a water bath at 40 ^{0}C for 5 min. Following this, 0.1 ml amyloglucosidase/α-amylase enzyme mixture (dissolved in sodium acetate buffer , 100 mM, pH 4.5) was added and the tube incubated at 40 ^{0}C for 30 min. The tube was then centrifuged at 2000 x *g* for 5 min. A 1 ml aliquot of the supernatant was mixed with 4 ml glucose oxidase/peroxidase (GOPOD) reagent in a test tube and incubated at 40 ^{0}C for 20 min.

3.2.4.4.2.2 Determination of total starch

For the determination of total starch, 0.5 ml solution A was mixed with 4 ml 100 mM sodium acetate buffer (100 mM, pH 4.5) and 100 µl amyloglucosidase/α-amylase enzyme mixture, and incubated at 40 ^{0}C for 10 min. A 1 ml aliquot of this solution was transferred to a test tube in duplicate, with addition of 4 ml GOPOD reagent, and incubated at 40 ^{0}C for 20 min concurrently with samples for amylose determination. Reagent blank (1 ml 100 mM sodium

acetate buffer and 4 ml GOPOD) and D-glucose controls (100 µl D-glucose standard solution (1 mg/ml), 900 µl 100 mM sodium acetate buffer and 4 ml GOPOD) were also incubated simultaneously. The absorbance of each sample and D-glucose controls were read at 510 nm against the reagent blank.

Amylose content (%) was determined by the calculation:

$$Amylose\ (\%) = \frac{Absorbance\ con\ A\ supernatant}{Absorbance\ Total\ starch\ aliquot}\ x\ 66.8$$

The absorbance ratio is multiplied by the value 66.8 to incorporate dilution factors for the Con A and Total starch extracts. Using the Total starch results from section 3.5.2, the amylose content (%) can be determined, and accordingly, the amylopectin content (%).

3.2.4.5 Glucose analyser

The glucose analyser (GM9; Analox Instruments Ltd) was used to measure the concentration of glucose in samples for starch analysis; including total starch and *in vitro* starch digestion methods. The GM9 is a fast, single channel analyser for glucose in liquids. Injection of relatively small sample volume (5-10 µl) gives results, expressed directly in concentration units (mg/dl), within 10 sec. Under suitable conditions, glucose oxidase catalyses the oxidation of glucose to gluconic acid, with the maximum rate of oxygen consumption being directly proportional to glucose concentration:

$$Glucose + oxygen \rightarrow \text{Gluconic acid} + \text{hydrogen peroxide}$$

The glucose analyser is calibrated against an 8 mmol/l (144.1 mg/dl) glucose standard in benzoic acid. Using a 10 µl sample, the reaction is linear to at least 30 mmol/l. When linearity limits are exceeded, a 5 µl samples is injected instead and the results multiplied by 2.

3.2.4.6 Wide angle X-ray diffraction (WAXD)

Wide-angle X-ray diffraction (WAXD) was performed on diets and *in vitro* digestion samples for determination of crystallinity. Samples were loaded onto a variable temperature stage and diffraction patterns recorded on a Bruker D5005 wide-angle diffractometer operating at 40 kV, 40 mA with CuKa radiation. The scanning regions of the diffraction angle, 2θ, were 3°-38°, with a step interval of 0.05, a 2.5 sec stay at each angle, at room temperature, for a total time of 29 min.

The degree of crystallinity of samples was estimated quantitatively. A linear baseline was drawn on the diffractograms, connecting the points of intensity at 2θ of 5° and 30°, to remove background. A smooth curve was then created to connect peak baselines. The area above the smooth curve was taken to correspond to the crystalline region and the area between the smooth curve and linear baseline was taken as the amorphous region. The ratio of the crystalline region to the total diffraction area was taken as the degree of crystallinity (Cheetham and Tao, 1998).

3.2.4.7 Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) was used for characterisation of cell wall arabinoxylan in diets and *in vivo* digesta. FTIR spectra were measured using a Bruker Tensor 27 FTIR. Data was recorded at room temperature in the spectral range of 550 and 4000 cm^{-1}, by accumulating 30 scans with a 4 cm^{-1} resolution. Spectra analysis was carried out using Opus 3.0 data collection software.

Substitution of the xylose backbone by arabinose side units was examined. Baseline correction was applied manually using a single point at 1900 cm^{-1}. Spectra were then normalised and cut to the region of 800-1020 $cm^{-1.}$ The ratio of absorbances at 958 cm^{-1}/984

cm^{-1} corresponding to the arabinose and xylose units, respectively, were used to determine the degree of substitution (Robert *et al.*, 2005).

3.2.5 Glucose transporter protein expression

3.2.5.1 RNA Extraction

3.2.5.1.1 Tissue crushing

Small intestine tissue fractions were crushed into smaller fractions before RNA extraction. During the process, tissue samples were stored in their collection bags within a liquid nitrogen filled dewar. It was essential that throughout the process tissue samples were kept frozen to avoid RNA degradation. Liquid nitrogen was poured into a metal crusher (Fig 3.5) near to the fill point. A tissue sample was then taken from the dewar and emptied onto a piece of aluminium foil. A metal hammer was then used to smash the tissue into smaller pieces. A couple of small tissue fractions were put into the crusher. The leftover tissue was folded up in the foil and put back into the dewar. Excess liquid nitrogen was poured off from the crusher before adding the lid and then a wooden hammer was used to hit the lid 3 times. Subsequently, an opened 2 ml Eppendorf tube (autoclaved, with a hole in the lid) was submersed in liquid nitrogen using tongs. The tube was allowed to fill with liquid nitrogen to keep it cold. The end of a spatula was also submersed liquid nitrogen before using it to transfer the pulverised sample from the crusher into the Eppendorf tube. This tube was then immediately placed into liquid nitrogen before storage at -80 °C.



Figure 3.5 Metal crusher used for tissue crushing

3.2.5.1.2 Total RNA isolation

3.2.5.1.2.1 Procedure

Total RNA was isolated from the tissue samples using an RNeasy Fibrous Tissue Mini Kit (Qiagen; 74704). Eppendorf tubes containing the crushed tissue were stored in a liquid nitrogen filled dewar. A hole was made in the lid of a 1.5 ml Eppendorf tube (autoclaved) before submersing it in liquid nitrogen using tongs. Excess liquid nitrogen was poured off and the tube tared on the weighing scales. Tongs were then used to pick out a tissue sample from the dewar, while a spatula was dipped into the liquid nitrogen, before transferring 20-25 µl of sample into the tube on the scales. The first tube containing the original crushed tissue was then placed back into the dewar while the second tube containing the weighed sample was put in a holding rack within a fume hood. Immediately, 300 µl lysis buffer (Buffer RLT with 1 % β-mercaptoethanol) was added to the tube. Samples were then homogenised using a pellet pestle (Sigma-Aldrich; Z359947-100EA), followed by repeated pipetting using a 1" needle (VWR International Ltd; TERUSG2-2525M) attached to a 1 ml syringe (Fisher Scientific

UK, 10142104). Following lysis and homogenisation, 590 µl RNase-free water was added to the lysate. Next, 10 µl proteinase K solution was added and mixed thoroughly by pipetting, and the lysate incubated at 55 °C for 10 min using a Dri-Block Heater (Techne; DB-2A). The samples were then centrifuged (Beckman Coulter Microfuge 22R centrifuge) at 20-25 °C for 3 min at 10000 x *g*. The supernatant was pipetted into a new 1.5 ml Eppendorf tube (autoclaved), 100 µl at a time, while avoiding taking up the white pellet. Next, 450 µl 100 % ethanol was added to the new tube and mixed immediately by pipetting. An RNeasy mini column was placed into a 2 ml collection tube and 700 µl of sample transferred into the column. The column was then centrifuged at 8000 x *g* for 15 sec. Liquid within the collection tube was discarded and the remaining sample transferred into the column, which was spun again at 8000 x *g* for 15 sec. Liquid within the collection tube was discarded and 350 µl buffer RW1 added to the column, followed by centrifugation at 8000 x *g* for 15 sec. Liquid within the collection tube was discarded and 80 µl DNase treatment added to the column. To make the DNase treatment, 10 µl DNase I stock was added to 70 µl Buffer RDD and mixed by gently inverting the tube. The contents were centrifuged at 8000 x *g* for 15 sec to allow collection of residual liquid from the sides of the tube. Once added, the column was incubated at room temperature for 15 min. Following incubation, 350 µl buffer RW1 was added to the column before centrifugation at 8000 x *g* for 15 sec. Liquid within the collection tube was discarded and 500 µl buffer RPE (with ethanol) was added to the column followed by centrifugation at 8000 x *g* for 15 sec. Liquid within the collection tube was discarded and 500 µl buffer RPE was added before centrifugation at 8000 x *g* for 2 min. The RNeasy column was then placed into new 2 ml collection tube and centrifuged at 10000 x *g* for 1 min. Finally, the RNeasy column was placed in a new 1.5 ml Eppendorf tube (autoclaved), 50 µl RNase-free water was added to the column, and the column centrifuged at 8000 x *g* for 1 min. The collected liquid was stored at -80°C.

3.2.5.1.2.2 RNA quantification and quality check

RNA concentration and quality was checked using a DS-11 spectrophotometer (DeNovix). Precisely 1 µl of RNA solution was used for measurements. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the RNA, with ratios of approximately 2.0 being accepted as "pure" RNA. Ratios lower than this may indicate contaminated samples. Once RNA concentration for all samples was determined, 10 µl of sample was diluted with RNase-free water to give a final concentration of 100 ng/µl, using the following calculation:

$$RNase\ free\ water\ required\ (\mu l) = \left(\frac{RNA\ concentration\ \left(\frac{ng}{\mu l}\right)\ x\ 10}{100} \right) - 10$$

The concentration of the diluted RNA sample, assumed to be 100 ng/µl, was checked using the DS-11 spectrophotometer.

3.2.5.2 Reverse transcription (RT) for cDNA synthesis

Synthesis of single-stranded complementary DNA (cDNA) from total RNA was performed using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific; K1622). Kit components were thawed, mixed and briefly centrifuged before use and stored on ice along with RNA samples (100 ng/µl). Each reaction was performed in duplicate: one containing the reverse transcriptase enzyme (+RT) and one where the enzyme was substituted with nuclease-free water (-RT). Another negative control was used in which total RNA was replaced with 3 µl nuclease-free water. This was to control for any genomic contamination in the RNA samples as well as other reaction components. Each reaction consisted of 3 µl total RNA, 1 µl random primer, 8 µl nuclease-free water, 4 µl 5X reaction buffer, 1 µl RiboLock RNase Inhibitor, 2 µl deoxynucleotide triphosphate (dNTP) mix and 1 µl RevertAid Reverse Transcriptase, for a total reaction volume of 20 µl. Reactions were run in a 96 well plate that was sealed with a film and placed into a Mastercycler Gradient thermal cycler (Eppendorf).

Conditions for reverse transcription were set for random hexamer primed synthesis, whereby samples were incubated at 25 °C for 5 min, followed by 42 °C for 60 min. The reaction was terminated by heating the samples to 70 °C for 5 min. The resulting cDNA products were stored at -20 °C.

3.2.5.3 Primer design

Primers were designed for the intestinal glucose transporter proteins GLUT2 (SLC2A2) and SGLT1 (SLC5A1). Ensembl was used to obtain the cDNA sequence showing exon boundaries for the GLUT2 gene. This sequence was copied into Primer Express 3.0.1 software for selection of suitable primer pairs (forward, 5′-CCCCGCAGAAGGTGATAGAA-3′; reverse, 5′- TGTCCCTGGAGGTGTTGGT-3′). Once selected, both forward and reverse primers were BLAST (NCBI) matched to confirm 100 % match solely to the GLUT2 gene. The expected product length for the GLUT2 target was 88 base pairs (bp). Forward and reverse primers for SGLT1 were designed by eye (forward, 5′-TTTGTATACAGATGTGGTGGC-3′; reverse, 5′- GCATAAGCTCCACTACCATT-3′), due to primers selected by Primer Express giving 100 % BLAST match to another gene. The expected product length for the SGLT1 target was 113 bp. Furthermore, the house-keeping gene, β-actin, was selected to allow for RT-qPCR data normalisation as this gene is assumed to maintain expression in all cells and conditions. Primers for β-actin were taken from the work of Gilbert *et al.* (2008) (forward, 5′-GTCCACCGCAAATGCTTCTAA-3′; reverse, 5′-TGCGCATTTATGGGTTTTGTT-3′). The expected product length for β-actin was 78 bp. All primers were designed to cross exon-exon boundaries thereby preventing amplification of DNA.

3.2.5.4 Quantitative polymerase chain reaction (qPCR)

3.2.5.4.1 Principle

Quantitative PCR of cDNA samples was performed using an Applied Biosystems StepOne Real-time PCR system. During PCR, temperature changes are used to control polymerase activity, primer and dye binding. To begin the reaction, the temperature is raised to 95 °C to 'melt' double stranded DNA (dsDNA) into single strands. The temperature is then lowered to 60 °C to allow primers to bind to the gene of interest. This also enables the polymerase to bind and begin extension of the DNA by incorporating dNTPs that are complementary to the template in a 5' to 3' direction to form dsDNA. At this point, a dsDNA-binding dye intercalates into the double-strands and begins to fluoresce. During each cycle of the amplification process fluorescence values are recorded.

When enough products accumulate, the fluorescence rises above the background level. The number of cycles required for the fluorescent signal to cross the threshold, thereby significantly exceeding background level, is known as the cycle threshold (Ct). Ct values are inversely proportional to the original expression level of the gene of interest; the higher the initial amount of cDNA, the earlier the accumulated product exceeds the threshold, and the lower the Ct value.

3.2.5.4.2 Procedure

PCR plates (Applied Biosystems MicroAmp® Fast Optical 48-Well Reaction Plate, 4375816) were designed to assay 4 +RT samples, including a sample from treatment group 1 (control sample). Each reaction was run in triplicate for each gene. PCR was also run on the -RT samples and a no template control (NTC) in which cDNA was replaced with nuclease-free water, to check for any contamination in the PCR components. The cDNA was diluted 1:3 with nuclease-free water to lower template concentration and to ensure there was enough

sample for any repeats. A mastermix for each gene was made consisting of 200 µl GoTaq qPCR Matermix (Promega, A6001), 4 µl gene specific forward primer, 4 µl gene specific reverse primer and 141.2 µl nuclease-free water. This mix was then pulse centrifuged for 10 sec using a ThermoScientific Espresso centrifuge. Into the appropriate wells, 18 µl of this mastermix and 2 µl cDNA were added. An optical adhesive film (Applied Biosystems MicroAmp®, 4311971) was applied to the plate before placing in the qPCR instrument. PCR was performed under the following conditions: 95 °C for 10 min and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. A melt curve was produced at the end of the run to determine single product amplification.

The comparative Ct method, also known as the 'delta-delta Ct' method, was used to quantify qPCR results. This method involved comparing the Ct values of the xylanase treated samples with the non-supplemented (control) samples. Both treated and control sample Ct values were normalised to the house-keeping gene.

$$\Delta\mathrm{Ct} = \mathrm{Ct\ value\ gene\ of\ interest} - \mathrm{Ct\ value\ control\ gene\ (\beta - actin)}$$

$$\Delta\Delta\mathrm{Ct} = \Delta\mathrm{Ct\ value\ gene\ of\ interest} - \mathrm{Average}\ \Delta\mathrm{Ct\ value\ control\ sample}$$

Fold change in gene expression was then calculated and averaged for the three replicates:

$$Fold\ change = 2\ \hat{}\ - \Delta\Delta\mathrm{Ct}$$

3.2.5.4.3 Target product confirmation

Agarose gel electrophoresis was used to confirm that qPCR amplified solely the gene of interest. PCR products were resolved on a 3 % agarose gel containing 5 % (v/v) ethidium bromide. One sample was selected from two different PCR plates that produced good melt curves. One replicate for each gene was selected and 4 µl 6X purple loading dye (New

England Biolabs, B7024S) was added. Each dyed product (10 µl) was placed in a separate well on the gel. The products were separated, alongside a 20 bp low ladder (Sigma-Aldrich, P1598), for approximately 1 h using 80 V. The gel was visualised and imaged under ultraviolet (UV) light using a transluminator. The product band size was then determined by comparing positions relative to the ladder. The expected band size of the GLUT2, SGLT1 and β-actin products were 88 bp, 113 bp and 78 bp, respectively.

3.2.6 Statistical analysis

Statistical significance was tested using IBM SPSS statistics 21 software. Multivariate analysis of variance (ANOVA) was used to compare means between groups, while univariate ANOVA was used to compare means within a group. For comparisons where only two bird replicates were available, an independent sample t-test was performed. For statistically significant results ($P < 0.05$), Tukey's HSD Post-hoc test was performed to determine which groups in the population differ.

CHAPTER 4

IN VITRO DIGESTION MODEL

In vitro digestion models are widely used to study the digestion of feedstuff under simulated gastrointestinal conditions. Animal trials are time consuming, costly and involve major ethical considerations. Therefore, development of an *in vitro* method to mimic the digestive process of birds as closely as possible will be hugely beneficial for testing novel ingredients to understand their digestive properties without the use of numerous animals. However, there is still no universally used *in vitro* model to simulate the digestive tract of birds. Also, much attention is focused on digestive processes that occur in the small intestine, as this is the major site of nutrient digestion and absorption. However, less is known about what role the gizzard plays in the digestion of starches, and what effect gastric conditions might have on starch digestion further down the tract. Consequently, a key part of this study was to develop an *in vitro* model that includes a gizzard simulation to best mimic the digestive tract of birds. This model was then used to compare starch digestion between different maize-based diets.

4.1 Diet characterisation

In this project, three main diets were selected to allow investigations into maize starch digestion under the influence of different food-matrices. These diets included native maize starch, maize grit and maize-soy (section 3.1.3). Essentially, all starches in these diets are native and come from the same botanical source, a description of which can be found in section 2.3.2.2. However, their surrounding matrices vary and this may affect the availability of these starches for digestion. In a standard broiler diet, starch is the major source of energy

for the bird, comprising around 50 % of the complete diet (Svihus, 2014b). Depending on the type of diet that is fed, the amount and composition of the starch will vary and this will inevitability affect digestion. Therefore, it is firstly important to evaluate the starch in diets to enable a better interpretation of *in vitro* digestion results.

4.1.1 Starch content and composition

The total starch content of diets was measured using the Megazyme total starch assay, as described in section 3.2.4.3. According to assay results, the native maize starch diet comprises approximately 92 % starch. Various processing steps are required to produce isolated native starch granules. These steps involve the removal of fibre, protein and lipid fractions from the kernel, leaving a purified starch milk that is then dried, producing a starch powder. However, complete removal of the other fractions is unachievable, and therefore a small percentage of the native starch diet will include other components. Starch comprises around 56 % of the maize grit diet. This mash diet contains all of the fractions that make up the corn kernel; the pericarp, germ and endosperm, as discussed in section 2.3.1. In the maize-soy diet, starch contributes even less to the total diet, at around 33 %. This is due to the addition of soybean meal, which increases the protein portion of the diet. How this affects the digestion of starch will be investigated later in this chapter.

The ratio of amylose to amylopectin in starches is an important determinant of starch digestibility. High amylose content has been shown to reduce the digestibility of starch, while high amylopectin demonstrates the contrary (Rooney and Pflugfelder, 1986). Native maize starches commonly consist of 25-30 % amylose and 70-75 % amylopectin. These values were supported in this work (Fig 4.1), with native maize starch having an amylose content of around 25 %. While both the maize grit and maize-soy diets had a slightly lower level of approximately 22 % amylose. The maize-soy diet also showed larger variation in mean

results. Considering that the same source of maize was used for both maize grit and maize-soy diets (section 3.1.3), this may simply be due to the variability of starches in the maize sample itself, or due to the presence of other feed components, such as the additional protein source, that might have affected the efficiency of the technique. Overall, amylose content was not significantly different between samples ($P > 0.05$) and therefore is unlikely to be the cause of any potential differences shown in the digestion of starches.

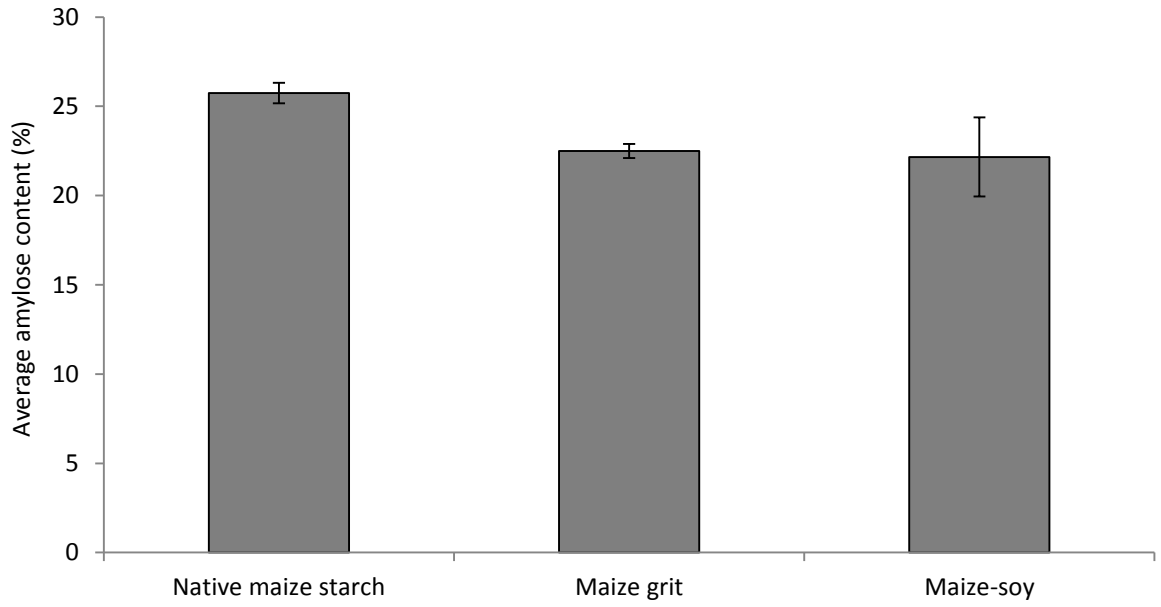


Figure 4.1 Amylose content of maize diets. Error bars denote ± standard error of the mean (from 2 replicates).

Particle size distribution of diets was determined using the method stated in section 3.2.4.1. As expected, the native maize starch diet showed a greater percentage of smaller particles, compared to the maize grit and maize-soy diets (Fig 4.2). However, in respect to maize starch granule size, it would be assumed that a greater distribution of particles would fall into the < 106 μm category. During the sieving process agglomeration of the starch powder was noted, and this may have affected the separation of particles through the sieves. This phenomenon was not observed for the other maize diets. Both maize grit and maize-soy diets were ground through a 5 mm screen by the supplier and thus showed a very similar particle size

distribution. It can therefore be assumed that any differences in the digestion of starches in these two diets are not the result of initial particle size.

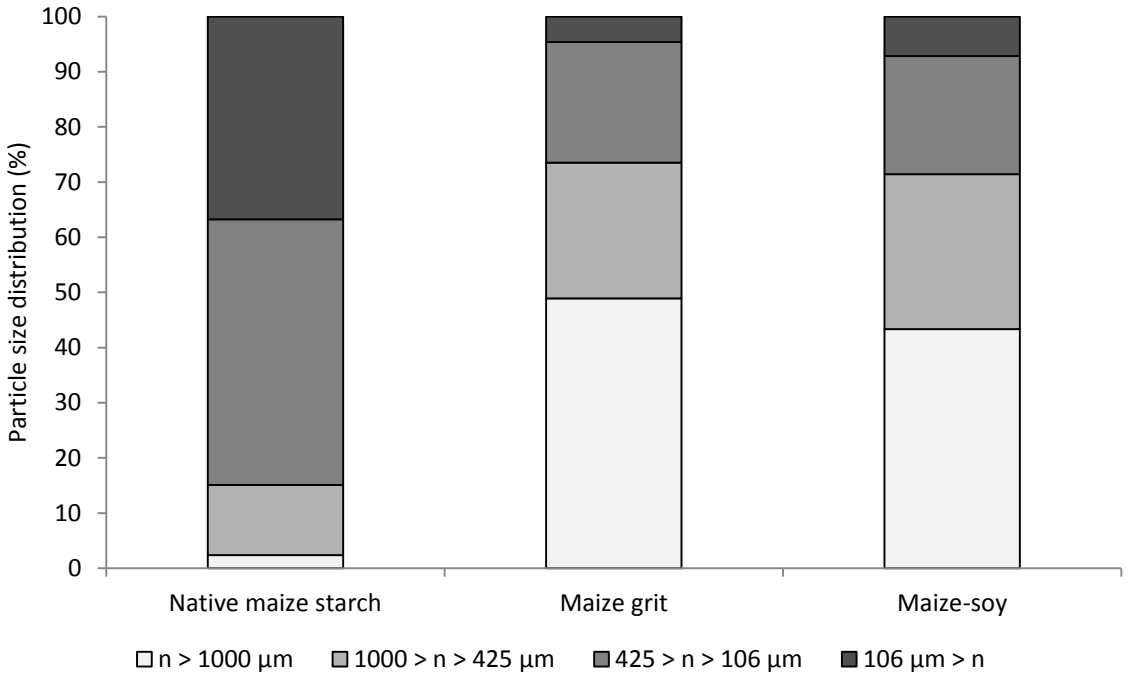


Figure 4.2 Particle size distributions of maize diets

Typical maize starch granules were observed under the SEM (Fig 4.3). Maize starch granules are spherical or polygonal in shape and have diameters ranging from 5-25 µm in the mature kernel. Starch granules in the native maize starch diet were highly dispersed with no signs of other feed components interacting with the granules (Fig 4.3a). Therefore, it can be assumed that the digestion of starches in this diet is solely influenced by enzyme-substrate interaction and not the presence of obstructing food-matrices. On the contrary, starch granules in the maize grit diet are bound together by plant cell wall and protein matrices (Fig 4.3b). This may limit enzyme access to the granules making them less available for digestion. This is also true for the maize-soy diet (Fig 4.3c). However, the inclusion of soybean meal into the maize diet will not only increase the proportion of protein in the feed, but also the NSP content. These components would be expected to create a physical barrier, thereby making the starch

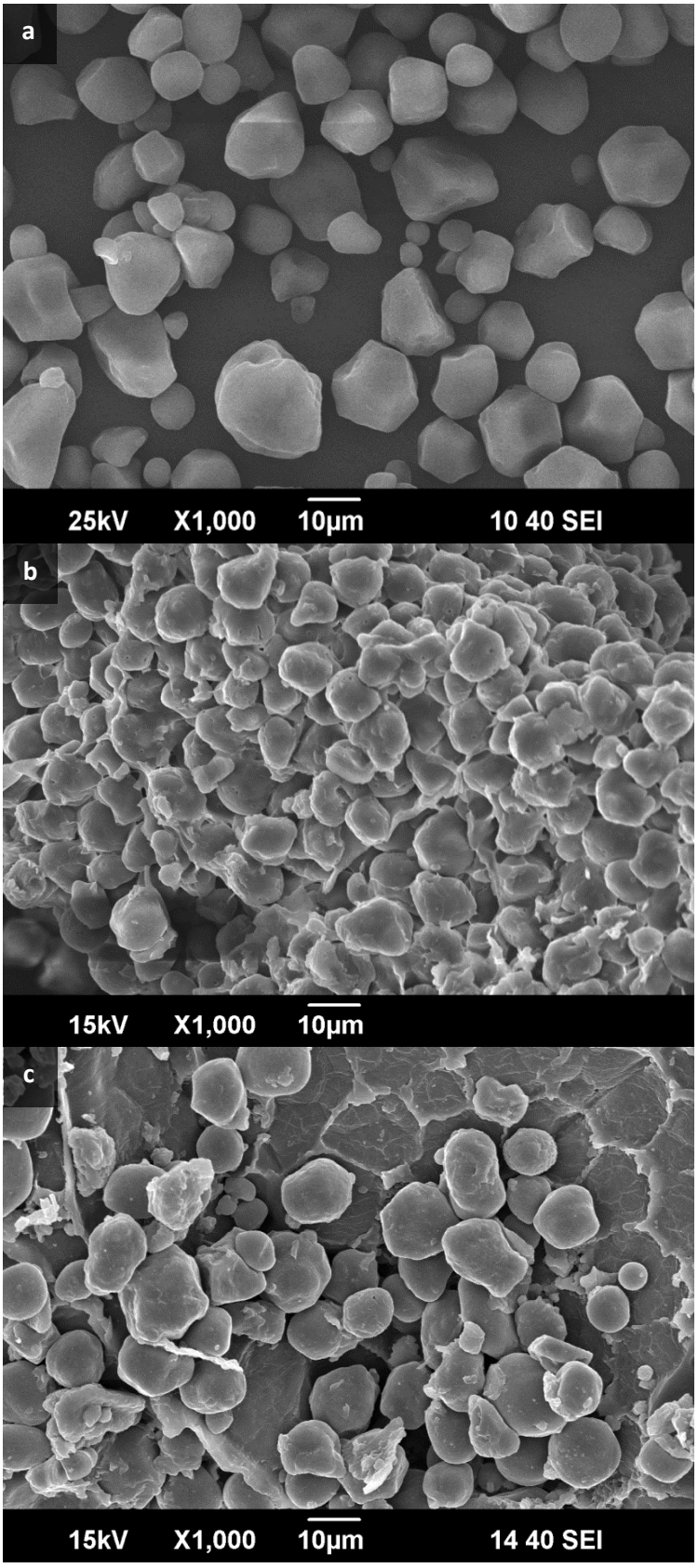


Figure 4.3 SEM images of native maize (a), maize grit (b) and maize-soy (c) starches

granules less available for enzymatic hydrolysis. Therefore, it is of interest to determine what influence these feed components have on the digestion of starches in a particular diet.

4.2 Digestion of different maize diets

4.2.1 Gizzard model

The gizzard is not generally considered an essential player in starch digestion compared to the small intestine. However, the gizzard is shown to affect other feed components, for example, through the digestion of proteins and particle size reduction. Therefore, the following experiments explored how gizzard conditions might affect starch characteristics.

4.2.1.1 Effect of buffer and processing on starch characteristics

In vitro models to simulate the digestive tract of birds have been previously designed. However, for many of these models, feed is initially ground to less than 1 mm to mimic the size of particles that would be leaving the gizzard. This ground feed would then be exposed to proventriculus and gizzard conditions. *In vivo*, feed would enter the proventriculus and gizzard the same particle size as ingested. In the gizzard, feed is ground in an acidic environment, until particles are small enough to enter the duodenum. The acidic conditions of the gizzard were replicated using 0.01 M HCl solution. Previous studies have used higher concentrations of acid of 0.1 M HCl (Walk *et al.*, 2012; Bedford and Classen, 1993). However, this was proven to give a particularly low pH of around pH 1. Measurements of gizzard digesta pH, during *in vivo* digestion studies, gave readings of approximately pH 2-3 and therefore a lower concentration of HCl was employed to reproduce these conditions. Taking this into account, a wet milling procedure was developed to better simulate gastric conditions in the broiler.

A ball milling technique was used in the current study, as described in section 3.2.1.1, to simulate the grinding action of the gizzard. To examine the effect that ball milling (representing gizzard grinding) has on starch characteristics, these attributes were compared to starches in feed that were roller mixed. Moreover, the effect of acidic conditions on starches was studied. Feed was either milled or mixed in HCl-pepsin solution or water. Feed was also dry milled to determine whether this might affect starches differently to wet milling.

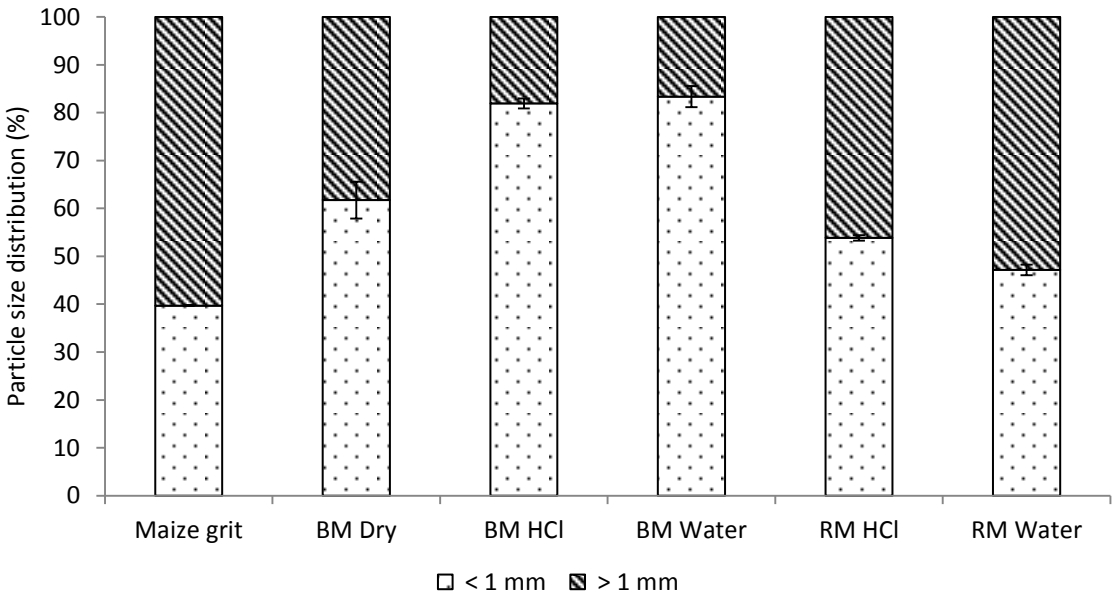


Figure 4.4 Effect of processing on the particle size distribution of a maize grit diet. Diet samples were ball milled (BM) or roller mixed (RM) in dry conditions, HCl-pepsin solution or water. Error bars denote ± standard error of the mean (from 2 replicates).

Particle sizing of the maize grit diet before and after different processing methods, according to the method in section 3.2.4.1, is shown in Figure 4.4. Ball milling increased the number of particles with sizes less than 1 mm, with greater increases observed when feed was milled in solution compared to dry milling. In wet milling, feed particles are dispersed in a liquid medium which can act to weaken the particles and reduce agglomeration. This can reduce particle sizes to levels lower than achieved by dry milling (Kotake *et al.*, 2011). Interestingly,

roller mixing in buffer increases particle size reduction compared to the unprocessed diet, with effects being greater for samples in HCl-pepsin solution than water. This would suggest that the addition of a liquid medium, particularly an acidic medium, to the feed has a notable influence on particle size reduction.

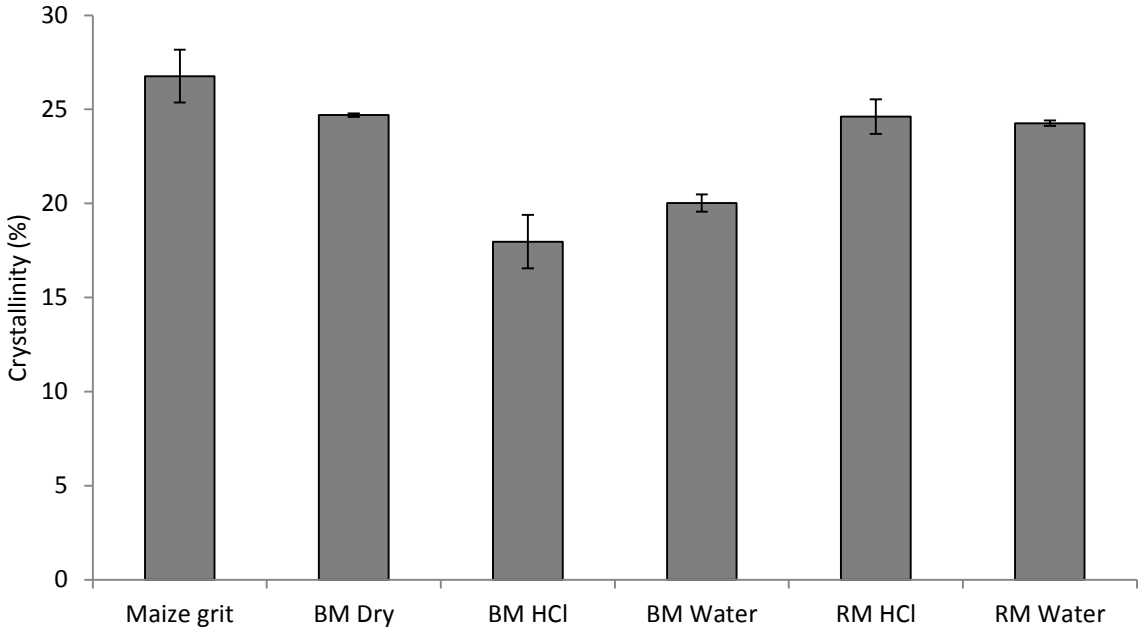


Figure 4.5 Effect of processing on starch crystallinity in a maize grit diet. Diet samples were ball milled (BM) or roller mixed (RM) in dry conditions, HCl-pepsin solution or water. WAXD measurements were taken from freeze-dried samples. Error bars denote ± standard error of the mean (from 2 replicates).

The milling of native maize starch has been shown to affect the physicochemical properties of starches through a reduction in crystallinity (He *et al.*, 2014). Therefore, the effect of ball milling on starch granule crystallinity was studied in this experiment using both WAXD (Fig 4.5) and microscopic techniques (Fig 4.6), as described in sections 3.2.4.6 and 3.2.3.1, respectively. Dry ball milling has been shown to increase the proportion of damaged starch granules compared to wet milling (Suksomboon and Naivikul, 2006). The lower degree of

recrystallization in dry milled samples would suggest a reduced overall crystallinity of these starches. However, this was not evident in this study, as demonstrated by WAXD results (Fig 4.5). It is possible that the milling time was not long enough or the rotation speed was not high enough to cause any significant impact on the starch granule structure. However, treatments of the starchy products in solution did influence the measured crystallinity of the starches with ball milling reducing crystallinity to a greater extent than roller mixing. Crystallinity was lowest for samples milled in HCl. This suggests that the grinding action of ball mill disrupts the particle and may allow moisture into the granule causing loss of crystalline order. The addition of an acidic environment may aid starch granule disruption through erosion of surface structures, thereby weakening the granule.

Microscopic imaging showed greater swelling of starch granules in diets milled or mixed in solution. Prior grinding of the maize grit diet by the supplier would have led to a number of damaged granules that are more readily hydrated in the presence of water. For the sample ball milled in HCl (Fig 4.6Bii), the polarised light image shows a “halo” effect indicating some change to the crystallinity of the sample compared to the native starches. Nonetheless, the majority of granules for all treatments still showed birefringence, indicating that the crystalline order had not been fully destroyed during the processing treatment.

4.2.2 Intestinal model

The aforementioned solutions and processing methods were then used to investigate how these conditions might affect the extent of starch digestion further down the digestive tract. The small intestine was modelled by incubating the previously processed diets at 40 °C in a sodium bicarbonate-pancreatin solution over a specified period of time.

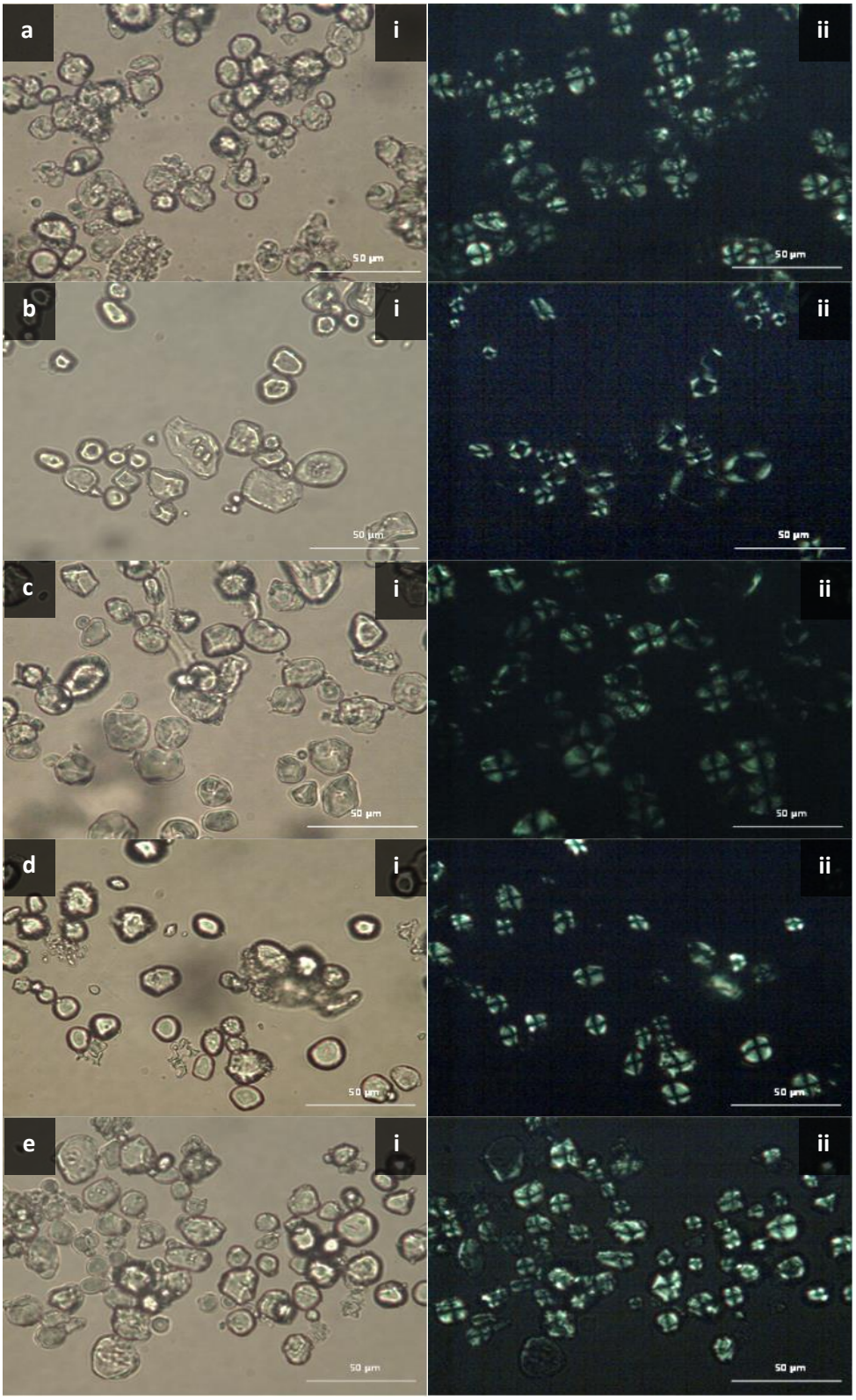


Figure 4.6 Micrographs of starch granules in a maize grit diet suspended in aqueous media. Diet samples were dry milled (a), ball milled in HCl-pepsin solution (b), ball milled in water (c), roller mixed in HCl-pepsin solution (d), roller mixed in water (e) and viewed under white light (i) and polarised light (ii).

4.2.2.1 Extent of starch digestion

The effect of ball milling on starch digestion following incubation with pancreatin is shown in Figure 4.7a. The extent of starch digestion was greater in ball milled samples compared to those that were roller mixed. This signifies the importance of feed particle size on the digestion of starches. For ball milled samples, solution type does seem to be important initially, with samples in HCl-pepsin solution having a constantly higher percentage of starch digested at each time point. However, the extent of starch digestion was not affected by solution type after 24 h of digestion. When considering roller mixed samples, solution type showed less of an effect, although samples in HCl-pepsin solution had a slightly higher level of glucose release at the endpoint. This might suggest that the grinding action of the ball mill and the acidic conditions of the HCl work collectively to enhance the digestion of starches in the diet.

The total amount of starch digested did not exceed 6 % for any of the samples. As amylase in pancreatin mainly produces maltoses and dextrins, measuring glucose content following this digestion process will underestimate starch digestion. Therefore, in the last experimental replicate, the supernatant produced by centrifugation was collected and the content digested with amyloglucosidase. This enzyme was used to digest the products of starch digestion by amylase, into glucose. Glucose concentration was then measured, and the total amount of glucose released from starch determined (Fig 4.7b). Similar to previous results, ball milling improved starch digestion compared to roller mixed samples. Moreover, samples processed in HCl-pepsin solution gave a slightly improved starch digestion compared to samples processed in water. The total amount of glucose released from starch was approximately 456 and 129 mg/g for ball milled and roller mixed samples, respectively. This implies that more than half of the starch in the diet remained undigested.

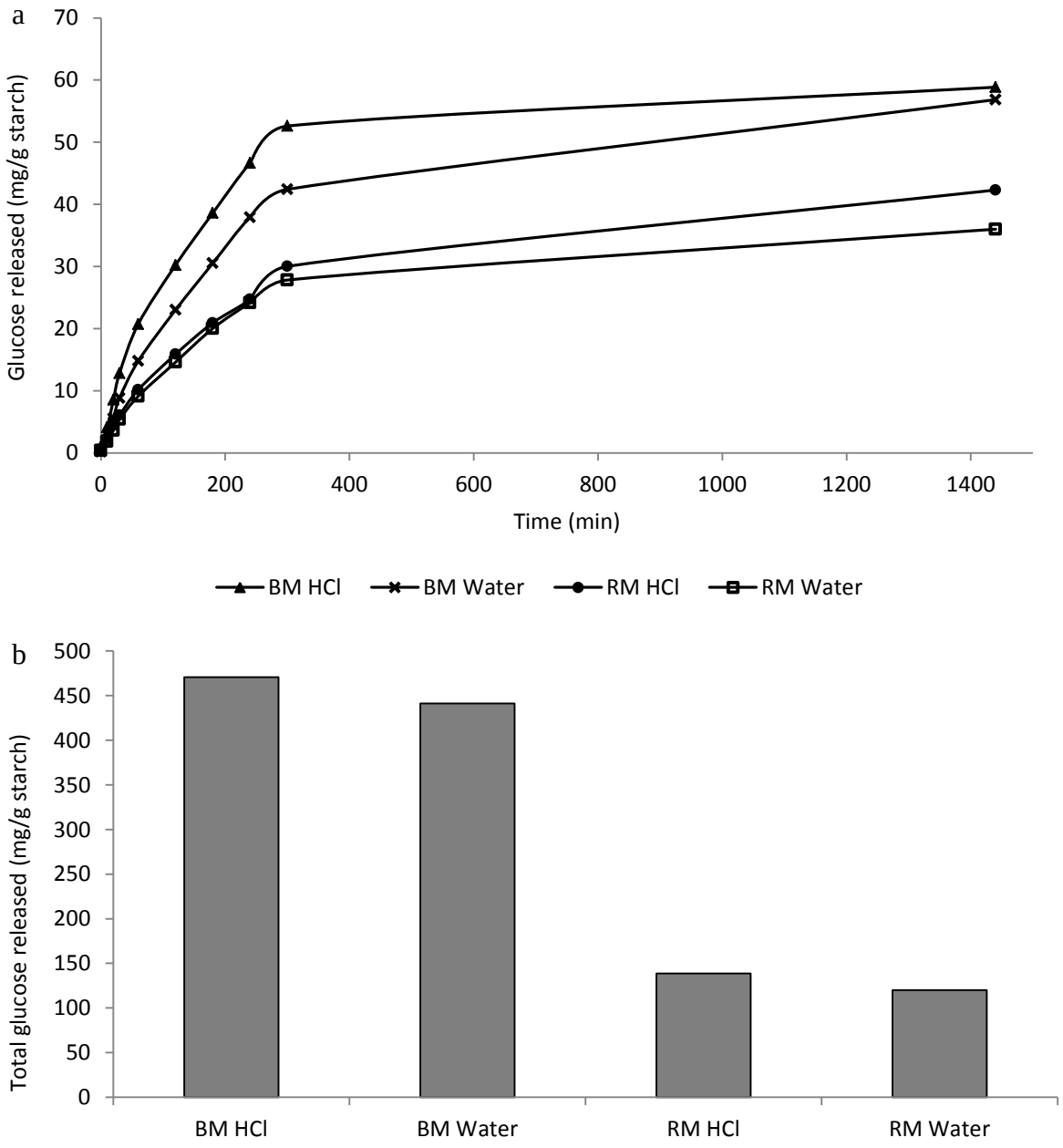


Figure 4.7 Glucose released from starch in a maize grit diet, processed under different gastric conditions, following *in vitro* digestion. Diets were processed by ball milling (BM) or roller mixing (RM) in HCl-pepsin solution or water. Data show glucose release over time during digestion with pancreatin (a) as well as total glucose release following supernatant incubation with amyloglucosidase (b).

Following processing and then digestion with pancreatin, starch crystallinity of samples was studied (Fig 4.8). A very similar pattern can be seen to previous crystallinity measurements for the different processing methods on the maize grit diet prior to digestion with pancreatin.

Ball milling decreased crystallinity to a greater extent than roller mixing compared to the unprocessed maize diet. Furthermore, samples processed in HCl-pepsin solution had a slightly lower crystallinity compared to samples in water, however, the variation in means was larger for these samples. Due to the greater particle size reduction and extent of starch digestion, the process of ball milling diets in HCl-pepsin was used to mimic gizzard conditions in all further experiments.

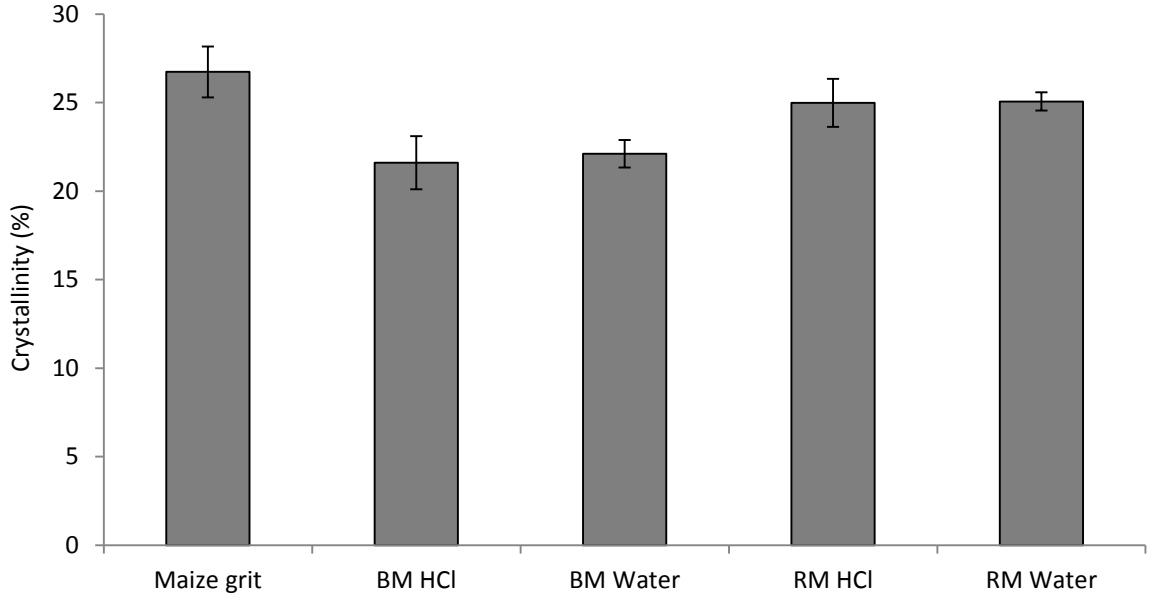


Figure 4.8 Effect of maize grit processing on the crystallinity of starch following digestion by pancreatin. Diets were processed by ball milling (BM) or roller mixing (RM) in HCl-pepsin solution or water. Error bars denote ± standard error of the mean (from 2 replicates).

Using ball milling and HCl-pepsin solution to mimic gizzard conditions, the extent of starch digestion was determined for different maize-based diets; native starch, maize grit and maize-soy with or without xylanase (Fig 4.9). This was to investigate the effect a food matrix has on native maize starch digestion. When looking at digestion by the amylase in pancreatin (Fig 4.9a), overall poor digestion of native maize starch was demonstrated. The initial rate of glucose release was higher than the other diets, although levels plateaued much earlier. This would be expected due to the nature of native granules being relatively resistant to digestion.

Moreover, the native starch diet would not have been previously ground as the other diets. The mechanical grinding of cereals causes damage to starch granules thereby enabling greater access of amylases to the starch. This would not have been the case for the native starch diet. The residual amylase in diets may also have initiated breakdown of the starch, although this was not assessed in this work.

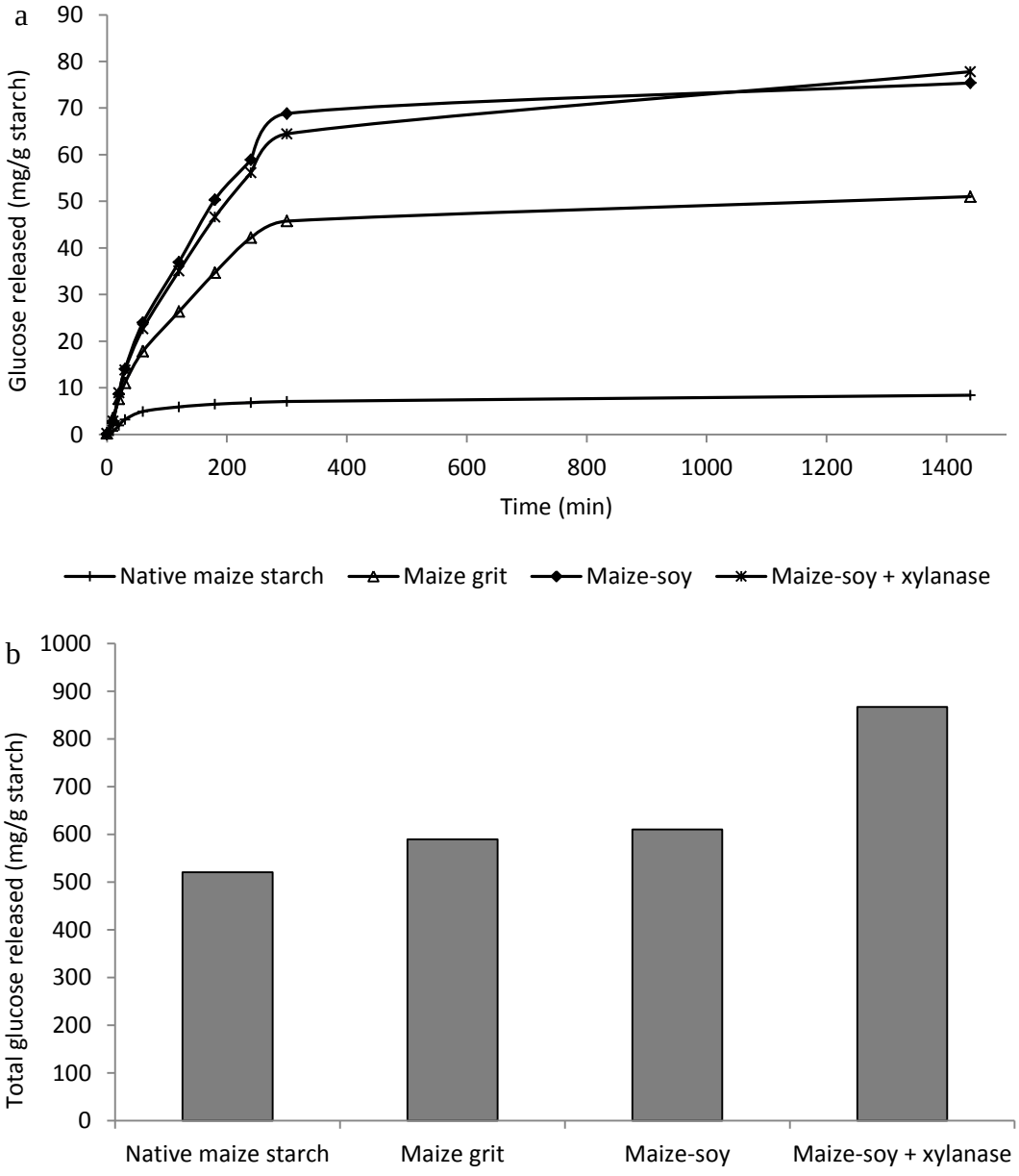


Figure 4.9 Glucose released from starch following *in vitro* digestion of different maize-based diets. Data show glucose release over time during digestion with pancreatin (a) as well as total glucose release following supernatant incubation with amyloglucosidase (b).

A greater extent of starch digestion was seen in the maize grit diet, potentially due to the process of milling prior to *in vitro* digestion. The largest proportion of starch was digested in the maize-soy diet with or without xylanase. This may be explained by the lower initial starch content of the maize-soy diet which would provide a higher enzyme to substrate ratio. Moreover, total starch measurements of the soybean in the diet revealed a content of approximately 7 % starch. Starch in soybean may be more easily digested due to their small particle size (Wilson *et al.*, 1978; Kong *et al.*, 2003), however its proportion in the diet is not great enough to account fully for the greater release of glucose. Amylase requirement for calcium and chloride for stabilisation and efficient activity has been recognised (Levitzki and Steer, 1974; Vallee *et al.*, 1959), with the removal of calcium leading to decreased enzyme activity and increased susceptibility to proteolytic degradation. The maize-soy diet also contains added calcium carbonate (limestone), monocalcium phosphate, and sodium chloride as part of the complete diet (see Table 3.2) and therefore the presence of these ingredients, supporting enhanced amylase activity, is the most likely explanation for the greater release of glucose in this diet. Following digestion of the collected supernatants with amyloglucosidase (Fig 4.9b), the results show a similar pattern. However, maize-soy diets supplemented with xylanase show a near 20 % improvement in starch digestion. This could be due to xylanase disruption of cell wall material which would lead to the release of starch granules from the endosperm, thereby making them more available for digestion (Masey O'Neill *et al.*, 2014b).

To confirm these results, repeat digestions of maize-soy diets with or without xylanase were performed and the results presented in Figure 4.10. The protocol for digestion was also modified to include a wash step every hour, with supernatant collection. Furthermore, as the extent of starch digestion did not greatly enhance from 5 h to 24 h of digestion, the period of intestinal digestion was completed at 5 h.

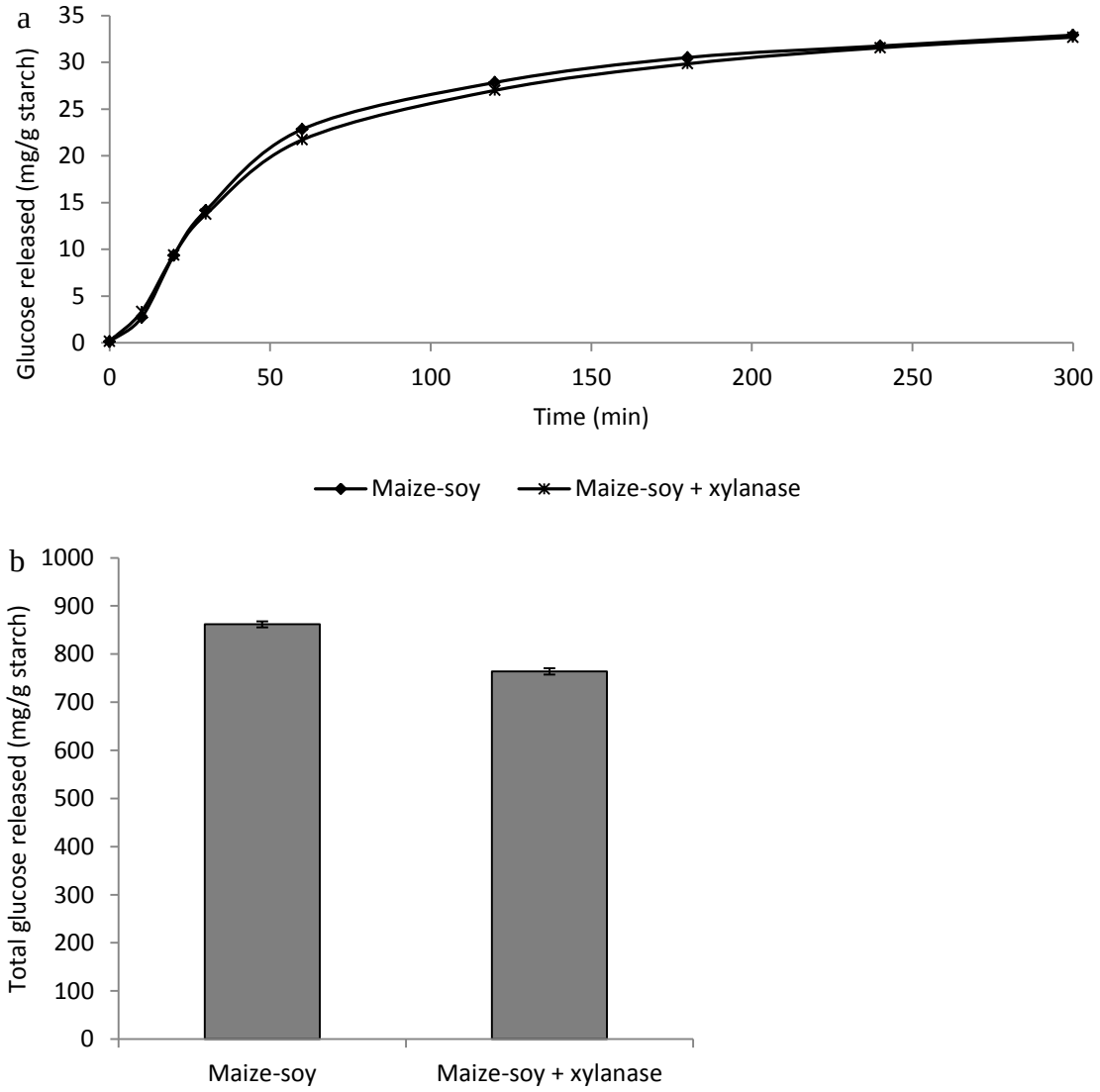


Figure 4.10 Glucose released from starch following *in vitro* digestion of a maize-soy diet with or without xylanase inclusion. Data show glucose release over time during digestion with pancreatin (a) as well as total glucose release following supernatant incubation with amyloglucosidase (b). Error bars denote ± standard error of the mean (from 2 replicates).

The results of this *in vitro* digestion showed no clear evidence that xylanase improved starch digestion in a maize-soy diet. One explanation for this difference could be the previous loss of maize-soy sample during supernatant decanting. Another possible explanation could be that the wash steps prevented the xylanase from acting on specific areas of the NSP thereby nullifying the effect. It is also interesting to note that the glucose released by amylase by the end of the experiment was lower in the repeated digestion. It is possible that more of the

sample was lost during the greater number of wash steps. However, digestion of the supernatant gave similar values for both experiments, with the amount of glucose released from starch reaching around 80 %. However, in the latter experiment, no obvious difference was observed between maize-soy diets with or without xylanase supplementation.

4.3 Conclusions

In vitro digestion models are widely used to study the structural changes, digestion, and release of food components under simulated gastrointestinal conditions. However, the results of *in vitro* digestion models are often different to those found using *in vivo* models because of the difficulties in accurately simulating the highly complex physicochemical and physiological events occurring in animal digestive tracts. However, this does not deflect from the importance of *in vitro* models to examine individual aspects of the digestive process as well as to draw comparisons between different feed ingredients.

From these results, it would appear that ball milling does affect starch crystallinity to an extent, yet does not cause granules to lose all integrity. Grinding action in acidic conditions is also important for particle size reduction and potentially preparing starch for digestion later in the digestive tract, for example, by removing proteins and HCl erosion of the granule surface. In support of this, ball milling of maize samples showed a greater extent of starch digestion compared to roller mixed samples. Samples mixed with HCl-pepsin solution revealed only a slight improvement in starch digestion compared to samples in water. This may be due to the use of a relatively weak concentration of acid to achieve the required pH. Therefore, this concentration may have not been high enough for a significant impact on the diet.

Using ball milling in HCl-pepsin as a model for gizzard action, the digestion of different maize diets was assessed. Following digestion of diets with pancreatin, digestion of the native

maize starch diet was relatively minimal. This reinforces the concept that native starches are highly resistant to digestion. This diet also had more particles in the smaller size range which may suggest that a smaller particle size does not necessarily mean greater potential for digestion. Moreover, the absence of other feed components would be expected to enhance the availability of the starch granules to enzymatic hydrolysis. The amount of enzyme was not considered to be limiting due to the re-addition of pancreatin following the wash steps. Maize grit and maize-soy diets both showed a greater extent of starch digestion. These diets were ground by the supplier prior to experimentation and therefore this process is likely to have damaged starch granules thereby making them more readily digested.

Maize-soy diets showed the greatest extent of starch digestion. This may have been due to the lower initial starch content which would signify a higher enzyme to substrate ratio. The addition of soybean meal would have increased the protein and NSP content in the diet and therefore might be expected to create a barrier for enzyme access to the starch granules. However, the mixing of samples before each glucose measurement would allow dispersion of the enzyme through the solution, thereby enabling contact with starch. Potentially, grinding of the maize-soy diet prior to and during ball milling was also enough to fractionate these components to a similar extent as those in the maize grit diet thereby minimising their coating effect. As a result, the difference in starch digestion between the diets is most likely due to the additional calcium and chloride in the maize-soy diet, enabling enhanced amylase activity. Supplementation of the maize-soy diet with xylanase did not prove to enhance the extent of starch digestion in the diet.

Even with fractionation of encapsulating plant material and disruption to granule integrity, it is important to note that only around 50 and 80 % starch in the maize grit and maize-soy diet, respectively, was digested. This implies that there was still a proportion of starch that remained undigested by the end of the *in vitro* digestion process. *In vivo* digestion studies

have reported up to 97 % starch digestion by the end of the lower small intestine. Therefore, it is clear that this digestion model does not truly replicate that of the bird. This represents the difficulties when trying to mimic highly complex biological mechanisms in *in vitro* conditions. Consequently, such models are useful for identifying differences between diets and predicting nutrient digestibility, however to gain a more accurate understanding of starch digestion in the bird *in vivo* models are necessary.

CHAPTER 5

BROILER DIGESTION OF MAIZE STARCHES

Maize starch is often used as the major energy ingredient in broiler diets. *In vitro* models to simulate starch digestion in the broiler have been designed, however starch digestion *in vivo* is still not fully understood, suggesting a need for additional information before a more detailed understanding of the mechanisms affecting starch digestion can be elucidated. Broiler digestion of a native maize starch and a maize grit diet was therefore investigated to determine the effect that feed matrices have on the digestion of starches *in vivo*.

5.1 Broiler digestion of native maize starch

This study investigated how and where native maize starch, without the influence of other feed components, is digested in the broiler. Male Ross 308 broiler chicks (40) were group housed until day 28 after which 24 birds were separated and caged individually; 24 cages in total. The experimental diet fed on day 29 was composed of a native maize starch crumb (as described in section 3.1.3). On day 29, following a 24 h starvation period, 2 control birds were slaughtered at 0 min before any experimental diet was offered. The remaining 22 birds were given 50 g pre-weighed experimental starch diet that was removed after 20 min. Only 3 out of the 22 birds were observed to ingest any of the diet offered and therefore these birds were slaughtered at 25, 55 and 95 min following the start of feeding and subsequent dissection carried out, as described in section 3.2.2.2.

5.1.1 Diet palatability

Preliminary tests for intake of the experimental starch crumb following 3 h and 17 h starvation periods indicated the need for a longer starvation time to increase appetite and thus encourage intake. However, this study demonstrated that even with a 24 h starvation period only 3 out of the 22 birds ingested some of the experimental starch crumb, suggesting poor palatability of the diet. Due to the limited number of birds ingesting the starch, replication was not viable. Observations of the duodenum revealed possible signs of tissue damage and bleeding. Furthermore, excreta produced from a control bird before slaughter showed contents of blood. There is a lack of understanding about the impact of feeding native maize starch in its pure form, such as in synthetic diets, to broilers. More apparent signs of duodenum tissue damage were observed in the bird slaughtered 95 min following feeding. This suggests that feeding pure starch disrupts intestinal milieu, although further tissue analysis would be needed to confirm these effects. Damage to the intestine may have also been contributed to by intestinal sloughing as a result of a long starvation period (Northcutt *et al.*, 1997). This imposes a problem for experimental trials involving starvation before re-feeding, as impaired intestinal function will affect rate and extent of nutrient digestion.

5.1.2 Blood glucose concentration

An increase in time interval following feeding correlated with an increase in blood glucose (Fig 5.1). This is indicative of starch digestion in the small intestine with the resulting glucose being absorbed into enterocytes and entering the blood. As time following feeding increases, more of the starch has reached the intestine thereby enhancing glucose production and raising its concentration in the blood. However, birds are also known to maintain their blood glucose concentrations at relatively constant levels (Braun and Sweazea, 2008), and therefore bird variation must also be taken into account. As only one bird replicate was taken at each time point, this data cannot be taken as representative of the population.

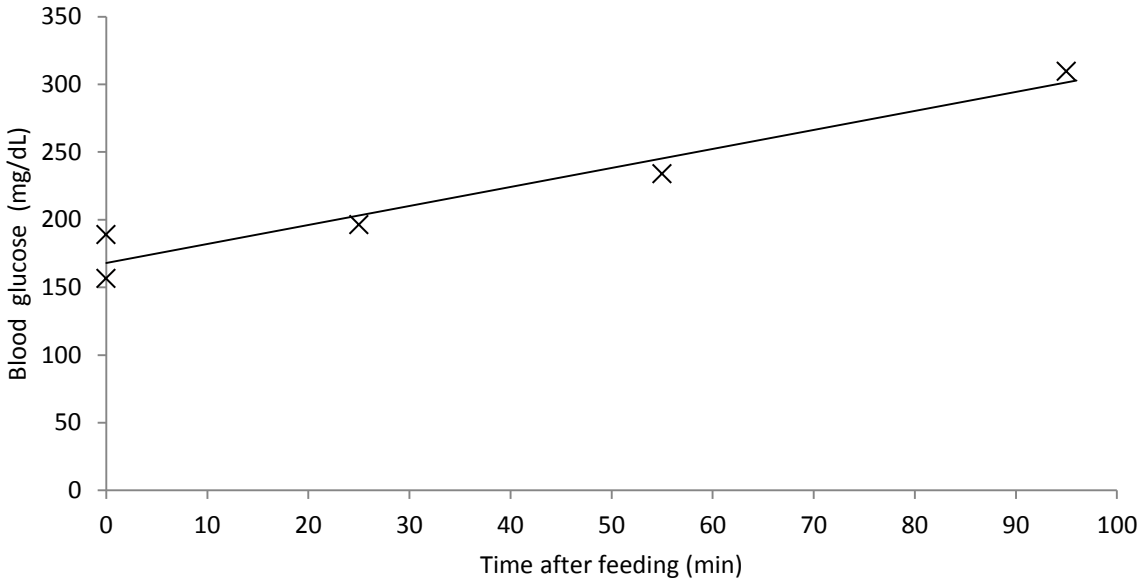


Figure 5.1 Blood glucose concentration of non-fed control birds and native starch fed birds. Data show blood glucose concentration (mg/dl) for birds at each time point (25, 55 and 95 min) following feeding as well as the non-fed controls (0 min). Solid line represents the trend in blood glucose concentration over the 95 min time period.

5.1.3 Digesta pH

Digesta pH for each tract section was measured, as described in section 3.2.2.2, to investigate what effect feeding solely native starch has on the digestive environment. Digesta pH increased from the gizzard to the upper and small intestine (Fig 5.2). Due to the lack of bird replicates, the change in digesta pH over time with feeding could not be determined. However, this information would be useful to understand how purified diets might affect digestive tract milieu and thus the digestive efficiency of the animal.

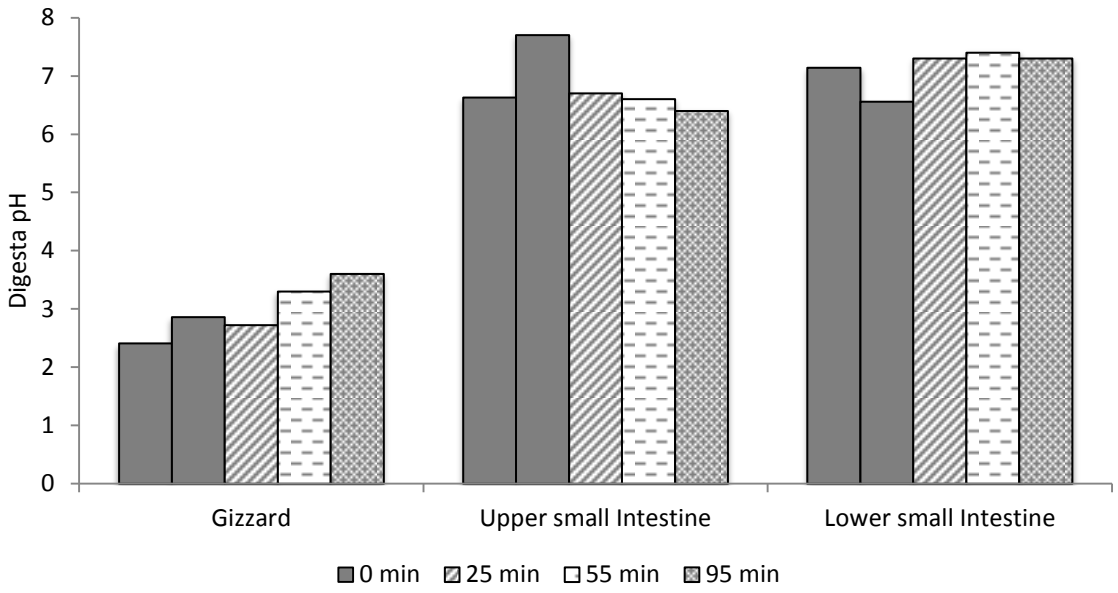


Figure 5.2 Digesta pH of birds fed a native starch diet. Data show the pH of digesta from distinct tract sections at 25, 55 and 95 min following feeding as well as the non-fed controls (0 min).

5.1.4 Starch digestion

Total starch assay results, according to the method in section 3.2.4.3, are presented in Figure 5.3. Samples with limited material were unable to be assayed and therefore the starch content of these samples is unknown. As only 1.15g, 7, 19g and 9.53 g of diet was ingested by birds slaughtered at 25, 55 and 95 min, respectively, these values for starch content cannot be taken as a true representation of native starch digestion through the tract of birds. However, these results were able to show the presence of starch in the digesta of control birds, indicating that starches were still remaining from the previous diet. The greatest amount of starch was found in the gizzard at 25 min, the upper small intestine at 55 min, and the lower small intestine at 95 min, following feeding, signifying digesta flow through the tract of birds over time. As the major site of starch digestion is considered to be in the more proximal intestine it is interesting to discover a large amount of starch remaining by the end of the intestinal tract. It is possible that the digestive tract cannot cope with the digestion of such large quantities of

starch at one time. Also, the absence of other plant material may reduce the retention of feed in the tract and therefore it can pass through relatively quickly leaving not enough time for enzyme digestion.

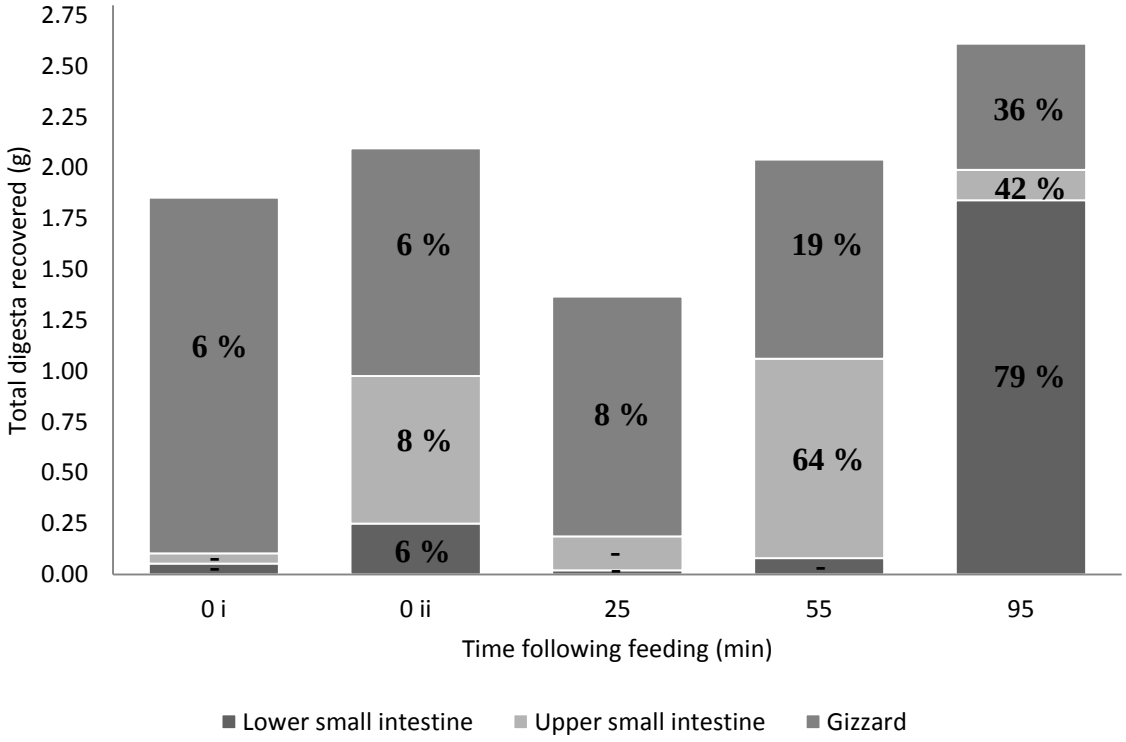


Figure 5.3 Starch content of digesta from distinct sections of the tract as a percentage of the total sample recovered from that section. The total weight (g) of digesta samples collected from each section of the tract, at 0 (two controls, i and ii), 25, 55 and 95 min following feeding, were recorded. Total starch for each digesta sample was determined and expressed as a percentage of the total sample recovered for the specific section. Samples with insufficient material to perform the total starch assay on are denoted with a (-).

Figure 5.4 shows SEM images of starch granules located in the digesta of control birds (Fig 5.4a) and birds slaughtered at 25, 55 and 95 min following feeding (Fig 5.4b-d). Supporting total starch assay results, SEM images revealed starch granules throughout the tract of control birds with varying degrees of hydrolysis. A food withdrawal period of 8-12 h is often used in the broiler industry to clear the tract of any feed residues (Northcutt *et al.*, 1997). However,

this study shows that even after a 24 h period of food withdrawal, starch is still present throughout the tract, imposing a problem for *in vivo* digestibility studies.

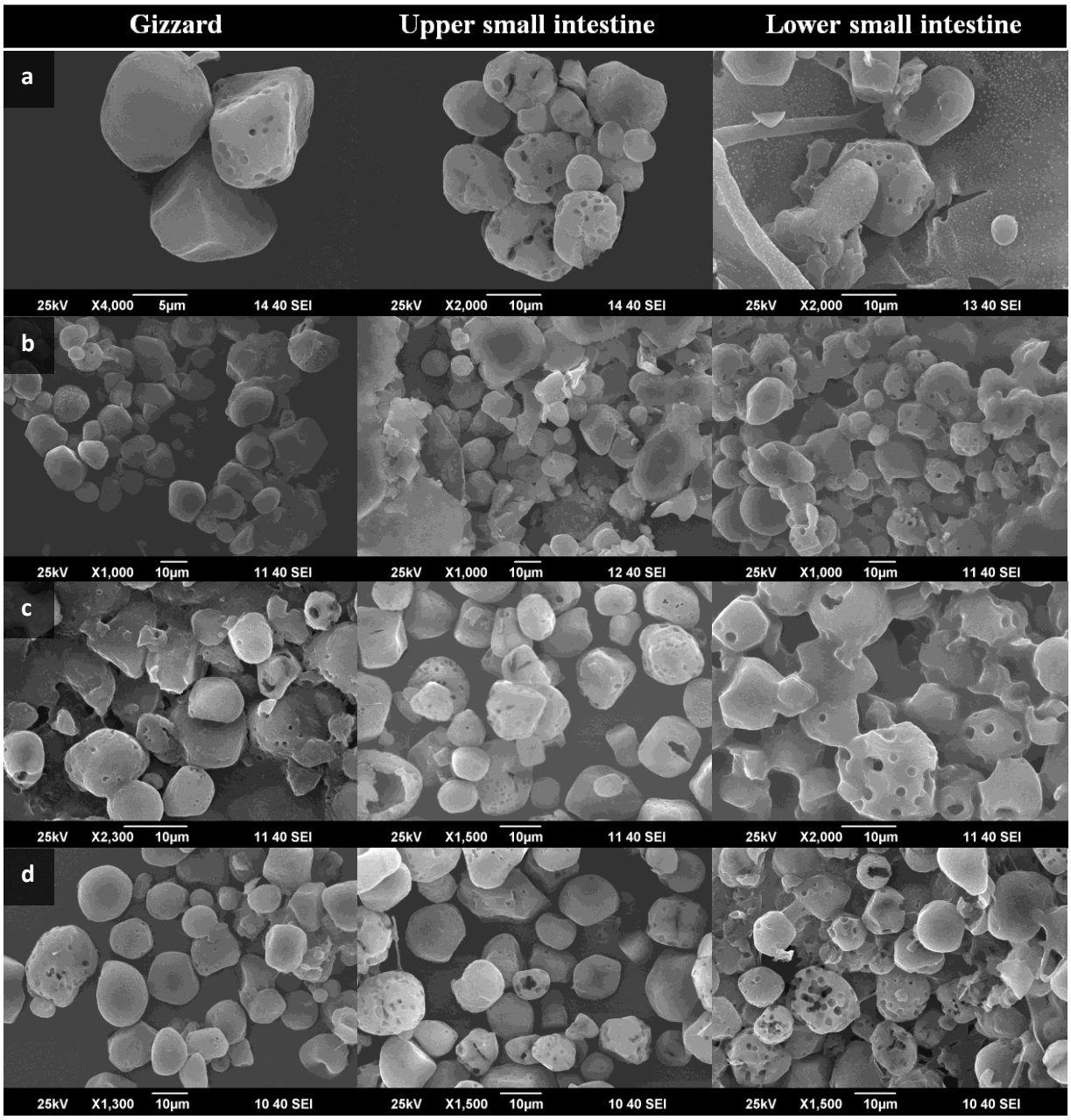


Figure 5.4 SEM images of starch granules from *in vivo* digesta. Images show starch granules in digesta taken from distinct sections of the gastrointestinal tract at 0 min (a), 25 min (b), 55 min (c) and 95 min (d) following feeding.

All birds slaughtered following feeding showed a slight increase in the proportion of damaged starch granules as the digesta moved from the gizzard through to the lower small intestine.

Starch granules with multiple holes, typical of amylase digestion, were seen in the gizzard of all birds. As α-amylase activity is limited in such acidic conditions it is likely that these damaged granules have been refluxed from the small intestine by means of anti-peristaltic movements. However, as native maize starch contains a small number of granules with signs of hydrolysis, presumably caused by amylase attack during germination, this may also explain the occurrence of some partially hydrolysed granules found in gizzard digesta. Starch recovered from the intestine showed that most of the granules were severely disrupted with signs of fragmentation. SEM images clearly suggest α-amylase attack through pores in the granules. In this way, α-amylase digests the amorphous regions first, leaving the more crystalline regions, as demonstrated by concentric ring structures observed in some partially hydrolysed granules. Interestingly, in every section of the tract some granules did not show any enzymatic breakdown, suggesting a population of starch granules resistant to digestion, despite no other coating materials being present in the feed. Due to the limited intake of the native starch diet, further investigation was required for a more comprehensive understanding of starch granule breakdown through the digestive tract of birds.

5.2 Digestion of a maize grit diet

Work continued into understanding native starch granule digestion through the feeding of maize to birds. Broiler digestion of a maize grit diet was investigated to determine what effect a food matrix has on starch digestion *in vivo*. Broiler chicks (50) were group housed until 28 days of age, after which 36 birds were caged individually. At 36 and 37 days of age, following a 17 h starvation period, 14 birds were offered 50 g maize grit diet for 20 min with 2 birds remaining unfed (control, 32 birds in total). Control birds were subsequently

euthanised and dissection was carried out. Once feed was withdrawn, the remaining birds were euthanised in duplicate every 10 min.

5.2.1 Blood glucose concentration

Fasting and non-fasting blood glucose levels were measured for indication of starch digestion and absorption (Fig 5.5), as described in section 3.2.2.2. Average blood glucose (mg/dl) for fed birds stayed within expected range of 210-250 mg/dl with non-fed control birds having around 20 % lower blood glucose levels. Chickens have been reported to maintain blood glucose levels during fasting for periods up to 72 h (Belo *et al.*, 1976) signifying the capability of chickens to avoid fasting hypoglycaemia. A 20 % increase in blood glucose concentration after 10 min following feeding is suggestive of starch entering the intestine and digestion taking place. Statistical analysis using univariate ANOVA revealed no significant difference ($P > 0.05$) between the blood glucose concentrations of fed birds slaughtered at each time point following feeding. However, the concentration of blood glucose at 20 min was significantly higher than that of control birds ($P < 0.05$). This initial peak in glucose concentration is suggestive of an insulin response to the elevated blood glucose level after a fast, as a result of starch digestion. However, bird to bird variation must also be taken into account. As glucose concentration did not significantly change over time, this may suggest a more consistent digestion of starches compared to the native starch diet. This may imply that a feed matrix gives a more balanced digestive process. However, the stress indicators, low bird numbers and small quantities of starch ingested in the first study must also be considered.

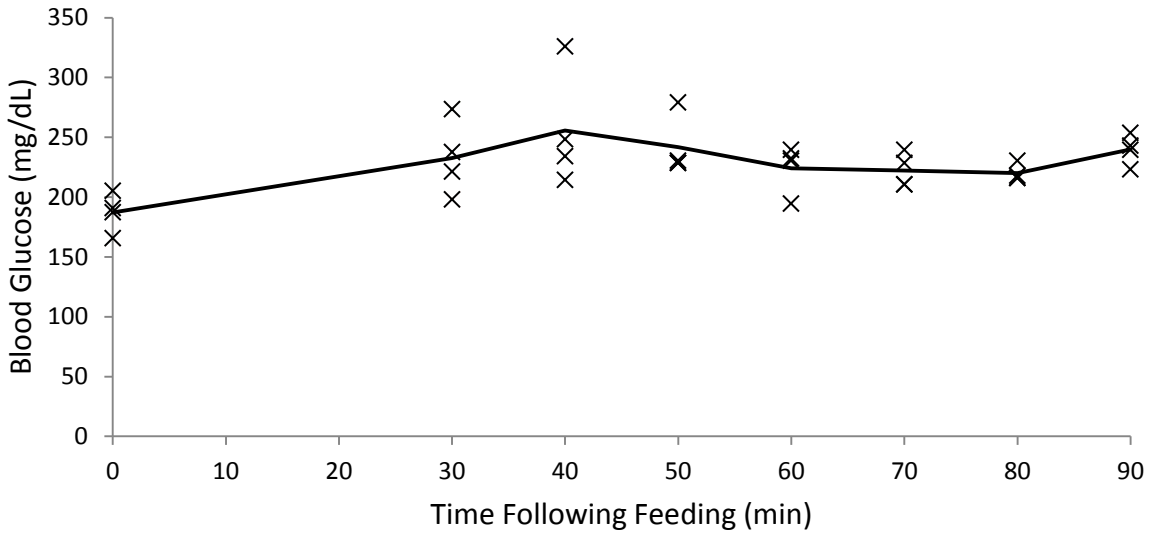


Figure 5.5 Blood glucose concentration of non-fed control and maize grit fed birds. Data show blood glucose concentration (mg/dl) for bird replicates at each time point following feeding as well as the non-fed controls (0 min). Solid line indicates average blood glucose over the 90 min time period.

5.2.2 Digesta pH

The pH of digesta taken from each tract section was recorded for birds fed a maize grit diet as well as non-fed control birds (Fig 5.6). Digesta pH significantly increased as gut contents passed through each tract section ($P < 0.05$), as determined by univariate ANOVA. Gizzard digesta pH was within the range of pH 2.2-3.0 due to the acidic conditions of the gizzard. As digesta entered the upper small intestine, alkaline pancreatic juices increased the pH to around pH 6.1-7.0 in the upper small intestine and pH 6.8-8.0 in the lower small intestine. The slightly lower pH in the upper small intestine is potentially due to the acidic digesta entering this section from the gizzard. By the time the digesta enters the lower small intestine, the alkaline fluid has generally maintained digesta pH. The pH of digesta taken from individual tract sections did not significantly change over the time period following feeding ($P > 0.05$), as determined by multivariate ANOVA. The results also suggest that a 17 h starvation period with re-feeding does not significantly affect pH of gizzard or small intestinal contents ($P > 0.05$).

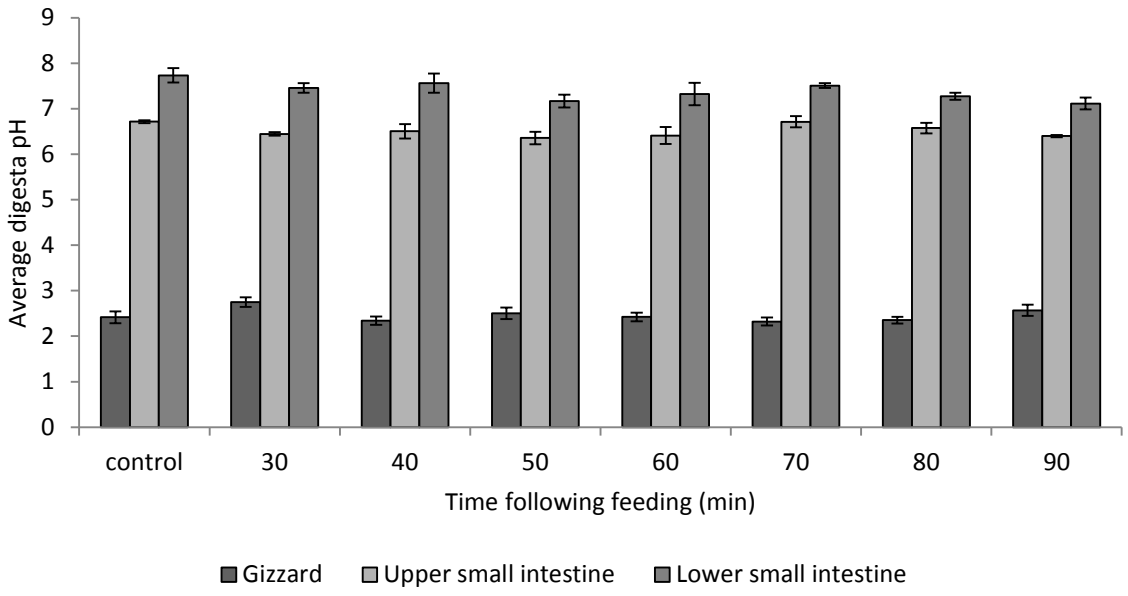


Figure 5.6 Digesta pH of birds fed a maize grit diet. Data show the average pH of digesta from distinct tract sections at 10 min intervals following feeding as well as the non-fed controls. Error bars denote ± standard error of the mean (from 4 bird replicates).

5.2.3 Starch digestion

The content of starch, dextrin and glucose in digesta, according to the total starch method in section 3.2.4.3, is shown in Figure 5.7a-c, respectively. Birds consumed approximately 10 g maize grit diet and therefore there was enough digesta collected to obtain measurements for all tract sections and time points. Univariate and multivariate ANOVA were used to test for significant differences between samples. Between 30-50 min after feeding there was a significantly higher amount of starch in gizzard digesta compared to control birds as well as other tract sections ($P < 0.05$). Mean retention time of feed in the proventriculus and gizzard has been estimated to be between 30 min and 1 h depending on the diet composition and processing method (Shires *et al.*, 1987; Svihus, 2014a). By 60 min, the content of starch in gizzard digesta decreased to levels comparable to that of control birds and upper small intestine starch content. From 70 min, the content of starch in digesta was not significantly

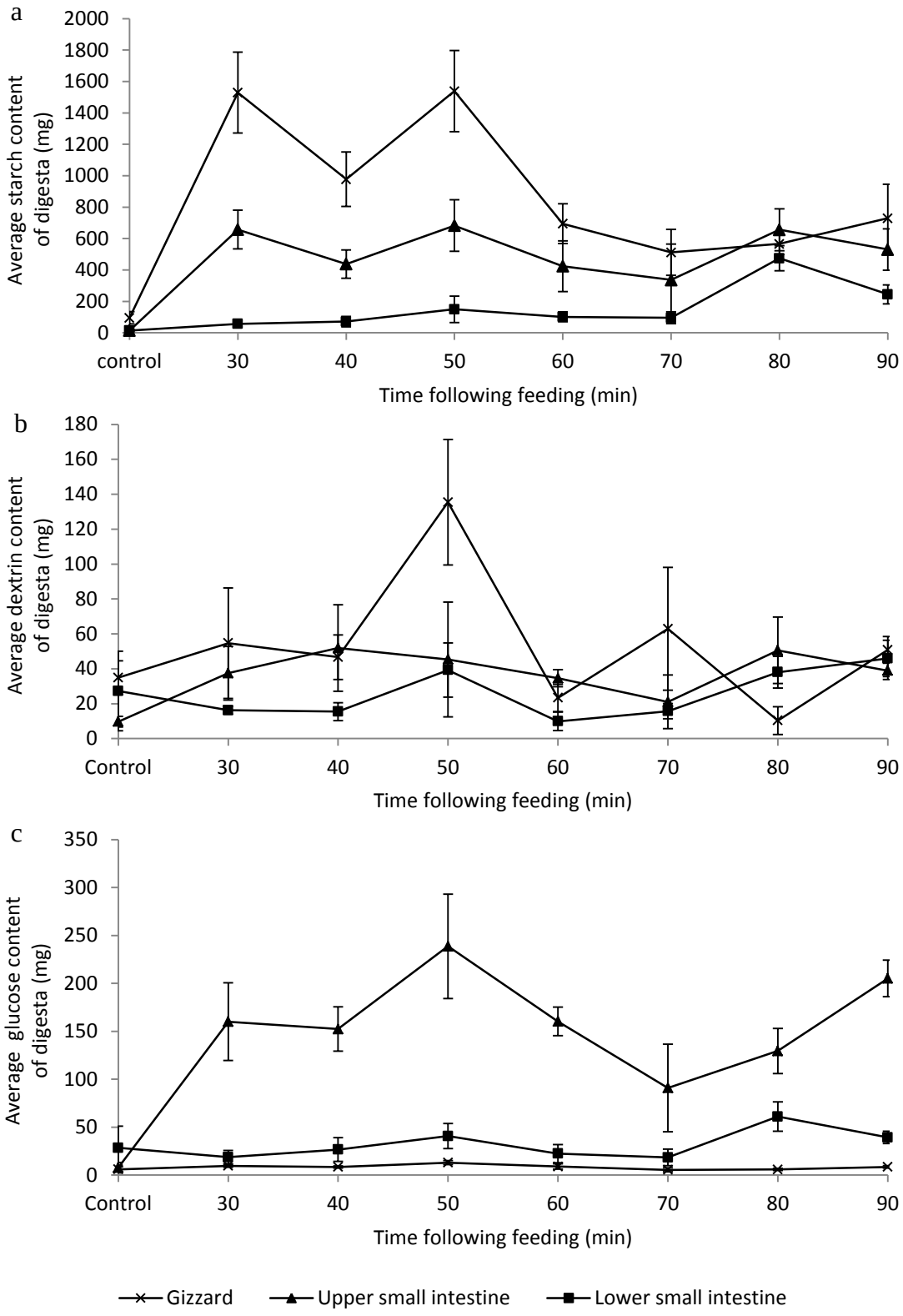


Figure 5.7 Digesta soluble and insoluble fractions of birds fed a maize grit diet. The average weight of starch (a), dextrin (b) and glucose (c) in the total digesta taken from distinct sections of the gastrointestinal tract at 10 min intervals over a 90 min time period following feeding is shown. Fractions measured in non-fed control birds are also presented. Error bars denote ± standard error of the mean (from 4 bird replicates).

different between each tract section ($P > 0.05$). Upper small intestine starch content was relatively maintained over time ($P > 0.05$), indicating continual filtration of feed from the gizzard and passage through the intestine. Results also show that a significant amount of starch did not enter the lower small intestine until 80 min after feeding.

Throughout the time period following feeding dextrin content of the upper and lower small intestine did not significantly change over time ($P > 0.05$). Gizzard dextrin content was also generally maintained, although dextrin content at 30 min was significantly higher than that at 60 and 80 min ($P < 0.05$). It could be assumed that the continual hydrolysis of starch into dextrin, followed by dextrin into glucose, would keep dextrin levels generally maintained with levels fluctuating according to the stage of digestion. As expected, the glucose content of gizzard digesta was minimal as starch digestion does not occur in this tract section. Conversely, the upper small intestine maintained a significantly higher glucose content throughout the time period after feeding compared to other tract sections and control birds ($P < 0.05$). Interestingly, it would be expected that the glucose released by starch digestion would be rapidly transported into enterocytes. However, it would seem from these results that glucose still lingers in the lumen for a short time before being absorbed as digesta reaches the lower small intestine.

Example SEM images of starch granules in digesta are shown in Figure 5.8. It is important to note that the amounts and digestion of starches differed between birds. Moreover, during SEM imaging, a number of micrographs were taken across the sample being analysed, and the micrographs chosen were selected to best represent the sample. Consequently, the subjective nature of the SEM must be taken into consideration when interpreting the images. SEM images revealed an unexpectantly large proportion of starch granules, mainly held within plant material, throughout the tract of control birds even after a 17 h starvation period (Fig 5.8a). This finding was also supported by total starch results. In fed birds, predominantly

undamaged granules are present in the gizzard with progressively more damaged granules being visible as the digesta moves through the tract (Fig 5.8b).

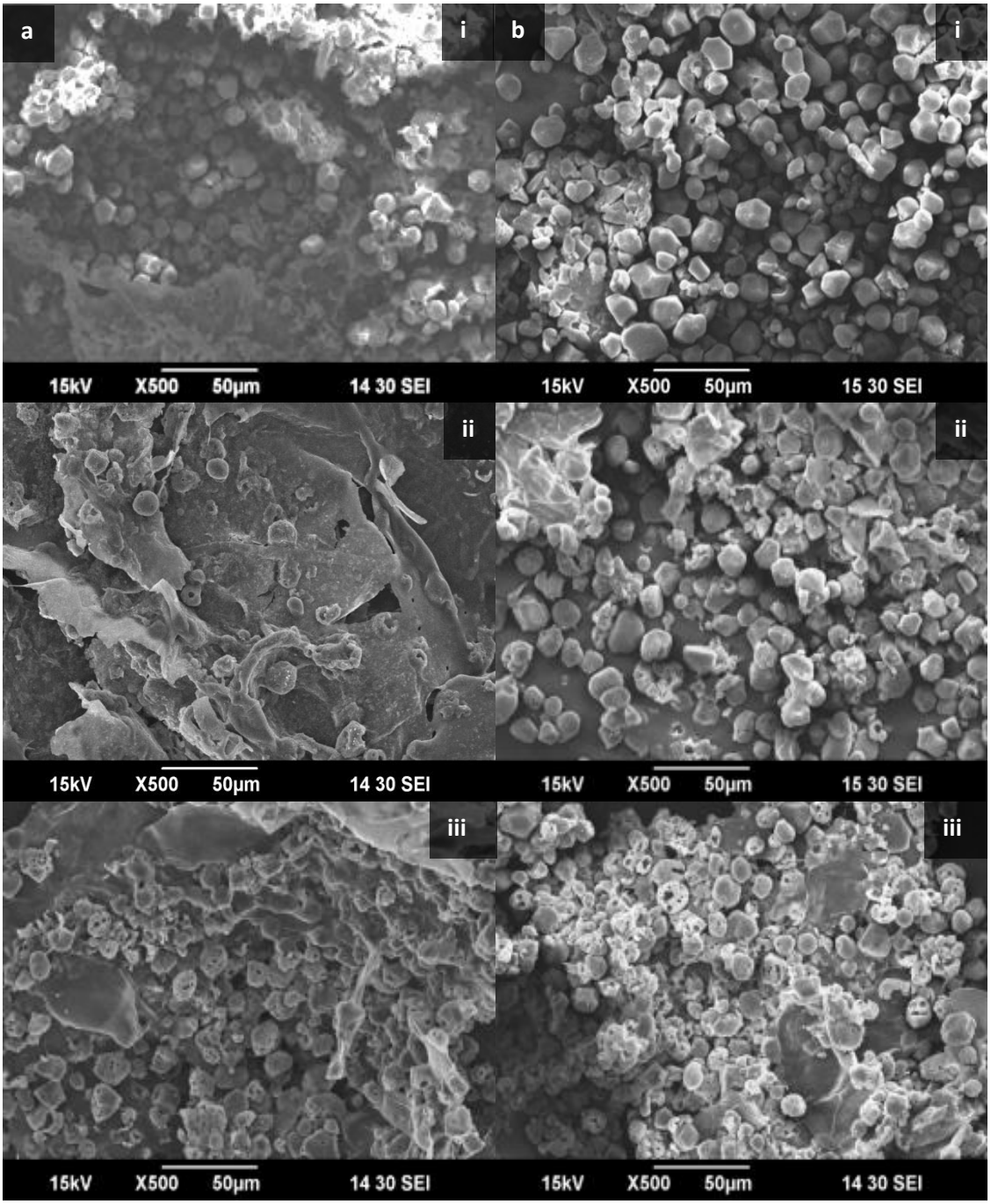


Figure 5.8 SEM images of starch granules in digesta taken from birds fed a maize grit diet. Images show starch granules in digesta taken from the gizzard (i), upper (ii) and lower small intestine (iii) of a control birds (a) and a bird slaughtered at 60 min following feeding (b).

Moreover, for the majority of starch granules that still remain in the lower small intestine, plant material seems to be entrapping the granules, thereby potentially limiting their digestion. The presence of large quantities of NSP in the small intestine has been shown to lead to fermentation in this section, a process which Choct *et al.* (1996) showed to be detrimental to bird health. Therefore, promoting starch release from physical matrices could be considered essential for improving broiler performance.

5.3 Conclusions

The purpose of the current studies was to investigate how and where the bird digests starches and to understand what influence a food matrix has on the digestive process. In the first study, it was assumed that native starches were rapidly digested due to the excessive increase in blood glucose concentration. However, a relatively large proportion of starch was still present by the lower small intestine. This could have been due to the rapid movement of starches through the tract as a result of a small particle size and lack of fibrous material to enhance feed retention. The major limitation to this study was the inadequate starch intake despite the addition of water to the starch, producing a more crumb-like consistency, and a starvation time of 24 h. Consequently, this resulted in a lack of sample replication and a few non-assayable samples. Therefore, the poor palatability of this starch crumb needs to be amended for increased intake. This would need to be accomplished without changing starch structure or interfering with the process of starch digestion.

To consider the effect that plant matrices might have on starch digestion, the second study was conducted. In this study, blood glucose concentrations were generally maintained over the course of feeding suggesting a consistent digestion of starch. Similarly to the native starch diet, the passage of feed through the tract could be followed using total starch measurements

along with SEM imaging. From this, it can be assumed that feed is held within the gizzard for approximately 50 min following feeding. During this time, feed is filtering into the upper small intestine, where starch is digested and its product of hydrolysis, glucose, is released. Digesta then enters the lower small intestine at around 80 min, for the last stages of digestion before the undigested feed leaves and enters the caeca/large intestine. It is important to note, that throughout this process feed is continuously being fluxed back and forth through the different tract sections by peristatic and retroperistaltic muscle movements. Therefore, these timings are only related to a significant change in starch content for any tract section.

In both studies, starch granules were still seen throughout the tract of starved control birds. Consequently, starches remaining from the previous diet would be included in total starch measurements leading to a slight over estimation of digesta starch content for fed birds. Moreover, a number of granules showed signs of enzyme attack, although digestion of these starches was not completed. This poses the question as to whether amylase activity in basal pancreatic secretions is sufficient for continued digestion or whether a physical or chemical barrier is preventing any further hydrolysis of the granule.

From these results, there is still a question as to whether feeding starches within a feed matrix aids or hinders digestion of these starches. Feeding a maize grit diet aided a steady digestion of starch and glucose release, thereby preventing glucose overload. Moreover, structural components may have supported feed retention resulting in more time for digestive processes. However, these materials can encapsulate starches thereby limiting enzyme access to the granules. Conversely, native starch diets are not encapsulated, however this may mean that starches pass quickly through the tract leaving minimal time for enzyme hydrolysis. In theory a balance is needed; a food matrix is required to support digestive processes, while also being easily removed to expose starch granules for digestion. This leads to the use of exogenous enzymes in feed to breakdown encapsulating matrices for an improved nutrient digestibility.

CHAPTER 6

EXOGENOUS ENZYME SUPPLEMENTATION

With an increasing demand from the biofuels industry for cereals such as maize to be used for ethanol production, the cost of such feed in recent years has risen. Consequently, considerable attention has been given to improve nutrient utilisation of these diets through the use of exogenous enzymes, including xylanases, to release more energy from the diet. Xylanase inclusion in maize-based diets has been shown to improve broiler performance (Cowieson, 2010). However, the mechanisms of xylanase action in such diets are still under debate. One mechanism is cell wall dissolution resulting in the release of encapsulated nutrients making them more readily available for digestion by endogenous enzymes. Another is a putative prebiotic mechanism, whereby AXOS released by xylanase hydrolysis of arabinoxylans leads to the colonisation of beneficial bacteria in the tract as well as increased VFA production. These VFA may stimulate PYY hormone secretion resulting in increased gastric retention and improved nutrient digestion. To investigate if the inclusion of a xylanase into a maize soy diet impacted on the digestive process a single feed trial was carried out. A second investigation fed the supplemented feed over a period of time to establish if the impact of the enzyme was some type of prebiotic mechanism.

6.1 Digestion of starch in birds fed xylanase on a single occasion

Xylanase supplementation of a maize-soy diet on a single occasion was investigated to determine what direct effect xylanase has on starch digestion in broilers. Male Ross 308 broiler chicks (32) were group housed until day 29, after which birds were separated and

caged individually; 32 cages in total. At 37 and 38 days of age, following a 17 h starvation period, 7 birds were offered 50 g maize-soy diet and a further 7 birds 50 g maize-soy diet plus xylanase (Econase XT 25 at 100 g/t; 18,100 BXU/kg), with 2 unfed control birds being euthanised. Feed was withdrawn after 20 min and birds euthanised in duplicate (1 maize-soy fed bird and 1 maize-soy plus xylanase fed bird) at 10 min intervals over a 90 min time period. Samples were collected and stored as described in section 3.2.2.2.

6.1.1 Blood glucose concentration

Blood glucose measurements were taken, as described in section 3.2.2.2, as an indicator of starch digestion (Fig 6.1). The average fasting blood glucose concentration for control birds was consistent with previous reports (Hazelwood and Lorenz, 1959) of birds fasted for 24 h. Average blood glucose for maize-soy fed birds with or without xylanase supplementation stayed within an expected range of 210-250 mg/dl, with non-fed control birds having around 10 % lower blood glucose levels.

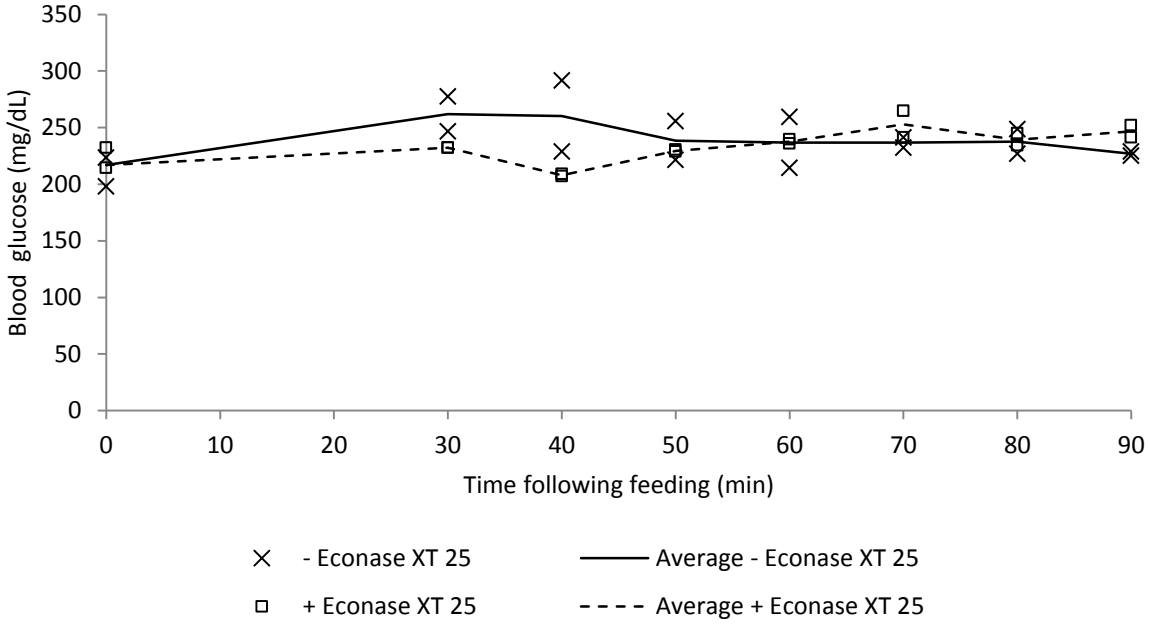


Figure 6.1 Blood glucose concentration of maize-soy fed birds, with (+Econase XT 25) and without (-Econase XT 25) xylanase supplementation. Data show blood glucose concentration (mg/dl) for bird replicates at each time point following feeding as well as for non-fed controls (0 min).

Xylanase supplementation did not significantly affect blood glucose concentration of maize-soy fed birds over the time period following feeding ($P > 0.05$), as determined using univariate ANOVA. However, the coefficient of variation was lower for xylanase supplemented birds (~2.6 %) than for non-supplemented groups (~8.46), suggesting less bird variation.

6.1.2 Digesta pH

Digesta from birds fed a maize-soy non-supplemented diet significantly increased in pH through the tract, from pH 3.0-4.2 in the gizzard, to pH 6.2-7.1 in the upper small intestine, and pH 7.3-8.0 in the lower small intestine (Table 6.1), as tested using univariate ANOVA. Statistical significance within each tract section over time was tested using multivariate ANOVA. The pH of gizzard digesta significantly increased at 30 min following feeding, when compared to the non-fed controls ($P < 0.05$). This is indicative of the buffering effect of feed on gastric juices. By 60 min, there was no significant difference in digesta pH between non-fed control birds and maize-soy fed birds ($P > 0.05$). At this time, more acid may have been produced due to the presence of feed in the gizzard, as well as contents of the gizzard exiting; this would result in the lowering of pH. The pH of digesta taken from the upper and lower small intestine of maize-soy fed birds did not significantly change over time or with feeding ($P > 0.05$) (Table 6.1). When compared to the results of Chapter 5, maize-soy fed birds had a higher digesta pH for all tract sections compared to maize grit fed birds (Table 6.2). The higher gizzard pH in maize-soy fed birds could be explained by the buffering effect of the soybean protein in ingested feed (Brooks, 1985). Moreover, the addition of calcium carbonate in the maize-soy diet will also contribute to the alkalinity of the diet.

For birds fed a maize-soy diet supplemented with xylanase, digesta pH ranged from pH 2.4-3.5 in the gizzard, to pH 6.2-6.9 in the upper small intestine, and pH 7.5-7.8 in the lower

small intestine (Table 6.1). The optimum pH of the xylanase used in this study is around pH 5. Therefore, enzyme activity may be limiting in the acidic conditions of the gizzard, while being more suited to conditions of the crop and small intestine (Masey O'Neill *et al.*, 2014b).

Table 6.1 Effect of maize-soy diet with or without xylanase supplementation on digesta pH

Time after feeding (min)	Gizzard				Upper small intestine				Lower small intestine			
	Minus XT		Plux XT		Minus XT		Plux XT		Minus XT		Plux XT	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	2.33[a]	0.10	2.33[a]	0.10	6.73[a]	0.10	6.70[a]	0.10	7.58[a]	0.12	7.58[a]	0.12
30	4.03[b]	0.18	3.42[a]	0.06	6.60[a]	0.08	6.47[a]	0.09	7.39[a]	0.10	7.53[a]	0.08
40	3.71[b]	0.25	2.89[a]	0.53	6.46[a]	0.24	6.31[a]	0.05	7.72[a]	0.16	7.68[a]	0.08
50	3.53[b]	0.07	3.04[a]	0.25	6.61[a]	0.02	6.61[a]	0.18	7.79[a]	0.09	7.64[a]	0.17
60	3.26[ab]	0.18	3.30[a]	0.17	6.61[a]	0.07	6.71[a]	0.17	7.48[a]	0.17	7.74[a]	0.08
70	3.27[ab]	0.27	2.84[a]	0.10	7.00[a]	0.13	6.27[a]	0.06	7.87[a]	0.12	7.46[a]	0.00
80	3.67[b]	0.23	3.17[a]	0.10	6.79[a]	0.02	6.39[a]	0.03	7.72[a]	0.06	7.61[a]	0.02
90	3.49[b]	0.27	2.78[a]	0.19	6.86[a]	0.00	6.49[a]	0.07	7.94[a]	0.01	7.72[a]	0.02

SEM = Standard Error of the Mean (from 2 bird replicates)
[a,b] Mean values not sharing a common superscript letter are significantly different at $P < 0.05$.

The pH of digesta taken from each tract section of birds fed a supplemented maize-soy diet did not significantly change over time ($P > 0.05$) (Table 6.1). However, xylanase supplementation significantly reduced the pH of digesta taken from the gizzard and upper small intestine ($P < 0.05$) (Table 6.2) compared to the non-supplemented diet. Xylanase addition may have liberated more protein from NSP encapsulation, increasing its availability for digestion (Cowieson *et al.*, 2010). Hsu *et al.* (1977) suggested that carboxyl groups released from hydrolysed peptides can overcome this buffering effect, thereby reducing digesta pH. This more acidic digesta would have then entered the upper small intestine,

resulting in the lower pH of digesta taken from this tract section, compared to birds fed a non-supplemented diet. However, no significant difference was shown for lower small intestine digesta samples ($P > 0.05$). Moreover, birds fed xylanase supplemented maize-soy diets had higher gizzard and lower small intestine digesta pH than maize grit fed birds. This can be explained once again by the buffering effect of the soybean protein in the diet. These results show how changing the diet can influence the environment of the digestive tract, an effect that could lead to differences in the digestibility of nutrients.

Table 6.2 Comparison of digesta pH measurements for each tract section for birds fed a maize grit diet and maize-soy diets with and without xylanase supplementation.

Tract section	Diet	Mean	P value[1]	SEM[2]
Gizzard	Maize grit	2.47		0.06
	Maize-soy	3.56	0.003	0.10
	Maize-soy + xylanase	3.06		0.09
Upper small intestine	Maize grit	6.49		0.05
	Maize-soy	6.70	0.024	0.07
	Maize-soy + xylanase	6.46		0.06
Lower small intestine	Maize grit	7.34		0.06
	Maize-soy	7.70	0.395	0.08
	Maize-soy + xylanase	7.62		0.04

[1]Independent-samples t-test on pH means for birds fed maize-soy diets with and without xylanase supplementation. Significant difference at $P < 0.05$.
[2]SEM = Standard Error of the Mean (Maize grit diet- 28 bird replicates; maize-soy diet with or without xylanase- 14 bird replicates).

6.1.3 Starch digestion

Following starvation, 20 min of feeding gave an average intake of approximately 27 g diet. The weight of digesta taken from each tract section is given in Table 6.3. As feed intake was

much higher than the weight of digesta collected it is possible that feed may have also been stored in the crop, a section where digesta was not collected. Starch, dextrin and glucose content of digesta (see methods in section 3.2.4.3) taken from control and maize-soy fed birds, with and without xylanase supplementation, are shown in Figure 6.2a-c, respectively. Statistical significance was tested using univariate and multivariate ANOVA. Throughout the time period following feeding, digesta starch and dextrin content was significantly higher in samples taken from the gizzard compared to the upper and lower small intestine, for birds fed diets including and excluding xylanase ($P < 0.05$). Xylanase supplementation did not significantly affect the amount of starch in digesta for any tract section ($P > 0.05$) (Table 6.4).

Table 6.3 Weight of digesta taken from each tract section over time.

Time after feeding (min)	Digesta weight (g DM)					
	Gizzard		Upper small intestine		Lower small intestine	
	Mean	SEM	Mean	SEM	Mean	SEM
Control	1.73^{a}	0.38	0.17^{a}	0.06	0.23^{a}	0.14
30	5.93^{b}	0.92	1.22^{b}	0.13	0.96^{abc}	0.29
40	5.16^{b}	0.29	1.73^{bc}	0.17	0.51^{ab}	0.08
50	6.11^{b}	0.48	1.92^{bc}	0.08	0.90^{abc}	0.20
60	5.72^{b}	0.36	2.42^{c}	0.22	1.32^{bcd}	0.09
70	4.38^{ab}	0.65	2.43^{c}	0.27	1.59^{cd}	0.13
80	5.06^{b}	0.38	2.78^{d}	0.20	2.09^{d}	0.22
90	5.02^{b}	0.88	2.22^{c}	0.26	1.83^{d}	0.15

SEM - Standard Error of the Mean

DM - dry matter

^{a,b,c,d} Mean values not sharing a common superscript letter are significantly different at $P < 0.05$.

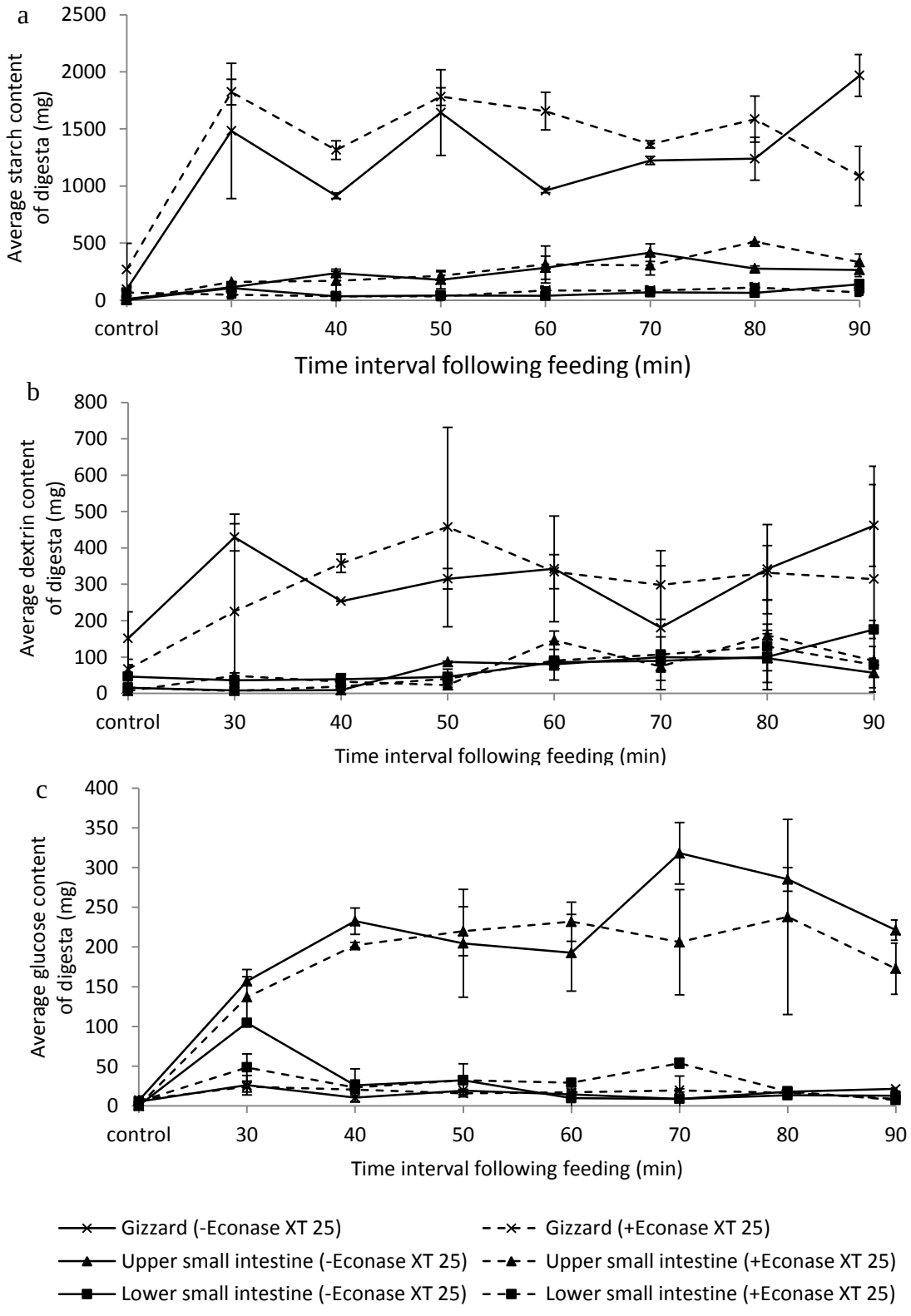


Figure 6.2 Digesta soluble and insoluble starch fractions of maize-soy fed birds with and without xylanase inclusion. The mean weight of starch (a), dextrin (b) and glucose (c) in the total digesta taken from distinct sections of the gastrointestinal tract at 10 min intervals over a 90 min time period following feeding is shown. Fractions measured in non-fed control birds are also presented. Error bars denote ± standard error of the mean (from 2 bird replicates).

Dextrin, being one of the products of starch digestion, would not be expected in the gizzard of birds as α-amylase activity is minimal in such acidic conditions (Pinchasov and Noy, 1994). However, the maize-soy diet was found to contain a higher initial dextrin content compared to the maize grit diet. Intestinal reflux of digesta from the small intestine back into the gizzard (Sklan *et al.*, 1978) may also explain the presence of dextrin in this section of the tract. Moreover, partial hydrolysis of starch in acid is known to produce dextrins, therefore the acidic environment of the gizzard may have initiated starch granule breakdown. Interestingly, xylanase supplementation significantly decreased the amount of dextrin in digesta taken from each tract section ($P < 0.05$) (Table 6.4). This implies that there is a greater hydrolysis of dextrin in xylanase supplemented birds, a response that may be linked to the degradation of cell wall material by xylanase, thereby allowing greater endogenous enzyme access to the dextrin for digestion.

Table 6.4 Effect of xylanase supplementation on starch, dextrin and glucose content (mg) of digesta taken from different tract sections over the entire time period following feeding.

		Starch			**Dextrin**			**Glucose**		
Tract section	Econase XT 25	Mean (mg)	SEM	P value[1]	Mean (mg)	SEM	P value[1]	Mean (mg)	SEM	P value[1]
Gizzard	-	928.01	121.5	0.922	449.78	40.2	0.001	18.58	2.4	0.263
	+	942.70	163.5		213.86	30.2		15.68	1.2	
Upper small intestine	-	478.57	61.5	0.320	98.4	17.8	0.002	197.63	12.8	0.140
	+	585.52	95.0		45.86	10.6		233.62	21.4	
Lower small intestine	-	149.40	40.9	0.363	96.91	16.4	0.014	28.30	8.1	0.729
	+	191.90	50.4		52.04	13.0		31.61	7.4	

SEM = Standard Error of the Mean (from 16 bird replicates- taken from across the time course following feeding).

[1] Multivariate ANOVA, blocked for time. Significant difference at $P < 0.05$.

The glucose content of digesta was significantly higher in the upper small intestine compared to the gizzard and lower small intestine ($P < 0.05$), for birds fed diets including and excluding xylanase. For birds fed non-supplemented diets, a significant amount of glucose entered the gizzard and lower small intestine at 30 min following feeding ($P < 0.05$), when compared to control birds. By 40 min, the amount of glucose reduced to levels comparable to that of control birds ($P > 0.05$). This initial increase in glucose content could be due to the rapid digestion of starch following fasting conditions. A significant amount of glucose was measured in the upper small intestine from 40 min following feeding ($P < 0.05$). This suggests that starches in the feed have entered the intestine at this time and are being digested by endogenous enzymes. For birds fed a xylanase supplemented diet, the glucose content of gizzard and lower small intestine digesta samples did not significantly change with feeding or over time ($P > 0.05$). Overall, xylanase supplementation did not significantly affect the amount of glucose in digesta for any tract section ($P > 0.05$) (Table 6.4).

For birds fed a maize-soy diet, starch seems to be held for a longer time in the gizzard compared to birds fed a maize grit diet. As both diets were ground through the same size screen (5 mm), the particle size of the diet may not be considered as the limiting factor. However, this prolonged retention of digesta in the gizzard may be attributed to acidic pectins, another form of NSP, in soybean meal which are known to increase viscosity and thus reduce feed passage rate (Yu *et al.*, 2002). This greater retention may also explain the lower levels of starch in digesta of the small intestine for maize-soy fed birds. Nonetheless, a reduction in starch by the small intestine would be expected as starch becomes hydrolysed by pancreatic and intestinal α-amylase into smaller molecules of maltotriose, maltose and α-limit dextrin (Gray, 1992; Osman, 1982). This may also explain why a significant amount of glucose did not enter the upper small intestine until 40 min following feeding, compared to 30 min as seen for maize grit fed birds.

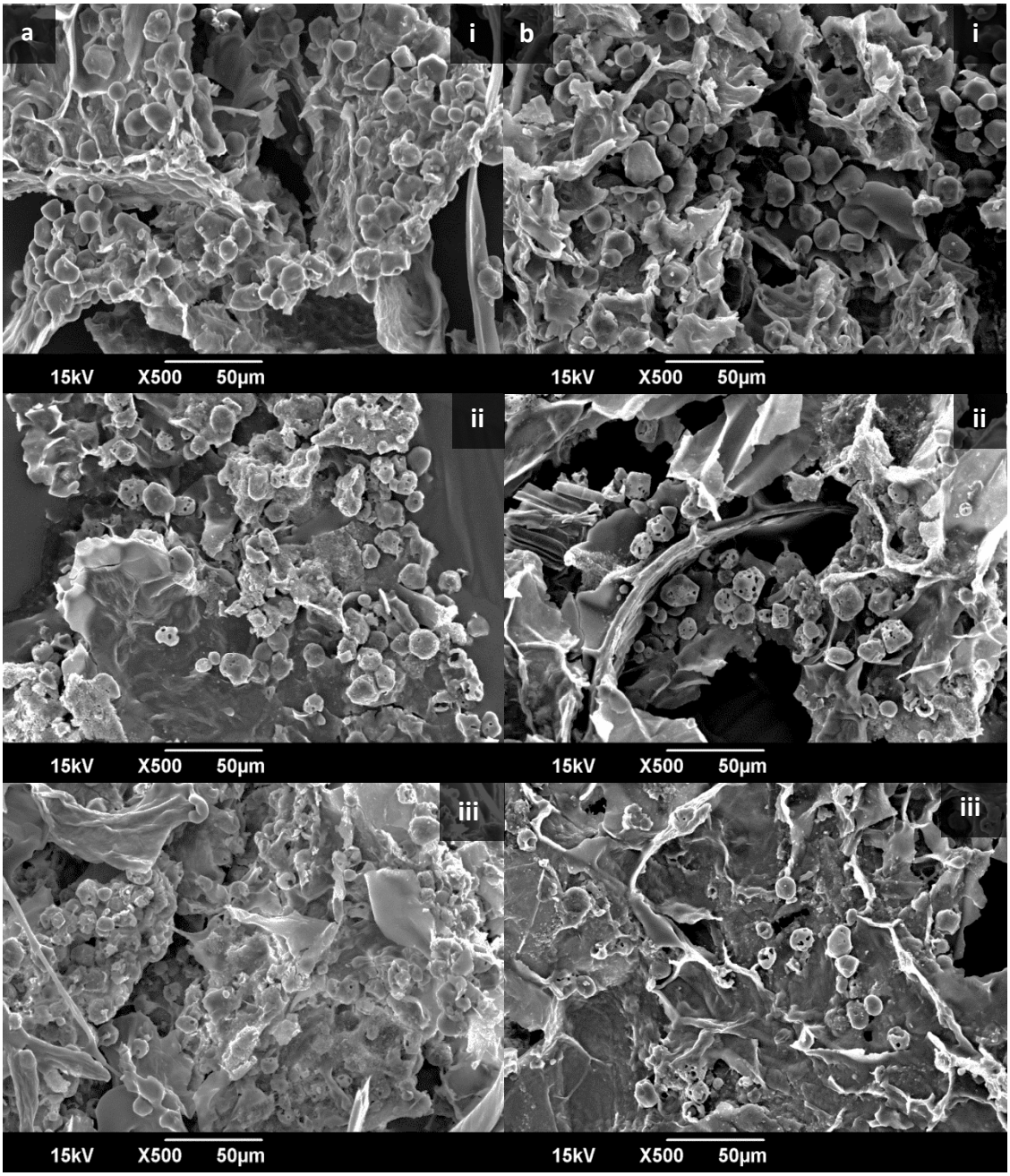


Figure 6.3 SEM images of starch granules in digesta taken at 60 min following feeding from the gizzard (i), upper (ii) and lower small intestine (iii) of birds fed a maize-soy diet excluding (a) and including (b) xylanase.

SEM images of starch granules in digesta are shown in Figure 6.3. Confirming previous results, starch granules are shown to remain in the tract even after a 17 h fast (control birds). These starch granules are still associated with plant material and show varying degrees of

enzyme attack. It is possible that feed becomes trapped in the muscular crevasses of the gizzard and through peristalsis, distributes throughout the tract (Duke, 1982). Kokue and Hayama (1972) reported an appreciable amount of digesta in the gizzard even up until 48 h of starvation in birds fed a mash diet. Therefore, it could be expected that a medium ground feed as presented in this study would be retained in the tract of fasting birds for a considerable amount of time. Birds fed either a maize-soy diet excluding xylanase (Fig 6.3a) or including xylanase (Fig 6.3b) in the diet similarly showed a greater number of enzyme attacked granules in the digesta from more distal tract sections. There was no evidence of any greater plant material breakdown in the digesta of birds supplemented with xylanase.

6.2 Effect of feeding xylanase over different time scales on starch digestion

To investigate the indirect mechanism of xylanase action, the current study explored the effect of supplementing maize-soy diets with xylanase over different time periods before slaughter on starch digestion in broilers. A total of 60 broilers were used in this study with 10 birds per treatment group; birds were group-housed in separate pens for a specific treatment group. Treatment groups (6) included a control group fed a maize-soy diet without xylanase, and groups fed maize-soy diets supplemented with xylanase (Econase XT 25 at 100 g/t; 18,100 BXU/kg) from 1, 2, 3, 4 or 5 weeks before slaughter. General observations of birds demonstrated good health, appetite and excreta consistency. On days 35 and 36 of age, birds were euthanised and dissection carried out as described in section 3.2.2.2. Birds slaughtered on day 35 of age were weighed before culling. Empty weight of the gizzard and lengths of the upper and lower small intestine as well as both caeca were recorded. Approximately two sections of around 2 cm in length were removed from the proximal upper small intestine, snap frozen in liquid nitrogen, and stored at -80 °C, for later RNA extraction.

6.2.1 Blood parameters

6.2.1.1 Blood glucose

Blood glucose concentration was measured according to the methods in section 3.2.2.2 and the results shown in Figure 6.4. When comparing between treatment groups, no significant effect of supplementing diets with xylanase for any length of time was observed ($P > 0.05$), as determined using univariate ANOVA. Other studies (Luo *et al.*, 2009; Gao *et al.*, 2007) have also reported no effect on blood glucose concentration with xylanase supplementation.

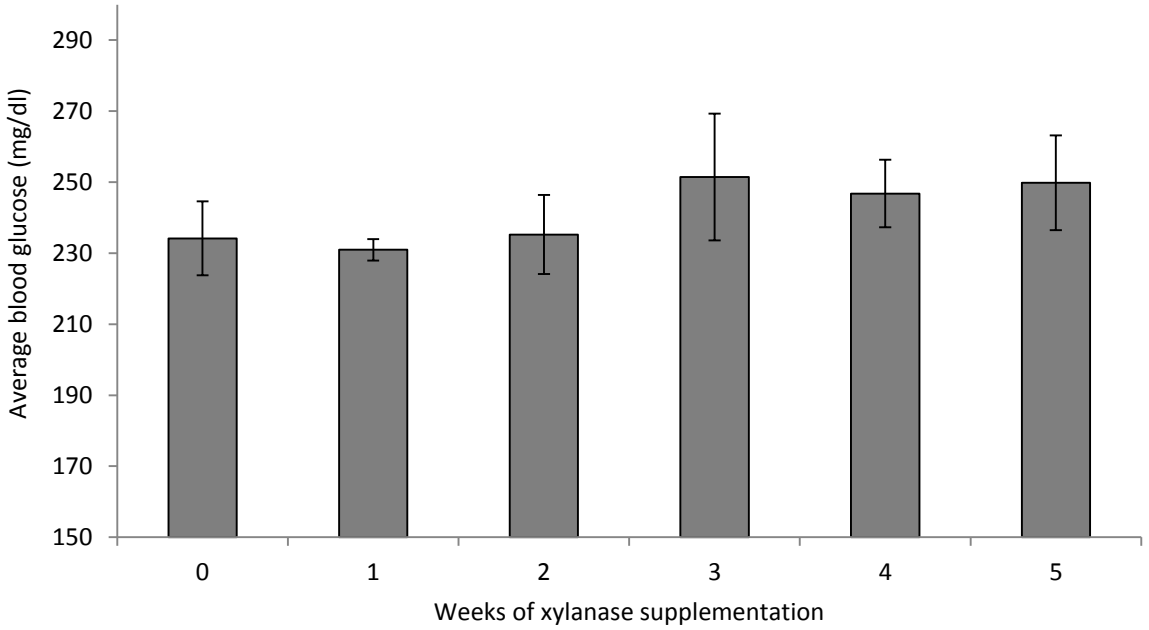


Figure 6.4 Blood glucose concentration of birds supplemented with xylanase for 0-5 weeks before slaughter. Error bars denote ± standard error of the mean (from 10 bird replicates).

However, when data was split between before and after 3 weeks of supplementation, a significant increase in glucose concentration was determined for birds fed diets supplemented with xylanase for 3 weeks or more ($P < 0.05$). Singh *et al.* (2012) reported an increase in serum glucose concentration of broilers fed a xylanase supplemented maize-soy diet for 42 days. Xylanase supplementation is assumed to improve nutrient digestion and absorption in

the small intestine of broilers (Choct *et al.*, 1999). Therefore, the rise in blood glucose level after 3 weeks of feeding xylanase could be due to greater starch digestion and glucose absorption. The mechanisms that may have led to this response will be discussed later in this chapter.

6.2.1.2 Blood PYY hormone

Changes in the circulating concentration of the gastrointestinal PYY hormone, as described in section 2.6.1.2, in response to xylanase supplementation was measured (Fig 6.5). Neyrinck *et al.* (2012) reported an increase in blood plasma PYY concentration with AXOS inclusion into the diet of mice, suggesting that products of NSP hydrolysis can have an effect on gastrointestinal hormone secretion. In the current study, when just the mean values are observed (Fig 6.5a), there seems to be an effect of supplementing diets with xylanase for a shorter period of time.

Birds supplemented for 3 weeks showed a significantly higher PYY concentration in the blood compared to the non-supplemented control birds ($P < 0.05$), as tested using an independent samples t-test . Feeding xylanase over a longer time period appears to alleviate this effect. It is possible that there is a spike in PYY secretion when xylanase is initially fed, before the bird acclimatises over longer periods of supplementation. However, when considering the raw data (Fig 6.5b), no significant effect was seen due to the large data variance ($P > 0.05$, R= 0.0931). Increasing the number of bird replicates per treatment group would be beneficial to elicit any treatment effect and give more statistical power to the data.

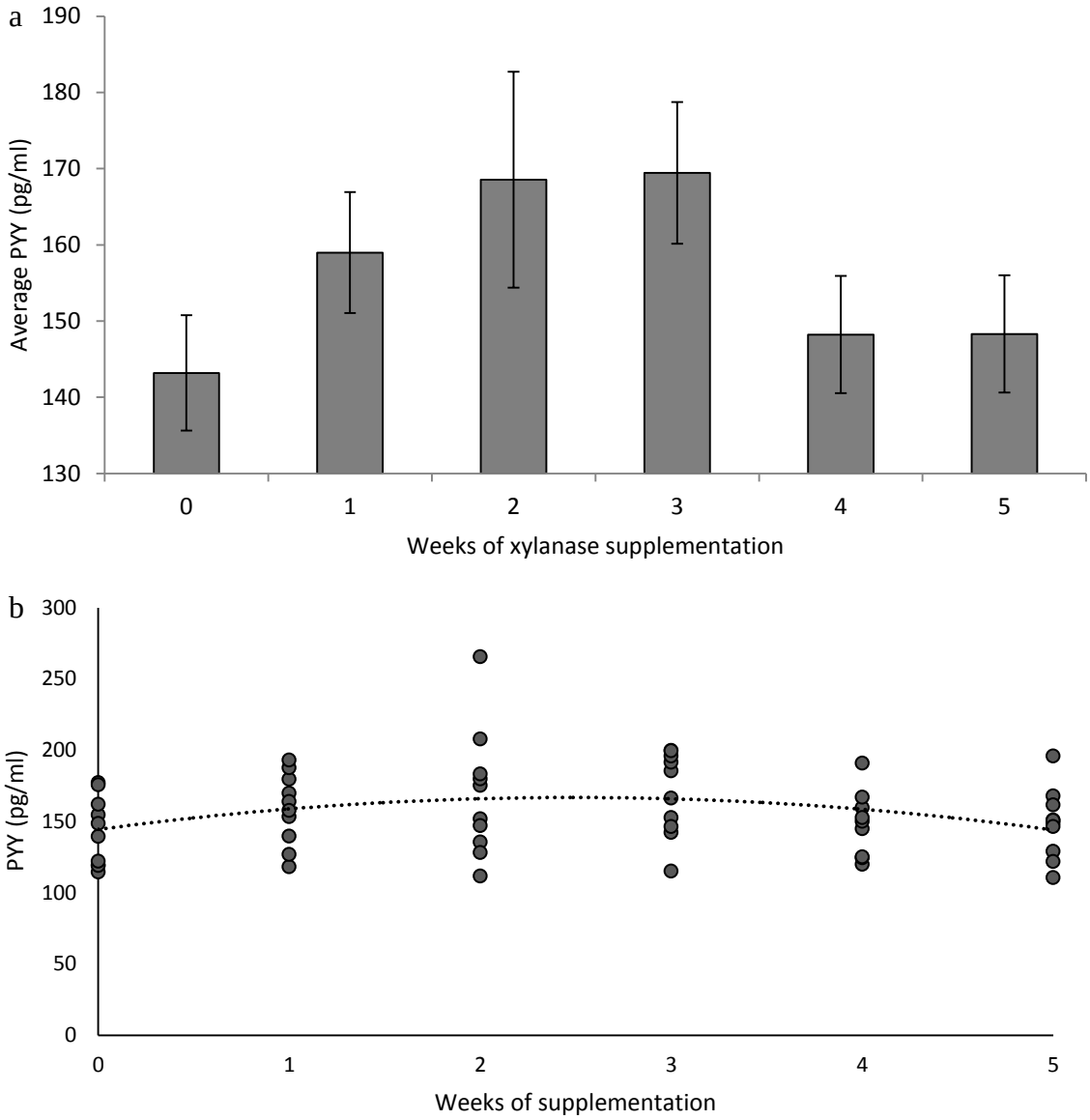


Figure 6.5 Peptide YY hormone concentration of birds supplemented with xylanase for 0-5 weeks before slaughter. Graphs show averaged data (a) and raw data (b) sets. Error bars denote ± standard error of the mean (from 10 bird replicates, except week 4 of supplementation which has 9 replicates).

6.2.2 Changes in gastrointestinal tract

Changes in the pH of digesta collected from different tract sections was measured (Fig 6.6). Statistical significance within and between tract sections was tested using multivariate and univariate ANOVA. For all treatment groups, the pH of digesta taken from the gizzard, pH 2.2-4.4, was significantly lower than that of the other tract sections ($P < 0.05$). The digesta

pH of the lower small intestine, pH 6.7-7.8, was significantly higher than the other tract sections ($P < 0.05$), while the digesta pH of the upper small intestine, pH 5.9-6.6, and both caeca, pH 5.7-7.3, did not significantly differ ($P > 0.05$). For each traction section, digesta pH did not significantly differ with xylanase treatment for any length of time ($P > 0.05$). Enhanced fermentation in the small intestine and caeca, due to changes in the microbial population, leads to greater production of VFA and thus lactic acid in these sections, resulting in a reduced pH (Gao *et al.*, 2008). Xylanase supplementation has been shown to reduce fermentation in the small intestine, while increasing caecal fermentation and VFA production (Choct *et al.*, 1999). As no change in intestinal or caecal pH was observed in the current study, this would suggest that there had not been any change in gut microflora due to xylanase supplementation. However, further microbial analysis would be needed to confirm these results.

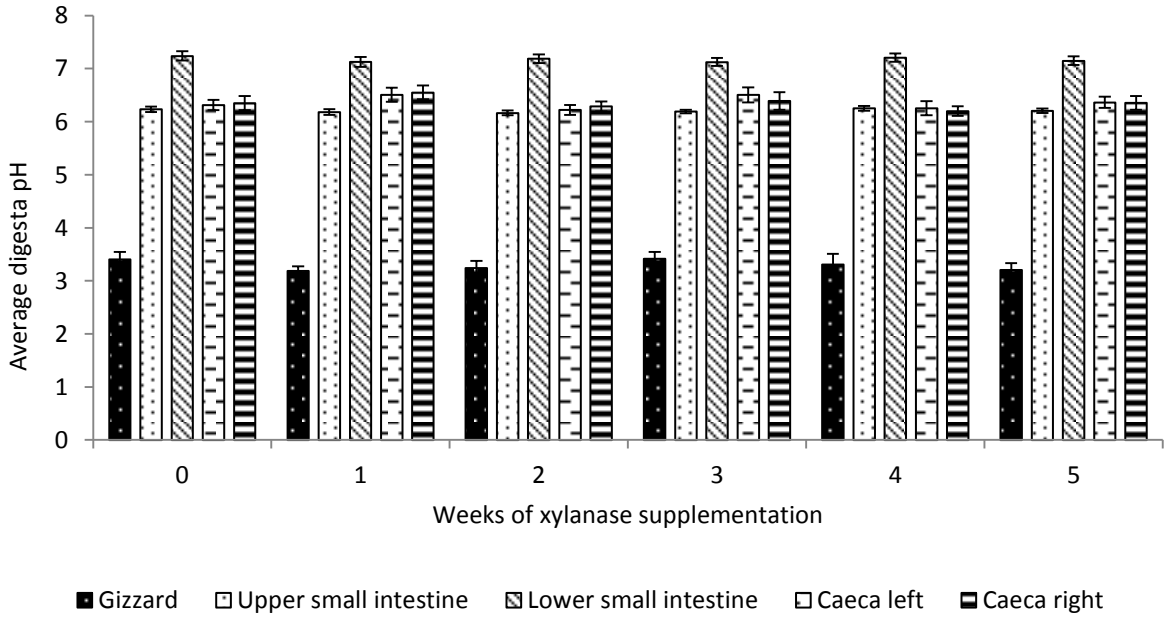


Figure 6.6 Digesta pH of distinct tract sections taken from birds supplemented with xylanase for 0-5 weeks before slaughter. Error bars denote ± standard error of the mean (from 10 bird replicates).

Gizzard weight as well as intestine and caeca length were measured (Table 6.5) to assess the potential effect of xylanase supplementation on the physiology of digestive tract organs. Statistical significance was tested using univariate ANOVA. The length of the upper small intestine and both caeca were not significantly affected by treatment ($P < 0.05$). However, the length of the lower small intestine significantly increased in birds fed a xylanase supplemented diet for 3 weeks before slaughter compared to the non-supplemented control birds ($P > 0.05$). Xylanase supplementation at 1000 XU/kg diet has been shown to reduce the relative weight and length of the small intestine (Wu *et al.*, 2004) in broilers fed a wheat-based diet for 21 days. As viscosity is less of a concern in a maize-based diet, this may be the reason why there is not a consistent change in intestinal length with enzyme addition.

Xylanase supplementation of a maize-soy diet has been shown to increase gizzard weight (Masey O'Neill *et al.*, 2014a). Through the ileal brake mechanism, xylanase is thought to induce greater feed retention in the gizzard, leading to a more developed gizzard. However, this was not supported by the current study. Although birds supplemented with xylanase for 3 weeks had the highest mean gizzard weight, overall gizzard weight was not significantly affected by treatment ($P < 0.05$).

Xylanase supplementation has also been shown to facilitate greater passage of digesta to the caeca (Singh *et al.*, 2012; Choct *et al.*, 1999). A weighted increase in digesta collected from the caeca of birds supplemented with xylanase for longer periods of time was shown (Table 6.6). Data was split between before and after 3 weeks of xylanase supplementation and significance was tested using an independent samples t-test. There was a significant increase in caecal digesta weight in birds fed xylanase diets for 3 or more weeks ($P < 0.05$). This may

suggest that following longer periods of enzyme feeding there is greater fractionation of the feed material thereby allowing greater quantities to enter the caeca.

Table 6.5 Effect of length of xylanase supplementation on gizzard weight, and intestinal and caecal length

Weeks of supplementation	Weight (g)		Length (mm)					
	Gizzard	SEM	USI	SEM	LSI	SEM	Caeca	SEM
0	42	1.49	632	20.78	669	14.02	154	3.91
1	43	0.89	711	21.66	727	30.34	159	4.40
2	41	1.68	663	15.00	686	18.03	160	4.90
3	45	1.12	691	15.25	754[a]	16.12	158	3.77
4	42	1.69	637	18.81	713	23.09	155	5.32
5	43	2.00	669	21.02	709	13.99	158	5.58

SEM = Standard Error of the Mean (from 10 bird replicates)

USI- upper small intestine; LSI- lower small intestine

[a] Mean values not sharing a common superscript letter are significantly different at $P < 0.05$.

Table 6.6 Effect of length of xylanase supplementation on the weight of digesta collected from each tract section.

Weeks of supplementation	Digesta weight (g DM)							
	Gizzard	SEM	USI	SEM	LSI	SEM	Caeca	SEM
0	6.35	0.68	3.08	0.28	3.21	0.22	0.31	0.03
1	6.28	0.45	3.12	0.39	2.74	0.34	0.30	0.03
2	6.78	0.27	3.18	0.22	2.66	0.36	0.27	0.03
3	6.56	0.30	3.50	0.33	3.17	0.22	0.34	0.05
4	5.74	0.50	2.63	0.34	2.48	0.21	0.34	0.04
5	6.57	0.55	3.14	0.33	3.16	0.19	0.41	0.07

SEM - Standard Error of the Mean (from 10 bird replicates)

DM - dry matter

USI - upper small intestine; LSI- lower small intestine

Although this was not a growth study, it was important to have an indication of the impact of the xylanase treatment on bird weight gain. Therefore, bird weights were recorded weekly to examine the effect of xylanase supplementation on live body weight (Fig 6.7). Unfortunately, birds euthanised on day 36 of age were not weighed and therefore only 6 out of the 10 birds were weighed at day 35 of age. However, their selection was random and therefore can be taken as representative of the group. Xylanase treatment for any length of time did not significantly affect the weight of birds from day 1-35 of age ($P > 0.05$).

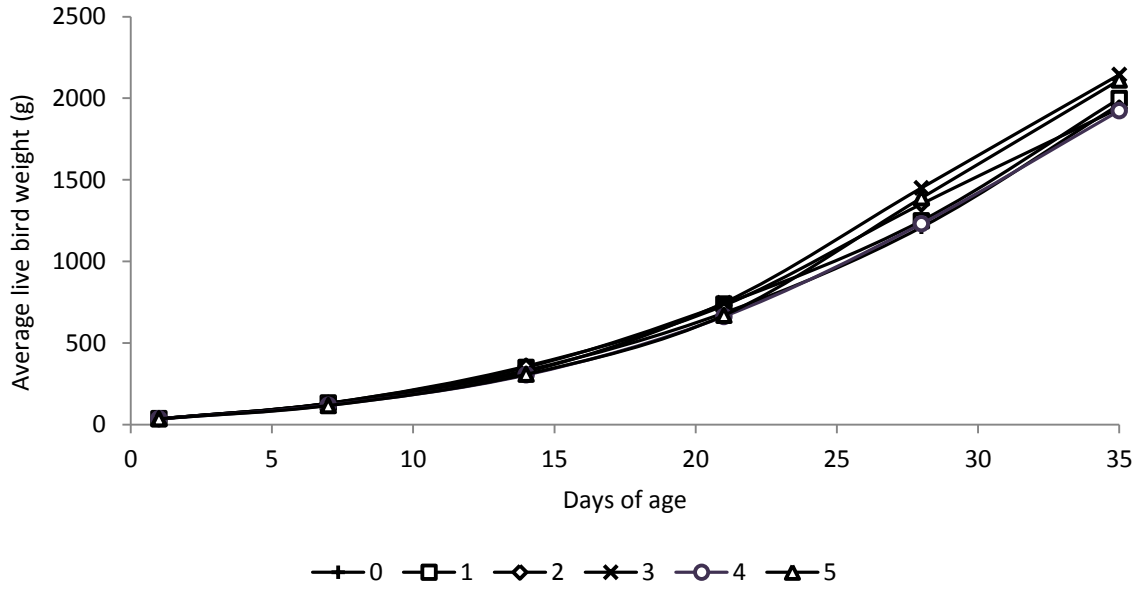


Figure 6.7 Live weight of birds supplemented with xylanase for 0-5 weeks before slaughter.

6.2.3 Starch digestion

Total starch results demonstrate the progression of starch granule digestion as digesta passes through the digestive tract of birds (Fig 6.8). Statistical significance was tested using univariate and multivariate ANOVA. Starch content was greatest in the gizzard (Fig 6.8a), with decreased levels within the upper small intestine and then less again in the digesta of the lower small intestine as starch becomes hydrolysed by endogenous amylases. The content of starch in any tract section was not significantly affected by treatment ($P > 0.05$).

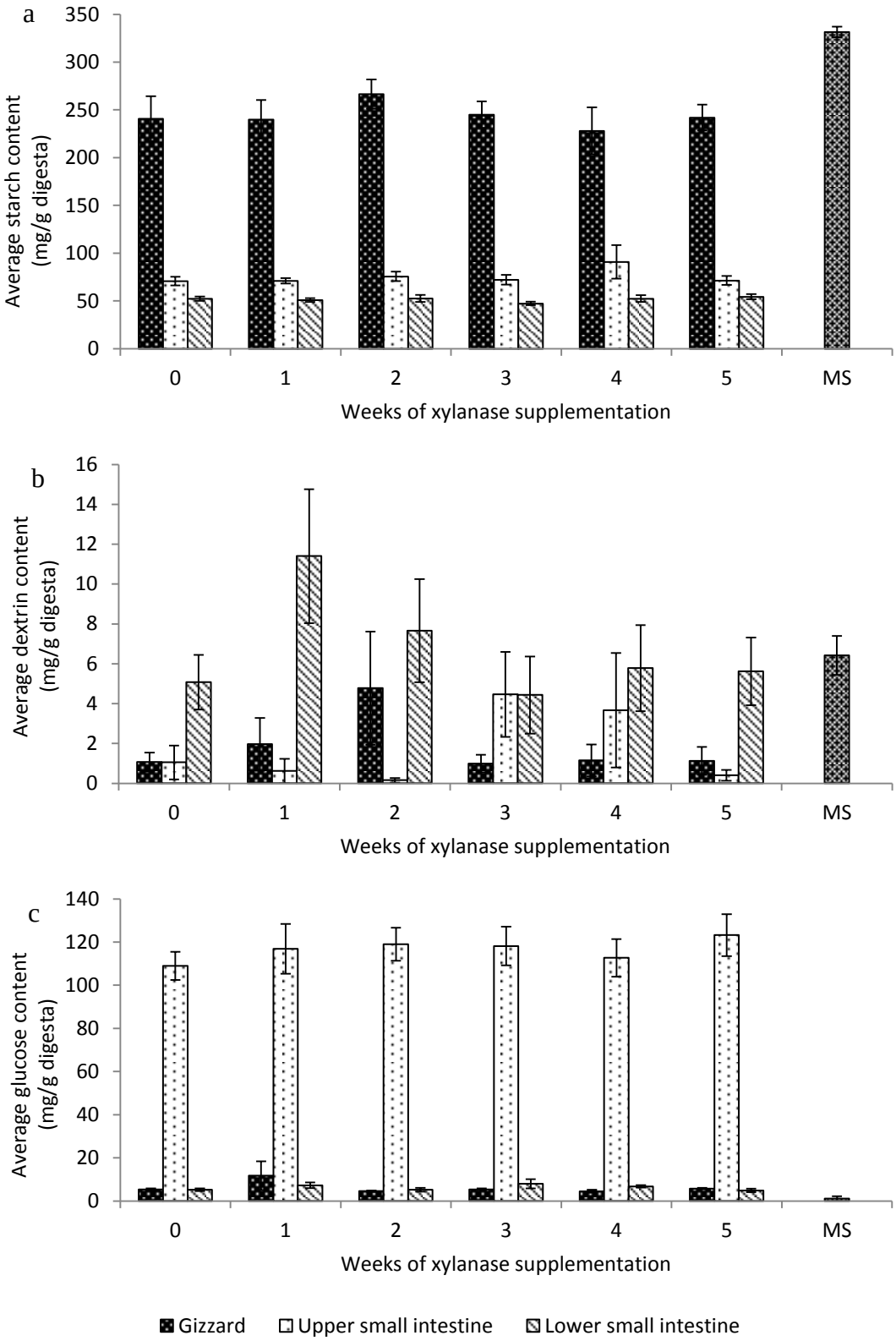


Figure 6.8 Soluble and insoluble starch fractions in digesta collected from birds supplemented with xylanase for 0-5 weeks before slaughter. Graphs show starch (a), dextrin (b) and glucose (c) content of digesta taken from the gizzard, upper and lower small intestine of birds fed a maize-soy diet (MS) supplemented for 0 (control), 1, 2, 3, 4 or 5 weeks before slaughter. Error bars denote ± standard error of the mean (from 10 bird replicates).

Dextrin content varied with tract section and treatment with no consistent trend (Fig 6.8b). There was no significant effect of xylanase treatment on dextrin content for any tract section ($P > 0.05$). As expected, the glucose content (Fig 6.8c) of gizzard digesta was minimal, while a higher glucose content was found in the upper small intestine, as this is the major site of starch digestion. The average glucose content (mg/g) of upper small intestine digesta samples numerically increased with xylanase treatment, with the highest concentration of glucose observed in birds exposed to xylanase for the full 5 weeks, although the difference was not significant ($P > 0.05$). By the lower small intestine, glucose levels fall again as monomers are absorbed into enterocytes.

6.2.4 Starch granule morphology

Total starch results were also supported by SEM imaging (Fig 6.9) where starch granules appear to become more hydrolysed and are fewer in number as digesta enters more distal tract sections. Interestingly, a small number of starch granules escaped digestion and entered the caeca (Fig 6.9d) for all treatment groups. Therefore, even when granules are released from their surrounding matrices some are still resistant to digestion. Weurding *et al.* (2001) reported the same total starch digestion values as ileal starch digestion, indicating that the undigested starch fraction was not fermented in the caeca. Carre (2004) suggested that while soluble molecules or free granules can enter the caeca, as shown in the present study, the majority are expected to bypass the caeca. Therefore, caecal fermentation of starches may not be influential for the completion of starch digestion.

Birds were fed *ad libitum* before slaughter and therefore samples from the digestive tract will show feed particles that had been ingested for different periods of time. All gizzard samples showed cell wall fractionation (Fig 6.10), however greater cell wall destruction with xylanase treatment was not evident. *In vitro* digestion of ground maize with xylanase has shown

greater starch granule release from cell wall material (Masey O'Neill *et al.*, 2014b) when compared to maize without xylanase inclusion. The environment of the digestive tract is much more complex than that of a test tube and therefore there are many other factors that will influence xylanase action. Moreover, the reasoning may not be solely down to plant cell wall material, but also protein matrices that can form a continuous network that encapsulates starch granules thereby preventing their release.

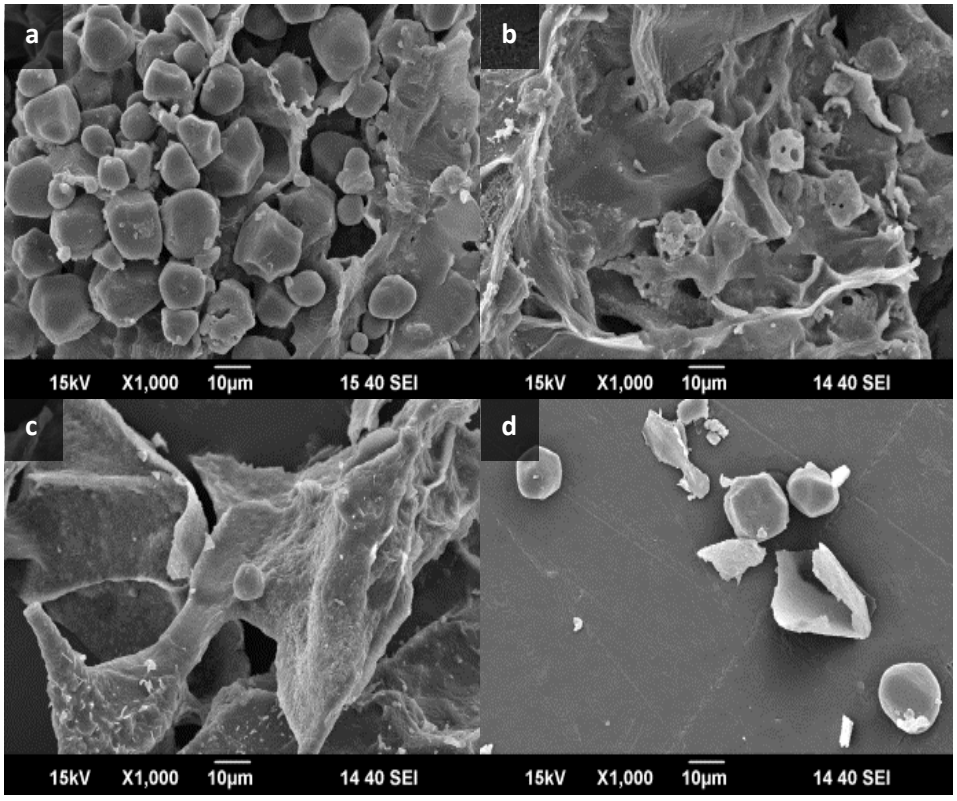


Figure 6.9 SEM images showing the progression of starch granule digestion through the tract of broilers. Starch granules in digesta taken from the gizzard (a), upper small intestine (b), lower small intestine (c) and caeca (d).

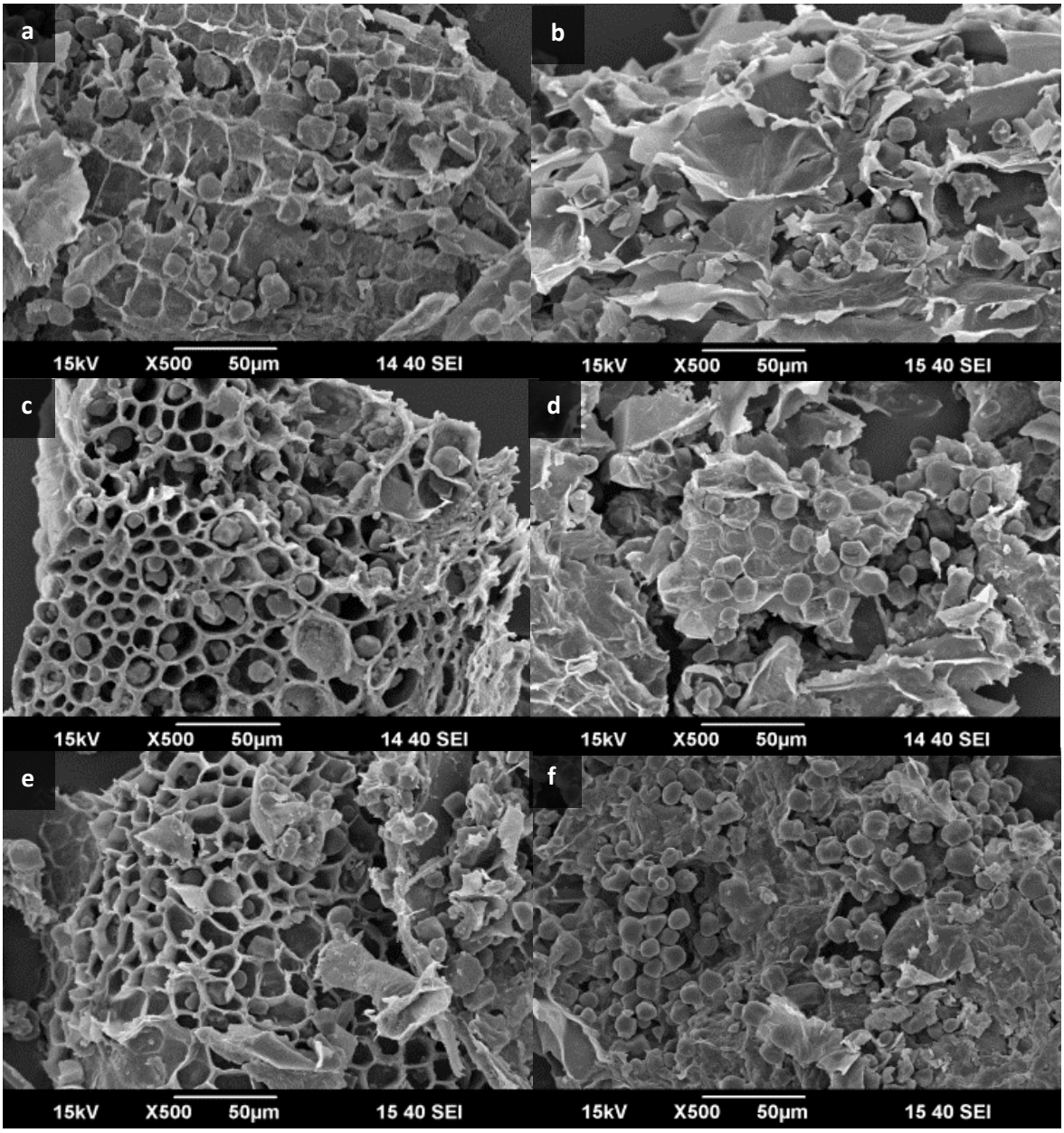


Figure 6.10 Starch granules in gizzard digesta taken from birds supplemented with xylanase for 0-5 weeks before slaughter. SEM images show control birds (a) and birds supplemented with xylanase 1 (b), 2 (c), 3 (d), 4 (e) and 5 (f) weeks before slaughter.

6.2.5 Arabinose/xylose (A/X) ratio

The degree of substitution of the xylose backbone by arabinose residues was studied using ATR-FTIR (see section 3.2.4.7) in the spectral region of 1020-800 cm-1 (Fig 6.11). Absorption bands at 958 and 984 cm-1 were taken to characterise arabinoxylan in the maize

diet and digesta samples. This method only indicates the ratio of the sugars and it is difficult to infer the total levels of the non-starch polysaccharide from the spectrograms due to other factors, such as particle size and the presence of other components. Zhang *et al.* (2003) reported the release of up to 10 % of the arabinose from corn arabinoxylan when incubated in gastric conditions (37 °C, pH 1-3). However, no oligosaccharides were detected suggesting that acidic conditions do not break the xylan backbone into shorter fragments.

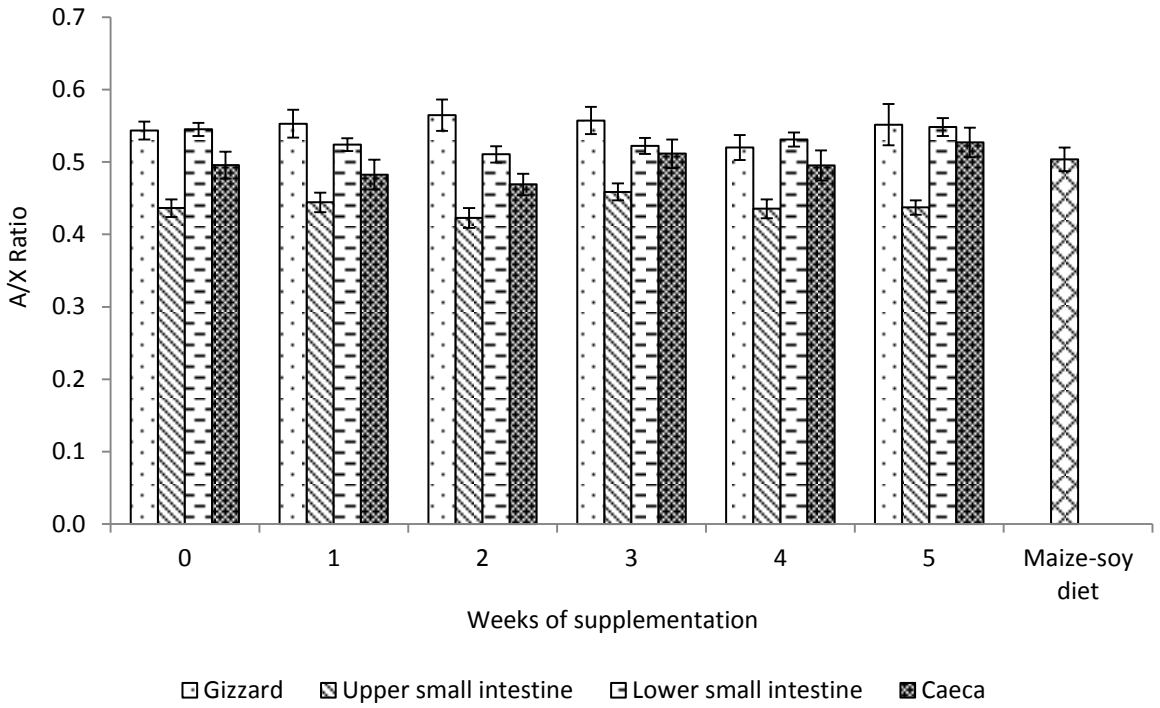


Figure 6.11 A/X ratio of digesta taken from distinct tract sections of birds supplemented with xylanase for 0-5 weeks before slaughter. Error bars denote ± standard error of the mean (from 10 bird replicates).

In the current study, the A/X ratio increased in the gizzard compared to the un-treated diet. This would suggest that the amount of arabinose associated with the xylan backbone increased in the gizzard. It is possible that bacteria in the crop acted on the arabinoxylan before the feed entered the gizzard. If so, some of the xylose units may have been removed thereby increasing the ratio of arabinose to xylose or that the arabinoxylan being assessed

after digestion was not the equivalent portion being measured in the diet. Proteins and other components could have been stripped away leaving other material for assessment by the FTIR, which is very much a measurement of what is at the surface of the sample.

A significant reduction in the A/X ratio was observed in digesta taken from the upper small intestine ($P < 0.05$), as determined by univariate ANOVA. Loss of the arabinose from the xylan backbone would lead to a reduction in the degree of substitution, correlating to a reduced A/X ratio. As this was evident in the upper small intestine following passage of digesta from the gizzard, it might suggest that this is the major site of arabinose loss and perhaps is an indication of the possible initial stages of xylan hydrolysis. If the xylose backbone is hydrolysed and removed the A/X ratio could then be expected to increase once more. This could explain the significant increases again of the A/X ratio by the lower small intestine ($P < 0.05$) although more work is needed to affirm such a speculative interpretation of the data. Again in the caeca, a significant reduction in A/X ratio ($P < 0.05$) occurs and this is most likely due to the presence of microflora in the caeca that can hydrolyse and ferment arabinoxylans. It must be taken into account that this is a crude method, useful for determining the A/X ratio of a sample, however it is most likely influenced by other factors such as feed components and diluents which can vary along the digestive tract. The results show obvious changes in the A/X ratio between different tract sections, with major changes seen in the upper small intestine. This is the predominant site for the digestion of feed and therefore changes to the chemical composition of digesta will be substantial. Although these results cannot be taken as absolute values, they can be used to show any significant change in the A/X ratio due to xylanase treatment. However, in this study, A/X ratio for any tract section was not significantly affected by xylanase treatment for any length of time ($P > 0.05$).

6.2.6 Glucose transporter expression in the small intestine

Xylanase regulation of the intestinal glucose transporters was investigated to determine the effect that supplementation might have on glucose uptake. RNA was extracted from upper small intestine tissue samples and expression of the GLUT2 and SGLT1 quantitatively measured using qPCR, as described in section 3.2.5. According to the delta-delta Ct method, gene expression was normalised to β-actin, before xylanase supplemented groups were compared to the non-supplemented control group to give fold change in gene expression (Fig 6.12). Melt curves were used to determine single product amplification. However, to confirm that the products of the PCR reaction were the genes of interest, products from two different samples were randomly selected and separated by gel electrophoresis (Fig 6.13). The expected band size of the GLUT2, SGLT1 and β-actin products were 88 bp, 113 bp and 78 bp, respectively. These were observed on the gel, indicating that the correct genes were amplified.

Due to the large variation in results, GLUT2 and SGLT1 gene expression was not shown to vary significantly with length of xylanase supplementation ($P > 0.05$), as tested using univariate ANOVA. However, results do show a trend towards an increased GLUT2 expression at 2 weeks of xylanase supplementation. GLUT2 expression reduced and plateaued as the length of xylanase supplementation increased. A similar trend was observed for SGLT1 expression. However the initial increase in expression started slightly later, at 3 weeks of xylanase supplementation. These results suggest that xylanase supplementation of a maize-soy diet may result in upregulation of glucose transporter expression, leading to enhanced glucose absorption. This work also indicates the importance of duration of enzyme inclusion into a diet, as upregulation of gene expression can take several days or weeks.

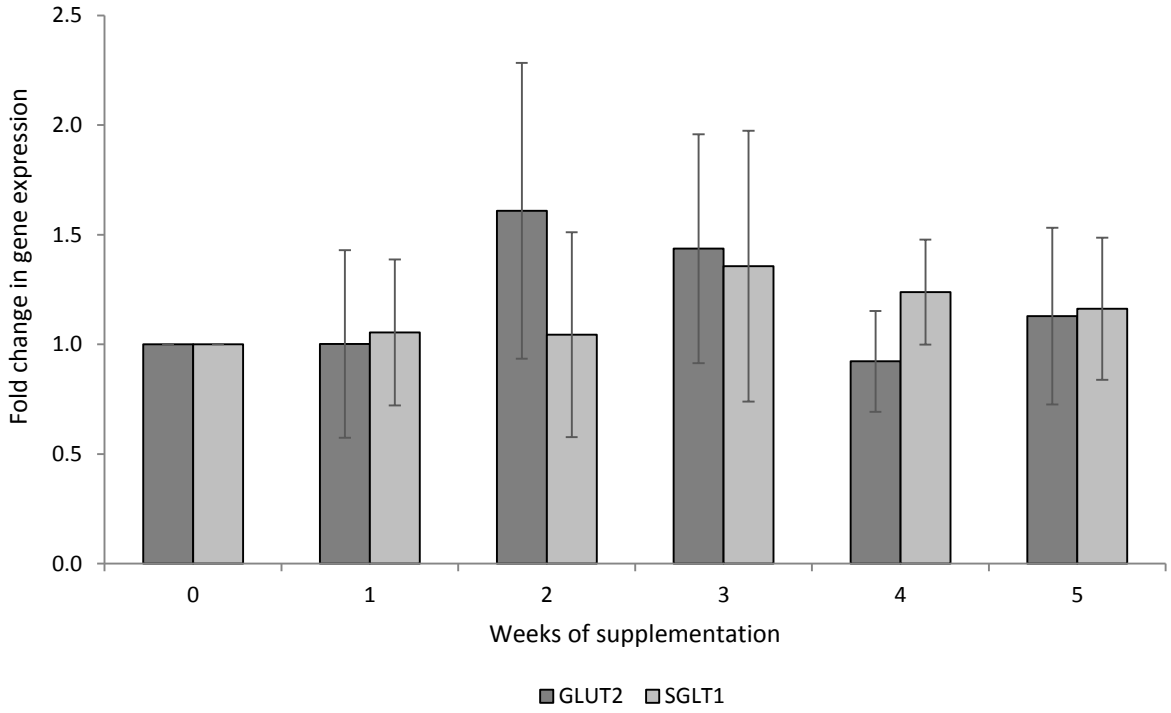


Figure 6.12 Expression of GLUT2 and SGLT1 gene in the small intestine of birds supplemented with xylanase for 1-5 weeks before slaughter. Data are presented as a fold change in gene expression relative to control (non-supplemented, 0 weeks) bird gene expression. Error bars denote ± standard error of the mean (from 10 bird replicates, except weeks 2 and 3 of supplementation which have 9 replicates).

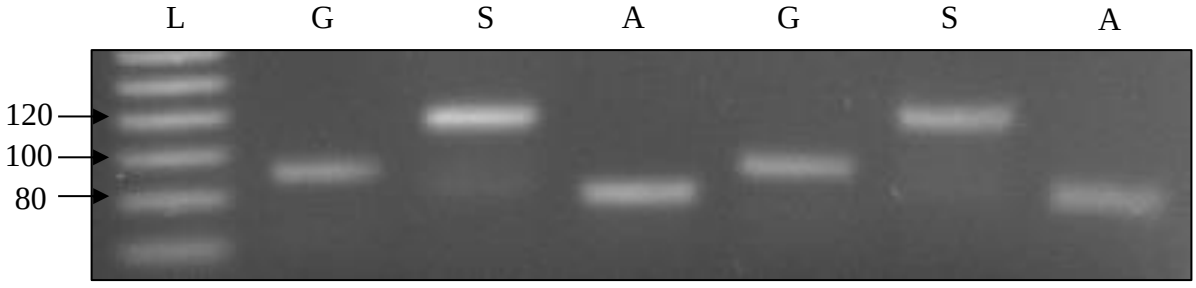


Figure 6.13 Agarose gel separation of qPCR reaction products. Products for the GLUT2 (G), SGLT1 (S) and β-actin (A) genes were run against a 20 bp ladder (L) for determination of product length.

6.3 Conclusions

The purpose of the present studies was to investigate broiler digestion of starches in a maize-soy diet and to determine how xylanase supplementation might affect this process. Feeding of a maize-soy diet showed an increase in digesta pH for all tract sections, when compared to the previously fed maize grit diet. This was most likely due to the buffering effect of the additional protein source and calcium carbonate. This is of importance when considering including additional ingredients to a diet, as it will not only change the nutritional value of the diet, but also the environmental conditions of the digestive tract. This can influence digestive efficiency, for example through altering feed retention time and enzyme activity in the tract. When xylanase was fed on a single occasion, no significant effect on starch or glucose content of digesta was observed. However, the dextrin content of digesta taken from each tract section was significantly reduced for xylanase supplemented birds. This may suggest greater accessibility of endogenous enzymes to dextrin through cell wall destruction. However, there was no apparent difference in the matrices surrounding the starch granules for diets with or without xylanase supplementation. This might imply that xylanase is not breaking down cellular material. However, there are limitations to using SEM such that interpretation is subjective and due to the complex nature of the digestive tract, these images may not be sufficient to show the complete picture of the processes occurring *in vivo*. From this trial, we can therefore assume that feeding xylanase on a single occasion does not show any noticeable effect on cell wall hydrolysis, although its potential impact on dextrin content should be noted. Feed was analysed for xylanase activity using an ELISA method thanks to ESC (Enzyme Services and Consultancy). A very good recovery of the enzyme was achieved, with an activity of 18,100 BXU/Kg, indicating that the enzyme was active in the feed. It is likely that inclusion of this enzyme into the diet on the day of slaughter does not give sufficient time to show any significant response and therefore the enzyme needs to remain in

the tract for a sufficient amount of time to elicit a significant response. This may suggest that the response is some physiological change in the digestion mechanism rather than direct action on the feed.

To test this hypothesis, another trial was designed to investigate the effect of supplementing xylanases for different lengths of time on starch digestion in broilers. Starch fraction measurements did not show any significant effect of xylanase supplementation on starch digestion. A/X ratio results suggest that the upper small intestine may be a major site of arabinoxylan digestion. However, SEM imaging did not reveal any greater dissolution of cell wall material when xylanase was included in the diet, but perhaps this was due to the birds being fed *ad libitum* before slaughter and therefore samples from the digestive tract will show feed particles that had been ingested for different periods of time. As a result, SEM imaging and total starch measurements may not give a full picture of the processes occurring *in vivo*.

Evidence of an indirect mechanism of xylanase action suggests that there is an interaction between blood PYY hormone level and xylanase supplementation (Singh *et al.*, 2012). This was moderately supported in the current study, whereby xylanase fed birds displayed an increase in blood PYY concentration, with the magnitude of response being greatest at 1-3 weeks of xylanase supplementation. This may also indicate an adaptive effect of enzyme supplementation, with birds acclimatising to these hormonal changes overtime.

While improvements in starch digestibility with xylanase inclusion have been well documented (Cowieson, 2010; Meng and Slominski, 2005; Zanella *et al.*, 1999), its effect on glucose absorption is less well defined. The current work has revealed another potential mechanism of xylanase action through regulation of glucose transporter expression in the small intestine of broilers. Although not significant, the numerical trend in results suggested an increase in the expression of the glucose transporters, GLUT2 and SGLT1 after 2 to 3

weeks of xylanase supplementation, respectively. Interestingly, this increase in expression coincided with an increase in blood glucose concentration at 3 weeks of supplementation. With further investigation into glucose transporter regulation, a link between glucose absorption and concentration in the blood may be made, signifying a potential mechanism for the reported improvement in broiler performance with xylanase supplementation. These results also justify the need for longer periods of enzyme inclusion into the diet to allow enough time to achieve the appropriate response.

CHAPTER 7

OVERALL DISCUSSION AND CONCLUSION

Cereals, such as maize, form the basis of poultry feed in many regions by providing the major energy-yielding component, starch. Starch in broiler diets is to a large extent present in the form of native granular starch. Despite the usual adequate levels of α-amylase (the principal enzyme involved in starch hydrolysis), the highly ordered, semi-crystalline structure of native starch proves a challenge for digestion, and will therefore be slowly and incompletely digested by many animals. Although properties of the starch granule such as composition, structure and size may affect digestibility (Moran, 1982), other components of the food matrix are also likely to interact and influence the digestion process. As part of the dietary fibre component of cereals, NSP form the major constituents of the cell walls that encapsulate starch granules within the endosperm of the maize kernel. Broilers do not produce NSP-degrading enzymes to breakdown these cell wall constituents enabling endogenous enzyme access to the granules. Therefore these cellular components can become a limiting factor for starch digestion. Inclusion of other ingredients such as soybean not only increases the protein content of the diet but also contributes to the NSP fraction (Choct *et al.*, 2010). Consequently, the addition of NSP-degrading enzymes, such as xylanase, into poultry diets has become commonplace, with supporting evidence of improvements in broiler performance. However, the mechanisms by which xylanases work in non-viscous diets, including maize, still remains unclear.

In the current project, digestion of maize starches over time was investigated *in vitro* and *in vivo*. This was to determine where and how native starches are digested, what influence a food matrix has on starch digestion and what might be the possible limitations to starch

digestion. The potential mechanisms of exogenous xylanase action in a maize-based diet were also studied to better understand how this enzyme might affect starch digestion and consequently lead to the reported improvement in broiler performance.

7.1 Gizzard action

While it is generally widely accepted that the main functional role of the chicken gizzard is to grind feed material and initiate protein digestion, the importance of the gizzard for starch digestion has not been given much consideration. It is well established that the major site of starch digestion is in the small intestine (Svihus, 2014a), with numerous *in vitro* and *in vivo* studies focusing solely on digestibility within this tract section. Release of starch granules from the endosperm of the maize kernel is important for enzyme accessibility to the granule (Bedford, 2002; Choct, 2006). This is partially achieved through grinding of the whole maize kernel during processing as well as through gizzard action. The grinding of feed not only fractionates cellular material, but also may damage starch granules thereby making them more easily digested (Tester *et al.*, 1994). As all ingested feeds are exposed to the conditions of the proximal tract, the physical and chemical changes that occur in these sections will undoubtedly impact digestibility of all feed components. Therefore, one of the objectives of the current project was to identify the role of the gizzard in the digestion of starch.

One way this was achieved was through designing an *in vitro* model to simulate the digestive tract of broilers. A ball milling technique was employed to mimic best the grinding action of the gizzard. The ceramic balls act in a similar manor to the hard grits retained in the gizzard, causing fractionation of the feed material through impact and attrition. This results in a reduced particle size, which is critical for feed exiting the gizzard and entering the duodenum (Moore, 1999). In support of this, SEM images of gizzard digesta showed fractionation of

plant material and the release of starch granules. This process is therefore not only vital for reducing particle size, it is also essential for making dietary components available for digestion. The contraction cycle of the gizzard occurs up to 4 times per minute (Svihus, 2014a), however there is still limited information on the grinding force and shearing action of the gizzard. Therefore, a relatively low rotational speed of 60 rpm was chosen to avoid overgrinding of the diet as well as allowing comparisons with the roller mixed samples to be made. With greater research into gizzard grinding forces, a more accurate representation of the gizzard can be made using the ball milling technique.

In the current work, the combination of ball milling and an acidic environment (0.01 M HCl) was shown to reduce starch crystallinity while not completely losing all granule integrity. Owing to the highly organised and complex structure of native starch granules, digestion is slow and often incomplete (Oates, 1997; Zhang *et al.*, 2006b). SEM images of gizzard digesta showed predominantly intact, whole starch granules. Amylase activity in this section is minimal due to the acidic environment and therefore enzymatic hydrolysis of starches is unlikely to occur in this tract section. However, the release of starch from within the cell matrix and disruption to the starch granule as a result of grinding as well as acidic erosion may be crucial for their digestibility in the more distal tract sections.

Diet composition and form has also been shown to affect digestive processes and the extent of digestion in chickens (Svihus, 2014a). Understanding the influence of a food matrix on the accessibility of starch to enzymatic degradation is essential when formulating diets to optimise diet utilisation. Broiler digestion of native starches with and without a food matrix was therefore investigated in the current work. Interestingly, even with a relatively small particle size, with a higher proportion of particles being less than 106 μm, and lack of a food matrix, starch from the native maize starch diet still remained in the gizzard 95 min after feeding. This could be explained by the intestinal reflux of feed from the small intestine back

into the proventriculus and gizzard. This theory is also supported by SEM images that show partially hydrolysed granules in the gizzard. However, the starting material also comprised a small number of granules showing signs of hydrolysis, presumably caused by amylase attack during germination which may also explain the occurrence of some partially hydrolysed granules found in gizzard digesta. It is likely that reflux of food between different tract sections has a significant role in the digestibility of feedstuff (Sacranie *et al.*, 2005). It allows for mixing of intestinal contents with digestive juices and movement of digesta back into proximal tract sections, thus allowing more time for digestion. This reflux of feed was not reproduced *in vitro* and thus could be one of the reasons for the lower digestibility of starches when compared to reported *in vivo* digestibilities.

Following ingestion, starch in the maize grit diet was retained in the gizzard for around 50 min. This is in agreement Shires *et al.* (1987), who showed that the mean retention time of feed in the gizzard was approximately 50 min in the broiler, although this will alter depending on diet type and form. Starches in the maize-soy diet were shown to remain in the gizzard for a longer period of time than starches the maize grit diet. This could be due to the additional NSP in this diet which can reduce feed passage rate (Yu *et al.*, 2002). This may also account for the increased content of dextrin in the gizzard, as reported in the current work. As feed is held in the gizzard for longer, this would allow extra time for acidic erosion of the starch granule which may lead to the production of dextrins (Evans and Wurzburg, 1967). The higher dextrin content of the maize-soy diet, implying contribution from soya, may also explain these findings. Increased feed retention in the gizzard is generally correlated to a decrease in pH due to greater HCl secretion. However, in the current work, the pH of gizzard digesta taken from maize-soy fed birds was higher (pH 3.56) than that of birds fed a maize grit diet (pH 2.47). This may be explained by the buffering effect of the additional protein source and calcium carbonate in the maize-soy diet. Potential dietary changes to digestive

tract pH should be evaluated, considering the influence of pH on enzyme and nutrient transporter activity of which will ultimately affect nutrient metabolism.

7.2 Starch digestion in the intestinal phase

The passing of feed material through the tract of birds has been shown within 5 hours of ingestion (Svihus *et al.*, 2002; Svihus *et al.*, 2010). Within this time, starch granules must be released from their cellular and protein matrices, become completely degraded by a number of endogenous enzymes and the resulting glucose absorbed. It is universally accepted that the small intestine is the predominant site of starch digestion and absorption in non-ruminants. Starches in the diet are hydrolysed by pancreatic α-amylase into shorter chain fractions, which are then digested further by brush border enzymes to release glucose monomers. Subsequent absorption of glucose into enterocytes and uptake into the blood allows the bird to utilise this energy source. Although this process is relatively well defined, the effect of feeding native starch granules within different food matrices on starch digestibility is less well understood. Therefore, part of the current project investigated how the composition of a maize diet affects the process and extent of starch digestion.

The progression of starch granule digestion was clear in the current work. Through the refeeding of starved birds, changes to the content of starch in digesta, and its products of hydrolysis, soluble dextrin and glucose, could be measured over time. This enabled an understanding of where starch is being digested in the tract and the time course of this digestion process. However, the limitations to this method should also be noted. Digestible markers are commonly used in feeding trials to estimate the digestibility of nutrients within the diet (Short *et al.*, 1996). The proportion of marker remaining in digesta or excreta in relation to that of the nutrients allows quantification of nutrient disappearance, and therefore digestibility. In the current study, the starch, dextrin and glucose content of digesta was

measured in each tract section. However, since the change in carbohydrate concentration in relation to feed intake and excreta was not measured, starch digestibility could not be determined. Therefore, these results can only be used to demonstrate the change in carbohydrate content of digesta for a particular tract section. A similar practice was achieved using an *in vitro* model, whereby the release of glucose was measured over time. Although there are limitations to *in vitro* and *in vivo* approaches, incorporating the results of both these methods allows for a more comprehensive understanding of starch digestion.

Amylase is considered to be the major enzyme for the hydrolysis of starch. In the bird, α-amylase is primarily secreted from the pancreas, although intestinal amylase has also been reported (Osman, 1982). These amylases digest starch to smaller molecules of maltose, maltotriose and α-limit dextrins. SEM images of starch granules in digesta taken from the upper small intestine show recognisable pores on the granule surface. This is where amylase is thought to bind and access the granule. Starch granules from maize and wheat have been shown to have pore like structures within the semi-crystalline granule and it is the entrance of these pores where the noticeable signs of enzyme attack are first noticeable. When starches in the various maize diets were digested with pancreatin, containing α-amylase, relatively small quantities of glucose were produced, with only approximately 6 % of the starch being digested. Amylase is an endo-acting enzyme that cannot cleave terminal glucose units. Therefore, other carbohydrases are required to hydrolyse further the products of amylase digestion into glucose monomers. These enzymes are found on the brush border of the small intestine of birds and were represented by amyloglucosidase *in vitro*. Addition of amyloglucosidase to the supernatant of pancreatin-digested samples showed that around 46 % and 13 % starch was digested for balled milled and roller mixed maize grit samples, respectively. This allowed the true extent of starch digestion to be determined. Amylase

therefore appears to have a more essential role for the initial stages of starch digestion, while other exo-acting carbohydrases are vital for completion.

Variations in the digestion of starches from different maize diets were assessed *in vitro*. The extent of starch digestion was shown to be dependent highly on diet composition. In opposition to what would be expected, a more complex food matrix appeared to result in a greater extent of starch digestion. Owing to the smaller particle size of the native maize starch diet, the initial rate of glucose release was higher than the other diets. A larger surface area per unit weight, due to a smaller particle size, allows for a greater interaction with digestive enzymes (Goodband *et al.*, 2002). Moreover, there is a lack of 'coating' material over the starch of which the enzyme would have to pass through before the starch could be attacked. This offers the enzyme more rapid access the granules. However, this release plateaued much earlier, with less than 1 % glucose being released from starch, potentially as a result of the robust nature of these granules. The addition of amyloglucosidase resulted in a similar glucose release to that of the maize grit diet, of approximately 50-60 % glucose release from starch. As previously discussed, it is likely amylase hydrolysed the starch into smaller molecular weight fractions that were then further digested by amyloglucosidase to release glucose monomers.

The feeding of native maize starches to broilers demonstrated that material of this low particle size was not acceptable to the birds and few would eat sufficient quantities for substantial analyses of the outcomes of starch digestion through the tract. However, it was clear from the three birds that ingested the feed, that there was a rapid movement of starch into the small intestine. This corresponded to a large glucose load whereby blood glucose levels were raised substantially with feeding, suggesting a rapid digestion of starch. However, the complete digestion of intact native starch granules, due to their robust nature, would be a fairly slow process (Butterworth *et al.*, 2011). Svihus and Hetland (2001) hypothesised that

an overload of starch in the intestine may reduce overall starch digestibility. In light of this, SEM images as well as total starch measurements also revealed a relatively high starch content of digesta collected from the lower small intestine at 95 min from feeding. Therefore, this high starch content and its rapid movement through the tract, along with an overall slow digestion rate, can lead to a number of granules that escape digestion. Due to the lack of bird replication in this experiment, these results should only be taken as a crude representation of native starch digestion *in vivo*.

On the contrary, amylase digestion of the maize grit diet *in vitro* released a noticeably higher concentration of glucose (51 mg/g starch) than the native maize starch diet (8 mg/ g starch). This might suggest differences in the way these starches were digested or that they were more easily degraded than the robust native maize starches. Although amylases are unable to hydrolyse terminal glucose units, a considerable amount of glucose can be produced following prolonged incubation with amylase (Kaczmarek and Rosenmund, 1977). This might explain the increase in glucose concentration of samples following incubation with pancreatin. Digestion of the maize grit diet *in vivo*, showed a more controlled release of granules and exposure to enzymatic digestion. This slower release of starch gave a steady and more controlled increase in blood glucose concentration in birds. The presence of other structural components, such as fibre, will also increase feed retention in the proximal tract (Hetland *et al.*, 2005) allowing more time for digestion. Therefore, this suggests that a food matrix may be beneficial to the digestion process. However, a large proportion of granules were shown to be entrapped within cell wall material by the end of the lower small intestine, a situation that can limit enzyme access to the granules.

Pancreatin digestion of the maize-soy diet with or without xylanase supplementation showed the greatest glucose release, with glucose concentration reaching around 76 mg/g starch. As the maize in the maize-soy diet used the same source as the maize grit it is unlikely that the

differences in granule composition or structure would be a contributing factor. Diet particle size was also found to be similar between the two diets. It is possible that starches in the soybean meal are more easily digested than those of maize. Wilson *et al.* (1978) reported starch granules in soybean with an average diameter of 3 μm. These particularly small granules may be more readily digested than the larger maize starch granules (Kong *et al.*, 2003) despite the soy starch being thought of as having C-type crystallinity, which is often associated with lower digestion rates. However, its proportion in the diet is not great enough to account fully for the greater release of glucose. Therefore, the primary influence is likely to be the presence of added calcium and chloride to the maize-soy diet. Amylase activity is known to be regulated by calcium and chloride binding to the enzyme (Vallee *et al.*, 1959; Levitzki and Steer, 1974) and thus it is possible that the amylase in pancreatin had increased activity in solution with this diet thereby enhancing starch digestion.

Higher digesta pH values were observed when birds were fed a maize-soy diet which gave pH 3.56, 6.70, 7.70 for the gizzard, and upper and lower small intestine, respectively, compared to pH 2.47, 6.49, 7.34 for the maize grit diet. This is in contrast to Mussehl *et al.* (1933), who concluded that the chicken is able to adapt to dietary changes without considerably altering digesta pH. Moreover, Hurwitz and Bar (1968) demonstrated that the intestine is able to regulate pH through adjustment to changes in dietary pH. However, this was not supported in the current work. The increase in intestinal pH may be due to the buffering effect of the additional protein source provided by the soybean, as well as the inclusion of calcium carbonate in this diet. This buffering effect may also have contributed to the differences in the digestion of starches shown in the *in vitro* digestion model. Although pH change was not measured *in vitro*, the potential increase in sample pH for the maize-soy diet may have provided a more optimum pH for enzyme activity thereby leading to greater starch digestion.

However, further experimental repeats are needed to gain a more representative and complete understanding of *in vitro* amylase digestion of starches in these different diets.

As ingested feed enters the small intestine, there is a significant reduction in starch content paralleled by a significant increase in glucose. Interestingly, it would be expected that the glucose released by starch digestion would be rapidly transported into enterocytes (Moran, 1985). However, it would appear from these results that glucose still lingers in the lumen of the upper small intestine for some time before being absorbed as digesta reaches the lower small intestine. It is possible that, after re-feeding, the rapid passage rate of finer feed particles may cause an overload of glucose in the small intestine which exceeds the bird's capacity to absorb it (Carre, 2004). Although, the greatest absorption of glucose occurs in the upper small intestine (Riesenfeld *et al.*, 1980), the lower small intestine seems to be highly capable of absorbing glucose that may not have been absorbed in the upper small intestine (Denbow, 2015).

7.3 Limitations to starch digestion

It is generally accepted that maize is a highly digestible cereal for broilers, with starch digestion reaching 97 % by the end of the small intestine (Riesenfeld *et al.*, 1980; Weurding *et al.*, 2001). However, other evidence presented by Noy and Sklan (1995) suggests that starch digestion may just exceed 85 %. Starch digestion is therefore incomplete, with undigested granules entering the hindgut for fermentation, at a lower energy yield. It is therefore essential to understand the limiting factors of starch digestion in order to be able to find means to alleviate them. This was consequently one of the main objectives of the current work.

Although the crop is known as a storage organ, feed content in this section was not considered. Birds were fed *ad libitum* and therefore were not adapted to being fed specific meals. As a result, prior to starvation it could be assumed that feed would not have been collected in the crop. It is thus unlikely that any significant amount of starch would be held in this section and that the tract would have been empty upon refeeding. However, in the current work, starch granules were still present along the tract of birds starved for 17-24 h. This may pose a problem when imaging or quantifying starch content of digesta, as the starch granules present due to ingestion of the experimental diet cannot be differentiated from those retained from the previous meal. This could be resolved by feeding birds prior to starvation a diet consisting of starch from another botanical source, such as potato, that can visually be distinguished under SEM. However, the total starch assay is unable to differentiate between starches from different botanical sources and therefore would only give a total value for starch content. The starch granules that remained in the tract showed varying degrees of degradation. Kokue and Hayama (1972) reported a continuous secretion of pancreatic juice in birds starved for 24 h. Accordingly, these remaining granules would have been exposed to digestive enzymes, yet complete digestion was still not possible.

Following refeeding, feed material passes straight into the proventriculus and gizzard of starved birds, bypassing the crop (Chaplin *et al.*, 1992). Consequently, the majority of ingested feed would be within the tract sections distal to the crop. When birds were fed for 20 min following a 24 hour starvation period, Kokue and Hayama (1972) reported an increase in pancreatic secretion after 3 min. Flow rate reached its maximum between 20 and 30 min, with levels returning to basal levels by around 120 min. In the current study, digesta was collected within a maximum of 90 min following feeding and therefore feed would have been exposed to pancreatic secretions above basal level. The amount of amylase secreted by the pancreas may also be considered in excess of requirements (Noy and Sklan, 1995). Therefore, it is

unlikely that pancreatic secretion or amylase content was a limiting factor for starch digestion. However, amylase adsorption and binding to the starch granule may be of greater importance. The presence of other feed components in the diet may act to impede enzyme-substrate complexing. Dhital *et al.* (2015) reported non-specific binding of amylase to cellulose under *in vitro* conditions, suggesting an inhibitory effect of cellulose in solution on amylase activity and availability for starch digestion. However, when considering the complexity of *in vivo* conditions, where protein and lipid interactions also occur, this inhibitory mechanism may not be as well defined.

The fact that starch granules were still present in the lower small intestine and in the caeca suggests the presence of RS. In this respect, RS was defined as the starch which escaped digestion in the small intestine. *In vitro* digestion of a maize-based diet reached a maximum of 80 % glucose release from starch. This process would therefore seem less efficient than the bird's digestive system which has been shown to digest starch to near 97 %. There are a number of digestive processes that are not included in the *in vitro* model, such as digesta reflux, microbial fermentation and hormonal signalling, for example. Therefore, these models may be more useful for understanding differences in digestion between samples than gaining actual digestibility values that can be achieved through *in vivo* studies. By the end of the lower small intestine, and in control birds, starch granules appear still to be held within plant material. It would seem that this is making the granules inaccessible to enzyme attack. The encapsulating effect of the food matrix has been well documented (Singh *et al.*, 2010) and is considered one of the major contributors to diet component digestibility.

7.4 Mechanisms of xylanase action

One of the major objectives of the current project was to investigate how xylanases might act in a diet that is low in soluble NSP. It is unclear how xylanases may affect FCR, although a number of theories have been put forward. Firstly, a reduction in digesta viscosity through xylanase hydrolysis of soluble NSP could allow for greater diffusion of enzymes. However, due to the relatively low soluble NSP content of maize, it is unlikely that this is the primary mechanism of xylanase action. Second, xylanases may enhance enzyme access to starch granules through degradation of the cell wall matrix. Lastly, xylanases may also be working through prebiotic mechanisms that lead to changes in digestive physiology or microflora of the bird. Consequently, experiments were set out to investigate the latter two potential mechanisms of xylanase action.

NSP may play an important role in the physical entrapment of starches and proteins in the cereal, thereby limiting their digestibility (Aftab, 2012). Disruption of the cell wall matrix through xylanase action is expected to release these entrapped components making them more available to endogenous enzymes (Bedford, 1996). This concept is supported by reports of improved starch, protein and fat digestibility in maize-soy diets when supplemented with exogenous xylanase containing enzyme cocktails (Cowieson *et al.*, 2010; Meng and Slominski, 2005; Zanella *et al.*, 1999). In the current work, xylanase supplementation was shown to significantly decrease the dextrin content of digesta taken from each tract section. This response might therefore support the cell wall dissolution theory. However, SEM images revealed no apparent difference in the matrices surrounding the starch granules for diets with or without xylanase supplementation, although the subjective nature of the SEM should be taken into account. A xylanase activity of 18,100 BXU/kg in feed was confirmed in the current study and with reports of fungal xylanase activity in digesta (Vahjen and Simon, 1999), although this was not measured in the current work, it can be assumed that the

xylanase was also active in the tract. It is likely that inclusion of this enzyme into the diet on the day of slaughter does not give sufficient time to show any notable effects. In general, feed trials include enzymes for several days or weeks before slaughter and therefore it is possible that the enzyme needs to remain in the tract for a sufficient amount of time to elicit a significant response. Figueiredo *et al.* (2012) reported a greater response to exogenous xylanase in older birds between 27-36 days of age, a response that may have been attributed to changes in microbial populations colonising the digestive tract (Bedford, 2000). Single feeding of the enzyme would not have given sufficient time for any changes in microbial populations and therefore any benefit would not be evident. Moreover, the crop may play an essential role in endogenous enzyme activity through moisturisation of the feed material (Svihus, 2014a). When birds were starved, feed material presumably bypassed the crop, as a result of an empty tract after fasting, thereby omitting this initial, and potentially crucial, stage of endogenous enzyme action. When birds were supplemented with xylanase over longer periods of time, still no considerable effect on cell wall degradation was observed. Birds were fed *ad libitum* prior to slaughter and therefore feed particles at various stages of digestion would be expected to be present along the tract.

Arabinoxylans comprise the largest proportion of the fibre fraction of the maize kernel, with insoluble arabinoxylans predominating. Endo-xylanases such as Econase XT 25 are able to degrade arabinoxylans by hydrolysing the xylan backbone, thereby reducing the molecular weight of the molecule to shorter chain oligosaccharides, AXOS. To investigate how and where xylanase might degrade arabinoxylan in the digestive tract of birds FTIR spectroscopy was employed. Results from this technique showed that arabinoxylan hydrolysis through the tract of birds was not significantly affected by xylanase inclusion into the diet. This may explain why xylanase supplemented birds did not show any greater cell wall degradation. In contrast to the reported release of starch granules from their cell wall matrices with addition

of xylanase to maize *in vitro* (Masey O'Neill *et al.*, 2012), the mechanism of xylanase action *in vivo* may not be as apparent due to the highly complex nature of the digestive tract.

In addition to a direct action cell wall material, xylanase may also play an indirect role on diet digestibility. Singh *et al.* (2012) reported increased secretion of PYY, an enterohormone responsible for controlling gastric emptying, with xylanase supplementation to a maize-soy diet. Caecal fermentation of AXOS produced from arabinoxylan breakdown by xylanase promotes PYY secretion leading to increased feed retention in the proventriculous and gizzard. This results in a 'finer grind' of feed material, with potential for greater gizzard development and improved protein digestion in the gastric phase. Since starch granules in the endosperm of the maize kernel are held within cellular and proteinaceous matrices, disruption of these materials can lead to granule release and enhancement of starch digestibility. In the current study, PYY secretion significantly increased when birds were fed maize-soy diets supplemented with xylanase for 1-3 weeks. This suggests that the response is greater when birds are supplemented with the enzyme for a shorter period of time compared to birds supplemented over the whole trial period, indicating an adaptive effect due to the age of birds when supplementation begins. When birds were exposed to the enzyme at a young age they are able to adapt to these changes, most likely through modification of the microflora population. However, laboratory conditions may have not allowed for a considerable change in microflora and therefore this could be the reason for limited enzyme response, particularly during short supplementation periods.

Greater feed retention in the gizzard due to the ileal brake mechanism has been linked to increased gizzard weight (Masey O'Neill *et al.*, 2014a). However, in the current work, empty gizzard weight was unaffected by xylanase supplementation. This may also be due to the physical form of the diet being fed, as mash diets, such as fed in this study, have already been shown to increase gizzard weight compared to pelleted diets (Nir *et al.*, 1995). Therefore the

change in gizzard weight may not be as noticeable when feeding a mash diet, when the gizzard is already well developed.

Previously, PYY administration has also been shown to increase active uptake of glucose in the small intestine of mice (Bird *et al.*, 1996). This was achieved without a significant change in energy expenditure, suggesting that PYY may also enhance the efficiency of glucose absorption in the small intestine. Interestingly, other reports have shown that PYY administration *in ovo* can improve growth and FCR of chicks during the first week post-hatch (Coles *et al.*, 1999; Coles *et al.*, 2001), an effect attributed to an enhanced absorptive capacity. Croom *et al.* (1999) postulated that *in ovo* PYY administration may increase glucose transporter maturation thereby enhancing glucose absorption during post-hatch growth when digestive and absorptive processes are not yet fully developed. Glucose transporter response to PYY in older birds has not yet received much attention. As xylanase supplementation has been shown to increase PYY secretion in birds, there is consequently a potential for an enhanced absorptive capacity in these birds.

Glucose is absorbed from the intestinal lumen into epithelial cells by the sodium-dependant transporter, SGLT1, which is located on the apical membrane of enterocytes. Glucose then leaves the enterocyte at the basolateral membrane by facilitated diffusion via GLUT2, before entering blood circulation. Guo *et al.* (2014) reported a decrease in SGLT1 expression in broilers fed a wheat-based diet when supplemented with xylanase. In contrast, in the current study, increased expression of the glucose transporters, GLUT2 and SGLT, although non-significant, may suggest a potential response to xylanase inclusion in maize-based diets. Miyamoto *et al.* (1993) reported an increase in SGLT1 and GLUT2 expression in the jejunum of rats fed a high-glucose diet compared to those fed a low-carbohydrate diet. This would suggest that a higher concentration of glucose in the intestine causes a response that elevates glucose transporter expression. As diet digestibility has been found to improve with xylanase

supplementation of wheat and maize-based diets (Choct *et al.*, 1999; Choct, 2006; Cowieson, 2005), this could increase glucose content of intestinal digesta. However in the current work, the glucose content of digesta taken from the upper small intestine was not significantly affected when birds were fed xylanase. Although the current study showed no significant effect of xylanase supplementation on glucose transporter regulation, a potential mechanism of xylanase action can be proposed. Xylanase induced secretion of PYY may enhance starch digestion, due to the 'ileal-brake mechanism', thereby increasing glucose concentration in the intestinal lumen. Subsequent promotion of glucose transporter expression could increase glucose absorption and thus concentration in the blood. With further investigation, this may give a potential mechanism for the improvement in performance seen in birds fed maize-based diets supplemented with xylanase.

7.5 Summary and outlook

The current project has given a novel insight into broiler digestion of native starches. An *in vitro* model was designed to simulate the digestive tract of broilers, which also aided our understanding of the gizzard's role in starch digestion. Differences in starch digestion between various maize diets has been shown, indicating the influence that other feed components have on the digestive process. The progression of starch granule digestion was followed through the digestive tract, giving an indication as to where and how starches are being digested. From this, limitations to starch digestion also became apparent. Furthermore, a potential mechanism of xylanase action was described that may explain the reported improvements in performance when birds are fed maize-based diets supplemented with xylanase. Therefore, this project has explored all of the set objectives and the results of which will contribute to the ongoing research into optimising nutrient utilisation of poultry feed.

Building on the aforementioned evidence collected in the current project, it is important to consider the need for additional work.

7.5.1 Future work

The *in vitro* model used in the current study incorporated a ball milling technique to mimic gizzard grinding action in acidic conditions. This method should be compared with others that ground samples to a specific particle size prior to exposure to gastric conditions. This may indicate whether the initial processing treatment of the diet affects starch digestion, and therefore this should be taken into account when using specific *in vitro* digestion models. Moreover, altering pH of the gastric and intestinal phases may indicate what influence environmental conditions have on the digestive process.

Nearer the end of the project, particles in feed were examined through X-ray micro-CT (Fig 7.1). Particle sieving was explored in gizzards taken from birds fed a commercial pelleted wheat diet (Fig 7.1a) and a maize-soy mash diet (Fig 7.1b). These images are in one plane, however the data, based on differential densities, was collected throughout the gizzards. Such information may be valuable for understanding how the bird separates different particle densities in the diet and this is thought to be the first example of the use of the micro-CT for this purpose. Therefore, further application of this X-ray CT technique may give a novel insight into the digestibility of different feedstuffs.

Work into improving the palatability of the native starch diet without changing starch structure or its digestibility would be beneficial. This could be achieved by cold pelleting of the diet as this may give a more acceptable feed form for birds to collect and ingest the feed. However, frictional forces can cause the temperature to increase during the pellet extrusion process which could alter structural properties of the starch and therefore this must be taken into consideration. Inclusion of other feed ingredients, such as fat or protein, into the diet may

also improve palatability. This would enable starch digestion without the influence of fibrous material to be studied, although the impact of these additional ingredients on starch digestibility must also be taken into account.

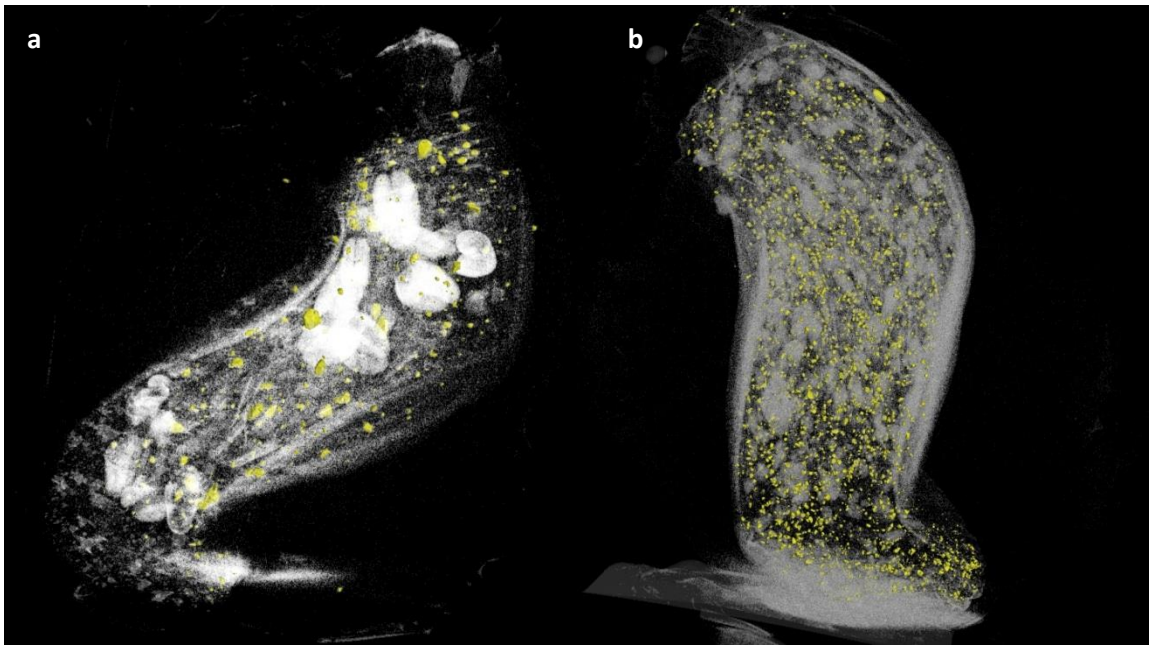


Figure 7.1 X-ray Micro-CT imaging of chicken gizzards. Gizzards were collected from birds fed a commercial pelleted wheat diet (a) and a maize-soy mash diet (b). Yellow particles indicate the highest density feed materials.

The potential mechanisms of xylanase action in a maize-based diet, as outlined in the current study, still needs further investigation. Increasing the number of bird replicates per treatment group will give greater statistical power to the data and will therefore increase the chances of finding a significant treatment effect. Collection of performance data, including bird weights and feed intake, will also indicate a response to xylanase supplementation.

The techniques used in the current study can be applied to other research investigating the digestibility of feedstuffs. SEM imaging of digesta has proven to be a useful tool for observing changes to the feed as it passes through the digestive tract of birds. Another

imaging technique, known as Raman microscopy, could be used to provide information about the chemical and physical structure of molecules in a sample. It could therefore be used to observe amylose and amylopectin distribution in digesta to show how starch granules are being digested through the tract. Combining this information with quantitative measurements will provide a more detailed understanding of the digestive process and how dietary changes can affect these mechanisms. Size exclusion, using techniques such as analytical ultracentrifugation, may also give a more detailed insight into the content of starch digestion products, such as maltose and dextrins, in digesta. This would provide a better understanding of how starches are being digested and what the main products of digestion might be.

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