THE ROLE OF XYLOOLIGOSACCHARIDES IN THE FEED EFFICIENCY OF NON-RUMINANTS.

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FOREWORD

This thesis contains original research in a style accepted for or suitable for publication. As such, the results chapters within are in the format of a manuscript each containing their own introduction, methods, results and discussion sections. Due to the succinctness of the methods sections prepared for manuscripts, a detailed methods section has been included which describes the methods of *in vitro* digestion in more detail.

ACKNOWLEDGEMENTS

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My special gratitude goes out to my parents, for their unwavering support throughout the past few years, their support and reassurance was essential to my completion of this project.

ABSTRACT

It has become increasingly common in non-ruminant animal production systems for the feed to be supplemented with exogenous fibrolytic enzymes, such as xylanase. This may help contribute to global food security by increasing the feed efficiency of livestock fed on poor quality diets. Fibrolytic enzymes, like xylanase, target the non-starch polysaccharide (NSP) fraction of feed. These have been reported to improve the growth characteristics of chickens, via various suggested mechanisms. Recently, there has been heightened interest in the xylooligosaccharides (XOS) that are generated by the hydrolysis of xylan with xylanase enzymes, which have been suggested to have prebiotic effects that ultimately may lead to improved bird health and growth characteristics. It is theorised that the XOS generated by enzymatic hydrolysis could be affected by the combination of enzymes and substrates present in different feed sources. Therefore, the aims of this thesis were as follows. 1) Identification of the xylo-oligosaccharide fingerprint of different feed sources digested with different exogenous xylanase enzyme products *in vitro.* 2) To further investigate and characterise the generation of XOS and monomeric substrates from different varieties of wheat when supplemented with an array of exogenous xylanase enzyme products. 3) To study the effects of xylanase supplementation on the growth performance, generation of XOS and related monomers in various locations of the gastrointestinal tracts of broilers fed a wheat-based diet *in vivo* and compare that to the *in vitro* digestion model used for previous works.

An *in vitro* study investigated the range of XOS and monosaccharides produced from four cereal samples (barley, maize, oats and wheat) over a 72 hour *in vitro* incubation using 3 commercially (AB Vista) available fibrolytic enzymes

Econase XT, Econase MP1000 and Barley P700, all containing endo-xylanase with other combinations of enzymes. There was a cereal x enzyme x incubation time 3-way interaction in the generation of XOS (xylotetraose, xylotriose and xylobiose) ($P<0.01$) indicating the generation of XOS varies dependent on both the cereal and the enzyme used. Econase XT generated the greatest quantity of xylose, with 38% of available xylose from wheat being released after 72 h, 11% from barley and 9% from oats.

In a second *in vitro* experiment, it was investigated how different exogenous enzymes interact with different varieties of wheat. The generation of NSP-derived substrates including XOS and monosaccharides from six wheat varieties (Maris Huntsman, Highbury, Paragon, Sinuelo, Chinese Spring and Pavon 76) were measured, over a 24 hour *in vitro* incubation, with different commercially available fibrolytic enzymes. There were significant variety x enzyme x incubation time interactions for the release of xylobiose, galactose and glucose (all $p<0.001$) and significant enzyme x variety interactions for the release of xylotriose ($p=0.022$), xylose ($p<0.001$) and arabinose ($p=0.028$).

A final study quantified xylanase-induced changes in soluble monosaccharides, XOS and volatile fatty acid (VFA) contents of the different sections of the poultry GIT and whether these relate to altered bird performance. To do this, an *in vitro* digestion of the wheat-based diet was carried out with xylanase (Econase XT at 16,000BXU/kg diet) to compare the *in vitro* and *in vivo* generation of these XOS and monosaccharides. For the *in vivo* study, 80 male Ross 508 broiler chicks were split into two groups fed a wheat-based diet with or without Econase XT (16,000BXU/kg diet) for 21 days. Although there were no effects of Econase XT inclusion on growth performance characteristics, supplementation increased

the xylotetraose (X4) content in the colon ($p=0.046$, enzyme x GIT section interaction) and the xylose contents in the colon and caeca ($p<0.001$, enzyme x GIT section interaction). There was also a trend for increased acetate proportion in the caeca of Econase XT treated birds ($p=0.062$).

These findings suggest that the fibrolytic enzymes tested in the present studies have some specificity for different cereals and for different varieties of wheat. Therefore, it may be possible to optimise the combinations of cereal varieties and enzymes used in animal feeds, to help maximise the feed efficiency of livestock. Also *in vivo,* the hydrolysis of wheat arabinoxylan in poultry is enhanced by xylanase supplementation, which may increase the production of beneficial VFA in the caeca, and thereby potentially modulate the caecal microbiome, although, this did not affect bird performance.

PUBLICATIONS

T Dale, T Parr, M R Bedford, J M Brameld and G Tucker. (April 2018). Examination of the effects of exogenous fibrolytic enzymes on the release of xylose from different cereal types *in vitro. British Society of Animal Science* **Oral Presentation**, Dublin, Ireland

T Dale, T Parr, M R Bedford, J M Brameld, J King and G Tucker. (April 2019). Differential effects of fibrolytic enzymes on the *in vitro* release of xylobiose from different cereal types *in vitro. British Society of Animal Science*

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Poster Presentation, Austin, Texas.

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1. INTRODUCTION

1.1 Overview

Globalisation has increased the need for greater farming intensity around the world. A substitution effect is taking place. Developing countries are replacing traditional carbohydrate rich diets of cereals, roots and tubers for meat, dairy and fish (Bruinsma, 2017). Animal feed production must be increased to meet animal product demands. Both political and societal pressure dictates this increasing food production must be done by both environmentally and socially sustainable means (Garnett et al., 2013). This underlines the need for research focused on increasing the supply of animal food products whilst decreasing the impacts on our climate and ecosystems. Livestock production efficiency is one area where progress could be made.

Xylanase has been used for over 25 years in non-ruminants as a supplement to break down the non-starch polysaccharide (NSP) fractions of commercial feeds and therefore increase feed efficiency (Annison and Choct, 1991).

This thesis will investigate the impact of xylanase and other fibrolytic enzymes on the release of monosaccharides and oligosaccharides that are released in the hydrolysis of NSP from a variety of cereals. The following literature review will address global food security, livestock production, our current knowledge of cereals fed to livestock and the exogenous fibrolytic enzymes that can be utilised to increase feed efficiency.

1.2 Global food security

Food security can be defined as when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life (FAO, 2016).

Global population is increasing, and food security is a growing concern. In 1950, the world population was 2.5 billion. Today, there are over 7.5 billion people. Projections by the UN suggest that in the year 2100, the population will surpass 10.1 billion (Economic and Division, 2010). With population growth comes a challenging dilemma: meeting the food production demands of a rising global population, from an increasingly limited supply of the resources, land and water.

How are we going to feed 10.1 billion people? To answer this, it should be noted that collaborative action will need to be taken from a wide range of stakeholders, who must coordinate efforts in a multi-faceted global approach to provide a sustainable and efficient level of global food security (Godfray et al., 2010).

Meat production is an energy inefficient task, and it has consequences on the environment and raises some ethical dilemmas. Yet, more people than ever are consuming meat or other animal products, and this requires more feed for these animals to be produced, shown by recent trends of increasing grain production for feeding livestock and the increases in poultry farming (Figure 1.1). This could have profound impacts on land use and the environment. The goal is to increase livestock production in a sustainable manner, environmentally,

socially and ethically. Land use and environmental effects will be discussed below.

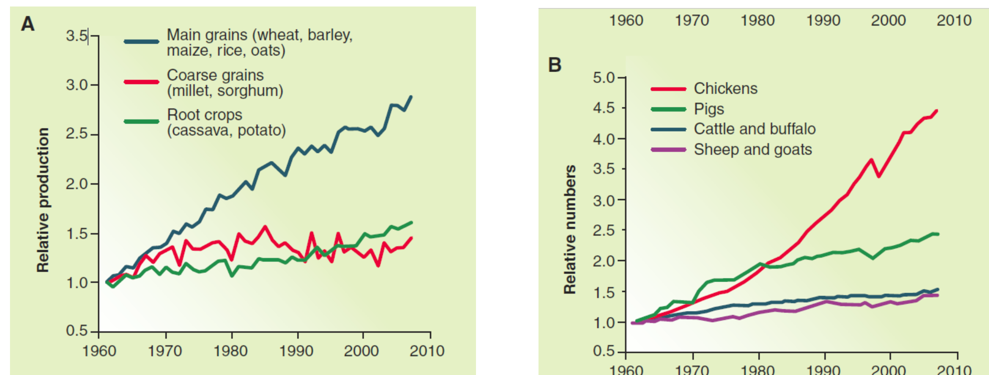

Figure 1. 1 The global production of animal feed and major livestock between 1960 – 2010 taken from Godfray et al., (2010).

1.3 Land use

With a rapidly expanding population, competition for land for residential and agricultural use is greater than ever. More livestock also means that more cereals must be sourced to feed them with. There is therefore a dilemma in that we must upregulate livestock production whilst simultaneously reducing the land used for this task. Farming intensity is defined by the FAO as producing larger amounts of the desired products per unit area of land already used for agriculture or forestry. Over 38% of land use on earth is for agricultural purposes, and the expansion of this would cause the loss of biodiversity, by the removal of important spaces, such as rainforests in the tropics (Foley et al., 2011).

Globally, 62% of crop production is used directly in the human diet (on a mass basis) and 3% is used for biofuels and other industries. The remaining 35% of crop production is used for animal feeds, which indirectly feeds the global population. Despite this, ~75% of agricultural land on earth is devoted to the production of animal products through pasture, grazing land or cropland

devoted to animal feed itself (Foley et al., 2011). This highlights the space for improvements in efficiency to be made in rearing livestock to produce animal products.

One singular process to increase the amount of meat produced per unit area of land, and thus increasing production efficiency, is the addition of exogenous enzymes to animal feeds. This process is additive to a range of interventions that increase efficiency further. There are various enzymes that can be utilised to increase feed efficiency, but fibrolytic enzymes will be the focus of this thesis. Fibrolytic enzymes such xylanase are conducive to global food security as they can reduce the environmental impact of many industrial processes, as they are used to recycle otherwise wasted xylan into new products (Collins et al., 2002).

1.4 Impacts on the environment

Increases in food production can have profound consequences on the environment. Therefore, there is pressure on global markets to lessen the negative effects of increased farming.

If we can reduce waste products generated from this process, we can protect our climate from damage. The challenge of feeding an ever-increasing global population is a difficult one, with many factors and players involved. If we can achieve food security ethically and sustainably, we will protect the world's ecosystems and biodiversity. The increase in farming means that inadvertently more fertilisers and pesticides will be used on agricultural land that is used to grow the cereals that are fed to livestock, resulting in a loss of biodiversity.

Greenhouse gases are also known to exacerbate global climate change, which is a critical issue in livestock farming (Robertson et al., 2000). Throughout the late 20th century and early 21st century, there has been sustained increases in greenhouse gases (GHGs). In the last 50 years the GHG emissions from beef have increased 59%, pork 89% and chicken 461%, when expressed as million metric tonnes of carbon dioxide equivalents (Mt CO_2e), largely due to increased consumption in developing countries (Caro et al., 2017). There is evidence that supplementation of fibrolytic enzymes such as xylanase can reduce GHG emissions (Hernández et al., 2017). Despite this, livestock production only represents around 9% of total GHG emissions globally (Caro et al., 2014). In 2010, due to ruminant enteric fermentation, beef production resulted in 54% of total agricultural GHG emissions alone, and 69% of those GHGs were methane (CH_4), which the Intergovernmental Panel on Climate Change (IPCC) deemed was 25 times more potent than carbon dioxide as a GHG during a 20 year study period (IPCC, 2013). This highlights the need for more sustainable farming methods moving forward. As cereals are so frequently used in animal feeds, increasing feed efficiency could result in a lesser impact on the environment as a whole through a reduction in these emissions.

1.5 Cereal carbohydrate structure

Cereal grains constitute an important source of energy and protein for humans, all farm livestock and especially non-ruminants (Placinta et al., 1999). They also contain large fractions of structural NSP within the cell wall which are indigestible to non-ruminants. These are known as dietary fibres, which can be classified based upon nature of action, physical origin of the fibre and the

chemical identity of the fibre (Ha et al., 2000). One such example is the solubility of dietary fibre, being either water-soluble or water-insoluble, which can affect the viscosity of a substrate such as digesta (Manthey et al., 1999).

Plant cell wall polysaccharides are found as a part of the cereal grain cell wall, where they provide structural support. A hemicellulose is a plant cell wall polysaccharide that is not soluble in water but is soluble in aqueous alkali (O'Neill and Selvendran, 1985). Components of hemicellulose will be discussed below.

1.5.1 Monosaccharides

Plant cell wall polysaccharides are predominately made up of the following monosaccharides: xylose, arabinose, galactose and glucose, with small amounts of mannose, seen in Figure 1.2 (Choct, 1997). They are found in chair and boat form confirmations, but more commonly chair form as this has been found to be the most stable form (Marszalek et al., 1998). Monosaccharides are the most basic form of carbohydrate, as they cannot be hydrolysed into another, simpler chemical compound.

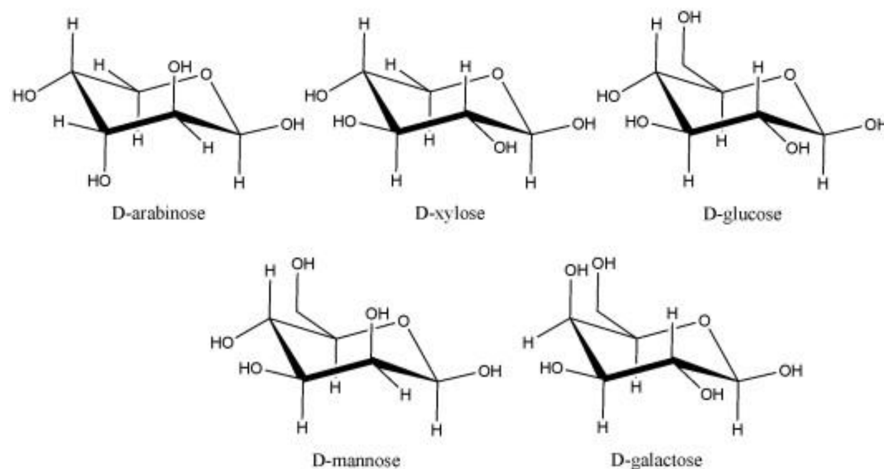

Figure 1. 2 Chair forms of the primary cell wall monosaccharides present in non-starch polysaccharide. Taken from Domingues et al. (2014).

Arabinose is an aldopentose, meaning it contains five carbon atoms and an aldehyde functional group, with a chemical formula of $C_5H_{10}O_5$ (Figure 1.2) (Dey and Harborne, 1997). It is a secondary constituent of the arabinoxylan polymer of plant cell walls, along with xylose (Izydorczyk and Biliaderis, 1992). Xylose is also an aldopentose and is primarily found in the hemicellulose, xylan. Its chemical formula is the same as arabinose (Kofod et al., 1995). Galactose is an aldohexose monosaccharide, with the same chemical formula as glucose ($C_6H_{12}O_6$). It also has a similar structure to glucose, except for the location of one hydroxyl group, making it an epimer of glucose (Kamide, 2005). Galactose is frequently found in soyabean meal (SBM) as part of rhamnogalacturonan, a staple of poultry diets around the world (Irish and Balnave, 1993). Glucose is an aldohexose, like galactose. It is a monosaccharide commonly found in starch, which is a large component of storage polysaccharides within plant cell walls and is the most abundant monosaccharide in cereals (Domb et al., 1998).

In wheat, overall monosaccharide levels have been shown to be around 78-83% amongst various varieties, with glucose comprising the largest fraction of this (69-75g/100g whole grain). Arabinose ranged from 2.4-2.9%, xylose 4-4.8% and galactose at 0.54-0.66% (Lafond et al., 2015).

The total plant cell wall monosaccharide composition has been shown to be around 80% glucose, 10% xylose, 7% arabinose and 1% galactose (Park et al., 2013).

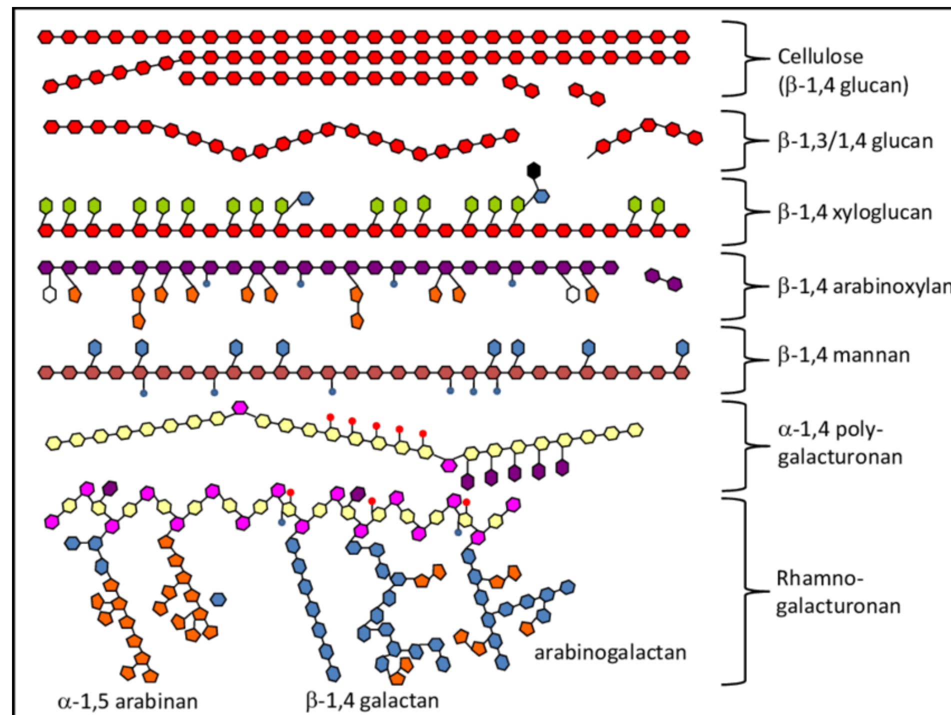

Figure 1. 3 Schematic representation of plant cell wall polysaccharides, taken from Phalip et al. (2012). Glucose residues shown in red, xylose residues in green, galactose in blue, β-1,4-arabinoxylan is a xylose backbone linked with arabinose in orange, mannan in dark red and glucuronic acid in white.

1.5.2 Non Starch Polysaccharides

NSP in the form of cellulose, arabinoxylan and β-glucan is the most prominent in grains which are routinely used in poultry feed (Knudsen, 1997). Those which are soluble in water are mostly NSP's such as arabinoxylan. The majority of variation in nutritive value of wheat is a direct result of the quantity and composition of NSP within the cereal grains, where larger levels of structural arabinoxylans contribute to increased digesta viscosity and a corresponding decrease in nutritive value of the feed due to a lack of mixing and absorption of nutrients trapped in the viscous digesta (Choct and Annison, 1992). In a study by Choct and Annison, when 35g/kg of (85% arabinoxylans)

were added to broiler feed, significant increases in digesta viscosity ($P<0.05$) resulted in significant decreases in bird performance ($P<0.05$). NSP is indigestible by poultry, pigs and humans, who lack the necessary endogenous enzymes to digest it. These carbohydrates have varying chemical and physical structures, which can affect feed efficiency.

Figure 1.3 shows how cellulose and β-1,3/1,4 glucans are comprised of repeating glucose units. B-1,4-xyloglucans are glucose-based polymers with xylose substitutions, which can also be linked to galactose. β-1-4-arabinoxylans are formed from a xylose backbone, linked with arabinose and/or glucuronic acid. β-1,4- mannans are units of mannose linked to galactose, that can be acetylated or not. β-1,4-polygalacturonan is made up of linear chains of galacturonic acid associated with rhamnose. Rhamno-galacturonan is made up of a rhamnose-galacturonic acid backbone, with non-linear, mixed side chains of arabinose, galactose.

1.5.3 Cellulose

Cellulose is the most abundant biomaterial on earth (Preston, 1975). It is the main constituent in plants and serves to maintain their structure. 150 years ago, Anselme Payen discovered and coined the name "Cellulose", after isolating it from green plants (Payen, 1839). Cellulose is a water-insoluble polymer, made up of linear d-anhydro-glucose units that are linked by β-1, 4-glucosidic bonds, with 15 to 14,000 degrees of polymerisation (Lin et al., 1981).

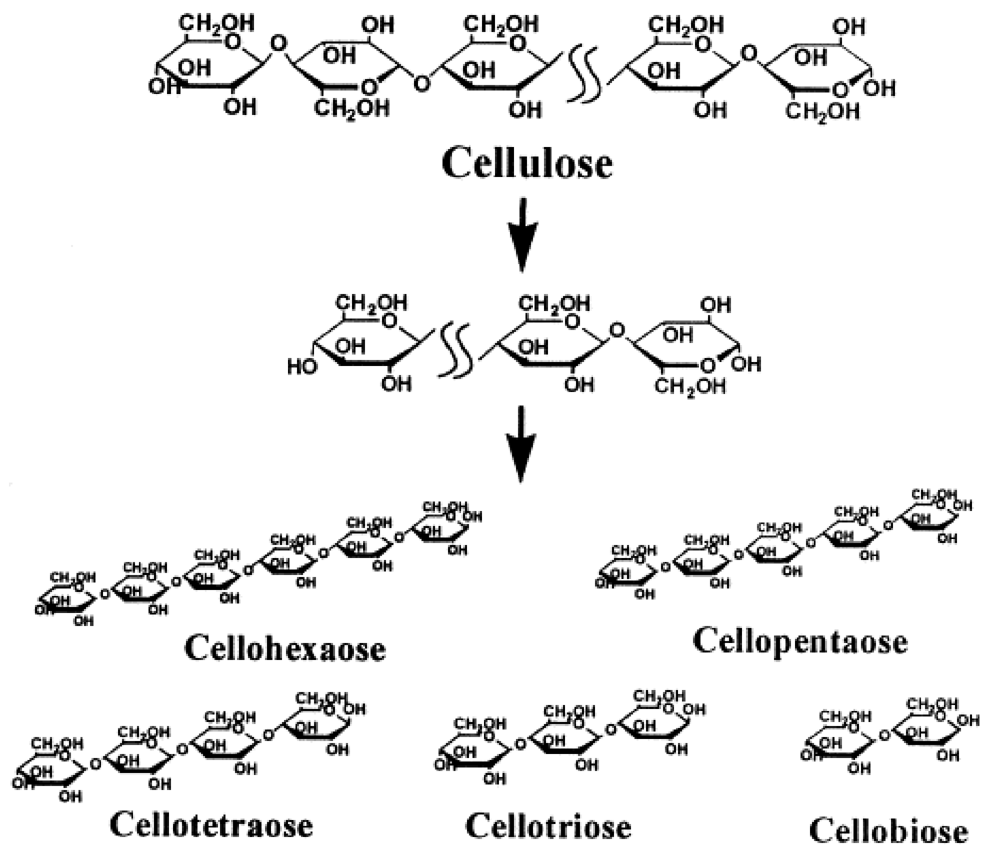

Figure 1. 4 A pathway of oligosaccharides generated from enzyme hydrolysis of cellulose. Taken from Sasaki et al., (1998).

1.5.4 β-glucans

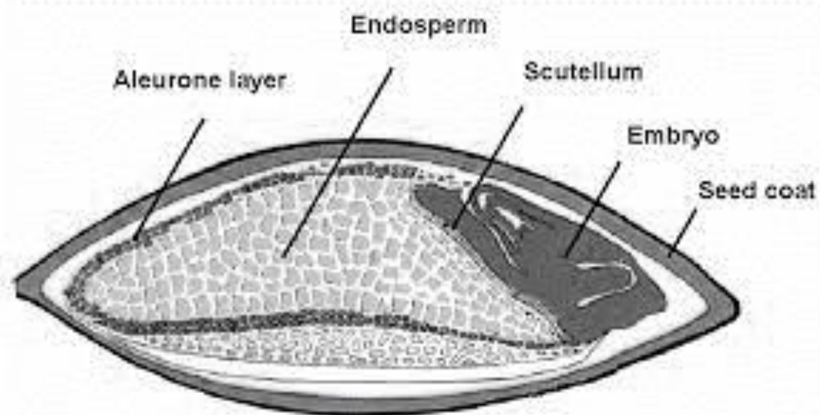

Figure 1. 5 The structure of barley seed. Taken from Shahpiri et al., (2008)

Cereal β-glucans are NSP's that are soluble in water and have mixed linkages of (1→3) and (1→4)-β-D-glucans. It is the (1→3)-β-D-glucans that make the

molecule flexible and soluble (Planas, 2000). They are the most abundant cell wall polysaccharides in barley and oats, present in both the aleurone layer and the endospermic cell walls which encapsulate the major carbohydrate stores of the grain in the starchy endosperm, made up of dead cells. Roughly 54% of barley β-glucans are water soluble, but over 80% is soluble in oats due to the varying chemical structure and molecular weight of different β-glucans (AMAN and GRAHAM, 1987).

1.5.5 β-Mannans

Mannans are present in plant NSPs primarily in the forms of galactomannan and glucomannan, as shown in Figure 1.6 (Tester and Al-Ghazzewi, 2013).

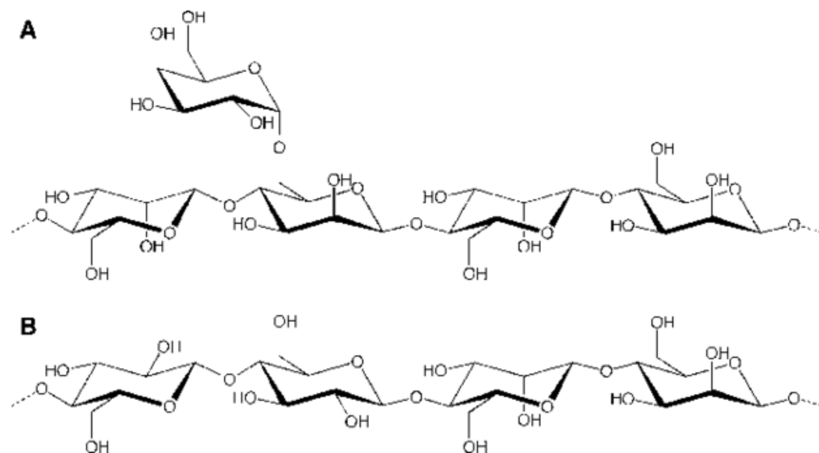

Figure 1. 6 The primary structure of two mannan hemicelluloses; A, galactomannans and B, glucomannans. Taken from Cunha and Gandini., (2010).

Mannans reside as part of the hemicellulose of legume plant cell walls (Reid, 1985). β-mannan is made up of repeating units of mannose attached to galactose with or without glucose (Figure 1.3) on the polysaccharide's backbone (Carpita et al., 2001).

β-mannans are found in most non-ruminant feeds as soybean is used almost universally as a protein source, containing around 8% β-mannan (Jackson et al., 2004). Barley, oats, wheat and maize contain relatively low levels of β-

mannans, from 0.09% in Maize to 0.49% in Barley (Vervaeke et al., 1989). β-mannans also increase digesta viscosity and have been shown to induce immune responses within rats (Ikegami et al., 1990).

1.5.6 Xylan

Xylans are hemicellulosic polysaccharides found in plants including trees and cereals (Aspinall, 1959). Xylans commonly act as structural units in plant tissues, associating closely with cellulose as shown in Figure 1.3 (Marchessault, 1994). Xylan is a heterogeneous polymer made up of 1,4-β-linked D-xylopyranose units in its main chain with branched chains of various sub-units. It is these subunits which give xylans their varying properties based upon chemical structure, degrees of branching and polymerisation (Bastawde, 1992). Xylans make up 30-35% of the cell wall material of cereals, second only to cellulose (Ebringerova and Heinze, 2000). This also means that xylan makes up a considerable portion of animal feeds.

1.5.7 Arabinoxylan

In cereals, arabinoxylan is the most common xylan, composed of repeating chains of arabinose and xylose pentosans (Moreira and Filho, 2016). It is present as endospermic and non-endospermic arabinoxylan, though is most abundant within the endosperm. Both forms have an L-arabinose group linked to the D-xylan backbone, usually at position 3, and less commonly 2, but

always furanose in form, where the ring structure of the carbohydrate has four carbon atoms and one oxygen atom, as seen in Figure 1.7 (Sinha et al., 2012).

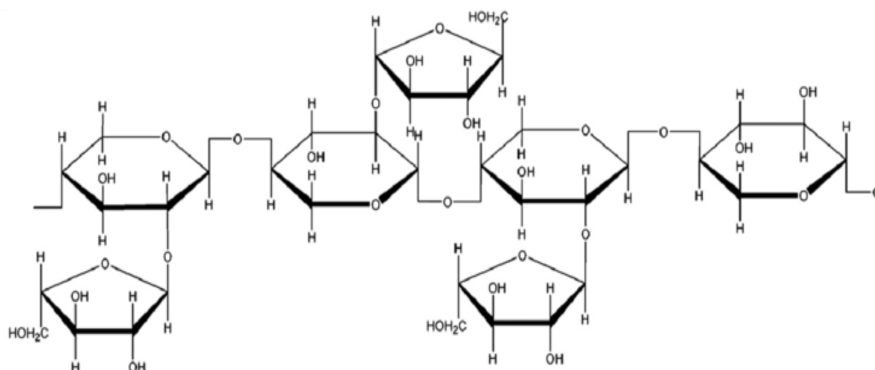

Figure 1. 7 Chemical structure of arabinoxylan taken from Sinha et al., (2012)

Non-endospermic arabinoxylan has an α-L-arabinofuranose group with a combination of 4-*O*-methyl-D-glucuronic acid and/or glucuronic acid plus acetyl and galactose as side groups positioned at the 3 and 2 carbons, as shown in Figure 1.7 (Aspinall, 1959). Endospermic xylans are the ones found in cereals. They are heavily branched and can be double substituted by α-L-arabinofuranose at 3 and 2 positions (Wilkie, 1979). It is rare to see the substitutions of uronic acid in endospermic arabinoxylan (Wilkie, 1979).

Arabinoxylans dominate as storage polysaccharides in cereals, and in wheat over a third of the arabinoxylan is soluble in water. They are located largely in both the endosperm and aleurone layers (Chesson et al., 1995).

1.5.8 Xylooligosaccharides (XOS)

Oligosaccharides are naturally occurring carbohydrates, comprising of 2-10 monosaccharide units, linked via β- glycosidic bonds (Figure 1.8). They can be either linear or branched and are connected by α- and/or β- glycosidic linkages (IUB-IUPAC nomenclature). There are numerous different subtypes of

oligosaccharide, including those based on the monosaccharides xylose, fructose, galactose and mannan (Zhao et al., 2017). XOS have various uses in the food industry and often used as sweeteners (Kumar et al., 2012).

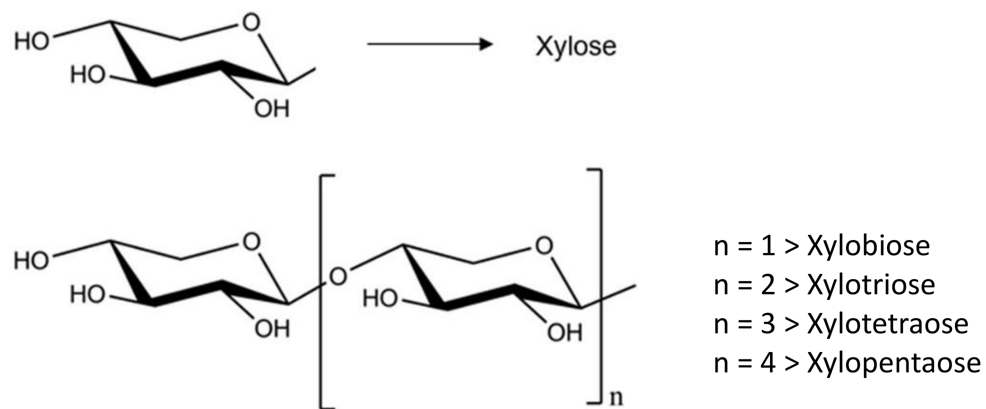

Figure 1. 8 Diagram showing the structural properties of linear xylooligosaccharides. Taken from De Freitas et al., (2019).

XOS are found in low amounts in various foods, such as bamboo shoots, fruits, vegetables, milk and honey (Vázquez et al., 2000), but are also the product of enzymatic hydrolysis of xylan. They are named based on the number of xylose units present in the backbone of each compound e.g., xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose (Figure 1.8).

XOS are safe for human consumption, are low calorie, low odour, and are stable in changing pH from 2.5-8.0 and temperatures up to 100°C (Vázquez et al., 2000). The global market of XOS is growing, as they can be readily incorporated into foods and supplements, are effective at low doses and show positive effects on health in various species. Japan is the current market leader in production.

There is an increasing interest in the farming industry for the potential beneficial effects of XOS when used as a feed supplement to help increase the growth performance characteristics of poultry (Ribeiro et al., 2018).

In humans, poultry and pigs, XOS are indigestible, so reach the hindgut for fermentation, as there is a lack of endogenous enzymes to break down β-glycosidic linkages. This means that when XOS are fermented in the hind-gut, increased acidity in the colon is observed, combined with greater production of VFA which can contribute to host energy supply (Aachary and Prapulla, 2011), giving XOS the ability to modulate the microbiome of the digestive tract, which can have numerous effects.

1.6 Digestive system of poultry

The gastrointestinal tract of poultry is the location of two important processes, the digestion and absorption of nutrients (Figure 1.9). Digestion involves both the mechanical breakdown of food and the chemical breakdown.

The digestive system pH can vary at different sections of the broiler digestive tract, from 3.8 – 6.7 in the crop, 1.6 – 4.8 in the gizzard and 5.7 – 7.7 in the intestines (Bedford and Partridge, 2011). Most NSP enzymes have optimum activity at 4.0 – 5.0 (de Vries and Visser, 2001) but it has been shown in xylanase that more than 50% of the enzymes maximum activity (at pH 6.0) is retained under conditions of 3.0 – 7.0 pH (Ding et al., 2008). The pH conditions must be considered when attempting to optimise enzyme efficiency.

Chemical breakdown begins in the beak, where food is exposed to mucus and salivary amylase, initiating enzymatic digestion, although only for a short period. The bolus then travels down the oesophagus to be stored in the crop.

The crop acts as a storage vessel for digesta in poultry and affects nutrient digestion by softening feed and exposing it to intital activity of both endogenous and exogenous enzymes (Classen et al., 2016). The food bolus is then slowly released from the crop to the proventriculus where muscular mixing of the food with hydrochloric acid and enzymes, such as pepsin, occurs.

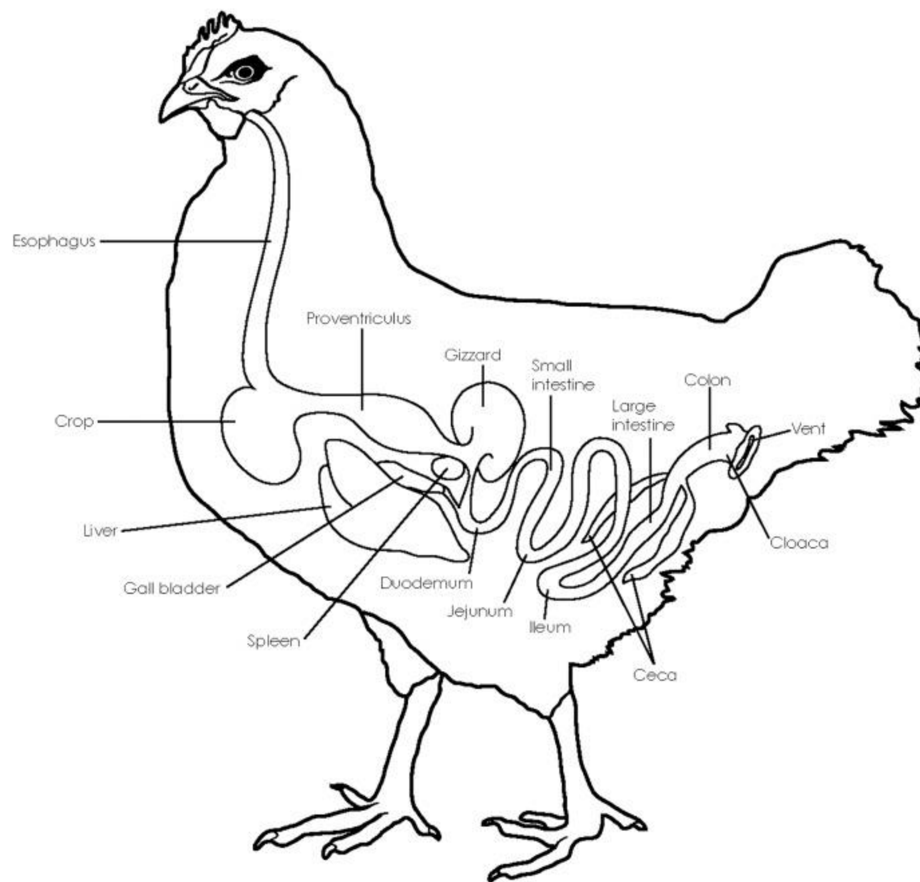

Figure 1. 9 Basic anatomy of the poultry digestive tract from the oesophagus to cloaca. Taken from Clavijo and Flórez., (2018).

Mechanical breakdown then continues once the food bolus reaches the gizzard, where accumulated grit and stone grinds food down via the strong muscles in this area.

Enzymes such as pancreatin, amylase and pepsin along with bile produced in the pancreas and liver then digest food further in the small intestine, where nutrients are absorbed (Bedford and Classen, 1993). Any food or water that is undigested at this point will reach the colon and caeca, where water is absorbed, and fermentation by the gut microbiome will occur. Excrement is then discharged from the cloaca. As previously mentioned, the endogenous enzymes present in poultry digestive tracts are unable to hydrolyse NSP, therefore, by incorporating exogenous enzymes into the diet, we can modulate NSP hydrolysis, to elicit various potential benefits.

1.7 History of enzyme usage in livestock

The first instance of enzymes being utilised in animal feeds goes back almost 100 years, where George W. Hervey (1925), conducted a 'Nutritional Study Upon a Fungus Enzyme". This pioneering work investigated the impact of supplementing poultry feed with 5%, 4%, 3%, 2%, 1% or 0% 'fungus enzymic material' as a percentage of total mash feed. After a 20-week experiment, the birds supplemented with the enzymatic material showed increases in growth in a dose-response relationship, with the 5% enzyme group showing a 22% increase in final body weight without any change in mean total grain consumption. Hervey concluded in his paper that increased starch and protein digestion in the enzyme treated groups was likely the mechanism of effect.

The use of fibrolytic enzymes have been well established over the past 100 years and the market share of enzymes is indeed growing, but there is still no consensus on how exactly some exogenous enzymes elicit their effects.

Only by understanding the mode of action can the true potential of fibrolytic enzymes be realised. This led to the introduction of fibrolytic enzyme use within these animal feeds, in order to release the nutrients encapsulated within the formerly indigestible NSP, with the aim of increasing non-ruminant feed efficiency.

1.8 Fibrolytic enzymes

All animals digest their food with enzymes, to break down what is consumed into smaller components that are available for absorption. These endogenous enzymes are normally produced by the animal internally and can also be generated by the microbiota of the gut, which may have a symbiotic relationship with the host.

Anti-nutritive factors, such as NSP, are present at significant levels in the gut and can increase digesta viscosity and encapsulate valuable nutrients within the feed which lead to nutrients passing through the gastrointestinal tract unabsorbed (Annison and Choct, 1991). Poultry do not possess the enzymes necessary to sufficiently hydrolyse NSP to alleviate the negative effects. This has repercussions on the farmers and the environment. The efficiency and profitability of poultry farms is dependent on the cost: gain of its livestock, so undigested material is considered wasted money and lowers feed efficiency.

Fibrolytic enzymes can target NSP, which reduce feed efficiency, to release encapsulated nutrients and to therefore increase the feed efficiency of animals (Aulrich and Flachowsky, 2001). They have been used in poultry systems since the 1980’s to increase feed efficiency (Bedford, 2018). They have been successful due to increasing the efficiency of poultry farming, whilst still

generating products safe for consumption, given that the enzymes are made up of various proteins that are either completely digested and recycled or excreted as waste products by the animal, meaning no residues are left over in the product (Bedford and Partridge, 2011).

Non starch polysaccharides are present in all the cereal-based feeds of poultry such as barley, oats, wheat and maize (Austin et al., 1999). Fibrolytic enzymes can break down NSP into its smaller constituents, dependant on the type of enzyme used. Due to the variability of NSP's physical and chemical characteristics, some enzymes may be highly effective in one feedstuff, yet ineffective in another feedstuff (Van Soest, 1967). There is, therefore, a need to characterise the structure, mode of action, target sites and specificity for different cereals and for different varieties within a cereal species of enzymes, in order to maximise their benefits.

1.8.1 Cellulases

Cellulase encompasses β-glucosidases, cellobiases, cellobiohydrolases and endoglucanases, that hydrolyse the cellulose backbone into oligomers (Figure 1.4) and glucose (Wu et al., 2018). β-glucosidases and cellobiases act mainly on cellobiose (an oligosaccharide consisting of two repeating units of β-glucose), cellobiohydrolases act on dense crystalline structures within cellulose and endoglucanases to cleave the unorganised amorphous regions of cellulose. Both 1,3 and 1,4- β-D-glucans are prevalent in animal feeds, especially those containing oats and barley and are composed mostly of cellotriosyl and cellotetraosyl residues (oligosaccharides consisting of three and four repeating

units of β-glucose, respectively), linked by single β-1,3-glycosidic bonds (Kamide, 2005).

1.8.2 β-glucanases

There are numerous classes of glucanase available to cleave β-glucan bonds sufficiently (Erfle et al., 1988). Numerous enzymes work synergistically to catalyse the depolymerisation of β-glucan, although the total hydrolysis of β-glucan is not required, or even desired to elicit beneficial effects in monogastrics. Endo-1,4 β-glucanases (laminarinase) cleave the (1 > 4) β-glucosidic links in cereal β-D-glucans and cellulose (Planas, 2000). 1,3(4)-β-glucanases (lichenase) hydrolyse (1>3) and (1>4)-linkages in β-D-glucans if the glucose's reducing group involved in the linkage is substituted at the carbon-3 location (Bairoch, 2000).

1.8.3 β –Mannanases

There has been particular interest in β –mannanase in feeds containing high soybean concentrations, where it has been shown that β –Mannanase increases the growth and feed efficiency of broilers (Jackson et al., 2004). The mechanism of action from β –mannanase is complex and there seems to be a relationship with insulin. β-galactomannan is known to inhibit the insulin secretion of pigs (Sambrook and Rainbird, 1985). Therefore, the addition of β –Mannanase to feeds may remove the insulin suppressive effect of β-mannans, allowing better utilisation of glucose within the animal.

1.9 Xylanases

Xylanases are important fibrolytic enzymes, frequently added exogenously to animal feeds. They also have a plethora of other uses, including waste management, renewable fuels and production of paper (Motta et al., 2013).

Commercial feed xylanases are utilised from both fungal and bacterial sources (Bastawde, 1992). There are an abundance of diverse xylanases due to the heterogeneity and complexity of xylan, which have all been classified into over 77 families (Henrissat and Coutinho, 2001) which mean we often rely on a mixture of xylanases in any enzyme product. Xylanases within a particular family have a similar structure, molecular mechanism and they may have a similar specificity of action on small, soluble, synthetic substrates (Collins et al., 2005). Xylanases used in animal feeds are often produced by *Trichoderma reesei* organisms belonging to xylanase families 10 and 11 (Tenkanen et al., 1992).

Xylanase, or more specifically, endo-1,4- β -xylanase acts by randomly cleaving xylan’s backbone (Polizeli et al., 2005). This results in a number of branched or unsubstituted xylooligosaccharides (XOS) being released as xylose or arabinose residues (Biely et al., 1997). It is not essential for the full hydrolysis of xylan to be achieved in the context of animal feeds, partial hydrolysis is enough to alleviate the problems with digesta viscosity and to initiate the dissolution of cell wall barriers and production of XOS (Bastawde, 1992).

To achieve full hydrolysis, a collaborative effort of numerous hemicelluloses is required to break down the complex xylan structure (Coughlan and

Hazlewood, 1993). 1,4- β –D xylosidase is the enzyme responsible for cleaving the XOS (xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose) into individual monomers of xylose (Coughlan and Hazlewood, 1993), so must be limited, but not completely excluded, when trying to produce XOS as an end product to examine their effects. The other essential enzymes for complete hydrolysis of arabinoxylan are α-L-arabinofuranosidase, feruloylesterase, acetylxylan esterase and α-D glucuronidase. There are 3 primary modes of action suggested for fibrolytic enzymes in non-ruminant feeds:

1. Viscosity reduction
2. Cell wall dissolution
3. Prebiotic mechanism

Table 1. 1 Studies that detailed the age, cereal, level of enzyme inclusion and whether or not feed conversion ratio (FCR) was improved in broilers under the dietary conditions outlined.

Study	Treatment (d)	Main cereal used in feed	Level of enzyme inclusion	FCR Improved?
(Selle et al., 2009)	1 - 21	Wheat	Xylanase (XU/kg): 0, 2000, phytase (FTU kg): 0, 500	Yes
(Cowieson et al., 2010)	1 - 42	Maize	Xylanase (BXU/kg): 0, 16 000, β-glucanase (BU/kg): 0, 30 000	Yes
(González-Ortiz et al., 2019)	0 - 42	Wheat	Xylanase (BXU/kg): 0, 16 000	Yes
(Ravn et al., 2018)	1 - 29	Maize	Xylanase, α-l-arabinofuranosidase: EP/kg 0,5,10	Yes
(Bedford and Classen, 1992)	1 - 19	Rye	Xylanase 2150U, β-glucanase 530U. g/kg: 0, 1, 2, 4, 8, 16	Yes
(Craig et al., 2019a)	0 - 21	Wheat + Barley	Xylanase (XU kg): 0,16 000,32 000, β-glucanase (BU/kg): 0, 16 000, 32 000	No
(Munyaka et al., 2015)	0 - 21	Maize + Wheat	Xylanase (XU/kg): 0, 2500 β-glucanase (BU/kg): 0, 250	Yes
(Zhang et al., 2014)	7 - 21	Wheat	Xylanase (g/kg): 0, 1	Yes
(Masey-O'Neill et al., 2014)	1 - 49	Maize + Wheat	Xylanase (XU kg): 0,16 000,32 000	Yes
(Craig et al., 2019b)	0 - 28	Wheat	Xylanase (XU kg): 0,16 000,32 000, XOS (g/kg): 0, 0.25, 1	No
(Singh et al., 2012)	0 - 42	Maize	Xylanase (XU kg): 0,16 000	No

XU, Xylanase Units, **BXU**, β-Xylanase Units, **FTU**, Phytase Units, **BU**, Beta-Units, **EP**, Enzyme Protein.

1.9.1 Viscosity Reduction

It is now well established that NSP can increase the viscosity of aqueous solutions, as with digesta (Choct and Annison, 1992). NSP's are most abundant in barley, wheat and rye cereal grains (Bedford and Classen, 1992, Choct et al., 1999), where an increase in digesta viscosity has been observed and repeated in numerous studies, as a result of the NSP fraction that is present in cereal grains. An increase in viscosity through soluble NSP ingestion also thickens the mucous layer lining the digestive tract, possibly to protect the lining of the digestive tract, which acts as a further physiochemical barrier to nutrient absorption in the gut, preventing the mixing of endogenous and exogenous enzymes reaching their target sites, decreasing feed intake and body weight gain (Hedemann et al., 2009). It has been estimated that around 400g of digestible energy per 1kg of corn-soyabean meal feed could remain unutilised as a result of the anti-nutritive effects of NSP (Cowieson, 2010).

When enzymes are provided to target NSP, degradation of this fraction leads to growth rate and efficiency increases in non-ruminants as a direct result of decreased digesta viscosity, via improvements in nutrient digestibility (Simon, 1998) – the more viscous the digesta is, the less mixing and subsequent absorption of nutrients occurs, and vice versa. Reduced viscosity through enzyme action then, can increase feed conversion ratio (FCR) (Masey-O'Neill et al., 2014) and apparent metabolisable energy (AME) of the feed (Masey O'Neill et al., 2012).

Not all cereals have high soluble NSP contents. Maize is an example of such a cereal grain (Cowieson, 2005). There have been numerous studies showing the

benefits of fibrolytic enzymes in maize based feeds (Masey O'Neill et al., 2012, Wyatt et al., 1999). This suggests that there are more mechanisms at play than simply the reduction of digesta viscosity

1.9.2 Cell wall dissolution

Plant cell walls are the physical barrier between nutrients encapsulated within cereal grains and the digestive tract. Non-ruminants do not possess the necessary endogenous enzymes to sufficiently break down this barrier, meaning the starch, protein and other important nutrients within the cell are protected from digestion. This is therefore known as the 'cage effect' (Bedford and Partridge, 2011).

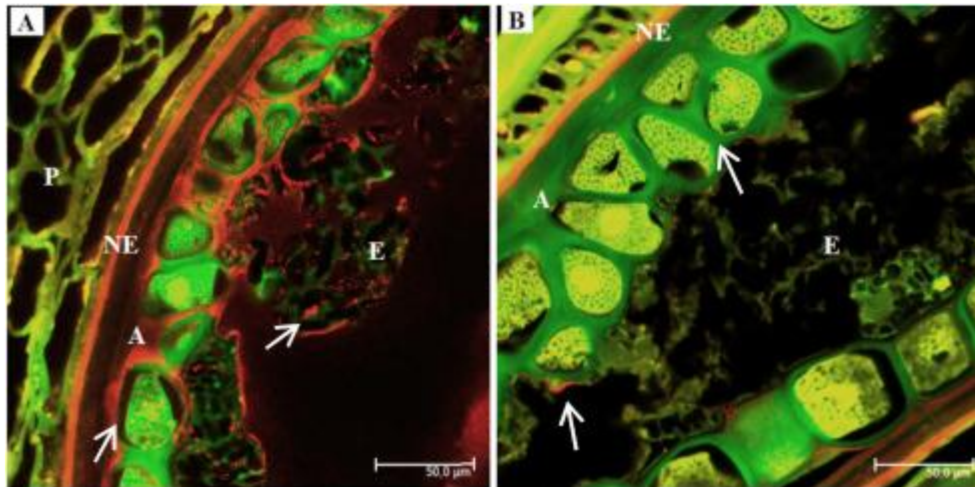

Figure 1. 10 The effect of xylanase on solubilisation of wheat bran AX, shown by confocal microscopy and indirect immunofluorescence. Image A) Control without xylanase. Image B) 10mg/kg xylanase treatment. Taken from Ravn et al. (2017).

If exogenous enzymes added to feeds have the capacity to puncture plant cell walls and release the nutrients trapped within this physical barrier, nutrient digestibility can increase, and animal performance along with it (Bedford, 2000). Bedford and Autio (1996) presented evidence via microscopy analysis of digesta, that cell walls were present and intact without enzyme addition, but

were disrupted at the proximal small intestine after fibrolytic enzymes were added to the feed. A further study in 2017 by Ravn et al, showed the destruction of arabinoxylans in wheat bran with 10mg/kg xylanase supplementation (Figure 1.10), where the red colour seen in A indicated binding of an antibody to arabinoxylan, with image B showing the solubilisation of this arabinoxylan after xylanase supplementation. This shows how NSP hydrolysis by fibrolytic enzymes also affects partially insoluble NSP that are contained within plant cell walls. These enzymes can lead to growth performance increases, highlighted in a study by Petterson and Åman (1989). In this particular study, xylanase supplementation for 15 days increased broiler bodyweight by 27% and feed conversion efficiency by 10% compared to controls who received no xylanase supplementation during the time course.

1.9.3 Prebiotic mechanism

Low NSP cereals such as maize are currently a focal point of research. Low NSP cereals do not increase digesta viscosity, so do not decrease performance in the theories previously mentioned, yet it has been shown that xylanase supplementation in low NSP cereals also results in performance increase. This suggests a different mechanism is occurring.

The presence of NSP within non-ruminant feeds can also induce gastrointestinal stressors which can result in wet litter which reduce growth performance, health and cost efficiency in farming (Teitelbaum and Walker, 2002). Soluble NSP can change the ecology of the gut from a more aerobic environment, to a more anaerobic one, through a decrease in oxygen tension within the small intestine (Simon et al., 2015). This is detrimental to

performance as anaerobic microflora will be developed leading to the production of pathologic components and toxins in the small intestine (Simon, 1998, Langhout, 2000). This can lead to decreased immune function and destruction of gut epithelial cells that leads to inflammatory bowel conditions and mucosal damage (Teirlynck et al., 2009). The use of xylanase and other fibrolytic enzymes can alleviate this cascade of events in the small intestine by hydrolysis of soluble NSP, ending in a modulation of the gut microbiome (Munyaka et al., 2015).

Degradation of NSP increases the generation of both oligo- and mono-saccharides present in the caeca (Cadogan and Choct, 2015). As XOS are indigestible by the endogenous enzymes in the broiler gut, they reach the hindgut where they are selectively and readily fermented by favourable bacteria in gut microbiome (Geraylou et al., 2013). The increase in fermentation in both the caeca and the colon seen with enzyme use (Marounek et al., 1999, Sanchez et al., 2009) has the potential to improve the overall digestibility of the feed. The fermentation of XOS in the caeca produces VFA that are known to lead to a healthier gut microflora (Jia et al., 2009). The main VFA produced are acetate, propionate and butyrate. These VFA lower intestinal pH, subsequently inhibiting the growth of potentially harmful bacterial populations. (Teitelbaum and Walker, 2002). They have specifically been shown to support the growth of microflora such as *Bifidobacterium* and *Lactobacillus* spp., whilst simultaneously suppressing the growth of pathogenic bacteria such as *Campylobacter, Clostridium, Salmonella,* and *E. coli* (Thammarutwasik *et al.,* 2009).

It is important to note that trials to date have used low levels (as low as 0.1g/kg) of inclusion of XOS, while still incurring benefits to broilers on wheat-based feeds (Ribeiro et al., 2018), so the benefits seen are not just the direct result of XOS providing a new energy substrate in the form of the VFA's produced after their fermentation, there are more complex mechanisms involved. The mode of action seems inherently linked to the XOS, though, as there seem to be relatively similar growth performance outcomes in trials that have either had isolated XOS incorporated into feed, or the fibrolytic enzymes themselves incorporated (Courtin et al., 2008).

The core ideas behind this prebiotic mechanism are that exogenous fibrolytic enzymes are producing XOS during hydrolysis of xylan, which are then utilised by favourable bacteria in the hindgut microbiome, to help them proliferate. This will presumably have downstream effects on the animal which ultimately lead to increased energy recovered from the feed and thus, greater feed efficiency.

1.10 How enzymes produce xylooligosaccharides

XOS are generated as a result of the breakdown of glycosidic linkages in the xylan chain. This can be achieved through subjecting xylan to high temperature, chemical reagents or enzymes (Aachary and Prapulla, 2011). For enzymatic hydrolysis to occur, there must be direct contact of substrate with enzyme, therefore the characteristics of the substrate itself will affect the interactions made with any enzyme used.

Endo-β-1,4-xylanase hydrolyse the xylan backbone, producing XOS with low degrees of polymerisation (Singh et al., 2015). It is important to limit β-

xylosidase activity, as this enzyme can further cleave XOS into monomeric xylose, which has been shown to be detrimental to feed efficiency (Schutte et al., 1992). This is difficult as animal feeds often contain endogenous β-xylosidases, which will interact with any exogenous enzymes supplemented (Cleemput et al., 1997).

It is difficult to control the degrees of polymerisation (DP) in any XOS produced during enzymatic hydrolysis (Akpinar et al., 2010) as xylan as a molecule has wide ranging variance in its bonds and types of branching and these are sometimes physical barriers to enzyme hydrolysis (Vangsøe et al., 2019). For example, xylanases from the GH10 family will produce more low DP XOS than those of the GH11 family, as GH10 xylanases have smaller active sites, preferring to cleave chains close to the substituent, as opposed to the GH11 xylanases which have large active sites and prefer to cleave main chains in unsubstituted regions (Vardakou et al., 2004).

1.11 XOS as a prebiotic

A prebiotic is an ingredient that is selectively fermented, resulting in specific changes to the composition and/or activity of gastrointestinal microbiota which then confers benefits to host health (Gibson et al., 2010). XOS have been studied since the 1990's.

XOS are selective in the microorganisms they proliferate. They increase levels of both *Bifidobacteria* spp. and *Lactobacillus* spp. which are major bacteria in human, poultry and pigs (Singh et al., 2015). The resulting shift in microbiota of the digestive tract has been associated with overall improved health, resistance to gut infections, better mineral absorption and reduced colonic

cancer rates (Wong et al., 2006). By increasing these beneficial bacteria, pathogenic bacteria and products of their fermentation are subsequently decreased (Samanta et al., 2015), which may all contribute to greater health and ultimately, growth of livestock.

1.12 Summary

There is an increasing demand for animal products globally, as a result of population increase and globalisation. The increase in supply must be met while decreasing the amount of land used for livestock production. Xylan is one of the most abundant plant cell wall polysaccharides (Diogo et al., 2015). It can be hydrolysed with exogenous fibrolytic enzymes into simple sugar monomers and oligomers (Yang et al., 2011). The production of enzyme cocktails to simultaneously degrade NSP whilst potentially producing as much XOS as possible could be advantageous to the animal feed industry, to reduce costs and improve animal performance. It is therefore vital to gain a deeper understanding of fibrolytic enzymes and their mode of action and specificity for different substrates, to develop bespoke products for the livestock feed industry.

1.13 Hypothesis, aims and objectives

This thesis aims to:

1. Evaluate the effects of different exogenous fibrolytic enzymes, firstly on different types of cereal, to characterise enzyme-cereal combinations which lead to optimal NSP degradation and the release of simple sugars and xylo-oligosaccharides *in vitro*.

2. Then, to evaluate if there are inter-variety differences within a cereal type in response to exogenous fibrolytic enzymes *in vitro* on the NSP degradation and release of simple sugars and xylo-oligosaccharides.
3. Finally, to investigate the role of xylo-oligosaccharides monosaccharides released by fibrolytic enzyme hydrolysis on the feed efficiency of broilers *in vivo*, by assessing the release of monosaccharides and XOS within different sections of the broiler gastrointestinal tract, to increase the understanding of xylo-oligosaccharides and their potential uses in feed efficiency.

The hypothesis for this body of work is that there will be identifiable cereal substrate-enzyme combinations that release significantly more monosaccharides and XOS through increased NSP degradation compared to other combinations *in vitro.* It is also hypothesised that there will be significant differences in the release of NSP degradation products *in vitro,* depending on the specific cereal variety, based upon structural and morphological qualities. Finally, it is hypothesised that during the *in vivo* study, broilers supplemented with a xylanase-based enzyme will increase their feed efficiency significantly during the study time-course and show greater xylooligosaccharide release in the GIT compared to control animals.

2. METHODS

2.1 Methodology of in vitro digests

Monosaccharide composition of cereals following enzymatic hydrolysis was carried out using an adaptation of a method described by Waldron (2017) and determined using High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection (HPAEC-PAD). Sample dilution factors were modified for the cereal substrates used in the present thesis, with 1:10, 1:50, 1:100, 1:200 all being assessed for the clearest peak separation of the monosaccharides assessed. 1:100 was subsequently used for the experiments that followed. XOS composition were determined using High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection (HPAEC-PAD) following the method described Xu et al (2013). The method was tested prior to experiments carried out in the following chapters to check that it performed well with the substrates and enzymes that will be used throughout the body of work.

Weighed out 200mg (+-1%) of cereals in three batches on three different days inside individual 50ml Falcon tubes (Falcon™ 50mL Conical Centrifuge Tubes, Thermo Fisher Scientific) and labelled them accordingly. One replicate of each substrate was labelled as a control sample without enzyme addition, and the other three labelled #1, #2 or #3 for batch number. After samples were weighed out, a 50 mM sodium citrate buffer was made. In the first experiment, there were 4 cereal combinations x 4 enzyme preparations subjected to enzyme hydrolysis with total 7 time points, carried out on different days for a total of three technical replicates for each combination, resulting in a total of 336 data

points. The second experiment used 6 cereal combinations x 4 enzyme preparations with 6 time points, repeated in three total batches on different days, resulting in a total of 432 data points.

First, a 1M sodium citrate stock was created using 294.1g sodium citrate monohydrate (Sigma Aldrich, CAS Number: 6132-04-3) made up to 1 Litre in an autoclaved Volumetric flask using Milli-Q water (Millipore, Bedford, MA, USA) in a volumetric flask, which was then shook and mixed well into solution using a Cole-Palmer magnetic stirrer (Thermo Fisher Scientific),. From the 1M sodium citrate solution, 25ml was taken using a glass bulb pipette (Fisher Scientific) into a previously autoclaved 500ml volumetric flask and mixed using a Cole-Palmer magnetic stirrer (Thermo Fisher Scientific), to create a 50mM sodium citrate solution.

This solution was then pH adjusted to 5.2 to represent a similar pH to that found in the small intestine, where most enzyme activity is thought to occur, as previously suggested by Bedford and Partridge (2011). The pH of the broiler GI tract has been observed as low as 1.6 in the gizzard and as high as 7.9 in the intestines, so this pH was comfortably within this range, though, there is an inherent variance of pH level in the broiler digestive tract. Addition of sodium azide (Thermo Fisher Scientific, CAS Number: 26628-22-8) at 0.05% to buffers was also used to effectively abolish the bacteria utilising the sugars in question for use as an energy substrate. This was a problem during initial experiments as it was observed that the monosaccharide levels during hydrolysis would increase up until the 24h mark before subsequently beginning to decline after this point, particularly noticeable with glucose. The addition of sodium azide was tested at 0.01%, 0.03% and 0.05% concentration

within the buffer and it was found that 0.05% was effective at preventing the observed increase followed by a decrease in monosaccharide levels following enzymatic hydrolysis *in vitro.* A stock enzyme solution was made fresh for each experiment using a xylanase enzyme supplied by AB Vista (Marlborough, UK). The enzyme preparation varied dependent on experiment. Enzyme solutions were added to the buffer at the recommended doses of the manufacturers. Although initial experiments were carried out with the enzymes to assess the monosaccharide release following enzyme inclusion at 1mg/kg, 10mg/kg, 100mg/kg and 1g/kg, the manufacturers dosage of ~100mg/kg was deemed to be the most applicable to real life scenarios.

After creating the buffer and enzyme solution, 40ml of buffer was added to each control sample, which were then shaken and vortexed using a variable speed SA8 Mixer (Thermo Fisher Scientific) for adequate mixing. Quickly after this, stock enzyme solution was added to the remaining sodium citrate buffer, this 500ml buffer now contained the appropriate concentration of enzyme per sample. 40ml of buffer with enzyme was added to each sample, except for controls, to provide the working activity of enzyme solution per 200 mg substrate in each sample. 0 hour sampling was then undertaken with each of the feed substrates. This was done by pipetting 1ml of each sample into a separate 15ml falcon tube containing 9ml of 10mM NaOH, which was then vortexed and frozen at -20°C, this created a 1:10 dilution factor, ready for sample for analysis on the HPAEC-PAD (dilutions are accounted for in later calculations).

After 0hr sampling, the samples contained within the 50ml falcon tubes were sealed taped to racks and placed in a shaking, rolling incubator at 150rpm. The

temperature used was 40.7°C, this was used because the internal body temperature of hatchlings has been observed at 39.7°C, which rises to 41.7°C in maturity (Aluwong et al., 2017), giving a suitable average of 40.7°C. Most enzymes have optimum activity at 45 – 65°C (Vahjen and Simon, 1999), but the changes in enzyme activity are very small when temperature is increased from 40°C to 50°C (Wu et al., 2018), thus temperature does not seem to be a crucial consideration in poultry.

Sampling was repeated in this manner at all of the relevant timepoints for the experiments undertaken. After all sampling was complete, all samples were removed from the freezer and thawed in a bath of warm water.

During the thawing period, fresh monosaccharide standards were made up at 2g/L, 1g/L, 0.5g/L and 0.25g/L for Arabinose, Galactose, Glucose and Xylose using serial dilutions and also for the pure XOS, X6-X2. Due to the peak intensity on the chromatogram, standards were diluted 1:100 with 10mM NaOH for analysis, and then accounted for in calculations later within excel.

2ml Amber Vials were labelled and prepared before vortexing each sample in their falcon tube and centrifuging at 5,000 RPM for 10 minutes. 1ml of each sample was then pipetted (Gilson, P1000) into their corresponding amber vial and sealed with a PTFE screw capped lid (PTFE). Mobile phase level was checked on the HPAEC-PAD, prior every run, as well as ensuring the waste bin was emptied. The run was then initialised and started on the HPAEC-PAD.

Results were processed in excel, where standard curves for the four monosaccharides and all of the XOS were created, utilising their Y value to determine the amounts of the corresponding sugar in each sample, represented

as % of total biomass of the substrate. Standards were placed at the beginning and end of each HPAEC-PAD run to help account for the inter-assay coefficients of variability (CV) and samples were repeated in triplicate to help account for intra-assay variation of CV.

2.2 Carbohydrate analysis via HPAEC-PAD

There has been a vast number of techniques utilised over the years to quantify the carbohydrates present in different foods. Chemical methods such as titration, gravimetric analysis and calorimetric analysis can be used to determine both monosaccharide and oligosaccharides, taking advantage of the fact that carbohydrates are often reducing agents that react with other compounds to produce different coloured complexes which can be quantified, alongside known standards (Biermann and McGinnis, 1988). Chromatographic techniques are currently the most popular. Gas chromatography can be used, but this requires volatile samples, so derivatization is often necessary during sample preparation (Churms, 2017). Liquid chromatography, however, makes it possible for the rapid, precise and sensitive measurements of carbohydrates to be taken.

High-performance anion exchange chromatography (HPAEC) is a technique that has been developed to separate carbohydrates, and in the past 30 years has become the routine tool used for quantification of oligomers (Cataldi et al., 2000). It uses pulsed amperometric design (PAD) to quantify nonderivatized carbohydrates with minimal sample preparation and clean up needed. Carbohydrates are weakly acidic; therefore, it is possible to separate them with

high selectivity under high pH conditions using a stationary phase composing anion-exchange.

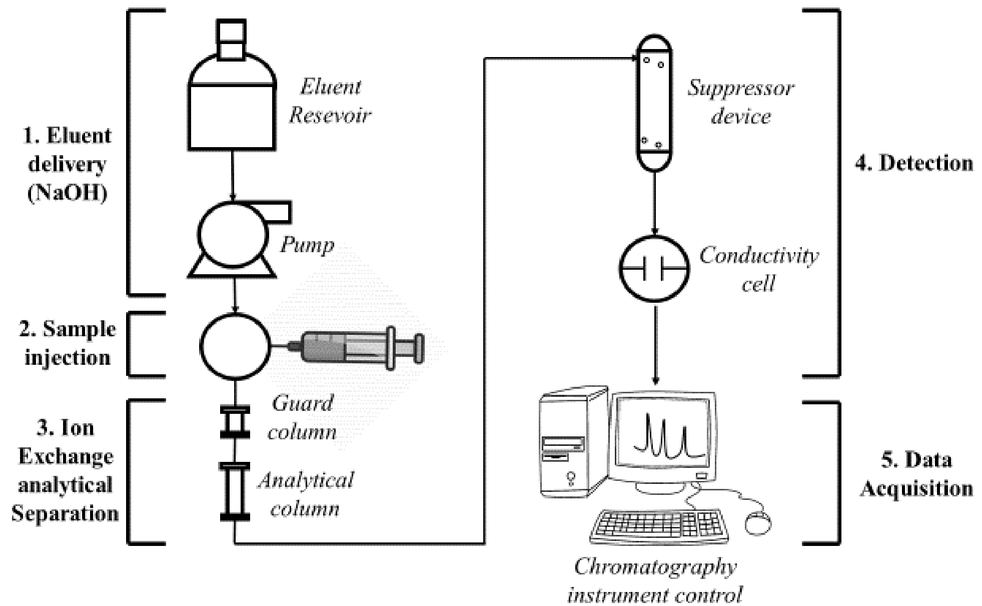

Figure 2. 1 A diagram showing the configuration of an anion exchange chromatography system, adapted from Xu et al., (2013).

The specificity of HPAE-PAD compared to other chromatography techniques is a result of how only the carbohydrates with functional groups oxidizable at the detection voltage utilised can be detected via PAD, and that neutral and cationic sample components present in the matrices do not interfere with analysis. This allows for high specificity and sensitivity in detection of oligomers and the highest resolution in XOS separation.

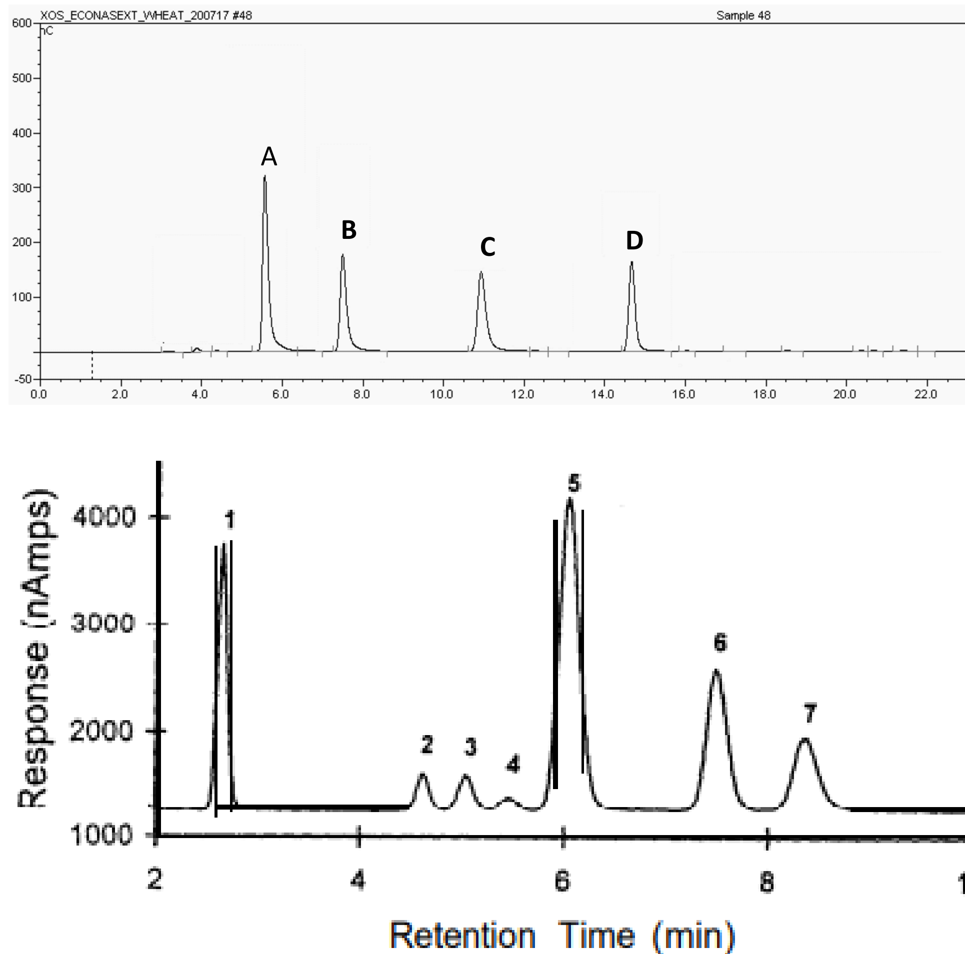

Figure 2. 2 A representative trace of four xylooligosaccharides commonly found resulting from hydrolysis of non-starch polysaccharides (top). A, Xylobiose, B, Xylotriose, C, Xylotetraose, D, Xylopentaose and a representative trace of seven monosaccharides commonly found in non-starch polysaccharides (bottom). Peaks 1-7 show, in order: Fucose, arabinose, galactose, rhamnose, glucose, xylose, and mannose. Xylooligosaccharide trace taken from Chapter 3. Monosaccharide trace taken from Davis (1998).

Carbohydrates are detected through measurement of the electrical current that is generated when they are oxidised at the surface of a gold electrode. Analysis is run in alkaline conditions to transform oligomers' hydroxyl groups into oxyanions, which are then captured in the HPAE column ready to be eluted via pushing ions, usually in the form of acetate. Analyte retention within the column is affected by degree of polymerisation, which is how many monomeric units an oligomer is comprised of, chemical structure, quantity of hydroxyl groups and molecular size.

Monosaccharide and XOS composition were therefore determined using High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection (HPAEC-PAD) following the methods described by Waldron (Waldron, 2017) for monosaccharides and Xu et al (2013) for XOS.

3. THE EFFECTS OF EXOGENOUS FIBROLYTIC ENZYMES ON THE *IN VITRO* GENERATION OF XYLOOLIGOSACCHARIDES AND MONOSACCHARIDES IS DEPENDANT UPON CEREAL TYPE

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3.1 Abstract

Fibrolytic enzymes are routinely added to non-ruminant livestock feeds to help degrade the non-starch polysaccharide (**NSP**) contents and thereby improve feed efficiency. This study investigated the range of xylooligosaccharides (**XOS**) and monosaccharides produced from four cereal samples (barley, maize, oats and wheat) over a 72 hour *in vitro* incubation using 3 commercially (ABVista) available fibrolytic enzymes Econase XT, Econase MP1000 and Barley P700, all containing endo-xylanase with other combinations of enzymes. Complete non-starch acid hydrolysis showed there were differences between cereals in the total monosaccharide quantity ($P<0.01$). There was a cereal x enzyme x incubation time 3-way interaction in the generation of XOS (xylotetraose, xylotriose and xylobiose) ($P<0.01$) indicating generation of XOS varies dependent on both the cereal and the enzyme used. The enzymes failed to generate any detectable xylose from maize. There was a significant 3-way interaction for xylose ($P<0.05$). Econase XT generated the greatest quantity of xylose, with 38% of available xylose from wheat being released after 72 h, 11% from barley and 9% from oats. For arabinose and galactose production there was a cereal x enzyme x incubation time 3-way interaction ($P<0.01$), whilst glucose release was only significantly affected by cereal ($P<0.05$) or time ($P<0.05$). These findings suggest that the fibrolytic enzymes tested have some specificity for certain cereals and therefore it might be possible to optimise the enzyme-cereal combinations used in animal feeds, to help maximise the feed efficiency of livestock.

Key words: Cereal. Xylanase. Xylooligosaccharide. Prebiotic. Feed.

Abbreviations: NSP, non-starch polysaccharide; XOS, xylooligosaccharide; AX, Arabinoxylan; SNSP, water soluble non starch polysaccharide; AXOS, Arabinoxylan oligosaccharides; TFA, trifluroacetic acid; ×g, times gravity; BXU/g, beta-xylanase units per gram; MNU/g, mannanase units per gram; BU/g, beta-units per gram, ECU/g**,** endo-cellulase units per gram; HPAEC-PAD**,** high-performance anion-exchange chromatography with pulsed amperometric design.

3.2 Introduction

There is an increasing literature on the use of non-starch polysaccharide (**NSP**) degrading enzymes in non-ruminant diets to improve feed efficiency in pigs and poultry (Classen et al., 1985, Wyatt et al., 1997, Bedford and Morgan, 2007). Cereals are one of the main feed ingredients used in non-ruminant diets, particularly wheat, maize, oats and barley. Their NSP content can vary widely between and within (varietal) cereals (Knudsen, 1997). Arabinoxylans (**AX**) are one of the major plant cell wall polysaccharides (Bastawde, 1992, Theander et al., 1993), with the major components being the pentose sugars, arabinose and xylose (Choct, 1997). The hydrolysis of AX requires β-1,4 endoxylanases to cleave linkages of the xylan backbone into polymeric and oligomeric XOS. Other enzymes such as arabino-furanosidases, cleave the substituent sugars, clearing the xylan backbone for greater access by the endo-xylanases.

High molecular weight polysaccharides such as some AX are anti-nutritive, as their high water-soluble fractions increase viscosity in the digestive tract and form a barrier between the substrate and enzyme, impairing digestibility (Misir and Marquardt, 1978, Bedford and Classen, 1992). More viscous feed ingredients such as barley, wheat and oats have larger concentrations of water soluble non starch polysaccharide (**SNSP**) and are therefore thought to respond well to carbohydrase supplementation. Indeed, xylanase supplementation of wheat-based diets released arabinose and xylose in the ileum, jejunum and duodenum of broiler chickens (Zhang et al., 2014), likely due to the hydrolysis of AX. Addition of xylanase has been shown to depolymerise high molecular weight arabinoxylans and thus reduce digesta viscosity. This results in

improved nutrient digestion, absorption and thus, animal performance (Zhang et al., 2014).

Maize is the most common feed ingredient in global poultry production, but the potential for xylanase enzymes to positively influence maize based feeds may be limited. This is because maize is considered highly digestible (Kocher et al., 2003) as there is 50% less total NSP in maize compared to wheat (Chesson, 1993), most of which is not soluble therefore not a viscosity risk. Despite this, several studies have reported benefits of incorporating xylanases and carbohydrase mixtures in maize based diets (Cowieson and Ravindran, 2008, Cowieson et al., 2010).

Interestingly, Bedford and Cowieson (2012) showed that the effects of xylanase on oligosaccharide size and quantity could differ if maize or wheat was used as the main substrate, given their different resistances to complete hydrolysis. In addition to viscosity considerations, the generation of fermentable oligosaccharides, including XOS, could be a key mechanism for the improved feed efficiency seen with enzyme supplementation (De Maesschalck et al., 2015). In agreement with this, arabinoxylan oligosaccharides (**AXOS**) derived from wheat-bran were shown to significantly improve feed efficiency of both wheat and maize-based diets (Courtin et al., 2008). Decreased feed intake and increased bodyweight gain were observed, resulting in improved feed efficiency.

These findings suggest a need to characterise the differential effects of exogenous enzymes on common cereal feed ingredients. Therefore this *in vitro* study determined the profiles of XOS, and monosaccharides released from 4

cereals (barley, maize, oats and wheat) when using 3 different commercial enzyme preparations over a 72h time course. A 72h time course was chosen because when dietary markers are ingested in poultry diets, most of the marker is excreted by 12h (Tuckey et al., 1958), but can still be detected up to 72h after feeding due to feed contents entering the caecum, which may then influence enzyme activity in this area of the digestive tract (Duke et al., 1968). More recent studies, however, have shown that the average retention time in the digestive tract of broilers (excluding the caecum) is 4 – 8 hours (Hetland and Svihus, 2001).

3.3 Materials and Methods

3.3.1 Total hydrolysis of non-cellulosic polysaccharides

Total sugar contents were determined in the 4 cereals (Barley, Maize, Oats and Wheat) by total hydrolysis of their non-cellulosic polysaccharides with Trifluroacetic acid (**TFA**), as previously described by Fry (1988). All cereals were obtained from a commercial feed manufacturer (Target Feeds). The release of 4 sugars (arabinose, galactose, glucose and xylose) was then quantified after acid hydrolysis.

For the enzyme hydrolysis experiment, the 4 cereals (barley, maize, oats and wheat) were ground to a fine powder (0.5mm) and suspended to 10mg/ml in 2M TFA as three technical replicates. Tubes were sealed and heated to 120°C for 1 hr in an autoclave, then allowed to cool to room temperature before being centrifuged at 2236×g for 10 min at room temperature. The supernatant was then diluted 1:100 with 10mM NaOH and transferred into 2ml clear vials for sugar analysis.

3.3.2 In vitro digestion of cereals with 3 different enzymes.

The following 3 commercial enzyme preparations were provided by AB Vista (Marlborough, UK) and used in digestions with the 4 cereals:

i. Econase XT – a xylanase with β-1,4 endo-xylanase activity (160,000 BXU/g). The enzyme was used at its suggested dose of 100g/tonne of feed (100µg/g cereal).

ii. Econase MP 1000 – a mannanase enzyme cocktail reported to contain mannanase (1,000,000 MNU/g), β-glucanase (300,000 BU/g) and β-1,4

endo-xylanase (200,000 BXU/g) activities. It was used at the recommended dose of 100g/tonne of feed (100µg/g cereal).

iii. Econase Barley P 700 - an enzyme cocktail prepared from a strain of *Trichoderma reesei*, designed for use in barley feeds. Its main activity is β-glucanase (700,000 BU/g), but it also contains endo-cellulase (165,000 ECU/g) and endo-xylanase (190,000 BXU/g) activities. It was used at the recommended dose of 40g/tonne of feed (40µg/g cereal).

The 4 cereals (barley, maize, oats and wheat) were individually ground to a fine powder (0.5mm), 0.2g of each cereal was suspended (in triplicate for each cereal triplicate) in 40mls 50 mM sodium citrate buffer (pH 5.2) containing one of the following enzymes:

a) Cereal samples only – no enzyme added.
b) Cereal samples + Econase XT at 100µg/g.
c) Cereal samples + Econase MP 1000 at 100µg/g.
d) Cereal samples + Econase Barley P 700 at 40µg/g.

The four enzyme preparations were added separately (in triplicate) along with 40ml of 50 mM sodium citrate buffer (pH 5.2). The buffer used has previously been described as representing the average pH of the broiler digestive tract (Mabelebele et al., 2014). Digestion reactions were then placed in a shaking incubator at 150RPM, with a temperature of 41°C for 72 h, with 1ml samples taken at 0, 6, 12, 24, 36, 48 and 72 h. At each time point, 1ml of each digest sample was removed and added to 9ml of 10 mM NaOH at room temperature, mixed, centrifuged and then frozen at -20°C prior to sugar analysis.

3.3.3 Identification and quantification of sugars using HPAEC-PAD

The sample concentrations of arabinose, galactose, glucose and xylose, as well as the XOS, were determined using High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection (HPAEC-PAD) following the method of Xu et al (Xu et al., 2013). Analysis was carried out using a Dionex ICS-3000 with a Dionex CarboPac PA20 Column (3mm x 150mm) and CarboPac PA20 Guard (3x30mm) for the monosaccharide analysis. A CarboPac PA200 column (3mmx250mm) and CarboPac PA200 guard (3mm x 50mm) were used for the oligosaccharide analysis. An injection volume of 10µl was used throughout for both samples and standard solutions. Monosaccharide standards (arabinose, galactose, glucose and xylose) were purchased from Sigma-Aldrich, UK and XOS standards (xylo-biose, -triose, –tetraose and - pentaose) from Megazyme, Ireland. Serial dilutions for each standard (2.0, 1.0, 0.5 and 0.25g/L for monosaccharides and 0.5, 0.25, 0.125 and 0.0625g/L for XOS) were made fresh for each batch of analyses.

For the monosaccharides, a single eluent, containing 10mM NaOH solution, was used as the mobile phase at 0.5ml/min for 14 min. For oligosaccharides, 2 eluents were used in a gradient for the mobile phase, 0.1M sodium hydroxide (Solution A) and 0.1M NaOH containing 0.5M sodium acetate (solution B) in standard quadruple waveform, as described by Xu et al (2013). The gradient program used for XOS determination was 100% solution A at 0 minutes, rising to 80% solution A and 20% Solution B at 25 minutes, before returning to 100% Solution A after 25 minutes elapsed. Both eluents were stored in plastic pressurised bottles with inert nitrogen gas at 6-9 psi. Data were collected with Dionex Chromeleon software (Version 6.7).

3.3.4 Data and Statistical analysis

One source was used for each cereal and all analyses (digestions and Dionex analysis) were carried out in batches of each enzyme-cereal combination in triplicate and the data processed in Excel (Microsoft, 2013) and expressed as means and standard deviations (SD). Using this level of replication the preliminary experiments which sought to determine monosaccharide concentrations gave an average co-efficient variance for detection of the monosaccharides of 3.3% (+/- 0.2 (standard deviation). Based on this experiment with ANOVA F-statistic of 9.75 it was calculated (G*Power3.1, University of Dusseldorf) that for 4 cereal groups the Power = 1 for total sample size of 8 (n=2 per group). Therefore, for it was deemed that an n=3 per group was sufficient.

Standards for the four monosaccharides or XOS were run at the start and end of each batch, standard curves generated from the areas under the curve, and results are presented as g/100g of cereal. Data was then analysed by one (cereal-only) or three (enzyme x cereal x time) way ANOVA, having accounted for repeated measures, as appropriate, using Genstat statistical software (17th Edition), with blocking for batch (each batch being a different technical replicated of each enzyme x cereal combination with a corresponding control for each enzyme x cereal combination) and digestion tube. A Tukey post-hoc test was used to identify significant differences between cereals following a significant 1-way ANOVA. No post hoc tests were possible for the enzyme digestion analyses since significant 3- or 2-way interactions were observed for all sugars. $P<0.05$ was taken as statistically significant.

3.4 Results

3.4.1 Total hydrolysis of non-cellulosic polysaccharides

The total sugar contents determined by TFA hydrolysis varied from ~52 – ~63g /100g cereal (Table 3.1). As expected, the main monosaccharide present in all 4 cereals was glucose, mainly from starch, and the order of glucose content was the same as that for total sugar content (maize> wheat= oats> barley) ($P<0.001$). It is important to note that the monosaccharide concentrations determined after total acid hydrolysis represent the total reduction of monosaccharide, oligosaccharide and polysaccharides present in the sample except for those from cellulose. As expected, the concentration of xylose, arabinose and galactose was much lower than glucose (Table 3.1).

Table 3. 1 Monosaccharide composition of various cereals after total hydrolysis of non-cellulosic polysaccharides

Cereal	Arabinose[1] (g/100g)	SD	Galactose (g/100g)	SD	Glucose (g/100g)	SD	Xylose (g/100g)	SD	Total (g/100g)	SD
Barley	3.15 [c]	0.08	0.00 [a]	0.00	43.40 [a]	1.53	5.22 [c]	0.17	51.76 [a]	1.78
Oats	1.17 [a]	0.02	1.03 [d]	0.02	49.34 [b]	0.92	3.54 [a]	0.06	55.08 [a]	1.02
Maize	2.63 [b]	0.10	0.64 [c]	0.03	55.58 [c]	1.81	4.46 [b]	0.14	63.31 [c]	2.08
Wheat	3.82 [d]	0.18	0.40 [b]	0.01	50.68 [b]	2.63	3.75 [a]	0.22	58.64[b]	3.05
P-Value[2]	**<.001**		**<.001**		**<.001**		**<.001**		**<.001**	

[1] Values are expressed as mean grams of each monosaccharide per 100g of cereal ± SD, standard deviation for technical replicates.
[2] One way ANOVA indicated significant differences between cereals for each sugar.
[a,b,c,d] Mean values within a column with different superscript letters were significantly different ($P<0.05$, Tukey's post hoc test).

It is assumed that the vast majority of xylose and arabinose were present as arabinoxylan, and as such the sum of the two monosaccharides and arabinose to xylose ratio of the arabinoxylan was determined. Barley had the highest xylose content (barley> maize> wheat= oats) ($P<0.001$), whereas wheat had the highest arabinose content (wheat> barley> maize> oats) ($P<0.001$). Galactose was only present in low amounts in 3 of the 4 cereals, but the content was significantly different ($P<0.001$) (oats> maize> wheat), with no detectable galactose found in barley. These total hydrolysis values for each sugar were subsequently used to calculate the proportion that was released during the *in vitro* digestions with and without the different enzymes, except in the case of galactose from barley digestions, where no comparison could be made as no galactose was detected after total non-cellulosic hydrolysis, but was present after enzyme digestion. This could be due to the fact that galactose is susceptible to acid hydrolysis which would lower the observed amount in the acid digestion but would still be released by the enzyme.

Total arabinoxylan (AX) content was highest in barley (Table 3.2, barley> wheat= maize> oats) ($P<0.001$), whereas the arabinose:xylose (A:X) ratio was highest in wheat (Table 3.2, wheat> barley= maize> oats) ($P<0.001$).

Table 3. 2 Total Arabinoxylan (AX) contents and Arabinose:Xylose (A:X) ratios of various cereals.

Cereal	Total AX[1] (g/100g)	SD	A:X Ratio [2]	SD
Barley	8.36 [c]	0.25	0.60 [b]	0.01
Oats	4.71 [a]	0.08	0.33 [a]	0.01
Maize	7.08 [b]	0.24	0.59 [b]	0.01
Wheat	7.57 [b]	0.40	1.02 [c]	0.01
P-Value[3]	**<.001**		**<.001**	

[1]Total AX values are expressed as mean grams of Arabinoxylan per 100g cereal ± SD, standard deviation for technical replicates.
[2]A:X values represent grams of Arabinose per 100g cereal divided by grams of xylose per 100g cereal ± SD, standard deviation for biological triplicates.
[3] One way ANOVA indicated significant differences between cereals for Total AX and A:X ratio.
[a,b,c] Mean values within a column with different superscript letters were significantly different ($P<0.05$, Tukey's post hoc test).

3.4.2 In vitro digestion of cereals with various enzymes – generation of xylooligosaccharides (XOS)

There were no XOS with 2-6 degrees of polymerisation observed from maize in any of the control incubations nor by any of the enzymes studied (data not shown). There was also no generation of xylopentaose from any of the cereals by any of the enzymes used (data not shown), but there were significant enzyme x cereal x time interactions (all $P<0.01$) for the generation of the other 3 XOS measured (xylotetraose, xylotriose and xylobiose).

For xylotetraose (Figure 3.1), Econase XT had a small effect on barley and wheat; Econase MP1000 had a much bigger effect, but only in barley; while Barley P700 had small effects on barley, oats and wheat (in that order) (enzyme x cereal x time 3-way interaction, $P=0.002$). Interestingly, a rapid initial generation, followed by decline was seen for barley-Econase MP1000, barley-Barley P700 and oats-Barley P700 combinations, suggesting initial generation then potential further digestion (Figure 3.1).

There was virtually no generation of xylotriose for most of the cereal-enzyme combinations observed at the timepoints used in this experiment (Figure 3.2). However, there was a significant 3-way enzyme x cereal x time interaction ($P=0.003$). Only oats incubated with Econase MP1000 resulted in generation of xylotriose above that of the control, with the peak again suggesting an initial generation followed by further digestion (Figure 3.2).

Although there was a significant 3-way enzyme x cereal x time interaction ($P=0.002$), there was very little xylobiose generated for most of the cereal-enzyme combinations (Figure 3.3). Econase XT generated xylobiose from

barley, wheat and oats (in that order), whereas Econase MP1000 had no effect on any of the cereals and Barley P700 only released small amounts from barley and wheat, but not oats (Figure 3.3).

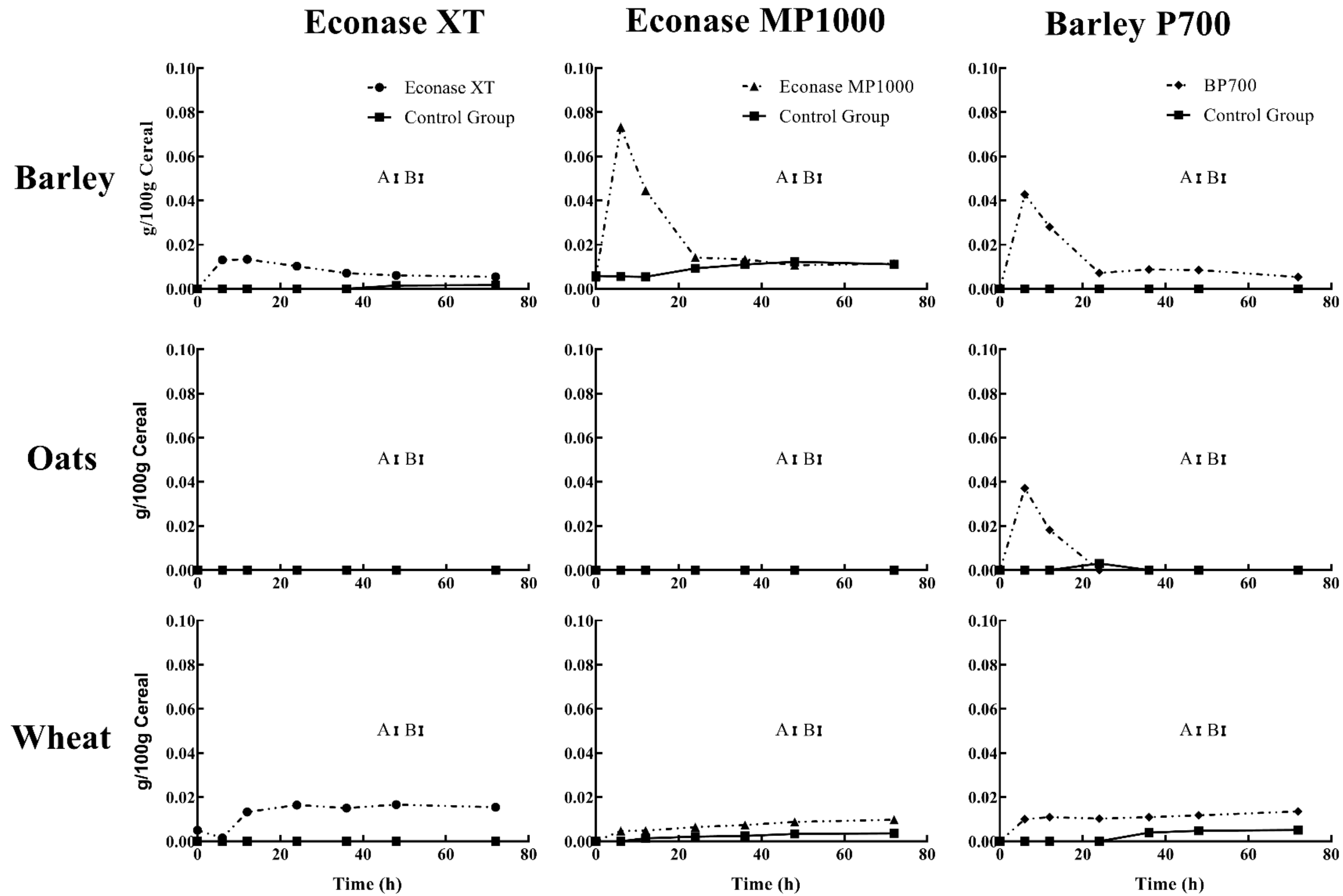

Figure 3. 1 Comparison of the effectiveness of different enzymes on the release of xylotetraose from different cereals. Mean values expressed as total xylotetraose g/100g biomass released during in vitro digestion over 72 h at 41°C. No xylotetraose was detected for maize in any of the control or enzyme-treated digestions. 'A' represents the standard error of the difference of the means (SED) for comparing control and enzyme treated mean values on the same graph. 'B' represents the SED for comparing different times within the same treatment (control or enzyme) on the same graph. Three-way ANOVA indicated a significant enzyme x cereal x time interaction (p=0.002).

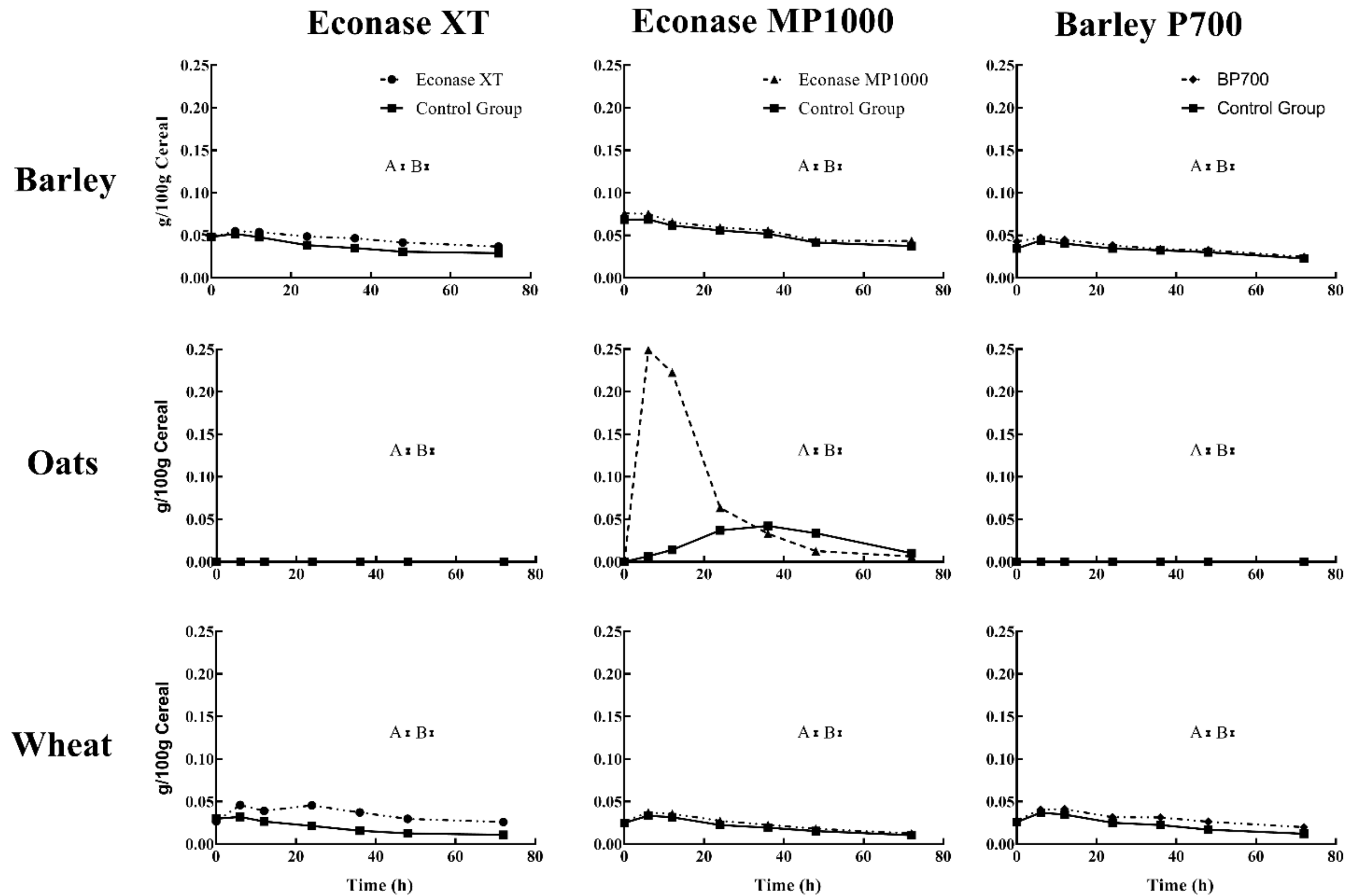

Figure 3. 2 Comparison of the effectiveness of different enzymes on the release of xylotriose from different cereals. Mean values expressed as total xylotriose g/100g biomass released during in vitro digestion over 72 h at 41°C. No xylotriose was detected for maize in any of the control or enzyme-treated digestions. 'A' represents the standard error of the difference of the means (SED) for comparing control and enzyme treated mean values on the same graph. 'B' represents the SED for comparing different times within the same treatment (control or enzyme) on the same graph. Three way ANOVA indicated a significant enzyme x cereal x time interaction (p=0.003).

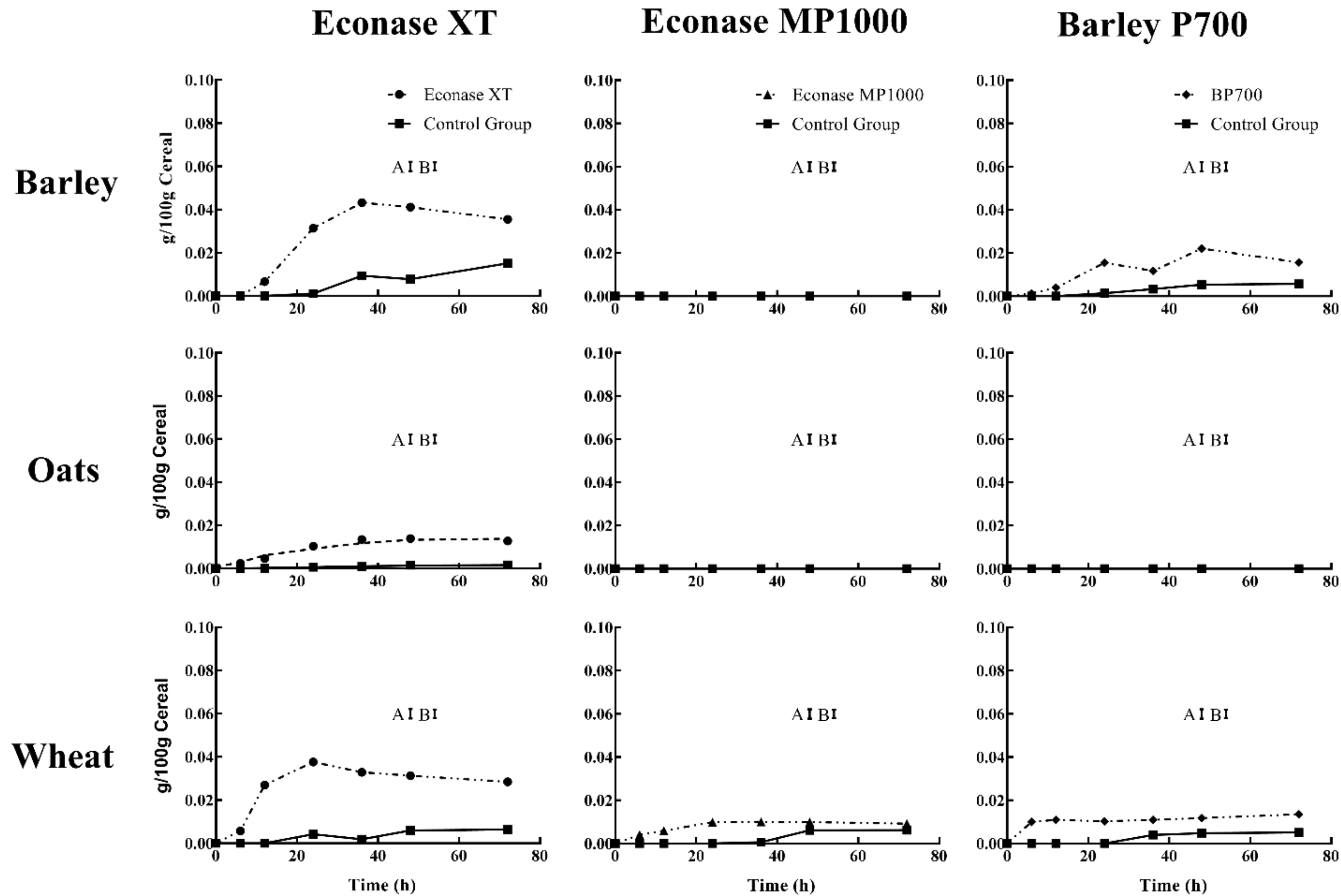

Figure 3. 3 Comparison of the effectiveness of different enzymes on the release of xylobiose from different cereals. Mean values expressed as total xylobiose g/100g biomass released during in vitro digestion over 72 h at 41°C. No xylobiose was detected for maize in any of the control or enzyme-treated digestions. 'A' represents the standard error of the difference of the means (SED) for comparing control and enzyme treated mean values on the same graph. 'B' represents the SED for comparing different times within the same treatment (control or enzyme) on the same graph. Three way ANOVA indicated a significant enzyme x cereal x time interaction (p=0.002).

3.4.3 In vitro digestion of cereals with various enzymes – release of monosaccharides

Surprisingly, there was virtually no release of xylose, galactose or arabinose from maize in any of the control incubations nor by any of the enzymes studied, with only glucose being released at the time points observed in the present study (0, 6, 12, 24, 48 & 72h).

There was a significant enzyme x cereal x time (P=0.042) interaction for the release of xylose (Figure 3.4). The greatest xylose release was when the 3 cereals (barley, oats and wheat) were incubated with Econase XT or Econase MP1000, whereas Barley P700 had a much smaller effect on barley and wheat and no effect on oats. The xylose release over time was highest for wheat (P=0.042, Figure 3.4).

The next greatest release of monosaccharide was arabinose (Figure 3.5). Once again there was no arabinose released from maize, but there was a significant enzyme x cereal x time interaction (P=0.007) indicating that the different enzymes had different substrate specificities. Econase XT increased the release of arabinose from barley and wheat to similar extents, but less so from oats (Figure 3.5). Econase MP1000 had similar effects on all 3 cereals, whereas Barley P700 released more arabinose from wheat than barley, with no effect on oats (Figure 3.5).

There was also a significant enzyme x cereal x time interaction (p=0.007) for the release of galactose (Figure 3.6). While small amounts were released from control incubations for all 3 cereals (Figure 3.6), only oats incubated with

Econase XT or Econase MP1000 enzymes showed consistent increases above the controls.

There were no significant interactions for glucose release. However, glucose release from the 4 cereals significantly increased with time ($P=0.036$) and there was a significant effect of cereal ($P=0.047$). There was a trend for an interaction between cereal and time ($P=0.09$). All 3 enzymes gave similar increases in glucose release from barley, whereas only Barley P700 increased glucose release above controls for barley and oats, with no apparent effect on wheat and maize, however this 2-way enzyme x cereal interaction was only a trend ($P=0.09$, Figure 3.7).

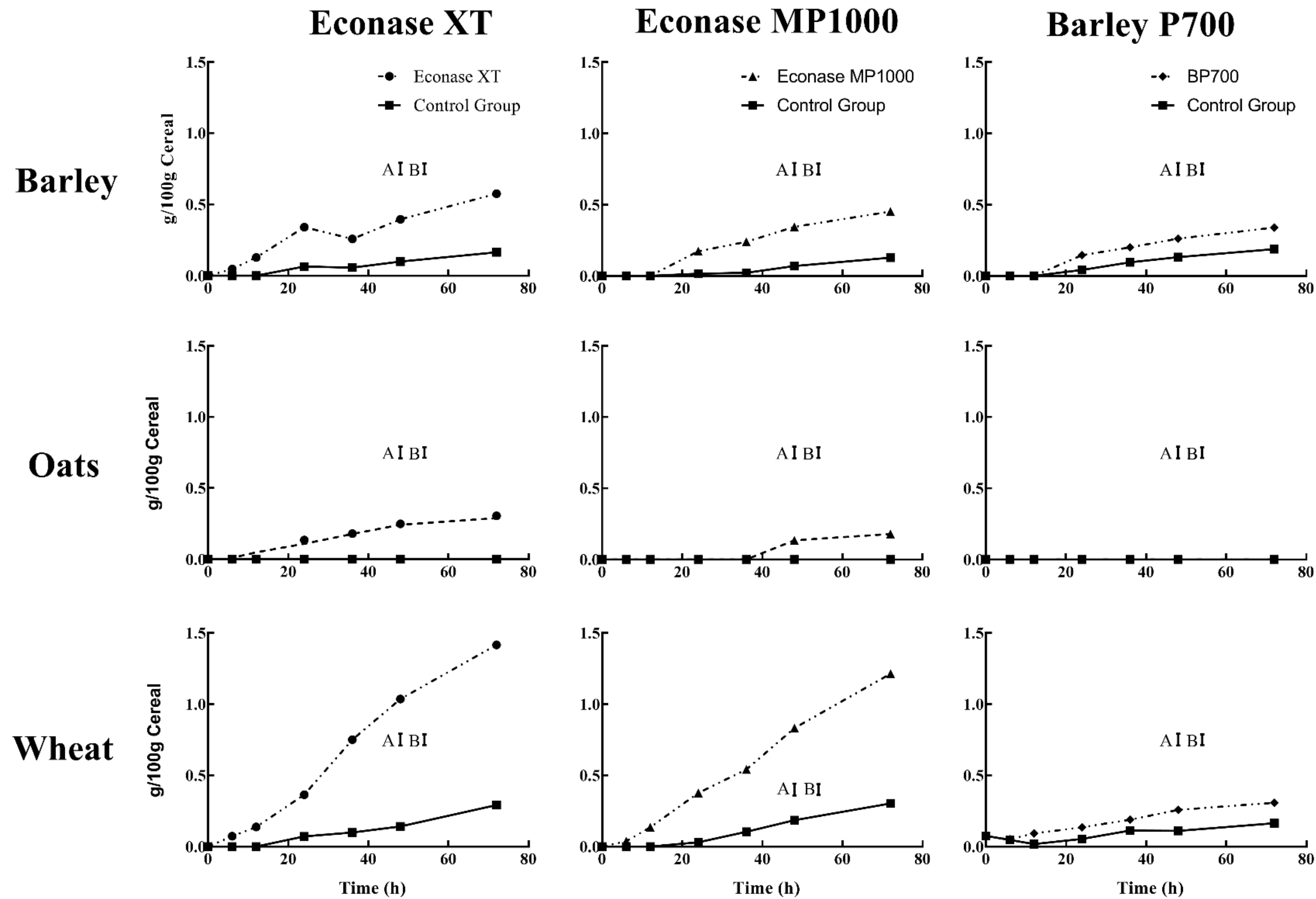

Figure 3. 4 Comparison of the effectiveness of different enzymes on the release of xylose from different cereals. Mean values expressed as total xylose g/100g biomass released during in vitro digestion over 72 h at 41°C. No xylose was detected for maize in any of the control or enzyme-treated digestions. 'A' represents the standard error of the difference of the means (SED) for comparing control and enzyme treated mean values on the same graph. 'B' represents the SED for comparing different times within the same treatment (control or enzyme) on the same graph. Three-way ANOVA indicated a significant enzyme x cereal x time interaction (P=0.042).

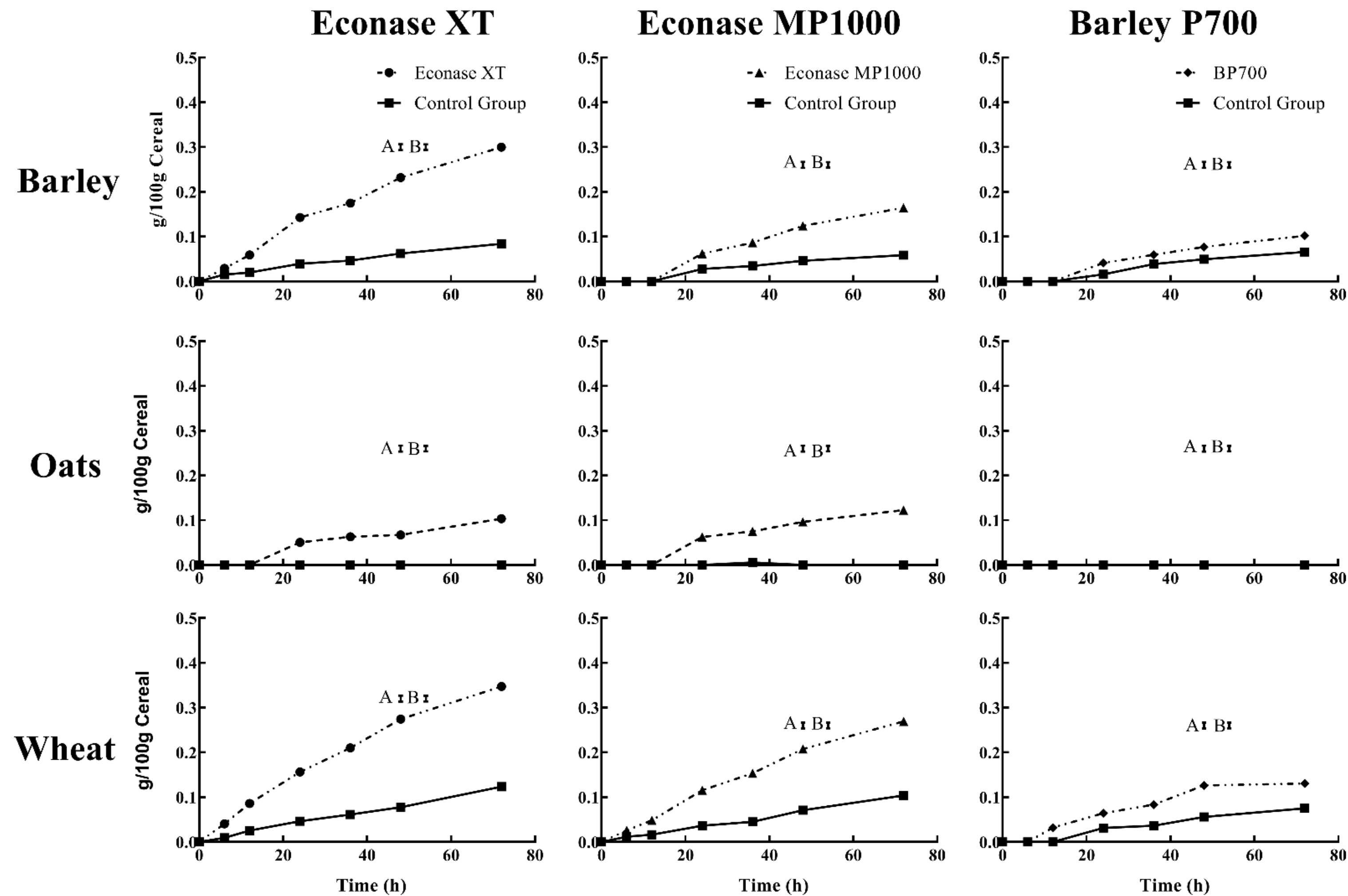

Figure 3. 5 Comparison of the effectiveness of different enzymes on the release of arabinose from different cereals. Mean values expressed as total arabinose g/100g biomass released during in vitro digestion over 72 h at 41°C. No arabinose was detected for maize in any of the control or enzyme-treated digestions. 'A' represents the standard error of the difference of the means (SED) for comparing control and enzyme treated mean values on the same graph. 'B' represents the SED for comparing different times within the same treatment (control or enzyme) on the same graph. Three-way ANOVA indicated a significant enzyme x cereal x time interaction (P=0.007).

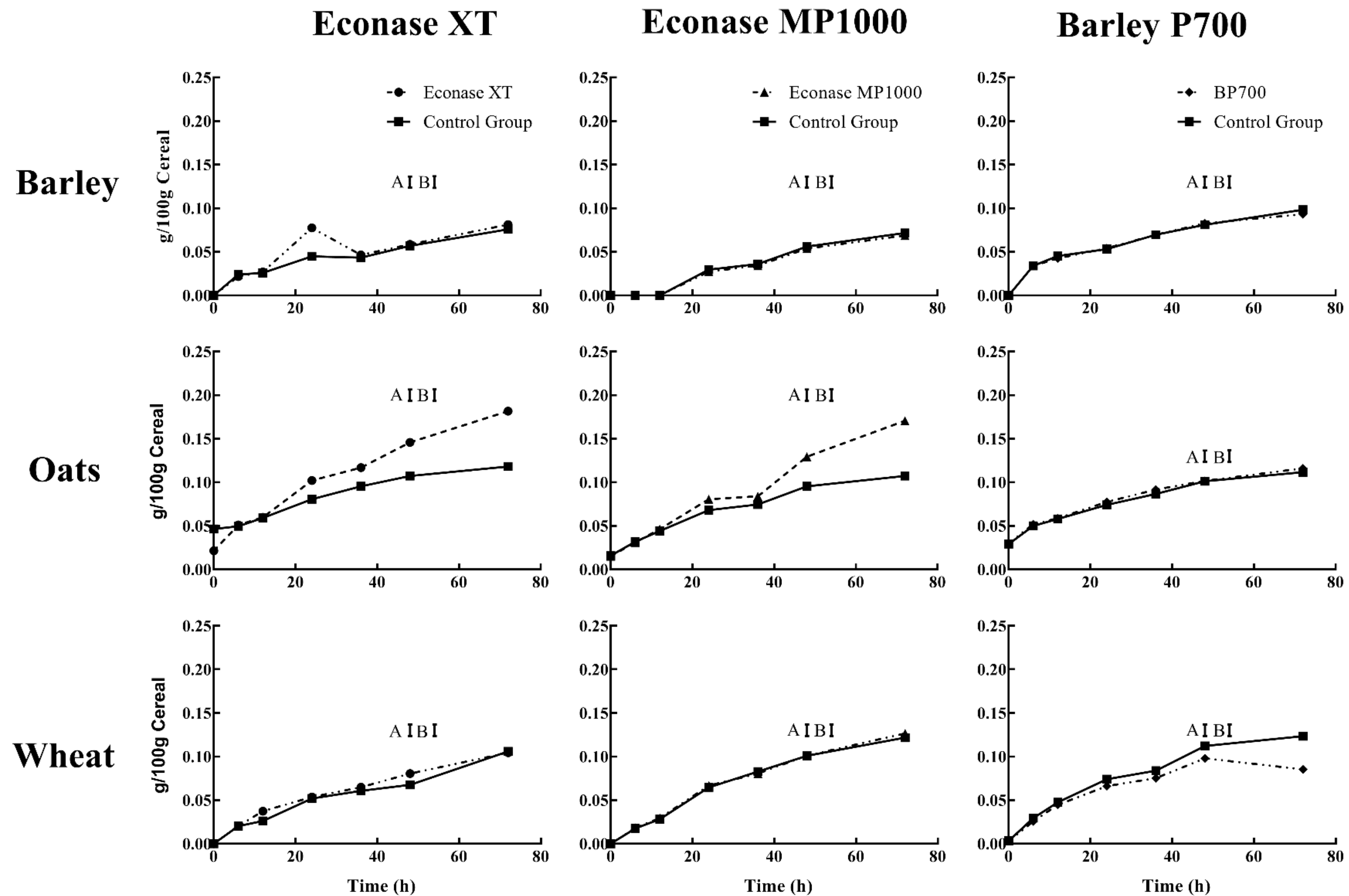

Figure 3. 6 Comparison of the effectiveness of different enzymes on the release of galactose from different cereals. Mean values expressed as total galactose g/100g biomass released during in vitro digestion over 72 h at 41°C. No galactose was detected for maize in any of the control or enzyme-treated digestions. 'A' represents the standard error of the difference of the means (SED) for comparing control and enzyme treated mean values on the same graph. 'B' represents the SED for comparing different times within the same treatment (control or enzyme) on the same graph. Three-way ANOVA indicated significant enzyme x cereal x time interaction (P=0.007).

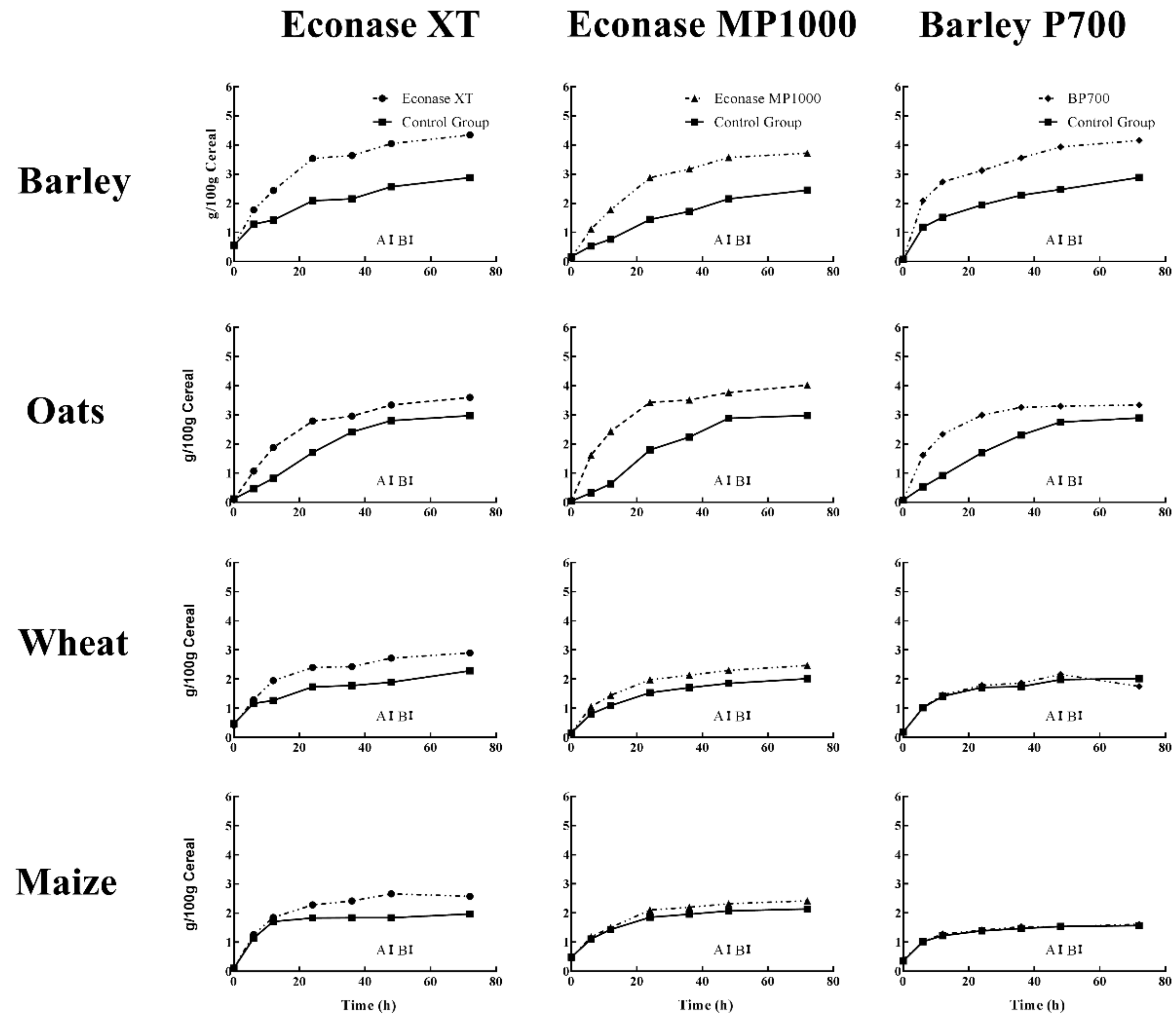

Figure 3. 7 Comparison of the effectiveness of different enzymes on the release of glucose from different cereals. Mean values expressed as total glucose g/100g biomass released during in vitro digestion over 72 h at 41°C. 'A' represents the standard error of the difference of the means (SED) for comparing control and enzyme treated mean values on the same graph. 'B' represents the SED for comparing different times within the same treatment (control or enzyme) on the same graph. Three-way ANOVA indicated a trend for enzyme x time (P= 0.09) and cereal x enzyme (P= 0.09) interactions, but significant effects of time (P= 0.036) and cereal (P=0.047) individually.

3.5 Discussion

To our knowledge, this is the first study to compare the effects of different enzymes on the release of mono- and oligo-saccharides from different cereals *in vitro* over time. Hence there is very little published literature to compare our results to. The main finding from this study is that the 3 enzymes appeared to have differing specificities for the 4 cereals, at least *in vitro*, suggesting that it might be possible to optimise the enzyme-cereal combination used in animal feeds. It was also interesting to see how the release of glucose went into asymptote over time in wheat and maize samples, suggesting all of the glucose available from these substrates was released during the time course regardless of enzyme addition, however this was not seen in barley and oats where presumably the larger amounts of glucose available via the hydrolysis of β -glucans within these cereals was more effectively released by enzyme addition.

3.5.1 Maize

Maize has often been perceived as being less responsive to NSPase treatments due to its low NSP content and low viscosity (Choct, 1997). In the present study, only glucose was detected following the *in vitro* digestions, most of which was released in the absence of any enzyme (Figure 3.7) and was likely due to starch dissolution more so than cellulose or β -glucan. Importantly, all 4 cereals released glucose to a similar extent in the absence of any enzyme and all 3 enzymes increased the glucose release to a similar extent from barley and oats, whereas there were no apparent effects of Barley P700 on glucose release from wheat or maize.

There was no release of any of the XOS from maize, nor any of the other monosaccharides measured (xylose, arabinose and galactose). This would therefore agree with the idea that maize is almost exclusively insoluble and relatively unresponsive to the exogenous enzymes (for anything other than glucose release). Importantly, this was not due to a lack of those sugars in maize, since complete TFA hydrolysis showed that the monosaccharide and total AX contents, as well as the A:X ratios for maize were all similar to the other 3 cereals studied (Tables 3.1 and 3.2). The total AX content of maize determined in this study is similar to that described previously(Choct, 1997); (Malathi and Devegowda, 2001). Despite the AX contents and A:X ratios found in maize in this study, these findings suggest that the structure and or accessibility of the AX in maize makes it less susceptible to degradation into lower molecular weight material observed in the present study (DP < 5). It could have been the case that larger molecular weight material (e.g. DP > 50) was released, but not processed down into oligosaccharides or monosaccharides. This larger DP material could still be fermented as a substrate by the gut microbiome in the caeca.

3.5.2 Barley

Interestingly, while barley had the lowest total glucose content after complete TFA hydrolysis, it had the highest xylose and total AX contents, but there was no galactose detected (Tables 3.1 and 3.2). This agrees with previous studies in terms of arabinose and xylose contents of barley. When 6 varieties of barley were assessed for monosaccharide contents (Åman and Newman, 1986), the average total arabinose and xylose contents were 2.5% and 3.6% of total biomass respectively, which is lower than the 3.2% and 5.2% in the present

study. This can be partially explained by the fact that two thirds of samples used in the Aman and Newman (1986) study were hulless, therefore lower in AX. AX is considered a good indicator for responsiveness to carbohydrase enzymes, although not in the case of maize. All 3 enzymes increased the release of most sugars from barley, with the only exceptions being xylotriose and galactose. However, despite barley having the highest total AX content, more arabinose and xylose was released from wheat, highlighting that the amount of sugars released by enzyme digestion does not necessarily relate to their inherent content, at least not *in vitro*, as observed with maize in the current experiment.

There were clear differential effects of the 3 enzymes on the generation of xylotetraose from barley (Figure 3.1), with a short, sharp generation observed around 12h that subsequently decreased, presumably due to it being rapidly broken down into smaller oligos and monomers. Econase MP1000 induced the biggest peak, followed by Barley P700, with Econase XT showing a smaller, but more prolonged release across the 72hr time course. There was very little effect of any of the enzymes on xylotriose release (Figure 3.2) and only Econase XT induced appreciable generation of xylobiose (Figure 3.3). All 3 enzymes increased the glucose generated from barley to a similar extent (Figure 3.7), despite there being appreciable glucose release in the absence of any enzyme. Similarly, some xylose and arabinose were released in the absence of any enzyme (Figures 3.4 and 3.5) and all 3 enzymes increased the release, although Econase XT tended to release the most and Barley P700 the least. Surprisingly, these findings suggest that Econase XT, and possibly Econase MP1000, might be better for use in barley-based diets, both of which

released more sugars than barley P700, although this obviously needs *in vivo* confirmation.

3.5.3 Oats

Of the 4 cereals studied, oats contained the lowest total AX content, but the most galactose. In a previous study, Westerlund et al (1993) showed the total AX content of flaked oats to be 7.4% of total dry mass, whereas the AX content in the present study was 4.71%. Some of this difference could be because the previous study used the dry weight, whereas we determined values as percentages of total biomass (on a wet weight basis), although this may likely only account for 10% of the variation seen.

Oats generated much smaller amounts of the XOS than either barley or wheat, and there were clear differential effects of the 3 enzymes. Only Barley P700 induced any generation of xylotetraose (Figure 3.1); while only the combination of Econase MP1000 and oats induced an appreciable generation of xylotriose (Figure 3.2) and there was virtually no xylobiose released from Barley (Figure 3.3), either in the absence or presence of any enzyme. Perhaps the addition of a mannanase enzyme in the oats sample gave a unique additive effect to the enzyme hydrolysis, bringing about a spike in xylotriose production. Similarly, only Econase XT and Econase MP1000 induced the release of xylose, arabinose and galactose (Figures 3.4-6), with no effects of Barley P700 over the release observed in the absence of any enzyme. It is therefore unclear which enzyme might be best in oats-based feeds, but Barley P700 is unlikely to have much effect, since it only increased the generation of

xylotetraose and glucose from oats. Once again this will need to be confirmed by *in vivo* studies.

3.5.4 Wheat

Of all the cereals studied, wheat contained the most arabinose and had the highest A:X ratio but was intermediate in terms of the other monosaccharide contents following TFA hydrolysis. These levels are in line with those published for a variety of wheat cultivars. For example, Pritchard et al (2011), quantified 211 varieties of wheat and showed that the total AX contents and A:X ratios ranged from 2.37-10.75% and 0.4-1.3 respectively, which agrees with the 7.57% and 1.02 in the present study. Similarly, Lafond et al (2015) characterised the total carbohydrate composition of 6 wheat cultivars and showed average contents of 2.65%, 4.57%, 0.59% and 7.22% for arabinose, xylose, galactose and total AX contents respectively, which are all similar to the levels observed in the present study. Lafond et al (2015) showed that the wheat cultivar with the highest AX content delivered significantly more arabinose and xylose into the ileum compared to the cultivar with the lowest AX content in the presence of enzyme addition. They also showed that the AX was not degraded in the absence of xylanase. This implies that high AX contents may result in greater digestion potential *in vivo* when a xylanase is fed.

In the present study, wheat samples generated all 3 XOS (xylotetraose, xylotriose and xylobiose), but in quantitively smaller amounts than the amount of xylose released through enzyme hydrolysis. Wheat was also the most responsive to enzyme supplementation in terms of release of both xylose

(Figure 3.4) and arabinose (Figure 3.5), with Econase XT releasing slightly more than Econase MP1000, and Barley P700 only releasing slightly more than the no enzyme controls. Wheat was the only cereal to release notably more xylotetraose in combination with Econase XT supplementation, suggesting the enzyme activity here is leading to greater production of DP4 XOS *in vitro.* In contrast, wheat didn't respond as much as barley or oats in terms of enzyme-induced release of glucose, probably due to the greater number of β-glucans present for hydrolysis into glucose in barley and oats (Figure 3.7). These results again indicate the importance of enzyme-cereal combinations in terms of the magnitude of the response seen, but it is important that these differences are confirmed *in vivo* before any recommendations are possible.

3.6 Summary

There were clear differences between cereals in terms of the effects of exogenous enzymes on the sugars generated and released *in vitro*. It is surprising that all of the enzyme mixes contain endo-xylanase activity yet gave differing results when other enzymes were added to the mix. For example, the generation of xylobiose was generally greatest with Econase XT addition but the addition of other enzymes in Econase MP1000 and Barley P700 mixes reduced xylobiose generation. This suggests that it might be possible to optimise the enzyme used for a diet containing a particular cereal, but also that the addition of enzymes to some diets may be ineffective due to the cereals used.

One criticism might be that we only used a single source of each cereal, hence it is important that future work determines the variation of response across different varieties of the same cereal, as well as confirming that similar effects are observed *in vivo* when other endogenous enzymes will also be present. Questions that remain unanswered include

1) What is be most beneficial XOS to generate? I.e. what DP and what A:X ratio
2) Is monomeric xylose release beneficial or detrimental?
3) Where (i.e. what point in the digestive tract) are these sugars most beneficial?

Improvements in current enzyme technologies may be achieved by targeting which XOS are produced as end products and ensuring the products do not further degrade these beneficial XOS to lower DP or even monosaccharides which may reduce the bioeffciacy of the enzyme.

Finally, while the use of exogenous enzymes in diets for pigs and poultry is relatively well established, there may be potential for their use in humans, in order to overcome gastrointestinal and digestive problems. For example, fibrolytic enzymes could potentially be used to improve digestibility of poor-quality diets and thereby help reduce malnutrition in poorer regions of the world.

3.7 Acknowledgements

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4. THE EFFECTS OF EXOGENOUS FIBROLYTIC ENZYMES ON THE *IN VITRO* GENERATION OF XYLOOLIGOSACCHARIDES AND MONOSACCHARIDES FROM SIX WHEAT VARIETIES

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4.1 Abstract

Wheat is a widely adopted cereal grain for poultry feeds globally. Despite this, there are noticeable differences in its nutritive value, potentially due to varying characteristics like inherent non-starch polysaccharide (**NSP**) content and composition. Fibrolytic enzymes help degrade NSP and thereby improve feed efficiency. However, it has been suggested that these enzymes have different effects dependent upon the characteristics of the wheat variety used in a feed. This study investigated the efficacy of different enzyme-wheat variety combinations, by quantifying the generation of NSP-derived substrates - xylooligosaccharides (**XOS**) and monosaccharides from six wheat varieties (Maris Huntsman, Highbury, Paragon, Sinuelo, Chinese Spring and Pavon 76) over a 24 hour *in vitro* incubation with different commercially available fibrolytic enzymes. Non-cellulosic acid hydrolysis showed there were differences in the total monosaccharide within varieties ($p<.001$). There were significant variety x enzyme x incubation time interactions for the release of xylobiose, galactose and glucose (all $p<0.001$) and significant enzyme x variety interactions for the release of xylotriose ($p=0.022$), xylose ($p<0.001$) and arabinose ($p=0.028$). Hence, there were differences between the different enzyme - variety combinations in the release of XOS, with Econase XT increasing xylotriose release from Highbury wheat, but increasing xylobiose release from Sinuelo, with both varieties subsequently showing comparable release of xylose. These findings suggest that the fibrolytic enzymes tested have some specificity for certain wheat varieties and therefore it might be possible to optimise the combinations of wheat and enzyme used in animal feeds, to help maximise the feed efficiency of livestock.

Key words: Cereal. Xylanase. Xylooligosaccharide. Prebiotic. Feed.

Abbreviations: NSP, non-starch polysaccharide; XOS, xylooligosaccharide; AME, apparent metabolisable energy; AX, Arabinoxylan; SNSP, water soluble non starch polysaccharide; AXOS, Arabinoxylan oligosaccharides; TFA, trifluroacetic acid; ×g, times gravity; BXU/g, beta-xylanase units per gram; MNU/g, mannanase units per gram; BU/g, beta-units per gram, ECU/g**,** endo-cellulase units per gram; HPAEC-PAD**,** high-performance anion-exchange chromatography with pulsed amperometric design.

4.2 Introduction

Wheat is the most common energy source used in European poultry feeds (Choct et al., 1999). This is in large part due to its high content of storage polysaccharides encapsulated within the cereal grain (del Alamo et al., 2008). Wheat contributes over 60% of starter feed formulations for broilers, and up to 75% in finisher feed formulations (Austin et al., 1999). There is, however, large variation in the apparent metabolisable energy (**AME**) of wheat-based feeds, largely attributable to their high non-starch polysaccharide (**NSP**) contents (12-18%) (Slominski et al., 2000).

NSP present in the wheat plant cell walls are known to be anti-nutritive. They increase the digesta viscosity of broilers *in vivo*, which results in inadequate mixing and movement of digesta, limiting the potential for both exogenous and endogenous enzymes to reach their target sites. A subsequent decrease in nutrient digestibility follows (Choct and Annison, 1992, Bedford, 2000).

Given that broilers do not possess the necessary endogenous enzymes to sufficiently break down plant cell walls, it is thought that the encapsulation of starch and proteins via these cell walls acts as a direct barrier to these nutrients being utilised (Bedford and Autio, 1996), further reducing the AME of wheat-based feeds.

Arabinoxylan is the predominant plant cell wall polysaccharide present in wheat, with a recent study showing that total arabinoxylans (**AX**) constituted 59% of NSP (De Keyser et al., 2018).

For these reasons, exogenous enzyme supplementation in the form of xylanases, β-glucanases and cellulases which target arabinoxylan, β-glucan and

cellulose, respectively, have the potential to increase digestibility of NSP in the intestinal tracts of broilers consuming wheat-based feeds (Masey O'Neill et al., 2014). Previous research (submitted for publication) showed that there were clear differences between different cereals in terms of the effects of exogenous enzymes on the sugars generated and released *in vitro.* The three enzymes studied varied in their dominant activity, but all contained a xylanase. These enzymes were then tested on three different cereals and yielded different results in terms of XOS release. This is likely due to the divergence in properties of the xylanases present in each preparation and shows cereal type and enzyme characteristics can interact to determine the XOS released.

An *in vitro* model has been developed to quantify the monosaccharides and XOS produced as a direct result of incubating different fibrolytic enzymes with different wheat varieties. This should inform future *in vivo* research to identify the optimal xylanase for wheat-based feeds (should the oligosaccharide mechanism prove to be significant), thereby tailoring the use of specific enzymes to specific wheat varieties (Maisonnier-Grenier et al., 2006, Lafond et al., 2011). Wheat was chosen as a substrate for its affinity to the enzymes on the generation and release of monosaccharides and XOS *in vitro* in a previous study (submitted for publication).

The aim of this study was to compare the effects of three different fibrolytic enzymes, on the *in vitro* hydrolysis of six different wheat varieties over 24 hours, focussing on the generation of XOS and monosaccharides, particularly the arabinoxylan (AX) components of wheat.

4.3 Materials and Methods

4.3.1 Wheat Varieties

The six wheat varieties used: Maris Huntsman, Highbury, Paragon, Sinuelo, Chinese Spring and Pavon 76 were obtained from the *BBSRC Wheat* Research Centre at the University of Nottingham and were selected for their differing characteristics and geographical location of use to act as a starting point for future work which may select varieties based on findings in the present study. Maris Huntsman (Foulkes et al., 2006) and Highbury are both winter wheat varieties with the latter being used for bread making (Payne et al., 1987); Paragon is a spring wheat variety which is also used in bread making (Milec et al., 2012). All of these varieties have been or are used in the UK. Sinuelo is a variety adapted for use in the harsh environment found in Brazil and shows resistance to mycotoxin growth (Fusarium) (Machado et al., 2017), whilst Pavon 76 is grown in Mexico and is relatively resistant to leaf rust (Puccinia) (Singh et al., 1998). Chinese Spring is a wheat variety which originates from China and can tolerate high levels of humidity and temperature (Warner et al., 2000).

4.3.2 Total hydrolysis of non-cellulosic polysaccharides

Total sugar contents were determined in the 6 wheat varieties by total hydrolysis of their non-cellulosic polysaccharides with Trifluroacetic acid (TFA), as previously described (Fry, 1988). Briefly, wheat samples were ground to a fine powder (0.5mm screen) and suspended at 10mg/ml in 2M TFA in triplicate. Tubes were sealed and heated to 120°C for 1 hr in an autoclave, then allowed to cool to room temperature before being centrifuged

at 2236*g* for 10 min at room temperature. The supernatant was then diluted 1:100 with 10mM NaOH and transferred to 2ml clear vials for sugar analysis. The release of 4 monosaccharides (arabinose, galactose, glucose and xylose) was quantified using HPAEC-PAD. The sum of these monosaccharides was taken to be the total sugar content, except for those from cellulose.

4.3.3 In vitro digestion of cereals with 3 different enzymes

The following 3 commercial enzyme preparations were provided by AB Vista (Marlborough, UK) and used in digestions with the 6 varieties:

i. Econase XT – a xylanase with β-1, 4 endo-xylanase activity (160,000 BXU/g). The enzyme was used at its suggested dose of 100g/tonne of feed (100µg/g cereal).
ii. Econase MP 1000 – a mannanase enzyme cocktail reported to contain mannanase (1,000,000 MNU/g), β-glucanase (300,000 BU/g) and endo-xylanase (200,000 BXU/g) activities. It was used at the recommended dose of 100g/tonne of feed (100µg/g cereal).
iii. Econase Barley P 700 - an enzyme cocktail prepared from a strain of *Trichoderma reesei*, designed for use in barley feeds. Its main activity is β-glucanase (700,000 BU/g), but it also contains endo-cellulase (165,000 ECU/g) and endo-xylanase (190,000 BXU/g) activities. It was used at the recommended dose of 40g/tonne of feed (40µg/g cereal).

The 6 varieties were individually ground to a fine powder (0.5mm) and 0.2g of each cereal was resuspended in 40mls 50 mM sodium citrate buffer (pH 5.2) containing one of the following enzymes (in triplicate):

a) Wheat samples only – no enzyme added.

b) Wheat samples + Econase XT at 100µg/g.

c) Wheat samples + Econase MP 1000 at 100µg/g.

d) Wheat samples + Econase Barley P 700 at 40µg/g.

The buffer was chosen to represent the average pH of the broiler digestive tract (Mabelebele et al., 2014). Digestion reactions were then placed in a shaking incubator at 150RPM and 41°C for up to 24 h. This time course differed from previous work done as it was considered more physiologically relevant in the context of the poultry digestive tract to take more measurements in the first 0-12h of digestion. At each time point (0, 3, 6, 9, 12 or 24h), 1ml of each digest was removed and added to 9ml of 10 mM NaOH at room temperature, mixed, centrifuged at 2236×g for 10 min at room temperature and then frozen at -20°C prior to sugar analysis.

4.3.4 Identification and quantification of sugars using HPAEC-PAD

The concentrations of arabinose, galactose, glucose and xylose, as well as the XOS (g/100g), were determined using High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection (HPAEC-PAD) following the method of Xu et al (Xu et al., 2013). Each sample was assayed in triplicate. Analysis was carried out using a Dionex ICS-3000 with a Dionex CarboPac PA20 Column (3mm x 150mm) and CarboPac PA20 Guard (3x30mm) for the monosaccharide analysis. A CarboPac PA200 column (3mmx250mm) and CarboPac PA200 guard (3mm x 50mm) were used for the oligosaccharide analysis. An injection volume of 10µl was used throughout for all standards and samples. Monosaccharide standards (arabinose, galactose, glucose and xylose) were purchased from Sigma-Aldrich, UK and XOS

standards (xylo-biose, -triose and –tetraose) from Megazyme, Ireland. Serial dilutions for each standard (2.0, 1.0, 0.5 and 0.25g/L for monosaccharides and 2, 1, 0.5 and 0.25g/L for XOS) were made fresh for each batch of analyses, diluted 1:100.

For the monosaccharides, a single eluent, containing 10mM NaOH solution, was used as the mobile phase at 0.5ml/min for 14 min. For oligosaccharides, 2 eluents were used in a gradient for the mobile phase, 0.1M sodium hydroxide (Solution A) and 0.1M NaOH containing 0.5M sodium acetate (solution B) in standard quadruple waveform, as described by Xu et al (2013). The gradient program used for XOS determination was 100% solution A at 0 minutes, rising to 80% solution A and 20% solution B at 25 minutes, before returning to 100% solution A after 25 minutes elapsed. Both eluents were stored in plastic pressurised bottles with inert nitrogen gas at 6-9 psi. Data were collected with Dionex Chromeleon software (Version 6.7).

4.3.5 Data and Statistical analysis

A single source was used for each wheat variety and all analyses (digestions and Dionex analysis) were carried out in batches consisting of every enzyme-cereal combination plus or minus controls per batch, repeated for a total of three batches. Standards for the four monosaccharides or XOS were run at the start and end of each batch and standard curves generated from the areas under the curve and presented as g/100g of wheat variety. Batches were undertaken due to constraints in the number of available digestion tubes and to account for inter-experiment variation. Data were processed in excel (Microsoft, 2013) and expressed as means ± standard error of differences (SED).

Data was then analysed by one (wheat variety, enzyme or time -only), two (enzyme x time, enzyme x variety or variety x time) or three (enzyme x variety x time) way ANOVA, using Genstat statistical software (17th Edition), with blocking for batch and digestion tube. A Bonferroni post-hoc test was used to identify significant differences between cereals following a significant 1-way ANOVA. Post hoc Bonferroni tests were used for the enzyme digestion analyses with significant interactions, with $p<0.05$ taken as statistically significant.

4.4 Results

4.4.1 Total hydrolysis of non-cellulosic polysaccharides

The total sugar contents were determined by TFA hydrolysis which results in complete hydrolysis of oligosaccharide and polysaccharides present in the sample to the constituent monosaccharide contents. The total sugar content was taken to be the sum of the arabinose, galactose, glucose and xylose monosaccharide contents of each wheat variety. This was significantly different between varieties, with Maris Huntsman containing the highest total sugars, and Paragon containing the lowest (Table 4.1, $p<.001$). As expected, the main sugar present in all four cereals was glucose, hence it is not surprising that the wheat varieties order of glucose (mostly from starch) content was the same as the total sugar content; Maris Huntsman > Highbury > Pavon 76 = Sinuelo = Chinese Spring > Paragon ($p<.001$). Galactose was present in low amounts in all cereals (0.6-1.0 g/100g) and followed a similar pattern to the glucose content, Maris Huntsman > Highbury > Sinuelo = Chinese Spring = Pavon 76 > Paragon ($p<.001$).

Table 4. 1 Monosaccharide composition of six wheat cultivars after total hydrolysis of non-cellulosic Polysaccharides.

Variety	Arabinose[1] (g/100g)	Galactose (g/100g)	Glucose (g/100g)	Xylose (g/100g)	Total [2] (g/100g)
Maris Huntsman	4.87 [a]	0.99 [a]	54.26 [a]	4.92 [a]	**65.04 [a]**
Highbury	3.52 [b]	0.71 [b]	46.39 [b]	3.75 [b]	**54.37 [b]**
Pavon 74	3.50 [b]	0.67 [bc]	45.18 [bc]	3.59 [bc]	**52.94 [bc]**
Sinuelo	3.18 [cd]	0.65 [bcd]	44.09 [c]	2.85 [d]	**50.77 [c]**
Chinese	2.95 [d]	0.61 [cd]	43.41[c]	3.34 [c]	**50.31 [c]**
Paragon	3.30 [bc]	0.59 [d]	34.67 [d]	3.33 [c]	**41.88 [d]**
SED[3]	**0.073**	**0.018**	**0.556**	**0.095**	**0.738**
P-Value[4]	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**

[1] Values are expressed as mean grams of each monosaccharide per 100g of cereal
[2] Total is the sum of the quantities of arabinose, galactose, glucose and xylose
[3] SED, Standard Error of Difference.
[4] One-way ANOVA indicated significant differences between cereals for each sugar.
[a,b,c,d] Mean values within a column with different superscript letters were significantly different ($P<0.05$, Bonferroni's post hoc test).

Maris Huntsman also had the highest xylose (Maris Huntsman > Highbury > Pavon 76 = Sinuelo = Chinese Spring > Paragon) and arabinose contents (Table 4.1, Maris Huntsman > Highbury = Pavon 76 = Paragon = Sinuelo = Chinese Spring). It is assumed that the vast majority of these two sugars are present as arabinoxylan. Hence the total arabinoxylan (AX) content was also highest in Maris Huntsman (Table 4.2, Huntsman > Highbury = Pavon 76 = Paragon = Chinese Spring > Sinuelo, $p<.001$). As well as the differences in the total AX content, the ratio of Arabinose: Xylose (A:X) in the wheat varieties was significantly different, with the highest being in Sinuelo (Table 2, Sinuelo > Maris Huntsman = Paragon = Pavon 76 > Highbury > Chinese Spring, $p<.001$).

Table 4. 2 Total Arabinoxylan (AX) contents and Arabinose: Xylose (A:X) ratios of six wheat cultivars.

Cereal	Total AX[1] (g/100g)	A:X Ratio [2]
Maris Huntsman	9.79 [a]	0.99 [b]
Highbury	7.27 [b]	0.94 [c]
Pavon 74	7.09 [bc]	0.98 [b]
Paragon	6.62 [cd]	0.99 [b]
Chinese	6.29 [de]	0.88 [d]
Sinuelo	6.03 [e]	1.09 [a]
SED[3]	**0.1673**	**0.005**
P-Value[4]	**<.001**	**<.001**

[1]Total AX values are expressed as mean grams of Arabinoxylan per 100g cereal
[2]A:X values represent grams of Arabinose per 100g cereal divided by grams of xylose per 100g cereal ± SD, standard deviation for technical replicates.
[3] SED, Standard Error of Difference.
[4] One-way ANOVA indicated significant differences between cereals for Total AX and A:X ratio.
[a,b,c,d,e] Mean values within a column with different superscript letters were significantly different ($P<0.05$, Bonferroni's post hoc test).

4.4.2 Release of XOS and monosaccharides from different wheat varieties in the absence of any exogenous enzymes

These incubations were carried out in a 50 mM sodium citrate buffer (pH 5.2) in the absence of exogenous enzymes, to represent an average pH of the broiler digestive tract (Mabelebele et al., 2014). Firstly, in the absence of exogenous enzymes, no xylotriose or xylotetraose was observed in the control incubations for any wheat variety. For the other sugars there was no significant variety x time two-way interactions, but they all had significant effects of wheat variety or time ($P<0.001$) (Figure 4.1). Xylobiose release was linear throughout the 24hr time course in all wheat varieties. Maris Huntsman and Paragon controls released the most xylobiose, with Highbury, Chinese Spring and Pavon 76 all releasing similar quantities of xylobiose, and Sinuelo gave the least xylobiose during control incubations. Sinuelo contained the lowest total quantity of xylose (Table 4.1) compared to the other varieties but it released the largest quantity of xylose in control incubations after a 24 h incubation. Paragon, Chinese Spring and Pavon 76 varieties all released similar amounts of xylose, whilst Highbury released the least. Generally, there was release of xylose during the 0-9hr period, which then tended to plateau from 9-24hr of incubation. Overall, although there was release of xylose from the wheat varieties following a 24hr incubation this was relatively small, approximately 1% of the total xylose available (Table 4.1).

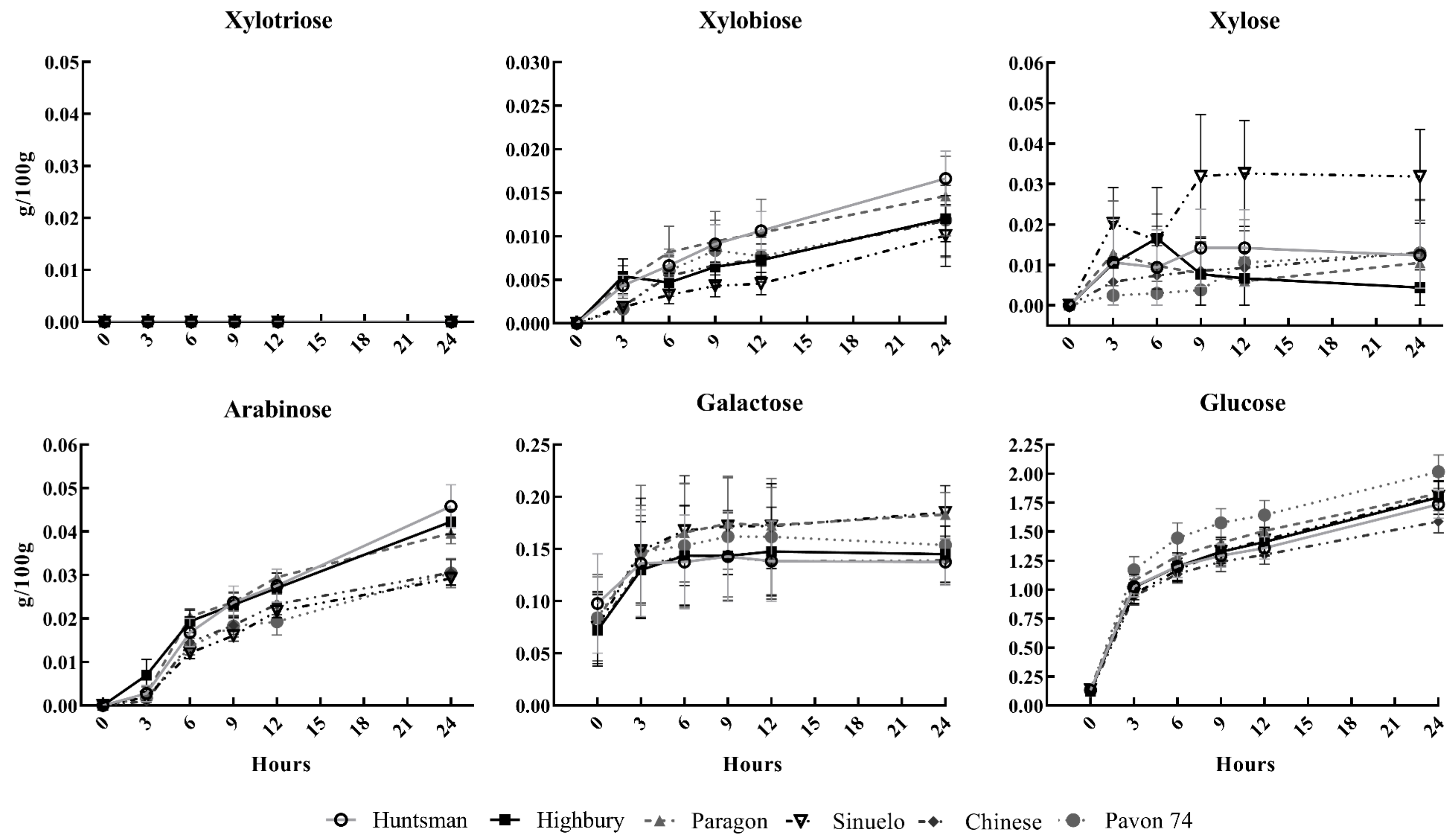

Figure 4. 1 Release of monosaccharides and XOS from different wheat varieties in the absence of any enzymes. Mean values (n=3) expressed as total g/100g biomass released during *in vitro* incubation in sodium citrate buffer (pH5.2) over 24 h at 41°C. Error bars represent the standard error of the mean (SEM). Two-way ANOVA indicated no significant interactions for variety x time for any sugar but did indicate significant effects of both variety and time ($p<0.001$).

As was found for total quantity of arabinose (Table 4.1) following TFA hydrolysis, Maris Huntsman released the most arabinose after the 24hr incubation. The Highbury and Paragon varieties were very similar but slightly lower than Huntsman. Sinuelo, Chinese Spring and Pavon 76 all released the lowest quantity of arabinose after the 24hr incubation. Arabinose release did not begin until 3hr and showed most release during the 3-6hr time period, before increasing linearly up to the end of digestion at 24hr. After the 24hr incubation, the release of arabinose was approximately 1% of the total available (Table 4.1).

Release of galactose was more variable (Figure 4.1), but the release was greatest from 0-3hr and remained static from 6-24hr. Galactose was released rapidly during 0-3hr and remained constant for the remainder of the 24hr, with Paragon and Sinuelo varieties releasing slightly more galactose than the other varieties, even though they tended to be the varieties with the lower total galactose following TFA hydrolysis (Table 4.1). Unlike xylose and arabinose, the proportion of total galactose (Table 4.1) released after 24hr incubation was much greater, at approximately 25% of the total available although the absolute amount of galactose released was low (Table 4.1).

Glucose release in controls was greatest at 0-3hr but continued to rise slowly to 24hr. The profile of glucose release followed a similar pattern in all varieties, although Pavon 76 released slightly more glucose than the other varieties. Although the release of glucose from the incubation was the greatest of all the monosaccharides examined, the quantity released after 24hr was only approximately 5% of the total glucose available (Table 4.1).

4.4.3 Release of monosaccharides and XOS from different wheat varieties by the different exogenous xylanase enzymes

There was no release of xylotetraose observed from any of the wheat varieties by any of the enzymes used at the time points used in the present study (data not shown).

For xylotriose there was no three-way interaction observed, but there were significant enzyme x variety ($p=0.022$), enzyme x time ($p<0.001$) and variety x time ($p=0.011$) interactions (Figure 4.2). Incubations in the presence of Econase XT had the greatest variation in its effect on the six wheat varieties, with the most variation seen at 24h compared to the other 2 enzymes. Sinuelo generated the most xylotriose with all three enzymes. There was a rapid initial generation, followed by decline for Econase Barley P700, whereas Econase MP1000 generated xylotriose linearly up to 12 hr, before plateauing for the remainder of the time course. There was a significant enzyme x variety x time interaction ($p<0.001$) for xylobiose. Econase Barley P700 and Econase MP1000 gave much more uniformity of release from the 6 wheat varieties, producing almost identical responses as well as around half the quantity of xylobiose compared to Econase XT (Figure 4.2). In contrast to the xylotriose results, Econase XT released the most xylobiose from Highbury and the least from both Paragon and Sinuelo, with the other varieties falling between these two extremes. For xylose there was only an enzyme x variety interaction ($p<0.001$). Econase XT released twice as much xylose from both Maris Huntsman and Highbury (Figure 4.2) compared to the other two enzymes, but a comparable release of xylose was seen from the other four varieties. The Econase XT gave a greater spread in the release of xylose between varieties

than the other two enzymes. Econase XT increased the release of xylose from some wheat varieties (Maris Huntsman and Highbury), releasing approximately 8% of the total xylose available (Table 4.1) in addition to that released in the controls, with other enzyme-variety combinations releasing an average of ~4% of the total available xylose in addition to that released in controls.

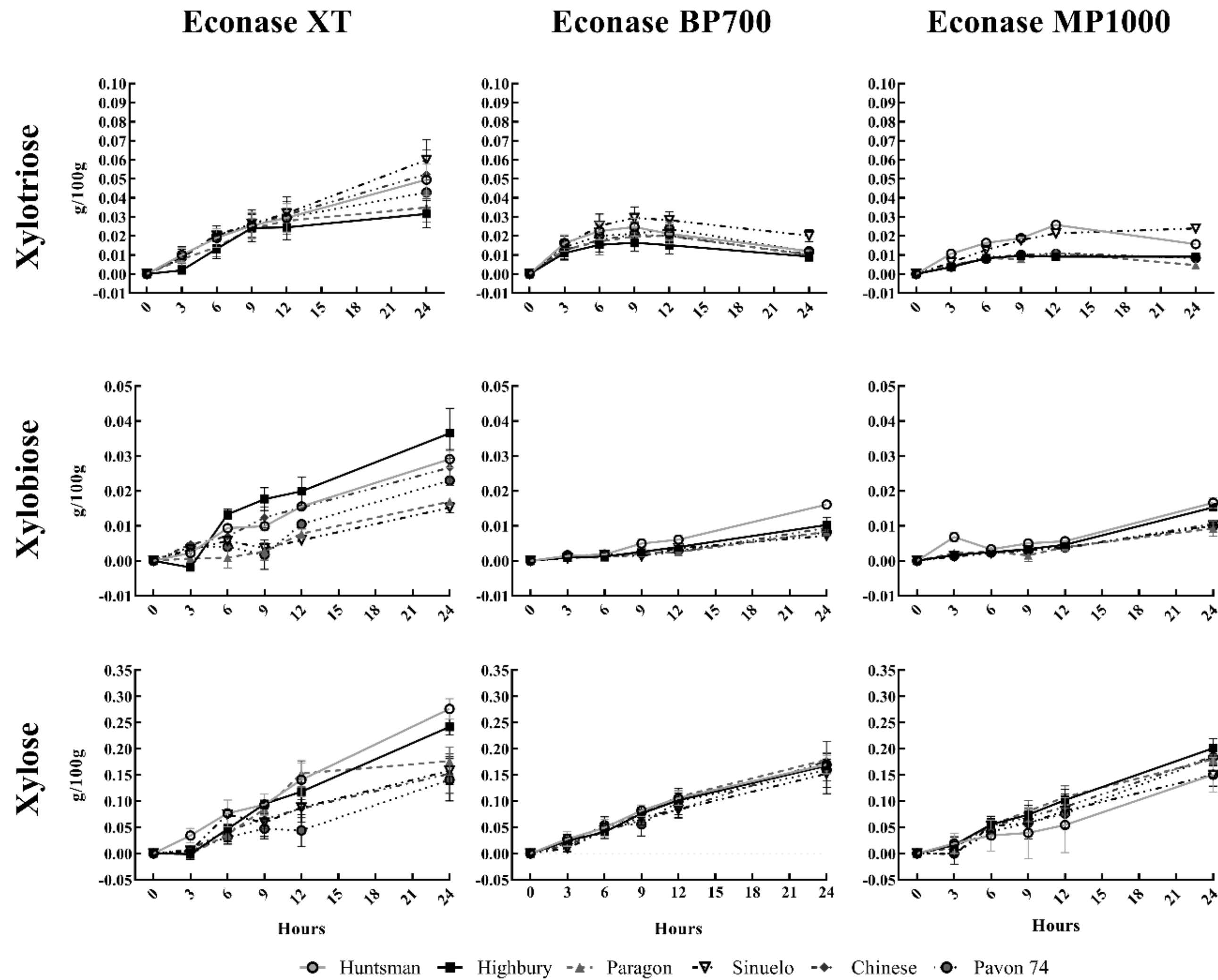

Figure 4. 2 Effect of different enzymes on the release of xylotriose, xylobiose and xylose from different wheat varieties. Mean values (n=3) expressed as total xylooligo- or mono- saccharide (g/100g biomass) released with enzyme minus the control (no enzyme) values during *in vitro* digestion in sodium citrate buffer (pH5.2) over 24 h at 41°C. Error bars represent the standard error of the mean (SEM). Three-way (enzyme x variety x time) ANOVA statistical analyses were performed for each sugar. For xylotriose there were significant two-way interactions for enzyme x variety ($p=0.022$), enzyme x time ($p<0.001$) and variety x time ($p=0.011$). For xylobiose there was a three-way interaction ($p<0.001$). For xylose there was only a two-way interaction for enzyme x variety ($p<0.001$).

For arabinose release there was no three-way interaction observed, but there were significant enzyme x variety ($p=0.028$), enzyme x time ($p<0.001$) and variety x time ($p<0.001$) interactions. As for xylose release, Econase XT showed more variation in response with each variety of wheat, Maris Huntsman and Highbury varieties giving the greatest release of arabinose, and Sinuelo and Paragon releasing the least. However, the enzyme incubation did not give as great an increase in arabinose release as was seen for xylose. Following the 24h incubation all enzymes released approximately 1.5% of the total arabinose available (Table 4.1) in addition to that released in the controls.

There was an enzyme x variety x time interaction ($p<0.001$) for galactose release. Incubations with Econase Barley P700 or Econase MP1000 were not different to controls, as shown by the flat line pattern of release (Figure 4.3). However, Econase XT did show some variation in galactose release, but the additional release of galactose was relatively small compared to that released in the control incubations (Figure 4.1).

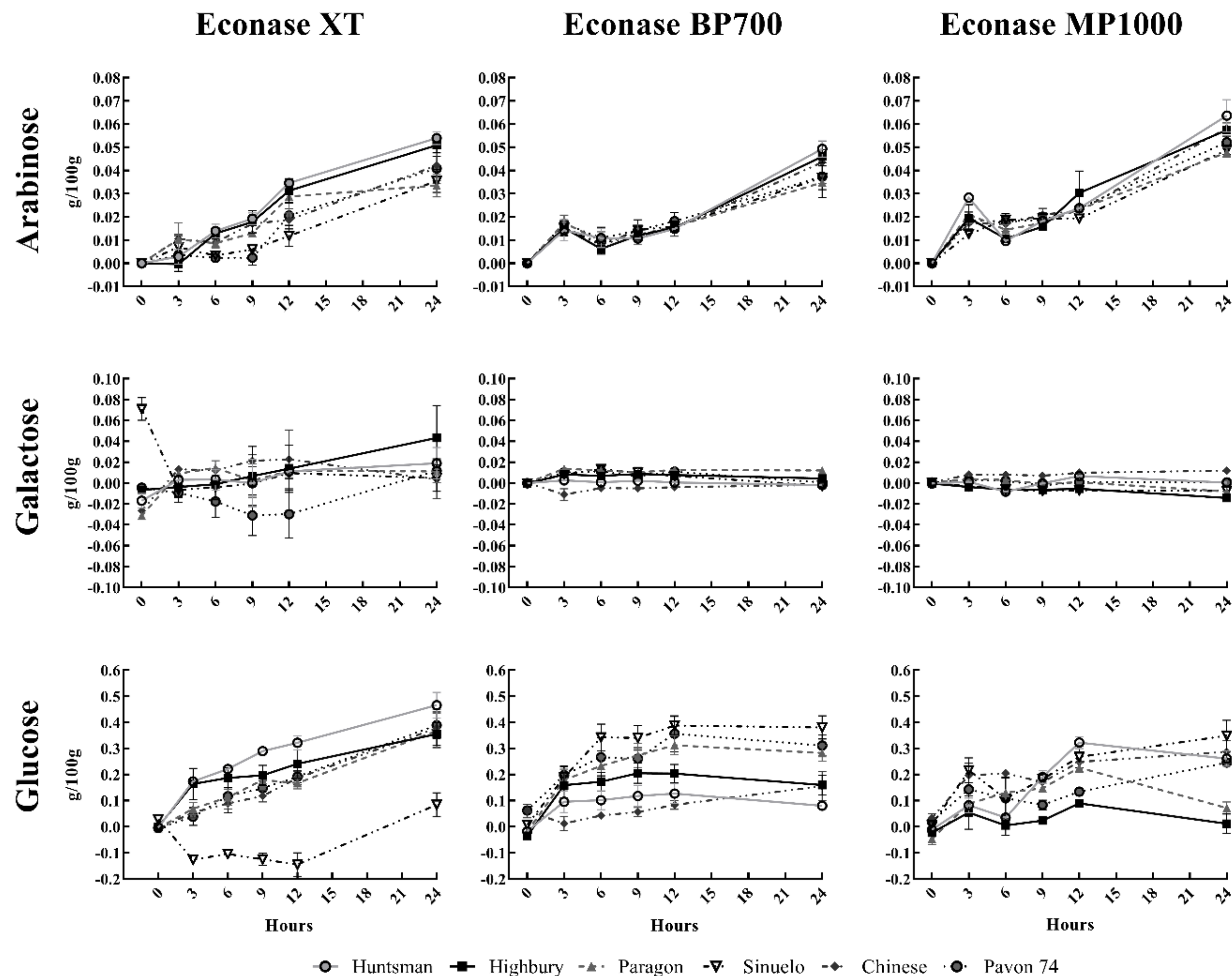

Figure 4. 3 Effect of different enzymes on the release of arabinose, galactose and glucose from different wheat varieties. Mean values (n=3) expressed as total monosaccharide (g/100g biomass) released with enzyme minus the control (no enzyme) values during in vitro digestion in sodium citrate buffer (pH5.2) over 24 h at 41°C. Error bars represent the standard error of the mean (SEM). Three-way (enzyme x variety x time) ANOVA statistical analyses were performed for each sugar. For Arabinose there were significant two-way interactions for enzyme x variety (p=0.028), enzyme x time and variety x time effects (p<0.001). For both Galactose and Glucose there were significant enzyme x variety x time interactions (p<0.001 for both).

For glucose there was an enzyme x variety x time interaction ($p<0.001$), with increased release of glucose following incubation with all 3 enzymes, but this was again relatively small at approximately 1% of the total glucose available (Table 4.1), in addition to that released in the controls. This was the only instance where all 3 enzymes resulted in obvious variations in sugar release. Econase XT gave fairly uniform release in most of the wheat varieties, but a markedly lower response in Sinuelo variety. Econase Barley P700 produced the most variation in release of glucose across the time course (Figure 4.3) giving the greatest release in Sinuelo, and the lowest release in Maris Hunstman. Importantly, this was the opposite of what was observed with Econase XT. Econase MP1000 released similar amounts of glucose in Sinuelo and Maris Huntsman, and the lowest in Highbury and Paragon varieties (Figure 4.3).

4.4 Discussion

This is one of the first studies is to compare the effects of different commercial exogenous feed enzymes on the release of mono- and oligosaccharides from different varieties of wheat *in vitro* over a 24-hour time course. Hence there is little to compare our results with. The main finding was that the three enzymes appeared to have very different specificities for the different varieties of wheat *in vitro*, with some enzymes producing more variation in release of monomeric products and XOS than others. This suggests that it might be necessary to optimise the enzyme mix dependant on the wheat variety used in animal feeds.

The present study found glucose to be 80-85% of the total non-cellulosic sugar contents observed (sum of arabinose, galactose, glucose and xylose) in the different wheat varieties, which agrees with previous findings (Knudsen, 1997). Total arabinoxylan contents and A:X ratio in the six wheat varieties determined in the current study and the wheat sample from chapter 3 were also in line with values obtained by Pritchard et al (2011). They quantified 211 wheat varieties and found AX to range from 2.37-10.75%, and A:X to be 0.4-1.3 (Pritchard et al.), but did not report whether there was a relationship between AX and A:X. Sinuelo wheat variety had the lowest total AX, yet the highest total A:X ratio due to a low xylose content. Given the A:X ratio can influence the susceptibility of an AX to hydrolysis by an endo-xylanase, the range in A:X ratios suggest potential differences in the arabinoxylan oligosaccharides that could be generated on application of a xylanase on these 6 wheat varieties.

All of the wheat varieties released both monosaccharides and XOS when incubated for 24h in the absence of exogenous enzymes. This may be due to the presence of enzyme activity inherent within wheat (Brijs et al., 2009). For most of the monosaccharides, the release was relatively low as a proportion of the total available. The exception to this was galactose which was presumably released due to endogenous galactosidase activity. Interestingly, although the Sinuelo variety had the lowest total xylose content it actually released the most xylose which may suggest that this variety was comprised of less arabinose substituted xylan than other the varieties or that this variety had large amounts of endogenous β- xylosidase activity, stimulating the release of monomeric xylose.

As expected, the most sugar released after incubation with the enzymes was glucose, as wheat is known to have a high starch content (Holm et al., 1986), highlighting the potential capacity for exogenous enzymes to improve the availability of nutrients. In the present study, Sinuelo released the least glucose with Econase XT supplementation, but had the greatest release of all varieties observed with Barley P700 + MP1000 enzymes. It is therefore suggested that the lack of glycosidase activity of Econase XT resulted in a lower glucose release, which was increased when glycosidase activity was introduced with the other fibrolytic enzyme products added.

In agreement with our findings, Lafond et al (2015) found that wheat varieties with the highest AX contents delivered significantly more arabinose and xylose in an *in vitro* digest, compared to the varieties with the lowest AX content. Similarly, the Maris Huntsman variety of wheat displayed the highest total AX along with the greatest release of arabinose and xylose when used in

combination with Econase XT. While the amount of arabinose and xylose released in the digest experiments was greater than the control incubations, it was relatively low compared to the total AX values obtained from TFA hydrolysis, which can in part be explained by the fact that the AX values represent a total of both soluble and insoluble fractions. However, the analysis in the present study was on the supernatant component of digests, and therefore only quantified the soluble arabinoxylans released. This leads to the question of whether the solubility of AX is an additional characteristic required for optimal enzyme efficacy prediction? It was clear in the present study that Econase XT was the superior enzyme product for the generation of XOS products *in vitro.* Both Econase Barley P700 and Econase MP1000 contain endo-xylanase activity. Their ability to generate xylose over the 24h was similar to Econase XT. However, there was a clear difference between these two enzymes and Econase XT in their interaction with different varieties. Econase XT gave a larger range of xylose release from the 6 wheat varieties. In addition, Econase XT also gave greater quantities of XOS release and this, like xylose release, was variable dependent on the wheat variety, especially for xylobiose. This suggests that the endo-xylanase activity present in Econase XT is more sensitive to the arabinoxylan structure within each wheat variety than that of the other enzyme products in the present study. Highbury had a relatively high total xylose content and one of the lowest A:X ratios in the study, but also had the lowest xylotriose release, and the most xylobiose release following incubation with Econase XT. This is in contrast to the findings for the Sinuelo variety, which had the lowest total xylose content and the highest A:X ratio, and released more xylotriose, but the least xylobiose

when incubated with Econase XT, the reverse of that seen with Highbury. Both varieties released comparable amounts of xylose though, which shows that complete degradation was similar, albeit through slightly different hydrolysis pathways, thereby generating different XOS products from the 2 varieties. This could be related to where arabinose was substituted on the AX backbone which should be a consideration of cereal varieties used in future work. A:X ratios suggest the likely number of arabinose units per xylose unit, but it is possible that there are regions of the backbone with no arabinose substitutions – and others which are heavily substituted. Therefore, it could be that Sinuelo had more regions with longer stretches of unsubstituted xylose, which allowed more xylotriose to be released. This could be an important finding, since XOS products are thought to regulate the gut microbiota, suggesting that optimum enzyme-wheat variety combinations may be possible.

The generation of monosaccharides and XOS increased over time, especially following incubation with Econase XT. This could be important, as different time points could, hypothetically, correspond to the delivery of these products to different sections of the birds GI Tract, with later timepoints relating to delivery to the hind gut, though there is considerable stasis in the crop. These soluble sugars and XOS may then be metabolised by the microflora in the caeca, producing volatile fatty acids (VFA) (Yang et al., 2009) which may be an important mechanism for increases in bird performance. Therefore, delivery of products to the hindgut may be beneficial for the microbiome composition of the caeca. However, it is unclear whether the quantities of XOS released in the present would fuel significant VFA production or to signal hind gut bacteria to change metabolism towards AX fibre degradation (Bedford, 2018).

Indeed, one hypothesis for the role of XOS suggests that supplementation with fibrolytic enzyme in the feed for broilers results in a cascade of events leading to XOS inducing an adaptation of the microbiome and greater fibre degradation. This may occur through increased endogenous xylanase production by the microbes, which leads to even greater AX degradation and increased substrate availability for fermentation in the hind gut. It is therefore the greater capacity for the fermentation of dietary fibre, rather than the XOS products generated by the enzyme that allows for arabinoxylan hydrolysis to increase. This challenges the traditional prebiotic hypothesis, where the XOS itself was considered a quantitative prebiotic (Bedford, 2018). Future studies could assess the level of endogenous xylanase produced in the broiler hind gut with and without fibrolytic enzyme supplementation to test this theory.

Previous studies have also shown how an *in vitro* digestion model can be used for rapid and cost effective screening for activity of NSP degrading enzymes on wheat varieties, where β1,4-xylanase plus β1,4-glucanase was shown to increase the release of both arabinose and xylose (Aulrich and Flachowsky, 2001). The release of arabinose, xylose and XOS above that of controls without enzyme supplementation in the present study reinforces the idea that previously indigestible plant cell wall polysaccharides are hydrolysed into soluble fractions by enzymatic action. It is worth considering that the incubation with enzymes may have produced other oligosaccharides / polymers that were of larger molecular weight or greater complexity of branching (e.g., Arabinoxylo-oligosaccharides), however we cannot comment on this, as only X1-X4 were quantified in the present study. These would then

be expected to enter the caeca *in vivo,* where they could be broken down and fermented by the animal.

However, it must also be stated that this might not be observed *in vivo.* An in vitro model of digestion shows the capability of the enzymes tested in generation of sugars of varying molecular mass in isolation only. Enzyme efficacy could be modulated by the varying conditions present in the digestive tract, e.g. endogenous enzyme activity, gut microflora composition, nutrient absorption / utilisation, differing pH levels and moisture content variances. It is important to consider that the intestinal arabinosidase, xylanases and acetyl esterases of microbial origin may interfere with or enhance exogenous enzyme activity. Hence comparisons between *in vitro* digestion models and *in vivo* measures of digestibility are required.

4.5 Summary

This study shows that different wheat varieties have varying levels of arabinoxylan and A:X ratios. Although all the commercial enzymes used had endo-xylanase activity, they had different effects on the 6 wheat varieties, especially Econase XT which only has endo-xylanase activity. The arabinoxylan contents of the 6 wheat varieties differed and the impact of exogenous enzymes on the release of XOS content appears to be wheat variety dependent. Therefore, it does appear that there may be significant variation in XOS production dependent on the combination of wheat variety and exogenous enzyme, which may subsequently have variable effects on the gut microbiome. Future research should examine the relationship between *in vitro* and *in vivo* digests that assess the XOS and monomeric products released both

with and without fibrolytic enzyme supplementation to assess the efficacy of further *in vitro* work in this area.

4.6 Acknowledgements

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5. THE EFFECTS OF EXOGENOUS XYLANASE SUPPLEMENTATION ON THE *IN VIVO* GENERATION OF XYLOOLIGOSACCHARIDES AND MONOSACCHARIDES IN BROILERS FED A WHEAT-BASED DIET

Dale T, Hannay I, Bedford M R, Tucker G, Brameld J M and Parr T (2020). The effects of exogenous xylanase supplementation on the *in vivo* generation of xylooligosaccharides and monosaccharides in broilers fed a wheat-based diet. *British Poultry Science*. DOI: 10.1080/00071668.2020.1751805.

5.1 Abstract

This study aimed to quantify xylanase-induced changes in soluble monosaccharides, xylooligosaccharides (XOS) and volatile fatty acid (VFA) contents of the different sections of the GIT and whether these relate to altered bird performance. An *in vitro* digestion of the wheat-based diet was carried out with the xylanase (Econase XT at 16,000BXU/kg diet) to compare the *in vitro* and *in vivo* generation of these XOS and monosaccharides. For the *in vivo* study, 80 male Ross 508 broiler chicks were split into two groups fed a wheat-based diet with or without Econase XT (16,000BXU/kg diet) for 21 days from d4 of age. There were no effects of Econase XT inclusion on growth performance characteristics, likely a result of the high-quality wheat diet and corresponding high performance of the control group (FCR average of 1.45 in controls on a mash diet), but also the relatively young age (from 4 to 26 days of age). Econase XT supplementation increased the xylotetraose (X_4) content in the colon ($p=0.046$, enzyme x GIT section interaction) and the xylose contents in the colon and caeca ($p<0.001$, enzyme x GIT section interaction). The trend for increased acetate proportion in the caeca of Econase XT treated birds ($p=0.062$) suggests that the XOS generated were subsequently fermented in the caeca, potentially impacting upon the types of microbiota present. The present study suggests that wheat arabinoxylan degradation is enhanced by xylanase supplementation, which may increase the production of beneficial VFA in the caeca, and thereby potentially modulate the caecal microbiome, but without affecting bird performance (at this early stage).

5.2 Introduction

Arabinoxylan (AX) is the most abundant hemicellulose in the endospermic cell wall of wheat (Mares and Stone, 1973). Non-starch polysaccharides (NSP) like AX are anti-nutritive in monogastrics, who lack the endogenous enzymes to break down plant cell walls (Choct and Annison, 1990). The result is an increase in digesta viscosity, which has negative effects on performance (Bedford, 2000). The supplementation of endo- β1, 4-xylanases effectively hydrolyse the xylan backbone of AX, generating arabinoxylan-oligosaccharides (AXOS) which encompasses both arabino-xylooligosaccharides and xylooligosaccharides (XOS) (Jommuengbout et al., 2009). Monosaccharides are generated if any contaminating β- xylosidase or arabinofuranosidase is present in an enzyme product or within the diet itself, cleaving β- xylosidic glycosidic linkages into the monomeric pentoses (e.g. xylose and arabinose) and hexoses (e.g. glucose and galactose). This results in a reduction in viscosity and improved growth rates and feed efficiency (Bedford and Classen, 1992). It is also hypothesised that the resulting short-chain AXOS from enzyme hydrolysis are utilised for fuel by the microbiota occupying the distal gastro-intestinal tract (GIT), which can have positive effects on the microbiome (Choct et al., 1999).

NSP account for 10-12% of the dry matter (DM) in wheat (Knudsen, 1997), of which 19-21% is soluble (Rodehutscord et al., 2016). The insoluble NSP in wheat mainly provides protection in the form of the cell wall (Simon et al., 2015). As per the cell wall degradation hypothesis, xylanase shows significant cell wall destruction *in vivo* (Bedford and Autio, 1996), which enhances access to cell contents by the endogenous enzymes, increasing their efficacy, thereby

increasing amino acid retention, modulating host pancreatic amylase and mucin secretion, all aiding digestion (Cowieson and Bedford, 2009).

There is also evidence that feeding xylanase influences the caecal microbiome, where beneficial bacteria ferment the end products of enzyme hydrolysis (McCracken et al., 2006, Masey-O'Neill et al., 2014). This is presumably from the generation of AXOS during hydrolysis, as feeding near-pure AXOS results in similar performance benefits to xylanase inclusion itself (Morgan et al., 2019). AXOS are thus displaying prebiotic effects, in that they are fermented into volatile fatty acids in the microbiome, particularly acetate and butyrate in the caeca and colon (Choct et al., 1999). Walker et al (2005) suggested that AXOS substrate fermentation in the colon significantly lowered the pH, which gave a boost to butyrate producing bacteria, whilst hampering the proliferation of *Bacteroides* spp. which have the capacity to cause harm to their host through amino acid fermentation which can produce ammonia (Rehman et al., 2007). This subsequently improves the gut environment and could also lead to better mineral absorption and immune response along with increasing gizzard grinding which can help the mechanical breakdown of digesta for greater susceptibility to enzyme activity, all leading to more efficient nutrient absorption (Kim et al., 2011).

One theory is that there is increased recovery of energy from the diet as volatile fatty acids (VFAs). The issue with this theory is that the AXOS studies so far used doses ranging from 0.1g – 10g per kilogram of diet (Ribeiro et al., 2018, Suo et al., 2015, Eeckhaut et al., 2008). The lower levels of inclusion are unlikely to provide enough substrate to generate significant amounts of energy in the form of VFA. The other issue is that in chickens, it is necessary to feed

xylanase over an extended period for a response to be observed. If the enzyme were generating significant quantities of AXOS instantaneously, which were then fermented, there would not be a delay in the response seen after enzyme inclusion. However, studies show performance benefits in wheat-based diets are often not seen until the birds reach 14d of age (Mendes et al., 2013).

Hence a new hypothesis (Bedford, 2018), suggests that the chicken microbiome is 'trained' over time as a result of xylanase supplementation. Rather than NSPases producing more fermentable sugars, it may be that NSPases produce AXOS that signal to the microbiome, thereby increasing the capacity to degrade fibre. This theory is supported by a recent study showing that chickens fed xylanase for 35d had greater fermentation of pentoses and AXOS in their caecum than controls (Bedford and Apajalahti, 2018).

Whether it is more efficacious that AX are hydrolysed into AXOS *in situ* via endogenous enzyme supplementation or to supplement diets with AXOS that has been prepared *in vitro* remains to be established.

Although AXOS are also likely released, the aim of this study was to quantify the XOS and monosaccharides generated when a commercially available xylanase (Econase XT) was fed to broilers on a wheat-based diet between d4 and d25/26 of age, to establish where the nutrients are generated and/ or utilised along the GIT. In addition, the *in vitro* digestion of the wheat-based diet was compared to the contents of the different sections of the GIT *in vivo*. Caecal VFA contents were also determined as an indication of possible effects of Econase XT on the activity of the microbiome and potential energy provision to the diet.

5.3 Materials and Methods

5.3.1 Diet and Enzyme used

The diet was formulated by a commercial feed manufacturer in mash form (Target Feed Ltd, England) and the ingredients and nutritional values are shown in Table 5.1. Econase XT 25 is a commercially available xylanase with reported β-1,4 endo-xylanase activity (160,000 BXU/g) provided by AB Vista (Marlborough, UK) and used at the recommended dose of 100µg/g diet, which provides 16,000 BXU/kg, where BXU represents the amount of enzyme required to release 1 µmol of reducing sugar per min from xylan under defined test conditions. This enzyme was chosen as it was shown to be most efficacious enzyme product when added to wheat in previous experiments.

5.3.2 Total hydrolysis of non-cellulosic polysaccharides in the diet

Total non-cellulosic sugar contents in the diet were determined by total hydrolysis of non-cellulosic polysaccharides using Trifluroacetic acid (TFA), as previously described (Fry, 1988). Briefly, the diet sample was ground to a fine powder (0.5mm) and suspended to 10mg/ml in 2M TFA in triplicate. Tubes were sealed and heated to 120°C for 1 hr in an autoclave, then allowed to cool to room temperature before being centrifuged at 2236×g for 10 min at room temperature. The supernatant was then diluted 1:100 with 10mM NaOH and transferred into 2ml clear vials for sugar analysis. The content of the 4 monosaccharides (arabinose, galactose, glucose and xylose) was quantified, but there were no measurable XOS.

5.3.3 *In vitro digestion of the wheat-based diet with Econase XT*

The diet samples were individually ground to a fine powder (0.5mm) and 0.2g was resuspended (in 4 replicates) in 40mls 50 mM sodium citrate buffer (pH 5.2) either without (Control – no enzyme) or with Econase XT 25 at 16,000 BXU/kg. The buffer was chosen to represent the average pH of the broiler digestive tract (Mabelebele et al., 2014). Digestion reactions were then placed in a shaking incubator at 150RPM and a temperature of 41°C for up to 24 h. At each time point (0, 3, 6, 9, 12 or 24h), 1ml of each digest was removed and added to 9ml of 10 mM NaOH at room temperature, mixed, centrifuged at 2236×g for 10 min at room temperature and then frozen at -20°C prior to sugar analysis.

5.3.4 *Chicken trial*

The trial was conducted at the University of Nottingham Bio-Support Unit using 80 one-day-old male Ross 308 broiler chickens (average body weight 42g), from a total batch of 95 birds, obtained from P D Hook Hatcheries Limited (Cote, Bampton OX18 2EG). Birds were housed and cared for according to the UK Animals (Scientific Procedures) Act 1986 (ASPA) Code of Practice for the care and accommodation of animals (February 2013), approval reference number 197. Birds were wing tagged for identification, acclimatised in one group, and fed the control diet (without enzyme) between days 1-4. On day 4, after acclimitisation, they were allocated to 20 pens in groups of 4 birds per pen, matched with birds of similar weights. Hence there were 10 replicate pens per diet (control and enzyme treated) each with four birds, all contained within the same room.

All birds were given *ad libitum* access to diet and water throughout the study and were raised under controlled conditions of light, temperature and humidity as recommended by the breeder. Temperature was maintained at 32° on arrival and reduced by approximately 1°C per day until 21°C was reached as per the Ross 308 Management Guidelines.

Body weight for individual birds was monitored and recorded following the start of access to the experimental diets on 4 (experimental diet d0), 11, 18 days of age and immediately following culling on days 25 or 26 (5 random pens per treatment were culled on 2 consecutive days). Diet consumption and body weight gain were measured between 4 and cull at 25 or 26 days of age to calculate feed conversion ratio (FCR). Birds from pens 1-10 were culled on 25 days of age and pens 11-20 on 26 days of age by Schedule 1 method (Animals (Scientific Procedures) Act 1986). Samples of intestinal digesta were collected from 5-cm segments of the mid-jejunum, proximal ileum and colon, as well as the caecal contents for each bird. Digesta samples were snap frozen using liquid nitrogen immediately after collection and stored at -80 °C prior to analysis. The gut contents of one representative bird closest to average pen weight from each pen was analysed for monosaccharide and XOS contents, whereas the first bird from each pen was selected for VFA analysis on the caecal contents and processed accordingly, before being sent to Alimetrics Ltd (Finland) for analysis.

5.3.5 Identification and quantification of sugars using HPAEC-PAD

The sample contents of arabinose, galactose, glucose and xylose, as well as the XOS (g/100g), were determined after being defrosted and diluted, using High-

Performance Anion-Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) following the method of Xu et al (Xu et al., 2013). Analysis was carried out using a Dionex ICS-3000 with a Dionex CarboPac PA20 Column (3mm x 150mm) and CarboPac PA20 Guard (3x30mm) for the monosaccharide analysis. A CarboPac PA200 column (3mmx250mm) and CarboPac PA200 guard (3mm x 50mm) were used for the oligosaccharide analysis. An injection volume of 10μl was used throughout for all standards and samples. Monosaccharide standards (arabinose, galactose, glucose and xylose) were purchased from Sigma-Aldrich, UK and XOS standards (xylo-biose, -triose and –tetraose) from Megazyme, Ireland. Serial dilutions for each standard (2.0, 1.0, 0.5 and 0.25g/L for monosaccharides and 2, 1, 0.5 and 0.25g/L for XOS) were made fresh for each batch of analyses, diluted 1:100.

For the monosaccharides, a single eluent, containing 10mM NaOH solution, was used as the mobile phase at 0.5ml/min for 14 min. For oligosaccharides, 2 eluents were used in a gradient for the mobile phase, 0.1M sodium hydroxide (Solution A) and 0.1M NaOH containing 0.5M sodium acetate (solution B) in standard quadruple waveform, as described by Xu et al (2013). The gradient program used for XOS determination was 100% solution A at 0 minutes, rising to 80% solution A and 20% Solution B at 25 minutes, before returning to 100% Solution A after 25 minutes elapsed. Both eluents were stored in plastic pressurised bottles with inert nitrogen gas at 6-9 psi. Data were collected with Dionex Chromeleon software (Version 6.7). Dry matter content of the digesta was determined by oven drying the digesta at 60°C for 36h. Monosaccharide

and XOS contents from *in vivo* digesta samples were then adjusted to dry matter contents.

5.3.6 Measurement of Volatile Fatty Acids & Lactic Acid

The VFAs in the caeca of one randomly selected bird from each pen were analysed as free acids by Alimetrics Ltd (Espoo, Finland), using gas chromatography, as described previously (González-Ortiz et al., 2019, Holben et al., 2002). In brief, 1g caecal contents were vigorously mixed with 1ml H_2O for 5 minutes, before 1ml of 0.8M perchloric acid was added and the mix shaken to extract the VFAs & lactic acid. The acids measured were acetic, butyric, lactic, propionic, valeric, and total branched chain fatty acids.

5.3.7 Data and Statistical analysis

For the *in vitro* digests, one source was used for the diet and all analyses (digestions and Dionex analysis) were carried out in four batches of control and Econase XT combinations. The data was then processed in excel (Microsoft, 2013) and expressed as means and standard error of the mean (SEM).

Standards for the four monosaccharides or XOS were run at the start and end of each batch, standard curves were generated from the areas under the curve and presented as g/100g of diet or digesta. Data was then analysed by one- (enzyme) or two-way (enzyme x time or enzyme x GIT section) ANOVA, as appropriate, using Genstat statistical software (19th Edition), with blocking for batch and tube in the *in vitro* digests. Bonferroni post-hoc tests were used to identify significant differences between groups following a significant ANOVA. $p<0.05$ was taken as statistically significant, and $p<0.10$ was described as a trend.

For average daily feed intake (AFDI), average daily gain (ADG) and feed conversion ratio (FCR), data was analysed by one-way ANOVA (Genstat statistical software, 19th Edition). For body weight, data were analysed by two-way (treatment x time) ANOVA with repeated measures (Genstat statistical software, 19th Edition). $p<0.05$ was taken as statistically significant in both instances.

For the correlations of XOS and monosaccharides across GIT sections, data were analysed by Pearson correlation coefficients, assuming gaussian distribution, using GraphPad Prism (Version 8.1.2). A two-tailed test was undertaken to determine statistical significance with a 95% confidence interval. $p<0.05$ was taken as statistically significant, and $p<0.10$ was described as a trend.

5.4 Results

5.4.1 Total hydrolysis of non-cellulosic polysaccharides in trial diet

The total sugar contents were determined by TFA hydrolysis of the control diet (Table 5.2). As expected, the highest monosaccharide in the diet was glucose, while the xylose and arabinose contents were similar (Table 5.2). It is assumed that the vast majority of these two monosaccharides are present as arabinoxylan (AX), and as such the total AX content and arabinose to xylose ratio were calculated. Galactose was present in fairly high amounts, presumably due to the use of soyabean meal in the diet, which is known to have high levels of galactose in various forms (Irish and Balnave, 1993). These total hydrolysis values for each monosaccharide were subsequently used to calculate the proportion that was released during the *in vitro* digestions and in the *in vivo* experiment with and without Econase XT supplementation.

Table 5. 1 Ingredient constituents and calculated nutritional values of the experimental diet.

% Ingredient unless otherwise specified			
Diet ingredient constituents [1]		**Calculated nutritive values [1]**	
Raw ground wheat	55	**Protein**	20.2
HiPro soya	23	**Energy ME, MJ**	12.96
Raw ground barley	9.37	**Oil**	6.36
Full fat soya	5	**Fibre**	3.07
Soya oil	4	**Ash**	5.35
Limestone flour	0.95	**Calcium**	0.80
Monocalcium phosphate	1.18	**Available P**	0.41
Vitamin and mineral premix[b]	0.4	**Sodium**	0.18
DL-Methionine	0.35	**Lysine[a]**	1.19
L-Lysine HCl	0.2	**Methionine[a]**	0.63
L-Threonine	0.15	**Methionine + Cysteine[a]**	0.94
Salt	0.25	**Threonine[a]**	0.85
Sodium bicarbonate	0.15	**Tryptophan[a]**	0.24

[1] The same diet was used for the 2 treatments: Control (diet as shown) and Econase XT (Control diet supplemented with 16,000 BXU/kg of Econase XT 25).

[a] Amino acid levels are expressed as standardised ileal digestible content.

[b] Supplying: retinoic acid 3mg/kg, cholecalciferol 125µg/kg, α-tocopherol 100mg/kg, thiamine 3 mg/kg, riboflavin 10 mg/kg, pyridoxine 3 mg/kg, cobalamin 30 µg/kg; nicotinic acid 60 mg/kg, pantothenic acid 15 mg/kg, folic acid 1.5 mg/kg, biotin 250 µg/kg, choline chloride 25 mg/kg, Fe 20 mg/kg, Cu 10 mg/kg, Mn 100 mg/kg, Co 1.0 mg/kg, Zn 80mg/kg, I 1 mg/kg, Se 0.25 mg/kg and Mo 0.5 mg/kg.

Table 5. 2 Monosaccharide composition, total Arabinoxylan (AX) content and Arabinose: Xylose (A:X) ratio of the wheat-based diet after Trifluroacetic acid hydrolysis of non-cellulosic polysaccharides.

Monosaccharide[1]	g/100g diet
Arabinose	9.21 ± 1.48
Galactose	6.22 ± 1.04
Glucose	18.11 ± 0.17
Xylose	10.59 ± 0.28
Total	44.16 ± 0.67
Total AX[2]	19.80 ± 1.63
A:X[3]	0.87 ± 0.13

[1]Values of monosaccharides are expressed as mean grams per 100g of diet ± SD of four replicates. [2]Total AX values are calculated as mean grams of Arabinose + Xylose per 100g cereal ± SD of four replicates.

[3]A:X ratio calculated as Arabinose (g/100g cereal) divided by Xylose (g/100g cereal) ± SD of four replicates.

5.4.2 In vitro digestion of the wheat-based diet with or without Econase XT – release of xylooligosaccharides (XOS) and Monosaccharides

There were significant enzyme x time interactions seen for the release of xylotetraose (X_4), xylotriose (X_3) and xylobiose (X_2, all $p<0.001$). X_4 was released from the diet in the absence of Econase XT but *post hoc* Bonferroni tests revealed that the release was greater with Econase XT at 3h, 9h, 12h and 24h ($p<0.001$, figure 5.1A). Similarly, X_3 was also released from the diet in the absence of Econase XT, peaking at 3h (Figure 5.1B) and then either declining (Control) or remaining flat (Econase XT). *Post hoc* Bonferroni tests, indicated greater concentrations with Econase XT only at 12h and 24h, with no differences at earlier timepoints. In contrast, there was no release of X_2 from the diet in the absence of Econase XT (Figure 5.1C), with significant release of X_2 only observed after 24h incubation with Econase XT.

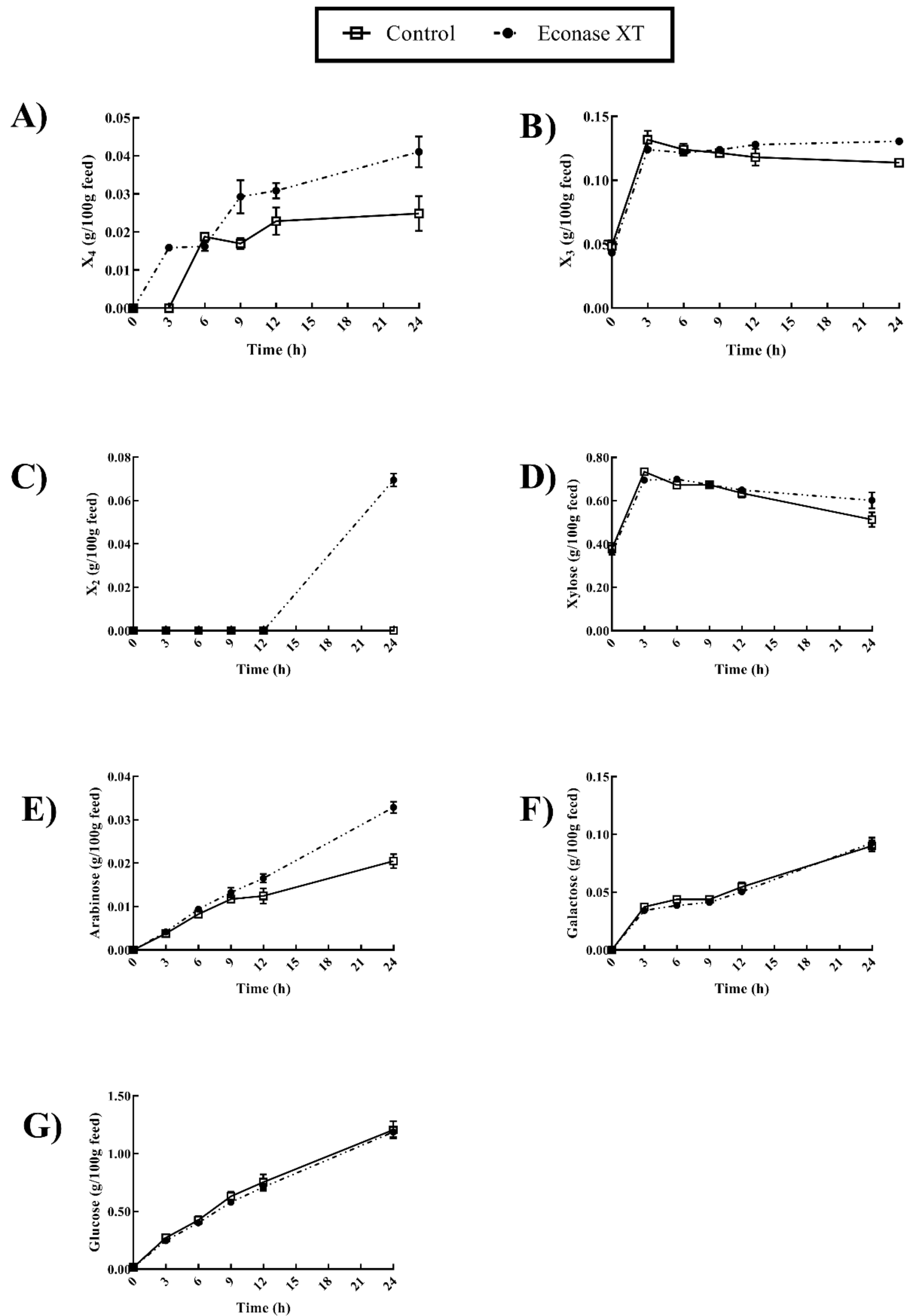

Figure 5. 1 Time-dependent release of Xylo-oligosaccharides and monosaccharides from the wheat-based diet during an *in vitro* incubation at 41°C in the presence or absence of Econase XT for 24 hours. A: Xylotetraose (X_4) B: Xylotriose (X_3) C: Xylobiose (X_2) D: Xylose, E: Arabinose, F: Galactose, G: Glucose. Data show mean values ± standard error of the mean (SEM). Two-way ANOVA indicated significant enzyme x time interactions for X_4, X_3, X_2 (all $p<0.001$), Xylose ($p=0.034$) and Arabinose ($p<0.001$). There were significant effects of time for Galactose ($p<0.001$) and Glucose ($p<0.001$), but no effects of enzyme.

There were significant enzyme x time interactions for the release of xylose ($p=0.034$) and arabinose ($p<0.001$). As observed for X_3, xylose was released in the absence of Econase XT and both groups peaked at 3h, before declining (Figure 5.1D). *Post hoc* Bonferroni tests indicated greater levels with Econase XT only at 24h, with no differences at earlier timepoints. In contrast, arabinose was released linearly from 0-9 hours, both with and without Econase XT (Figure 5.1E). *Post-hoc* Bonferroni tests showed significantly more was released with Econase XT at 12h and 24h. Unlike the release of xylose, there were no significant enzyme x time interactions for the release of galactose or glucose (Figures 5.1F and G respectively), nor were there any effects of enzyme, but the release of both increased with time (both $p<0.001$).

5.4.3 The effect on Broiler performance of Econase XT in a wheat-based diet

There were no differences in average daily feed intake (ADFI), average daily gain or feed conversion ratio (FCR) of broiler chickens fed the diets for 3 weeks (Table 5.3). There were no mortalities.

Table 5. 3 Effect of inclusion of Econase XT in diet on broiler performance.

	Control[1]	Econase XT[1]	SED [2]	P-Value
ADFI [3] (d4-cull) (g/bird/d)	61	68	14.171	0.11
ADG [4] (d0-cull) (g/bird/d)	42	43	9.093	0.67
FCR [5] (d0-cull)	1.45	1.61	0.126	0.23

[1]Data are means for ten replicate pens per treatment, with four birds per pen.
[2]SED, Standard error of the difference of the means;
[3]ADFI, average daily feed intake;
[4]ADG, average daily gain;
[5]FCR, feed conversion ratio.

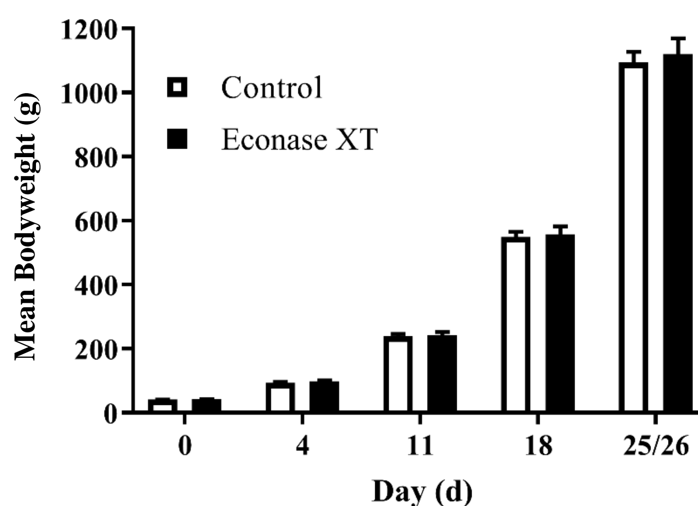

Figure 5. 2 Effect of inclusion of Econase XT in diet on broiler bodyweight. Data show mean values ± standard error of the mean (SEM). Data represent ten replicate pens per treatment, with four birds per pen. There was no enzyme x time interaction (p=0.716), nor any effect of enzyme (P=0.686).

5.4.4 The effect on release of xylooligosaccharides (XOS) and monosaccharides at different sections of the broiler gastro-intestinal tract (GIT) of Econase XT in a wheat-based diet

There was a significant enzyme x GIT section interaction for X_4 release ($p=0.038$, Figure 5.3A), such that X_4 concentration was highest in the colon of the birds supplemented with Econase XT, but there was no X_4 observed in the caeca. There was no enzyme x GIT section interaction for X_3 release (Figure 5.3B) and no effect of Econase XT, but there was a highly significant effect of GIT section ($p<0.001$). In agreement with in vitro findings, the X_3 content was highest in the ileum followed by the jejunum and colon, with the lowest concentrations observed in the caeca. As for X_3, there was no enzyme x GIT section interaction nor any effect of Econase XT for X_2 release (Figure 5.3C), but there was a highly significant effect of GIT section ($p<0.001$). The concentration of X_2 declined down the GIT, although the concentrations in the ileum and colon were not significantly different nor were those in the colon and caeca.

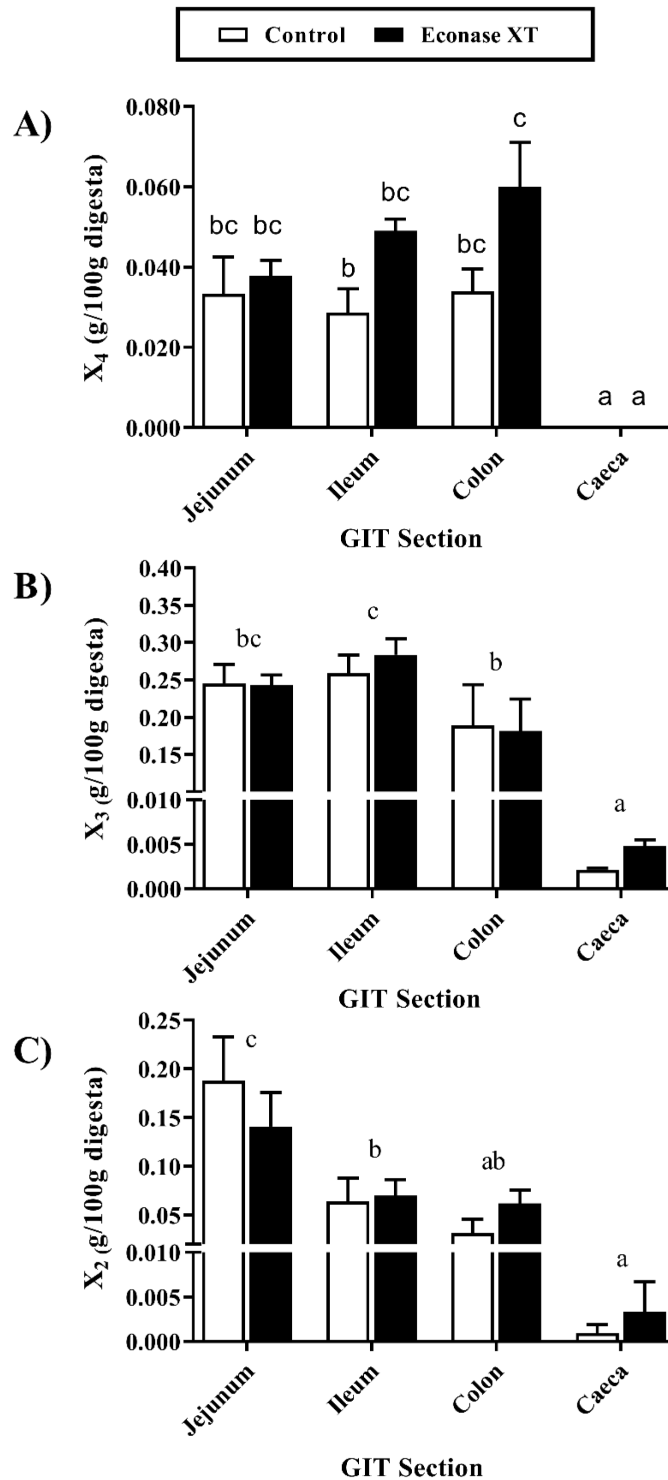

Figure 5. 3 Effects of Econase XT inclusion in the diet on release of Xylooligosaccharides at different sections of the broiler gastro-intestinal tract (GIT). Data show mean g/100g of digesta dry matter ± standard error of the mean (SEM) for 10 replicates per treatment (1 bird per pen per treatment) for A: Xylotetraose (X_4) B: Xylotriose (X_3) C: Xylobiose (X_2). Two-way ANOVA indicated a significant enzyme x gut section interaction for X_4 ($p=0.038$) and significant effects of gut section for both X_3 and X_2 (both $p<0.001$). [a,b,c,d] Columns with different superscript letters were significantly different ($p<0.05$, Bonferroni *post-hoc* test). In B and C *post-hoc* Bonferroni tests were for effects of gut section.

As observed for X_4, there was a significant enzyme x GIT section interaction for xylose ($p<0.01$, Figure 5.4A). Econase XT increased xylose content in the colon and caeca, with no detectable xylose in the jejunum or ileum of any of the birds irrespective of whether the enzyme was present. Unlike xylose release, there were no significant enzyme x GIT section interactions for arabinose, galactose or glucose (Figure 5.4B, C and D respectively). Econase XT significantly increased the release of arabinose ($p=0.012$) and tended to increase galactose concentration ($p=0.071$) in all sections of the GIT. There were significant differences between the sections of the GIT for release of arabinose, galactose and glucose ($p<0.001$). Arabinose concentrations were larger in the colon than in the jejunum and ileum, with the caeca being intermediate (Figure 5.4B), whereas galactose was highest in the colon (Figure 5.4C). In contrast, glucose concentrations declined down the GIT but with no difference between colon and caeca (Figure 5.4D).

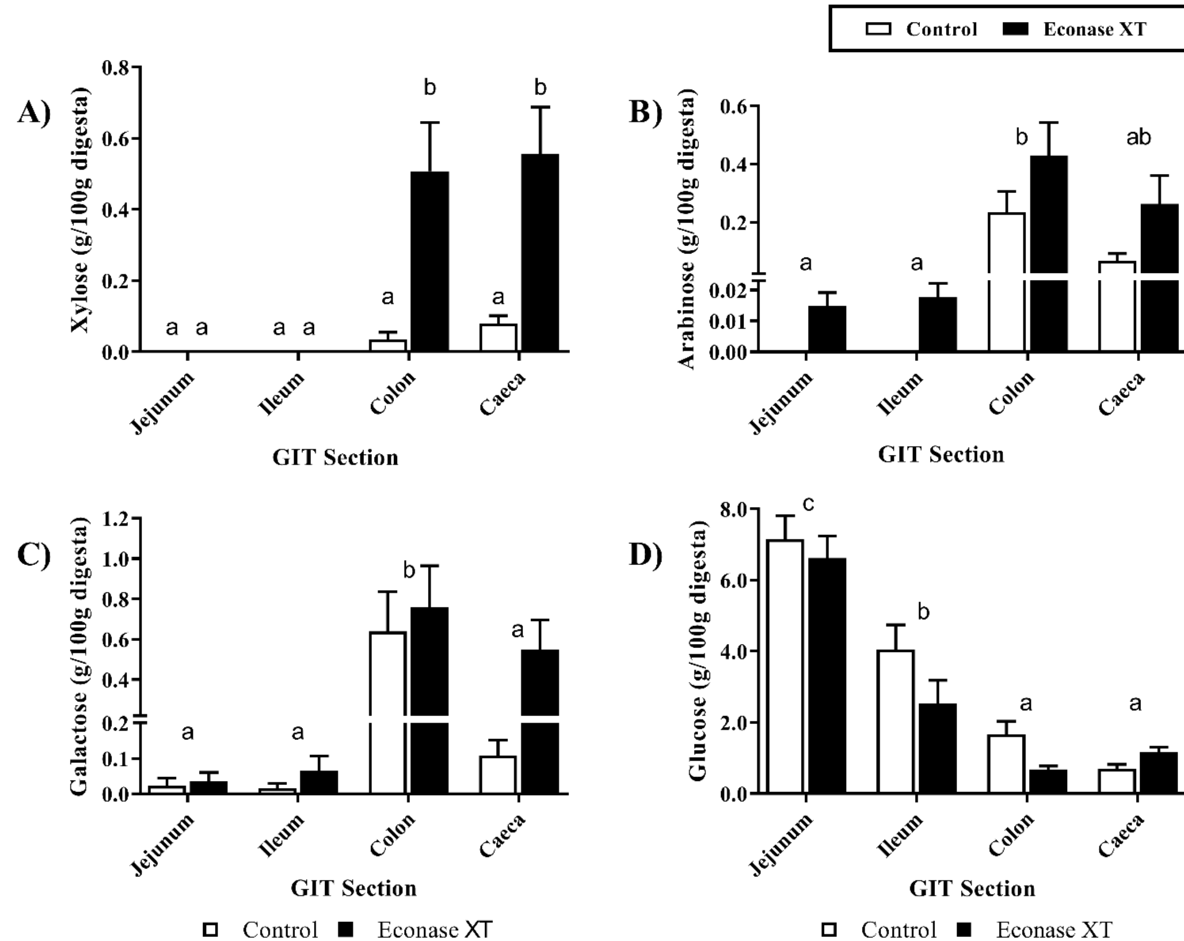

Figure 5. 4 Effects of Econase XT inclusion in the diet on release of Monosaccharides at different sections of the broiler gastro-intestinal tract (GIT). Data show mean g/100g of digesta dry matter ± standard error of the mean (SEM) for 10 replicates per treatment (1 bird per pen per treatment) for A) Xylose, B) Arabinose, C) Galactose and D) Glucose. Two-way ANOVA indicated a significant enzyme x gut section interaction for Xylose ($p<0.001$) and significant effects of gut section for Arabinose, Galactose and Glucose (all $p<0.001$). There was also a significant effect of enzyme for Arabinose ($p=0.012$) and a trend for Galactose ($p=0.071$). [a,b,c,d] Columns with different superscript letters were significantly different ($p<0.05$, Bonferroni post-hoc test). In B, C and D *post-hoc* Bonferroni tests were for effects of gut section.

As xylanase increased X_4 in the colon (but not in the caeca) and xylanase increased xylose in the caeca, correlations were done between colonic X_4, X_3 or X_2 contents and caeca xylose or other monosaccharides. The correlations for X_3 or X_2 were not significant (Supplemental 1). There appeared to be positive relationships between X_4 and both xylose and arabinose contents, but negative relationships with galactose and glucose contents. However only colonic X_4 and caecal arabinose contents showed a trend for positive correlation ($p=0.089$, Figure 5.5B), whereas none of the other correlations were significant (Figure 5.5).

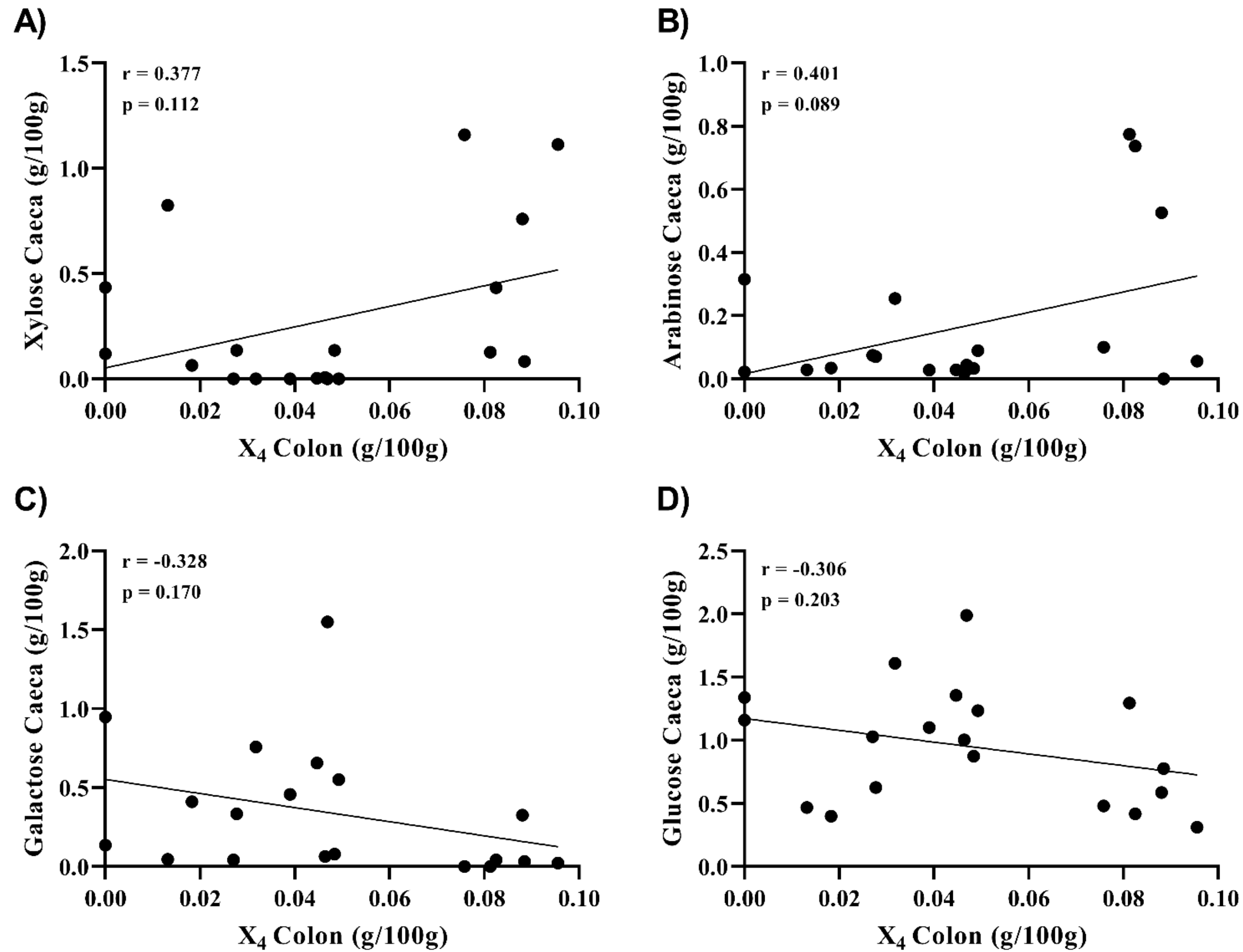

Figure 5. 5 Correlations between colonic xylotetraose (X_4) contents and caecal monosaccharide contents. Data show colonic X_4 contents (g/100g of digesta dry matter) against caecal contents for A) Xylose, B) Arabinose, C) Galactose and D) Glucose (all g/100g of digesta dry matter). Pearson correlation coefficients indicated a positive trend for colonic X_4 against caecal arabinose (p=0.089), but not for xylose (p=0.112), galactose (p=0.170) or glucose (p=0.203).

5.4.5 Proportions of Volatile Fatty Acids & Lactic Acid in the Caeca

Acetic acid was the most abundant VFA in the caeca (Figure 5.5). Total concentration of acetic acid was not statistically different between control birds (91.7mM) and Econase XT birds (97.8mM, p= 0.338). However, control birds had a slightly lower (p= 0.062) proportion of acetic acid (74%) than birds supplemented with Econase XT (78%). Others were in the order Butyric acid > Lactic acid > Propionic acid > BCFAs > Valeric acid (Figure 5.6), but there were no significant differences in proportions in the caeca of birds fed the Control and Econase XT supplemented diets.

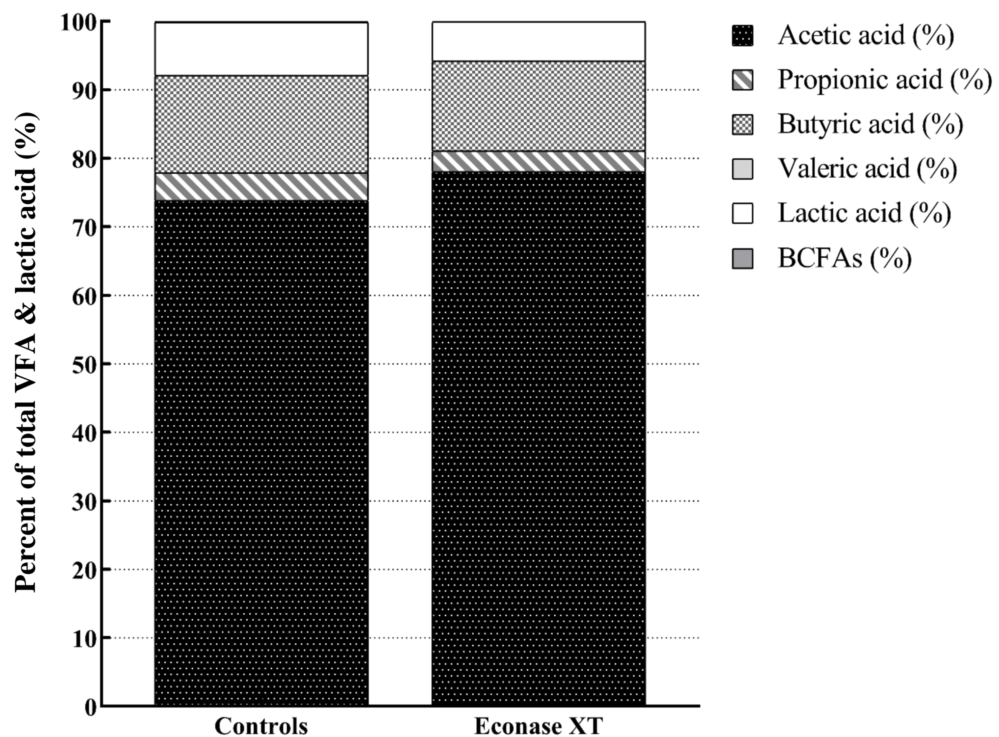

Figure 5. 6 Effects of Econase XT inclusion in the diet on the proportions of Volatile Fatty Acids (VFA) and lactic acid in the caeca of male Broilers. Data are means of 10 randomly selected birds (one from each pen) expressed as % of the total VFA/SCFA content. There was a trend for Econase XT supplementation to increase the proportion of acetic acid in the caeca (p=0.062).

5.5 Discussion

To our knowledge, this is the first *in vivo* study to quantify the generation of XOS and release of monosaccharides through the GIT of broilers and to compare that to an *in vitro* model of digestion for the same enzyme and diet combination. The main findings of this study are that Econase XT supplementation of a wheat-based diet increased the X_4 content in the colon, the arabinose content throughout the GIT and the xylose content in the colon and caeca of male broilers. Although other XOS and monosaccharides were detected in the GIT, they were unaffected by Econase XT supplementation.

There was a larger quantity of X_4 in the colon of broilers fed Econase XT, but this was lost in the caeca, where X_4 was undetectable in both treatment groups. Econase XT increased the generation of X_4 along the GIT from jejunum to ileum before peaking in the colon and vanishing in the caeca. There are currently no reports of X_4 transporters and XOS are resistant to digestion via saliva, gastric juices, pancreatin and the intestinal mucosa, limiting their absorption (Fujikawa et al., 1991). Therefore, the reduced X_4 content between colon and caeca was likely a result of factors acting on AXOS in the GIT, potentially the microbiome. These factors may also be responsible for the increased xylose contents seen in the colon and caeca. AXOS are known to be readily fermented by the microbial populations that inhabit the caeca (Kiriyama et al., 1992). Their fermentation, along with that of xylose, can lead to greater acetate production, so the increased proportion of acetate observed with Econase XT potentially indicates greater fermentation of AX products in the caeca (Johnson et al., 2006).

Interestingly there were increases in X_4 generation associated with Econase XT, but not X_2 or X_3. This might suggest that the endogenous xylanase enzymes present in both the wheat source used and the broilers GIT were able to hydrolyse bonds in the AX chain associated with X_2-X_3, but the addition of Econase XT was necessary to create AXOS products with more than 3 degrees of polymerisation. Since X_2, X_3 and X_4 are not thought to be absorbed due to a lack of transporter system, then concentrations of each XOS might be expected to increase down the GIT, before reaching the caeca where they may be metabolised by the microbiome. This was true for X_4, but X_2 declined and X_3 remained constant across the GIT, suggesting that X_2 may be more easily degraded to xylose by pre-caecal microbiome activity or that there are differential specificities for oligosaccharide fermentation with different degrees of polymerisation. It is important to remember that the concentrations along the GIT reflect a single time point, that reflect a balance between generation of XOS and monosaccharides by enzymatic action and disappearance by absorption/utilisation *in vivo*, whereas there was no disappearance mechanism with the *in vitro* model. Therefore, *in vitro,* in the situation where no detectable molecules are observed, it can not be directly assessed if there has been no release or whether these further molecules have been further metabolised or absorbed. Perhaps there could be a shift towards the measurement of unreleased components in future works, where starting point is measured against a measurement differential as another method to inform XOS release potential.

Comparing the *in vitro* data to the *in vivo,* the X_4 generation *in vitro* was most like the *in vivo* findings. At the 9h time point *in vitro* there was a clear increase

in X_4 associated with Econase XT addition to the diet. The X_4 contents in the colon were also increased by Econase XT *in vivo* by Econase XT. There were no effects of Econase XT on the *in vitro* levels of X_3, which was also reflected *in vivo.* In contrast there was an increase in X_2 *in vitro,* but only at the 24h time point, which did not reflect *in vivo* digests. Although broiler GIT transit time can lost for longer periods of time, it is known to be rapid, with marker first appearing in the faeces 90 minutes after consumption and a peak at 220 minutes (Summers and Leeson, 1986).

The monosaccharides arabinose and galactose, both increased linearly over 24 hours during *in vitro* digests, with arabinose release being greater with Econase XT during the final 12h of digestion than controls. This contrasts with the *in vivo* profiles, where arabinose and galactose were low in the jejunum and ileum, yet markedly greater in the colon, before decreasing again in the caeca. This could be because enzyme hydrolysis of arabinose and galactose does not occur before reaching the colon, or because arabinose and galactose are being rapidly removed from the GIT by their corresponding transporter proteins in the jejunum and ileum. Arabinose and galactose appear to be well utilised in the early stages of GIT, based on data from other species (Schutte, 1990, Csáaky and Ho, 1965, Wagh and Waibel, 1966).

Glucose release also increased linearly throughout the 24h time course *in vitro*, contrasting with *in vivo,* where glucose levels decreased down the GIT, with no effect of Econase XT both *in vitro* and *in vivo*. The gradual decrease down the GIT is likely due to glucose being released from the starch component of the diet followed by rapid absorption in the jejunum and ileum (Klasing, 1998), via both glucose transporter (GLUT) and sodium-glucose cotransporter

(SGLT) proteins (Braun and Sweazea, 2008), resulting in very little getting through to the colon and caeca.

There were increases in xylose release due to Econase XT supplementation only at 24h *in vitro*, whereas xylose contents were significantly larger in the colon and caeca of Econase XT supplemented birds. The lack of xylose seen in the jejunum and ileum was probably due to rapid absorption in the chicken small intestine as previously described (Schutte et al., 1991, Longstaff et al., 1988). The increase in xylose in the colon and caeca could result from the colonic and caecal bacteria producing their own xylanases after being activated by Econase XT, which then more aggressively attack the fibre component of the diet. This suggests that Econase XT supplementation may be inducing adaptations to the microbiome in the colon and caeca over time, rather than providing an acute effect of increasing AX hydrolysis. This is supported by the ratio of free arabinose and xylose being closer to one, much higher than would be susceptible to Econase XT attack, suggesting activity other than the added Econase XT may be responsible for the release of these monomers.

One question is why X_3 and X_2 concentrations along the GIT were not different between controls and Econase XT treated birds? It has previously been shown that wheat can contain endogenous xylanase activity (Dornez et al., 2008), suggesting that the wheat-based diet used may also contain endogenous xylanase activity that generated XOS without inclusion of exogenous Econase XT. The generation of XOS without any exogenous enzyme may also be due to the birds being fed a mash rather than a pelleted diet. Pelleting requires high temperature and high pressure, which would be expected to denature the proteins present, thereby reducing any endogenous

xylanase activity. The results from the *in vitro* digests of the same diet would appear to confirm this, with similar release of xylose, galactose and glucose observed with or without Econase XT addition. This suggests endogenous xylanase causes the release of certain monosaccharides *in vitro*, but this is not enhanced further by exogenous xylanase. We therefore suggest there might be greater effects of Econase XT in broilers fed a pelleted diet or a mash diet where the wheat contains either less endogenous xylanase activity or more xylanase inhibitors (Juge et al., 2004). Despite this, there were still significant effects of Econase XT on the generation of X_4, xylose and arabinose, particularly in the colon and caeca. We propose that the Econase XT produces more X_4 in the colon, thereby activating the gut microbiome through provision of substrate, leading to further enzyme attack to produce the larger levels of xylose and arabinose seen in the colonic digesta and caeca. This appeared to be supported by the correlation analysis, where there tended ($p=0.089$) to be more arabinose in the caeca of broilers with the highest X_4 contents in the colon, with a similar positive relationship seen for caecal xylose and colonic X_4 although this was not significant ($p=0.112$). The fact that similar relationships were not seen for X_3 and X_2 suggests that undigested X_4 produced in the colon is being utilised in the caeca (Svihus et al., 2013), potentially resulting in the increased xylose and proportion of acetate seen in the caeca of Econase XT supplemented broilers. This increase in proportion of acetate in the caeca with Econase XT supplementation agrees with previous studies (Kabel et al., 2002, Ravn et al., 2018), showing that acetate and butyrate are produced by the fermentation of pure AXOS or via xylanase action. However, there was no effect of Econase XT on butyrate in the present study. Whether the observed

increase in acetate represents a change in the microbiome is unclear but does appear to support the hypothesis that the chicken microbiome is ‘trained’ or adapted over time as a potential mechanism for the effects of xylanase supplementation.

The lack of effect on bird performance could be due to the limited time frame, and late exposure to Econase XT, as many studies have supplemented xylanase from day of hatch, when the microbiome is rapidly developing. Birds were given Econase XT supplementation from 4 days of age for a total of 21d. This relatively short exposure implemented from d4 may be too short or may be before the birds’ microbiome had established or had completely adapted but was carried out to allow birds to acclimatise before experimental diets began. A recent study (Figueiredo et al., 2012) showed that xylanase supplementation from d1 of age only had significant effects on FCR at 28 days of age, though, as previously mentioned, other studies have observed significant effects after 14 days (Mendes et al., 2013), highlighting the need for more research in this area.

The present study shows that there was degradation by Econase XT of AX in the wheat-based diet, as indicated by larger xylose, arabinose and X_4 levels present, particularly in the colon and caeca of male broilers. This may then be responsible for the observed increase in acetate production by the caecal microbiome and may be indicative of the microbiome adapting. Although these effects did not result in improved performance of the birds, this could be explained by a) the presence of endogenous xylanase activity in the diet, particularly as it was not pelleted, and b) the relatively short exposure time to

Econase XT at such an early stage of development, due to constraints of the present trial on diet type & total time of treatment.

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6. GENERAL DISCUSSION

Prior to the work carried out in the present thesis, there was little known about the size or quantity of xylooligosaccharides (XOS) generated from different cereals, including within the broiler GI tract, after supplementation with xylanase and other fibrolytic enzymes. Though, it had previously been alluded to that there may be beneficial effects of XOS generation on broiler feed efficiency (Suo et al., 2015), potentially by modification of the caecal microbiome (Courtin et al., 2008), identification of which XOS were responsible for these positive effects was lacking, mainly due to a lack of research on the characterisation of XOS generated from different cereals after xylanase hydrolysis. Consequently, the body of work presented here was intended to bridge this knowledge gap, elucidating the effects of exogenous xylanases on the generation of xylan hydrolysis products, with emphasis on XOS, due to the availability of pure standards for carbohydrate analysis, although it is important to note that AXOS are also likely produced in xylan hydrolysis and should be considered in future works. These experiments were first carried out *in vitro*, before continuing to an *in vivo* experiment in broiler chickens.

The present thesis profiled the monosaccharide and xylooligosaccharide products of xylanase mediated hydrolysis in a variety of cereals (wheat, barley, maize and oats), as well as comparing different varieties of wheat. *In vitro* digests were utilised as they are fast, cost-effective and have the potential to be more controlled than *in vivo* studies, as well as reducing the number of animals needed for scientific research. In the final experiment, the effects observed

using the *in vitro* model utilised were directly compared to *in vivo* findings, to help assess any potential relationships between the two techniques.

With regards to the hypothesis of the present thesis, it was indeed possible to identify substrate-enzyme combinations *in vitro* that released significantly more monosaccharides and XOS than other combinations via increased NSP degradation. In the present thesis, a novel finding was that xylanase alone in the form of Econase XT produced the greatest effects on wheat when compared to other cereals, though there is clear variation in the amount of AX between wheat varieties which may affect the potential XOS resulting from enzymatic hydrolysis. It was also hypothesised that there would be significant differences in the release of NSP degradation products *in vitro,* depending on the specific variety of wheat used. In the present thesis, there were clear differences between different enzyme – wheat variety combinations in the generation of XOS, with the novel findings that xylanase generated more X2 in some varieties, yet more X3 in others, but all generating similar amounts of the monosaccharide, xylose. The present study (chapter 5) was also the first to quantify the generation of XOS and release of monosaccharides through the GIT of broilers fed a wheat-based feed containing exogenous enzymes and to compare that to an *in vitro* model of digestion for the same enzyme and diet combination. The results did not match the hypothesis for this experiment; although there was greater monosaccharide and xylooligosaccharide released in the GIT compared to control animals, feed efficiency was not improved by this change. Perhaps this was due to a combination of the time constraints leading to relatively low total exposure to enzyme supplementation along with the use of a mash diet in the current experiment. Another factor worth

mentioning is the measurement of feed intake being notoriously difficult with mash diets, with spills being troublesome to account for, especially as the feed that is retrieved from pen floors being mixed with the shavings that cover all pens.

It was shown in the present thesis that Econase XT had the highest affinity for wheat, releasing the most XOS and xylose, followed by barley and oats respectively. Econase XT had the lowest affinity for maize and produced little measurable XOS during enzymatic hydrolysis *in vitro*. These findings are consistent with the literature which has repeatedly shown beneficial effects of xylanase in non-ruminant diets high in NSP's contained within the viscous cereals such as wheat and barley (Craig et al., 2019a, Owusu-Asiedu et al., 2010, Gao et al., 2008, Masey-O'Neill et al., 2014). An interesting finding was how maize showed little response to xylanase supplementation *in vitro.* Other studies have shown beneficial effects of xylanase supplementation on growth performance in maize-based diets (Cowieson and Ravindran, 2008, Masey O'Neill et al., 2012), so it is possible that the effects of endogenous enzymes seen *in vitro* are different to the effects observed during enzymatic hydrolysis with xylanase *in vivo* or perhaps another mechanism is responsible for the increases in growth performance.

The second study in the present thesis showed the diversity of XOS production across six varieties of wheat, especially when xylanase was present in the form of Econase XT. Other studies have also shown strong variances between varieties in both chemical composition and nutritive value (Maisonnier-Grenier et al., 2006). This is one reason why there are inconsistencies in results from

experiments undertaken in the field, where the variety of wheat can differ greatly in characteristics, from one trial to the next.

Future studies should continue to assess the potential relationship between A:X ratio and subsequent XOS production from wheat varieties, however, in the present study, correlational analysis showed no strong correlations between A:X ratio of cereal varieties and XOS release.

The present thesis has also not characterised the variability that may be observed across the same variety, obtained from different environments, where there may be geographical and / or weather variations. It was interesting how in the first study of the present thesis, the presence of X_4 was observed *in vitro,* however, in the second study, the presence of X_4 was not observed. It is possible that the presence of X_4 was not observed in the second study as the *in vitro* digestions were undertaken for only 24h, as opposed to 72h in the first study. However, a 24h digestion was chosen in chapter 4 to allow for more frequent observations to be taken in earlier and potentially more physiologically relevant timepoints in broilers, whilst considering restraints of sample storage, the number of digestions that could be carried out in one batch and the amount of samples that could be analysed by HPAEC-PAD per run.

One important point to note is that *in vitro* digests, like those used in the present thesis, are a closed, static system and cannot completely mimic the conditions that would be found in a broilers GI tract. The conditions present *in vivo* cannot currently be exactly replicated *in vitro,* which may impact the efficacy of xylanase acting on xylan and eliciting its effects. However, *in vitro* models, for this same reason, may be more sensitive in judging the

effectiveness of an exogenous enzyme alone, due to less variables being present which can impact the results, such as the presence of intestinal fibrolytic enzymes of microbial origin. Therefore, I believe that *in vitro* studies will support future research in creating bespoke enzymes for the animal feed and livestock industries during the early stages of product development. However, future *in vitro* work could more accurately replicate the conditions seen in the broiler GIT by adjusting the PH and the presence of digestive enzymes throughout digestion to better mirror the conditions found *in vivo.*

The present findings further support the theory of a prebiotic mechanism, where the Econase XT supplementation did indeed generate XOS by hydrolysis of xylan, which were then utilised by bacteria in the hindgut, resulting in a shift towards acetate production in the caecal microbiome. Though, in the present thesis, this did not lead to increased energy recovery from the feed and thus, did not affect feed efficiency. This was possibly a result of the relatively short exposure time to enzyme supplementation as a result of experiments being undertaken during a holiday period, making bird care a factor that needed to be considered, along with the relatively late introduction of these enzymes (d4) that was necessary for ethical approval in the current experiment.

It is also important to note that the cereals and oilseeds used in the present thesis will be contributing to other prebiotics within the diet which can arise from fructooligosaccharides (FOS) and galactooligosaccharides (GOS) that can be found in barley, wheat and soyabean meal (Poeker et al., 2018). The contribution of these prebiotics should be assessed in the future as they could dilute or even mask the possible effects from in situ generated XOS.

Based on the present findings and others, it seems these bespoke xylanase enzymes should aim to hydrolyse xylan, but only to the extent that they produce large amounts of low DP XOS, and as little xylose as possible, through the restriction of β-xylosidase within enzyme products. This will help alleviate the anti-nutritive effects of xylan and xylose whilst still stimulating the prebiotic mechanisms seen with the generation of XOS, to their maximum potential.

Previous studies have shown that the presence of XOS in a feed ration can improve the feed efficiency of broilers (Zhenping et al., 2013). Other studies have shown that incorporating XOS into feeds has the potential to increase NSP concentrations in the caeca and subsequent VFA production, than with xylanase supplementation alone (Craig et al., 2019b). However, it was noted by the authors that these increases did not result in significant differences in broiler growth performance, although feed intake was lowered in birds supplemented with xylanase or XOS, compared to control. This was further elucidated in another study by the same group, where XOS supplementation decreased feed intake in broilers fed a wheat based diet, compared to xylanase supplementation alone, potentially due to the increased viscosity that arises when xylanase is not added to the feed ration. However, this did not result in differences in feed conversion ratio (Craig et al., 2019a). This suggests an increase in nutrient utilisation occurred, without affecting feed efficiency. Other studies have eluded to how XOS produced during hydrolysis of NSP have potential as emerging prebiotics (Courtin et al., 2008), where they may influence the microbiota and immune system of the gastrointestinal tract by reducing inflammatory cytokines that can upregulate intestinal inflammation

(Jung et al., 2008). There is conflicting research in this area presently, highlighting a need for further research (Arsi et al., 2015, Gao et al., 2007).

The results from the present thesis showed xylanase supplementation significantly altered the concentrations of some XOS, mainly X_4, in the colon and caeca yet had no effects on the growth performance of the broilers studied. Perhaps future experiment should begin supplementation from d0 rather than d4 and utilise pelleted diets rather than mash diets once feasible in the growing broiler.

There was also a trend for increased acetate proportion in the caeca of birds supplemented with xylanase, suggesting that XOS were being generated and subsequently, fermented, in the caeca, which can impact the types of microbiota present. This is shown in Figure 6.1, which highlights the main findings of the present thesis, and how each experiment lead to the next one, from comparing four cereals with three different enzyme products, to comparing six varieties of wheat with those same three enzymes, before finally using Econase XT supplementation *in vivo* and assessing the effects on feed efficiency, XOS generation and the modulation of the caecal microbiome.

The results from other studies have shown significant increases in feed conversion ratio after XOS supplementation, which would be detrimental to broiler performance (De Maesschalck et al., 2015), which again highlights the inconsistencies in the field. It is clear from the present thesis that wheat arabinoxylan degradation is enhanced by xylanase supplementation and that this may increase the production of both prebiotic XOS and beneficial VFA in

the caeca, thereby potentially modulating the caecal microbiome, but without affecting subsequent bird performance in the present study.

Aside from the varying levels of enzyme and XOS inclusion in current studies, one major factor that might be responsible for the inconsistencies in results between studies, is the different varieties of cereals and other feed ingredients used. One important area to consider is how XOS should be incorporated into the diet. Is it better to supplement XOS directly within the feed, or to supplement specific cereal substrates with xylanase which will then generate XOS during xylan hydrolysis, or a combination of the two? As the present thesis and work of others has shown, the efficacy of xylanase supplementation can vary greatly with feed composition, whereas XOS that is added independently can be pre-measured by feed & enzyme manufacturers, however the optimal dosage and composition of these XOS has not yet been elucidated. There are still unique benefits to xylanase supplementation with specific cereal substrates in terms of the fibre degrading capacity of the microbiome, that mean a combination of both xylanase (and other fibrolytic enzymes?) and added XOS, may produce the best results. It is still unclear whether a ceiling at exists at which XOS concentration may begin to produce undesirable outcomes, or if there is a linear dose-response relationship in their inclusion.

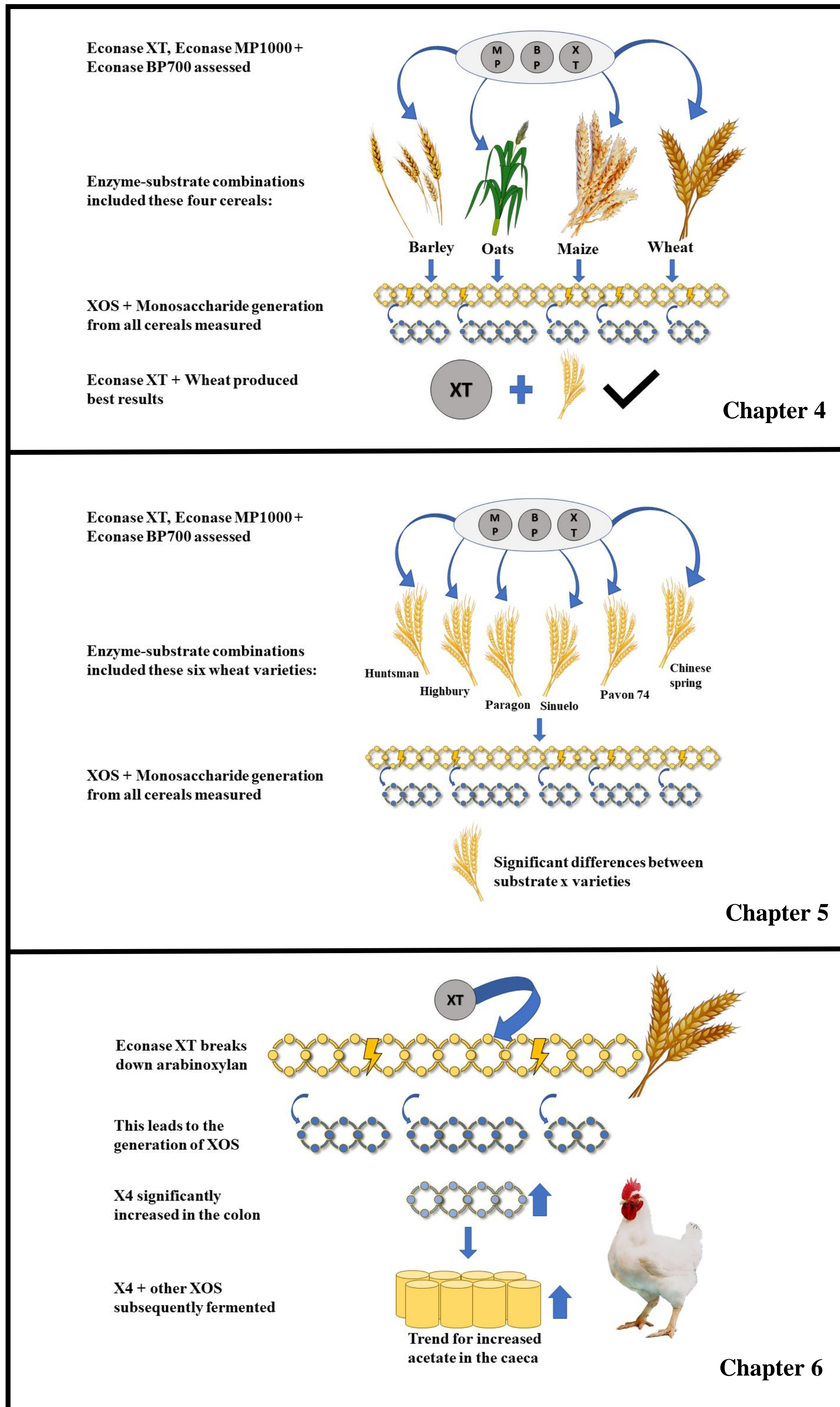

Figure 6. 1 A summary of the findings of chapters 3, 4 and 5.

6.1 Main Conclusions of the Present Thesis

In summary the current thesis has added to the previous body of work by finding:

- Fibrolytic enzymes appear to have some specificity for different cereals, and it will therefore be prudent to optimise the combinations used in animal feeds, in order to maximise the feed efficiency of non-ruminant livestock.
- Fibrolytic enzymes also have some specificity for different wheat varieties. It may then be possible to optimise the combination of wheat variety to a specific enzyme in order to maximise the feed efficiency of non-ruminant livestock.
- The impact of exogenous enzymes on the release of XOS content appeared to be wheat variety dependent. Therefore, it does appear that there may be significant variation in XOS production dependent on the combination of wheat variety and exogenous enzyme, which may subsequently have effects on the gut microbiome.
- Xylanase supplementation to broilers from 4-26d of age did not result in any changes in growth performance, but the concentrations of both X_4 in the colon and xylose in the colon and caeca were increased, as were arabinose concentrations throughout the GI tract. Other XOS and monosaccharides were detected, but not affected by xylanase supplementation.
- It is unlikely that VFA production with xylanase supplementation increased additional energy to the diet as overall VFA levels were not

affected. However, there was a trend for increased acetate proportions in the caeca of xylanase supplemented birds, suggesting greater XOS fermentation in the caeca of birds supplemented with xylanase, which may impact the caecal microbiome, but without affecting bird performance (at this stage of growth).

6.2 Future Work

Future work that should be considered in order to further knowledge in this area include:

- It is important to continue to explore the characterisation of XOS generated from exogenous fibrolytic enzyme hydrolysis, both *in vivo* and *in vitro.* The unanswered question is which XOS are the most beneficial to increase feed efficiency. Future research should attempt to isolate specific XOS and supplement broilers a range of diets with a range of specific XOS, for example, using different varieties of wheat with the individual XOS in isolation to help ascertain which combinations are most effective for increasing feed efficiency and at what quantities. It is possible that different cereals (and varieties within those cereals), may produce different XOS following hydrolysis by the same enzyme.
- Further research should be undertaken to investigate the effects of supplementation of low levels of xylose within a feed ration to broilers on the resulting feed efficiency. Increasing xylose levels are known to negatively affect feed efficiency in a dose-response manner from 2.5% inclusion in the diet (Schutte et al., 1992), but it is unclear whether the

lower levels of xylose that were generated during exogenous xylanase supplementation in the present experiments are beneficial or detrimental.

- There should be more studies undertaken to isolate where in the GI tract these XOS are most potent. Is there a way that XOS can be delivered safely to this area for optimal substrate utilisation?
- Future enzyme products could be developed to target the production of XOS. It should also be considered how the production of monosaccharides can be optimised during hydrolysis to produce the largest amount of XOS possible, both *in vitro* and *in vivo*.
- Continue to examine the relationship between *in vitro* findings and *in vivo* findings, by assessing the XOS and monomeric products released both with and without fibrolytic enzyme supplementation.
- Finally, the use of exogenous enzymes is well established in both pig and poultry nutrition. There may be potential for their use in humans, in order to help alleviate gastrointestinal and digestive problems, such as irritable bowel syndrome / disorder. Another example where they could be utilised is in poorer regions of the world where poor-quality diets could be supplemented with exogenous enzymes to increase nutrient utilisation and thereby decrease the prevalence of problems associated with malnutrition in these regions. There will need to be a greater body of research into supplementation in humans to assess the efficacy of such ideas.

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Appendix

STATEMENT OF AUTHORSHIP OF PUBLICATIONS

This statement confirms the contribution made by Tom Dale to jointly authored work

I confirm that Tom Dale contributed **80%** to the paper:

> **Dale, T**, Bedford, MR, Tucker, GA, Brameld, JM & Parr, T. The effects of exogenous fibrolytic enzymes on the in vitro generation of xylooligosaccharides and monosaccharides is dependent upon cereal type

I confirm that Tom Dale contributed **80%** to the paper:

> **Dale, T**, Bedford, King, J, MR, Tucker, GA, Brameld, JM & Parr, T. The effects of exogenous fibrolytic enzymes on the in vitro generation of xylooligosaccharides and monosaccharides from six wheat varieties

I confirm that Tom Dale contributed **80%** to the publication:

> **Dale T,** Hannay I, Bedford M R, Tucker G, Brameld J M and Parr T (2020). The effects of exogenous xylanase supplementation on the *in vivo* generation of xylooligosaccharides and monosaccharides in broilers fed a wheat-based diet. *British Poultry Science*. DOI: 10.1080/00071668.2020.1751805.

Signature:

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