School of Animal, Rural and Environmental Sciences

The efficacy of xylo-oligosaccharides in supporting the gut health, oxidative status and performance of broilers

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Copyright statement

Abstract

Population growth and associated food security has led to pressure on the livestock production industries to increase both production output and production sustainability. The 2006 EU ban on the use of non-therapeutic antibiotics had created a need for alternative measures to support the health and growth of livestock. In recent years there has been increasing evidence that broilers may be experience high levels of oxidative stress. This has been linked with an emerging problem with poultry muscle meat myopathies such as woody breast, white stripe and spaghetti meat.

Pre-biotics are one area being explored to fill this void. Xylanase is an exogenous enzyme routinely used in broiler production to reduce the anti-nutritional effects of non-starch polysaccharides present in viscous feed grains. It has been speculated that xylanase may also act as a prebiotic by hydrolysing long-chain polysaccharides in the diet into oligosaccharides that can be utilized for fermentation by microbiota in the hind gut. Subsequently, fermentation products can have antimicrobial and energy sparing effects that may be able to enhance broiler performance and gut health. Oligosaccharides have also been shown to have antioxidant properties.

A series of bird trials and in vitro studies were conducted to investigate the effect of xylanase, arabinoxylans and xylo-oligosaccharides on the performance, gut health and oxidative status of broilers. . Initially a bird trial investigated the effect of an arabinoxylan extract, with and without the addition of xylanase. This study showed performance benefits of supplementing broilers with an arabinoxylan extract with birds consistently gaining more weight than the other treatments (significant during days 21-28) and finally weighing on average over 100g more than the other treatments. Pre-treating the AX extract seemed to negate any performance benefits, possibly due to a mixing or distribution effect of the pre-treatment process.

A second bird trial investigated the effect of two XOS products derived from different base materials with and without the addition of xylanase in a 3x2 factorial study. This study revealed very few performance effect but did show that xylanase inclusion increased the tibial breaking strength at day 21 and increased calcium digestibility at day 42.

An in vitro study investigated novel antioxidant assays as a way to measure the oxidative status of broilers and the antioxidative properties of broiler feed and various commercial oligosaccharides. This study provided initial indications for optimizing the use of this assay in broilers and showed that all of the XOS products investigated had some antioxidative capacity.

A final bird trial investigated the dose response of Signis®, a commercial XOS and xylanase supplement. This study compared a commercially recommended dose of 0.1g/kg Signis® with a dose of 0.2g/kg. This study showed significantly improved body weight gain, average bird weight for both doses of Signis® compared to the control and significantly improved feed conversion ratio for the 0.2g/kg dose compared to the control and 0.1g/kg dose. This study did not find any significant difference in digesta viscosity, suggesting that the performance enhancing effects of XOS/xylanase may be due to alternative mechanisms such as the production of xylanase by bacteria in the GIT.

This project demonstrated that broilers can gain performance benefits from the supplementation of an AX extract without the addition of xylanase, suggesting that broilers may be able to ferment AX independently and that although broilers may not be able to produce endogenous xylanase, the microbiota within the intestinal tract may be able to and that viscosity and cell wall effects may not be responsible for all beneficial effects of XOS/xylanase supplementation.

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List of abbreviations

AX – Arabinoxylan

AXOS - Arabinoxylan oligosaccharides

BWG – Body weight gain

BW – Bird weight

Ca – Calcium

DDGHS – Distillers dried grains with solubles

EPEF – European Poultry Efficiency Factor

FCR – Feed Conversion Ratio

FD – Freeze dried

FI – Feed intake

ICP-OES – Inductively coupled plasma-optical emission spectrometer

KS – Kolmogorov Smirnoff

ANOVA – Analysis of variance

NSP Non-starch polysaccharide

NDO – Non digestible oligosaccharide

VFA – Volatile fatty acid

XYL – xylanase

XOS – xylo-oligosaccharide

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Chapter 1 Review of the literature

1.1 Introduction

Global population growth and associated food insecurity has increased demand for sustainable, inexpensive meat protein. This has placed pressure on the poultry industry to meet this demand. In 2015 global poultry production stood at 111,000 thousand metric tons, however the industry is predicted to reach over 131,255 metric tons by 2025. 1Historically, the poultry industry has supported production with the use of non-therapeutic anti-biotics for growth promotion and disease prevention (Onrust *et al.,* 2015). Following the 2006 EU ban on the use of non-therapeutic antibiotics, the industry has been driven to seek alternative approaches to enhance bird health and support growth performance. Gut health and oxidative status are two inextricably linked areas of broiler health that have been found to have a profound effect on health and performance (Enberg *et al.*,1996; Sihvo *et al.,* 2013; Ismail *et al.,* 2013; Sugiharto, 2014; Estevez, 2015).

Identifying ways to improve oxidative status and gut health is vital for the rapid growth of the industry. One approach to improving gut health is the provision of pre-biotics. Pre-biotics are substances that provide a nutrient source for selected beneficial microbes within the gut. Encouraging proliferation of beneficial bacteria can have a protective and restorative effect on the host's immune system, potentially preventing pathogenic disease and therefore improving production performance (Enberg *et al*., 1996).

Improving the oxidative status of broilers can also have wide reaching benefits. One of the main detrimental effects of oxidative stress in broilers is the loss of integrity of tight junctions within the intestinal epithelium (Gangwar *et al.,* 2017). When these tight junctions are damaged, leakage can occur, translocating gut microbiota, potentially leading to systemic infection. As such, ensuring the microbial population of the gut is optimized, can also have a protective effect on the host by preventing systemic pathogenic infection (Latorre *et al.,* 2018). Additionally, both poor gut health and

oxidative stress are likely to impinge on animal welfare, potentially causing pain and suffering at an individual level.

Oligosaccharides are compounds that have recently been investigated for their pre-biotic properties (Wichienchot and Chinachoti, 2011). Oligosaccharides have also been confirmed to possess antioxidant properties (Hou *et al.,* 2015; Zhou *et al.,* 2018). As such, oligosaccharides could represent a viable supplement to support two key areas of poultry health.

The overarching aim of this project is to investigate the efficacy of xylo-oligosaccharides and/or xylanase in supporting the performance, gut health, and oxidative status of broilers.

1.2 Population growth

Between 1950 and 2017 the global population rose by around 5 billion people (Population Reference Bureau, 2019) and is expected to continue this exponential growth, with some predications estimating the global population will top 10 billion by the end of the century. This vast increase in population naturally creates an increased need for food, especially protein sources. It is becoming widely recognized that the population increase, extended life expectancy and the associated food insecurity is a major global concern (FAO, 2015).

1.3 Growth of the Poultry Industry

In response to the need for cost efficient and abundant protein, global poultry production increased by 53% between 1995 and 2005 (Scanes, 2007). This increase was achieved due to several factors, including genetic manipulation, selective breeding, improved management and a better understanding of optimal nutrition for broilers. This has led to a 30% reduction in the feed conversion ratio of the modern broiler chicken when compared to its 1957 equivalent (Havenstein *et al,* 2003) (figure 1.1).

Figure 1.1 A comparison of broiler chicken development from 1957-2005 (Zuidhof *et al.,* 2014)

The UK alone currently slaughters over 20 million broilers a week (DEFRA, 2020). Poultry production has also been streamlined, with modern intensive systems requiring 30% less land, and a 30% lower carbon footprint and energy usage than 50 years ago (Burton *et al*., 2016).

Poultry meat continues to grow in popularity (Magdelaine *at al.,* 2008), due to cultural acceptability, low price points and the nutritional advantages of poultry meat over other meats (Magdelaine *et al.,* 2008). In the last 15 years, poultry meat production has risen by nearly 44 million metric tons, reaching production of over 125 million metric tons by 2019 (Shahbandeh, 2019). In the UK alone, consumption of poultry meat doubled in the 20 years between 1987 and 2007 (Foods ethics Council, 2007). Reasons for this include increased awareness of nutrition for health, benefits of low-fat white meat and reduced price and availability of meat (Magdelaine *et al.,* 2008).

In the quest for sustainable agriculture and protein production, species such as cattle and sheep are considered to be less sustainable and possibly even damaging to the environment due to the methane production resulting from enteric fermentation as well as the large time, space and resources required to raise them to slaughter age (Desjardins *et al*., 2012). Recent years have seen a rise in the popularity of vegan diets, whilst this market is growing it is unlikely to replace meat protein due to a large majority of consumers being averse to meat-free diets. Research into alternative protein sources for the human food market is now also prevalent, with increased interest in insect and plant-based proteins. Novel proteins are often unpopular with consumers, with many people being unwilling or unable to give up traditional meat protein (Burton *et al.,* 2016).

Chicken is one of the most viable options for a sustainable animal protein, which can be produced in large quantities (Neeteson-van Nieuwenhoven *et al*., 2016). This is due to several factors. Broilers (meat chickens) are bred to reach slaughter weight 35-42 post hatch, and as such, are incredibly efficient at turning feed into muscle, thus maximising the feed investment. Broilers also have minimal space requirements and produce far less hazardous waste than other production species.

There is also growing use of chicken manure as a commercial fertilizer due to its high nitrogen, potassium and phosphorus content (Vandecasteele *et al.,* 2013), therefore improving industry sustainability. In recent years it is becoming more recognized that the industry is utilizing high quality diet ingredients that could be utilized by the human sector. As omnivores, chickens can and will eat a

very wide range of food (Sturkie, 1965). This has led to increased research into alternative poultry diets, that utilize ingredients that are not suitable or palatable to humans such as industrial by-products and insect proteins. This is particularly useful in improving the sustainability of the industry, allowing high quality meat protein for human consumption whilst reducing industrial waste (Burton *et al.,* 2016).

1.4 The Avian Digestive Tract

Chickens share many similarities in digestive strategy with other monogastrics such and pigs and humans. The avian digestive tract is a relatively simple, but highly efficient structure. As a result of evolution, avian species developed lightweight, fast transit systems that allowed flight and thus predator escape, while maximising utilisation of a wide range of nutritional materials.

1.4.1 The Alimentary canal

The alimentary canal is the entire digestive organ of the chicken. It runs from the beak, where ingestion occurs, down to the cloaca, where waste is excreted. The entire alimentary canal can be up to 6 times the length of the total bird length (Scholey, 2012). The length of the individual tract components varies by age and can be dependent upon variables such as diet. For example, Sturkie (1965) reports an association between fibrous and viscous diets and larger alimentary tracts.

As a precocial species, chickens have highly developed digestive systems at point of hatch. The alimentary tract can account for 25% of the total live weight at hatch, reducing to only 5% by 56 days of age (Larbier and Leclercq, 1994).

The mechanical, enzymatic and chemical processes of digestion occur throughout the entire tract, however, the process of nutrient absorption occurs only in the small intestine where nutrients are absorbed through the wall of the tract into the circulatory system for transport throughout the body (Larbier and Leclercq, 1994; Scholey, 2012). The majority of the tract is lined with mucus membranes, layers of muscle that run lengthwise and glandular tissue that secretes digestive enzymes. Digesta can transit the tract in as little as 2-4 hours but can take up to 10 hours depending on feed composition and form. This allows highly efficient uptake of nutrients (Scholey, 2012).

1.4.2 The Mouth Structure

Food is taken into the alimentary canal via the beak of the bird. In the absence of lips and cheeks, chickens possess dense horny skin that covers the mandible and incisive bones (Lorenzoni, 2010). This provides structure to the mouth. Chicken do not possess teeth, with the exception of a small bony ridge on the beak that only remains for a few days post hatch and aids exit from the egg. There is no specific distinction between the mouth and pharynx of the chicken and there is no soft palate present (Duke, 1986; Lorenzoni, 2010). As such, the combination of the mouth and pharynx is sometimes referred to as the oropharynx (Lorenzoni, 2010; Jacobs and Pescatore, 2013). The dorsal surface of the oropharynx contains a common orifice for the two Eustachian tubes (Bradley, 1960).

The dense, hard palate that forms the mouth has a long narrow median slit that intersects the nasal cavity (Larbier and Leclercq, 1994). The hard palate has five rows of hard conical papillae that point backwards. A number of salivary glands line the hard palate, secreting limited quantities of digestive enzymes including amylase.

The majority of feed is ingested whole and very little maceration occurs in the beak. Feed is transferred to the pharynx via mechanical movement of the head in an upward and forward direction (Hill, 1976). Water is ingested in a similar manner, but with the beak closed. Water flows directly into the oesophagus when the head is raised.

Situated in the beak, the tongue contains few intrinsic muscles and is ridged and narrow. In cross section, the tongue is triangular and pointed. The tongue is attached to the hyoid bone, which providing the framework for the tongue to transfer ingested substances to the oesophagus (Hill, 1976).Chickens possess very few taste buds and as such taste is likely to play only a very small role in feed consumption (Scott *et al*., 1976).

1.4.3 Salivary Glands

A thick layer of stratified squamous epithelium covers the surface of the mouth structure. The hard palate has salivary gland running its entire length and the maxillary, palatine and sphenopterygoid glands lead to an area on the dorsal palate at the bottom of the papillae (Lorenzoni, 2010; Kleyn, 2013). The combined glands form a mass of glandular tissue beneath the epithelium. Most of this glandular tissue contains lymphoid tissue (Husveth, 2011). The ventral and dorsal palates meet in an area known as the lateral cheek where the buccal gland is located (Denbow, 2000). Saliva acts as a lubricant to ingested feed. Unlike many other species, only minimal amylase is secreted with the saliva (Denbow, 2000; Lorenzoni, 2010; Kleyn, 2013).

1.4.4 Pharynx and Tongue

The roof of the pharynx houses more salivary glands (Kleyn, 2013). These glands mainly secrete mucus, containing muco-proteins and both sulphated and non-sulphated muco-polysaccharides to lubricate ingested feed(Kleyn, 2013).During swallowing, feed is grabbed by the beak and the tongue used to manoeuvre the feed to the roof of the mouth where mucus is added to form a sticky bolus (Kleyn, 2013).The tongue is used to manoeuvre the ingested feed towards the oesophagus (Lorenzoni, 2010; Kleyn, 2013).

1.4.5 Oesophagus and Crop

The oesophagus does not possess a sphincter and the walls of the oesophagus are very thin. The oesophagus is split into two indistinct regions – cervical (upper section) and thoracic (lower) (Lorenzoni, 2010; Kleyn, 2013). The cervical section dilates and opens into the bird's crop. The crop is an expanding diverticulum structure that acts as a storage vessel for ingested feed (Lorenzoni, 2010; Kleyn, 2013). Mucous is produced in the oesophagus and crop to provide lubrication for ingested matter (Scholey, 2012). From the crop, digesta is passed to the proventriculus.

1.4.6 Proventriculus

The proventriculus is the glandular stomach of the chicken (Lorenzoni 2010; Kleyn, 2013). The organ is lined with papillae and multi-lobular glands which give the proventriculus its granular appearance. These papillae possess oxynticopeptic cells that produce and secrete hydrochloric acid and proteolytic enzymes in the form of pepsin (Lorenzoni, 2010; Kleyn, 2013). The proventriculus leads directly to the ventriculus or gizzard. The gizzard represents the muscular stomach, and here mixing and grinding occurs. The external surface of the gizzard is composed of smooth muscle (Kleyn, 2013). The internal wall of the gizzard is covered with a thick cuticle membrane that is secreted by mucosal glands beneath the surface. This cuticle is folded longitudinally and provides protection to the organ from mechanical friction created by the grinding of digesta (Lorenzoni, 2010). Grinding the digesta is achieved by two pairs of opposing muscles that contract in turn to create a grinding motion. There is a small pyloric section of the gizzard that again contains mucosal glands as well as villi. Here mucus is secreted to lubricate the passage of digesta from the gizzard to the duodenum, and passage of digesta controlled by the pyloric sphincter.

1.4.7 Small intestine

The small intestine is composed of three distinct parts, the duodenum, jejunum and ileum. The small intestine of an adult broiler would measure approximately 120-140cm in length from the gizzard to ileal-caecal junction.

The duodenum forms the cranial portion of the small intestine (Kleyn, 2013) and the gizzard-duodenal junction forms an active filter that prevents larger ingested particles from entering the duodenum. It is compiled of a descending and ascending loop that surround the pancreas. The pancreas is a secretory organ. Both pancreatic and bile ducts open into the top of the duodenal loop. Pancreatic secretions can be broken down into two components: aqueous and enzymatic. Aqueous secretions

contain high concentrations of bicarbonate ions that neutralise chime and optimize pancreatic enzymatic activity (Scholey, 2012). Secretions contain enzymes including peptidases, amylases, saccharidases and lipases for the enzymatic degradation of proteins, carbohydrates, and lipids respectively. Digestive enzymes are secreted as proenzymes, where the presence of ingesta converts proenzymes to their active status.

Secretin is a peptide hormone that influences the secretion of pancreatic juice. Secretin is produced in the intestinal wall along with cholecystokinin-pancreozyme (CCK-PZ). Secretin release is stimulated by gastric hydrochloric acid and food reaching the duodenum. CCK-PZ is then released following stimulation from amino acids, peptides and fats from the ingesta into the duodenal loop (Duke, 1986b). The presence of secretin triggers an initial secretion of aqueous and enzymatic components from the pancreas.

Enterocytes line the entire intestinal lumen. These are arranged into villi and microvilli - finger like projections that are continually replaced by cells migrating from the crypts of Lieberkühn (situated between villi) throughout the lifespan of the chicken (Lorenzoni, 2010). The crypts of Lieberkühn also secrete mucus, as Brunner's glands (found in humans) are absent in birds. These villi create a very large surface area which confers increased absorptive ability to uptake nutrients and water (Lorenzoni, 2010).

Liver bile enters the duodenum via two ducts (from the caudal end) at a pH of 6. This acts as an emulsifier and facilitates the action of pancreatic lipase and lipid absorption. Synthesis and secretion of bile develops with age and therefore lipid digestion improves throughout the lifespan of the bird (Larbier and Le Clercq, 1994).

The pancreatic and bile ducts enter the caudal end of the ascending loop of the duodenum and marks the start of the jejunum, which runs until the Meckel's diverticulum where the jejunum nominally becomes referred to as the ileum. The jejunum and ileum are similar in function, length and structure. These two portions of the small intestine are the major absorptive sites of the intestinal tract (Larbier

and LeClercq, 1994). Digesta is propelled through the small intestine via peristalsis and segmentation movement. As in the duodenum, the jejunum and ileum are lined with villi, covered with delicate absorptive tissue. The absorptive cells are large, cylindrical structures containing a basal nuclei and an apical membrane covered in microvilli (Hill, 1976). Villi contain undifferentiated cells that have the ability to undergo mitotic division and form the precursors to absorptive cells. These can in turn migrate either up the villus or down into the neighbouring crypts of Lieberkühn as required by the bird.

1.4.8 Large Intestine – Caeca and Colon

The large intestine of an adult broiler is approximately 20cm in length (Calhoun, 1954). The caeca are a pair of blind ended sacs that sit at the junction of the small and large intestine. The caeca provide a home to a host of microorganisms which enjoy a symbiotic relationship with the bird. The microbial population degrade undigested nutrients via fermentation and produce metabolites during this process that can in part be absorbed by the caeca. Metabolites can also play a vital role in the balance and maintenance of the microbial population of the gut as a whole. Metabolites such as butyrate are available as a nutrient source for bacterial populations which are thought to be beneficial to the health of the bird (Bedford and Gong, 2018).

The ileo-caecal-colonic junction is a sphincter that controls the flow of chime between the colon and caeca. Retro peristalsis is utilized. The sphincter relaxes when the ileum distends to allow colonic movement. Once movement is possible, the colon distends and contracts to move digesta towards the caeca or cloaca (depending on peristaltic direction). As commercial broilers have constant access to food and water, the caeca replenish frequently with evacuation occurring from a strong distal end contraction. The frequency of caecal emptying varies with pH, electrolyte activity, diet and caecal distention (Scholey, 2012). Nutrient digestion is minimal in the large intestine (Larbier and Le Clercq, 1994). A schematic of the chicken gastrointestinal tract is shown in figure 1.2.

Figure 1.2 – Gastrointestinal tract of the chicken

1.5 Antibiotic growth promotors

In recent decades the broiler production industry has routinely utilized antimicrobial compounds at sub therapeutic doses as both a prophylaxis for disease and as a growth promoter (Singer and Hofacre, 2006). This usage has allowed the industry to improve production output and reduce disease related losses. However, in 2006, the EU enacted a ban on the use of antibiotics for any purpose other than therapeutic uses (European Commission, 2005) due to concerns surrounding widespread antimicrobial cross resistance and multiple antibiotic resistance to pathogenic bacteria in both humans and animals (Hajati and Rezaei, 2010).

The historical use of antimicrobials allowed the industry to utilize sub-optimal ingredients and ration formulations without negative effects on performance or liveability. Studies from the 1960's showed that maize inclusion in broiler diets could be replaced with barley and rye, if the birds were supplemented with sufficient levels of antibiotics (Bedford, 2019) . The addition of antibiotic growth promotors (AGP) compensated for numerous anti-nutritional factors and often sub-optimal management and environmental conditions (Enberg *et al.,* 2000; Mehdi *et al.,* 2018). The EU ban forced producers to seek alternative strategies to support the health and growth of broilers. One of the main benefits of AGP was considered to be the modulation of intestinal microbiota. The exact mechanism remains unclear, but Dibner and Richards, (2005) suggest that AGP work by modulating microbiota diversity and relative abundance in the GIT to provide optimal conditions for bird growth (Bedford, 2000; Fung *et al.,* 2013).

The ban, and the widespread opinion that the current ban is likely to be extended to prevention of any antibiotic use, has led to an exponential growth in research looking at the health and microbial population of the GIT.

1.6 Gut Health

Ensuring effective poultry production relies on a number of factors including; genetic potential of the birds, optimal nutrition, good management and environmental conditions and disease prevention (Szollosi *et al*, 2014; Sugiharto, 2016). However, the genetic potential of the birds can only be achieved by the efficient conversion of fuel into its key components for maximum nutrient absorption. One method of optimising this conversion is to maximise gut health potential (Sugiharto, 2014).

The gut is a pivotal, multi-faceted organ. It regulates the uptake of nutrients and how these nutrients are metabolised and utilised, as well as being the largest immunological organ in the body of most animals (Kogut, 2013). Paradoxically, the gut is also one of the main sites for exposure to external and environmental pathogens (Yegani and Korver, 2014). It has been understood for some time now that the microbial population of the gut can play a substantial role in the performance and health status of animals (Choct, 2007; Awad *et al.*, 2009), with associated financial implications on the poultry sector. The gut has been shown to be involved in body weight and energy homeostasis and on the development and maturation of both the adaptive and innate immune systems (Kogut, 2008, Sugiharto, 2014, Muir *et al.*, 2000, Brisbin *et al.*, 2008). The microbial populations of the gut are highly diverse in chickens, with each species producing different metabolic products such as fermentation acids and various gases (Onrust *et al.,* 2015).

As such, the development of strategies to manipulate the microbial community of the gut are key to supporting gut health.

The gut has three primary interactions

1. The intestinal epithelium and associated neuroendocrine connections
2. The immune system
3. Commensal microbiota

The study of gut health has its origins in human medicine. Studying health of the avian gut is a highly complex amalgam of disciplines including microbiology, nutrition, immunology, and physiology. The results of poor gut health may be detrimental, impacting not only on the welfare of the bird, but also on the efficacy of production (Yegani and Korver, 2008). Reasons cited for poor gut health include stress (oxidative and environmental), dietary protein sources which are poorly digestible, diseases such as coccidiosis, and the routine use of antimicrobial drugs such as antibiotics (Dibner and Richards, 2005). The mitigation of these issues can only be achieved by a holistic approach which has to be a combined effort from farmers, scientists, nutritionists, and commercial feed companies.

Of additional note is the impact that the gut health of broilers can have on human health. Pathogenic gastrointestinal diseases of the chicken such as *Salmonella, Campylobacter, Listeria, and Clostridium* can also infect humans, causing severe gastroenteritis and even death. As such, preventing the colonisation of these pathogens in the gut of the chicken can also prevent human disease (Eeckhaut *et al*., 2008). Gastrointestinal pathogens such as these can often survive in the chicken during life without causing outward signs of illness Post-mortem, pathogens can easily transfer into the human food chain.

An influx of poultry gut health research has been seen in recent years, mainly due to the perceived impact on production. However, more recently research has shifted toward examining the role of poultry gut health as a limiting factor in the sustainable production of food to feed an exponentially expanding population (Burton *et al*, 2016; Roosendaal and Wahlstrom, 2016; Van der Aar et al., 2016)

1.7 Gastrointestinal dysbiosis

The historical use of antibiotic growth promoters and subsequent ban in 2006 has led to gut health problems in broilers such as gastrointestinal dysbiosis - an imbalance of gastrointestinal bacteria (Onrust *et al.,* 2015). Controlling this dysbiosis without the use of antimicrobials is difficult and

requires thorough knowledge and understanding of the microbial populations within the gut, and the interactions between these microbes and the gut mucosa of the host (Onrust *et al.,* 2015).

The microbial composition of the gastrointestinal tract is diverse and variable. Variation can occur for numerous reasons including host diet, health status, biosecurity, housing and environmental conditions (Onrust *et al.,* 2015). Dysbiosis occurs when there is an overgrowth of a given microorganism, causing competitive exclusion of other microbial species (Belisarius and Faintuch, 2018). This causes an imbalance of microbiota and therefore microbial function. Gut microbiota exert vital functions that in combination maintain homeostasis (Peterson *et al.,* 2015; Belizario and Faintuch, 2018). These vital functions include regulation of innate and adaptive immune function, digestion, xenobiotic metabolism and numerous signalling pathways (Peterson *et al*., 2015). There can be a number of causative factors that affect dysbiosis including the use of antibiotics, antifungals, poor diet, disease, and oxidative status (Peterson *et al*., 2015; Belizario and Faintuch, 2018; Dumitrescu *et al*., 2018).

1.8 The role of the gut epithelial in gut health

The internal gut wall (luminal side) is covered with epithelial cells with absorptive properties that facilitate both nutrient and water uptake (Eeckhaut *et al*., 2008). This provides a semipermeable wall between the lumen and the body cavity. This wall is comprised of epithelial cells, intersected with tight junctions that serve as a connection between the epithelial cells (Eeckhaut *et al*., 2008).

The permeability of the epithelial lining can be affected by things such as luminal signalling and cell death. Luminal signalling can increase permeability by loosening the tight junctions which can adversely affect the host. The loss of integrity caused by this increased permeability can allow an efflux of host proteins into the gut, potentially leading to toxins and microorganisms reaching the luminal side of the gut epithelium, resulting in a condition termed “leaky gut” (Eeckhaut *et al*., 2008). Oxidative stress has been shown to damage tight junctions (Anderson and Itallie, 1995). Studies

suggest that interaction between the mucosa and enteric microbes can result in the production of reactive oxygen species and consequently lead to oxidative stress (Naidoo *et al.*, 2008). Damage caused to the intestinal epithelium can deleteriously affect and destroy the barrier function and tight junctions, leaving the gut vulnerable to pathological disease, and the host open to systemic infection from the microbial population of the gut (Mishra and Jha, 2019).

1.9 The Gut-brain axis

It has become recognized recently that a vital pathway exists between the gut and the brain (Calefi *et al.,* 2016; Dumitrescu *et al*., 2018). This so-called "gut-brain axis" is responsible for physiological regulation and signalling pathways. Communication between the gut microbiota, enteric nervous system and the brain acts as an interface between the host and its environment (Carabotti *et al.,* 2015) (Figure 1.3). The gut-brain axis is a humeral pathway between the intestinal epithelium, portal and systemic circulations, the blood-brain barrier, neuroendocrine-immune network and nervous system (Dumitrescu *et al*., 2018). In broilers the gut-brain axis has been found to mediate and modulate behavioural and immune responses, meaning that the effects of environmental stressors such as heat stress and stocking density can ultimately affect gut immune and gut health parameters (Calefi et al., 2016; Rana, 2018). It is therefore possible that modulating the GIT with pre or probiotics may have more implications than just improving gut health.

Figure 1.3 - Schematic of the gut-brain axis (Rana, 2018)

1.10 Gut permeability

The GIT plays a key role in protecting the host from enteric pathogens (Vicuna *et al.,* 2015). Therefore, integrity of this barrier function is vital to prevent disease and maintain optimal production. The mucosal layer comprises of tight junctions situated between epithelial cells and gut-associated lymphoid tissue. This barrier maintains homeostasis between dietary antigens, enteric pathogens and beneficial microbiota (Van Der Hulst *et al.,* 1998). Damage or disruption to barrier integrity can result in increased permeability and loss of barrier function. Yarandi *et al.,* (2016) reported that increased gut permeability can facilitate systemic translocation of gut bacteria and/or the metabolites of gut microbiota from the intestinal lumen. The exposure of epithelial or mucosal cells to this bacteria or metabolic products can trigger an immune response and subsequent production of pro-inflammatory cytokines. Stress (including oxidative) can lead to the hypothalamus-pituitary axis being activated and subsequent excessive release of corticotropin-releasing factor. Consequently, this hormone can

modulate local activation of mast cells in the intestinal wall, releasing cytokines that cause increased permeability of the gut epithelium (Yarandi *et al.,* 2016) (Figure 1.4). Increasingly, there is a link being made between the gut's microbiota and oxidative stress.

Figure 1.4 Two-way interaction between gut microbiota, gut permeability and the central nervous system (adapted from Yarandi *et al.,* 2016)

1.11 Oxidative stress

1.11.1 Free radicals and reactive oxygen species

Oxidative stress can be defined as an imbalance in the production of reactive oxygen species (ROS) and free radicals and the antioxidant defences of the body (Betteridge, 2000). Free radicals are naturally occurring, highly unstable molecules that are formed during normal aerobic metabolism (Sies, 1996). They are a chemical species that possess an outer electron orbital and a single unpaired electron that increase the reactivity of an atom or molecule (Dugan and Choi, 1999; Betteridge, 2000). Most ROS are unstable and will donate the unpaired electron to neighbouring molecules or seek a hydrogen atom in order to pair it with their free electron (Dugan and Choi, 1999). However, some ROS can persist for extended periods of time. The process of reactivity allows oxygen to participate in high-energy electron transfer and production of adenosine-5-triphosphate (ATP) via oxidative phosphorylation. Whilst this process is vital in the evolution of multicellular organisms, it

also has the propensity to attack biological molecules such as lipids, proteins, and DNA (Burton and Jauniaux, 2011). Exposure to exogenous free radicals is also possible through environmental contact with air pollution, sunlight and substances such as cigarette smoke (Burton and Jauniaux, 2011).

The production of endogenous free radicals is intrinsic to normal metabolic process such as cell respiration, phagocytosis of foreign bodies, lipid synthesis and metal metabolism and in low and moderate numbers, they actually play a number of beneficial roles (Gagne, 2014; Pizzion *et al*., 2017). Free radicals are required in order to synthesize various cellular structures and are also used by the host's immune system to mount a defence (Pizzion *et al.,* 2017). Phagocytes both synthesize and store free radicals and use them to overcome invading pathogens (Pizzion *et al.,* 2017). Some cellular signalling pathways are also regulated by free radicals including endothelial cells, fibroblasts, vascular smooth muscle cells, thyroid cells and cardiac myocytes (Pizzion *et al*., 2017). Conversely, free radicals play a vital role in the innate immune system, effectively mitigating inflammation (Dumitrescu *et al*., 2018). One of the most elucidated free radical signalling molecules is nitric oxide. Nitric oxide acts as a cell-to-cell messenger that modulates blood flow and is vital for normal neural activity (Pizzion *et al.,* 2017). Additionally, nitric oxide is involved in the elimination of intra-cellular pathogens and tumour forming cells. As such, free radicals remain vital for the health and wellbeing of the host, however in excess they can be highly damaging, and this balance needs to be maintained.

1.11.2 Oxidative Stress

When free radical production overwhelms intrinsic anti-oxidative defences, oxidative stress may occur. Oxidative stress is a damaging process that effects a number of cellular structures including membranes, lipids, proteins, lipoproteins and DNA (Pizzino *et al.,* 2017). Polyunsaturated fatty acids (PUFA) are at particular risk. Oxidative damage to PUFA is classified as lipid peroxidation, and this process is particularly damaging as it triggers a self-perpetuating chain reaction. At higher levels, indiscriminate damage to biological molecules including cytotoxicity, genotoxicity, carcinogenesis and cell death (tissue damage) may occur (Burton and Jauniaux, 2011; Gagne, 2014). An example of this

process is when hydroxyl radicals and peroxynitrite arise in excess causing lipid peroxidation. This damages cell membranes and lipoproteins and subsequently leads to the formation of malaondialdehyde (MDA) and conjugated diene compounds. These are known to be mutagenic and cytotoxic. Due to this process being a radical chain reaction, lipid peroxidation will promptly spread to affect large quantities of lipidic molecules (Pizzino *et al*., 2017). Likewise, proteins damaged by oxidative stress have been found to undergo morphological and conformational changes that can cause loss and/or damage to their enzyme production and activity (Pizzino *et al*., 2017).

DNA is particularly at risk to lesions caused by oxidative stress. Nishida *et al.,* (2013), found that oxidative stress can be responsible for mutagenesis and loss of epigenetic tagging or information. If oxidative stress is not detected and controlled it has been found to be a causative factor in a number of degenerative and chronic diseases in humans such as cancer, cardiovascular disease, various neurological diseases such as Alzheimer's, Parkinson's, multiple sclerosis, rheumatoid arthritis, kidney disease, and depression (Pizzino *et al.,* 2017).

Conversely, oxidative stress also plays a role in maintaining homeostasis of the central nervous system (Dumitrescu *et al.,* 2018). Oxidative stress levels are monitored and when present in excess, trigger a negative feedback reaction to attempt to return to manageable levels. The level of free radicals circulating in the body is controlled by antioxidants, originating both from internal (produced in response to the presence of ROS)and external processes (such as heat stress) and sources such as oxidised lipids in the food supply.(Mironczuk-Chodakowska *et al.,* 2018).

1.11.3 The effect of oxidative stress in broilers

The main causes of oxidative stress in broilers appear to be heat stress and diet composition although there is also a pre-disposition in some birds as a result of heavy genetic selection (Fellenberg and Speisky, 2006; Sihvo *et al.,* 2013). However, the challenging conditions of commercial production such as growth rate, stocking density, heat requirements, handling, pre-slaughter stress and the provision

of oxidized oils within the ration all increase the possibility of oxidative stress occurring (Enberg *et al*., 1996; Sihvo *et al.,* 2013; Ismail *et al.,* 2013; Estevez, 2015). Oxidative stress not only effects the bird, but also negatively impacts the quality, palatability and nutritional of the meat (Estevez, 2015; Grady *et al.,* 1996). It is also of importance to the producers as it as has been shown to cause inhibited growth in broilers (Fellenberg and Speisky, 2006).

Heat stress is widely documented to increase lipid oxidation and downstream negative effects on meat (Fellenberg and Speisky, 2006; Zhang *et al.,* 2011; Sihvo *et al.,* 2013; Ismail *et al.,* 2013). However, reducing the ambient temperature of animal houses and reducing stocking density are not generally practical due to the loss of growth performance from the increased energy required for thermoregulation.

Diet composition is the other well-cited cause of oxidative stress (Manilla and Husveth, 1999). The dietary inclusion of either fish meal or oil has historically been used for poultry diets as a protein and energy source due to its superior amino acid profile and lack of anti-nutritive factors (Frempong *et al*., 2019). However, the oil component of broiler diets is the most prone to oxidation and therefore the most prominent dietary cause of oxidative stress.

1.11.4 The effect of oxidative stress on poultry gut health

The continual genetic selection of broilers has enabled modern birds to absorb substantial quantities of nutrients via the intestinal epithelium (Mishra and Jha, 2019). Reactive oxygen species are generated by intestinal epithelial cells as a by-product of normal oxygen metabolism and are also produced in direct response to enteric commensal bacteria. Oxidative stress in broilers can be a result of nutritional, environmental or pathological factors, all of which can negatively impact performance and health as well as meat and egg quality (Fellenburg and Speisky, 2006).

Tight junctions are situated between intestinal epithelial cells acting as a barrier to prevent microorganism invasion (Anderson and Itallie, 1995). Studies suggest that interaction between the mucosa and enteric microbes can result in the production of reactive oxygen species and consequently oxidative stress (Naidoo *et al*., 2008). Damage caused to the intestinal epithelium can damage or destroy the barrier function and tight junctions, leaving the gut vulnerable to pathological disease and the host open to systemic infection from the microbial population of the gut (Mishra and Jha, 2019). It is also suggested that oxidative stress can affect the composition and functionality of the microbial community in the gut (Dumitrescu *et al.,* 2018). As anaerobes thrive in the presence of electron acceptors (such as free radicals), it can increase the possibility of potentially pathogenic bacteria reaching systemic circulation (Dumitrescu *et al.,* 2018).

1.11.5 Oxidative effects on chicken meat

Oxidative stress is also thought to have profound effects on meat quality and shelf-life. Post-mortem, chicken meat is particularly vulnerable to oxidative processes due to the degree of unsaturated lipids within the muscle. Post-mortem oxidation (lipid and protein) dramatically affects the shelf life and quality of the final meat product. Modern production means that meat is often transported large distances for sale and as such, reduced shelf life represents significant financial losses. A number of external factors can also affect the levels of oxidative reactions. Physiological damage and psychological stress prior to and during the slaughter process may have an effect on the oxidation of poultry meat (Min and Ahn, 2005). Likewise, meat handling (aging, tenderizing, shortening, deboning), storage (temperature, time and oxygen exposure) and preparation (deboning, adding additives and cooking) can accelerate this process. Rancidity and poor flavour are generally attributed to the effects of lipid and protein oxidation (Min and Ahn, 2005, Lund *et al.,* 2011) and these are obviously undesirable to the end consumer.

White striping, spaghetti breast and woody breast are emerging challenges facing the broiler industry. These novel myopathies started to appear around 10 years ago and until that point were entirely unknown (Petracci *et al*., 2019). The financial impact of these are expected to result in $200 million to $1 billion economic losses to the US broiler industry alone (Huang and Ahn, 2018), as they make the meat unacceptable and it is downgraded for pet food use. Whilst the mechanism for these conditions is largely still poorly understood, all three conditions have been linked to the oxidative status of the bird (Petracci *et al.,* 2019).

1.11.6 White striping

White striping the presence of distinctive white striation throughout the breast meat. The pectoralis major is most commonly affected, but white striping is sometimes seen in the pectoralis minor (Petracci *et al.,* 2019). White striping has been widely associated with increased growth rate and body weight (Kuttappan *et al*., 2012). Under microscopic examination breast meat affected with white stripe reveal accumulation of lipids, lysis of muscle fibres, inflammation, loss of cross striation and proliferation of connective tissue (Kuttappan *et al*., 2013; Huang and Ahn, 2018). Affected meat has been found to show reduced marinade uptake, increased water loss during cooking and reduced consumer acceptance (Kuttappan *et al*., 2013; Mudalai *et al*., 2014). Whilst there is no threat to human health from consumption of affected meat, the tabloids and other press have published reports of affected meat containing up to 224% more fat than unaffected, further reducing consumer acceptance. White striping has been reported to affect up to 50% of British breast meat (Petracci *et al*., 2019). Causative factors are thought to include rapid growth and body weight gain, heavy genetic selection for fast growing strains, genetic mutations and oxidative stress (Petracci *et al.,* 2019).

1.11.7 Spaghetti meat

It is still debated whether spaghetti breast/meat is related to white striping (Petracci *et a*l., 2019). However, this myopathy is characterized by a separation of bundles of muscle fibres, generally located in the in the cranial region of the breast muscle (Baldi *et al*., 2018; Soglia *et al.,* 2018) (figure 1.6). Intramuscular connective tissue comprises of three sections; endomysium, perimysium and epimysium that cover individual muscle fibres and the entire muscle (Huang and Ahn, 2018). In the presence of spaghetti meat, the connective tissue of the endomysium and perimysium progressively decrease in density. As such, the bundles of muscle fibres disintegrate and become soft with the appearance of spaghetti (Huang and Ahn et al., 2018). It is still unclear whether this condition occurs pre or post-mortem but is thought to effect around 20% of British chicken breast (Petracci *et al*., 2019). As with white stripe, the causative factors are generally considered to be rapid growth, genetic selection/changes and/or oxidative stress (Petracci *et al*., 2019; Kuttappan *et al.,* 2012).

Figure 1.6 – The spaghetti breast myopathy found in broiler meat

1.11.8 Woody breast

As with white stripe and spaghetti meat, woody breast is generally considered to be caused by heavy genetic selection for fast growing strains, and by oxidative stress (Huang and Ahn, 2018). Woody breast is characterized by hard and rigid muscle that can appear as bulged and display a surface haemorrhage and/or a pale-yellow viscous exudate on the surface of the muscle (Mazzoni *et al*.,

2015). The pH of the meat is also higher than found in a normal muscle tissue and extensive lipidosis and fibrillar collagen deposition is present (Chatterjee *et al*., 2016). Woody breast begins as focal legions before spreading as a diffuse form throughout the breast as the age of the bird increases (Soglia *et al.,* 2016).

1.12 Nutritional interventions to improve gut health and oxidative status

1.12.1 Antioxidants

Antioxidants are substances or compounds that prevent or inhibit the damaging effect of excess free radicals (Gagne, 2014). Antioxidants can be categorized in several ways. Firstly, antioxidants can be of either endogenous or exogenous origin. Can be either naturally occurring or synthetic (Fellenburg and Speisky, 2006). However, the most common method of categorization is either enzymatic or non-enzymatic. Enzymatic antioxidants work by degradation and removal of free radicals (Nimse and Pal, 2015). They convert reactive oxygen species to hydrogen peroxide and then to water during a multi-step process utilizing cofactors including copper, zinc, iron and manganese (Dugan and Choi 1999, Nimse and Pal, 2015). Non-enzymatic antioxidants include substances such as vitamin C, E, plant polyphenols, carotenoids and glutathione (Nimse and Pal, 2015). These work by interrupting the chain reactions of free radicals.

Alternatively, antioxidants can be categorized their solubility in either water or lipids. The water-soluble antioxidants such as vitamin C are predominantly found within cellular fluids including cytosol or cytoplasmic matrix. Whilst the lipid-soluble antioxidants such as carotenoids, vitamin E and lipoic acid are generally found within the cell membrane (Nimse and Pal, 2015; Mironczuk-Chodakowska *et al.,* 2018).

Finally, antioxidants can be categorized by molecular size. Small-molecule antioxidants such as vitamin C and E, carotenoids and glutathione are responsible for neutralizing radical oxygen species (scavenging) and then removing them. Whereas large molecule antioxidants (the enzymes and some

sacrificial proteins such as albumin) absorb reactive oxygen species, preventing the attack of essential proteins (Nimse and Pal., 2015; Mironczuk-Chodakowska *et al.,* 2018) (Figure 1.8).

Figure 1.8 - The biologic mechanisms of antioxidant protection (Source: de la Vega-Monroy, 2013)

Picture not included due to lack of permission

1.12.2 The effect of dietary antioxidants on broilers

As dietary antioxidants are absorbed in the gut, they have the potential to assert a systemic effect on the bird (Fellenburg and Speisky, 2006). Supplementing broilers with antioxidants has been found to have a number of beneficial effects, not only on the birds, but also on the meat entering the human food chain (Fellenburg and Speisky, 2006). When looking at the literature for broilers, vitamin E is possibly the most heavily studied exogenous antioxidant. Deficiencies of vitamin E have been shown to cause a large number of negative effects, including breast meat being more susceptible to membrane lipo-peroxidation Supplementing broilers with the antioxidant glutathione peroxidase

and Selenium has been shown to protect capillary cells from the potential damage of lipo-peroxyl radicals (Noguchi *et al.,* 1973). Vitamin E residing within the membrane can then serve as a second line of antioxidant defence, averting propagation of the lipo-peroxidative chain. If the bird is deficient in either vitamin E or Se, antioxidant mechanisms fail to work and could therefore allow lipo-peroxidation and its deleterious disease effects to ensue (Noguchi *et al*., 1973).

Of the synthetic antioxidants ethoxyquin, butylated hydroxytolulene (BHT) and butylated hydroxyanisole are probably the most commonly used in the animal feed industry (Fellenburg and Speisky, 2006; Salami *et al.,* 2015). Ethoxyquin has been demonstrated to produce significant increases in BWG before 4 weeks of age (Waldroup *et al.,* 1961, Wang *et al.,* 1997), and reduced level of tissue lipid peroxidation. Dibner *et al*.,(1996) demonstrated the benefits of supplementing broilers with ethoxyquin, showing increased villus length and surface area in the small intestine of birds fed oxidised fat. Likewise, ethoxyquin is believed to prevent the oxidation of the lipids and lipid soluble portion of the feed ration (Cabel and Waldroup, 1989).

BHT is a monophenolic fat-soluble antioxidant that is stable at high temperatures. BHT has been shown to have numerous beneficial effects, including chemoprevention (Hocman, 1988),protective effects against hepacarcinogenocity (Williams and Latropoulos, 1996) and protection against some classes of mycotoxins (Larsen *et al*., 1985) Singh *et al.* (2019) demonstrated the efficacy of BHT for ameliorating aflatoxicosis. These authors fed broilers fed a maize-rice-soy diet with maize infected with aflatoxin (form of mycotoxin). It was found that birds given BHT at 1000ppm and 2000ppm showed significantly increased BWG, FCR and improved metabolic responses to aflatoxin exposure. BHT is often fed in combination with BHA as they work synergistically together (Salami *et al.,* 2015). In combination BHT and BHA act as chain reaction terminators and are particularly effective at preventing the lipid oxidation of fats/oils present in broiler diets. BHT and BHA has been demonstrated to have protective effect the final meat product (Fellenburg and Speisky, 2006). Lin *et al.* (1989) demonstrated that birds fed diets supplemented with a mixture of BHT and BHA showed

significantly increased BWG during production and the chicken meat had better oxidative stability compared to a control diet without antioxidants.

1.12.3 Probiotics

A normal gastrointestinal tract has a mix of bacteria, both beneficial and potentially pathogenic in nature. In optimum conditions, this balance would be tipped towards a higher percentage of beneficial microbes. Unfortunately, in modern commercial broiler production, conditions are rarely optimal, and therefore a number of challenges exist leading to sub-optimal gut health. Chicks begin life with a sterile GIT and bacterial colonisation occurs post hatch. Commercial broilers are hatched in a hatchery, away from the parent birds. Due to stringent hygiene standards in a commercial hatchery and the fumigation or washing of eggs, there is limited contact and exposure to the commensal bacteria of the parent that would naturally start the initial colonization of the chicks GIT (Varmuzova *et al.,* 2016). Likewise, it has been shown that the stress of transport and move to new housing can negatively affect the microbial balance of the gut. Research has shown that exposing chicks to probiotic bacteria can aid optimal bacterial colonisation of the GIT (Pan and Yu, 2014).

The term probiotic is derived from the Greek 'for life'. Currently pro-biotic is used to cover any bacteria deemed to provide a positive effect on health.

The World Health Organization defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"(WHO, 2002) ; in order to be labelled a probiotic, scientific evidence for health benefit has to be documented. Probiotics are often a mono or mixed culture of live microorganisms, which are often of lactic acid bacteria descent. These include *bifido* bacteria, *lactobacilli* or *lactococcus*, *streptococcus* and a variety of yeast species. These indigenous bacteria are considered beneficial to gut health due to their ability to inhibit growth of pathogenic bacteria (Patterson and Burkholder, 2003). Mechanisms by which this is achieved include; outcompeting pathogenic bacteria for resources such as nutrients and space, decreasing the pH of

the gut causing environmental acidification via rapid production of volatile fatty acids (VFAs) such as acetate or lactate, or by producing compounds which can act as an antimicrobial, such as bacitracin (Hill *et al*, 2014), thereby inhibiting the growth of potentially pathogenic bacteria. It has also been found that administration of probiotics can also stimulate the immune system (Dong *et al.,* 2012). Alternative strategies to stimulate these indigenous beneficial bacteria are by providing a substrate to essentially selectively provide nutrients to the beneficial bacteria, thus improving their chances of increased colonization. This can be achieved through the use of prebiotics.

1.12.4 Prebiotics

Prebiotics are one of the primary groups of compounds that are being considered as potential alternatives to antimicrobials. Prebiotics have been defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Roberfroid *et al*., 2010).

Unlike probiotics, prebiotics are compounds that can be metabolized by one or a limited number of beneficial bacteria in the gut, inferring a competitive advantage to the given bacteria. It has been suggested that providing probiotics alone may be less successful, as without nourishment the live microorganisms may not survive well enough to infer any benefit. One option identified is to supplement the diet with both pro and prebiotics in the form of a symbiotic. Thus, providing some of the nutritional requirements of the microorganisms at the same time as adding the bacteria, in order to aid their colonization in the hindgut.

Prebiotics are thought to have the following modes of action;

1. Lower the intestinal pH by stimulating bacterial production of VFAs (Chio *et al,* 1994)
2. Inhibiting and/or preventing colonization of pathogenic bacteria (Bengmark, 2001).
3. Modifying metabolic activity of indigenous intestinal flora.
4. Stimulation of the immune system

Prebiotics provide a selective substrate for microflora in the gastrointestinal tract. This substrate provides nutrients that can be metabolized by selected organisms considered beneficial to gut health, thus allowing beneficial gut micro flora to flourish and aid their colonization in the hindgut (Hajati and Rezaei, 2010). Microbes that are considered to be beneficial to the host enjoy a mutualistic relationship with the host providing anaerobic shelter and nutrients for the bacteria. In return, these beneficial bacteria produce enzymes that aid degradation of various complex molecules including polysaccharides (Onrust, 2015).

Prebiotics are generally carbohydrate based, including inulin, lactulose and oligosaccharides such as fructo-oligosaccharides and galacto-oligosaccharides (Hernandez-Hernandez *et al.,* 2012). These prebiotics have the ability to aid the proliferation of lactobacilli and bifido bacteria. These bacterial species are of particular interest due to their metabolites which include an abundance of short chain fatty acids such as lactate and butyrate (Kanauchi *et al.,* 1999).

1.12.5 Exogenous enzymes

One strategy for supporting the growth and performance of broilers is the use of exogenous enzymes. This practice is the act of supplementing broilers with enzymes that the birds cannot produce independently. Exogenous enzymes literally break down larger dietary components into smaller pieces. They can allow producers to improve the availability and utilization of nutrients, improve intestinal health, gut function, litter quality and reduce anti-nutritional factors and the environmental impact of animal production (Kleyn, 2013). Exogenous enzymes used in broiler production include carbohydrases such as xylanase, beta-glucanase that are used to degrade the carbohydrate fraction of the feed ration and Phosphatases such as phytase, that is used to release dietary phosphorus, bound within phytate in the diet. There are a wide range of different enzymes on the market for use in production animals which serve a variety of purposes. For the purpose of this project only xylanase will be discussed in detail.

1.12.6 Fibre

All plant derived animal feed ingredients contain fibre. Dietary fibre is defined as “carbohydrate polymers with three of more monomeric units”. These polymers cannot be digested nor absorbed in the small intestine of mammals. There are several types of fibre that can all act differently when ingested (table 1.1).

Table 1.1 Types of fibre and dietary sources (adapted from the British Nutrition Foundation, 2020)

Fibre component	Description	Dietary sources
Cellulose	Polysaccharides of up to 10,000 closely packed glucose units arranged linearly	Grains, vegetables, fruit, cereal grains
Hemicellulose	Polysaccharides containing sugars other than glucose	Cereal grains, legumes
Lignin	A non-carbohydrate component associated with plant cell walls	Outer cell walls of cereal grains
Beta-glucans	Glucose polymers that have branched structure (as opposed to linear like cellulose)	Cell walls of oats and barley
Pectins	Non-starch polysaccharides common to all cell walls	Grains, fruit. Vegetables legumes and potatoes
Gums and mucilages	Non-starch polysaccharides that are gel-forming and help keep the structure of plants	Seeds, seaweed extracts
Resistant starch	Starch and the products of starch digestion, not absorbed by the small intestine	Legumes, potatoes, cereal grains
Oligosaccharides	Short chain carbohydrates of 3-9 monomers.	Fruits, vegetables, xylan in cereal grains

Historically fibre has been avoided in broiler diets due to the perception that it has a diluting effect on nutrient availability, reducing feed intake, digestibility and growth performance. However, in recent years there has been a paradigm shift towards the industry recognising the value of certain types and quantities of fibre in the diet. Choct (2009) demonstrated the value of insoluble dietary fibre as a structural component to increase gizzard development and function and a number of other studies have demonstrated enhanced nutrient digestibility in birds fed structural components such as fibre, whole or coarsely ground grains (Xu *et al.,* 2017; Kheravii *et al.,* 2017; Kimiaeitalab *et al.,* 2017).

2018. However, not all fibre is beneficial to broilers. Pectins, gums and mucilages are all non-starch polysaccharides that tend to have a gel-forming effect in the GOT, increasing the viscosity of digesta and reducing nutrient availability (Kimiaeitalab *et al.,* 2017).

1.12.7 Non-starch polysaccharides

Cereal grains and legumes form the basis of feed rationsof most poultry diets. The fibre component of many grains can be comprised mainly of non-starch polysaccharides (NSP) that have been shown to elicit significant anti-nutritive effects on monogastric species such as pigs and poultry (Choct, 1997; Stef *et al.,* 2010). NSP levels have been shown to vary from 0.1% in corn flour, up to 37% in wheat bran. NSP are derived from the plant cell wall and account for approximately 10% of the whole grain in wheat (Pirgozliev *et al.,* 2015). NSP also have a role in the storage of energy (Choct, 1997). NSP can be either soluble or insoluble. Both types can have detrimental effects on monogastric livestock (Choct, 1997).

Polysaccharides are polymers of monosaccharides attached by glycoside linkages (Choct, 1997). NSP cover a vast range of polysaccharide molecules which comprise of a number of chemical compounds including cellulose, arabinoxylans, beta glucans and pectic polysaccharides containing mannans and gallactans (*Stef et al*., 2010) (figure 1.9).

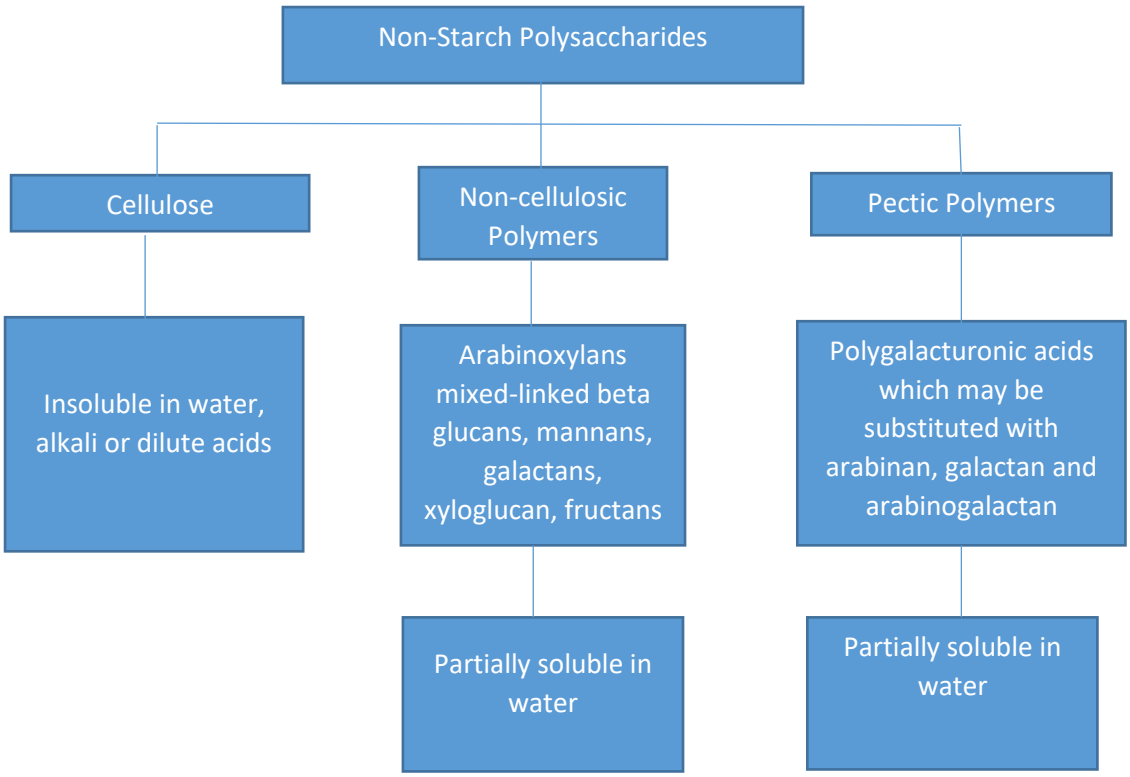

Figure 1.9 – The classification of non-starch polysaccharides (Choct *et al.,* 2010,)(Diagram made by Desbruslais, 2020)

1.12.8 The anti-nutritive effect of NSP

The anti-nutritional effects of NSP can be diverse and at time severe (Choct, 1997). The majority of detrimental effect of NSP are associated with the viscosity, modulation of the intestinal microbiota and physical and morphological effects on the intestinal tract.

Non-starch polysaccharides can be viscous by nature, with viscosity varying by solubility and molecular weight (Choct, 1997). Viscosity effects are mainly caused by the uptake of water in the digestive tract by soluble NSP, causing them to become gelatinous and highly viscous (Rosicka-Kaczmarek *et al.*, 2017). This can in turn reduce the rate of diffusion between endogenous digestive enzymes and digesta, reducing active interaction on the mucosal surface (Edwards *et al.*, 1988). The glycocalyx of the intestinal brush boarder can interact with NSP, causing thickening to the rate-limiting unstirred water layer of the intestinal mucosa, which directly inhibits nutrient absorption via

the intestinal wall (Johnson and Gee, 1981). This in turn can decrease feed intake and body weight gain. Cowieson, (2010) suggests that up to 400-450kcal (1.67-1.88 MJ) of digestible energy per kg of feed can be lost due to the effects of NSP in corn/soy-based diets. The increased viscosity of the digesta reduces the transit time through the gut, consequently increasing the time in which undigested nutrients can be utilized for pathogenic microbial propagation (Raza *et al.,* 2019)

Prolonged consumption of soluble NSP has been associated with increased secretion of digestive juices and an enlargement of digestive organs, generally convoyed with reduced nutrient digestibility (Choct, 1997). Insoluble NSP can also prevent the contents of plant cells from being accessed by endogenous enzymes, consequently reducing the digestibility of nutrients such as protein and amino acids (Halas and Babinszky, 2014). The insoluble NSP can also be responsible for reducing transit time in the large intestine, preventing optimal water resorption (Halas and Babinszky, 2014)

1.12.9 Xylanase

Xylanases are hydrolytic exogenous enzymes from the glycanase family (Xylanases and B-glucanases). Xylanases cleave the β1,4 backbone of the complex plant cell wall polysaccharide xylan (Collins *et al*., 2005). Xylanases are found in a number of forms, which vary in mode of action, substrate specificities and hydrolytic activities. Xylanases catalyse the endohydrolysis of 1,4 –β-D- xylosidic linkages in xylan and are involved in the production of xylose, one of the primary carbon sources for plant metabolism (Collins *et al*., 2005). Xylanases can be derived from a number of sources including bacteria, algae, fungi, protozoa, arthropods and gastropods.

Exogenous xylanase has been routinely added to poultry diets for the last 20 years (Cowieson and Masey O'Neil, 2013). The addition of xylanase has been demonstrated to improve performance by between 2-4% when compared to un-supplemented birds. It has been shown that these improvements are consistent when used in diets high in viscous grains such as wheat, oats, barley, rye and triticale. However, improvements are less consistent in diets containing sorghum and maize (Cowieson and Masey O'Neil, 2013, Bedford and Cowieson, 2012).

The mode of action for exogenous xylanase has been extensively studied, but still not fully elucidated. Four potential mechanisms have been suggested for the beneficial effects of xylanase on reducing the apparent anti-nutritional effects of viscous grain-based diets (Bedford and Schulze, 1998; Wu *et al.*, 2004 Bedford, 2018).

1. Viscosity effect – xylanase reduces the viscosity of digesta by degrading long chain polysaccharides via hydrolysis of the soluble high molecular weight pentosans found in highly viscous cereal grains (Gonzalez-Ortiz, 2016)
2. Cage effect (cell wall mechanism) – xylanase degrades the cell wall of cereal grains releasing encapsulated nutrients. This increases the diffusion of nutrients, substrates and enzymes enabling the host better utilize nutrients in the ration (Bedford and Autio, 1996)
3. Pre-biotic effect – xylanase degrades AX present in the diet into oligosaccharides which can be used a nutrient source by saccharolytic microbes in the gut (Bedford, 2018,Craig, 2019)
4. Signalling effect – xylanase stimulates microbes in the gut to produce xylanase *in situ* which can subsequently increases the hydrolysis of AX into AXOS and XOS which can then be used as a nutrient source by saccharolytic microbes in the gut (Bedford, 2018).

It is widely agreed that xylanase plays an active role in cleavage and degradation of the cell wall matrix in the NSP fraction of viscous grains, reducing xylans in the diet down to xylose (figure 1.10).

Probably the most demonstrated mechanism of xylanase is the reduction of digesta viscosity (Choct, 1998). Xylanase aids the degradation of long chain polysaccharides by way of hydrolysis of the soluble high molecular weight pentosans found in highly viscous cereal grains. This therefore improves the rate of diffusion between substrates and enzymes, allowing endogenous digestive enzymes to access nutrients more readily (Bedford, 2000, Cowieson and Massey O'Neill, 2013). This reduction in digesta viscosity has been found to stimulate gut motility, increasing the rate of passage, consequently allowing birds to consume more ration, and accelerate growth. (Choct, 1997).

Figure 1.10: The structure of xylan and its digestion to xylose (Biotech, 2020).

The second mechanism is a reduction in cell wall effect – also known as a cage factor. This mechanism describes how xylanase degrades the plant cell walls, increasing the availability of nutrients previously encapsulated in the cell wall matrix (Bedford and Schulze, 1998).

The third potential mechanism of xylanase explores the potential of xylanase to modulate the microbial population of the hindgut. Bedford and Cowieson, (2012) report that when structural fibre is hydrolysed by exogenous xylanase, it yields a reduced molecular weight end product that is rich in short-chain xylo-oligomers of 2-5 monomers in length. It has been shown that provision of these xylo-oligomers into the gastrointestinal tract (GIT) of the bird increases the number of *bifido* bacteria in the caeca (Cowieson and Masey O'Neill, 2013; Courtin *et al*., 2008). When these oligosaccharides are fermented by micro-flora in the caeca and colon, the microbes rapidly produce volatile fatty acids (VFA) that cause changes to the short-chain fatty acid profile in the distal GI tract (Cowieson and Masey O'Neill, 2013). The additional VFA reduce the environmental pH and inhibit proliferation of putrefactive organisms, allowing beneficial microbes to outcompete and flourish (Bedford, 2000).

Additionally, the reduction in pH encourages proliferation of enterocytes and it has been suggested it may also be directly linked to the mediation of gastric emptying, potentially increasing transit time (Choct, 2015, Cowieson and Masey O'Neill, 2013).

Bedford, (2000) describes the function of xylanase as working in two phases. The ileal phase allows enzymes to remove fermentable substrates from the ileum. The caecal phase then degrades the sugar products such as xylose and xylo-oligomers, which are the fermented by the caecal bacteria. This stimulates volatile fatty acids (VFA) production and the growth of specific bacteria such as *lactobacilli* and *bifido bacteria* which can be considered beneficial to the bird (Bedford, 2000). The final potential mechanism is highly related to the third mechanism and explores the possibility of microbiota within the gut producing xylanase *in situ* , so consequently enhancing the potential effects as described above. This has recently been demonstrated by Bautil *et al*. (2019). These authors demonstrated that broilers develop the ability to hydrolyse arabinoxylan with age, and that by 35 days of age, broilers could achieve up to 24% total tract digestibility for arabinoxylan in the diet.

1.12.10 Xylanase-inhibitors

Xylanase-inhibitors are substances that prevent the function of xylanase (Debyser *et al.,* 1999)

Two distinct classes of proteinaceous endoxylanase have been identified in cereal grains (Juge *et al.*, 2004), Triticum aestivum xylanase inhibitor (TAXI) and xylanase inhibitor protein (XIP)

(Perez-Vendrell and Brufau De Barbera, 2007). XIP has been shown to impose significant inhibition on fungal derived xylanases compared to those of bacterial derivation. Whereas TAXI are able to inhibit the function of both bacterial and fungal derived xylanase (Juge *et al.,* 2004). The quantity and the level of activity of xylanase inhibitors has been shown to vary with different grain varieties and cultivars with Rialto and Cadenza wheat both containing high levels of xylanase inhibitors (Perez-Vendrell and Brufau De Barbera, 2007).

In broilers the presence of xylanase inhibitors has been shown to reduce growth performance (Madsen *et al.,* 2018). Again, these authors report variation in xylanase inhibitors between different wheat varieties.

1.12.11 Xylans

Xylans are the main structural polysaccharides within plant cells. Xylans account for around one-third of the earth's renewable organic carbon and are one of the most abundant non starch polysaccharides (NSP) in nature (Busse-Wicher *et al.,* 2014; Collins *et al*., 2005). Xylans are one of the main components of hemicellulose, an organic complex of polymeric carbohydrates; they are comprised mainly of the pentose sugars, xylose and arabinose (Collins *et al*., 2005).

Xylan, cellulose and lignin form the main polymeric constituents of the plant cell wall (Busse-Wicher *et al.,* 2014 Collins *et al.,* 2005; Wong *et al*., 1988). These three constituents interact via covalent and non-covalent linkages. Xylan sits the interface between lignin and cellulose, where its main function is thought to be that of plant cell wall elasticity, strength, integrity and fibre cohesion (Busse-Wicher *et al.,* 2014; Collins *et al*., 2005; Wong et al., 1988). The structure of xylan varies between plant species with various substitutions to the homopolysaccharide backbone chain. The purpose and pattern of decorations upon the xylan backbone are still to be fully elucidated and vary based upon the species and cell wall type (Busse-Wicher *et al.,* 2014). However, it is widely thought that one of the main functions of the decorations is the prevention of digestion by microbial enzymatic degradation (Busse-Wicher *et al.,* 2014).

Arabinoxylan is one of the common forms of xylan and is the main NSP present in wheat (Immerseel *et al.,* 2014) **-** Arabinoxylans are made up of α-L-arabinofuranose residues that attach at branch joints to β-(1$\longrightarrow$4)-linked D-xylopyranose polymeric backbone chains. It is possible for 2- or 3-substituted or 2- and 3- di-substituted. These substitutions are not random, however, the distribution of

substituted residues along the chain does appear random/ Arabinose residues can also be connected to other attached groups such as glucuronic acid residues, acetyl groups and ferulic acid cross links (Figure 1.11). Figure 1.12 illustrates the most stable conformations of α-L-arabinofuranose (top) and β-(1→4)-linked D-xylopyranose residues. The furanose can, take up other conformations with similar energy whereas the chair conformation of the pyranose residue remains fixed.

Wheat is reported to contain 5-8% arabinoxylans pentosans consisting of arabinose and xylose linked with B 1-4 linkages with arabinose side chains at the 02 and/or 03 positions on a xylopyranosyl backbone (Choct *et al*., 2004; Scheller and Ulvskov, 2010). The quantity of arabinose associated with the xylose backbone can be expressed as the Ara/Xyl ratio (Immerseel *et al.,* 2014). For wheat this is reported to be between 0.47 and 0.56.

Due to being non-starch polysaccharides, Xylans act as an anti-nutritional factor to some species including monogastric agricultural production animals such as pigs and poultry (discussed in section 1.13.8).

Figure 1.11 – The structure of Arabinoxylans

1.12.12 Arabinoxylans

Arabinoxylans (AX) are a soluble fraction of NSP found in many cereal grains. AX are pentosans composed of two pentoses (arabinose and xylose) (Choct, 1997).

AX are abundant in many plants as a constituent part of hemicellulose, one of the components of the plant cell wall (Ramkrishna *et al.*, 2015). The molecular structure of AX consists of a linear xylan backbone with substituents attached through 02 and 03 atoms of xylosyl residues (Perlin, 1951). There are several substituents, the main one being single arabinose residues although hexoses and hexuronic acid can also be found (Fincher, 1975). The majority of arabinoxylans in grains are not soluble in water due to being attached to cell walls by alkali-labile ester-like cross links as opposed to basic physical attachment (Mares and Stone, 1973). However, free arabinoxylans that are not attached to the cell wall are able to absorb up to ten times their weight in water, forming highly viscous solutions. When exposed to oxidative agents such as H_2O_2/peroxidase, arabinoxylans can form a gel network due to rapid re-establishment of cross-links. It is this reaction that causes the main anti-nutritional effect on poultry (Choct, 1997). This exerts an anti-nutritional effect on monogastric species such as poultry and pigs causing highly viscous conditions in the small intestine.

1.12.13 Oligosaccharides

Oligosaccharides are carbohydrates and as such can be categorized by degree of polymerization (quantity of monosaccharides units combined). Oligosaccharides are defined by IUB-IUPAC nomenclature (IUPAC-IUB, 1980) to possess between 3 and 10 sugar moieties. Other sources classify oligosaccharides as having anything up to 19 moieties, however, there appears to be no rationale for these differing limits (Voragen, 1998). Non-digestible oligosaccharides (NDOs) are classed as such due to the fact that the anomeric C atom (C1 or C2) of the monosaccharide units has a unique configuration that renders their osidic bonds unaffected by hydrolytic activity of digestive enzymes (Roberfroid and Slavin, 2000). NDOs that have been shown to enhance beneficial bacterial growth in the gut are derived from carbohydrates where the monosaccharide unit is glucose, galactose,

fructose and xylose (Sako *et al.,* 1999) (Fig.6). As such, these NDOs are considered to have prebiotic potential (Hernandez-Hernandez *et al.,* 2012). Oligosaccharides have also been shown to have antioxidant properties as discussed in section 1.13.1 (Hou *et al*., 2015)

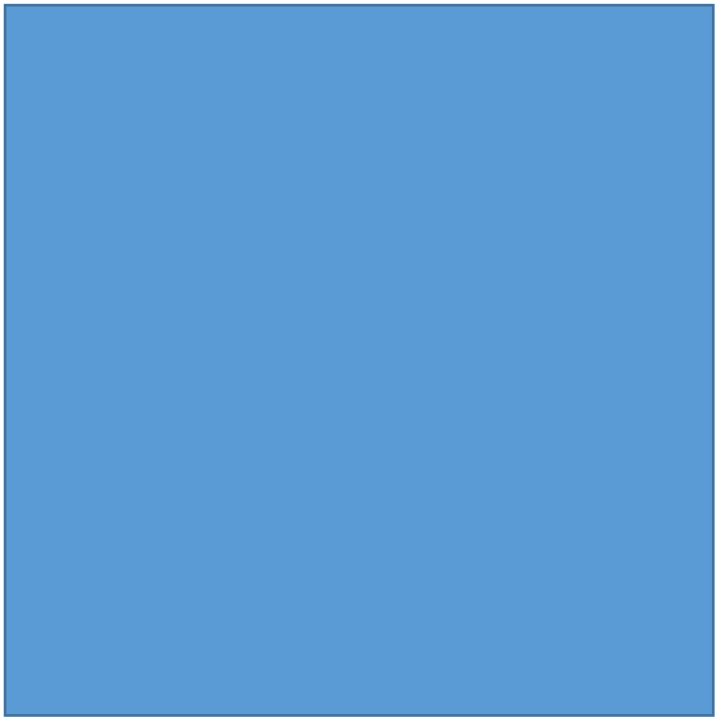

Figure 1.12 - Monosaccharide components of non-digestible oligosaccharides (Mussatto and Mancilha, 2007)

1.12.14 Xylo-oligosaccharides

Xylo-oligosaccharides (XOS) are a class of oligosaccharides that are currently undergoing an increase in research output in numerous species, from humans to chickens (Maesschalck *et al.,* 2015; Mussatto and Mancilha, 2007; Selle *et al.,* 2003; Sun *et al*., 2013).

As previously mentioned, a XOS is a sugar oligomer comprised of xylose units. It is present in numerous natural sources including fruits, vegetables and cereal crops (Vazquez *et al.,* 2000).

Arabinoxylans have been identified in all major cereal grains including wheat, rye, barley, oats, sorghum maize and millet (Izydorczyk, 2009). When the heteropolymer AX is exposed to hydrolytic

degradation, the result is a combination of arabinose-substituted xylo-oligosaccharides (AXOS) and non-substituted xylo-oligosaccharides (XOS) (Maesschalck *et al.,* 2015).

XOS are oligomers made up of xylose units, joined by β-(1-4) linkages (Aachary and Prapulla, 2008). Industrial production of XOS is carried out from lignocellulosic materials (LCMs) from sources such as forestry, agriculture, industry and urban solid waste (Vazquez *et al.,* 2000). In addition to non-structural components, LCMs comprise of three polymers: cellulose, lignin and hemicelluloses, including xylose, arabinose and mannose (Vazquez *et al.,* 2000). Polymers of xylose (xylan), arabinose (arabinan) or mannose (mannan) will account for a substantial portion of the hemicellulose depending on the source of the LCM. This portion of the hemicellulose can then be substituted via ether or ester bonds. XOS is typically extracted from raw materials including corncobs, sugarcane bagasse, straw and DDGS (Isao *et al*., 1989; Suwa *et al*., 1999; Vazquez *et al.,* 2000). As these materials are by-products of other processes, XOS production can represent increased value as part of these processes.

When XOS is selectively fermented in the hind gut of monogastrics, it has been shown to modulate both the composition and activity of the host's microbiota (Maesschalck *et al.,* 2015). Lactate and short-chain fatty acid (SCFA) production following fermentation has been confirmed both in a number of studies in both pigs and poultry (Broekaert *et al.,* 2011; Scott *et al.,* 2014).

1.12.15 Oligosaccharides and their use in broilers

Originally commercially produced as fillers for human food and used as artificial sweeteners, oligosaccharides have more recently been utilised in livestock for their prebiotic function. The fibres of cereal grains are comprised of carbohydrate polymers that remain resistant to digestive enzymes in the small intestine of monogastric species and so reach the hind-gut intact. (Maesschalck *et al.,* 2015). However, some or all these polymers are fermented in the distal gut and as such are thought to have

beneficial implications on the gut health of broilers due to the provision of a selective substrate for beneficial microbes in the GIT (Teng and Kim, 2018).

Various oligosaccharides have been used in poultry diets including manno-oligosaccharides, galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS) and xylo-oligosaccharides. All of these have been found to infer beneficial results on the gut health of broilers, though to a greater or lesser extent.

Mannan-Oligosaccharides

Mannan-oligosaccharides (MOS) (present in the cell wall of yeast), have been found to alter the gut's bacterial population due to the yeast cell wall having high affinity ligand for bacteria which then offers a competitive binding site for bacteria (Biggs *et al.*, 2007). Pathogenic bacteria that possess mannose-specific type-1 fimbriae attach to the MOS instead of binding to the intestinal epithelial allowing the bacteria to move through the gut without colonization. It is also thought that oligosaccharides have a positive effect on immune function. MOS has been shown to increase Immunoglobulin-A production. This production inhibits the attachment and penetration of bacteria within the lumen as well as increasing the amount of intestinal mucus present (Biggs *et al.*, 2007). Immunoglobulin A has also been shown to prevent inflammation (Biggs *et al.,* 2007) that has the potential to cause damage to epithelial tissue. Ao and Choct, (2013) investigated the effect of MOS and FOS on broiler performance and gut development. When birds were fed a sorghum and soy-based ration supplemented with either MOS or FOS at 1kg per tonne in the starter, and 0.5kg per tonne in the grower and finisher, these authors found that both oligosaccharides significantly improved body weight and FCR and birds fed MOS showed a trend towards increased bursa weight. MOS supplementation also significantly improved flock uniformity. Likewise, Shendare *et al.,* (2008), explored the performance effect of MOS and beta-glucans with a maize-based die. These authors found MOS increased body weight by 5.37% compared to a control and enhanced FCR by 8.45%. Iji *et al*., (2001) studied the intestinal structure and function of broilers fed sorghum and lupin-based diets supplemented with MOS. This study

showed that when supplemented at 5g/kg, MOS significantly increased jejunal villi length and L-tryptophan uptake by the jejunal brush-border membrane vesicles. These authors also reported small increases in body weight in supplemented birds, but no improvement in FR.

Isomalto and fructo oligosaccharides

Isomalto-oligosaccharides (IMO), when supplemented at 0.3% significantly increased the quantity of butyrate and isobutyrate concentrations in the jejunum (Zhang *et al.,*2003), However, performance was not significantly altered by isomalto-oligosaccharides supplementation compared to a control. Chung *et al.*, (2004) compared the growth of broiler caecal isolates grown with either IMO and FOS as a sole carbon source. These authors report that FOS significantly increased caecal isolate growth compared to IMO. When the caecal isolates and *Salmonella* were grown in a mixed culture on IMO, the salmonella population was decreased with IMO. Likewise, when the caecal isolates were grown with *Bifido* bacteria, FOS significantly increased the growth of the isolates and the *Bifido* bacteria. This study concludes that FOS and IMO show promise for supporting beneficial broiler intestinal microflora modification by aiding the proliferation of *Bifido* bacteria.

1.12.16 The Effect of XOS in broilers

XOS supplementation has been shown to have numerous benefits for broiler production, on both performance and physiological parameters. In 2006, Wu *et al*. found that XOS inclusion (1,2 and 4 g/kg) significantly reduced cumulative mortality and feed conversion ratio over a 7-week broiler study. These authors also found that when fed at 2g/kg, XOS increased the number of lactobacillus in the ileum. More recently, Maesschalck *et al*. (2015) found that birds fed a wheat/rye-based diet supplemented with XOS at 0.2% over 39 days showed significantly improved FCR and increased numbers of *lactobacilli* present in the hind gut as well as significantly increased the length of ileal villi,

thus increasing the surface area and absorptive capacity of the ileum compared to the control birds. Subsequently, this led to increased concentrations of SCFA.

A number of studies report that XOS inclusion shows numerical increases in performance parameters such as weight gain, feed conversion ratio (FCR) and feed intake (Suo *et al*., 2015; Dang, 2004, Craig, 2019). However, improvements are not always statistically significant. Suo *et al.* (2015) fed a corn/soy diet supplemented with XOS at 25, 50, 75 or 100mg/kg. The authors found that 75 and 100mg/kg of XOS did improve FCR, but only significant between days 22-42 and 0-42, suggesting that XOS may only be having a significant affect during the later periods of broiler production. Likewise, Craig *et al.* (2020) compared supplementing broilers with various carbohydrase enzymes (xylanase, xylanase/ß-glucanase) to a diet containing XOS (0.025%) or a control. In a 21-day study feeding wheat/soy/corn diets, these authors found increased BWG and reduced FCR in the XOS treatment compared to the control. The same authors conducted an earlier study (2019) comparing XOS or xylanase supplementation to a control. The study used a control diet (wheat/soy) to compare to a low and high dose of xylanase (16000BXU and 32,000BXU) and low and high dose of XOS (0.25g/kg and 1g/kg) . The authors found reductions in FCR for both the xylanase and XOS treatments compared to the control (Craig, et al., 2019). Ribeira *et al*., (2018) found that in a 0-28-day study feeding wheat-based diets, XOS (Corn cob derived) supplementation at 0.06g/kg significantly increased the final body weight of broilers and reduced the cumulative FCR compared to the control, but feed intake and digesta viscosity were not affected. This may suggest that the mode of action for XOS may purely be microbial modulation, rather than an enzymatic effect on NSPs in this study. However, Ribeira *et al.,* (2018), only looking at days 0-28, there may have been effects during the latter stages of broiler production as seen in the work of Sue *et al. (*2015). Ribeira *et al*., (2018) went on to conduct a second 0-42 day study utilizing XOS at two inclusion amounts (0.1g/kg and 1g/kg) with a wheat-based diet. Here the authors found that final body weights were significantly increased in both experimental groups supplemented with XOS, and that the lower inclusion level produced higher body weights. Feed intake was also significantly improved in both XOS groups, with the lower inclusion rate again

highest, but only in the latter stages of production (D35-42), which agrees with the results of *Suo et al.,* 2015). FCR was also significantly improved in the lower inclusion rate group when compared to the control. The majority of papers which investigate the microflora of the host agree that XOS supplementation has a positive effect on the microbial population of the gut in broilers. Courtin *et al.* (2008) supplemented a wheat-based diet with XOS at 0.25 and 0.5g/kg and found that two weeks of XOS supplementation (extracted from wheat flour) resulted in significantly increased numbers of *bifido* bacteria in the caeca of broilers, however the authors did not find any significant difference in the numbers of *lactobacilli.* A study by Pourabedin *et al.* (2015) found similar results with significant increases in beneficial bacteria in the caeca of birds supplemented with XOS at 2g/kg, however, in this case it was *lactobacillus* genus bacterium that flourished. In some cases, both *bifido* and *lactobacillus* bacteria have been found to proliferate with XOS inclusion. Ribeira *et al.* (2018) found that XOS supplementation at 0.1g/kg increased the microbial populations of *bifido bacterium* and *lactobacilli*. As was found in the performance data from this study, a lower XOS inclusion rate (0.1g/kg) produced higher levels of beneficial bacterium. The results of Ribeira *et al.* (2018) suggest that there is likely to be an optimal dosage when supplementing broilers and that overdosing can reduce or eliminate the beneficial effects of supplementation.

XOS supplementation has also been shown to increase the production of volatile fatty acids. De Maesschalck et al., (2015) showed that supplementing wheat/rye-based diets with XOS at 0.5% directly increased production of both butyrate and lactate by the microbial population of the distal gut. Likewise, Craig *et al*., 2020, reported that XOS supplemented at 0.25g/kg and 1g/kg significantly increased production of iso-butyric, acetic and iso-valeric acids in the caeca of broilers. A study looking at the effect of XOS on broilers showed that supplementing corn/soy diets with 0.2g/kg XOS significantly increased the presence of butyrate and acetate in the caeca (Yuan *et al.,* 2018).

1.13 Methods for measuring levels of oxidative stress/damage

Accurate measuring of ROS can be difficult due to their short life span and high reactivity with other components. Peroxyl radicals and hydro peroxide are considered relatively stable with a half-life of a few seconds to a maximum of a few minutes. However, hydroxyl radicals are highly reactive and have a half-life of less than a nanosecond (Pryor, 1986). Due to this it is generally considered more accurate to assess the damage caused as a direct result of ROS to lipids, proteins and nucleic acids as described in the following sections.

1.13.1 Measuring protein damage

Protein damage due to ROS is one of the main concerns, as this is the key component in ROS-mediated tissue damage. Protein carbonyl are produced as a by-product of oxidation of protein backbones and amino acid residues, including lysine, arginine, and threonine by ROS (Berlett and Stadtman, 1997). As such, protein carbonyl is often used as a marker for oxidative changes. Protein carbonyl can be measured in two ways. The first uses 2, 4-dinitrophenylhydrazine (DNPH) assay developed by Levine *et al*., (1990). Here DNPH reacts with protein carbonyls to form a Schiff base that can produce dinitrophenylhydrazone products. The levels of these products can then be measured using spectrophotometry at 375 nm and correlated to levels of oxidized proteins. The second method uses 2D gel electrophoresis and western blot as described by Marco *et al.,* (2012). Another approach to measuring protein oxidation is to detect advanced oxidation proteins products (Witko-Sarsat *et al.,* 1996). Advanced oxidation protein products are produced via a reaction of plasma proteins and chlorinated oxidants such as chloramines. This assay uses either plasma or serum, calibrated with chloramine-T. This is mixed with potassium iodide and acetic acid before the absorbance is measured using spectrophotometry at 340nm. The main problem with these assays is that they only measure one type of oxidative reaction and as such, are unlikely to fully elucidate the full oxidative process,

1.13.2 Measuring lipid damage

Lipid peroxidation is of particular interest to the poultry sector as it thought to be the main contributor to breast tissue myopathies such as woody breast, spaghetti meat and white stripe (as described in sections 1.10.6, 1.10.7 and 1.10.8)(Salles *et al*., 2019). Lipid peroxidation is also the process responsible for meat rancidity and reduced shelf-life and is believed to be compounded by increased levels of oxidative stress during life of the bird (Abasht *et al*., 2016). Rancidity occurs as a result of metabolic transformations of fatty acids and subsequent oxidation of the polyunsaturated fat within the meat (Bigolin et al., 2013). Damage to cell membranes by ROS is commonly assessed by measuring lipid peroxidation (Katerji *et al.,* 2019). There are numerous assays that can be used to assess lipid peroxidation, however, the most commonly used assay in the poultry industry is quantifying malondialdehyde (MDA) (Esterbauer *et al.,* 1990).

Malondialdehyde is one of the most studied end-products of peroxidation of polyunsaturated fatty acids (Esterbauer *et al.,* 1990). In addition to measuring lipid peroxidation, measuring MDA can be used to estimate levels of oxidative stress. Typically, this is done using thiobarbituric acid reactive substances (TBARS) and can be performed using blood or homogenised tissue. In this assay, MDA reacts with TBARS in acid at 100°C to create pink/red-coloured product that can be extracted with butanol, then measured using a spectrophotometer at 520-535 nm. Whilst the TBARS method is relatively simple, it is possible that aldehydes other than MDA could react with TBARS, generating derivatives that could absorb light in the same wavelength range, thus skewing the result (Giustarini *et al.,* 2009). MDA can also be measured via gas chromatography, which removes this risk, however, the analytical kit required is more substantial and costly.

In poultry, drip loss tests are often used to measure the lipid peroxidation of meat (typically breast meat). A sample of breast meat is taken immediately post slaughter, weighed individually and placed on a grid. After a period of time (24-72 hours) samples are dried with a paper towel and re-weighed. Drip loss is expressed as a percentage of the initial sample (Correa *et al.,* 2007). Whilst this method is

regularly used by the meat quality industry, it is often considered inaccurate and requires high accuracy balances to measure with precision.

1.13.3 Measuring nucleic acid damage

8-Hydroxy-2' –deoxyguanosine (8-OHdG) is amongst the major oxidative changes seen in DNA, produced by hydroxylation of the deoxyguanosin residues. These residues can be excised from the DNA by enzymatic repair systems, which leads to systemic circulation via the blood stream and subsequent urinary excretion (Ock *et al*., 2012). Due to this, 8-OHdG can be measured in either blood or urine as a marker for oxidative damage to DNA (Keterji *et al.,* 2019). This can be performed by ELISA and immunohistochemical analysis or by oxyDNA-FITC conjugate binding followed by flow cytometry with fluorescence (Katerji *et al.,* 2019). Due to birds producing combined urine and faeces (excreta), blood must be used to measure thi8s parameter in poultry.

1.13.4 Assessing antioxidant status

The homeostatic system of the body is equipped with an antioxidant system to regulate and counter-balance oxidation/ROS and their deleterious effects. This system is referred to as redox homeostasis (Katerji *et al*., 2019). Oxidative stress can occur when levels of ROS and antioxidants become unbalanced. The imbalance of pro and antioxidants can be caused by excess free radical production or by excessive antioxidant consumption. As such, antioxidant status can be correlated to the levels of oxidation found in clinical samples (Katerji *et al.,* 2019). Essentially, most methods for assessing oxidative/ antioxidant status are looking at the capacity of the individual to quench ROS and thereby maintain homeostasis. To date, there no assays that have been specifically validated in broilers to assess total antioxidant capacity, however, a number of assays can be used to assess the capacity of the blood to quench various individual reactive oxygen species. Unfortunately, this makes assessing the true, overall oxidative status of a bird impossible.

1.14 Aims and objectives

The overarching aim of this project was to explore ways of supporting the gut health and production parameters of broilers via pre-biotic supplementation. Specifically, the project aims to determine if XOS and/or xylanase can support the gut health, oxidative status and performance of broilers. The following specific objectives were set to achieve this aim:

1 – To determine if exogenous xylanase and/or xylo-oligosaccharides have a positive effect on the production performance of broilers.

2 – To elucidate the mechanism via which XOS and/or xylanase may improve the performance of broilers

3 – To determine whether xylo-oligosaccharides and or xylanase may improve gut health parameters of broilers.

4 – To determine whether the material from which the XOS is derived influences its efficacy

5 – To develop and assess a novel method for measuring the oxidative status of broilers and broiler feed

6 - To determine the efficacy of XOS and xylanase in improving the oxidative status of commercial broilers

Chapter Two: Materials and Methods

2.1. Introduction

This chapter provides an overview of the materials and methods used throughout this thesis. Three animal trials and one *in vitro* study were conducted as part of this body of work and are summarised in the table below with associated chapters where results are presented (table 2.1).

Study 1 evaluated an arabinoxylan extract derived from distillers wet grains with solubles, made by the University of Huddersfield. The study used three diets (control, Control + AX and control + AX pre-treated with xylanase) to evaluate the effect of the product on broiler chickens. Study 2 compared two different xylo-oligosaccharides derived from different base materials with and without commercial xylanase in a 3x2 factorial study to evaluate the general effect of XOS inclusion upon both performance and physiological parameters. Study 3 evaluated novel oxidation assays and validated these assays in broilers. Study 4 was a dose response trial looking at the effect of increasing the dose of a commercial corn XOS + xylanase product. All animal trials were conducted at the Poultry Research Unit at Nottingham Trent University's Brackenhurst campus, UK. Ethical approval was granted via the university ethics committee and approval numbers are provided in the individual material and methods sections for each study.

Table 2.1 - Outline of studies conducted

Study	Investigation	Chapter
1	The effect of feeding a distillers dried grains with solubles AX extract upon the performance and gut health of broilers	3
2	A comparison of two XOS products derived from different base materials	4
3	Validation of two novel methodologies for assessing the oxidative status of broilers and broiler feed	5
4	Investigation of dose response for a corn based XOS +Xyl	6

2.2 Birds and Management

Institutional and UK national NC3R ARRIVE guidelines for the care, use and reporting of animals in research (Kilkenny et al., 2010) were followed at all times. The University's College of Arts and Science ethical review committee approved all experimental procedures utilizing animals.

For all studies, day old, male Ross 308 broiler chicks were obtained from PD Hook Hatcheries Ltd, Oxfordshire. Upon arrival at the Poultry Research Unit (PRU) the chicks were allocated pens approximately 0.64m^2. The chicks were raised in a thermostatically controlled poultry room with an initial temperature of 32°C, which was reduced daily to 21°C by day 21. The room was then maintained at 21°C for the remainder of each trial. Lighting was controlled with 1 hour of darkness from day 1, which was then increased by 1-hour increments until day 6. 6 hours of darkness was then maintained until the end of the trial with 15-minute dusk and dawn periods. Wood shavings were provided as a substrate in the pens (3cm thick). Additional shavings were added as required throughout the trials. Water was continually available via nipple drinker lines and the experimental diets were provided *ad libitum* in open troughs throughout the trial. The bird room was checked twice daily to monitor bird welfare and environmental conditions. Environment was altered according to bird behaviour. Bird mortality was recorded daily in addition to the bird weight and reason if culled (for example due to deformities or illness).

2.3 Diet Formulation and Feed Preparation – study 1, 2 and 4

For study 1, mash diets were manufactured on-site by PRU staff. Three phases of diet were used, starter from day 1-14, grower, day 14-21 and finisher day 21-35. Diet formulation was undertaken by a commercial nutritionist and diets manufactured by NTU. Dry ingredients were weighed in a top pan balance (Mettler, Toledo, Leicester, UK) as per the diet production specifications sheet. Dry ingredients were mixed for 5 minutes in a ribbon mixer (Regal Bennet, Goole, UK) in batches of no more than 100KG. Oil was added, and the diet was mixed for a further five minutes before being

weighed into individual bags for each pan. Diet specification and calculated nutritional content are presented in the relevant chapters. 1kg samples of each diet treatment were reserved for proximate analysis and stored at -20°C. Nutritional composition was confirmed by laboratory analysis and are described later in this chapter.

The basal mash diets for study 2 and 4 were produced by Target feeds, Shropshire UK. The diets were formulated by a commercial nutritionist to meet the age and strain of the birds.

2.4 Sampling and Preparation Methods

2.4.1 Bird Performance Measurements – study 1, 2 and 4

In all studies, feed was weighed into pre-weighed bags that were labelled with designated pen number. Each pen of birds was fed from the designated bag. Additional feed of the same formulation was added if required throughout the trial. Feed intake was recorded weekly, by tipping back remaining feed into the original bag and weighing. Feed intake per bird was calculated from the difference between the starting amount and the amount remaining on each weigh day divided by the number of birds in the pen to give an average feed intake per bird.

Birds were individually weighed at the start of trial to ensure equal distribution between treatments. Birds were then weighed by pen at the start of the study (d0) and then weekly to calculate average bird weight (BW) (total pen weight/ number of birds). Body weight gain (BWG) and feed conversion ratio (FCR) were calculated weekly.

Birds were sampled according to trial needs, (as detailed in chapter 3,4 and 6) on days 7, 21, 35and 42. Prior to sampling birds were humanely euthanized via cervical dislocation by trained operatives in a separate room in line with the guidelines of the Scientific procedures Act (ASPCA, 1986). Birds were weighed individually. Feed conversion ratio (FCR) was determined by pen by dividing the total feed intake by the total weight gain.

2.4.2 Blood Sampling – Studies 1, 2, 3&4

Immediately post-mortem the bird was dissected, and a blood sample was collected from the cavity (surrounding the heart muscle) in a clean syringe and placed in a blood tube containing EDTA as an anti-coagulant. The samples were centrifuged at 3000RPM for 10 minutes (Thermo Scientific, Megafuge 8, Fisher, UK), before the plasma was removed and collected into clean tubes. Samples were stored at -20°C for analysis.

2.4.3 Digesta Sampling – Studies 1, 2 & 3

Prior to digesta collection, birds were sequentially fed in order to stimulate eating and had access to feed for 1 hour before sampling to ensure adequate gut fill. After euthanasia, digesta was collected by careful digital pressure along the distal end of the small intestine (identified as the portion of gut between the Meckel's diverticulum and the ileal-caecal-colonic junction). Digesta was pooled per pen (when multiple birds sampled) into labelled pots and weighed. Digesta samples were stored at -20° until required. Samples were later freeze dried (LTE Scientific, Oldham, UK) for 7 days before being re-weighed and then ground manually with a pestle and mortar to homogeneity fine powder. Ground samples were analysed for digestibility analysis described later in this chapter.

2.4.4 Bone Samples – Study 2

Tibia bones were excised from the feet at the tibial-tarsal junction, and from the femur at tibio-femoral junction using a scalpel. The cartilage caps were retained on the tibia. The tibias were de-fleshed using a scalpel and they were stored individually in labelled bags at -20°C until analysis could take place. Prior to further analysis, bones were thawed to room temperature to prevent false readings from bones being partially frozen and therefore more brittle.

2.4.5 Histology Samples – Study 2

The ileum was excised from the bird from the ileo-caecal-colonic junction to the Meckel's diverticulum as described in section 2.5.18. A 3cm section was taken from the end proximal to Meckel's diverticulum and fixed in a pot containing approximately 20ml of Bouin's fixative (Fisher Scientific, UK) for 8 hours. After 8h the samples were transferred into 70% Industrial methylated spirit and stored at room temperature until processing.

2.5 Analytical procedures

2.5.1 Dry matter

Dry matter was analysed for diet as follows. 5-10g of diet was weighed on an analytical balance into pre-weighed crucibles and dried in a forced air oven at 105°C until constant weight. The dried samples were reweighed, following cooling in a desiccator. Dry matter content was then calculated with the following formula.

$$\text{Moisture content (\%)} = \frac{\text{fresh sample weight (g)} - \text{dry sample weight (g)}}{\text{Fresh sample weight}} \times 100$$

Dry Matter (%) = 100 – moisture content (%)

2.5.2 Crude Protein – Study 1, 2 and 4

Diets were analysed for nitrogen content using a Dumatherm Nitrogen analyser (Gerhardt, UK). AS nitrogen is a compound unique to protein, it can be used as a direct measure of amino acid (protein status). Diets were weighed and pelleted in foil. The Dumas method of combustion for nitrogen analysis was used. The unknown sample is burned in an oxygen rich atmosphere at high temperature.

The machine then analyses the resulting gases. Results are expressed as mg of nitrogen, then converted into percentage of protein present in the sample.

2.5.3 Crude Fat (ether extraction) - study 1, 2 and 4

A SOXTHERM® fat crude fat analyser (Gerhardt, UK) was used to quantify the fat content of the diets. Extraction flasks containing boiling stones were pre-weighed on an analytical balance. 5g of diet was weighed into extraction thimbles (Whatman, USA) which were then placed inside the flasks and petroleum ether added as a solvent. A 2-hour cycle was used. Following the extraction cycle the flasks were heated in a fume hood to ensure all solvent had evaporated. Flasks were then placed in an oven (105°C) for 2 hours until a constant weight was achieved. Flasks containing the extracted fat and boiling stones were cooled in a desiccator and then re-weighed. Fat content was calculated using the following formula:

% extractable fat = [M2-M1÷ M0] X 100

Where:

M0 = sample weight (g)

M1 = weight of flask + boiling stones (g)

M3 = weight of flask= fat + boiling stones (g)

2.5.4 Ash Content study 1,2 and 4

Ash content of diets and digesta was measured by weighing 5-10g of dried samples into pre-weighed crucibles. Crucibles were then placed in a muffle furnace (Nabertherm, Germany) for 14 hours at 650°C. Samples were then cooled in a desiccator and re-weighed . Ash weight was calculated by subtracting ashed weight from the original weight then expressing this as a percentage. Results were corrected for dry matter.

2.5.5 Titanium Dioxide Analysis – study 1,2 and 3

Titanium dioxide was added at 0.5% to all diets as an inert marker for digestibility measures. TiO_2 was measured in the diets and digesta using a UV spectrophotometer following the methodology of Short *et al.,* (1996). Standard titanium solution (0.5mg ml) was made by dissolving 250mg TiO_2 in 100ml concentrated H_2SO_4 (Fisher Scientific, UK) before adding 500ml of distilled water. 1-10ml of the TiO_2 standard was placed in pre-labelled 100ml volumetric flasks. Concentrated H_2SO_4 was then added to each flask to bring the total volume to 10ml. 10ml of 30% hydrogen peroxide (Fisher Scientific, UK) was added before the flasks were brought to volume with distilled water. Samples were measured on a UV spectrophotometer (Cecil, CE 1011, USA) at 410nm to confirm standard curve. The standard solution was stored in amber bottles in darkness until required.

0.3-0.5g of feed or digesta (freeze dried) were weighed in triplicate (diet) and duplicate (digesta) into ceramic crucibles and ashed in a muffle furnace (SNOL, Germany) for 14 hours at 650°C. Once cooled the samples had 10ml 7.4M H_2SO_4 added and were boiled on a hotplate for 2 hours. An additional 5ml 7.4M $H_2SO_4$was added to each crucible and the samples were continually heated until completely dissolved.

Once cooled the samples diluted with 10ml of distilled water then quantitatively filtered into 100ml volumetric flasks through Whatman 541 hardened ashless filter papers. 10ml of 30% hydrogen peroxide was added to each flask which was then brought to volume with distilled water. The absorbance of the samples was measured after thorough mixing at 410nm using a UV spectrophotometer (Cecil, CE 1011, USA). A standard curve was measured each day from the standard solutions previously described. The coefficient used to determine TiO_2 concentration was derived from the regression analysis of the standard curve . The amount of TiO_2 in the solutions was calculated by:

$$TiO2/mg = \frac{\text{Absorbance x 10}}{\text{Coefficient x sample weight (mg)}}$$

2.5.6 Calcium and Phosphorus Analysis– study 1,2 and 4

Calcium and phosphorus content of diets and digesta was analysed via inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 2100 DV, Perkin Elmer, USA). Glass wear was acid washed in 1% nitric acid overnight to remove mineral contamination. 0.5g of sample was incubated at room temperature in duplicate with 10ml aqua regia (1-part HNO_3 - 3 parts HCl) in 50ml conical flasks for 16h. Samples were then heated for 90 minutes before a further 5ml aqua regia was added to each flask. The samples were heated for a further 60 minutes. Once cooled the samples were filtered through Whatman 541 hardened ashless filter papers into 50ml volumetric flasks. Ultrapure water was then used to bring flasks to volume. Samples were mixed then decanted into 15ml tubes.

Ca standards (10-1000ppm) and P standards (0-350ppm) were prepared by diluting 1000ppm ICP-OES standards (Fisher Scientific, UK) with ultra-pure water before being analysed. Ca and P contents were measured at wavelengths of 317.933nm (Ca) and 213.617nm (P). The emission intensities of both samples and standards were recorded. The standard curves were determined by plotting Ca and P emission intensities against the standards (figures 2.1, and 2.2). The concentration of Ca and P (ppm) was calculated in the following way:

$$\text{Mineral conc. (ppm)} = \frac{\text{Dilution volume (ml)} \times \text{MEI-Intercept}}{\text{Sample weight} \quad \text{Gradient}}$$

$$\text{Mineral conc. (g/kg)} = \text{Mineral conc. (ppm)} \div 1000$$

Where:

Mineral conc. = mineral concentration of Ca or P

Dilution volume = final volume in which samples were dissolved. i.e. 50ml

Sample weight = weight of sample analysed

MEI = Ca or P mineral emission intensity analysed by ICP-OES

Intercept = intercept of the standard curve of Ca or P

Gradient = gradient of the standard curve of Ca or P

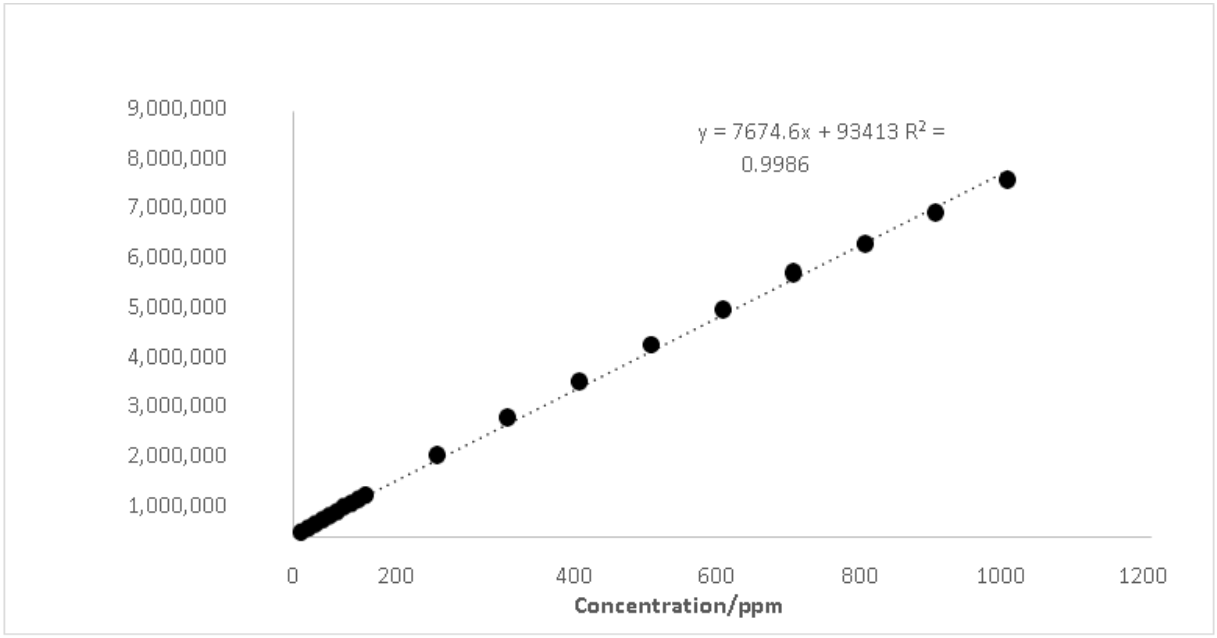

Figure 2.1 – Example of a standard curve for Ca emission intensity

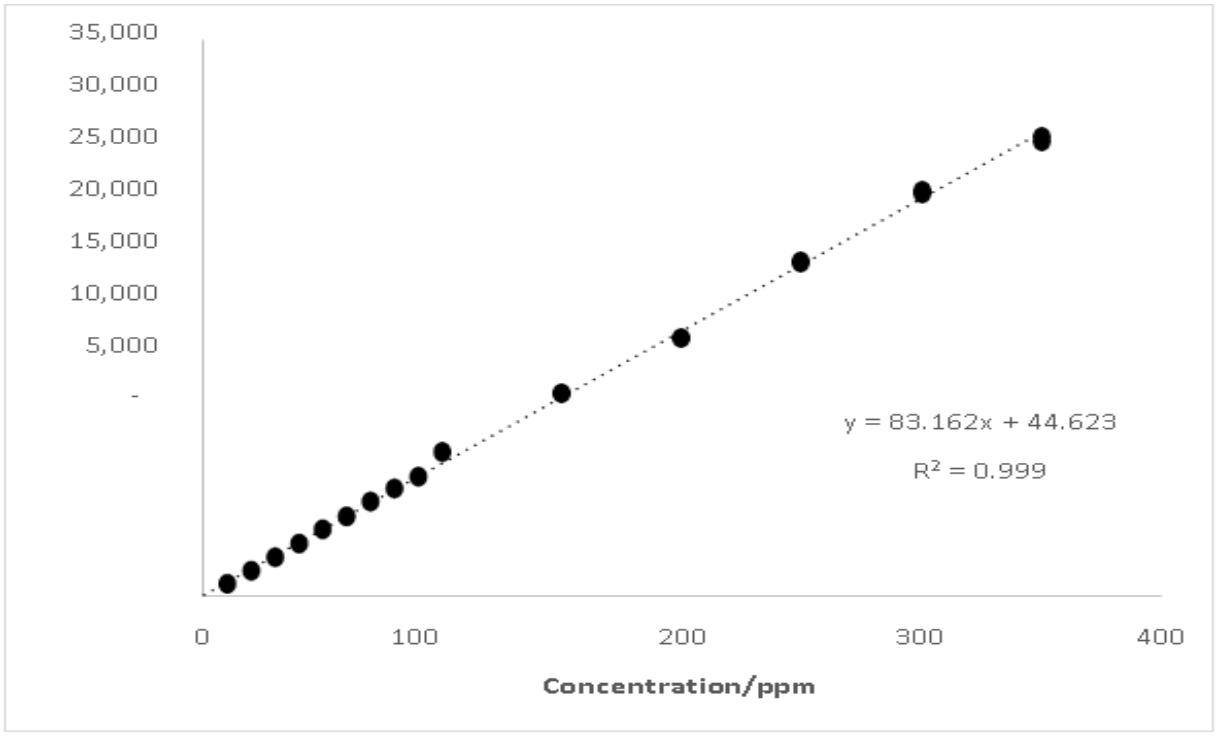

Figure 2.2 - Example of a standard curve for P emission intensity

2.5.7. Apparent Metabolizable Energy

Gross energy (GE) of the feed and excreta was sent to a commercial lab and measured using bomb calorimetry. Excreta was manually collected from depopulated pens, with care taken to obtain clean, bedding free samples. Titanium content of the diet and ileal digesta was determined as described in section 2.5.5. Apparent metabolizable energy (AME) was calculated by: GE diet-(GE digesta*(TiO2 in the diet/TiO2 in excreta)).

2.5.8 Nitrogen retention

Nitrogen content of diet and digesta was analysed by Dumas as described in section 2.5.2. Nitrogen retention was calculated using the following equation. Nitrogen per g of diet – Nitrogen per g of digesta * (Diet TiO2/Digesta TiO2)

2.5.9 Apparent metabolizable energy corrected for nitrogen

Both apparent metabolizable energy (AME) and nitrogen were analysed on a dry matter basis. AME corrected for nitrogen (AMEn) was calculated by the method of JHill and Anderson (1958) using 34.4MJ per g of dietary nitrogen.

2.5.10 Nutrient Digestibility – Study 1, 2 and 3

Titanium dioxide concentration in the diets and digesta was determined by UV spectrophotometry as described in section 2.5.5, after the method of Short *et al*., (1996). Ca and P content of diets and digesta was determined by ICP-OES as described in the previous section. The following calculations were utilized to determine an ileal digestibility coefficient and quantity of nutrient digested, following the methodology of Mutucumarana *et al,* (2014).

The apparent ileal digestibility coefficient = 1 - $\frac{\text{(Ca or P in digesta x } TiO_2 \text{ in diet)}}{(TiO_2 \text{ digesta x Ca/P diet})}$

Ca or P digested = apparent ileal digestibility coefficient x Ca or P in diet

2.5.11 Gastrointestinal pH – Study 1

Birds were fed sequentially to ensure adequate gut fill and no differences in intestinal pH resulting from periods of empty tract prior to measurement of gut pH. Immediately post-mortem birds were dissected And a digital pH meter (fully calibrated to manufactures calibration specifications) (Mettler-Toledo, UK) with piercing tip attachment was inserted into the ileum at the Meckel's diverticulum and into the centre of one caeca (from the ileal-caecal-colonic junction) in the method described by Morgan *et al*., (2014). The measurement was repeated in triplicate for each site. In-between birds, the tip was rinsed in ultra-pure water to reduce cross contamination. The mean of the three measurements per section of tract was calculated.

2.5.12 Mucin Layer Thickness Analysis

Mucin layer thickness was measured as described by Smirnov et al. (2004). A 10mm section of jejunum was incised from the Meckel's end of the tract and weighed (4D.P). The tissue was gently flushed with distilled water and then placed in 10g/l Alcian blue (160mmol/l sucrose with 50mmol sodium acetate) for 2 hours. The tissue was then washed in 250mmol/l sucrose to remove excess dye. The bound dye was then extracted using 10g/l docusate sodium salt overnight at room temperature. The supernatant was centrifuged at 700 x g for 10 minutes to remove particulate matter. The absorbance was then read at 620nm on a spectrophotometer against a standard curve of diluted Alcian blue. (Jenway 7315, Bibby Scientific Ltd, UK). Results are expressed as µg Alcian blue released per gram of tissue.

2.5.13 Immunoglobulin Analysis – study 1,2 and 3

IGA and IGM quantification ELISA kits (Bethyl, UK) were used as per the manufacturer's instructions.

A standard curve is prepared by diluting a IgM/IgA standard in set dilutions. IgM or IgA present in the unknown sample was captured by anti-chicken IgM/IgA that has been pre-adsorbed on the surface of the microtiter wells on the ELISA plates. Once any IgM/IgA present in the unknown sample had bound to the surface anti-bodies, any unbound proteins were washed away. A biotinylated detection antibody was added to each well to bind with the captured immunoglobulin. A streoavidin-conjugated horseradish peroxidase was added to catalyse a colorimetric reaction. A blue product was produced that then changes to yellow when a stop solution of dilute sulfuric acid was added. Plates were read in a plate reader (thermo fisher, UK) at 390nm(IgA) and 450nm (IgM). The absorbance measured is proportional to the amount of IgM/IgA present in the sample. Results were calculated using the standard curve. The amount of immunoglobulin present was calculated by comparing the absorbance results of the unknown samples with the values on the standard curve (figure 2.5 and 2.6).

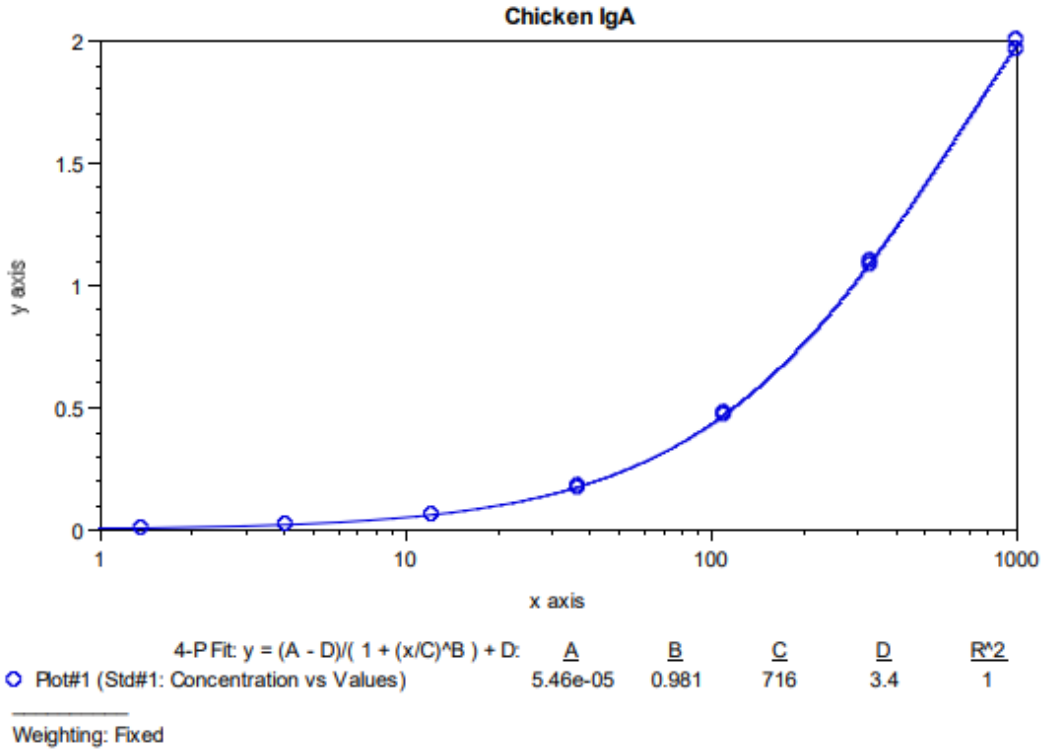

Figure 2.3 - Example of typical standard curve for IgA

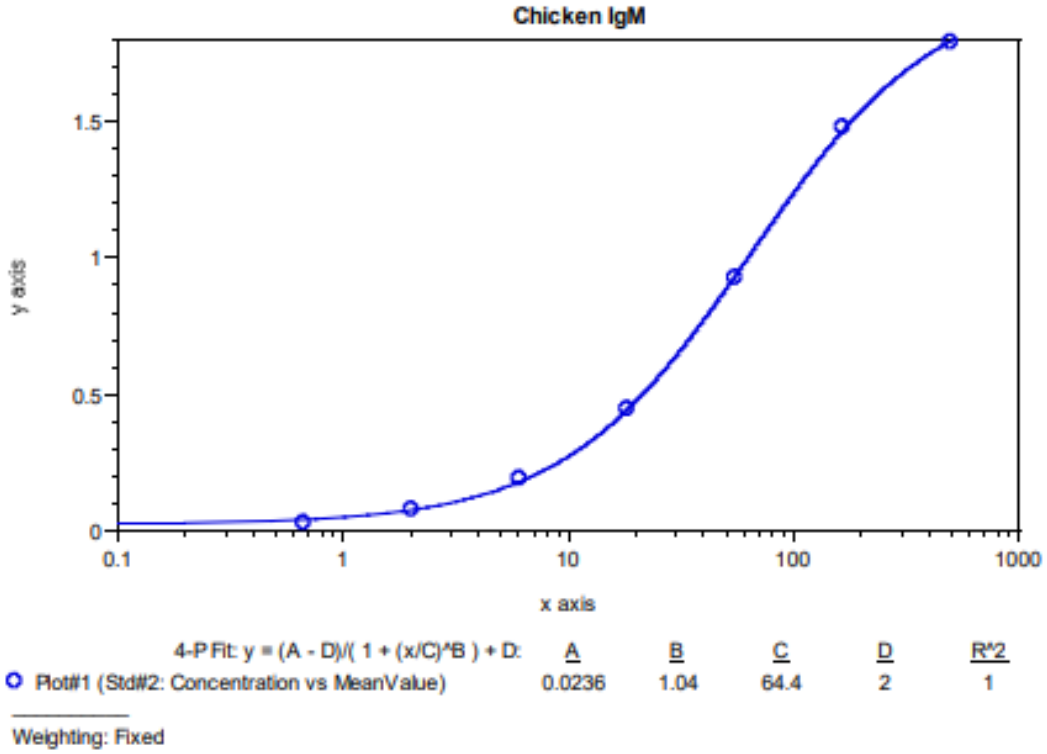

Figure 2.4 - Example of standard curve for IgM

2.5.14 Bone morphometry and strength – study 2

Tibias were fully defrosted and then manually de-fleshed. Tibia's were then weighed on an analytical balance (Sartorius, UK) to determine bone weight. Tibial length and width were then measured using Vernier callipers. Tibial strength was the measured using a 3-point bend TA-XT Plus Texture Analyser (Stable Micro System, UK), formatted with a 50kg load cell and test speed of 1.0mm/s following the method of Shaw *et al.* (2010). Bones were placed on a frame adjusted to bone size which varied by age (d21 and d42). Identical orientation was always ensured. Peak force was measured in newtons and then recorded.

2.5.15 Ileal weight full and empty – study 1 and 2

Immediately post-mortem the birds were dissected, and the ileum incised from the ileo-caecal-colonic junction and Meckel's diverticulum. Care was taken to ensure the ileal content was retained.

The ileum was weighed on a 4d.p balance (Acculab, UK) and recorded. The ileal contents were then removed as described in section 2.4.3. The empty tract was then re-weighed.

2.5.16 Caeca weight – study 1, 2 and 3

The caeca were removed as a pair. They were then carefully separated at the ileo-caecal-colonic junction and weighed individually. The caeca weights were then combined to give both individual and pair weights per pen.

2.5.17 16S Sequencing study 2 and 4

The V4 hypervariable region of the 16S rRNA gene was amplified from genomic DNA using forward primer 515F: GTGCCAGCMGCCGCGGTAA and reverse primer 806R: GGACTACHVGGGTWTCTAAT (Earth Microbiome, 2015), using the 2X KAPA HiFi Hotstart ReadyMix and primers.

PCR amplification was performed using 25 µl reaction mixtures of 2.5 µl microbial DNA (5ng/ul); 5 ul Amplicon PCR Forward Primer (1uM); 5ul Amplicon PCR Reverse Primer (1 µm) and 12.5 µl 2X KAPA HiFi HotStart ReadyMix (KAPA-Germany). This was added into a 96 well plate fully sealed. PCR was performed in a thermal cycler (Techne, TC-512, UK) using the following program: 95°C for 3 minutes; 25 cycles of :95°C for 30 seconds; 55°C for 30 seconds;72°C for 30 seconds 72°C for 5 minutes. The PCR products were run through 1.5% agarose gel electrophoresis and bands analysed to check the amplicon quality. Gels were run at 100 volts for 45 minutes after loading 2 µl of loading dye. (Cleaver, MP-250v, UK), and 5 µl of amplicon. Gels were viewed under UV light (Syngene, G: Box, USA). The 16S V4 amplicon was prepared using 20 µl of AMPure XP beads and then incubated at room temperature for 5 minutes. The 96 well plate was then placed on a magnetic stand (FastGene MagnaStand, YS, Germany) for 120 seconds or until the supernatant had cleared. The supernatant was then discarded, and the beads washed twice with 80% ethanol. The beads were then allowed to air-dry at room

temperature for 10 minutes. The amplicon was then suspended in 52.5 µl of 10mM Tris pH 8.5 and gently mixed. The sample was then incubated at room temperature for 2 minutes. 50 µl of the clear supernatant was then transferred onto a fresh 96 well PCR plate.

Index PCR was performed as follows: 5 µl of DNA was moved on to a fresh plate and the index 1 and 2 primers added. The plate containing the re-suspended PCR product was placed in a TruSeq index plate fixture (Illumina, USA). 5 µl of DNA amplicon, 5 µl of Nextera XT Index Primer 1 (N71-12) horizontally, 5 µl Nextera XT Index primer 2 (s51-8) vertically, 25 µl 0f 2x KAPA HiFi HotStart ReadyMix, and 10 µl PCR grade water were gently mixed then the plate covered with Microseal. This was then centrifuged at 1000 x g at 20°C for 1 minute then PCR performed again on a thermo cycler: 95°C for 3 minutes, 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 72°C for 5 minutes, then finally held at 4°C. The index PCR product was then cleaned up as before and 56 µl of AMPure XP beads added to each well. Each well was then mixed by gently pipetting the mixture up and down with a multi-channel pipette. This was incubated at room-temperature for 5 minutes. Once again, the plate was placed on a magnetic stand for 2 minutes or until the supernatant had cleared. The bead was then removed, and the supernatant discarded. Beads were washed with 80% ethanol twice, then the excess ethanol removed, and the beads air-dried. 27.5 µl 0f 10 mM Tris (pH8.5) was added to each well and incubated at room temperature for 2 minutes. 25 µl of the supernatant from each well was then carefully moved to a fresh plate. For validation purposes 1 µl of the library product was run on a tape-station DNA 1000 (Agilent, USA) to verify the size. Quantification, normalization, and pooling were the performed.

DNA concentration was calculated in nM based on the size of DNA amplicons as determined by the Agilent Tape Station 1000. The library sample was then diluted to 4 nM using Tris pH (8.5). 5µl of diluted DNA from each library sample was then pooled. The MiSeq reagent cartridge was removed from -15°C storage and thawed at room temperature. DNA was then denatured by combining 5 µl of 4nM pooled library sample and 5µl 0.2 N NaOH. This was then briefly vortexed and centrifuged at 280

X g at 20°C for 60 seconds. The sample was then incubated at room temperature for 5 minutes. 990 µl of pre-chilled hybridization buffer HT1 was added to a tube containing 10 µl of denatured DNA. 5 µl of 4 nM phiX library sample and 5 µl of 0.2 n NaOH were combined in a micro centrifuge tube and briefly vortexed before incubating for 5 minutes at room temperature to denature the phiX library sample into individual strands. 990 µl of pre-chilled HT1 (20pM) phiX library was added to the tube containing 10 µl denatured phiX library to result in 20 pM phiX. This was then diluted to the same loading concentration as the amplicon library sample to equal 8mM by mixing 20 pM denatured library and pre-chilled HT1 (360 µl). The amplicon library sample was then set aside on ice until it was time to heat denature the mixture immediately prior to loading it into the MiSeq v3 reagent cartridge. The mixture was once again incubated at 96°C for 2 minutes using a heat block. The tube was then mixed and placed in an ice-water bath. The template allocations of samples were set up in the illumina sheet then the combined library and phiX sample was loaded into the Miseq cartridge then into the illumine machine using version 3 (300x2) chemistry on the Miseq instrument (illumine Inc., USA) according to manufacturer's instructions.

Results were then analysed through microbiome analyst software that performed comparative diversity analysis and statistical analysis of any differences by either treatment or age. The significance level was set to p=>0.05 to match the rest of the statistical analysis used in this thesis.

2.5.18 Peroxynitrite Analysis

ABEL-TAC™ kits were purchased from Knight Scientific, Plymouth, UK. This assay measures the ability of a substance to quench peroxynitrite radicals to determine oxidative status or antioxidant capacity. The kit can be used on numerous substances, but for the purpose of this project it was used to measure the antioxidant properties of feed and the oxidative status of birds (serum).

The kit makes peroxynitrite from the reaction between nitric oxide and superoxide which is released continually from a 2.5 mmol L^{-1} solution of 3-morpholino-sydnonimine HCL; $C_6H_{10}N_4O_2$. HC1 (SIN-1). A

0.5 mmol Vitamin E Analogue (VEA) standard curve is prepared using the reconstituted VEA standard provided by the manufacturer.

A test run was performed to determine a suitable range for the VEA standards using 3 high range standards and 3 low range standards, as described in the kit, together with 3 samples to get an indication of where the samples fall. Once the standard range has been identified, this is used to provide vitamin E equivalent units, to which the samples are compared.

In brief, 50 µl Pholasin™, 100 µl peroxynitrite, 5 µl of serum sample and 95 µl of kit buffer are added to each well of a 96 well microplate with the samples analysed in duplicate. This was placed in the luminescent plate reader (Fluostar Omega plate reader, BGM Labtech, UK), which subsequently injects 50µl if SIN-1 into each well. The SIN-1 continually releases peroxynitrite. Causing the Pholasin™ to glow. The Pholasin™ will emit gradually increasing light that will reach a peak after a few minutes. If the sample has any ability to scavenge peroxynitrite radicals it will compete with the Pholasin™ for peroxynitrite, resulting in a peak of lower intensity. The point at which peak luminescence occurs following the injection of SIN-1 is expressed as a vitamin E analogue equivalent units. The sample results are then compared to the curve and linear regression can then be used to convert the times to peak luminescence to VEA equivalent units.

2.5.19 ABEL test for Halogenated Oxygen

This kit measures the ability of a given substance to quench radicals of the halogenated oxygen species. This method was used to determine the antioxidant properties of various feed stuffs. The kit uses chloramine-T to create halogenated oxygen radicals and the unknown samples can then be assessed by measuring the ability of the sample to quench the radicals and comparing to a known standard.

This test is performed on liquid samples, so initially a suitable solvent must be identified. The kit suggests ultra-pure water, acetone or ethanol at either 100% or 50% with 50% ultra-pure water. These are recommended as the antioxidant potential of these solvents is known and taken into consideration within the kit. The suitability of each solvent was assessed by adding 10 mg of feed sample to 1 ml of solvent (or any amount in these ratios). The samples are then placed on rollers for 20 minutes. After 20 minutes the samples are visually assessed to determine the optimal solvent according to how much of the feed has not dissolved.

On a microplate, 700 µl of assay buffer, 200 µl Pholasin™ and 50 µl of sample were added in duplicate. The plate was then placed in the luminescent plate reader (Fluostar Omega plate reader, BGM Labtech, UK), which subsequently injected chloramine-T to initiate the reaction. The Pholasin™ glows when it comes into contact with the chloramine-T. If the samples have any ability to quench halogenated oxygen radicals, the sample competes with the Pholasin™ for the chloramine-T, causing the sample to emit less light. The antioxidant capacity of a given sample is expressed in relative light units (light emitted per second (mV)). No sample controls are run on each plate.

2.5.20 ABEL Test for Singlet Oxygen Species

This kit measures the ability of a feed sample to quench singlet oxygen species.

Chloramine-T and urea peroxide are used to create singlet oxygen radicals *in situ.* Pholasin™ then reacts to the radicals and emits light. If the unknown sample has any capacity to quench singlet oxygen species it will compete with the Pholasin™ for radicals, reducing the light emitted. Results are expressed as a percentage inhibition of light.

To perform the test, 30 µl assay buffer, 40 µl sample, 50 µl Pholasin™ and 40 µl of urea peroxide were pipetted onto a microplate. Samples were run in. The plate was loaded into a luminescence plate

reader (Fluostar Omega plate reader, BGM Labtech, UK), which subsequently injected 40 µl of Chloramine-T in to each well.

2.5.21 Histology – Jejunum and breast tissue

A 2cm section of jejunum (taken from the Meckel's end) or a cm cube of breast tissue was dissected from 1 bird per pen. The tissue was carefully flushed with distilled water (pH7.4) to avoid damaging intestinal architecture then placed in an individual pot of Bouin's fixative for 6 hours (for birds up to 14 days old) or 8 hours (for birds over 14 days). Following fixation in Bouin's, the tissue was transferred into 70% IMS and stored at room temperature until processing.

Samples were removed from the IMS, cut with a scalpel to ensure straight ends and remove any damaged areas, and placed into a disposable histology cassette (Thermo Fisher Scientific, UK). The cassettes were then placed in a tissue processor (Leica ASP300). The processor sequentially submerges the samples as follows (table 2.2)

Table 2.2 – Tissue processor chemical procedure

Chemical	Duration
70% IMS	Overnight
Fresh 70% IMS	1 hour
90% IMS	1 hour
90% IMS	1 hour
100% IMS	1 hour
100% IMS	1 hour
Histoclear	1 hour
Histoclear	1 hour
wax 60°C	1 hour
wax 60°C	1 hour

Once removed from the processor, the samples were embedded in paraffin wax using an embedding machine (Leica embedding station). The samples were then placed on a cold plate to set and stored in the fridge until cutting.

The wax blocks were trimmed with a single edge razor blade and placed on ice to keep cold. a rotary manual microtome (Leica RM2235) at 10 microns thickness into a ribbon of sections. Ribbons were placed directly onto the surface of a warm water bath to remove creases. A slide was then dipped below the surface to slide the sample onto it. 4 sections of the same sample were added to each slide.

Once dry the jejunum samples were stained using haematoxylin and eosin staining using staining troughs in the order below:

Xylene – 5 minutes

1. Histoclear – 5 minutes
2. 100% alcohol - 2 minutes
3. 95% alcohol - 2 minutes
4. 70% alcohol – 2 minutes
5. Running tap water
6. Haematoxylin – 10 minutes
7. Running water until water runs clear
8. 2 dips in 1% acid IMS
9. Running tap water
10. 2 dips in ammoniated water
11. Running tap water
12. Eosin – 5 minutes
13. Running tap water until water runs clear
14. 70% alcohol – 6x 4 second dips
15. 95% alcohol – 6 x 4 second dips
16. 100% alcohol – 1 minutes
17. Histoclear – 5 minutes
18. Xylene – 5 minutes

Finally, in a fume hood, slides were mounted with DPX mountant and covered with a cover slip.

Analysis of villi height and crypt depth was done on an Olympus BX51 microscope fitted with an Olympus DP71 camera that had an internal measurement function (freshly calibrated). From each sample, a villi and crypt were measured (eight Villi and eight crypts per pen). An average of the eight measurements was calculated and used for statistical analysis.

2.5.22 Viscosity

Viscosity of jejunum digesta was measured using a Brookfield cone and plate viscometer (Brookfield Engineering laboratories, INC. USA) maintained at chick body temperature (41^{o}C) using a circulating water bath. Jejunum digesta was collected post mortem. This was then split into multiple Eppendorf tubes and spun at 15,000 RPM for 5 minutes (Sigma 7 Microcentrifuge, Sigma-Aldrich, UK). The supernatant was then removed and used for analysis. The viscometer was calibrated with distilled water (viscosity 0.66cP). 0.5ml of sample was then pipetted into the cup and the spindle started at 30rpm. Each sample was repeated 3 times and recorded. The average value for each pen was used for statistical analysis.

2.5.23 Texture Analysis

Breast meat texture was assessed using a TA.XT Plus texture analyser (Stable Microsystems, Guildford, UK), set up with a 50KG load cell and compression fixture (Park *et al.,* 2003, Taylor *et al.,* 2003, Shaw *et al.,* 2010). The texture analyser was set up to measure force (N) required to maximum compression. A 4cm square chunk of fresh (unfrozen) breast meat was placed on the texture analyser plate and the compression fixture applied. The machine recorded the maximum compression force over three tests per sample. An average was used for statistical analysis.

Following this test, the meat was cooked in an oven at 180°C for 15 minutes, until the juices ran clear. The cooked breast meat was then re-tested on the texture analyser with identical settings as described in the previous paragraph.

2.5.24 Drip Loss

The quantity of drip loss from a sample of breast or thigh tissue was measured by weighing a 4cm square chunk of meat on the day of slaughter, after 48 hours and again 5 days later. Samples were placed on a grid made of plastic pond netting, inside a sealed seed propagator to allow the liquid to fall away from the sample. Samples were identified with a cocktail stick with a sticker. The seed propagators were sealed with parcel tape and placed in a fridge at 4°C for 5 days. After 48 hours the samples were removed and weighed then placed back in the fridge for the remainder of the 5 days. The weight lost from each sample was recorded so a drip loss percentage of the original weight could be calculated.

2.6 Data Analysis

Outliers were removed from each data set if they fell above or below 2x the standard deviation of the mean. All data was analysed using SPSS software, version 23 for Windows (IBM Statistics, 2018). KS testing was applied to determine normality, then either a one-way ANOVA or Kruskal Wallis was applied to determine significance. Univariate analysis was used to determine interactions between analysed factors. Correlations were analysed by bivariate correlation using Pearson correlation, chosen because it computes based on true values and depicts linear relationships. Multiple linear regressions were used to determine the unique contribution and relatedness of factors. Interpretations of the strength between relationships was based on those of Cohen (1988): small r = 0.1-0.29, medium r = 0.30-0.39 and large r = 0.50 to 1.0. Statistical significance was declared at $p<0.05$. Statistical significance was declared at $p<0.05$.

Chapter 3 – The effect of XOS from a distiller's wet grain source on the gut health parameters and performance of broilers

3.1 Introduction

This chapter investigates the effect of a novel arabinoxylan (AX) extract on the performance and gut health of broilers.

Pre and probiotics are one of the key areas being investigated in the effort to support gut health of broilers without the use of antimicrobials. Oligosaccharides are a potential prebiotic but as the type of oligosaccharide and substance from which an oligosaccharide is extracted can vary hugely, identifying specific efficacy from the literature can be difficult.

Reduced broiler performance due to increased digesta viscosity is commonplace when diets contain high levels of non-starch polysaccharides (NSP) due to reduced transit time and subsequent reduced feed intake (Jia *et al.,* 2009; Chen *et al.*, 2017). To prevent this, exogenous xylanase is routinely fed to improve nutrient utilisation and increase performance. Xylanase hydrolyses the NSP fraction of the diet, cleaving the xylan backbone and breaking it into smaller oligosaccharides. The routine use of exogenous xylanase in commercial broiler production has been widely shown to improve performance parameters by 2-4% (Enberg *et al.,* 2004; Owens *et al*., 2008; Wu and Ravindran, 2004). Xylanase is considered to have four potential modes of action (Bedford, 2018).

1. Viscosity effect – xylanase reduces the viscosity of digesta (Gonzalez-Ortiz, 2016)
2. Cage effect (cell wall mechanism) – xylanase degrades the cell wall of cereal grains releasing encapsulated nutrients. This increases the diffusion of nutrients, substrates and enzymes enabling the host better utilize nutrients in the ration (Bedford and Autio, 1996)
3. Pre-biotic effect – xylanase degrades AX present in the diet into oligosaccharides that can be used a nutrient source by saccharolytic microbes in the gut (Bedford, 2018; Craig, 2019)

4. Signalling effect – xylanase stimulates microbes in the gut to produce xylanase *in situ* (Bedford, 2018).

Distillers dried grains with solubles (DDGS) is a major by-product of cereal-based bio-refineries (Alyassin, 2019). During bioethanol production, wheat is ground, mixed with water then heated in a high temperature cooking step (Scholey, 2012). Once the resulting mash is cooled to 90-100°C, amylase is added to breakdown the starch and reduce the viscosity of the solution. Glucoamylase is then added to remove glucose. A fermentation step follows where the mash is further cooled, and yeast is added. This is left to ferment for 48-72 hours at 30-35°C. This is then distilled to produce a 95% ethanol and 5% water solution. The remaining stillage is separated by either centrifugation, or a pressing and extrusion process into wet grain and a thin stillage. DDGS is then produced by drying the thin stillage to a syrup to remove some of the water content, then remixed with the grain (Scholey, 2012). In commercial bio-ethanol plants, the distillers wet/dried grains cannot be sampled, or separated from the solubles due to a closed production process.

Chemical engineers at the University of Huddersfield have developed a method using a GUNT CE-640 (GUNT Technology, Hamburg, Germany) bio-ethanol unit to produce distiller's wet grains with dried solubles, therefore allowing the value of the wet grains and the solubles to be assessed independently. The AX manufacturing process is described in detail in the material and methods section of this chapter.

This study was designed to determine whether an AX extract could be manufactured and fed to broilers to enhance performance and/or gut health. The rational for this was that supplying an AX extract (as opposed to the AX present in the grain) would increase the availability of the AX for degradation. The study was designed to use a control treatment to compare to a treatment containing AX and a treatment containing AX pre-treated with xylanase.

The key aims of this study were as follows:

- To investigate the effect of a DWGS derived AX on the gut health and performance parameters of broilers
- To determine whether pre-treating the DWGS AX extract with exogenous xylanase increased efficacy of the AX extract in terms of performance and gut health of the birds

3.2 Trial procedure

3.2.1 Husbandry Conditions

336-day-old male Ross 308 chicks were sourced from PD Hook Cote hatchery from a flock aged 57 weeks. Birds were feather sexed on the day of hatch and any poor birds were discarded on arrival. Chicks were individually weighed on arrival, then randomly assigned to 48 mesh sided pens, littered with a wood shavings substrate. Food and water were provided *ad libitum* and care taken to ensure chicks were eating and drinking as soon as possible. If chicks had not started to eat within 60 mins chick paper was added to stimulate food consumption. Husbandry guidelines were followed as described in chapter 2 and adhered to the institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986). Ethical approval was obtained and is recorded as project ARE551.

3.2.2 Diet formulation and condition of animals

AX extract was manufactured and provided dried and ground by the University of Huddersfield. Exogenous xylanase (Econase XT) was provided by A.B. Vista. Doubled-milled wheat was sourced from Target Feeds (Shropshire, UK) and passed through a 1.4mm sieve to remove any larger particles. 6kg of wheat was added into the mash tank of the bio-ethanol unit with 15 litres of water. This was heated to 85°C. by direct steam injection. 1.2ml of α-amylase and 3.6ml of gluco-amylase was added to induce starch hydrolysis. pH was adjusted to 4.5 with sulphuric acid and the mash was stirred continually for 2 hours. Upon completion of the stirring time, the mash was pumped into the fermentation tank and 6g of yeast and 0.9ml of anti-foaming agent was added to initiate fermentation. The mash was fermented at 25-28°C. for 72 hours. The fermented mash was then pumped into the distillation column and distilled for 4 hours to separate the ethanol. The resulting slurry was filtered through a cloth to obtain the distillers wet grains (DWG), and the filtrate (solubles). The solubles were dried in an oven to produce dry solubles. A single fermentation batch of 6kg of

wheat yielded on average 1275g of wet grains with 16% AX content and 800g of dried solubles with 11.4% AX content. Therefore, the wet grains were selected for further processing. The wet grains were enzymatically treated with xylanase by adding 50ml per kg of wet grains and incubating in a shaking water bath at 55°C. for 1 hour (Econase XT, A.B Vista, UK). At the end of this time, the sample was submerged into an ice bath to stop enzyme activity. This yielded less than 3% oligosaccharides. AX polysaccharides were then extracted via alkaline oxidation with enzymatic purification, yielding a 44% AX (figure 3.1). AX extract was freeze dried before adding to poultry diets.

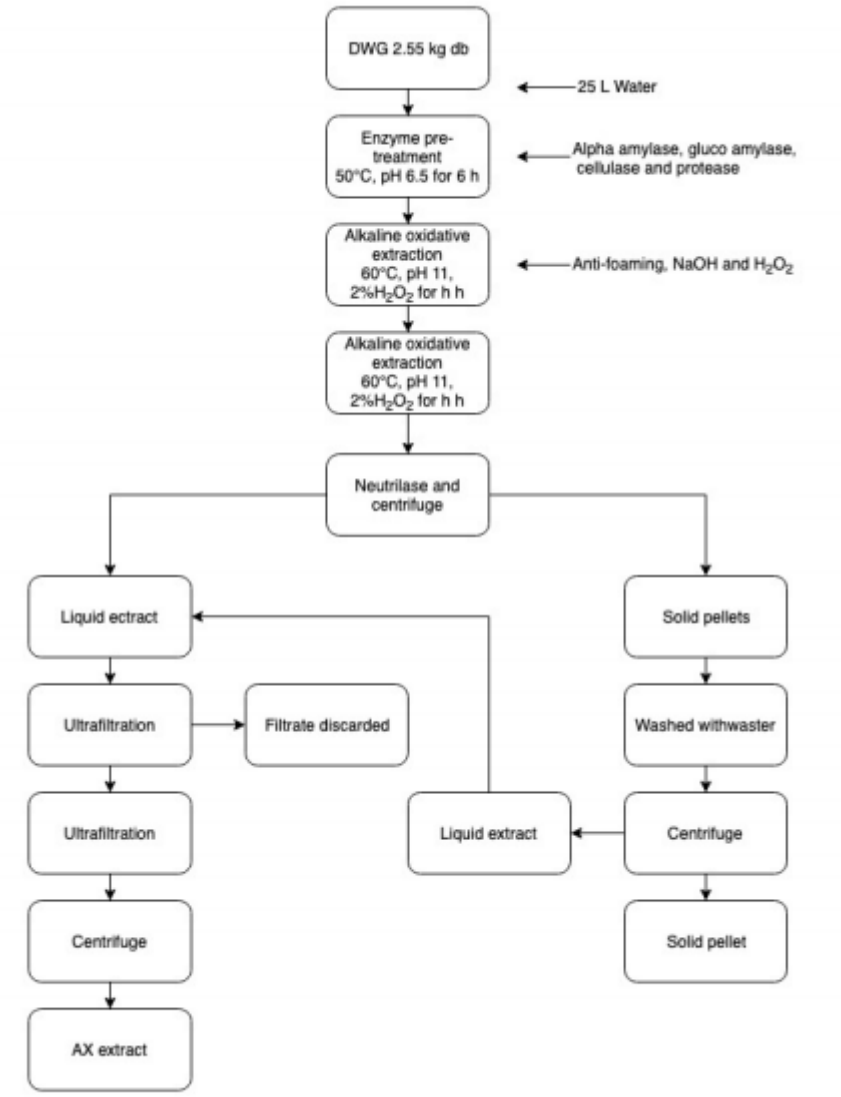

Figure 3.1 Chemical extraction of AX extract from distillers' wet grains, Alyassin, 2019, used with permission, A.B. Vista, 2020

The four diets were manufactured in-house as a mash diet as per the method in section 2.3.5, with treatments detailed in table 3.1. Diets were formulated by a commercial nutritionist (table 3.2) in 3 phases – starter, grower, and finisher. Analysed values for the individual diets are shown in table 3.3. Quantum Blue 5G phytase was added at 0.01% to all treatments.

Table 3.1 – Dietary treatments for AX extract trial

Diet	Treatment
Con	Standard broiler diet
AX	Standard broiler diet + 100g/t DDGS 44% AX extract
AX + Xy	Standard broiler diet + 100g/t DDGS 44% AX extract Pre-treated with xylanase at 1g/kg

Table 3.2 – Diet Formulation and rate of inclusion for AX extract trial (%)

Ingredient	Starter	Grower	Finisher
Wheat	35	35	35
Corn	23.91	26.58	29.18
Soybean meal	34.37	31.06	27.55
Soya oil	2.76	3.85	4.96
Salt	0.3	0.31	0.31
Limestone	0.1	0.06	0.03
Dicalcium Phos, 18%P	2.26	2	1.84
Sodium Bicarbonate	0.1	0.1	0.1
Lysine HCl	0.23	0.18	0.18
DL-Methionine	0.31	0.27	0.26
Threonine	0.13	0.09	0.08
Vitamin & Mineral premix	0.5	0.5	0.5
Quantum Blue 5G Phytase	0.01	0.01	0.01

Table 3.3 - Diet proximate analysis for AX extract trial

Diet	Starter Con	Starter AX	Starter AX+Xy	Grower Con	Grower AX	Grower AX+ Xy	Finisher Con	Finisher AX	Finisher AX+Xy
DM (g/kg)	873.1	873.1	876.4	872.2	872.2	874.7	871.3	871.3	870.7
Ash (g/kg)	5.37	5.51	5.95	5.03	4.68	5.05	4.58	4.96	4.97
Protein (g/kg DM)	21.42	21.76	22.16	20.21	20.04	20.81	19.02	18.55	20.72
Total P (g/kg DM)	8.80	7.93	8.39	7.29	8.06	7.58	6.88	6.45	6.72
Total Ca (g/kg DM)	9.84	9.28	9.03	8.03	8.83	8.61	7.43	6.90	6.93
Fat (g/kg)	4.41	4.21	4.04	5.48	5.83	5.01	5.85	5.69	5.66
Ti (g/kg)	4.98	4.95	4.72	N/A	N/A	N/A	5.01	4.84	4.40
GE (MJ/kg)	16.27	16.30	16.20	16.55	16.45	16.31	16.69	17.03	16.97
Phytase FTU/kg	612	990	857	794	501	261	615	1010	989

3.2.3 Pre-treatment of AX

The AX+Xyl was pre-treated with exogenous xylanase as follows; 30.77g of AX and 123.08g of distilled water were weighed accurately to 4dp. 0.003g of xylanase was weighed and added to the distilled water and mixed. The distilled water and xylanase were then added to the AX and placed on a heated magnetic stirrer for 1 hour at 41 degrees C°. with constant stirring. Following this treatment was added to the basal diet and mixed in the ribbon mixer to attempt to ensure even distribution.

3.2.4 Treatment schedule / randomisation plan / condition of animals

A replicate consisted of a pen containing 7 birds, with only birds weighing between 38g and 46g placed. The weight of each pen was recorded on day 0 and treatments randomly around the trial

room using an online randomiser allocated (by non-involved individual to prevent bias), to reduce any possible effects of ventilation and room placement. Pen layout is shown in figure 3.2 and pen allocation is shown in table 3.4.

Figure 3.2 - Room plan for AX extract trial

19		20	41		42
18		21	40		43
17		22	39		44
16		23	38		45
15		24	37		46
14		25	36		47
13		26	35		48
12		27	34		
11		28	33		
10		29	32		1
9		30	31		2
8					
	7	6	5	4	3

Table 3.4 - Diet allocation for AX extract trial

pen	diet allocation		pen	diet allocation
pen 1	D		pen 25	D
pen 2	C		pen 26	C
pen 3	B		pen 27	B
pen 4	A		pen 28	A
pen 5	D		pen 29	D
pen 6	C		pen 30	C
pen 7	B		pen 31	B
pen 8	A		pen 32	A
pen 9	D		pen 33	D
pen 10	C		pen 34	C
pen 11	B		pen 35	B
pen 12	A		pen 36	A
pen 13	D		pen 37	D
pen 14	C		pen 38	C
pen 15	B		pen 39	B
pen 16	A		pen 40	A
pen 17	D		pen 41	D
pen 18	C		pen 42	C
pen 19	B		pen 43	B
pen 20	A		pen 44	A
pen 21	D		pen 45	D
pen 22	C		pen 46	C
pen 23	B		pen 47	B
pen 24	A		pen 48	A

3.2.6 Study observations

Bird observations were performed a minimum of twice daily to ensure bird welfare and environmental conditions were maintained. Temperature and/or ventilation were adjusted depending on bird behaviour. Dead birds were removed and weighed, and any unhealthy birds were culled and recorded. Bird feed intake was calculated as per the method detailed in section 2.4.2. Birds were weighed weekly by pen on days 0, 7, 14, 21, 28 and 35, as per section 2.4.3. Each week, remaining feed was weighed by pen to calculate feed intake. Feed intake and bird weight were used to calculate weekly feed conversion ratio (FCR).

3.2.7 Sample Collection

On day 7, three birds per pen (collected to remove outliers where possible) were euthanized. Post mortem, blood samples were collected (as per section 2.4.2) and placed into tubes containing EDTA as an anticoagulant. The blood was centrifuged at 3000RPM for 10 minutes and plasma removed and stored at -20°C. Ileal digesta (defined as gut content located between the Meckel's diverticulum and ileal-caecal-colonic junction) was collected (as per section 2.4.3) from all three birds, pooled per pen, and frozen for later analysis. A 1 cm section of ileal tissue was collected of mucin layer thickness as described in section 2.5.9. One set of caeca per pen was removed at the ileal-caecal-colonic junction, weighed as a pair and snap frozen on dry ice.

On day 35, four birds per pen were euthanized. A post mortem blood sample was collected into EDTA tubes as previously described and plasma removed for future immunoglobulin analysis (2.5.10). The pH of the ileum and caeca were measured using a spear piercing pH probe (as per section 2.5.8). One full ileum per pen was weighed (defined as the section of intestine from the Meckel's diverticulum to the ileal-caecal-colonic junction. Ileal content was then collected in to 100ml pots (as previously described) and frozen for further analysis. The empty ileum weighed on a 4dp balance and a 1 cm

section of ileal tissue was collected of mucin layer thickness as described in section 2.5.9. One set of caeca per pen were collected and weighed

Digesta was freeze dried and ground with a pestle and mortar before being analysed for titanium quantification (as described in section 2.5.5) and digestibility of apparent metabolizable energy, apparent metabolizable energy corrected for nitrogen and nitrogen retention (section 2.5.8).

Diets and ileal digesta were analysed for gross energy by bomb calorimetry and protein content (via Dumas) by external labs. Titanium dioxide content of the diet was measured using the method of Short *et al*., 1996 (section 2.5.5). Calcium and phosphorus content of the diets and digesta was determined by ICP-OES (section 2.5.6). Ash analysis was determined by ashing a sample in a muffle furnace (2.5.4). Dry matter was calculated by drying in an oven for 5 days (section 2.5.1). Digestibility of apparent metabolizable energy, apparent metabolizable energy corrected for nitrogen and nitrogen retention were calculated using the formulas shown in section 2.5.8.

3.2.8 Statistical analysis of data

Outliers were removed from data if they fell either two standard deviations above or below the mean. Statistical analysis was performed using SPSS v.23. KS testing was used to determine data normality, followed by one-way *ANOVA* analysis as appropriate. *Tukey post hoc* tests were used to elucidate differences in treatment. Correlations were analysed by bivariate correlation using Pearson correlation, chosen because it computes based on true values and depicts linear relationships. Multiple linear regressions were used to determine the unique contribution and relatedness of factors. Interpretations of the strength between relationships was based on those of Cohen (1988): small r = 0.1-0.29, medium r = 0.30-0.49 and large r = 0.50 to 1.0. Statistical significance was declared at $p<0.05$.

3.3 Results

3.3.1 Environment

No environmental abnormalities occurred during this trial.

3.3.2 Health and Condition

Mortality data shown in table 3.5 demonstrates that there were no significant concerns regarding the health of the birds. There was no statistical significance in mortality between treatments or at any age. Total flock mortality for the study was 2.6%, which is considered standard for trials conducted at the NTU unit and significantly lower than would be expected in a commercial setting.

Table 3.5 - Bird mortality for the AX extract trial by week and treatment (per bird)

Treatment	d0-7	d7-14	d14-21	D21-35	Total
Con	0	0	1	0	1
AX	0	0	0	0	0
AX + Xy	0	1	0	0	1

3.3.3 Bird Uniformity

The mean start weights are shown in table 3.6. There was no statistical significance in the start weight of the chicks between treatments.

Table 3.6 - Average start weights for chicks for the AX extract trial (±SE).

Treatment	d0 BW/bird (g)
Con	45.9 (0.19)
AX	45.9 (0.32)
AX + Xyl	45.3 (0.43)
P value	0.469

3.3.4 Cumulative Performance

Table 3.7 shows that there was cumulatively no significant difference in BWG and FCR for d0-d35. However, there was significantly increased feed intake for the AX treatment compared to the control and AX + Xyl treatments over the course of the trial. Although not statistically significant, the AX treatment also produced numerically heavier birds (126g and 128g) and a reduction in FCR compared both the control and AX + Xyl treatments.

Table 3.7 - Cumulative performance results for birds supplemented with AX or AX pre-treated with xylanase compared to a control diet mean (±SE)

Diet	D0-35 BWG (g/bird)	D0-35 FI/bird (g)	D0-35 FCR
Con	1862 (48.1)	3577 (63.6)[b]	1.85 (0.035)
AX	1988 (52.5)	3794 (72.0)[a]	1.83 (0.087)
AX + Xyl	1860 (44.2)	3516 (47.1)[b]	1.90 (0.032)
P value	0.117	0.008	0.406

[a-b] Means within the same column that do not share a common superscript are significantly different ($p<0.05$)

3.3.5 Weekly Performance

There was no significant difference in average body weight at day 7. By day 14 the average body weight is numerically increased, with a trend ($p=0.056$; average extra weight 31g) that the AX treatment was higher than the AX + Xyl and the control (33g extra BW). At D21 there was no significant difference in average bird weight, however, once again the AX treatment was numerically higher. This pattern continues for the remainder of the trial, with the AX treatment consistently performing numerically better than the other two treatments (a trend for increased body weight was recorded at D28) (table 3.8).

Table 3.8 - Weekly bird weights for birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (±SE)

Diets	D7 BW (g/bird)	D14 BW (g/bird)	D21 BW (g/bird)	D28 BW (g/bird)	D35 BW (g/bird)
Con	137 (2.6)	356 (11.4)	735(21.8)	1254 (29.3)	1908 (48.2)
AX	145 (2.4)	388 (9.3)	770 (19.6)	1343 (30.9)	2034 (52.4)
AX+Xyl	140 (2.9)	357 (9.8)	738 (20.6)	1250 (30.4)	1906 (44.2)
P Value	0.130	0.056*	0.449	0.063*	0.116

Trend AX > (p=0.06) day 14 and 28

[a-b] Means within the same column that do not share a common superscript are significantly different (p<0.05)

Body weight gain results reflect the average body weight results with the AX treatment consistently performing better than the other two treatments, although BWG was only significantly increased in the fourth week (table 3.9).

Table 3.9 - Body weight gain by week results for birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (± SE)

Diets	D0-7 BWG (g/bird)	D7-14 BWG (g/bird)	D14-21 BWG (g/bird)	D21-28 BWG (g/bird)	D28-35 BWG (g/bird)
Con	91 (2.7)	221 (9.9)	371 (11.1)	520[b] (14.2)	654 (25.1)
AX	99 (2.4)	244 (7.6)	391 (11.0)	560[a] (14.1)	709 (21.0)
AX+Xyl	94 (2.9)	221 (7.6)	372 (10.3)	511[b] (11.4)	656 (16.5)
P Value	0.139	0.112	0.856	0.033	0.140

[a-b] Means within the same column that do not share a common superscript are significantly different (p<0.05)

There was no significant difference in feed intake between treatments during the first three weeks of the study. At day 28 there was a significant increase in feed intake for the AX treatment compared to the AX + Xyl treatment (p<0.001) (table 3.10). There was also a trend towards increased feed intake

for this same diet during the period of day 28-35. There was no significant diet effect on FCR at any point of the trial (table 3.11).

Table 3.10 – Weekly feed intake of birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (± SE)

Diet	D0-7 FI (g/bird)	D7-14 FI (g/bird)	D14-21 FI (g/bird)	D21-28 FI (g/bird)	D28-35 FI (g/bird)
Con	207 (12.4)	517 (21.6)	666 (27.7)	921 [ab] (23.3)	1257 (30.50)
AX	205 (8.7)	539 (15.3)	687 (28.8)	1033[a] (35.4)	1341 (33.3)
AX+Xyl	222 (7.2)	516 (16.0)	685 (10.3)	876[b] (11.3)	1226 (37.3)
P value	0.420	0.601	0.846	<0.001	0.062

[a-b] Means within the same column that do not share a common superscript are significantly different (p<0.05)

Table 3.11 – Weekly feed conversion ratio of birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (± SE)

Diet	D0-7 FCR	D7-14 FCR	D14-21 FCR	D21-28 FCR	D28-35 FCR
Con	2.2 (0.15)	2.3 (0.13)	1.7 (0.05)	1.8 (0.08)	1.9 (0.09)
AX	2.1 (0.14)	2.2 (0.12)	1.8 (0.08)	1.9 (0.09)	1.9 (0.05)
AX+Xyl	2.2 (0.13)	2.3 (0.11)	1.8 (0.08)	1.7 (0.02)	1.9 (0.08)
P value	0.860	0.763	0.567	0.362	0.970

[a-b] Means within the same column that do not share a common superscript are significantly different (p<0.05)

3.4.6 Ileal weight

There was no significant difference in ileum weight between treatments when full or empty (p=0.862 and p=0.791) (table 3.12)

Table 3.12 - Day 35 Ileal weight full and empty for birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (± SE)

Diet	Ileal Weight Full (g)	Ileal Weight Empty (g)
Con	39.1 (1.6)	20.5 (0.7)
AX	38.9 (1.6)	20.3 (0.7)
AX+Xyl	41.6 (1.6)	20.7 (0.7)
p value	0.862	0.791

3.3.7 Caeca weight

There was no significant difference in the caecal weights between treatments on either day 7 or 35 (see table 3.13).

Table 3.13 - Day 35 caeca weight (mean of one ceca/bird) for birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (± SE)

Diet	D7 Mean caeca weight (g)	D35 Mean caeca weight (g)
Con	3.6 (0.70)	9.4 (0.70)
AX	3.7 (0.06)	9.6 (0.76)
AX+Xyl	3.6 (0.03)	8.4 (1.60)
p value	0.733	0.429

3.3.8 Measurement of the thickness of the mucin layer

There was no significant difference in the thickness of the mucin layer between treatments (table 3.14)

Table 3.14 - Mucin Adherence D7 (µg of alcian blue released per gram of tissue) for birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (± SE)

Diet	D7 Mucin (µg/g)
Con	22.4 (0.03)
AX	21.8 (0.05)
AX+Xyl	22.0 (0.02)
p value	0.228

3.3.9 Measurement of Intestinal pH

pH of the jejunum (taken at the jejunal side of the Meckel's diverticulum) and caeca were measured on day 35 of the trial by taking 3 individual readings then calculating the mean per bird, and this mean was used for statistical analysis. There were no significant differences between treatments in either the caeca or the ileal pH measurements (table 3.15)

Table 3.15 - Day 35 Caeca pH and Gut pH for birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (+/- SE)

Diet	Caeca pH D35	Jejunum pH D35
Con	5.53 (0.113)	5.29 (0.060)
AX	5.56 (0.135)	5.35 (0.098)
AX+Xyl	5.54 (0.144)	5.26 (0.109)
p value	0.979	0.773

3.3.10 Digestibility of AME, AMEn and Ni retention

There was no significant difference between treatments in apparent metabolizable energy (AME) of the diet, apparent metabolizable energy corrected for nitrogen (AMEn) or nitrogen retention (Ni ret) at d7 (table 3.16). At day 35 there was no significant difference in AME, however, there was a significant decrease in the nitrogen retention of the birds fed the AX treatment, as shown in table 3.17. Interestingly, this did not have a significant effect on the AMEn result, as there was no significant difference in AMEn on day 35.

Table 3.16 – Day 7 digestibility for birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (+/- SE)

Diet	AME D7 (MJ/kg)	Ni ret D7 (g/kg diet)	AMEn D7 (MJ/kg)
Con	4.9 (0.50)	25.3 (0.48)	4.0 (0.49)
AX	5.2 (0.50)	23.9 (0.54)	4.3 (0.50)
AX+Xyl	5.3 (0.36)	24.4 (0.40)	4.4 (0.35)
p Value	0.790	0.126	0.756

[a-b] Means within the same column that do not share a common superscript are significantly different (p<0.05)

Table 3.17 - Day 35 digestibility for birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (+/- SE)

Diet	AME D35 (MJ/kg)	Ni ret D35 (g/kg diet)	AMEn D35 (MJ/kg)
Con	9.9 (0.66)	24.4[b] (0.32)	9.1 (0.65)
AX	10.1 (0.50)	21.6[a] (0.30)	9.4 (0.40)
AX+Xyl	9.9 (0.48)	25.3[b] (0.29)	9.1 (0.47)
p Value	0.967	<0.001	0.862

[a-b] Means within the same column that do not share a common superscript are significantly different (p<0.05)

3.3.11 Immunoglobulins

There was no significant difference in either Immunoglobulin A or M between treatments (table 3.18).

Table 3.18 – Plasma immunoglobulin for birds supplemented with AX or AX pre-treated with xylanase pre-treated xylanase compared to a control diet (+/- SE).

Diet	D7 IGA (mg/ml)	D35 IGA (mg/ml)	D7 IGM (mg/ml)	D35 IGM (mg/ml)
Con	0.12 (0.012)	0.35 (0.064)	2.55 (0.137)	2.09 (0.255)
AX	0.12 (0.007)	0.44 (0.080)	2.06 (0.105)	2.10 (0.307)
AX+Xyl	0.16 (0.019)	0.40 (0.040)	2.06 (0.047)	2.11 (0.127)
P Value	0.078	0.740	0.955	0.399

3.4 Discussion

The aim of this study was to investigate the effect of a DDGS derived AX extract in supporting the performance and gut health of broilers and to investigate whether pre-treating the AX with xylanase influenced efficacy. This study originally had four treatments, with the fourth treatment being AX + xylanase added directly into the diet, rather than as a pre-treatment. Unfortunately, following the trial it was found that this diet was lacking phytase in the starter diet. Lack of phytase is widely recognized to inhibit bird growth and development as chicks are unable to access nutrients such as calcium, phosphorus and amino acids bound up in the phytate molecule (Oluyinka *et al*., 2007; Lelis *et al.,* 2012). The poor start that this treatment received makes it not comparable to the other treatments. Therefore, this treatment was removed from the analysis.

Despite the difficulties experienced, there are some interesting results. Overall, the final bird weight at d35 was lower than a typical mash trial at NTU (1.952kg compared to 2.181kg). The reason for this is unclear as the birds had constant access to feed that contained sufficient protein, energy, and essential nutrients for the duration of the trial. The study birds were from a 57-week flock, which has been shown to produce more bird weight variability and decreased bird performance, which may account for some of the decreased performance (Joseph and Moran, 2005). Extremes of temperature and humidity have been shown to negatively affect broiler performance, however there was little change in environmental conditions during the trial, which would therefore be unlikely to produce a reduction in body weight.

The AX extract continually performed at least as well as the control treatment, so it can be deduced that it did not have any deleterious effect on bird performance or health, and in fact showed positive effects at a number of measurement timepoints. During the first week of the trial both the experimental treatments showed numerical improvements in body weight gain, with the AX treatment gaining nearly 6% more weight than the control. Following the first week, the AX pre-treated with Xyl no longer shows any improvements and performs in a very similar way to the control.

At day 14 the AX treatment continues to show increased performance compared to the other treatments, with the average bird weight (BW) becoming almost significantly increased at day 14 (p=0.056). Likewise, at day 14 the body weight gain, although not significantly different, is 10% higher than the other two treatments. This effect seems to continue with the AX treatment consistently showing numerically increased BWG and BW compared to the other treatments. At day 28 the body weight gain becomes significantly increased for the AX treatment compared to the other two treatments (p=0.033) and there is a trend towards increased BW (p=0.063).

The increased growth performance of the AX treatment shows that AX alone appears to be having a beneficial effect on bird growth compared to the other two treatments. The lack of enhanced performance for the AX+Xyl treatment was surprising as it could have been expected that the improvements seen for AX supplementation would be reflected in this treatment. It appears that either the pre-treatment process or the addition of xylanase acts to negate any benefit from AX supplementation.

The routine use of exogenous xylanase in commercial broiler production has been widely shown to improve performance parameters by 2-4% (Enberg *et al.,* 2004; Owens *et al*., 2008; Wu and Ravindran, 2004). Xylanase is considered to have four potential modes of action (Bedford, 2018).

5. Viscosity effect – xylanase reduces the viscosity of digesta (Gonzalez-Ortiz, 2016)
6. Cage effect (cell wall mechanism) – xylanase degrades the cell wall of cereal grains releasing encapsulated nutrients. This increases the diffusion of nutrients, substrates and enzymes enabling the host better utilize nutrients in the ration (Bedford and Autio, 1996)
7. Pre-biotic effect – xylanase degrades AX present in the diet into oligosaccharides that can be used a nutrient source by saccharolytic microbes in the gut (Bedford, 2018; Craig, 2019)
8. Signalling effect – xylanase stimulates microbes in the gut to produce xylanase *in situ* (Bedford, 2018).

However, the addition of distilled water during the pre-treatment process diluted the xylanase to the point where an enzyme recovery test could not determine a difference between the AX pre-treated with xylanase and the two treatment diets that did not contain xylanase. Therefore, it seems unlikely that there was enough xylanase present in the diet to exert any effect.

Xylanase inhibitors are chemicals that inhibit the xylanase activity. Xylanases can also be subject to product inhibition, by some hydrolysis products of AX degradation, including xylose, arabinose and xylotrose (Dekker and Richards, 1976). Currently, three proteinaceous xylanase inhibitors have been identified in wheat. These include *Triticum aestivum* xylanase inhibitor (TAXI) (Debyser *et al.,* 1999), xylanases inhibiting protein, (XIP) (McLauchlan, 1999), and thaumatin like xylanase inhibitor (TLXI) (Fierens, 2007). The quantity of these xylanase inhibitors is considered high in wheat compared to other cereal grains (Gebruers *et al.,* 2010). Likewise, the quantity and type of xylanase inhibitors can vary by cultivar. Soerensen *et al.,* (2004) demonstrated that endogenous xylanase inhibitors can reduce the effectiveness of xylanase inclusion in pig and poultry diets. Therefore, it is possible that the wheat used in this study may have been particularly high in xylanase inhibitors, thus accounting for the lack of xylanase effect.

AX on its own would generally be considered deleterious to broiler production, as the larger polysaccharides would generally be thought to form gels and increase viscosity (Bedford, 2002). In commercial production, exogenous xylanase is fed with diets containing high levels of AX to counteract its effects (Bedford 2018). However, in the current study the AX treatment fed on its own seems to improve most performance parameters, this may support the theory that xylanase is being produced in vivo by the microbial population of the intestinal tract. It is generally believed that broilers do not possess the ability to produce endogenous xylanase (Bedford, 2018; Salah *et al.,* 2019). However, studies have shown that a number of bacterial species often found in the intestinal tract of broilers are capable of producing xylanase (Asem *et al.,* 2017; Bhardwaj *et al.,* 2019). Asem *et al.* (2017) demonstrated that of 29 bacterial isolates collected from the intestinal tract of a pig, 13

species had the capacity to produce xylanase with the capacity to hydrolyse xylan. Likewise, Latorre *et al.* (2015) demonstrated the ability of three different *Bacillus.spp* strains to produce xylanase in an *in vitro* digestive model. Bedford, (2018) suggests that when saccharolytic microbes produce xylanase, the AX present in the diet is degraded, providing more fermentable substrate for the caeca. VFA's are the primary products of caecal fermentation (Annison *et al.,* 1968). From the caeca, VFA's are transferred to the blood and can contribute energy to the host (Annison *et al.,* 1968; Svihus *et al*., 2013). From the studies reviewed, there seems to be a consensus that broiler caeca generally contain acetic acid, butyric acid and propionic acid in varying amounts and on some occasions a small amount of lactic acid was detected (Choct *et el*., 1999; Jamroz *et al*., 2002; Jozefiak *et al*., 2006;, Jozefiak *et al*., 2011). VFA production in the caeca is associated with an increase in the population of beneficial bacteria in the GIT (Enberg *et al.,* 2004). Volatile fatty acids have been shown to enhance the performance of broilers, with butyrate in particular resulting in increased energy availability for the bird (Ravangard *et al.,* 2017). Butyrate has also been shown to have anti-inflammatory properties and act as fuel for epithelial cells – consequently improving the integrity of the epithelial barrier (Duncan *et al.,* 2004; De Maesschalck *et al.,* 2015).

When trying to determine possible reasons for the lack of AX effect in the AX pre-treated with xylanase treatment, there are several possibilities. One possibility is the effect of the method used for pre-treating the AX with xylanase. Distilled water was added to the powdered xylanase and the AX extract before this mixture was mixed with the diet. Arabinoxylans have been shown to have the ability to absorb up to 10 times their weight in water, forming a highly viscous, gel-like solution (Choct, 1997). It is possible that the AX extract when mixed with water became bound up in the viscous solution and the amount of xylanase present was insufficient to decrease this viscosity. If this occurred, the increased viscosity may have cancelled out any of the positive effect of the AX.

In vitro pre-treatment with xylanase was performed in the hope of initiating the AX to break down into oligosaccharides prior to feeding the diet, rather than *in situ.* However, as xylanase is typically

added to the diet as a dry powder as part of the vitamin and mineral premix to ensure adequate mixing, another possibility is that the wet addition caused the AX to cluster, preventing even distribution and potentially making the diet unpalatable to the birds. Particle size has been shown to have a profound effect on palatability in broilers, and although they typically prefer the particle size to be around 800-1000µm (Mingbin *et al*., 2015), wet clusters of feed are likely to exceed this. Another possibility is that if the AX coagulated, it may have produced clusters of AX that would have been limited in number. This could mean that some birds in the pen were able to consume AX and others not. Finally, there is a possibility that adding exogenous xylanase directly to the AX extract may have depolymerised the AX into monosaccharides, rather than the desired, xylobiose and xylotriose (Kiran et al., 2013). Biose and triose are of particular interest as they are readily utilized by probiotic microorganisms (Bailey *et al.,* 1992; Yamada *et al.,* 1993). XOS with a degree of polymerisation of (2-3) present faster fermentation kinetics and therefore contribute to a favourable microbiome (Gullon *et al.,* 2011).

The cumulative feed intake for the AX treatment is significantly increased compared to the other two treatments. When looking at the weekly feed intake, there is no significant difference until D28 ($p<0.001$) and by D35 there was only a trend that birds fed the AX treatment ate more ($p=0.062$). As the AX treatment birds were also larger at these time points, it could be expected that FI would increase. A number of authors that reviewed broiler feed intake have deduced that feed intake is inextricably linked to growth rate (Nitsan, 1995; Ferket, 2002; Ferket and Gernat, 2006). Likewise, Ferket and Gernat (2006) state that digesta motility is a key influence on feed intake. As before, if the bacteria in the gut are producing *in situ* xylanase, it is plausible that the digesta viscosity may have been reduced and therefore gut motility was increased allowing increased feed intake. Unfortunately, digesta viscosity was not measured on this trial, so it is not possible to confirm that this affected feed intake. It has also been suggested that birds, rather than eating to satisfy hunger, just consume enough to satisfy their energy and protein requirements (Kamran *et al*., 2008). Therefore, it could be expected that larger birds would eat more to fulfil these requirements. However, there was no

significant difference in ileal weight when full between treatments, this suggests that there may not have been a difference in digesta viscosity as if one treatment produced decreased digesta viscosity, a difference in ileal weight when full, may have been expected.

The digestibility results show that there was no significant difference in AME and AMEn between treatments at either day 7 or 35. There was no significant difference between treatments for nitrogen retention at day 7, but by day 35, the AX treatment shows significantly reduced nitrogen retention. This directly contrasts with other studies that found feeding xylose (the pentose sugar portion of xylans) caused increased nitrogen retention (Peng *et al.*, 2004). As the AMEn was not affected, it is unlikely that any reduction in nitrogen retention is affecting the bird to any substantive degree. Furthermore, Morgan *et al.,* (2019), found that supplementing broilers with AXOS significantly increased nitrogen retention when fed a wheat/soy diet and supplemented AXOS at a 2% inclusion rate. It is possible that this result may just be a statistical error, however, if it is a genuine reduction in nitrogen retention, this raises the concerns of protein or amino acid utilisation and additional excretion of nitrogenous waste.

No significant differences were found between treatments in analysed plasma immunoglobulin G or M. It is possible that the birds in this trial were not placed under any immune challenge. Immunoglobulin levels have been found to increase under immunological challenge. This could potentially be due to the nature of the experimental unit at NTU. As the unit is kept very clean and a high level of biosecurity is observed, the chances of pathogenic challenge are greatly reduced. Likewise, environmental stress is likely to be lower due to reduced stocking density, easier access to feed and water and less social hierarchy (Marsteller *et al.,* 1980; Estevez *et al.,* 1997; Marino, 2017). Immunoglobulin levels rise in line with antibody production when the host undergoes an immune response (Painter, 1998). If the birds were not immunologically challenged, levels typically remain unchanged. The IgM results concur with that of Alsudani, (2018), where no difference was found when supplementing broilers with lactobacilli probiotic. The levels of IgM also concur with Lebacq *et*

al. (1974) who performed the original quantification and distribution of chicken immunoglobulins and found IgM levels to be an average of 2.55mg/ml and IgA to be an average of 0.33mg/ml.

There was no significant difference in the mucin layer thickness between treatments, and the levels reported on this study are substantially (50%) lower than other authors have found. The mucin adherent layer lines the intestinal tract, providing a vital barrier function, lubrication and protection to the mucosal surface from mechanical injury and enteric pathogen entry (Tsirtsikos *et al.,* 2012). Secreted from goblet cells in the lining of the gut epithelium, mucin is thought to effect nutrient absorption, if the adherent layer is too thick. If the layer is not thick enough, barrier integrity can be compromised. This may imply that intestinal barrier function was impaired for all treatments, potentially causing systemic infiltration of gut microbiota. The lack of difference between treatments seen in the current study, imply that any effect of mucin layer thickness was universal across all treatments. Impaired intestinal barrier function, also called leaky gut, has been widely reported to inhibit broiler performance (Bischoff *et al.,* 2014; Gilani *et al*., 2016; Barekatain *et al.,* 2019). Therefore, it is possible that all treatments were experiencing a degree of leaky gut, consequently reducing the overall performance seen in this study.

There was also no difference in caeca weight or intestinal pH. This may suggest that there were not substantial differences in the bacterial make-up of the caecal contents. Jozefiak *et al*. (2007) looked at the effect of xylanase supplementation on diets of either, triticale, rye, or wheat. The authors found a significant increase in the weight of the caeca from birds fed rye-based diets, but despite this could not detect a significant difference in caecal pH between treatments. The same study analysed caeca content and found a significant increase in acetate and lactic acid production in the triticale and wheat treatments, despite the caecas for this treatment weighing significantly less. This may suggest that caecal pH measurement is not sensitive enough to detect differences in VFA production and that changes in caecal weight may not always indicate changes in microbial activity. The work of Olnood *et al*., (2015) seems to support this theory. These authors looked at supplementing broilers with different

strains of *lactobacillus* as a potential probiotic. When feeding a wheat, sorghum and mung bean-based ration, these authors found no significant differences in caeca pH or concentrations of VFA between treatments. As no pH differences were seen in either of these studies although VFA concentrations were altered it seems likely that either caecal pH is not substantially changed by differences in VFA concentration, or that the measuring procedure is not precise enough to detect differences, or that the dissection process and exposure to the environment affects the caecal pH. Therefore, looking at VFA concentration and 16S metagenomic profiling should be considered as a more accurate method to quantify changes in future studies.

3.5 Conclusion

The AX extract produced from distillers' wet grains does seem to have a beneficial effect on performance when fed without any xylanase pre-treatment. However, the mode of action is still unclear from the parameters investigated in this study.

Pre-treating the AX extract appears to negate any performance improvements and does not seem to have any effect on other measures. It is unclear whether the benefits seen for AX supplementation are negated by the addition of xylanase or the pre-treatment process not allowing sufficient incorporation of the supplement into the diet.

Unfortunately, from the parameters measured on this trial, pinpointing the mechanism of AX benefits is difficult. However, this study adds further weight to the suggestion that whilst broilers cannot produce endogenous xylanase, bacteria present in the gut, may be able to do so. In future studies, measuring the viscosity of digesta and exploring the potential of a microbial effect in the caeca may help to further determine the mechanism for the beneficial effects of AX supplementation.

Chapter 4 - Comparing the efficacy of a corn derived XOS to a sugar cane bagasse XOS on the performance and gut health of broilers

4.1 Introduction

As the AX extract used in chapter 3 was successful in supporting bird performance, a second trial was designed to try to determine whether specifically manufacturing a xylo-oligosaccharide rather than simply an AX extract would enhance the performance benefits seen in the first study.

XOS supplementation has been shown to have numerous benefits for broiler production, on both performance and physiological parameters. In 2006, Wu *et al*. found that XOS inclusion (1,2 and 4 g/kg) significantly reduced cumulative mortality and feed conversion ratio over a 7-week broiler study. These authors also report that supplementing at 2g/kg, XOS increased the quantity of lactobacillus in the ileum. Furthermore, Maesschalck *et al*. (2015) demonstrated that birds fed a wheat/rye-based diet supplemented with XOS at 0.2% over 39 days experienced significantly ireduced FCR and increased numbers of *lactobacilli* present in the hind gut as well as significantly increased the length of ileal villi, thus increasing the surface area and absorptive capacity of the ileum compared to the control birds. Subsequently, this led to increased concentrations of SCFA. Ribeira *et al.* (2018) found that XOS supplementation at 0.1g/kg increased the microbial populations of *bifido bacterium* and *lactobacilli*.This study demonstrated that a lower XOS inclusion rate (0.1g/kg) produced higher levels of beneficial bacterium.

The chemists at The University of Huddersfield developed an arabino-xylan oligosaccharide derived from sugar cane bagasse. It was proposed to compare this novel bagasse XOS to a commercial corn based XOS (Longlive Biotechnology, Shandong, China) to elucidate if the material from which a XOS was extracted played a role in its efficacy as a feed supplement.

Global sugar cane production is approximately 1.9 billion tonnes a year (Campbell *et al.,* 2019) and results in around 570 million tonnes of waste product wet bagasse. Therefore, bagasse represents a major waste stream. Bagasse is the fraction of biomass resulting from the extraction of sugar cane juice (Santos *et al.,* 2015). Bagasse is primarily fibre based and has three main components: cellulose (30-40%), hemicellulose (25-35%) and lignin (20-30%). With a xylose content of approximately 10.5% and an arabinose content of approximately 1.5%, it is a viable base for deriving arabino xylo-oligosaccharides. As a low value by-product, it has the potential to be a sustainable source of XOS. The University of Huddersfield tested a number of base materials before performing a comparison study on wheat bran and bagasse. The AX from both substances was extracted via both chemical and enzyme extraction (figure 4.1). Bagasse was chosen as most appropriate because although bagasse has a comparable quantity of AX to wheat bran (20-30%), bagasse has a lower arabinoxylan substitution on the xylan backbone (A:X ratio of 0.2 compared with 0.6 for wheat bran). Therefore, the bagasse derived AX is likely to be less soluble and more susceptible to enzyme activity (Campbell *et al.,* 2019). Additionally, bagasse can produce a wider range of molecular weight sugars (Campbell *et al*., 2019).

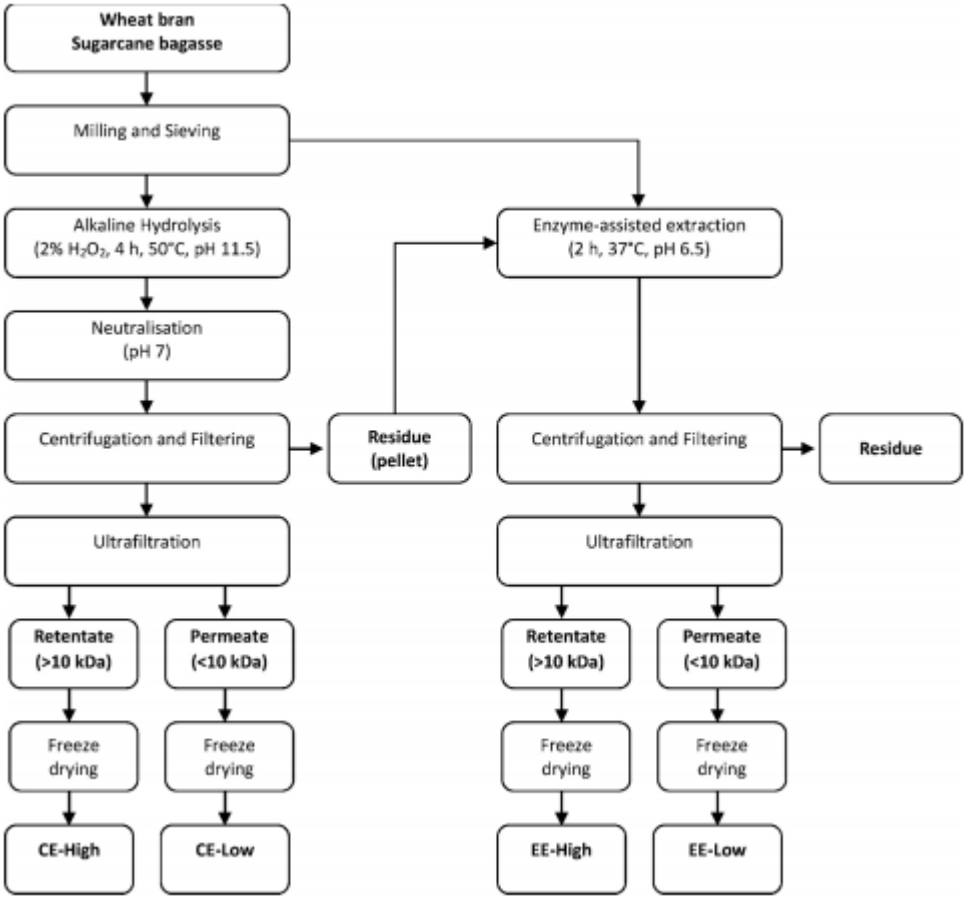

Figure 4.1 - Procedure for chemical extraction of AX from wheat bran or sugarcane bagasse, yielding either high or low cut-off fraction following either chemical or enzyme-assisted extraction (used with permission fromA.B. Vista 2020).

The corn XOS was derived from corn cobs, contained 35% oligosaccharides and at the time of the study was readily commercially available as a functional feed ingredient for monogastrics.

As the xylanase pre-treatment used in the first study had not been successful, this study was designed to examine the effect of both XOS products both with and without exogenous xylanase added directly to the diet to determine whether this affected the efficacy of the XOS.

The key aims of this study were as follows:

- To directly compare the efficacy on bird performance of two XOS products derived from different plant materials (corn and sugar cane bagasse) compared to a control
- To examine the effect of the two XOS products on bird performance both with and without an exogenous xylanase added directly to the diet compared to a control.
- To determine whether any XOS and/or xylanase addition has any effect on anatomical or physiological parameters associated with gut health

4.2 Trial Procedure

4.2.1 Husbandry Conditions

400 day old male Ross 308 broilers were sourced from PD Hook from a flock aged 43 weeks. Birds were feather sexed on the day of hatch and any poor birds were discarded on arrival. Chicks were individually weighed on arrival, then randomly assigned to 48 mesh sided pens, littered with a wood shavings substrate. Food and water were provided *ad libitum* and care taken to ensure chicks were eating and drinking as soon as possible. Husbandry guidelines were followed as described in chapter 2 and adhered to the institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986). Ethical approval was obtained as is recorded as NTU school ethics committee approval ARE798.

4.2.2 Diet formulation and condition of animals

6 dietary treatments were utilized in this study (table 4.1) with three phases (starter d0-14, grower d14-28 and finisher d28-42). The bagasse XOS was manufactured by chemists at The University of Huddersfield and the corn XOS was commercially manufactured by Longlive Biotechnology, Shandong, China. The Longlive XOS is a mixed product containing 35% XOS with a degree of polymerisation between 2 and 7 and contains 65% maltodextrin.

As in the previous chapter, the Huddersfield team used the GUNTCE-640 bio-ethanol unit to produce the xylan extract. The bagasse AX was subsequently treated with xylanase to produce xylo-oligosaccharides from the xylan fraction. Following the previous production, the Huddersfield team developed a method to measure the oligosaccharide content of raw materials. The bagasse XOS was found to only contain 10% oligosaccharides, so the quantity fed was multiplied up to balance the oligo content of the commercial XOS (35% measured oligosaccharides), to ensure a like for like comparison.

Exogenous xylanase (Econase XT) was provided by A.B. Vista and added at 100g per tonne (16000 BXU). Quantum Blue 5G phytase was added to all diets at 0.01% inclusion rate to provide approximately 500FTU. Diets were formulated by a commercial poultry nutritionist (table 4.2) and proximate analysis was performed to confirm composition (Table 4.3).

Table 4.1 - Study Treatments for XOS comparison trial

Diet	Treatment
Con	Control – standard broiler diet
Corn XOS	Control + Corn derived XOS (100g/t)
Bag XOS	Control + bagasse derived XOS (402 g/t)
Con + Xyl	Control + xylanase (100g/tonne)
Corn + Xyl	Control + corn XOS (100g/t) + xylanase (100g/tonne)
Bag + Xyl	Control + bagasse (402g/t) + xylanase (100g/tonne)

Two basal mash diets for each phase were manufactured by RDS in The Netherlands, based on a wheat, soy corn base. The first basal was a standard broiler diet (table 4.2) and the second was an identical standard diet with the addition of xylanase at 100g/t. 40kg of each starter diet was mixed and weighed into 4kg, individually labelled bags. The XOS products were added to the micro ingredients then sequentially mixed with increasing volumes of feed to ensure homogenous mixing. Individual bags were then weighed into labelled bags for each pen to allow feed intake to be calculated weekly. 90kg of each grower and 140kg of each finisher were mixed and weighed into individual pen bags. Birds were fed from their designated bag throughout to ensure they only consumed their correct treatment.

Table 4.2 - Control Diet formulations for XOS comparison trial

Ingredient	Starter	Grower	Finisher
Wheat	35	35	35
Corn	23.91	26.58	29.18
Soybean meal	34.37	31.06	27.55
Soya oil	2.76	3.85	4.96
Salt	0.3	0.31	0.31
Limestone	0.1	0.06	0.03
Dicalcium Phos, 18%P	2.26	2	1.84
Sodium Bicarbonate	0.1	0.1	0.1
Lysine HCl	0.23	0.18	0.18
DL-Methionine	0.31	0.27	0.26
Threonine	0.13	0.09	0.08
Vitamin & Mineral premix	0.5	0.5	0.5
Quantum Blue 5G	0.01	0.01	0.01
TOTAL	100	100	100

Table 4.3 - Diet proximate analysis for the two basal diets (standard broiler diet and standard broiler diet + xylanase) for XOS comparison trial

Diet	Starter	Starter + Xyl	Grower	Grower +Xyl	Finisher	Finisher + Xyl
DM (%)	87.37	87.62	87.34	87.34	87.14	87.07
Ash (g/kg	5.91	4.95	5.69	5.93	4.50	5.12
Protein (g/kg DM)	21.60	22.48	21.53	22.45	21.67	20.14
Total P (g/kg DM)	7.45	7.55	7.58	7.58	6.78	7.39
Total Ca (g/kg DM)	8.40	8.28	8.16	8.11	6.00	7.32
Fat (g/kg)	2.80	2.79	3.88	4.25	4.49	4.62
Ti (g/kg)	4.37	4.95	4.72	4.93	4.31	4.40
GE (mj/kg)	16.27	16.30	16.20	16.28	16.31	16.47
Phytase FTU/kg*	716	678	819	651	477	489
Xylanase BXU/kg*	<2000	14900	<2000	15000	<2000	16700

*Enzymes were analysed by an external lab (ESC, Cardiff, UK)

DM = dry matter, P=phosphorus, Ca = calcium, Ti = titanium, GE = gross energy

4.2.3 Treatment Schedule / randomisation plan / condition of animals

A replicate consisted of a pen containing 8 birds (individually weighed) and only birds weighing between 40g and 50g were placed on trial. The combined weight of each pen was recorded on day 0 and randomly allocated around the trial room to reduce any possible effect of ventilation and room differences. Pen allocation is shown in table 4.4 and a plan of the experimental room is shown in appendix B.

Table 4.4 - Diet allocations for the XOS comparison trial

Pen	Diet	Pen	Diet
1	A	25	E
2	B	26	F
3	C	27	A
4	D	28	B
5	E	29	A
6	F	30	B
7	A	31	C
8	B	32	D
9	C	33	E
10	D	34	F
11	E	35	A
12	F	36	B
13	A	37	C
14	B	38	D
15	A	39	E
16	B	40	F
17	C	41	A
18	D	42	B
19	E	43	A
20	F	44	B
21	A	45	C
22	B	46	D
23	C	47	E
24	D	48	F

4.2.4 Study observations

Bird observations were performed a minimum of twice daily to ensure bird welfare and environmental comfort. Temperature and/or ventilation were adjusted accordingly. Bird and room observations were recorded by unit staff twice daily in the trial diary. Dead birds were removed and weighed, and any unfit birds were culled and removed from trial. Bird feed intake was calculated as per the method detailed in section 2.4.1. Birds were weighed weekly by pen on days 0, 7, 14, 21, 28, 35 and 42, as per section 2.4.1. Each week, remaining feed was weighed by pen to calculate feed intake. Feed intake and bird weight were used to calculate weekly feed conversion ratio (FCR).

4.2.5 Sample Collection

On day 7, birds were sequentially fed fresh diet for a minimum of 30 minutes to ensure adequate gut fill prior to euthanasia. Two birds per pen were humanely euthanized by cervical dislocation. Post mortem blood samples were collected into tubes containing EDTA as an anti-coagulant (as per section 2.4.2) for post-trial immunoglobulin analysis (section 2.5.10). The blood was centrifuged at 3000RPM for 10 minutes and plasma removed and stored at -20°C. Ileal digesta (defined as gut content located between the Meckel's diverticulum and the ileal-caecal-colonic junction) was collected (as per section 2.4.3) from all three birds and pooled per pen.

Digesta was freeze dried and ground with a pestle and mortar. Diets and ileal digesta were analysed for gross energy by bomb calorimetry and protein content by external labs (PAS, Shropshire, UK and DM Scientific, Thirsk, UK respectively). Titanium dioxide content of the diet and digesta was measured using the method of Short *et al*., 1996 (section 2.5.5). Calcium and phosphorus content of the diets and digesta was determined by ICP-OES (section 2.5.6). Ash and dry matter were analysed as per sections 2.5.4, and 2.5.1. Digestibility of calcium and phosphorus were calculated using the formulas shown in section 2.5.7.

Both caeca were removed and weighed as a pair then individually snap frozen for potential VFA and 16S analysis at a later date (as per section 2.5.14). 1cm of ileal tissue was removed from the Meckel's

end of the ileum for mucin analysis (see section 2.5.9). 2cm of jejunum (taken from directly proximal to the Meckel's diverticulum) was removed and placed in Bouin's fixative for 6 hours, before being moved into 70% IMS for later histology analysis (as per section 2.5.18).

On day 21, and 42 the following measures were taken from a single weighed bird. One bird per pen was culled, though only if six birds remained in the pen, to ensure balanced performance data was collected. The culled bird was individually weighed.

Blood samples were collected (as per section 2.4.2) and placed into tubes containing EDTA as an anticoagulant. The blood was centrifuged at 3000RPM for 10 minutes and plasma removed and stored at -20°C . The left tibia from each bird was excised and stored at -20C for bone morphometric and strength analysis (see section 2.5.11).

On d42, one bird per pen was culled and individually weighed. Blood was taken (as described above) for immunoglobulin A analysis (section 2.5.13). Ileal digesta was taken and stored in the manner described above for Ca digestibility analysis. A 1cm section of ileum was removed closest to the Meckel's diverticulum was taken for mucin analysis (section 2.5.9). 2cm of jejunum (taken from proximal to the Meckel's diverticulum) was removed and placed in Bouin's solution for 8 hours, before being moved into 70% IMS for later histology analysis (section 2.5.18).

4.2.5 Statistical Analysis of data

Outliers were removed from data if they fell either two standard deviations above or below the average. Statistical analysis was performed using SPSS v.23. KS testing was used to determine data normality, followed by one-way ANOVA, Kruskal Wallis or Univariate analysis as appropriate. Tukey post hoc tests were used to elucidate differences in treatment. Correlations were analysed by bivariate correlation using Pearson correlation, chosen because it computes based on true values and depicts linear relationships. Multiple linear regressions were used to determine the unique contribution and relatedness of factors. Interpretations of the strength between relationships was

based on those of Cohen (1988): small r = 0.1-0.29, medium r = 0.30-0.39 and large r = 0.50 to 1.0. Statistical significance was declared at $p<0.05$.

4.3 Results

4.3.1 Environment

No environmental abnormalities occurred during this trial in terms of temperature or weather changes.

4.3.2 Health and Condition

During the whole 42d study, mortality was 4.17% which is considered standard for trials conducted at the NTU unit and approximately the same as might be expected in a commercial setting. There was no apparent effect of treatment on mortality as can be seen in table 4.5 which splits the mortality by week and dietary treatment.

Table 4.5 - Mortality for XOS comparison trial

Treatment	d0-7	d7-14	d14-21	d21-28	d28-35	d35-42	total
A Con	0	0	0	1	0	0	**1**
B) Corn XOS	2*	1	0	0	0	1	**4**
C) Bag XOS	1	2	0	0	0	0	**3**
D) Con + Xyl	1	0	0	0	1	2	**4**
E) Corn + Xyl	0	0	1	0	1	1	**3**
F) Bag + Xyl	0	1	0	0	0	0	**1**

*runt bird culled

4.3.3 Bird Uniformity

Birds were individually weighed and only birds between 40 to 50g were placed. Start weights for each treatment are shown in table 4.6. There was no significant difference in start weights between treatments.

Table 4.6 - Start weights for chicks for XOS comparison trial (+/- S.E)

Diet	D0 bird wt (g)
A) Con	44 (0.6)
B) Corn XOS	44 (0.7)
C) Bag XOS	44 (0.8)
D) Con + Xyl	44 (0.8)
E) Corn + Xyl	44 (0.6)
F) Bag + Xyl	44 (0.6)
p value	0.978

4.3.4 Performance

Cumulatively, there was no significant difference in body weight gain (p=0.828), feed intake (p=0.413), or feed conversion ratio (p=0.828) between treatments for the duration of the trial (table 4.7). Likewise, there was no significant difference for any individual week of the trial for any performance parameter (table 4.8). There was no interaction seen between XOS and xylanase.

Table 4.7 - Cumulative performance for XOS comparison trial (+/- S.E)

Diet	D0-42BWG (g/bird) (±SE)	D0-42 FI (g/bird) (±SE)	D0-42 FCR (±SE)
Con	3022 (31.0)	4938 (49.3)	1.63 (0.065)
Corn XOS	3007 (40.2)	4864 (68.8)	1.61 (0.078)
Bag XOS	2997 (42.1)	4966 (73.8)	1.66 (0.064)
Con + Xyl	3003 (37.4)	5055 (95.7)	1.69(0.081)
Corn + Xyl	2985 (68.6)	4867 (65.6)	1.62 (0.029)
Bag + Xyl	3077 (37.6)	5008 (58.8)	1.65 (0.051)
P value*	0.885	0.337	0.720
P-Xyl	0.778	0.357	0.503
P-XOS	0.768	0.133	0.477
P-Interaction	0.599	0.708	0.673

*p-value from ANOVA analysis

Table 4.8 - Weekly body weight gain for XOS comparison trial (g/bird, +/- S.E.)

Diet	D0-7 BWG/bird	D7-14 BWG/bird	D14-21 BWG/bird	D21-28 BWG/bird	D28-35 BWG/bird	D35-42 BWG/bird
Con	88 (2.8)	243 (5.8)	467 (8.5)	670 (11.8)	740 (9.0)	815 (44.2)
Corn XOS	86 (3.1)	231 (9.3)	482 (10.6)	663 (17.0)	772 (19.2)	798 (32.9)
Bag XOS	90 (2.2)	254 (7.3)	477 (10.6)	655 (16.5)	757 (24.0)	770 (77.7)
Con + Xyl	88 (2.3)	250 (9.3)	454 (13.9)	681 (20.1)	779 (14.5)	751 (33.7)
Corn + Xyl	88 (2.2)	249 (7.8)	452 (10.4)	659 (10.0)	750 (23.1)	788 (44.8)
Bag + Xyl	89 (2.9)	255 (10.3)	463 (21.7)	672 (10.0)	786 (14.5)	812 (23.7)
P value*	0.973	0.408	0.627	0.958	0.590	0.795
P-Xyl	0.704	0.236	0.110	0.642	0.358	0.718
P-XOS	0.762	0.253	0.781	0.778	0.830	0.956
P-interaction	0.929	0.640	0.819	0.859	0.300	0.360

*p-value from ANOVA analysis

There was no significant difference in feed intake between treatments until week 5 of the trial when the birds fed bagasse + Xyl consumed significantly more than those fed the other treatments (table

4.9). By week 6 there was once again no significant difference in feed intake between treatments. There is a significant increase in feed intake for the diets containing XOS (p=0.030). There is also a trend towards an interaction between XOS and xylanase (p=0.059). When xylanase is added to the bagasse XOS the feed intake increase, however when xylanase is added to the corn XOS the feed intake decreases.

Table 4.9 - Weekly feed intake for XOS comparison trial (g/bird, +/- S.E.)

Treatment	D0-7 FI/bird (g) (±SE)	D7-14 FI/bird (g) (±SE)	D14-21 FI/bird (g) (±SE)	D21-28 FI/bird (g) (±SE)	D28-35 FI/bird (g) (±SE)	D35-42 FI/bird (g) (±SE)
Con	158 (7.0)	368 (11.5)	650 (11.0)	1020 (16.4)	1268[b] (13.2)	1474 (17.6)
Corn XOS	169 (10.3)	367 (17.0)	692 (46.4)	962 (56.0)	1284[b] (25.5)	1469 (26.1)
Bag XOS	148 (6.7)	384 (14.4)	650 (15.0)	967 (39.8)	1303[ab] (21.2)	1505 (24.2)
Con + Xyl	139 (5.0)	390 (12.5)	709 (38.3)	947 (38.5)	1356[ab] (53.2)	1515 (56.7)
Corn + Xyl	151 (5.6)	382 (11.4)	680 (40.5)	944 (23.0)	1235[b] (70.6)	1469 (24.9)
Bag + Xyl	147 (8.4)	400 (14.7)	690 (40.0)	973 (35.0)	1391[a] (47.0)	1421 (26.3)
p-value*	**0.099**	**0.495**	**0.767**	**0.528**	**0.017**	**0.408**
p-Xyl	**0.038**	**0.115**	**0.295**	**0.263**	**0.132**	**0.584**
p-XOS	**0.184**	**0.414**	**0.987**	**0.605**	**0.030**	**0.560**
p-Interaction	**0.403**	**0.962**	**0.691**	**0.407**	**0.059**	**0.162**

[a-b] Means within the same row that do not share a common superscript are significantly different by one-way ANOVA (p=<0.005)

*p-value from ANOVA analysis

There was a trend towards decreased feed conversion ratio for the control +xyl treatment in the first week (table 4.10). There is also a significant decrease in FCR for the diets containing xylanase during the first week, compared to those without. There were no other significant differences between treatments for the remainder of the trial.

Table 4.10 - Feed Conversion Ratio by week D0-D42 for XOS comparison trial (+/- S.E.)

Treatment	D0-7 FCR	D7-14 FCR	D14-21 FCR	D21-28 FCR	D28-35 FCR	D35-42 FCR
Con	1.82	1.51	1.39	1.53	1.73	1.84
	(0.111)[b]	(0.040)	(0.012)	(0.036)	(0.069)	(0.074)
Corn XOS	1.97	1.59	1.45	1.44	1.67	1.86
	(0.116)[b]	(0.061)	(0.097)	(0.084)	(0.017)	(0.073)
Bag XOS	1.66	1.52	1.36	1.48	1.74	1.98
	(0.076)[ab]	(0.046)	(0.015)	(0.058)	(0.110)	(0.777)
Con + Xyl	1.58	1.57	1.49	1.39	1.74	1.93
	(0.085)[a]	(0.066)	(0.015)	(0.037)	(0.064)	(0.161)
Corn + Xyl	1.72	1.56	1.50	1.44	1.64	1.75
	(0.083)[ab]	(0.073)	(0.110)	(0.035)	(0.111)	(0.140)
Bag + Xyl	1.65	1.48	1.50	1.49	1.79	1.79
	(0.093)[ab]	(0.127)	(0.079)	(0.126)	(0.057)	(0.031)
P Value	0.069	0.767	0.544	0.736	0.452	0.378
p-Xyl	0.037	0.905	0.084	0.426	0.843	0.299
p-XOS	0.122	0.440	0.779	0.773	0.127	0.514
p-Interaction	0.364	0.637	0.857	0.465	0.780	0.194

4.3.5. Mucin layer thickness

There was no significant difference in mucin adherence between treatments at days 7, 21 or 42 (table 4.10) There was no significant interaction between XOS and xylanase.

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Table 4.11 - Mucin layer thickness D7, D21 and D42 for XOS comparison trial (µg of alcian blue released per gram of tissue, +/- S.E.)

Treatment	D7 Mucin (µg/g)	D21 Mucin (µg/g)	D42 Mucin (µg/g)
Con	58.32 (12.888)	33.10 (8.150)	46.27 (7.029)
Corn XOS	57.93 (13.946)	35.28 (4.857)	40.71 (7.029)
Bag XOS	46.41 (7.774)	37.22 (5.802)	54.16 (15.004)
Con + Xyl	41.39 (4.990)	44.06 (6.155)	64.20 (6.760)
Corn + Xyl	76.79 (13.270)	40.59 (5.143)	52.91 (7.503)
Bag + Xyl	75.20 (28.541)	38.51 (5.919)	52.76 (6.587)
p-Value	0.486	0.849	0.093
p-Xyl	0.422	0.270	0.596
p-XOS	0.525	0.992	0.187
p-XOS/Xyl	0.309	0.731	0.501

Table 4.12 - Mean Mucin layer thickness across all treatments by age for XOS comparison trial (±SE

Age	Mean Mucin Layer Thickness (µg/g)
7 days	53.02 (28.49)
21 days	35.31 (15.30)
42 days	47.05 (27.50)
p-value age	0.006

4.3.6 Caeca weight

There was no significant difference in caeca weight at day 21 or 42, however, the addition of xylanase seems to numerically increase the weight of the caeca at day 42 (table 4.13).

Table 4.13 - Caeca Weight (Pair) D21 and D42 for XOS comparison trial (g +/- S.E.)

Treatment	D21 Caeca weight (g)	D42 Caeca Weight (g)
Con	7.02 (0.641)	18.22 (2.037)
Corn XOS	6.41 (0.393)	16.95 (2.314)
Bag XOS	7.07 (1.033)	16.35 (1.625)
Con + Xyl	6.62 (0.698)	20.14 (3.383)
Corn + Xyl	7.20 (1.146)	19.74 (0.957)
Bag + Xyl	5.53 (0.668)	22.49 (2.974)
p- Value	0.787	0.488
p-Xyl	0.615	0.100
p-XOS	0.826	0.912
p-Interaction	0.471	0.726

4.3.7 Immunoglobulin A

There was no significant difference in the IgA levels between treatments on D7, D21 and D42 (Table 4.14). IgA levels for all treatments increase with age from day 7 to day 21 and then seem to maintain at around that level until day 42.

Table 4.14 - IgA D7, D21 and D42 for XOS comparison trial (ng/ml of plasma, +/- S.E.)

Treatment	D7 (ng/ml)	D21 (ng/ml)	D42 (ng/ml)
Con	0.535 (0.0116)	1.771 (0.2535)	2.052 (0.4512)
Corn XOS	0.691 (0.0252)	2.692 (0.6482)	1.731 (0.4467)
Bag XOS	0.564 (0.0911)	1.975 (0.3684)	2.011 (0.5959)
Con + Xyl	0.462 (0.0121)	1.765 (0.2268)	2.161 (0.3696)
Corn + Xyl	0.494 (0.0119)	2.149 (0.3222)	1.875 (0.4101)
Bag + Xyl	0.677 (0.032)	2.845 (0.5252)	2.148 (0.3189)
p-Value	0.349	0.236	0.916
p-Xyl	0.105	0.405	0.562
p-XOS	0.684	0.714	0.826
p-Interaction	0.395	0.527	0.464

4.3.8 Bone parameters

There was no significant difference between individuals diets for tibial morphometrics at either day 21 (Table 4.15). However, there is a significant increase in tibial strength for the diets containing xylanase at day 21 (p=0.037). There were no significant differences in tibial morphometrics on day 42, however there is a statistical trend towards increased tibial length at day 42 (p=0.084) (table 4.16).

Table 4.15 - Tibia Morphometrics at d21 for XOS comparison trial +/- SE

Treatment	Tibial Strength (N/kg of BW)	Tibial Weight (g)	Tibial Width (mm)	Tibial Length (mm)
Con	193.87 (33.265)	6.60 (0.160)	5.97 (0.110)	74.32 (0.960)
Corn XOS	164.42 (37.435)	6.60 (0.125)	5.90 (0.216)	74.27 (1.382)
Bag XOS	114.03 (44.074)	7.12 (0.434)	6.07 (0.130)	74.86 (1.462)
Con + Xyl	199.22 (30.273)	6.66 (0.262)	5.71 (0.124)	74.27 (0.929)
Corn + Xyl	222.55 (11.426)	6.39 (0.264)	5.81 (0.109)	73.10 (0.899)
Bag + Xyl	222.45 (11.345)	6.89 (0.187)	5.90 (0.137)	75.14 (0.797)
p-Value	0.121	0.500	0.657	0.782
p-Xyl	0.037	0.665	0.193	0.732
p-XOS	0.572	0.283	0.407	0.503
p-Interaction	0.187	0.708	0.910	0.805

Table 4.16 - Tibia Morphometrics D42 for XOS comparison trial

Treatment	Tibial Strength (N/kg BW+/- SE)	Tibial Weight (g +/- SE)	Tibial Width (mm+/- SE)	Tibial Length (mm+/- SE)
Con	128.20 (9.212)	23.64 (0.803)	7.83 (0.304)	109.80 (1.171)
Corn XOS	127.94 (9.941)	22.78 (0.583)	7.91 (0.240)	110.05 (0.884)
Bag XOS	113.05 (9.622)	23.59 (0.543)	8.13 (0.203)	112.28 (0.884)
Con + Xyl	120.55 (7.816)	22.61 (0.675)	7.81 (0.215)	110.22 (1.251)
Corn + Xyl	107.47 (7.718)	22.09 (0.354)	7.79 (0.227)	109.07 (1.447)
Bag + Xyl	112.47 (3.234)	23.65 (0.938)	8.39 (0.239)	111.01 (1.109)
p-Value	0.157	0.641	0.415	0.471
p-Xyl	0.617	0.175	0.843	0.356
p-XOS	0.369	0.164	0.210	0.084
p-Interaction	0.308	0.154	0.443	0.147

4.3.9 Calcium and phosphorus digestibility – Day 42

There was no significant difference in the quantity of calcium or phosphorus digested between treatments, however, there was a significant increase in the amount of calcium digested for the treatments containing xylanase compared to those without (Table 4.17).

Table 4.17 – Day 42 Calcium and phosphorus digestibility for XOS comparison trial (±SE)

	Apparent ileal digestibility coefficient		Amount of Mineral digested	
Treatment	Calcium	Phosphorus	Calcium (g/day)	Phosphorus (g/day)
Con	0.61 (0.032)	0.84 (0.023)	0.03 (0.001)	0.07 (0.008)
Corn XOS	0.58 (0.030)	0.82 (0.024)	0.03 (0.002)	0.07 (0.008)
Bag XOS	0.52 (0.042)	0.78 (0.032)	0.023 (0.004)	0.07 (0.009)
Con + Xyl	0.60 (0.042)	0.81 (0.028)	0.036 (0.005)	0.06 (0.034)
Corn + Xyl	0.61 (0.045)	0.81 (0.028)	0.044 (0.004)	0.08 (0.085)
Bag + Xyl	0.55 (0.076)	0.82 (0.058)	0.037 (0.007)	0.07 (0.012)
P Value	0.712	0.851	0.14	0.115
p-Xyl	0.637	0.808	0.039	0.690
p-XOS	0.308	0.806	0.222	0.722
p-Interaction	0.885	0.510	0.549	0.872

4.3.10 16S Sequencing - Microbiota Diversity

There was no significant difference in the taxonomic diversity of microbiota between dietary treatments at either day 7 or day 42 (p= 0.260 and p=0.325) (figure 4.2 and 4.3). However, there was a significant composition change in the type of microbes present between day 7 and day 42 (p=0.018) (figure 4.4, 4.4 and 4.6)

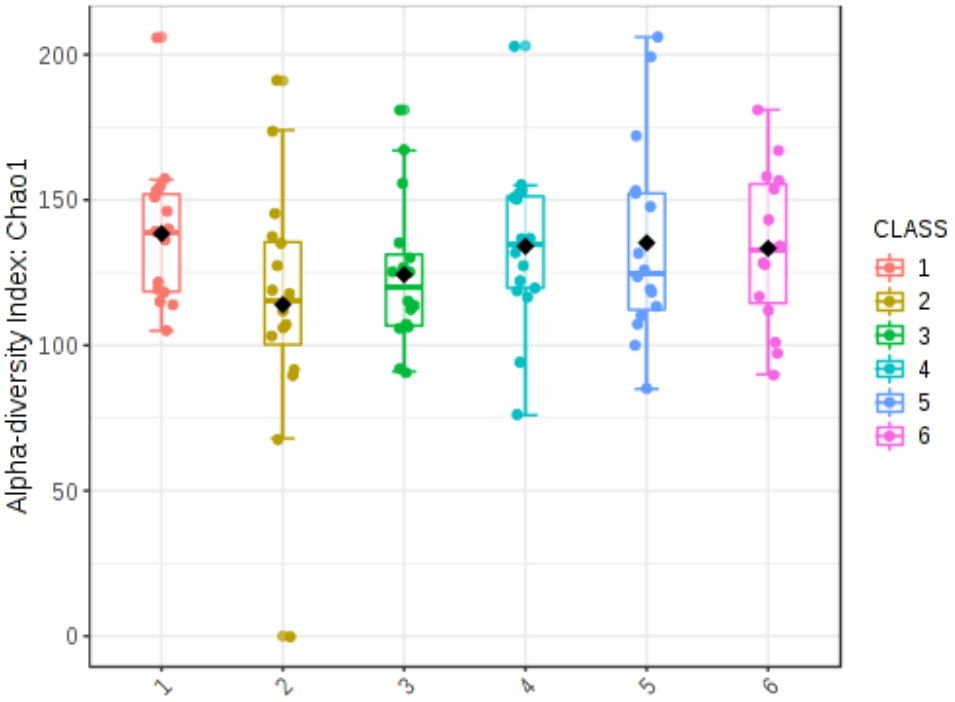

Figure 4.2 - Taxonomic diversity between dietary treatments D7 as follows; 1= Control, 2= Corn XOS, 3= Bagasse XOS, 4= Con+ Xyl, 5= Corn XOS + XYL, 6= Bagasse + Xyl

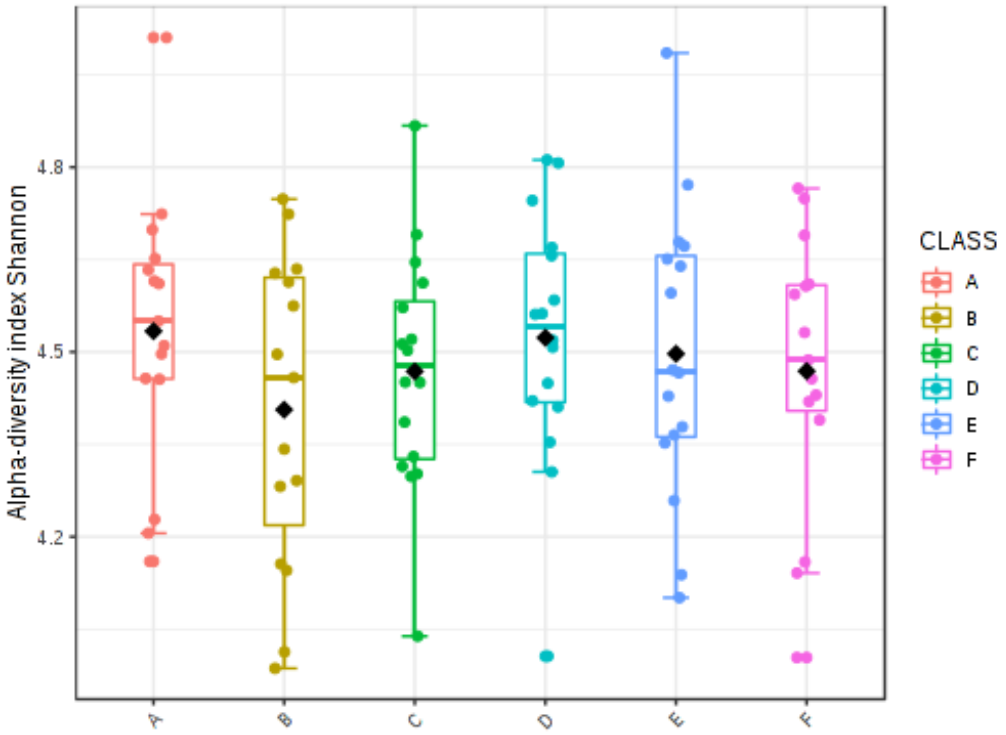

Figure 4.3 - Taxonomic diversity between dietary treatments D42 (A-F represents treatments 1-6 as follows) treatments as follows; 1= Control, 2= Corn XOS, 3= Bagasse XOS, 4= Con+ Xyl, 5= Corn XOS + XYL, 6= Bagasse + Xyl Day 42

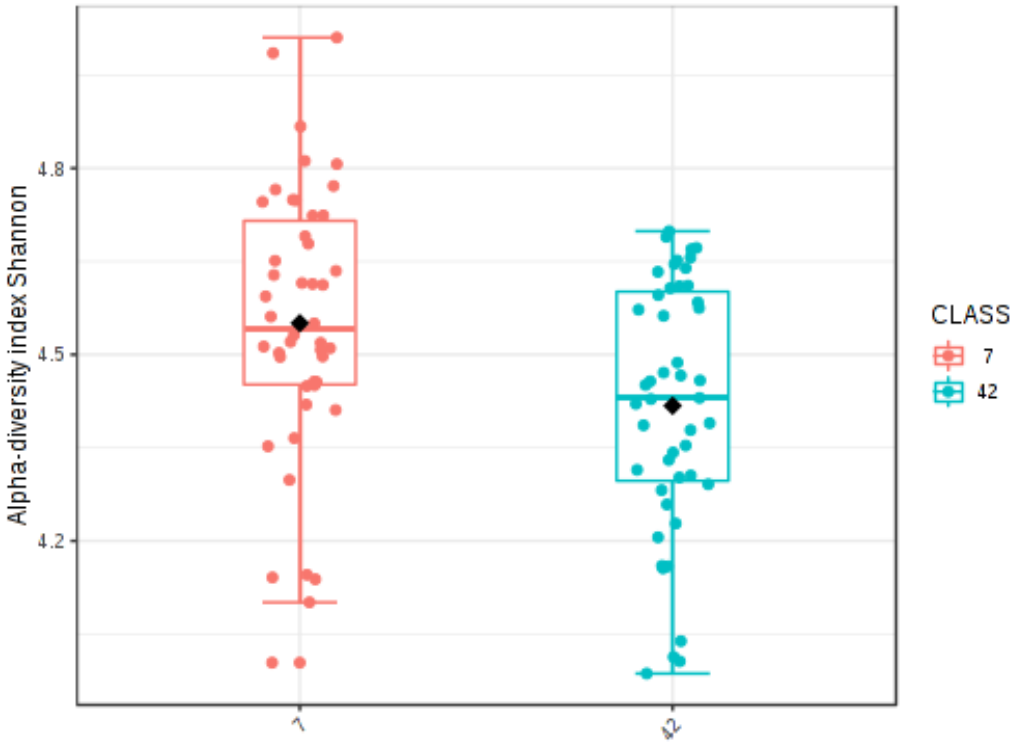

Figure 4.4 - Taxonomic diversity of microbiota for XOS comparison trial days 7 and 42

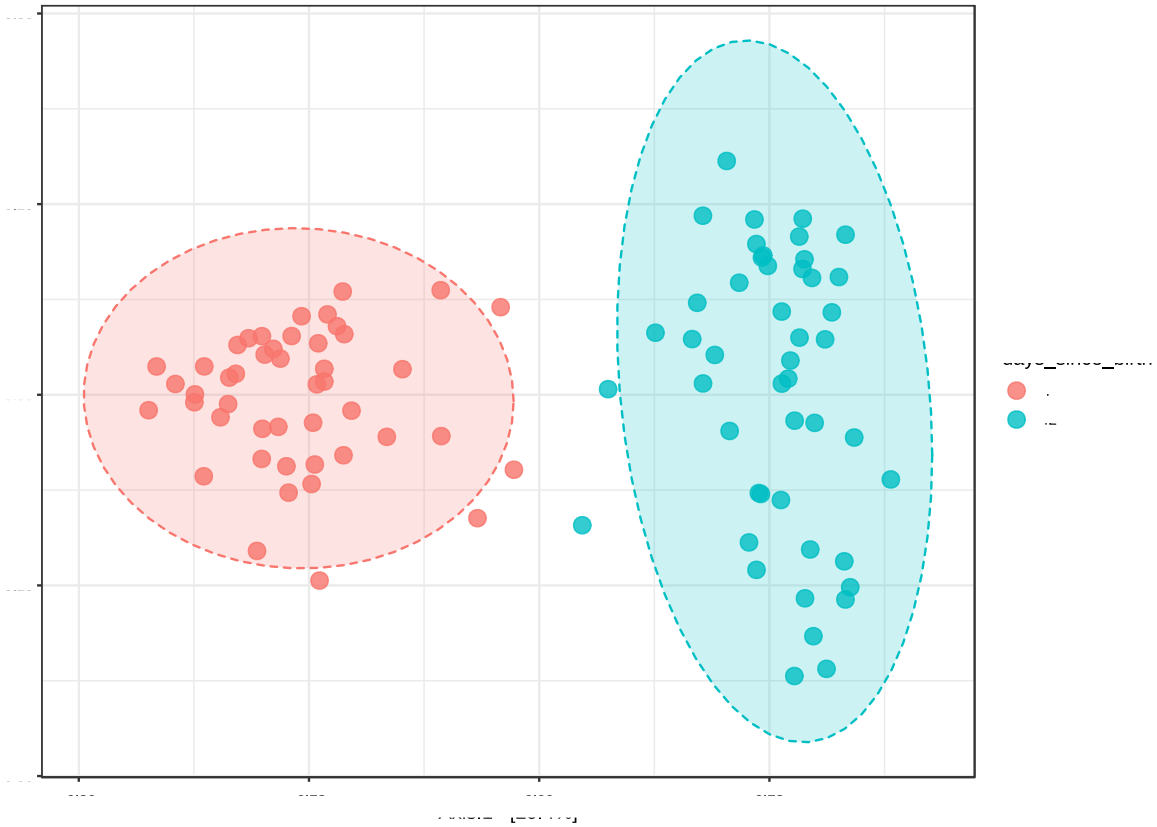

Figure 4.5 - Taxonomic diversity of microbiota for XOS comparison trial days 7 and 42

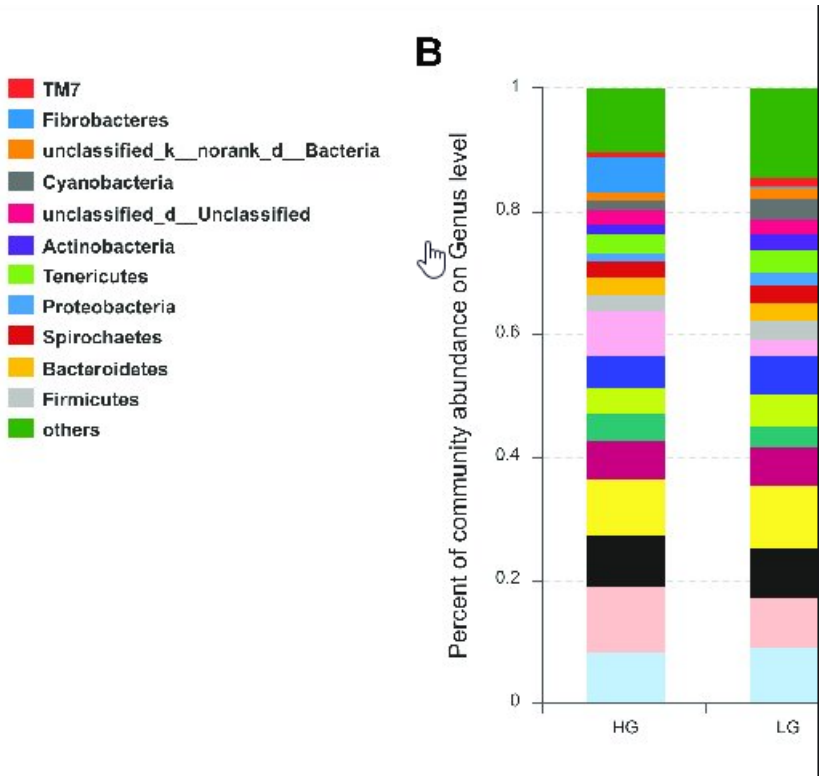

Figure 4.6 Taxonomic diversity by class for XOS comparison trial days 7 and 42

4.3.11 Gut Histology

There were no significant differences in either villus height or crypt depth in the jejunum between treatments (Table 4.18)

Table 4.18 – Gut Histology for XOS comparison trial- day 7 and 42

Treatment	D7 Villi Height (µm)	D7 Crypt Depth (µm)	D42 Villi Height (µm)	D42 Crypt Depth (µm)
Con	1488.8 (45.17)	68.0 (5.41)	2588.4 (73.51)	57.9 (6.25)
Corn XOS	1415.3 (58.23)	69.8 (4.10)	2595.7 (65.7)	60.5 (6.33)
Bag XOS	1427.6 (32.32)	73.3 (3.16)	2625.7 (91.52)	74.1 (16.66)
Con + Xyl	1507.0 (39.50)	75.9 (1.03)	2610.4 (70.77)	54.6 (3.73)
Corn + Xyl	1482.1 (55.26)	66.6 (3.081)	2662.8 (114.39)	51.3 (2.47)
Bag + Xyl	1483.1 (53.91)	68.6 (2.86)	2482.4 (80.77)	58.15 (5.22)
p-Value	**0.719**	**0.448**	**0.757**	**0.465**
p-Xyl	**0.238**	**0.993**	**0.619**	**0.233**
p-XOS	**0.541**	**0.573**	**0.681**	**0.327**
p-Interaction	**0.868**	**0.175**	**0.475**	**0.649**

4.4 Discussion

The aims of this study were to directly compare the efficacy of two XOS products derived from different plant materials in supporting the performance and health of broilers. Additionally, the study aimed to determine whether the addition of xylanase would affect efficacy of these XOS products. Due to structural differences in the xylan backbone, it was hypothesised that the plant matter from which a XOS was derived would affect the efficacy in supporting broiler performance, as one plant XOS maybe more accessible to gut microbiota than another. It was expected that xylanase might enhance any beneficial effects of the XOS as xylanase would hydrolyse AX /X present in the diet and potentially therefore increase the levels of XOS.

The performance results of this trial are essentially unremarkable with the cumulative results showing no significant differences. Numerically there is a 54g (nearly 2%) increase in body weight gain for birds fed the Bag + Xyl diet at day 42 compared to the other treatments. Both the Corn and Corn + Xyl treatments produced the lowest feed intake and FCR, which could suggest that the corn XOS is improving the efficiency of converting feed into body weight (Figure 4.4 and 4.5). Whilst these results are not statistically significant, these small improvements may represent financial gains – particularly on a commercial scale where margins are tight and tiny savings per bird are meaningful.

When considering weekly performance, the feed intake for the Bag + Xyl diet is significantly increased ($p=0.017$) during week 5. This result is unexpected as this increase cannot be seen either before or after this point, even numerically. It is possible therefore, that a feed spillage or weighing error occurred at this time point, accounting for the loss of feed, rather than a true effect of the birds consuming more. The only other significant performance effect is the decrease in week 1 FCR for the Con + Xyl diet. Unfortunately, this result is almost certainly due to feed spillage as the feed type (mash) and feed system (open troughs) encourages the chicks to climb in the feeders, spill feed and dust bathe. The Ross 308 performance objectives state that typical FCR for a day 7 male Ross 308 should be in the region of 0.821 (Aviagen, 2020) (Figure 4.19). The results of the current study are

approximately 8% lower than The Ross 308 performance objectives, likely due to the provision of a mash diet. It is of note that a day 7 FCR for a good trial at NTU is around 1.2. As the day 7 FCR's of the current trial are far above this any significance should probably be discounted.

Table 4.19 - Comparison of Ross 308 Broiler objectives FCR with the mean results of the XOS comparison trial.

Age	Ross 308 objective for FCR	Current study FCR
Day 7	0.821	1.733
Day 14	1.057	1.538
Day 21	1.201	1.448
Day 28	1.336	1.462
Day 35	1.473	1.718
Day 42	1.611	1.858

Having found a positive effect for AX supplementation in the previous trial, it was expected that this trial may show similar effects. The AX content of bagasse has a low ratio of arabinose to xylose units (A:X =0.2:0.6) compared to wheat (A:X=0.5:0.6) and corn (A:X=0.59:0.6) (Kurakake *et al.,* 2005). This "cleaner" xylan backbone with less arabinose substitutions are thought to make the AX less soluble (more likely to transit the GIT intact) and potentially more susceptable to enzyme activity (Campbell *et al*., 2019). Therefore, it was expected that the bagasse XOS used in the current study would be more effective than the wheat derived DDGS AX extract used in the previous study. The latter supplemented the birds with whole arabinoxylans (long-chain polysaccharides), whereas this study investigated the effect of hydrolysing the AX and depolymerising the polysaccharides down into oligosaccharides prior to feeding. This approach was taken as there was robust literature demonstrating the benefits of directly supplementing broilers with shorter-chain polysaccharides (De Maesschalck *et al.,* 2015). Therefore, it was quite disappointing that there was very little to be seen in terms of performance effects. However, other authors have found similar results.

The performance effects of XOS appear to be variable with some studies reporting performance enhancement (De Maesschalck *et al.,* 2015; Ribeiro *et al*., 2018), and others reporting little or no performance effects at all (Li *et al.,* 2017; Craig *et al.,* 2019; Morgan *et al.,* 2019). This may be due to differences in the base products from which XOS supplements were derived. As previously mention, the substitution of arabinose and xylose on the xylan backbone varies both by plant type and variety. Likewise, external factors such as housing, welfare, and pathogenic challenge may all play significant roles in affecting efficacy.

Both De Maesschalck *et al*. (2015) and Craig *et al*. (2019) performed studies using the same commercial XOS as used in this study, though the results were conflicting between these two studies. Craig *et al.* (2019) performed a 29-day study with Ross 308 broilers fed a wheat soy diet. The diets were slightly deficient in protein (by 20g/kg) and energy (by 1MJ/kg) and supplemented with Longlive XOS (Corn XOS in the study in this chapter) at 0.25 g/kg and 1g/kg. No performance effects were found over the duration of the study. The authors suggest that the lack of growth performance may have been due to the use of low protein or energy diets. As the current study fed wheat/corn/soy diets that were sufficient in essential nutrients, to the same strain of birds, it seems unlikely that this was a factor in the lack of XOS response found in this study.

De Maesschalck *et al.,* (2015), studied Ross 308 broilers fed a wheat and rye diet for 39 days. They supplemented the birds with 0.2% XOS from day 0-13 followed by 0.5% XOS from day 14-39. This study showed significant improvements in FCR for the birds supplemented with XOS compared to a control diet. The authors attribute the positive performance effect of XOS to a microbial shift in the GIT, leading to increased production of beneficial VFA's including butyrate. Exogenous butyrate has been shown to enhance broiler growth performance, and to modulate intestinal microbiota and the metabolic activity of the microbiota (De Maesschalck *et al.,*2015). In addition to the regulatory role that butyrate plays in trans-epidermal fluid transport, amelioration of mucosal inflammation and oxidative stress (Canani *et al.,* 2011), butyrate has been shown to fuel epithelial cells, increasing

intestinal epithelium integrity and consequently preventing leaky gut (Guilloteau *et al.,* 2010; Canani *et al.,* 2011).

De Maesschalck *et al.,* (2015) also performed some in vitro work as part of the same study and demonstrated that when the Longlive corn XOS used in this study was fermented *in vitro,* cross-feeding between *L.crispatus* and *A.butytaricus* occurred. L.crispatus produced lactate as a fermentation product, that was consequently used by butyrate producing *anaerostipes* species, thus increasing butyrate production. Butyrate has also been shown to enhance non-specific intestinal defence mechanisms against pathogens such as *clostridium perfringens* (Willemsen *et al.,* 2003; Gantois *et al.,* 2006; Timbbermont *et al.,* 2010). This raises the possibility that the reduced performance of the birds in the current study may have been due to a *clostridium perfringens* overgrowth in the GIT, leading to subclinical necrotic enteritis. Necrotic enteritis is an enteric condition of broilers that can lead to malabsorption and nutrient deficiencies (Klyne, 2013). The subclinical form of this condition is highly prevalent in broilers, characterized by poor digestion, body weight gain and increased FCR. The birds on this study were unfortunately not scored for lesions in the GIT, so it impossible to rule this out, although it is of note, that no lesions were noted during the sampling process. In future work, it is important to measure fermentation products in the caeca to elucidate whether butyrate production was increased, but unfortunately this was beyond the scope of this project.

Hydrolyses of AX into XOS has been shown to vary both by raw material derivation and by different xylanases. Kiran *et al.,* (2013), showed that XOS production from various lignocellistic waste biomasses varied according to the type of xylanase used for hydrolyses. Likewise, these authors found that different xylanases produced varying quantities of products with differing degrees of polymerisation. Xylobiose and triose are considered preferable as they can be readily utilized by pro-biotic microbiota, consequently proliferating these beneficial microbes and improving gut health (Bailey *et al.,* 1992; Yamada *et al.,* 1993). As such it is possible that the lack of performance effect

seen was due to the production of hydrolyses products that were either too small or not small enough. Interestingly, Craig *et al.* (2019), used both the same corn XOS and xylanase (Econase XT) used on this study and likewise showed no performance effect.

There was no significant effect of xylanase on growth performance on this study. The effect of xylanase seems to be variable with some studies demonstrating significant performance improvements (Enberg *et al.,* 2004; Owens *et al*., 2008; Wu and Ravindran, 2004; Masey O'Neil *et al.,* 2012; Zhang *et al.,* 2014) and other studies showing nothing (Singh *et al.,* 2012.; Azhar *et al.,* 2018). The type of diets used seems to be one factor that may affect the efficacy of xylanase (Cardoso *et al.,* 2018; Azhar *et al.,* 2018). Of the 3 or 4 suggested mechanisms of xylanase, the reduction of digesta viscosity is probably the most demonstrated (Bedford, 2019). However, Cardoso *et al*., (2018) reported that exogenous xylanase only reduced the viscosity of digesta in wheat-based diets, if the wheat used had high levels of extract viscosity (>14.8cP). Similarly, Azhar *et al.,* (2018) reports that xylanase only improved AME in wheat cultivars that had a high level of NSP to act as a substrate for enzyme activity. Unfortunately, the viscosity of digesta was not measured on this study as it was felt that the literature conclusively demonstrated that xylanase reduced digesta viscosity in wheat/soy diets (Enberg *et al.,* 2004; Owens *et al*., 2008; Wu and Ravindran, 2004). Therefore, it is possible that the experimental diets did not have sufficient NSP content to provide a substrate for xylanase activity. Alternatively, as mentioned above, it is also possible that the xylanase may have either not hydrolysed the AX sufficiently, or indeed have hydrolysed the AX into undesirable monosaccharides (Bailey *et al.,* 1992; Yamada *et al.,* 1993).

The increased calcium digestibility for the xylanase treatments seems to suggest that xylanase is increasing the availability of calcium in the diet. As the calcium coefficients of digestibility do not show any differences between treatments, the increase in digested calcium appears to occur *in vivo.* A plausible explanation of how calcium digestibility is being increased is possibly via reduction of a cell wall effect, whereby the hydrolysis of the non-starch-polysaccharide fraction of the diet in the upper

digestive tract, results in the release of nutrients that had previously been encapsulated in the endosperm cell wall of the grain structure (Choct *et al.,* 2004; Vandeplas *et al.,* 2010; Knudsen, 2014). This theory is supported by Dong *et al.* (2018) who reported similar results from xylanase supplementation in pigs. The authors report that a 28 study of weanling pigs fed a wheat-based diet with varying amount of xylanase demonstrated increased calcium digestibility in pigs supplemented at 500 and 1000BXU/kg compared to pigs supplemented at 0, 2000 and 4000BXU/kg. These authors suggest that increased calcium digestibility may be due to xylanase decreasing the cell wall effect and releasing trapped nutrients. Li *et al.* (2018) found increased calcium digestibility in laying hens supplemented with XOS. This is interesting as the current study found no effect for XOS on calcium digestibility. Li et al., (2018) attribute the increased calcium digestibility to the XOS altering mineral absorption rates by altering the metabolic activity of microbiota. Roberfroid (2000), describes an osmotic mechanism, whereby the provision of non-digestible carbohydrates such as inulin-type fructans, trigger the transfer of water into the hindgut, this increase in fluid allows minerals to further dissolve.

Xylanase has long been suspected of having a potential prebiotic effect, whereby xylanase hydrolyses AX in to XOS and AXOS (Craig *et al*., 2019). Consequently, it is possible that the increased calcium digestibility seen for the xylanase treatments on this trial are a result of xylanase acting as a prebiotic and hydrolysing enough AX to trigger an osmotic response.

Interestingly, the current trial showed increased tibial strength at day 21 for all the treatments containing xylanase, however, by day 42 there was no significant difference in tibia strength between treatments. This raises the possibility that the increased calcium digestibility seen for the xylanase treatments, may have contributed to increased mineralisation of the tibias at day 21. Unfortunately, calcium digestibility was not measured at 21, so it is impossible to know if calcium digestibility was increased for the entire duration of the study, or only towards to the end. However, this theory is supported by Muszynski *et al.,* (2019), who found similar results and report increased tibial strength, ash percentage and tibial mineralisation at day 42 with xylanase supplementation. Unfortunately,

bone mineralisation was not measured on this study, so increased mineralisation cannot be confirmed. This result is in agreement with Latorre *et al.,* (2014) who showed that feeding turkey poults with a *Bacillus Subtilis* direct fed microbial that produced significant levels of xylanase increased bone mineralization in diets high in NSPs. Neither author explains a mechanism for this, but once again, it is possible that a reduction in the cell wall mechanism as a result of xylanase supplementation could be increasing the availability of encapsulated minerals (Bedford, 2019). It is interesting that the increased tibia strength does not continue to day 42 as it did in the study of Muszyynski *et al.* (2019), and the reason for this is unclear. These authors supplemented xylanase at 200mg/kg (15% inclusion rate) compared to the current study that used 100g/t (100mg/kg). It is therefore possible that the xylanase inclusion rate was insufficient in the current study to exert a significant effect by day 42. It is also possible that when measuring bone strength at day 21 differences between treatments are able to be detected due to being in an active growth phase, and the xylanase is increasing the rate of bone maturity. Therefore, as all treatment groups increase in body mass, bones are strengthened in response to body weight, causing all treatments to have similar bone breaking strength.

Whilst this study did not show any significant differences in mucin layer thickness between dietary treatments at any time point, it did show a significant effect by age. For the majority of treatments, the mucin layer seems to drop at day 21 then start to recover by day 42. When compared to the findings of other trials (table 4.20), the results are variable. The day 7 and 42 results from the current study appear to be in the same range as the other studies. Alsudani (2018), shows a similar mean thickness at day 28 to the day 21 results for the current study. Afrin *et al.*, (2018), are also roughly similar to the current study, in that the mucin layer thickness seems to start thicker and initially decrease with age. It is unfortunate that Afrin *et al.,* did not measure at day 42, to assess whether their results would have started to increase again by day 42.

The varying results of xylanase and XOS trials within the literature make drawing conclusions from the evidence difficult. This is potentially due to the different types of xylanase and/or XOS used as well as variation in dose. However, the weight of the literature reviewed suggests that more beneficial effects may be obtained from xylanase supplementation, as opposed to XOS. This however, does not consider the potential effect of combining both additives and whether this may have a synergistic effect.

Table 4. 20 - Mean Mucin layer thickness across various studies by age (Mean mucin layer thickness (µg Alcian Blue/g tissue) (adapted from Alsudani, 2018 and Amir *et al.,* 2018)

Age	Current trial	Alsudani (2018)	Afrin *et al.,* (2019)
7 days	53.02	N/A	66
21 days	35.31	N/A	41
28 days	N/A	38.04	30
42 days	47.05	N/A	N/A

The mucin layer that covers the lining of the intestinal tract is an essential component of gut barrier function, acting as a lubricant and providing protection to the mucosal surface from physical injury and enteric pathogen entry (Tsirtsikos *et al*., 2012). Likewise, the mucus layer represents the primary barrier limiting contact between the host tissue and commensal microbiota and preventing the translocation of microbes (McGuckin *et al.,*2011). Mucin is secreted from the goblet cells lining the gut epithelium and the thickness of the mucin layer is generally expected to affect nutrient absorption and gut barrier function (Tsirtsikos *et al*., 2012). As such a balance must be achieved, if the mucin layer is too thick, nutrient absorption can be inhibited, but if it is not thick enough, barrier integrity can be compromised. The results of the current study did not show any difference in mucin layer thickness at and time point (D7, 21 and 42). Other authors have found mixed mucin responses.

Tsirtsikos *et al.,* (2012), did not find any difference in mucin layer thickness when supplementing broilers with a dietary phytogenic. However, Alsudani, (2018), showed significant changes in mucin layer thickness when supplementing broilers with a pro-biotic. One possibility is that the birds were not immunologically challenged. Whilst mucin is continually secreted to support normal gut function, the quantity and composition of the mucin changes in response to pathogenic challenge (McGuckin *et al*., 2011). Antimicrobial peptides and glycoproteins are produced throughout the intestinal tract. These molecules target different classes of pathogens in an effort to maintain the sterility of the mucus layer (McGuckin *et al.,* 2011). Therefore, if the birds in the current study were not exposed to pathogenic challenge, it is logical that there may not have been differences in mucin layer thickness.

There was also no significant difference in the immunoglobulin A levels between treatments. As before, we know that immunoglobulins increase in line with antibody production when the host undergoes an immune/inflammatory response (Painter, 1998). IgA is a class of antibody that is of particular importance in diseases involving mucus membrane lined tissues (Cotter, 2007). IgA originates as a secretary product of B-cells in the bursa of Fabricius. It is produced with the aid of intestinal dendritic cells that sample microbiota associated with the epithelium and interact with B and T cells in Peyer's patches to produce specific IgA for microbiota-derived antigens (Macpherson and Uhr, 2004). As such, if the birds were not immunologically challenged, they may not have increased production of IgA. The lack of differences between dietary treatments for mucin layer thickness also reflect this.

The blood IGA levels found in this study are in the same range as other broilers studies (Zhang *et al*., 2015, Alsudani, 2018). Whilst there is not any specific literature that looks at mucin or IgA levels of broilers supplemented with XOS, Komura *et al.,* (2014), looked at the effect of fructo-oligosaccharides (FOS) on mucin and IgA levels in rats. These authors found that FOS supplementation increased mucin and IgA concentrations in rat caeca. Zhang *et al.,* (2015), studied the development with age of mucin and IgA in broilers and pekin ducks.

It is of note that other studies have measured secretory IgA, which is the prevalent form of IgA found in mucin and levels highly correlates to mucin production. Secretory IgA is a dimer of two IgA bound by a J chain and attached to a secretory piece (Genova Diagnostics, 2008). As such, measuring secretary IgA is considered more accurate and maybe something to consider in future studies. The current trial took place under experimental conditions, that whilst trying to emulate commercial conditions, lack the high stocking density that can allow pathogenic disease to spread quickly and easily.

16S analysis was undertaken to determine whether the microbial diversity of the caeca was affected by XOS/xylanase supplementation. The results showed that there was no significant difference in the microbial diversity at either day 7 or 42 between treatments, but that there was a significant ($p=0.018$) change in the microbial composition between time points. These results reflect those reported by Glendinning *et al*., (2019), who similarly found a profound change in the diversity of the microbiota in broiler caeca with age. These authors extracted microbial DNA from various points (including the caeca) of Ross 308 broilers at day 1, 3, 7, 14, and 35. Glendinning *et al.,* (2019) do not provide any information on what the birds were fed or how they were housed. The authors found significant changes in composition, diversity, and richness at every sampling point. Awad *et al*., 2016 looked at age related microbiota changes in broilers from day 1-28 post-hatch. Again, these authors found significant age-related changes in microbiota composition. In birds less than 14 days of age, the most predominant phyla were *Proteobacteria*, however birds over 14 days of age showed predominantly *Firmicutes* and *Tenericutes.*

Whilst in mammals, the microbiota can be transmitted to the foetus via the maternal placenta, uterus and vagina, in avian species the embryo develops in isolation. Ding *et al.* (2017), explored the establishment of chicken microbiota. The authors performed 16S microbial sequencing on embryonic chickens. The study showed that by as early as 4 days of incubation, gut microbiota were present in chicken embryos. They also observed that the composition of the embryonic chick's microbiota

changed fundamentally with age. Whilst in the early embryonic stages, proteobacteria were most abundant (86%) followed by firmicutes (5%). However, by day 19 of incubation firmicutes became most prevalent (88%) (Ding *et al.,* 2017).

The reason for the lack of differences in microbial diversity by treatment is not clear. The population of commensal microbiota within the gut is thought to play a profound role in regulating the immune system (Belkaid and Hand, 2014). The microbiota interacts with both the adaptive and innate immune systems, selecting, calibrating and terminating immune responses (Kamada *et al.*, 2013). As both pathogens and commensal microbiota require similar conditions and ecological niches in order to colonize and proliferate in the GIT, a competition mechanism has evolved, allowing microbes to compete for colonization space in the gut (Kamada *et al.,* 2013). Therefore, as a direct consequence of pathogenic infection, the microbiota responds with protective mechanisms such as the production of bacteriocins, to try and inhibit growth of pathogenic microbes. The lack of differences in the microbial diversity seen in this study, could therefore suggest that the birds were either not exposed to pathogenic challenges and therefore, the microbiota was able to remain relatively stable. Likewise, this could also indicate that all the birds were all exposed to the same pathogenic challenges and therefore experienced similar shifts in microbiota diversity. The experimental setting used for this study, has the benefit of allowing numerous dietary treatments to be explored in one room and therefore, one environment. This of course has a number of limitations. The proximity of birds on different treatments means that if one set of birds was exposed to pathogenic infection, then all treatments are likely to become infected. At the same time, this also ensures that all the subjects are kept in like-for-like conditions and are therefore comparable. The nature of an experimental setting and the regulations they must adhere to, also means that by definition these facilities have to be kept clean and biosecure, potentially cleaner than a commercial broiler setting. This obviously reduces the bird's exposure to environmental pathogens. Repeating this study in a commercial setting in the future would be very interesting to see if differences in microbial diversity could be detected.

Initially, there was concern over the validity of the 16S results for this trial as there were no Bacteroidetes microbes present. However, a second NTU trial revealed identical findings. Glendinning *et al.,* (2019), also found no *Bacteroidetes,* which coupled with similar findings in the microbiota change with age adds weight to the findings of this study.

By day 42 the microbes found in this study are predominantly firmicutes, which again agrees with the findings of Glendinning *et al.* (2019). Interestingly, low levels of *Bacteroidetes* and predominant *Firmicutes* are associated with obesity in humans and mice due to Firmicutes ability to harvest energy from the diet (Ley *et al.,* 2005; Faintuch and Faintuch, 2019). *Firmicutes* can be split into two major groups: *clostridium coccides* (*Clostridum* cluster XIVa) and *Clostridium leptum* (*Clostridium cluster* IV). The bacteria in the *Clostridum Coccoides* groups play a large part in the fermentation of carbohydrates within the gut (Faintuch and Faintuch, 2019). Butyrate is the primary metabolite of *firmicutes* (Besten *et al.,* 2013). It therefore seems possible that the microbial diversity results of this trial (and others) would show predominant *Firmicutes* and lower levels of *Bacteroidetes* as high levels of phyla that are proficient in extracting energy from the diet would be beneficial, if not vital to maintain the high levels of growth efficiency required of modern broilers.

The histology results of this study revealed no significant differences between treatments at either day 7 or days 42. This is somewhat unexpected as De Maesschalck *et al.,* (2015), found that supplementing broilers with the same corn XOS used in this study (0.2% day 0-13 and 0.5% day 14-39) resulted in significantly longer villi in the ileum (section closest to the Meckel's diverticulum), at 26 days of age. In the current study the intestinal samples were taken from the jejunum, however the samples were collected from the section closest to the Meckel's diverticulum, so similar results may have been expected. As the current study measured at day 7 and day 42, it remains possible that taking measurements at different time points could have accounted for this lack of differences. Luo *et al.,* (2009), demonstrated increased villus height in the jejunum of birds fed wheat-based diets, supplemented with xylanase at 1000BXU/kg. These authors showed that supplementing broilers with

either no xylanase or xylanase at 5000BXU/kg significantly reduced villus height in comparison to the 1000BXU/kg dose. As the birds in the current study were supplemented with xylanase at either 0 or 1600BXU/kg it is possible that the dose was either insufficient or too large to see increased villus height. When the villus height is correlated to mucin (day 42), there is no positive correlation (R^2=0.0221) (Figure 4.6). When a Pearson correlation was performed the relationship is r=0.141 showing there is no indication that longer villi are related to increased mucin secretion/mucin layer thickness.

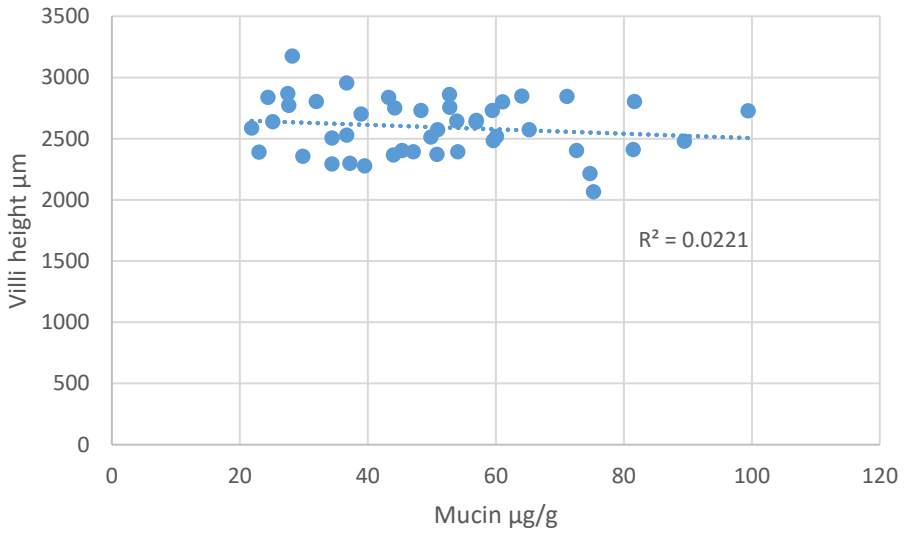

Figure 4.6 – Correlation between villi height and mucin layer thickness at day 42

4.5 Conclusion

The results of this trial were unexpected, as following the initial study it was thought that directly supplying a XOS product might enhance the beneficial effects seen with AX supplementation. The results of this study showed no real effect of XOS supplementation; therefore, it is difficult to draw conclusions as to whether the base material from which a XOS is extracted can affect efficacy.

This study did, however, show a number of beneficial effects for xylanase supplementation. The treatments containing xylanase showed increases in tibial strength at day 21 and calcium digestibility at day 42. The mechanism by which xylanase is improving these parameters remains unclear, but a reduction in the cell wall effect, increasing the availability of encapsulated nutrients seems plausible.

The lack of differences in immune and gut health parameters seems to suggest that the birds were not exposed to any immune challenges and therefore no response was observed. Upscaling this study to a commercial would be interesting, as the birds are more likely to be exposed to environmental and pathogenic challenges, due to increased stocking density, reduced environmental control and lack of experimental legislation. It would also be interesting to examine other areas of bird health, to determine whether differences between treatments can be detected in an experimental setting. Oxidative stress is an emerging field in animal production that has been shown to have profound effects on downstream meat quality. Looking at whether differences in oxidative stress can be detected between dietary treatments may be a more precise measure of bird health. Looking at the dose of XOS and/or xylanase may also further elucidate their effect. When comparing the results of this study to others in the literature, there was a broad variation in dose of both XOS and xylanase. Therefore, a dose response study may

provide further information into the mode of action and efficacy of XOS and xylanase supplements.

Chapter 5 was appended during the viva and can be found at the end of this document

Chapter 6 – Optimizing the dose of a commercial xylo-oligosaccharide/xylanase supplement

6.1 Introduction

After the initial work on this project the corn based XOS used in chapter 4 was marketed as a supplement containing both the corn XOS and xylanase (Signis ®). As the previous studies had shown results for both AX and xylanase independently, but had raised questions over whether the dose of xylanase in particular, may have affected efficacy, it was decided that a dose response study may further elucidate both the mode of action and effects of XOS and xylanase when provided together.

As other studies had provided varied results in response to the dose of XOS supplied, gaining some more insight on this seemed important. Courtin *et al.* (2008) complemented a wheat-based diet with XOS at 0.25 and 0.5g/kg and reports that fourteen days of XOS supplementation (extracted from wheat flour) resulted in significantly increased quantities of *Bifido* bacteria inbroiler ceacas, however the authors did not find any significant changes in the numbers of *Lactobacilli* present. Furthermore, a study by Pourabedin *et al.* (2015) reports similar results with significant increases in beneficial bacteria in the caeca of birds supplemented with XOS at 2g/kg, however, in this case it was *Lactobacillus* genus bacterium that flourished. In some cases, both *Bifido* and *Lactobacillus* bacteria have been found to proliferate with XOS inclusion. Ribeira *et al.* (2018) found that XOS supplementation at 0.1g/kg increased the microbial populations of *Bifido bacterium* and *Lactobacilli*. As was found in the performance data from this study, a lower XOS inclusion rate (0.1g/kg) produced higher levels of beneficial bacterium. The results of Ribeira *et al.* (2018) suggest that there is likely to be an optimal dosage, however, whether this may vary by the base material from which the XOS is extracted is unclear.

As such, a 35-day bird trial was designed to compare a control diet to Signis® at both the commercially recommended dose and at double the recommended dose. Several studies had used considerably higher doses of both XOS and xylanase with no ill effects to the birds (Courtin *et al.,* 2008; Zhenping *et al.*, 2012; Ribeira *et al.,* 2018) and therefore no negative effects of doubling the dose were expected.

The results of the previous studies in chapters 3 and 4, had revealed some benefits to feeding an AX supplement in terms of significantly improving body weight gain during the finisher phase and numerically increasing final bird weight by 6%. Likewise, feeding xylanase revealed increased bone breaking strength at day 21 and increased calcium digestibility at day 42. As such, the previous results had not shown a clear effect of the combination of XOS and xylanase, so measures were repeated to try and obtain more conclusive data.

The main aims of the trial were as follows:

- Investigate the effect of Signis® (XOS/Xyl) on the performance of broilers
- Determine whether any effects of Signis® were altered by doubling the recommended dose
- Determine whether any performance enhancement derived from the supplementation of XOS/Xyl is down to a viscosity effect
- Undertake a range of analyses to add information into how this product may be working mechanistically.

6.2 Trial procedure

6.2.1 Husbandry Conditions

240-day old male Ross 308 broilers were sourced from PD Hook Cote hatchery from a flock aged 44 weeks. Birds were feather sexed on the day of hatch and any poor birds were discarded on arrival. Chicks were individually weighed on arrival, then randomly assigned to 48 mesh sided pens (5 birds per pen), littered with a wood shavings substrate. Food and water were provided *ad libitum* and care taken to ensure chicks were eating and drinking as soon as possible. Husbandry guidelines were

followed as described in chapter 2 and adhered to the institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986). Ethical approval was granted by the University ARES Ethics Committee and was logged as project ARE906.

6.2.2 Diet formulation and condition of animals

Signis® was provided by A.B. Vista. The trial used three treatments (table 6.1) with three phases (starter, grower, and finisher). Three basal diets (one per phase) were commercially manufactured by Target Feeds (Shropshire, UK). Diets were formulated by a commercial nutritionist to meet the age and strain of the bird (table 6.2). Each basal diet was divided into three treatments inhouse and Signis® was added to treatments B and C at either 0.1g/kg (diet B) or 0.2g/kg (diet C). Quantum Blue 5G phytase was added to all diets at the time of manufacture at 0.01% inclusion rate to provide approximately 500FTU. Proximate analysis was performed to confirm composition (table 6.3)

Table 6.1 – Dietary treatments for dose response trial

Diet	Treatment
Control	Standard broiler diet
Control + 0.1g/kg Signis®	Standard broiler diet + 0.1g/kg Signis®
Control + 0.2g/kg Signis®	Standard broiler diet + 0.2g/kg Signis®

Table 6.2 – Diet Formulations and rate of inclusion (Roi) for dose response trial (g/kg)

Ingredient	RoI Starter	Roi Grower	Roi Finisher
Wheat	615	635	670
Extruded Soy A	324.3	291.3	252.9
Soya oil	15.8	29.4	38.9
Salt	3.8	5	3.8
Limestone	1.6	3.8	1
Dicalcium Phos, 18%P	21.7	19.1	17.4
Lysine HCl	2.8	2.2	2.3
DL-Methionine	3.2	2.7	2.6
Threonine	1.6	1.2	1.2
Vitamin & Mineral premix	5	5	5
Quantum Blue 5G	0.1	0.1	0.1
Signis®	0	0	0
Titanium	5	5	5

Table 6.3 - Proximate analysis of basal diets for dose response trial

Diet	Starter	Grower	Finisher
DM (g/kg)	879	874	875
Ash (g/kg)	58	46	52
Protein (g/kg DM)	224	186	208
Total P (g/kg DM)	55	60	78
Total Ca (g/kg DM)	49	47	67
Fat (g/kg)	24	33	48
Ti (g/kg)	4.96	4.02	5.15
GE (MJ/kg)	15.91	16.24	16.40
Phytase (FTU/kg)	491	617	981
Xylanase (BXU/kg) (0.1g/kg)	13900	14800	16400
Xylanase (BXU/kg) (0.2g/kg)	24200	30600	28700

6.2.3 Treatment Schedule / randomisation plan / condition of animals

A replicate consisted of a pen containing 5 birds (individually weighed) and only birds weighing between 40g and 50g were placed on trial. The combined weight of each pen was recorded on day 0. Treatments were randomly allocated via, an online randomisation tool (random.org) by block (one block of 3 pens) to reduce any effect of ventilation and temperature differences within the room. Pen allocation is shown in table 6.4 and a plan of the experimental room is shown in appendix B

Table 6.4 - Diet allocation for dose response trial

pen	diet allocation		pen	diet allocation
pen 1	A		pen 25	B
pen 2	B		pen 26	C
pen 3	C		pen 27	A
pen 4	B		pen 28	A
pen 5	A		pen 29	B
pen 6	C		pen 30	C
pen 7	C		pen 31	C
pen 8	A		pen 32	B
pen 9	B		pen 33	A
pen 10	B		pen 34	A
pen 11	C		pen 35	C
pen 12	A		pen 36	B
pen 13	C		pen 37	B
pen 14	B		pen 38	A
pen 15	A		pen 39	C
pen 16	C		pen 40	A
pen 17	A		pen 41	B
pen 18	B		pen 42	C
pen 19	A		pen 43	B
pen 20	C		pen 44	C
pen 21	B		pen 45	A
pen 22	A		pen 46	C
pen 23	B		pen 47	A
pen 24	C		pen 48	B

6.2.4 Study observations

Bird observations were performed a minimum of twice daily (generally more) to ensure bird welfare and environmental conditions were maintained. Temperature and/or ventilation were adjusted depending on bird behaviour. Dead birds were removed and weighed, and any unhealthy birds (defined as any bird displaying discomfort or distress) were culled and recorded. Bird feed intake was calculated as per the method detailed in section 2.4.1. Birds were weighed weekly by pen on days 0, 7, 14, 21, 28 and 35, as per section 2.4.1. Additionally, birds were weighed individually on days 28 and 35 to assess flock variability. Feed intake and bird weight were used to calculate weekly feed conversion ratio (FCR).

On day 35, birds were sequentially fed fresh diet for a minimum of 30 minutes to ensure adequate gut fill prior to euthanasia. One bird per pen was humanely euthanized by cervical dislocation. Post mortem blood samples were collected and split into tubes containing EDTA as an anti-coagulant for post-trial immunoglobulin analysis (as per section 2.4.2) or into serum tubes for antioxidant analysis (section 2.5.10). The blood was centrifuged at 3000RPM for 10 minutes (Thermo Scientific, Megafuge 8, Fisher, UK) and plasma/serum removed and stored at -20°C.

The ileum and jejunum were excised from the end of the duodenal loop to the ileal-caecal-colonic junction then split at the Meckel's diverticulum. Ileal and jejunal digesta were collected from each bird by gentle digital pressure. Jejunal digesta was aliquoted into Eppendorf tubes and immediately centrifuged at 15000RPM for 5 minutes (Sigma 1-14 micro centrifuge, Sigma-Aldrich, UK) for viscosity analysis as described in section 2.5.22.

Ileal digesta was pooled into one pot per pen. Digesta was frozen at -20^{o}C before being freeze dried and ground with a pestle and mortar. Diets and ileal digesta were analysed for gross energy by bomb calorimetry at an external lab. Protein was analysed via Dumatherm (Gerhardt, UK) as described in section 2.5.2. Titanium dioxide content of the diet and digesta was measured using the method of Short *et al*., 1996 (section 2.5.5). Calcium and phosphorus content of the diets and digesta was

determined by ICP-OES (section 2.5.6). Ash and dry matter were analysed as per sections 2.5.4, and 2.5.1. Digestibility of calcium and phosphorus were calculated using the formulas shown in section 2.5.7. Both caeca were removed and weighed as a pair then individually snap frozen for potential VFA and 16S analysis in a later project (as per section 2.5.14). The bursa of Fabricius was excised from each bird and weighed. The left breast fillet and thigh were excised and later split for drip loss (section 2.5.24) and texture analysis, both fresh and cooked(section 2.5.23). Variation was assessed using the percentage coefficient of variation (C.V). This was calculated using the following equation:

$$\frac{\text{Standard deviation of treatment body weight}}{\text{Average body weight of treatment}} \times 100$$

6.2.5 Statistical analysis of data

Outliers were removed from data if they fell either two standard deviations above or below the mean. Statistical analysis was performed using SPSS v.23 (IBM statistics). KS testing was used to determine data normality, followed by one-way ANOVA and Univariate analysis as appropriate. Bonferroni post hoc tests were used to elucidate differences in treatment. Correlations were analysed by bivariate correlation using Pearson correlation, chosen because it computes based on true values and depicts linear relationships. Interpretations of the strength between relationships was based on those of Cohen (1988): small r = 0.1-0.29, medium r = 0.30-0.39 and large r = 0.50 to 1.0. Statistical significance was declared at $p<0.05$.

6.3 Results

6.3.1 Environment

This trial was carried out in winter, during a cold and wet period of weather. On Day 29 of the trial, a power cut caused loss of heating, causing temperatures to drop to 11°C. The system reset and raised temperatures to 14°C. The following day, the system failed again, causing temperatures to drop to 10°C. The room was immediately heated up using portable heaters and the extraction system disabled to aid heating. By the afternoon check the room was up to temperature and the extraction system was restarted. Due to the heat fluctuations combined with high external humidity and low temperatures, the bedding quality was recorded as poor, and was capped by day 34 (figure 6.1).

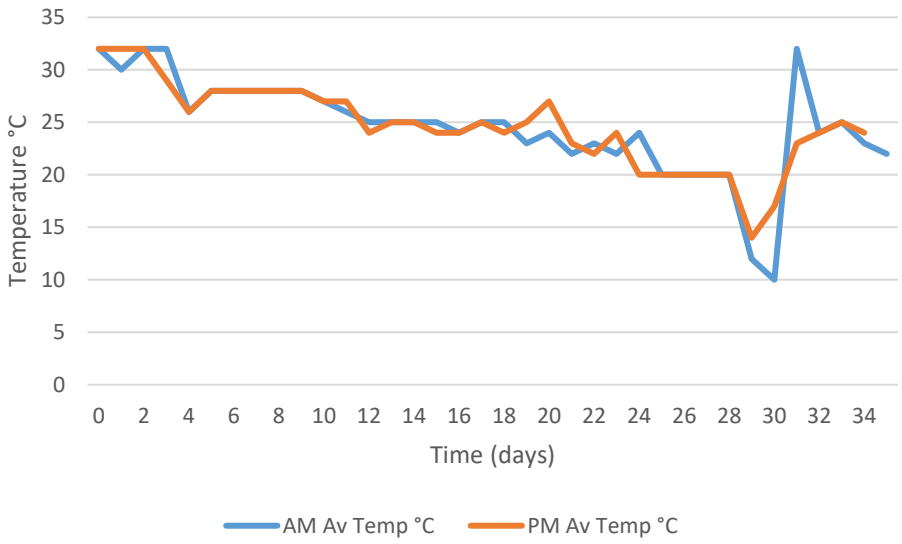

Figure 6.1- Temperature record for dose response trial

6.3.2 Health and Condition

Mortality data is shown in table 6.8. During the whole 35d study, mortality was 3.3% which is considered standard for trials conducted at the NTU unit and lower than would be expected in a commercial setting which would be typically in excess of 4%. There was no apparent effect of treatment on mortality as can be seen in table 6.5 which splits the mortality by week and dietary treatment.

Table 6.5 - Bird mortality for dose response trial

Treatment	d0-7	d7-14	d14-21	D21-28	D28-35	Total
Control	2	0	0	1	1	4
0.1g/kg Signis®	1	0	0	1	1	3
0.2g/kg Signis®	1	0	0	0	0	1

6.3.3 Bird Uniformity

Upon arrival at the research unit, all chicks were individually weighed and only birds weighing between 38 and 46g were placed. The mean start weights for each treatment are shown in table 6.5. There was no statistical difference in the start weight of the chicks between treatments.

Table 6.6 - Average start weight for chicks for dose response trial

Treatment	d0 BW/bird (g)
Control	41 (0.7)
0.1g/kg Signis®	41 (0.8)
0.2g/kg Signis®	41 (0.7)
P value	0.469

6.3.4 Cumulative Performance

Cumulatively there was a significant increase in body weight gain during the periods of day 0-7, 0-14, and 0-21 for the higher dose of Signis® (0.2g/kg) compared to the other two treatments. From d0-28 there was a significant increase in body weight gain for the higher dose of Signis compared to the lower dose and also a significant increase body weight gain for the low dose compared to the control. Between day 0 and35 there was a significant increase in body weight gain for both Signis® treatments compared to the control (table 6.6).

Table 6.7 - Cumulative body weight gain for dose response trial (g +/- SE)

Treatment	D0-7 BWG	D0-14 BWG	D0-21 BWG	D0-28 BWG	D0-35 BWG
Control	72 (2.9)[b]	266 (11.3)[b]	633 (24.4)[b]	1161 (35.4)[ab]	1819 (54.1))[b]
0.1g/kg Signis®	74 (2.5)[b]	280 (8.7)[b]	685 (16.6)[b]	1282 (27.1)[b]	1987 (53.8)[a]
0.2g/kg Signis®	83 (3.3)[a]	321 (8.3)[a]	750 (15.1)[a]	1350 (22.8)[a]	2089 (31.9)[a]
P Value	0.019	<0.001	<0.001	<0.001	0.001

[a-b] Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p<0.05)

From days 0-7, 0-14, and 0-21 there was no significant difference in feed intake between treatments and between day 0-28 and 0-35 there was a significant increase in feed intake for both Signis® treatments compared to the control (table 6.7).

Table 6.8 - Cumulative feed intake for dose response trial (+/- S.E.)

Treatment	D0-7 FI (g)	D0-14 FI (g)	D0-21 FI (g)	D0-28 FI (g)	D0-35 FI (g)
Control	246 (12.9)	458 (22.5)	1022 (34.5)	1932 (34.2)[b]	3046 (50.8)[b]
0.1g/kg Signis®	254 (16.09)	438 (50.6)	1013 (54.3)	2033 (35.4)[a]	3214 (47.7)[a]
0.2g/kg Signis®	259 (18.3)	418 (22.5)	1003 (24.2)	2055 (39.9)[a]	3234 (51.6)[a]
P Value	0.850	0.709	0.945	0.050	0.020

[a-b] Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

During day 0-7 there was no significant difference in FCR between treatments. During day 0-14, 0-21, 0-28, and 0-35 there was a significant reduction in FCR for the birds fed the higher dose of Signis® compared to the other two treatments (table 6.8). These FCR values are high due to feed spillages typically seen in the first weeks of bird trials at NTU.

Table 6.9 - Cumulative feed conversion ratio for dose response trial +/- SE

Treatment	D0-7 FCR	D0-14 FCR	D0-21 FCR	D0-28 FCR	D0-35 FCR
Control	2.19 (0.125)	1.79 (0.131)[b]	1.68 (0.046)[b]	1.65 (0.086)[b]	1.68 (0.033)[b]
0.1g/kg Signis®	2.19 (0.123)	1.56 (0.155)[b]	1.59 (0.030)[b]	1.48 (0.060)[b]	1.63 (0.040)[b]
0.2g/kg Signis®	2.08 (0.158)	1.31 (0.080)[a]	1.52 (0.020[a]	1.34 (0.040)[a]	1.55 (0.020)[a]
P Value	0.812	0.036	0.007	0.008	0.002

[a-b] Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

6.3.5 Weekly bird body weight gain

By day 7, there was a significant increase in bodyweight for the birds fed the higher dose of Signis®, with birds weighing on average over 10g more than the control (table 6.10). For the next two weeks (day 14 and 21) this trend continues, with the higher dose of Signis® consistently showing a significant increase in average bird weight. At day 14, the higher dose treatment weigh 22.7% more than the control and by day 21 this treatment weighs 22.3% more than the control (p= 0.001 and p <0.001). However, by day 28 both Signis treatments showed a significant increase in average bird weight compared to the control. Whilst the higher dose has a mean average bird weight of nearly 70g more than the lower dose, this difference was not statistically significant (p=0.985). During the final week of the trial, both Signis® treatments significantly increase average bird weight compared to the control birds. There was no significant difference between the two Signis® treatments at day 35 although numerically, the higher dose yields birds at slaughter age that weigh 100g (5%) more than the lower dose and nearly 15% higher than the control.

Table 6.10 - Weekly Body Weight Gain (g) for dose response trial (g) (+/- S.E.)

Diet	D0-7 BWG/bird	D7-14 BWG/bird	D14-21 BWG/bird	D21-28 BWG/bird	D28-35 BWG/bird
Control	72[b] (3.0)	194[b] (9.0)	367[b] (15.5)	529[b] (18.0)	657 (30.4)
0.1g/kg Signis®	74[b] (2.5)	206[b] (7.7)	404[b] (10.4)	597[a] (13.6)	705 (35.6)
0.2g/kg Signis®	83[a] (3.3)	238[a] (5.4)	429[a] (9.4)	600[a] (10.7)	739 (18.7)
P value	0.019	<0.001	0.003	0.001	0.148

[a-b] Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

6.3.6 Weekly average bird weight

By day 7, there was a significant increase in bodyweight for the birds fed the higher dose of Signis®, with birds weighing on average over 10g more than the control (table 6.10). For the next two weeks (day 14 and 21) this trend continues, with the higher dose of Signis® consistently showing a significant increase in average bird weight. At day 14, the higher dose treatment weigh 22.7% more than the control and by day 21 this treatment weighs 22.3% more than the control (p= 0.001 and p <0.001). However, by day 28 both Signis treatments showed a significant increase in average bird weight compared to the control. Whilst the higher dose has a mean average bird weight of nearly 70g more than the lower dose, this difference was not statistically significant. During the final week of the trial, both Signis® treatments significantly increase average bird weight compared to the control birds. There was no significant difference between the two Signis® treatments at day 35 although numerically, the higher dose yields birds at slaughter age that weigh 100g (5%) more than the lower dose and nearly 15% higher than the control.

Table 6.11 - Weekly Average Bird Weight for dose response trial (g ±SE)

Diet	D0 BW	D7 BW	D14 BW	D21 BW	D28 BW	D35 BW
Control	42 (0.7)	113[b] (3.2)	307[b] (11.6)	674[b] (24.6)	1208[b] (37.7)	1860[b] (54.3)
0.1g/kg Signis®	42 (0.8)	116[b] (2.6)	322[b] (8.9)	727[b] (16.9)	1323[a] (27.4)	2029[a] (54.0)
0.2g/kg Signis®	41 (0.7)	125[a] (3.4)	363[a] (8.3)	791[a] (15.2)	1392[a] (22.9)	2131[a] (32.0)
P value	0.469	0.029	0.001	<0.001	<0.001	0.001

[a-b] Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

6.3.7 Weekly Feed Intake

There was no significant difference in feed intake between treatments during the first 4 weeks of the study. During the final week, the two Signis treatments both show significantly increased feed intake compared to the control diet (table 6.11)

Table 6.12 - Weekly feed Intake for dose response trial (±SE)

Diet	D0-7 FI (g)	D7-14 FI (g)	D14-21 FI (g)	D21-28 FI (g)	D28-35 FI (g)
Control	246 (3.2)	326 (11.8)	554 (19.1)	826 (17.8)	1114 (27.8)
0.1g/kg Signis®	254 (64.0)	356 (8.9)	566 (15.0)	857 (14.9)	1181 (23.2)
0.2g/kg Signis®	259 (18.3)	349 (11.7)	576 (14.1)	872 (15.1)	1179 (16.7)
P value	0.85	0.248	0.639	0.126	0.075

[a-b] Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

6.3.8 Weekly Feed Conversion Ratio

There was no significant difference in FCR by D7 (table 6.12), However, on D14 and 21 the higher dose of Signis® has a significantly reduced FCR of 1.46 and 1.35 respectively (D14, p=0.02,day 21 p=0.039) when compared to the other two treatments. By D28, the FCR for the lower dose of Signis®

is no longer significantly different to the higher dose, and both Signis® treatments have significantly reduced FCR compared to the control. During the final week of the trial, there was no significant difference in FCR between treatments, though the numerical difference between the control diet and 1g.kg Signis and the 2g/kg Signis diet is substantial (14 points of FCR).

Table 6.13 - Weekly FCR for dose response trial (+/- S.E.)

Diet	D0-7 FCR	D7-14 FCR	D14-21 FCR	D21-28 FCR	D28-35 FCR
Control	2.18 (0.125)	1.72[b] (0.078)	1.54[b] (0.081)	1.58[b] (0.052)	1.75 (0.084)
0.1g/kg Signis®	2.19 (0.123)	1.75[b] (0.095)	1.41[b] (0.04)	1.44[a] (0.027)	1.74 (0.110)
0.2g/kg Signis®	2.08 (0.158)	1.46[a] (0.044)	1.35[a] (0.04)	1.45[a] (0.017)	1.61 (0.031)
P value	**0.812**	**0.02**	**0.039**	**0.013**	**0.384**

[a-b] Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

6.3.9 Variability

There was a large difference in the standard deviation of weights between the three treatments, with variability being considerably lower for the higher dose of Signis® (table 6.13). The highest dose of Signis® produced the lowest coefficient of variation (12.4%) (table 6.14).

Table 6.14 – Flock Variability (standard deviation from individual bird weights (S.D.)) for dose response trial

Treatment	Min wt (g) D28	Max wt (g) D28	Min wt (g) D35	Max wt (g) D35	S.D. (g) D28	S.D. (g) D35
Control	567	1669	1009	2555	249	217
0.1g/kg Signis®	750	1756	1181	2727	223	216
0.2g/kg Signis®	954	1774	1476	2641	192	128

Table 6.15 - Coefficient of variation percentages for each treatment for the dose comparison trial

Treatment	Coefficient of variation D28	Coefficient of variation D35
Control	20.6%	17.6%
0.1g/kg Signis®	16.9%	15.9%
0.2g/kg Signis®	13.8%	12.4%

6.3.10 Digesta Viscosity

There was no significant difference in the viscosity of the jejunal digesta on day 35 (p=0.103), though numerically, the viscosity reduced with Signis inclusion (table 6.15).

Table 6.16 - Digesta viscosity (cP) for dose response trial (±SE)

Treatment	Digesta Viscosity (cP)
Control	1.869 (0.1719)
0.1g/kg Signis®	1.537 (0.0979)
0.2g/kg Signis®	1.485 (0.1238)
P Value	0.103

6.3.11 Bursa Weight

There was no significant difference in bursa weights on day 35 between treatments (p=0.815; table 6.16)

Table 6.17 - Bursa weight D35 for dose response trial (±SE)

Treatment	Bursa Weight (g/kg bird wt)
Control	2.16 (0.111)
0.1g/kg Signis®	2.01 (0.131)
0.2g/kg Signis®	1.95 (0.130)
P Value	0.815

6.3.12 Caeca Weight

There was no significant difference in caeca weight between treatments on day 35 (p=0.496) (table 6.17).

Table 6.18 - Mean caeca weight per treatment on day 35 for dose response trial (per pair - g/kg bird wt; ±SE)

Treatment	Caeca weight (pair)
Control	12.40 (1.455)
0.1g/kg Signis®	12.21 (2.285)
0.2g/kg Signis®	10.97 (0.836)
P- Value	0.496

6.3.13 Texture Analysis

There was no significant difference between treatments in the force required to depress breast meat by 10mm when uncooked (p=0.682), though there was a numerical decrease with increasing SIgnis® dose. There was no significant difference between treatments in the force required to depress meat by 10mm when cooked (p=0.776) (table 6.18).

Table 6.19 - Texture analysis for breast meat for dose response trial (±SE)

Treatment	Force required to depress 10mm (N) Raw	Force required to depress 10mm (N) Cooked
Control	47.73 (11.246)	13.63 (4.080)
0.1g/kg Signis®	35.36 (5.501)	13.31 (3.446)
0.2g/kg Signis®	33.69 (7.061)	18.62 (5.103)
P Value	0.682	0.776

6.3.14 Drip Loss

There was no significant difference in breast meat drip loss percentage between treatments at either 48 hours or 120 hours post slaughter (p=0.811 and p=0.824) (table 6.19). There was no significant

difference in thigh meat drip loss percentage between treatments at either 48 hours or 5 days post slaughter (p=0.286, p=0.711).

Table 6.20 - Drip loss values between treatments on D35 for dose response trial (±SE)

Treatment	Breast drip loss 48 h (%)	Breast drip loss 120 h (%)	Thigh drip loss 48 h (%)	Thigh drip loss 120 h (%)
Control	0.14 (0.009)	3.35 (0.316)	0.10 (0.006)	0.19 (0.018)
0.1g/kg Signis®	0.14 (0.016)	3.36 (0.262)	0.13 (0.014)	0.23 (0.024)
0.2g/kg Signis®	0.14 (0.018)	3.59 (0.360)	0.11 (0.020)	0.22 (0.029)
P Value	0.811	0.824	0.286	0.711

6.3.15 Total Antioxidant Capacity

There was no significant difference in the total antioxidant capacity of the blood between treatments (table 6.21)

Table 6.21 - Total Antioxidant capacity for dose response trial

Treatment	Total antioxidant capacity (VEA µmol/L (±SE)
Control	755.71 (35.192)
Control + 0.1g/kg Signis®	799.87 (29.076)
Control + 0.2g/kg Signis®	748.89 (28.342)
P- Value	0.457

6.3.16 Day 35 Digestibility

There was a significant decrease in apparent metabolizable energy, apparent metabolizable energy corrected for nitrogen and nitrogen retention between the higher dose of Signis and the other two treatments (table 6.22). When AME is corrected for feed intake by dividing AME per kg feed intake, there is still a significant difference between each of the treatments (table 6.23).

Table 6.22 - Day 35 Digestibility for dose response trial

Treatment	AME (MJ/kg)	AMEn (MJ/kg)	Ni ret (g/kg)
Control	16.57 (0.500)[b]	15.73 (0.516)[b]	28.13 (0.165)[b]
0.1g/kg Signis®	15.19 (0.605)[b]	14.24 (0.599)[b]	27.57 (0.231)[b]
0.2g/kg Signis®	13.71 (0.413)[a]	12.95 (0.398)[a]	26.88 (0.241)[a]
P Value	0.001	0.001	0.002

[a-b] Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

6.3.17 Calcium and phosphorus digestibility

There were no significant differences in either the apparent ileal digestibility coefficient for calcium or phosphorus between treatments, or between the amount of calcium or phosphorus digested between treatments (table 6.23).

Table 6.23 - Calcium and Phosphorus digestibility for dose response trial (+/- S.E.)

	Apparent ileal digestibility coefficient		Amount of Mineral digested (g/)	
Treatment	Calcium	Phosphorus	Calcium	Phosphorus
Control	0.69(0.01)	0.79 (0.019)	0.04 (0.003)	0.06 (0.001)
0.1g/kg Signis®	0.69 (0.00)	0.78 (0.340)	0.04 (0.004)	0.06 (0.002)
0.2g/kg Signis®	0.68 (0.02)	0.75 (0.027)	0.04 (0.003)	0.06 (0.002)
P Value	0.948	0.274	0.571	0.386

6.3.18 Immunoglobulin Y

There was no significant difference in the quantity of immunoglobulin Y present in the blood between the dietary treatments (table 6.24). However, there was an apparently linear numerical decrease in IgY (including a decrease in standard error) in line with increase in Signis dose.

Table 6.24 - Levels of immunoglobulin Y present in the blood on day 35 (±SE)

Treatment	Immunoglobuline Y (ug/ml)
Control	1151.4 (197.26)
0.1g/kg Signis®	1113.6 (101.60)
0.2g/kg Signis®	953.1 (85.80)
P Value	0.553

6.4 Discussion

The main aim of this study was to determine whether Signis® (a combined corn XOS and xylanase supplement) was beneficial in supporting the performance of broilers and elucidating the mechanisms behind its action. A second aim was and to try and determine to investigate the effect of increasing the commercially recommended dose.

The performance data from this trial indicates that, from the parameters measured, there appears to be no deleterious effect of feeding Signis® at either 0.1g/kg or 0.2g/kg as mortality was not significantly different between treatments. As birds were fed identical basal diets, it can be assumed that any beneficial effects seen are a result of the Signis® supplement and not due to discrepancies in nutritional provision.

All cumulative measuring points up to day 28 show significantly increased BWG for the higher dose of Signis® compared to the other two treatments. From days 0-35 (the full trial length) both Signis® treatments show significantly increased BWG compared to the control.

Interestingly, there was no significant difference in cumulative feed intake between treatments until day 28. However, from day 0-28 and day 0-38 there was a significant increase in feed intake for both Signis® diets. This may be explained by the fact that the birds are over 15% (high dose) and 9% (low dose) heavier respectively and therefore need more energy to support their growth and maintenance (Kleyn, 2013). The higher dose of Signis® shows a significantly reduced FCR for days 0-7, 0-14, 0-21, 0-28 and 0-35 compared to both other treatments. In the first two weeks of the study the lower dose of Signis® and control treatments produced similar FCR results, however, from the third week onwards, the lower dose of Signis start to improve and the FCR decreases until the end of the study.

During the first week, the higher dose of Signis® produced a significant increase in body weight gain, average bird weight and significantly reduced FCR compared to the other two treatments. The first week BWG is increased by 12g (almost 15%) on average compared with the control treatment and increased by 9g compared to the lower dose of Signis®. This differs from the results of Craig *et al*.

(2019) who studied the effects of XOS and xylanase individually. These authors fed a wheat and soya-based diet with the addition of wheat bran (to increase AX levels) over 29 days. The study only found BWG increases at day 14 and 21 with xylanase and the inclusion of XOS showed no performance enhancing effects. It is possible that it is the combination of XOS and xylanase together that is required in order to produce a performance effect to the level seen in the current study. This may be due to the provision of xylanase stimulating hydrolysis of AX in the diet, in addition to the provision of XOS already in a fermentable form for the gut bacteria.

By day 14, birds fed the higher dose treatment gained significantly more weight than those fed the other two treatments and on average has gained 45g (nearly 20%) more than the control and 32g (6%) more than the lower dose Signis®. Likewise, average bird weight is significantly increased by approximately 15% and FCR is significantly reduced. There was no significant difference in feed intake at this point. Whilst these FCR results are still slightly higher than the Aviagen Ross 308 objectives, the birds in the current study were fed mash diets as opposed to pelleted diets used for the Aviagen objectives, possibly accounting for the difference. The FCR results for the current study are also considerably improved compared to the two previous animal studies in this project (table 6.24).

Table 6.25 - Comparison of Aviagen Ross 308 FCR objectives with the FCR of the 0.2g/kg Signis® treatment in the dose response trial

Age	Ross 308 objective for FCR	Current study FCR (0.02g/kg Signis®)
Day 7	0.821	2.08
Day 14	1.057	1.31
Day 21	1.201	1.52
Day 28	1.336	1.34
Day 35	1.473	1.55

On day 21, the higher dose treatment shows significantly increased BWG, higher average bird weight and significantly reduced FCR compared to both the other diets. By this point the high dose birds weigh approximately 20% more than the control and 10% more than the lower dose birds. Once again, there was no significant difference in feed intake between treatments. By day 28, both Signis® treatments show significantly increased BWG and average bird weight and significantly reduced FCR compared to the control. During the final week of the study, there was no significant difference in BWG, however, both Signis® treatments still show significantly increased average bird weight and feed intake compared to the control. There was no significant difference in FCR between treatments at day 35.

The results of this study seem to indicate that Signis® is not merely causing the birds to eat more feed in order to gain extra body weight. With the exception of the final weeks of the study, there is a significant improvements in performance for Signis® fed birds, but no difference in feed intake (Table 6.8, 6.9 and 6.10). This suggests that Signis® is causing the birds to convert feed into energy more efficiently than the control. Whilst we do see an increase in feed intake towards the end of the study, it is likely that by this stage the supplemented birds were so substantially bigger, that they actually required additional feed purely for maintainance.

Unfortunately, there was a campus wide power cut on day 28 of the trial which caused room temperature to fall to 10°C (figure 6.2) overnight and the birds were chilled and demonstrating huddling behaviours. Unfortunately, the next day, the environmental controls reset to the start of trial setting and the room heated overnight to over 30°C. The following morning the birds were heat stressed. Extremes of temperature have been reported to negatively affect bird performance (Olanrewaju *et al*., 2010). As far back as 1968, Deaton *et al.,* demonstrated a negative effect of temperature on broiler performance, reporting that 8-week-old broilers kept at average temperatures over 27°C showed significantly reduced growth compared to equivalent birds kept at average temperature below 27°C. Lott *et al.* (1998) looked the effect of temperature and drinker type

on broiler performance. This study showed that birds kept at temperatures over 30°C demonstrated significantly reduce body weight gain. These authors suggested that birds may be utilizing energy to reduce their core body temperature via cooling behaviours such as panting, thus reducing available energy for growth. Likewise, Abu-Dieyeh (2006) ran a study that kept half the birds at 35°C and half at 20°C and found that body weight gain, feed intake and feed conversion ratio were negatively impacted by keeping birds at 35°C.

Cold stress can have equally profound effects on performance. Ipek and Sahan (2006) report that birds exposed to cold stress (29°C at placement, 26.4°C week two then 21°C to 6 weeks of age) displayed significantly reduced bird weight, body weight gain and significantly increased FCR and feed intake. These authors suggest that energy is being expended to retain heat, thus reducing performance. Therefore, it is possible that the lack of significant BWG and FCR improvement between treatments recorded in the final week of the study could be due to the severe fluctuations in temperature deleteriously effecting feed intake and weight gain.

However, it is also possible that the lack of significant differences for the performance parameters in week 5 could be due to the supplement being more beneficial during the early stages of production. Rubio (2019) reports in a review paper that early stimulation of beneficial bacteria is vital as it profoundly affects the health and performance throughout the life of the bird. Kim *et al.* (2011) looked at supplementing broilers with fructo-oligosaccharides (FOS) and mannan-oligosaccharides (MOS) for 4 weeks. They found that 0.25 g/kg of FOS and MOS produced significant increases in BWG and numeric reductions in FCR compared to the control diet in the first two weeks of the study. However, by the third week and for the remainder of the study, there were no significant differences between treatments. These authors suggested that early benefits may have been due to stimulation and establishment of beneficial microbiota in the GIT. Contrary to this, Bautil *et al.* (2019) studied the ability of broilers to ferment AX in wheat-based diets. These authors found that although within the first two weeks of life broilers were able to partially ferment a small amount of the AX present, this

ability increased with age and age-related establishment of a more mature microbial community. This seems to suggest that the drop-in performance results during the last week of the current study, may be down to the temperature fluctuations rather than an early fermentation effect. Although this would need to be confirmed by further studies, it is possible that under different environmental circumstances that the longevity of the beneficial response may have continued.

The differences in uniformity of body weight seen between treatments is very interesting. Uniformity is the measure of the amount of body weight variation within a flock. At day 28 of the current study the standard deviation in mean body weight for the high dose of Signis® is nearly 60g lower (25%) than the control and the reduction shows a linear response in line with Signis® dose (figure 6.2). Likewise, at day 35, this result is more pronounced with the higher dose reducing the standard deviation by approximately 90g - (40%) lower than the control. This suggests that the inclusion of Signis® is having a profound positive effect on the uniformity of growth of the birds in line with dose rate.

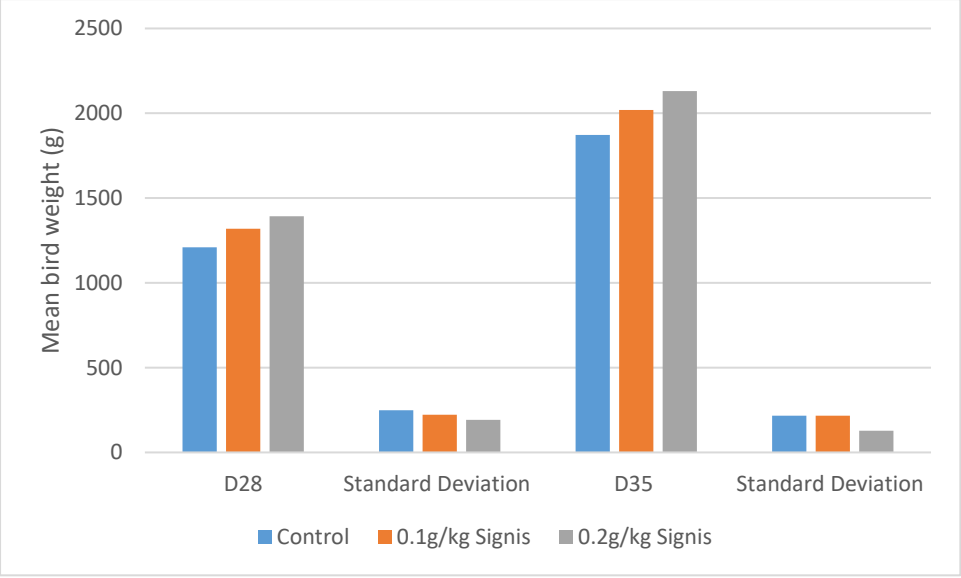

Figure 6.2 - Flock uniformity for Signis® dose response trial

Flock uniformity is vital for optimal production, and poor uniformity is correlated to delayed growth, increased rejects and down-grading at processing, and poor FCR results (Corzo *et al.,* 2004; Toudic, 2007). Vasdal *et al.* (2019) suggests that flock uniformity (measured as co-efficient of variability) can be a reliable indicator of animal welfare. In a study of 45 commercial flocks of Ross 308 broilers, the authors found that poor flock uniformity was highly associated with first week mortality, increased total mortality, increased FCR, reduced growth rates and increased rejection rate at slaughter.

Modern broiler processing requires uniform flocks with as little deviation as possible to conform to the distribution network. Uniformity is considered an important measure of performance as it allows optimization of feed and feeding programmes for a given population of broilers, allowing the profit margins to be enhanced (Berhe and Gous, 2008). If Signis® supplementation can improve uniformity profoundly, the benefits to the broiler industry, both in terms of animal welfare and reduced financial losses are likely to be substantial. This could also potentially improve the sustainability of the industry by reducing the number of birds lost to downgrading and rejections at the processing plant, consequently improving food security by reducing waste.

There was no significant difference in feed intake between treatments until day 28, which suggests that birds were not simply eating additional feed to gain more weight and that they were potentially more efficient than the control. The increased feed intake seen in the final two weeks was expected as the treatment birds weighed around 20% more than the control, so would require more feed for maintenance. The lack of difference in feed intake between treatments seen in the final week, is likely to be an artefact of the temperature fluctuations.

Whilst Signis supplementation indisputably improved growth performance in this study, identifying the mode/modes of actions is still difficult. Xylanase fed at the recommended inclusion rate (16000 BXU/kg) has been shown to improve performance by 2-4% (Enberg *et al.,* 2004; Owens *et al.*, 2008; Wu and Ravindran, 2004; Masey O'Neill *et al.,* 2012; Zhang *et al.,* 2014). However, previous xylanase results have been variable, with some studies showing no effect (Singh *et al.,* 2012; Azhar *et al.,*

2018). Possibly the most demonstrated effect of xylanase is a reduction in digesta viscosity from hydrolysis of the NSP fraction of the grain (Bedford, 2019). In the current study it appears that this may not be involved as there was no significant difference in digesta viscosity between treatments. The type of diet and indeed the cultivar of grain used in the ration has been shown to affect the efficacy of xylanase. Cardoso *et al*. (2018) report that exogenous xylanase only reduced digesta viscosity in wheat-based diets, if the wheat used had high levels of NSP and therefore, extract viscosity (>14.8cP). Similarly, it is reported that Azhar *et al.,* (2018) found that xylanase only improved AME in wheat cultivars that had a high level of NSP to act as a substrate for enzyme activity. As such, it supports the theory that the wheat included in the diets of the current study were likely to be very low in NSP content and that a viscosity effect does not seem to be the mechanism for improved bird performance.. The control diet measured 1.869cP which is low, but similar to some other studies. Wheat viscosity was measured in vitro by Ayers *et al.,* (2019), who report that their wheat sample measured 2.02cP following grinding and digestion simulation. Likewise, Saki and Alipana (2005) report measuring jejunal digesta viscosity ranging from 1.5-1.7cP with wheat-based diets. However, other authors have reported considerably higher viscosity. Cowieson *et al.,* 2002 report a wheat viscosity of 13.7cP following in vitro digestion simulation. Likewise, Bedford, 1995 found the viscosity of foregut digesta of broilers fed a wheat diet without NSPases to be in the region of 12cP. Dusel *et al.,* (1997) compared the viscosity of different wheat varieties and found that viscosity varied from 4-22cP in jejunal digesta. As these values are so much higher than found in the current study, it must be considered that the viscometer may have been incorrectly calibrated and potentially reading low. The varied results of these studies suggest a wide variation in the digesta viscosity of wheat-based diets.

It is also possible that the xylanase (whether supplied with the diet, or produced by endogenous bacteria) could have reduced a potential cell wall effect, allowing incapsulated nutrients to be released from within plant cells and consequently metabolized by the birds (as discussed in detail in chapter 3), or, a potential signalling effect may have occurred on this study. Bedford, (2018) reports that xylanase supplementation can stimulate the microbes in the gut in produce xylanase *in situ.* As

described in the previous chapters, a number of studies have demonstrated the ability of various microbial species found in the GIT to produce endogenous xylanase (Asem *et al.,* 2017; Bhardwaj *et al.,* 2019). Bedford, (2018) suggests that when saccharolytic microbes produce xylanase, the AX present in the diet is degraded, providing more fermentable substrate for the caeca. This in turn enables the production of fermentation products (VFAs) within the caeca (Annison *et al.,* 1968). VFAs subsequently enter the blood stream via active diffusion and provide energy sources for a number of bodily functions (Annison *et al.,* 1968; Svihus *et al*., 2013). VFAs produced in the chicken caeca include butyrate, which is the primary energy source of colonocytes and epithelial cells, consequently improving the integrity of the gut epithelium (Duncan *et al.,* 2004; Ravangard *et al.,* 2017; De Maesschalck *et al.,* 2015). Likewise, acetate has been shown to be the primary energy source for the liver. Ruminants obtain approximately 75% of their energy from VFA produced by bacteria in the rumen, which demonstrates the potential value of increasing VFA production in other species (Penn, 2014).

Energy harvested from sources other than the primary feed can produce an energy sparing effect, providing additional energy for growth and other essential functions (Penn, 2014). As such, if a signalling effect did occur in this study, it could account for the increased performance and particularly the improved feed conversion efficiency. This theory is supported by the work of Bautil *et al.* (2019), who performed a study looking at the ability of broilers to digest AX from wheat-based diets. The study collected digesta on days 5, 10, 15, 21, and 35 of age and analysed the digesta for AX content, AV digestibility and microbial xylanase production. This study showed that by 2 weeks of age, birds had some ability to solubilize AX and although xylose units were able to enter the caeca, only very limited quantities were fermented. However, by day 35 the birds were able to achieve a total tract digestion of 24%. Bedford, (2019) suggests that the presence of xylanase can stimulate further microbial production, thus increasing AX digestibility and subsequent fermentation, with the down-stream benefits of VFA production.

Increased hydrolysis of AX present in the diet is also another possible reason for the performance effects found in this trial. When xylanase hydrolyses AX, it decreases the degree of polymerisation, producing AXOS, xylobiose and xylose (Courtin *et al.,* 1999). Hydrolysis products can vary both by raw material (plant type) and depending on variety of xylanase used. Kiran *et al.* (2013) demonstrated this by *in vitro* hydrolysis of different waste biomasses using various types of xylanase. The results showed significantly different quantities of products with varying degrees of polymerisation. The degree of polymerisation has been shown to have a significant effect on whether the hydrolysis products can be fermented by microbiota in the gut. Several authors report that xylo-biose and xylo-triose are preferable as they can be readily fermented by pro-biotic microbiota, resulting in proliferation of these beneficial species and consequently improving gut health and maximising the nutritive value of the ration (Bailey *et al.* 1992; and Yamada *et al.,*1993) . Therefore, it is plausible that some of the performance effects seen on this study are a result of successful depolymerisation of AX in the ration, subsequently providing a nutrient source for beneficial bacteria. This project had intended to measure both VFAs and carry out 16s metagenomic profiling for this study, but unfortunately this was not possible in the timeframe of the project due to external influences which limited the project scope. It is important that these measures are completed in the future to further elucidate the performance enhancing mechanism seen in this study.

The linear increase in performance effect, seen in line with the increase in dose in the early weeks, suggest that the dose of supplement is significant, and that performance improves in line with dose until day 28. This is in agreement with Craig *et al*. (2019), who compared Ross 308 broilers supplemented with xylanase at different doses. The study ran for 29 days and compared a control diet to 16000BXU/kg xylanase and 32,000BXU/kg xylanase. The authors found significantly reduced FCR at day 14 and 29 for the high dose of xylanase compared to the control and low dose of xylanase. However, other studies have found contrasting results. Kolln *et al.* (2017) investigated the effect of xylanase at different inclusion rates in broilers. They used doses varying from 0-250g/t in a wheat and soya diet. Whilst these authors did find a significant decrease in digesta viscosity at the highest dose

of xylanase, they did not observe performance effects at any inclusion rate. Yan *et al.* (2016) investigated powdered xylanase at 250BXU/kg and 500BXU/kg and liquid xylanase supplemented at 250BXU/kg and 500BXU/kg in a wheat-based diet. Whilst these authors did find increased body weight at day 21 for all xylanase treatments, the dose did not alter the response. Likewise, Luo *et al.,* 2009) supplemented broilers with doses of 0, 500, 1000 and 5000U/kg of xylanase, fed in a wheat-based diet. These authors found a significant improvement in FCR for all doses of xylanase compared to the control, but no differences between doses. They also found that the 500U/kg and 1000U/kg doses increased Villus height and crypt depth in the small intestine. A dose of 5000U/kg significantly increased the height of villi in the ileum; however, this dose also showed a tendency to induce proliferation of *E. coli* and total aerobes. The authors conclude that supplementing boilers with xylanase at 500U/kg and 1000U/kg is beneficial for broilers, but excess xylanase does not produce further improvements and may have negative effects on microbiota.

Dose responses to xylanase can seem to be highly variable, and in future it would be interesting to add more treatments to investigate the effects of xylanase at both high and low doses without XOS to allow the beneficial mechanisms to be further clarified, as currently it is impossible to discern the effect of xylanase, from XOS and XOS + xylanase together. Other studies have demonstrated varying performance effects with XOS supplementation. De Maesschalck *et al.* (2015) investigated the effect of a corn derived XOS without the addition of xylanase. This study used Ross 308 broilers fed a wheat and rye-based diets over 39 days. Study results showed significantly improved FCR, increased villus height in the ileum and the number of gene copies encoding the key bacterial enzyme for butyrate production were significantly increased in the caeca. A further study looked at supplementing Arbor Acres broilers with either XOS or flavomycin (an antibiotic complex) (Yuan *et al.* 2018). The authors found that XOS significantly improved performance compared to the control and produced performance results comparable to the flavomycin. XOS supplementation also significantly increased production of acetate and butyrate in the caeca. Likewise, Vasudevan *et al.,* (2019) compared the effect of XOS to a control. The study used Cobb 400 broilers fed a maize and soya-based diet and

compared doses of 0.2, 0.5, 0.75 and 1.00% XOS. The author found significantly increased body weight for 0.5 and 0.75% doses of XOS compared to the other doses and the control at both 35 and 42 days of age. Interestingly, this study found similar results to the current study in that during the first 3 weeks body weights increased in line with the inclusion of XOS, but the lower dose made considerable gains by week 4 and 5 nearing the body weights for the higher dose. This suggests that increased levels of XOS in the first 3-4 weeks of production improves BWG, however by the end of production, lower doses of XOS achieve the same benefit. As such it may be financially beneficial to feed the lower dose rate in practice to reduce supplement costs for the same ultimate effect.

In addition to performance, this study looked at several anatomical and metabolic parameters to try and elucidate any mechanisms of Signis® action. The bursa of Fabricius is a lymphoid organ found in birds that plays a primordial role in poultry immunity. A number of papers have suggested that the bursa weight reflects the hosts ability to provide lymphoid cells during an immune response and that it increases in line with response to a specific immune challenge (Heckert *et al.,* 2002; Al-Mayah *et al.,* 2010). McMullin (2004) suggests that a typical bursa should weigh approximately 0.2- 0.3% of the bird's total body weight, which agrees with the measures taken in the current study. Bursas weighing less than 0.1% of total bodyweight are thought to be indicative of infectious bursa disease, a highly contagious immunosuppressive disease (Kasanga *et al.,* 2008; Al-Mayah *et al.,*2010). Rathgeber *et al.,* (2008) looked at the effect of feeding beta-glucan and virginiamycin (antimicrobial) on bursa weight. Their results were in a similar weight range to the current study but showed that bursa was significantly smaller in birds fed an antibiotic. This supports the theory that bursa size increases with exposure to pathogenic challenge, as the birds fed a prophylactic antibiotic were given significant protection from infection. The results of the current study seem to suggest that the bursas were within normal weight range, but that the birds were not subject to an immune challenge. Due to legislation and restrictions surrounding animal experiments, the trial facility used for the current study is kept clean, reducing the potential for pathogen exposure.

The IgY results are in the same range as other broiler studies (Kulkarni et al., 2007; Agrawal *et al.*, 2016). As with the bursa weight, the lack of significant differences seen in IgY levels between dietary treatments suggests that the birds are not under immunological stress. Kulkarni *et al.* (2007) performed a study looking at IgY antibody titres in response to vaccination for necrotic enteritis caused by *C.Perfringens* . That study showed that IgY levels increased in line with pathogenic exposure to mount a protective response. The results of the current study agree with a number of other studies where livestock were supplemented with either pre or pro-biotics (Franklin *et al.*, 2002; Huang *et al.,* 2004; Midilli *et al.*, 2008) who also found no significant differences in immunoglobulin levels.

When bursa weight is correlated to Immunoglobulin Y (IgY) via Pearson correlation, there is no positive correlation (r=0.083) (figure 6.5).

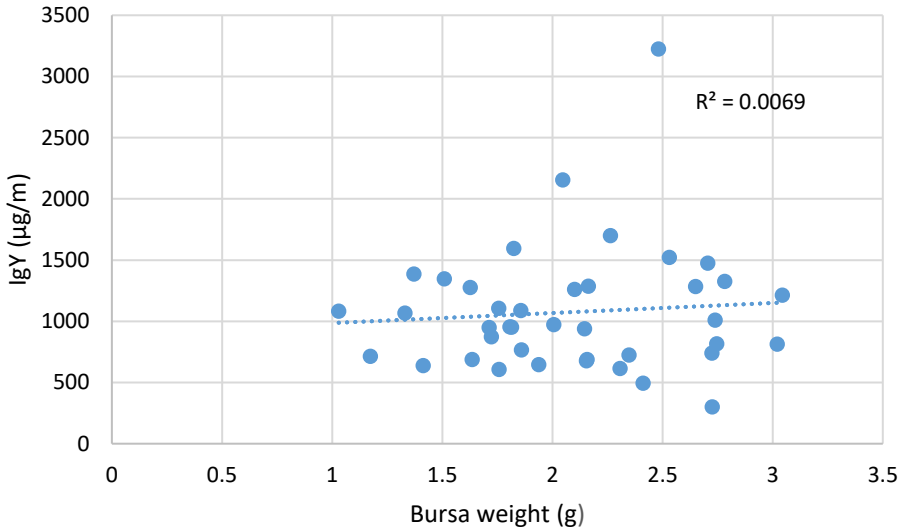

Figure 6.3- Correlation between bursa weight (g/kg of bird weight) to immunoglobulin Y for XOS dose response trail

The results for apparent metabolizable energy (AME), apparent metabolizable energy corrected for nitrogen (AMEn) and nitrogen retention appears to suggest that increasing SIgnis has a detrimental effect on these measures. However, it may be that AME is reduced due to increased feed intake. There was no significant difference in weekly feed intake between the treatments with the exception

of the final week, when both Signis® treatments show significantly increased feed intake. Increased feed intake has been associated with increased intestinal transit time (Watson *et al.,* 2006). This in turn may reduce the amount of time that the digesta is available in the GIT for uptake of nutrients, thus reducing AME and also having a similar detrimental effect on AMEn and Ni retention. The level of titanium (the inert marker) recovered from the ileal digesta was lower than expected in the 2g/kg SIgnis treatment, which may suggest that there was increased density of feed material at the end of the ileum, consequently diluting the marker. This may in turn suggest reduced digestion or increased feed passage, leading to reduction in nutrient disappearance. The high temperatures experienced during the final week are likely to have increased water intake. Increased water intake has also been shown to increase tract transit time (Johnson, 2019) which may have exacerbated this effect. As there was no difference in analysed gross energy between diets, it seems unlikely that the birds fed 0.2g/kg of Signis® would grow significantly better than the other treatments, and yet report significantly reduced levels of AME and AMEn. Titanium oxide (Ti) was used as an inert digestibility marker in all treatments. As the AME, AMEn and NI all reduce in a linear fashion in line with Signis®, it may be possible that there is some type of detrimental interaction between the Ti and the Signis®. If the Ti somehow binds to the Signis®, it could potentially be producing an artificial reduction in these parameters.

Breast meat texture analysis, drip loss and the peroxynitrite TAC (total antioxidant capacity) assays were performed to explore the effect of Signis® on oxidative stress measures, particularly in respect to the muscle meat myopathies such as white stripe and woody breast. Chapter 5 had demonstrated that the corn XOS included in Signis® had substantial antioxidant properties, so it was hoped differences between treatments would be measured in these assays. It is possible that the birds from the current trial were not mature enough to show muscle effects. Woody breast is particularly prevalent in larger birds (Lorenzi *et al.,* 2014). Likewise, Bauermeister *et al.,* (2009) report that a higher incidence in muscle meat myopathies in broilers processed at 8 weeks of age compared to 6 weeks of age. As the birds in the current study were only 5 weeks old, it is possible that had the study

continued, we may have seen increased incidence of muscle myopathies. There were no significant differences between treatments in the total antioxidant capacity either, however, the peroxynitrite assay results agreed with the results of the previous trial, with no differences being seen between dietary treatments. This may suggest that the birds were not experiencing sufficient levels of oxidative stress to be detected, or that there was no antioxidant effect of the supplement.

The TAC for peroxynitrite assay had been chosen as it was a precursor to lipid peroxidation (Knight, 2002), which is thought to be a contributory factor in the muscle meat myopathies. However, the antioxidant value of the corn XOS was measured for its capacity to quench singlet and halogenated oxygen species. It is therefore possible that the XOS may not be a good antioxidant against peroxynitrite radicals, and therefore no differences in TAC scores were found.

There were no significant differences between treatments for drip loss percentage after either 24 or 120 hours. After 24 hours breast meat drip loss was 0.14% and after 120 hours was around 3.5% for all dietary treatments. Drip loss is essentially a measurement of the water loss of meat over time in storage. It is commercially important as the visual appearance of meat seeping is undesirable to consumers and implies an inferior product (Warner, 2017).

Suo *et al.,* 2015 looked at the effect of Longlive XOS on Arbor Acre broilers fed a corn-soya diet over 42 days. Whilst they didn't find any performance or breast meat drip loss effect from supplementing XOS at 25, 50, 75 and 100 mg/kg, they did report a linear reduction in thigh drip loss percentage in line with quantity of XOS inclusion after 24 hours. In the current study there was a small linear increase in drip loss percentage in line with Signis® dose for the breast meat at 5 days. However, this result was not statistically significant and drip loss was identical for breast meat at 24 hours. Likewise, there was no significant difference in thigh meat drip loss at either time point, so it seems unlikely that there is cause for concern. Once again, the study by Suo *et al.*, (2015) was 42 days long. As previously mentioned, muscle meat myopathies have been reported to increase with age and size of

the birds (Bauermeister *et al.,* 2009; Lorenzi *et al.,* 2014). Therefore, it is possible that had the current trial ran for an extra week, more differences may have been detected.

The percentage of drip loss found after 24 hours on this trial are similar to other studies in the literature. Albrecht *et al*., (2019), investigated the effect of supplementing Ross 308 broilers fed a corn, soy, and pea-based diet with different sources and concentrations of methionine. This study showed drip loss percentage after 24 hours to range from 0.175% to 0.34%. A further study looked at the effect of combined BHT (200ppm /0.02%) and vitamin E (500Iu) compared to a diet containing oxidised oil and a control (Zhang *et al*., 2011). This study showed the breast meat drip loss ranged from 0.42% for the control to 0.6% for the oxidised oil at 24 hours. As the results of the current study fall on the low side of this range, it may suggest that the birds were not experiencing high levels of oxidative stress, consequently accounting for the lack of differences observed in all the oxidation measures explored in this study.

6.5 Conclusion

The results of this study clearly show enhanced bird performance with Signis® supplementation, with birds fed a dose of 0.2g/kg weighing approximately 20% more than the control and 5% more than the lower dose (0.1g/kg). Whilst there were no significant differences in BWG and BW between the two Signis® treatments by the end of the trial, the higher dose does show significantly reduced cumulative FCR (1.55) compared to both the other treatments. This reduction in FCR as well as the substantial improvement in bird uniformity seen are likely to represent substantial financial gains on a commercial scale, indicating the potential for use of Signis® at 0.2g/kg compared to the 0.1g/kg currently recommended dose.

The significant reduction in AME, AMEn and nitrogen retention, need further investigation, as although these results may be explained to an extent by the increased feed intake seen with the higher dose of Signis®, the negative implications of increased nitrogen excretion and subsequent environmental effects must be explored.

Due to external influences preventing the VFA and 16s metagenomic analysis, the mechanism behind the performance enhancement observed in this study is still unclear, however the results of this study seem to suggest that performance enhancement is not purely due to a reduction in digesta viscosity. Likewise, the lack of significant differences in calcium and phosphorus digestibility may suggest that an increase in the availability of encapsulated nutrients is also not entirely responsible for performance effects observed. Therefore, investigating a potential microbial effect is indicated in future work.

Chapter 7: Conclusion, and recommendations

7.1 Introduction

This chapter has been split into four sections to discuss the potential benefits of AX, xylanase and xylo-oligosaccharides as supplements to support the performance and physiological function of broiler chickens. Initially, the results of the studies undertaken as part of this project will be discussed alongside their key findings. Secondly, the impacts of these conclusions on global poultry production and food security will be considered. Subsequently, key areas for future research and development are outlined and finally, key recommendations based on this work will be provided.

Global population growth and its impact on food insecurity places intense pressure on the poultry industry to optimize production (Shahbandeh, 2019). The 2006 EU ban on the use of non-therapeutic antimicrobials due to concerns over antibiotic resistance has increased the challenge of producing plentiful chicken meat, while supporting health and boosting bird performance (Hajati and Rezaei, 2010). This has led to increased research into alternative methods of supporting bird performance and health, including understanding that managing the microbiome could have beneficial effects. In the absence of antibiotics, producers must try and minimise the possibility of pathogenic infection by optimising the hosts microbiome. Likewise, there is not a universal solution of an ideal microbiome as this will vary depending on the environmental conditions in which a bird is raised (Bedford, 2020).

This project began in response to a theory that exogenous xylanase may have some pre-biotic mechanisms due to hydrolysis of arabinoxylans (AX) present in feed materials. This would produce to xylo-oligosaccharides (XOS) that could be used as a potential nutrient source for beneficial bacteria in the gastro-intestinal tract (Bedford, 2019). Prebiotic, probiotic and symbiotic supplements have all demonstrated the ability to provide a beneficial effect both in humans and animals. One of the primary benefits of these supplements is the capacity to modulate gut microbiota. In broiler chickens, proliferation of bacteria that can ferment non-digestible carbohydrates from the diet can be highly

beneficial for growth performance and bird health. Fermentation products of these bacteria (VFA) can directly control pathogenic growth due to the reduced pH resulting from increased production of VFAs. However, almost more importantly, some VFAs can have antimicrobial and/or anti-inflammatory properties or be used as energy sources within the body. As such, proliferation of these bacteria can maximise the nutritional value of the diet, gaining nutritional benefit from feed fractions that the animal would otherwise be unable to utilize. This not only reduces waste in terms of excreted nutrients, but also allows low value waste and by-products to be profitably utilized by the industry, thus increasing sustainability. The hypothesis that exogenous xylanase was reducing the degree of polymerisation of AX into AXOS and XOS led to the investigation of whether the direct provision of an AX extract or XOS supplement could enhance this potential pre-biotic effect.

Through the course of this project the industry began to focus on the issue of poultry muscle meat myopathies such as woody breast and spaghetti meat. Preliminary work indicated that lipid peroxidation and oxidative stress could be involved in the aetiology of these syndromes (Petracci *et al.,* 2019). The genetic selection for growth performance and efficiency has led to birds that have improved feed conversion efficiency, reach slaughter weight at an earlier age and are subject to more intensive production processes than their counterparts from as little as 50 years ago (Bradshaw *et al.,* 2002; Julian, 1998; Havenstein *et al.,* 2003). This trajectory is predicted to continue with some experts predicting that broilers will be reaching slaughter weight by 29 days of age by the year 2034 (Tavarez and Solis, 2016). This all raises the likelihood of broilers to be subjected to oxidative stress (Fellenburg and Speisky, 2006), increasing the possibility of these muscle meat myopathies. As there was evidence that oligosaccharides could have antioxidative properties, this project developed to included looking at some measures of oxidative stress. Therefore, the overarching objectives of this project were:

1. To determine whether AX, XOS and/or xylanase can effectively support the performance of broilers

2. Investigation of the effect of AX, XOS and/or xylanase on various anatomical or physiological parameters of broilers
3. To validate and assess a novel assay for measuring oxidative stress in broilers and to investigate the potential antioxidant properties of AX, XOS and/or xylanase
4. To gather evidence to further elucidate the mode(s) of action of AX, XOS and/or xylanase

7.2 Conclusions and Critiques

7.2.1 Effect of AX, XOS and/or xylanase on broiler performance

The results of the studies undertaken as part of this thesis show that AX, XOS and/or xylanase can all have beneficial effects on the performance of broilers. However, there was some variation within the results. The first animal study looked at the effect of a novel DDGS derived AX extract (chapter 3). The results showed that the AX extract treatment consistently numerically improved body weight gain compared to the control and a pre-treated AX treatment. Body weight gain was significantly improved for the AX during days 21-28 of the study. At the end of the study, the AX fed birds weighed on average 100g more than birds fed either of the other treatments.

Pre-treating the AX extract with xylanase seemed to negate any of the performance benefits seen with AX supplementation alone. It is unclear whether the benefits seen for AX supplementation are negated by the addition of xylanase or by the pre-treatment process. It may be that the liquid mixing and homogenising of this treatment may have caused problems when combining into the basal diet. Whether this issue was particle size related or due to over/under hydrolysation of the AX extract is still unclear. This study had to discount one treatment due to incorrect diet formulation. This treatment was the AX extract with xylanase added directly to the diet (rather than pre-treated). Had this data been comparable, it would have clarified the effect of XOS + xylanase and determined whether the lack of performance response for the AX pre-treated with xylanase was due to the process of pre-treating, or the synergistic relationship between the AX extract and xylanase. It was

originally thought that a negative control was not necessary, as the effects of xylanase in a wheat/soy diet was well established. However, with hindsight, this study would have been more robust with the addition of a negative control treatment (control + xylanase). This would have allowed the effect of the xylanase to be assessed in direct comparison to the other treatments and would have added evidence to clarify why the AX pre-treated with xylanase did not perform as well as the AX alone.

The second study compared two different novel XOS supplements derived from different base materials (corn and sugar cane bagasse), both fed with and without the addition of exogenous xylanase (chapter 4). This study showed very little difference in performance effects between treatments, which was not expected as it was felt that directly supplying the shorter chain polysaccharides as a XOS product would have enhanced the beneficial effects seen with AX supplementation in the previous study. There could be several reasons for this. It is possible that the hydrolysis products of the xylanase activity were either too large or too small to be fermentable by gut bacteria. Likewise, the cereals used in the ration may have been high in xylanase inhibitors, preventing enzyme activity. Finally, there is the possibility that the birds were sub-clinically pathogenically challenged, resulting in poor performance.

The final animal study (chapter 6) explored the response to different doses of a commercial corn based XOS combined with xylanase (commercially available as Signis®). Cumulatively, this study showed significantly increased body weight gain for both doses of Signis® (1 and 2g/kg) compared to the control, with the higher dose birds weighing over 20% more than the control and 5% more than the lower dose. Cumulatively, the FCR was significantly reduced for birds fed 0.2g/kg Signis® compared to the other two treatments. This study also revealed that Signis® inclusion was significantly beneficial in reducing flock variability, particularly in the final week of the study, where the standard deviation of bodyweight is almost halved in the higher dose treatment.

In conclusion, the quantitative analysis of these studies shows that AX extract and Signis® (XOS + xyl) are both effective at improving broiler performance to varying degrees. There are however factors

that may have prevented optimal information gathering in these studies, including incorrect diet manufacture and possible homogenization issues when attempting to pre-treat XOS with xylanase.

7.2.2 Effect of AX, XOS and/or xylanase on gut health, immune, or physiological parameters of broilers

The initial study (chapter 3) showed very little effect on any of the physiological parameters examined. However, in the second study, where very little performance effect was observed, there were physiological differences. Xylanase inclusion increased tibial bone breaking strength at day 21 and calcium digestibility at day 42, though neither of these measures were assessed in the first study. Improved calcium digestibility and bone mineralisation have both been reported when supplementing xylanase or XOS and is generally attributed to a reduction in cage effect, increasing the availability of encapsulated nutrients (Nortey *et al.,*2008; Li *et al.,* 2016; Muszynski, 2019). Interestingly, the final study in this project (chapter 6) did not find any significant difference in calcium digestibility at day 35. As the final study included a treatment with double the dose of xylanase used in chapter 4, a calcium digestibility response may have been expected. Therefore, the mechanism for improved digestibility may be multifactorial and not as simple as a reduction in cage effect alone.

The final animal study (chapter 6) revealed reduced AME, AMEn and nitrogen retention for the higher dose of Signis®. As a reduction in nitrogen retention was also seen in chapter 3 when birds were supplemented with an AX extract, there is a strong indication that this area requires further investigation. Whilst to an extent these results may be explained by the increased feed intake observed in both these treatments, consequently increasing gut throughput and reducing the time available for digestion. If AX or XOS are indeed genuinely increasing nitrogen excretion, there are environmental concerns regarding nitrogenous waste and pollution. Unfortunately, the bone parameters measured in chapter 4 were not analysed until after the final study was completed, otherwise they would have been included in the analysis for chapter 6.

Throughout this project there has been a possibility that the experimental facility in which these studies were conducted may have precluded an immune or gut health response to the various treatments. The enhanced biosecurity and husbandry are one of the accepted shortcomings of using an experimental setting compared to a commercial facility. Although this enables precise measurements to be obtained, ensures optimal welfare and allows preliminary work to be performed inexpensively, there are likely to be substantial differences between experiments this setting compared with a commercial barn. The sheer quantity of birds raised in a single commercial facility provide huge statistical power that can enable small differences to be detected. However, a commercial setting can also increase the exposure to disease challenge, as there may be a risk of cross contamination between flocks and poor biosecurity between houses can promote the spread of pathogenic disease. Likewise, the higher stocking density, reduced access to food and water and proximity in which commercial birds are kept can all increase the incidence of physiological stress and disease. Therefore, the environmental conditions of a study and the particular effects being investigated should be considered, both when undertaking an animal study, or indeed, extrapolating results.

7.2.3 Effect of XOS on the microbiome of broilers

In chapter 4 it was shown that the microbial community of the gastrointestinal tract changes significantly with age, but no differences were found in microbial diversity between dietary treatments at either day 7 or 42. As such a profound performance effect was seen in chapter 6, VFA and 16S analysis may have contributed to explaining the mechanism behind the performance effect. Unfortunately, this was outside the scope of this project, however, biological samples were collected for this purpose in a future project, which may fully elucidate the mode of action of XOS.

7.2.4 Validation and assessment of novel oxidative assays

The pilot studies for the novel oxidative assays investigated in chapter 5 revealed a number of preliminary recommendations. The results suggest that running the TAC for peroxynitrite assay on broiler serum, rather than plasma collected with either EDTA or heparin, allows higher antioxidant capacity to be detected from the sample. It also showed that the assay should be run using fresh blood rather than blood stored at -20°C. When looking at the euthanasia method, the results were less clear. The birds euthanised via cervical dislocation showed greater capacity to scavenge peroxynitrite radicals compared to those dispatched via captive bolt. However, it is still unclear whether either of these results equate to the levels of antioxidative capacity experienced during life.

When examining the antioxidant capacity of feed and feed ingredients, the results showed that the broiler diets used in chapter 4, demonstrated very limited antioxidant capacity, however, they were not prooxidant and therefore were not increasing potential oxidative stress. The various commercial XOS products investigated all showed some ability to quench singlet and halogenated oxygen species. The corn XOS used in chapter 4 and later included in the Signis® product showed moderate antioxidant ability to quench singlet and halogenated oxidative species. Again, none of the XOS products showed pro-oxidative properties for the species of reactive oxygen investigated.

Considerably more work is required to fully determine the optimal methodologies required for these assays and to clarify the usefulness of these methods in broiler production, and whether they correlate to health of the birds and meat quality

7.2.5 Investigating the mode of action for AX, XOS and/or xylanase

The possible modes of action for xylanase have been suggested to include:

9. Viscosity effect – xylanase reduces the viscosity of digesta (Gonzalez-Ortiz, 2016)
10. Cage effect (cell wall mechanism) – xylanase degrades the cell wall of cereal grains releasing encapsulated nutrients. This increases the diffusion of nutrients, substrates and enzymes enabling the host better utilize nutrients in the ration (Bedford and Autio, 1996)
11. Pre-biotic effect – xylanase degrades AX present in the diet into oligosaccharides that can be used a nutrient source by saccharolytic microbes in the gut (Bedford, 2018; Craig, 2019)
12. Signalling effect – xylanase stimulates microbes in the gut to produce xylanase *in situ* (Bedford, 2018).

Whilst this project has not further elucidated the mode of action for AX, XOS and/or xylanase, the studies have added weight to various theories surrounding their mechanisms. The initial study showed a beneficial performance effect of feeding AX without xylanase, particularly in terms of body weight gain. This may suggest that the birds develop the ability to ferment xylan with age, which supports the findings of Bautil *et al*., (2019) who demonstrated that broilers develop the ability to ferment AX with age and that by day 35, broilers could ferment up to 24% of xylan in the diet. This study provides evidence that whilst broilers may not be able to produce endogenous xylanase themselves, microbes residing within the GIT may well be able to.

Whilst the second study (chapter4) did not reveal any performance effect of XOS and/or xylanase, it showed that xylanase inclusion increased the mineral digestibility and bone strength, which may indicate that xylanase is reducing the cell wall effect and releasing encapsulated nutrients.

Chapter 6 revealed the most substantial performance effect with a combined XOS and xylanase inclusion. The lack of effect on digesta viscosity suggests that other mechanisms may be responsible

for this effect. Likewise, no significant differences were found for calcium or phosphorus digestibility, which suggest that a potential cell wall effect may not be entirely responsible for the enhanced performance. This indicates the mechanism may involve either a potential prebiotic or signalling effect. Therefore, it was unfortunate that the VFA and 16S analysis were outside the scope of this project as they may have been able to elucidate this effect.

7.3 Future work

This project has highlighted the need for future work. All of these studies could be upscaled to a more commercial setting as this is more representative of industry and birds are likely to be exposed to increased physiological and pathogenic challenges. This may provide further confidence in the results and ensure that results are repeatable in a non-research setting. However, there is also substantial potential for future work in both commercial and experimental settings, expanding on the current findings, the key areas of which are explained below.

7.3.1 Performance effects of arabinoxylans, xylanase and/or xylo-oligosaccharides

When looking at the performance effects of these studies, separating the performance effects of AX, XOS and xylanase both independently and in combination is the next logical step. Repeating the initial study from chapter 3 with the addition of an AX treatment where xylanase is included in the diet and with a negative control would provide more information in regard to the mechanisms of action. It would also be useful to include some of the other measures in this trial, such as digesta viscosity, VFA and microflora analysis.

Although the industrial partner for this project purchased the corn XOS from chapter 4 for use in Signis®, the AX extract used in the initial study could still merit further investigation. The reduced processing required to manufacture an AX extract verses a XOS product (no enzyme extraction required), may possibly reduce the production costs making a low cost AX supplement viable –

particularly as the AX is derived from a low value by-product so could have a potential sustainability angle for bioethanol or sugarcane production.

It would be necessary to repeat the dose response study from chapter 6 due to the environmental issues experienced and extend it to 42 days or beyond to large bird size (usually 49 days). From the results of the current study it looks as if the lower dose of Signis® begins to show similar growth benefits to the higher dose, which may be an artefact from the conditions, so extending this study may allow any effects to be clarified. The flock variability measures were undertaken in response to a visual difference between pens. Although the process was labour intensive and time consuming, it would be interesting to see whether these beneficial results are repeatable on a commercial scale. Likewise, it would be interesting to look at individual bird weights over an entire study. Following this study, it has become routine practice to individually weigh birds at NTU and exploring the degree of variation over all age ranges would provide insight into the point at which Signis® starts to reduce flock variability.

7.3.2 Other effects of arabinoxylans, xylanase and/or xylo-oligosaccharides

It was unfortunate that the scope of the project meant that two of the animal studies (ones which showed significant performance results) were unable to look at gut microbial effects or VFA production. Performing 16S metagenomic profiling on a repeat of the AX extract study and Signis ® study would provide information on whether a microbial effect was the mechanism for improved performance. Likewise, VFA analysis of the caeca contents would allow the fermentation products to be quantified and further elucidate the mechanism(s) behind XOS and/or xylanase supplementation.

As previously mentioned, the lack of immune response seen during this study, may be due to a lack of physiological or pathogenic challenge. Testing these supplements during exposure to pathogenic challenge may elucidate any effect on the immune response.

In the second animal study, there was a significant increase in tibial breaking strength found at day 21 and significantly increased calcium digestibility found at day 42. Measuring the calcium digestibility at

day 21 in future studies would be informative to look at whether increased calcium digestibility may play a role in the mechanism for improving tibial breaking strength. Likewise, upscaling sampling in terms of tibia collection from trials would improve statistical power and potentially reveal further effects.

In future work it would be enlightening to look at the arabinoxylan content, hydrolysis, and fermentation within the GIT of the AX extract used in chapter 3. Bautil *et al.* (2019) measured the total and water-unextractable AX content of feed and digesta by gas chromatography in a method described by Olaerts *et al*. (2016). This allowed the authors to measure hydrolysis and fermentability of AX at various points in the GIT and may elucidate the mechanism of breakdown of the AX extract.

Outside the poultry sector, it is possible that XOS may have applications for human or other species. Other oligosaccharides have been shown to have pre-biotic properties in humans and pigs (Tian *et al.,* 2018; Yang *et al.,* 2020), so it is possible that XOS may have beneficial effects on the microbiome in other species. In the current global situation of food insecurity maximising the nutritive value of food is becoming more important. Whilst hypothetical, if XOS products can improve the nutritive value of plant-based feed for monogastrics such as chickens and pigs, it may do the same for humans. With more of the population moving over to plant-based diets for, optimizing the nutritional value of these diets will become increasingly important. XOS has been shown to proliferate *bifidobacteria* in the human gut by as much as 8-18% after two weeks, improving gut motility and immune function (Flavia *et al.,* 2013). Proliferation of beneficial bacteria can aid recovery from gastrointestinal dysbiosis, particularly following antibiotic treatment. Likewise, galacto-oligosaccharides have been used as a pre-biotic in human infant formula to improve gut health and reduce diarrhoea and infant colic (Vandenplas, 2003). Therefore, XOS has potential cross-over value in both human, livestock and pet markets which could be explored in further work.

Oligosaccharides have been used as artificial sweeteners and sugar replacers. XOS have a sweetness level around 50% of sucrose (Belorkar and Gupta, 2016), however, only a very small proportion of XOS

is digested, so is much less calorific. As such, there may be potential for XOS to be used in diabetic and low-calorie foods as an alternative sweetener.

The increased bone strength and calcium digestibility seen in chapter 4 has potential in a number of species. If the mechanism of how these are being improved can be fully understood, this could have far reaching benefits in supporting bone health. Both meat and egg laying chickens are prone to skeletal issues; whilst broilers are prone to leg abnormalities, laying strains suffer from osteomalacia and osteoporosis. Human and animal athletes such as horses and agility dogs could also benefit from improved mineral digestibility and bone strength. The incidence of microfractures from heavy exercise strain, and recovery from bone injuries are important areas which could be considered in the future. Likewise, these ingredients may aid post-menopausal women suffering from osteoporosis. Osteoporotic fractures cost the NHS over £2.1 billion per year - a figure that is likely to increase without an effective preventative intervention (Burge *et al*., 2001).

7.3.3 Further oxidative stress work

Further work is required in validating the novel antioxidant assays used in chapter 5. The assays need to be tested on birds that are habituated to blood samples to try and gain an accurate baseline for broilers. Once this is complete, capture, restraint and euthanasia method can be quantified with more precision. The blood storage also needs further investigation. Storing blood at -80°C is recommended for any work looking at DNA/RNA, and therefore a lower temperature may be more suitable for storage of blood samples for these assays. It is clear from the initial validation that the sample tubes effect the results in these assays and care needs to be taken to be consistent in sample collection going forward.

7.4 Contribution to knowledge

The results of this project contribute to current knowledge in a number of ways. The results of the initial AX study demonstrate that broilers can gain beneficial effects from AX supplementation, which adds weight to the suggestions that broilers can ferment AX and whilst broilers may not be able to produce endogenous xylanase, the microbiota within the gut may be able to.

XOS combined with xylanase (Signis®) is a new product to market and this project has shown that it can significantly enhance broiler performance, improving body weight gain, average bird weight and feed conversion ratios. Whilst a dose of 0.1g/kg does significantly improve the later performance, a dose of 0.2g/kg is indicated to be optimal for optimal final body weight and feed conversion ratio. Likewise, a dose of 0.2g/kg substantially increases the uniformity of a flock compared to a dose of 0.1g/kg or a control. The performance effects observed in the final study may not be due to decreased digesta viscosity or a cell wall effect and as such a potential prebiotic or signalling effect is indicated.

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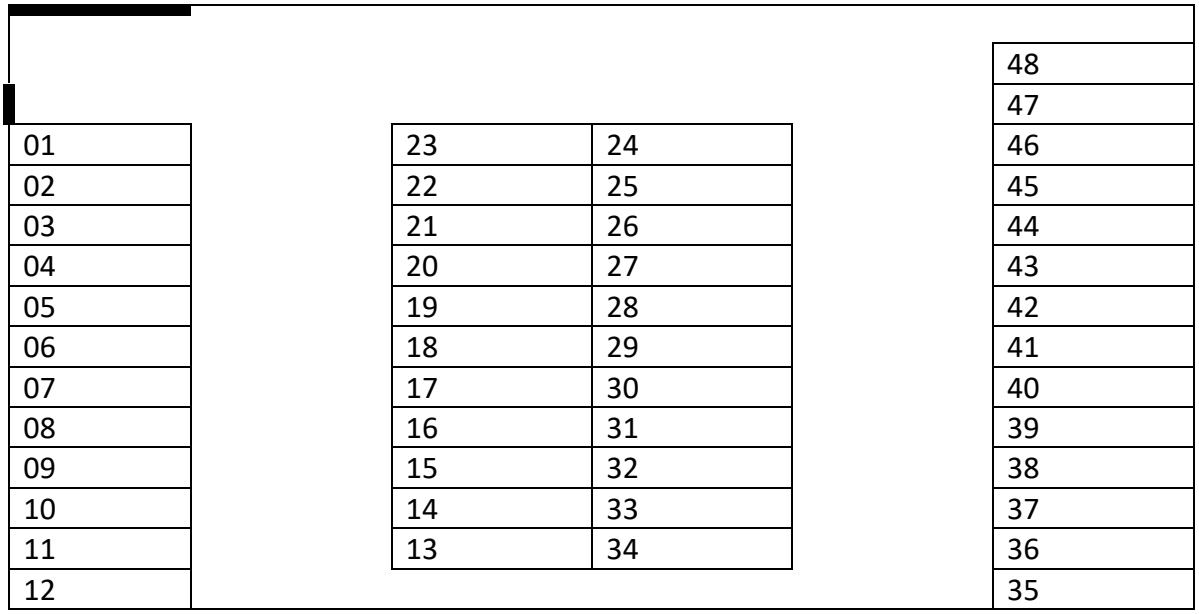
Appendix

Appendix A: Room plan for study 1

19		20	41		42
18		21	40		43
17		22	39		44
16		23	38		45
15		24	37		46
14		25	36		47
13		26	35		48
12		27	34		
11		28	33		
10		29	32		1
9		30	31		2
8					
(pilot birds)	7	6	5	4	3

Appendix B: Room plan for study 2 and 3



Appendix C: Vitamin and mineral pre-mix formulation for all studies

CONSTITUENT	VOLUME/KG DIET
Calcium	10g
Phosphorus	4.5g
Sodium	1.5g
Potassium	4.0g
Chloride	1.5g
Magnesium	0.6g
Manganese	60mg
Zinc	50mg
Iron	80mg
Copper	6mg
Iodine	0.5mg
Molybdenum	0.2mg
Selenium	0.15mg
Retinol	2.25mg
Cholecalciferol	37.5µg
Tocopherol	10mg
Menadione	3.0mg
Thiamin	3.0mg
Riboflavin	5.0mg
Pantothenic acid	10mg
Pyroxidine	4.0mg
Niacin	30mg
Cobalamin	10µg
Folic acid	1.5mg
Biotin	0.15mg
Choline	1.3g
Amprolium	125mg
Antioxidant	125mg

Appendix D: Diet allocation for study 1

pen	diet allocation		pen	diet allocation
pen 1	D		pen 25	D
pen 2	C		pen 26	C
pen 3	B		pen 27	B
pen 4	A		pen 28	A
pen 5	D		pen 29	D
pen 6	C		pen 30	C
pen 7	B		pen 31	B
pen 8	A		pen 32	A
pen 9	D		pen 33	D
pen 10	C		pen 34	C
pen 11	B		pen 35	B
pen 12	A		pen 36	A
pen 13	D		pen 37	D
pen 14	C		pen 38	C
pen 15	B		pen 39	B
pen 16	A		pen 40	A
pen 17	D		pen 41	D
pen 18	C		pen 42	C
pen 19	B		pen 43	B
pen 20	A		pen 44	A
pen 21	D		pen 45	D
pen 22	C		pen 46	C
pen 23	B		pen 47	B
pen 24	A		pen 48	A

Appendix E: Diet allocations for Study 2

Pen	Diet		Pen	Diet
1	A		25	E
2	B		26	F
3	C		27	A
4	D		28	B
5	E		29	A
6	F		30	B
7	A		31	C
8	B		32	D
9	C		33	E
10	D		34	F
11	E		35	A
12	F		36	B
13	A		37	C
14	B		38	D
15	A		39	E
16	B		40	F
17	C		41	A
18	D		42	B
19	E		43	A
20	F		44	B
21	A		45	C
22	B		46	D
23	C		47	E
24	D		48	F

Appendix F: Diet allocation for study 3

pen	diet allocation		pen	diet allocation
pen 1	A		pen 25	B
pen 2	B		pen 26	C
pen 3	C		pen 27	A
pen 4	B		pen 28	A
pen 5	A		pen 29	B
pen 6	C		pen 30	C
pen 7	C		pen 31	C
pen 8	A		pen 32	B
pen 9	B		pen 33	A
pen 10	B		pen 34	A
pen 11	C		pen 35	C
pen 12	A		pen 36	B
pen 13	C		pen 37	B
pen 14	B		pen 38	A
pen 15	A		pen 39	C
pen 16	C		pen 40	A
pen 17	A		pen 41	B
pen 18	B		pen 42	C
pen 19	A		pen 43	B
pen 20	C		pen 44	C
pen 21	B		pen 45	A
pen 22	A		pen 46	C
pen 23	B		pen 47	A
pen 24	C		pen 48	B

Appendix G: Original tables for study 1 with Removed diet and outliers included

Table 3.1 – Dietary treatments for XOS/xylanase study

Diet	Treatment
Control	Standard broiler diet
Control + XOS	Standard broiler diet + 100g/t DDGS AX
Control + XOS pre-treated with Xyl	Standard broiler diet + 100g/t DDGS XAX Pre-treated with xylanase at 1g/kg
Control + XOS + Xyl	Standard broiler diet 100g/t DDGS AX + xylanase added at 1g/kg directly to diet

Table 3.4 Diet proximate analysis for AXOS 1

Diet	Starter A	Starter B	Starter C	Starter D	Grower A	Grower B	Grower C	Grower D	Finisher A	Finisher B	Finisher C	Finisher D
DM (%)	87.31	87.31	87.64	87.64	87.22	87.22	87.47	87.47	87.13	87.13	87.07	87.07
Ash (g/kg	5.37	5.51	5.95	5.75	5.03	4.68	5.05	4.78	4.58	4.96	4.97	4.99
Protein (g/kg DM)	21.42	21.76	22.16	22.48	20.21	20.04	20.81	22.60	19.02	18.55	20.72	19.00
Total P (g/kg DM)	8.80	7.93	8.39	8.32	7.29	8.06	7.58	7.50	6.88	6.45	6.72	7.43
Total Ca (G/KG DM)	9.84	9.28	9.03	9.06	8.03	8.83	8.61	8.24	7.43	6.90	6.93	7.91
Fat (g/kg)	4.41	4.21	4.04	4.11	5.48	5.83	5.01	4.93	5.85	5.69	5.66	5.87
Ti (g/kg)	4.98	4.95	4.72	4.94	N/A	N/A	N/A	N/A	5.01	4.84	4.40	4.27
GE (mj/kg)	16.27	16.30	16.20	16.28	16.55	16.45	16.31	16.47	16.69	17.03	16.97	17.05

Table 4 – Performance results for the effect of birds supplemented with XOS either with or without xylanase added at 1g/kg compared to a control diet

Diet	D0-35 BWG (g/bird)	D0-35 FI/bird (g)	D0-35 FCR
Control	1917 (42.8)	3499 (72.5)[b]	1.83 (0.035)
Control + 100G/t XOS	1860 (44.2)	3533 (52.7)[b]	1.91 (0.087)
Control + 100g/t XOS pre-treated with Xyl	1988 (52.5)	3873 (95.4)[a]	1.97 (0.087)
Control +100g/t XOS +Xyl in diet	1862 (48.1)	3624 (69.9)[ab]	1.96 (0.077)
P value	0.195	0.004	0.406

Values in the table denote mean (standard error)

Table 5 - Body weight gain by week results for the effect of birds supplemented with XOS either with or without xylanase compared to a control diet

Diets	D0-7 BWG (g/bird)	D7-14 BWG (g/bird)	D14-21 BWG (g/bird)	D21-28 BWG (g/bird)	D28-35 BWG (g/bird)
Control	85 (206)[b]	222 (8.6)	380 (14.7)	533 (17.1)	696 (25.1)
Control + XOS	92 (3.3)[ab]	228 (10.1)	372 (10.3)	511 (11.4)	656 (25.9)
Control + XOS pre-treated Xyl	100 (2.7)[a]	248 (8.4)	389 (14.4)	559 (14.1)	691 (16.5)
Control + XOS + Xyl	89 (3.1)[ab]	220 (9.9)	380 (13.3)	519 (14.2)	654 (13.6)
P Value	0.007	0.140	0.856	0.103	0.335

Trend control + 100g/t AXOS > (p=0.06)

Values in the table denote mean (standard error)

Table 6 – Weekly feed intake of birds supplemented with XOS either with or without xylanase added at 1g/kg compared to a control diet

Diet	D0-7 FI (g/bird)	D7-14 FI (g/bird)	D14-21 FI (g/bird)	D21-28 FI (g/bird)	D28-35 FI (g/bird)
Control	200 (13.4)	512 (25.9)	654 (21.9)	895 (22.7)[b]	1224 (40.1)
Control + 100G/T XOS	213 (10.8)	535 (23.3)	685 (29.2)	876 (11.3)[b]	1225 (37.3)
Control + 100g/t XOS pre treated with Xyl	213 (10.5)	557 (22.3)	687 (28.8)	1032 (35.4)[a]	1341 (33.5)
Control +100g/t XOS +Xyl in diet	214 (12.3)	517 (21.6)	687 (33.1)	921 (23.3)[b]	1286 (40.5)
P value	0.800	0.530	0.801	<0.001	0.116

Values in the table denote mean (standard error)

Table 7 – Immunoglobulin results for of birds supplemented with XOS either with or without xylanase added at 1g/kg to a control diet

Diet	D7 IGA	D35 IGA	D7 IGM	D35 IGM
Control	0.12 (0.012)	0.35 (0.064)	0.05 (0.000)	0.09 (0.005)
Control + XOS	0.12 (0.007)	0.44 (0.080)	0.06 (0.005)	0.10 (0.007)
Control + XOS pre-treated with Xyl	0.16 (0.019)	0.40 (0.040)	0.06 (0.007)	0.11 (0.007)
Control +XOS +Xyl in diet	0.21 (0.050)	0.40 (0.050)	0.06 (0.007)	0.10 (0.004)
P Value	0.078	0.740	0.955	0.399

Values in the table denote mean (standard error)

at 1g/kg compared to a control diet

Diet	AME diet D35	Ni ret diet D35	AMEn D35	AME D7	Ni ret diet D7	AMEn D7
Control	10.3 (0.72)	25.5 (0.27)	9.4 (0.71)	6.1 (0.81)	24.6 (0.57)	5.3 (0.79)
Control + XOS	11.1 (0.45)	23.0 (0.25)	10.4(0.44)	6.2 (0.55)	25.3 (0.47)	5.3 (0.55)
Control + XOS pre treated with Xyl	10.9 (0.65)	25.9 (0.26)	9.9(0.64)	4.6 (0.54)	23.5 (0.62)	3.8 (0.52)
Control +XOS +Xyl in diet	11.3 (0.60)	24.9 (0.32)	10.4(0.59)	4.9 (0.41)	24.4 (0.27)	4.1 (0.40)
p Value	0.713	0.000	0.668	0.145	0.134	0.145

Values in the table denote mean (standard error)

Table 9 - D35 Ileum and caeca weights of birds supplemented with XOS either with or without xylanase added at 1g/kg compared to a control diet

Diet	Illeal Weight Full (g)	Illeal Weight Empty (g)	Caeca weight Full (g)
Control	39.1 [ab] (2.03)	20.5 (0.72)	8.9 (0.54)
Control + 100G/t XOS	38.9 [ab] (1.74)	20.3 (0.66	8.9 (0.42)
Control + 100g/t XOS pre-treated with Xyl	43.8 [a] (1.39)	22.6 (0.85)	9.7 (0.58)
Control +100g/t XOS +Xyl in diet	37.1 [b] (1.64)	20.7 (0.91)	8.9 (0.43)
P value	0.049	0.187	0.554

Values in the table denote mean (standard error)

Chapter 5 was appended to a supplementary chapter at the viva

Chapter 5 - Novel antioxidant methodologies

5.1 Introduction

It has been demonstrated that some oligosaccharides may have antioxidant properties (Zhao *et al.,* 2020). Emerging literature has indicated that oxidative stress, and particularly lipid peroxidation may be responsible for damaging the integrity of the tight junctions in the gut epithelium (Rao, 2008). In addition to this, an ongoing problem with breast muscle myopathies has been identified which may be linked to oxidative stress. Following an approach from Knight Scientific, the use of novel antioxidant assays was discussed in order to quantify any antioxidant properties of the XOS products used in this project. Knight Scientific have developed a number of assays for quantifying the total antioxidant capacity of a substance, or the ability of a substance to quench various reactive oxygen species (singlet and halogenated oxygens and peroxynitrite radicals).

These assays were demonstrated to be highly sensitive and accurate in humans and were entirely novel in the poultry sector. These assays use Pholasin™, a substance extracted from the light organs of a marine mollusc. Pholasin™ emits light when exposed to free radicals. This can then be used to compare the quenching ability of unknown substance as the quenching reduces light emitted and/or time to maximum light peak. The assays use the reaction between super oxide and nitric oxide released simultaneously and continually from a solution of 3-morpholino-sydnonimine HCl to release reactive oxygen species *in situ*, thus allowing changes in the light emitted by the Pholasin™ to be measured by a chemiluminescent plate reader. These novel methods had been validated in horses (Knight and Larkins, 2003), humans (Knight *et al.*,2002) and wild birds (Tobler and Sandell, 2009; Tobler *et al.*, 2013), but to date had not been assessed in broilers.

Initially, the aim was to determine the oxidative status of Ross 308 commercial broilers and to compare this to a backyard ‘pet’ bird to determine from the relative levels of oxidative stress present. The hypothesis was that the commercial birds would be experiencing high levels of oxidative stress

compared to an average backyard housed bird. The intense genetic selection for enhanced increased growth rate and enhanced commercial performance have been reported to place the birds under high levels of oxidative stress (Fellenburg and Spiesky, 2006). This together with the environmental conditions that broilers experience (high stocking density and high ammonia levels) are reported to increase the risk of oxidative stress (Fellenburg and Speisky, 2006). For the first part of this study, blood samples analysed were from the broiler trial described in chapter 4. These were used to determine a base level of oxidative stress in broilers and compare it to that of a backyard raised pet chicken. Secondly, the feed samples from the broiler trial described in chapter 4 and a number of commercially available XOS samples were analysed to assess their antioxidant capacity. A pilot collection study was performed to ensure that the optimal method of blood collection and storage were determined for the peroxynitrite assay. Additionally, we hypothesized that the method of euthanasia may affect the levels of peroxynitrite that could be detected, due to potential stress to the bird during this process, so this was also investigated. Peroxynitrite was selected as an appropriate measure for these assessments it is the major pre-cursor to lipid peroxidation. Lipid peroxidation is of particular relevance to the poultry industry as it is thought to be the primary cause of the common poultry muscle meat myopathies such as woody breast (Huang and Ahn, 2018). It was hoped that this particular assay would be a good prediction marker for these muscle meat myopathies. The antioxidant properties of broiler diets and commercial XOS products were also investigated to assess whether an in vitro measure of antioxidant status could be valid for feed and supplements, to allow selection of new antioxidant feed ingredients in the future.

Exogenous Antioxidants

Antioxidants have been defined as “substances that, when present in low concentrations compared to an oxidizable compound (e.g., DNA, proteins, lipids or carbohydrates), delay or prevent oxidative damage caused by the presence of reactive oxygen species” (Gutteridge, 1990). Endogenous and exogenous antioxidants are able interact synergistically in order to maintain homeostasis (Bouayed

and Bohn, 2010). Exogenous antioxidants are found in numerous foods and as such, intake can aid the prevention of oxidative stress. Antioxidants exist as nutrients such as vitamins or phytochemicals such as polyphenols. Dietary antioxidants contribute to the reinforcement of the human/animal antioxidant systems by removing excess oxygen metabolites. Complete foods containing naturally occurring antioxidants have been widely recommended for humans and animals in an effort to prevent the deleterious effect of oxidative stress (Bouayed and Bohn, 2010, The department of Health, 2008). Common dietary antioxidants include fruits vegetables and food preservation substances. Most synthetic antioxidants are derived from phenolic structures such as butylated hydroxyanisol, butylated hydroxytoluene. (Fellenburg and Speisky, 2006). Whilst many fruits and vegetables do indeed contain antioxidants, unfortunately they are not always bioavailable to animals and often cannot be synthesized (Bouayed and Bohn, 2010). Likewise, some plant-based antioxidants such as quercetin, when delivered in high quantities potentate super oxide radical production within isolated mitochondria and cultured cells (De Marchi *et al*., 2009, Bouayed and Bohn, 2010). As such the actual total antioxidant capacity for a given species must be considered in order to prevent/control oxidative stress.

The key aim of this work was to investigate the informative ability of novel antioxidant assays to accurately assess the oxidative status of broilers and antioxidant capacity of broiler feed. Secondly, this work aimed to assess the most appropriate sampling procedures for their use in broilers. The objectives were as follows :

- To begin to quantify the oxidative status of Ross 308 commercial broiler
- To determine optimal blood collection, storage and euthanasia methods for optimal sample analysis using the peroxynitrite assay
- To determine whether a number of commercial xylo-oligosaccharides possess the ability to quench singlet or halogenated oxygen species

5.2 Materials and Methods

The samples used in this chapter are from control birds taken from commercial studies. All birds were raised under trial conditions at the PRU therefore all husbandry information is as previously stated in section 2.2. All birds were fed commercial style mash diets formulated for the age and strain of the bird, and samples taken in addition to the required trial procedures. All studies were approved by the college ethics committee.

5.2.1 Pilot study to determine whether euthanasia method affects the peroxynitrite levels in blood

Twenty Ross 308, 35 day old broilers were euthanized by either cervical dislocation (10) or captive bolt using a 0.22mm pellet to fire (Cash small animal tool, Frontmatec, UK) (10). The captive bolt was operated by a trained user and two handlers were used to restrain the bird in order to minimise stress. Blood samples were collected immediately post-mortem (as described in section 2.4.2) and split in to either a serum, heparin or EDTA tube and serum or plasma analysed for peroxynitrite (as described in section 2.5.15). Results were also adjusted for body weight of the birds and calculated as VEA per kg BW (µmol/L).

5.2.2 Pilot study to determine whether the type of blood tube/anticoagulant used to collect the blood sample effects the levels of peroxynitrite detected in the blood.

Blood samples were collected from twenty Ross 308 male birds (all 35 days of age) and split in to three tubes containing, heparin, EDTA or plain serum tubes, respectively. The samples were centrifuged immediately after the final sample had been collected as described in section 2.4.2 and the plasma or serum aliquoted into fresh tubes. Each sample was analysed according to the procedure for the peroxynitrite assay (section 2.5.15). Results were also adjusted for body weight of the birds and calculated as VEA per kg BW (µmol/L).

5.2.3 Pilot study to determine whether freezing the samples effects the levels of peroxynitrite present in the sample.

The 20 samples used in the previous pilot study (section 5.2.2) were analysed fresh on the same day as collection, then the samples were frozen at -20°C for 1 week. Samples were then thawed at room temperature prior to reanalysis to determine whether freeze thaw cycles detrimentally affected the assay results. Results were also adjusted for body weight of the birds and calculated as VEA per kg BW (µmol/L).

5.2.4 Pilot study to determine whether diet effects peroxynitrite levels in the blood

The twenty birds used in sections 5.2.1, 5.2.2 and 5.2.3 were all taken from the control treatment of a commercial trial Birds were all fed a balanced, wheat/corn/soy diet fed in three phases formulated for the age and strain of bird. Results were adjusted for body weight of the birds and calculated as VEA per kg BW (µmol/L).

5.2.5 Determination of oxidative status – ABEL TAC™ (peroxynitrite assay) - Blood

The blood samples taken for animal study 2 (chapter 4) were used to determine oxidative status of birds fed trial diets. On day 42, blood samples from 48 birds (1 bird per pen) were centrifuged in serum tubes and the serum retained for the analysis. An ABEL TAC™ kit was used to analyse the serum fresh as described in section 2.5.16. Results were also adjusted for body weight of the birds and calculated as VEA per kg BW (µmol/L).

5.2.6 Determination of antioxidant capacity – ABEL TAC™ - Diet samples

The treatment and control diets from the study described in chapter 4, and the vitamin and mineral premix and xylanase used in both previous studies (chapters 3 and 4) were assessed for their antioxidant capacity. This was to try and quantify the antioxidant value of these dietary components, and to ensure that these components were not pro-oxidant and therefore inhibiting any antioxidant activity of the diets or XOS. As the kit requires liquid samples (section 2.5.16) solubility was first assessed in ultra-pure water, 50% and 100% ethanol and 50% and 100% acetone. Solubility was visually determined by the quantity of substance that remains in dissolved in a vessel following 30 minutes agitation on a roller. It was determined that maximum solubility was achieved with 50% acetone for the complete diets and with 50% ethanol for the premix and with ultra-pure water for the xylanase. The diets had all been stored at -20°C to prevent lipid oxidation, avoid bacterial overgrowth and maintain the nutritional profile as fed.

5.2.7 ABEL test for halogenated oxygen

The ABEL kit for halogenated oxygen was used as described in section 2.5.18 to quantify the ability of five commercially available XOS products (including the corn XOS used in chapter 4) to quench halogenated oxygen radicals (see table 5.1 for details of each product). Solubility was again assessed prior to analysis, as described in section 5.2.6.

Table 5.1 Commercial XOS products used in comparison of antioxidant properties assay

Product	Material extracted and/or manufacturer
Corn XOS (Longlive)	Corn cob, Shandong Longlive Bio-Technology Co (China)
Yuan Long	Corn cob, corn straw, cotton seed hull and peanut hull, Henan Biotechnology (China)
Yatai	Source material unknown, Yibin Yatai Biotechnology (China)
Taixin	Source material corn cob, Hebi Taixin Technology Co (China)
Heagreen	Source material unknown*, Henan Heagreen Biotechnology (China)

* Derived from hemicellulose in biomass and is composed of pentose glycosyl

5.2.8 ABEL test for singlet oxygen species

The ABEL Tac™ kit for singlet oxygen species was performed as described in section 2.5.19 to measure the capacity of five commercial XOS products (table 5.1). As with the halogenated test, solubility was assessed and the XOS products were analysed in ultra-pure, 50% ethanol and 100% ethanol to ensure a complete picture was obtained.

5.3 Results

5.3.1 Euthanasia method

There was a significant increase in the total antioxidant capacity (TAC) for peroxynitrite for birds euthanised via cervical dislocation compared with those euthanised by captive bolt. When the TAC for peroxynitrite levels were adjusted for bird weight there was still a significant increase in TAC for peroxynitrite levels for the CD culled birds (Table 5.2)

Table 5.2 TAC for Peroxynitrite scores for birds euthanised in different ways (mean VEA µmol/L ±SE)

Euthanasia method	VEA µmol/L	VEA adjusted for body weight µmol/L
Cervical dislocation	986.9[a] (35.84)	481.7[a] (21.89)
Captive bolt	808.5[b] (35.28)	416.0[b] (22.90)
p-value	0.002	0.015

[a-b] Means within the same row that do not share a common superscript are significantly different (p=<0.005)

5.3.2 Blood collection comparison

There was a significant increase in the TAC for peroxynitrite levels detected in blood collected into tubes containing no anti-coagulant (Table 5.3), however, it is of note that the standard error is substantially higher for this treatment.

Table 5.3 Comparison of TAC for peroxynitrite scores for blood collected into different anticoagulant tubes VEA (vitamin E analogue) µmol/L (S.E.)

Anticoagulant	VEA µmol/L	VEA per kg BW µmol/L
EDTA	805.7[b] (29.17)	403.2[b] (20.40)
Heparin	894.2[b] (30.51)	448.3[b] (22.02)
None	1095.0[a] (66.49)	533.1[a] (38.92)
p-value	0.001	0.004

[a-b] Means within the same row that do not share a common superscript are significantly different (p=<0.005)

5.3.3 Blood sample storage

There was a significant increase in the levels of peroxynitrite that could be detected in fresh blood samples compared to serum that had been frozen prior to analysis (Table 5.4).

Table 5.4 Comparison of TAC for peroxynitrite scores detected in either fresh or frozen blood

Storage method	VEA µmol/L	VEA adjusted for body weight µmol/L
Fresh	1029.2[a] (41.59)	550.6[a] (24.24)
Frozen	863.2[b] (34.59)	405.6[b] (20.39)
p-value	**0.003**	**<0.001**

[a-b] Means within the same row that do not share a common superscript are significantly different (p=<0.005)

5.3.4 Peroxynitrite levels of birds fed different diets

There was no significant difference between TAC for peroxynitrite in the blood (P=0.519) of the birds sampled in the XOS trial describe in chapter 4 (Table 5.5). Although not statistically significant, the control + corn XOS and control + bagasse XOS did produce a TAC score almost 20% higher than the other treatments. This suggests these diets may be conferring a 20% improved capacity to scavenge peroxynitrite radicals.

(Table 5.5)

Table 5.5 TAC for Peroxynitrite scores detected in the birds used for chapter 4 (VEA µmol/L +/- SE)

Treatment	Peroxynitrite (VEA µmol/L +/- SE)
Control	863.20 (117.090)
Control + Corn XOS	1029.38 (102.186)
Control + bag XOS	1028.35 (129.307)
Control + Xyl	816.49 (108.700)
Control + corn XOS + Xyl	877.83 (122.790)
Control + bag XOS + Xyl	848.62 (81.709)
P Value	0.519

[a-b] Means within the same row that do not share a common superscript are significantly different (p=<0.005)

5.3.5 Antioxidant properties of diets and diet components

All the broiler diets tested did have some degree of capacity to quench halogenated oxygen species. Likewise, both the xylanase and the vitamin and mineral pre-mix also possess a degree of antioxidant capacity (figure 5.2). However, when compared to grape seed extract – a known excellent quencher of halogenated oxygen radicals, the feed ingredients the feed did not have significant antioxidant capacity (figure 5.3).

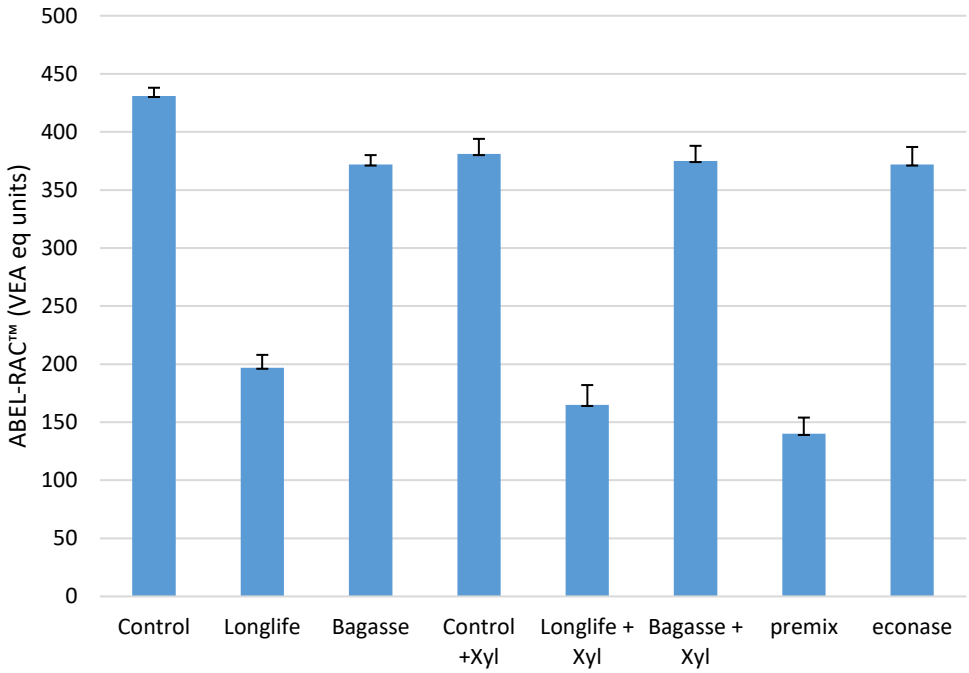


Figure 5.2 - Comparison of the capacity of broiler diets used in chapter 4 and diet components to quench halogenated oxygen species

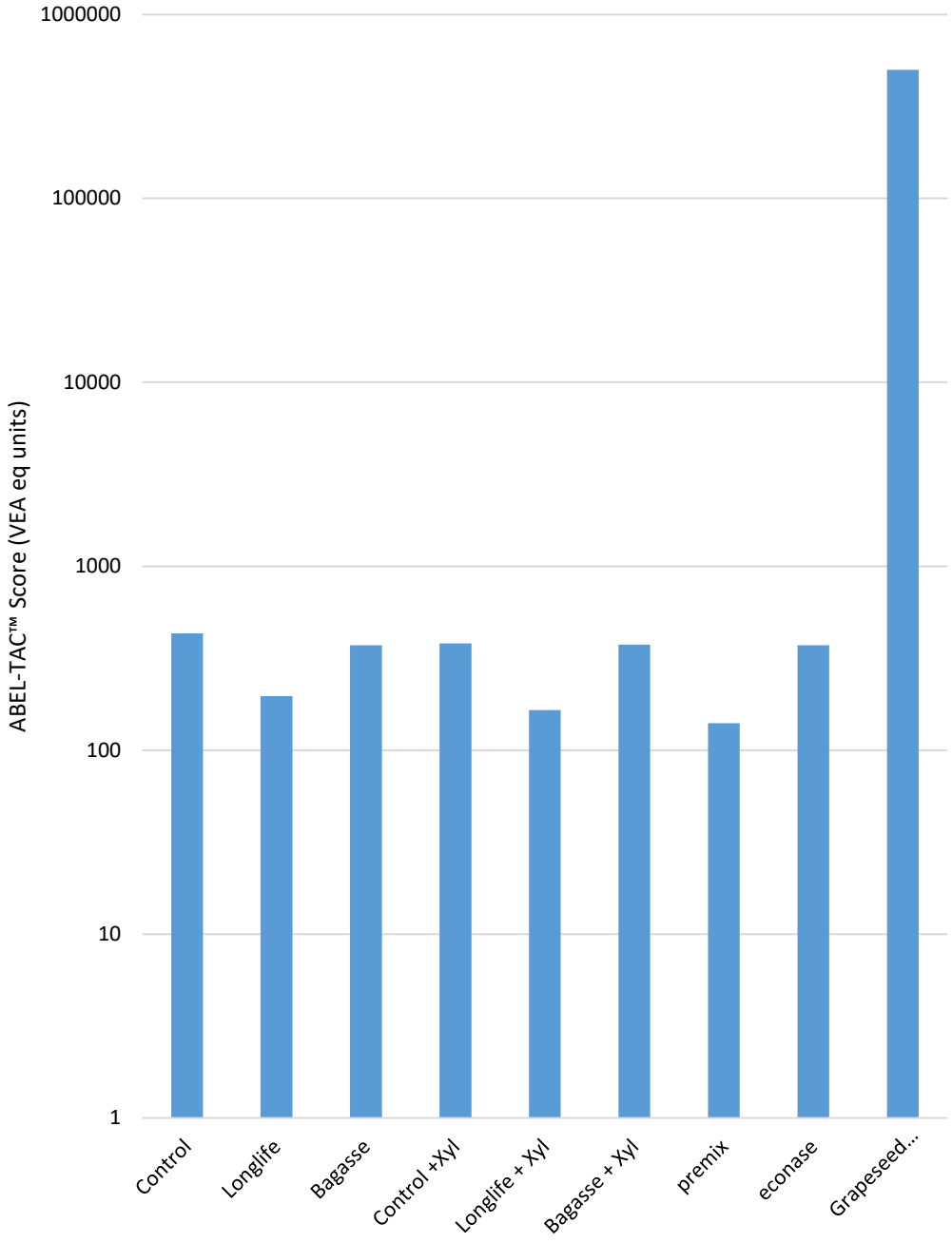


Figure 5.3 - The capacity of broiler diets used in chapter 4 and diet components to quench halogenated oxygen species compared to grapeseed oil as a known antioxidant

5.3.6 Antioxidant properties of commercial XOS products

When analysed for the ability to quench singlet oxygen radicals, three of the five XOS products showed significant antioxidant properties. The Taixin showed outstanding ability to quench singlet oxygen with a score of ABEL score of 114966 (Figure 5.4). Both the corn XOS used in chapter 4 and Yuan Long displayed very good levels of antioxidant activity against singlet oxygen. Yaitai XOS did not exhibit any antioxidant activity and Heagreen XOS only showed very low levels of activity. The ABEL-RAC score of ascorbic acid is shown as a comparison, being a known good antioxidant (figure 5.4).

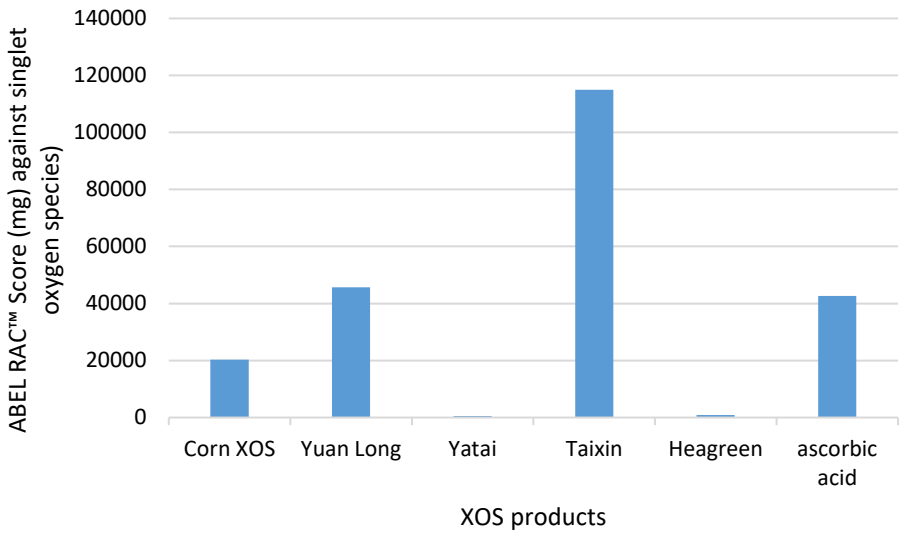


Figure 5.4 - Comparison of the ability of commercial XOS products to quench singlet oxygen species

When analysed for antioxidant activity against halogenated oxygen species, results for the XOS products were very similar to those for singlet oxygen. The Taixin XOS achieved a score of 51,207 compared to ascorbic acid that scored 21,000. The corn XOS showed reasonable ability to quench halogenated oxygen compared to the ascorbic acid. The Yuan Long XOS also performed moderately well, though it was consistently inferior to both the corn XOS and the Taixin. Both the Yatai and the Heagreen showed very little antioxidant activity against halogenated oxygen species (figure 5.5).

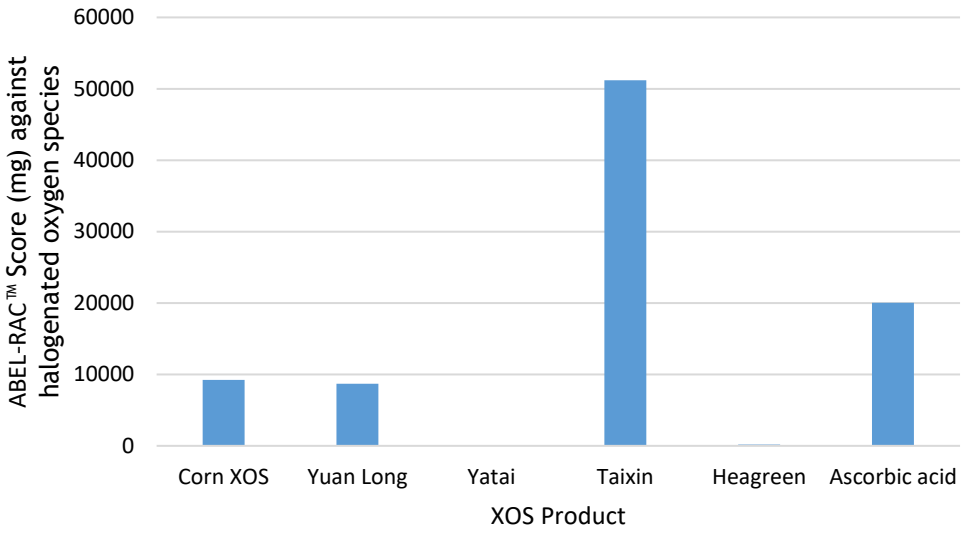


Figure 5.5 - Comparison of the ability of commercial XOS products to quench halogenated oxygen species

5.4 Discussion

The assays used in this chapter are all commercially available from Knight Scientific, (Plymouth, UK) and validation procedures in broilers were carried out under their supervision and advice as experts in this field. The TAC assay for peroxynitrite had been validated in horses (knight and Larkins, 2003), humans (Knight *et al.,* 2002), and wild birds (Tobler et al., 2013) but had never been used for broilers. In humans and horses (Knight and Larkins, 2003) the assay was validated to use either serum or plasma. Tobler and Sandell (2009) used plasma (unidentified anticoagulant) from Zebra Fiches to run the TAC assay, although the publication did not describe any particular rationale for this. As the assay had not previously been used in broilers, the initial pilot study looked at comparing the total antioxidant capacity for peroxynitrite levels detectable in both serum and plasma (using two common anticoagulants). The results showed that significantly higher capacity to scavenge peroxynitrite could be detected in serum compared to either of the two plasma samples (EDTA and heparin tubes). This result was unexpected as both serum and plasma had been shown to be equally accurate in other species (Knight, 2019). As the study by Tobler and Sandell (2009) did not provide any reason for using plasma, it is possible that this parameter was not considered. As they were using the assay as a direct comparison from one bird to another, it is possible that plasma may produce repeatable results, and that serum samples are just able to detect a higher level for antioxidant capacity. With limited information from other studies, the fact that serum could repeatedly detect higher levels of antioxidant capacity was used as the rationale for selecting this method for further testing.

Serum is defined as the fluid and solute component of blood without the fibrinogen (Reece *et al*., 2014). Serum contains all blood proteins that do not play a role in clotting. As such it is possible that something in the clotting factors present in plasma, reduce the detectability of capacity to scavenge peroxynitrite. Zhonghao *et al.,* (2011), reports that both collecting procedures and the coagulation cascade can influence the concentration of proteins and metabolites present in a human blood sample. Their study showed that metabolite concentration was generally higher in a serum samples

than in a corresponding plasma sample and that consequently, serum consistently showed higher sensitivity in biomarker detection. These authors suggest that this may be due to the volume displacement effect, whereby deproteinization of serum eliminates the volume fraction of proteins and consequently distributes any remaining low molecular weight constituents in a smaller volume, thus increasing the concentration. Likewise, Dettmer *et al*., (2010) found that some metabolites (citrate and lactate) in the cow blood differed significantly between serum and plasma samples.

It is also possible that differences in avian blood physiology may cause reactions with the heparin or EDTA, resulting in lower detectable biomarkers. There are a few fundamental differences in avian and mammalian blood that may account for this result. Like humans, avian blood contains erythrocytes, however, unlike humans, avian erythrocytes are larger and possess a nucleus and mitochondria (Sturkie,2000). Whilst many other assays successfully use plasma, it is possible that peroxynitrite specifically is affected by the anticoagulants, and that the oxidation products are partially neutralized or changed by contact with them. Papp and Smits (2007) investigated another chemical luminescence assay which used lucigenin to assess the innate immune function of broilers and other animals. These authors found that consistent luminescence responses were found when blood was collected in heparin or EDTA, but when blood exposed to heparin, was mixed with EDTA, the chemical luminescence was dramatically inhibited. However, these authors did not assess serum for the assay, so no direct comparison can be made. This does suggest that collection of blood samples is important when carrying out this type of analysis and therefore at least consistency of tube type should be incorporated into future work.

When the TAC for peroxynitrite (TAC) scores for broilers are compared to other species, there is some variation. Human TAC scores collected by the assay manufacturers varied from 275 to 625 (figure 5.6). Whereas the TAC scores for horses are lower and vary between 80 and 300 (figure 5.7). The TAC levels for Zebra Finches aged 35 days were higher than humans and very similar to the levels found for broilers in this study, ranging between 758 and 840 (Tobler and Sandell, 2009). The variation by

species and the similarity of zebra finches to broilers suggests that the total antioxidant capacity for peroxynitrite of avian species may be similar, however, more avian species would need to be assessed to confirm this.

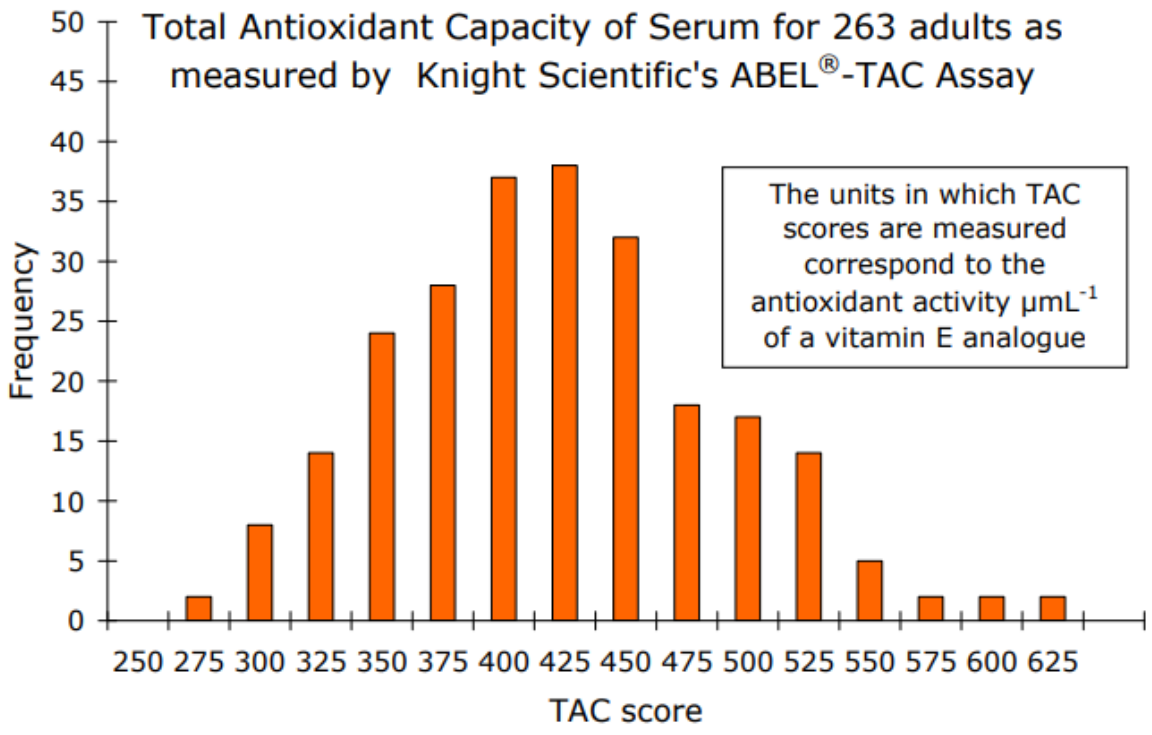


Figure 5.6 - TAC for peroxynitrite scores of 263 human subjects (Source, Knight Scientific, used with permission, 2019).

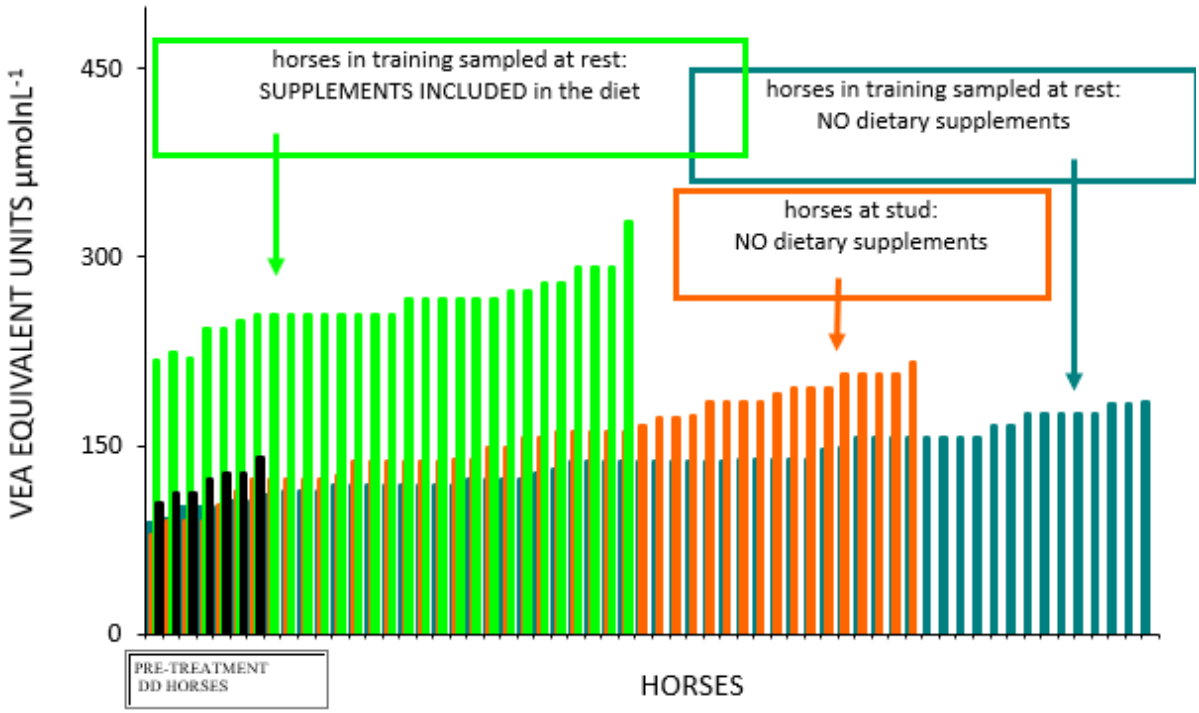


Figure 5.7 – TAC for peroxynitrite scores in horses (Source: Knight Scientific, used with permission 2019)

The method of euthanasia was assessed as it was hypothesised that the reflex flapping after cervical dislocation may alter the biochemistry and effect results, or that the procedure and handling of the bird may cause stress. Unfortunately, live blood samples could not be collected to compare results. However, as a captive bolt is one of the approved methods of humane slaughter, and that total brain decimation by the captive bolt may potentially stop metabolic and biochemical changes rapidly, it was used to compare to cervical dislocation. The results showed that there was a significant increase in the TAC for peroxynitrite score detected in birds that were euthanized via cervical dislocation compared to captive bolt. This may suggest that cervical dislocation produces results closer to those experienced during life. As the birds were taken from a control treatment and had experienced identical diets and lives until euthanasia, the loss of antioxidant capacity for the captive bolt group is more likely due to the euthanasia process, rather than oxidative status prior to death.

This was quite unexpected as it had been assumed that the complete decimation of the brain, caused by the captive bolt strike, may stop biochemical reactions quicker than just severing the spinal cord during cervical dislocation. However, as two people are required to restrain birds during captive bolt euthanasia, and restraint must be firm, to ensure both human and animal safety, it is possible that this may increase stress on the bird, subsequently causing a high TAC score.

Cartner *et al*., (2008), report that cortical function was disrupted within 10 seconds post-cervical dislocation. This suggests that biochemical changes may occur post-mortem, potentially skewing results. Donaldson and Lamont (2013) assessed post-mortem biochemical changes in humans, pigs and rats. The authors found that biochemical changes varied post-mortem between species. Martin *et al*., (2019), assessed different methods of euthanasia, time to unconsciousness, and the rate at which flapping, and leg paddling reflexes stopped. These authors found that manual cervical dislocation achieved unconsciousness more quickly, in an average of 2.6 seconds compared to captive bolt device (3.5 seconds). Reflex flapping was slightly shorter in the manual cervical dislocation treatment (134.8 seconds compared to 138.0 seconds for captive bolt) and leg paddling reflex was also slightly reduced in the manual cervical dislocation group compared to captive bolt.

Therefore, it is difficult to discern the results of the current study. On the one hand, cervical dislocation could genuinely cause less stress and produce a result closer to what the result may have been during the life of the bird. However, it is also possible, as discussed above, that in response to increased oxidative stress the birds produced increased levels of circulating antioxidants, thus increasing the capacity of the plasma to scavenge peroxynitrite radicals. It is also of note that the capture and restraint of the birds could also induce biochemical changes. This is very difficult to assess, as even collecting a live blood sample would involve capture and restraint of the bird. Without keeping a bird and habituating it to restraint and blood sampling, it is almost impossible to remove this bias. Although the birds used in this study were captured in the same manner, cervical dislocation only needs one operative, whilst captive bolt requires two handlers and an operative. It is also of note

that cervical dislocation is the standard method of euthanasia at NTU and as such, operatives are highly trained and rapid in dispatching birds. Captive bolt was a new method for the team and in ensuring it was carried out humanely, the dispatching process did take substantially longer than cervical dislocation. Therefore, the necessary variation between treatments makes forming conclusions difficult in this regard.

As the birds used in this study were captured and restrained in the same manner prior to euthanasia, this study can at least compare like with like. Additionally, as results were consistent, over all replicates it can be concluded that birds euthanized via cervical dislocation had increased ability to scavenge peroxynitrite radicals. However, repeating this study under a Home Office Licence with live blood samples prior to euthanasia may help to confirm whether changes blood biochemistry are genuine and at what point peroxynitrite levels may change.

The results of freezing broiler blood serum for a week showed that the TAC for peroxynitrite score was significantly reduced by freezing the serum sample at -20°C, compared to analysing a fresh sample. This result is not unexpected as the manufacturer's instructions recommend storage at -80°C. However, there are a number of publications that investigate the effect of freezing on blood samples. Briard *et al.* (2016) report that ice recrystallization is a major cause of damage to proteins in frozen blood serum samples. Lee *et al.* (2015) looked at the effect of freezing and thawing plasma and serum and found that the stability of circulating proteins can be significantly affected by the freezing and thawing process. These authors found multiple biomarkers that were significantly affected by frozen storage including Interferon-γ and tumour necrosis factor-α. Work in sheep also found that the effect of storage on ovine serum and plasma and also found significant changes in energy metabolites after frozen storage at -20°C (Morris *et al*., 2002). It is of note that some of these studies found that biomarkers were more stable when stored at -80°C rather than -20°C. Whilst some change still occurred, the changes were significantly smaller. Therefore, in cases where samples must be frozen,

freezing at -80^{o}C may provide an alternative solution to allow this analysis to be carried out with least variability.

Following the initial validation work on the TAC method, dietary effects were investigated using the TAC for peroxynitrite scores of the birds from the trial described in chapter 4. These samples however, had been stored for a number of months at -20°C, as originally no antioxidant analysis had been planned for this trial. Although this analysis did not show any significant differences in peroxynitrite levels between the 6 treatments used, there was a 20% increase in the ability of the birds fed the control + corn XOS and control + bagasse XOS treatments to scavenge peroxynitrite radicals compared to the other treatments. The TAC scores of these birds were still in the same range as the other broiler results from section 5.3.2, so the long-term storage did not seem to profoundly affect the TAC score, although subtle differences between treatments may be lost during storage.

Knight scientific had previously validated an assay for measuring the ability of feed ingredients and nutraceuticals to quench peroxynitrite reactive oxygen species (Knight, 2005). Initially the diets used for the trial described in chapter 4 were assessed. These were commercial wheat/soy/corn-based broiler diets with the addition of two different XOS products both with and without xylanase. (Figure 5.2). Whilst there were some apparent differences in antioxidant capacity between diets, when these diet scores were compared to a known antioxidant (grape seed oil), the antioxidant capacity of the diets was negligible. Figure 5.8 shows the ABEL RAC™- peroxynitrite scores for various other known antioxidant substances for comparison.

SOME ABEL-RAC peroxynitrite scores determined for particular samples tested

Berberis	6300	Ginger	4000
Bee pollen	9520	Grapeseed extract sample 1	390000
Bilberry	7100	Grapeseed extract sample 2	350000
Broccoli extract	6600	Hawthorn	2600
Cayenne	850	Mint	16000
Curcumin	43000	Picrorhiza	18181
Dandelion	19100	Red sage root	20400
Gallic Acid	122160	Rosemary (second extract)	21500
Gingko leaves	4900	Rosemary (first extract)	819100
Gingko extract	100632	Urtica	30075

Figure 5.8 - Examples of ABEL RAC™ scores from other substances (adapted from Knight Scientific website –2020)

What is interesting, is the repeatability in scores between the diets (figure 5.2). Both the control and the control + xylanase have very similar scores (431 and 381) likewise, the two corn XOS and the two bagasse XOS diets were also similar to one another. This does seem to infer a good level of precision and repeatability for this procedure; however, this also seems to suggest the corn XOS is somehow reducing the ability of that diet to quench peroxynitrite radicals. As the diets were manufactured as two basal diets (control and control + xylanase) then each split into three treatments before the various XOS products added; the two basal diets could only be detrimentally affected by the addition of XOS. The reduced anti-peroxynitrite capacity of the corn XOS treatments suggest this product is a potential pro-oxidant and therefore reducing the antioxidant capacity of the basal diet in the treatment. This result was unexpected as other authors have found alternative oligosaccharides to be

antioxidants (Zhao *et al*., 2020). However, some studies have found known antioxidants to be pro-oxidants in some circumstances.

Podmore *et al.,* (1998), investigated the antioxidant properties of vitamin C (ascorbic acid) and found that, although vitamin C was an excellent antioxidant against some reactive oxygen species, it was actually pro-oxidative in some circumstances and could cause damage to lymphocyte DNA. Mandal, (2019) reports that although vitamin C is excellent at scavenging hydrogen peroxide, it also reduces metal ions that consequently generate free radicals. It is therefore possible that the corn XOS may have triggered a pro-oxidant effect, either directly, or indirectly. Likewise, this TAC assay measures the ability of a substance to scavenge peroxynitrite radicals specifically. Reactive oxygen species (ROS) can be split into two categories. Radical ROS possess an unpaired electron and therefore try to seek another electron (Lagunes and Trigos, 2015). Non-radical ROS have their full complement of electrons but possess very high energy making them extremely reactive. Most antioxidants act by donating an electron to stabilize the molecule, however as non-radical species cannot accept an electron, the only way for an antioxidant to stabilize them is by quenching – or trapping the non-radical. Therefore, it is possible that the corn XOS may be capable of quenching non-radical ROS but is not able to donate electrons to radical species (Lagunes and Trigos, 2015). This is supported by the results of the vitamin and mineral pre-mix. The ingredients specifically list an “antioxidant” in the ingredients list (appendix C). Whilst this is not identified by the commercial premix supplier, it would be expected to display measurable antioxidant activity. As the peroxynitrite assay found very little antioxidant activity in the premix, it again appears that whilst the pre-mix may possess antioxidant properties against some ROS, it appears to have little activity against peroxynitrite radicals. The xylanase showed numerically more peroxynitrite scavenging ability than the pre-mix. However, as the xylanase is added to the diet at a very low inclusion rate (0.01%), it was expected that the diets containing xylanase did not perform better than the diets without.

The results of the peroxynitrite assay led to further exploration of the corn XOS to attempt to determine any antioxidant properties. As part of this investigation, , four commercially available XOS products were purchased to act as direct comparisons. The ability of the five XOS products (including the corn XOS) to quench singlet and halogenated oxygen species was measured. These assays were selected as they measure the ability of the XOS to quench non-radical ROS, rather than the radical species tested previously. The results showed highly significant differences between the five XOS products in their ability to quench non-radical singlet and halogenated ROS. Whilst two of the commercial products showed very little quenching ability, the Taixin XOS demonstrated exceptional ability to quench both singlet and halogenated oxygen species. This XOS product (Taixin) is also produced from corn manufacturing, but the process used is not openly available, so may be a different product source to the other materials tested. The corn XOS used in the trial described in chapter 4 achieved an ABEL RAC™ score of 20288 for singlet ROS and 9224 for halogenated oxygen species. When compared with ascorbic acid which scored 42619 for singlet ROS and 20023 for halogenated ROS, the corn XOS demonstrates approximately 50% of the ability to quench these non-radical ROS. As mentioned previously ascorbic acid is considered an excellent antioxidant (Padayatty *et al.,* 2003), despite potentially negative side effects due to the reduction of metal ions increasing the production of free radicals (Mandal, 2019). This suggests that the corn XOS may have the added benefit of acting as a quencher of non-radical ROS. However, the inclusion rate in the complete diet may negate any major benefits. Further research on in vivo inclusion of corn XOS with monitoring of antioxidant effects both in the bird and in terms of meat quality is required to further assess any potential antioxidant effect.

5.5 Conclusion

In conclusion, the methodology pilot study revealed that the TAC for peroxynitrite could detect higher levels of antioxidant capacity in broiler serum than in plasma collected using either heparin or EDTA tubes. Cervical dislocation demonstrated the highest antioxidant capacity; however, it is unclear if this really does provide a true reflection of the levels of oxidative stress a bird experiences during its life. The storage of blood samples clearly shows that the assay can detect higher levels of antioxidant scavenging ability in fresh samples, rather than those stored at -20°C for one week.

The commercial broiler feed used as the control diet for chapter 4, demonstrates limited ability in scavenging free radicals and as such, supplementary antioxidants are indicated. Some commercial XOS products do have the ability to quench non-radical ROS. However, when the level of dietary inclusion is considered, the antioxidant benefits of XOS are unlikely to be significant.