Prebiotics in weaner and grower pigs

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Declaration of Authorship

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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TS designed the experiment, conducted the research, analysed the data and wrote the manuscript. Co-authors assisted in designing the experiment, advised on analysis and revised the manuscript.

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Chapter 2

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Chapter 3

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Abstract

Alongside increased pressure to reduce antimicrobial usage, the UK pig industry is facing an imminent ban on therapeutic levels of Zinc oxide. Hence, seeking suitable feeding concepts that promote health and prevent disease is of utmost importance for the industry's sustainability and profitability. Prebiotics positively influence gut bacterial community composition, thus improve host health and the nutritive value of feedstuffs. New-generation prebiotics like xylo-oligosaccharides (XOS) are gaining attention for monogastric nutrition. The research in this thesis aimed to determine the effect of XOS, with or without alternative additives, on weaner and grower pig growth, bacteria modulation and fibre digestibility. In addition, faecal L-lactate concentration and inert markers for fibre digestibility analysis were investigated. Results showed XOS had limited effects on pig performance but altered ileal bacterial community composition of weaner pigs, including beneficial *Lactobacillus* stimulation. XOS increased ileal fermentative activity and non-starch polysaccharide (NSP) digestibility, indicating a stimulatory effect on increasing the bacteria's fibre-degrading capacity. The effects of XOS were lost when combined with long-chain inulin, demonstrating incompatibility of the products. XOS and fructans increased the faecal digestibility of cellulose and NSPs. Furthermore, L-lactate was detected in piglet faeces at low levels, faecal bacteria metabolism of L-lactate was rapid, and a handheld device was found to be a suitable real-time method for L-lactate analysis. Due to ileal fractionation, titanium dioxide (TiO_2) was shown to be an unsuitable inert marker for NSP digestibility whilst cellulose showed realistic initial results, yet further validation is required. TiO_2 fractionation was rectified at the faecal level, whilst post-ileal degradation rendered cellulose infeasible as a marker for total tract NSP digestibility. In conclusion, XOS affected bacterial community composition and increased NSP digestibility but did not translate into improved growth,

with a relatively short feeding period being the likely explanation. Further work is required to expand on these findings.

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Abbreviations

ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
AHDB	Agriculture and Horticulture Development Board
AIA	Acid Insoluble Ash
AID	Apparent Ileal Digestibility
AMR	Antimicrobial Resistance
ANOVA	Analysis Of Variance
ara	Arabinose
ATTD	Apparent Total Tract Digestibility
AX	Arabinoxylans
AXOS	Arabinoxylan-oligosaccharides
BW	Body Weight
BXU	Birch Xylan Units
CON	Control
Cr_2O_3	Chromic Oxide
DDGS	Distillers Dried Grains with Solubles
DM	Dry Matter
DNA	Deoxyribonucleic Acid
DP	Degree of Polymerisation
E. coli	*Escherichia coli*

EU	European Union
FAO	Food and Agricultural Organisation
FCR	Feed Conversion Ratio
FOS	Fructo-oligosaccharides
fuc	Fucose
G:F	Gain to Feed ratio
gal	Galactose
galA	Galacturonic Acid
GC	Gas-liquid Chromatography
GE	Gross Energy
GF_2	Kestose
GF_3	Nystose
GF_4	Fructosylnystose
GIT	Gastro-Intestinal Tract
glcA	Glucuronic Acid
glu	Glucose
GOS	Galacto-oligosaccharides
HPIC	High Pressure Ion Chromatography
HPLC	High Performance Liquid Chromatography
INU	Inulin
LAB	Lactic Acid Bacteria

LDH	L-Lactate Dehydrogenase
man	Mannose
MOS	Mannan-oligosaccharides
NCP	Non-Cellulosic Polysaccharides
NGP	Non-Glucosyl Polysaccharides
NMDS	Non-Metric Multidimensional Scaling
NSP	Non-Starch Polysaccharides
OTUs	Operational Taxonomic Units
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis Of Variance
PYY	Peptide tyrosine
rha	Rhamnose
rRNA	Ribosomal Ribonucleic Acid
RSM	Rapeseed Meal
RUMA	Responsible Use of Medicines in Agriculture alliance
SBS	Short-Bowel Syndrome
SCFAs	Short-Chain Fatty Acids
SCT	L-Lactate Scout device
SD	Standard Deviation
SEM	Standard Error of the Mean

TCA	Trichloroacetic Acid
TiO_2	Titanium Dioxide
TOS	Trans-galactooligosaccharides
TP	Timepoint
UK	United Kingdom
XOS	Xylo-oligosaccharides
XYL	Xylanase
xyl	Xylose
ZnO	Zinc Oxide

Chapter 1

General introduction

1.1 United Kingdom (UK) pig industry

The UK pig industry comprises 5.1 million pigs and produces 922,000 tonnes of pork each year with a value of £1.32 billion (DEFRA, 2019). Pig production in the UK consists of ~60 % indoor and ~40 % outdoor production, with varying systems in place including slatted, partially slatted or straw based accommodation. The volume of animal feed supplied to the UK agricultural industry equates to 30.6 million tonnes and has a value of £5.5 billion per year (DEFRA, 2019). Of the 13.2 million tonnes of compound feed supplied, approximately 2.1 million tonnes are used in the pork supply chain (DEFRA, 2019). For producers to make a profit in pig farming, the cost to produce each pig must be lower than the total amount received for the carcass.

1.2 Challenges in the pig industry

With the global population forecasted to increase to 9.1 billion people by 2050 and with over one-third more mouths to feed, the Food and Agricultural Organisation (FAO) have predicted that 70 % more food will need to be produced to meet this demand (FAO, 2009). Regarding meat production, this equates to a requirement for an extra 200 million tonnes to reach a total of 470 million tonnes by 2050. This heightened demand for human food production naturally increases the demand for sustainable raw material supply and feed production. Increasing the efficiency in which food is produced is therefore paramount to supply this demand. Efficient meat production requires a holistic approach by improving a multitude of factors including animal nutrition, biosecurity, on-farm management, genetics, animal welfare and animal health. To this point, with feed accounting for up to 60 % of pig production costs, improving feed efficiency is one of the biggest drivers to improve the scale and profitability of the pig industry, particularly with volatile prices.

However, maximisation of feed efficiency is only possible in healthy animals, hence improving and maintaining the health of production animals is fundamental to sustainable pig production.

Antimicrobial drugs are medicines that are active against various infections, including those caused by bacteria, fungi, viruses and parasites, and are used in human and veterinary medicine to treat and prevent disease (O'Neill, 2016). Historically, antimicrobials were routinely included in pig feed as a method of reducing pathogenic bacteria and associated disease, thus consequently resulting in improved pig growth performance. However, on the 1st January 2006, the use of antibiotics in animal feed for non-medicinal growth promoting effects were banned in the European Union (EU). Prophylactic or metaphylactic antimicrobial treatment is still common with the intention that medication is only administered for a short period of time when there is a risk of an infectious outbreak (Barton, 2014). However, overuse or extended use of antimicrobials is common in animal production to prevent disease from reoccurring. Overuse of antimicrobials is a major driver for the emergence and spread of resistant bacteria, consequently threatening the treatment of human and animal infections (Barton, 2014). Antimicrobial resistance (AMR) occurs when microorganisms like bacteria survive exposure to a medicine that would usually stop their growth or kill them. This therefore allows resistant strains to grow and spread due to a lack of competition from other strains, which has ultimately led to the emergence of ‘superbugs’ like methicillin-resistant *Staphylococcus aureus*. ‘Superbugs’ are extremely difficult or even impossible to treat and claim the lives of 700,000 people each year globally (O'Neill, 2016). Indeed, resistance to antimicrobials is a natural process which has been observed since the initial discovery of antibiotics, yet AMR is increasing at a worryingly fast rate due to overuse of antimicrobials in both human and livestock medicine, with no new drugs for treatment.

Moreover, there are environmental concerns regarding residual antimicrobials and AMR genes entering the environment via the land application of animal waste, which can further contaminate surface and groundwater (Joy et al., 2013). Due to these concerns, the agricultural industry is under heightened pressure to reduce the use of antimicrobials. Associations in the UK like the Responsible use of medicines in agriculture alliance (RUMA) and the Agriculture and Horticulture Development Board (AHDB) are driving this change by providing reduction targets, practical strategies to reduce the need for antimicrobials and reporting systems to aid the accurate recording of usage. Since this task force was launched, great progress has been made in reducing, refining and replacing antimicrobials in UK farming, whereby overall sales in 2019 were 50 % lower (31 mg/kg) than in 2014 (RUMA, 2020). Despite this, further reductions are required, and the UK government has launched a 5-year national action plan to tackle AMR (HM Government, 2019).

Although great strides forward have been made in antimicrobial reduction, the pig industry faces further challenges in maintaining pig health. Weaning is a stressful period in a pig's life. A variety of stressors are responsible including a change in diet composition from the sow's milk to solid feed, internal physiological and immunological changes, social changes as well as environmental and bacterial challenges (Campbell, J.M. et al., 2013; Kim, J. et al., 2012). The combined effect of these factors makes the young pig vulnerable to disease, hence weaning is often associated with infection, disease, diarrhoea, high mortality and reduced performance (Bosi et al., 2004). Since the antibiotic growth promoter ban in 2006 , the UK pig industry has relied on feeding therapeutic levels of Zinc oxide (ZnO; 2500 ppm) for 14 days post-weaning to reduce the need for in feed antimicrobials, prevent diarrhoea and improve health and growth. However, there are increasing concerns regarding the low absorption of ZnO in the body

and its consequent excretion via the faeces (Milani et al., 2017). Concerns therefore relate to environmental pollution and its toxic effects on microorganisms and plants via soil accumulation, as well as its potential role in bacterial resistance (Gräber et al., 2005; Yazdankhah et al., 2014). Due to these concerns, the EU has stated that the use of therapeutic ZnO will be phased out by 2022 (EU Commission, 2017), meaning alternatives need to be sought. Coupled with the requirement to reduce antimicrobial usage, the ZnO ban has increased the demand for alternative feeding concepts to improve gut health, enhance disease resistance and support growth performance in pigs.

1.3 Gut health

The term ‘gut health’ is increasingly used in medical and veterinary literature, as well as in the food industry (Bischoff, 2011). This term covers multiple aspects of the gastro-intestinal tract (GIT), including the effective digestion and absorption of food, a normal and stable intestinal microbiome, the absence of GIT illness, effective immune status and a state of well-being (Bischoff, 2011). It is however still very unclear exactly what good gut health is, how it can be defined and how it can be measured, especially as great variation exists in what is considered a normal and healthy GIT (Bischoff, 2011; Cummings et al., 2004).

To sustain life, the GIT must function first and foremost as a means of digesting food and absorbing nutrients that are vital for maintenance and growth of the host. To enable efficient digestion and absorption, the GIT must first breakdown ingested food via physical and chemical processes, before transporting luminal nutrients, water and electrolytes across the gut wall and into the circulation (Moeser et al., 2017). At the same time, large influxes of toxins, antigens and pathogens into the lumen means the gut

epithelium is constantly exposed to potential pathogenic components (Moeser et al., 2017). Hence, the gut wall must act as a barrier to these negative components to prevent infectious, inflammatory and functional GIT disease (Bischoff, 2011). Concurrently, selective uptake of microbial antigens is important in the development of the mucosal immune system (Moeser et al., 2017). Thus, the function of the gut wall is very complex and multiple barrier mechanisms exist to enable the GIT to perform such a wide range of functions (Moeser et al., 2017).

The GIT bacterial community has been studied for many decades due to its importance in the health and wellbeing of animals and is defined as 'the totality of the microbes, their genetic elements and the environmental interactions in a particular environment' (Kim, H B and Isaacson, 2015). The bacterial content of the mammalian GIT consists of ~10^{14} bacteria and has a collective genome of 3 million genes, creating a diverse bacterial community that has the potential to provide vast biological activities that the host lacks (Isaacson and Kim, 2012). The GIT bacterial community maintains a symbiotic relationship with the gut mucosa and provides substantial metabolic, immunological and protective functions in healthy individuals (Jandhyala et al., 2015). The GIT bacterial community ferment substrates that escape endogenous digestion and reach the hindgut, whereby fermentation end-products provide 5-20 % of the hosts energy requirement (Kim, Hyeun Bum et al., 2011). Hence, the structure and composition of the bacterial community reflects natural selection at two levels; firstly, the host, whereby sub-optimal fermentation and functionality of the GIT bacterial community can reduce energy provision, host growth and fitness (Bäckhed et al., 2005). Secondly, at the microbial level, whereby the growth rate and substrate utilisation efficiency affects the fitness of the individual bacteria in a competitive environment (Bäckhed et al., 2005).

Before bacterial stability is reached at ~ 5 weeks of age, the GIT bacterial community is relatively dynamic and fluid in its response to various factors including time of day, microbe exposure, diet, environment and stress (Kim, Hyeun Bum et al., 2011; Thompson et al., 2008). For example, animals are born with a lack of a microbial community in the GIT, but during birth and thereafter, exposure to microbes increases and a process of bacterial succession occurs (Isaacson and Kim, 2012). Succession of bacterial populations occurs as the animal ages, with dietary adaptations and as the GIT tract moves to an anaerobic state; this succession continues until a relatively stable community is established (Isaacson and Kim, 2012). It has been shown that as young broilers grow and a stable bacterial community is established, the ability of the bacteria to ferment arabinoxylans (AX) increases with age, providing beneficial improvements in digestibility and energy provision (Bautil et al., 2019). However, large disruptions in the GIT bacterial community can allow pathogenic bacteria to thrive and ultimately cause disease, hence the GIT ecosystem is complex and dynamic in phases when the bacterial community is not stable.

The ability of the bacterial community to change and respond to dietary adaptations has gained much attention in the literature for the potential to positively shift the bacterial community and metabolic capacity to the benefit of the host. This hypothesis suggests that changes in diet should result in bacterial community composition and metabolic changes that can improve substrate utilisation and energy provision, inhibit digestive diseases, enhance beneficial bacterial balance as well as improving host growth (Kim, H B and Isaacson, 2015; Isaacson and Kim, 2012). To this point, the role of many functional ingredients and additives have been considered including acids, probiotics, prebiotics, enzymes, plant extracts and essential oils. Among others, prebiotics have shown

promising results in this area of bacterial community composition and metabolic capacity manipulation to improve host growth and health.

1.4 Prebiotics

In 1995, the prebiotic concept was introduced, and prebiotics were defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the gut, and thus improving host health" (Gibson and Roberfroid, 1995). For compounds to classify as a prebiotic they must meet various criteria. Firstly, prebiotics should be resistant to the acidic environment of the stomach, must not be hydrolysed by endogenous enzymes, and not be absorbed in the GIT (Gibson et al., 2004). Secondly, the prebiotic must be fermented by GIT bacteria, and finally, the growth and/or activity the GIT bacteria must be selectively stimulated by the prebiotic to provide benefits to the health of the host (Gibson et al., 2004). An improvement in pig health through the beneficial activities of its own microflora results in reduced use of antimicrobials, hence prebiotics are considered as potential antimicrobial alternatives.

1.4.1 Sources, types and production

Dietary fibre is defined as plant-based carbohydrates that cannot be broken down by digestive enzymes, but instead are fermented in the large intestine. Non-starch polysaccharides (NSP) are complex carbohydrates that form the main part of dietary fibre and include cellulose, pectins, glucans, gums, mucilages, inulin and chitin (Bender and Cunningham, 2021). The largest constituent of the plant cell wall is cellulose (40 – 45 %), a water-insoluble, linear polymer of glucose monomers which are rotated 180 ºC in respect to the adjacent moiety and linked by β-1,4-glycosidic bonds (Bedford, M.R. and

Partridge, 2010). The second largest structural polysaccharide (30-35%) of the plant cell wall is hemicellulose, which is structurally associated with cellulose in the walls and is found in all terrestrial plants (Bedford, M.R. and Partridge, 2010). Hemicelluloses' are named according to the main sugar residue in the polymer backbone, hence, xylans are polymers with D-xylose units, mannans with D-mannose units, galactans with D-galactose units or arabinans with L-arabinose units (Bedford, M.R. and Partridge, 2010). Xylan is the major component of hemicellulose, and after cellulose is the second most abundant polysaccharide in nature, hence due to the high presence of xylans in plants it is a major component in animal feed. There are various types of prebiotics with the majority being a subset of carbohydrate groups classed as non-digestible oligosaccharides (Davani-Davari et al., 2019). Non-digestible oligosaccharides are low molecular weight carbohydrates between simple sugars and polysaccharides (Mussatto and Mancilha, 2007). Commercial production of prebiotic oligosaccharides involves either creation from simple sugars by enzymatic transglycosylation, or formation by controlled enzymatic hydrolysis reactions of polysaccharides like xylan (Mussatto and Mancilha, 2007). The most commonly known prebiotics include galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS) and inulin (INU) but there are different prebiotic types as summarised in Table 1.1 (Al-Sheraji et al., 2013). This thesis will focus mainly on xylo-oligosaccharides (XOS) and fructans, including short-chain FOS and long-chain INU.

Table 1.1 Types, sources and example inclusions of prebiotics in pigs diets, adapted from Al-Sheraji et al. (2013).

Type of prebiotic	Sources of prebiotic	Example inclusion levels in pig diets (g/kg)
Fructans [Fructo-oligosaccharides (FOS) and inulin (INU)]	Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato and rye (Sangeetha et al., 2005).	FOS = 0 - 6 g/kg (Xu, Z.R. et al., 2002) INU = 0 – 5 g/kg (He et al., 2002)
Xylo-oligosaccharides	Bamboo shoots, fruits, vegetables, milk, honey and wheat bran (Vazquez et al., 2000).	0.2 g/kg (Liu, J. et al., 2018)
Galacto-oligosaccharides (GOS) or t*rans*-GOS (TOS)	Human's milk and cow's milk (Alander et al., 2001).	0-20 g/kg (Houdijk, JGM et al., 1998)
Raffinose oligosaccharides	Seeds of legumes, lentils, peas, beans, chickpeas, mallow composite, and mustard (Johansen et al., 1996).	2 – 5 g/kg (Zeng et al., 2021)
Stachyose oligosaccharides	Soybean (Mussatto and Mancilha, 2007).	0 - 20 g/kg (Liying et al., 2003)

1.4.1.1 XOS

XOS are sugar oligomers made up of xylose (xyl) monomers linked together by β-(1→4)-linkages. The structure of commercial XOS varies by degree of polymerisation (DP) and type of linkages depending on the source of xylan that is used for production (Aachary and Prapulla, 2011). In general, XOS consists of mixtures of xyl residues from 2 to 10 units, known as xylose, xylobiose, xylotriose, and so forth as seen in Figure 1.1 (Vazquez et al., 2000). In addition to xyl residues, xylan often has associated side groups such as α-D-glucopyranosyl, uronic acid or its 4-O-methyl derivative, acetyl groups, or arabinofuranosyl residues (Aachary and Prapulla, 2011). Hence, these side groups create a branched XOS molecule such as arabinoxylan-oligosaccharides (AXOS). Commercial XOS are produced from xylan containing raw materials like corncobs by chemical methods (Nabarlatz et al., 2007), direct enzymatic hydrolysis from raw materials (Katapodis and Christakopoulos, 2005) or a combination of both chemical and enzymatic methods (Yang, R. et al., 2005).

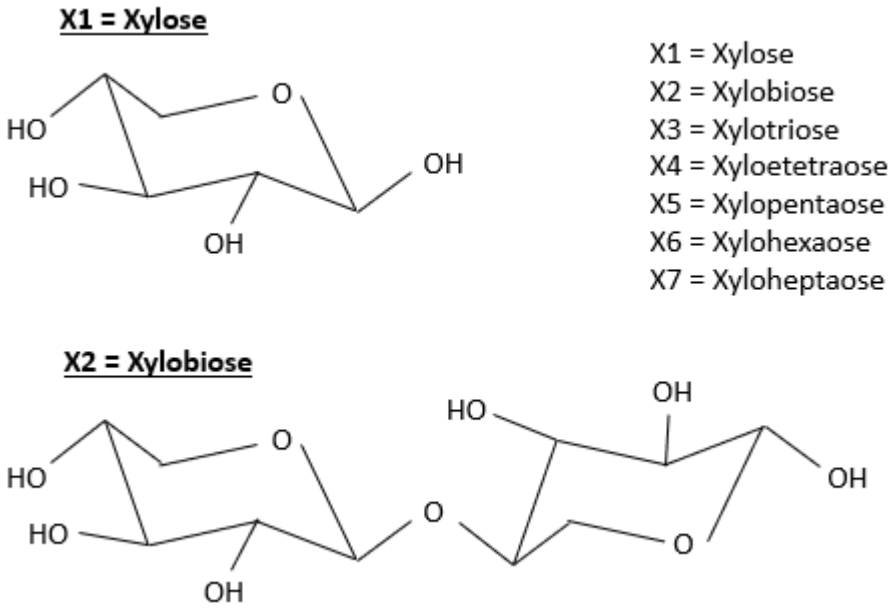

Figure 1.1: Schematic structure of xylose and xylo-oligosaccharides (XOS).

1.4.1.1.1 XOS production from xylanase (XYL)

Plants contain a wide variety of polysaccharides which are separated into two groups according to structure and function; such that starches are polymers of glucose (glu) with α-glycosidic linkages, whilst the remaining polysaccharides are referred to as NSPs containing β-linkages (Englyst, H.N. et al., 1994). Xylans, β-glucans and cellulose are the most prominent type of NSPs in cereal grains but the quantity of each type varies (Choct, 1997). Large amounts of soluble and insoluble NSPs are found in wheat and rye, with arabinoxylan being the main soluble component (Choct, 1997). Soluble xylans are anti-nutritive in the GIT as their higher molecular weight fractions (i.e. larger than oligomers) can dissolve and form viscous aggregates within the digesta. Increased viscosity traps nutrients leaving them inaccessible to digestive enzymes, hence reduces nutrient digestibility and slows the rate of passage through the GIT, which can ultimately reduce feed intake and host growth (Olukosi, O.A. et al., 2015). The combination of a slower

rate of passage and an increased level of undigested nutrients can also cause the proliferation of adverse pathogenic bacteria (Masey O'Neill et al., 2014). Further to this, the majority of energy in cereals comes from intracellularly-stored starch, but along with other nutrients like protein, they are trapped within cells rendering them inaccessible to the host due to a lack of endogenous enzyme production to degrade plant cell walls (Masey O'Neill et al., 2014). For the reasons described above, NSPs are often perceived as an anti-nutritional factor in monogastric nutrition. However, addition of exogenous β-1,4-XYL can alleviate the anti-nutritive effects described above. XYL hydrolyses the xylan polysaccharide, hence decreases digesta viscosity, releases entrapped nutrients and improves the nutritive value of feed and host growth (Masey O'Neill et al., 2014). Yet, a less well-studied mechanism of XYL and its benefits on performance is the indirect provision of fermentable AXOS and XOS from the hydrolysis of the xylan backbone and its potential prebiotic effect. Hence, provision of AXOS and XOS to the hind gut can be indirect via the supplementation of XYL and production of AXOS and XOS *in situ*, or direct via the addition of commercially produced XOS.

1.4.1.2 Fructans

Fructan is a general term used for any carbohydrate where one or more of the fructosyl-fructose links makes up the majority of the osidic bonds (Roberfroid and Delzenne, 1998). Fructans are sugar oligomers and polymers made up of β-D-fructofuranosyl monomers linked together by β-(2→1) linkages, usually with a terminal glu unit (Roberfroid and Delzenne, 1998; Davani-Davari et al., 2019). FOS consists of mixtures of fructose residues with varying numbers of fructose monomers, for example, kestose (GF_2), nystose (GF_3), fructosylnystose (GF_4) and so forth, as seen in Figure 1.2. Further fructose addition to the molecule creates long-chain INU (Figure 1.2). INU-type fructans naturally occur in many plants but are mainly extracted from sources such as the agave plant, chicory

root, or Jerusalem artichoke for commercial production (Grela et al., 2014). The DP of fructans differs greatly between plant origins. INU extracted from chicory roots typically has a DP between 3 and 60 (Van De Wiele et al., 2007) whereas for native Jerusalem artichoke the average DP is 6 (De Leenheer, 1994). Chemical degradation or controlled enzymatic hydrolysis of INU produces oligofructose compounds with an average DP between 2 and 20 (Van De Wiele et al., 2007). Alternatively, short-chain FOS can be produced by transfructosylation and typically have a DP of between 2 and 4 (Van De Wiele et al., 2007).

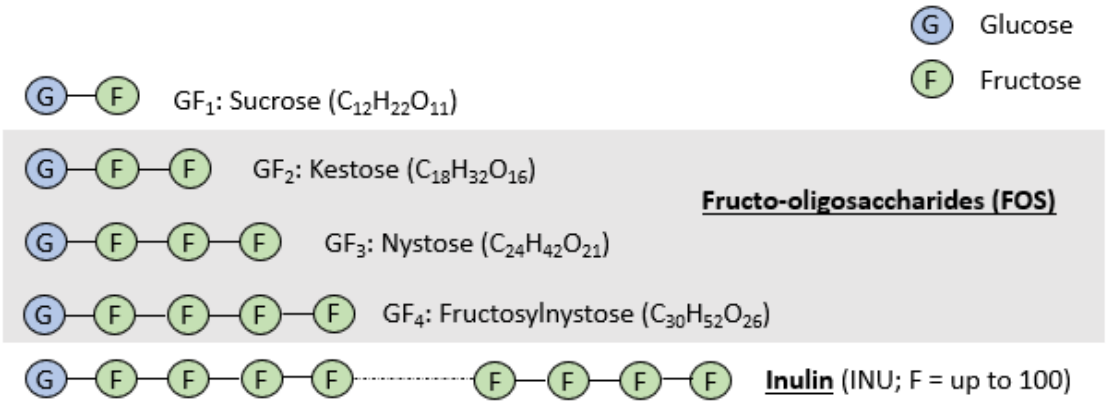

Figure 1.2: Schematic of fructo-oligosaccharides (FOS) and inulin (INU).

1.4.2 Prebiotic Functionality

1.4.2.1 Bacterial modulation

Glycosidic bonds that contain β conformation of the C2 atom cannot be digested by mammalian endogenous enzymes (Csernus and Czeglédi, 2020). Hence, these carbohydrates survive digestion and reach the hind GIT where they act as substrates for fermentation by the GIT bacterial community. By provision of an energy source for the bacteria, prebiotics have the ability to modulate the composition and functionality of the GIT bacterial community (Davani-Davari et al., 2019). Modulation of the bacterial

community to selectively promote the growth and activity of beneficial bacteria and inhibit pathogenic bacteria is of importance to maintain and improve the health of pigs.

Lactic acid bacteria (LAB) are a group of gram-positive, acid-tolerant bacteria that are associated by their common metabolic characteristics, such as the production of lactic acid as the major metabolic end-product of carbohydrate fermentation (Yang, F. et al., 2015). LAB such as *Lactobacillus* spp and *Bifidobacterium* spp, have shown beneficial effects on the regulation of the intestinal microflora, inhibition of GIT pathogens, improving intestinal mucosal immunity and maintaining intestinal barrier function, all of which contributes to the health and growth of the host (Yang, F. et al., 2015). Contrary to beneficial bacteria, pathogenic bacteria like *Escherichia coli* (*E. coli)* and *Salmonella enterica* exploit sources of carbon and nitrogen in the GIT environment as nutrients and regulatory signals to promote their own virulence and growth (Bäumler and Sperandio, 2016). Release of cytokines during an inflammatory response triggers the release of reactive oxygen and nitrogen species from the intestinal epithelium, which react to form nitrates (Bäumler and Sperandio, 2016). Elevated levels of nitrates drive the growth of bacteria like Enterobacteriaceae as they are able to produce nitrate reductase which couples the reduction of nitrate to energy-conserving electron transport systems for respiration (Bäumler and Sperandio, 2016). Hence, a niche is created in the lumen of the intestines that supports the uncontrolled expansion of the pathogenic bacteria like Enterobacteriaceae which ultimately leads to disease. On the contrary, obligate anaerobic bacteria lack the conserved ability to produce nitrate reductase hence do not thrive. Common pathogenic bacteria in pigs include *E. coli*, *Clostridium perfringens*, *Clostridium difficile* and *Salmonella enterica* (Li, 2017). Typical symptoms of these bacterial infections include intestinal lesions, diarrhoea, host growth reduction, necrohaemorrhagic enteritis and high mortality (Li, 2017). To this point, prebiotics that

selectively promote the growth and activity of beneficial bacteria whilst reducing pathogenic bacteria are of great importance.

1.4.2.1.1 *In vitro* fermentation

The fermentation of prebiotics in the GIT depends on a multitude of factors including the sugar composition, DP, type of linkages, structure complexity, as well as the presence of substrate specific degrading bacteria and access to the substrate itself within the GIT (Houdijk, Jos, 1998). Hence, together these factors determine the site and rate of fermentation as well as the bacterial stimulation effect. The effect of these factors on substrate fermentability have mainly been studied *in vitro.*

In vitro fermentability studies investigating XOS have previously shown that it is a good substrate for the growth of many different *Bifidobacterium* species and specific species like *Lactobacillus brevis* (Wang et al., 2010; Crittenden et al., 2002). However, it is clear that bacterial preference exists whereby certain species of *Bifidobacterium* such a *B. breve* showed no growth with XOS (Wang et al., 2010) along with the majority of other *Lactobacillus* species studied (Crittenden et al., 2002). XOS has also been shown to be fermented by some *Bacteroides* isolates but not by classic pathogenic bacteria such as *E. coli, Enterococcus, Clostridium difficile* or *Clostridium perfringens* (Crittenden et al., 2002). Another *in vitro* study found that *B. adolescentis* and *L. brevis* had the highest growth and fermentation of XOS compared to other strains (Moura, Patrícia et al., 2007), agreeing with the results from Crittenden et al. (2002) and Wang et al. (2010).

Given that prebiotics differ in their structural composition logically means GIT bacterial species will have varying abilities to utilise different prebiotic substrates according to

their specific polysaccharide degrading machinery. When comparing the *in vitro* fermentation of both XOS and FOS by the same human inoculum, XOS showed the highest increases in numbers of *Bifidobacterium* whilst FOS produced the highest numbers of *Lactobacillus* (Rycroft et al., 2001). Moreover, another study showed that both short-chain FOS (DP 2-3) and XOS (DP 2-7) have previously shown no growth of *Salmonella typhimurium* (De Figueiredo et al., 2020). In addition, bacterial strains again showed preferences for different substrates such that *Bifidobacterium breve* and *Lactobacillus brevis* preferred XOS, disagreeing with the results from Wang et al. (2010) and agreeing with the results from Crittenden et al. (2002). Whilst *Bifidobacterium lactis* and *Lactobacillus acidophilus* preferred FOS (De Figueiredo et al., 2020). The only tested strain to grow on both XOS and FOS was *B. longum* whilst *B. animalis* was unable to ferment any of the substrates. This highlights the highly selective nature of GIT bacteria and explains the variability of results in the literature given that only a small number of targeted species are usually investigated in prebiotic studies.

The DP of oligomers can influence bacterial substrate preference and utilisation. For example, *B. adolescentis* has been shown to mainly ferment XOS mixtures consisting of xylobiose, xylotriose and xylotetraose, with an increase in DP to 5-6 resulting in a reduction in the degree of fermentation (Moura, Patrícia et al., 2007). In contrast, *L. brevis* prefers short-chains where the XOS mixture mainly consisted of xylobiose (Moura, Patrícia et al., 2007). For fructans, *in vitro* fermentability studies have shown that short-, medium- and long-chain fructans are fermented by human (Hernot et al., 2009) and swine (Smiricky-Tjardes et al., 2003a) faecal inoculum. The growth of *Lactobacillus* increased similarly among fructan substrates of differing length, whilst the greatest increase in *Bifidobacterium* was seen with short-chain fructans (Hernot et al., 2009). To this point, it has previously been reported that the ability to simulate the growth of *Bifidobacterium* by

short-chain carbohydrates (DP 2-10) is approximately an order of magnitude higher than that of substrates with a DP > 10 (Roberfroid et al., 1998). Hence, the utilisation patterns of prebiotic substrates also depend on the bacterial preference for differing DP.

The presence of bacterial species capable of utilising differing oligomers dictates the rate of fermentation and consequent location of GIT fermentation. For example, short-chain fructans are more rapidly fermented than those with a greater DP (Hernot et al., 2009; Van De Wiele et al., 2007). When investigating the *in vitro* fermentability of XOS with varying DP by piglet ileal, caecal and distal colonic digesta, short-chain (DP = 2-5), medium-chain (DP = 2-14) and long-chain (DP = 2-25) XOS were all extensively fermented (Moura, P. et al., 2008). However, the rate of fermentation of medium- and long-chain XOS in the ileum was reduced compared to short-chain XOS, indicating an improved fermentation efficiency of the foregut microbiota to utilise XOS with a low DP. In contrast, another study found that the rate of *in vitro* fermentation of short-chain XOS by swine faeces was slower when compared to GOS and soy solubles, leading to the conclusion that XOS was a good substrate for the distal portion of the GIT (Smiricky-Tjardes et al., 2003a). However, there were differences in the studies, including different inoculum and different ages of pigs for sample collection. Moreover, another *in vitro* model showed that both FOS and INU stimulated LAB in the proximal and distal sections of the colon, with longer-chain INU having more pronounced beneficial effects in the colonic microbiota compared to short-chain FOS (Van De Wiele et al., 2007).

Hence, in order to stimulate a wide range of probiotic bacteria it is evident that a mixture of different prebiotics is required. To this point, an *in vitro* study found that mixing FOS and INU together was effective at reducing the amount of gas produced whilst maintaining or increasing the growth of *Bifidobacterium* as the highest prebiotic index

was obtained with FOS alone and FOS and INU combined (Ghoddusi et al., 2007). Albeit, there has been minimal focus on investigating the effect of combining prebiotic substrates and their mode of action within the GIT.

1.4.2.1.2 *In vivo* fermentation

The effect of XOS and fructans on their ability to selectively promote the growth and activity of probiotic bacteria has been studied *in vivo* in many species. In rats, both XOS and FOS have been shown to increase the population of *Bifidobacterium* in the colon, with XOS having a greater effect than FOS (Hsu et al., 2004). In mice, XOS has been shown to increase *Lactobacillus* by 10-fold in the large intestine and produced the highest counts of *Bifidobacterium,* whilst also reducing *Clostridium* (Santos et al., 2006). Whilst another study showed that XOS was the preferential substrate for beneficial bacteria, mainly *Bifidobacterium,* in the caecum of broilers (Ribeiro et al., 2018).

In vivo studies with weanling pigs have shown that supplementation of XOS decreased faecal *E. coli* counts and increased *Lactobacillus* (Liu, J. et al., 2018). Similarly, XOS has been shown to increase the abundance of the *Lactobacillus* genus in the ileum and caecum of piglets, whilst also reducing the level of *Clostridium_sensu_stricto_1* and *Escherichia-Shigella* (Chen, Y. et al., 2021). The effects of fructans on gut bacterial composition in pigs has been extensively reviewed by Csernus and Czeglédi (2020). Some studies have shown no effect of short-chain FOS (Farnworth et al., 1992) or long-chain INU (Branner et al., 2004) on the GIT bacterial profile of pigs. However, other studies have shown increases in levels of beneficial LAB when feeding fructans of differing lengths (Paßlack et al., 2012; Zhao et al., 2013; Xu, Z.R. et al., 2002). Hence, it is clear that both XOS and fructans of differing lengths promote the levels of beneficial LAB in many species.

1.4.2.2 Fermentation metabolites

1.4.2.2.1 Short-chain fatty acids (SCFAs)

Substrates that reach the hindgut are fermented into several metabolites by GIT bacteria, such as SCFAs primarily constituting of acetate, propionate and butyrate. These SCFAs generally occur in ratios ranging from 75:15:10 to 40:40:20 (Trachsel, 2017). Other SCFAs are also produced in small quantities like valerate and caproate, or are classed as metabolic intermediates like lactate and succinate as they are rapidly converted to other SCFAs by GIT bacteria (Trachsel, 2017). SCFAs are readily absorbed, and 95 % are absorbed by the colonocytes in the caecum and large intestine (den Besten et al., 2013). SCFA production is thought to provide ~10 % and ~30 % of human and swine daily energy requirements and colonocytes derive 60-70 % of their energy from SCFA oxidation, with butyrate being the preferential source (den Besten et al., 2013; Bergman, 1990). Once absorbed, acetate acts as an energy source in the tricarboxylic acid cycle and is also utilised in the production of fats and lipids (Trachsel, 2017). Propionate is largely utilised as a substrate for gluconeogenesis in the liver but has also been shown to be an energy source for immune cells (Trachsel, 2017). Whilst the main role of butyrate is as an energy source for colonocytes, with any remaining butyrate being oxidised by hepatocytes to prevent toxic concentrations (Trachsel, 2017). Oxidising butyrate also consumes large amounts of oxygen which reduces the quantity of electron acceptors available for microbial respiration. This shift consequently favours species that use fermentative metabolism and prevents the overgrowth of facultative anaerobic bacteria like pathogenic *Salmonella* and *E. coli.* Butyrate has also been shown to induce transcriptional changes in the gut epithelium, for example by inducing expression of antimicrobial peptides in colonic mucosa (Campbell, Y. et al., 2012). Moreover, butyrate has been shown to increase the expression of tight junction proteins and decrease epithelial permeability, all of which contributes to improved gut barrier function. Butyrate

also plays important roles in anti-inflammatory effects whereby inflammation is reduced by down-regulating the activity of pro-inflammatory mediators such as interleukin-6 and interleukin-12 (Chang et al., 2014). Taken together, all of these butyrogenic effects have a positive effect on gut function and homeostasis.

1.4.2.2.2 pH

The presence of SCFAs in the GIT lumen creates an acidic environment such that the pH of the gut lumen decreases. A reduction in pH can alter the GIT bacterial community composition, prevent the overgrowth of pH-sensitive pathogenic bacteria like *Enterobacteriaceae* and *Clostridium*, and aid effective mineral solubilisation (den Besten et al., 2013; Csernus and Czeglédi, 2020). It has previously been shown that butyrate-producing bacteria such as *Roseburia* spp. and *Faecalibacterium prausnitzii* occupy an environment around pH 5.5, whereas butyrate-producing bacteria almost disappear and acetate- and propionate-producing bacteria become dominate at ~ pH 6.5 (den Besten et al., 2013). Hence, the fermentation of prebiotic substrates that produce SCFAs and reduce pH, therefore promotes the growth of beneficial butyrate-producing bacteria. To this point, a lower pH in the ileum of weaner pigs has been demonstrated with the addition of FOS, along with an increase in propionic and lactic acid (Houdijk, J.G.M. et al., 2002). Whilst *in vitro* studies have confirmed production of SCFAs during the fermentation of XOS (Wang et al., 2010; Moura, P. et al., 2008) and fructans (Van De Wiele et al., 2007; Stewart et al., 2008; Houdijk, J.G.M. et al., 2002). Similarly, i*n vivo* supplementation of XOS and FOS have been shown to increase the SCFA concentrations in the caecum of weaned piglets (Chen, Y. et al., 2021; Liu, L. et al., 2020).

1.4.2.2.3 Lactate and bacterial-cross feeding

Prebiotic effects can be enhanced by bacterial cross-feeding, which is defined as the metabolic product of one species which can be consumed by another (Davani-Davari et al., 2019). For example, bacterial species that are known to be involved in the fermentation of resistant starch include *Bifidobacterium, Prevotella,* and *Mitsuokella* (Trachsel, 2017). These organisms degrade resistant starch into small polysaccharides or other metabolic intermediates such as lactate, the latter of which then subsequently acts as a carbon source for other bacteria (Trachsel, 2017). Consequently, numbers of these bacteria, such as *Veillonella, Megasphaera elsdenii,* and *Anaerostipes ceccae,* naturally increase in order to utilise the lactate substrate (Muñoz-Tamayo et al., 2011; Duncan et al., 2004). *Megasphaera elsdenii* and *Anaerostipes ceccae* also produce butyrate by utilising the lactate, thus conferring additional benefits to the host, which highlights the importance of cross-feeding in butyrate production (Muñoz-Tamayo et al., 2011).

The extensive fermentation of XOS and fructans by *Bifidobacterium*, *Lactobacillus* and *Bacteroides* likely leads to the accumulation of metabolic intermediates like lactate which become available for cross-feeding by non-degrading species (Santos et al., 2006). The conversion of lactate to other SCFAs has been demonstrated in the caecum of growing pigs whereby the content of lactate linearly decreased as concentrations of acetate, propionate and butyrate increased (Brestenský et al., 2017). Due to this rapid conversion, intermediate metabolites like lactate are seldom detected in the faeces of healthy subjects (Duncan et al., 2004). However, lactate has been shown to accumulate in the faeces up to ~100 mM in individuals who have undergone gut resections (short-bowel syndrome; SBS) or who are sufferers of ulcerative colitis (Hove et al., 1994; Kaneko et al., 1997; Vernia et al., 1988). In SBS patients, an increased load of undigested carbohydrates leads to increased fermentation and production of metabolites like lactate which exceeds the

amount that can be metabolised or absorbed, hence an accumulation of lactate occurs and pH reduces (Kowlgi and Chhabra, 2015). The more acidic environment leads to further growth of lactate-producing species and contributes further to the lactate pool. Colonic infusions of donor human intestinal bacterial communities have been reported to permanently reverse the condition in certain cases, hence microbial balance is a likely contributing factor (Borody et al., 2003). To this point, an increase in the abundance of lactate-producing bacteria, and/or the reduction of lactate-utilising bacteria leads to lactate accumulation and is a potential risk for diarrhoea (Saunders and Sillery, 1982). Hence, bacterial lactate production in the GIT is mainly beneficial, providing the balance of lactate producers and utilisers are in equilibrium.

1.4.2.3 Digestibility

1.4.2.3.1 Nutrient digestibility

Many studies have shown that the supplementation of prebiotics results in improved nutrient digestibility in pigs (Liu, J. et al., 2018; Liu, P. et al., 2008; Zhao et al., 2013; Zhao et al., 2012; Shim, 2005), whilst others have found no improvement in dogs (Swanson et al., 2002; Propst et al., 2003) or in pigs (Smiricky-Tjardes et al., 2003b). The reason for an improvement in nutrient digestibility is multi-factorial including an effect on host health via the stimulation of beneficial bacteria, reduction of pathogenic bacteria, improved intestinal morphology, lower intestinal pH, stimulation of mucosal immune system and bacterial enzyme production (Liu, J. et al., 2018).

The villus height to crypt depth ratio in the small intestine is a useful indicator to estimate the capacity of nutrient absorption as it has been shown that maximal absorption in newly weaned pigs occurs as the villus height to crypt depth ratio is increased (Pluske et al.,

1996). This is explained by a larger surface area for nutrient uptake which therefore leads to increased nutrient absorption, highlighting the benefit of feeding concepts that increase these gut morphology indicators. To this point, supplementation of XOS has been shown to increase the villus height and villus height to crypt depth ratio in the ileum of weanling pigs (Chen, Y. et al., 2021), whilst chito-oligosaccharides have shown the same effect in the ileum and jejunum (Liu, P. et al., 2008). This latter response was coupled with an increase in apparent total tract digestibility (ATTD) of dry matter (DM), gross energy (GE), crude protein, crude fat, calcium and phosphorus, as well as host growth (Liu, P. et al., 2008). Similar results have been seen in piglets with XOS supplementation whereby villus height to crypt death ratio increased in the jejunum as well as ATTD of DM, nitrogen and GE (Liu, J. et al., 2018). The authors attributed these results to increased beneficial and decreased pathogenic GIT bacteria, as well as an increased production of SCFAs by microbial fermentation causing a proliferation of enterocytes and hence increasing the absorption capacity in the gut. Increased epithelial cell proliferation is associated with decreased mucosal atrophy and accounts for increases in caecal and colonic weights (Hsu et al., 2004). Supplementation of XOS has been shown to increase the relative colonic wall and caecal wall weights of rats more effectively than FOS, indicating that XOS has a larger effect on epithelial cell proliferation than FOS (Hsu et al., 2004; Howard et al., 1995).

1.4.2.3.2 Fibre digestibility

Prebiotics like INU have been shown to increase the pre-caecal digestibility of crude fibre, indicating an increased degradation by the ileal bacterial communities (Böhmer et al., 2005). Similar results were also found by Kumprecht and Zobac (1998) whereby a combination of mannan-oligosaccharides (MOS) and *Enterococcus faecium* had the highest fibre digestibility compared to unsupplemented pigs. Moreover, trans-

galactooligosaccharides (TOS) have been shown to increase the ATTD of non-detergent fibre and hemicellulose (Mountzouris, K. et al., 2006), whilst other studies have shown no effect of FOS or TOS on the apparent ileal digestibility (AID) or ATTD of crude fibre (Houdijk, J.G. et al., 1999).

As previously discussed, XOS acts as a substrate for saccharolytic bacterial fermentation and produce SCFAs as main fermentation end-products. XOS has been shown to improve animal performance (+ 320g from day 0-42) by increasing feed digestion, feed intake and by stimulating the modulation of the bacterial community to a more favourable composition (Ribeiro et al., 2018). However, it has been demonstrated that the amount of substrate used to elicit these responses are relatively low (Courtin et al., 2008; Morgan, Natalie K et al., 2019; Ribeiro et al., 2018). An inclusion of 0.1 g/kg of XOS would equate to < 0.3 Kcal/kg, which even if converted to SCFAs with 100 % efficiency, would not be enough energy for the scale of performance response observed in broilers (Ribeiro et al., 2018). Hence, the authors concluded that XOS acts as a signal to encourage xylan-degrading bacteria to increase in activity, digest the xylan more efficiently and interact with the GIT in such a way that improves overall efficiency of digestion (Ribeiro et al., 2018). This concept has recently been termed ‘stimbiotic’ and is defined as ‘fermentable additives that stimulate fibre fermentability but at a dose that is too low that a stimbiotic itself could contribute to a meaningful level of SCFA production’ (Cho et al., 2020). To further study this concept, the direct measurement of NSPs provides a good index of the plant cell-wall material present in samples and the gut. The procedure for measuring NSPs was developed by Englyst in 1994, and involves the enzymatic removal of starch and summation of the constituent sugars released from acid hydrolysis (Englyst, H.N. et al., 1994). Individual monosaccharides can be measured by gas-liquid chromatography (GC)

or by high-performance liquid chromatography (HPLC), alternatively a single value for total sugars can be obtained using spectrophotometry (Englyst, H.N. et al., 1994).

There is a clear effect of age on the ability of the GIT bacteria to hydrolyse fibrous fractions like NSP, with the hydrolysing ability increasing as age increases and a more developed bacterial community is established. A recent study has shown that AX solubilisation occurs in the small intestine of young broilers, and the ability of the hindgut microbiota to ferment the AX substrates increased as the broiler aged (Bautil et al., 2019). This was demonstrated by increased AID, caecal digestibility and ATTD, coupled with an increase in AX-degrading enzyme activity with age. Hence, this highlights a window of opportunity to speed up the fibre degrading capability of the bacteria and provide benefits to the host from a younger age. To this point, compared to control (CON) fed broilers, the addition of AXOS has been shown to increase the solubilisation of insoluble AX into soluble fractions in the ileum (Bautil et al., 2020). This effect was not associated with increases in AX-hydrolysing enzymes as expected, but caution was raised over the accuracy of the enzyme results. Furthermore, the ATTD of total-AX was increased when AXOS was supplemented, resulting in a greater ability of the intestinal microbiota to break down the insoluble AX fraction. It was therefore postulated that AXOS had a clear stimulating effect on the dietary fibre-degrading capacity of the hindgut bacterial community in young broilers. This kick-starter effect was likely due to a combination of a quicker emergence of an AX-degrading microbiota and a microbial-modulated effect of decreasing the digesta transit rate allowing for greater dietary fibre hydrolysation.

As previously stated, provision of XOS can be direct via the addition of commercially produced oligomers or via the supplementation of XYL and production of AXOS and XOS *in situ*. Hence, XYL could exert similar kick-starter effects on increasing the fibre

degrading ability of the bacterial community. Both bran AXOS and XYL supplementation have been shown to improve feed utilisation in broilers, suggesting that the AXOS released *in situ* by the action of XYL contributes to the beneficial effects of feed supplemented XYLs (Courtin et al., 2008). Moreover, AXOS increased the abundance of caecal *Bifidobacterium*, an effect which was not seen with XYL addition, indicating that the mode of action of XYL and AXOS are not identical and suggests the latter has a larger microbiota-modulating effect. Another study investigated the effects of AXOS or AX plus XYL in broilers, results indicated that feed conversion ratio (FCR) was numerically lowest and metabolizable energy intake higher in birds fed the AXOS treatment (Morgan, N.K. et al., 2017). It was suggested that the depolymerisation of NSPs *in situ* was not instantaneous, hence AXOS generation in the gut via the use of enzymes was not as efficient as feeding AXOS directly. Taken together, these studies suggest that the kick-starter effects may be greater with XOS compared to *in situ* XYL-produced XOS, albeit the combination of XYL and XOS together has received little attention to date. One study that has investigated the effect of feeding both XYL and XOS together, showed a shift in the intestinal microbiome to favour fibre fermentation by increasing the abundance of fibrolytic species such as *C. cellobioparum, B. crossotus, I. butyriciproducens*, C. *ruminocola, F. intestinalis, P. ruminis and F. prausnitzii* (Cho et al., 2020). Although fibre fermentation was not measured it was stated that most of these bacterial species have genes encoding for XYL production, postulating that fibre digestion could have been increased.

The ability to kick-start the metabolic activity of fibre-fermenting bacteria would be accompanied by an increase in activity of bacterial enzyme production. Little work has been done on this concept *in vivo* for stimbiotics. Yet, a combination of XOS and *Saccharomyces cerevisiae* has been shown to increase the microbial xylanolytic activity

in the small intestine of piglets, whilst feeding the additives alone increased the caecal cellulolytic activity (Marinho et al., 2007). This indicates a greater microbial activity and potential for increasing NSP digestibility. Moreover, the effect of insoluble AX fractions on the fermentability and modulation of gut bacterial hydrolase activity has been investigated in humans (Vardakou et al., 2008). When added to a pH-controlled anaerobic fermentation vessel, AX had a prebiotic index of 2.03, but when AX were pre-treated with XYL, the prebiotic index significantly increased to 3.48. This indicates that the XYL-treated AX provided shorter-chain oligomers that were better utilised by the gut bacteria compared to the longer-chain untreated AX. Moreover, addition of AX to a 24-hour batch culture resulted in a 2-fold increase of bacterial XYL production, but a 120-fold increase for XYL-treated AX. Thus, the production of XYL by GIT bacteria was induced by the addition of AX, but even more so by the shorter-chain XYL-treated substrates highlighting the potential of short-chain oligomers to kick-start fibre-degradation. Furthermore, both FOS and TOS have been shown to increase the β-galactosidase activity in the caeca of pigs, whilst TOS also showed increases in the colon and rectum compared to CON fed pigs (Mountzouris, K.C. et al., 2006). The production of microbial β-galactosidase hydrolyses products of plant glycosides and is usually produced by LAB like *Bifidobacterium* and *Lactobacillus*. Hence, this increase along the GIT suggests a stimulatory effect of TOS on LAB, their associated enzyme activity and potential for fibre hydrolysis.

Overall, it is postulated that the provision of a highly fermentable fibre substrate that has structural similarities to the main dietary fibre source tends to train and imprint the metabolic activity of the hindgut bacterial communities enabling the hydrolysis and fermentation of fibre fractions (Bautil, 2020). However, it has also been shown that supplementing a different source of dietary fibre like β-glucans can also alter the digestion

of other fibre fractions (de Vries et al., 2016). Provision of β-glucans increased the ATTD of non-glucosyl polysaccharides (NGP) compared to CON diets, which was mainly attributed to the increased degradation of xylosyl-polysaccharides. The increased ATTD of NGP in diets containing corn distillers dried grains with solubles (DDGS) supplemented with β-glucans was attributed to a better degradability of barley NGPs rather than the NGPs from DDGS itself. However, ATTD of NGPs in rapeseed meal (RSM) diets were still increased by β-glucans even after correction for the presence of barley NGPs. This was explained by the presence of β-glucans potentially specifically stimulating the colonisation and activity of bacteria that are capable of degrading RSM NGPs, particularly xyloglucans. Hence, the fermentation of fibre substrates is not only due to fibre characteristics but also the presence of other fibres in the diet.

Prebiotics selectively stimulate specific GIT bacteria; hence the bacterial community modulation effect will vary between different prebiotics along with their associated metabolic effects. This therefore indicates that the fibre degrading capacity of the GIT bacteria depends on which bacteria are stimulated. Taking the stimbiotic concept into account, addition of substrates like XOS should increase the abundance and metabolic activity of xylan-degrading bacteria leading to higher xylan digestibility, whilst supplementation of fructans should increase the digestibility of fructans. There is little evidence to date regarding the ability of substrates like XOS or FOS to increase the digestibility of other types of fibre fractions. However, the combination of prebiotics should theoretically broaden the number of different bacteria that are stimulated, leading to a wider range of microbial fibre-degrading capabilities, hence improving overall fibre digestion if the prebiotics work in synergy. Moreover, site of fermentation will play a role, for example it is unknown whether the provision of 2 short-chain oligomers would be preferential over 2 long-chain oligomers, or a combination of different chain lengths.

1.4.3 Animal performance

All the above-mentioned functionalities of prebiotic substrates should theoretically lead to an improvement in host growth. Supplementation of a synthetic short-chain FOS mixture (Meioligo-P; GF_2 = 40 %, GF_3 = 50 %, GF_4 = 7 %)) at 0, 2, 4 and 8 g/kg diet was investigated in broilers (Xu, Z. et al., 2003). Addition of 4 g/kg of FOS significantly increased average daily gain (ADG) of broilers, with a significant FCR improvement with 2 and 4 g/kg addition. A similar study was conducted in growing pigs, where FOS was added at 0, 2, 4 and 6 g/kg of diet (Xu, Z.R. et al., 2002). Similar to the broiler study, supplementation with 4 and 6 g/kg diet of Meioligo-P FOS significantly improved ADG and FCR of pigs, as well as enhancing the growth of *Bifidobacterium* and *Lactobacillus*, and inhibiting *E. coli* and *Clostridium* (Xu, Z.R. et al., 2002). This agrees with Xu, C. et al. (2005) who found 4 g/kg of an alternative FOS product (GF_2 = 47 %, GF_3 = 39 %, GF_4 = 11 %) also improved piglet performance compared to a positive Auromycin CON. Regarding heavier pigs, supplementation of FOS at 2.5 g/kg, 5.0 g/kg, and 7.5 g/kg increased ADG by 4.0 %, 9.7 % and 10.7 % respectively, whilst 5.0 g/kg and 7.5 g/kg % also reduced FCR by 8.2 % and 7.6 % (Xu, Z.-r. and Hu, 2003). However, not all studies with short-chain FOS have shown beneficial effects. For example, addition of Jerusalem artichoke flour (GF_{1-2} = 33.3 %, GF_{3-4} = 46.4 % and $GF_{>5}$ = 20.3 %) or Neosugar (GF_2 = 28 %, GF_3 = 60 %, GF_4 = 12 %) at 15 g/kg had no significant effect on weaning pig performance, SCFA production or bacterial composition, but there was a confounding replicate effect (Farnworth et al., 1992). Albeit, similar effects were seen in a weaned piglet study where supplementation of 5 g/kg FOS (Raftilose P95®) reduced growth and insulin-like growth factor I (Estrada et al., 2001). It is not clear why some studies show beneficial effects and other show neutral or negative effects of FOS on performance, but it is likely due to differences in inclusion rate, product concentration and composition, differing feed formulations and different aged pigs. However, from the above literature,

supplementation of a short-chain FOS mixture at 4 g/kg seems to be the optimum inclusion for piglets.

Regarding longer chain linear INU, the literature reports both positive and neutral effects on weaner pig performance. In a weaner study, supplementation of INU in water and feed improved ADG and FCR compared to the CON (He et al., 2002). Similarly, 40 g/kg of dried artichoke or chicory powder supplementation has been reported to improve ADG and FCR (Grela et al., 2014). However, 80 g/kg of INU from chicory showed no effect on weaner pig performance (Halas et al., 2009). There is little in the literature on INU from agave which have a different linear and branched structure with β-(2,6)-linked fructose units. Yet one study used a synthetic mixture of FOS components with an average DP of 10 and β-(2,6)-linkages (Zhao et al., 2013). Results of this study showed that supplementation of 10 and 20 g/kg increased ADG, improved FCR and increased the ATTD of DM and GE in finishing pigs.

The majority of research on XOS has been conducted in broilers, whereby promising effects with inclusions at 0.06 g/kg, 0.10 g/kg and 10 g/kg all showed improvements in broiler performance (Suo et al., 2015; Zhenping et al., 2013; Ribeiro et al., 2018). In weanling pigs, Liu, J. et al. (2018) showed a 17 % improvement in ADG and 14 % improvement in FCR when supplemented with 0.2 g/kg of XOS (50 % purity). Likewise, Chen, Y. et al. (2021) showed that 0.5 g/kg of XOS (95 % purity) increased ADG and improved FCR in weaned pigs compared to a CON diet. However, Yin, J. et al. (2019) found no performance benefits when using a lower inclusion rate of XOS at 0.1 g/kg (40 % purity) in weaned piglets. Yet, there were differences between the studies such as diet raw material basis, piglet age and XOS purity. Taken together, these studies suggest 0.10 – 0.48 g/kg of pure XOS improves the performance of young pigs and highlights that 0.04

g/kg of pure XOS may not be a high enough concentration to elicit performance benefits in piglets.

Individually, the supplementation of fructans and XOS tends to improve the performance of monogastric animals. It is however unknown whether combining different prebiotics together would improve piglet performance above and beyond that of feeding them individually. Indeed, the combination of INU and FOS has been found to reduce gas production whilst maintain the growth of *Bifidobacterium* in a faecal batch culture, with the highest prebiotic index being achieved with FOS alone and a mixture of FOS and INU (Ghoddusi et al., 2007). However, to the author's knowledge there is no literature focusing on the effect of combining prebiotic substrates *in vivo*. In theory, combining different prebiotics together could increase the substrate availability for a wider range of resident probiotic bacteria. In turn, this could increase fermentative activity, SCFA production, energy provision for the host and potential host growth. However, given the plasticity of the pig GIT bacterial community, it is unknown whether prebiotic combinations would create excessive competition in the GIT ecosystem and hence be of detriment to the host. Hence, further research in this area is required.

1.4.4 Aims and objectives

There is a lack of peer reviewed literature on the effects of XOS in pigs, with most of the research having been performed in poultry. Moreover, the effect of XOS alone or in combination with alternative additives in pigs is a new area of research which has not been reported in the literature before.

Specific objectives:

1. To determine the effect of XYL and XOS supplementation on grower pig performance and their impact on faecal bacterial community composition over time.
2. To determine whether L-lactate could be detected in piglet faeces over the weaning period and whether concentrations changed with age.
3. To determine how quickly a known concentration of L-lactate would be metabolised by pig faecal bacteria, and whether this differed between solutions, sample fractions or analytical methods.
4. To evaluate titanium dioxide (TiO_2) and intrinsic cellulose as inert markers for AID and ATTD of NSPs in piglets.
5. To determine the effect of XOS with or without fructans on piglet performance and various GIT functions, including bacterial community composition, microbial endo-XYL activity, fermentation activity and NSP digestibility.

Chapter 2 will investigate the effect of XYL and XOS supplementation on growth performance and faecal bacterial community composition in growing pigs. Following this, Chapter 3 will investigate if L-lactate can be detected in piglet faeces and if concentrations change with age. Moreover, the rate of metabolism of a known concentration of L-lactate by pig faecal bacteria will be explored. Chapter 4 will investigate the effect of XOS with or without fructans on growth performance, gut bacterial community composition and microbial endo-XYL activity in weaned piglets. Whilst Chapter 5 will evaluate TiO_2 and intrinsic cellulose as inert markers for NSP digestibility. Using the results from Chapter 5, Chapter 6 will investigate the effect of XOS with or without fructans on gut bacterial fermentation activity and NSP digestibility in weaned piglets. Conclusions of the thesis will be made in Chapter 7.

Chapter 2

Effect of xylanase and xylo-oligosaccharide supplementation on growth performance and faecal bacterial community composition in growing pigs

2.1 Abstract

Feeding concepts to improve pig health and performance are of upmost importance to the swine industry. This study was conducted to investigate the effects of XYL and XOS supplementation on the growth performance and faecal bacterial community composition in growing pigs over time. In this 35-day trial, a total of 464 grower pigs with an average initial body weight (BW) of 14.5 kg (Standard deviation; SD ±1.56 kg) were blocked into mixed sex pens of 4–5 pigs balanced for BW, sex and litter origin. Pens were randomly allocated to 1 of 4 dietary treatments in a 2 × 2 factorial treatment arrangement with 2 levels of supplementary XYL (0 and 0.15 g/kg) and XOS (0 and 0.20 g/kg). Every week, pen feed intake and pigs were weighed to calculate pig performance. Faecal samples from 32 male pigs were collected on days 1, 14 and 35 of the trial and analysed to profile the bacterial communities through 16S ribosomal ribonucleic acid (rRNA) sequencing of the V4 region on the MiSeq platform (Illumina). There was no effect of XYL on pig performance, nor was there an interaction between XYL and XOS. Pigs supplemented with XOS had a poorer gain to feed ratio (G:F) during the first week (Day 1–7) of the trial ($P < 0.002$). During the second week of the trial (Day 8–14), pigs fed XOS showed an improved ADG compared to those without XOS ($P < 0.003$), but there were no performance effects in the overall trial period (Day 1–35). Alpha diversity increased over time ($P < 0.05$), and as an index of bacterial community compositions, beta diversity also changed over time ($P < 0.001$), but there was no overall effect of treatment on alpha or beta diversity. Despite no overall treatment effect, certain operational taxonomic units (OTUs) associated with *Muribaculaceae_ge* and *Prevotellaceae NK3B31 group* were

higher in all 3 dietary treatments compared to the unsupplemented CON diet ($P < 0.05$). In summary, XYL and XOS had limited effect on pig performance in this trial. Faecal bacterial communities significantly changed over time but despite influencing certain OTUs, treatment had no overall effect on faecal bacterial community composition. Supplementation of XYL or XOS, individually or simultaneously, increased the abundance of OTUs belonging to the *Muribaculaceae* and *Prevotellaceae* families associated with carbohydrate metabolism, indicating that these bacteria are likely involved in the mechanistic pathways of XYL and XOS.

2.2 Introduction

The fibre component of cereals is primarily composed of complex carbohydrates found in plant cell walls called NSPs. The amount and type of NSPs vary among cereal grains, with xylans, β-glucans and cellulose being the most prominent (Choct, 1997). Cereals such as wheat and rye contain large amounts of soluble and insoluble NSPs, with the main soluble component being xylan (Choct, 1997). Within the GIT of monogastrics, solubilised xylans are anti-nutritive as they increase digesta viscosity which in turn reduces nutrient absorption and host growth (Olukosi, O.A. et al., 2007). Further to this, valuable nutrients are trapped within cells rendering them inaccessible to the host due to a lack of endogenous enzyme production to degrade plant cell walls (Masey O'Neill et al., 2014). Supplementation of exogenous β-1,4-XYL can alleviate these antinutritive effects by hydrolysing the xylan polysaccharide, thus decreasing digesta viscosity, releasing entrapped nutrients and improving the nutritive value of feed and host growth (Masey O'Neill et al., 2014). A less well-known mechanism of XYL and its benefits on performance, is the indirect provision of fermentable XOS from the hydrolysis of the xylan backbone (Masey O'Neill et al., 2014). These are short-chain xylo-oligomers that resist digestion and are fermented in the hindgut where they have prebiotic effects by

selectively stimulating beneficial bacteria like *Bifidobacterium* or *Lactobacillus*, and reducing pathogenic bacteria like *E. coli* (Hsu et al., 2004; Liu, J. et al., 2018). Furthermore, XOS can also be manufactured commercially via the hydrolysis of corncobs and can be formulated directly into the host's diet. As an emerging new-generation prebiotic, studies using XOS have shown promising improvements in performance, nutrient digestibility, gut structure and gut bacterial community composition in broilers and weanling pigs (Liu, J. et al., 2018; Ribeiro et al., 2018; Chen, Y. et al., 2021). However, little attention has been given to growing pigs; hence this study investigated the effect of XYL and XOS supplementation on the performance and faecal bacterial community composition of growing pigs over time.

2.2.1 Study aims

At present, there is a lack of information in the literature on the effect of XOS in growing pigs. Hence, this study aimed to determine the effect of XYL and XOS supplementation on grower pig performance and their impact on faecal bacterial community composition over time.

2.2.2 Hypotheses

- Hypothesis 1: XYL and XOS supplementation will improve grower pig performance compared to the CON. When XYL and XOS are fed in combination, the performance of grower pigs will be improved compared to when XYL and XOS are provided independently.
- Hypothesis 2: Faecal bacterial community composition (alpha and beta diversity) will differ between dietary treatment and over time.

- Hypothesis 3: Compared to the CON, the supplementation of XYL and XOS, independently or in combination, will increase the abundance of beneficial bacteria like *Bifidobacterium* and *Lactobacillus* and decrease pathogenic bacteria such as *Escherichia coli*.

2.3 Materials and methods

Study protocols were approved by the University of Leeds Pig Research Centre and ethical approval was granted by the Animal Welfare and Ethical Review Body as described in Appendix A.1.

2.3.1 Animals and housing

At 7 weeks of age, 464 grower pigs ((Large White x Landrace females) x JSR Pietrain-based Geneconverter 900 sire line) with an average initial BW of 14.5 kg (SD ±1.56 kg) were used in this randomised complete block design with 24 replicates and 4–5 mixed-sex pigs per pen for a 35-day feeding study. All pigs were weighed at the start of the trial and blocked into pens balanced for litter origin, sex and BW. Pens within each replicate were randomly allocated to 1 of 4 dietary treatments described below. The trial was conducted over 2 batches with 12 replicates in each batch. Pigs were housed in conventional fully slatted weaner-grower facilities where each pen (155 × 129 cm) had 2 nipple drinkers and 1 single-space feeding trough (Appendix A.2).

2.3.2 Experimental design and dietary treatments

Pigs were fed with a 1-phase feeding program from day 1–35 of the trial and had *ad libitum* access to pelleted feed and water. All dietary treatments were formulated to meet or exceed the National Research Council nutrient recommendations for 11–50 kg pigs (NRC, National Research Council. 2012). The basal diet was prepared as a single batch of feed at Roslin Nutrition Ltd. (Scotland) before additive addition and pelleting. Dietary treatment compositions with calculated and analysed nutrient concentrations are presented in Table 2.1. All diets included 0.10 g/kg phytase [Quantum® Blue, AB Vista, Marlborough, UK; 5000 phytase units (FTU)/g] to give an expected activity of 500 FTU/kg of feed. Inclusion of XYL [endo-1,4-β-XYL, Econase® XT, AB Vista, Marlborough, UK; 160000 Birch Xylan Units (BXU)/g)] in the XYL and XYL + XOS dietary treatment groups provided an expected activity of 24000 BXU/kg of feed. The XOS product was manufactured from the hydrolysis of corncobs and had a DP of 2–7. Supplementation of XOS (XOS 35, 35 %, Longlive Biotechnology Corporation, China) in the XOS and XYL + XOS dietary treatment groups provided 0.07 g/kg of pure XOS in the feed. Inclusion rates of XYL and XOS were according to the supplier's recommendations at the time of the trial. Dietary treatments were arranged in a 2 × 2 factorial treatment arrangement with 2 concentrations of supplementary XYL (0 and 0.15 g/kg) and 2 concentrations of XOS (0 and 0.20 g/kg) to give 4 experimental treatments; CON (0 g/kg XYL and XOS), XYL (0.15 g/kg), XOS (0.20 g/kg) and XYL + XOS (0.15 and 0.20 g/kg, respectively).

Table 2.1 Dietary treatment composition with calculated and analysed nutrient levels.

Raw material	Control	XYL [a]	XOS [b]	XYL + XOS
Ingredient, g/kg				
Wheat	719.9	719.7	719.7	719.5
Soybean meal	225.0	225.0	225.0	225.0
Soya oil	19.8	19.8	19.8	19.8
Dicalcium phosphate	11.1	11.1	11.1	11.1
Vitamin-mineral premix [c]	5.0	5.0	5.0	5.0
Limestone	4.5	4.5	4.5	4.5
L-Lysine HCl, 784 g/kg	4.4	4.4	4.4	4.4
Sodium bicarbonate	3.7	3.7	3.7	3.7
Salt	3.6	3.6	3.6	3.6
Threonine, 980 g/kg	1.3	1.3	1.3	1.3
DL-Methionine, 980 g/kg	0.90	0.90	0.90	0.90
XYL	0.00	0.15	0.00	0.15
XOS	0.00	0.00	0.20	0.20
Phytase [d]	0.10	0.10	0.10	0.10
L-Tryptophan, 980 g/kg	0.07	0.07	0.07	0.07
L-Valine, 965 g/kg	0.06	0.06	0.06	0.06
Calculated nutrient composition				
Net energy (MJ/kg)	10.43	10.43	10.43	10.43
Standardised ileal digestible lysine (g/kg)	11.0	11.0	11.0	11.0
Apparent total tract digestible phosphorus (g/kg)	3.8	3.8	3.8	3.8
Analysed nutrient composition				
Dry matter (g/kg)	875.0	873.0	876.0	876.0
Ash (g/kg)	45.0	42.0	43.0	44.0
Crude protein (g/kg)	183.0	188.0	187.0	187.0
Crude fibre (g/kg)	26.0	23.0	23.0	21.0
Calcium (g/kg)	6.30	5.90	5.70	6.20

[a] XYL; xylanase - endo-1,4-β-xylanase, Econase® XT, AB Vista, Marlborough, UK. [b] XOS; xylo-oligosaccharide - XOS 35, Longlive Biotechnology Corporation, China. [c] Vitamin premix, active substance per kg of diet: 10,000 IU Vitamin A (retinyl acetate), 2250 IU Vitamin D3 (cholecalciferol), 75 mg Vitamin E (alpha tocopheryl acetate), 0.91 mg Vitamin K3 (menadione), 1.6 mg Vitamin B1 (thiamine mononitrate), 4 mg Vitamin B2 (riboflavin), 8.9 mg Pantothenic acid (calcium-D-pantothenate), 2.4 mg Vitamin B6 (pyridoxine hydrochloride), 25 ug Vitamin B12 (cyanocobalamin), 30 mg Nicotinic acid, 0.5 mg Folic acid, 100 ug Biotin, 100 mg Iron (sulphate monohydrate), 15 mg Copper (sulphate pentahydrate), 45 mg Manganese (sulphate monohydrate), 80 mg Zinc (sulphate monohydrate), 1 mg Iodine (calcium iodate anhydrous), 0.25 mg Selenium (selenite) and 1500 mg Magnesium phosphate. [d] Phytase; Quantum® Blue, AB Vista, Marlborough, UK.

2.3.3 Measurements and sampling

2.3.3.1 Pig performance and health observations

Every week (Days 1, 7, 14, 21, 28, 35) all pigs were weighed individually and ADG was calculated. Weekly feed disappearance was recorded on a pen basis and pen average daily feed intake (ADFI) was calculated. ADG and ADFI data were used to calculate the weekly average G:F ratio. The timing of any pig mortality or removal from the trial was recorded, along with the pig BW to allow for ADFI and G:F adjustments.

Health condition as indicated by digestive function was assessed by recording daily pen faecal scores on a scale of 1–4 by the same personnel (1 = firm faeces, 2 = soft faeces, 3 = mild diarrhoea, 4 = severe diarrhoea). Pen health scores were recorded daily on a scale of 1–4 by the same personnel (1 = no signs of ill health, 2 = some signs of ill health, 3 = clear indications of ill health, 4 = seriously ill pigs). Pen cleanliness scores were recorded daily on a scale of 1–4 by the same personnel (1 = clean pigs, 2 = light contamination with faecal material, 3 = contamination with faecal material, 4 = heavy contamination with faecal material).

2.3.3.2 Faecal collection

Faecal samples were collected from 1 individual male pig per pen on days 1, 14 and 35. Faeces were collected in a 30 ml universal container immediately after defecation and placed on ice before being stored frozen (–80°C) until analysis. Of the collected samples, 32 were selected for bacterial community analysis (8 replicates per treatment). Those chosen for analysis had a BW close to that of the pen average, had not received antibiotic treatment and were littermates within replicates.

2.3.4 Analysis

2.3.4.1 Phytase and XYL recovery

Phytase and XYL recovery were determined at ESC (Ystrad Mynach, Wales, UK) according to the manufacturer's enzyme-linked immunosorbent assay for Quantum® Blue and Econase® XT. All diets were analysed for standard nutrients at DM Scientific (East Lothian, Scotland, UK).

2.3.4.2 Deoxyribonucleic acid (DNA) extraction and bacterial community analysis

Total bacterial DNA was extracted from the faecal samples (*ca* 0.2 g) using the QIAamp DNA Stool Mini Kit (QIAGEN®, Hilden, Germany) as per the manufacturer's protocol, with 2 modifications. To maximise cell lysis, the faecal samples underwent bead beating (Tissue Lyser LT, Qiagen; 0.2 g of 0.1 mm silica beads) for 5 min at a maximum speed of 50 rps and were incubated at a higher temperature of 95°C. Extracted DNA quantity and quality were measured spectrophotometrically (NanoDrop ND-1000).

Extracted DNA was sent to the Environmental Genomics Facility at the University of Southampton for next-generation sequencing following the Illumina 16S Metagenomic sequencing library preparation protocol. Polymerase chain reactions (PCR) were used to amplify the V4 region of the bacterial 16S rRNA gene using the modified 515F (Parada et al., 2016) (*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*GTGYCAGCMGCCGCGGTAA) and 806R (Apprill et al., 2015) (*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GGACTACNVGGGTWTCTAAT) primer set (overhang sequences correspond to Illumina adapters shown in italics). The 25 µl PCR reaction consisted of 2.5 µl microbial DNA (5 ng/ul), 5 µl forward primer (515

F), 5 µl of reverse primer (806R) and 12.5 µl KAPA HiFi HotStart ReadyMix. The amplification was performed using the following program: 95ºC for 3 min, 25 cycles of (30 s at 95ºC, 30 s at 55ºC, 30 s at 72ºC), 72ºC for 5 min, before being held at 4ºC. Amplification was confirmed using a Bioanalyser 1000 chip. AMPure XP beads were used to purify the 16S V4 amplicon away from the primers and primer dimer species. Nextera XT v2 index adaptors were attached using a further 8 cycles of PCR. AMPure XP beads were used to clean the final library before quantification. All AMPure clean-up steps and the setup of the indexing PCR were carried out on a liquid handling robot (Biomek 4000). Libraries were quantified by a fluorometric quantification method using double-stranded DNA binding dyes, normalised and pooled. Pooled libraries were denatured with NaOH and diluted with hybridisation buffer before heat denaturation and MiSeq sequencing.

2.3.4.3 Bioinformatics

Mothur (v.1.41.1) was used to process the sequence reads and the MiSeq standard operating procedure was followed (Kozich et al., 2013). Briefly, contigs were created by combining the forward and reverse reads, any ambiguous bases or contigs smaller or larger than 200–300 base pairs were removed. Duplicate sequences were merged, and unique sequences were aligned to the SILVA reference database (v.132). Only contigs that aligned between position 11894 and 25319 were selected with a maximum homopolymer length of 8. Sequences were pre-clustered, allowing for 1 difference in every 100 base pairs of sequence. Chimeras and sequences that aligned to Archaea, Eukaryota, chloroplasts or mitochondria were removed from the dataset. Sequences were clustered into OTUs with 97 % similarity, before quantifying the number of OTUs within each group and their taxonomy. A BIOM file was then created to transfer the data into R (v. 1.1.463).

2.3.5 Statistical analysis

2.3.5.1 Pig performance and health observations

The pen served as the experimental unit for all growth performance data (BW, ADG, ADFI, G:F). G:F ratio was calculated as a ratio of weight gain to feed intake according to the following equation:

$$Gain\ to\ Feed\ (G{:}F) = \frac{Average\ daily\ gain\ (kg\ per\ day)}{Average\ daily\ feed\ intake\ (kg\ per\ day)} \quad \textbf{Equation 2.1}$$

Data were tested for normality by visualisation of histograms and the Shapiro-Wilk test for normality, while the Levene's test was used to assess the homogeneity of variance. Any data showing non-normal distribution or unequal variance were inversely transformed prior to analysis. Transformed data were back transformed for inclusion into the data tables. Performance data were analysed as a 2-way analysis of variance (ANOVA) using the statistical package JMP® (Version *14.1*. SAS Institute Inc., Cary, NC, 1989 - 2019) (SAS, 2020). The statistical model included the fixed effects of XYL, XOS and their interaction, and replicate and batch as random variables. The initial BW of pigs was included as a covariate for BW and ADG analysis. Main effects were analysed individually when interactions were non-significant. Average pen faecal, health and cleanliness scores were analysed by the non-parametric Kruskal-Wallis one-way ANOVA. Significant differences were classed as $P < 0.05$ and trends as $P < 0.10$.

2.3.5.2 Bacterial community composition analysis

The software R was used to analyse the microbiome data (v. 1.1.463) statistically. Individual pigs served as the experimental unit for the bacterial community composition analysis. A general linear model was used to determine the effects of treatment and time

on bacterial abundance at the phylum and genus level. *Post-hoc* differences were identified using a Tukey's test (JMP® v.14.1). The number of OTUs, Chao1 (Chao, 1984) and Shannon-Weiner (Shannon, 1948) alpha diversities were measured using the Phyloseq package (v.1.22.3) in R (McMurdie and Holmes, 2013). A general linear model (lme4) was used to determine the effects of treatment and time on alpha diversity and the number of OTUs. Models were reduced using analysis of deviance. Beta diversity was analysed using the packages Vegan (v.2.5.3) and DESeq2 (v.1.18.1). A permutational multivariate ANOVA (PERMANOVA - adonis) was used to assess community similarities across treatment and time. A non-metric multidimensional scaling (NMDS; axis = 2) plot using Bray-Curtis distances was used to plot beta diversity. DESeq2 analysis identified the fold change of OTUs, which differed significantly between 2 groups. DESeq2 was performed on un-rarefied data and *P* values presented were corrected for multiple testing (Benjamin-Hochberg correction).

2.4 Results

2.4.1 Phytase and XYL recovery

The analysed phytase activity in the feed (FTU/kg) of the CON, XYL, XOS and XYL + XOS dietary treatments were 457, 658, 561 and 613, respectively. The analysed XYL activity in the feed (BXU/kg) in the CON, XYL, XOS and XYL + XOS supplemented diets were < 2000, 19700, < 2000 and 20800, respectively. Hence, the XYL dietary treatment had a recovery of 82 %, while the XYL + XOS treatment had a recovery of 87 %. Recovery was lower than expected but similar in both XYL treatments.

2.4.2 Pig performance and health observations

Pig growth performance and health scores are presented in Table 2.2. There was no effect of XYL on any of the performance parameters throughout the trial, nor was there an interaction between XYL and XOS, hence only the main effects are presented. However, XOS supplementation increased ADFI ($P < 0.010$) and decreased G:F ratio ($P < 0.002$) during the first week of the trial (Day 1–7). During the second week of the trial (Day 8–14), XOS supplementation increased ADFI compared to treatments without XOS ($P < 0.040$), which led to a higher ADG ($P < 0.003$) and BW at day 14 ($P < 0.040$) but G:F ratio was not affected. There was also a trend for XOS supplemented pigs to have a higher ADFI between day 15–21 ($P = 0.087$). There was a trend for XYL fed pigs to have a higher G:F ratio between day 29–35 compared to those without XYL ($P = 0.085$). There were no significant main or interactive effects on overall performance (Day 1–35) of either XYL or XOS. There was no difference between treatments for average pen faecal, health or cleanliness scores from day 1–35.

Table 2.2 Main effects of XYL and XOS on grower pig performance and health observations [a]

	Treatments					*P*-Value		
	XYL[b]		XOS[c]		SEM[k]			
Inclusion (g/kg)	0.0	0.15	0.0	0.20		XYL	XOS	XYL + XOS
BW[d] (kg)								
Day 1	14.5	14.6	14.5	14.6	0.31	0.470	0.640	0.360
Day 7	17.2	17.2	17.3	17.2	0.08	0.590	0.220	0.630
Day 14	20.7	20.6	20.5	20.8	0.17	0.620	0.040	0.800
Day 21	24.9	24.6	24.6	24.9	0.22	0.210	0.180	0.480
Day 28	29.8	29.2	29.4	29.6	0.31	0.130	0.590	0.290
Day 35	35.6	35.2	35.2	35.6	0.32	0.310	0.390	0.640
ADG[e] (kg/d)								
Day 1–7	0.38	0.37	0.38	0.37	0.01	0.640	0.380	0.420
Day 8–14	0.52	0.51	0.48	0.54	0.02	0.630	0.003	0.750
Day 15–21	0.60	0.55	0.58	0.57	0.02	0.110	0.560	0.550
Day 22–28	0.70	0.66	0.69	0.67	0.03	0.180	0.390	0.220
Day 29–35	0.81	0.83	0.81	0.83	0.02	0.500	0.750	0.160
Day 1–35	0.60	0.59	0.59	0.60	0.01	0.250	0.460	0.640
ADFI[f] (kg/d)								
Day 1–7	0.76	0.75	0.73	0.78	0.04	0.820	0.010	0.820
Day 8–14	1.08	1.02	1.00	1.11	0.04	0.320	0.040	0.730
Day 15–21	1.08	1.05	1.04	1.09	0.02	0.400	0.090	0.570
Day 22–28	1.37	1.33	1.35	1.35	0.02	0.250	0.750	0.440
Day 29–35	1.46	1.42	1.44	1.44	0.01	0.130	0.920	0.600
Day 1–35	1.15	1.12	1.12	1.15	0.02	0.300	0.250	0.900
G:F[g]								
Day 1–7	511	513	542	482	19.70	0.910	0.002	0.310
Day 8–14	511	522	507	527	31.00	0.620	0.350	0.530
Day 15–21	552	528	552	528	20.60	0.280	0.300	0.670
Day 22–28	516	501	521	496	20.80	0.440	0.210	0.500
Day 29–35	559	589	572	576	14.90	0.090	0.800	0.220
Day 1–35	531	535	538	528	11.70	0.730	0.390	0.830
Faecal score - day 1–35[h]	2.47	2.49	2.41	2.49	0.02		0.570	
Health score - day 1–35[i]	0.10	0.10	0.08	0.11	0.01		0.920	
Cleanliness score – day 1–35[j]	1.13	1.14	1.16	1.17	0.01		0.590	

[a] Data are means of 24 replicate pens of 4–5 pigs. [b] XYL; xylanase. [c] XOS; xylo-oligosaccharide. [d] BW; body weight. [e] ADG; average daily gain. [f] ADFI; average daily feed intake. [g] G:F; gain to feed ratio. [h] Faecal score; 1 = firm faeces, 2 = soft faeces, 3 = mild diarrhoea, 4 = severe diarrhoea. [I] Health score; 1 = no signs of ill health, 2 = some signs of ill health, 3 = clear indications of ill health, 4 = seriously ill pigs. [j] Cleanliness score; 1 = clean pigs, 2 = light contamination with faecal material, 3 = contamination with faecal material, 4 = heavy contamination with faecal material. [k] SEM: Standard error of the mean.

2.4.3 Bacterial community composition analysis

Most faecal bacteria belonged to the phyla Firmicutes (51 %) and Bacteroidetes (40 %), jointly making up 91 % of the bacterial community (Table 2.3). Changes in the bacterial community over time and between dietary treatments were estimated at the phylum and genus level. There were 6 phyla with a relative abundance greater than 1 % in a minimum of one treatment or time group (Table 2.3). There was no effect of treatment on the relative abundance of OTUs at the phyla level, nor was there an interaction between dietary treatment and timepoint (TP; Table 2.3). There was a trend for the abundance of Tenericutes to be lowest in the CON group and highest in the XOS group ($P = 0.059$). The abundance of Tenericutes also decreased from day 1–14, and then preceded to increase from day 14–35 ($P < 0.001$). Spirochaetes tended to follow the same trend as that of Tenericutes over time ($P = 0.091$).

There were 33 genera with a relative abundance greater than 1 % in a minimum of one treatment or time group (Table 2.3). Of the 33 genera, 17 were from the phylum Firmicutes, 11 from Bacteroidetes, 2 from Actinobacteria, 1 from Tenericutes, 1 from Spirochaetes and 1 from Epsilonbacteraeota. The abundance of *Prevotellaceae_NK3B31_group* was highest in the XOS group ($P < 0.04$). Of the 33 genera, the abundance of 21 significantly changed over time ($P < 0.05$). Many genera decreased in abundance from day 1–14, namely, *Phascolarctobacterium, Rikenellaceae_RC9_gut_group, Mollicutes_RF39_ge, Ruminococcaceae_unclassified, Prevotellaceae_NK3B31_group, Ruminococcaceae_NK4A214_group* and *Prevotella_1*. Moreover, the abundance of many genera also increased from day 1–14, namely, *Prevotella_7, Dialister, uncultured bacteria, Acidaminococcus, Mitsuokella, Oribacterium* and *Streptococcus*.

Table 2.3 The average relative abundance (> 1 %) at the Phyla and Genera level in pig faeces over time and between diets (n = 32).

Relative abundance (%)		Day			SEM	Diet				SEM	*P* Value		
Phylum[1]	**Genus[1]**	**1**	**14**	**35**		**CON[a]**	**XYL[b]**	**XOS[c]**	**XYL + XOS**		**Day**	**Diet**	**Day × Diet**
Firmicutes		49.86	52.33	49.8	1.06	51.14	51.31	50	50.21	1.23	0.163	0.835	0.528
Bacteroidetes		39.4	39.69	40.25	0.93	38.9	39.08	39.99	41.14	1.07	0.806	0.439	0.175
Tenericutes		2.63^{a}	1.59^{b}	2.45^{a}	0.21	1.8	2.24	2.73	2.12	0.24	<0.001	0.059	0.902
Spirochaetes		2.11	0.96	1.78	0.38	1.47	1.97	2	1.02	0.43	0.091	0.338	0.779
Proteobacteria		2.06	1.89	1.98	0.19	2.21	1.93	1.88	1.87	0.22	0.809	0.654	0.937
Actinobacteria		1.62	1.9	1.17	0.67	1.75	1.74	1.22	1.55	0.31	0.155	0.593	0.356
Bacteroidetes	*Prevotella_9*	12.59	14.04	11.25	0.92	12.43	13.42	10.56	14.09	1.06	0.107	0.109	0.904
Firmicutes	*Lactobacillus*	7.24	7.55	7.45	1.00	7.16	8.39	6.71	7.4	1.16	0.976	0.769	0.072
Bacteroidetes	*Muribaculaceae_ge*	6.54^{a}	5.4^{a}	8.60^{b}	0.60	6.44	6.38	6.93	7.64	0.69	<0.001	0.544	0.854
Firmicutes	*Lachnospiraceae_unclassified*	5.69	5.26	4.28	0.50	5.54	4.36	5.28	5.12	0.58	0.130	0.511	0.680
Firmicutes	*Megasphaera*	5.46	6.02	6.27	0.60	6	6.6	5.83	5.24	0.69	0.616	0.579	0.987
Firmicutes	*Phascolarctobacterium*	4.17^{a}	1.43^{b}	1.63^{b}	0.32	1.93	2.64	2.52	2.54	0.37	<0.001	0.529	0.682
Bacteroidetes	*Rikenellaceae_RC9_gut_group*	3.46^{a}	1.68^{b}	2.39^{c}	0.21	2.6	2.46	2.72	2.25	0.24	<0.001	0.554	0.995
Bacteroidetes	*Alloprevotella*	2.91	3.9	2.98	0.36	3.44	3.19	3.01	3.42	0.42	0.103	0.874	0.616
Tenericutes	*Mollicutes_RF39_ge*	2.59^{a}	1.56^{b}	2.41^{a}	0.21	1.77	2.21	2.67	2.1	0.24	<0.001	0.070	0.863
Firmicutes	*Ruminococcaceae_UCG-002*	2.56^{ab}	1.85^{a}	2.64^{b}	0.22	2.02	2.22	2.85	2.3	0.25	0.020	0.116	0.950
Firmicutes	*Ruminococcaceae_unclassified*	2.35^{a}	1.73^{b}	2.05^{ab}	0.13	2	1.88	2.25	2.04	0.15	0.004	0.369	0.928
Firmicutes	*Subdoligranulum*	2.3	2.38	1.6	0.24	2.08	2.41	1.8	2.08	0.27	0.040*	0.488	0.205
Bacteroidetes	*Prevotella_7*	2.28^{a}	4.17^{b}	3.70^{ab}	0.44	2.94	3.37	3.83	3.39	0.51	0.010	0.678	0.614
Bacteroidetes	*Prevotellaceae_unclassified*	2.12	2.35	2.02	0.28	2.06	1.85	2.65	2.09	0.32	0.684	0.333	0.754

Relative abundance (%)		Day			SEM	Diet				SEM	*P* Value		
Phylum[1]	**Genus[1]**	**1**	**14**	**35**		**CON[a]**	**XYL[b]**	**XOS[c]**	**XYL + XOS**		**Day**	**Diet**	**Day × Diet**
Bacteroidetes	*Prevotellaceae_NK3B31_group*	2.05[a]	0.92[b]	0.80[b]	0.19	1.09	1.04	1.83	1.08	0.22	<0.001	0.040*	0.182
Firmicutes	*Dialister*	2.01[a]	5.54[b]	3.70[c]	0.45	4.05	4.25	2.94	3.76	0.52	<0.001	0.297	0.488
Bacteroidetes	*Prevotella_2*	1.96	2.08	1.92	0.22	1.96	2.13	1.93	1.94	0.25	0.870	0.933	0.846
Spirochaetes	*Treponema_2*	1.9	0.76	1.59	0.36	1.24	1.74	1.81	0.88	0.42	0.077	0.353	0.726
Firmicutes	*Ruminococcaceae_ge*	1.88	1.81	2	0.17	1.8	1.95	1.96	1.88	0.19	0.702	0.932	0.063
Firmicutes	*Ruminococcaceae_UCG-014*	1.7	1.44	1.73	0.14	1.67	1.34	1.81	1.67	0.16	0.247	0.195	0.856
Firmicutes	*Faecalibacterium*	1.16[ab]	1.65[a]	0.89[b]	0.20	1.52	1.22	1.11	1.09	0.23	0.028	0.519	0.912
Bacteroidetes	*Uncultured*	1.06[a]	1.60[b]	1.17[a]	0.12	1.15	1.28	1.34	1.34	0.13	0.003	0.708	0.895
Firmicutes	*Ruminococcaceae_NK4A214_group*	0.87[a]	0.42[b]	0.58[b]	0.08	0.57	0.56	0.71	0.65	0.09	<0.001	0.611	0.605
Bacteroidetes	*Bacteroidales_unclassified*	0.86	0.8	0.97	0.18	1.05	0.75	1.05	0.65	0.21	0.783	0.395	0.272
Actinobacteria	*Olsenella*	0.82[a]	0.59[ab]	0.39[b]	0.10	0.65	0.78	0.39	0.58	0.12	0.014	0.117	0.240
Firmicutes	*Acidaminococcus*	0.79[a]	2.39[b]	2.28[b]	0.35	1.82	1.61	1.8	2.05	0.40	0.002	0.902	0.723
Bacteroidetes	*Prevotella_1*	0.79[a]	0.20[b]	0.42[ab]	0.11	0.58	0.44	0.46	0.42	0.13	<0.001	0.821	0.358
Firmicutes	*Clostridiales_unclassified*	0.71[ab]	0.45[a]	0.92[b]	0.10	0.74	0.49	0.84	0.69	0.12	0.007	0.207	0.873
Firmicutes	*Mitsuokella*	0.63[a]	2.15[b]	1.39[ab]	0.28	1.21	1.6	1.12	1.63	0.32	<0.001	0.581	0.712
Firmicutes	*Oribacterium*	0.53[a]	1.07[b]	0.75[a]	0.09	0.96	0.72	0.72	0.74	0.11	<0.001	0.323	0.152
Epsilonbacteraeota	*Campylobacter*	0.48	0.45	0.3	0.19	0.84	0.21	0.36	0.23	0.22	0.777	0.160	0.849
Actinobacteria	*Collinsella*	0.23	0.66	0.22	0.15	0.59	0.3	0.22	0.36	0.17	0.060	0.461	0.612
Firmicutes	*Streptococcus*	0.03[a]	0.74[b]	0.94[b]	0.15	0.84	0.3	0.64	0.49	0.17	<0.001	0.156	0.501

[a] CON; Control. [b] XYL; Xylanase. [c] XOS; Xylo-oligosaccharides. [1]Phyla and genera with > 1 % abundance in a minimum of one treatment or time group.
* No significant post-hoc (Tukey). [a-c] Means within a row that do not share a common superscript are significantly different ($P < 0.05$).

The number of OTUs and alpha diversity (Chao1 and Shannon) are presented in Table 2.4. The number of OTUs and Chao1 diversity was higher at day 35 compared to day 1 and 14 ($P < 0.001$). Shannon indices were greater at day 35 than day 14, but not day 1 ($P < 0.006$). Dietary treatment did not affect the number of OTUs. However, XOS supplementation tended to have higher numbers of OTUs than the other dietary treatments ($P = 0.078$). Chao1 diversity was not significantly different between dietary treatments, but Shannon indices tended to be the lowest in the XYL treatment group and highest in the XOS treatment group ($P = 0.089$). There was no time × diet interaction for the number of OTUs or Shannon indices. However, there was a trend for Chao1 ($P = 0.064$), such that the CON and XYL treatments increased in diversity over time, whereas a reduction in diversity was observed at day 14, with a subsequent increase at day 35 for the XOS and XYL + XOS treatments.

There was no diet × TP interaction for beta-diversity. The beta diversity of bacterial communities changed over time ($P < 0.001$; Figure 2.1) but was unaffected by treatment. An NMDS plot of the similarity of bacterial communities at each TP shows the divergence of day 14 samples from day 1 samples, whilst the samples at day 35 cluster more closely together than other time points (Figure 2.1).

Table 2.4 Number of OTUs and alpha diversity measures of bacterial community across time and diet [a]

	Number of OTUs [b]	Chao1	Shannon
Day			
1	838.6[a]	1380.1[a]	4.6[ab]
14	813.8[a]	1346.1[a]	4.5[a]
35	940.8[b]	1572.1[b]	4.7 [b]
Diet [c]			
CON	845.6	1389.4	4.6
XYL	836.2	1387.2	4.5
XOS	916.5	1505.8	4.7
XYL + XOS	859.4	1448.7	4.6
***P* value**			
Day	< 0.001	< 0.001	0.006
Diet	0.078	0.115	0.089
Day × Diet	0.262	0.064	0.975

[a-b] Means within a column that do not share a common superscript are significantly different ($P < 0.05$). [a] Data are means of 8 replicates pigs. [b] OTU; operational Taxonomic Unit. [c] CON; Control diet, XYL; Xylanase, XOS; xylo-oligosaccharides.

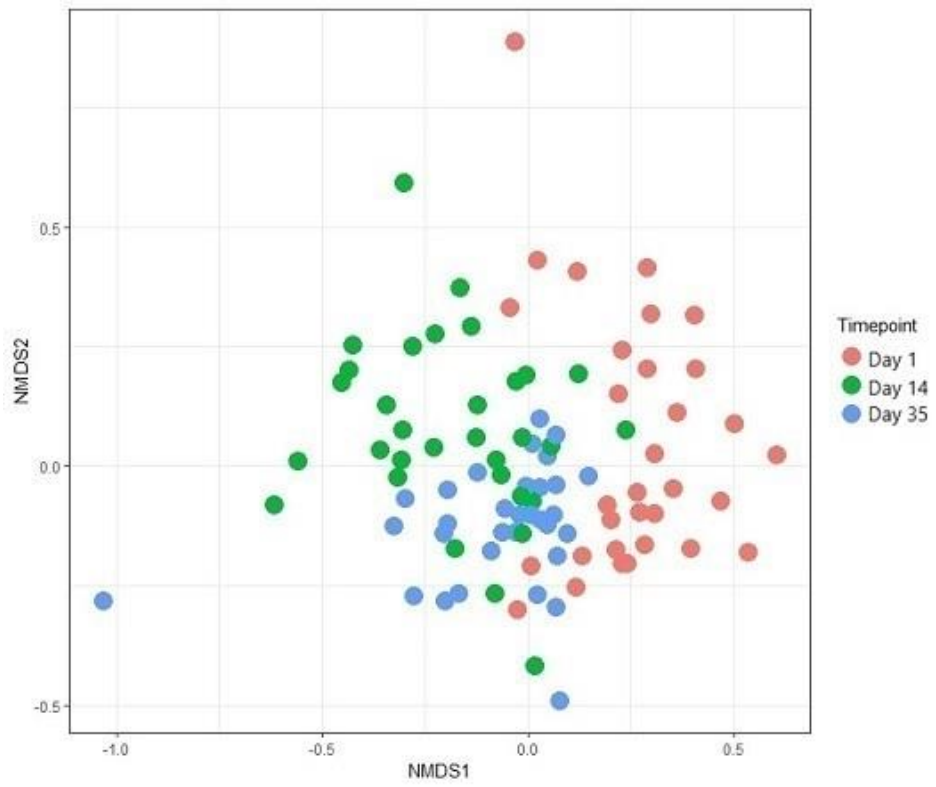

Figure 2.1 Non-metric multidimensional scaling (NMDS) plot of Bray-Curtis similarity coefficients from 16S rRNA gene sequence data from individual pigs over time (n = 32, *P* < 0.001).

Due to the significant effect of time on bacterial community composition, DESeq2 was used to identify individual OTUs which showed the greatest change in abundance between TP. Of interest, from day 1–14 (Figure 2.2), OTUs associated with *Veillonella* and *Megamonas* from the *Veillonellaceae* family increased by 24.2 and 10.0 log2 fold from a base mean of 17.61 and 74.12, respectively (base mean; mean counts of all samples normalised for sequencing depth; $P < 0.001$). The greatest decrease in abundance from day 1–14 was for OTUs associated with the genus *Prevotella_2*, where abundance decreased by 23.7 log2 fold from a base mean of 9.14. Of the top 10 decreases in abundance from day 1–14, all genera were from the *Prevotellaceae*, *Muribaculaceae* and *Rikenellacea*e families, which all belong to the order Bacteroidales.

Although there was no overall treatment effect for beta diversity, DESeq2 analysis between the CON group and the 3 dietary treatments was conducted to identify if specific OTUs were affected. Each dietary treatment (XYL, XOS and XYL + XOS) showed an increased abundance of OTUs associated with *Muribaculaceae_ge* ($P < 0.05$) and *Prevotellaceae_NK3B31_group* ($P < 0.001$) compared to the CON diet (Table 2.5). Moreover, all 3 dietary treatments showed a reduction in OTUs associated with *Prevotella_9* ($P < 0.001$) and *Alloprevotella* ($P < 0.001$) compared to the CON diet.

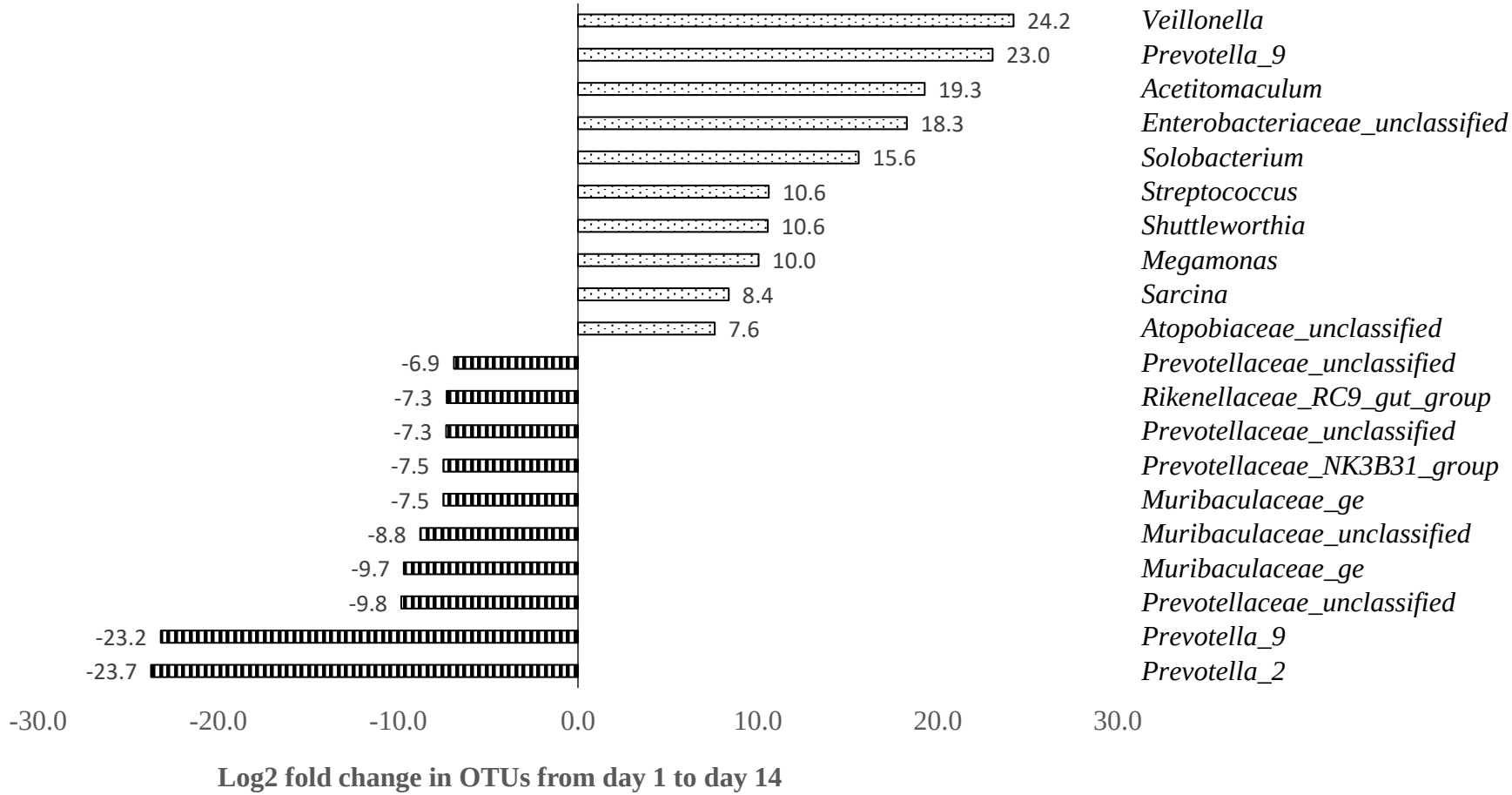

Figure 2.2 DESeq2 analysis of the operational taxonomic units (OTUs) from day 1 to 14 that showed the greatest change in abundance (n = 32).

Table 2.5 DESeq2 analysis of the OTUs in XYL, XOS and XYL + XOS treatment groups compared to the dietary CON group (n = 32).

OTU[a] Number	Genus	Base mean[b]	Log 2 fold change	*P* - Value	OTU Number	Genus	Base mean	Log2 fold change	*P* - Value
Increased from CON[c] to XYL[d]					**Decreased from CON to XYL**				
544	*Prevotellaceae_NK3B31_group*	20.9	20.26	<0.001	223	*Prevotella_9*	58.0	–27.08	<0.001
270	*Muribaculaceae_ge*	2.9	14.89	0.022	452	*Alloprevotella*	5.3	–18.28	<0.001
					4219	*Ruminococcaceae_UCG-004*	0.3	–16.27	0.034
					428	*Veillonellaceae_unclassified*	14.9	–6.73	<0.001
					41	*Campylobacter*	426.3	–2.53	0.039
Increased from CON to XOS[e]					**Decreased from CON to XOS**				
544	*Prevotellaceae_NK3B31_group*	20.9	22.93	<0.001	354	*Prevotella_9*	27.3	–25.58	<0.001
317	*Prevotella_7*	10.2	22.79	<0.001	652	*Prevotellaceae_NK3B31_group*	9.3	–24.22	<0.001
255	*Prevotella_9*	10.4	21.35	<0.001	408	*Alloprevotella*	5.1	–21.19	<0.001
609	*Dorea*	6.3	21.28	<0.001					
270	*Muribaculaceae_ge*	2.9	15.14	0.002					
353	*Treponema_2*	27.7	10.97	<0.001					
Increased from CON to XYL + XOS					**Decreased from treatment CON to XYL + XOS**				
544	*Prevotellaceae_NK3B31_group*	20.9	22.83	<0.001	286	*Prevotella_7*	33.3	–25.56	<0.001
255	*Prevotella_9*	10.4	22.66	<0.001	698	*Prevotella_9*	9.2	–23.93	<0.001
609	*Dorea*	6.3	21.86	<0.001	408	*Alloprevotella*	5.1	–21.35	<0.001
317	*Prevotella_7*	10.2	21.61	<0.001	303	*Bacteria_unclassified*	47.2	–6.41	0.003
270	*Muribaculaceae_ge*	2.9	21.37	<0.001					

[a] OTU; operational Taxonomic Unit. [b] Base mean; mean counts of all samples normalised for sequencing depth. [c] CON; Control diet. [d] XYL; Xylanase. [e] XOS; Xylo-oligosaccharides.

2.5 Discussion

2.5.1 Pig performance

This study aimed to investigate the effects of XYL and XOS supplementation on the growth performance and faecal bacterial community composition of growing pigs over time. The nutritive value of cereals can be improved with XYL supplementation by increasing nutrient digestibility via the degradation of the plant cell walls and release of trapped nutrients and reducing digesta viscosity (Passos et al., 2015). Furthermore, the degradation of plant cell walls produces short-chain oligomers called XOS as an end-product of xylan degradation *in vivo*. The oligomers produced during the hydrolysis of plant cell walls reach the hindgut and exert prebiotic effects by acting as substrates for selective bacteria, thus influencing the GIT bacterial community composition and subsequent energy provision for the host (Courtin et al., 2008; Ribeiro et al., 2018). Provision of XOS to the hindgut can be indirect via the supplementation of XYL or direct via the addition of commercially produced XOS into the diet. While it was expected that the supplementation of XYL, XOS and their combination would improve pig performance, there were no effects on overall growth performance in the current study.

The effect of XYL supplementation on pig performance is inconsistent in the literature, most likely due to differences in the duration of the studies, age of pigs, XYL concentration and the type and quantity of dietary substrates (Barrera et al., 2004). A recent study found that 0.05 and 0.10 g/kg of XYL supplementation in a corn-soybean meal-based diet linearly increased ADG and G:F in weanling pigs (Lan et al., 2017). Moreover, others have found a higher concentration of XYL (0.50 g/kg) in a corn-soybean meal-based diet improved ADG and FCR in heavier pigs of 27–68 kg (Fang et al., 2007). However, in the current study, despite a trend for improved G:F efficiency in the final

week of the trial (Day 29–35), overall, pigs fed XYL supplemented diets showed a similar performance to those without XYL and therefore had no overall beneficial effects on growth performance. These findings agree with a study where weanling pigs (10–24 kg) receiving diets composed of corn, rye, wheat and soybean meal supplemented with 5 different concentrations of a *Bacillus circulans* XYL between 0 and 32 000 U kg^{-1} did not show any improvements in growth performance (Olukosi, O.A. et al., 2007). Albeit, there were differences in the trials, for instance, the current study used a wheat-soybean meal diet and a *Trichoderma reesei* XYL. The age of the animal studied can also affect the pig performance response to XYL, for instance, including 0.10 g/kg of XYL in the first 2 weeks post-weaning (3–5 weeks of age) has been shown to decrease BW, ADG and feed efficiency (Lu et al., 2019). However, XYL supplementation from 2 weeks post-weaning led to an improved final BW and overall ADG up to 6 weeks post-weaning (5–9 weeks of age). While the current study used pigs of 7–12 weeks of age, similar benefits of XYL inclusion were not shown despite this older age.

The majority of research on XOS has been conducted in broilers where some studies have shown no effect on bird growth performance (Craig et al., 2019), while others have demonstrated beneficial effects on growth and immunity (Zhenping et al., 2013; Suo et al., 2015; Ribeiro et al., 2018). Despite this, there is a scarcity of research focusing on the effect of XOS in pigs. In the current study, XOS fed pigs had a higher ADFI but similar ADG compared to those without XOS, leading to a poorer G:F ratio in the first week of the trial (Day 1–7). However, during the second week of the trial from day 8–14, XOS increased ADFI by 0.11 kg/d which lead to an extra 60 g/d of growth and an increased BW of 0.35 kg at day 14, but this benefit was not maintained throughout the trial. It is sufficient to conclude that XOS had a limited effect on overall pig performance in the current study, hence disagrees with hypothesis 1. These findings agree with a recent

weanling pig study (Yin, J. et al., 2019) which reported no performance benefits when using a lower concentration of XOS at 0.10 g/kg (40 % purity XOS, 0.10 g/kg supplied 0.04 g/kg of pure XOS). However, beneficial effects of XOS supplementation occurred in piglets where ADG increased by 17 % and G:F by 14 % compared to unsupplemented diets (Liu, J. et al., 2018). Likewise, another study showed XOS increased ADG and improved FCR in weaned pigs compared to a CON diet (Chen, Y. et al., 2021). However, there were considerable differences between the studies. For instance, the current study used a wheat-soybean meal diet, 7-week-old pigs and 0.20 g/kg of a 35 % XOS product supplying 0.07 g/kg of pure XOS. In comparison, a corn-soybean meal diet was used, 3–4 week-old piglets and either 0.20 g/kg of a 50 % purity XOS or 0.50 g/kg of a 95 % purity XOS product providing 0.10 or 0.48 g/kg of pure XOS (Liu, J. et al., 2018; Chen, Y. et al., 2021). Together, this indicates that a concentration of 0.10 – 0.48 g/kg of pure XOS improves the performance of young pigs but highlights that concentrations of 0.04 g/kg (Yin, J. et al., 2019) and 0.07 g/kg of pure XOS may not be a high enough concentration to elicit these benefits. Furthermore, the performance differences reported could be because the microbial community of the 7-week-old pigs in the current trial was more stable and less susceptible to change under the influence of XOS compared to a newly weaned pig with a more plastic microbiota, resulting in XOS having more of an effect in the younger pig.

There was no difference between dietary treatments for average pen faecal, health or cleanliness scores from day 1–35. The average pen faecal score from day 1–35 across all treatments was 2.47, thus between the observations of ‘soft faeces’ and ‘mild diarrhoea’, which may have contributed to the slightly lower growth performance expected for this unit.

2.5.2 Bacterial community composition

To investigate the prebiotic effect of XYL and XOS in pigs, faecal bacterial community composition was studied by sequencing the V4 region of the 16S rRNA gene between dietary treatment groups and over time. Over 90 % of faecal bacteria belonged to the Firmicutes and Bacteroidetes phyla. Similar bacterial compositions to those found in this study have been observed in the literature (Kim, Hyeun Bum et al., 2011; Holman et al., 2017). An interesting observation was the abundance of Tenericutes. The phylum Tenericutes consists of the class Mollicutes and are bacteria that lack a cell wall (Zhan et al., 2017). Tenericutes have been identified as an opportunistic phylum, and for example, a study showed that Tenericutes tended to increase with the inclusion of dietary flavonoid supplementation in dairy cows (Zhan et al., 2017). The abundance of Tenericutes was lower at day 14 compared to day 1 or 35 in the current study and showed a tendency to be lower in the CON group than the XOS group. Interestingly, Tenericutes were also one of the most dominant phyla after dietary XOS intervention for 6 months in pigs (Pan et al., 2019). At the genus level, most of these changes can be explained by the change in abundance of *Mollicutes_RF39_ge* which belongs to the Tenericutes phyla. This may indicate that the abundance of bacterial competitors of *Mollicutes_RF39_ge* was highest at day 14 and in the CON group, leading to the lower abundance observed. However, the functional roles of *Mollicutes_RF39_ge* remain unclear (Turnbaugh, 2017).

Of the 33 genera identified to have an abundance > 1 %, the only genus affected by dietary treatment was the *Prevotellaceae_NK3B31_group* which was higher in the XOS group compared to the CON, XYL or XYL + XOS groups. Belonging to the *Prevotellaceae* family, the abundance of *Prevotellaceae_NK3B31_group* has been enriched in low FCR pigs compared to high FCR pigs (Quan et al., 2019), albeit there were no efficiency improvements with XOS supplementation in the current study. The abundance of 21

genera changed from day 1–14. These results may be explained by a change in diet since the pigs transitioned from a highly digestible weaner diet at day 1 to a more indigestible cereal-based grower diet, possibly explaining the flux in bacterial abundance while the bacterial community adjusted.

Alpha diversity defines the diversity within a particular ecosystem and is commonly used as an indicator of species richness and evenness using Chao1 and Shannon indices measures, respectively (Pan et al., 2019). Disagreeing with hypothesis 2, dietary treatment had no significant effect on alpha diversity. This agrees with other studies investigating XOS (Pan et al., 2019; Pourabedin et al., 2017) and alternative prebiotics (Berding et al., 2016; Li, 2017). XOS did show a trend for an increased number of OTUs and species evenness, indicating there is potential for XOS to increase alpha diversity. To this point, XOS has been shown to increase species richness (Chao1) in weanling pigs (Yin, J. et al., 2019). Early bacterial colonisation and succession in the GIT are vital for establishing specific bacterial community compositions and subsequent host health. The age of the host has a notable effect on the diversity and bacterial community of the microbiome, with stability generally reached after 5 weeks of age (Thompson et al., 2008). Supporting this, in a trial with 3-month-old pigs, time had no effect on alpha or beta diversities over a 12-week sampling period, indicating that the bacterial communities had stabilised by this later age (Umu et al., 2015). Agreeing with hypothesis 2, the current study demonstrated that time influenced alpha diversity, particularly the number of OTUs and species richness, which was greater at day 35 compared to day 1 and 14. This indicates that the bacterial communities may have been continuing to adapt over time.

Beta diversity defines the heterogeneity of species composition between different communities along the environmental gradient, thus reflecting the species diversity between communities (Pan et al., 2019). In contrary to hypothesis 2, the beta diversity of bacterial community composition was not affected by dietary treatment; yet, it did change over time as hypothesised (no. 2). As shown in the NMDS graph, the communities at day 14 diverged from day 1, while the communities at day 35 clustered more tightly, indicating a more homogenous bacterial community composition at the end of the trial. A change in diet may explain these results since the pigs transitioned from a highly digestible weaner diet at day 1 to a more indigestible cereal-based grower diet. These changes in raw material content could have altered the quantity and type of material that reached the hindgut, highlighting the importance of diet in shaping gut bacterial communities (Frese et al., 2015). This, in turn, likely disrupted the bacterial community. By day 35, it is likely the community had specialised in fermenting more indigestible materials. Similar results occurred in weanling pigs, where the composition of bacterial communities significantly diverged over two weeks after weaning, demonstrating bacterial community composition change over time irrespective of treatment (Looft et al., 2012). Moreover, clustering tendencies have been observed between different doses of XOS in pigs. However, the growth stage at which XOS was added was postulated to have been more of a driving force to shape the gut microbiota structure than XOS dosage, which played a comparable insignificant role (Pan et al., 2019).

To explore the time effect further, DESeq2 analysis was conducted to identify the greatest changes in abundance between day 1 and 14. Both *Veillonella* and *Megamonas* from the *Veillonellaceae* family increased with time. *Veillonellaceae* are gram-negative bacteria known for lactate fermentation (Bonder et al., 2016). *Veillonellaceae* is considered a pro-inflammatory family of bacteria as sufferers of irritable bowel disease and irritable bowel

syndrome increase their abundance (Gevers et al., 2014; Shukla et al., 2015), thus possibly indicating some gut dysfunction at day 14 in the current study. To this point, the abundance of the genera *Megasphaera*, *Dialister* and *Mitsuokella* which belong to the *Veillonellaceae* family were all identified as having a > 1 % relative abundance in the current study, with the latter 2 genera showing a significant increase in abundance at day 14. With the primary function of the *Veillonellaceae* family being lactate utilisation (Daly et al., 2012), it is reasonable to assume that an increase of lactate could have been present in the gut at day 14, hence explaining the large increase in lactate-utilising bacterial abundance in the faeces. A potential reason for lactate presence could include an increased level of starch fermentation, whereby bacteria degrade starch into small polysaccharides or other metabolic intermediates such as lactate, thus increasing the abundance of lactate-utilising bacteria to prevent its accumulation (Trachsel, 2017; Duncan et al., 2004). Interestingly, the dietary switch from the digestible weaner diet to a more indigestible grower diet at day 1 resulted in a 28.6 % increase in dietary starch levels, from 350 to 450 g/kg. Most starch is usually digested in the upper GIT of monogastrics. Still, an overload of dietary starch into an immature digestive system or an increased level of resistant starch may lead to increased starch fermentation in the hindgut. Dissections and consequent GIT sample collection were not within the scope of this study. However, investigating starch digestion and lactate concentration along the GIT and its interplay with lactate-utilising bacteria and other metabolites in pigs would be interesting for future studies. This is of particular importance when considering cross-feeding, as bacteria like *Prevotella* spp. or *Bifidobacterium* spp. may produce lactate as a metabolic intermediate of starch fermentation (Trachsel, 2017; Duncan et al., 2004), while species like M*egasphaera elsdenii* and *Anaerostipes caccae* utilise the lactate and produce butyrate, thus conferring additional health and energy benefits to the host (Muñoz-Tamayo et al., 2011).

Members of the *Prevotellaceae*, *Muribaculaceae* and *Rikenellacea*e families, which all belong to the order Bacteroidales, showed the greatest decline in abundance from day 1–14. *Prevotella* spp. are adapted to metabolise a wide range of complex carbohydrates, and therefore provide benefits to the host via the production of SCFAs (Dou et al., 2017; De Filippo et al., 2010). Moreover, *Muribaculaceae* are involved in complex carbohydrate degradation (Lagkouvardos et al., 2019; Ormerod et al., 2016) and *Rikenellacea*e ferment carbohydrates and proteins (Su et al., 2014; Xin et al., 2019). As pigs age, the gut matures and becomes more efficient at degrading less digestible material. It is therefore expected that the abundance of bacteria capable of degrading complex carbohydrates would increase. With the change to a more indigestible cereal-based grower diet occurring at day 1, the microbiota was likely in a state of flux at day 14 while adapting to the change in quantity and different substrates reaching the hindgut.

Although there was no overall treatment effect on bacterial community structure, disagreeing with hypothesis 3, there were some treatment effects worth mentioning. Supplementation of XYL decreased OTUs associated with *Veillonellaceae_unclassified* when compared to the CON group. Interestingly, similar results have also been reported in a weanling pig study (Lu et al., 2019). XYL decreased the abundance of *Veillonellaceae* and tended to decrease *Megasphaera*, both of which are members of the lactate-utilising *Veillonellaceae* family (Daly et al., 2012). This repeated observation may indicate that the mechanistic pathway of XYL could suppress the growth of *Veillonellaceae* families.

Furthermore, OTUs classified as *Prevotella_9* and *Alloprevotella* declined in all 3 dietary treatments compared to the CON, while the same OTUs associated with

Muribaculaceae_ge and the *Prevotellaceae NK3B31 group* increased. *Muribaculaceae_ge* and the *Prevotellaceae NK3B31 group* belong to the *Muribaculaceae* and *Prevotellaceae* family, both of which have been associated with the degradation of complex carbohydrates, including xylan (De Filippo et al., 2010; Ormerod et al., 2016; Quan et al., 2019; Xin et al., 2019). This indicates that specific OTUs associated with both *Muribaculaceae_ge* and the *Prevotellaceae NK3B31 group* may be involved in the mechanistic pathways of XYL and XOS in the gut. Also, the combination of XYL and XOS would be expected to have an enhanced effect via a dual approach. The increase in *Muribaculaceae_ge* abundance (21.37 log2 fold) in the XYL + XOS treatment group was indeed higher than the increases seen in the single XYL and XOS treatment groups (14.89 and 15.14 log2 fold, respectively), albeit no performance benefits were seen.

A series of experiments investigating the prebiotic activity of XOS in broilers showed improvements in bird performance and shifts in microbial populations in the upper GIT tract (Ribeiro et al., 2018). The authors postulated that even if all the supplemented XOS was converted to SCFAs at 100 % efficiency, this would not solely be responsible for the improvements observed. Hence, it is possible that XOS can act as a signal to the xylan-degrading bacteria to increase in abundance and activity, thus improving xylan digestibility and efficiency of overall digestion. This stimulatory concept has been shown in broilers (Bautil et al., 2020) and described elsewhere in the literature (Bedford, Michael R, 2018; Petry and Patience, 2020). Despite the supplements in the current trial appearing to increase the abundance of some bacterial families associated with carbohydrate metabolism, xylan degradation along the GIT was not measured within this project's scope and no performance benefits were observed. A longer feeding period or an earlier introduction might allow for a longer ‘training’ period, in which the bacterial

communities would become xylan-degrading specialists and ultimately confer performance benefits to the host.

2.5.3 Conclusion

Overall, XYL and XOS supplementation had a limited effect on pig performance and faecal bacterial community composition. Results of this trial and comparison with the literature suggest a higher concentration of pure XOS (≥ 0.10 g/kg) in younger pigs may be necessary to observe performance benefits (assessed in Chapter 4). The degradation of xylan along the GIT coupled with bacterial community composition analysis would be of interest for future similar trials (assessed in Chapter 6).

Chapter 3

L-lactate concentration and metabolism in pig faeces

3.1 Abstract

Lactate is a metabolic intermediate that is quickly utilised by bacteria in the GIT, thus it is seldom detected in the faeces of healthy subjects or at low concentrations (< 3 mM). Accumulation of faecal lactate indicates the inability of the GIT bacterial community to clear hyper-lactate concentrations and could act as an indicator of bacterial imbalance and gut health status. This chapter presents a series of pilot studies with the aims of identifying (i) if L-lactate can be detected in piglet faeces and if concentrations change with age; and (ii) how quickly a known concentration of L-lactate is metabolised by pig faecal bacteria. In experiment 1, faecal samples from 5 piglets were collected on a weekly basis from a week before weaning until 20 days post-weaning for faecal L-lactate analysis, as well as 4 grower and finisher samples at one point in time. Samples were thawed, deproteinised and L-lactate concentrations were measured using a colorimetric assay kit. In experiment 2, a faecal sample was taken from a finisher pig and immediately aliquoted into 1 of 2 extraction solution types, distilled water (n = 14) or phosphate buffer solution (PBS; n = 14). The samples were then equally split between 2 sample fraction groups; 'Whole' unseparated samples or separated 'Supernatant' samples. Samples were then spiked with 5 mM of L-lactate at 0 min, and L-lactate concentration was measured at 7 TPs; 0, 20, 30, 60, 120, 240 and 360 min using a colorimetric assay or handheld L-lactate Scout (SCT) device. L-lactate concentrations were also measured with the SCT immediately before and after freezing to assess the effect of freeze-thawing. Data were analysed using repeated measures mixed analysis (JMP-v14). Experiment 1 showed that L-lactate concentrations were low or below the minimum detectable range of the assay (< 0.02 mM) in piglet faeces, with only 20 % of samples having detectable concentrations, the majority of which were immediately post-weaning. L-lactate was not detected in grower

or finishing pig faeces. Experiment 2 showed a 3-way interaction between TP, sample fraction and analytical method ($P < 0.001$). At 0 min, the SCT device correctly measured the spiked ~5 mM L-lactate concentration in both the 'Whole' and 'Supernatant' samples but the assay measured lower concentrations, suggesting elements within the faecal matrix may have interfered with the assay. L-lactate concentration in the 'Whole' samples decreased to ~0.60 mM after 360 min when measured by the SCT device and was undetectable after 120 min with the assay, indicating L-lactate metabolisation by pig faecal bacteria. L-lactate concentration in the 'Supernatant' samples, where bacteria were separated via centrifugation prior to lactate addition, remained stable over time when measured by both the SCT and assay. There was no effect of solution type on lactate concentrations ($P > 0.050$), and the freeze-thaw cycle reduced L-lactate concentrations ($P < 0.020$). In summary, L-lactate can be detected in piglet faeces albeit at low levels, whilst 5 mM of L-lactate was metabolised by pig faecal bacteria in 360 min. The SCT device is a suitable real-time method for measuring L-lactate concentrations compared to the colorimetric assay which failed to measure the spiked concentration. Faecal collection for L-lactate analysis must be rapid and the use of a buffer to halt microbial activity may be required to ensure true values are obtained. Further research and validation are required.

3.2 Introduction

GIT health, or gut health, is a key topic of interest in the animal nutrition field. The term 'gut health' lacks clear definition in the scientific literature, although it is commonly used in both human medicine and in animal health (Bischoff, 2011). Gut health covers multiple aspects of the GIT, such as effective digestion and absorption of food, the absence of GIT illness, normal and stable intestinal microbiota, effective immune status and a state of well-being (Bischoff, 2011). The function of the GIT is not restricted to food digestion and nutrient absorption but is also integral to host health. The GIT communicates with bacteria that support digestion via their enzymatic capabilities (Blaut and Clavel, 2007) and regulate major immune functions vital for gut and general health (Chung and Kasper, 2010). Moreover, the gut-brain axis transmits information from the GIT to the brain to feedback detection of dietary nutrients and other conditions that might affect mood and general well-being (Tsurugizawa et al., 2009). The assessment of gut health status is important but remains a challenge as it is difficult to evaluate the condition of the gut non-invasively. Rapid, accurate and non-invasive diagnostics to assess intestinal condition on-farm are required to act as a diagnostic tool and determine the effectiveness of potential treatments of gut disorders (Derikx et al., 2010).

Various non-invasive markers of gut health have been studied but none provide instantaneous results and are not commercially practical, available or used. This is mainly due to the need for scientific or veterinary intervention, requirement of a home office license or impracticality in a farm setting. The measurement of lactate in faecal samples has the potential to act as a marker of microbial imbalance, albeit this is a new research area for the pig sector. Lactate is present in isoforms in the body, D- and L-lactate. Usually, lactate exists entirely in the L-lactate form as mammalian cells almost exclusively produce this isomer (Kowlgi and Chhabra, 2015) and it is rapidly metabolised

to pyruvate by L-lactate dehydrogenase (LDH) (Ewaschuk et al., 2005). In comparison, D-lactate is formed in miniscule concentrations from methylglyoxal via the glyoxalase pathway in mammalian organisms (Kowlgi and Chhabra, 2015). Mammals lack D-LDH, and instead it is thought to be metabolised to pyruvate by the enzyme D-α-hydroxy acid dehydrogenase, which metabolises D-lactate at about one-fifth of the rate that L-LDH metabolizes L-lactate (Ewaschuk et al., 2005).

Both L- and/or D-Lactate can be produced by the bacterial communities in the GIT in varying amounts depending on the relative concentration and biological activity of different bacterial species (Kowlgi and Chhabra, 2015). L-lactate is the major fermentation end-product of LAB such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus* and *Streptococcus* (Duncan et al., 2004). The lactate isomer produced by bacteria depends on the genus, species and strain as seen in Table 3.1 (Blake, 2017). The production of lactate and associated reduction in pH of the gut environment can be beneficial as it restricts the growth of potentially pathogenic bacteria like *Escherichia coli* and *Clostridium perfringens* (Bakker-Zierikzee et al., 2005). Furthermore, bacteria such as *Megasphaera*, *Anaerostipes*, *Selenomonas* and *Veillonella* are capable of utilising lactate and converting it mainly to acetate and propionate, and in the case of *Megasphaera* and *Anaerostipes* also butyrate (Counotte et al., 1981; Hashizume et al., 2003; Muñoz-Tamayo et al., 2011). Butyrate acts as an energy source for gut epithelial cells and is generally deemed beneficial for host growth, gut health and inflammation (Guilloteau et al., 2010). Therefore, lactate plays important roles within the gut ecosystem and cross-feeding of bacteria.

Table 3.1 Lactate isomers produced by bacteria in the gastrointestinal tract (Blake, 2017).

L-Lactate	D-Lactate	Both	Reference
Aerococcus, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Tetragenococcus*, *Streptococcus*, *Vagococcus*	*Leuconostoc*, *Oenococcus*	*Lactobacillus*, *Pediococcus*, *Weissella*	(Liu, S.-Q., 2003)
Bifidobacterium, *Lactococcus*, *Enterococcus*	*Leuconostoc*	*Lactobacillus*, *Pediococcus* (except *L.dextrinicus*)	(Stiles and Holzapfel, 1997)
		Enterococcus faecalis (primarily D-lactate), *Streptococcus sanguinis* (primarily D-lactate), *Escherichia coli* (primarily L-lactate)	(Sheedy et al., 2009)

Lactate is rarely detected as a major fermentation product of mixed anaerobic bacterial communities in human faeces or gut contents due to its rapid utilisation by other bacterial species (Duncan et al., 2004). One study assessed the content of lactate and SCFA concentrations along the GIT of growing pigs and found a high lactate concentration at the start of the GIT in the jejunum (69.1 mM) and lower concentrations in the caecal digesta (36.3 mM) and faeces (2.5 mM) (Brestenský et al., 2017). Results showed that lactate concentration decreased linearly with an increasing content of other SCFAs, which is explained by the large microbial population in the caeca that metabolise lactate and convert it to other SCFAs. This utilisation means that faecal lactate concentrations are usually < 5 mM in healthy individuals (Duncan et al., 2004). Any lactate that is unused by bacteria is either absorbed by the host or excreted in the faecal matter (Blake, 2017). However, lactate can accumulate in the faeces up to ~100 mM in individuals who have undergone gut resections, like SBS, or who are sufferers of ulcerative colitis (Hove et al.,

1994; Kaneko et al., 1997; Vernia et al., 1988). Some report this to be due to poor carbohydrate digestion, increased fermentative activity and a relative increase in lactate-producing species (Ewaschuk et al., 2005), whilst others suggest that once the pH reduces past a certain point (pH 5.2) lactate production is maintained but lactate utilisation decreases (Belenguer et al., 2007).

In SBS patients where the small intestine is absent or reduced in length, an increased load of undigested carbohydrates reach the colon (Kowlgi and Chhabra, 2015). As a result, the number of metabolites produced exceeds the amount that can be metabolised or absorbed, leading to the accumulation of SCFAs and lactate, subsequently leading to a reduction in pH. As previously mentioned, a reduction in gut pH can be beneficial in restricting the growth of potentially pathogenic bacteria, yet the more acidic environment also favours the growth of lactate-producing species as they are acid-resistant. This results in a cascade where further lactate is produced, further decreases in pH are seen and more lactate accumulates (Kowlgi and Chhabra, 2015; Mayeur et al., 2013). In particular, the concentration of D-lactate accumulation in SBS patients can be serious and lead to neurotoxicity and cardiac arrythmia in humans (Chan et al., 1994; Vella and Farrugia, 1998). This highlights the vital role of the GIT microbiota in the production and utilisation of lactate under disease conditions. In comparison, the mechanism of lactate accumulation and disease development of ulcerative colitis is uncertain. However, colonic infusion of donor human intestinal bacterial communities from healthy individuals has been reported to permanently reverse the condition in some cases, indicating that microbial imbalance may be a contributing factor (Borody et al., 2003).

A rapid and large influx of readily fermentable substrates, such as starch, can induce lactate accumulation in the large intestine or faeces of non-ruminant mammals. In rats, the ingestion of certain oligosaccharides induced an accumulation of lactate and succinate up to ~70 mM in the caecum and reduced the pH to 5.5 (Hoshi et al., 1994). In pigs fed a low digestible diet, increased levels of undigested and unabsorbed food residues acted as substrates for fermentation by the GIT bacterial community which lead to increased faecal lactate concentrations (Etheridge et al., 1984). This could be caused by a rapid increase in abundance of amylolytic lactate-producing bacteria, and/or the reduction of lactate-utilising bacteria. In the absence of lactate-utilising bacteria, lactate accumulation is rapid as it is very slowly absorbed by epithelial cells (Umesaki et al., 1979). An *in vitro* model of pig caecal fermentation showed that lactate accumulation can be avoided if digesta pH is maintained > 6 when oligosaccharides were provided as a substrate (Ushida and Sakata, 1998). This is further supported by a dairy calf study which demonstrated the majority of high lactate concentrations were observed in faeces with a pH < 6 (Shimomura and Sato, 2006).

Luminal accumulation of lactate can lead to diarrhoea as it causes an osmotic pressure which results in water secretion from the mucosa into the large intestine (Saunders and Sillery, 1982). One study investigated pigs' faecal organic acid profiles with pathogenic and non-pathogenic diarrhoea (Tsukahara and Ushida, 2001). While diarrhoetic piglets receiving antibiotic treatments and those with dyspepsia (non-pathogenic) exhibited high faecal lactate concentrations, this was not so for piglets with colibacillosis. It was concluded that the rapid quantitative analysis of lactate in diarrhoetic faeces would provide a possible method for differentiating between colibacillosis and non-pathogenic diarrhoea in piglets. Differing lactate isomers and concentrations have also been seen with different GIT diseases in canines (Blake, 2017). Due to its rapid metabolism, lactate is

seldom detected in faeces. The detection of lactate in faecal matter may therefore indicate a gut microbial imbalance where lactate has been able to accumulate either due to an increase in lactate-producing bacteria and/or a reduction in lactate-utilising bacteria.

In a previous trial (Chapter 2), the abundance of bacteria from the *Veillonellaceae* family were shown to increase in pig faecal matter over time. *Veillonellaceae* are gram-negative lactate-utilising bacteria (Bonder et al., 2016). Although lactate was not measured, it is reasonable to assume that an increase of lactate could have been present in the gut, explaining the large increase in lactate-utilising bacteria abundance. This may have been due to a microbial imbalance or the dietary switch from a digestible weaner diet to a more indigestible grower diet that was accompanied by a 28.6 % increase in dietary starch and possible increase of starch fermentation. The overall aim of the research reported in this chapter is to determine if lactate can be measured in pig faeces and potentially act as a non-invasive and rapid marker of microbial imbalance.

3.2.1 Study aims

Coupled with the stress of weaning that encompasses abrupt dietary, social, bacterial and environmental changes, young piglets have an underdeveloped and immature GIT system making them vulnerable to diseases and dysbiosis. The dietary switch from easily digestible sows' milk to a more indigestible solid feed has a large impact on the GIT bacterial community as it abruptly adapts to utilise different feed materials that reach the hindgut. Therefore, the piglet is at increased risk of lactate accumulation and inability to clear hyper-lactate concentrations when the microbiota is unstable and underdeveloped.

The aim of experiment 1 was to investigate whether L-lactate could be detected in piglet faeces over the weaning period using a colorimetric assay kit and to determine whether faecal concentrations changed with age. The aim of experiment 2 was to decipher how quickly a known concentration of L-lactate would be metabolised by pig faecal bacteria; this was compared between different testing solutions, sample fractions and using different analytical methods. The analytical methods tested were a colorimetric assay kit and a handheld L-lactate SCT device. The SCT device was tested for its suitability as an on-site real-time detection method, with the overall aim of determining best practise for measuring L-lactate as an indicator of gut health in pigs on farm. A series of pilot trials were conducted to assess the study aims.

3.2.2 Hypotheses

3.2.2.1 Experiment 1

1. L-lactate will be detected in piglet faeces.
2. Faecal L-lactate concentrations will be higher immediately after weaning than before weaning.
3. After weaning, faecal L-lactate concentrations will decrease over time as the piglet ages and the GIT bacterial communities become more developed and established.

3.2.2.2 Experiment 2

4. Metabolism of L-lactate by faecal bacteria will be rapid and full utilisation of a known concentration will occur within 6 hours as seen in the literature.
5. L-lactate will be metabolised when kept in contact with faecal bacteria (‘Whole’ samples), but to a lesser extent when the L-lactate is added to the ‘Supernatant’ after centrifugation and bacterial separation.

6. L-lactate concentration will be similar when measured by the colorimetric assay or handheld L-lactate SCT device.
7. L-lactate concentration will be similar before and after freeze-thawing.

3.3 Materials and methods

3.3.1 Animals

The animals used in experiments 1 and 2 were from the University of Leeds' Spen Farm breeding herd ((Large White × Landrace females) × JSR Pietrain-based Geneconverter 900 sire line).

3.3.1.1 Animals - experiment 1

A week before weaning, 5 piglets were randomly selected from different sows in their respective farrowing crates for faecal sampling. They were followed throughout the 4-week trial at Spen Farm, Leeds, UK. Faecal samples were taken weekly, starting from 7 days before weaning until 20 days post-weaning, referred to as TP 1–5 as described below.

- TP 1: 7 days before weaning
- TP 2: Immediately post-weaning
- TP 3: 7 days post-weaning
- TP 4: 14 days post-weaning
- TP 5: 20 days post-weaning

Selected piglets were weighed at TP 1, 2 and 5 to determine piglet growth rate. In addition, at TP 1, 4 grower pigs (42 kg ± 3.3 kg) and 4 finisher pigs (106 kg ± 3.9 kg) were also randomly selected from the herd for faecal sampling to determine whether L-lactate could be detected at different life stages in commercially reared pigs.

3.3.1.2 Animals – experiment 2

A finisher pig with a BW of 96 kg was randomly selected for faecal sampling from within the indoor finishing herd at Spen Farm, Leeds, UK.

3.3.2 Sample collection and processing

3.3.2.1 Sample collection and processing - experiment 1

Faecal samples were collected immediately after defecation and placed on ice before further processing. A 2.5 g homogenised sub-sample of each faecal sample was weighed into a 15 ml falcon tube filled with 10 ml of distilled water [adapted protocol from (Shimomura and Sato, 2006)]. After thorough homogenisation by manual shaking, samples were centrifuged for 10 min at 2000 × g at room temperature. The supernatant (2 – 5 ml) was collected and immediately placed on dry ice. Samples were transferred to the laboratory and stored at –80 ºC until analysis.

3.3.2.2 Sample collection and processing - experiment 2

A schematic diagram for the methodology of experiment 2 is shown in Figure 3.1. A large faecal sample (*ca* 100 g) from the randomly selected finisher pig was collected immediately after defecation and placed on ice before further processing. Within minutes of collection, 2.5 g of the homogenised faecal sample was weighed into a 15 ml falcon tube containing either 10 ml of distilled water (n = 14) or 10 ml of PBS (n = 14) and thoroughly homogenised. The samples were equally split between 2 groups for processing ('Whole' or 'Supernatant'; sample fraction).

- 'Whole' samples (Water, n = 7; PBS, n = 7) were spiked with 5 mM of L-lactate (Supelco; 46937; 100 % purity) at 0 min. At 7 TPs, 0, 20, 30, 60, 120, 240 and

360 min, the sample was centrifuged for 5 min at 2000 × g and the supernatants were collected before being immediately frozen on dry ice and transported to a –80 ºC unit until analysis.

- ‘Supernatant’ samples (Water, n = 7; PBS, n = 7) were first centrifuged for 5 min at 2000 × g and the supernatant collected into a new falcon tube. The supernatants were then spiked with 5 mM of L-lactate. Samples were kept at room temperature and then frozen on dry ice at each TP (as described for the ‘Whole’ samples above) and transported to a –80ºC unit until analysis.

L-lactate concentrations were measured in duplicate at each TP using 2 analytical methods; a handheld L-lactate SCT device (EKF-Diagnostics) was used to measure real-time L-lactate concentrations immediately before freezing on dry ice or a L-lactate colorimetric assay was used after thawing in the laboratory (described below).

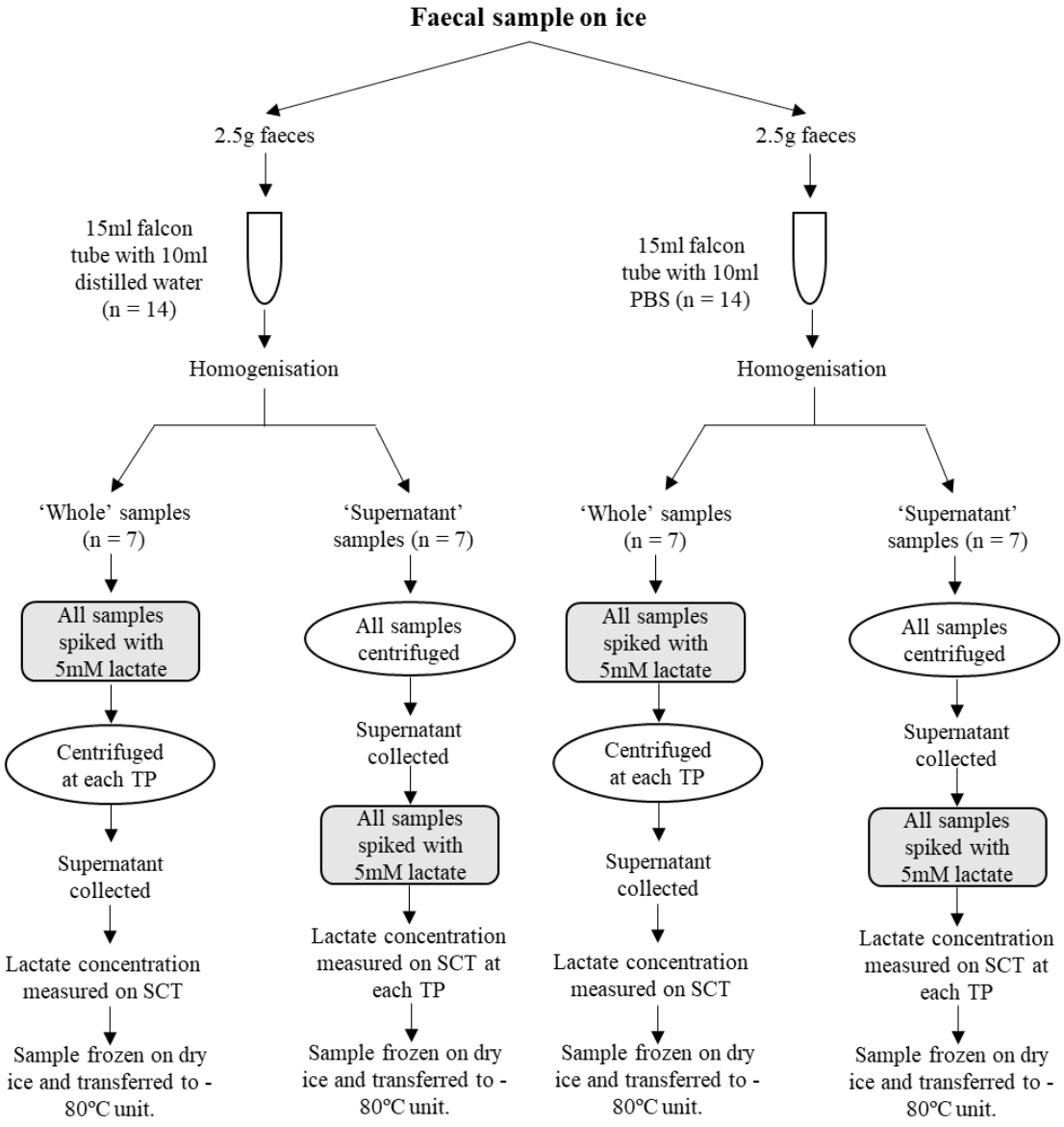

Figure 3.1 Schematic methodology of experiment 2 (Timepoint, TP; L-lactate Scout device, SCT).

3.3.3 L-lactate analysis

3.3.3.1 L-lactate analysis - experiment 1

Samples were thawed and deproteinised prior to analysis using the Trichloroacetic acid (TCA) method according to the kit manufacturers' protocol (Biovision, K823-200). Briefly, 100 µl of sample was mixed with 15 µl of cold (4 ºC) TCA in a 1.5 ml microcentrifuge tube. The sample was kept on ice for 15 min before centrifugation at 12000 × g for 5 min and collecting the supernatant. To neutralise excess TCA, 10 µl of cold (4 ºC) neutralisation solution was mixed with the supernatant and placed on ice for a further 5 min. The resulting sample was used directly in the L-lactate assay.

A colorimetric assay kit (Biovision, K627-100) was used to measure L-lactate concentration in the faecal samples according to the kit manufacturers' protocol. To ensure the readings were within the standard curve range, 4 dilutions were tested including undiluted, 1:1, 1:10 and 1:20, of which the undiluted samples were the best fit. Test samples were prepared in duplicate at 50 µl/well. A reaction mix was prepared containing 46 µl of lactate assay buffer, 2 µl of lactate substrate mix and 2 µl of lactate enzyme mix. In addition, 2 samples were tested for nicotinamide adenine dinucleotide + hydrogen and nicotinamide adenine dinucleotide phosphate background levels in the absence of the lactate enzyme mix (adjusted 48 µl lactate assay buffer and 2 µl lactate substrate mix). The reaction mix was added to each well and incubated at room temperature for 30 min before being measured on a microplate reader at 450 nm. Sample concentrations were corrected for assay background levels, deproteinisation dilution (0.8), initial sample dilution (0.2) at collection and were calculated back to mM per g of faeces. Duplicate readings were averaged.

3.3.3.2 L-lactate analysis - experiment 2

Initial L-lactate concentrations were measured in duplicate using the SCT device immediately prior to freezing. The SCT device works by enzymatic amperometry where L-lactate oxidase coats the electrode and reacts with lactate, this produces hydrogen peroxide which then generates an electrical current that is proportional to the amount of L-lactate in the sample. L-lactate sensors were kept at 4 ºC and allowed to come to room temperature 20 min before use. As seen in Figure 3.2, to take a reading, a sensor was inserted into the handheld device and then placed into the media. This was repeated with a new sensor to take duplicate readings.

Figure 3.2: Handheld L-lactate Scout (SCT) device with sensor.

After thawing, L-lactate concentrations were re-measured in all samples using the lactate SCT device to assess the impact of freeze-thawing. Following this, samples were deproteinised and L-lactate concentrations were measured in duplicate using a colorimetric assay, as per experiment 1 described above. To ensure the readings were within the standard curve range, 3 dilutions were tested according to the initial SCT results of each sample. Samples with a reading of > 2 mM on the SCT device were diluted

at 1:10, whilst readings at < 2 mM were diluted at 1:1, and those with non-detectable readings below the 0.5 mM detection range of the SCT device were left undiluted. Sample concentrations were corrected for assay background levels, deproteinisation dilution (0.8) and sample dilutions as described above. SCT results were corrected for the constant automatic haematocrit adjustment made by the handheld device (0.2), as suggested by the supplier. Duplicate readings were averaged.

3.3.4 Statistical analysis

Statistical analysis was not possible on data from experiment 1 due to the small sample size and non-detectable concentrations. Data from experiment 2 were analysed using a repeated measure mixed analysis (JMP-v.14), with TP, analytical method (SCT and assay), sample fraction ('Whole' and 'Supernatant') and solution (Distilled water and PBS) as factors. The effect of freezing on L-lactate concentration in each sample fraction was assessed using a repeated measure mixed analysis (JMP-v.14), with TP and pre- or post-thaw as factors.

3.4 Results

3.4.1 Results - experiment 1

The post-weaning growth performance of the study piglets was similar to the average performance of the unit, with an ADG of 0.35 kg/d (± 0.06 kg/d) from TP 2–5. All piglets remained healthy throughout the sampling period, had normal faecal consistency and received no antibiotics.

Experiment 1 investigated whether L-lactate could be detected in piglet faeces and whether faecal concentrations changed with age. L-lactate concentrations were low or below the minimum detectable range of the assay (< 0.02 mM), as shown in Table 3.2. Only 20 % of samples showed concentrations that were detectable, ranging from 0.03 to 0.34 mM per g of faeces, with the majority of these measurable concentrations being at TP 2, which was immediately post-weaning. L-lactate concentrations were undetectable at TP 4 and 5, or in the grower and finisher pig samples (data not shown).

Table 3.2 L-Lactate concentration (mM per g of faeces) in piglet faeces over time.

Piglet	L-Lactate concentration (mM per g of faeces)				
	TP 1[a]	**TP 2[b]**	**TP 3[c]**	**TP 4[d]**	**TP 5[e]**
1	ND	0.18	ND	ND	ND
2	ND	0.25	ND	ND	ND
3	ND	0.16	0.03	ND	ND
4	ND	ND	ND	ND	ND
5	0.34	ND	ND	ND	ND

ND: Non-detectable. [a] TP; Timepoint 1: 7 days before weaning. [b] TP 2: Immediately post-weaning. [c] TP 3: 7 days post-weaning. [d] TP 4: 14 days post-weaning. [e] TP 5: 20 days post-weaning.

3.4.2 Results - experiment 2

Experiment 2 aimed to decipher how quickly a known concentration of L-lactate would be metabolised by pig faecal bacteria between different testing solutions, sample fractions and using different analytical methods. There was a significant 3-way interaction between TP, sample fraction and analytical method (Figure 3.3; $P < 0.001$). At 0 min (baseline), the SCT device correctly measured the spiked L-lactate concentration (~5 mM) in both the 'Whole' and 'Supernatant' samples. However, the colorimetric assay yielded concentrations much lower at the same TP in both the 'Whole' and 'Supernatant' samples. L-lactate concentration in the 'Whole' samples decreased to ~0.60 mM over 360 min when measured by the SCT device and was undetectable after 120 min with the colorimetric assay. L-lactate concentration in the 'Supernatant' samples remained stable over time when measured by both the SCT and assay. There was no significant effect of solution type (distilled water vs PBS) on L-lactate concentrations ($P > 0.050$).

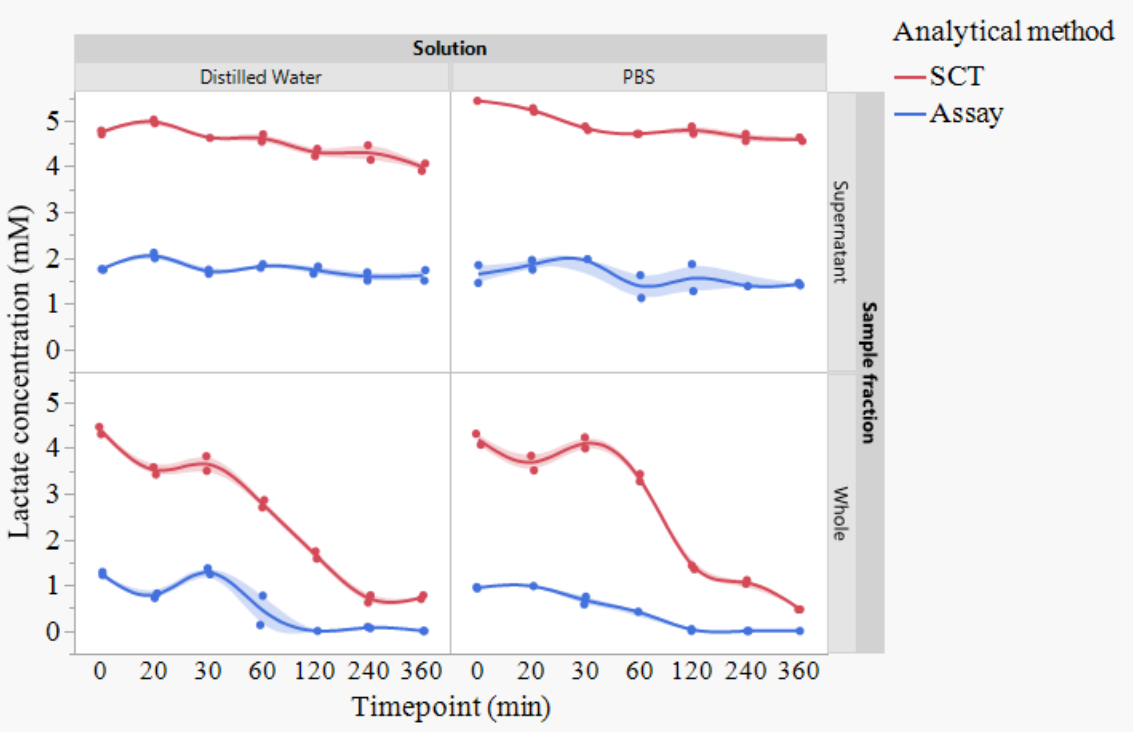

Figure 3.3 L-lactate concentration (mM) in pig faeces over time in different solutions, different sample fractions and measured by different analytical methods ($P < 0.001$. SCT; L-Lactate Scout device).

There was a significant effect of the freeze-thaw process on L-lactate concentrations in the 'Whole' samples ($P < 0.020$; Figure 3.4) and a trend for the 'Supernatant' samples ($P = 0.053$; Figure 3.5). Over time, the average L-lactate concentration in the 'Whole' samples in distilled water and PBS were –0.18 mM and –0.27 mM lower, respectively, after freeze-thawing compared to real-time analysis before freezing. Over time, the average L-lactate concentration in the 'Supernatant' samples in distilled water and PBS were –0.32 mM and –0.45 mM lower, respectively, after freeze-thawing compared to real-time analysis before freezing.

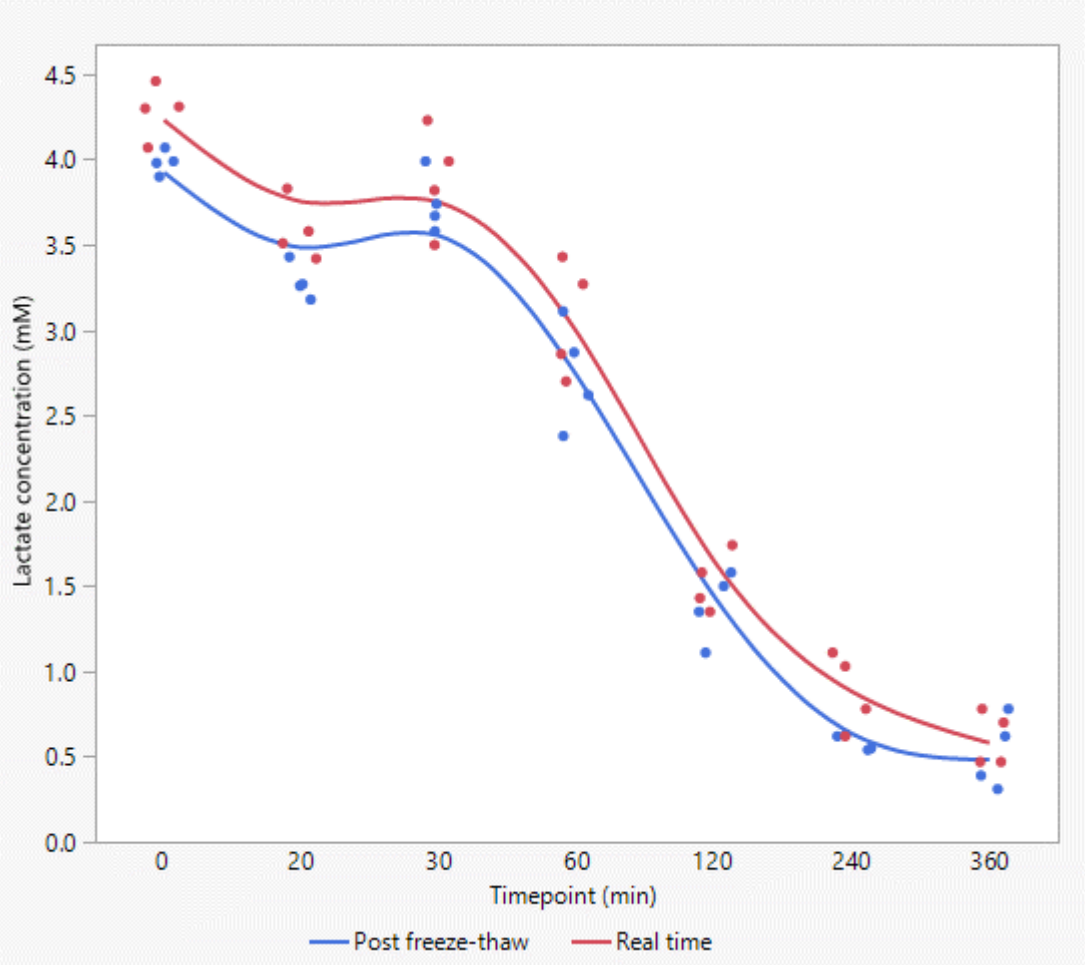

Figure 3.4 L-lactate concentration (mM) over time in 'Whole' samples measured in real time (before freezing) and after freeze-thawing by the L-lactate Scout (SCT) device ($P < 0.02$).

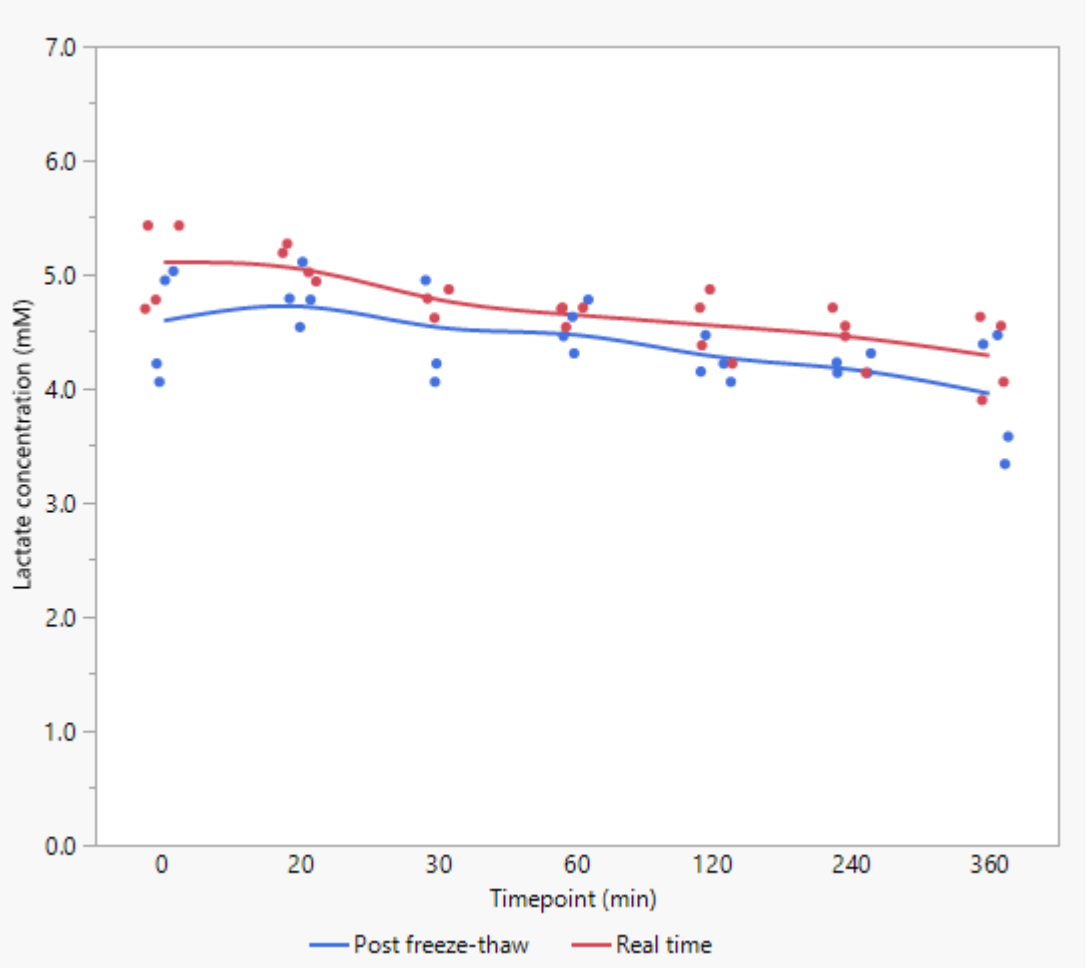

Figure 3.5 L-lactate concentration (mM) over time in 'Supernatant' samples measured in real time (before freezing) and after freeze-thawing by the L-lactate Scout (SCT) device (*P* = 0.053).

3.5 Discussion

Lactate is seldom detected in the faeces of healthy subjects as bacteria rapidly metabolise it in the GIT. However, lactate can accumulate in the faeces of individuals suffering from certain GIT disorders (Hove et al., 1994). Lactate accumulation can be caused by various gut disorders and an overload of undigested carbohydrates to the hindgut. An over-production of lactate by lactate-producing bacteria or a reduction in lactate metabolism by lactate-utilising bacteria can cause lactate to accumulate in the GIT environment. For this reason, the measurement of lactate in pig faeces has the potential to act as a marker of microbial imbalance and an indicator of gut health.

Experiment 1 investigated whether L-lactate could be detected in piglet faeces around weaning and whether faecal concentrations changed with age. Results showed that L-lactate concentrations were low or below the minimum detectable range of the colorimetric assay, hence partially agreeing with hypothesis 1. Only 20 % of samples showed detectable concentrations, ranging from 0.03 – 0.34 mM per g of digesta. In faeces from healthy dogs, L-lactate concentrations were reported to be around ~0.60 mM (Blake, 2017). In growing pigs (~25 kg), faecal lactate concentrations of ~0.80 – 2.80 mM have been reported, although it was not stated whether the D- or L- isomer was measured or if the values represented total lactate (Brestenský et al., 2017). The L- and D-lactate concentrations in the stomach, duodenum, ileum, caecum and colon of weanling pigs have also been investigated (Mathew et al., 1998). Although faecal lactate concentrations were not measured, L-lactate was not detectable in the colon of CON fed pigs, and D-lactate was only detectable at 0.10 – 0.30 mM, indicating that very low or no lactate would have been detected in faeces. Hence, the L-lactate concentrations found in the current study were low but not dissimilar from the literature.

Moreover, 3 of the detectable L-lactate readings were at TP 2, which were taken immediately post-weaning, partially supporting hypothesis 2. Weaning is an extremely stressful time for the piglet with abrupt changes to diet, social, bacterial and environmental life conditions (Kim, J. et al., 2012). These stressors can affect the gut microbiome, physiology and ultimately increase susceptibility to enteric disease (Pajarillo et al., 2014). The presence of faecal lactate around the weaning period is most likely multifactorial. Firstly, although not instantaneous, weaning is associated with marked changes to the histology and biochemistry of the small intestine such as villous atrophy and crypt hyperplasia, leading to an overall reduction in digestive and absorptive capacity, and subsequent increase in substrate fermentation (Pluske et al., 1997). Secondly, weaning stress causes abrupt changes to the microbiome often leading to dysbiosis in bacterial communities (Gresse et al., 2017). Suppose the GIT bacterial communities were in a state of flux and adaption. In that case, the abundance of lactate-producing and utilising bacteria may have been unbalanced leading to the presence of lactate in the faeces. By the week after weaning (TP 3), L-lactate was either non-detectable or had greatly reduced. This suggests either a possible increase in digestive and absorptive capacity leading to less substrate fermentation or stabilisation of the bacterial communities. L-lactate concentration did not show a decreasing relationship with age as expected, hence disagrees with hypothesis 3.

Lactate concentration in faeces has been measured successfully over time in dairy calves (Shimomura and Sato, 2006). Elevated total faecal lactate concentrations were observed within the first 4 weeks of life but then remained low (~ < 5 mM) after this age (Shimomura and Sato, 2006). Lactate is considered the main anion in the immature gut flora of the colonic lumen in early life. It is thereafter replaced by replaced by SCFAs with advancing age and development of GIT bacterial communities (Shimomura and

Sato, 2006). The processing of calf faeces involved homogenising 5 g of faeces with 20 ml of water, separating the sample via centrifugation and freezing the supernatant before sample analysis (Shimomura and Sato, 2006). Before analysing D- and L-lactate concentrations with a UV method using an LDH assay kit, the thawed supernatant was deproteinized with zinc sulfate and potassium hydroxide. A similar methodology was used in the current study, but slightly adapted with half the faecal matter quantity due to the difference in animal size and amount of faeces produced in a single defecation. Furthermore, samples were deproteinised using the TCA method as recommended by the colorimetric assay kit used to measure L-lactate concentrations via LDH. In summary, experiment 1 showed that L-lactate can be detected in piglet faeces but at very low or non-detectable concentrations.

Experiment 2 was designed to investigate the metabolism of L-lactate by pig faecal bacteria over time, and whether this differed between solutions and sample fractions. This experiment also compared a colorimetric assay with a real-time handheld device to determine its potential as an on-farm rapid analysis technique. The concentration of lactate in blood is one of the most commonly measured parameters taken during clinical exercise testing or performance analysis of athletes (Goodwin et al., 2007). Small handheld analysers like the SCT device that measure blood L-lactate are available on the marketplace and are used by athletes to monitor their blood lactate concentrations with the aim of improving their fitness and endurance training. When compared to a spectrophotometric analyser method, the SCT device has also proved a reliable alternative method when assessing blood L-lactate concentrations in dogs (Ferasin et al., 2007). Similar accurate and reliable blood L-lactate results using the SCT device have been obtained in ovine, bovine and porcine medicine (Kaynar et al., 2015; Burfeind and

Heuwieser, 2012; Rocha et al., 2015). The handheld analysers are based on enzymatic amperometry, whereby L-lactate oxidase coats an electrode and reacts with lactate, this produces hydrogen peroxide which then generates an electrical current that is proportional to the amount of L-lactate in the sample (Pang and Boysen, 2007; Allen and Holm, 2008). However, handheld L-lactate analysers like the SCT device are modified for use in blood and make automatic compensations for haematocrit levels (Blake, 2017), making their use sub-optimal in other biological samples unless the correction factor is known and adjusted for. To the authors knowledge, the SCT device has not previously been tested on faecal matter.

Results showed that the SCT device correctly measured the spiked L-lactate concentration of ~ 5 mM at 0 min (baseline) in both the 'Whole' and 'Supernatant' samples, indicating the device can measure L-lactate in faecal matter. Regardless of solution, the initial SCT reading at 0 min in the 'Supernatant' samples was very close to the spiked concentration at 5.08 mM. In comparison, regardless of solution, in the 'Whole' samples, the initial SCT reading at 0 min was 4.28 mM which was slightly lower than the 5 mM expected. This indicates that some L-lactate metabolism may have occurred during sample preparation. Similar results have been found in a study using ^{13}C-labeled lactate to study the butyrate formation from lactic acid by pig caecal bacteria (Ushida and Hoshi, 2002). Results highlighted the importance of *Megasphaera elsdenii* in lactate conversion in the pig caecum and demonstrated its ability to remove hyper-lactate concentrations. Labelled lactate was introduced into incubation vessels to obtain 20 mM as the final concentration after the inoculation of digesta. The labelled lactate was fully metabolised within 6 hours of incubation, with the concentration of acetate being highest amongst the produced acids, followed by propionate and butyrate. However, lactate concentration at 0 hours was less than the intended 20 mM at approximately 14

mM. This lower concentration was reported to be caused by the metabolism of lactate during the preparation of the samples. The authors stated that placing the samples in a crushed-ice bath followed by centrifugation was insufficient to stop fermentation promptly, highlighting that lactate fermentation is rapid and protocols need to account for this.

Enzymatic assays are another method of lactate measurement, and have been used previously with human faeces (Mayeur et al., 2013), bovine faeces (Shimomura and Sato, 2006) and murine faeces (Rul et al., 2011). An enzymatic assay for the measurement of D-, L- and total lactate has been tested and successfully established for canine faeces (Blake, 2017). Assays are a good option for lactate analysis and certain kits can distinguish between D- and L-lactate concentrations, albeit they do not produce instantaneous results. However, the results from the colorimetric assay used in this study generated lower concentrations than the expected spiked level (5 mM) at the same TP in both 'Whole' and 'Supernatant' samples. This may have been because certain elements within the faecal matrix interfered with the assay, despite sample deproteinisation. Supporting this proposition, a CON sample (without faecal matter) of distilled water or PBS spiked with 5 mM L-lactate was tested in the assay at the same time. Results showed that the L-lactate concentration was 4.5 mM in distilled water and 4.4 mM in PBS, further supporting the theory that elements within the faecal matter interfered with the assay. Furthermore, when the SCT device tested these samples, results were similar at 4.6 mM in distilled water and 4.4 mM in PBS. In summary, the results show that the specific assay used in this study was not a suitable method to measure L-lactate in faecal medium, whereas the SCT was, and this may have given false low readings in experiment 1. Hence, hypothesis 6 was not supported.

When faecal bacteria remained in contact with the L-lactate in the ‘Whole’ samples the majority of L-lactate was utilised by 360 min (~0.60 mM remained) when measured by the SCT, but L-lactate was undetectable after 120 min with the assay. This indicates that the faecal bacteria successfully metabolised the majority of the added L-lactate within 360 min, thus agreeing with hypothesis 4. This is slower than in the referenced *in vitro* study (Ushida and Hoshi, 2002) whereby ~14 mM was metabolised within 360 min, but caecal digesta was used instead of faeces. The microbial community in faecal matter is likely to represent only a discrete population of the intestinal microbiota (Quan et al., 2019). Moreover, the differences in microbiota distribution along the GIT suggests that different intestinal niches have different roles. This may explain the difference in the rate of lactate metabolism between the two studies (Gresse et al., 2019). As hypothesised (no. 5), when L-lactate was added to the ‘Supernatant’ samples the concentration did not decrease over time and remained around the initial spiked level when measured by both the SCT and assay. These results demonstrate that centrifugation separated the majority of lactate-utilising bacteria as the L-lactate was not metabolised.

Chemicals like sulfuric acid and ethanol can halt fermentation and help preserve samples for metabolite analysis (Ushida and Hoshi, 2002), although these can have undesired effects in further analysis. Prior to experiment 2 being conducted, faecal matter suspended in ethanol and spiked with 5 mM of L-lactate were tested on the SCT device. However, the SCT could not measure the L-lactate as the ethanol interfered with the enzymatic amperometric detection method. PBS was selected as an alternative solution as recommended for faecal analysis by a different Porcine LDH ELISA Kit (MBS042294). Homogenising the faecal matter in PBS instead of distilled water did not affect the bacterial fermentation of L-lactate.

The lactate SCT device can measure L-lactate concentrations in real-time, whereas the samples were frozen for preservation purposes before being thawed and analysed by the assay. The effect of the freeze-thaw process on L-lactate concentrations was assessed by analysing the samples in real-time before freezing compared to after freeze-thawing, both measured by the SCT device. Results showed that average L-lactate concentrations over time were significantly lower after freeze-thawing compared to real time analysis, such that 'Whole' samples in distilled water and PBS were –0.18 mM and –0.27 mM lower after freeze-thawing respectively. Moreover, 'Supernatant' samples in distilled water and PBS tended to be –0.32 mM and –0.45 mM lower after freeze-thawing than real-time analysis. This indicates deterioration of the L-lactate molecule during the freeze-thaw process, hence disagrees with hypothesis 7. These results highlight the importance of real-time analysis as a loss of –0.18 to –0.45 mM is a large deterioration, especially if the concentration is already low such as in experiment 1. However, it has been shown that once deproteinised, lactate in faecal extracts from dogs can remain stable for 24 hours of storage at 4 ºC and 28 days of storage at –80 ºC (Blake, 2017).

3.5.1 Conclusion

To conclude, this series of pilot studies has highlighted various benefits and limitations of measuring lactate concentrations in pig faecal matter. Firstly, faecal L-lactate concentrations are generally low or undetectable in healthy piglets and growing/finishing pigs. The SCT device successfully measured the spiked L-lactate concentration whereas the assay failed to do so, indicating the specific assay used in this study was not a suitable method of L-lactate analysis in pig faeces and casts doubts on the results of experiment 1. The SCT provides real-time results which is a positive attribute if faecal L-lactate

concentrations become a useful non-invasive gut assessment tool in the future, but the SCT is limited to the analysis of the L-lactate isoform and has a relatively high minimum detection limit of 0.50 mM. In comparison, the assay kit used in these trials was not suitable, but other kits have proved accurate for faecal analysis (Blake, 2017) and these should be further validated for pig faecal samples. Moreover, both D- and L-lactate isoforms can be measured in certain assays, but results are not instantaneous, and the freeze-thaw process may lead to lower results than true. When faecal bacteria remain in contact with the spiked L-lactate, the majority of L-lactate was utilised within 360 min, but when the bacteria were separated, the L-lactate was not metabolised. Lactate metabolism is relatively fast and the process of collecting and preparing faecal samples for lactate analysis must be rapid; the use of a buffer to halt microbial activity may be required to ensure true values are obtained. The freeze-thaw process has a negative effect on L-lactate concentrations, highlighting the benefits and importance of real-time analysis to prevent this deterioration. Further research and validation are required.

Further research is required to establish the normal range of lactate concentrations in pig faecal matter at different ages in healthy and diseased individuals. Future work in this area could include the examination of faecal lactate and bacterial communities to investigate the interplay between the microbiota and metabolites. Furthermore, research on the D/L faecal lactate ratio is required as this has been described as the most relevant index for assessing imbalanced microbiota in SBS patients (Mayeur et al., 2013). It would be interesting to investigate faecal lactate concentrations in a variety of GIT diseases that have different characteristics and symptoms to obtain a broader understanding of the role of lactate in gut health. Finally, it would be interesting to assess the effect of feeding prebiotics that promote LAB and thus lactate production to young piglets and assess their effect on the capability of the bacterial community to deal with an accumulation of lactate.

If more lactate is present in the GIT when supplementing diets with prebiotics, the abundance of lactate-utilising bacteria should theoretically be increased and therefore the gut would be more primed to remove hyper-lactate concentrations from a young age.

Chapter 4

Investigating the effect of xylo-oligosaccharides with or without fructans on growth performance, gut bacterial community composition and microbial endo-xylanase activity in weaned piglets.

4.1 Abstract

Seeking suitable alternatives to antibiotics that promote gut health and prevent disease in pig production is of great importance for the sustainability and profitability of the industry. Compared to feeding a single prebiotic, feeding a combination of different prebiotics could upregulate the metabolic capability of a wider range of gut bacteria, hence improving overall fibre digestion if the prebiotics work in synergy. This study investigated the effect of XOS with or without short and long-chain fructans on growth performance, gut bacterial community composition and microbial endo-XYL activity in weaned piglets. In this 28-day trial, a total of 474 weaner piglets with an average initial BW of 7.88 (SD ± 1.51 kg) were blocked into mixed sex pens of 4–5 piglets. Pens were randomly allocated to 1 of 6 dietary treatments in a 2 × 3 factorial treatment arrangement with 2 levels of XOS (0 and 0.286 g/kg) and 3 levels of fructans [(0 g/kg) and 6.50 g/kg of short-chain FOS and 4.08 g/kg of INU to supply 3.8 g/kg of pure fructans)]. Piglet BW and feed intake were recorded throughout the study to calculate piglet performance. At the end of the experiment, 48 piglets were euthanised for the collection of ileal and colonic digesta to profile the bacterial communities through 16S rRNA sequencing of the V4 region and colonic microbial endo-XYL activity. In the overall trial period, piglets supplemented with fructans (FOS and INU) had a lower ADFI compared to unsupplemented CON fed piglets. When supplemented alone, XOS also reduced ADFI compared to the CON, but intake increased to levels not different from the CON group when XOS was fed in combination with FOS and INU, resulting in a significant

interaction ($P < 0.018$). There was no overall effect of treatment on piglet ADG, but at the end of the trial piglet BW tended to follow a similar interaction pattern to ADFI ($P = 0.088$). FCR tended to be higher when piglets were supplemented with FOS and INU compared to the CON ($P = 0.086$). Ileal and colonic bacterial diversity was lower for FOS fed piglets compared to the CON ($P < 0.050$), whilst overall bacterial community structure differed between treatments in the ileum ($P < 0.026$) but not the colon. In the ileum, XOS increased the abundance of OTUs associated with *g__Helicobacter*, *g__Prevotella_7*, *g__Prevotella_9*, *g__uncultured (Prevotellaceae Family)* and reduced *g__Actinobacillus* ($P < 0.050$). There was a XOS × fructan interaction for ileal abundance of OTUs associated with *g__Lactobacillus*, whereby supplementation of XOS alone increased abundance above all treatment groups apart from the FOS group ($P < 0.038$). There was no effect of treatment on colonic microbial endo-XYL activity ($P > 0.050$). In summary, prebiotic supplementation supressed overall ADFI, whilst fructans tended to increase FCR. Bacterial community structure differed with prebiotic supplementation in the ileum but not the colon. Despite the enrichment of beneficial *Lactobacillus* abundance in the ileum with XOS, no overall performance benefits were seen, likely because of the reduction in ADFI and the need for a longer feeding period.

4.2 Introduction

The GIT constitutes an environment for the activity and development of the largest microbiota population in the body and has been the subject of study for many years due to its importance in the health and well-being of animals and humans (Barszcz et al., 2016; Isaacson and Kim, 2012). The GIT bacterial community confers a range of benefits to the host including improved disease resistance, GIT function, health status and animal performance (Isaacson and Kim, 2012; Barszcz et al., 2016). For this reason, research focused on nutritional strategies to improve animal health and performance through the

modulation of the GIT microbiota is timely. One such strategy is the supplementation with feed additives such as prebiotics.

Prebiotics are defined as ‘non-digestible food ingredients that survive digestion and reach the large intestine, where they act as substrates for selective bacteria, consequently influencing their growth and activity which provides health benefits to the host’(Gibson and Roberfroid, 1995). Prebiotics selectively stimulate beneficial bacteria like *Bifidobacterium* or *Lactobacillus*, and reduce pathogenic bacteria like *Escherichia coli* (Hsu et al., 2004; Liu, J. et al., 2018). The consequent shift in the bacterial community composition is associated with improved overall health, increased SCFA production, and reduced gut infection and inflammation (Macfarlane et al., 2006). The most commonly studied prebiotics include GOS and fructans such as FOS and INU. However, new-generation prebiotics like XOS are gaining attention for monogastric nutrition. Fructan supplementation, in the form of FOS (Xu, C. et al., 2005; Xu, Z.R. et al., 2002) and INU (Grela et al., 2014; He et al., 2002) can improve pig performance and gut health parameters. Similarly, supplementation of XOS has shown promising improvements in growth performance, gut structure, and gut bacterial community composition in broilers and weaner pigs (Liu, J. et al., 2018; Ribeiro et al., 2018; Chen, Y. et al., 2021).

Fructans occur naturally in many plants and are mainly extracted from sources such as the agave plant, chicory root or Jerusalem artichoke for commercial production (Grela et al., 2014). Fructose units are linked together by β-(2-1) glycosidic bonds which are resistant to endogenous digestive enzymes, hence survive transit to the distal portion of the GIT (Grela et al., 2014). INU extracted from chicory roots typically have a DP of 3–60 (Van De Wiele et al., 2007), whereas for native Jerusalem artichokes the average DP is ~6 (De Leenheer, 1994). Moreover, short-chain FOS can be produced by enzymatic transfructosylation from sugar beet and typically have a DP of 2–4. In contrast, XOS

consists of 2–7 xyl units linked by β-(1-4) linkages and can be commercially produced via the hydrolysis of corncobs (Aachary and Prapulla, 2008).

The site of fermentation of prebiotics in the GIT depends on a multitude of factors including the sugar composition, the DP, the type of linkages, the structure complexity, as well as the presence of substrate specific degrading bacteria and access to the substrate itself within the GIT (Houdijk, Jos, 1998). Measuring the metabolic activity of substrate specific degrading bacteria, like microbial endo-XYL activity, is key to understanding the mode of action of prebiotics. Substrates with a longer DP are fermented at a slower rate than those with a shorter DP due to the number of linkages that need to be broken (Roberfroid et al., 1998; Houdijk, Jos, 1998). To this point, fructans like FOS with a DP < 10 have been shown to be fermented approximately twice as quickly *in vitro* as those such as INU that have a longer DP (Roberfroid et al., 1998). Therefore, the rate of fermentation naturally dictates the location of fermentation along the GIT, such that longer chains with a higher DP are more resistant to saccharolytic activity and fermentation takes place more slowly and distally in the GIT (Hughes and Rowland, 2001). However, differences in rate of fermentation still exist between prebiotics with a similar DP, such that short-chain FOS and GOS have been shown to be rapidly fermented so likely function as a substrate in the terminal small intestine and proximal large intestine (Smiricky-Tjardes et al., 2003a). In comparison, short-chain XOS are slowly fermented and may be more suitable substrates for bacterial communities inhabiting the distal large intestine (Smiricky-Tjardes et al., 2003a). Hence, a complex relationship exists between substrate structure and fermentative activity.

Furthermore, the bacterial community composition along the GIT varies by location (Kim, H B and Isaacson, 2015), thus, the location of prebiotic fermentation will influence different bacterial communities, their associated metabolic activities and consequent

benefits to the host. The ability of different prebiotics to selectively stimulate different bacteria may provide further benefits to the host if fed in combination. An *in vitro* study confirmed the resistance of FOS and XOS to GIT enzymes and gastric juices, and the ability of *Lactobacillus* and *Bifidobacterium* to ferment these substrates (De Figueiredo et al., 2020). However, preferences for FOS or XOS were shown between different strains of bacteria, such that *Bifidobacterium breve* and *Lactobacillus brevis* preferred XOS, whilst *Bifidobacterium lactis* and *Lactobacillus acidophilus* showed a preference for FOS. These differences highlight a potential synergy between the prebiotics if fed in combination to stimulate a more representative population of beneficial GIT bacteria. In this study, we investigated the difference in prebiotic activity between short-chain (low DP) and long-chain (high DP) fructans, and their synergy with XOS compared to without XOS. Both the effects on piglet performance and bacterial community composition in the ileum and colon were assessed, along with the microbial endo-XYL activity in the colon.

4.2.1 Study aims

There is a lack of information in the literature on the fermentative activity and potentially synergistic effects of feeding different prebiotics in combination. The aim of this study was to determine the effect of XOS with or without short-chain (low DP) and long-chain (high DP) fructans on piglet performance, bacterial community composition and microbial endo-XYL activity.

4.2.2 Hypotheses

1. Supplementation of prebiotics will improve piglet performance; those fed FOS, INU and XOS will have improved performance compared to the CON group,

whilst piglets fed FOS or INU in combination with XOS will outperform both the CON and individual prebiotic supplementation.

2. Supplementation of FOS will increase alpha diversity and alter bacterial community composition in the ileum of piglets.
3. Supplementation of XOS or INU will increase alpha diversity and alter bacterial community composition in the colon of piglets.
4. Addition of FOS and XOS together will increase alpha diversity and alter bacterial community composition in the ileum and colon of piglets, whilst addition of XOS and INU together will affect the colon.

4.3 Materials and methods

Study protocols were approved by the University of Leeds Pig Research Centre and ethical approval was granted by the Animal Welfare and Ethical Review Body as described in Appendix A.1.

4.3.1 Animals and housing

At an average age of 26 days (SD ± 1.6 days), 474 weaner piglets with an average initial BW of 7.88 kg (SD ± 1.51 kg) were weaned on this 28-day feeding trial (day 0–27) until ~54 days of age. All piglets were weighed at the start of the trial (day 0) and blocked into mixed-sex pens of 4–5 piglets, balanced for litter origin, sex and BW. Pens within each of the 16 replicates were randomly allocated to 1 of 6 dietary treatments and piglet weight difference between pens was kept to a minimum (< 0.3 kg). The trial was conducted over 2 batches, with 234 piglets in batch 1 and 240 piglets in batch 2 (8 replicates per treatment). Piglets were housed in conventional weaner-grower accommodation as described in Appendix A.2.

4.3.2 Experimental design and dietary treatments

Piglets were fed with a 2-phase feeding program from day 0 to day 27 of the trial and had *ad libitum* access to pelleted feed and water. The first phase diet was fed from day 0–12, whilst the second phase diet was fed from day 13–27. Diets were formulated to meet or exceed the National Research Council (NRC, National Research Council. 2012) nutrient recommendations for 7–11 kg and 11–25 kg pigs. Basal dietary treatment compositions with calculated and analysed nutrient concentrations (Sciantec, York, UK) are presented in Table 4.1.

All diets included 5 g/kg of TiO2 as an indigestible marker and 0.10 g/kg phytase [Quantum® Blue, AB Vista, Marlborough, UK; 5000 phytase units (FTU)/g] to give an expected activity of 500 FTU/kg of feed. Supplementation of XOS (XOS 35, 35 % purity, Longlive Biotechnology Corporation, China; same product as in Chapter 2) at 0.286 g/kg in the XOS dietary treatment groups provided 0.10 g/kg of pure XOS in the feed. A short-chain FOS product (58.7 % purity; BETAFOS60 provided by Betalia, part of Azucarera, Spain) was included at 6.50 g/kg to provide 3.82 g/kg of pure FOS. The FOS product contained differing amounts of fructans attached to a terminal glu unit, whereby 39.2 % was GF_2, 18.4 % was GF_3 and 1.15 % was GF_4, hence had a DP of 2–4. The FOS product was extracted by an enzymatic process from sugar beet at a temperature of 50–60ºC. A long-chain INU product (93.5 % purity; Orafti® IPS provided by Beneo, Germany) was included at 4.08 g/kg to provide 3.82 g/kg of pure long-chain INU. The INU product had a DP of 2–60, with ~40 % having a DP < 10 and 60 % with a DP > 10. Orafti® IPS was produced by a hot-water extraction process from chicory roots, whereby crude chicory INU was then purified and concentrated.

Dietary treatments were arranged in a 2 × 3 factorial arrangement with 2 levels of XOS (0 and 0.286 g/kg) and 3 levels of fructans [(0 g/kg) and 6.50 g/kg of short-chain FOS and 4.08 g/kg of INU to supply 3.8 g/kg of pure fructans)] to give 6 dietary treatments, as follows:

- CON, 0 = CON without XOS
- FOS, 0 = Short-chain FOS without XOS
- INU, 0 = Long-chain INU without XOS
- CON, 1 = CON with XOS
- FOS, 1 = Short-chain FOS with XOS
- INU, 1 = Long-chain INU with XOS

The basal diets were weighed, mixed and prepared as a single batch of meal at Target Feeds Ltd (Shropshire, UK) before being split into 6 separate dietary batches for additive addition. Analysis of fructans or XOS recovery in feed was not possible at the time of the study, therefore the products were mixed with TiO_2 to act as a carrier and marker before being added to the basal diet. The meal was pelleted at Primary Diets (Ripon, UK) and packaged into 20 kg bags at Target Feeds Ltd, before delivery to Spen farm.

Table 4.1 Basal dietary treatment composition with calculated and analysed nutrient levels.

Raw material	Phase 1 diet (day 0–12)	Phase 2 diet (day 13–27)
Ingredient, g/kg		
Barley	100.0	100.4
Wheat	205.5	502.4
Micronised wheat meal	100.0	0.0
Micronised oats	100.0	0.0
Wheatfeed	0.0	36.7
Fishmeal (69 %)	72.5	25.1
Soybean meal	159.9	228.1
Full fat soybean meal	30.0	25.1
Vitamin and mineral premix [a]	5.0	5.0
Dried skimmed milk	40.0	0.0
Whey powder	114.1	36.4
L-Lysine HCL, 785 g/kg	3.2	3.7
DL-Methionine, 990 g/kg	1.82	1.43
L-Threonine, 980 g/kg	1.87	1.63
L-Tryptophan, 980 g/kg	0.31	0.00
L-Valine, 965 g/kg	0.69	0.45
Vitamin E	0.31	0.21
Phytase [b]	0.10	0.10
Flavouring	0.15	0.15
Dicalcium phosphate	1.00	7.83
Salt	0.00	3.18
Titanium dioxide	5.00	5.00
Soya oil	58.50	17.07
Calculated nutrient composition		
Net energy (MJ/kg)	11.44	10.20
Standardised ileal digestible lysine (g/kg)	14.00	12.40
Apparent total tract digestible phosphorus (g/kg)	4.50	4.00
Analysed nutrient composition		
Dry matter (g/kg)	903.0	887.5
Ash (g/kg)	53.9	50.7
Crude protein (g/kg)	219.8	209.5
Crude fibre (g/kg)	20.2	27.3
Calcium (g/kg)	6.6	5.4

[a] Vitamin premix per kg of diet: 12,500 IU Vitamin A, 2000 IU Vitamin D3, 250 mg Vitamin E, 4.2 mg Vitamin B1, 5.6 mg Vitamin B2, 5.0 mg Vitamin B6, 50 ug Vitamin B12, 4.4 mg Vitamin K hetrazeen, 40 mg Nicotinic acid, 20 mg Pantothenic acid, 1 mg Folic acid, 150 ug Biotin, 250 mg Choline Chloride, 200 mg Iron, 62 mg Manganese, 140 mg Copper, 100 mg Zinc, 2.2 mg Iodine, 0.35 mg Selenium. [b] Phytase; Quantum® Blue, AB Vista, Marlborough, UK.

4.3.3 Measurements and sampling

4.3.3.1 Piglet performance and health observations

Individual piglet weights were recorded on days 0, 13 and 27 of the trial to determine average pen BW and ADG. Feed troughs were weighed back daily for the first stage diets and second stage diets from day 0–20 to determine ADFI per pen. Diets were weighed back on day 20 at trough changeover and again at the end of the trial on day 27 to calculate pen ADFI. These measurements were used to calculate weekly pen FCR. Faecal, health and cleanliness scores were recorded by the same personnel daily, as described in section 2.3.3.1.

4.3.3.2 Digesta collection

At the end of the experiment, in accordance with the Animals (Scientific Procedures) Act 1986, 48 mixed-sex piglets from 8 replicates (24 piglets per batch) (20.44 kg ± SD 3.0 kg) were killed via captive bolt penetration, followed by exsanguination to confirm death (schedule 1 method). Dissections took place over 2 consecutive days for sample collection (days 28 and 29). Selected piglets had a BW close to the pen average, were littermates within a replicate and had received no antibiotic treatment. The order of slaughter was random within and between replicates.

A ventral incision was made into the piglets' abdomen and the GIT was identified. The start of the GIT was clamped at the pyloric junction where the stomach connected to the duodenum and the end of the GIT was clamped ~10–15 cm prior to the end of the rectum, before removing the GIT from the body cavity. Clamps were placed at various points along the GIT to prevent movement of digesta. For digesta collection, the terminal ileum was identified as ~90 cm prior to the ileocecal valve and the proximal colon was identified

as ~30 cm after the caecum. Digesta were gently squeezed from each section into 30 ml universal containers, then apportioned and used in immediate further processing or stored frozen (-80 ºC) pending further analysis. Apportioned digesta samples were freeze dried under vacuum at -35ºC for 24 hours before being ground and homogenised using a pestle and mortar.

4.3.4 Analysis

4.3.4.1 DNA extraction and bacterial community analysis

Total bacterial DNA was extracted from the ileal and colonic samples (*ca* 0.2 g) using the QIAamp Fast DNA Stool Mini Kit (QIAGEN®, Hilden, Germany). The manufacturers protocol was followed, with minor modifications as described in section 2.3.4.2.

Extracted DNA was sent to Novogene (China) for next generation sequencing on the Ilumina platform. PCR (200ng DNA) was used to amplify the V4 region of the bacterial 16S rRNA gene using the 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primer set. Each primer set was ligated with a unique barcode set. All PCR reactions were conducted with Thermo Scientific Phusion High-Fidelity PCR Master Mix (New England Biolabs). PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen, Germany). The same amount of PCR product from each sample was pooled, end polished, A-tailed, and ligated with adapters. Sequencing libraries were generated using NEBNext ® Ultra™ IIDNA Library Pre-Kit (Cat No. E7645) for Illumina following the manufacturers recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was then

sequenced on the NovaSeq 6000 SP flowcell Illumina platform and 250 bp paired-end reads were generated.

4.3.4.2 Bioinformatics

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (v1.2.7) (Magoč and Salzberg, 2011) and then filtered according to the QIIME process (v1.7.0) (Caporaso et al., 2010). Unique sequences were aligned to the GreenGene Database (v.2.2). Chimeras and sequences that aligned to Archaea, Eukaryota, chloroplasts or mitochondria were removed from the dataset. Sequences were clustered into OTUs with 97 % similarity, before quantifying the number of OTUs within each group and their taxonomy. A BIOM file was then created to transfer the data into R (v. 1.3.1093).

4.3.4.3 Microbial endo-XYL activity

There was not enough material to analyse microbial endo-XYL activity in the ileum, hence this analysis was only conducted on colonic samples. Microbial endo-XYL activity of the colonic samples were analysed using a Megazyme endo-XYL assay (Megazyme, Bray, Ireland; Kit XylX6-2V) and the kit protocol was followed with a few alterations. The principle of the assay was based on a colorimetric substrate (XylX6) and β-xylosidase. Incubation with the endo-XYL in the samples generated a colorimetric oligosaccharide that was rapidly hydrolysed by the β-xylosidase to quantitatively release 4-nitrophenol. The reaction was then terminated, and the absorption of the colorimetric group released was measured at 400 nm. The rate of release of 4-nitrophenol was therefore directly related to the rate of hydrolysis of XylX6 by the microbial endo-XYL.

One unit of activity was defined as the amount of enzyme required to release 1 micromole of 4-nitrophenol from the XylX6 substrate in 1 minute. Endo-XYL activities were expressed as endo-XYL units (EU) per g of DM.

The endo-XYL was extracted and diluted from freeze-dried colonic samples by adding 0.2 g of sample to 10 ml of 100 mM phosphate extraction/dilution buffer (pH 6.5) and gently stirred until dispersed. The solution was clarified by centrifugation (4000 × g) for 10 min. For analysis, 0.05 ml aliquots of XylX6 reagent solution were added to 2 ml microcentrifuge tubes. To each tube containing the XylX6 solution, 0.05 ml of endo-XYL solution was added and vortexed before incubation at 40ºC for 30 min. After incubation, 1.5 ml of stopping reagent was added and mixed. The absorbance of the reaction solution and the reagent blank were read at 400 nm on a benchtop spectrophotometer.

4.3.5 Statistical analysis

4.3.5.1 Piglet performance and health observations

The pen served as the experimental unit for all growth performance data (BW, ADG, ADFI, FCR). FCR was calculated as a ratio of feed intake to weight gain according to the following equation:

$$Feed\ conversion\ ratio\ (FCR) = \frac{Average\ daily\ feed\ intake\ (kg\ per\ day)}{Average\ daily\ gain\ (kg\ per\ day)} \qquad \textbf{Equation 4.1}$$

Normality of data was tested by visualisation of histograms and the Shapiro-Wilk test, whilst homogeneity of variance was assessed using the Levene's test. Any data showing non-normal distribution or unequal variance were inversely transformed prior to analysis.

Transformed data were back-transformed for inclusion into the data tables. The statistical package JMP® (Version *15.1*. SAS Institute Inc., Cary, NC, 1989 - 2019) (SAS, 2020) was used to analyse the performance data as a 2-way ANOVA. The statistical model included the fixed effects of XOS, fructans and their interaction, and replicate and batch as random variables. The initial BW of piglets was included as a covariate for BW and ADG analysis. When interactions were not significant, main effects were analysed individually. A 2-way ANOVA was used to analyse the colonic microbial endo-XYL activity data. A non-parametric Kruskal-Wallis one-way ANOVA was used to analyse average pen faecal, health and cleanliness scores. Significant differences were classed as $P < 0.05$ and trends as $P < 0.10$.

4.3.5.2 Bacterial community composition analysis

The software R was used to statistically analyse the microbiome data (v. 1.3.1093). Individual piglets served as the experimental unit for the bacterial community composition analysis. A general linear model was used to determine the effects of XOS and fructans on bacterial abundance at the level of the phylum and genus, with all data meeting the necessary assumptions. *Post-hoc* differences were identified using a Tukey's test (JMP® v.*15.1*). Number of OTUs, Chao1 (Chao, 1984) and Shannon-Weiner (Shannon, 1948) alpha diversities were measured using the Phyloseq package (v.1.22.3) in R (McMurdie and Holmes, 2013). To determine the effects of XOS and fructans on alpha diversity and number of OTUs, a general linear model was used (lme4) and models were reduced using analysis of deviance. The package Vegan (v.2.5.7) was used to assess beta diversity. To determine community similarities between XOS and fructan groups, a PERMANOVA was used. An NMDS plot (axis = 2) plot using Bray-Curtis distances was used to plot beta diversity.

4.4 Results

4.4.1 Piglet performance and health observations

The effect of fructans and XOS on piglet growth performance are presented in Table 4.2. There was a trend for an interaction between XOS and fructans on day 13 piglet BW (P = 0.052) and ADG between day 0–13 (P = 0.054), whilst there was a significant interaction for ADFI between day 0–13 (P < 0.017). A similar interaction pattern was seen across all 3 key performance indicators and was driven by ADFI (Figure 4.1). Piglet ADFI between day 0–13 was lower with fructan (FOS and INU) supplementation compared to the CON fed piglets. When XOS was supplemented alone or in combination with INU, ADFI was also reduced compared to the CON, but not dissimilar from the FOS + XOS group. There was no effect of treatment on FCR between day 0–13 (P > 0.05).

Table 4.2 Effect of XOS and fructans (FOS and INU) on piglet growth performance [a]

	Fructan			SEM	XOS[e]		SEM	CON	FOS	INU	CON	FOS	INU	SEM	*P* value		
	CON[b]	FOS[c]	INU[d]		0	1		0	0	0	1	1	1		Fructan	XOS	Fructan × XOS
BW[f] (kg)																	
Day 0	7.91	7.86	7.85	0.003	7.87	7.88	0.003	7.90	7.84	7.85	7.86	7.92	7.86	0.130	0.954	0.979	0.998
Day 13	10.16	9.99	10.03	0.078	10.08	10.03	0.064	10.29	9.93	10.04	9.92	10.07	10.10	0.111	0.628	0.549	0.052
Day 27	18.18	17.70	17.68	0.184	17.94	17.77	0.150	18.52	17.67	17.63	17.68	17.77	17.84	0.260	0.263	0.402	0.088
ADG[g] (kg/d)																	
Day 0–13	0.172	0.163	0.167	0.006	0.169	0.166	0.005	0.185	0.157	0.165	0.158	0.169	0.170	0.008	0.605	0.647	0.054
Day 14–27	0.572	0.548	0.543	0.010	0.558	0.550	0.008	0.586	0.550	0.539	0.554	0.547	0.549	0.015	0.169	0.480	0.329
Day 0–27	0.380	0.364	0.363	0.007	0.372	0.366	0.006	0.394	0.362	0.360	0.363	0.366	0.368	0.010	0.235	0.424	0.104
ADFI[h] (kg/d)																	
Day 0–13	0.210	0.204	0.202	0.005	0.207	0.204	0.004	0.224[a]	0.198[b]	0.202[b]	0.197[b]	0.211[ab]	0.202[b]	0.007	0.437	0.414	0.017
Day 14–27	0.652	0.652	0.657	0.012	0.652	0.655	0.010	0.675	0.645	0.643	0.629	0.659	0.674	0.017	0.902	1.000	0.060
Day 0–27	0.439	0.433	0.433	0.007	0.435	0.435	0.006	0.457[a]	0.424[b]	0.427[b]	0.421[b]	0.442[ab]	0.441[ab]	0.011	0.818	0.842	0.018
FCR[i]																	
Day 0–13	1.24	1.29	1.24	0.015	1.26	1.25	0.013	1.21	1.29	1.28	1.26	1.29	1.21	0.022	0.271	0.967	0.336
Day 14–27	1.15[a]	1.19[b]	1.21[b]	0.008	1.18	1.19	0.007	1.15	1.18	1.19	1.14	1.21	1.23	0.012	0.001	0.264	0.362
Day 0–27	1.16	1.19	1.19	0.008	1.17	1.19	0.007	1.16	1.18	1.19	1.16	1.21	1.20	0.865	0.086	0.261	0.578

[a] Data are means of 16 replicate pens of 4–5 piglets. [b] CON; Control. [c] FOS; Fructo-oligosaccharides.[d] INU; Inulin. [e] XOS; Xylo-oligosaccharides, 0 = No XOS; 1= XOS. [f] BW; Body weight. [g] ADG; Average daily gain. [h] ADFI; Average daily feed intake. [I] FCR; Feed conversion ratio. [a-b] Means within a row that do not share a common superscript are significantly different ($P < 0.05$).

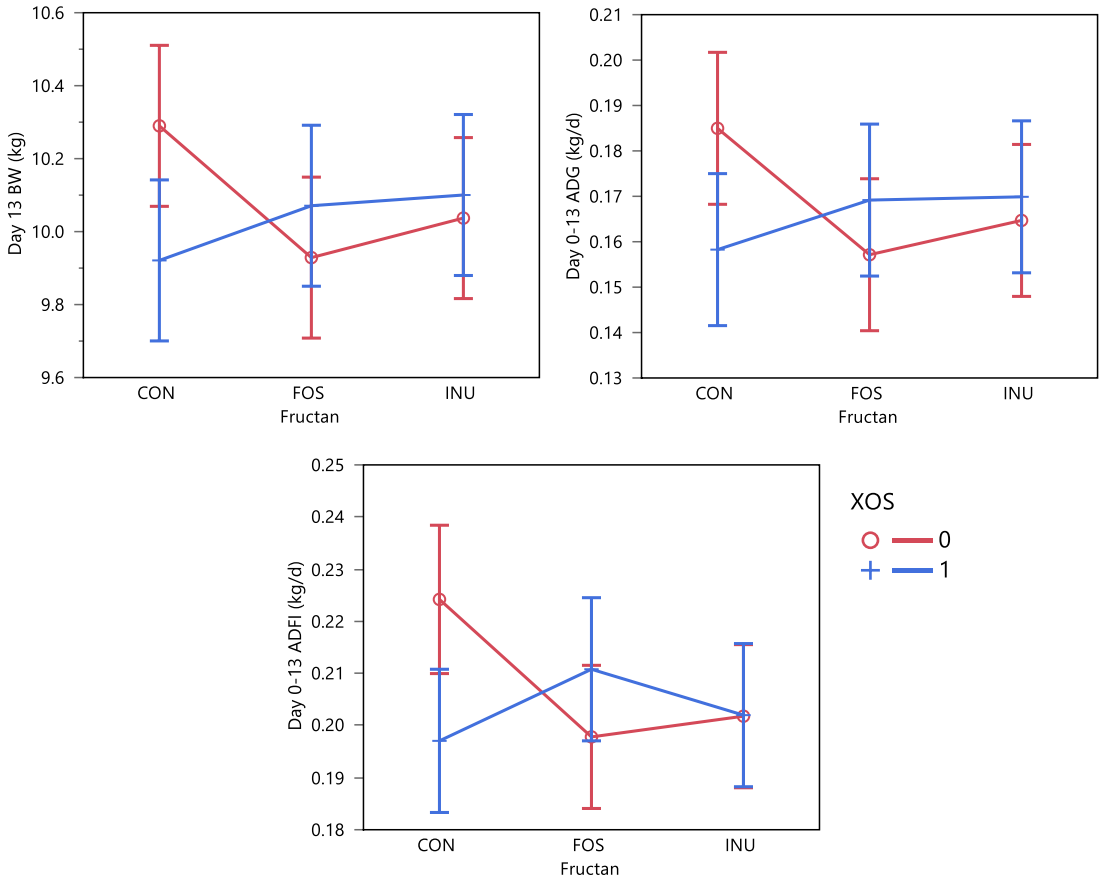

Figure 4.1 Effect of xylo-oligosaccharides (XOS) and fructans (Fructo-oligosaccharides (FOS)) and (Inulin (INU)) on day 13 body weight (BW; *P* = 0.052), day 0–13 average daily gain (ADG; *P* = 0.054) and day 0–13 average daily feed intake (ADFI; *P* < 0.017).

As for the first 2 weeks of the trial, a similar interaction trend was observed for day 27 piglet BW (*P* = 0.088) and ADFI between days 14–27 (*P* = 0.06). Piglet BW and ADFI tended to be lower with FOS and INU inclusion compared to the CON. When supplemented alone XOS tended to reduce ADFI and BW, but they increased when XOS was fed in combination with FOS and INU. There was no effect of treatment on ADG between days 14–27 (*P* > 0.05). FCR was higher between days 14–27 (*P* < 0.001) when piglets were supplemented with FOS and INU compared to the CON.

There was a significant interaction between fructans and XOS on ADFI for the overall trial period between days 0–27 ($P < 0.018$; Figure 4.2). Again, ADFI was lower with FOS and INU inclusion compared to the CON fed piglets. When supplemented alone, XOS also reduced ADFI compared to the CON, but intake increased to levels not dissimilar from the CON fed piglets when XOS was fed in combination with FOS and INU. There was no effect of treatment on ADG between days 0–27 ($P > 0.05$). FCR tended to be higher between days 0–27 when piglets were supplemented FOS and INU compared to the CON ($P = 0.086$; Figure 4.2).

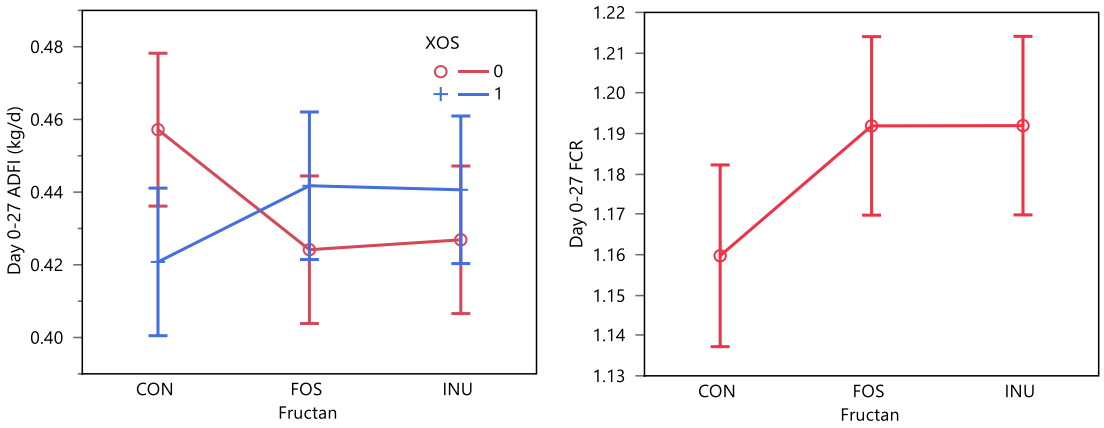

Figure 4.2 Effect of xylo-oligosaccharides (XOS) and fructans (Fructo-oligosaccharides (FOS)) and (Inulin (INU)) on day 0–27 average daily feed intake (ADFI; $P < 0.018$) and main effect of fructans on FCR ($P = 0.086$).

There were no effects of treatment on health, faecal or cleanliness scores throughout the trial (Table 4.3; $P > 0.05$). There was a trend for an increased health score value (more observations of ill health) between days 0–13 of the trial, whereby both INU treatments with and without XOS had a higher score compared to the other treatments ($P = 0.063$).

Table 4.3 Effect of XOS and fructans (FOS and INU) on average piglet faecal, health and cleanliness scores.

Fructan XOS[d]	Treatment						SEM	*P* Value
	CON[a] 0	FOS[b] 0	INU[c] 0	CON 1	FOS 1	INU 1		
Faecal score[e]								
Day 0–13	2.17	2.17	2.25	2.13	2.20	2.16	0.071	0.174
Day 14–27	2.13	2.11	2.15	2.19	2.10	2.08	0.051	0.634
Day 0–27	2.15	2.14	2.20	2.16	2.15	2.12	0.047	0.276
Health score[f]								
Day 0–13	0.05	0.12	0.26	0.09	0.08	0.22	0.077	0.063
Day 14–27	0.03	0.05	0.11	0.10	0.04	0.06	0.058	0.523
Day 0–27	0.04	0.08	0.18	0.09	0.06	0.14	0.055	0.199
Cleanliness score[g]								
Day 0–13	1.03	1.06	1.04	1.03	1.05	1.07	0.029	0.647
Day 14–27	1.12	1.15	1.1	1.14	1.17	1.10	0.055	0.842
Day 0–27	1.07	1.10	1.07	1.09	1.11	1.09	0.032	0.816

[a] CON; Control. [b] FOS; Fructo-oligosaccharides.[c] INU; Inulin. [d] XOS; Xylo-oligosaccharides, 0 = No XOS; 1= XOS. [e] Faecal score; 1 = firm faeces, 2 = soft faeces, 3 = mild diarrhoea, 4 = severe diarrhoea. [f] Health score; 1 = no signs of ill health, 2 = some signs of ill health, 3 = clear indications of ill health, 4 = seriously ill pigs. [g] Cleanliness score; 1 = clean pigs, 2 = light contamination with faecal material, 3 = contamination with faecal material, 4 = heavy contamination with faecal material.

4.4.2 Bacterial community analysis

4.4.2.1 Ileal bacterial community composition

The majority of ileal bacteria belonged to the phyla Firmicutes (72.7 %), Bacteroidetes (13.2 %) and Proteobacteria (9.68 %), which jointly make up 95.6 % of the bacterial community (Table 4.4). Changes in the bacterial community between dietary treatments were estimated at the phylum and genus level. There were 5 phyla with a relative abundance greater than 1 % in a minimum of 1 sample. There was no effect of fructans on the relative abundance of OTUs at the phyla level, nor was there an interaction between

fructans and XOS ($P > 0.050$). There was a trend for the abundance of Bacteriodetes to be higher ($P = 0.094$) and Proteobacteria to be lower ($P = 0.070$) when XOS was included. The abundance of Epsilonbacteraeota was higher when XOS was included ($P < 0.019$).

There were 29 genera with a relative abundance greater than 1 % in a minimum of 1 sample and the inclusion of fructans and XOS affected the abundance of several genera. Supplementation of XOS significantly decreased the abundance of *g__Actinobacillus* and increased the abundance of *g__Helicobacter, g__Prevotella_7, g__Prevotella_9 and g__uncultured* (Prevotellaceae Family) in the ileum ($P < 0.050$). There was a trend for an interaction between fructans and XOS on the abundance of *g__Escherichia-Shigella* in the ileum ($P = 0.084$), whereby the abundance was similar with and without XOS in the CON and INU groups but was higher without XOS and lower with XOS in the FOS group (Figure 4.3). There was a significant interaction between fructans and XOS on the abundance of *g__ Lactobacillus* in the ileum ($P < 0.038$), whereby the abundance in the XOS group was higher than all other treatment groups but was not dissimilar from the FOS group (Figure 4.3). There was a significant interaction between fructans and XOS on the abundance of *g__Terrisporobacter* in the ileum ($P < 0.020$), whereby the abundance was similar with and without XOS in the FOS and INU groups but was higher without XOS and lower with XOS in the CON group (Figure 4.3).

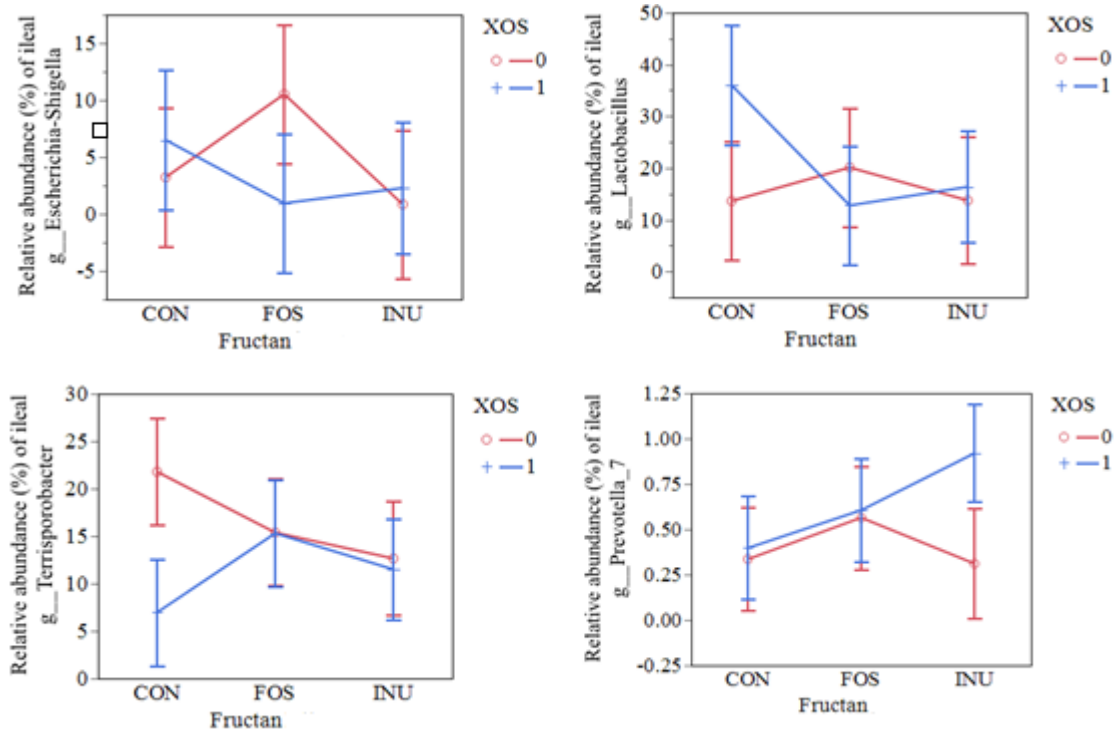

Figure 4.3: Effect of xylo-oligosaccharides (XOS) and fructans (Fructo-oligosaccharides (FOS)) and (Inulin (INU)) on the relative abundance of *g_Escherichia_shigella* (P = 0.084), *g__ Lactobacillus* (P < 0.038), *g__Terrisporobacter* (P < 0.020) and *g__Prevotella_7* (P = 0.087) in the ileum.

Table 4.4 Effect of XOS and fructans (FOS and INU) on average relative abundance (> 1 %) at the Phyla and Genera level in the ileal digesta of piglets.

Ileal relative abundance (%)	Fructan				XOS[d]			CON	FOS	INU	CON	FOS	INU		P value		
	CON[a]	FOS[b]	INU[c]	SEM	0	1	SEM	0	0	0	1	1	1	SEM	Fructan	XOS	Fructan × XOS
Phylum[1]																	
p__Firmicutes	74.56	71.32	72.21	3.797	73.15	72.24	3.101	71.21	72.74	75.51	77.92	69.90	68.91	5.370	0.824	0.837	0.450
p__Bacteroidetes	11.66	13.94	15.40	1.936	11.75	15.58	1.581	12.04	11.06	12.17	11.29	16.83	18.63	2.738	0.397	0.094	0.357
p__Proteobacteria	11.48	9.07	8.48	3.190	13.10	6.25	2.605	15.47	13.87	9.98	7.49	4.28	6.99	4.512	0.782	0.070	0.749
p__Epsilonbacteraeota	1.75	5.19	3.44	1.372	1.53[a]	5.39[b]	1.120	0.70	1.95	1.95	2.80	8.43	4.93	1.940	0.219	0.019	0.496
Other	0.54	0.47	0.47	0.046	0.46	0.53	0.038	0.58	0.39	0.41	0.51	0.55	0.54	0.066	0.46	0.16	0.17
Genus[1]																	
g__Acidaminococcus	0.25	0.35	0.24	0.060	0.23	0.32	0.049	0.21	0.35	0.15	0.29	0.34	0.33	0.089	0.366	0.219	0.510
g__Actinobacillus	6.04	2.87	6.73	2.361	8.19[a]	2.25[b]	1.958	11.58	3.15	9.82	0.51	2.59	3.64	3.542	0.470	0.034	0.293
g__Alloprevotella	2.53	2.39	2.56	0.356	2.12	2.86	0.295	2.65	1.80	1.92	2.40	2.98	3.20	0.534	0.935	0.081	0.244
g__Anaerovibrio	0.45	0.39	0.42	0.060	0.37	0.48	0.050	0.47	0.30	0.33	0.43	0.49	0.50	0.090	0.804	0.118	0.344
g__Campylobacter	0.11	0.12	0.82	0.478	0.05	0.65	0.396	0.07	0.03	0.06	0.16	0.21	1.58	0.717	0.489	0.283	0.498
g__Clostridium_sensu_stricto_1	23.81	26.71	30.36	4.017	26.25	27.67	3.331	23.69	24.04	31.03	23.94	29.39	29.70	6.025	0.515	0.759	0.828
g__Dialister	0.76	0.84	0.93	0.121	0.73	0.95	0.101	0.73	0.76	0.71	0.78	0.91	1.15	0.182	0.608	0.132	0.509
g__Escherichia-Shigella	4.87	5.74	1.58	2.157	4.88	3.25	1.788	3.24	10.53	0.86	6.51	0.95	2.30	3.235	0.361	0.516	0.084
g__Faecalibacterium	0.65	0.67	0.71	0.112	0.58	0.77	0.093	0.69	0.51	0.54	0.60	0.82	0.88	0.168	0.917	0.154	0.322
g__Helicobacter	1.72	5.25	2.60	1.362	1.52[a]	4.86[b]	1.129	0.67	1.97	1.93	2.77	8.52	3.28	2.043	0.172	0.039	0.352
g__Ignatzschineria	0.10	0.01	0.01	0.042	0.07	0.01	0.035	0.17	0.01	0.02	0.02	0.01	0.00	0.064	0.247	0.246	0.406

Ileal relative abundance (%)	Fructan				XOS[d]			CON	FOS	INU	CON	FOS	INU		P value		
	CON[a]	FOS[b]	INU[c]	SEM	0	1	SEM	0	0	0	1	1	1	SEM	Fructan	XOS	Fructan × XOS
g__Lactobacillus	24.84	16.46	15.07	4.052	15.84	21.73	3.359	13.65[b]	20.12[ab]	13.76[b]	36.03[a]	12.79[b]	16.39[b]	6.077	0.191	0.213	0.038
g__Megasphaera	0.45	0.68	0.57	0.112	0.47	0.65	0.093	0.41	0.67	0.34	0.49	0.68	0.79	0.167	0.359	0.174	0.346
g__Phascolarctobacterium	0.26	0.35	0.23	0.101	0.22	0.34	0.084	0.28	0.20	0.19	0.25	0.50	0.26	0.152	0.677	0.316	0.504
g__Prevotella_2	1.09	1.22	1.53	0.208	1.16	1.40	0.173	1.19	1.13	1.14	0.98	1.31	1.92	0.312	0.307	0.307	0.245
g__Prevotella_7	0.37	0.59	0.62	0.100	0.40[a]	0.64[b]	0.083	0.34	0.57	0.31	0.40	0.61	0.92	0.150	0.169	0.046	0.087
g__Prevotella_9	4.08	6.68	6.94	1.121	4.38[a]	7.42[b]	0.930	4.02	4.84	4.28	4.13	8.52	9.60	1.682	0.145	0.023	0.252
g__Prevotellaceae_NK3B31_group	0.84	0.73	0.78	0.137	0.68	0.88	0.114	0.91	0.54	0.60	0.77	0.92	0.96	0.205	0.853	0.213	0.319
g__Rikenellaceae_RC9_gut_group	0.76	0.52	0.53	0.108	0.60	0.61	0.089	0.92	0.40	0.48	0.60	0.65	0.57	0.162	0.206	0.969	0.162
g__Romboutsia	2.40	1.52	1.48	0.650	1.90	1.70	0.539	2.11	1.43	2.17	2.69	1.62	0.79	0.975	0.528	0.783	0.531
g__Ruminococcaceae_UCG-002	0.38	0.40	0.32	0.055	0.33	0.40	0.046	0.40	0.33	0.27	0.35	0.47	0.38	0.083	0.605	0.273	0.455
g__Ruminococcaceae_UCG-014	0.36	0.37	0.38	0.049	0.35	0.39	0.040	0.38	0.35	0.32	0.34	0.39	0.44	0.073	0.974	0.436	0.525
g__Sarcina	0.54	2.43	0.76	1.253	2.18	0.30	1.039	0.94	4.37	1.23	0.13	0.49	0.29	1.880	0.506	0.199	0.618
g__Streptococcus	0.90	0.73	4.96	2.043	3.55	0.84	1.694	0.92	0.47	9.26	0.87	1.00	0.66	3.064	0.263	0.255	0.219
g__Subdoligranulum	0.53	0.47	0.52	0.067	0.44	0.57	0.056	0.57	0.33	0.43	0.49	0.61	0.62	0.101	0.783	0.105	0.159
g__Terrisporobacter	14.38	15.35	12.08	1.988	16.61[a]	11.26[b]	1.649	21.79[a]	15.39[ab]	12.66[ab]	6.97[b]	15.31[ab]	11.51[ab]	2.982	0.495	0.024	0.020
g__uncultured (Prevotellaceae Family)	0.45	0.51	0.53	0.068	0.42[a]	0.58[b]	0.056	0.43	0.48	0.34	0.47	0.55	0.72	0.102	0.681	0.041	0.170
g__uncultured_Porphyromonadaceae_bacterium	0.53	0.47	0.50	0.080	0.46	0.53	0.066	0.59	0.41	0.39	0.47	0.52	0.61	0.120	0.845	0.449	0.303
Other	5.57	5.19	5.25	0.533	4.99	5.68	0.442	5.975	4.52	4.46	5.17	5.86	6.03	0.799	0.860	0.261	0.228

[1]Phylum and genus with > 1 % abundance in a minimum of 1 sample. [a] CON; Control. [b] FOS; Fructo-oligosaccharides. [c] INU; Inulin. [d] XOS; Xylo-oligosaccharides. [a-b] Means within a row that do not share a common superscript are significantly different ($P < 0.05$).

4.4.2.2 Colonic bacterial community composition

The majority of colonic bacteria belonged to the phyla Bacteroidetes (56.7 %) and Firmicutes (37.8 %), jointly making up 94.5 % of the bacterial community (Table 4.5). There were 7 phyla with a relative abundance greater than 1 % in a minimum of 1 sample. There was no effect of fructans or XOS on the relative abundance of OTUs at the phyla level.

There were 44 genera with a relative abundance greater than 1 % in a minimum of 1 sample and the inclusion of fructans and XOS affected the abundance of several genera (Table 4.5). FOS reduced the abundance of OTUs associated with *g_(Muribaculaceae* Family*)* ($P < 0.048$) and *g__Oscillospira* compared to the CON group but not the INU group ($P < 0.025$). The abundance of OTUs associated with *g__Prevotellaceae_NK3B31_group* ($P = 0.060$), *g__Prevotellaceae_UCG-003* ($P = 0.061$) and *g__uncultured_rumen_bacterium* ($P = 0.054$) tended to be lowest in the FOS group. The abundance of OTUs associated with *g__Clostridium_sensu_stricto_1* ($P = 0.055$) and *g__Terrisporobacter* ($P = 0.081$) tended to be highest in the FOS group. The abundance of OTUs associated with *g__Phascolarctobacterium* ($P = 0.094$) tended to be highest in the INU group. The abundance of OTUs associated with *g__Mitsuokella* ($P < 0.016$) and *g__Ruminococcaceae_UCG-014* ($P < 0.039$) were lower in the XOS group compared to the CON group, whilst *g__Subdoligranulum* tended to be higher in the XOS group compared to the CON group ($P = 0.085$). The abundance of OTUs associated with *g__Sphaerochaeta* were no different with or without XOS in the FOS and INU groups but were higher without XOS and lower with XOS in the CON group ($P < 0.038$). There was a trend for an interaction between fructans and XOS on the abundance of OTUs associated with *g__Romboutsia* ($P = 0.089$). There was no difference in the CON group, but abundance was higher with XOS in the FOS group but lower with XOS in the INU group.

Table 4.5 Effect of XOS and fructans (FOS and INU) on average relative abundance (> 1 %) at the Phyla and Genera level in the colonic digesta of piglets.

Colonic relative abundance (%)	Fructan				XOS[d]			CON	FOS	INU	CON	FOS	INU		P value		
	CON[a]	FOS[b]	INU[c]	SEM	0	1	SEM	0	0	0	1	1	1	SEM	Fructan	XOS	Fructan × XOS
Phylum																	
p__Bacteroidetes	58.85	55.23	55.89	1.909	57.03	56.28	1.559	59.29	58.13	53.67	58.40	52.33	58.11	2.700	0.370	0.737	0.178
p__Firmicutes	35.46	39.45	38.43	1.762	37.60	37.96	1.438	35.32	36.98	40.52	35.60	41.92	36.35	2.491	0.262	0.862	0.200
p__Proteobacteria	3.34	3.34	3.45	0.362	3.19	3.56	0.296	2.97	3.05	3.55	3.71	3.63	3.35	0.512	0.970	0.378	0.615
p__Epsilonbacteraeota	0.86	0.89	0.56	0.167	0.64	0.89	0.136	0.67	0.75	0.50	1.04	1.02	0.61	0.236	0.314	0.199	0.850
p__Spirochaetes	0.81	0.45	0.95	0.172	0.80	0.68	0.141	0.93	0.41	1.06	0.69	0.49	0.85	0.244	0.119	0.550	0.769
p__Tenericutes	0.23	0.20	0.21	0.059	0.24	0.19	0.048	0.29	0.27	0.16	0.17	0.13	0.27	0.083	0.903	0.475	0.246
Other	0.46	0.45	0.50	0.047	0.50	0.44	0.038	0.54	0.41	0.55	0.38	0.48	0.46	0.066	0.670	0.243	0.229
Genus																	
g__(Muribaculaceae Family)	0.84[a]	0.28[b]	0.75[ab]	0.166	0.51	0.73	0.138	0.75	0.29	0.49	0.93	0.27	1.00	0.249	0.048	0.252	0.520
g__ (Mollicutes_RF39 Order)	0.17	0.19	0.20	0.058	0.20	0.18	0.048	0.17	0.27	0.14	0.16	0.12	0.25	0.087	0.932	0.760	0.298
g__Acidaminococcus	0.88	1.00	0.83	0.239	1.02	0.78	0.198	0.89	1.34	0.84	0.86	0.66	0.82	0.359	0.876	0.384	0.531
g__Actinobacillus	0.59	0.82	0.54	0.167	0.50	0.79	0.138	0.51	0.54	0.45	0.66	1.10	0.62	0.250	0.440	0.134	0.611
g__Alloprevotella	11.98	11.46	12.22	1.380	11.05	12.73	1.144	10.00	11.37	11.79	13.97	11.56	12.66	2.070	0.923	0.297	0.587
g__Anaerovibrio	1.28	1.20	1.56	0.245	1.42	1.27	0.203	1.46	1.27	1.54	1.11	1.12	1.58	0.367	0.557	0.585	0.849
g__Asteroleplasma	0.28	0.14	0.16	0.079	0.24	0.16	0.066	0.42	0.14	0.14	0.15	0.14	0.18	0.119	0.393	0.386	0.335
g__Campylobacter	0.21	0.24	0.16	0.073	0.17	0.24	0.060	0.22	0.21	0.07	0.19	0.26	0.25	0.109	0.744	0.401	0.576
g__Clostridium_sensu_stricto_1	4.42	7.01	5.00	0.776	5.24	5.71	0.643	4.43	5.89	5.41	4.42	8.13	4.59	1.164	0.055	0.601	0.356
g__Desulfovibrio	0.48	0.35	0.54	0.071	0.46	0.45	0.059	0.46	0.38	0.56	0.50	0.33	0.53	0.106	0.163	0.905	0.883
g__Dialister	2.37	3.64	2.19	0.530	2.67	2.79	0.440	2.23	3.36	2.41	2.50	3.91	1.96	0.795	0.118	0.843	0.789
g__Erysipelotrichaceae_UCG-004	0.44	0.31	0.35	0.061	0.38	0.35	0.050	0.45	0.28	0.40	0.42	0.33	0.30	0.091	0.316	0.695	0.668
g__Escherichia-Shigella	0.95	0.83	0.78	0.206	0.81	0.90	0.171	1.03	0.83	0.57	0.86	0.84	1.00	0.309	0.849	0.722	0.583
g__Faecalibacterium	1.61	1.89	1.98	0.254	1.85	1.80	0.211	1.69	1.95	1.90	1.53	1.82	2.05	0.381	0.574	0.880	0.890
g__Helicobacter	0.76	0.75	0.45	0.160	0.55	0.75	0.133	0.54	0.62	0.49	0.97	0.87	0.41	0.241	0.326	0.281	0.525
g__Lactobacillus	3.91	4.85	4.35	0.668	4.07	4.67	0.554	3.54	4.04	4.63	4.28	5.67	4.07	1.002	0.607	0.435	0.510
g__Megasphaera	1.67	1.80	1.54	0.423	1.63	1.71	0.351	1.71	2.00	1.17	1.63	1.59	1.91	0.634	0.913	0.861	0.610

Colonic relative abundance (%)	Fructan				XOS[d]			CON	FOS	INU	CON	FOS	INU		P value		
	CON[a]	FOS[b]	INU[c]	SEM	0	1	SEM	0	0	0	1	1	1	SEM	Fructan	XOS	Fructan × XOS
g__metagenome	0.24	0.12	0.23	0.053	0.24	0.16	0.044	0.30	0.16	0.27	0.19	0.09	0.19	0.080	0.228	0.156	0.973
g__Mitsuokella	0.30	0.43	0.36	0.062	0.45[a]	0.27[b]	0.051	0.40	0.56	0.39	0.20	0.30	0.32	0.093	0.316	0.016	0.565
g__Oscillospira	0.71[a]	0.34[b]	0.57[ab]	0.092	0.46	0.62	0.077	0.63	0.34	0.43	0.79	0.35	0.71	0.139	0.025	0.156	0.592
g__Phascolarctobacterium	0.79	0.62	1.11	0.158	0.85	0.83	0.131	0.61	0.67	1.28	0.98	0.57	0.95	0.238	0.094	0.913	0.293
g__Prevotella_1	1.22	1.10	0.90	0.195	1.10	1.05	0.161	1.26	1.11	0.94	1.19	1.10	0.86	0.292	0.495	0.815	0.992
g__Prevotella_2	5.64	6.29	6.01	0.759	6.27	5.68	0.630	6.42	6.55	5.84	4.86	6.03	6.17	1.139	0.831	0.506	0.677
g__Prevotella_7	2.20	2.24	2.35	0.441	2.21	2.32	0.366	2.23	2.63	1.78	2.18	1.86	2.92	0.662	0.971	0.837	0.312
g__Prevotella_9	24.86	27.59	23.26	2.003	27.02	23.45	1.661	25.80	29.69	25.58	23.92	25.49	20.94	3.004	0.310	0.128	0.871
g__Prevotellaceae_NK3B31_group	6.77	2.49	5.28	1.263	4.50	5.19	1.047	8.02	2.65	2.83	5.53	2.33	7.72	1.895	0.060	0.635	0.116
g__Prevotellaceae_UCG-003	0.57	0.29	0.55	0.091	0.39	0.54	0.076	0.51	0.26	0.42	0.64	0.32	0.67	0.137	0.061	0.165	0.771
g__Rikenellaceae_RC9_gut_group	2.72	1.98	2.74	0.481	2.39	2.58	0.399	2.45	1.75	2.96	2.99	2.22	2.52	0.721	0.452	0.732	0.722
g__Romboutsia	0.45	0.55	0.53	0.123	0.51	0.51	0.102	0.45	0.36	0.73	0.45	0.74	0.33	0.185	0.831	0.974	0.089
g__Roseburia	0.30	0.26	0.46	0.094	0.29	0.39	0.078	0.34	0.20	0.33	0.26	0.31	0.59	0.141	0.283	0.376	0.422
g__Ruminococcaceae_UCG-002	1.11	0.81	1.22	0.142	1.12	0.98	0.118	1.13	0.91	1.32	1.10	0.72	1.11	0.213	0.118	0.391	0.884
g__Ruminococcaceae_UCG-005	0.59	0.36	0.63	0.095	0.50	0.55	0.079	0.58	0.38	0.55	0.60	0.34	0.71	0.143	0.107	0.672	0.750
g__Ruminococcaceae_UCG-014	0.63	0.59	0.66	0.048	0.68[a]	0.57[b]	0.039	0.69	0.67	0.70	0.57	0.50	0.63	0.071	0.511	0.039	0.770
g__Sphaerochaeta	0.25	0.11	0.19	0.057	0.20	0.17	0.047	0.38[a]	0.11[b]	0.11[b]	0.13[b]	0.11[b]	0.28[ab]	0.086	0.239	0.702	0.038
g__Streptococcus	0.73	0.71	0.96	0.333	0.90	0.70	0.277	0.57	0.71	1.43	0.90	0.72	0.48	0.500	0.848	0.600	0.380
g__Subdoligranulum	1.36	1.15	1.49	0.129	1.20	1.46	0.107	1.25	1.16	1.20	1.48	1.13	1.77	0.194	0.177	0.085	0.266
g__Succinivibrio	0.38	0.63	0.26	0.194	0.36	0.49	0.161	0.17	0.61	0.30	0.59	0.65	0.23	0.292	0.401	0.564	0.637
g__Sutterella	0.39	0.39	0.48	0.052	0.38	0.46	0.043	0.42	0.33	0.40	0.36	0.44	0.56	0.078	0.374	0.213	0.310
g__Terrisporobacter	3.06	4.90	3.58	0.585	3.73	3.96	0.485	2.68	4.36	4.16	3.43	5.44	3.00	0.878	0.081	0.740	0.351
g__Treponema_2	0.66	0.39	0.93	0.202	0.71	0.61	0.168	0.68	0.34	1.10	0.65	0.44	0.75	0.303	0.183	0.686	0.715
g__uncultured	2.13	2.00	2.27	0.314	2.26	2.01	0.260	2.43	1.91	2.44	1.83	2.09	2.10	0.471	0.834	0.486	0.674
g__uncultured_Porphyromonadaceae_bacterium	2.58	1.67	2.55	0.348	2.11	2.43	0.288	2.22	1.53	2.57	2.94	1.81	2.53	0.521	0.118	0.430	0.743
g__uncultured_rumen_bacterium	0.55	0.31	0.83	0.146	0.56	0.57	0.121	0.41	0.34	0.92	0.69	0.29	0.73	0.219	0.054	0.935	0.508
Other	5.98	4.93	6.03	0.362	5.83	5.46	0.301	6.52	4.95	6.03	5.44	4.91	6.04	0.544	0.061	0.381	0.488

[1]Phylum and genus with > 1 % abundance in a minimum of 1 sample. [a] CON; Control. [b] FOS; Fructo-oligosaccharides. [c] INU; Inulin. [d] XOS; Xylo-oligosaccharides. [a-b] Means within a row that do not share a common superscript are significantly different ($P < 0.05$).

4.4.2.3 Alpha and Beta diversity

The number of OTUs and alpha diversity (Chao1 and Shannon) are presented in Table 4.6. There was no significant interaction between fructans and XOS on alpha diversity measures in the ileum or colon (Table 4.6). The number of OTUs ($P < 0.026$) and Chao1 diversity ($P < 0.027$) indices in the ileum were significantly lower with FOS inclusion compared to the CON, but not the INU treatment. Dietary treatment did not affect the Shannon-Wiener diversity index in the ileum ($P > 0.05$). The number of OTUs were significantly lower in the colon with FOS inclusion compared to the CON and INU treatments ($P < 0.017$). The Chao1 diversity index was significantly lower in the colon with FOS inclusion compared to the INU treatment, but not the CON treatment ($P < 0.038$). The Shannon-Wiener diversity index was significantly lower in the colon with FOS inclusion compared to the CON treatment, but not the INU treatment ($P < 0.011$).

Table 4.6 Effect of XOS and fructans (FOS and INU) on number of OTUs, Chao1 diversity and Shannon-Weiner diversity in the ileum and colon of piglets.

	Fructan				XOS[d]			CON	FOS	INU	CON	FOS	INU		*P* value		
	CON[a]	FOS[b]	INU[c]	SE	0	1	SE	0	0	0	1	1	1	SE	Fructan	XOS	Fructan × XOS
Ileum																	
Number of OTUs[e]	623[a]	562[b]	602[ab]	15.91	597	596	12.85	640	567	585	606	556	619	23.24	0.026	0.858	0.294
Chao1 diversity	678[a]	613[b]	658[ab]	17.24	652	650	13.92	692	620	643	664	605	674	25.18	0.027	0.851	0.431
Shannon-Weiner diversity	2.98	2.83	2.99	0.10	2.85	3.02	0.08	3.00	2.80	2.76	2.96	2.87	3.21	0.14	0.439	0.150	0.171
Colon																	
Number of OTUs	691[a]	643[b]	697[a]	13.81	677	677	11.28	702	646	683	681	640	710	19.53	0.017	0.996	0.459
Chao1 diversity	743[ab]	699[b]	750[a]	14.82	730	731	12.10	751	699	740	736	698	760	20.96	0.038	0.943	0.703
Shannon-Weiner diversity	4.08[a]	3.82[b]	4.02[ab]	0.06	3.92	4.02	0.05	4.10	3.79	3.88	4.06	3.85	4.17	0.09	0.011	0.148	0.159

[a] CON; Control. [b] FOS; Fructo-oligosaccharides. [c] INU; Inulin. [d] XOS; Xylo-oligosaccharides. [e] OTUs; Operational taxonomic units. [a-b] Means within a row that do not share a common superscript are significantly different ($P < 0.05$).

There was a significant interaction between fructans and XOS on beta diversity in the ileum ($P < 0.026$). An NMDS plot of the similarity of bacterial communities in each treatment group is shown in (Figure 4.4). When looking at the effect of fructans on bacterial community structure with and without XOS, there was no difference between fructans in the absence of XOS (CON vs FOS, CON vs INU and FOS vs INU), but in the presence of XOS, both FOS and INU were different to the CON (CON vs FOS + XOS, and CON vs INU + XOS) but not each other (FOS + XOS vs INU + XOS). When looking at the effect of XOS on each fructan, bacterial community structure differed when XOS was included compared to the CON (CON vs CON + XOS), but there was no effect on FOS (FOS vs FOS + XOS) or INU (INU vs INU + XOS) with or without XOS. There was no effect of fructan and XOS on beta diversity in the colon ($P > 0.05$).

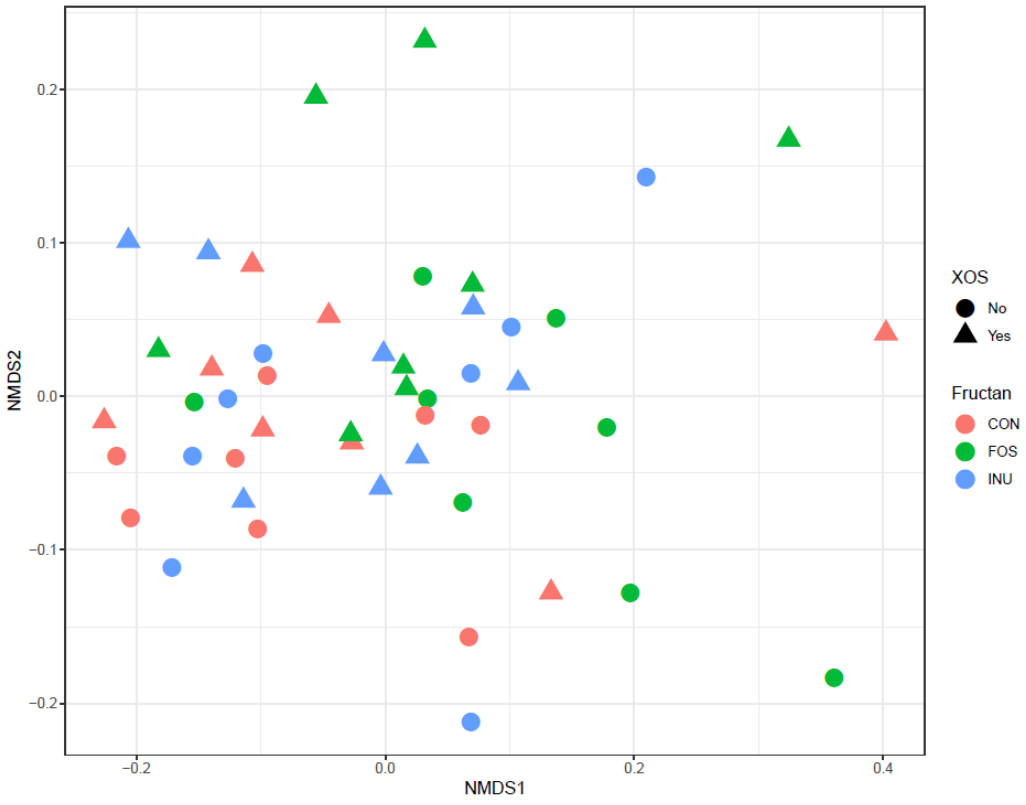

Figure 4.4 Non-metric multi-dimensional scaling (NMDS) plot of the bacterial community composition between xylo-oligosaccharides (XOS) and fructans (Fructo-oligosaccharides (FOS)) and (Inulin (INU)) in piglet ileal digesta samples.

4.4.3 Microbial endo-XYL activity

There was no effect of treatment on colonic microbial endo-XYL activity (Figure 4.5; $P > 0.05$).

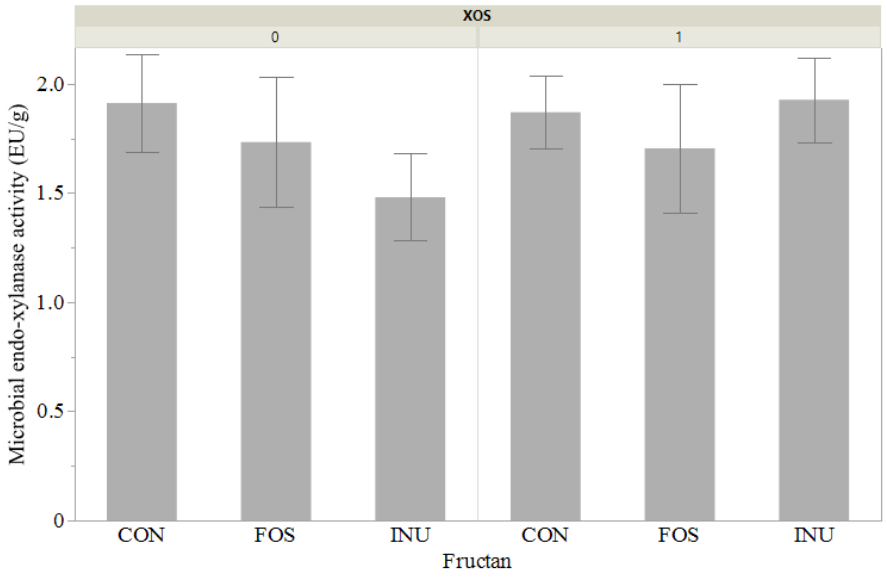

Figure 4.5: Effect of xylo-oligosaccharides (XOS) and fructans (Fructo-oligosaccharides (FOS)) and (Inulin (INU)) on piglet colonic microbial endo-xylanase activity ($P > 0.05$).

4.5 Discussion

4.5.1 Piglet performance

There is increasing demand to find alternative feeding concepts to improve gut health, enhance disease resistance and support growth performance in pigs. This study aimed to investigate the effect of short or long DP fructans with or without XOS on piglet performance, bacterial community composition and microbial endo-XYL activity. In the absence of XOS, piglets receiving the FOS and INU treatments had a reduced ADFI. In the presence of XOS alone, piglet ADFI was similar to that of the FOS and INU fed piglets, yet when XOS was fed in combination with FOS and INU, ADFI increased to a level similar to the CON treatment. The interaction between fructans and XOS on ADFI resulted in similar interaction trends for BW. Similar results have been reported in the literature whereby FOS was shown to reduce ADFI and ADG in young growing pigs (Houdijk, JGM et al., 1998). In contrast, FOS has also been shown to have no effect on ADFI, but increase ADG and improve FCR in both weaning piglets and growing pigs (Xu, C. et al., 2005; Xu, Z.R. et al., 2002). Improvements in pig growth performance with INU supplementation have been reported up until 84 days of age, but, INU was in fact shown to reduce ADFI and subsequent ADG immediately after weaning (Grela et al., 2014), agreeing with the current trial results. Moreover, the reduction in ADFI and growth performance when XOS was supplemented independently disagrees with other studies whereby a significant improvement in ADG was observed in the first 2- (Liu, J. et al., 2018) and 4 weeks after weaning (Chen, Y. et al., 2021), and an increased ADFI was seen from 7–8 weeks of age (Chapter 2).

A possible reason for the reduction in ADFI and consequent reduction in BW observed with FOS, INU and XOS supplementation could be partially due to the ileal brake mechanism. This concept is based upon the theory that partially digested nutrients and

their microbial fermentation products stimulate the release of peptides from the GIT epithelium which affect digestion and ultimately influence feed consumption (Dunshea, F.R. et al., 2018). The release of GIT hormone peptides such as peptide tyrosine tyrosine (PYY) and glucogon-like peptide 1 creates a feedback mechanism which slows gastric emptying, gastric acid production and the intensity of peristaltic contractions, all of which reduce feed intake (Black et al., 2009). Commencement of the ileal brake mechanism via GIT nutrient infusion has been shown to reduce feed intake by up to 60 % in humans and rodents (Dunshea, F.R. et al., 2018). In pigs, feeding albus lupins that are high in oligosaccharide content reduced feed intake by ~15–25 % and subsequently reduced ADG (Dunshea, F. et al., 2001), with similar results found by Moore et al. (2021). Throughout the current trial, the largest reduction in ADFI was 12 % in XOS fed piglets within the first 2 weeks of the trial (day 0–13), hence are not dissimilar from the levels reported in the literature. Interestingly, other studies have shown a reduction in ADFI with both INU and FOS supplementation in young pigs when initially fed. However, the reduction was only temporary and was compensated for in later growth stages (Grela et al., 2014; Houdijk, JGM et al., 1998). The current trial was concluded at 28 days post-weaning, hence data past this point was not obtainable.

The extent of the ileal brake mechanism and its consequent effect on ADFI tends to increase relative to the amount of stimulating substrate within the GIT (Meyer et al., 1998; Van Avesaat et al., 2015). To this point, it would be expected that feeding XOS in combination with either FOS or INU would have a larger ileal brake effect due to increased fermentation and SCFA production, thus have a greater reduction on ADFI compared to when fed independently. In fact, the results of this trial showed the opposite, such that in the last 2 weeks of the trial the combination of XOS + INU somewhat increased ADFI compared to the INU group. Although not expected, this could be

explained by a lower level of fermentative activity whereby less microbial fermentation products were produced, hence less GIT hormones were released to initiate the ileal brake mechanism. This therefore would have lessened the effect on reducing feed intake, thus explaining the higher ADFI observed. This theory is supported by the fermentative activity data (pH and lactate concentration) reported in section 6.4.1, whereby lactate concentration was lower, and pH was higher with XOS + INU compared to when INU was fed alone. This suggests that XOS and INU stimulate lactate-producing bacteria which somehow compete with each other when combined.

Despite the interaction effect of fructans and XOS on ADFI, there was no overall effect on BW or ADG, hence disagrees with hypothesis 1. One possible reason for the lack of growth response is that the piglets were of a good weight at weaning (7.88 kg) and observations of performance benefits were therefore less likely, hence is a limitation of this study. Piglets fed the CON diet tended to have a higher BW at the end of the trial compared to all test treatments. The ADG of XOS fed piglets was reduced numerically by 14.6 % in the first 2 weeks of the trial, but only by 5.5 % in the last 2 weeks of the trial. This indicates the piglets were able to cope better with XOS as they aged, suggesting a longer feeding period may have been required to observe benefits in performance. Furthermore, supplementing fructans (FOS and INU) increased piglet FCR in the final 2 weeks of the trial (day 14–27) and tended to have the same effect in the overall trial period (day 0–27). This reduction in efficiency is explained by a similar feed intake but lower ADG. Overall, there was no beneficial effect of fructans and XOS on piglet performance, hence disproving the initial hypotheses. This agrees with another study that investigated the effect of FOS and TOS whereby lower ADFI and ADG were reported compared to the CON (Houdijk, JGM et al., 1998). Furthermore, increased observations of ill health tended to be seen between days 0–13 of the current trial, whereby both INU treatments

with and without XOS tended to have a higher score compared to the other treatments, suggesting incompatibility of INU within the first 2 weeks post-weaning. This could have been due to INU favouring the growth of undesirable bacteria in this particular study.

4.5.2 Bacterial community analysis

4.5.2.1 Ileal bacterial community composition

To investigate the prebiotic effect of fructans and XOS, ileal bacterial community composition was studied by sequencing the V4 region of the 16S rRNA gene between dietary treatment groups. Over 95 % of the ileal bacteria belonged to the phyla Firmicutes, Bacteroidetes and Proteobacteria, agreeing with other studies (Isaacson and Kim, 2012; Holman et al., 2017). The increase in the abundance of the phylum Epsilonbacteraeota in the ileum of XOS fed piglets was explained by an increased abundance of OTUs associated with *g__Helicobacter* at the genus level. *Helicobacter* is a genus of gram-negative curved rod bacteria which thrive in the acidic environment of the stomach (Christakopoulos et al., 2003). The most well-known species of this genus is *Helicobacter pylori* which causes chronic gastritis and stomach ulceration in humans, thus thrives in the very acidic and low pH environment of the stomach (Ansari and Yamaoka, 2017). Encouragingly, XOS has been reported to show antimicrobial activity against *H. pylori in vitro* (Christakopoulos et al., 2003). The current study showed an increase in the abundance of OTUs associated with *g__Helicobacter* with XOS fed piglets, albeit the species was unknown. Given that the ileal pH was 6.75 (section 6.4.1) it is unlikely that the *Helicobacter* species measured in the current study was *H. pylori*, suggesting XOS was a substrate for other species within the genus.

Moreover, the tendency for the abundance of Proteobacteria to decrease in the ileum of XOS fed piglets can be explained by a reduction in the abundance of OTUs associated with *g__Actinobacillus* at the genus level. Proteobacteria are a major group of bacteria including a wide variety of pathogens such as *Escherichia* and *Salmonella*. Species of the *Actinobacillus* genus, family *Pasteurellaceae*, are gram negative facultative anaerobic bacteria that range from commensal species to pathogenic members (Vanni et al., 2012). One well known species is *Actinobacillus pleuropneumoniae* which causes pleuropneumonia, a severe respiratory disease in pigs (Vanni et al., 2012). Although the species was unknown in the current study, XOS reduced the relative abundance of OTUs associated with *g__Actinobacillus*. To the author's knowledge, this effect of XOS has not been reported in the literature to date, but the abundance of *Lactobacillus* has been shown to have a significant negative correlation with the abundance of *Actinobacillus* in piglet ileal digesta (Tian et al., 2019). This agrees with the current study whereby an increase in the abundance of *Lactobacillus* and a reduction in the abundance of *Actinobacillus* was seen in XOS fed piglets.

Furthermore, the tendency for the abundance of Bacteroidetes to increase in XOS fed piglets can be explained by an increase in the abundance of OTUs associated with *g__Prevotella_7, g__Prevotella_9 and g__uncultured* (Prevotellaceae Family) at the genus level. Prevotella species are known to possess genes for cellulose and xylan hydrolysis (Ivarsson et al., 2014), hence the presence of XOS in the ileum seemed to stimulate the increase in abundance of OTUs associated with the *Prevotellaceae* family and carbohydrate metabolism. Unfortunately, the analysis of microbial endo-XYL activity in the ileum was not possible in the current study which would have supported this theory. However, similar results were found in Chapter 2, whereby an increase in the abundance of OTUs associated with *Prevotellaceae_NK3B31_group, Prevotella_7 and*

Prevotella_9 were seen in the faeces of XOS fed piglets compared to the CON. Hence, this family of bacteria are likely involved in the mechanistic pathways of XOS. It is important to note that there was a trend for an interaction between XOS and fructans on the abundance of OTUs associated with *g__Prevotella_7,* whereby increases with XOS were only seen in the INU group. This indicates the significant main effect stemmed mostly from this trend for an interaction in the INU group.

The abundance of OTUs associated with *g__Escherichia-Shigella* in the ileum tended to be similar with and without XOS in the CON and INU groups but were higher without XOS and lower with XOS in the FOS group. This suggests a potential advantage of feeding XOS and FOS in combination to prevent the growth of pathogenic *g__Escherichia-Shigella* in the ileum. The supplementation of XOS alone has been shown to reduce faecal *Escherichia coli* counts in weanling pigs (Liu, J. et al., 2018), and similarly, FOS has been to shown to reduce *Escherichia coli* in the caecum (Liu, L. et al., 2020). However, to the author's knowledge, the combination of XOS and FOS on the abundance of *Escherichia coli* in the ileum has not been reported elsewhere and is likely due to competitive exclusion.

Lactobacillus is the largest genus of LAB and mainly prominent in the small intestine (Yu et al., 2018). *Lactobacillus* have been shown to affect intestinal physiology, regulate the immune system and maintain intestinal homeostasis of the host, all of which improves overall health and growth performance (Valeriano et al., 2017). In the current study, the abundance of OTUs associated with *g__ Lactobacillus* in the ileum was highest in the XOS group compared to all other treatment groups but was not dissimilar to the FOS group. This indicates that XOS was a good substrate for *g__ Lactobacillus* with

fermentation taking place in the ileum. XOS has been shown to increase the abundance of *Lactobacillus* in the ileum and caecum (Chen, Y. et al., 2021), colon (Pan et al., 2019) and faeces (Liu, J. et al., 2018) of pigs, hence supports the current trials results. An *in vitro* study suggested that the slower rate of XOS fermentation compared to GOS or soy solubles may indicate that XOS is a better substrate for bacteria in the distal portions of the GIT such as the transverse and descending regions of the large intestine (Smiricky-Tjardes et al., 2003a). However, given the clear effect of XOS on *Lactobacillus* in the ileum in the current study, coupled with the absence of a similar effect in the colon, indicates that XOS is a readily fermentable substrate for the bacteria in the small intestine and perhaps the caecum (not measured) *in vivo*. Hence, the hypothesis of XOS mainly being fermented in the lower gut is rejected. Moreover, piglets with a 'good' ADG have been found to have higher abundance of *Lactobacillus* and unclassified *Prevotellaceae* compared to piglets with a poor ADG (Gaukroger et al., 2020). However, despite the increase in abundance of beneficial *Lactobacillus* and unclassified *Prevotellaceae* in XOS fed piglets in the current study, this did not translate into a performance benefit for the host and in fact the opposite was observed.

Furthermore, the abundance of OTUs associated with *g__Terrisporobacter* was similar with and without XOS in the FOS and INU groups but was higher without XOS and lower with XOS in the CON group. Similar results have been found in the ileum and caecum of weaner pigs, whereby the abundance of *Terrisporobacter* was lower with XOS than without (Chen, Y. et al., 2021). In humans, infants that were fed on a formula diet showed the lowest microbial diversity and highest relative abundance of *Terrisporobacter* compared to those fed other diets (Cai et al., 2019). Moreover, correlation network analysis revealed that the abundance of *Terrisporobacter* was positively correlated with an oxidative stress marker (urinary F2-isoprostane) (Cai et al., 2019). Hence, the

reduction in abundance of OTUs associated with *g__Terrisporobacter* in XOS fed piglets could indicate the potential for XOS to reduce oxidative stress.

4.5.2.2 Colonic bacterial community composition

Over 94 % of the colonic bacteria belonged to the phyla Bacteroidetes and Firmicutes, which agrees with other studies (Isaacson and Kim, 2012; Holman et al., 2017). The abundance of OTUs associated with an unknown genus from the *Muribaculaceae* family and *g__Oscillospira* were reduced in FOS fed piglets compared to the CON but not the INU group. Little has been reported on the role of these bacteria in animals, but supplementing raw potato starch, a form of resistant starch, in pigs also reduced the relative abundance of OTUs associated with *Oscillospira* and S24-7 group (now known as *Muribaculaceae*), possibly indicating similarities between the mode of action of resistant starch and FOS (Sun et al., 2015). Furthermore, a meta-analysis of 5 microbiota human studies revealed a significant reduction in the abundance of *Oscillospira* in patients suffering with inflammatory bowel conditions like Crohn's disease (Walters et al., 2014), indicating a possible association with a degree of dysbiosis in FOS fed piglets.

Compared to the CON group, XOS fed piglets had a lower abundance of OTUs associated with *g__Mitsuokella* and *g__Ruminococcaceae_UCG-014*. In the ileum of XOS fed piglets, the abundance of OTUs associated with lactate producing g__ *Lactobacillus* increased, hence it was expected that lactate utilising bacteria like *Mitsuokella* (Newman et al., 2018) would increase in abundance to utilise the free lactate. However, this was not the case and a reduction in the abundance of *g__Mitsuokella* was seen in the colon of XOS fed piglets compared to the CON. This indicates that the majority of lactate was absorbed or utilised after the terminal ileum and before the proximal colon, meaning less

lactate was present in the colonic samples, hence explaining the reduction of lactate utilising bacteria. Moreover, species in the *Ruminococcaceae* family are known for having XYL and cellulase gene activity, thus are well equipped to break down plant material (Biddle et al., 2013). As seen in the ileum with members of the *Prevotellaceae* family, it was expected that the presence of XOS in the colon would stimulate the abundance of xylan-degrading bacteria and their activity. However, a reduction in the abundance of *g__Ruminococcaceae_UCG-014* was seen in the colon. Moreover, there was no difference in the colonic microbial endo-XYL activity, hence disagrees with hypotheses 2-4. The reduction of lactate utilising *g__Mitsuokella* and xylan degrading *g__Ruminococcaceae_UCG-014* abundance in the colon, coupled with a large increase in the abundance of g__ *Lactobacillus* and members of the *Prevotellaceae* family in the ileum suggests that the fermentative activity of XOS is mainly in the ileum, hence further up the gut than initially expected.

Finally, *g__Sphaerochaeta* belong to the family *Spirochaetaceae*; little is known about this genus, but isolates have been found in freshwater sediments and from the hindgut contents of termites (Ritalahti et al., 2012). One study showed that the abundance of *Sphaerochaeta* in the duodenum were increased in low FCR pigs compared to high FCR pigs (Tan et al., 2018). This somewhat agrees with the current study, as the colonic abundance was highest in the CON fed piglets which had the lowest FCR, whilst the abundance was lowest in the FOS and INU group which had significantly higher FCRs. Albeit, the location along the GIT was different and it does not explain the increase in abundance when XOS and INU were fed in combination. Furthermore, there was a trend for an interaction between XOS and fructans on the abundance of OTUs associated with *g__Romboutsia*. Again, little is known about this bacterial genus but it has been shown to utilise an array of carbohydrates and grow on arabinose (ara) and glu substrates, as well

as having genes encoding for the specific carbohydrate degrading enzymes (Gerritsen, 2015). Despite this, no difference was seen in the colonic endo-XYL activity with treatment, although other enzyme activities like arabinofuranosidase were not studied. With the caecum being a highly active fermentation site, average caecal levels of endo-XYL activity (7.41 EU/g DM) have been reported to be 18 times that of the average activity in the ileum of broilers (0.43 EU/g DM) (Bautil, 2020). In the current study, the average colonic activity was 1.77 EU/g DM, hence in between the ileal and caecal figures reported for broilers. With dietary treatment having a larger effect on bacterial community composition in the ileum compared to the colon in the current study, a difference in endo-XYL activity with treatment would have been more likely in the ileum than the colon. This effect was seen in piglets, whereby XOS increased the xylanolytic activity in the ileum but not the caecum or colon (Marinho et al., 2007).

4.5.2.3 Alpha and Beta diversity

Alpha diversity was estimated based on the Chao1 index as an indicator of species richness, whilst the Shannon-Wiener index was measured as an indicator of species evenness (richness and abundance). There was no effect of XOS on alpha diversity in the ileum or colon, which disagrees with hypothesis 3 and a study where XOS increased Chao1 and Shannon index (Chen, Y. et al., 2021), albeit XOS was included at a higher inclusion rate (0.50 g/kg) than the current study. Contrary to hypothesis 2, lower numbers of OTUs and species richness were found in the ileum of piglets supplemented with FOS compared to those fed the CON diet, but not the INU diet. One study found no effect of FOS on species richness in the ileal digesta or mucosa of broiler chickens (Shang et al., 2018), whilst another found no effect of alternative prebiotics like GOS on alpha diversity (Berding et al., 2016; Tian et al., 2019). A high diversity is generally considered beneficial for host health and helps maintain resistance and stability in an ecosystem after a

challenge (Konopka, 2009; Chen, L. et al., 2017). Alpha diversity tends to increase with age as the bacterial community becomes more developed (Chen, L. et al., 2017). However, it has been shown to reduce immediately after stressful events and periods of reduced growth such as after weaning (Hu et al., 2016). Others have shown low alpha diversity to be associated with bacterial community instability, as less diverse communities are more prone to the introduction of foreign species due to a lesser coverage of ecological niches (Frost et al., 2021). A good example of how low bacterial diversity can negatively affect the gut bacterial balance is *Clostridium difficile,* whereby the proliferation of this pathogenic bacteria increases when co-cultured with a low diverse dysbiotic microbiota compared to a healthy community (Horvat and Rupnik, 2018). Supporting this, in the current study, FOS fed piglets tended to have a higher abundance of OTUs associated with *g__Clostridium_sensu_stricto_1* in the colon, coupled with a low alpha diversity. The reduction in alpha diversity when piglets were supplemented with FOS indicates a degree of bacterial community imbalance which may explain the poorer efficiency of the FOS fed piglets.

There was a significant interaction between fructans and XOS on beta diveristy in the ileum, but not the colon hence disagreeing with hypothesis 3-4. Despite the lower ileal bacterial diversity of FOS fed piglets, there was no difference in ileal bacterial community structure between fructans or compared to the CON in the absence of XOS. However, in the presence of XOS, both FOS and INU were different to the CON. There was weak visual clustering in the NMDS plot, yet the FOS and INU samples were higher up the NMDS2 axis compared to the CON samples in the presence of XOS. Similarly, although no clear visual clustering was seen, the bacterial community structure differed when XOS was included compared to the CON, indicating that supplementation of XOS altered the

bacterial community structure in the ileum. Similarly, clustering tendencies have been observed with different doses of XOS (Pan et al., 2019).

4.5.3 Conclusion

Overall, supplementation of XOS with or without short and long-chain fructans had no beneficial effect on piglet growth performance, likely because of the large reduction in ADFI. The ADG of XOS fed piglets was reduced in the first 2 weeks of the trial, but to a much lesser extent in the last 2 weeks of the trial, suggesting piglets adjusted to XOS with age and a longer feeding period may have been required to observe performance benefits. Bacterial community structure differed with prebiotic supplementation in the ileum but not the colon, indicating the fermentation of the products may be more suitable for bacteria inhabiting the distal small intestine compared to the large intestine. Dietary treatment had no effect on colonic microbial endo-XYL activity. Positive bacterial abundance changes were observed with XOS supplementation, for example the stimulation of beneficial *Lactobacillus* in the ileum. Hence, despite the lack of performance benefits observed, the bacterial modulation effect of XOS was positive and the piglets likely needed longer for these benefits to reflect in increased growth performance. In summary, results of the current study and comparison with the literature suggest a longer feeding period may be necessary to allow the gut bacteria a longer time to adapt to the substrates and to elicit benefits in performance.

Chapter 5

Evaluation of titanium dioxide and intrinsic cellulose as inert markers for non-starch polysaccharide digestibility.

5.1 Abstract

The accuracy of the inert marker technique for digestibility studies relies on the marker being homogenously distributed throughout the digesta with the nutrients to facilitate calculation of accurate and precise digestibility values. However, numerous studies have shown separation of inert markers and digesta when investigating fibre fraction digestibility, resulting in negative or unexpectedly high digestibility values. Hence, this study aimed to evaluate TiO_2 and intrinsic cellulose as inert markers for NSP digestibility in piglets. A total of 474 weaner piglets (7.88 ± 1.51 kg) were blocked into mixed sex pens of 4–5 piglets and assigned to 1 of 6 dietary treatments in a 2 × 3 factorial treatment arrangement. Diets contained 2 levels of XOS (0 and 0.286 g/kg) and 3 levels of fructans [(0 g/kg) and 6.50 g/kg of short-chain FOS and 4.08 g/kg of INU to supply 3.8 g/kg of pure fructans)]. TiO_2, cellulose and NSP concentrations of the feed, ileal digesta and faeces were determined to calculate the AID and ATTD of NSPs using both TiO_2 and cellulose as comparative inert markers. Results showed highly negative AID of NSP when using TiO_2 as an inert marker, indicating separation of the marker and NSP. In contrast, using cellulose as an inert marker yielded more realistic, less negative and less variable AID of NSP. There was a negative correlation between AID of total NSP when using TiO_2 as a marker compared to cellulose ($P < 0.021$). At the faecal level, positive and more similar ATTD of NSPs were observed between the markers compared to AID, but no positive correlation was seen between the markers. Post-ileal cellulose degradation caused the average ATTD for total NSPs, obtained using cellulose as a marker, to be 15.1 % lower compared to when using TiO_2 as a marker, and cellulose fermentation was also affected by dietary treatment. In conclusion, this study has shown that TiO_2 is an

unsuitable inert marker for AID of NSPs, with cellulose yielding more realistic initial results. In contrast, TiO_2 should be used for ATTD of NSPs, as post-ileal cellulose degradation rendered the use of cellulose as an inert marker infeasible.

5.2 Introduction

Digestibility studies are an important method of evaluating the nutritive value of feed materials or additives in monogastric nutrition. The digestibility of diets can be measured by total collection of faeces after a known quantity of feed has been consumed or by using an inert marker substance (Kavanagh et al., 2001). The marker method allows digestibility to be calculated using the ratio of inert marker to the nutrient of interest in the feed and digesta/excreta, and is a more efficient and less laborious method than total collection (de Vries, 2014). Chromic oxide (Cr_2O_3) is commonly used as an inert marker and has shown good recovery rates in some studies (Bakker and Jongbloed, 1994), but low recoveries in other studies (Yin, Y.-L. et al., 2000; Jagger et al., 1992). Likewise, TiO_2 is also commonly used as a marker with good recovery rates and is suggested as the most appropriate inert marker for digestibility studies (Jagger et al., 1992).

The accuracy of the marker technique relies on the marker being homogenously distributed throughout the digesta with the nutrients to facilitate calculation of accurate and precise digestibility values (Choct et al., 1996). The most common inert markers are insoluble and associated with the solid phase of the digesta throughout the GIT, but separation of the solid and liquid phase of the digesta can occur which complicates digestibility results (Choct et al., 1996; Van der Klis and Van Voorst, 1993). For example, peristaltic and anti-peristaltic contractions cause pressure at the ileocaecal junction, which can force more of the liquid fraction contents into the caeca compared to the solid fraction

(Fenna and Boag, 1974). Cr_2O_3 is a fine particulate material and is likely transported with the liquid phase at a higher rate than larger solid components, with differences in water intake only increasing the discrepancy (Van der Klis and Van Voorst, 1993). To this point, Oberleas et al. (1990) found that Cr_2O_3 was carried more readily by the liquid rather than the solid phase of the digesta in rats, hence concluded that it was a more useful marker for studying the flux of water rather than dietary DM. In a well digested diet, transit times are fairly short in the lower ileum and the distribution of nutrients and marker are relatively uniform, but the presence of large amounts of soluble NSPs can exacerbate differences in transit times and separation of nutrients (Choct et al., 1996). As such, the suitability of traditional inert markers for calculating digestibility in high fibre diets is questionable and the basal diet components should be considered (Prawirodigdo et al., 2019; Prawirodigdo et al., 2021). To this point, numerous studies have shown negative or unexpectedly high digestibility values for fibre fractions using Cr_2O_3 (Jamroz et al., 2002; Brenes et al., 2003) or TiO_2 (Bautil et al., 2019) with the overall concluding reason being that this was due to separation of the indigestible marker and digesta. When estimating the AID of NGP with Cr_2O_3, separation of the marker and digesta resulted in unrealistically high estimates (54 to 66 %) in broilers, infeasibly exceeding ATTD values by 16 to 42 % units (de Vries, 2014). This further supports that the marker method can yield erroneous results, especially in high fibre diets, ultimately leading to mis-estimation and confounded digestibility results.

Minimal work has been conducted in finding suitable alternatives to the traditional markers for use in digestibility studies on fibre, but an ideal marker would be intrinsic to the diet and have a similar structure to fibre. Intrinsic, naturally occurring, acid-insoluble ash (AIA) has been examined and deemed a reliable marker for energy and DM digestibility in comparison to Cr_2O_3 and labour-intensive total collection in metabolism

crates for pigs (Kavanagh et al., 2001). However, it cannot simply be applied to analyse the digestibility of other dietary components, like fibre fractions. Cellulose is not hydrolysed by endogenous enzymes and is therefore considered relatively indigestible prior to the hindgut, making it a possible candidate as an inert marker for ileal digestibility. Any fermentation of cellulose by the GIT bacterial community mainly occurs in the distal part of the colon, hence using cellulose as a marker in the small intestine is theoretically plausible, whilst its use at the faecal level would require correction (Jha and Berrocoso, 2015). Indeed, indigestible cellulose has been shown to provide a better estimate of organic matter digestibility over AIA in ruminants (Penning and Johnson, 1983). By comparison, there is a lack of data in the monogastric field. This study aimed to evaluate TiO_2 and intrinsic cellulose as inert markers for AID and ATTD of NSPs in piglets.

5.2.1 Study aims

A number of studies have shown separation of inert markers and digesta when investigating fibre fraction digestibility, resulting in negative or unexpectedly high digestibility values. This study therefore aimed to evaluate TiO_2 and intrinsic cellulose that has a more similar structure to NSPs, as inert markers for AID and ATTD of NSPs in piglets.

5.2.2 Hypotheses

1. As seen in the literature, separation of TiO_2 and digesta will occur in the ileum leading to mis-estimation of AID of NSPs, whereas cellulose will not separate from the digesta and thus provide correct estimations of AID of NSPs.

2. Fractionation of the markers and digesta will be corrected at the faecal level, leading to a positive correlation between ATTD of NSPs using TiO_2 or cellulose as inert markers.

5.3 Materials and methods

Study protocols were approved by the University of Leeds Pig Research Centre and ethical approval was granted by the Animal Welfare and Ethical Review Body as described in Appendix A.1.

5.3.1 Animals, housing, experimental design and dietary treatments

The animals, housing, experimental design and dietary treatments used in this study were as described in (section 4.3.1 and 4.3.2).

5.3.2 Measurements and sampling

5.3.2.1 TiO_2 analysis

TiO_2 in the feed, ileal digesta and faecal samples were analysed according to the method of Short et al. (1996). Briefly, a known weight (~ 0.2 g) of freeze-dried (section 4.3.3.2) sample was weighed into a 28 ml glass tube and ashed at 580 ºC for 13 hours. Once cooled, 10 ml of 7.4 M sulphuric acid was added to the ash, and the tubes were transferred to a hot plate at 150 ºC for 1 h to enable ash dissolution. The cooled tube contents were poured into a small beaker containing 10 ml of deionised water before filtration through Whatman 541 filter paper into a 100 ml volumetric flask. 10 ml of hydrogen peroxide were added, and the solution was brought up to 100 ml with deionised water. A yellow/orange colour was formed, which correlated to the amount of TiO_2 in the sample. 250 ul aliquots

were transferred into a 96 well plate and the absorbance was measured at 408 nm using a spectrophotometer. The concentration of TiO_2 in each sample was calculated by plotting the absorbance value on a calibration curve of known standards.

5.3.2.2 Carbohydrate content

5.3.2.2.1 Total NSP, NSP constituent sugars and cellulose analysis

Total NSP, NSP constituent sugars, and cellulose content of the feed, ileal digesta, and faeces were determined in duplicate by GC following the methods of Englyst, H.N. et al. (1994) at Englyst Carbohydrates Ltd, Southampton, UK. Briefly, starch was dispersed with dimethylsulfoxide and enzymatically hydrolysed before being removed along with sugars by washing with either 80 % ethanol or pH 7 buffer for the total and insoluble NSP fractions respectively. The dried residues were treated with 12 M sulphuric acid at 35 ºC to disperse the cellulose followed by 2 M sulphuric acid treatment at 100 ºC to complete the hydrolysis of the neutral sugar cell-wall polysaccharides to their constituent monosaccharides and de-sulphate them.

A portion of the hydrolysate was buffered to pH 4.5 and pectinase was added to complete the hydrolysis of pectin to galacturonic acid (galA). The neutral sugars were reduced to their alditols, and uronic acids to their aldonic acids with alkaline sodium borohydride. The aldonic acids were converted to their lactones by heating in acidified ethanol and derivatised with propylamine to their propylaldonamides. All sugars were then acetylated with acetic anhydride in the presence of methylimidazole as catalyst. A GC system with flame ionization detection (GC2010, Shimadzu, Japan) was used to measure the resulting alditol acetates of the neutral sugars and N-acetyl propylaldonamides of the uronic acids. NSP constituent sugars analysed included rhamnose (rha), fucose (fuc), ara, xyl, mannose

(man), galactose (gal), glu, glucuronic acid (glcA) and galA. Total NSP content was calculated according to the sum of the constituent sugars as shown in Equation 5.1. Cellulose was calculated by the difference between total NSP glu and non-cellulosic polysaccharide (NCP) glu, as shown in Equation 5.2.

$$Total\ NSP = rha + fuc + ara + xyl + man + gal + glu + glcA + galA \quad \textbf{Equation 5.1}$$

$$Cellulose = total\ NSP\ glu - NCP\ glu \quad \textbf{Equation 5.2}$$

5.3.2.3 Inert marker ratios and apparent NSP digestibility

The TiO_2 : cellulose ratio in the feed, ileal digesta and faeces was calculated by dividing the concentration of TiO_2 by the concentration of cellulose in each medium. The AID and ATTD of NSPs were calculated according to Equation 5.3 and Equation 5.4 respectively with each of the inert markers tested, TiO_2 and cellulose. The ATTD of cellulose was calculated using TiO_2 as an inert marker only.

$$AID = 1 - \left(\frac{Feed\ inert\ marker\ (\%)\ \times\ Ileal\ nutrient\ (\%)}{Ileal\ inert\ marker\ (\%)\ \times\ Feed\ nutrient\ (\%)}\right) \quad \textbf{Equation 5.3}$$

$$ATTD = 1 - \left(\frac{Feed\ inert\ marker\ (\%)\ \times\ Faecal\ nutrient\ (\%)}{Faecal\ inert\ marker\ (\%)\ \times\ Feed\ nutrient\ (\%)}\right) \quad \textbf{Equation 5.4}$$

5.3.3 Statistical analysis

The individual piglet served as the experimental unit for all data in this chapter. Normality of data and homogeneity of variance were assessed as per section 2.3.5.1. The statistical package JMP® (Version *15.1*. SAS Institute Inc., Cary, NC, 1989 - 2019) (SAS, 2020) was used to analyse marker concentrations, marker ratios, AID and ATTD of NSP data as a 2-way ANOVA. The statistical model included the fixed effects of XOS, fructans and their interaction. A linear regression was used to assess the relationship between AID and ATTD of NSPs using different inert markers. Significant differences were classed as $P < 0.05$ and trends as $P < 0.10$.

5.4 Results

5.4.1 Marker concentration in diet, ileum and faeces

Analysed TiO_2 concentrations in the experimental diets were close to the expected added marker concentrations (0.50 %; Table 5.1). The analysed cellulose concentrations and calculated TiO_2 : cellulose ratio in the experimental diets were not different between the dietary treatments. There was a trend for an interaction between XOS and fructans on the ileal concentration of TiO_2, whereby compared to the CON, concentrations were higher in the FOS and INU groups without XOS, but not different with XOS (Table 5.1; P = 0.060). There was no effect of XOS, fructans or their interaction on the cellulose concentration or the TiO_2 : cellulose ratio in the ileum, nor on the TiO_2 or cellulose concentration in the faeces (P > 0.05). There was an interaction between XOS and fructans on the TiO_2 : cellulose ratio in the faeces, whereby the CON diet had a lower ratio compared to all other treatments other than FOS + XOS (Table 5.1; P < 0.029).

Table 5.1: Effect of XOS and fructans (FOS and INU) on feed, ileal digesta and faecal concentrations of TiO_2, cellulose and TiO_2 : cellulose ratios [a].

	Fructan				XOS[e]			CON	FOS	INU	CON	FOS	INU		*P* value		
	CON[b]	FOS[c]	INU[d]	SEM	0	1	SEM	0	0	0	1	1	1	SEM	Fructan	XOS	Fructan × XOS
Diet																	
TiO_2[f] (g/100g DM)	0.51	0.49	0.50	-	0.50	0.49	-	0.50	0.48	0.52	0.51	0.49	0.48	-	-	-	-
Cellulose (g/100g DM)	1.87	1.90	1.89	-	1.93	1.84	-	1.94	1.97	1.87	1.79	1.83	1.91	-	-	-	-
TiO_2 : Cellulose	0.27	0.26	0.27	-	0.26	0.27	-	0.26	0.24	0.28	0.28	0.27	0.25	-	-	-	-
Ileum																	
TiO_2 (g/100g DM)	1.26	1.38	1.54	0.083	1.36	1.42	0.067	1.11	1.32	1.67	1.42	1.44	1.41	0.113	0.072	0.552	0.060
Cellulose (g/100g DM)	6.95	6.72	6.40	0.379	6.65	6.73	0.306	6.51	6.76	6.69	7.40	6.69	6.10	0.553	0.572	0.860	0.372
TiO_2 : Cellulose	0.19	0.23	0.26	0.024	0.21	0.23	0.019	0.18	0.21	0.25	0.20	0.24	0.26	0.034	0.132	0.532	0.941
Faeces																	
TiO_2 (g/100g DM)	3.20	3.26	3.31	0.113	3.23	3.29	0.092	2.98	3.32	3.40	3.43	3.20	3.23	0.159	0.791	0.658	0.111
Cellulose (g/100g DM)	9.04	8.60	8.57	0.240	8.92	8.55	0.196	9.48	8.57	8.72	8.59	8.64	8.41	0.340	0.311	0.182	0.369
TiO_2 : Cellulose	0.36	0.38	0.39	0.014	0.37	0.39	0.012	0.32[b]	0.39[a]	0.40[a]	0.40[a]	0.37[ab]	0.39[a]	0.020	0.308	0.222	0.029

[a] Data are means of 8 replicate samples per treatment. [b]CON; Control. [c]FOS; Fructo-oligosaccharides.[d] INU; Inulin. [e] XOS; Xylo-oligosaccharides, 0 = No XOS; 1= XOS. [f] TiO_2; Titanium dioxide. [a-c] Means within a row that do not share a common superscript are significantly different.

5.4.2 AID of NSPs using TiO_2 and cellulose as inert markers

When using TiO_2 as an indigestible marker for NSP digestibility profiling, highly negative AID values were obtained, indicating proportionately higher NSP levels in the ileum to the feed compared to their TiO2 content (Table 5.2 and Figure 5.1a + b). In comparison, when using cellulose as an inert marker for NSP digestibility profiling, the AID values were mainly positive and much less variable (Table 5.2 and Figure 5.1a+b). There was a negative correlation between AID of total NSP when using TiO_2 as a marker compared to cellulose ($P < 0.021$; Figure 5.1c). Dietary treatment effects are discussed in detail in Chapter 6.

Table 5.2: Effect of XOS and fructans (FOS and INU) on the AID of NSP constituent sugars and total NSP using TiO_2 and cellulose as inert markers [a].

	Fructan			SEM	XOS[e]		SEM	CON	FOS	INU	CON	FOS	INU	SEM	*P* value		
	CON[b]	FOS[c]	INU[d]		0	1		0	0	0	1	1	1		Fructan	XOS	Fructan × XOS
AID[f] of NSP[g] using TiO_2[h] (%)																	
Ara	-44.97	-27.66	-12.04	9.862	-31.16	-25.29	7.883	-50.52	-31.70	-11.27	-39.43	-23.63	-12.80	13.947	0.069	0.601	0.890
Fuc	-97.56	-75.64	-91.62	16.120	-80.93	-95.62	12.885	-95.56	-62.90	-84.32	-99.57	-88.38	-98.92	22.797	0.601	0.425	0.892
Gal	-31.17	-16.23	-6.34	9.189	-17.04	-18.79	7.345	-33.27	-16.06	-1.78	-29.06	-16.41	-10.90	12.995	0.163	0.867	0.869
GalA	-1.85	11.32	18.90	8.439	8.57	10.34	6.745	-0.29	8.34	17.64	-3.41	14.29	20.15	11.934	0.217	0.853	0.927
Glu	-34.95[b]	-16.08[ab]	2.00[a]	10.107	-18.18	-14.51	8.079	-36.88	-20.43	2.78	-33.02	-11.72	1.22	14.294	0.042	0.750	0.934
Man	-6.01	-0.68	-5.21	6.818	0.76	-8.69	5.449	-17.13[ab]	3.17[ab]	16.25[a]	5.10[1b]	-4.53[ab]	-26.66[b]	9.642	0.830	0.227	0.006
Rha	-16.34	-18.88	1.48	10.000	-1.90	-20.59	7.995	-8.86	-2.65	5.81	-23.83	-35.10	-2.85	14.145	0.283	0.107	0.671
Xyl	-47.53[b]	-28.54[ab]	-6.36[a]	10.130	-35.06	-19.89	8.097	-54.18	-42.53	-8.48	-40.88	-14.56	-4.23	14.327	0.021	0.193	0.692
Total NSP	-38.02	-20.64	-5.08	9.381	-24.11	-18.37	7.499	-42.09	-26.34	-3.92	-33.95	-14.94	-6.23	13.267	0.053	0.592	0.857
AID of NSP using cellulose (%)																	
Ara	5.74	0.63	0.46	2.407	-0.81[a]	5.36[b]	1.943	-0.54[abc]	-6.48[c]	4.6[abc]	12.02[a]	7.75[ab]	-3.68[bc]	3.516	0.217	0.029	0.002
Fuc	-34.75	-46.97	-67.54	12.900	-45.26	-54.25	10.415	-37.61	-39.03	-59.13	-31.9	-54.9	-75.95	18.842	0.192	0.540	0.780
Gal	14.52	9.71	3.95	4.235	9.89	8.89	3.419	10.75	7.49	11.43	18.28	11.94	-3.54	6.185	0.210	0.836	0.126
GalA	34.38	32.42	28.23	2.820	30.21	33.15	2.277	33.59	27.94	29.09	35.17	36.9	27.38	4.119	0.285	0.361	0.375
Glu	13.43[ab]	10.24[b]	13.93[a]	0.949	9.78[a]	15.28[b]	0.766	9.12[b]	2.22[c]	17.99[a]	17.74[a]	18.25[a]	9.86[b]	1.385	0.014	<.0001	<.0001
Man	28.86[a]	17.03[ab]	5.43[b]	5.202	21.42	12.79	4.200	20.24[a]	16.24[a]	27.79[a]	37.47[a]	17.82[a]	-16.94[b]	7.598	0.009	0.149	0
Rha	24.42[a]	7.94[b]	12.98[ab]	3.570	20.77[a]	9.46[b]	2.882	28.34	17.03	16.94	20.51	-1.15	9.02	5.214	0.006	0.008	0.485
Xyl	4.74	-0.58	6.19	2.378	-3.63[a]	10.54[b]	1.920	-3.18[bc]	-15.65[c]	7.93[ab]	12.67[a]	14.50[a]	4.44[ab]	3.473	0.104	<.0001	<.0001
Total NSP	10.56	5.85	6.82	1.997	4.60[a]	10.89[b]	1.612	5.00 [abc]	-2.48[c]	11.26[ab]	16.12[a]	14.19[a]	2.37[bc]	2.917	0.217	0.008	<.0001

[a] Data are means of 8 replicate samples per treatment. [b]CON; Control. [c]FOS; Fructo-oligosaccharides.[d] INU; Inulin. [e] XOS; Xylo-oligosaccharides, 0 = No XOS; 1= XOS. [f] AID; Apparent ileal digestibility. [g] NSP; Non-starch polysaccharides. [h] TiO_2; Titanium dioxide. [a-c] Means within a row that do not share a common superscript are significantly different.

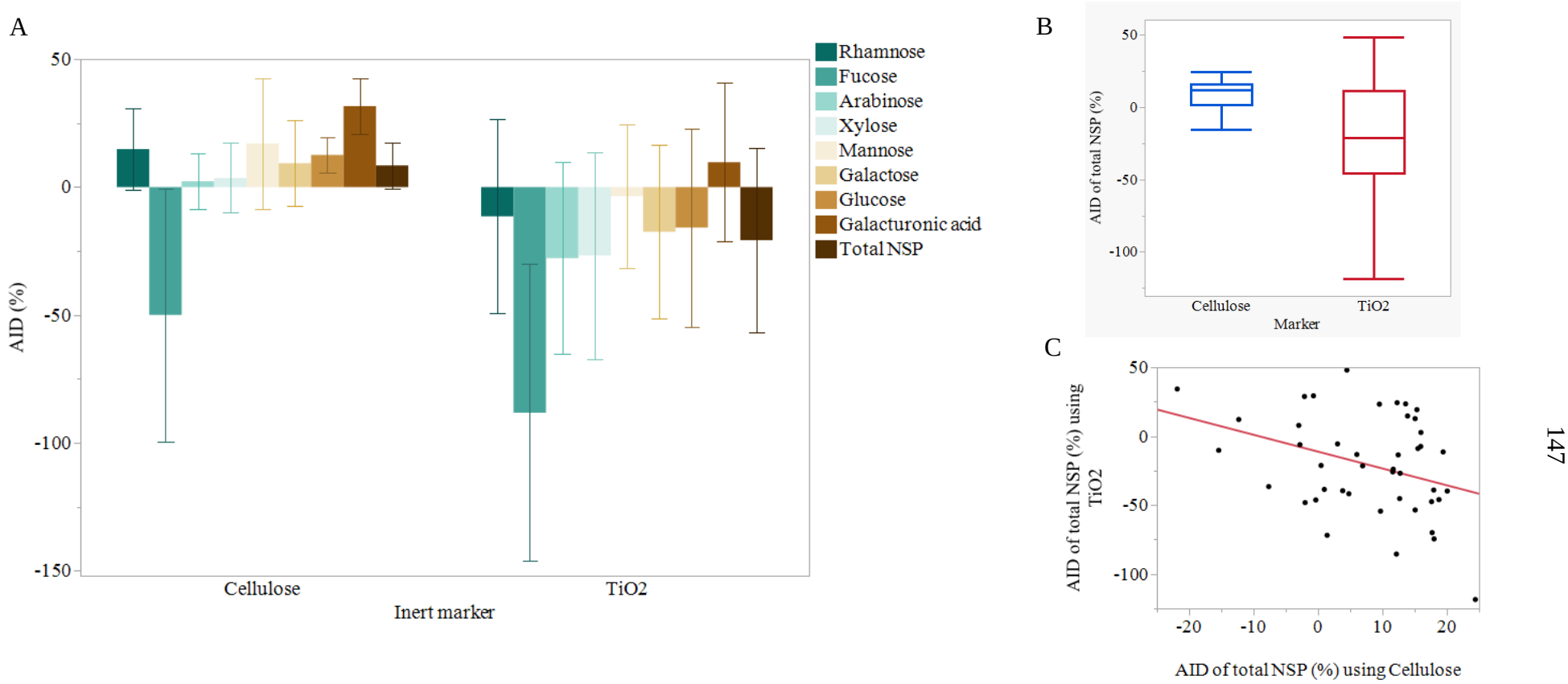

Figure 5.1: (A) Apparent ileal digestibility (AID) of non-starch polysaccharide (NSP) constituent sugars and total NSP using cellulose or titanium dioxide (TiO_2) as an inert marker. (B) AID of total NSP using cellulose or TiO_2 as an inert marker. (C) Correlation between AID of total NSP when using TiO_2 or cellulose as an inert marker ($P < 0.021$).

5.4.3 ATTD of NSPs using TiO_2 or cellulose as inert markers

The average ATTD results obtained when using TiO_2 and cellulose as inert markers were similar for NSP constituent sugars and total NSP (Figure 5.2a). However, the average total NSP values were lower when using cellulose (47.4 % ± 3.17) as a marker compared to TiO_2 (62.5 % ± 5.20) (Figure 5.2b and Table 5.3). There was no correlation between the TiO_2 and cellulose for ATTD of total NSP (Figure 5.2c; $P > 0.05$). Average ATTD of cellulose was 28.4 % (± 11.28 %; Table 5.3). There was a significant interaction of XOS and fructans on the ATTD of cellulose, whereby the CON group had lower digestibility compared to all other treatment groups ($P < 0.038$). Dietary treatment effects are discussed in detail in Chapter 6.

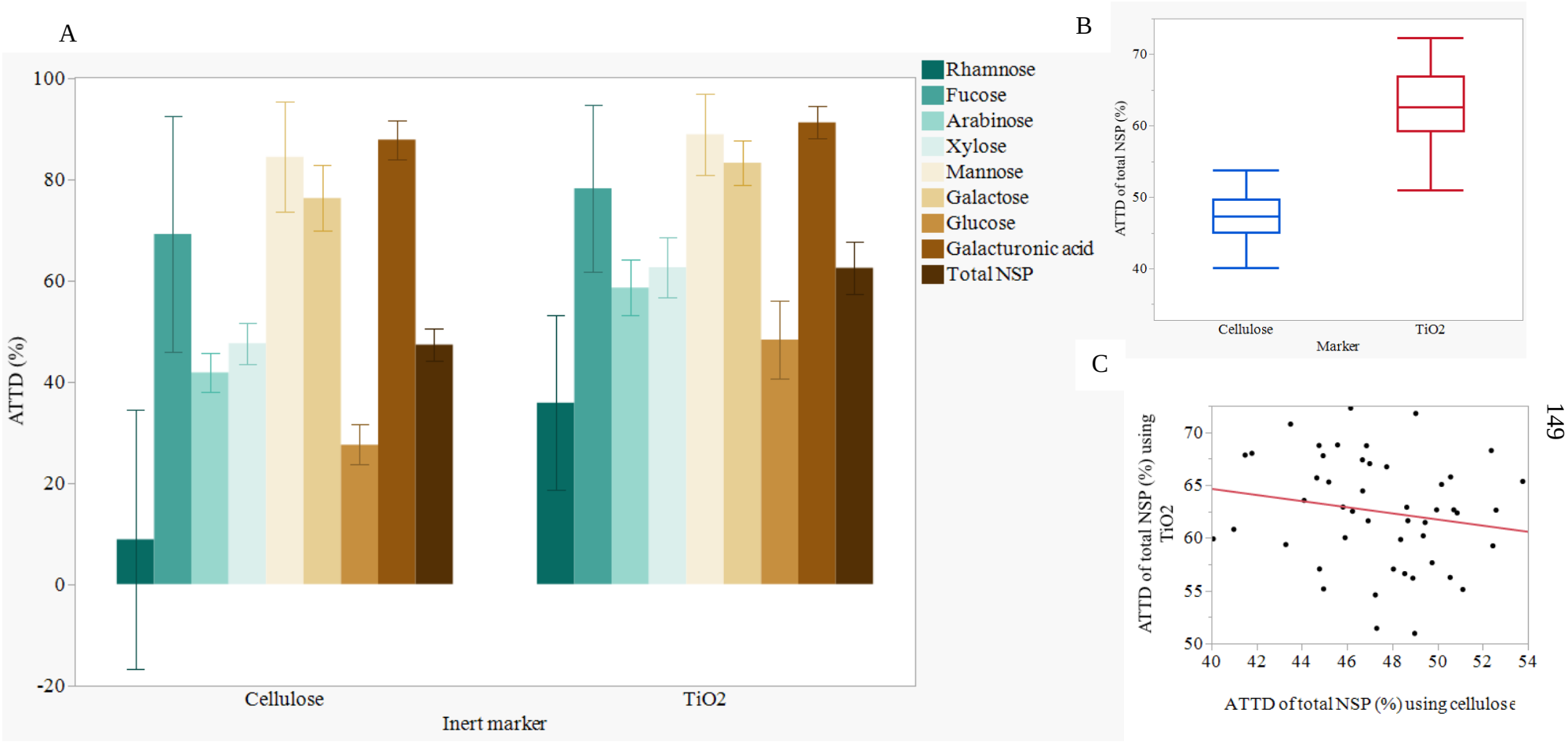

Figure 5.2: (A) Apparent total tract digestibility (ATTD) of non-starch polysaccharide (NSP) constituent sugars and total NSP using cellulose or titanium dioxide (TiO_2) as an inert markers. (B) ATTD of total NSP between dietary treatments when using cellulose or TiO2 as inert markers. (C) Correlation between ATTD of total NSP when using TiO_2 or cellulose as an inert marker ($P > 0.05$).

Table 5.3: Effect of XOS and fructans (FOS and INU) on the ATTD of NSP constituent sugars and total NSP using TiO_2 and cellulose as inert markers [a]

	Fructan			SEM	XOS[e]		SEM	CON	FOS	INU	CON	FOS	INU	SEM	*P* value		
	CON[b]	FOS[c]	INU[d]		0	1		0	0	0	1	1	1		Fructan	XOS	Fructan × XOS
ATTD[f] of NSP[g] using TiO_2[h] (%)																	
Ara	55.47[a]	60.14[b]	59.14[b]	1.25	57.44	59.07	1.02	52.49	59.72	60.1	58.45	60.56	58.19	1.767	0.029	0.265	0.089
Fuc	79.17	82.98	72.13	4.146	81.81	74.37	3.385	82.29	86.27	76.88	76.05	79.68	67.37	5.864	0.184	0.127	0.954
Gal	82.81	83.93	82.71	1.127	83.25	83.04	0.92	82.51	83.58	83.67	83.1	84.28	81.76	1.594	0.699	0.872	0.653
GalA	90.91	91.2	91.52	0.801	90.65	91.78	0.647	88.89	91.3	91.77	92.93	91.11	91.28	1.094	0.856	0.22	0.082
Glu	45	48.44	50.09	1.816	46.4	49.28	1.482	40.35	47.17	51.68	49.65	49.7	48.5	2.568	0.143	0.176	0.063
Man	90.4	87.62	88.21	2.098	89.69	87.8	1.713	90.75	87.63	90.68	90.05	87.62	85.74	2.967	0.619	0.441	0.670
Rha	41.62	30.78	32.91	4.1	41.65[a]	28.56[b]	3.347	41.61	39.01	44.32	41.63	22.55	21.51	5.798	0.153	0.008	0.140
Xyl	59.62	63.53	63.81	1.314	60.29[a]	64.36[b]	1.073	55.96	61.53	63.37	63.28	65.54	64.25	1.859	0.052	0.01	0.235
Total NSP	60.14	63.28	63.11	1.214	61.26	63.09	0.991	57.07	62.55	64.15	63.21	64.01	62.06	1.717	0.134	0.198	0.067
Cellulose	18.08	26.53	26.04	2.752	20.76	26.34	2.247	9.68[a]	28.68[b]	23.92[b]	26.48[b]	24.39[b]	28.16[b]	3.893	0.062	0.086	0.038
ATTD of NSP using cellulose (%)																	
Ara	41.86	43.09	40.57	0.76	41.12	42.56	0.621	42.16[b]	39.40[b]	41.80[b]	41.56[b]	46.79[a]	39.35[b]	1.075	0.076	0.107	<.0001
Fuc	72.17	75.92	59.56	5.764	73.62	64.81	4.707	77.87	78.05	64.94	66.46	73.78	54.18	8.152	0.122	0.193	0.890
Gal	77.21	76.99	74.74	1.635	76.75	75.87	1.335	78.41	75.36	76.48	76	78.61	73	2.313	0.504	0.644	0.305
GalA	88.36	87.57	87.57	0.991	87.21	88.46	0.8	86.39	87.08	88.16	90.34	88.05	86.99	1.448	0.798	0.272	0.179
Glu	28.46[a]	26.40[b]	27.86[a]	0.273	26.02[a]	29.12[b]	0.223	27.44[c]	20.55[e]	30.09[b]	29.48[b]	32.26[a]	25.63[d]	0.386	<.0001	<.0001	<.0001
Man	87.78	82.22	83.42	2.794	85.13	83.82	2.281	88.68	81.08	85.62	86.88	83.36	81.21	3.951	0.343	0.687	0.697
Rha	22.47[a]	1.28[b]	2.80[b]	5.673	17.56[a]	0.15[b]	4.632	27.55	7.47	17.65	17.39	-4.91	-12.04	8.023	0.019	0.011	0.419
Xyl	47.5	47.8	47.6	0.593	45.19[a]	50.09[b]	0.485	46.50[b]	42.09[c]	46.97[b]	48.50[b]	53.52[a]	48.23[b]	0.839	0.934	<.0001	<.0001
Total NSP	48.04	47.55	46.52	0.505	46.45[a]	48.29[b]	0.412	47.70[bc]	43.64[d]	47.99[bc]	48.38[b]	51.46[a]	45.04[cd]	0.714	0.105	0.003	<.0001

[a] Data are means of 8 replicate samples per treatment. [b] CON; Control. [c] FOS; Fructo-oligosaccharides.[d] INU; Inulin. [e] XOS; Xylo-oligosaccharides, 0 = No XOS; 1= XOS. [f] ATTD; Apparent total tract digestibility. [g] NSP; Non-starch polysaccharides. [h] TiO_2; Titanium dioxide. [a-c] Means within a row that do not share a common superscript are significantly different.

5.5 Discussion

5.5.1 Marker concentration in diet, ileal digesta and faeces

Well-researched inert markers such as TiO_2 and Cr_2O_3 are routinely used in digestibility studies for monogastric animals, with the majority of studies focusing on the digestibility of costly nutrients such as energy and amino acids (Kong and Adeola, 2014; Jagger et al., 1992; Olukosi, O. et al., 2012). However, when using these markers for profiling NSP digestibility, previous studies have shown negative or unrealistically high coefficients (Bautil et al., 2019; de Vries, 2014). An ideal marker for digestibility studies has previously been described as being totally indigestible and unabsorbable, inactive within the GIT, to pass through the GIT at a uniform rate, be easily determined chemically and preferably be a substance that is intrinsically present in the feed (Jagger et al., 1992). The recovery of a marker in faecal matter relative to the proportion that is consumed indicates its efficacy in regard to its indigestible properties. However, this assessment requires a known quantity of feed to be ingested before total collection of excreta. This was not possible in the current study as the piglets were housed in groups of 5 and individual monitoring of feed intake and excreta collection was not possible. However, the efficacy of alternative markers can be estimated by comparing digestibility values against a marker with well-known recovery, such as TiO_2 (Jagger et al., 1992).

When comparing inert markers, calculating the ratio of one marker to another in the same GIT location indicates the relative differences in the transit time between markers. For example, if the ratio of TiO_2 : cellulose remains constant throughout GIT sections then the transit time of markers would be assumed to be similar, but if they differ, the transit time likely differs between the markers. For example, de Vries (2014) found a low Cr_2O_3 : co-EDTA ratio in the caeca of chickens demonstrating that limited amounts of Cr_2O_3

were detected in the caeca compared to co-EDTA which was detected abundantly, hence proving separation of the marker and digesta in the caecum. The same principal can be applied to the effect of diets, such that if the marker ratio differs between treatments, then it can be assumed that dietary treatment affects the transit time of the marker or digesta. For example, de Vries (2014) found that the Cr_2O_3 : co-EDTA ratios throughout the GIT were differently affected in birds fed a low-fibre diet compared to those fed a RSM diet, highlighting that dietary fibre content affected the flow and transit time of the marker or digesta. In the current study, the TiO_2 : cellulose ratios in the ileum were not affected by dietary treatment, suggesting that the transit times of the marker or digesta were similar between treatments in the ileum. However, the concentration of TiO_2 in the ileum tended to be affected by dietary treatment, whereby in the absence of XOS, addition of FOS and INU resulted in higher concentrations compared to the CON but were no different in the presence of XOS. As explained above, this trend cannot be explained by differences in the transit time of the marker given that the relative amounts of TiO_2 to cellulose were not different between treatments. There was also no correlation between ileal DM and ileal TiO_2 concentrations (data not shown). Hence, the reason for this trend is unknown but potentially could be due to separation and poor distribution of the marker in the ileal digesta samples meaning the analysed TiO_2 concentrations were not homogenously representative or precise. To this point, the SEM relative to the means for ileal TiO_2 concentration differed by 33.7 % between diets, whereas cellulose concentration only differed by 17.5 % between treatments. Hence, variation between diets was almost twice as high for the ileal TiO_2 concentrations compared to the cellulose concentrations, highlighting the trend for the treatment effect on the former could have been due to the precision of the TiO_2 analysis.

At the faecal level, variation between diets was lower than in the ileum and there was similar variation between diets for TiO_2 concentrations (13.2 %) and cellulose concentrations (11.4 %). The faeces are the final destination of any GIT matter, hence although separation in the ileum is likely, at the faecal level these issues would reach an equilibrium, such that the amount of undigested TiO_2 relative to cellulose is a true ratio between the markers. There was an interaction between XOS and fructans on the TiO_2 : cellulose ratio in the faeces, such that the CON diet had a lower ratio compared to all other treatments apart from FOS + XOS. This indicates that there was more TiO_2 relative to cellulose with prebiotic addition, hence suggests an increased degradation of cellulose with prebiotics. To this point, although there was an insignificant dietary effect on individual faecal marker concentrations, the prebiotic treatments tended to have numerically lower faecal cellulose concentrations compared to the CON group. One likely explanation for this result is that the addition of prebiotics to the diet altered the bacterial community composition whereby cellulose-degrading bacteria were stimulated which consequently increased cellulose fermentation.

5.5.2 AID and ATTD of NSPs using TiO_2 and cellulose as inert markers

Supporting hypothesis 1 and the literature, the results from this study showed highly negative AID of NSPs when using TiO_2 as an inert marker, indicating proportionately higher NSP levels in the ileum to the feed compared to their TiO2 content. A negative correlation existed between TiO_2 and cellulose for AID of total NSPs, whereby digestibility using TiO_2 decreased as digestibility using cellulose increased. Indeed, negative digestibility figures for soluble NSP fractions are plausible as the breakdown of insoluble fractions results in the accumulation of soluble fractions (Bautil et al., 2019). This accumulation therefore results in higher concentrations of soluble fractions in the

GIT relative to the diet, thus produces negative digestibility coefficients (Bautil et al., 2019). However, negative digestibility figures are implausible for total NSP as it encompasses both soluble and insoluble fractions which can only be hydrolysed through microbial fermentation and not formed like soluble fractions (Bautil et al., 2019). Hence, the negative NSP digestibility values observed in the current study can only be explained by fractionation of the TiO_2 and digesta in the GIT or as an effect of differing transit rates.

It has previously been noted that differences in transit times and the partitioning of nutrients into aqueous and solid phases in the digesta compared to the phase where the marker situates can complicate analysis of fibre digestibility (Choct et al., 1996; de Vries, 2014). Negative digestibility results have been found when using TiO_2 for total AX digestibility in broilers with the same conclusions drawn (Bautil et al., 2019). Calculating the AID of NSPs is naturally more complicated compared to other macronutrients as they are not simply digested by endogenous GIT processes and are therefore much more sensitive to marker transit issues. Taking starch as an example, if ileal starch recovery was 3 % and a marker transit issue caused a 50 % variation in recovery, then the SD would be 1.5 %, hence AID would be 97 % ± 1.5 %. However, ileal NSP recovery could be much higher at 90 %, meaning the same marker transit issue would cause 50 % variation in recovery such that AID would be 10 % ± 45 %, hence NSP digestibility would be much more affected by marker transit issues. Moreover, a sufficient adaptation feeding period is required before ileal digesta collection to ensure a constant marker concentration in the digesta - a period of 3 days has previously been suggested (Kim, B.G. et al., 2020). In the current study, the piglets received the TiO_2 throughout the 28-day trial period hence had a sufficient adaption period. It is clear that the AID results obtained for NSP

constituent sugars and total NSPs when using TiO_2 as a marker are not correct and that the separation of TiO_2 from the NSP fraction confounded the digestibility results.

There is a lack of information on cellulose as an intrinsic indigestible marker in swine, and to the author's knowledge there is no literature available comparing markers specifically for NSP digestibility. Supporting hypothesis 1, when using cellulose as an inert marker in the current study, the AID values were mainly positive as expected and much less variable compared to when using TiO_2. A positive AID of NSPs can be interpreted as a loss of NSP through fermentation or due to an impact on transit times of different NSP fractions. It is therefore important that markers of fermentation are measured alongside NSP digestibility data to aid explanations of the results. In the current study, fermentation markers correlated well with AID of NSPs (section 6.4.1) hence the positive AID results were explained by fermentation. Indeed, there was at least one treatment mean with negative AID for rha, ara, xyl and gal, suggesting there was still a degree of fractionation of cellulose and sugars in the ileum, but to a lesser extent than for TiO_2.

AID of fuc remained highly negative when both cellulose and TiO_2 were used as markers. Mucin polysaccharides are rich in fuc and can be used as a marker of endogenous losses via mucus secretion, hence highly negative values suggest increased mucus secretion and endogenous losses (Cadogan and Choct, 2015). However, the AID of fuc was not affected by dietary treatment. The lack of dietary treatment effect on cellulose concentration and TiO_2 : Cellulose ratio in the ileum indicates that the proportionate transit time of cellulose and TiO_2 were similar in the ileum, and cellulose concentration remained unaffected by diet, unlike TiO_2. Hence, taken together, the results from the current study indicate that

cellulose is a potentially more reliable inert marker for NSP digestibility analysis in the ileum compared to TiO_2.

At first view, the ATTD of NSPs were much closer when using TiO_2 and cellulose as inert markers compared to the AID results. The pattern of digestibility of each NSP constituent sugar was also encouragingly similar across the markers. However, the average ATTD for total NSP was 15.1 % lower when using cellulose as a marker compared to TiO_2, this was explained by the average ATTD of cellulose being 28.4 %. In a study using growing pigs (~29 kg), the ATTD of cellulose has been reported to be ~57 % which was higher than the current study at 28.4 %. However, this is not surprising as piglets in the current study were younger and would have had a relatively less developed microbiota to cope with cellulose degradation (Le Sciellour et al., 2018). If TiO_2 and cellulose were working in tandem, a positive correlation with an R^2 of 1 would have been expected and observed, yet there was no correlation between the markers, rejecting hypothesis 2. Moreover, there was a significant effect of dietary treatment on the ATTD of cellulose, indicating prebiotic addition increased the fermentation of cellulose. Taken together, this therefore renders the use of cellulose as an inert marker for ATTD of NSPs implausible. In summary, fractionation of TiO_2 and NSP occurred in the ileum, making cellulose a more promising inert marker for AID of fibre fractions. Whilst the fractionation and consequent mis-estimation of AID when using TiO_2 was rectified at the faecal level, but post-ileal cellulose degradation rendered the use of cellulose as an inert marker infeasible.

5.5.3 Conclusion and future work

This study, along with others (de Vries, 2014; Jamroz et al., 2002; Brenes et al., 2003; Bautil et al., 2019), has highlighted the care that needs to be taken when inert markers are used to estimate the digestibility of NSPs. Highly negative AID values for NSPs were found when using TiO_2 as an inert marker, indicating separation of the marker and NSP fraction within the GIT. In contrast, using cellulose as an inert marker yielded more realistic, less negative and less variable AID of NSP. Moreover, there was a negative correlation between AID of total NSP when using TiO_2 as a marker relative to cellulose. At the faecal level, positive and more similar ATTD of NSPs were observed between the markers but no positive correlation was seen. Post-ileal cellulose degradation caused the average ATTD for total NSPs to be 15.1 % lower compared to when using TiO_2 as a marker, and cellulose fermentation was also affected by dietary treatment. Overall, this study has shown that TiO_2 is an unsuitable inert marker for AID of NSPs, whilst cellulose showed more realistic initial results as an alternative marker, but further validation work is required. On the contrary, the fractionation and consequent mis-estimation of AID when using TiO_2 was rectified at the faecal level, but post-ileal cellulose degradation rendered the use of cellulose as an inert marker for ATTD infeasible.

Future work into inert markers like cellulose is timely as the safety of TiO_2 has recently been questioned for its use as a food or feed additive due to concerns over its accumulation in the body and potential genotoxicity effect (EFSA, 2021). To the authors knowledge, there is no literature available on the use of intrinsic cellulose as markers for NSP digestibility studies, with the current study showing encouraging initial results at the ileal level. Albeit, further work is required to investigate the suitability, reliability and accuracy of cellulose or other intrinsic indigestible dietary components as markers for NSP

digestibility analyses. Key to this future work is the comparison and validation of well-known markers like Cr_2O_3 and potential alternative intrinsic markers like AIA or lignin against the gold standard method of total collection for different fibre fractions. Comparative total collection and marker studies should ensure that digestibility is compared along all GIT sections and in combination with digesta flow rates due to their complex interaction.

Chapter 6

Investigating the effect of xylo-oligosaccharides with or without fructans on gut bacterial fermentation activity and non-starch polysaccharide digestibility in weaned piglets.

6.1 Abstract

Supplementation of prebiotics can positively influence the gut bacterial community composition, increase fermentative activity and fibre degradation, consequently improving the health of the host and nutritive value of feedstuffs. This study aimed to investigate the effect of XOS and fructans (FOS and INU) on piglet gut pH, lactate concentration and NSP digestibility. A total of 474 weaner piglets (7.88 ± 1.51 kg) were blocked into mixed sex pens of 4–5 piglets and assigned to 1 of 6 dietary treatments in a 2 × 3 factorial treatment design. Diets contained 2 levels of XOS (0 and 0.286 g/kg) and 3 levels of fructans [(0 g/kg) and 6.50 g/kg of short-chain FOS and 4.08 g/kg of INU to supply 3.8 g/kg of pure fructans)]. At the end of the experiment, 48 piglets were euthanised for the collection of ileal and colonic digesta to analyse the pH and lactate concentration as indicators of fermentative activity. AID and ATTD of NSPs were also analysed. Compared to the CON group, XOS reduced ileal digesta pH and increased ileal lactate concentration ($P < 0.001$), indicating increased bacterial fermentative activity. The AID of NSP ara, glu, xyl and total NSP was higher for the CON and/or FOS groups with XOS compared to without, indicating XOS stimulated an increase in NSP constituent sugar digestibility in the ileum ($P < 0.050$). However, the effects of XOS on fermentative activity and AID of NSPs were lost when combined with INU, indicating incompatibility of the products. Compared to the CON, both XOS and INU increased the AID of stachyose ($P < 0.047$). All prebiotics and their combinations increased the ATTD of cellulose compared to the CON ($P < 0.038$), whilst similar interaction trends were seen for NSP ara ($P = 0.089$), glu ($P = 0.063$) and total NSP ($P < 0.067$). Dietary treatment

also affected the selective fermentation of remaining cellulose and NSP fractions at the faecal level. In summary, XOS increased ileal fermentative activity and the AID of NSP fractions, suggesting XOS has a stimulatory effect on increasing the fibre-degrading capacity of the ileal bacteria. Both XOS and fructans when offered separately, as well as in combination, increased the ATTD of cellulose and NSP fractions, indicating beneficial effects on fibre digestibility with oligomer supplementation.

6.2 Introduction

Alongside increased pressure to reduce antimicrobial usage, the UK pig industry is facing an imminent ban on therapeutic levels of ZnO. As such, seeking suitable alternatives that promote health and prevent disease is of utmost importance for the industry. Prebiotics are defined as 'non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the gut, thus improving host health' (Gibson and Roberfroid, 1995). The majority of prebiotics are differing lengths of carbohydrate chains that are non-digestible by endogenous GIT enzymes, hence survive host digestion and are instead fermented by resident bacteria in the GIT (Cummings et al., 2001). The fermentability characteristics of different prebiotics depends on their monomeric unit composition, linkage type, DP and bacterial communities residing in the GIT (Sako et al., 1999). As previously discussed in section 4.2, substrates with a longer DP are generally fermented at a slower rate than those with shorter a DP due to the number of linkages that need to be broken. Yet differences still exist between prebiotics with a similar DP such that short-chain XOS is fermented at a slower rate than short-chain GOS (Smiricky-Tjardes et al., 2003a). Well-researched prebiotics like fructans have received much attention in both human and animal nutrition, but new-generation prebiotics like XOS are of increasing interest. Fructan supplementation, in the form of FOS (Xu, C. et al., 2005; Xu, Z.R. et al., 2002) and INU (Grela et al., 2014; He et al., 2002) have been shown to improve pig performance and gut health parameters. Similarly, XOS has shown promising improvements in growth performance, gut structure, and gut bacterial community composition in broilers and weaner pigs (Liu, J. et al., 2018; Ribeiro et al., 2018). Both XOS and fructans have previously been shown to selectively increase the abundance of beneficial bacteria like *Lactobacillus* whilst decrease pathogenic bacteria like *Escherichia coli*, hence have a bacterial community modulating effect (Liu, J. et al., 2018; Ribeiro et al., 2018; Xu, Z.R.

et al., 2002). The end-products of fermentation are SCFAs such as acetate, propionate, butyrate and lactate which act as an energy source for the host or cross-feeding substrate for other resident bacteria. The presence of SCFA in the GIT reduces the pH of the gut environment which prevents the growth of pH sensitive pathogenic bacteria. Hence, an increase in the concentration of SCFAs and a reduction in gut pH is an indicator of increased fermentative activity.

Compared to other well-studied prebiotics like FOS and INU, relatively low levels of XOS (0.2 g/kg and 1.0 g/kg) or AXOS (2.5 g/kg) have been shown to stimulate the abundance of beneficial bacteria (Ribeiro et al., 2018; Liu, J. et al., 2018; Courtin et al., 2008). Courtin et al. (2008) showed that FOS failed to increase caecal levels of *Bifidobacterium* in broilers at inclusion rates of 10 g/kg, yet AXOS did so at levels four times lower (2.5 g/kg), highlighting the higher prebiotic potential of AXOS despite its low inclusion rate. However, the fermentation of these substrates cannot solely explain the performance results observed. For example, supplementation of XOS at 0.10 g/kg has previously been shown to improve broiler growth performance by +320g from day 0-42. However, even if the substrate was converted to SCFAs with 100 % efficiency, it would only equate to < 0.3 Kcal/kg of energy provision which is not enough to explain the growth response reported. The classical definition of a prebiotic describing the quantitative fermentation of a substrate eliciting SCFAs as a sole mode of action is therefore questionable for XOS (Bedford, Michael R, 2018; Bautil et al., 2020).

An alternative hypothesis for the mechanism of XOS has been proposed, whereby XOS acts as a signal to encourage fibre-degrading bacteria to increase in activity, ferment fibre more efficiently and interact with the GIT in such a way that improves overall efficiency

of digestion and growth (Ribeiro et al., 2018). This mechanism has recently been investigated in broilers, whereby the supplementation of AXOS was shown to increase the ATTD of wheat AX in young birds (Bautil et al., 2020). The author concluded that there was a 'kick-start' effect of AXOS on the development of the fibre-degrading bacterial community and consequent increased AX digestibility. To this point, the term 'stimbiotic' has recently been given to 'non-digestible but fermentable additives that stimulate fibre fermentability but at a dose that is too low that the stimbiotic itself could contribute in a meaningful manner to SCFA production' (González-Ortiz et al., 2019). Ultimately, this stimulatory effect could improve the nutritive value and energy utilisation of fibrous feed ingredients via their increased fermentation, which could potentially lead to increased growth performance and health benefits (Bautil et al., 2020; Morgan, N. et al., 2021).

The majority of work on this new concept has been conducted in broilers, with little focus on pigs. However, one piglet study has shown that provision of a stimbiotic (XYL + XOS) altered the intestinal bacterial community composition to favour fibre fermentation which likely contributed to improved performance, particularly in poor sanitary conditions (Cho et al., 2020). However, to the author's knowledge, there has been no investigation into the stimulatory effect of XOS specifically on fibre fraction digestibility in pigs. Furthermore, different prebiotics selectively stimulate different bacterial species, hence upregulate a diverse range of bacterial metabolic activities and functions within the GIT. It is unknown whether feeding a combination of substrates with differing DP and monomer composition would increase fermentative activity and NSP digestibility over and above feeding them alone. This study aimed to investigate the effect of XOS and fructans (FOS and INU) on piglet gut pH, lactate concentration and NSP digestibility.

6.2.1 Study aims

There is a lack of information in the literature on the effect of feeding different prebiotics alone or in combination on gut bacterial fermentative activity and NSP digestibility. The aim of this study was to determine the effect of XOS with or without fructans (FOS and INU) on gut bacterial fermentation activity and NSP digestibility to decipher if they have a stimulatory effect as described in the literature for broilers.

6.2.2 Hypotheses

1. Due to differing DP, supplementation of FOS will have a greater fermentative activity (higher lactate concentration and lower pH) in the ileal digesta of piglets, whereas XOS and INU will have a greater fermentative activity in the colonic digesta.
2. Combining FOS and XOS together will increase fermentative activity in the ileal and colonic digesta of piglets, whilst addition of XOS and INU together will increase fermentative activity in the colonic digesta.
3. Despite its low inclusion, feeding XOS alone or in combination with FOS and INU will increase the digestibility of NSP fractions compared to treatments without XOS.

6.3 Materials and methods

Study protocols were approved by the University of Leeds Pig Research Centre and ethical approval was granted by the Animal Welfare and Ethical Review Body as described in Appendix A.1.

6.3.1 Animals, housing, experimental design and dietary treatments

The animals, housing, experimental design and dietary treatments used in this study were as described in section 4.3.1 and 4.3.2.

6.3.2 Measurements and sampling

6.3.2.1 Digesta pH and lactate

At the end of the experiment, 48 piglets were euthanised for the collection of ileal and colonic digesta as described in section 4.3.3.2. Once collected, the pH was measured immediately using a handheld electrode (Extech SDL100), rinsed in distilled water and re-measured for duplicate readings. The electrode was calibrated using pH 4 buffer.

From previous testing and review of the literature, it was identified that the lactate concentration of ileal digesta was higher than that for colonic digesta, hence ileal digesta was diluted to ensure the readings were within the measuring device range, whilst the colonic digesta remained undiluted.

- Ileal digesta: 0.3 g of ileal digesta was weighed into a 2 ml Eppendorf and 1.2 ml of distilled water was added. The sample was homogenised by vortexing, followed by centrifugation at 14,000 × g for 2 minutes. The supernatant was collected in a 2 ml Eppendorf and the lactate concentration was measured in duplicate using a handheld lactate device (Lactate Scout, EKF Diagnostics, UK). As described and pictured in section 3.3.3.2, to take a reading, a sensor was inserted into the handheld device and then placed into the media. This was repeated with a new sensor to take duplicate readings.

- Colonic digesta: 1 g of colonic digesta was weighed into a 2 ml Eppendorf. The sample was homogenised by vortexing, followed by centrifugation at 14,000 × g for 2 minutes. The supernatant was collected in a 2 ml Eppendorf and the lactate concentration was measured in duplicate using the same method as described for the ileal digesta.

Lactate concentrations were corrected for any sample dilution, for the constant automatic haematocrit adjustment made by the handheld device (0.2 – as described in section 3.3.3.2) and were back calculated to mM per g of digesta. Duplicate readings were averaged.

6.3.2.2 DM determination

Ileal digesta was collected as described in section 4.3.3.2, whilst a faecal sample was collected in a 30 ml universal container from each selected piglet in the holding pen before euthanasia and stored at -80 °C pending analysis. Ileal and faecal samples were weighed, freeze dried (as per section 4.3.3.2), and then re-weighed to determine DM. The samples were then ground and homogenised using a pestle and mortar.

6.3.2.3 NSP content

6.3.2.3.1 Total NSP, NSP constituent sugars and cellulose content

Total NSP, NSP constituent sugars and cellulose content of the feed, ileal digesta and faeces were determined as per section 5.3.2.2.1.

6.3.2.3.2 Other carbohydrates: fructan, starch and alpha-galactoside content

Other carbohydrates present in the feed and ileal digesta were determined by a combined approach applying enzymatic hydrolysis and high pressure ion chromatography (HPIC). The initial step involved a heat dispersion of the sample in phosphate buffer pH 7.0 and an internal standard. An aliquot was taken for direct determination by HPIC of sugars (gal, glu, fructose and sucrose) and alpha-galactosides (raffinose and stachyose). A second aliquot was treated with fructanase and alpha-glucosidase for the hydrolysis of fructans and starch respectively, with the released fructose and glu quantified by HPIC. The following calculations (Equation 6.1 and Equation 6.2) were applied to correct for other sources of glu and fructose. The glu component of the fructans was not taken into consideration for this application where the emphasis was on changes observed in the ileal samples compared to the diets.

$$Fructans = Fructose\ (enzyme\ treatment) - (sugar\ fructose) - (0.525\ \times sucrose) - (0.333\ \times raffinose) - (0.25\ \times stachyose)$$ **Equation 6.1**

$$Starch = glucose\ (enzyme\ treatment) - (sugar\ fructose) - (0.525\ \times sucrose)$$ **Equation 6.2**

6.3.2.4 AID and ATTD of DM and NSPs

As explained in Chapter 5, the AID of DM and NSPs were calculated using cellulose as an inert marker, whilst the ATTD of DM and NSPs were calculated using TiO_2 as an inert marker. AID and ATTD were calculated using Equation 5.3 and Equation 5.4 respectively, as detailed in section 5.3.2.3.

6.3.3 Statistical analysis

The individual piglet served as the experimental unit for all data in this chapter. Lactate concentrations were calculated back to mM per g of digesta before analysis. Normality of data and homogeneity of variance were assessed as per section 2.3.5.1. The statistical package JMP® (Version *15.1*. SAS Institute Inc., Cary, NC, 1989 - 2019) (SAS, 2020) was used to analyse the digesta pH, digesta lactate concentrations, AID and ATTD of DM and NSP data as a 2-way ANOVA. The statistical model included the fixed effects of XOS, fructans and their interaction. Significant differences were classed as $P < 0.05$ and trends as $P < 0.10$.

6.4 Results

6.4.1 Digesta pH and lactate concentration

The effect of XOS and fructans on ileal and colonic digesta pH and lactate concentrations are shown in Table 6.1 and Figure 6.1. There was a significant interaction between XOS and fructans on ileal pH ($P < 0.001$) and lactate concentration ($P < 0.001$) whereby there was no difference in the FOS or INU group with or without XOS, but in the CON group pH was significantly higher and lactate was significantly lower without XOS than with XOS (Table 6.1 and Figure 6.1). The interaction pattern for pH and lactate concentration between treatments was the same but opposite, such that lactate concentration was highest when pH was lowest, and lactate concentration was lowest when pH was highest. There was no effect of treatment on colonic pH or colonic lactate concentration ($P > 0.05$).

Table 6.1: Effect of XOS with or without short-chain (FOS) and long-chain (INU) fructans on ileal and colonic pH and lactate concentration.

	Fructan			SEM	XOS		SEM	CON	FOS	INU	CON	FOS	INU	SEM	*P* value		
	CON	FOS	INU		0	1		0	0	0	1	1	1		Fructan	XOS	Fructan × XOS
pH																	
Ileum	6.94	6.93	6.95	0.050	7.00	6.88	0.040	7.12[a]	7.02[ab]	6.85[ab]	6.75[b]	6.84[ab]	7.06[a]	0.070	0.944	0.054	0.001
Colon	5.77	5.70	5.65	0.059	5.67	5.74	0.048	5.71	5.68	5.61	5.83	5.71	5.68	0.083	0.350	0.275	0.848
Lactate concentration (mM per g of digesta)																	
Ileum	51.82	33.59	36.04	8.510	32.49	48.48	6.948	19.53[b]	22.93[b]	55.02[ab]	84.12[a]	44.26[ab]	17.05[b]	12.035	0.272	0.113	0.001
Colon	0.75	0.85	0.84	0.048	0.85	0.78	0.039	0.77	0.94	0.83	0.73	0.76	0.85	0.068	0.281	0.243	0.325

[a] CON; Control. [b] FOS; Fructo-oligosaccharides. [c] INU: Inulin. [d] XOS; Xylo-oligosaccharides.

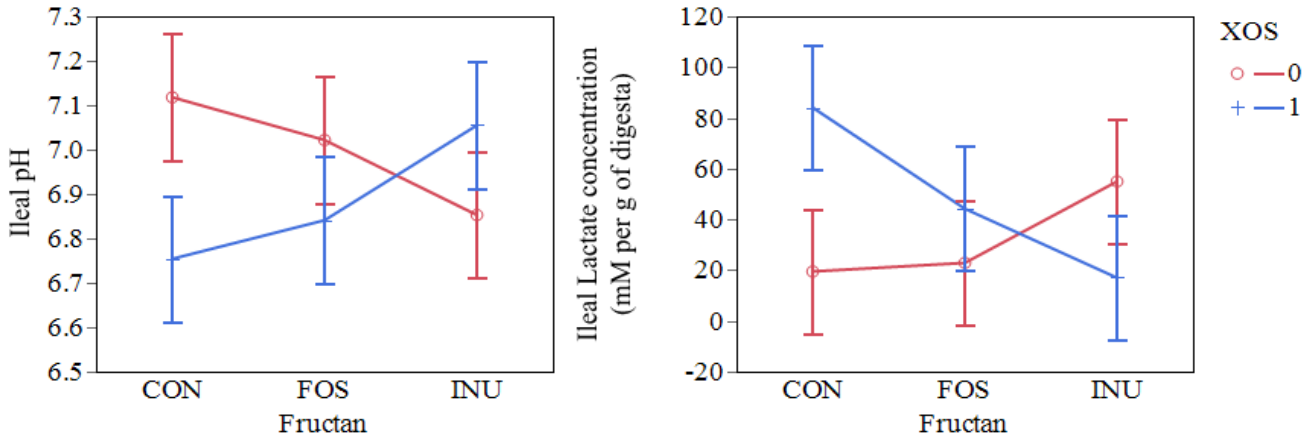

Figure 6.1: Effect of xylo-oligosaccharides (XOS) with or without short-chain (Fructo-oligosaccharides (FOS)) and long-chain (Inulin (INU)) fructans on ileal digesta pH and lactate concentration ($P < 0.05$).

6.4.2 Ileal and faecal NSP and other carbohydrate content

The main NSP constituent sugars available for fermentation in the ileal digesta were ara, glu and xyl (Table 6.2); the same was true in the faeces (Table 6.3). Although the lowest NSP constituent sugar, ileal rha content were similar in the CON and FOS groups with and without XOS, values tended to be higher without XOS and lower with XOS in the INU group ($P = 0.079$; Table 6.2). The main other carbohydrate available for fermentation in the ileal digesta was starch (Table 6.2). There was a significant interaction between XOS and fructans on total alpha-galactoside ($P < 0.028$) and stachyose content ($P < 0.022$) in the ileal digesta, whereby there was no difference in the FOS and INU groups with or without XOS, but content was higher in the CON without XOS than with XOS (Table 6.2). There was a trend for the same interaction pattern for alpha-galactoside raffinose content ($P = 0.084$) and total fructans ($P = 0.084$).

Table 6.2: Effect of XOS and fructans (FOS and INU) on ileal NSP constituent sugars, total NSP and other carbohydrate content (g/100g DM)[a].

	Fructan			SEM	XOS[e]		SEM	CON	FOS	INU	CON	FOS	INU	SEM	*P* value		
	CON[b]	FOS[c]	INU[d]		0	1		0	0	0	1	1	1		Fructan	XOS	Fructan × XOS
Ileal content (g/100g DM)																	
Ara	6.74	6.85	6.54	0.286	6.69	6.73	0.231	6.48	6.88	6.72	7.00	6.82	6.36	0.417	0.725	0.918	0.538
Fuc	0.37	0.38	0.42	0.020	0.38	0.40	0.016	0.34	0.36	0.44	0.40	0.39	0.40	0.028	0.130	0.552	0.186
Gal	4.07	4.18	4.10	0.175	4.04	4.19	0.141	3.81	4.14	4.18	4.33	4.23	4.01	0.255	0.889	0.460	0.366
GalA	1.43	1.44	1.40	0.080	1.42	1.42	0.065	1.32	1.48	1.45	1.53	1.40	1.34	0.117	0.911	0.972	0.302
Glu	9.16	9.07	8.43	0.492	8.90	8.86	0.398	8.78	9.26	8.67	9.53	8.88	8.18	0.719	0.506	0.944	0.610
Man	0.88	0.89	0.90	0.032	0.89	0.89	0.026	0.87	0.89	0.91	0.88	0.89	0.88	0.047	0.901	0.937	0.900
Rha	0.33	0.33	0.31	0.017	0.32	0.32	0.014	0.30	0.33	0.34	0.35	0.34	0.28	0.025	0.707	0.910	0.079
Xyl	9.86	9.92	9.18	0.490	9.75	9.55	0.396	9.59	10.27	9.41	10.13	9.58	8.95	0.716	0.482	0.720	0.636
Total NSP[f]	33.02	33.25	31.59	1.417	32.65	32.59	1.144	31.69	33.82	32.43	34.35	32.69	30.75	2.069	0.652	0.974	0.498
Other carbohydrate content (g/100 DM)																	
Alpha-galactosides – raffinose	0.37	0.31	0.29	0.104	0.40	0.25	0.085	0.64	0.31	0.25	0.10	0.32	0.33	0.147	0.844	0.219	0.084
Alpha-galactosides – stachyose	1.18	0.69	0.62	0.321	1.09	0.57	0.262	2.18^{a}	0.68^{ab}	0.40^{ab}	0.17^{b}	0.69^{ab}	0.84^{ab}	0.454	0.417	0.166	0.022
Alpha-galactosides - Total	1.55	1.00	0.91	0.418	1.49	0.82	0.341	2.82^{a}	0.99^{ab}	0.64^{ab}	0.27^{b}	1.01^{ab}	1.17^{ab}	0.591	0.509	0.171	0.028
Total fructans	1.92	1.87	2.07	0.354	2.11	1.79	0.289	2.72	1.87	1.76	1.11	1.87	2.38	0.501	0.913	0.428	0.084
Starch	4.30	5.15	4.06	0.600	4.55	4.46	0.490	3.93	5.65	4.07	4.67	4.66	4.04	0.848	0.405	0.898	0.599
Total carbohydrates excl NSP	9.58	10.52	9.38	0.790	10.37	9.28	0.645	11.23	10.96	8.91	7.93	10.08	9.84	1.117	0.555	0.242	0.177

[a] Data are means of 8 replicate samples per treatment. [b]CON; Control. [c]FOS; Fructo-oligosaccharides.[d] INU; Inulin. [e] XOS; Xylo-oligosaccharides, 0 = No XOS; 1= XOS. [f] NSP; Non-starch polysaccharide. [a-c] Means within a row that do not share a common superscript are significantly different ($P < 0.05$).

Table 6.3: Effect of XOS and fructans (FOS and INU) on faecal NSP constituent sugars and total NSP content (g/100g DM)[a].

	Fructan			SEM	XOS[e]		SEM	CON	FOS	INU	CON	FOS	INU	SEM	*P* value		
	CON[b]	FOS[c]	INU[d]		0	1		0	0	0	1	1	1		Fructan	XOS	Fructan × XOS
Faecal content (g/100g DM)																	
Ara	5.49	5.09	5.31	0.141	5.32	5.27	0.115	5.56	5.01	5.38	5.43	5.16	5.23	0.200	0.135	0.792	0.699
Fuc	0.10	0.08	0.13	0.019	0.09	0.12	0.016	0.09	0.07	0.12	0.12	0.09	0.14	0.027	0.206	0.288	0.946
Gal	1.41	1.40	1.47	0.089	1.42	1.43	0.072	1.36	1.43	1.48	1.47	1.36	1.47	0.125	0.817	0.942	0.774
GalA	0.34	0.35	0.33	0.030	0.36	0.32	0.024	0.41	0.34	0.33	0.27	0.35	0.33	0.044	0.916	0.210	0.145
Glu	9.92	9.51	9.48	0.239	9.83	9.44	0.195	10.36	9.49	9.64	9.47	9.52	9.32	0.339	0.362	0.163	0.403
Man	0.21	0.25	0.21	0.038	0.23	0.22	0.031	0.19	0.26	0.23	0.23	0.24	0.20	0.054	0.721	0.896	0.789
Rha	0.43	0.46	0.45	0.020	0.45	0.45	0.016	0.44	0.46	0.44	0.43	0.46	0.46	0.028	0.673	0.986	0.842
Xyl	7.18	6.67	6.94	0.206	7.02	6.84	0.168	7.43	6.52	7.11	6.94	6.82	6.78	0.292	0.224	0.466	0.373
Total NSP[f]	25.31	24.00	24.57	0.580	24.91	24.34	0.474	26.03	23.77	24.94	24.59	24.24	24.20	0.821	0.289	0.396	0.505

[a] Data are means of 8 replicate samples per treatment. [b]CON; Control. [c]FOS; Fructo-oligosaccharides.[d] INU; Inulin. [e] XOS; Xylo-oligosaccharides, 0 = No XOS; 1= XOS. [f] NSP; Non-starch polysaccharide. [a-c] Means within a row that do not share a common superscript are significantly different ($P < 0.05$).

6.4.3 AID and ATTD of DM

There was a trend for an interaction between XOS and fructans on AID of DM; AID tended to be higher for the CON group with XOS compared to without, whereas for the INU group, AID tended to be higher without XOS compared to with XOS (Figure 6.2; *P* = 0.100). This similar interaction trend was seen for fermentative activity (pH and lactate concentration; Figure 6.1). There was no effect of treatment on ATTD of DM (*P* > 0.05).

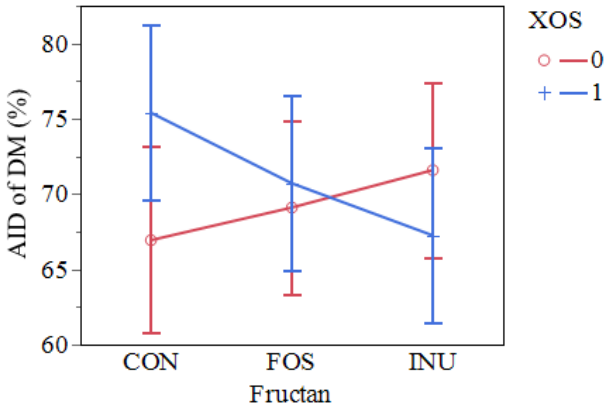

Figure 6.2 Effect of xylo-oligosaccharides (XOS) and fructans (Fructo-oligosaccharides (FOS)) and (Inulin (INU)) on apparent ileal digestibility (AID) of dry matter (DM) (*P* = 0.100).

6.4.4 AID of NSP

There was a significant interaction between XOS and fructans on the AID of NSP ara (*P* < 0.002), glu (*P* < 0.001), xyl (*P* < 0.001) and total NSP (*P* < 0.001;Table 6.4). The interaction pattern was similar for the digestibility of the above-mentioned NSP constituent sugars and total NSP as seen in Figure 6.3 A-D. Taking all the interaction patterns together, AID tended to be higher for the CON and FOS groups with XOS compared to without, whereas for the INU group, AID tended to be higher without XOS compared to with XOS. There was no difference in the AID of NSP ara and total NSP in

the CON and INU groups with or without XOS, but AID was significantly higher in the FOS group with XOS compared to without XOS. Similarly, there was no difference in the AID of NSP xyl in the INU group with or without XOS, but AID was significantly higher in the CON and FOS group with XOS compared to without XOS. For NSP glu, AID in the CON and FOS groups was significantly higher with XOS compared to without XOS, whilst in the INU group, glu AID was significant lower with XOS compared to without XOS. There was a significant interaction between XOS and fructans on AID of NSP man; there was no difference in the CON and FOS group with or without XOS, whereas in the INU group AID was higher without XOS compared to with XOS (Table 6.4E; $P < 0.001$).

There was a significant interaction between XOS and fructans on AID of the alpha-galactoside stachyose; XOS and INU independently increased the AID of stachyose compared to the CON, but reduced the AID when combined to levels not dissimilar from the CON (Table 6.4; Figure 6.3F; $P < 0.047$). Similar interaction trends were seen for the AID of alpha-galactoside raff ($P = 0.079$), total alpha galactosides ($P = 0.052$), fructans ($P = 0.055$), and total carbohydrates excluding NSP ($P = 0.070$).

Table 6.4: Effect of XOS and fructans (FOS and INU) on AID of NSP constituent sugar, total NSP and other carbohydrates [a].

	Fructan			SEM	XOS[e]		SEM	CON	FOS	INU	CON	FOS	INU	SEM	*P* value		
	CON[b]	FOS[c]	INU[d]		0	1		0	0	0	1	1	1		Fructan	XOS	Fructan × XOS
AID[f] of NSP[g] (%)																	
Arabinose	5.74	0.63	0.46	2.407	-0.81[a]	5.36[b]	1.943	-0.54[abc]	-6.48[c]	4.6[abc]	12.02[a]	7.75[ab]	-3.68[bc]	3.516	0.217	0.029	0.002
Fucose	-34.75	-46.97	-67.54	12.900	-45.26	-54.25	10.415	-37.61	-39.03	-59.13	-31.90	-54.90	-75.95	18.842	0.192	0.540	0.780
Galactose	14.52	9.71	3.95	4.235	9.89	8.89	3.419	10.75	7.49	11.43	18.28	11.94	-3.54	6.185	0.210	0.836	0.126
Galacturonic acid	34.38	32.42	28.23	2.820	30.21	33.15	2.277	33.59	27.94	29.09	35.17	36.90	27.38	4.119	0.285	0.361	0.375
Glucose	13.43[ab]	10.24[b]	13.93[a]	0.949	9.78[a]	15.28[b]	0.766	9.12[b]	2.22[c]	17.99[a]	17.74[a]	18.25[a]	9.86[b]	1.385	0.014	<.0001	<.0001
Mannose	28.86[a]	17.03[ab]	5.43[b]	5.202	21.42	12.79	4.200	20.24[a]	16.24[a]	27.79[a]	37.47[a]	17.82[a]	-16.94[b]	7.598	0.009	0.149	0.000
Rhamnose	24.42[a]	7.94[b]	12.98[ab]	3.570	20.77[a]	9.46[b]	2.882	28.34	17.03	16.94	20.51	-1.15	9.02	5.214	0.006	0.008	0.485
Xylose	4.74	-0.58	6.19	2.378	-3.63[a]	10.54[b]	1.920	-3.18[bc]	-15.65[c]	7.93[ab]	12.67[a]	14.50[a]	4.44[ab]	3.473	0.104	<.0001	<.0001
Total NSP	10.56	5.85	6.82	1.997	4.60[a]	10.89[b]	1.612	5.00 [abc]	-2.48[c]	11.26[ab]	16.12[a]	14.19[a]	2.37[bc]	2.917	0.217	0.008	<.0001
AID of other carbohydrates (%)																	
Alpha-galactosides – raffinose	65.50	71.94	73.08	11.707	63.74	76.60	9.452	37.90	71.10	82.23	93.09	72.77	63.93	17.099	0.883	0.336	0.079
Alpha-galactosides - stachyose	70.43	81.21	81.49	10.352	72.00	83.42	8.358	44.28[b]	80.24[ab]	91.49[a]	96.58[a]	82.18[ab]	71.49[ab]	15.120	0.687	0.334	0.047
Alpha-galactosides - Total	69.30	79.05	79.56	10.594	70.11	81.83	8.553	42.84	78.12	89.37	95.77	79.97	69.75	15.473	0.739	0.333	0.052
Total Fructans	36.86	40.87	46.24	13.652	35.15	47.49	11.023	4.68	40.03	60.75	69.04	41.71	31.73	19.940	0.884	0.428	0.055
Starch	96.77	96.02	96.91	0.407	96.36	96.77	0.329	96.51	95.57	97.00	97.03	96.48	96.82	0.595	0.246	0.371	0.618
Total carbohydrates excl NSP	93.49	92.32	93.38	0.821	92.54	93.59	0.663	91.57	91.85	94.19	95.40	92.80	92.58	1.199	0.527	0.261	0.070

[a] Data are means of 8 replicate samples per treatment. [b]CON; Control. [c]FOS; Fructo-oligosaccharides.[d] INU; Inulin. [e] XOS; Xylo-oligosaccharides, 0 = No XOS; 1= XOS. [f] AID; Apparent ileal digestibility. [g] NSP; Non-starch polysaccharide. [a-c] Means within a row that do not share a common superscript are significantly different ($P < 0.05$).

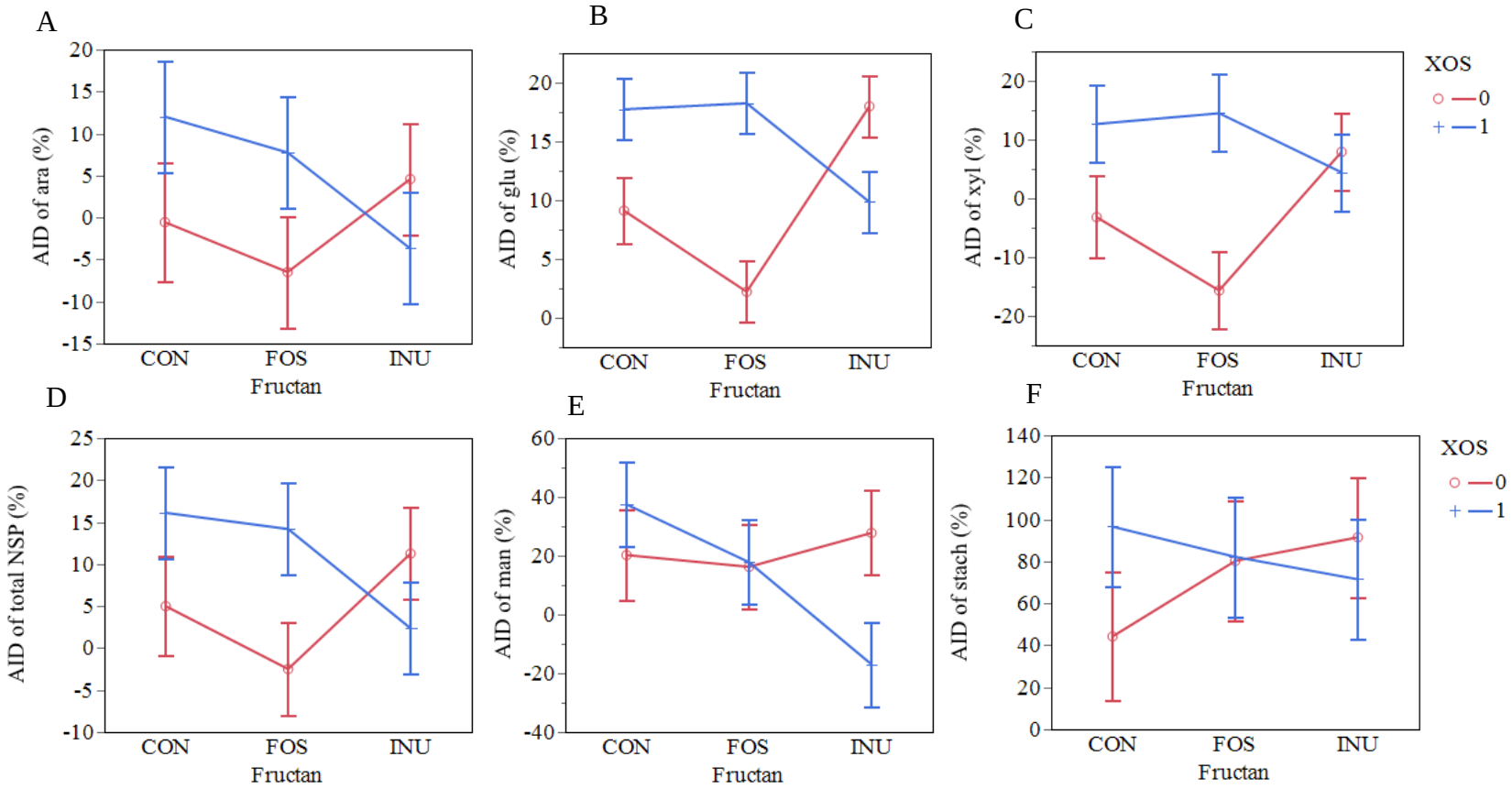

Figure 6.3: Effect of xylo-oligosaccharides (XOS) and fructans (Fructo-oligosaccharides (FOS)) and (Inulin (INU)) on apparent ileal digestibility (AID) of NSP arabinose (ara; A), glucose (glu; B), xylose (xyl; C), total non-starch polysaccharide (NSP) (D), mannose (man; E) and stachyose (stach; F) ($P < 0.05$).

6.4.5 ATTD of NSP

As seen in Table 6.5 and Figure 6.4D, there was an interaction between XOS and fructans on ATTD of cellulose; the digestibility was lower in the CON group compared to all other dietary treatments ($P < 0.038$). There was a similar interaction trend for NSP ara ($P = 0.089$; Figure 6.4A), glu ($P = 0.063$; Figure 6.4B) and total NSP ($P = 0.067$; Figure 6.4C), whereby ATTD tended to be higher for all prebiotic dietary treatments compared to the CON. There was a trend for an interaction between XOS and fructans on NSP Gal A, whereby there was no difference in the FOS and INU group with or without XOS, but ATTD was higher with XOS compared to without XOS in the CON group (Figure 6.4E; $P = 0.082$). There was a trend for a main effect of fructans (Figure 6.4F; $P = 0.052$) on NSP xyl whereby ATTD was higher with FOS and INU compared to the CON. There was a significant effect of XOS (Figure 6.4G; $P < 0.010$), whereby the ATTD of xyl was increased with XOS compared to without. The ATTD of NSP rha was lower with XOS compared to without ($P < 0.008$; Figure 6.4H).

6.4.6 Faecal NSP constituent sugar ratios

Faecal ara : cell ratio was higher in the INU group compared to the FOS group, but no different from the CON ($P < 0.029$), whilst the ratio was higher with XOS compared to without XOS ($P < 0.032$; Table 6.6). Faecal ara : glu ratio was higher in the CON and INU groups compared to the FOS group ($P < 0.035$; Table 6.6), whilst the ratio tended to be higher with XOS compared to without XOS ($P = 0.055$). Faecal xyl : cell ratio ($P < 0.017$) and xyl : glu ($P < 0.029$) was higher in the INU group compared to the FOS group, but no different from the CON (Table 6.6). A positive correlation was found between ATTD of cellulose and ara : cell content ($P < 0.001$), ara : glu content ($P < 0.003$), glu : cell content ($P < 0.003$) and ara : xyl content ($P < 0.001$; Figure 6.5 A-C & E), whilst there was a trend for a correlation for xyl : cell content ($P < 0.060$; Figure 6.5D).

Table 6.5: Effect of XOS and fructans (FOS and INU) on ATTD of NSP constituent sugars and total NSP [a].

	Fructan			SEM	XOS[e]		SEM	CON	FOS	INU	CON	FOS	INU	SEM	*P* value		
	CON[b]	FOS[c]	INU[d]		0	1		0	0	0	1	1	1		Fructan	XOS	Fructan × XOS
ATTD[f] of NSP[g] (%)																	
Arabinose	55.47[a]	60.14[b]	59.14[b]	1.250	57.44	59.07	1.020	52.49	59.72	60.10	58.45	60.56	58.19	1.767	0.029	0.265	0.089
Fucose	79.17	82.98	72.13	4.146	81.81	74.37	3.385	82.29	86.27	76.88	76.05	79.68	67.37	5.864	0.184	0.127	0.954
Galactose	82.81	83.93	82.71	1.127	83.25	83.04	0.920	82.51	83.58	83.67	83.10	84.28	81.76	1.594	0.699	0.872	0.653
Galacturonic acid	90.91	91.20	91.52	0.801	90.65	91.78	0.647	88.89	91.30	91.77	92.93	91.11	91.28	1.094	0.856	0.220	0.082
Glucose	45.00	48.44	50.09	1.816	46.40	49.28	1.482	40.35	47.17	51.68	49.65	49.70	48.50	2.568	0.143	0.176	0.063
Mannose	90.40	87.62	88.21	2.098	89.69	87.80	1.713	90.75	87.63	90.68	90.05	87.62	85.74	2.967	0.619	0.441	0.670
Rhamnose	41.62	30.78	32.91	4.100	41.65[a]	28.56[b]	3.347	41.61	39.01	44.32	41.63	22.55	21.51	5.798	0.153	0.008	0.140
Xylose	59.62	63.53	63.81	1.314	60.29[a]	64.36[b]	1.073	55.96	61.53	63.37	63.28	65.54	64.25	1.859	0.052	0.010	0.235
Total NSP	60.14	63.28	63.11	1.214	61.26	63.09	0.991	57.07	62.55	64.15	63.21	64.01	62.06	1.717	0.134	0.198	0.067
Cellulose	18.08	26.53	26.04	2.752	20.76	26.34	2.247	9.68[a]	28.68[b]	23.92[b]	26.48[b]	24.39[b]	28.16[b]	3.893	0.062	0.086	0.038

[a] Data are means of 8 replicate samples per treatment. [b]CON; Control. [c]FOS; Fructo-oligosaccharides.[d] INU; Inulin. [e] XOS; Xylo-oligosaccharides, 0 = No XOS; 1= XOS. [f] ATTD; Apparent total tract digestibility. [g] NSP; Non-starch polysaccharide. [a-c] Means within a row that do not share a common superscript are significantly different.

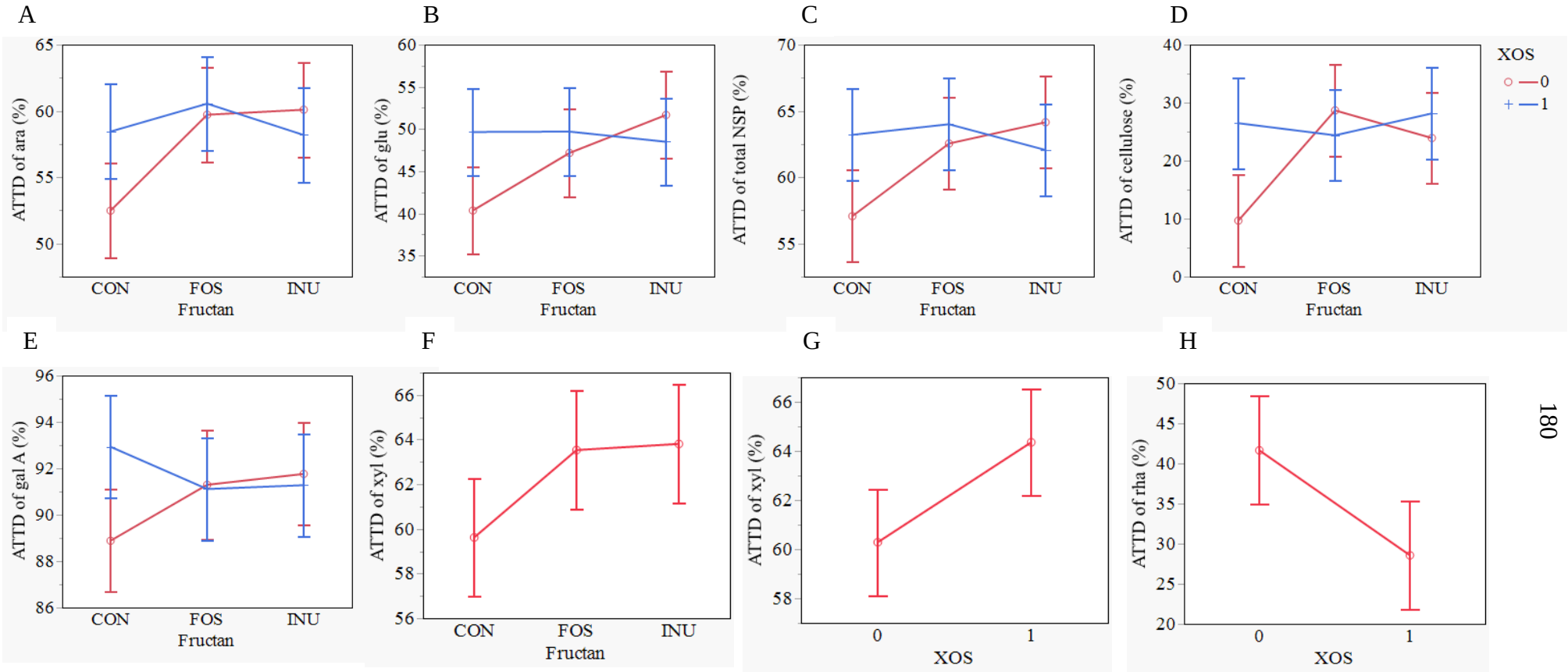

Figure 6.4: Effect of xylo-oligosaccharides (XOS) and fructans (Fructo-oligosaccharides (FOS)) and (Inulin (INU)) on apparent total tract digestibility (ATTD) of NSP arabinose (ara; A; *P* = 0.089), glucose (glu; B; *P* = 0.063), total NSP (C; *P* = 0.067), cellulose (D; *P* < 0.038), galacturonic acid A (gal A; E; *P* = 0.082). Main effect of fructans and XOS on ATTD of xylose (xyl; F; *P* = 0.052; G; *P* < 0.010) and main effect of XOS on ATTD of rhamnose (rha; H; *P* < 0.05).

Table 6.6: Effect of XOS and fructans (FOS and INU) on faecal NSP constituent sugar ratios.

	Fructan			SEM	XOS[e]		SEM	CON 0	FOS 0	INU 0	CON 1	FOS 1	INU 1	SEM	*P* value		
	CON[b]	FOS[c]	INU[d]		0	1									Fructan	XOS	Fructan × XOS
Faecal NSP[f] fraction ratios																	
Ara : cell	0.61[ab]	0.59[b]	0.62[a]	0.008	0.60[a]	0.62[b]	0.007	0.59	0.58	0.62	0.63	0.60	0.62	0.011	0.029	0.032	0.138
Ara : glu	0.56[a]	0.53[b]	0.56[a]	0.007	0.54	0.56	0.006	0.54	0.53	0.56	0.57	0.54	0.56	0.010	0.035	0.055	0.247
Ara : xyl	0.77	0.76	0.77	0.008	0.76	0.77	0.007	0.75	0.77	0.76	0.78	0.76	0.77	0.012	0.924	0.253	0.195
Glu : cell	1.10	1.11	1.11	0.004	1.10	1.11	0.003	1.09	1.11	1.11	1.10	1.10	1.11	0.006	0.196	0.599	0.490
Xyl : cell	0.79[ab]	0.76[b]	0.81[a]	0.009	0.79	0.80	0.007	0.78	0.76	0.82	0.81	0.79	0.81	0.012	0.017	0.186	0.204
Xyl : glu	0.72[ab]	0.70[b]	0.73[a]	0.008	0.71	0.72	0.007	0.71	0.69	0.74	0.73	0.72	0.73	0.012	0.029	0.208	0.211

[a] Data are means of 8 replicate samples per treatment. [b]CON; Control. [c]FOS; Fructo-oligosaccharides.[d] INU; Inulin. [e] XOS; Xylo-oligosaccharides, 0 = No XOS; 1= XOS. [f] NSP; Non-starch polysaccharide. [a-c] Means within a row that do not share a common superscript are significantly different.

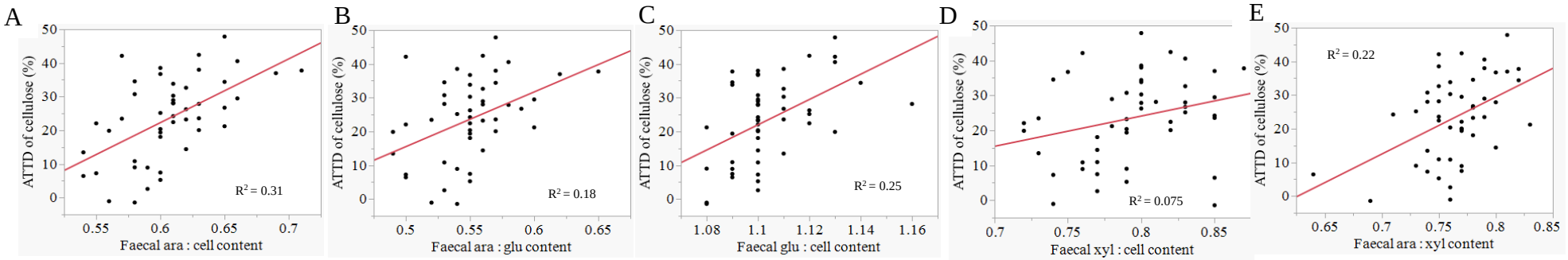

Figure 6.5: Correlation between ATTD of cellulose (%) and faecal NSP arabinose : cellulose content (ara: cell; A; *P* < 0.001), ara : glucose (glu; B; *P* < 0.003) content, glu : cell content (C; *P* < 0.003), xylose : cell (xyl; D; *P* < 0.060) content and ara : xyl content (E; *P* < 0.001).

6.5 Discussion

6.5.1 Digesta pH and lactate concentration

SCFAs including acetate, propionate and butyrate, are the main metabolites produced by bacterial fermentation in the GIT. Lactate is an intermediate metabolite which is rapidly converted to acetate, propionate and butyrate by intestinal bacteria. An increase in the production of SCFAs and lactate lowers the GIT pH which often increases nutrient digestibility by stimulating intestinal peristalsis and enzyme activity. A more acidic environment also affects the gut bacterial composition by preventing the growth of pH sensitive pathogenic bacteria like *Enterobacteriaceae* and *Clostridiaceae*.

In the current study, as expected, higher ileal lactate concentrations lowered gut pH, whilst lower lactate concentrations increased pH. Lactate was likely the dominate SCFA present in the ileum and thus had a major role in determining pH. Supplementation of XOS reduced ileal pH compared to the CON and INU + XOS groups, and increased lactate concentration compared to the CON, INU + XOS and FOS groups. Hence, these results indicate that XOS fed piglets had a higher level of ileal fermentative activity than the CON, INU + XOS and FOS groups. This can be explained by an increased abundance of OTUs associated with lactate producing *g__Lactobacillus* in the ileum of XOS fed piglets (section 4.4.2.1). This indicates that XOS is a good fermentative substrate for *Lactobacillus* species and general stimulator of fermentative activity. Supporting this, *in vitro* growth experiments have shown XOS to grow on *Lactobacillus* species and produce lactate (Moura, P. et al., 2008; De Figueiredo et al., 2020), whilst *in vivo* work has also shown that XOS increased the abundance of *Lactobacillus* in the ileum, colon (Chen, Y. et al., 2021) and faeces (Liu, J. et al., 2018; Zhang et al., 2002) of weaned piglets. Increasing the abundance of LAB like *Lactobacillus* that are perceived to provide health-

promoting properties are deemed beneficial in improving the health of the host (Gibson and Roberfroid, 1995). Moreover, an important underlying cross-feeding network exists which often explains the butyrogenic effect of certain dietary substrates and the beneficial effects obtained from increased butyrate concentrations in the GIT. Bacteria capable of fermenting oligosaccharides are often lactate producers, whilst bacteria capable of utilising lactate are often butyrate producers, hence although not measured, a higher concentration of lactate could lead to a higher level of beneficial butyrate production (Duncan et al., 2004).

The benefit of XOS described above was lost in the presence of INU such that ileal digesta lactate concentration was reduced and pH increased to levels not dissimilar from the CON. This indicates that the combination of INU and XOS together counteracted each other and reduced fermentative activity. The reason for this effect is unknown and the 16S rRNA data does not provide any further clarity. However, one possible reason could be that the combination of INU and XOS increased the transit rate of the digesta, leading to less time for bacterial fermentation and measured activity. Alternatively, as explained in section 4.5.1, XOS and INU could stimulate lactate-producing bacteria which somehow compete with each other when combined. There was no effect of treatment on colonic pH or lactate concentration, indicating the majority of fermentative activity of the supplemented prebiotics took place in the ileum, hence rejecting hypothesises 1 and 2.

6.5.2 Ileal and faecal NSP content

NSPs are polysaccharides containing β-linkages and they vary in their constituent sugar composition (Englyst, K. et al., 2007). Large amounts of soluble and insoluble NSPs are found in wheat and rye, with xylan being the main soluble component (Choct, 1997). The

diets used in the current study were wheat based, hence the main NSP constituents in the diet were ara, xyl and glu. As expected, the NSP constituent sugar profile available in the ileal digesta and faeces reflected that of the diet, such that ara, xyl and glu were the main NSP components measured. Other carbohydrates like starch and alpha-galactosides are well digested in the small intestine, hence were not analysed in the faeces. Starch is the main storage carbohydrate in wheat making up 60-75 % of the grain, hence the presence of some starch in the ileum is expected despite the fact it is highly digestible simply due to its preponderance in the diet (Shevkani et al., 2017). Alpha galactosides, such as stachyose and raffinose are low molecular weight sugars that are commonly found in seeds, roots and tubers of many legumes like soya (Martínez-Villaluenga et al., 2008). The content of stachyose and total alpha-galactosides were very similar between dietary treatments, yet despite this, the content in the ileum was affected by dietary treatment, indicating a digestibility effect which will be discussed below.

6.5.3 AID and ATTD of DM

A positive correlation between DM digestibility and passage rate of digesta has been shown to exist in pigs, whereby the higher the DM digestibility, the slower the rate of total passage (longer retention time) (Kim, B. et al., 2007). This relationship is explained by increased time available for enzymatic digestion, intestinal absorption and bacterial fermentation leading to higher DM digestibility. In the current study, AID of DM tended to be higher for the CON group with XOS compared to without, whereas for the INU group, AID tended to be higher without XOS compared to with XOS. Although transit time was not measured in this study, if the same concept was applied it would indicate that supplementation of XOS tended to slow the rate of passage and increase AID of DM compared to the CON. It is well-known that the addition of XYL to diets reduces digesta

viscosity by hydrolysing xylan into low molecular weight XOS. As such, direct inclusion of XOS is not capable of creating a viscous digesta and therefore would not explain the potential slower rate of passage. It is therefore proposed that the presence of XOS in the ileum greatly increased fermentative activity which is supported with the observed higher abundances of *Lactobacillus,* a lower pH and higher lactate concentration. In turn, this high fermentative activity increased the presence of microbial fermentation products which caused the release of hormone peptides from the gut to activate the ileal brake mechanism which slowed the rate of passage, reduced feed intake and increased AID of DM. This concept is supported by the ADFI data described in section 4.5.1, whereby the intake of piglets in the last two weeks (day 14-27) before sample collection showed a similar but opposite interaction trend to AID of DM and fermentative activity. Similar reductions in ADFI have been seen when feeding albus lupins that are high in oligosaccharide content to pigs (Dunshea, F. et al., 2001; Moore et al., 2021). In comparison, the lower fermentative activity observed in the CON and INU + XOS group likely had an insignificant effect on the ileal brake mechanism, hence passage rate would have been relatively quicker than the XOS group, thus explaining the reduced AID of DM and higher ADFI.

6.5.4 AID of NSPs

The predominant site of fermentation is in the large intestine of monogastrics, but it is now recognised that fermentation can also occur within the stomach and small intestine, particularly the terminal ileum (Williams et al., 2017). Quantitively, the digestibility of NSP fractions at the terminal ileum were relatively low as expected, with the maximum AID of total NSPs reaching 16 %. Others have shown results not too dissimilar from the current trial in growing pigs (~ 39 kg) ranging between 11 and 33 % (Hogberg and

Lindberg, 2004), or weaner pigs (~15 kg) between 28 and 32 % (Yin, Y.-L. et al., 2001) and 19.4 and 28 % (Yin, Y. et al., 2001). The AID of fuc remained highly negative which was discussed in section 5.5.2.

Supplementation of AXOS in broilers has previously been shown to have a stimulating effect on the dietary degrading capacity of the hindgut microbiota (Bautil et al., 2020). It is suggested that providing a highly fermentable substrate which is similar to the main dietary fibre source can train the metabolic activity of resident GIT microbiota to ferment dietary fibre. Bedford, M. and Apajalahti (2018) showed that feeding XYL to chickens increased the ability of the caecal bacteria to ferment xyl, XOS, AXOS and wheat bran, further suggesting a degree of bacterial modulation towards a greater fibre degrading capacity that would otherwise not occur. Moreover, adding an extra source of dietary fibre like β-glucans has also been shown to alter the fermentation and digestibility of alternative dietary fibres other than the main source, hence has an effect on overall fibre digestibility (de Vries et al., 2016). The current study supports this concept and hypothesis 3, as addition of XOS to the CON and FOS group increased the AID of NSP xyl from the main dietary fibre constituent xylan (compared to in the absence of XOS). Furthermore, the addition of XOS to the FOS group increased the AID of NSP ara and total NSP, whilst addition of XOS to both the CON and FOS group increased the AID of NSP glu. Moreover, XOS increased the AID of total fructans to a numerically greater extent than FOS. Hence, XOS not only increased the AID of substrates similar to the main dietary fibre source but also other dietary fibre fractions. The reason for this is not clear but likely due to a broader stimulation of fibre-degrading bacteria in the GIT via cross-feeding and consequent upregulation of their fibre-degrading activity such as enzymes or membrane transport proteins. To this point, genome prediction of microbial communities have

shown that XOS affects carbohydrate metabolism, cell motility, cellular processes and signalling, lipid metabolism and metabolism of amino acids, hence likely has a broad mode of action (Yin, J. et al., 2019). Interestingly the inclusion rate of XOS (0.10 g/kg of pure XOS) was much lower than that of FOS and INU (3.82 g/kg of pure FOS or INU), yet XOS had the largest and most consistent effect on increasing NSP digestibility. This suggests that only small amounts of XOS are required to stimulate the GIT bacteria towards an increased fibre-degrading capacity.

In a study using growing pigs, diets containing high levels of NSP have been shown to increase the AID of total sugars, ara, xyl, total NSPs and dietary fibre compared to CON rations (Hogberg and Lindberg, 2004). The increase in AID was suggested to be due to adaptation of the gut microflora to high fibre diets, such that they increased the microbial activity in the distal small intestine. To this point, the AID results of the current study support the data obtained for the ileal fermentative activity and AID of DM. An increase in the abundance of *g_Lactobacillus* and lactate concentration in the ileum along with a reduction in pH indicates increased fermentative activity, which helps explain the increased AID of DM and NSPs for piglets receiving XOS.

Analysing the bacterial community composition helps provide explanations for digestibility results in terms of identifying which bacteria were upregulated or downregulated by XOS. For example, Cho et al. (2020) showed that supplementation of a stimbiotic increased the abundance of specific fibre-degrading bacteria. Indeed, an increase in the abundance of *g_Lactobacillus* was observed in the current study which agrees with others (Chen, Y. et al., 2021), but the specific species was unknown. Previous studies have shown *Lactobacillus brevis* to be the main species to utilise XOS (Crittenden

et al., 2002; Moura, Patrícia et al., 2007), whilst the majority of other *Lactobacillus* species showed no growth on XOS (Crittenden et al., 2002). Despite this utilisation of XOS, various *Lactobacillus* species have been shown not to utilise β-glucans, xylan or AX (Crittenden et al., 2002), hence the increase in the abundance of *g_Lactobacillus* in the current study is unlikely to be the direct or single reason for the increase in AID of NSPs with XOS. Other bacterial changes with the XOS group included a decrease in *g__Actinobacillus and g__Terrisporobacter* from the CON (section 4.4.2.1), neither of which explains the current results. Hence, it is instead proposed that XOS could have had minimal effect on measurable fibre-degrading bacterial abundance, but could have still increased bacterial metabolic activity, for example fibre-degrading enzyme production, which led to increased AID of NSPs. Alternatively, the increased production of metabolic end-products like lactate in the ileum of XOS fed piglets were likely utilised by other species as their preferred source of energy (Smith et al., 2019). Hence, this could have created a cross-feeding network which ultimately increased overall NSP digestibility without observing direct and specific bacterial abundance effects.

Addition of INU increased the AID of NSP glu to the same extent as XOS and FOS + XOS, but the combination of INU + XOS was significantly lower, hence these results did not support hypothesis 3. Similar increases of AID with INU were seen for other fibre fractions, though they were not significantly different from the CON or INU + XOS group. This highlights that the positive effects of XOS on the AID of NSPs were numerically confounded when combined with INU, once again agreeing with the reduced fermentative activity data and lower AID of DM in the INU + XOS group. As previously explained, this could be due to an increased transit rate of the digesta consequently leading to less time for bacterial fermentation of NSP fractions. Alternatively, linking the AID of

NSPs to the 16S rRNA bacterial community data, there was a trend for an interaction between XOS and fructans on the abundance of ileal *g__Prevotella 7*. The abundance was similar with or without XOS in the CON and FOS group, but abundance tended to be higher with XOS compared to without XOS in the INU group (P = 0.087; Table 4.4). Similar numerical increases in the INU + XOS vs INU group were seen for *g__Prevotella 2*, *g__Prevotella 9* and *g__Prevotellaceae_NK3B31_group* but these were not significant or identified as a trend. Prevotella species are known to possess genes for fibre hydrolysis (Ivarsson et al., 2014), hence an increase in the AID of NSPs would be expected with an increased abundance in the INU + XOS group. However, this was not the case, and reductions in fermentative activity, AID of DM and AID of NSPs were seen with these elevated abundances. Interestingly certain species of Prevotella have been shown to produce extracellular viscous material that appears as meshwork-like structures under a microscope (Yamanaka et al., 2006). The viscous material has been shown to contain neutral sugars, uronic acids and amino acids, but with 83 % being composed of man. To this point, the AID of man in the XOS + INU group was indeed negative and significantly lower compared to all other treatment groups, indicating man was higher in the ileum compared to the feed and therefore produced in the ileum. This could indicate that the higher abundance of Prevotella species may have increased the amount of extracellular matrix produced by the bacteria, which in turn limited the interaction between digestive enzymes and dietary substrates. This could have therefore reduced fermentative activity and as such explained the lower AID of DM and NSPs. The reason as to why this may have only occurred when XOS + INU were combined over fed alone is unknown, but perhaps likely due to increased competition or a degree of bacterial community imbalance with an overload of fermentative substrate which allowed Prevotella species to thrive. Regardless, the combination of INU + XOS proved negative for NSP digestibility at the ileal level.

The AID of ‘other carbohydrates’ in the current study were relatively high, for example the average AID of starch was 96.6 %. This agrees with other studies whereby the AID of starch has been shown to be almost complete at 99.4 % in growing pigs. Supplementation of all prebiotics numerically increased the AID of stachyose, but only XOS and INU independently increased the AID of stachyose compared to the CON, with their combination showing numerically lower AID. This suggests that independently XOS and INU promoted the fermentation of stachyose, but their combination tended to be less effective, agreeing with the results for the AID of NSPs. Similar trends were also seen for raffinose, total alpha-galatcosides, total fructans and total carbohydrates excluding NSP. Again, the AID data followed similar interaction trends to the AID of NSPs and ileal fermentative activity results, however there were differences. For example, there was a clear negative effect of feeding FOS alone on the AID of NSP ara, glu, xyl and total NSPs compared to when FOS + XOS were fed together, and in the case of glu, also compared to the CON diet. In comparison, FOS tended to numerically increase the AID of raffinose, stachyose, total alpha-galactosides and fructans compared to the CON. This therefore indicates that FOS demoted the fermentation of NSP fractions in the ileum whilst it promoted the fermentation of ‘other carbohydrates’. The abundance of pathogenic *g__E . coli* tended to be higher in the FOS group compared to the FOS + XOS group (section 4.4.2.1) coupled with a main fructan effect for lower bacterial diveristy (section 4.4.2.3), likely suggesting a degree of dysbiosis. To this point the NSP fibre-degrading capacity of the bacterial community could have been compromised, coupled with a relative preference for simpler sugar structures. Moreover, the independent addition of FOS and INU tended to increase the AID of total fructans as expected, yet the largest increase from the CON group was with XOS. The combination of FOS and XOS was not dissimilar from feeding FOS alone, yet the combination of INU

and XOS reduced AID of fructans by almost half again indicating a negative effect when fed in combination.

6.5.5 ATTD of NSPs

Quantitively, the ATTD of NSP fractions were relatively high as expected, with the average ATTD of total NSPs reaching 62.2 % across treatments. Similar digestibility coefficients have been seen in the literature whereby the average ATTD of total NSPs in growing pigs has been reported at 59 % (Hogberg and Lindberg, 2004). The AID of NSPs described above were from the terminal ileum, hence the ATTD results represents any fermentation throughout the caecum, large intestine and faeces. The negative effects of combining INU + XOS on fermentative activity and AID of NSPs were no longer present at the end of the GIT. Interestingly, the addition of all individual prebiotics and their combination increased the ATTD of cellulose compared to the CON. Similar trends were seen for other fibre fractions like NSP ara, glu, total NSP and main effects for xyl. This is likely due to a stimbiotic effect whereby the prebiotics stimulated fibre-degrading bacteria and their metabolic activities. The bacterial communities in the faeces were not analysed in this study, hence only colonic data could be inferred to these results, albeit there were no clear bacterial abundance effects to explain these results. Supporting this, there was also no effect of treatment on colonic pH, colonic lactate concentration or colonic microbial endo-XYL production (section 4.4.3). Hence, taken together this suggests the stimbiotic effect on GIT bacteria to cause the increase in ATTD of NSPs likely occurred in the ileum and caecum. To this point, future studies investigating the stimbiotic mode of action of XOS should analyse the bacterial community compositions in all GIT sections. Further to this, a wider range of microbial enzyme activities such as

cellulase, arabinofuranosidase, β-glucanases and feruloyl esterase should be analysed in each GIT section to aid explanations of NSP digestibility results.

Supplementation of XOS tended to increase the ATTD of gal A compared to the CON group, whilst the ATTD of rha was lower with XOS compared to without. This indicates that XOS selectively stimulated the utilisation of gal A whilst it reduced the utilisation of rha. Moreover, as described above, in the ileum FOS tended to demote the fermentation of NSP fractions whilst it promoted the fermentation of 'other carbohydrates'. However, at the end of the GIT, this demotion effect was no longer present, and FOS tended to increase the ATTD of NSP fractions similar to the other prebiotics. Hogberg and Lindberg (2004) reported similar concepts whereby the pattern of fibre fraction digestion was different at the ileum compared to total tract and did not follow the same pattern with dietary treatment. For example, the AID of ara, xyl, total NSP and dietary fibre increased with high fibre diets compared to the CON diet, whereas the ATTD showed more extensive digestion in diets of low fibre compared to the CON diets.

Despite these overall improvements in the ATTD of cellulose and NSP fractions, no beneficial effect on piglet growth performance was observed. This effect was not expected as an increase in fibre digestibility means greater amounts of energy were derived from the diet. It would be expected that this increased energy derivation would be utilised for extra lean tissue growth and thus improve host growth, however this was not observed. One possible explanation for this result is the reduced ADFI seen across the trial period in prebiotic supplemented pigs compared to the CON, meaning relatively less digestible DM would have been consumed. This feed intake effect likely outweighed the benefits seen in NSP digestibility. Hence, the proposed ileal brake mechanism reduced

ADFI to a greater extent than it improved NSP digestibility, consequently resulting in poorer performance. Moreover, the bacterial modulation effect of XOS was positive and the reduction in ADG was lessened in the second half of the trial. This suggests a longer feeding period was required for the benefits in NSP digestibility to surpass the ileal brake mechanism on ADFI.

As previously discussed, the ATTD of cellulose and other NSP fractions were increased for all dietary treatments compared to the CON group, this therefore meant that relatively less cellulose and NSP substrate was present in the faeces of the prebiotic groups. The faecal NSP constituent sugar ratios therefore indicate the relative selective fermentation of the remaining fibre fraction substrates. The faecal ara : cell ratio was higher in the INU group compared to the FOS group, and higher with XOS compared to without XOS, indicating that cellulose was more selectively fermented relative to NSP ara with INU or XOS supplementation. This therefore suggests that the faecal bacterial community with INU or XOS had a higher capability or preference to ferment cellulose over NSP ara. To the author's knowledge this effect has not been shown before. Similarly, the faecal ara : glu ratio was lower with FOS compared to the CON and INU groups, suggesting that the faecal bacterial community with FOS had a lower capability or preference to ferment NSP glu over ara. Moreover, the ara : glu ratio tended to be increased with XOS compared to without XOS indicating NSP glu was more selectively fermented relative to NSP ara with XOS. Finally, the xyl : cell and xyl : glu ratio were higher in the INU group compared to the FOS group, indicating that NSP cell and glu were more selectively fermented relative to NSP xyl with INU supplementation. Again, this therefore suggests that the faecal bacterial community with INU had a higher capability or preference to ferment NSP cell and glu compared to NSP xyl. The general higher faecal NSP constituent ratios with INU

and XOS suggest their prebiotic effects remain throughout the GIT and are not isolated to just the ileum, coupled with a low preference for NSP ara relative to other NSP fractions.

Furthermore, there was a positive correlation between ATTD of cellulose and faecal NSP constituent sugar ratios (ara : cell, ara : glu, glu : cell, and ara : xyl), and a trend for xyl : cell. For example, as the ATTD of cellulose increased, the faecal ara : glu ratio increased, hence as less cellulose substrate was present, more NSP glu was fermented compared to ara. In summary, the ATTD of cellulose and consequent remaining substrates available for fermentation affected the selective fermentation of different fibre fractions.

6.5.6 Conclusion

XOS increased ileal fermentative activity, the AID of DM and AID of NSP fractions, suggesting it is a suitable fermentative substrate for the bacteria inhabiting the small intestine and had a stimulatory effect on increasing the bacteria's' fibre-degrading capacity. However, these effects of XOS were lost when combined with INU indicating incompatibility of the products. At the end of the GIT both XOS and fructans, along with their combination, increased the ATTD of cellulose and NSP fractions, indicating beneficial effects on fibre digestibility with oligomer supplementation. Despite this improvement, supplementation of XOS with or without fructans had no beneficial effect on piglet growth performance. This was likely explained by reduced feed intake outweighing the benefits of NSP digestibility.

Chapter 7
General discussion

The main socio-economic factors that drive increased demand for food production are population growth, increasing urbanisation and rising incomes, with uncertainty surrounding the magnitude, nature and regional patterns of the first two factors (FAO, 2009). Based on the projected growth of these socio-economic factors, it has been predicted that future global food demand can be met if certain conditions are addressed such as strong global economic growth, expansion of food supplies, food production growth in developing countries, global trade aiding low income countries and higher animal productivity (FAO, 2009). The latter factor relates to greater priority being given to agricultural research and development in order to achieve the yield and productivity gains required to feed the world in 2050 (FAO, 2009). Hence, improving the efficiency of animal production is of utmost importance to meet global food security challenges, yet this is only possible in healthy animals that are free of disease and able to express their maximum genetic potential. Improving animal health is therefore fundamental for sustainable meat production.

Modulation of the GIT bacterial community via use of prebiotics to selectively promote the growth and activity of beneficial bacteria and inhibit pathogenic bacteria is one feeding concept to improve the health and growth of animals. New-generation prebiotics like XOS are gaining attention for monogastric nutrition, but most of the research has been conducted in chickens with little focus on pigs. This research therefore set out to determine the effects of XOS alone or in combination with alternative additives on pig performance, and with a view of providing more clarification on the underlying

mechanisms of XOS within the pig GIT. The main objectives of this research were set out in section 1.4.4.

7.1 Objective 1

As described in Chapter 2, provision of AXOS and XOS can be direct via the addition of commercially produced oligomers or via the supplementation of XYL and production of AXOS and XOS *in situ*. In broilers, both XYL and AXOS improved feed utilisation in broilers suggesting that the AXOS released *in situ* by the action of XYL contributed to the beneficial effects of feed supplemented XYLs (Courtin et al., 2008). AXOS increased the abundance of caecal *Bifidobacterium*, an effect which was not seen with XYL addition. This indicated that the mode of action of XYL and AXOS were not identical and suggests the XYL did not release much AXOS with the direct provision having a larger microbiota-modulating effect. Hence, a dual combination of XYL and XOS may synergise by having a greater stimulatory effect on the GIT bacterial community whilst also eliciting the traditional mechanisms of XYL such as viscosity reduction and increased nutrient digestibility (González-Ortiz et al., 2019). Hence, the first objective of this research was to determine the effects of XYL and XOS on grower pig performance and faecal bacterial community composition. In Chapter 2, results showed that XYL had no effect on grower pig performance, nor was there an interaction between XYL and XOS. Pigs supplemented with XOS were less efficient in the first week of the trial, whilst ADG was improved in the second week; but there were no performance effects in the overall trial period. Given the scarcity of research of XOS in pigs these results can only be compared to current literature available. The data from the current thesis agreed with a weaner pig study that showed no effect of XOS (Yin, J. et al., 2019), but was contrary to other studies which have shown improved performance with XOS or XOS + XYL

addition (Chen, Y. et al., 2021; Liu, J. et al., 2018). Hence, the effect of XOS on pig growth performance is inconsistent. As described in Chapter 2, results of this trial and comparison with the literature suggest a higher concentration of pure XOS (≥ 0.10 g/kg) in younger weaned pigs may be necessary to observe performance benefits. An initial study investigating a dose response effect of XOS in different aged pigs would have been beneficial and is an important learning of this thesis. Despite a lack of performance benefits, supplementation of XYL or XOS, individually or in combination, increased the abundance of OTUs belonging to the *Muribaculaceae* and *Prevotellaceae* families. These bacteria are associated with carbohydrate metabolism, hence are likely involved in the mechanistic pathways of XYL and XOS and suggest the start of a bacterial modulation effect of the supplemented products. Future work needs to focus on the bacterial modulation effect of XOS along the entire GIT and not just the faeces as this only provides a limited view of the mechanism of XOS. This is an important learning of this thesis as Chapter 6 revealed the main effect of XOS was at the ileal level.

7.2 Objective 2 & 3

In Chapter 2, the abundance of lactate-utilising bacteria from the *Veillonellaceae* family were shown to increase in pig faecal matter over time; although not measured, this was likely explained by an increased level of lactate within the GIT. As described in Chapter 3, lactate is a metabolic intermediate that is quickly utilised by bacteria in the GIT, thus it is seldom detected in the faeces of healthy subjects or at low concentrations (< 3 mM) (Hove et al., 1994). However, accumulation of faecal lactate indicates the inability of the GIT bacterial community to clear hyper-lactate concentrations and could therefore act as an indicator of bacterial imbalance and gut health status, albeit this is a new research area for the pig sector. Hence, the secondary objective of this research was to determine

whether L-lactate could be detected in piglet faeces over the stressful weaning period and whether concentrations changed with age as the bacterial community developed and became more established (Chapter 3). Results showed that L-lactate concentrations were low or below the minimum detectable range of the assay (< 0.02 mM) in piglet faeces, with only 20 % of samples having detectable concentrations, the majority of which were immediately post-weaning. The relative absence of L-lactate in the faeces may have been due to it being metabolised by faecal bacteria before analysis. Hence, the third objective of this research was to determine how quickly a known concentration of L-lactate would be metabolised by pig faecal bacteria, and whether this differed between sample collection solutions, sample fractions (whole or supernatant) or analytical methods (Chapter 3). A large learning from this experiment was that the assay incorrectly measured lower baseline measurements of the spiked L-lactate concentration (~ 5 mM at 0 min), indicating the specific assay used in this study was not a suitable method of L-lactate analysis in pig faeces. The reason for this is unknown, but it is proposed that elements within the faecal matrix may have interfered with the assay since a blank spiked sample without faecal matter correctly measured the spiked concentration. Hence, the failure of the assay to correctly measure the spiked L-lactate concentration casts doubt over the results reported for the secondary objective; it is therefore suggested that the secondary objective is repeated in more depth with future work. The suitability of a real-time L-lactate detection method was investigated with a handheld SCT device which is commonly used by human athletes to measure blood L-lactate (Goodwin et al., 2007). To the authors knowledge, the SCT device has not previously been tested on faecal matter. If successful, this novel approach would allow for on-site real-time detection of L-lactate in biological samples. Results showed that the handheld L-lactate SCT device correctly measured the spiked L-lactate concentration at baseline measurements, proving the device can measure L-lactate in faecal matter. L-lactate concentration in the ‘Whole’

samples decreased to ~0.60 mM after 360 min when measured by the SCT device, indicating rapid L-lactate metabolisation by pig faecal bacteria as expected. Similar results have been seen in the literature whereby 20 mM of lactate was fully metabolised within 6 hours by pig caecal bacteria (Ushida and Hoshi, 2002). However, when bacteria were separated via centrifugation prior to L-lactate addition, the concentration remained stable over time proving that L-lactate was not metabolised which was likely due to separation of lactate-utilising bacteria. Chapter 3 concluded that the SCT device was a suitable real-time method for measuring L-lactate concentrations. L-lactate metabolism was rapid and therefore faecal collection and processing must be rapid. This data has not been shown in the literature before and is a novel method of on-farm L-lactate analysis, but as described in section 3.5.1, further research and validation are required to identify the potential of faecal L-lactate concentration as a non-invasive marker of gut health. In particular, the normal range of L-lactate concentration in pig faecal matter at different ages, in healthy and diseased individuals is important. Furthermore, there is also great potential for the handheld SCT device to provide further insight into the fermentative activity of bacteria from different GIT sections and provides a useful real-time method for L-lactate analysis during dissections. Hence, this is a new, insightful and rapid tool to use in future animal studies.

7.3 Objective 4

Digestibility studies are an important method of evaluating the nutritive value of feed materials or additives in monogastric nutrition. As described in section 5.2, the gold standard traditional total collection method is time consuming and laborious compared to the commonly used inert marker method. However, the accuracy of the marker technique relies on the marker being homogenously distributed throughout the digesta with the

nutrients to facilitate calculation of accurate and precise digestibility values. Numerous studies have shown negative or unexpectedly high digestibility values for fibre fractions using Cr_2O_3 (Jamroz et al., 2002; Brenes et al., 2003) or TiO_2 (Bautil et al., 2019) with the overall concluding reason being due to separation of the indigestible marker and digesta. This highlights that the marker method can yield erroneous results, especially in high fibre diets, ultimately leading to mis-estimation and confounded digestibility results. Therefore, the fourth objective of this research was to evaluate TiO_2 and intrinsic cellulose as inert markers for AID and ATTD of NSPs in piglets. Results of Chapter 5, indeed supported the literature in that highly negative AID of NSPs were found when using TiO_2 as an inert marker, indicating separation of the marker and NSP fraction. In contrast, using cellulose as an inert marker yielded more realistic, less negative and less variable AID of NSP. At the faecal level, positive and more similar ATTD of NSPs were observed between the markers compared to AID, yet no positive correlation was seen between the ATTD of the NSP fractions determined using the different markers. The lack of a positive correlation between the markers proves that the TiO_2 and cellulose were not working in tandem. The lack of a positive correlation can be explained by post-ileal cellulose degradation causing the average ATTD for total NSPs, obtained using cellulose as a marker, to be 15.1 % lower compared to when using TiO_2 as a marker. Hence, Chapter 5 concluded that TiO_2 is an unsuitable inert marker for AID of NSPs, with cellulose yielding more realistic initial results. Whilst, TiO_2 should be used for ATTD of NSPs, as post-ileal cellulose degradation rendered the use of cellulose as an inert marker infeasible. These learnings were applied in Chapter 6. Homogenous distribution and transit rates of inert markers within GIT digesta are fundamental for accurate and correct digestibility estimates, but this data proves that this criterion cannot be met when analysing fibre fractions. This work therefore demonstrates the need for a re-evaluation of current and alternative inert markers for fibre digestibility work as described in section 5.5.3. This

chapter provides the baseline knowledge in this complex area; however, a more in-depth series of studies are required to address this knowledge gap. Key to this future work is the comparison and validation of well-known markers like Cr_2O_3 and potential alternative intrinsic markers like AIA or lignin against the gold standard method of total collection for different fibre fractions along the entire GIT. Nutritive fibre is a vast topic area and advancing the methodologies to accurately measure its degradation within monogastric animals will advance this field greatly and ensure mis-estimation does not occur in future work.

7.4 Objective 5

Chapter 2 concluded that 0.07 g/kg of pure XOS may not have been enough to elicit performance benefits in 7-week old pigs, and that younger piglets may have responded better to XOS supplementation. Hence, a higher inclusion of pure XOS (0.10 g/kg) and younger piglets (4-weeks of age) were used in Chapter 4 and Chapter 6.

The mechanism of XOS in the GIT is multi-factorial and is not yet completely understood. As described in Chapter 6, the amount of XOS required to elicit measurable bacterial modulation effects and performance improvements is relatively low compared to conventional prebiotics like FOS and INU. An alternative hypothesis for the mechanism of XOS has therefore been proposed, whereby XOS acts as a signal to encourage fibre-degrading bacteria to increase in activity, ferment fibre more efficiently and interact with the GIT in such a way that improves overall efficiency of digestion and growth. Moreover, the combination of different prebiotic substrates should theoretically broaden the number of different GIT bacteria that are stimulated, leading to a wider range of microbial fibre-degrading capabilities, hence improving overall fibre digestion if the

prebiotics work in synergy. Hence, the fifth objective of this research was to determine the effect of XOS with or without fructans on piglet performance and various GIT functions, including bacterial community composition, microbial endo-XYL activity, fermentation activity and NSP digestibility (Chapter 4 and Chapter 6). The fermentative activity was assessed by measuring digesta pH and L-lactate concentration using the SCT device from Chapter 3.

Overall, supplementation of XOS with or without short and long-chain fructans had no beneficial effect on piglet growth performance, likely because of the large reduction in ADFI. The ADG of XOS fed piglets was reduced in the first 2 weeks of the trial compared to the CON, but to a much lesser extent in the last 2 weeks of the trial, suggesting piglets adjusted to XOS with age and a longer feeding period may have been required to observe performance benefits. Regarding piglet ADFI, an interaction between XOS and fructans existed, whereby XOS, FOS and INU reduced ADFI compared to the CON, whilst XOS + FOS and XOS + INU had intermediate ADFI levels. This feed intake effect was proposed to be due to the ileal brake mechanism. As described in Chapter 4, this concept is based upon partially digested nutrients and their microbial fermentation products stimulating the release of hormone peptides from the GIT epithelium. This effect slows gastric emptying, gastric acid production and the intensity of peristaltic contractions, all of which reduce feed intake (Dunshea, F.R. et al., 2018). Supporting this theory, as reported in Chapter 6, the fermentative activity in the ileal digesta of XOS fed piglets was indeed increased (lower pH and higher lactate concentration) which would explain the commencement of the ileal brake mechanism and reduction in ADFI. Moreover, Chapter 4 showed that bacterial community structure differed with prebiotic supplementation in the ileum but not the colon; indicating that the prebiotic substrates were more suitable for

bacteria inhabiting the distal small intestine compared to the large intestine. This was an unexpected finding as initially it was hypothesised that XOS and long-chain INU would mainly be fermented in the large intestine given the slower rate of fermentation (Smiricky-Tjardes et al., 2003a) or longer chain length (Paßlack et al., 2012). Moreover, addition of XOS to diets stimulated beneficial *Lactobacillus* in the ileum, hence despite the lack of performance benefits observed, the bacterial modulation effect of XOS was positive and a longer feeding period was likely required for these bacterial benefits to reflect in increased growth performance.

Regarding NSP digestibility, the learnings from Chapter 5 were applied, whereby cellulose was used as an inert marker for AID of NSPs, whilst TiO_2 was used as an inert marker for ATTD of NSPs. XOS and XOS + FOS increased the AID of DM and NSP fractions suggesting that XOS had a stimulatory effect on increasing the bacteria's' fibre-degrading capacity. Moreover, the independent addition of FOS and INU tended to increase the AID of total fructans as expected, but the largest increase from the CON group was with XOS. This highlights that XOS is capable of improving the AID of fibre fractions different from its own structure and composition above and beyond that of substrates with similar structure. To the authors knowledge, this effect has not been shown in the literature before. There was no clear effect on bacterial community composition to explain these results at the ileal level. It was therefore proposed that XOS likely had minimal effect on measurable fibre-degrading bacterial abundance, but could have still increased bacterial metabolic activity, for example fibre-degrading enzyme production, which led to increased AID of NSPs. Moreover, an interesting but unexpected observation across the performance, fermentative activity and AID data was that the effect of XOS was lost in the presence of INU, indicating incompatibility of the fermentative substrates.

To the authors knowledge, this effect has not been shown in the literature before and the reason for this interaction is unclear. However, possible explanations could include an increased transit rate when the products were combined leading to less time for bacterial fermentation of NSP fractions or it could be associated with an increase in Prevotella species as described in section 6.5.4. The fact that INU increased the AID of NSPs to similar levels as XOS also suggests the substrates stimulated certain ileal bacteria when fed alone, which somehow negatively competed when combined.

At the faecal level, the negative effects of combining INU + XOS on fermentative activity and AID of NSPs were no longer present at the end of the GIT, suggesting this negative competition and effect on NSP digestibility was mainly driven at the ileal level. Both XOS and fructans, as well as their combination, increased the ATTD of cellulose and NSP fractions compared to the CON. Albeit, there was no benefit of combining the prebiotics together over and above feeding them independently, leading to the conclusion that feeding multiple prebiotics is not required. The increase in fibre digestibility suggests a greater energy provision from the dietary fibre fractions ingested that the host would not have received without oligomer supplementation. Despite this improvement in digestibility, no beneficial effect on piglet growth performance was observed. The individual ADFI of dissected piglets was not measured as the piglets were housed in groups of 5. However, the reduced ADFI seen across the trial period would have meant relatively less digestible DM was consumed. This intake effect likely outweighed the benefits seen in NSP digestibility. Hence, the ileal brake mechanism reduced ADFI to a greater extent than it improved NSP digestibility, consequently resulting in poorer performance. Given that the bacterial modulation effect of XOS was positive and the reduction in ADG was lessened in the second half of the trial suggests a longer feeding

period was required for the benefits in NSP digestibility to surpass the ileal brake mechanism on ADFI. Moreover, with the negative performance effect lessened in the second half of the trial coupled with the positive effects of XOS on NSP digestibility at the end of the trial suggests there may be an optimal time to introduce XOS post-weaning. New to the literature, the effect of XOS in this study was mainly observed at the ileal level at the end of trial. Perhaps feeding XOS immediately after weaning overloaded the ileal bacteria with fermentable substrate that could not be utilised at that point in time and instead primed the caecal and colonic bacteria. Then, as the pig aged, the ileal bacterial community developed and were able to utilise XOS more efficiently, hence essentially starved the caecal and colonic bacteria from XOS. It could therefore be a combination of overloading the ileum with fermentable substrate immediately after weaning with a subsequent starving effect on the caecal and colonic bacteria as the pig aged that caused this performance drop in the first 2 weeks. Hence, further work on the optimal timing of XOS introduction is required.

Chapter 6 provided insights into the mechanism of XOS within the GIT of piglets, however further work is required to expand on this knowledge. Firstly, the complex interaction between fermentation and transit time upon addition of prebiotic substrates warrants further research. Combined with this, the effect of XOS on bacterial fermentative activity and consequent regulation of appetite via GIT hormone mechanisms is important. Likewise, identifying the main bacteria responsible for increased fermentation of specific substrates is key in furthering knowledge on targeted bacterial modulation effects. As described in Chapter 6, one important learning from this thesis was that bacterial abundance changes may not show significant changes but improvements in NSP digestibility may still be observed, it is therefore critical to analyse

bacterial activity as well as abundance. All of these factors are closely intertwined, hence focusing on one individual aspect can lead to extrapolation and mis-interpretation of results. It is therefore recommended that in future similar studies a minimum of bacterial abundance and activity along the GIT, fermentative activity and GIT hormone concentrations are measured to provide sufficient information to help explain results.

7.5 Conclusions

This research set out to determine the effects of XOS alone or in combination with alternative additives on pig performance, and with a view of providing more clarification on the underlying mechanisms of XOS within the pig GIT. From this work it can be concluded that XOS has an inconsistent effect on pig growth performance and there was no benefit of combining different prebiotic substrates. Compared to the literature, the lack of performance benefits seen in this thesis stem from not enough XOS inclusion, age of pig and duration of the feeding period. It is clear from the literature and this thesis that a minimum inclusion of pure XOS for piglets should be 0.10 g/kg and supplementation from a young age after weaning increases the likelihood of observing performance effects. It is suggested that introduction of prebiotic substrates like XOS from a young age is required to prime the GIT bacterial community and the feeding period needs to be longer than 4 weeks to provide sufficient time for performance benefits to be observed. Furthermore, this work has shown a clear effect of XOS and other prebiotic substrates on ADFI, likely due to the ileal brake mechanism. Coupled with this, XOS increased ileal fermentative activity, positively altered the ileal bacterial community and increased the AID of DM and NSPs, indicating XOS increased the fibre degrading capacity of the ileal bacteria despite its low inclusion rate. Whilst at the faecal level, all prebiotic substrates increased the ATTD of cellulose and NSP fractions. Overall, this suggests that the large

effect of XOS observed in the ileum was met by other prebiotic substrates at the faecal level. Despite these beneficial effects on fibre digestibility and energy provision, they did not translate into growth performance benefits. This was likely due to a complex relationship between the ileal brake effect outweighing the positive effect of NSP digestibility, with a longer feeding period required. In summary, based on the findings of the current work and those of others, it is clear that there are considerable opportunities to improve the health and performance of pigs through prebiotic supplementation. However, the optimal inclusion rate, age of introduction and feeding period in piglets need identifying to maximise the potential benefits of new-generation prebiotics like XOS.

References

Aachary, A.A. and Prapulla, S.G. 2008. Corncob-induced endo-1, 4-β-D-xylanase of Aspergillus oryzae MTCC 5154: production and characterization of xylobiose from glucuronoxylan. *Journal of agricultural and food chemistry.* **56**(11), pp.3981-3988.

Aachary, A.A. and Prapulla, S.G. 2011. Xylooligosaccharides (XOS) as an Emerging Prebiotic: Microbial Synthesis, Utilization, Structural Characterization, Bioactive Properties, and Applications. *Comprehensive Reviews in Food Science and Food Safety.* **10**(1), pp.2-16.

Al-Sheraji, S.H., Ismail, A., Manap, M.Y., Mustafa, S., Yusof, R.M. and Hassan, F.A. 2013. Prebiotics as functional foods: A review. *Journal of functional foods.* **5**(4), pp.1542-1553.

Alander, M., Mättö, J., Kneifel, W., Johansson, M., Kögler, B., Crittenden, R., Mattila-Sandholm, T. and Saarela, M. 2001. Effect of galacto-oligosaccharide supplementation on human faecal microflora and on survival and persistence of Bifidobacterium lactis Bb-12 in the gastrointestinal tract. *International Dairy Journal.* **11**(10), pp.817-825.

Allen, S.E. and Holm, J.L. 2008. Lactate: physiology and clinical utility. *Journal of Veterinary Emergency and Critical Care.* **18**(2), pp.123-132.

Ansari, S. and Yamaoka, Y. 2017. Survival of Helicobacter pylori in gastric acidic territory. *Helicobacter.* **22**(4), pe12386.

Apprill, A., McNally, S., Parsons, R. and Weber, L. 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology.* **75**(2), pp.129-137.

Bäckhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. and Gordon, J.I. 2005. Host-bacterial mutualism in the human intestine. *science.* **307**(5717), pp.1915-1920.

Bakker-Zierikzee, A.M., Alles, M.S., Knol, J., Kok, F.J., Tolboom, J.J. and Bindels, J.G. 2005. Effects of infant formula containing a mixture of galacto-and fructo-

oligosaccharides or viable Bifidobacterium animalis on the intestinal microflora during the first 4 months of life. *British Journal of Nutrition.* **94**(5), pp.783-790.

Bakker, G.C. and Jongbloed, A.W. 1994. The effect of housing system on apparent digestibility in pigs, using the classical and marker (chromic oxide, acid-insoluble ash) techniques, in relation to dietary composition. *Journal of the Science of Food and Agriculture.* **64**(1), pp.107-115.

Barrera, M., Cervantes, M., Sauer, W.C., Araiza, A.B., Torrentera, N. and Cervantes, M. 2004. Ileal amino acid digestibility and performance of growing pigs fed wheat-based diets supplemented with xylanase. *Journal of Animal Science.* **82**(7), pp.1997-2003.

Barszcz, M., Taciak, M. and Skomiał, J. 2016. The effects of inulin, dried Jerusalem artichoke tuber and a multispecies probiotic preparation on microbiota ecology and immune status of the large intestine in young pigs. *Archives of animal nutrition.* **70**(4), pp.278-292.

Barton, M.D. 2014. Impact of antibiotic use in the swine industry. *Current opinion in microbiology.* **19**, pp.9-15.

Bäumler, A.J. and Sperandio, V. 2016. Interactions between the microbiota and pathogenic bacteria in the gut. *Nature.* **535**(7610), pp.85-93.

Bautil, A. 2020. *Arabinoxylan digestion and endoxylanase functionality in ageing broilers fed wheat-based diets*. Doctor of Bioscience Engineering (PhD) thesis, KU Leuven University.

Bautil, A., Verspreet, J., Buyse, J., Goos, P., Bedford, M. and Courtin, C. 2019. Age-related arabinoxylan hydrolysis and fermentation in the gastrointestinal tract of broilers fed wheat-based diets. *Poultry science.* **98**(10), pp.4606-4621.

Bautil, A., Verspreet, J., Buyse, J., Goos, P., Bedford, M. and Courtin, C. 2020. Arabinoxylan-oligosaccharides kick-start arabinoxylan digestion in the aging broiler. *Poultry Science.* **99**(5), pp.2555-2565.

Bedford, M. and Apajalahti, J. 2018. Exposure of a broiler to a xylanase for 35d increases the capacity of cecal microbiome to ferment soluble xylan. In: *Proc. Poultry Science Association 107th Annual Meeting, San Antonio, Texas, US*, pp.98-99.

Bedford, M.R. 2018. The evolution and application of enzymes in the animal feed industry: the role of data interpretation. *British poultry science.* **59**(5), pp.486-493.

Bedford, M.R. and Partridge, G.G. 2010. *Enzymes in farm animal nutrition.* 2 ed.

Belenguer, A., Duncan, S.H., Holtrop, G., Anderson, S.E., Lobley, G.E. and Flint, H.J. 2007. Impact of pH on lactate formation and utilization by human fecal microbial communities. *Applied and environmental microbiology.* **73**(20), pp.6526-6533.

Bender, D.A. and Cunningham, S.M. 2021. *Introduction to nutrition and metabolism.* 6 ed. New York: CRC Press.

Berding, K., Wang, M., Monaco, M.H., Alexander, L.S., Mudd, A.T., Chichlowski, M., Waworuntu, R.V., Berg, B.M., Miller, M.J. and Dilger, R.N. 2016. Prebiotics and bioactive milk fractions affect gut development, microbiota, and neurotransmitter expression in piglets. *Journal of pediatric gastroenterology and nutrition.* **63**(6), pp.688-697.

Bergman, E. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological reviews.* **70**(2), pp.567-590.

Biddle, A., Stewart, L., Blanchard, J. and Leschine, S. 2013. Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. *Diversity.* **5**(3), pp.627-640.

Bischoff, S.C. 2011. 'Gut health': a new objective in medicine? *BMC medicine.* **9**(24), pp.1-14.

Black, J.L., Williams, B. and Gidley, M. 2009. Metabolic regulation of feed intake in monogastric mammals. *Voluntary feed intake in pigs.* Wageningen Academic Publishers Wageningen, pp.187-211.

Blake, A.B. 2017. *Fecal Lactate Concentrations in Dogs with Gastrointestinal Disease*. Master of science thesis, Texas A&M University.

Blaut, M. and Clavel, T. 2007. Metabolic diversity of the intestinal microbiota: implications for health and disease. *The Journal of nutrition.* **137**(3), pp.751S-755S.

Böhmer, B., Branner, G. and Roth-Maier, D. 2005. Precaecal and faecal digestibility of inulin (DP 10–12) or an inulin/Enterococcus faecium mix and effects on nutrient digestibility and microbial gut flora. *Journal of animal physiology and animal nutrition.* **89**(11-12), pp.388-396.

Bonder, M.J., Tigchelaar, E.F., Cai, X., Trynka, G., Cenit, M.C., Hrdlickova, B., Zhong, H., Vatanen, T., Gevers, D. and Wijmenga, C. 2016. The influence of a short-term gluten-free diet on the human gut microbiome. *Genome medicine.* **8**(45), pp.1-11.

Borody, T.J., Warren, E.F., Leis, S., Surace, R. and Ashman, O. 2003. Treatment of ulcerative colitis using fecal bacteriotherapy. *Journal of clinical gastroenterology.* **37**(1), pp.42-47.

Bosi, P., Casini, L., Finamore, A., Cremokolini, C., Merialdi, G., Trevisi, P., Nobili, F. and Mengheri, E. 2004. Spray-dried plasma improves growth performance and reduces inflammatory status of weaned pigs challenged with enterotoxigenic K88. *Journal of animal science.* **82**(6), pp.1764-1772.

Branner, G.R., Böhmer, B.M., Erhardt, W., Henke, J. and Roth-Maier, D.A. 2004. Investigation on the precaecal and faecal digestibility of lactulose and inulin and their influence on nutrient digestibility and microbial characteristics. *Archives of animal nutrition.* **58**(5), pp.353-366.

Brenes, A., Slominski, B., Marquardt, R., Guenter, W. and Viveros, A. 2003. Effect of enzyme addition on the digestibilities of cell wall polysaccharides and oligosaccharides from whole, dehulled, and ethanol-extracted white lupins in chickens. *Poultry Science.* **82**(11), pp.1716-1725.

Brestenský, M., Nitrayová, S., Bomba, A., Patráš, P., Strojný, L., Szabadošová, V., Pramuková, B. and Bertková, I. 2017. The content of short chain fatty acids in the jejunal digesta, caecal digesta and faeces of growing pigs. *Livestock Science.* **205**, pp.106-110.

Burfeind, O. and Heuwieser, W. 2012. Validation of handheld meters to measure blood L-lactate concentration in dairy cows and calves. *Journal of dairy science.* **95**(11), pp.6449-6456.

Cadogan, D.J. and Choct, M. 2015. Pattern of non-starch polysaccharide digestion along the gut of the pig: contribution to available energy. *Animal Nutrition.* **1**(3), pp.160-165.

Cai, C., Zhang, Z., Morales, M., Wang, Y., Khafipour, E. and Friel, J. 2019. Feeding practice influences gut microbiome composition in very low birth weight preterm infants and the association with oxidative stress: A prospective cohort study. *Free Radical Biology and Medicine.* **142**, pp.146-154.

Campbell, J.M., Crenshaw, J.D. and Polo, J. 2013. The biological stress of early weaned piglets. *Journal of animal science and biotechnology.* **4**(1), p19.

Campbell, Y., Fantacone, M.L. and Gombart, A.F. 2012. Regulation of antimicrobial peptide gene expression by nutrients and by-products of microbial metabolism. *European journal of nutrition.* **51**(8), pp.899-907.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K. and Gordon, J.I. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature methods.* **7**(5), pp.335-336.

Chan, L., Slater, J., Hasbargen, J., Herndon, D.N., Veech, R.L. and Wolf, S. 1994. Neurocardiac toxicity of racemic D, L-lactate fluids. *Integrative Physiological and Behavioral Science.* **29**(4), pp.383-394.

Chang, P.V., Hao, L., Offermanns, S. and Medzhitov, R. 2014. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proceedings of the National Academy of Sciences.* **111**(6), pp.2247-2252.

Chao, A. 1984. Nonparametric estimation of the number of classes in a population. *Scandinavian Journal of statistics.* **11**(4), pp.265-270.

Chen, L., Xu, Y., Chen, X., Fang, C., Zhao, L. and Chen, F. 2017. The maturing development of gut microbiota in commercial piglets during the weaning transition. *Frontiers in microbiology.* **8**, p1688.

Chen, Y., Xie, Y., Zhong, R., Liu, L., Lin, C., Xiao, L., Chen, L., Zhang, H., Beckers, Y. and Everaert, N. 2021. Effects of Xylo-Oligosaccharides on Growth and Gut Microbiota as Potential Replacements for Antibiotic in Weaning Piglets. *Frontiers in microbiology.* **12**, p355.

Cho, H.M., González-Ortiz, G., Melo-Durán, D., Heo, J.M., Cordero, G., Bedford, M.R. and Kim, J.C. 2020. Stimbiotic supplementation improved performance and reduced inflammatory response via stimulating fiber fermenting microbiome in weaner pigs housed in a poor sanitary environment and fed an antibiotic-free low zinc oxide diet. *Plos one.* **15**(11), pe0240264.

Choct, M. 1997. Feed non-starch polysaccharides: chemical structures and nutritional significance. *Feed milling international.* **191**(June issue), pp.13-26.

Choct, M., Hughes, R.J., Wang, J., Bedford, M., Morgan, A. and Annison, G. 1996. Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. *British poultry science.* **37**(3), pp.609-621.

Christakopoulos, P., Katapodis, P., Kalogeris, E., Kekos, D., Macris, B., Stamatis, H. and Skaltsa, H. 2003. Antimicrobial activity of acidic xylo-oligosaccharides produced by family 10 and 11 endoxylanases. *International Journal of Biological Macromolecules.* **31**(4-5), pp.171-175.

Chung, H. and Kasper, D.L. 2010. Microbiota-stimulated immune mechanisms to maintain gut homeostasis. *Current opinion in immunology.* **22**(4), pp.455-460.

Counotte, G., Prins, R., Janssen, R. and DeBie, M. 1981. Role of Megasphaera elsdenii in the fermentation of DL-[2-13C] lactate in the rumen of dairy cattle. *Appl. Environ. Microbiol.* **42**(4), pp.649-655.

Courtin, C.M., Broekaert, W.F., Swennen, K., Lescroart, O., Onagbesan, O., Buyse, J., Decuypere, E., Van de Wiele, T., Marzorati, M. and Verstraete, W. 2008. Dietary inclusion of wheat bran arabinoxylooligosaccharides induces beneficial nutritional effects in chickens. *Cereal chemistry.* **85**(5), pp.607-613.

Craig, A.D., Bedford, M.R., Hastie, P., Khattak, F. and Olukosi, O.A. 2019. The effect of carbohydrases or prebiotic oligosaccharides on growth performance, nutrient utilisation and development of small intestine and immune organs in broilers fed nutrient-adequate diets based on either wheat or barley. *Journal of the Science of Food and Agriculture.* **99**(7), pp.3246-3254.

Crittenden, R., Karppinen, S., Ojanen, S., Tenkanen, M., Fagerström, R., Mättö, J., Saarela, M., Mattila-Sandholm, T. and Poutanen, K. 2002. In vitro fermentation of cereal dietary fibre carbohydrates by probiotic and intestinal bacteria. *Journal of the Science of Food and Agriculture.* **82**(8), pp.781-789.

Csernus, B. and Czeglédi, L. 2020. Physiological, antimicrobial, intestine morphological, and immunological effects of fructooligosaccharides in pigs. *Archives Animal Breeding.* **63**(2), pp.325-335.

Cummings, J.H., Antoine, J.-M., Azpiroz, F., Bourdet-Sicard, R., Brandtzaeg, P., Calder, P.C., Gibson, G.R., Guarner, F., Isolauri, E. and Pannemans, D. 2004. PASSCLAIM 1—gut health and immunity. *European Journal of Nutrition.* **43**(2), pp.ii118-ii173.

Cummings, J.H., Macfarlane, G.T. and Englyst, H.N. 2001. Prebiotic digestion and fermentation. *The American journal of clinical nutrition.* **73**(2), pp.415s-420s.

Daly, K., Proudman, C.J., Duncan, S.H., Flint, H.J., Dyer, J. and Shirazi-Beechey, S.P. 2012. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. *British Journal of Nutrition.* **107**(7), pp.989-995.

Davani-Davari, D., Negahdaripour, M., Karimzadeh, I., Seifan, M., Mohkam, M., Masoumi, S.J., Berenjian, A. and Ghasemi, Y. 2019. Prebiotics: definition, types, sources, mechanisms, and clinical applications. *Foods.* **8**(3), p92.

De Figueiredo, F.C., De Barros Ranke, F.F. and De Oliva-Neto, P. 2020. Evaluation of xylooligosaccharides and fructooligosaccharides on digestive enzymes hydrolysis and as a nutrient for different probiotics and Salmonella typhimurium. *Lwt.* **118**, p108761.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S., Collini, S., Pieraccini, G. and Lionetti, P. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America.* **107**(33), pp.14691-14696.

De Leenheer, L. 1994. Production and use of inulin: industrial reality with a promising future. *Carbohydrates as organic raw materials.* **3**, pp.67-92.

de Vries, S. 2014. *Fiber fermentation in pigs and poultry: sense and nonsense of its manipulation*. PhD thesis, Wageningen University.

de Vries, S., Gerrits, W.J., Kabel, M.A., Vasanthan, T. and Zijlstra, R.T. 2016. β-Glucans and resistant starch alter the fermentation of recalcitrant fibers in growing pigs. *PLoS One.* **11**(12), pe0167624.

den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.-J. and Bakker, B.M. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of lipid research.* **54**(9), pp.2325-2340.

Department for Environment Food and Rural Affairs (DEFRA). 2019. *Agriculture in the United Kingdom.* [Online]. [Accessed 21st June 2021]. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/895106/AUK-2019-25jun20.pdf

Derikx, J.P., Luyer, M.D., Heineman, E. and Buurman, W.A. 2010. Non-invasive markers of gut wall integrity in health and disease. *World journal of gastroenterology: WJG.* **16**(42), pp.5272-5279.

Dou, S., Gadonna-Widehem, P., Rome, V., Hamoudi, D., Rhazi, L., Lakhal, L., Larcher, T., Bahi-Jaber, N., Pinon-Quintana, A. and Guyonvarch, A. 2017. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PloS one.* **12, 1**(1).

Duncan, S.H., Louis, P. and Flint, H.J. 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Applied and Environmental Microbiology.* **70**(10), pp.5810-5817.

Dunshea, F., Gannon, N., Van Barneveld, R., Mullan, B., Campbell, R. and King, R. 2001. Dietary lupins (Lupinus angustifolius and Lupinus albus) can increase digesta retention in the gastrointestinal tract of pigs. *Australian Journal of Agricultural Research.* **52**(5), pp.593-602.

Dunshea, F.R., Bittner, E.P., Pluske, J.R. and Black, J.L. 2018. Role of the gut, melanocortin system and malonyl-CoA in control of feed intake in non-ruminant animals. *Animal Production Science.* **58**(4), pp.627-639.

Englyst, H.N., Quigley, M.E. and Hudson, G.J. 1994. Determination of dietary fibre as non-starch polysaccharides with gas–liquid chromatographic, high-performance liquid chromatographic or spectrophotometric measurement of constituent sugars. *Analyst.* **119**(7), pp.1497-1509.

Englyst, K., Liu, S. and Englyst, H. 2007. Nutritional characterization and measurement of dietary carbohydrates. *European journal of clinical nutrition.* **61**(1), pp.S19-S39.

Estrada, A., Drew, M.D. and Van Kessel, A. 2001. Effect of the dietary supplementation of fructooligosaccharides and Bifidobacterium longum to early-weaned pigs on performance and fecal bacterial populations. *Canadian Journal of Animal Science.* **81**(1), pp.141-148.

Etheridge, R., Seerley, R. and Huber, T. 1984. The effect of diet on fecal moisture, osmolarity of fecal extracts, products of bacterial fermentation and loss of minerals in feces of weaned pigs. *Journal of Animal Science.* **58**(6), pp.1403-1411.

European Commisson. 2017. *Commission implementing decision concerning, in the framework of Article 35 of Directive 2001/82/EC of the European*

Parliament and of the Council, the marketing authorisations for veterinary medicinal

products containing "zinc oxide" to be administered orally to food producing species [Online]. [Accessed August]. Available from: https://ec.europa.eu/health/documents/community-register/2017/20170626136754/dec_136754_en.pdf

European Food Safety Authority (EFSA), Bampidis, V., Azimonti, G., Bastos, M.d.L., Christensen, H., Dusemund, B., Fašmon Durjava, M., Kouba, M., López-Alonso, M. and López Puente, S. 2021. Safety and efficacy of a feed additive consisting of titanium dioxide for all animal species (Titanium Dioxide Manufacturers Association). *EFSA Journal.* **19**(6), pe06630.

Ewaschuk, J.B., Naylor, J.M. and Zello, G.A. 2005. D-lactate in human and ruminant metabolism. *The Journal of nutrition.* **135**(7), pp.1619-1625.

Fang, Z.F., Peng, J., Liu, Z.L. and Liu, Y.G. 2007. Responses of non-starch polysaccharide-degrading enzymes on digestibility and performance of growing pigs fed a diet based on corn, soya bean meal and Chinese double-low rapeseed meal. *Journal of Animal Physiology and Animal Nutrition.* **91**(7-8), pp.361-368.

Farnworth, E., Modler, H., Jones, J., Cave, N., Yamazaki, H. and Rao, A. 1992. Feeding Jerusalem artichoke flour rich in fructooligosaccharides to weanling pigs. *Canadian Journal of Animal Science.* **72**(4), pp.977-980.

Fenna, L. and Boag, D. 1974. Filling and emptying of the galliform caecum. *Canadian journal of zoology.* **52**(4), pp.537-540.

Ferasin, L., Dodkin, S.J., Amodio, A., Murray, J.K. and Papasouliotis, K. 2007. Evaluation of a portable lactate analyzer (Lactate Scout) in dogs. *Veterinary clinical pathology*. **36**(1), pp.36-39.

Food and Agricultural Organisation (FAO). 2009. *How to feed the world in 2050.* [Online]. [Accessed June 2021]. Available from: http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf

Frese, S.A., Parker, K., Calvert, C.C. and Mills, D.A. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome*. **3**(28).

Frost, F., Kacprowski, T., Rühlemann, M., Pietzner, M., Bang, C., Franke, A., Nauck, M., Völker, U., Völzke, H. and Dörr, M. 2021. Long-term instability of the intestinal microbiome is associated with metabolic liver disease, low microbiota diversity, diabetes mellitus and impaired exocrine pancreatic function. *Gut.* **70**(3), pp.522-530.

Gaukroger, C.H., Stewart, C.J., Edwards, S.A., Walshaw, J., Adams, I.P. and Kyriazakis, I. 2020. Changes in faecal microbiota profiles associated with performance and birthweight of piglets. *Frontiers in microbiology*. **11**, p917.

Gerritsen, J. 2015. *The genus Romboutsia: genomic and functional characterization of novel bacteria dedicated to life in the intestinal tract*. PhD thesis, Wageningen University.

Gevers, D., Kugathasan, S., Denson, L.A., Vázquez-Baeza, Y., Van Treuren, W., Ren, B., Schwager, E., Knights, D., Song, S.J. and Yassour, M. 2014. The treatment-naive microbiome in new-onset Crohn's disease. *Cell host & microbe.* **15**(3), pp.382-392.

Ghoddusi, H., Grandison, M., Grandison, A. and Tuohy, K. 2007. In vitro study on gas generation and prebiotic effects of some carbohydrates and their mixtures. *Anaerobe.* **13**(5-6), pp.193-199.

Gibson, G.R., Probert, H.M., Van Loo, J., Rastall, R.A. and Roberfroid, M.B. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition research reviews.* **17**(2), pp.259-275.

Gibson, G.R. and Roberfroid, M.B. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of nutrition.* **125**(6), p1401.

González-Ortiz, G., Gomes, G., Dos Santos, T. and Bedford, M. 2019. Chapter 14 New strategies influencing gut functionality and animal performance. *The value of fibre: Engaging the second brain for animal nutrition.* Wageningen Academic Publishers, pp.19-26.

Goodwin, M.L., Harris, J.E., Hernández, A. and Gladden, L.B. 2007. Blood lactate measurements and analysis during exercise: a guide for clinicians. *Journal of diabetes science and technology.* **1**(4), pp.558-569.

Gräber, I., Hansen, J.F., Olesen, S.E., Petersen, J., Østergaard, H.S. and Krogh, L. 2005. Accumulation of copper and zinc in Danish agricultural soils in intensive pig production areas. *Geografisk Tidsskrift-Danish Journal of Geography.* **105**(2), pp.15-22.

Grela, E.R., Sobolewska, S., Kowalczuk-Vasilev, E. and Krasucki, W. 2014. Effect of dietary inulin source on piglet performance, immunoglobulin concentration, and plasma lipid profile. *Bulletin of the Veterinary Institute in Pulawy.* **58**(3), pp.453-458.

Gresse, R., Chaucheyras-Durand, F., Fleury, M.A., Van de Wiele, T., Forano, E. and Blanquet-Diot, S. 2017. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. *Trends in microbiology.* **25**(10), pp.851-873.

Gresse, R., Chaucheyras Durand, F., Dunière, L., Blanquet-Diot, S. and Forano, E. 2019. Microbiota composition and functional profiling throughout the gastrointestinal tract of commercial weaning piglets. *Microorganisms.* **25**(9), p343.

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R. and Van Immerseel, F. 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutrition research reviews.* **23**(2), pp.366-384.

Halas, D., Hansen, C.F., Hampson, D.J., Mullan, B.P., Wilson, R.H. and Pluske, J.R. 2009. Effect of dietary supplementation with inulin and/or benzoic acid on the incidence

and severity of post-weaning diarrhoea in weaner pigs after experimental challenge with enterotoxigenic Escherichia coli. *Archives of animal nutrition.* **63**(4), pp.267-280.

Hashizume, K., Tsukahara, T., Yamada, K., Koyama, H. and Ushida, K. 2003. Megasphaera elsdenii JCM1772T normalizes hyperlactate production in the large intestine of fructooligosaccharide-fed rats by stimulating butyrate production. *The Journal of nutrition.* **133**(10), pp.3187-3190.

He, G., Baidoo, S., Yang, Q., Golz, D. and Tungland, B. 2002. Evaluation of chicory inulin extracts as feed additive for early-weaned pigs. *J. Anim. Sci.* **80**(S1), p81.

Hernot, D.C., Boileau, T.W., Bauer, L.L., Middelbos, I.S., Murphy, M.R., Swanson, K.S. and Fahey Jr, G.C. 2009. In vitro fermentation profiles, gas production rates, and microbiota modulation as affected by certain fructans, galactooligosaccharides, and polydextrose. *Journal of Agricultural and Food Chemistry.* **57**(4), pp.1354-1361.

HM Government. 2019. *Tackling antimicrobial resistance 2019–2024.* [Online]. [Accessed June]. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/784894/UK_AMR_5_year_national_action_plan.pdf

Hogberg, A. and Lindberg, J.E. 2004. Influence of cereal non-starch polysaccharides on digestion site and gut environment in growing pigs. *Livestock Production Science.* **87**(2/3), pp.121-130.

Holman, D.B., Brunelle, B.W., Trachsel, J. and Allen, H.K. 2017. Meta-analysis to define a core microbiota in the swine gut. *mSystems.* **2**(3).

Horvat, S. and Rupnik, M. 2018. Interactions Between Clostridioides difficile and fecal Microbiota in In Vitro batch model: Growth, sporulation, and microbiota changes. *Frontiers in microbiology.* **9**, p1633.

Hoshi, S., Sakata, T., Mikuni, K., Hashimoto, H. and Kimura, S. 1994. Galactosylsucrose and xylosylfructoside alter digestive tract size and concentrations of cecal organic acids

in rats fed diets containing cholesterol and cholic acid. *The Journal of nutrition.* **124**(1), pp.52-60.

Houdijk, J. 1998. *Effects of non-digestible oligosaccharides in young pig diets*. PhD thesis, Wageningen University.

Houdijk, J., Bosch, M., Verstegen, M. and Berenpas, H. 1998. Effects of dietary oligosaccharides on the growth performance and faecal characteristics of young growing pigs. *Animal Feed Science and Technology.* **71**(1-2), pp.35-48.

Houdijk, J.G., Bosch, M.W., Tamminga, S., Verstegen, M.W., Berenpas, E.B. and Knoop, H. 1999. Apparent ileal and total-tract nutrient digestion by pigs as affected by dietary nondigestible oligosaccharides. *Journal of animal science.* **77**(1), pp.148-158.

Houdijk, J.G.M., Hartemink, R., Verstegen, M.W.A. and Bosch, M.W. 2002. Effects of dietary non-digestible oligosaccharides on microbial characteristics of ileal chyme and faeces in weaner pigs. *Archives of Animal Nutrition.* **56**(4), pp.297-307.

Hove, H., Nordgaard-Andersen, I. and Mortensen, P.B. 1994. Faecal DL-lactate concentration in 100 gastrointestinal patients. *Scandinavian journal of gastroenterology.* **29**(3), pp.255-259.

Howard, M.D., Gordon, D.T., Garleb, K.A. and Kerley, M.S. 1995. Dietary fructooligosaccharide, xylooligosaccharide and gum arabic have variable effects on cecal and colonic microbiota and epithelial cell proliferation in mice and rats. *The Journal of nutrition.* **125**(10), p2604.

Hsu, C.-K., Liao, J.-W., Chung, Y.-C., Hsieh, C.-P. and Chan, Y.-C. 2004. Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats. *Journal of nutrition.* **134**(6), pp.1523-1528.

Hu, J., Nie, Y., Chen, J., Zhang, Y., Wang, Z., Fan, Q. and Yan, X. 2016. Gradual changes of gut microbiota in weaned miniature piglets. *Frontiers in microbiology.* **7**, p1727.

Hughes, R. and Rowland, I. 2001. Stimulation of apoptosis by two prebiotic chicory fructans in the rat colon. *Carcinogenesis.* **22**(1), pp.43-47.

Isaacson, R. and Kim, H.B. 2012. The intestinal microbiome of the pig. *Animal Health Research Reviews.* **13**(1), pp.100-109.

Ivarsson, E., Roos, S., Liu, H. and Lindberg, J. 2014. Fermentable non-starch polysaccharides increases the abundance of Bacteroides–Prevotella–Porphyromonas in ileal microbial community of growing pigs. *Animal.* **8**(11), pp.1777-1787.

Jagger, S., Wiseman, J., Cole, D. and Craigon, J. 1992. Evaluation of inert markers for the determination of ileal and faecal apparent digestibility values in the pig. *British journal of nutrition.* **68**(3), pp.729-739.

Jamroz, D., Jakobsen, K., Knudsen, K.E.B., Wiliczkiewicz, A. and Orda, J. 2002. Digestibility and energy value of non-starch polysaccharides in young chickens, ducks and geese, fed diets containing high amounts of barley. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology.* **131**(3), pp.657-668.

Jandhyala, S.M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M. and Reddy, D.N. 2015. Role of the normal gut microbiota. *World journal of gastroenterology: WJG.* **21**(29), p8787.

Jha, R. and Berrocoso, J. 2015. Dietary fiber utilization and its effects on physiological functions and gut health of swine. *Animal.* **9**(9), pp.1441-1452.

Johansen, H.N., Glitsø, V. and Bach Knudsen, K.E. 1996. Influence of extraction solvent and temperature on the quantitative determination of oligosaccharides from plant materials by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry.* **44**(6), pp.1470-1474.

Joy, S.R., Bartelt-Hunt, S.L., Snow, D.D., Gilley, J.E., Woodbury, B.L., Parker, D.B., Marx, D.B. and Li, X. 2013. Fate and transport of antimicrobials and antimicrobial resistance genes in soil and runoff following land application of swine manure slurry. *Environmental science & technology.* **47**(21), pp.12081-12088.

Kaneko, T., Bando, Y., Kurihara, H., Satomi, K., Nonoyama, K. and Matsuura, N. 1997. Fecal microflora in a patient with short-bowel syndrome and identification of dominant lactobacilli. *Journal of clinical microbiology.* **35**(12), pp.3181-3185.

Katapodis, P. and Christakopoulos, P. 2005. Xylanases as a tool for the production of novel phytopharmaceuticals. *NutraCos.* **4**(2), pp.17-21.

Kavanagh, S., Lynch, P., O'mara, F. and Caffrey, P. 2001. A comparison of total collection and marker technique for the measurement of apparent digestibility of diets for growing pigs. *Animal feed science and technology.* **89**(1-2), pp.49-58.

Kaynar, O., Karapinar, T., Hayirli, A. and Baydar, E. 2015. Reliability of the Lactate Scout point-of-care instrument for the determination of blood l-lactate concentration in sheep. *Veterinary clinical pathology.* **44**(4), pp.559-563.

Kim, B., Lindemann, M., Cromwell, G., Balfagon, A. and Agudelo, J. 2007. The correlation between passage rate of digesta and dry matter digestibility in various stages of swine. *Livestock Science.* **109**(1-3), pp.81-84.

Kim, B.G., Lee, S.A., Park, K.R. and Stein, H.H. 2020. At least 3 days of adaptation are required before indigestible markers (chromium, titanium, and acid insoluble ash) are stabilized in the ileal digesta of 60-kg pigs, but values for amino acid digestibility are affected by the marker. *Journal of animal science.* **98**(2), pskaa027.

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J. and Isaacson, R.E. 2011. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Veterinary microbiology.* **153**(1-2), pp.124-133.

Kim, H.B. and Isaacson, R.E. 2015. The pig gut microbial diversity: understanding the pig gut microbial ecology through the next generation high throughput sequencing. *Veterinary microbiology.* **177**(3-4), pp.242-251.

Kim, J., Hansen, C.F., Mullan, B. and Pluske, J. 2012. Nutrition and pathology of weaner pigs: nutritional strategies to support barrier function in the gastrointestinal tract. *Animal feed science and technology.* **173**(1), pp.3-16.

Kong, C. and Adeola, O. 2014. Evaluation of amino acid and energy utilization in feedstuff for swine and poultry diets. *Asian-Australasian Journal of Animal Sciences.* **27**(7), p917.

Konopka, A. 2009. What is microbial community ecology? *The ISME journal.* **3**(11), pp.1223-1230.

Kowlgi, N.G. and Chhabra, L. 2015. D-lactic acidosis: an underrecognized complication of short bowel syndrome. *Gastroenterology research and practice.* **2015**.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. and Schloss, P.D. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miSeq illumina sequencing platform. *Applied and environmental microbiology*. **79**(17), pp.5112-5120.

Kumprecht, I. and Zobac, P. 1998. The effect of a combined preparation containing Enterococcus faecium M-74 and mannan-oligosaccharides in diets for weaning piglets. *Animal Science-UZPI (Czech Republic).*

Lagkouvardos, I., Lesker, T.R., Hitch, T.C., Gálvez, E.J., Smit, N., Neuhaus, K., Wang, J., Baines, J.F., Abt, B. and Stecher, B. 2019. Sequence and cultivation study of Muribaculaceae reveals novel species, host preference, and functional potential of this yet undescribed family. *Microbiome.* **7**(28).

Lan, R., Li, T. and Kim, I. 2017. Effects of xylanase supplementation on growth performance, nutrient digestibility, blood parameters, fecal microbiota, fecal score and fecal noxious gas emission of weaning pigs fed corn-soybean meal-based diet. *Animal Science Journal* **88**(9), pp.1398-1405.

Le Sciellour, M., Labussière, E., Zemb, O. and Renaudeau, D. 2018. Effect of dietary fiber content on nutrient digestibility and fecal microbiota composition in growing-finishing pigs. *PLoS One.* **13**(10), pe0206159.

Li, Y. 2017. *Effects of Prebiotics and Probiotics on Gut Health, Immunity, and Growth of Weanling Pigs*. PhD thesis, University of Nebraska - Lincoln.

Liu, J., Cao, S., Liu, J., Xie, Y. and Zhang, H. 2018. Effect of probiotics and xylo-oligosaccharide supplementation on nutrient digestibility, intestinal health and noxious gas emission in weanling pigs. *Asian-Australasian journal of animal sciences.* **31**(10), pp.1660-1669.

Liu, L., Chen, D., Yu, B., Yin, H., Huang, Z., Luo, Y., Zheng, P., Mao, X., Yu, J. and Luo, J. 2020. Fructooligosaccharides improve growth performance and intestinal epithelium function in weaned pigs exposed to enterotoxigenic Escherichia coli. *Food & Function.* **11**(11), pp.9599-9612.

Liu, P., Piao, X., Kim, S., Wang, L., Shen, Y., Lee, H. and Li, S. 2008. Effects of chito-oligosaccharide supplementation on the growth performance, nutrient digestibility, intestinal morphology, and fecal shedding of Escherichia coli and Lactobacillus in weaning pigs. *Journal of animal science.* **86**(10), pp.2609-2618.

Liu, S.-Q. 2003. Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. *International journal of food microbiology.* **83**(2), pp.115-131.

Liying, Z., Li, D., Qiao, S., Johnson, E., Li, B., Thacker, P. and Han, I.K. 2003. Effects of stachyose on performance, diarrhoea incidence and intestinal bacteria in weanling pigs. *Archives of Animal Nutrition.* **57**(1), pp.1-10.

Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul, W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., Hashsham, S.A., Tiedje, J.M. and Stanton, T.B. 2012. In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences of the United States of America.* **109**(5), pp.1691-1696.

Lu, H., Yan, H., Masey O'Neill, H., Bradley, C., Bedford, M., Wilcock, P., Nakatsu, C., Adeola, O. and Ajuwon, K. 2019. Effect of timing of postweaning xylanase supplementation on growth performance, nutrient digestibility, and fecal microbial composition in weanling pigs. *Canadian Journal of Animal Science.* **100**(1), pp.27-36.

Macfarlane, S., Macfarlane, G. and Cummings, J.t. 2006. Prebiotics in the gastrointestinal tract. *Alimentary pharmacology & therapeutics.* **24**(5), pp.701-714.

Magoč, T. and Salzberg, S.L. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics.* **27**(21), pp.2957-2963.

Marinho, M.C., Lordelo, M.M., Cunha, L.F. and Freire, J.P.B. 2007. Microbial activity in the gut of piglets: I. Effect of prebiotic and probiotic supplementation. *Livestock Science.* **108**(1-3), pp.236-239.

Martínez-Villaluenga, C., Frias, J. and Vidal-Valverde, C. 2008. Alpha-galactosides: antinutritional factors or functional ingredients? *Critical Reviews in Food Science and Nutrition.* **48**(4), pp.301-316.

Masey O'Neill, H.V., Smith, J.A. and Bedford, M.R. 2014. Multicarbohydrase enzymes for non-ruminants. *Asian-Australasian Journal of Animal Sciences.* **27**(2), pp.290-301.

Mathew, A., Chattin, S., Robbins, C. and Golden, D. 1998. Effects of a direct-fed yeast culture on enteric microbial populations, fermentation acids, and performance of weanling pigs. *Journal of animal science.* **76**(8), pp.2138-2145.

Mayeur, C., Gratadoux, J.-J., Bridonneau, C., Chegdani, F., Larroque, B., Kapel, N., Corcos, O., Thomas, M. and Joly, F. 2013. Faecal D/L lactate ratio is a metabolic signature of microbiota imbalance in patients with short bowel syndrome. *PLoS One.* **8**(1), pe54335.

McMurdie, P.J. and Holmes, S. 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one.* **8**(4).

Meyer, J., Hlinka, M., Khatibi, A., Raybould, H.E. and Tso, P. 1998. Role of small intestine in caloric compensations to oil premeals in rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology.* **275**(4), pp.R1320-R1333.

Milani, N., Sbardella, M., Ikeda, N., Arno, A., Mascarenhas, B. and Miyada, V. 2017. Dietary zinc oxide nanoparticles as growth promoter for weanling pigs. *Animal Feed Science and Technology.* **227**, pp.13-23.

Moeser, A.J., Pohl, C.S. and Rajput, M. 2017. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. *Animal Nutrition.* **3**(4), pp.313-321.

Moore, K.L., Loudon, E. and Dunshea, F.R. 2021. Increasing the Dietary Concentration of Lupinus albus L. Decreased Feed Intake and Daily Gain of Immunocastrated Male Pigs. *Animals.* **11**(7), p1866.

Morgan, N., Gomes, G. and Kim, J. 2021. Comparing the efficacy of stimbiotic and a combination of xylanase and beta-glucanase, in broilers fed wheat-barley based diets with high or low AME. *Poultry Science.* p101383.

Morgan, N.K., Keerqin, C., Wallace, A., Wu, S.-B. and Choct, M. 2019. Effect of arabinoxylo-oligosaccharides and arabinoxylans on net energy and nutrient utilization in broilers. *Animal Nutrition.* **5**(1), pp.56-62.

Morgan, N.K., Keerqin, C., Wu, S.B. and Choct, M. 2017. Effect of arabinoxylans and xylo-oligosachharides on net energy in broilers. *Australian Poultry Science Symposium.* **28**, pp.120-123.

Mountzouris, K., Xypoleas, I., Kouseris, I. and Fegeros, K. 2006. Nutrient digestibility, faecal physicochemical characteristics and bacterial glycolytic activity of growing pigs fed a diet supplemented with oligofructose or trans-galactooligosaccharides. *Livestock Science.* **105**(1-3), pp.168-175.

Mountzouris, K.C., Balaskas, C., Fava, F., Tuohy, K.M., Gibson, G.R. and Fegeros, K. 2006. Profiling of composition and metabolic activities of the colonic microflora of growing pigs fed diets supplemented with prebiotic oligosaccharides. *Anaerobe.* **12**(4), pp.178-185.

Moura, P., Barata, R., Carvalheiro, F., Gírio, F., Loureiro-Dias, M.C. and Esteves, M.P. 2007. In vitro fermentation of xylo-oligosaccharides from corn cobs autohydrolysis by Bifidobacterium and Lactobacillus strains. *LWT-Food Science and Technology.* **40**(6), pp.963-972.

Moura, P., Cabanas, S., Lourenco, P., Girio, F., Loureiro-Dias, M.C. and Esteves, M.P. 2008. In vitro fermentation of selected xylo-oligosaccharides by piglet intestinal microbiota. *Lwt-Food Science and Technology.* **41**(10), pp.1952-1961.

Muñoz-Tamayo, R., Laroche, B., Walter, É., Doré, J., Duncan, S.H., Flint, H.J. and Leclerc, M. 2011. Kinetic modelling of lactate utilization and butyrate production by key human colonic bacterial species. *FEMS microbiology ecology.* **76**(3), pp.615-624.

Mussatto, S.I. and Mancilha, I.M. 2007. Non-digestible oligosaccharides: A review. *Carbohydrate Polymers.* **68**(3), pp.587-597.

Nabarlatz, D., Ebringerová, A. and Montané, D. 2007. Autohydrolysis of agricultural by-products for the production of xylo-oligosaccharides. *Carbohydrate Polymers.* **69**(1), pp.20-28.

Newman, M.A., Petri, R.M., Grüll, D., Zebeli, Q. and Metzler-Zebeli, B.U. 2018. Transglycosylated starch modulates the gut microbiome and expression of genes related to lipid synthesis in liver and adipose tissue of pigs. *Frontiers in microbiology.* **9**, p224.

NRC. National Research Council. 2012. *Nutrient requirements of Swine.* National Academies Press, Washington DC, USA.

O'Neill, J. 2016. *Tackling drug-resistant infections globally: final report and recommendations.* [Online]. [Accessed August]. Available from: https://apo.org.au/node/63983

Oberleas, D., Li, Y.-C., Stoecker, B.J., Henley, S.A., Keim, K.S. and Smith Jr, J.C. 1990. The rate of chromium transit through the gastrointestinal tract. *Nutrition Research.* **10**(10), pp.1189-1194.

Olukosi, O., Bolarinwa, O., Cowieson, A. and Adeola, O. 2012. Marker type but not concentration influenced apparent ileal amino acid digestibility in phytase-supplemented diets for broiler chickens and pigs. *Journal of animal science.* **90**(12), pp.4414-4420.

Olukosi, O.A., Bedford, M.R. and Adeola, O. 2007. Xylanase in diets for growing pigs and broiler chicks. *Canadian journal of animal science. Ottawa ON.* **87**(2), pp.227-235.

Olukosi, O.A., Beeson, L., Englyst, K. and Romero, L.F. 2015. Effects of exogenous proteases without or with carbohydrases on nutrient digestibility and disappearance of non-starch polysaccharides in broiler chickens. *Poultry Science.* **94**(11).

Ormerod, K.L., Wood, D.L., Lachner, N., Gellatly, S.L., Daly, J.N., Parsons, J.D., Dal'Molin, C.G., Palfreyman, R.W., Nielsen, L.K. and Cooper, M.A. 2016. Genomic characterization of the uncultured bacteroidales family S24-7 inhabiting the guts of homeothermic animals. *Microbiome.* **4**(36).

Pajarillo, E.A.B., Chae, J.-P., Balolong, M.P., Kim, H.B. and Kang, D.-K. 2014. Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. *Journal of General and Applied Microbiology.* **60**(4), pp.140-146.

Pan, J., Yin, J., Zhang, K., Xie, P., Ding, H., Huang, X., Blachier, F. and Kong, X. 2019. Dietary xylo-oligosaccharide supplementation alters gut microbial composition and activity in pigs according to age and dose. *AMB Express.* **9**(134).

Pang, D.S. and Boysen, S. 2007. Lactate in veterinary critical care: pathophysiology and management. *Journal of the American Animal Hospital Association.* **43**(5), pp.270-279.

Parada, A.E., Needham, D.M. and Fuhrman, J.A. 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental microbiology.* **18**(5), pp.1403-1414.

Paßlack, N., Al-Samman, M., Vahjen, W., Männer, K. and Zentek, J. 2012. Chain length of inulin affects its degradation and the microbiota in the gastrointestinal tract of weaned piglets after a short-term dietary application. *Livestock Science.* **149**(1-2), pp.128-136.

Passos, A.A., Park, I., Ferket, P., von Heimendahl, E. and Kim, S.W. 2015. Effect of dietary supplementation of xylanase on apparent ileal digestibility of nutrients, viscosity of digesta, and intestinal morphology of growing pigs fed corn and soybean meal based diet. *Animal Nutrition.* **1**(1), pp.19-23.

Penning, P. and Johnson, R. 1983. The use of internal markers to estimate herbage digestibility and intake: 1. Potentially indigestible cellulose and acid insoluble ash. *The Journal of Agricultural Science.* **100**(1), pp.127-131.

Petry, A.L. and Patience, J.F. 2020. Xylanase supplementation in corn-based swine diets: a review with emphasis on potential mechanisms of action. *Journal of Animal Science.* **98**(11), pskaa318.

Pluske, J.R., Hampson, D.J. and Williams, I.H. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livestock production science.* **51**(1-3), pp.215-236.

Pluske, J.R., Thompson, M.J., Atwood, C.S., Bird, P.H., Williams, I.H. and Hartmann, P.E. 1996. Maintenance of villus height and crypt depth, and enhancement of disaccharide digestion and monosaccharide absorption, in piglets fed on cows' whole milk after weaning. *British Journal of Nutrition.* **76**(3), pp.409-422.

Pourabedin, M., Chen, Q.L., Yang, M.M. and Zhao, X. 2017. Mannan- and xylooligosaccharides modulate caecal microbiota and expression of inflammatory-related cytokines and reduce caecal Salmonella Enteritidis colonisation in young chickens. *Fems Microbiology Ecology.* **93**(1).

Prawirodigdo, S., Gannon, N.J., Leury, B.J. and Dunshea, F.R. 2019. Basal diet and indigestible marker influence apparent digestibilities of nitrogen and amino acids of cottonseed meal and soybean meal in pigs. *Animal Nutrition.* **5**(3), pp.234-240.

Prawirodigdo, S., Gannon, N.J., Leury, B.J. and Dunshea, F.R. 2021. Acid-insoluble ash is a better indigestible marker than chromic oxide to measure apparent total tract digestibility in pigs. *Animal Nutrition.* **7**(1), pp.64-71.

Propst, E.L., Flickinger, E., Bauer, L., Merchen, N. and Fahey Jr, G. 2003. A dose-response experiment evaluating the effects of oligofructose and inulin on nutrient digestibility, stool quality, and fecal protein catabolites in healthy adult dogs. *Journal of animal science.* **81**(12), pp.3057-3066.

Quan, J., Cai, G., Yang, M., Zeng, Z., Ding, R., Wang, X., Zhuang, Z., Zhou, S., Li, S., Yang, H., Li, Z., Zheng, E., Huang, W., Yang, J. and Wu, Z. 2019. Exploring the fecal microbial composition and metagenomic functional capacities associated with feed efficiency in commercial DLY pigs. *Frontiers in Microbiology.* **10**(52).

Responsible use of medicines in agriculture alliance (RUMA). 2020. *Targets task force report 2020.* [Online]. [Accessed June]. Available from: https://www.ruma.org.uk/wp-content/uploads/2020/11/SO-469-RUMA-REPORT-021220.pdf

Ribeiro, T., Cardoso, V., Ferreira, L., Lordelo, M., Coelho, E., Moreira, A., Domingues, M., Coimbra, M., Bedford, M. and Fontes, C. 2018. Xylo-oligosaccharides display a prebiotic activity when used to supplement wheat or corn-based diets for broilers. *Poultry science.* **97**(12), pp.4330-4341.

Ritalahti, K.M., Justicia-Leon, S.D., Cusick, K.D., Ramos-Hernandez, N., Rubin, M., Dornbush, J. and Löffler, F.E. 2012. Sphaerochaeta globosa gen. nov., sp. nov. and Sphaerochaeta pleomorpha sp. nov., free-living, spherical spirochaetes. *International journal of systematic and evolutionary microbiology.* **62**(1), pp.210-216.

Roberfroid, M.B. and Delzenne, N.M. 1998. Dietary fructans. *Annual review of nutrition.* **18**(1), pp.117-143.

Roberfroid, M.B., Van Loo, J.A. and Gibson, G.R. 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. *The Journal of nutrition.* **128**(1), pp.11-19.

Rocha, L., Dionne, A., Saucier, L., Nannoni, E. and Faucitano, L. 2015. Hand-held lactate analyzer as a tool for the real-time measurement of physical fatigue before slaughter and pork quality prediction. *animal.* **9**(4), pp.707-714.

Rul, F., Ben-Yahia, L., Chegdani, F., Wrzosek, L., Thomas, S., Noordine, M.-L., Gitton, C., Cherbuy, C., Langella, P. and Thomas, M. 2011. Impact of the metabolic activity of Streptococcus thermophilus on the colon epithelium of gnotobiotic rats. *Journal of Biological Chemistry.* **286**(12), pp.10288-10296.

Rycroft, C., Jones, M., Gibson, G.R. and Rastall, R. 2001. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *Journal of applied microbiology*. **91**(5), pp.878-887.

Sako, T., Matsumoto, K. and Tanaka, R. 1999. Recent progress on research and applications of non-digestible galacto-oligosaccharides. *International Dairy Journal.* **9**(1), pp.69-80.

Sangeetha, P., Ramesh, M. and Prapulla, S. 2005. Recent trends in the microbial production, analysis and application of fructooligosaccharides. *Trends in food science & technology*. **16**(10), pp.442-457.

Santos, A., San Mauro, M. and Díaz, D.M. 2006. Prebiotics and their long-term influence on the microbial populations of the mouse bowel. *Food Microbiology*. **23**(5), pp.498-503.

SAS Institute Inc. 2020. Cary, NC, 1989-2019.

Saunders, D. and Sillery, J. 1982. Effect of lactate and H+ on structure and function of rat intestine. *Digestive diseases and sciences.* **27**(1), pp.33-41.

Shang, Y., Kumar, S., Thippareddi, H. and Kim, W.K. 2018. Effect of dietary fructooligosaccharide (FOS) supplementation on ileal microbiota in broiler chickens. *Poultry science.* **97**(10), pp.3622-3634.

Shannon, C.E. 1948. A mathematical theory of communication. *Bell system technical journal*. **27**(3), pp.379-423.

Sheedy, J.R., Wettenhall, R.E., Scanlon, D., Gooley, P.R., Lewis, D.P., Mcgregor, N., Stapleton, D.I., Butt, H.L. and De Meirleir, K.L. 2009. Increased d-lactic acid intestinal bacteria in patients with chronic fatigue syndrome. *in vivo.* **23**(4), pp.621-628.

Shevkani, K., Singh, N., Bajaj, R. and Kaur, A. 2017. Wheat starch production, structure, functionality and applications—a review. *International Journal of Food Science & Technology*. **52**(1), pp.38-58.

Shim, S. 2005. *Effects of prebiotics, probiotics and synbiotics in the diet of young pigs*. PhD thesis, Wageningen University.

Shimomura, Y. and Sato, H. 2006. Fecal D-and L-lactate, succinate, and volatile fatty acid levels in young dairy calves. *Journal of veterinary medical science.* **68**(9), pp.973-977.

Short, F., Gorton, P., Wiseman, J. and Boorman, K. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. *Animal feed science and technology.* **59**(4), pp.215-221.

Shukla, R., Ghoshal, U., Dhole, T.N. and Ghoshal, U.C. 2015. Fecal microbiota in patients with irritable bowel syndrome compared with healthy controls using real-time polymerase chain reaction: an evidence of dysbiosis. *Digestive diseases and sciences.* **60**(10), pp.2953-2962.

Smiricky-Tjardes, M., Flickinger, E., Grieshop, C., Bauer, L., Murphy, M. and Fahey, G. 2003a. In vitro fermentation characteristics of selected oligosaccharides by swine fecal microflora. *Journal of animal science.* **81**(10), pp.2505-2514.

Smiricky-Tjardes, M., Grieshop, C., Flickinger, E., Bauer, L. and Fahey Jr, G. 2003b. Dietary galactooligosaccharides affect ileal and total-tract nutrient digestibility, ileal and fecal bacterial concentrations, and ileal fermentative characteristics of growing pigs. *Journal of animal science.* **81**(10), pp.2535-2545.

Smith, N.W., Shorten, P.R., Altermann, E., Roy, N.C. and McNabb, W.C. 2019. The classification and evolution of bacterial cross-feeding. *Frontiers in Ecology and Evolution.* **7**, p153.

Stewart, M.L., Timm, D.A. and Slavin, J.L. 2008. Fructooligosaccharides exhibit more rapid fermentation than long-chain inulin in an in vitro fermentation system. *Nutrition Research.* **28**(5), pp.329-334.

Stiles, M.E. and Holzapfel, W.H. 1997. Lactic acid bacteria of foods and their current taxonomy. *International journal of food microbiology.* **36**(1), pp.1-29.

Su, X.-L., Tian, Q., Zhang, J., Yuan, X.-Z., Shi, X.-S., Guo, R.-B. and Qiu, Y.-L. 2014. Acetobacteroides hydrogenigenes gen. nov., sp. nov., an anaerobic hydrogen-producing bacterium in the family Rikenellaceae isolated from a reed swamp. *International journal of systematic and evolutionary microbiology.* **64**(9), pp.2986-2991.

Sun, Y., Zhou, L., Fang, L., Su, Y. and Zhu, W. 2015. Responses in colonic microbial community and gene expression of pigs to a long-term high resistant starch diet. *Frontiers in microbiology.* **6**, p877.

Suo, H.-q., Lu, L., Xu, G.-h., Xiao, L., Chen, X.-g., Xia, R.-r., Zhang, L.-y. and Luo, X.-g. 2015. Effectiveness of dietary xylo-oligosaccharides for broilers fed a conventional corn-soybean meal diet. *Journal of Integrative Agriculture.* **14**(10), pp.2050-2057.

Swanson, K.S., Grieshop, C.M., Flickinger, E.A., Bauer, L.L., Healy, H.-P., Dawson, K.A., Merchen, N.R. and Fahey Jr, G.C. 2002. Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. *The Journal of nutrition.* **132**(5), pp.980-989.

Tan, Z., Wang, Y., Yang, T., Ao, H., Chen, S., Xing, K., Zhang, F., Zhao, X., Liu, J. and Wang, C. 2018. Differences in gut microbiota composition in finishing Landrace pigs with low and high feed conversion ratios. *Antonie Van Leeuwenhoek.* **111**(9), pp.1673-1685.

Thompson, C.L., Wang, B. and Holmes, A.J. 2008. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *The ISME Journal.* **2**, pp.739-748.

Tian, S., Wang, J., Yu, H., Wang, J. and Zhu, W. 2019. Changes in Ileal Microbial Composition and Microbial Metabolism by an Early-Life Galacto-Oligosaccharides Intervention in a Neonatal Porcine Model. *Nutrients.* **11**(8), p1753.

Trachsel, J.M. 2017. Defining and Modulating the Butyrate-Producing Microbial Community in the Swine Gut. *Graduate Theses and Dissertations. 16529.*

Tsukahara, T. and Ushida, K. 2001. Organic acid profiles in feces of pigs with pathogenic or non-pathogenic diarrhea. *Journal of veterinary medical science.* **63**(12), pp.1351-1354.

Tsurugizawa, T., Uematsu, A., Nakamura, E., Hasumura, M., Hirota, M., Kondoh, T., Uneyama, H. and Torii, K. 2009. Mechanisms of neural response to gastrointestinal nutritive stimuli: the gut-brain axis. *Gastroenterology.* **137**(1), pp.262-273.

Turnbaugh, P.J. 2017. Microbes and diet-induced obesity: fast, cheap, and out of control. *Cell host & microbe.* **21**(3), pp.278-281.

Umesaki, Y., Yajima, T., Yokokura, T. and Mutai, M. 1979. Effect of organic acid absorption on bicarbonate transport in rat colon. *Pflügers Archiv.* **379**(1), pp.43-47.

Umu, Ö.C.O., Frank, J.A., Fangel, J.U., Oostindjer, M., da Silva, C.S., Bolhuis, E.J., Bosch, G., Willats, W.G.T., Pope, P.B. and Diep, D.B. 2015. Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations. *Microbiome.* **3**(16).

Ushida, K. and Hoshi, S. 2002. 13 C-NMR studies on lactate metabolism in a porcine gut microbial ecosystem. *Microbial ecology in health and disease.* **14**(4), pp.242-247.

Ushida, K. and Sakata, T. 1998. Effect of pH on oligosaccharide fermentation by porcine cecal digesta. *Animal science and technology.* **69**(2), pp.100-107.

Valeriano, V., Balolong, M. and Kang, D.K. 2017. Probiotic roles of Lactobacillus sp. in swine: insights from gut microbiota. *Journal of applied microbiology.* **122**(3), pp.554-567.

Van Avesaat, M., Troost, F., Ripken, D., Hendriks, H. and Masclee, A. 2015. Ileal brake activation: macronutrient-specific effects on eating behavior? *International Journal of Obesity.* **39**(2), pp.235-243.

Van De Wiele, T., Boon, N., Possemiers, S., Jacobs, H. and Verstraete, W. 2007. Inulin-type fructans of longer degree of polymerization exert more pronounced in vitro prebiotic effects. *Journal of Applied Microbiology.* **102**(2), pp.452-460.

Van der Klis, J. and Van Voorst, A. 1993. The effect of carboxy methyl cellulose (a soluble polysaccharide) on the rate of marker excretion from the gastrointestinal tract of broilers. *Poultry Science.* **72**(3), pp.503-512.

Vanni, M., Merenda, M., Barigazzi, G., Garbarino, C., Luppi, A., Tognetti, R. and Intorre, L. 2012. Antimicrobial resistance of Actinobacillus pleuropneumoniae isolated from swine. *Veterinary microbiology.* **156**(1-2), pp.172-177.

Vardakou, M., Palop, C.N., Christakopoulos, P., Faulds, C.B., Gasson, M.A. and Narbad, A. 2008. Evaluation of the prebiotic properties of wheat arabinoxylan fractions and induction of hydrolase activity in gut microflora. *International journal of food microbiology.* **123**(1-2), pp.166-170.

Vazquez, M., Alonso, J., Domınguez, H. and Parajo, J. 2000. Xylooligosaccharides: manufacture and applications. *Trends in Food Science & Technology.* **11**(11), pp.387-393.

Vella, A. and Farrugia, G. 1998. D-lactic acidosis: pathologic consequence of saprophytism. *Mayo Clinic Proceedings.* **73**(5), pp.451-456.

Vernia, P., Caprilli, R., Latella, G., Barbetti, F., Magliocca, F.M. and Cittadini, M. 1988. Fecal lactate and ulcerative colitis. *Gastroenterology.* **95**(6), pp.1564-1568.

Walters, W.A., Xu, Z. and Knight, R. 2014. Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS letters.* **588**(22), pp.4223-4233.

Wang, J., Sun, B., Cao, Y. and Wang, C. 2010. In vitro fermentation of xylooligosaccharides from wheat bran insoluble dietary fiber by Bifidobacteria. *Carbohydrate polymers.* **82**(2), pp.419-423.

Williams, B.A., Grant, L.J., Gidley, M.J. and Mikkelsen, D. 2017. Gut fermentation of dietary fibres: physico-chemistry of plant cell walls and implications for health. *International journal of molecular sciences.* **18**(10), p2203.

Xin, J.W., Chai, Z., Zhang, C., Cao, H., Zhu, Y., Zhang, Q., Zhong, J. and Ji, Q. 2019. Comparing the microbial community in four stomach of dairy cattle, yellow cattle and three yak herds in Qinghai-Tibet Plateau. *Frontiers in microbiology.* **10**.

Xu, C., Chen, X., Ji, C., Ma, Q. and Hao, K. 2005. Study of the application of fructooligosaccharides in piglets. *Asian-australasian journal of animal sciences.* **18**(7), pp.1011-1016.

Xu, Z.-r. and Hu, C.-h. 2003. Effects of Fructooligosaccharide on Growth Performance, Intestinal Micro flora and Immune Function in Finishing Pigs [J]. *Chinese Journal of Veterinary Science.* **1**.

Xu, Z., Hu, C., Xia, M., Zhan, X. and Wang, M. 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. *Poultry science.* **82**(6), pp.1030-1036.

Xu, Z.R., Zou, X.T., Hu, C.H., Xia, M.S., Zhan, X.A. and Wang, M.Q. 2002. Effects of Dietary Fructooligosaccharide on Digestive Enzyme Activities, Intestinal Microflora and Morphology of Growing Pigs. *Asian-Austr. J. Anim. Sci.* **15**(12), pp.1784-1789.

Yamanaka, T., Sugimori, C., Furukawa, T., Mashimo, C., Yoshida, M., Baba, T., Matsuura, O., Mizukawa, K., Ichii, M. and Sumita, Y. 2006. Viscous material production by clinical isolates of Prevotella intermedia under ethanol and salt stress. *Journal of Osaka Dental University.* **40**(2), pp.113-118.

Yang, F., Hou, C., Zeng, X. and Qiao, S. 2015. The use of lactic acid bacteria as a probiotic in swine diets. *Pathogens.* **4**(1), pp.34-45.

Yang, R., Xu, S., Wang, Z. and Yang, W. 2005. Aqueous extraction of corncob xylan and production of xylooligosaccharides. *LWT-Food Science and Technology.* **38**(6), pp.677-682.

Yazdankhah, S., Rudi, K. and Bernhoft, A. 2014. Zinc and copper in animal feed–development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. *Microbial ecology in health and disease.* **25**(1), p25862.

Yin, J., Li, F., Kong, X., Wen, C., Guo, Q., Zhang, L., Wang, W., Duan, Y., Li, T. and Tan, Z. 2019. Dietary xylo-oligosaccharide improves intestinal functions in weaned piglets. *Food & function.* **10**, pp.2701-2709.

Yin, Y.-L., Baidoo, S.K., Jin, L., Liu, Y., Schulze, H. and Simmins, P. 2001. The effect of different carbohydrase and protease supplementation on apparent (ileal and overall) digestibility of nutrients of five hulless barley varieties in young pigs. *Livestock Production Science.* **71**(2-3), pp.109-120.

Yin, Y.-L., McEvoy, J., Schulze, H. and McCracken, K. 2000. Studies on cannulation method and alternative indigestible markers and the effects of food enzyme supplementation in barley-based diets on ileal and overall apparent digestibility in growing pigs. *Animal science.* **70**(1), pp.63-72.

Yin, Y., Baidoo, S.K., Schulze, H. and Simmins, P. 2001. Effects of supplementing diets containing hulless barley varieties having different levels of non-starch polysaccharides with β-glucanase and xylanase on the physiological status of the gastrointestinal tract and nutrient digestibility of weaned pigs. *Livestock Production Science.* **71**(2-3), pp.97-107.

Yu, M., Mu, C., Zhang, C., Yang, Y., Su, Y. and Zhu, W. 2018. Marked response in microbial community and metabolism in the ileum and cecum of suckling piglets after early antibiotics exposure. *Frontiers in microbiology.* **9**, p1166.

Zeng, Z., Zhang, Y., He, J., Yu, J., Mao, X., Zheng, P., Luo, Y., Luo, J., Huang, Z. and Yu, B. 2021. Effects of soybean raffinose on growth performance, digestibility, humoral immunity and intestinal morphology of growing pigs. *Animal Nutrition.* **7**(2), pp.393-399.

Zhan, J., Liu, M., Wu, C., Su, X., Zhan, K. and qi Zhao, G. 2017. Effects of alfalfa flavonoids extract on the microbial flora of dairy cow rumen. *Asian-Australasian journal of animal sciences.* **30**(9), pp.1261-1269.

Zhang, Y., Li, D., Fan, S., Piao, X., Wang, J. and Han, I.K. 2002. Effects of casein and protein-free diets on endogenous amino acid losses in pigs. *Asian-australasian journal of animal sciences.* **15**(11), pp.1634-1638.

Zhao, P., Jung, J. and Kim, I. 2012. Effect of mannan oligosaccharides and fructan on growth performance, nutrient digestibility, blood profile, and diarrhea score in weanling pigs. *Journal of Animal Science.* **90**(3), pp.833-839.

Zhao, P., Wang, J. and Kim, I. 2013. Evaluation of dietary fructan supplementation on growth performance, nutrient digestibility, meat quality, fecal microbial flora, and fecal noxious gas emission in finishing pigs. *Journal of animal science.* **91**(11), pp.5280-5286.

Zhenping, S., Wenting, L., Ruikui, Y., Jia, L., Honghong, L., Wei, S., Zhongmie, W., Jingpan, L., Zhe, S. and Yuling, Q. 2013. Effect of a straw-derived xylooligosaccharide on broiler growth performance, endocrine metabolism, and immune response. *Canadian Journal of Veterinary Research.* **77**(2), pp.105-109.

Appendix

A.1 General ethics statement

The University of Leeds Animal Welfare and Ethical Review Body approved all experimental protocols prior to their commencement. As set out in the Directive 2010/63/EU, the principles of the '3Rs'of replacement, reduction and refinement were applied throughout all experimental designs. Pig husbandry practices and housing were compliant with the Council Directive 2008/120/EC standards and the Welfare of Farmed Animals (England) Regulations 2007. Moreover, all scientific procedures carried out during the experiments were compliant with the Animals (Scientific Procedures) Act 1986, as revised by the Directive 2010/63/EU.

A.2 Standard Spen farm practice

All animal experiments were conducted on the indoor pig production system at the University of Leeds farm, Spen Farm, Leeds, UK. All experiments used crossbred pigs that were sourced directly from Spen Farm's breeding herd. All pigs used throughout this research were treated according to standard Spen Farm practice before and after the experiments. Spen Farm is a 200-sow herd that operates on an indoor 3-week batch farrowing system. Progeny pigs were reared on site from birth to slaughter. After farrowing, sows remained in their farrowing crates with their litter for approximately 4 weeks. During this time, piglets suckled on the sow but also had access to a standard commercial creep feed and water. Within the first 24 hours of birth, all piglets had their teeth clipped, tails docked, and ears tagged. Piglets were weaned from their mother at approximately 4 weeks of age and sows were returned to the main herd before being served 4 days later. At weaning, piglets received an intra-muscular injection of Suvaxyn

Circo + MH RTU for the prevention of Porcine Circovirus and the bacteria *Mycoplasma hyopneumoniae* which causes enzootic pneumonia. After vaccination, piglets from different litters were mixed according to size into pens (155 × 129 cm) in a conventional fully slatted weaner-grower facility. Each pen had 2 nipple drinkers and 1 multi-space feeding trough for the first 3 weeks (from 4–7 weeks of age) where a standard commercial piglet diet was fed. Following this, a standard grower diet was fed in a single-spaced feeding trough for the remaining 5 weeks (from 7–12 weeks of age) until exit from the building. After 8 weeks in the weaner-grower facility, pigs were moved into finisher accommodation where they remained until they were sent to the abattoir for slaughter at ~110 kg. Individual pig weights were recorded weekly to determine average pig performance. Dependant on BW, pigs were either lifted and placed into a weighing cage on a stationery trolley or walked out of their pen into a corridor floor weigher.

Throughout the experiments, all pigs had *ad libitum* access to feed and water. Feed troughs were topped up with a known quantity of feed when required and all feed data was recorded. Diets were formulated with the assistance of Associated British Nutrition (ABN, Peterborough, UK), a leading British pig feed manufacturer in the UK and industry sponsor of this research. Details of dietary specifications are provided in the relevant chapters. All feed additives were incorporated into the pelleted diets at the time of feed manufacture. Representative samples of all experimental diets were collected weekly from a mixture of feed bags on farm and stored at –20ºC prior to analyses.