



**University of
Reading**

**Exploring the Relationship between the Structure of Wheat
Dietary Fibre and Prebiotic Activity**

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Suzanne Harris

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List of Abbreviations

AG	Arabinogalactan
AGP	Arabinogalactan-peptide
ANOVA	Analysis of variance
AX	Arabinoxylan
A: X	Arabinose: xylose ratio
AXOS	Arabinoxylan oligosaccharides
CAC	Codex Alimentarius Commission
CD	Crohns' disease
CVD	Cardiovascular disease
DF	Dietary fibre
DP	Degree of polymerisation
FA	Ferulic acid
FISH	Fluorescence in-situ hybridisation
FOS	Fructo-oligosaccharides (oligo fructose)
GH	Glycoside hydrolase
GT61	Wheat line with reduced activity of glycosyl transferase family 61
HPAEC	High performance anion exchange chromatography
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
kDa	Kilo Daltons
LDL	Low-density lipoprotein

MALDI-TOF	Matrix assisted laser desorption/ionization- time of flight
MM	Molecular Mass
NDNS	The National Diet and Nutrition Survey of the UK
NSP	Non-starch polysaccharides
PBS	Phosphate Buffered Saline
RNA	Ribonucleic acid
RNAi	RNA interference
SCFA	Short chain fatty acid
SEC	Size-exclusion-chromatography
tFA	Trans-ferulic acid
UC	Ulcerative colitis
WE-AX	Water-extractable arabinoxylan

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Abstract

The relationships between wheat dietary fibre (DF) and prebiotic effects were investigated by testing how various DF types in different combinations and how the structure, of the major DF component of wheat, arabinoxylan (AX) modulate prebiotic activity. It was hypothesised that DFs could act synergistically resulting in different prebiotic activities when fermented *in vitro* at different ratios. The DF, arabinogalactan-peptide (AGP) was isolated from wheat flour and characterised, AX and mixed-linked β -glucan were commercially bought. The three fractions were tested singly and in combination in *in vitro* fermentation studies to assess their prebiotic activity when used individually and in different combinations. SCFAs produced from *in vitro* fermentation were measured using HPLC; flow-FISH was used for bacterial enumeration. Increases in bifidobacteria and acetate were observed with combinations of AGP and AX fractions. There were no additive effects on prebiotic activity when AGP and AX were combined; the greatest increases in total SCFAs, bifidobacteria and total bacteria were observed when AX and β -glucan were combined at a 3:1 ratio. The importance of AX structure was investigated using AX isolated from transgenic wheat with an altered arabinosylation pattern and pre-incubation of AX fractions in a simulated gastric digest prior to *in vitro* colonic fermentation. AX isolated from flour of homozygous TaXAT1 RNAi transgenic wheat showed lower prebiotic activity compared to AX isolated from flour of the corresponding azygous control wheat. Pre-incubation of AX in an *in vitro* gastric digest resulted in smaller AXOS, a lower A:X ratio, improved bacterial composition and slower fermentation compared to the control. Based on these results, three soluble arabinoxylan (AX) fractions from wheat flour with different average molecular mass (MM) and one insoluble AX fraction were subjected to *in vitro* gastric and duodenal digestion prior to *in vitro* fermentation, resulting in an increase in smaller AXOS, decreases in MM, viscosity and A:X ratio and a reduction in bound ferulic acid. Differences in prebiotic activity were observed between AX samples of different MM after changes in structure caused by *in vitro* digestion. The AX fraction of 323kDa gave the greatest increases in SCFA production, while the insoluble AX fraction showed the smallest increases in SCFA production.

1. General Introduction

1.1. Human digestive system and health

4.4.1. Gut microbiota

The human body is colonised by a vast number of microbes, collectively known as the microbiota. Bacteria have co-evolved with us and colonise almost every available space within and on our bodies (Costello et al., 2009). It is only in more recent years that we have become aware of the importance of our microbiota and the relationships between them and human health. It is now well established that a healthy gut flora is very important for the overall health of the 'host' (Prescott et al., 1996; Aune et al., 2011).

The largest concentration of microbiota is found in our colon. The adult human large intestine is about 150 cm long with a surface area of 1300 cm². The average total colonic transit is 30.7h for men and 38.3h for women (Beck et al., 2014). The colon contains between 58-908 g of contents (Banwell et al. 1981; Cummings et al. 1990) and microbiota are a major component, comprising 40-45% of faecal solids in people eating a western diet (Stephen & Cummings 1980; Cabotaje et al. 1990). Microbiota refers to the entire population of colonizing microorganisms including fungi, archaea, viruses, and protozoans as well as bacteria. The vast majority of all microbial cells in the colon are bacteria and therefore interest has focussed on the contribution of bacteria to health and literature on the other microbiota is limited.

Research is increasingly linking the importance of the gut microbiota to many aspects of human health, from gut physiology to depression using signalling referred to as the gut-brain axis, the bidirectional communication between the central and enteric nervous systems, linking the brain with the gut (Carabotti et al., 2015).

The colonic microbiota can be thought of as a metabolic organ due to its essential functions including: providing energy from fermentation of indigestible carbohydrates and proteins; synthesis of vitamins B and K (Hill, 1997) and regulating mucosal physiology (Tlaskalová-Hogenová et al., 2011); liver function (Björkholm et al. 2009); immune response (Round and Mazmanian, 2014); obesity and satiety (DiBaise et al., 2008) and gene expression (e.g. activation of g-protein coupled receptors that induce cell derived hormones that regulate gut motility (Samuel et al., 2008)). It also provides protection against infections from invading pathogens and chronic diseases such as some cancers, inflammatory conditions and heart disease by release

of metabolites and competition with pathogenic bacteria for space and nutrients (Fukuda et al., 2011; Aune et al., 2006).

The composition of the colonic microbiota is usually stable but can fluctuate under some circumstances including acute diarrhoeal illnesses, antibiotic treatment, or by dietary interventions (Simon and Gorbach, 1984; Moore and Moore, 1995; Musso et al., 2010; Turnbaugh et al., 2009). Maintenance of a stable and diverse population of commensal bacterial groups contributes greatly towards improved colonisation resistance and protection against gastrointestinal disorders. Many studies have now shown that diet plays an important role in the composition of the gut microbiome (Gibson, 1999; Wu et al., 2011).

The large intestine can contain more than 10^{11} bacterial cells/g of luminal content (Simon and Gorbach, 1984), and is thought to host between 400-500 different species of indigenous bacteria, (Moore et al., 1975) although this is difficult to determine as many species are impossible to culture outside of the colon and remain unknown. It has been estimated based on genetic analysis that the collective human gut microflora is composed of over 35000 bacterial species (Frank et al., 2007). Each person has a different combination of gut microbiota and these different combinations can contribute to health and disease. Figure 1.1. shows the distribution of microbiota in the human digestive tract.

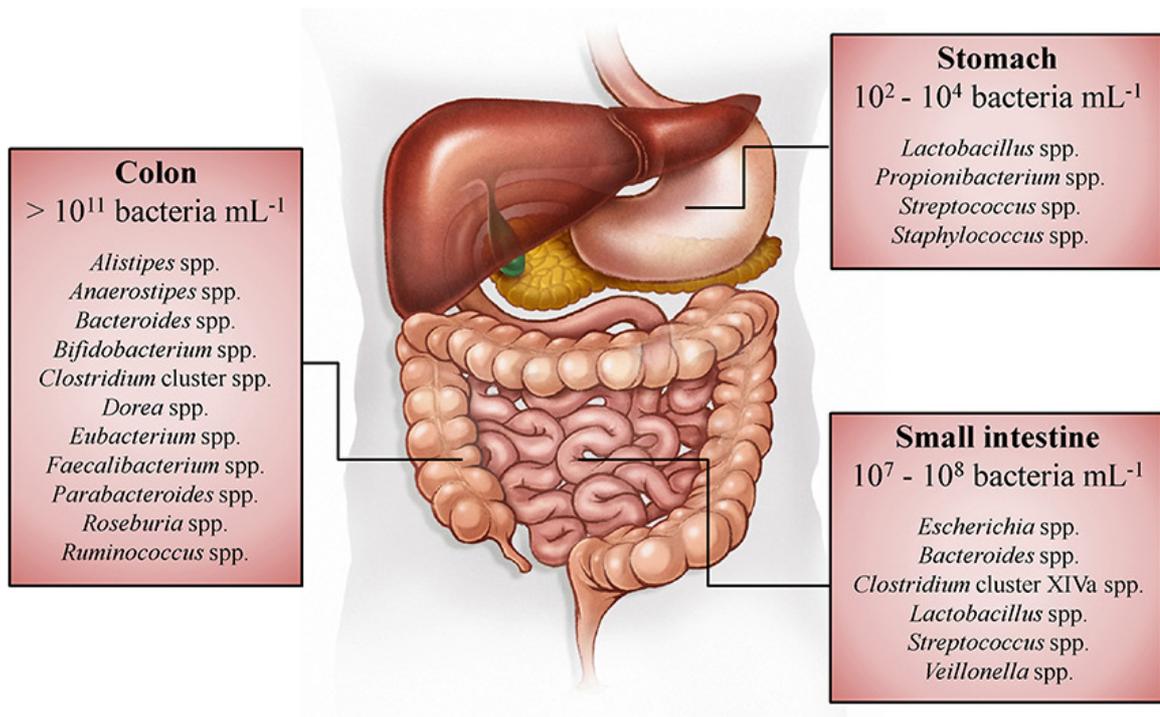


Figure 1.1: The distributions of different bacteria in the gastro-intestinal tract of humans. Image from Riviere et al., 2016 citing Tap et al., 2009; Zoetendal et al., 2012; Delgado et al., 2013; Walker et al., 2014).

4.4.2. Composition of colonic microbiota

The composition of the colonic microbiota is usually stable and only changes drastically at the extremes of life or due to antibiotic application or illness. Although there are large individual differences in microbial composition the two dominant bacterial phyla in the gut are always Firmicutes (which contains over 200 genera, including *Lactobacillus*, *Eubacterium*, *Faecalibacterium* and *Clostridium*) and Bacteroidetes (*Bacteroides* and *Prevotella*). The next most abundant are Actinobacteria (*Bifidobacterium* and *Atopobium*), and Proteobacteria (*Proteus*, *Salmonella*, *Escherichia*). Most colonic luminal bacteria are thought to belong to the genera: *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Enterobacteriaceae*, *Enterococcus*, *Clostridium*, *Lactobacillus* and *Ruminococcus*. *Clostridium*, *Lactobacillus*, *Enterococcus* and *Akkermansia* are the predominant mucosal associated genera (Swidsinski et al., 2005).

The majority of the Firmicutes in the colon are split into two main groups, the *Clostridium* *coccoides* group (also known as *Clostridium* cluster XIVa) and the *Clostridium* *leptum* group (also referred to as *Clostridium* cluster IV) (Collins et al. 1994; Mariat et al. 2009). Both groups contain members of the genera *Clostridium*, *Eubacterium* and *Ruminococcus*. The *Clostridium* *coccoides* group

are a major group, representing almost 75% of the Firmicutes, including many butyrate producers like *Eubacterium rectale* (Kelly, Conway and Aminov, 2005).

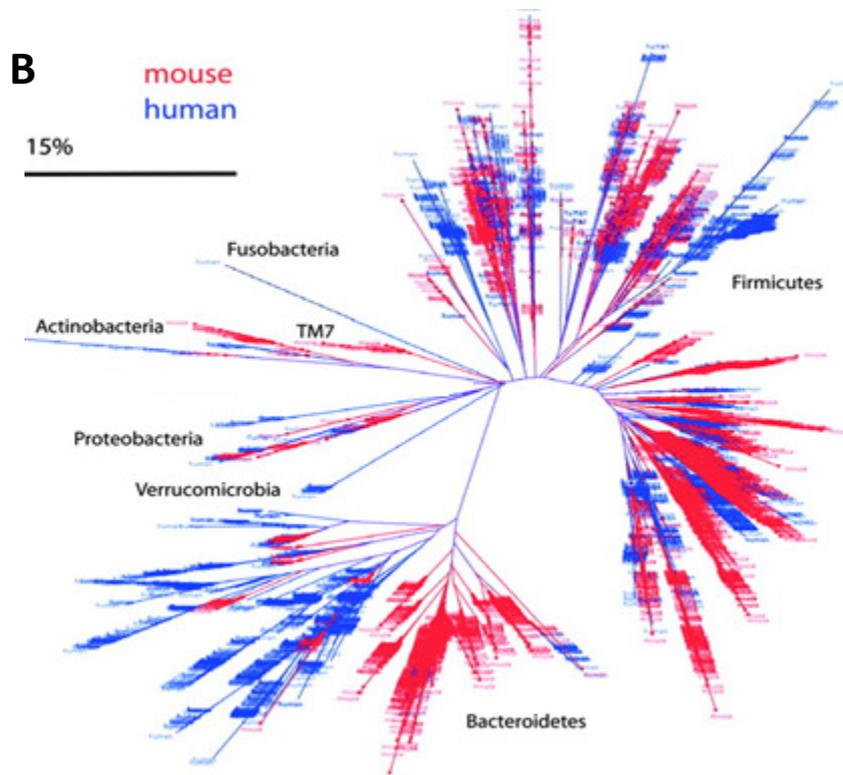
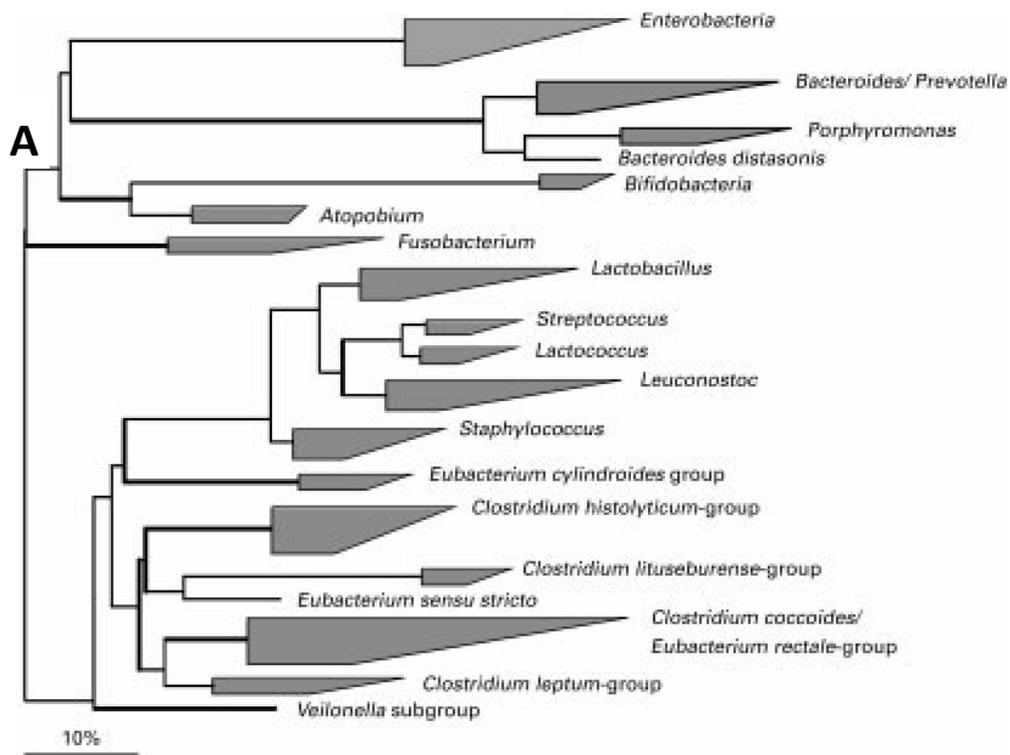


Figure 1.2: Bacterial diversity A) Phylogenetic tree of major phylogenetic groups of intestinal bacteria (Blaut et al., 2002). B) Phylogenetic tree of human and mouse colonic bacteria obtained using 16S rRNA sequencing (Ley et al., 2005).

Figure 1.2A shows the large diversity of colonic bacteria using a phylogenetic representation of groups of important known colonic bacteria. The species of resident colonic bacteria vary greatly between individuals (Simon and Gorbach, 1984; Moore and Moore, 1995). Figure 1.2B shows the phylogenetic tree of a human compared to a mouse, there are large amounts of overlapping species but humans have many more Bacteroidetes bacteria, shown by the blue area, demonstrating that mouse models cannot be used as direct comparisons to human reactions.

A study by Arumugam et al (2013) identified three distinct clusters of microbial populations (not specific to nation or continent), which were named enterotypes and suggested that gut microbiota are stratified. The enterotypes were hypothesised to be driven by groups of species that together contribute to the preferred community compositions and the three enterotypes are identifiable by the variation in the levels of one of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3). Dietary habits are considered one of the main factors contributing to the diversity of human gut microbiota and the maintenance of these enterotypes (Backhed et al., 2005), diet has a dominant role over other possible variables such as ethnicity, sanitation, hygiene, geography, and climate in determining the gut microbiota (De Filippo et al., 2010).

4.4.3. Fermentation

Most bacteria in the colon are strict anaerobes, deriving energy from fermentation (Macfarlane et al., 1999). Dietary fibres (non-digestible carbohydrates) reaching the large intestine are converted to monosaccharides via fermentation by bacterial enzymes and metabolised to short chain fatty acids (SCFAs). Bacterial fermentation involves a wide variety of enzymes and biochemical pathways. This capacity is estimated to be encoded by 2-4 million microbial genes (Possemiers et al., 2011). Digestion of polysaccharides requires specific glycosyl hydrolase enzymes, some bacteria can produce a wide variety of these whilst others are more specialised. In 1991, Cummings and Macfarlane hypothesized that the breakdown of highly polymerized

materials in the gut is a co-operative activity, with enzymes from many different species participating in the process.

4.4.4. Important colonic bacteria

Bifidobacteria are an important class of saccharolytic Actinobacteria. They are at their highest proportion, in the colon, just after birth when they constitute about 90% colonic bacteria due to their ability to use fucosylated oligosaccharides in human milk (Yu et al., 2012) however, they decrease in number with the cessation of breastfeeding. In adults, they compose only about 5% of the total colonic bacteria, and even less in the elderly or those with Inflammatory Bowel Disease (IBD) which includes Crohn's disease (CD) and ulcerative colitis (UC) or in individuals with irritable bowel syndrome (IBS), or after antibiotic use. Bifidobacteria provide B vitamins, antioxidants and polyphenols through fermentation and are involved in maturation of the immune system during early life, preservation of colonic barrier functions and protection from pathogens by reducing the luminal pH through the release of SCFA (mainly acetate) and blocking pathogen adhesion to the endothelium (Riviere et al., 2016).

Lactobacillus is a Gram-positive facultative anaerobe and is part of the lactic acid bacterial group which produces lactate as the sole product of fermentation (Walter, 2008). The ability to ferment galactose is a major characteristic which can be used to differentiate *Lactobacillus helveticus* (galactose-positive) from *Lactobacillus lactis* and *Lactobacillus bulgaricus* (galactose-negative) (Turner and Martley, 1983). *Lactobacillus* were theorised by Metchnikoff to "arrest intestinal putrefaction and postpone and ameliorate old age" (Metchnikoff, 1907), which kick-started work in to their use as probiotics (defined by the FAO/WHO as live microorganisms that when administered in adequate amounts confer a health benefit on the host). Lactobacilli comprise a small proportion of the colonic microbiota, rarely being cultured at population levels exceeding 10^8 CFU per gram accounting for only 0.01% of the total cultivatable bacteria (Walter, 2008).

Faecalibacterium prausnitzii is an important butyrate producing bacterium which belongs to the clostridial cluster IV (the most abundant gut bacterial group). Notably, faecalibacteria are present at low numbers in patients with IBD and display significant anti-inflammatory effects in mouse colitis models (Sokol et al., 2008).

The most predominant anaerobic bacteria in the colon are *Bacteroides* species (around 25%), these bacteria colonize the gut in early life and maintain a complex and mostly beneficial

relationship with the host when maintained in the gut, however they can be pathogenic when transferred away from the gut. *B. fragilis* is a potent pathogen and the most commonly isolated anaerobic pathogen despite accounting for only 0.5% colonic microbiota (Wexler, 2007). Several species of *Bacteroides* can utilize fucosylated oligosaccharides as carbon sources suggesting that their early colonization of the gut is aided by properties of human milk (Marcobal et al., 2011).

Bacteroides are the principal polysaccharide degrading bacteria (Salyers and Leedle, 1983) and are able to grow on a wide variety of polysaccharides through their ability to produce a large variety of polysaccharide depolymerases and glycosidases (Salyers and Leedle, 1983; Macfarlane et al., 1990).

Clostridium histolyticum is a highly proteolytic cluster II bacterium that produces acetate as the major end product of metabolism (Collins et al., 1994). Members of the *C. histolyticum* group are recognized toxin-producers and may contribute towards gut dysfunction. Populations have been shown to be increased in children with autism, indicating a possible link (Parracho et al., 2005). Some *Bacteroides* and *Clostridium* cause increased production of electron sink products such as succinate and lactate, which are used for methanogenesis instead of formation of SCFA at high growth rates, or during growth in the presence of excess substrate (Allison and Macfarlane, 1989) resulting in fewer SCFA formed from the carbohydrate substrate.

Desulfovibrionales (DSV) are a bacterial group in the *Fusobacterium* genus that metabolise the sulphate moiety of sulphated mucins and are important contributors to sulphide formation in the gut (Fite et al., 2004). DSVs oxidize lactate to acetate and carbon dioxide. The end product of sulphate reduction during anaerobic respiration is hydrogen sulphide (H₂S) (Widdel, 1992) which is toxic to the colonic mucosa. Increased numbers of DSVs have been linked with acute ulcerative colitis (Rowan et al., 2010).

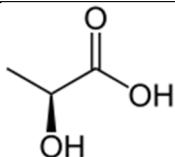
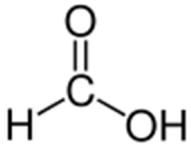
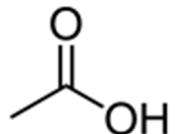
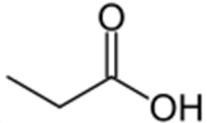
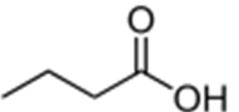
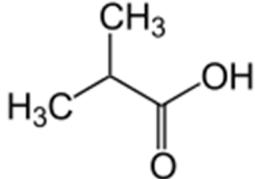
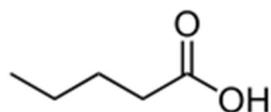
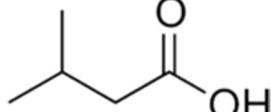
Some beneficial bacteria can be ingested directly to improve health; these are known as probiotics. Probiotics are defined as ‘microbial organisms that remain viable and stable after culture, manipulation, and storage before consumption; survive gastric, biliary, and pancreatic digestion; are able to induce a host response once they enter the intestinal microbial ecosystem (by adhering to gut epithelium or other mechanisms); and yield a functional or clinical benefit to the host when consumed’ (Savvedra, 2007). Some beneficial bacteria can be used as probiotics, mainly the *Lactobacillus* and *Bifidobacterium* species including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum* and *Bifidobacterium longum*, and the yeast *Saccharomyces*

boulardii. These microorganisms are generally well-accepted and tolerated and rarely cause negative effects in humans (Goldin, 1998; Taibi and Comelli, 2014).

4.4.5. Short Chain Fatty Acids

SCFAs are volatile fatty acids consisting of a straight-chain, aliphatic tail of fewer than six carbon atoms and consist of mainly acetate, propionate and butyrate (95%) (Cummings, 1981) but also include formate and valerate. Lactate is also formed from fermentation, lactate is a monocarboxylic acid. SCFA are mostly produced from fermentation of carbohydrates but branched amino acids leucine, isoleucine and valine can be utilised creating branched SCFAs Isobutyrate and isovalerate. An average of 400 mmol/day (range of 150–600 mmol/day) of SCFAs is produced in the colon (Beck et al., 2014) and more than 95% are immediately absorbed by the colon, contributing towards about 10% of the energy requirements of the host (Cummings, 1981; Vogt and Wolever, 2003; McNeil, 1984). They occur in a ratio of: butyrate [15 %], propionate [25 %], and acetate [60 %] (Beck et al., 2014). The concentration of total SCFAs in humans is reported to be $\approx 50\text{--}100\ \mu\text{M}$ in peripheral blood and $\approx 300\text{--}450\ \mu\text{M}$ in portal blood (Cummings et al., 1987). Table 1.1 shows the chemical formulas of SCFAs and lactic acid and Figure 1.3 shows the chemical pathways that generate them.

Table 1.1: Table of SCFAs and branched SCFAs showing structure and chemical formulas.

Name		Formula
Lactate		$\text{CH}_3\text{CH}(\text{OH})\text{CO}_2\text{H}$
Formate		HCOOH
Acetate		CH_3COOH
Propionate		$\text{CH}_3\text{CH}_2\text{COOH}$
Butyrate		$\text{CH}_3(\text{CH}_2)_2\text{COOH}$
Isobutyrate		$\text{C}_4\text{H}_8\text{O}_2$
Valerate		$\text{C}_5\text{H}_{10}\text{O}_2$
Isovalerate		$\text{C}_5\text{H}_{10}\text{O}_2$

The most abundant SCFAs, acetate, propionate and butyrate, have been shown to have multiple beneficial effects for the host, for example, providing dietary energy for the host and suppressing the growth of pathogens by decreasing the pH of the intestinal lumen (Blaut, 2002). These SCFAs were also found to have anti-inflammatory effects in rats (Vinolo, et al., 2011)

and influence intestinal motility in rats via G-protein coupled receptor activation, with acetate being the most effective, followed by propionate and butyrate (Dass et al, 2007).

Acetate is utilised systemically by skeletal muscle and liver cells and can be used by adipocytes for lipogenesis. Acetate has been found to have a major role in the ability of bifidobacteria to inhibit toxicity from enteropathogens by inhibiting the transport of toxins from the gut lumen to the blood (Fukuda et al., 2011).

Propionate may help to inhibit the synthesis of LDL cholesterol in animals and humans (Wolever et al., 1991) and propionate has also been linked with regulation of adipose tissue deposition in rats (Reshef et al, 1967) via an increase in circulating leptin levels (Xiong et al 2004).

Butyrate is the preferred energy substrate for the colonic epithelium and is preferentially transported by gut epithelial cells (Ritzhaupt et al., 1998). Rectally administered butyrate was also shown to relieve subjects from IBD symptoms (Scheppach, 1996). Butyrate has been linked with protection from initiation of colon cancer by many different pathways including: promoting cell differentiation, cell-cycle arrest and apoptosis of transformed colonocytes and a reduction of transformation of primary to secondary bile acids by reducing luminal pH (Cummings, 1995, Williams et al., 2003). Primary bile acids are steroid carboxylic acids derived from cholesterol, synthesized in the liver and conjugated with taurine or glycine before secretion from the gall bladder into the intestine. Cholic acid and chenodeoxycholic acid are major primary bile acids synthesised in humans (Chiang, 2013). Transformation to secondary bile acids occurs by partial dihydroxylation and the removal of glycine and taurine groups by colonic bacteria, allowing them to re-enter the circulation back to the liver for re-conjugation (Chiang, 2013; Ajouz et al., 2014). Table 1.2 summarises the principal health benefits of the main SCFAs, acetate, propionate and butyrate.

Table 1.2: Health benefits of the most abundant SCFAs. (Hamer et al., 2008 ; Al-Lahham et al., 2010; Havenaar, 2011 ; Macfarlane and Macfarlane, 2012 ; Chang et al., 2014 ; Louis et al., 2014; Tralongo et al., 2014 ; Chambers et al., 2017).

SCFA	Physiological effect
Acetate	<p>Reaches the portal vein and is metabolised in various tissues</p> <p>Is a minor energy source for colonic epithelial cells</p> <p>Decreases the pH of the colon (decreasing bile salt availability and increasing colonic calcium, magnesium, zinc, and iron absorption, decreasing ammonia absorption and inhibiting growth of pathogens)</p> <p>Has anti-inflammatory properties. Acts on leukocytes, inhibiting release of inflammatory cytokines (TNF-α, IL-2, IL-6)</p> <p>Increases colonic blood flow and oxygen uptake</p> <p>Is an energy source for muscle and brain tissue</p> <p>Is used by cross feeding species as a co-substrate to produce butyrate</p>
Propionate	<p>Reaches the portal vein and is subsequently taken up by the liver</p> <p>Is a minor energy source for colonic epithelial cells</p> <p>Decreases the pH of the colon (decreasing bile salt availability, increasing colonic calcium, magnesium, zinc, and iron absorption, decreasing ammonia absorption and inhibiting growth of pathogens)</p> <p>Interacts with the immune system</p> <p>Has anti-inflammatory properties. Acts on leukocytes, inhibiting release of inflammatory cytokines (TNF-α, IL-2, IL-6)</p> <p>Promotes satiety by increasing colonic cell secretion of gut hormones peptide YY and glucagon like peptide-1 (GLP-1) that regulate satiety in the brain</p> <p>Reduces circulating cholesterol</p> <p>Decreases liver lipogenesis</p> <p>Improves insulin sensitivity</p> <p>Prevents proliferation and induces apoptosis of colorectal cancer cells</p>
Butyrate	<p>Is mainly taken up by colonic epithelium, small amounts reach portal vein and systemic circulation</p> <p>Is the preferred energy source for colonic epithelial cells</p> <p>Stimulates the proliferation of normal colonic epithelial cells</p> <p>Prevents proliferation and induces apoptosis of colorectal cancer cells</p>

	<p>Affects gene expression of colonic epithelial cells</p> <p>Improves the gut barrier function by stimulation of the formation of mucin, anti-microbial peptides and tight junction proteins</p> <p>Interacts with the immune system</p> <p>Has anti-inflammatory properties. Acts on leukocytes, inhibiting release of inflammatory cytokines (TNF-α, IL-2, IL-6)</p> <p>Stimulates the absorption of water and sodium</p> <p>Reduces oxidative stress in the colon</p> <p>Promotes satiety by increasing colonic cell secretion of gut hormones peptide YY and glucagon like peptide-1 (GLP-1) that regulate satiety in the brain</p> <p>Decreases the pH of the colon (decreasing bile salt availability, increases mineral absorption, decreases ammonia absorption and inhibits growth of pathogens)</p>
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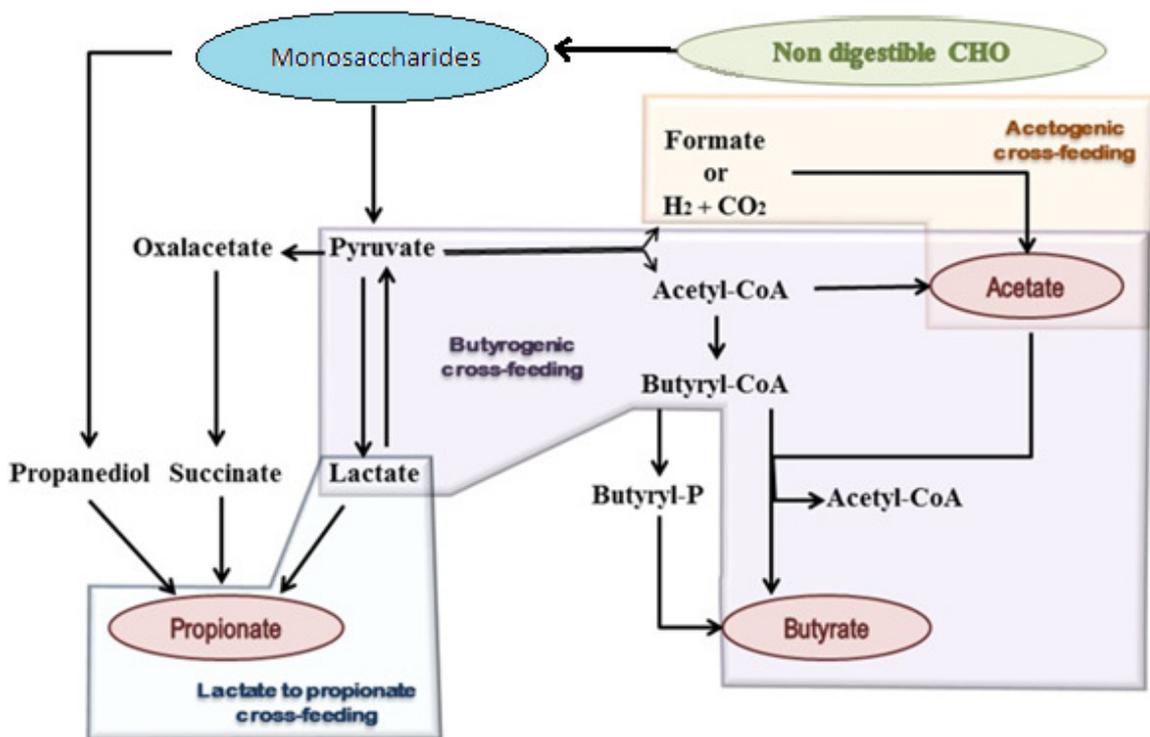


Figure 1.3: Schematic representation of microbial metabolic pathways and cross-feeding mechanisms, contributing to SCFA formation in the human gut. Shaded geometric shapes summarize routes of formation for each of the three main SCFAs: acetate, propionate, butyrate. (Image adapted from Ríos-Covián et al., 2016)

4.4.6. Bacterial cross-feeding

There is a large diversity in polysaccharide substrates that reach the colon, creating a vast array of ecological niches that are exploited by gut bacteria. Although some species can utilize different substrates, (e.g. substrates from the diet and host origin (Sonnenburg et al., 2005; Scott et al., 2006)), other species, are far more specialized. The ability to produce hydrolytic enzymes and transport systems determines the specificities of a bacterium's feeding profile. Figure 1.3 shows cross feeding mechanisms of metabolic pathways and Figure 1.4 shows examples of cross-feeding mechanisms between bifidobacteria and butyrate producing bacteria, utilizing different polysaccharides as carbon sources.

Acetate, propionate and butyrate can be generated through cross feeding and different bacteria can utilize different pathways. For example, three different pathways are used by bacteria to produce propionate: the succinate pathway, where succinate is the substrate is present in some *Firmicutes* and some *Bacteroidetes*; the acrylate pathway, where lactate is utilised is present in a few members of the families *Veillonellaceae* and *Lachnospiraceae* (Flint et al., 2015) and the propanediol pathway where deoxy-sugars are used. The succinate pathway is thought to be dominant (Salonen et al., 2014).

A greater diversity of bacteria in the colon provides more opportunity for breakdown and fuller utilization of different dietary polysaccharides. For example, the ability of bifidobacteria to break down arabinoxylan-oligosaccharides (AXOS) is strain dependant (Riviere et al., 2014) due to the heterogeneous nature of AXOS and AX, their complete hydrolysis requiring both debranching and depolymerizing enzymes. A study of AXOS consumption amongst members representing eleven different bifidobacterial species found different clusters who preferentially metabolise different AXOS, with some unable to utilise the xylan backbone but able to use arabinose, some able to utilise the xylan backbone only up to xylotetraose whilst not using the arabinose and some that can utilise both the xylan backbone and the arabinose sidechains, suggesting that AXOS metabolism is strain dependant and rather complex (Riviere et al., 2014).

Most butyrate-producing bacteria belong to the *Clostridium* cluster XIVa (~80%) (Barcenilla et al., 2000) and use acetate to produce butyrate via the butyryl- CoA: acetate CoA pathway (Figure 1.3), utilising butyryl- CoA: acetate CoA transferase enzyme which is present in the most abundant bacteria in the human colon, such as *Faecalibacterium prausnitzii* (~14%), *Eubacterium rectale* (~13%), and other bacterial groups such as *Roseburia* spp. (Riviere et al., 2015). Lactate is

formed from pyruvate through the action of lactate dehydrogenase in the homofermentative pathway (Figure 1.3) by many common gut bacteria including lactobacilli, bifidobacteria, enterococci, and streptococci and *Eubacterium* spp. (Barcenilla et al., 2000). However, under normal conditions, lactate is not present at high levels in mixed microbial communities such as faecal samples, possibly due to lactate utilization by different bacterial species. In normal individuals, faecal lactate is usually at a concentration of less than 5 mM but has been reported to accumulate in faeces from individuals who are suffering from ulcerative colitis, at concentrations up to 100 mM (Hove et al., 1994; Tsukahara et al., 2002).

Cross-feeding by colonic bacteria has a huge impact on the microbial populations and resultant SCFA production as well as the efficient use of fermentable polysaccharides (Ríos-Covián et al., 2016), and helps to explain the reported butyric effect of some polysaccharides like oligofructose (FOS), which are not known to be fermented by butyrate producing bacteria (shown in figure 1.4- cross feeding 2). For example, some undefined bacterial species related to *Eubacterium hallii* were shown to use lactate to produce butyrate. The abundance of *E. hallii* in the gut suggests an important role in preventing lactate accumulation and butyrate production for these species (Duncan et al., 2004).

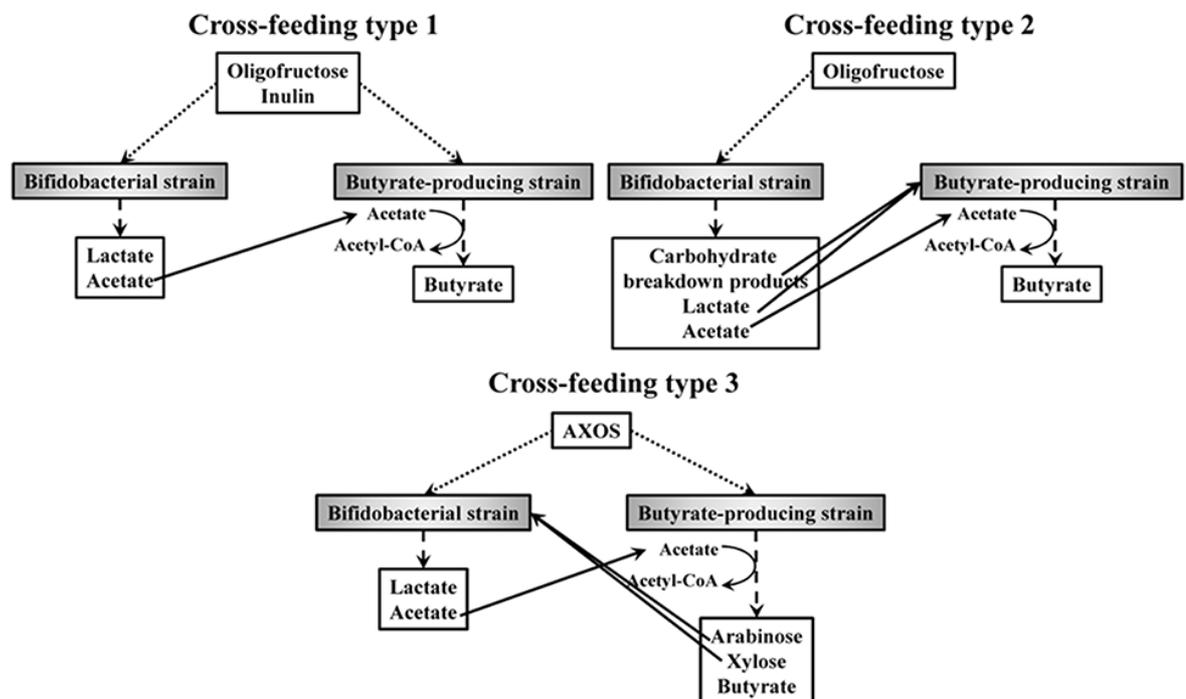


Figure 1.4: Different types of cross-feeding that can take place between *Bifidobacterium* spp. and species of butyrate-producing colon bacteria in the human colon. Arrows indicate consumption of oligofructose, inulin, and AXOS (.....), production of carbohydrate breakdown

products and/or metabolic end-products (- - -), and cross-feeding interactions between the bifidobacterial and butyrate-producing strains (—). Image from Riviere et al. (2016).

4.4.7. Protein fermentation

Proteins that have not been digested in the upper digestive tract can also be fermented by colonic bacteria into branched fatty acids such as isobutyrate, isovalerate and other nitrogen and sulphur containing compounds. Because carbohydrate fermentation is more energetically favourable, there is depletion of fermentable carbohydrates in the distal regions of the colon, making proteins and amino acids a more dominant metabolic energy source for bacteria in this area of the gut (Macfarlane et al., 1992). Some end products of protein fermentation can be harmful to the host e.g. amines and ammonia, and a shift to protein fermentation has been linked with increases in diseases such as IBS and colonic cancers, which occur more often in the distal regions of the gut (Roberfroid et al., 2010).

4.4.8. Gut dysfunction

Inflammatory bowel disease is a result of a lack of immune tolerance to commensal gut microbiota. It is estimated to be present in around 0.8% people in Europe (Molodecky et al., 2012) and most often presents as Crohn's disease (CD) or ulcerative colitis (UC). Under normal conditions the mucosal layer in the gut covers the colonic epithelial cells and provides a first line of defence against microorganisms. Usually only specific types of bacteria can adhere to the mucus layer, but in sufferers of IBD, the mucus layer is damaged or discontinuous, becoming more permeable and allowing other microorganisms to reach the colonic epithelium. This results in immune recognition of antigens on the surfaces of the microorganisms and results in inflammation of the epithelium.

CD presents as discontinuous inflammation which may extend deeply into the submucosal regions and can occur anywhere along the colon. In UC, inflammation involves only the superficial layers of the intestinal mucosa and is localised to regions of the gut most highly colonized by bacteria, beginning distally and moving proximally along the colon (Cho, 2008). Irritable bowel syndrome (IBS) is recognized by chronic abdominal pain and discomfort associated with alterations in bowel habit and does not present with altered colonic physiology, but low-grade inflammation and immunological alterations believed to be associated with an activated adaptive immune response which is thought to be involved in the pathogenesis.

Unfavourable alteration of gut microbiota composition, known as dysbiosis, has been implicated in the pathogenesis of inflammatory bowel diseases (IBD), and other gastrointestinal disorders, including gastritis, peptic ulcer, IBS and even gastric and colon cancer (Swidinski et al., 2002; Frank et al., 2007; Sartor, 2008). Low counts of *F. prausnitzii* have been correlated with CD (Sokol et al., 2009; Willing et al., 2009) and low *F. prausnitzii* numbers and *Roseburia spp.* counts were seen in people with UC (Vermeiren et al., 2012). Both bacterial groups are butyrate producers and the enzyme responsible for butyrate production, butyryl-CoA: acetate CoA transferase, is also decreased in both the lumen and mucosa of those with UC. Butyrate has been linked with a reduction in inflammatory responses key to the pathogenesis of CD and UC (Segain et al., 2000; Scheppach et al., 1992). It is becoming clear that microbial alterations and resulting butyrate deficiency have a role in the pathogenesis of IBD but it is not known if this is cause or effect.

1.2. Diet and health

4.4.9. Dietary fibre

Increasing morbidity from chronic diseases such as cardiovascular disease (CVD), type II diabetes and colonic cancers, is occurring not only in wealthy but also, disproportionately, in low and middle-income countries. The increase in non-communicable diseases such as those described above, have been linked with rapid urbanisation and changes to a more sedentary lifestyle, coupled with a move away from traditional high-fibre foods to increased consumption of processed foods including highly refined cereals with a lower intake of plant dietary fibre and whole grains cereals.

Dietary fibre in food consists of plant cell walls which are not digested by human digestive enzymes and are instead partially or completely fermented by the microflora in the colon.

Dietary fibre has been historically difficult to define, with many different definitions in use around the world. The Codex Alimentarius Commission (CAC) has developed a definition that can be used universally.

“Dietary fibre means carbohydrate polymers with 10 or more monomeric units (Andersson et al., 2013), which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories:

1. Edible carbohydrate polymers naturally occurring in the food as consumed.
2. Carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities,
3. Synthetic carbohydrate polymers, which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.

Footnote 1 states, “when derived from a plant origin, dietary fibre may include fractions of lignin and/or other compounds associated with polysaccharides in the plant cell walls. These compounds also may be measured by certain analytical method(s) for dietary fibre.

Footnote 2 states that, “Decision on whether to include carbohydrates of 3 to 9 monomeric units should be left up to national authorities.”

Footnote 1 aligns with most definitions and recognizes ‘associated substances’ and lignin when part of, but not isolated from, the DF complex”.

Table 1.3. Description and sources of the main dietary fibre fractions captured by current definitions. Table edited from Buttriss and Stokes (2008) original data from Gray (2006).

Fibre component	Description
Cellulose	Polysaccharides comprising up to 10 000 closely packed glucose units, arranged linearly, making cellulose very insoluble and resistant to digestion by human enzymes. Principal component of cell walls of most plants. Forms about 25% fibre in grains and fruit.
Hemicelluloses	Polysaccharides containing sugars other than glucose. Associated with cellulose in cell walls and present in both water soluble and insoluble forms. Main dietary sources are cereal grains.
Pectins	Polysaccharides comprising galacturonic acid and a variety of sugars; soluble in hot water and forms gels on cooling. Mostly found in fruit but also present in vegetables.
Beta-glucans	Glucose polymers that, unlike cellulose, have a branched structure enabling them to form viscous solutions. Major cell wall component in oats and barley.
Resistant starch	Starch and starch degradation products that are not absorbed in the small intestine. Four classes have been identified: physically inaccessible starch, RS1; native starch granules, RS2; retrograded starch, RS3; and chemically modified starch (Englyst & Cummings 1987). RS1 is present in legumes and grains, RS2 in unripe bananas, RS3 is produced during cooking, cooling and storage of foods.
Other synthetic carbohydrate compounds	Synthetic derivatives of cellulose (for example, methyl cellulose and hydroxypropylmethyl cellulose) are non-digestible and, unlike their parent (cellulose), are soluble. But they are hardly fermented by microflora. Polydextrose has an average degree of polymerisation of 12 and is synthesised from glucose and sorbitol. It is partially fermented in the colon (~50% in humans) and has bulking and prebiotic properties.
Gums and mucilages	Gums are hydrocolloids derived from plant exudates. Mucilages are present in the cells of the outer layers of seeds of the plantain family, for example psyllium. Both are used as gelling agents, thickeners, stabilisers and emulsifying agents.
Lignin	Not a polysaccharide but chemically bound to hemicelluloses in plant cell walls.
Other minor components	Phytic acid (inositol hexaphosphate) is associated with fibre in some foods, especially cereal grains. May reduce mineral absorption in the small intestine as it binds strongly. Other compounds associated with fibre include tannins, cutins and phytosterols.

Dietary fibre includes a range of substances including polysaccharides, oligosaccharides, lignin and associated plant substances including the waxes cutin and suberin (indigestible fatty acid derivatives) which are bound to dietary fibre polysaccharides (noted in foot note 1), often forming cross links between other components and increasing resistance to digestion (DeVries, 2003). The principal types of dietary fibre are the non-starch polysaccharides (NSP) which constitute the major proportion of dietary fibre, resistant starches, defined as “the starch and products of starch digestion that are not absorbed in the small intestine of healthy individuals” (Englyst and Cummings, 1982) and non- α -glucan oligosaccharides- short chain carbohydrates. Table 1.3 provides a description and main sources of DF according to current definitions.

The National Diet and Nutrition Survey of the UK showed that cereal products contributed 29/30% of the total daily energy intake of adult males/females, 22/21% of the intake of protein and 39/37% of the intake of non-starch polysaccharides (the major components of dietary fibre, DF) (NDNS, 2011). Some are almost completely fermented by gut bacteria, and others are less fermentable.

The average fibre intake by UK adults falls far short of dietary recommendations, contributing to increasing health problems. The average intake is 12.8g/day for women and 14.8g/day for men. The recommended average intake for UK adults was 18g (NSP) per day (Department of health, 1991). This was increased in July 2015 by The Scientific Advisory Committee on *Nutrition* recommendation on carbohydrates, to a recommended 30g/ day for adults which is in line with the FAO/WHO opinion and more similar to recommendations for the rest of the world.

4.4.10. Dietary fibre and health

Dietary fibre is usually found in the diet as a mixture of different polysaccharides from different plant based sources. Those eating diets high in dietary fibre have been shown to have reduced risk of many chronic diseases such as cancers, cardiovascular diseases including coronary heart disease, obesity and type II diabetes. A meta-analysis by (Threapleton et al. 2013) showed an association with greater intake of dietary fibre (including cereal, fruit and vegetable fibre) and reduced cardiovascular disease and coronary heart disease, highlighting the case for increased intake of DF.

The more soluble and easily fermentable dietary fibres have been shown to reduce the effect of carbohydrates on increases in blood sugar (glycaemic index), reduce the amount of insulin required to reduce blood sugar (insulin sensitivity) and decrease cholesterol absorption (Hallfrisch and Behall, 2000; Marlet, 1997). Dietary fibre can improve general health by helping to regulate satiety and diluting the energy density of food. The addition of insoluble fibre to the diet helps to increase stool weight not only from fibre bulk but from increases in bacteria and water holding capacity (Harvey et al., 1973; Cummings, 1980).

Fibre intake has also been linked to reductions in colorectal cancer risk (Aune et al., 2011; Trichopoulou et al., 2003). The European Prospective Investigation on Cancer study reported a 40% reduction in risk of colorectal cancer between the lowest (15 g/day) and the highest (35 g/day) intakes based on incidences of colon cancer (Trichopoulou et al., 2003). The protective effect was greatest for the proximal colon, and lowest for the distal regions. The mechanism of risk reduction from dietary fibre has been suggested to be due to: a reduction in exposure of the colonic mucosa to carcinogens by dilution from additional stool bulk (Reddy et al., 1989); reduction in contact time from decreased transit time (Harris and Ferguson, 1993); decreased binding of carcinogens (Ryden and Robertson, 1995a) and reduction of colonic luminal pH by SCFAs produced from bacterial fermentation and modulation of cell proliferation by butyrate (Clausen, 1995).

Studies on dietary fibre have often focussed on singular types of fibre e.g. resistant starch and inulin, (Englyst & Cummings 1987; Tarini and Wolever, 2010), however more relevant *in vivo* results are obtained from using real life scenarios such as a combination of different DF from whole grain intake (Schatzkin et al., 2007).

1.3. Prebiotics

Some DFs are classed as prebiotic or have prebiotic activity. Prebiotics have been defined as “non-digestible food ingredients that selectively stimulate growth or activity of gastro-intestinal microflora that confers benefits upon the host in terms of well-being and health” (Gibson and Roberfroid, 1995). This usually involves an increase of bifidobacteria and lactobacilli. A more recent definition of prebiotic activity is “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host well-being and health” (Gibson et al., 2004). Not all dietary fibres are prebiotics, to qualify they must resist gastric digestion by acidity and mammalian enzymes and

gastrointestinal absorption. Recognized prebiotics consist mainly of glucose, galactose, xylose and fructose oligomers/polysaccharides and have monomers linked by β -configuration linkages which are not digestible by human digestive enzymes (e.g. Cellulose consists of glucose monomers linked via β -1-4 glycosidic linkages whereas starch consists of glucose monomers linked via α -1-4 glycosidic linkages).

Differences in the structure or sizes of polysaccharides, such as changes in molecular mass (MM), type of bond between monomers or the presence of substitutions along a backbone of sugar units can cause significant changes in prebiotic properties (Kabel et al., 2002a; Kabel et al., 2002b; Holck et al., 2011).

The proximal colon contains more saccharolytic bacteria and thus more sugars are fermented there. The more distal regions are where most dysfunctions arise and this is due to more proteolytic fermentation resulting in free amines (Macfarlane et al., 1992). Thus, it has been suggested that the presence of prebiotics in the distal region are likely to prove beneficial (Roberfroid, 2007). Most currently available prebiotics are derived from disaccharides and simple polysaccharides, for example fructo-oligosaccharides and galacto-oligosaccharides, with chain lengths (or degree of polymerization (DP) of 2-10 sugar residues. More complex structures may increase resistance to fermentation and allow a polysaccharide to persist further in to the distal colon.

1.4. The wheat grain

4.4.11. Structure of the wheat grain

The mature wheat grain is composed of three major tissues, the endosperm (which is the major storage organ), the embryo, and the protective outer layers. The endosperm is made up of mainly starch, protein and cell wall polysaccharides and comprises two tissues. The major tissue is the starchy endosperm which is rich in starch ($\sim 80\%$ w/w). However, the outer cells of this tissue have higher contents of protein (up to 40%) and are termed sub-aleurone cells. Surrounding the starchy endosperm is a single layer of cells with thick walls, high contents of protein and minerals and little or no starch. These are called the aleurone layer. On milling the starchy endosperm cells form the white flour fraction with the germ, aleurone and outer layers forming the bran fractions.

Proteins are the second most abundant storage compounds in the starchy endosperm, representing about 10-15% (w/w) (Toole et al., 2009), and are deposited in protein bodies which become compressed between the starch granules in the mature grain.

Cell wall polysaccharides in the starchy endosperm account for only 2-3% dry weight, with the major components in wheat grain being arabinoxylan (AX) (about 70%) and (1→3,1→4)-β-D-glucan (about 20%), with small amounts of cellulose ((1→1→4)-β-D-glucan) (about 2%) and glucomannan (about 7%) (Mares and Stone, 1973). Figure 1.5 is a schematic of a wheat grain, with the structures of the wheat grain and the constituents of each structure labelled.

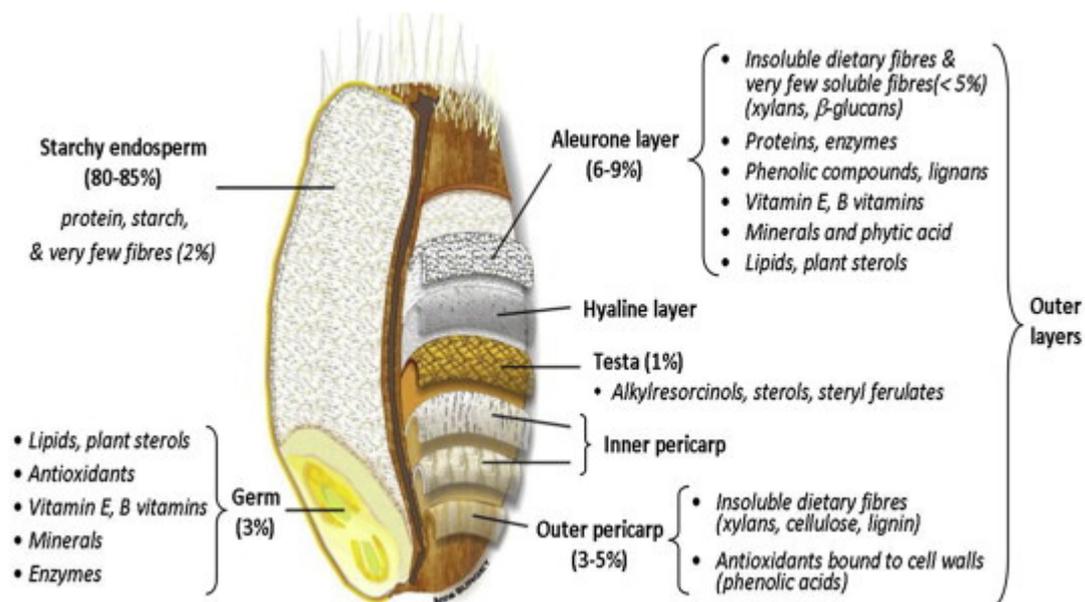


Figure 1.5: Schematic representation of transverse section through a wheat grain showing components. Image from Braun et al. 2003, (adapted) from Barron et al. (2007). The starchy endosperm is the largest tissue, containing mostly starch.

White flour, made from the wheat starchy endosperm, is used to make most food products derived from wheat, including white bread. About 60% of flour produced in the UK is used in the manufacture of bread products, with the remaining 40% being used in a huge diversity of other food products including use as thickeners, batters and for brewing. Bread is bought by 99% of British households, and the equivalent of nearly 12 million loaves are sold each day (Zhong et al., 2000), median bread consumption per person is approximately 90 grams per day,

higher for men (113g) than for women (76g). Approximately 75% of the bread we eat is white and sandwiches are thought to account for 50% of overall bread consumption (Swan, 2004). Therefore, white bread is a useful vehicle with which to deliver dietary health benefits to a large proportion of the population.

Arabinoxylans from the cell walls of the wheat grain have been reported to exhibit various biological activities, such as lowering serum cholesterol, having antioxidant activity; causing post-prandial glycaemic response reduction and anti-inflammatory and immune modulatory effects (e.g. enhancing natural killer cell activity). Additionally, they have been linked to an ability to reduce the risk of coronary heart disease and applications in weight management by increasing satiety (Ghoneum et al., 1998; Zhong et al., 2000; Lu et al., 2004; Stone and Morell, 2009).

4.4.12. Arabinoxylan (AX)

Hemicelluloses are branched polymers formed by combinations of various monosaccharides including, xylose, arabinose, galactose, mannose and fucose, with a linear backbone composed of monosaccharide subunits. In the 1920s a high viscosity hemicellulosic 'pentosan' polysaccharide, composed of xylose and arabinose (pentose sugars), was extracted from wheat flour by Hoffman and Gortner (1927). This was the first extraction of arabinoxylan (AX) from wheat flour. Subsequently AX polysaccharides were discovered in the outer layers of cereal grains such as wheat, maize, rye and their structures have since been extensively studied (Freeman and Gortner 1932; Fincher and Stone, 1986; Izydorczyk, and Biliaderis, 1995; Swennen et al., 2005; Toole et al., 2009).

Wheat endosperm contains arabinoxylan in low but significant amounts, in soluble (0.7% w/w) and insoluble (1.2% w/w) forms.

1.4.1.1. The structure of AX

Arabinoxylan is the most abundant polysaccharide present in wheat grain, it consists of a chain of β -1,4-linked D-xylopyranosyl units which may be unsubstituted or mono-substituted with *O*-2- or di-substituted with *O*-2- and *O*-3- α -L-arabinofuranose units (Gruppen et al., 1992) (Fig 1.6). The structure of AX differs between tissues. The outer layers contain more highly substituted xylan chains which have more arabinose residues esterified to a phenolic acid called

ferulic acid, which is involved in cross-linking of polysaccharide chains., The starchy endosperm and aleurone have more mono- or disubstituted xylan chains and fewer ferulic acid substitutions.

The AX structure is determined by several factors: the degree of polymerisation, the arabinose:xylose (A/X) ratio and the arabinose substitution pattern creating much heterogeneity and resulting in different physiochemical and biological properties.

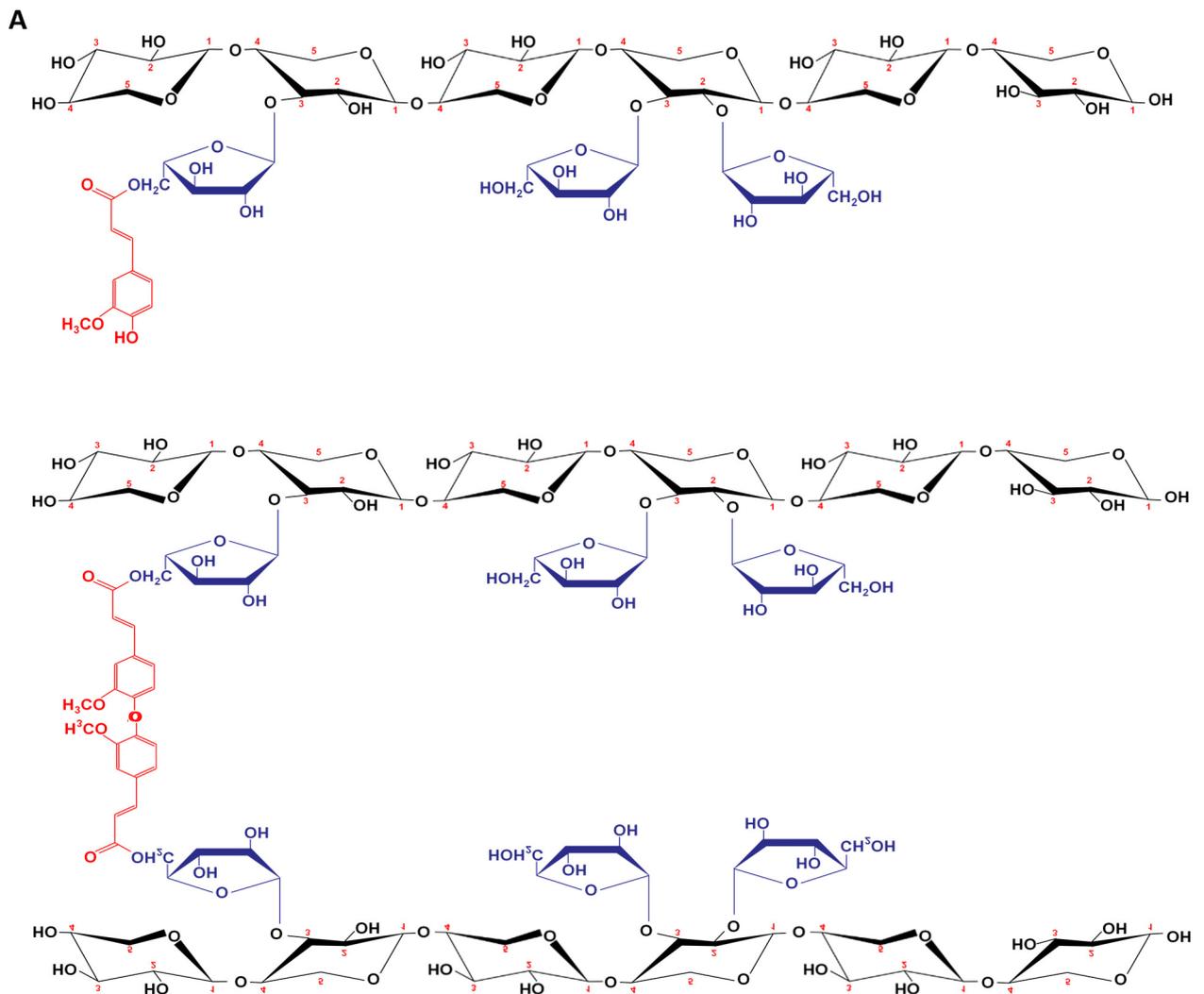


Figure 1.6: A) structure of AX with ferulic acid (FA) esterified to one arabinose monomer. B) structure of AX with FA esterified to one arabinose monomer cross linked with a second AX and FA.

In wheat endosperm about 25% of AX is water extractable (WE-AX) and soluble with a molecular mass between 200-300 kDa (Saulnier et al., 2007). WE-AXs have high heterogeneity but are characterised by their proportions of unsubstituted xylose residues; 60–65% mono-substituted xylose residues (12–20%) and di-substituted xylose residues (15–30%) (Andersson et al., 1994; Cleemput et al., 1993; Delcour et al., 1999; Izydorczyk and Biliaderis, 1995). AX that is not extractable with water is called ‘water-unextractable’ (WU-AX) or ‘alkali-extractable’ (AE-AX). The structure of AE-AX is very like WE-AX, but it has a larger average molecular mass (MM) and a slightly higher A/X ratio due to the higher proportion of arabinose side branches (Gruppen et al., 1991; Gruppen et al., 1992a; Gruppen et al., 1992b and Gruppen et al., 1993).

The ratio of arabinose to xylose (A:X or average degree of arabinose substitution) in cereal AX ranges from 0.10 to over 1.0, depending on tissue and plant species. A value of around 0.6 is common for wheat WE-AX, although WE-AX sub-fractions from flour have been shown to have values ranging from 0.31-1.06 (Dervilly et al., 2000 and Dervilly-Pinel et al., 2001).

The arabinose at the *O*-3 position of mono-substituted xylose residues can also be substituted with ferulic acid at the *O*-5 position, allowing the formation of cross-links by oxidation of ferulate present on adjacent AX chains (Bunzel et al., 2005) and creating ferulate dimers or tri-ferulate residues which are stable to acid hydrolysis (Lafiandra et al., 2014). The ferulic acid substitutions can also cross link with lignin monomers to generate ferulate-arabinoxylan-lignin complexes, strengthening the cell wall (Fincher and Stone 1986).

The A:X ratio influences solubility of AX as arabinose substitutions sterically inhibit aggregation of the (1,4)- β -D-xylan chains and lead to the formation of an extended, asymmetrical polysaccharide, thus, highly substituted AX is more soluble. For example, when a water-soluble arabinoxylan from wheat flour was treated with α -L-arabinofuranosidase, the resulting products had fewer arabinofuranosyl substituents and more readily aggregated into insoluble complexes (Marcotuli et al., 2016).

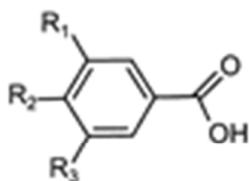
4.4.13. Phenolic acids bound to AX

Phenolic acids are a group of secondary metabolites which possess one aromatic ring bearing one or more hydroxyl groups, found in plants and are involved in defence against pathogens

and ultraviolet radiation (Poquet et al., 2008). They are present in cereals in three forms: as soluble free acids; soluble conjugates, esterified to sugars and other low molecular mass components and insoluble bound forms. Around 90% phenolic acids in wheat are bound (Saulnier et al., 2007).

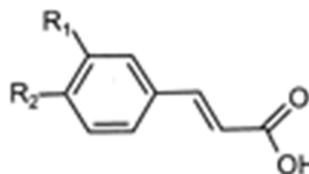
Phenolic acids can be divided into two groups and are derivatives of either hydroxycinnamic acid or hydroxybenzoic acid depending on their derivative acid (Figure 1.7). Hydroxybenzoic acid derivatives include p-hydroxybenzoic, protocatechuic, vanillic, syringic, and gallic acids. These acids are commonly present in insoluble bound forms and are typically components of more complex tannins and lignins. Hydroxycinnamic acid derivatives include p-coumaric, caffeic and ferulic acids. These acids are also present mainly in the bound forms, linked to cell wall structural components such as cellulose, lignin and AX through ester bonds (Li et al., 2008).

Hydroxybenzoic acids



$R_1 = R_2 = OH, R_3 = H$: Protocatechuic acid
 $R_1 = R_2 = R_3 = OH$: Gallic acid

Hydroxycinnamic acids



$R_1 = OH$: Coumaric acid
 $R_1 = R_2 = OH$: Caffeic acid
 $R_1 = OCH_3, R_2 = OH$: Ferulic acid

Figure 1.7: Hydroxybenzoic acids and hydroxycinnamic acids are the two forms in which phenolic acids occur. Image edited from Manach et al. (2004).

The most abundant phenolic compounds in cereals belong to the class of hydroxycinnamic acids and are mainly ferulic, diferulic, vanillic, p-coumaric, and syringic acids (Sosulski et al., 1982). Among the phenolic compounds found in wheat grain, ferulic acid is by far the most abundant and is strongly correlated with the antioxidant capacity of different wheat fractions (Mateo Anson et al., 2008). The trans isomer of FA accounts for about 90% of the phenolic acids found in wheat flour (Fulcher, 1982).

Ferulic acid is concentrated in the outer parts of the grain. The aleurone layer and the pericarp of wheat grain contain 98% of the total ferulic acid. Figure 1.8 shows how ferulic acid is linked

to AX. One of the main food sources of ferulic acid is wheat bran (5 mg/g) (Kroon et al. 1997). Wheat bran is rich in ferulic acid; however, its bio-accessibility is very low because ferulic acid is covalently bound to the indigestible polysaccharides of the cell walls (Mateo Anson et al., 2009).

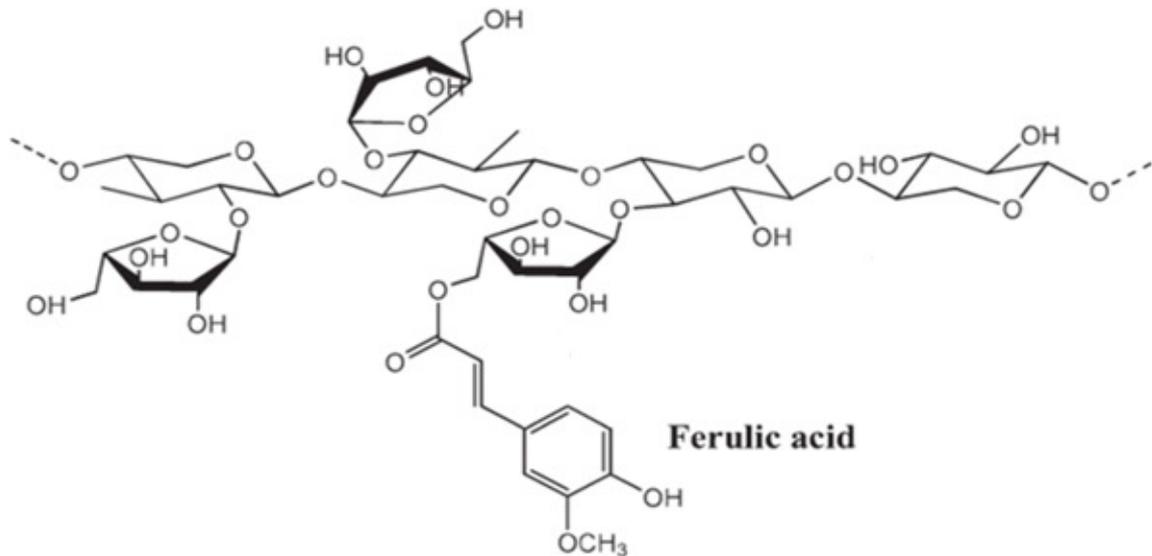


Figure 1.8: Ferulic acid attached to AX. Ferulic acid is bound to the arabinoxylans via the acid group acetylating the primary hydroxyl at the C5 position of α -L-arabinofuranosyl residues (Hatfield et al., 1999). Image from Martins et al., 2011

Although daily intake of phenolic acids varies geographically due to differences in diet, it is estimated that daily intake ranges between 20mg-1g (Luximon-Ramma et al., 2005). Although the dosage of phenolic acids required to protect against inflammatory diseases has not been determined it was shown that 50mg/kg/day ferulic acid protected diabetic rats by altering oxidative stress, expression of pro-inflammatory cytokines and apoptosis (Roy et al., 2013). However, the plasma concentration of any individual molecule rarely exceeds 1 μ M after the consumption of 10–100 mg of a single compound (Hulthe and Fagerberg, 2002) due to the limiting step of bioavailability of phenolic acids including FA so differences in dietary intake may not affect bioavailability and therefore physiological effects. The *in vivo* activity of phenolic acids strongly depends on their absorption, further metabolism, and tissue distribution. Ferulic acid was efficiently transported as the free form through an *in vitro* model for the colonic epithelium consisting of cocultured Caco-2 and mucus-producing HT29-MTX cells. Transport was shown to be strongly linked to the hydrophobicity of the different phenolic acids tested, suggesting a transcellular rather than a paracellular transport (Poquet et al., 2008).

Ferulic acid (FA) has been shown to be a potent anti-oxidant and this activity is more potent when the FA is conjugated (found as a ferulic acid β -glucuronide which contains FA and a sugar moiety- e.g. AX) (Ohta et al., 1997). FA has been linked with prevention of various diseases associated with oxidative stress, such as cancers, cardiovascular diseases and inflammation by termination of oxidation reactions leading to formation of free radicals and cell damage (Castelluccio et al., 1995; Hudson et al., 2000). For example, oxidised LDL-cholesterol is a key antigen in the generation of atherosclerosis, but not regular LDL-cholesterol (Ohta et al., 1997). Oxidised LDL appears to be able to initiate and affect inflammatory mediators such as C-reactive protein, interleukin (IL)-6, and tumour necrosis factor (TNF)- α (Hulthe and Fagerberg, 2002). Polyphenols are the most abundant antioxidants in our diets.

4.4.14. Bacterial hydrolysis of AX

For microbiota to break down arabinoxylan during fermentation, many different bonds need to be hydrolysed, requiring different enzymes, and both the yield and the structure of the resulting AX are dependent on the substrate and the specificity of the enzymes used. Glycoside hydrolases (GHs) are enzymes that hydrolyse glycosidic bonds between two or more sugars and are produced by some gut bacteria. They are currently classified into 133 families based on their amino acid sequence similarities. The most important GH enzymes involved in AX degradation are the endo- β -(1,4)-xylanases (Henrissat 1991). These enzymes randomly cleave internal β -1,4-linkages in the xylan backbone resulting in AX with a reduced MM and unsubstituted xylo-oligosaccharides. There are two major families of xylanase, GH10 and GH11, which differ in structure and in catalytic properties (Biely et al., 1997). GH10 xylanases have a MM of about 40kDa and acts preferentially on substituted xylan, whereas GH11 are about 20kDa and act preferentially on unsubstituted xylan (Vardakou et al. 2004, Vardakou et al. 2005). GH11 xylanases will not catalyse hydrolysis in the presence of (1 \rightarrow 2) and/or (1 \rightarrow 3) linked arabinose substitutions on the xylose residues present in the (-1) and (+1) or (-2) subsite, whereas GH10 enzymes appear to tolerate arabinose-decorated xylose residues in either the (-3), (-2), or (+1) subsites. (Beugrand et al., 2004).

Arabinofuranosidases are required to cleave α -L-arabinofuranosyls that are linked to singly and doubly substituted xyloses and xylosidases hydrolyse 1-4 β -D-xylans from the non-reducing termini. Feruloyl esterases, also known as ferulic acid esterases, are the enzymes involved in the release of phenolic compounds, such as ferulic, *p*-coumaric, caffeic, and sinapic acids from polysaccharides (Benoit et al., 2008). α -L-Arabinofuranosidases (EC 3.2.1.55) catalyse the

hydrolysis of the bond between arabinose and the xylan backbone, thereby leaving behind a polysaccharide with longer stretches of unsubstituted xyloses, which can in turn be more easily degraded by xylanases (Figueroa-Espinoza et al. 2002). Ferulic acid esterases (EC 3.1.1.73) hydrolyse the ester linkage between ferulic acid and arabinose residues (Petit-Benvegnen et al. 1998) (Figueroa-Espinoza et al. 2002), while β -D-xylosidases (EC 3.2.1.37) release terminal xylose residues from the non-reducing end (Tenkanen et al. 1996).

The β -glucuronidase enzyme catalyses hydrolysis of β -D-glucuronic acid from polysaccharides and is associated with production of toxic metabolites (McBain and Macafarlane, 1998).

Synergistic effects have been described between xylanases, α -L-arabinofuranosidases, and β -D-xylosidases with respect to AX degradation into monomers which occurs in the gut (Sørensen et al. 2007). The presence of arabinose substitutions, ferulic acid side chains and diferulate cross linkages between ferulic acid side chains in arabinoxyylan can significantly hinder the action of bacterial xylanase enzymes by physically inhibiting access to the β -1,4-linkages in the xylan backbone. Figure 1.9 illustrates where the various enzymes act to breakdown an AX polysaccharide.

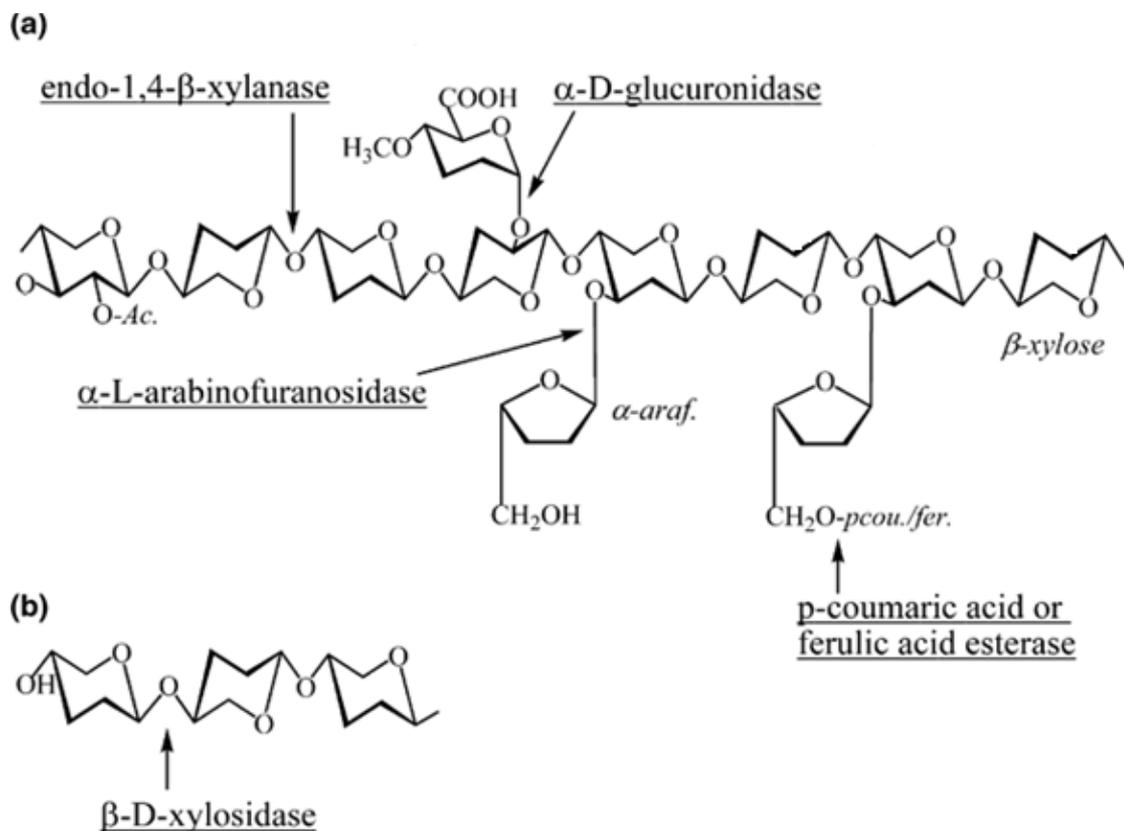


Figure 1.9: Structure of xylan and the sites of its attack by xylanolytic enzymes. The backbone of the substrate is composed of 1,4- β -linked xylose residues. α -araf., α -arabinofuranose; pcou., *p*-coumaric acid; fer., ferulic acid. (b) Hydrolysis of xylo-oligosaccharide by β -xylosidase. Adapted from Collins et al. (2005).

1.4.1.2. The effect of AX in the colon

The physiological effects of AX on gut microbiota and host health have been extensively studied and AX has been shown to provide health benefits when fermented (Englyst et al., 1987; Grootaert et al., 2007; Van Craeyveld et al., 2008; Hughes et al., 2007). The bifidogenic effect of AX has been demonstrated in a number of *in vitro* (Van Laere et al., 2000; Crittenden et al., 2002) and *in vivo* studies (Neyrinck et al., 2011; Van Craeyveld et al., 2008) where introduction of AX with different cereal source/ dp/ substitution in to the diet of rats was shown to increase SCFA concentration, increase ceacal *Bifidobacterium* populations and reduce branched SCFA production (the result of protein fermentation). *In vivo* fermentation of AX by humans has been linked with increases in acetate, propionate and butyrate after consumption of AX enriched bread (Grasten et al., 2003). Fermentation of AX *in vitro* has also been shown to increase propionic acid production, which can decrease circulating LDL-cholesterol (Amrein et al., 2003; Grootaert et al., 2009).

Fermentation of wheat endosperm AX results in the production of mainly acetate and propionate (Hopkins et al., 2003), although higher MM AX has been shown to increase concentrations of butyrate (Hughes et al., 2007).

4.4.15. Beta-Glucan

Cereal-derived β -glucans are unbranched hemicellulosic polymers (shown in Figure 1.10), similar to cellulose (β (1 \rightarrow 4) glucan), but with β (1 \rightarrow 3)-linked glucose residues every 3-4 units (Staudte et al., 1983). β -glucans have received much attention due to a beneficial effect as dietary fibre in cereals, for example in barley (Aman, 2006; Cleary and Brennan, 2006; Keenan et al., 2007). They are extended molecules comprising up to about 250,000 glucose units.

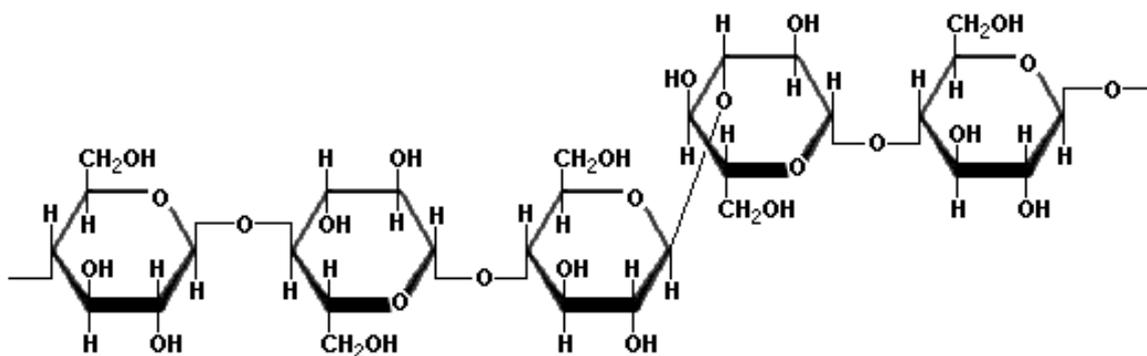


Figure 1.10: The structure of cereal beta-glucans. Cereal-derived β -glucans are unbranched (β (1 \rightarrow 4) glucan), with β (1 \rightarrow 3)-linked glucose residues every 3-4 units (Staudte et al., 1983).

Cereal derived β -glucans have been recognised to have health benefits including the maintenance of normal serum LDL cholesterol concentrations (EFSA health claim ID 1236, 1299), increase in satiety leading to a reduction in energy intake (EFSA health claim ID 851, 852), reduction of post-prandial glycaemic responses (EFSA health claim ID 821, 824) and digestive function (EFSA health claim ID 850). A 4-week crossover trial found a diet enriched with barley foods was successful in reducing total and LDL-cholesterol in men, whilst a diet enriched in wheat products had no effect (McIntosh et al., 1991).

Although most studies involving β -glucan have used oat sources, β -glucan is also found in barley and to a lesser degree in wheat. A study found β -glucan from oat and barley sources to have similar antiatherogenic effects on hamsters (Delaney et al., 2003). However, barley β -glucan is much less well researched than oat β -glucan. Structure appears to be important in regulation of physiological effect of β -glucans, fungal β -glucans with a β -(1, 3) chain with β -(1, 6) branching were found to be more effective than linear β -(1, 3) linear chain at immune modulation (Bohn and BeMiller, 1995).

Various mechanisms have been suggested to explain the reduction of cholesterol resulting from the consumption of β -glucan: Prevention of bile salt reabsorption from the small intestine, leading to increased bile salt excretion; a reduced glycaemic response leading to lower insulin stimulation of hepatic cholesterol synthesis or via microbial fermentation in the gut, releasing SCFA including propionate, which has been linked with reductions in circulating LDL cholesterol concentrations in rats (Chen et al., 1984; Kishimoto et al., 1995) and humans (Wolever et al., 1991). Regarding the latter, other studies found that rat hepatic cells were far more sensitive to propionate hypocholesterolaemia than humans and human hepatic cells

required a far larger dose of propionate than would be biologically available (Lin et al., 1995). Hughes et al (2008) found an increase in propionate after using β -glucans in *in vitro* fermentation studies and attributed the increased production to increased numbers of *Clostridium histolyticum*. Some studies have linked β -glucans with prebiotic effects on single strains of *Lactobacillus plantarum* (Russo et al., 2012) but others have shown β -glucans to have little effect on mixed bacterial colonies (Hughes et al., 2008). It is not known if β -glucan can interact with other dietary fibres to create modulate prebiotic effects through cross feeding.

4.4.16. Arabinogalactan-Peptide (AGP)

AGPs are small, water extractable proteoglycans, containing glycans consisting of a (1 \rightarrow 6)- β -D-galactopyranosyl backbone substituted in the O-3-position with a single α -L-arabinofuranosyl or a single β -D-galactopyranosyl residue (which can also be substituted in the O-3-position with a single α -L-arabinofuranosyl residue) (Van den Bulck et al., 2005). The wheat AGP peptide core is encoded by the *Gyp-1* gene and comprises 15 amino acids including three highly conserved hydroxyprolines (Hyp). Each peptide is O-glycosidically linked to a carbohydrate chain through the Hyp residue (Van den Bulck et al., 2005) (shown in Figure 1.11). Only one species of water-extractable AGP occurs in wheat flour, of which, 90% is reported to comprise the AG module, suggesting that this is the functional group and not the peptide (Tryfona et al., 2010).

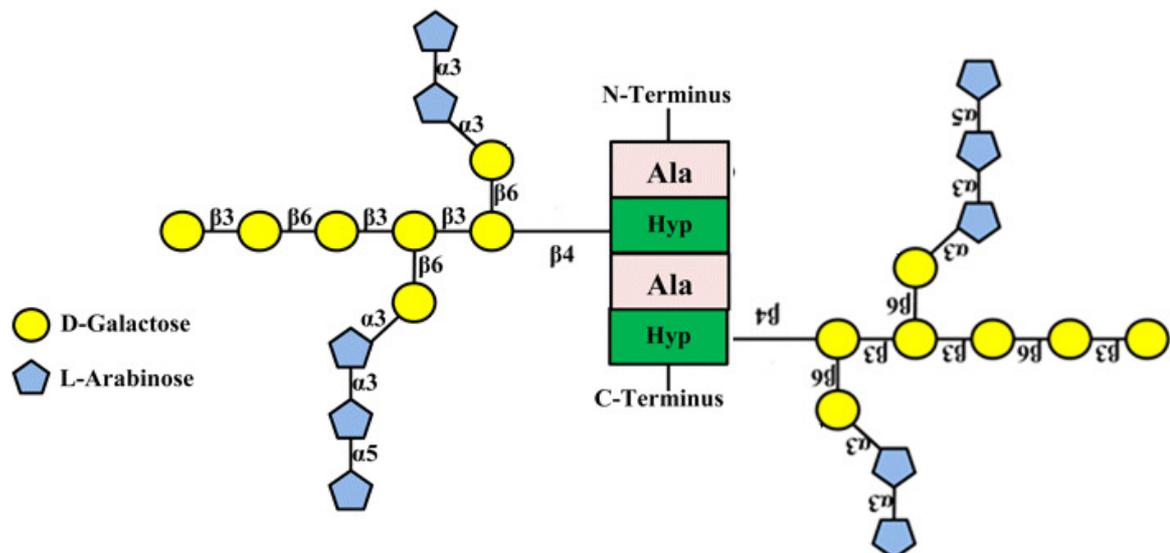


Figure 1.11: The structure of an AGP polysaccharide showing galactan backbone with arabinose side chains and amino acid residue. Modified from Tan et al. (2004).

Wheat endosperm AGP have been found in two different wheat cultivars with an arabinose to galactose (A/G) ratio of 0.66–0.73 (Loosveld *et al.*, 1998) and reported apparent molecular mass ranging from 22-70 kDa (Fincher *et al.*, 1974; Loosveld *et al.*, 1998). The galactose content of the starchy endosperm non-starch polysaccharides is almost entirely derived from AGP and accounts for approximately 10% of the non-starch derived monosaccharides (Pellny *et al.*, 2012). Several studies have elucidated the structure and plant function of wheat AGP (Fincher and Stone, 1974; Fincher *et al.*, 1974; Tryfona *et al.*, 2010; Loosveld *et al.*, 1998; Letarte *et al.*, 2006), but there have been no studies on the effect of AGP as a component of food. AGP has been estimated to be present in wheat flour at 0.27-0.38% flour dry weight and shown to influence bread making properties (Courtin and Delcour, 2002), but it is not known if AGP has prebiotic activity or any other physiological effects.

A study on arabinogalactan-protein from white-skinned sweet potato, a polysaccharide with a similar structure to AGP but with (1→3)- β -d-galactopyranosyl linkages rather than (1→6)- β -d-galactopyranosyl linkages found in AGP, showed it to cause a decrease in insulin resistance in mice by decreasing elevation of plasma glucose levels (Oki *et al.*, 2011). This suggests that AGP could have beneficial effects which should be examined.

1.5. Aims

The aim of this work is to determine how the structures of dietary fibre components and proportions of the various DF components found in wheat endosperm affect prebiotic activity.

- To determine the prebiotic activity of the three most abundant dietary fibre components of wheat endosperm, arabinoxylan, β -glucan and arabinogalactan-peptide (chapter 3).
- To determine whether DF components act synergistically and whether the ratio of polysaccharides affects prebiotic activity *in vitro* (chapter 3).
- To determine if the structure of arabinoxylan influences prebiotic activity and whether differences in the degree of polymerization and arabinose substitution result in differences in bacterial fermentation (chapter 4).
- To determine if simulated gastric digestion of isolated AX results in altered structure and effects prebiotic activity of arabinoxylan fractions of different molecular mass (chapter 5).

These aims were addressed through the following research plans:

- AGP was extracted from wheat flour and tested by *in vitro* fermentation to determine prebiotic activity. The *in vitro* fermentation of AGP was then compared to that of AGP and AX combined to determine any modulation of prebiotic activity.
- B-glucan and AX were tested for prebiotic activity by *in vitro* fermentation and the relative levels of β -glucan and AX altered to explore effects on *in vitro* fermentation.
- Commercial AX of different molecular masses were subjected to simulated gastric and duodenal digestion, prior to *in vitro* fermentation. The effect of simulated gastric digestion on polysaccharide structure was determined
- AX was isolated from a transgenic wheat line with an altered arabinose substitution pattern. This was compared to AX from control lines to determine if specific modification of arabinose substitution had significant effects on prebiotic activity

In all cases, prebiotic activity was determined by the enumeration of important bacterial groups using Flow- FISH and measurement of SCFA production.

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2. Materials and Methods

2.1. Carbohydrates

Wheat arabinoxylan of low (Polysaccharide-Wheat Arabinoxylan- Low(P-WAXYL)), medium (P-WAXYM) and high (P-WAXYH) molecular mass and insoluble arabinoxylan (P-WAXYI), high viscosity barley β -glucan (P-BHBH) and FOS with dp 2-8 (P-FOS28) were purchased from Megazyme (Co. Wicklow, Ireland).

Plant Material

Plant material was grown prior to commencement of this thesis and therefore was not done personally.

Triticum aestivum cv. Yumai-34 (a Chinese winter wheat), Transgenic wheat line TaXAT1 (TaGT61_1) (hereafter referred to as GT61) was grown according to Sparks and Jones, (2009). Construct preparation and generation of transgenic material was done at Rothamsted Research as detailed in Anders et al. (2012).

The azygous control wheat was grown at Rothamsted Research in temperature-controlled glasshouse rooms with 18°C to 20°C day and 14°C to 16°C night temperatures with a 16-h photoperiod provided by natural light supplemented with banks of Son-T 400 W sodium lamps (Osram, Ltd.) giving 400 to 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation.

White Flour

Seed moisture was measured using NMR (Bruker Minispec, Billerica, Massachusetts, US) and adjusted to 16.5% by addition of sterile distilled water at a volume of 1mL per 100g grain per 1% moisture increase before milling with Chopin CD1 mill (Calibre Control International Limited, Warrington, UK). First break and first reduction flours were combined to give white flour for analysis.

2.2. Extraction of arabinogalactan-peptide (AGP) and WE-AX from white flour

The method was adapted from Loosveld et al, (1997) based on Fincher and stone, (1974), based on Preece and MacDougall (1958).

Two hundred g flour was heated at 130 °C for 90 min to inactivate endogenous enzymes then 20g (repeated ten times) was weighed into an extraction thimble (Whatman, 22mm x 80mm) then refluxed twice with 80% ethanol for 30mins using Soxhlet apparatus (shown in figure 2.1). The ethanol insoluble residue was kept and extracted with deionized water (1:5 w/v) for 60min at 40°C then centrifuged at 3000 x *g* for 15 minutes. The supernatant was retained and 0.4 mL (7000 units) of α -amylase (A7095, Sigma-Aldrich) solution added to hydrolyse residual starch, the solution was heated to 50°C for 24h and to deactivate the amylase heated to 100 °C for 30 min.

Amyloglucosidase (PRO E0057, Prozomix) (120 units) was added and the solution heated to 60 °C for 12 h before centrifugation at 3000g for 15 mins. The supernatant was dialysed using Snakeskin dialysis tubing (3.5k molecular Weight cut off (MWCO) (Thermo Scientific) for 48h at 4°C with three changes of water. Water extractable arabinoxylan (WE-AX) and arabinogalactan-peptide (AGP) were precipitated by stepwise addition of aliquots of ethanol (95%) to a final concentration of 80%. The solution was stirred for 30 min then incubated at 4 °C overnight before centrifugation at 10,000 x *g* for 30 min at 4°C.

Precipitates were dissolved in 300mL of deionized water for 60 min at room temperature with shaking and 95% ethanol was added to a final concentration of 65% (v/v) to separate WE-AX from AGP. AGP was recovered from the supernatants by vacuum rotary evaporation at 50 °C. Precipitated AX was lyophilised.

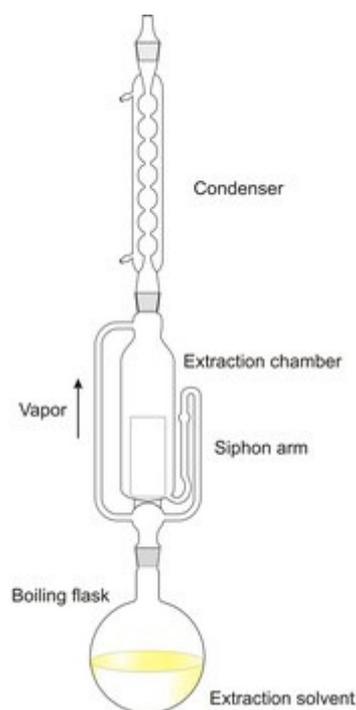


Figure 2.1: Soxhlet apparatus used to reflux WF.

2.3. Analysis of AX structure using high performance-anion exchange chromatography with pulsed amphoteric detection (HP-AEC-PAD) - 'enzymatic fingerprinting'

The protocol was adapted from Ordaz-Ortiz et al. (2005). One hundred mg AX was suspended in 0.9mL MilliQ water (18.2M ohms) and 0.1mL enzyme solution containing 16U xylanase (PRO-E0062 Prozomix) to hydrolyse β -(1 \rightarrow 4)-D-xylosidic linkages and/or 2U lichenase (PRO-E0017 Prozomix) to hydrolyse β -(1 \rightarrow 4) and β -(1 \rightarrow 3) glycosidic linkages were added. The sample was incubated at 40°C for 16 hours under continuous rotation, then centrifuged for 5 minutes at 16100 \times g and 0.6mL of supernatant removed to a fresh tube and heated to 95°C for 10 minutes in a water bath. The sample was then centrifuged at 16100 \times g for 2 minutes and the supernatant filtered using a 0.45 μ m Millex-HV syringe driven filter using a Terumo 2mL syringe. The filtrate was diluted 1 in 20 in water (50ul sample plus 950ul water) mixed thoroughly and placed in a HPLC vial with internal standard (melibiose). Twenty μ l of the diluted sample was injected onto a CarboPak PA1 (Thermo) anion exchange column on a Dionex ICS-3000 (running at 250 μ L/min, in a constant 20mM NaOH environment with a sodium acetate gradient increasing from 0-27% between 0 and 40 minutes, then increasing from 27-80% between 41-46 minutes before decreasing to 0% at 47 minutes).

2.4. Polysaccharide Analysis using Carbohydrate gel Electrophoresis (PACE)

The polyacrylamide gel consisted of a 19.7 % (w/v) resolving gel (20.2mL water, 5mL Tris-Base Borate pH8.2, 24.6mL 40% (w/v) acrylamide, 20 μ L Tetramethylethylenediamine (Temed), 200 μ L 10% (w/v) APS) and 10.5% (w/v) stacking gel (10.2mL water, 1.5mL Tris-Base Borate pH8.2, 4.2mL 40% (w/v) acrylamide, 12 μ L Temed, 60 μ L 10% (w/v) APS).

ANTS labelling: 8- Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) was used to fluorescently label the oligosaccharides released following digestion with specific endohydrolases xylanase GH11 (PRO-E0062 Prozomix) and β -1,3-1,4 glucanohydrolase lichenase (PRO-E0017 Prozomix) as described in 2.3. Fifty μ L of 1mg/mL AX digest was dried down by speed vac. Ten μ L DMSO buffer (30 μ L Acetic acid, 170 μ L water, 200 μ L DMSO), 5 μ L ANTS (0.2M molecular probes, A350 in water: acetic acid 17:3) and 5 μ L 1M NaCNBH₃ in DMSO were added to the dried AX digest. Samples were incubated at 37°C in a water bath for 16 hours, centrifuged for 30 seconds at 2,000 $\times g$ then dried under vacuum before being re-suspended in 100 μ L of 3M urea. ANTS labelled samples were loaded on to the polyacrylamide gel (3 μ L/lane). The gel was run at 200 mV for 30 minutes then 1,000 mV for 110 minutes, while cooled to 7-10°C. The PACE gel was then viewed in Gel-Doc IT TS2 310 imager using UV plate and GFP emission filter (513-557 nm).

2.5. Monosaccharides analysis by high-performance anion exchange chromatography (HPAEC)

Fifty μ L of a solution of 1mg/mL AX was dried under vacuum to which was added 400 μ L of 2M trifluoroacetic acid (TFA) and incubated at 120°C for 1 h in a heating block to hydrolyse samples. Hydrolysed samples were cooled on ice and dried in speed-vac at 30°C (overnight). 500 μ L of water was added to remove any remaining TFA and the sample was dried again in the speed-vac. The sample was finally resuspended in 400 μ L of MilliQ water. The hydrolysate was diluted further 1:1 with water (100 μ L of each sample and 100 μ L of water was mixed and pipetted into glass vials with insert. Standard curves were constructed for fucose, rhamnose, arabinose, galactose, glucose, xylose and mannose using monosaccharide standards at concentrations of 2-100 μ M prepared from stock solutions of 1mM. All samples and standards were run under the same conditions as described below. Twenty μ L was injected onto a CarboPac PA20 column (running at 500 μ L/min, eluents water, 0.1M KOH, 1M NaOH. With a NaOH gradient of

10mM for 7 minutes, 10- 20mM for 8 minutes, 800mM for 25 minutes, 200mM for 0.5 minutes then returning to starting conditions of 10mM for 13.5 minutes to equilibrate the column) on a Dionex 5000 IC HPLC equipped with disposable gold electrode.

2.6. HP-SEC-MALLS analysis of AX

Ten mg of each commercial AX fraction were weighed into an Eppendorf tube with 1mL MilliQ water and heated to 60°C for one hour to dissolve. Samples were filtered through a 0.45µM filter and pipetted into a glass vial with insert. One hundred µL was injected onto an OHPak SB 802.5 HQ column on an Agilent 1260 infinity LC system, measuring UV, LS, dRI and dp. Samples were analysed using ASTRA software. The eluent was 0.1M NaNO₃ with 0.02% NaN₃ with a flow rate of 0.5mL/min⁻¹. Average MM (kDa) and intrinsic viscosity were measured (mL/g).

2.7. MALDI-TOF-MS

MALDI-ToF-MS method was adapted from Marsh et al., (2011) using a Micromass MALDI-LR mass spectrometer (Waters, Manchester, U.K.) using a standard peptide mass fingerprinting method and mass acquisition between m/z 400 and 2400 (The laser firing rate was 5 Hz, 40 random aims per spot, 10 shots per spectrum, 10 spectra per scan, 10 scans combined, 10% adaptive background subtracted, smoothed (SavitzkyGolay), and centroided to obtain the MS spectra). The MALDI-MS was tuned to 10000 fwhm and calibrated with a tryptic digest of ADH as detailed in 2.7.1.

4.4.17. ADH digest for calibration

An alcohol dehydrogenase (ADH) digest was prepared to calibrate MALDI-Tof mass spectrometer (to generate peptides of known mass). Four mg ammonium hydrogen carbonate was added to 1mL water (AMBIC buffer). 1mg ADH was dissolved in 300µL AMBIC buffer to make the ADH solution. One mg trypsin was dissolved in 500µL AMBIC buffer. Five µL of trypsin solution was added to the ADH solution and incubated at 37°C for 90 minutes to make tryptic peptides digest. Ten µL tryptic peptides digest was added to 990µL in 0.1% TFA (10µL TFA in 9990µL water) to produce 1pmol/µL tryptic peptides.

An α -cyano matrix of 2mg/mL α -cyano-4-hydroxycinnamic acid was made using 1mg/500 μ L of 49.5% ACN, 49.5% EtOH, 1% TFA). The α -cyano matrix was mixed with ADH digest in 1:1 ratio to provide 500fmol/ μ l on target. One μ L was spotted on to MALDI plate multiple times, other dilutions were also used, down to 50fmol/ μ l.

4.4.18. Methylation

Five NaOH pellets were ground into fine powder in a pre-warmed pestle. Three mL dimethyl sulphoxide (DMSO) was added. One mL of NaOH/DMSO slurry was added to 20mg lyophilised AGP sample with 0.5mL iodomethane and sample was capped and mixed using vortex for ten minutes. The reaction was quenched using dropwise addition of 1mL water and mixed immediately after each addition. Two mL CHCl₃ was added, the sample was vortexed and left to settle in to two phases. The upper aqueous phase was removed and discarded and the lower layer was washed with water until the upper phase became clear. The lower CHCl₃ layer was dried with N₂ 20ul DHB matrix (10mg/mL Dihydroxybenzoic acid (DHB solution in 50% methanol) was added to each experimental sample.

For samples, 20 μ L DHB solution were added to methylated samples and 1 μ L spotted on to MALDI target plate multiple times. Samples were air dried before running on MALDI, spectra were analysed using Mass Lynx software as described above (2.7).

Experimental masses obtained from MALDI-ToF spectra, were matched to glycans using internet tools designed to find all possible compositions of glycan structures for their experimentally determined masses using GlycoMod (ExPaSy) (<http://www.expasy.ch/tools/glycomod/>), a table of these masses is shown in table 2.1.

Table 2.2: Molecular masses of most common monosaccharide residues and their methylated forms.

	native	methylated
Monosaccharide masses (average)		
Pentose (Arabinose, Xylose)	132.1161	160.1699
Deoxyhexose (Fucose)	146.1430	174.1968
Hexose (Glucose, Mannose, Galactose)	162.1424	204.2230
N-acetylhexosamine (N-acetylglucosamine)	203.1950	245.2756
N-acetylneuraminic acid (Sialic acid)	291.2579	361.3923
Sum of terminal group masses for sodiated <i>N</i> -glycans	41.0141	69.0679

2.8. Analysis of phenolic acid content

4.4.19. Extraction of Phenolic acids

Twenty mg samples were weighed (in triplicate) for each sample and suspended in 1mL 80% ethanol (v/v) and extracted twice, for free and conjugated phenolics followed by bound phenolics (giving a total of six extracts per total phenolic measurement). To extract free and free-conjugated phenolics, 5µL internal standard B (IS B) was added (IS B was a 20 x dilution of IS A: 1.5 mg.ml⁻¹ 3,5-dichloro-4-hydroxybenzoic acid in 80:20 (v/v) ethanol). The 20mg sample was mixed with 1mL 80% ethanol, vortexed, heated to 80°C for 10 minutes, sonicated

for 10 minutes and centrifuged at 5000 x g for 15 minutes. This was repeated twice and the supernatants combined and dried in a centrifugal evaporator. Four hundred μL 2M NaOH was added to the dried supernatant and kept in the dark for 4 hours (free and conjugated). For the extraction of bound phenolics 20 μL IS A was added to the wet pellet (from the free and conjugated extraction) and 800 μL of 2M NaOH added. Samples were left in the dark to hydrolyse for 16 hours. All extracts were acidified to pH 2 by addition of 125 μL 12M HCL and pH tested with pH paper (Fisherbrand pH-fix). The phenolic acids were extracted three times with ethyl acetate (800, 700 then 600 μL respectively) by phase partitioning. Samples were vortexed after each addition of ethyl acetate and centrifuged. The combined upper phases were dried by centrifugal evaporator. One hundred μL of 2% (v/v) acetic acid was added to each dried sample, and vortexed.

4.4.20. HPLC analysis of phenolic acid extracts

HPLC analysis of phenolic acid extracts from was carried out on a Shimadzu HPLC. 40 μL of each sample was injected on to a Kinetex 5 μm Pheny-Hexyl 100 A 150mm x 4.6 mm column and run for 18minutes. The eluent was acetonitrile (A) and 2% acetic acid (B) with the following stepped gradient: 0min 0% A, 100% B, 0.1-14min gradient to 70% A, 30% B, 14.1-18 min 100% A, 0% B. The flow rate was 1mL/ minute using a column temperature of 30°C and detection of 280/320 nm. Standards of trans-ferulic acid (tFA) at 10, 25, 50, 75, 100, 125 and 150 m[μg] were run under the same conditions with internal standard to obtain a standard curve. Sample data were compared to the standard curve to calculate the amounts of pCa and tFA.

2.9. *In vitro* gastric and duodenal digestion

In vitro gastric and duodenal digestion was done using the protocol devised by Minekus et al. (2014). Five hundred mg of each commercial AX sample (low, medium, high molecular weight and insoluble arabinoxylan fractions from wheat flour (Megazyme Co. Wicklow, Ireland) were added to 5mL water and mixed using a magnetic stirrer. Five mL simulated salivary fluid (SSF) (described in table 2.1) and 375 μL salivary amylase (A0521 Sigma-Aldrich) were added, vortexed for 2 mins, and 10 mL simulated gastric fluid (SGF) and Pepsin (P0525000 Sigma-Aldrich) equivalent to 4000U added. The pH was checked and adjusted to pH 3 by dropwise addition of 6M HCL. The digest was incubated on a shaker at 250 rpm at 37°C for two hours (simulated gastric digest) based on a gastric emptying time between water (1h) and solid meal (3-4h)

(Minekus et al., 2014). Twenty mL simulated intestinal fluid (SIF), 40mg porcine pancreatin (P7545 Sigma-Aldrich) and 19.4mg bile salts were added and samples were incubated at 37°C on a shaker at 250 rpm for two hours (simulated duodenal digest) based on times provided by Versantvoort et al., (2005). Samples were snap frozen in liquid nitrogen and then freeze dried (Lyodry compact, Mechatech systems, Bristol, UK).

Table 2.1: Preparation of stock solutions of simulated digestion fluids. Volumes are calculated for a final volume of 500mL.

	SSF	SGF	SIF
	pH 7	pH 3	pH 7
Constituent	Conc. in SSF	Conc. in SGF	Conc. in SIF
	Mmol ⁻¹	Mmol ⁻¹	Mmol ⁻¹
KCl	15.1	6.9	6.8
KH ₂ PO ₄	3.7	0.9	0.8
NaHCO ₃	13.6	25	85
NaCl	-	47.2	38.4
MgCl ₂ (H ₂ O) ₆	0.15	0.1	0.33
(NH ₄) ₂ CO ₃	0.06	0.5	-

2.10. *In vitro* fermentation in static batch culture

Batch culture vessels were autoclaved and 45mL medium was added. The medium contained per litre: 2 g of peptone water (Oxoid Ltd., Basingstoke, United Kingdom), 2 g of yeast extract (Oxoid), 0.1 g of NaCl, 0.04 g of K₂HPO₄, 0.01 g of MgSO₄·7H₂O, 0.01 g of CaCl₂·6H₂O, 2 g of NaHCO₃, 0.005 g of haemin (Sigma), 0.5 g of l-cysteine HCl (Sigma), 0.5 g of bile salts (Oxoid), 2 mL of Tween 80, 10 µL of vitamin K (Sigma).

Polysaccharide samples (AX/Pre-digested AX/AGP/ β -glucan depending on experimental set up) were added to each to give a final concentration of 1% (w/v). Each vessel was inoculated with 5 mL of faecal slurry, which was prepared by homogenizing fresh human faeces (10%, w/v) in phosphate-buffered saline (PBS; 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na₂HPO₄, and 0.2 g/L KH₂HPO₄), pH 7.3 (Oxoid), with a manual homogenizer (Fisher, Loughborough, United Kingdom) (Three faecal donors were used per experiment, between 23-59 years of age and on a normal diet without any special dietary requirements and that had not taken antibiotics, or prebiotic or probiotic supplements in the previous three months). Vessels were kept anaerobic with N gas and incubated at 37°C with a water jacket for up to 48h. Three mL samples were removed at different time points for analysis. Samples of 750 μ L were pipetted into sterile Eppendorf tubes in duplicate and centrifuged at 12045 x g for 5 minutes (Eppendorf Minispin, Eppendorf, Germany). The supernatants were removed and stored at -20 °C for SCFA analysis (2.11).

2.11. Quantification of SCFA using HPLC

The supernatants (from 2.10) that had been stored at -20°C were thawed and filtered using 0.2 μ M nitrocellulose filter and 20 μ L was injected on to a Phenomenex Rezex ROA Organic Acid H⁺ (8%) HPLC column (Watford, UK) at 50°C on a Shimadzu HPLC with 0.0025 M H₂SO₄ eluent at a flow rate of 0.6 mL min⁻¹. SCFA (lactate, formate, acetate, propionate and butyrate) were quantified using standard calibration curves from 5-50mM.

2.12. Fluorescence *in-situ* hybridisation coupled with flow cytometer (Flow-FISH)

The targeted bacterial groups were enumerated by FISH using 16S rRNA targeted oligonucleotide probes labelled with fluorescent dye. Bacteria that were counted were known to be present in human microflora and belonged to the following groups: *Bifidobacterium* genus, *Lactobacillus*/*Enterococcus* group, *Bacteroides*-*Prevotella* group, *Clostridium coccoides*/*Eubacterium rectales* groups, *Roseburia*, *Atopobium* cluster, *Clostridium cluster IX*, *Fusobacterium prausnitzii*, *Desulfovibrionales*, *Clostridium-cluster I* and *II*.

4.4.21. Fixation of samples for Flow-FISH

750 μ L samples from batch culture fermentations were pipetted into sterile Eppendorf tubes in duplicate and centrifuged at 12045 x g for 5 minutes (Eppendorf Minispin, Eppendorf, Germany). The supernatants were removed and stored at -20 °C for SCFA analysis (2.11). The pellets were resuspended in 375 μ L 1M PBS and 1125 μ L 4% (w/v) paraformaldehyde solution (2g of paraformaldehyde, 30ml of ddH₂O and 100 μ l of 1M NaOH added to a Duran bottle and incubated at 50°C in a water bath with constant stirring every 15 minutes until all the powder had dissolved. One hundred μ l 1M HCl and 16.6ml 3M PBS was added to the bottles and the solutions made up to 50ml, followed by filter sterilisation through 0.22 μ m filter into a sterile container and stored at 4°C.)

Samples were incubated at 4°C for four hours to fix, then spun as before and the supernatants discarded. The pellets were resuspended in 1mL 1M PBS and then centrifuged at 12045 x g for 5 minutes. This step was repeated once more to remove residual paraformaldehyde. PBS was removed using a pipette and samples were resuspended in 300 μ L 1M PBS and 300 μ L EtOH and stored at -20°C.

4.4.22. Addition of fluorescent probes and hybridisation

Following thawing at room temperature each sample was vortexed and 100 μ l was added to 500 μ l of 1M PBS in a 1.5mL Eppendorf tube. Samples were mixed via pipetting and then centrifuged for 3 min at 12045 x g (Eppendorf Minispin, Eppendorf, Germany). The supernatants were discarded and the pellets were resuspended in 100 μ l of TE-FISH buffer (0.1M Tris-HCl, pH 8, 0.05M EDTA, pH 8) containing lysozyme (1mg/ml). The samples were incubated in the dark for 10 min at room temperature, vortexed and centrifuged as before. The supernatants were discarded and the pellets washed with 500 μ l of 1M PBS, mixed by pipetting, vortexed and centrifuged as before.

The supernatants were discarded and the pellets were resuspended in 150 μ l of hybridization buffer (per 1mL: 180 μ L 5M NaCl, 20 μ L 1M Tris/HCl (ph 8.0), 300 μ L formamide, 499 μ L distilled H₂O 1 μ L 10 % sodium dodecyl sulphate), mixed by pipetting, vortexed and centrifuged as before. The supernatants were discarded and the pellets resuspended in 1ml of hybridization buffer, homogenized thoroughly by pipetting and 50 μ l of each sample, in hybridisation buffer, was aliquoted into Eppendorf tubes.

Four μl of the appropriate probe (MWG Biotech, Ebersberg, Germany) was added to each Eppendorf tube. Probes are shown in Table 2.2. Eub338 I-II-III is a universal bacterial probe, which binds with the all types of bacterial cells and was used as a positive control in addition to each sample probe to measure the total number of bacterial cells in each sample. Non-Eub338, which does not bind to any bacterial cells, was used as a negative control. The sample probes used were Bif164 (Langendijk et al., 1995), Bac303 (Manz et al., 1669), Lab158 (Harmsen et al., 1999), Ato291 (Harmsen et al., 2000), Prop853 (Walker et al., 2005), Erec482 (Franks et al., 1998), Rrec584 (Walker et al., 2005), Fprau655 (Hold et al., 2003), Chis150 (Franks et al., 1998) all linked to fluorescent Alexa647) and mixed Eub338 I-II-III (linked to Alexa488). Four μl of Eub338 I-II-III was added to all samples. Samples were gently vortexed and incubated in a heating block for 16 hours at 35°C covered with aluminium foil.

One hundred and fifty μl of hybridization buffer were added to each tube and vortexed gently. Samples were centrifuged for 3min at 12045 x g and the supernatants removed and discarded. The pellets were washed with 200 μl of washing buffer (per 1mL: 12.8 μL 5M Na Cl, 20 μL 1M Tris/HCl (pH 8.0), 10 μL 0.5 M EDTA (pH 8.0), 956.2 μL ddH₂O, 1 μL 10 % sodium dodecyl sulphate), vortexed gently and incubated for 20 min at 37°C in a heating block. After incubation, the samples were centrifuged as before and the supernatants discarded. Three hundred μL PBS was added to each sample tube. Samples were vortexed and screened using a flow cytometer (Accuri C6, BD Biosciences, USA) with Accuri CFlow software. Samples were run under the following conditions: 100,000 events, ungated sample, medium fluidics speed. Control samples Non EUB FL1, EUB FL1, Non-EUB FL4, EUB FL4 were run to determine autofluorescence and total bacteria. Individual bacterial group probes were calculated based on these values. Values were adjusted according to dilution factor by multiplication by the amount of sample taken to analyse/amount of PBS added to the sample (100*300).

Table 2.2. Oligonucleotide probes used during Flow-FISH analysis of batch culture samples. (50ng/ml) * These probes are used together in equimolar concentrations)

Probe Name	Sequence (5' to 3')	Fluorescence	Targeted Bacteria
Non Eub	ACTCCTACGGGAGGCAGC	Alexa-488	None
Eub338I*	GCTGCCTCCCGTAGGAGT	Alexa-488	All
Eub338II*	GCAGCCACCCGTAGGTGT	Alexa-488	All
Eub338III*	GCTGCCACCCGTAGGTGT	Alexa-488	All
Non Eub	ACTCCTACGGGAGGCAGC	Alexa-647	None
Eub338I	GCTGCCTCCCGTAGGAGT	Alexa-647	All
Eub338II	GCAGCCACCCGTAGGTGT	Alexa-647	All
Eub338III	GCTGCCACCCGTAGGTGT	Alexa-647	All
Bif164	CATCCGGCATTACCACCC	Alexa-647	<i>Bifidobacterium</i>
Lab158	GGTATTAGCAYCTGTTTCCA	Alexa-647	<i>Lactobacillus</i> and <i>Enterococcus</i>
Bac303	CCAATGTGGGGGACCTT	Alexa-647	<i>Bacteroides</i>
Erec482	GCTTCTTAGTCARGTACCG	Alexa-647	<i>Eubacterium rectale</i> / <i>Clostridium</i> <i>coccoides</i> cluster
Rrec584	TCAGACTTGCCGYACCGC	Alexa-647	<i>Roseburia</i>
Ato291	GGTCGGTCTCTCAACCC	Alexa-647	<i>Atopobium</i>
Prop853	ATTGCGTTAACTCCGGCAC	Alexa-647	<i>Clostridium</i> cluster IX
Fprau655	CGCCTACCTCTGCACTAC	Alexa-647	<i>Faecalibacterium prausnitzii</i>
DSV687	TACGGATTTCACCTCT	Alexa-647	<i>Desulfonivirionales</i>
Chis150	TTATGCGGTATTAATCTYCCTTT	Alexa-647	<i>Clostridium histolyticum</i>

2.13. Statistical analysis

Statistical analysis was undertaken by S.J. Powers (Rothamsted Research). One-way analysis of variance (ANOVA) and F-test were applied to determine differences between treatments. Differences were deemed significant when $P < 0.05$. The Genstat (2015, 18th edition, © VSN International Ltd, Hemel Hempstead, UK) statistical package was used for all analysis.

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3. Do Combinations of different Dietary Fibres from wheat flour affect Prebiotic Activity?

3.1. Abstract

The main components of the non-starch polysaccharide (NSP) fraction of wheat flour are AX, β -glucan and a water-extractable arabinogalactan-peptide (AGP). These components occur in different amounts and ratios in different cereals. Therefore, it was hypothesised that these NSPs could act synergistically resulting in different prebiotic activities when fermented *in vitro* at different ratios. AGP was isolated from wheat flour and characterised, whereas AX and mixed-linked β -glucan used in this series of experiments were purchased from Megazyme. The three fractions were then tested singly and in combination in *in vitro* fermentation studies to assess their prebiotic activity when used individually and or in different combinations and ratios. SCFAs produced from *in vitro* fermentation were measured using HPLC; flow-FISH was used for bacterial enumeration. Increases in beneficial bacteria, bifidobacteria were observed with combinations of AGP and AX fractions after 8h. Acetic acid production increased after 24h but no other SCFA increased significantly compared to the negative control treatment (no polysaccharide). There were increases in the beneficial bacterial group *Atopobium* and decreases in the sulphate reducing bacterial group *Desulfovibrionales* when AGP and AX were combined compared to when used individually, demonstrating additive effects. When AX and β -glucan were combined at different ratios in *in vitro* fermentation the greatest increases in total SCFAs (20.29M at 0h to 72.93 mM at 24h), bifidobacteria (\log_{10} 7.33 to \log_{10} 9.15) and total bacteria (\log_{10} 8.8 to \log_{10} 9.8) were observed when AX and β -glucan were combined at a 3:1 ratio.

3.2. Introduction

The major components of non-starch polysaccharide (NSP) of wheat flour are AX, β -glucan and water-extractable AGP, all are included in the CODEX Alimentarius Commission (CAC) definition of dietary fibre. Although there has been much research on the effects of AX and β -glucan in isolation, the prebiotic activity of these components when used in combination has not been investigated. This should be investigated as it is unlikely that these DFs would be found separately in foods. As AX and β -glucan are present in cereals together with AGP and have been part of the human diet for thousands of years, it is possible that our gut microbiota have co-evolved with the presence of these fibres resulting in a greater 'prebiotic effect' for fibre mixtures than for single individual types of fibre. As these NSPs are present in different ratios in different foods, for example, in wheat the ratio of AX: β -glucan is 3:1 whereas in barley the ratio is 1:3 (i.e. the reverse), different combinations could stimulate the growth of different bacteria and result in different SCFA production. AGP comprises about 0.4% of the dry weight of the wheat endosperm (Mares and Stone, 1973b) which is about equal to the amount of WE-AX (Loosveld et al., 1997; Wilkinson et al., 2017). It is composed mainly of carbohydrate (92%) with a A: G ratio of 0.67 (Fincher and Stone, 1974) attached to a 15-amino acid peptide. The structure of wheat AGP has been determined (Tryfona et al., 2010) but nothing is known about whether it acts as a prebiotic.

An experiment was therefore designed to determine whether there are synergistic effects of the major DF components and if so, which ratio of components might provide the greatest beneficial effect as determined by prebiotic activity.

3.3. Materials and Methods

4.4.23. Polysaccharides

AGP and AX were extracted from white flour isolated from white flour produced from wheat cv. Yumai-34 using the method described in 2.2.

Commercially available AX and barley β -glucan preparations from Megazyme (Bray, co. Wicklow, Ireland) was used for this series of experiments, as the *in vitro* fermentation system used was with large-scale vessels requiring 500mg of each polysaccharide. A medium MM AX fraction was selected for these experiments as it had previously been shown to have the greatest prebiotic activity of the four commercially available AX preparations.

4.4.24. Characterisation of polysaccharides

The polysaccharides were characterised prior to use, in terms of monosaccharide composition, structure and size.

AGP was also characterized using MALDI-TOF-MS after per-methylation as detailed in 2.7. Monosaccharide, structural analysis and size determination was as detailed in 2.5 and 2.6.

4.4.25. *In vitro* fermentation of AX and β -glucan

In vitro fermentation was performed as described in 2.10. AX and β -glucan were added to 100mL vessels in the following amounts 1g AX alone; 666mg AX: 333mg β -glucan; 500mg AX: 500mg β -glucan; 333mg AX: 666mg β -glucan; 1g β -glucan alone. These were compared to FOS (1g) and a negative control, in triplicate, using faecal samples obtained from three healthy donors. Polysaccharide was added to basal media at 1% (w/v). Samples were inoculated with 10% faecal slurry, as before. Sub-samples were taken at 0h, 4h, 8h and 24h.

4.4.26. *In vitro* fermentation of AGP and AX

Small scale *in vitro* fermentation was performed as described in 2.10. Smaller scale fermentation was used due to the small amounts of AGP and AX extorted from WF. AGP (100mg), AX

(100mg) or AGP+AX (50mg of each) were compared to FOS (100mg) and a negative control (no polysaccharide) in 10mL vessels in triplicate for each sample, using faecal samples obtained from three healthy donors. Polysaccharide was added to basal media at 1% (w/v). Samples were inoculated with 10% faecal slurry, and sub-samples were taken at 0h, 8h and 24h.

4.4.27. Enumeration of bacteria by flow-FISH

Bacteria were enumerated from samples taken at 0h, 8h and 24h using flow-FISH as detailed in 2.12.

4.4.28. SCFA analysis

SCFA were quantified using HPLC as detailed in 2.11.

4.4.29. Statistical analysis

The Genstat (2015, 18th edition, © VSN International Ltd, Hemel Hempstead, UK) statistical package was used for all analysis. ANOVA data was supplied by SJ Powers (Statistical department, Rothamsted Research) and applied as detailed in 2.13.

3.4. Results and Discussion

4.4.30. Polysaccharide characterisation

Table 3.1 shows that all polysaccharides have a purity of 91% or above. Previous studies have used samples containing polysaccharides of 90% purity, therefore it can be concluded that >91% is sufficient purity for reliable results (Van Laere et al., 2000; Hughes et al., 2007).

Commercially available AX was compared to commercially available β -glucan and extracted AX compared to extracted AGP to reduce extraneous variables by reducing variation from DF source.

Table 3.1: Characterisation of AGP, AX and β -glucan: ¹ data provided by Megazyme (Co. Wicklow, Ireland); ² isolated from white flour and determined by monosaccharide determination), described in Figure 3.1.

Polysaccharide	Purity	MM (kDa)
Extracted AX ²	91 % ²	n/d
Extracted AGP	96.73% ²	n/d
Commercial Beta-glucan	94% ¹	495 ¹
Commercial AX ¹	95% ¹	323.9

3.4.1.1. Monosaccharide analysis

Figure 3.1 shows monosaccharide analysis of AGP and AX isolated for the study from wheat flour (*Triticum aestivum* cv. Yumai-34), samples were purified with isolated polysaccharide comprising >91% of the total monosaccharides present. Arabinose and galactose comprised 96.73% (± 0.18) of total sugars in AGP, with other monosaccharides detected, glucose and xylose comprising 2.36% for glucose and 1.74% for xylose. The A: G ratio for AGP was 0.48.

This is lower than the average ratio of 0.66–0.73 found by Loosveld *et al.* (1998) which could be related to the wheat cultivar.

For isolated AX, the arabinose and xylose content was 91% (± 0.05), with galactose 5% and glucose 4%. The A:X ratio for AX was 0.62.

Figure 3.2 shows monosaccharide analysis of commercial β -glucan and commercial AX. No glucose or galactose were detected.

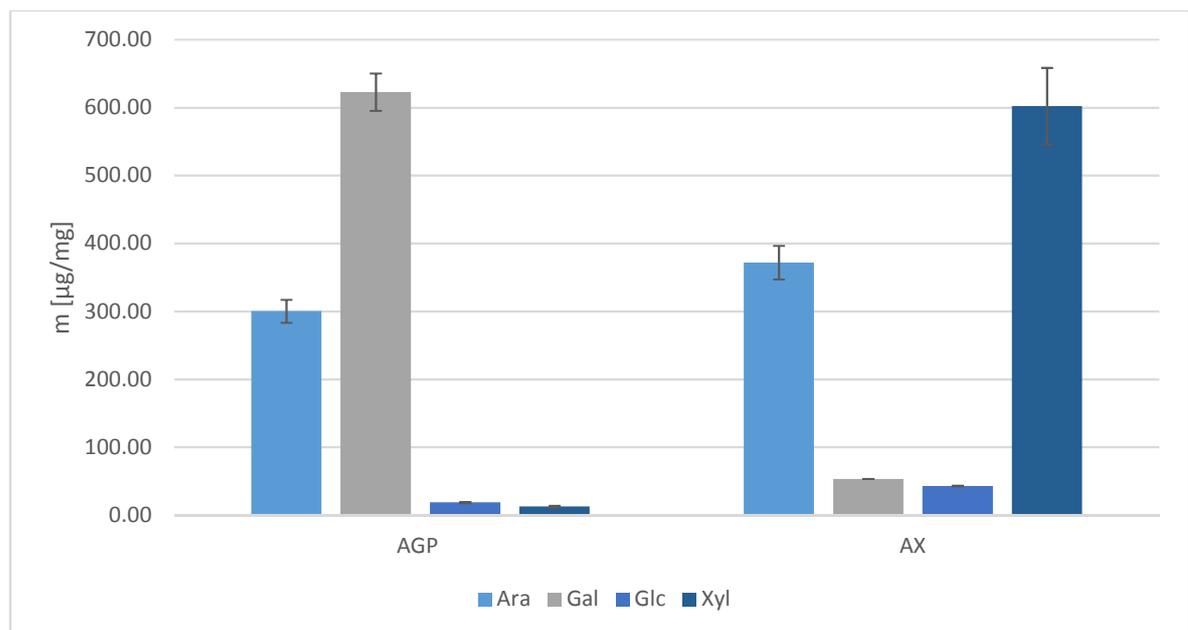


Figure 1.1: Monosaccharide analysis of extracted AGP and AX using a Carbopac PA20 column. (N=3) Error bars are SD. AGP is composed of mostly arabinose and galactose, with small amounts of glucose and xylose, whilst AX is composed mostly of arabinose and xylose with small amounts of glucose and galactose.

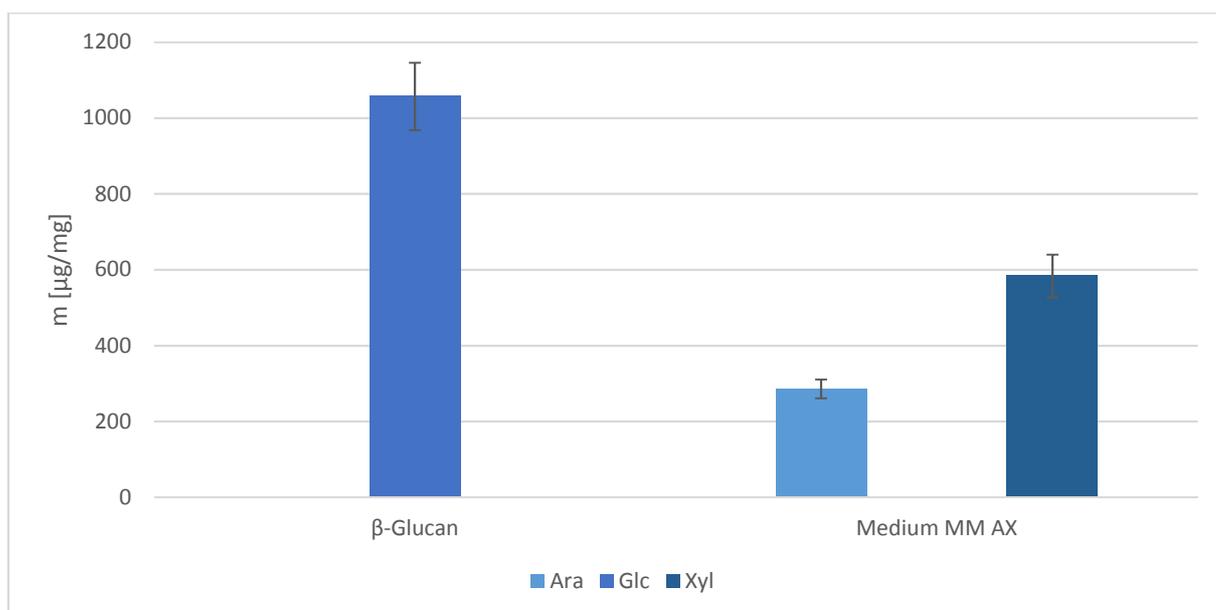


Figure 3.2: Monosaccharide analysis of commercial β -glucan and Medium MM AX using a Carbowac PA20 column. (N=3) Error bars are SD. B-glucan is entirely composed of glucose whilst AX is composed only of arabinose and xylose.

3.4.1.2. Analysis of AGP by MALDI-TOF-MS

MALDI-TOF-MS was used to confirm the structure of the carbohydrate moiety of the AGP, based on the molecular masses of the oligosaccharides released by the exo-b-(1- \rightarrow 3)-galactanase, as described in Tryfona et al (2011). Figure 3.3 shows the spectrum from 400-2400 m/z and Figure 3.4 shows magnification of the spectra from 400-1300 m/z. for greater clarity. Oligosaccharide composition is indicated by Hex (hexose residue) or Pent (pentose residue) and subscript indicates the number of residues present. The most dominant ion was 'Pent₄', at 637 m/z which is predicted to be four arabinose subunits (chapter 2.7 shows details of ion identification).

Figures 3.3 and 3.4 show similar results to the spectra for AGP obtained from *Triticum aestivum* cv. Cadenza white flour found by Tryfona et al. (2010). Thus, the monosaccharide composition (Fig 3.1) and the mass spectra obtained for AGP confirmed the purity and identity of the AGP isolated and used in the *in vitro* fermentation experiments.

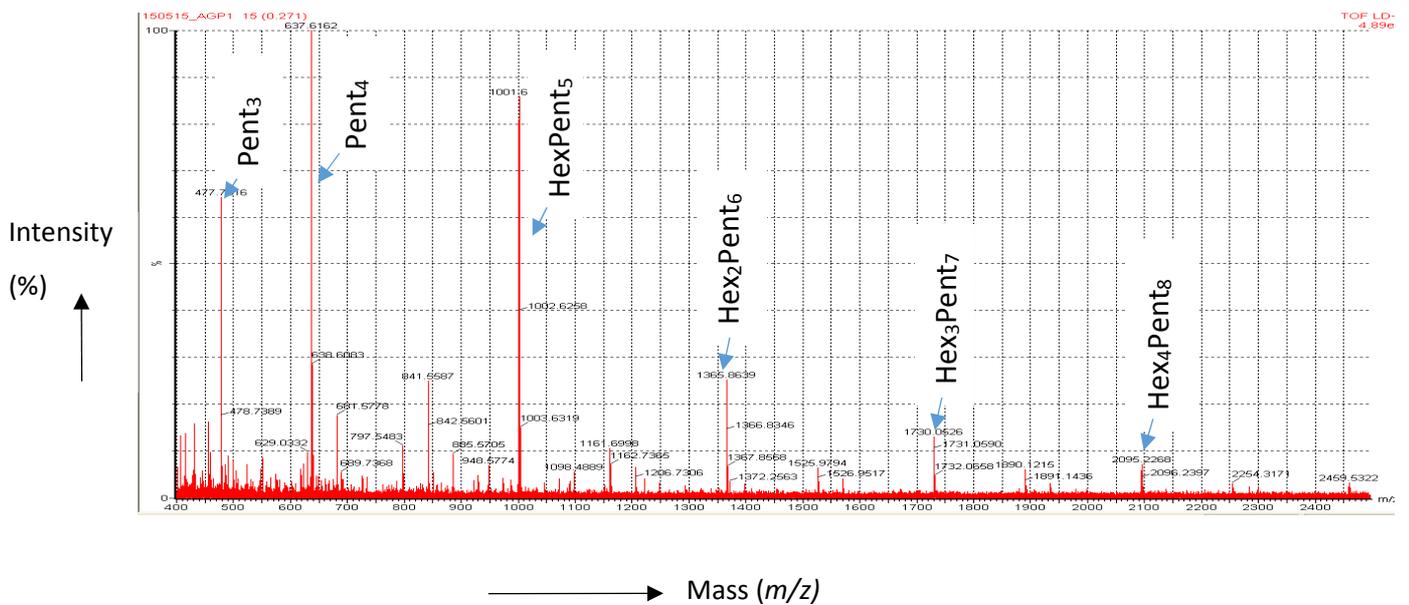


Figure 3.3: MALDI-ToF MS spectra showing ions of m/z diagnostic of methylated oligosaccharides released from AGP by exo-B-(1->3) galactanase digestion. AGP was isolated from *Triticum aestivum* cv. Yumai-34 white flour. Spectra shows 400-2400 m/z

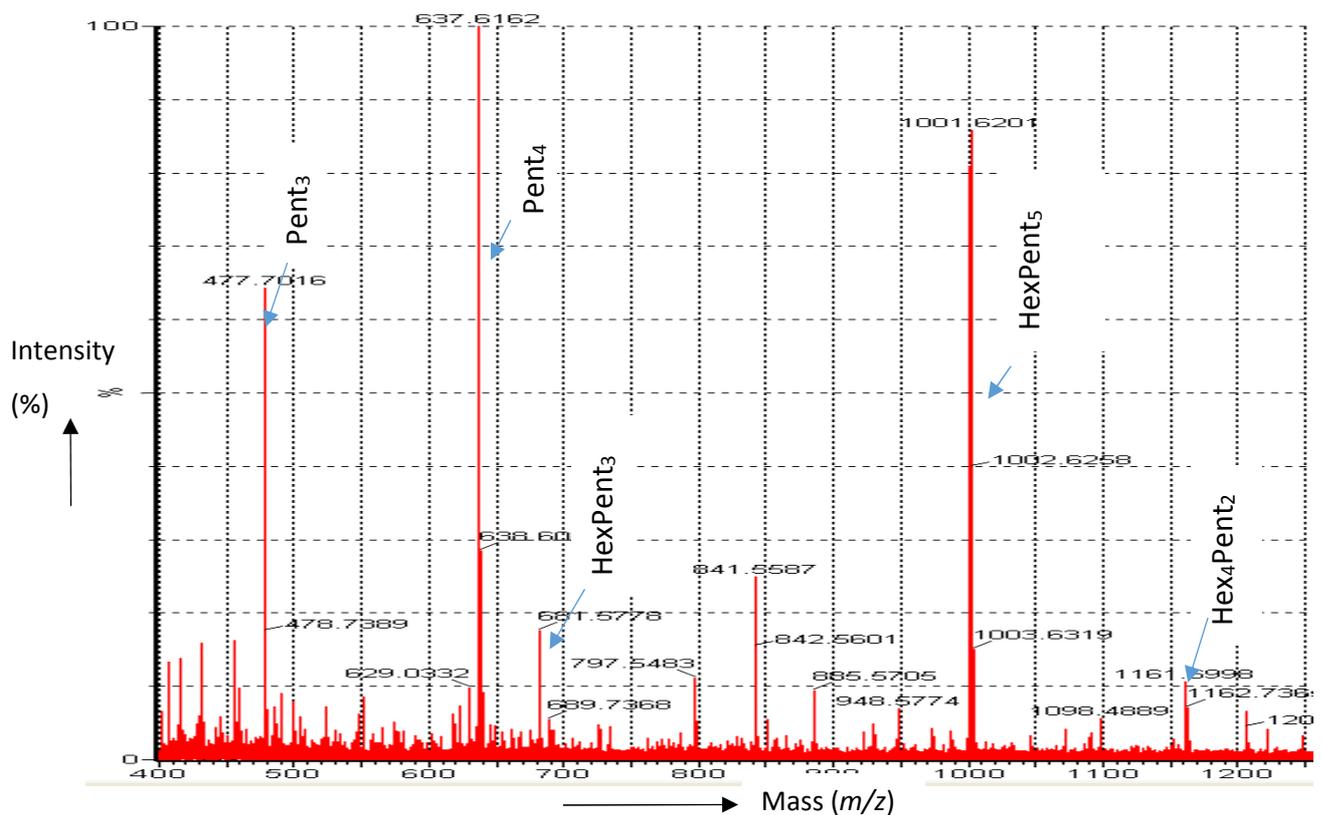


Figure 3.4: Zoomed in and labelled MALDI-ToF MS spectra showing ions from methylated oligosaccharides released by exo-B-(1->3) galactanase digestion of AGP isolated from *Triticum aestivum* cv. Yumai-34 white flour.

4.4.31. Analysis of *in vitro* fermentation of AGP and AX by changes in SCFA concentrations and composition of bacterial populations

The concentration of SCFA and lactate after fermentation of AGP, AX and AGP+AX are shown in Table 3.2. and the numbers of bacterial populations after fermentation of AGP, AX and AGP+AX are shown in Table 3.3. All substrates caused increases in SCFAs and beneficial bacterial groups concomitant with prebiotic activity. AX singularly has previously been shown to have prebiotic activity (Englyst et al., 1987; Grootaert et al., 2007; Van Craeyveld et al., 2008; Hughes et al., 2007) and data has been included from a separate experiment with a different negative control to observe whether AGP +AX in combination have greater prebiotic activity due to synergistic effects than AX or AGP singularly.

All substrates AGP, AX and AGP+AX caused significant ($p>0.95$) increases of beneficial bacteria *Bifidobacterium* from 8-24h compared to the negative control. The AGP+AX sample gave increases similar to FOS (the positive control), whilst AGP alone gave still significant but smaller increases. The *Bifidobacterium* populations peaked at 8h for all samples and began to decrease after. This indicates that AGP and AX singly or in combination can be fermented quickly- at a similar rate to FOS.

Bifidobacteria are known acetate producers (Fukuda et al., 2011; Bindels et al., 2015), so an increase in bifidobacteria may result in increased acetate production, which was observed after fermentation of all substrates, AGP, AX and AGP+AX together, with AGP and AGPP+AX showing larger increases, although all increases in acetate cannot be attributed to bifidobacteria as they contribute a relatively small proportion of colonic bacteria. The faster increase in acetate shown in Table 3.2 for AGP than AGP+AX might suggest that AGP alone can be fermented faster by the bacterial populations present than AGP when mixed with AX. Presumably this could be because the two dissimilar structures would require a wider variety of hydrolytic enzymes to break them down or because the bacteria present were able to break down AGP faster than AX.

Fermentation of AGP and AX together showed different responses to AGP alone. Fermentation of the two DFs AGP+AX together caused increased growth of *Clostridium coccoides*- *Eubacterium rectale* ($p>0.95$), plus significant ($p>0.95$) reductions in sulphate reducing

group *Desulfovibrionales* and proteolytic group *Clostridium cluster I* and *II*. These data show that AGP+AX appear to have greater prebiotic activity than AGP alone.

The AGP+AX sample increased beneficial groups *Clostridium coccoides*/*Eubacterium rectale* increased significantly ($P>0.95$) compared to the negative control at 8h from 23% to 37% bacteria. This increase was not sustained until 24h, demonstrating a short fermentation. This bacterial group was also increased with AX. *Eubacterium rectale* is one of the most frequently recovered bacterial species from human faeces and is usually present in large numbers. It produces mixtures of SCFAs from fermentation including acetate, butyrate, lactate and formate but not propionate (Krumholz and Bryant, 1986) and so could also be responsible for large increases in acetate shown in Table 3.2. A significant ($p>0.95$) increase in *Clostridium coccoides*/*Eubacterium rectale* compared to the negative control could explain the increase in formate observed after fermentation with AX. There were no significant ($p>0.95$) changes to formate concentrations in any other sample.

The *Atopobium* group decreased with the AGP+AX substrate compared with the negative control, as did *Desulfovibrionales* and *Clostridium cluster I* and *II*. These groups are associated with pathogenic behaviour, *Clostridium cluster I* and *II* are associated with protein fermentation, and so reductions in these groups show AX+AGP have higher selectivity than AX alone or FOS (the positive control). *Atopobium* can be a butyrate producing group, reductions in this group may help to explain the lack of increase in butyrate in the AX+AGP sample.

Significant differences ($p>0.95$) compared to the negative control, were seen in the concentration of acetic acid, which increased to a similar level as the positive control (FOS) for AGP and AGP+AX together after 24h, although increases were significant ($p>0.95$) after 8h fermentation of FOS but only after 24h fermentation of AGP+AX and AX, indicating a quicker fermentation of FOS than AX and AX+AGP.

After 8h fermentation, average acetate concentration had increased significantly ($p>0.95$) to 23.83 mM in the AGP samples, and to 21.12mM in the AX sample, but only 17.05mM in the AGP plus AX sample which was not significant ($p>0.95$), however by 24h this difference had disappeared. This might suggest that AGP or AX alone can be fermented faster by the bacterial populations present than AGP when mixed with AX. Presumably this could be because the two dissimilar structures would require a wider variety of hydrolytic enzymes to break them down or because the bacteria present were able to break down AGP faster.

There was a large decrease in lactate concentration in the AGP, AX and AGP+AX samples after 24h compared with the negative control. Lactate is formed from pyruvate through the action of lactate dehydrogenase in the homofermentative pathway by many common gut bacteria including *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Streptococcus* and *Eubacterium* spp. (Barcenilla et al., 2000). An increase in bifidobacteria was shown (Table 3.3), which could explain the concomitant decrease in lactate observed, as the lactate is used by cross-feeding bacteria. Under healthy gut conditions lactate is usually found in low concentrations in faeces (<5mM) (Duncan et al., 2004) because bacterial breakdown of lactate markedly exceed production (Belenguer et al., 2011). Therefore, decreases in lactate over the course of the fermentation in both AX samples reflect healthy colonic conditions.

Table 3.2: SCFA and lactate concentration in batch cultures at 0, 4, 8 and 24 hours' fermentation comparing no substrate, FOS, AGP and AGP+AX. Negative control is no added carbohydrate and positive control is FOS. One way ANOVA was applied to the data to test the interaction between treatments. Standard error of the mean (SEM) is shown in brackets. Significant interaction between treatments and negative control are denoted * p= 0.05 (F-test) and are shown in bold.

	Time	Lactate (μM)	Formate (μM)	Acetate (μM)	Propionate (μM)	Butyrate (μM)	Total (μM)
Negative control	0	2.28 (1.20)	2.79 (4.97)	3.13 (1.05)	3.52 (0.62)	2.86 (1.08)	21.61 (1.48)
	8	6.30 (1.79)	1.58 (5.21)	8.31(1.84)	5.10 (0.97)	1.57 (0.65)	27.12 (5.41)
	24	10.17 (2.42)	4.31 (5.06)	8.28 (2.04)	10.25 (5.19)	3.32 (1.24)	36.33 (8.49)
FOS	0	7.22 (3.51)	3.57 (4.88)	9.16 (1.09)	5.27 (1.80)	2.33 (0.28)	27.55 (8.28)
	8	12.61 (8.95)	9.91 (3.91)	30.57* (4.77)	11.27 (5.59)	3.09 (1.98)	67.45 (17.12) *
	24	10.16 (5.53)	7.46 (4.64)	33.35* (6.69)	13.03 (7.03)	8.36 (6.59)	72.36 (32.92) *
AGP	0	7.86 (5.12)	1.61 (5.59)	6.80 (3.25)	3.49 (1.52)	5.78 (1.67)	25.54 (5.97)
	8	5.75 (2.37)	6.24 (2.24)	23.83* (5.95)	12.33 (5.09)	2.81 (1.07)	50.96 (11.40) *
	24	3.48* (1.65)	5.33 (3.68)	39.34* (4.53)	8.60 (6.90)	5.20 (3.53)	61.95 (9.94) *
AGP + AX	0	10.01* (2.58)	10.95 (10.65)	6.95 (3.49)	5.48 (1.37)	3.53 (0.84)	36.92 (9.28)
	8	4.50 (1.98)	7.30 (3.80)	17.05 (8.52)	12.86 (5.25)	2.37 (1.94)	44.08 (15.81)
	24	2.38* (1.03)	10.52 (5.62)	38.91* (9.58)	15.61 (6.47)	2.95 (1.74)	67.42 (10.47) *
Negative control for AX	0	11.02 (3.68)	0.00 (0.00)	4.21 (1.22)	4.36 (1.10)	2.26 (0.47)	21.85 (3.15)
	8	5.66 (0.43)	0.62 (0.28)	9.05 (2.40)	8.77 (1.41)	2.14 (0.28)	26.24 (3.97)
	24	6.45 (0.16)	3.71 (0.93)	9.83 (2.72)	9.33 (1.84)	1.48 (0.19)	30.08 (1.97)
AX	0	16.39 (3.37)	0.00 (0.00)	4.93 (1.01)	5.05 (0.60)	1.54 (0.20)	27.91 (3.04)
	8	10.19* (2.86)	6.63* (2.30)	21.12* (4.46)	8.09 (0.48)	1.76 (1.76)	47.79 (4.99) *
	24	2.56* (1.31)	15.90* (4.17)	27.64* (4.85)	9.76 (2.77)	1.29 (0.12)	57.15 (12.25) *

Table 3.3: Bacterial enumeration of *in vitro* batch culture fluid after fermentation with AGP or AGP plus AX. Negative control is no added carbohydrate and positive control is FOS. Values are mean log₁₀ bacterial numbers/mL found using flow FISH. One-way AVONA was applied to the data to test (F-test) the main interaction between treatments. Values in brackets are SEM. Significant interaction between treatments and negative control are denoted * (p < 0.05) and shown in bold.

	Time (h)	<i>Bifidobacterium</i> genus	<i>Lactobacillus</i> Enterococcus group	<i>Bacteroides-Prevotella</i> group	<i>Clostridium</i> <i>coccoides-Eubacterium rectale</i> group	<i>Roseburia</i>	<i>Atopobium</i> cluster	<i>Clostridium</i> cluster IX	<i>Faecalibacterium prausnitzii</i> group	<i>Desulfovibrionales</i>	<i>Clostridium</i> -cluster I and II	Total
Negative	0	7.96 (0.26)	6.80 (0.38)	7.14 (0.031)	8.53 (0.33)	6.78 (0.75)	7.20 (0.58)	7.35 (0.36)	8.28 (0.25)	7.85 (0.47)	6.86 (0.40)	8.97 (0.33)
	8	7.72 (0.11)	7.16 (0.12)	7.43 (0.22)	8.37 (0.25)	7.38 (0.02)	7.20 (0.10)	7.42 (0.27)	8.06 (0.24)	7.69 (0.30)	7.31 (0.17)	8.85 (0.22)
	24	7.68 (0.66)	7.25 (0.73)	7.36 (0.68)	8.00 (0.49)	7.46 (0.71)	7.51 (0.52)	7.53 (0.60)	7.49 (0.58)	7.38 (0.46)	7.12 (0.73)	8.62 (0.57)
FOS	0	7.88 (0.27)	7.01 (0.46)	7.57 (0.43)	8.23 (0.27)	7.41 (0.50)	7.01 (0.49)	7.45 (0.35)	8.22 (0.25)	7.75 (0.46)	7.11 (0.59)	8.87 (0.30)
	8	9.83* (0.15)	7.32 (0.60)	8.20* (0.40)	9.70* (0.17)	8.16 (0.70)	8.02 (0.46)	8.22 (0.38)	8.31 (0.41)	7.88 (0.55)	7.69 (0.48)	10.26* (0.09)
	24	8.95* (0.21)	7.13 (0.72)	7.43 (0.66)	8.73 (0.20)	7.29 (0.72)	7.17 (0.54)	7.72 (0.60)	7.89 (0.58)	7.61 (0.60)	7.16 (0.60)	9.36 (0.27)
AGP	0	7.96 (0.25)	7.02 (0.26)	7.23 (0.10)	8.59 (0.19)	7.66 (0.24)	7.27 (0.30)	7.41 (0.17)	8.19 (0.24)	8.04 (0.19)	7.16 (0.19)	9.00 (0.20)
	8	8.89* (0.45)	6.87 (0.51)	6.74 (0.70)	8.92 (0.36)	7.89 (0.22)	7.23 (0.14)	7.32 (0.30)	8.47 (0.16)	8.06 (0.08)	7.18 (0.35)	9.49 (0.31)
	24	8.70* (0.78)	6.57 (0.33)	6.98 (0.83)	8.66 (0.47)	6.77 (0.30)	6.57 (0.37)	7.20 (0.78)	7.34 (0.17)	6.49 (0.35)	5.54* (0.76)	9.14 (0.55)
AGP+AX	0	7.98 (0.18)	7.06 (0.36)	7.20 (0.46)	8.63 (0.29)	7.37 (0.46)	7.12 (0.46)	7.20 (0.41)	8.38 (0.19)	7.95 (0.36)	7.04 (0.40)	9.06 (0.26)
	8	9.35* (0.46)	7.22 (0.44)	7.74 (0.28)	9.36* (0.38)	7.61 (0.78)	7.00 (0.12)	7.63 (0.31)	8.59 (0.23)	8.00 (0.35)	6.94 (0.30)	9.89 (0.33)
	24	8.82* (0.28)	6.88 (0.76)	6.73 (0.67)	8.82 (0.19)	6.64 (0.75)	6.27* (0.28)	6.47 (0.53)	7.13 (0.30)	6.07* (0.45)	5.62* (0.43)	9.25* (0.13)
Negative for AX	0	8.53 (0.33)	7.65 (0.13)	7.95 (0.12)	9.11 (0.04)	8.63 (0.11)	7.60 (0.16)	8.20 (0.09)	9.07 (0.04)	8.61 (0.06)	7.75 (0.15)	9.70 (0.03)
	8	8.58 (0.32)	7.90 (0.03)	8.55 (0.08)	9.01 (0.08)	8.25 (0.13)	7.70 (0.09)	8.70 (0.06)	8.90 (0.02)	8.54 (0.10)	8.20 (0.12)	9.67 (0.07)
	24	8.76 (0.30)	7.77 (0.18)	8.45 (0.13)	8.92 (0.07)	7.74 (0.20)	7.85 (0.10)	8.76 (0.08)	8.54 (0.06)	8.12 (0.12)	7.93 (0.12)	9.58 (0.10)
AX	0	8.52 (0.41)	9.84 (0.14)	8.11 (0.17)	9.13 (0.01)	8.65 (0.06)	7.88 (0.05)	8.28 (0.12)	8.89 (0.09)	8.58 (0.03)	8.00 (0.16)	9.67 (0.07)
	8	9.23* (0.19)	7.92 (0.20)	8.84 (0.23)	9.09 (0.38)	8.32 (0.52)	7.65 (0.37)	8.93 (0.24)	9.00 (0.19)	8.61 (0.19)	7.81 (0.40)	9.94 (0.24)
	24	9.84* (0.09)	7.67 (0.22)	8.55 (0.44)	9.57* (0.18)	8.42* (0.40)	8.45* (0.53)	8.63 (0.44)	9.06* (0.17)	8.26 (0.20)	8.11 (0.42)	10.29* (0.05)

4.4.32. *In vitro* fermentation of AX and β -glucan, analysis of changes in SCFA concentrations and composition of bacterial populations

Table 3.4 shows the SCFA and lactate concentration of samples incubated with: AX alone, AX: β -glucan (3:1), AX: β -glucan (1:1), AX: β -glucan (1:3), β -glucan alone and FOS after 24 hours' fermentation and Table 3.5 shows the bacterial populations. In this experiment, larger 100mL vessels were used, requiring 1g substrate, because the substrate was commercially bought and therefore readily available.

Differences were observed between the prebiotic activities of AX and β -glucan fractions. Fermentation of AX, AX: β -glucan (3:1), AX: β -glucan (1:1) and FOS increased total SCFA concentration significantly ($p > 0.95$) compared to the negative control. This increase was mainly due to production of acetate. Acetate and butyrate concentrations increased significantly for AX alone; AX: β -glucan (3:1) and AX: β -glucan (1:1) compared to the negative control. Neither acetate or butyrate were significantly increased compared to the negative control for AX: β -glucan (1:3) or β -glucan alone, however no increases in bacterial groups were observed for these samples, supporting this finding.

The data from Table 3.4 and Table 3.6 showing acetate concentration and the *Bifidobacterium* populations support each other as bifidobacteria are known producers of acetate (Fukuda et al., 2011; Bindels et al., 2015) and the increases in acetate and bifidobacteria are observed with the same samples (AX, AX: β -glucan (3:1), AX: β -glucan (1:1) and FOS). The largest increase in bifidobacteria resulted from fermentation of AX: β -glucan in a ratio of 3:1. Only fermentation vessels containing AX at a concentration of least 50% showed an increase in bifidobacteria, showing a bifidogenic effect for AX but not β -glucan and supports previous studies where oat and barley β -glucans did not affect bifidobacteria numbers but AX did result in an increase in bifidobacteria (Hughes et al., 2007; Hughes et al., 2008; Kim and White, 2009). Thus, the results of the present study confirm that AX is the preferred substrate for bifidobacteria. The increases in bifidobacteria were as sustained for FOS as for AX, with all increases lasting from 8-24h.

Table 3.5 shows the ratios of SCFA after 24 hours' *in vitro* fermentation with the different ratios of polysaccharides. The highest proportion of propionate is seen with the β -glucan sample alone at 44.9%, just above the negative control at 37%. Butyrate remains low for all samples, peaking in the AX: β -glucan (1:3) sample at just 17.7%. Acetate is the highest contributor of total SCFA for all samples. The addition of larger amounts of AX appears to drive the concentration

towards greater acetate production, whereas greater β -glucan appears to favour propionate production. Two studies found fermentation of β -glucan to favour production of propionate and fermentation of AX to encourage acetate (Hughes et al., 2007; 2008). Table 3.6 shows a significant ($p > 0.95$) increase in the *Bacteroides-Prevotella* group at 24h compared to the negative control after fermentation of β -glucan, which supports this theory as microbiota from the *Bacteroides-Prevotella* group are known producers of propionate (Macy and Probst, 1979). It is proposed that three colonic *Bacteroides* species: *Bacteroides thetaiotaomicron*, *Bacteroides distasonis* and *Bacteroides fragilis* are responsible for the majority of β -D-(1 \rightarrow 3)-glucanase activity responsible for β -glucan hydrolysis (Salyers et al., 1977a).

The greatest increases in bacterial numbers were seen for the AX: β -glucan in a (3:1) sample. Fermentation of AX alone showed the next highest increase while the smallest increase in bacterial numbers occurred when β -glucan alone was used. In fact, the proportion of β -glucan in the *in vitro* fermentation was inversely correlated with the increase in total bacteria. This suggests that AX is more readily used as a substrate for growth by bacteria than β -glucan. As the ratio of AX: β -glucan is greater in wheat (3:1) than barley (1:3), this data agrees with a study which showed that a wheat based diet in pigs resulted in a greater increase in gut bacteria than a barley based diet (Garry et al., 2007). The increase in total bacteria with higher amounts of AX is more sustained than with FOS as it continues in these samples for 24h whereas fermentation of FOS increases total bacteria only up to 8h.

Fermentation of β -glucan alone caused the largest increases in *Lactobacillus* compared to the negative control after 24h, implying that β -glucan was the preferred substrate for these bacteria. Crittenden et al. (2002) found that β -glucan was fermented by *Bacteroides* and *Clostridium beijerinckii* but not fermented by *Lactobacillus*, *Bifidobacterium* or *Enterococci*. In this study, there is no difference in the number of bifidobacteria after incubation with β -glucan, showing that bifidobacteria are not increasing due to fermentation of β -glucan, but the *Lactobacillus/Enterococci* group did increase. There are two possibilities for these results, the study by Crittenden et al. (2002) used single bacterial species which were cultured on agar, whereas this study used populations of bacteria obtained from faecal samples which likely included obligate anaerobic species which are not currently able to be cultured. Therefore, it may be that some species of *Lactobacillus/Enterococci* found in the colon can ferment β -glucan. It is also possible that other bacterial species were fermenting the β -glucan and *Lactobacillus* species were proliferating as the result of cross feeding, which would not have occurred in the single-culture experiments done previously.

Fermentation of AX alone and AX: β -glucan (1:1) caused a large increase in *Roseburia* after 24h which implies that AX is also the preferred substrate for this bacterial genus, however, the AX: β -glucan (3:1) sample would also be expected to increase *Roseburia* if this was the case, because it has a large proportion of AX, but this was not the case. This may be the result of competition of different bacteria.

The *Clostridium coccooides*-*Eubacterium rectale* group were significantly different from the negative control at 8h and 24h with AX alone, and for AX: β -glucan (3:1) and at 24h for AX: β -glucan (1:1). These bacterial groups appear to strongly favour fermentation of AX.

Thus, it appears that AX is readily used as a substrate for fermentation in these experiments, more so than β -glucan, and that the bifidobacteria, *Roseburia* and *Clostridium coccooides*-*Eubacterium rectale* group bacterial groups show preferential growth with AX as a substrate. This is reflected in the SCFA profiles produced by these bacteria, namely increases in acetate and butyrate.

Figure 3.5. shows the change in bacterial populations after 24 hours' fermentation of AX, AX: β -glucan (3:1), AX: β -glucan (1:1), AX: β -glucan (1:3), β -glucan and FOS. It shows that with no substrate (negative control) most bacterial groups decrease, which is to be expected. There are no significant ($p > 0.05$) increases over the course of the fermentation. Fermentation of FOS causes selective increases in beneficial groups *Bifidobacterium*, *Clostridium coccooides*/*Eubacterium rectales*, and *Atopobium*, whilst decreasing pathogenic groups such as *Clostridium histolyticum* and *Desulfovibrionales*. Bifidobacteria have long been associated with prebiotic activity (as described in 1.1.2.), *Eubacterium rectales* is a butyrate producer and has been linked with improvements in host health (Barcenilla et al., 2000), although in this case there was a small but not significant ($p > 0.05$) concomitant increase in butyrate. The AX: β -glucan sample with the best apparent prebiotic activity is AX: β -glucan (3:1) as it causes increase of beneficial groups *Bifidobacterium* and *Clostridium coccooides*/*Eubacterium rectales*, like FOS but also increases *Lactobacillus*. It does not cause increases in pathogenic groups *Clostridium histolyticum* and *Desulfovibrionales* which was seen in the other treatments containing AX at different ratios.

After fermentation of AX: β -glucan (1:3) there was no beneficial and selective bacterial growth.

Despite increases of *Bacteroides* spp. which are known to produce propionate (Macy and Probst, 1979) after 24h fermentation of β -glucan alone, no differences were observed between that or any other sample and the negative control. Propionate production peaked in this sample after 24 hours so it is possible that longer fermentation may have provided a significant increase in propionate.

Table 3.4: SCFA and lactate concentration in batch cultures at 0, 4, 8 and 24 hours' fermentation comparing no treatment, FOS and different ratios of AX and β -glucan. Negative control is no added carbohydrate and positive control is FOS. One-way ANVOA was applied to the data to test (F-test) the main effect of treatment. Values are μM . SEM is shown in italics. Significant interaction between treatments and negative control are denoted * ($p < 0.05$) and shown in bold.

	Time	Lactate (μM)	Formate (μM)	Acetate (μM)	Propionate (μM)	Butyrate (μM)	Total (μM)
No treatment	0	0.93 (0.40)	4.55 (0.68)	4.01 (0.45)	6.56 (1.81)	2.22 (0.19)	18.27 (0.26)
	4	4.79 (1.41)	5.23 (0.44)	6.72 (0.95)	13.46 (6.90)	2.56 (0.53)	32.77 (1.10)
	8	3.57 (1.50)	6.63 (1.39)	7.58 (2.02)	14.26 (6.37)	2.96 (0.61)	34.99 (0.91)
	24	1.72 (0.50)	5.27 (1.33)	9.66 (2.77)	7.58 (1.59)	3.22 (0.84)	27.45 (0.35)
FOS	0	3.75 (2.53)	3.74 (1.05)	3.29 (0.83)	5.67 (1.33)	2.11 (0.38)	18.56 (0.32)
	4	6.17 (2.76)	5.06 (0.52)	7.86 (1.21)	8.67 (2.56)	3.70 (0.96)	31.56 (0.40)
	8	21.62 (6.52)	19.10 (6.42)	18.65* (6.27)	7.05 (1.46)	5.31 (1.25)	71.73* (1.11)
	24	7.43 (3.96)	12.41 (6.84)	28.16* (3.23)	6.20 (1.73)	5.86 (1.86)	60.06* (0.83)
AX	0	5.01 (4.03)	4.77 (1.34)	4.28 (0.31)	7.55 ^a (2.45)	2.28 (0.30)	23.89 (0.63)
	4	9.84 (4.93)	6.54 (1.08)	9.82 (1.35)	12.88 (5.52)	3.61 (0.63)	42.69 (0.93)
	8	6.28 (2.59)	10.79 (2.14)	18.61* (3.46)	8.66 (1.33)	3.39 (1.40)	47.73 (0.35)
	24	2.12 (0.62)	13.82 (7.13)	35.59* (7.80)	7.46 (1.73)	5.38 (2.06)	64.36* (1.33)

	Time	Lactate (μM)	Formate (μM)	Acetate (μM)	Propionate (μM)	Butyrate (μM)	Total (μM)
AX 3:1 B glucan	0	1.96 (0.94)	5.27 (1.15)	4.34 (0.43)	7.27 (2.09)	2.06 (0.41)	20.89 ^a (0.27)
	4	10.41(4.73)	6.92 (1.55)	8.56(1.16)	11.53 (5.32)	2.70 (0.27)	40.12(0.27)
	8	11.43(5.63)	13.02(2.45)	24.94* (7.91)	9.13 (1.85)	3.10 (0.82)	61.52* (1.18)
	24	3.97 (0.94)	10.72 (4.92)	43.63* (6.16)	9.01 (2.73)	5.60 (1.61)	72.93* (0.88)
AX 1:1 B glucan	0	5.18 (5.25)	3.38 (1.16)	3.57 (0.92)	5.69 (1.75)	2.79 (0.61)	20.61(0.76)
	4	6.50 (4.62)	3.43 (1.23)	6.74 (2.31)	10.21(3.83)	3.26 (0.95)	30.15 (0.64)
	8	7.15 (5.85)	8.05 (3.98)	7.86 (2.99)	12.10(5.01)	3.53 (1.05)	38.69 (0.75)
	24	1.24 (0.60)	10.41 (7.31)	31.77* (11.15)	5.31 (1.62)	7.03 (2.37)	55.77* (1.79)
AX 1:3 B glucan	0	2.67 (1.24)	6.87 (2.18)	5.44 (1.21)	7.15 (2.31)	3.29 (1.28)	25.45 (0.22)
	4	5.47 (3.40)	5.51 (1.02)	7.16 (2.29)	12.15 (6.35)	1.86 (0.24)	32.16 (0.96)
	8	5.42 (3.41)	9.39 2.68)	7.25 (0.90)	14.29 (3.46)	2.91 (0.78)	39.25 (0.53)
	24	1.68 (0.48)	7.10 1.28)	15.96 (4.18)	4.88 (1.45)	4.48 (1.15)	34.11 (0.57)
B glucan	0	0.87 (0.41)	4.35 (0.82)	3.70 (0.43)	5.64 (1.36)	2.15 (0.44)	16.81 (0.16)
	4	2.78 (1.22)	3.28 (1.24)	4.09 (0.27)	3.80 (0.58)	1.29 (0.15)	15.25 (0.21)
	8	2.78 (1.02)	3.70 (1.02)	5.65 (0.80)	5.31 (1.06)	1.49 (0.33)	18.93 (0.12)
	24	2.72 (0.97)	5.36 (0.73)	9.77 (2.21)	9.69 (3.77)	2.09 (0.29)	29.63 (0.56)

Table 3.5: % SCFA produced in *in vitro* colonic fermentation vessels at 24 hours containing AX and β -glucan alone and combined in different ratios. FOS is the positive control, and no added polysaccharide (no treatment) is the negative control.

	SCFA (%)		
	Acetate	Propionate	Butyrate
No treatment	47.2	37.0	15.7
FOS	70.0	15.4	14.6
AX	73.5	15.4	11.1
AX 3:1 β -glucan	74.9	15.5	9.6
AX 1:1 β -glucan	72.0	12.0	15.9
AX 1:3 β -glucan	63.0	19.3	17.7
B glucan	45.3	44.9	9.7

Table 3.6 Bacterial enumeration (mean log₁₀ bacterial numbers / mL) of samples taken from static batch cultures after 0, 4, 8 and 24 hours' fermentation comparing no substrate, FOS and different ratios of AX and β-glucan Negative control is no added carbohydrate and positive control is FOS. Values are mean log₁₀ bacterial numbers/mL found using flow FISH. One-way ANOVA was applied to the data to test (F-test) the main interaction between treatments. Values in brackets are SEM. Significant interaction between treatments and negative control are denoted * (p < 0.05) and shown in bold.

Time (h)	<i>Bifidobacterium</i> genus	<i>Lactobacillus</i> Enterococcus group	<i>Bacteroides-Prevotella</i> group	<i>Clostridium</i> <i>coccoides-Eubacterium rectale</i>	<i>Roseburia</i>	<i>Atopobium</i> cluster	<i>Clostridium</i> cluster IX	<i>Faecalibacterium prausnitzii</i> group	<i>Desulfovibrionales</i>	<i>Clostridium</i> -cluster I and II	Total	
Negative	0	7.54 (0.32)	7.32 (0.10)	7.66 (0.14)	8.43 (0.20)	7.63 (0.09)	7.41 (0.06)	8.04 (0.02)	8.30 (0.13)	7.79 (0.20)	7.14 (0.34)	9.00 (0.14)
	4	7.76 (0.39)	6.91 (0.12)	7.92 (0.11)	8.31 (0.10)	6.81 (0.16)	7.04 (0.26)	7.90 (0.19)	8.33 (0.10)	8.05 (0.10)	7.28 (0.11)	8.99 (0.10)
	8	7.27 (0.51)	6.83 (0.21)	7.49 (0.56)	8.00 (0.40)	6.95 (0.16)	7.14 (0.21)	7.60 (0.49)	7.89 (0.45)	7.57 (0.38)	7.03 (0.31)	8.63 (0.41)
	24	7.56 (0.37)	7.18 (0.02)	7.27 (0.35)	8.05 (0.29)	7.20 (0.06)	7.51 (0.14)	7.66 (0.14)	7.65 (0.26)	7.73 (0.12)	7.34 (0.10)	8.74 (0.17)
FOS	0	7.48 (0.16)	7.39 (0.09)	7.86 (0.08)	8.32 (0.04)	7.73 (0.05)	7.60 (0.08)	8.26 (0.06)	8.34 (0.05)	7.82 (0.01)	7.30 (0.20)	9.01 (0.02)
	4	8.12 (0.17)	7.48 (0.20)	7.85 (0.14)	8.30 (0.11)	7.39 (0.03)	8.06 (0.07)	8.26 (0.08)	8.51 (0.10)	8.25 (0.03)	7.63 (0.10)	9.15 (0.04)
	8	9.42* (0.27)	6.97 (0.11)	8.29 (0.26)	8.89 (0.03)	6.94 (0.29)	8.31 (0.35)	8.52 (0.25)	8.78 (0.22)	8.47 (0.26)	7.95 (0.15)	9.80* (0.21)
	24	9.07* (0.23)	7.14 (0.13)	7.71 (0.18)	8.66 (0.04)	6.68 (0.15)	7.91 (0.26)	7.47 (0.13)	6.84 (0.32)	7.68 (0.25)	7.13 (0.36)	9.37 (0.14)
AX	0	7.66 (0.23)	7.42 (0.06)	7.84 (0.17)	8.47 (0.19)	7.75 (0.17)	7.43 (0.09)	8.07 (0.11)	8.28 (0.09)	7.91 (0.15)	7.20 (0.16)	9.02 (0.10)
	4	8.49 (0.37)	6.65 (0.03)	7.92 (0.04)	8.56 (0.15)	7.13 (0.36)	6.73 (0.35)	7.60 (0.42)	8.24 (0.04)	7.93 (0.02)	6.91* (0.18)	9.23 (0.11)
	8	8.86* (0.15)	6.60 (0.23)	7.62 (0.49)	8.95* (0.12)	7.70 (0.52)	6.78 (0.19)	7.32 (0.61)	7.66 (0.70)	6.80 (0.59)	6.51* (0.01)	9.45* (0.09)
	24	8.63* (0.28)	8.04* (0.13)	8.50 (0.11)	9.44* (0.14)	8.59* (0.01)	7.93* (0.05)	8.50* (0.10)	7.94 (0.28)	8.12* (0.06)	8.01* (0.19)	9.77* (0.01)

	Time (h)	<i>Bifidobacterium</i> genus	<i>Lactobacillus</i> <i>Enterococcus</i> group	<i>Bacteroides</i> - <i>Prevotella</i> group	<i>Clostridium</i> <i>coccoides</i> - <i>Eubacterium</i> group	<i>Roseburia</i>	<i>Atopobium</i> cluster	<i>Clostridium</i> cluster IX	<i>Faecalibacterium</i> <i>prausnitzii</i> group	<i>Desulfovibrionales</i>	<i>Clostridium</i> -cluster I and II	Total
AX 3:1 B glucan	0	7.33 (0.33)	7.17 (0.11)	7.55 (0.19)	8.18 (0.14)	7.55 (0.15)	7.25 (0.04)	7.80 (0.02)	8.12 (0.11)	7.68 (0.23)	7.02 (0.27)	8.80 (0.12)
	4	8.27(0.41)	6.77 (0.26)	8.24 (0.25)	8.24 (0.22)	7.25 (0.08)	7.72 (0.27)	8.28 (0.08)	8.42 (0.04)	8.11 (0.02)	7.17 (0.14)	9.23 (0.13)
	8	8.98* (0.52)	7.16 (0.39)	8.56 (0.27)	8.90* (0.10)	7.77* (0.10)	8.06* (0.22)	8.55 (0.13)	8.68 (0.05)	8.25 (0.11)	7.50 (0.16)	9.76* (0.23)
	24	9.15* (0.35)	7.91* (0.27)	8.37* (0.17)	9.23* (0.19)	7.52 (0.50)	7.78 (0.65)	8.19 (0.38)	8.17 (0.17)	7.79 (0.46)	7.54 (0.43)	9.80* (0.16)
AX 1:1 B glucan	0	7.45 (0.33)	7.17 (0.08)	7.50 (0.09)	8.24 (0.19)	7.44 (0.07)	7.25 (0.04)	7.87 (0.04)	8.11 (0.12)	7.65 (0.20)	7.06 (0.27)	8.82 (0.12)
	4	8.29 (0.39)	6.78 (0.17)	7.89 (0.19)	8.44 (0.12)	7.27 (0.29)	7.36 (0.41)	8.13 (0.08)	8.23 (0.09)	7.92 (0.06)	7.09 (0.17)	9.18 (0.11)
	8	8.57* (0.49)	6.88 (0.23)	7.85 (0.15)	8.73 (0.21)	7.34 (0.50)	7.48 (0.45)	8.00 (0.09)	8.14 (0.18)	7.83 (0.21)	7.28 (0.24)	9.45* (0.20)
	24	8.95* (0.12)	8.05* (0.31)	8.40* (0.10)	9.02* (0.11)	8.35* (0.16)	8.27 (0.29)	8.35 (0.13)	8.35 (0.11)	8.31 (0.24)	7.92 (0.45)	9.66* (0.05)
AX 1:3 B glucan	0	7.47 (0.35)	7.24 (0.16)	7.62 (0.20)	8.20 (0.31)	7.45 (0.18)	7.24 (0.11)	7.92 (0.09)	8.06 (0.23)	7.74 (0.26)	7.18 (0.19)	8.82 (0.21)
	4	7.45 (0.26)	6.75 (0.20)	7.18 (0.28)	7.79 (0.15)	6.85 (0.15)	7.04 (0.22)	7.38 (0.19)	7.35 (0.15)	7.18 (0.11)	6.87 (0.09)	8.42 (0.09)
	8	7.82 (0.53)	6.88 (0.32)	7.60 (0.72)	7.83 (0.49)	7.06 (0.31)	7.30 (0.04)	7.88 (0.64)	7.98 (0.50)	7.89 (0.40)	7.43 (0.33)	8.84 (0.47)
	24	8.61 (0.43)	7.88* (0.08)	8.43 (0.60)	8.46 (0.36)	7.67 (0.20)	7.64 (0.25)	8.33 (0.53)	7.77 (0.56)	8.06 (0.38)	7.95 (0.18)	9.36 (0.38)
B glucan	0	7.21 (0.33)	6.93 (0.18)	7.33 (0.23)	8.04 (0.27)	7.29 (0.21)	7.05 (0.13)	7.65 (0.09)	7.89 (0.17)	7.46 (0.26)	6.99 (0.18)	8.59 (0.22)
	4	7.26 (0.36)	6.65 (0.22)	7.41 (0.54)	7.91 (0.35)	6.98 (0.40)	7.11 (0.41)	7.46 (0.39)	7.38 (0.37)	7.21 (0.26)	6.97 (0.29)	8.47 (0.35)
	8	7.54 (0.46)	7.07 (0.34)	7.78 (0.69)	8.01 (0.56)	7.38 (0.58)	7.36 (0.53)	7.72 (0.62)	7.42 (0.60)	7.56 (0.53)	7.41 (0.44)	8.73 (0.55)
	24	7.90 (0.24)	8.32* (0.30)	7.53 (0.41)	8.09 (0.36)	7.51 (0.39)	7.20 (0.52)	7.58 (0.35)	6.86 (0.74)	7.20 (0.54)	8.11* (0.04)	9.10 (0.25)

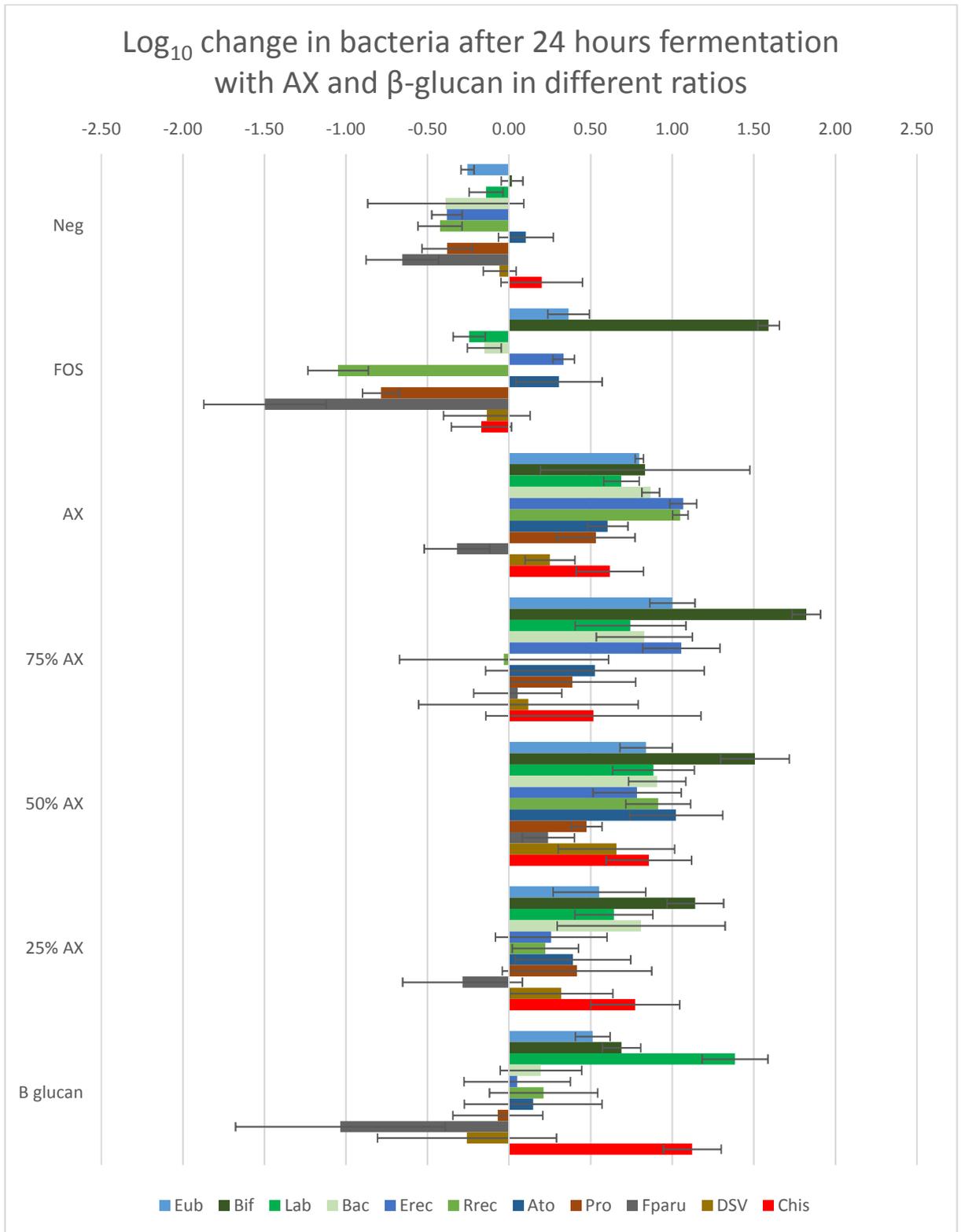


Figure 3.5: The change in mean log₁₀ bacterial numbers / mL after 24h fermentation found using Flow-FISH on samples from batch fermentation comparing different ratios of AX and β-glucan. Error bars are SEM.

3.5. Conclusions

Wheat is widely consumed for human nutrition, and has been for thousands of years. Consequently, the ratio of the DF polysaccharides present in wheat may have influenced the activities and populations of human gut microbiota, in healthy individuals. The results reported here provide support for this hypothesis,

Fermentation of AGP+AX showed somewhat increased prebiotic effectiveness by increasing the growth of beneficial bacterial groups *Clostridium coccoides- Eubacterium rectale* group and reducing proteolytic groups *Desulfonibrionales* and *Clostridium cluster I and II* compared to fermentation of AGP alone and AX alone. This supports the hypothesis that DF can act synergistically and this seems plausible as DFs are usually found in combinations. A greater array of substrates would provide a wider choice of fermentable oligosaccharides to support a more diverse bacterial population, creating a healthier and more resilient microbiome.

In 3.4.2. it was hypothesised that AGP is faster to ferment than AGP+AX as acetate concentration increased at a faster rate with fermentation of AGP alone, however in 3.4.3. it was noted that there was no change in the time of peak numbers of different bacterial populations, which would be a result of faster fermentation. A further study may be required with time points in between 8 and 24 hours to determine if AGP is faster to ferment than AGP+AX.

Beneficial bacteria were shown to preferentially ferment AX compared to β -glucan with the preferred ratio of AX: β -glucan being that present in wheat (3:1). This makes sense as we are exposed to high levels of AX in wheat as a staple food world-wide, while β -glucan (alone, or at ratios greater than AX) in the diet is rather rare.

This study showed that *Bifidobacterium* species preferentially fermented AX rather than β -glucan, and *Lactobacillus* species preferentially fermented β -glucan. This is supported by a study by Crittenden et al (2002) who observed bifidobacteria fermented AX and not β -glucan.

A summary of the conclusions is shown in table 3.7.

Table 3.7: Summary of prebiotic effects

AGP+AX	AX+ β-glucan
<ul style="list-style-type: none">• AGP has prebiotic activity, which is shown by increase in SCFA and modulation of bacterial compositions after fermentation• AGP+AX causes increase in acetate at the same rate as AGP alone• Prebiotic activity of AGP and AX in conjunction is greater than AGP alone due to beneficial changes in bacterial populations.	<ul style="list-style-type: none">• AX and β-glucan have different prebiotic effects when fermented in different ratios• AX, AX: β-glucan (3:1), AX: β-glucan (1:1) increased total SCFA concentration.• Bifidobacteria favour AX fermentation

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4. *In vitro* gastric digestion of AX and transgenic AX with altered structure. Does arabinosylation substitution pattern influence prebiotic activity?

4.1. Abstract

AX has previously been shown to have prebiotic activity, however, the relationship between AX structure and prebiotic activity has yet to be characterised. The importance of AX structure was investigated in two ways: using AX isolated from transgenic wheat with a specific reduction in O-3 linked arabinose and secondly, using a pre-incubation of AX fractions in a simulated gastric digest prior to *in vitro* colonic fermentation to explore any potential modification of AX structure in the stomach. AX isolated from homozygous TaXAT1 RNAi transgenic wheat flour showed lower prebiotic activity compared to AX isolated from the corresponding azygous control wheat flour. Total bacteria and bifidobacteria increased to a greater extent in cultures containing the control AX fraction compared to AX from the TaXAT1 transgenic flour. Pre-incubation of AX in a static *in vitro* gastric digest solution resulted in smaller AXOS and a lower A:X ratio. AX exposed to simulated gastric digestion resulted in slower fermentation compared to control samples; at 8h 15.68mM acetic acid was produced compared to 30.83mM for the untreated AX. As with the AX isolated from TaXAT1, differences observed in SCFA production were not maintained throughout the time course of fermentation, and by 24h there was no difference between treatments. Propionate production, in control samples was 22.93mM compared to 12.49mM in gastric-digest treated samples, at 8h. By 24h there was no significant difference between treatments. Total SCFA, butyrate and lactate concentrations were also greater for untreated AX samples. AX with a higher A: X ratio appeared to be fermented faster than AX with a lower A:X ratio and resulted in microbiota with a more favourable composition and produced more SCFAs.

4.2. Introduction

Previous research has shown that AX has prebiotic activity and that the size of the AX is of importance (Hughes et al., 2007). It has been hypothesised that the structure of AX may also be important and that this can be altered by low pH, such as that found in the stomach, it has been proposed that the acid present in the stomach causes hydrolysis of the arabinose to xylose bonds (Whistler and Corbett 1955; Zhang et al., 2003). The aim of the present study was to determine whether the structure of AX is an important factor in modulating prebiotic activity of AX. This was investigated this in two ways. Determination of whether AX structure is altered from passage through the acid environment of the stomach to the colon, and uniquely, by use of AX isolated from transgenic wheat flour with a known alteration in arabinosylation pattern.

It has been reported that under gastric acidities up to 10% L-arabinose can be released from arabinoxylan of corn hull, larch wood and banana peel hemicellulose (Zhang et al., 2003). Therefore, it was hypothesized that a similar release of arabinose from arabinoxylan fractions isolated from wheat flour could occur. Commercially available AX samples from wheat flour were analysed biochemically, before and after *in vitro* gastric and duodenal digestion, before being incubated in *in vitro* colonic batch cultures to determine whether prior gastric and duodenal conditions would result in subsequent differences in prebiotic activity.

AX in wheat is composed of β -1,4-linked-D-xylopyranose units forming a backbone with α -L-arabinofuranose decorations at the O-2 or/and O-3 positions of xylose units. Glycosyl transferase enzymes in the GT61 family are responsible for attaching arabinose to the xylose backbone. A study by Anders et al. (2012) showed that suppression of the gene TaXAT1 resulted in a 70–80% decrease in the amount of α -(1,3) linked arabinofuranosyl in AX, altering the substitution pattern of AX and reducing the A: X ratio.

A homozygous RNAi transgenic wheat line described by Anders et al., (2012), hereby referred to as RNAi GT61, produces AX with an altered substitution pattern caused by a reduction in O-3 linked arabinose substitutions. RNAi GT61 was therefore used, along with its azygous control line, for experiments designed to explore the importance of arabinose substitution on prebiotic activity. An altered substitution pattern and reduced A: X ratio, as is present in AX isolated from RNAi GT61 could affect the rate of AX fermentation. A similar reduction in arabinosylation might also occur to AX that had undergone prior simulated *in vitro* gastric digestion (before *in vitro* colonic fermentation), although in this case the loss of arabinose units may be more random than in the case of AX isolated from RNAi GT61.

Prebiotic activity was tested by comparing bacterial numbers and bacterial activity (in terms of SCFA production). Numbers of different bacterial groups were counted using Flow-FISH and the metabolites of fermentation, SCFAs, were measured using HPLC.

4.3. Materials and methods

4.3.1. Arabinoxylan

AX extracted from white flour from homozygous RNAi GT61 and an azygous control line were as described in 2.2.

A commercial AX preparation (P-WAXYM) (Megazyme, Bray, co. Wicklow, Ireland) was used for investigation of the effect of prior exposure of AX to gastric conditions before *in vitro* colonic fermentation. A Medium MM AX fraction was selected for these experiments as they had been shown to have the greatest prebiotic activity of the commercially available AX preparations (chapter 5).

4.3.2. AX characterisation

AX isolated from white flour from homozygous RNAi GT61 and azygous control wheat lines were characterised using monosaccharide analysis as described in 2.5, PACE, as described in 2.4 and HPAEC, for determination of effects on arabinose substitution patterns as described in 2.3. AX fractions before and after *in vitro* gastric/duodenal digestion were also analysed by HPAEC as described in 2.3 following specific endoxylase treatment, to determine effects of the simulated gastric/duodenal environment on AX structure. HPSEC-MALS, as described in 2.6 was used to determine whether there had been any change in size brought about by exposure to the simulated gastric conditions prior to *in vitro* colonic fermentation studies.

4.3.3. *In vitro* gastric digestion of AX

In vitro gastric and duodenal digestions were carried out as described in 2.9. using the protocol devised by Minekus et al. (2014).

4.3.4. *In vitro* fermentation of AX

In vitro fermentation was performed as described in 2.10. AX isolated from white flour from RNAi GT61 and the control line were compared as were pre-digested (simulated gastric/duodenal digest) untreated AX (not previously exposed to simulated gastric/duodenal digestive environment). Both sets of samples were added to 10ml vessels containing basal media and positive control (FOS) and a negative control (no carbohydrate) were set up as previously described. *In vitro* colonic fermentation cultures were set up in triplicate, using faecal samples obtained from three healthy donors. AX was added to basal media at 1% (w/v). Samples were inoculated with 10% faecal slurry. Samples were taken at 0h, 8h and 24h.

4.3.5. Enumeration of bacteria by flow-FISH

Bacteria were enumerated from samples taken at 0h, 8h and 24h as detailed in 2.12. As described in 2.12, bacteria that were counted were known to be present in human microflora and belonged to the following groups: *Bifidobacterium* genus, *Lactobacillus/Enterococcus* group, *Bacteroides-Prevotella* group, *Clostridium coccoides/ Eubacterium rectales* groups, *Roseburia*, *Atopobium* cluster, *Clostridium cluster IX*, *Fusobacterium prausnitzii*, *Desulfovibrionales*, *Clostridium-cluster I* and *II*.

4.3.6. SCFA and lactate analysis

SCFA and lactate were quantified by HPLC as detailed in 2.11.

4.3.7. Statistical analysis

The Genstat (2015, 18th edition, © VSN International Ltd, Hemel Hempstead, UK) statistical package was used for all analysis. ANOVA data was supplied by SJ Powers (Statistical department, Rothamsted Research) and applied as detailed in 2.13.

4.4. Results

4.4.1. AX characterisation

4.1.1.1. Monosaccharide analysis of RNAi GT61 AX compared to azygous control AX

Monosaccharide analysis of AX from RNAi GT61 AX was done to determine if the A:X ratio was reduced compared to the azygous control AX. Monosaccharide analysis showed a reduction in both arabinose and xylose residues (Figure 4.1), however there was a greater reduction in arabinose monosaccharides, providing a lower A:X ratio (Table 4.1). A lower A:X ratio confirmed the published data (Anders et al., 2012), indicating a reduction in arabinosylation in the RNAi GT61 AX.

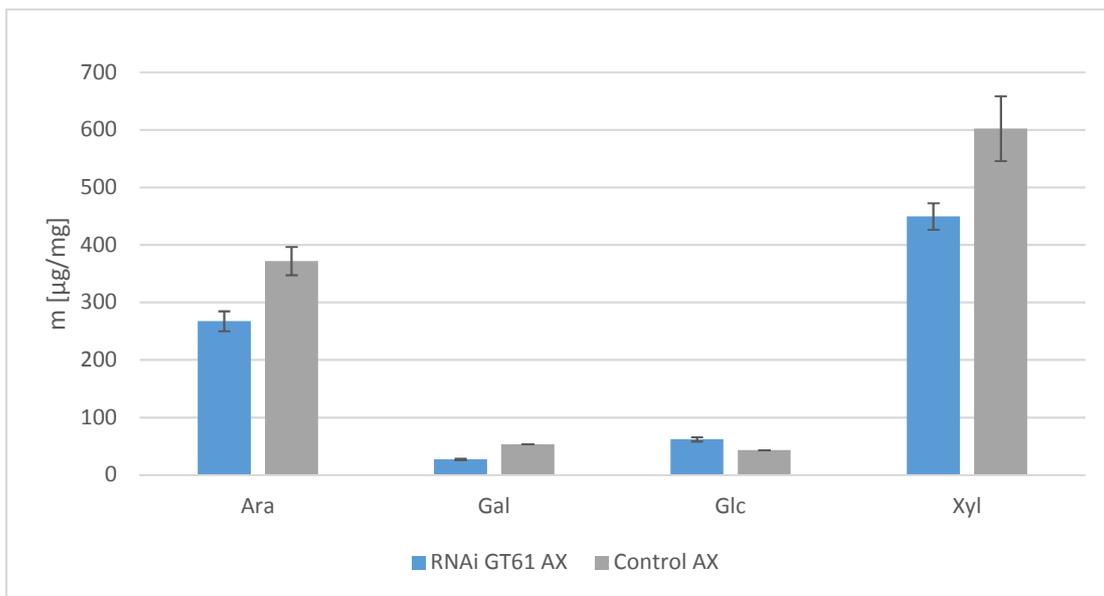


Figure 4.1: Monosaccharide analysis of RNAi GT61 AX and azygous control AX using a Carbopac PA20 column. N=3 Error bars show standard deviation.

Table 4.1: Arabinose to xylose ratio of AX fractions after monosaccharide analysis using a Carbopac PA20 column. Values are average m [$\mu\text{g}/\text{mg}$] where N=3, values in brackets are SD. Adjusted A:X ratio is according to Loosveld et al., (2010).

	Arabinose	Xylose	Galactose	Glucose	Adjusted A:X ratio
RNAi GT61 AX	267.1 (± 17.44)	449.2 (± 23.01)	26.8 (± 1.28)	61.8 (± 3.67)	0.52
Azygous control AX	371.8 (± 4.27)	602.2 (± 45.82)	53.4 (± 5.75)	42.9 (± 4.56)	0.54

4.1.1.2. HPAEC of AX from RNAi GT61 and azygous control

AX from RNAi GT61 was compared to the azygous control using HPAEC to provide a ‘fingerprint’ of the AXOS fragments. AX vary in structure, including the distributions of unsubstituted, monosubstituted and disubstituted xylose residues and so after digestion with endoxylase to release AX oligosaccharides (AXOS), the AXOS peaks were assigned as described by Ordaz-Ortiz et al. (2004, 2005); Saulnier et al., 2009). An example of identified AX oligosaccharides from a standard wheat are shown in Figure 4.2. When using HPAEC the carbohydrates with a higher degree of substitution and polymerisation (DP) elute later due to increased interactions with the column.

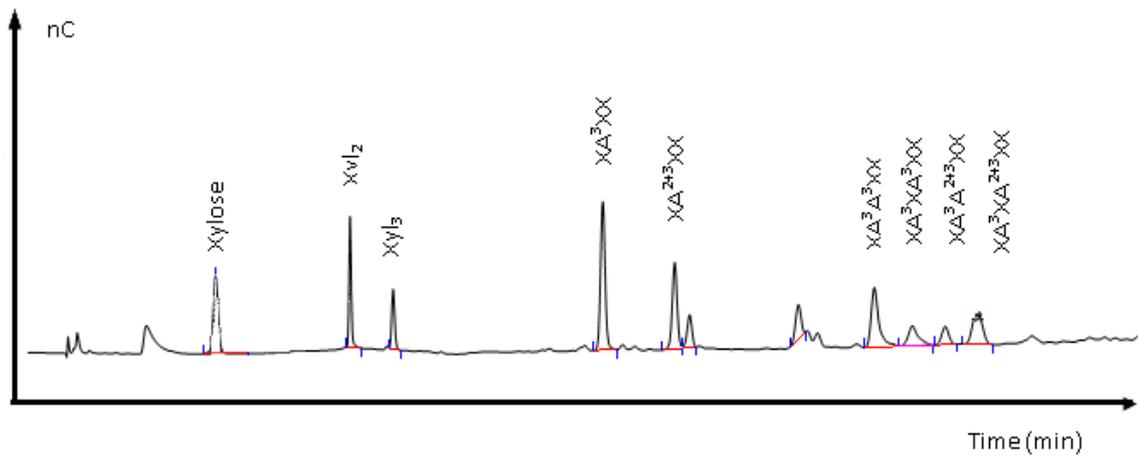


Figure 4.2: Representative HPAEC chromatogram indicating identities of AXOS peaks following endoxylanase (GH11) digestion of low MM wheat AX obtained from Megazyme (Co. Bray, Ireland).

Shown in Table 4.2, the knock down of the glycosyl transferase (GT 61) in the RNAi GT61 wheat line results in a reduction in the total arabinosylation of AX but notably a reduction in O-3 linked arabinose (XA^3XX , XA^3A^3XX , XA^3XA^3XX , $XA^3XA^{2+3}XX$) compared to the control line, (as described by Anders et al., 2012) which is as expected as the GT61_1 enzyme is responsible for the addition of monosubstituted arabinofuranose on the O-3 position of the xylose backbone. The largest reduction in AXOS is in the O-3 linked AXOS (shown in Figure 4.3 in red). There are reductions in the mixed linked (O-2+3 linked) AXOS in purple and the unsubstituted AXOS in green are also reduced but not as much.

Table 4.2 and Figure 4.3 below show the AXOS fractions obtained from HPAEC analysis of AX from the RNAi GT61 and the azygous control AX. It can be seen that in the RNAi GT61 AX, all AXOS fragments apart from the mixed linkage $XA^3A^{2+3}XX$ are reduced compared to the control line. This supports the monosaccharide data in Figure and Table 4.1, which showed a reduction in xylose and a larger reduction in arabinose and confirms that the structure of the isolated AX used in the *in vitro* fermentation studies is modified in arabinosylation pattern, as observed by Anders et al. (2012).

Table 4.2: AXOS fractions of AX extracted from RNAi GT61 WF compared to AX from azygous control. Analysed by HPAEC using Carbobac PA20 column. Values are means where N=3, values in brackets are SD. Control AX has greater amounts of all AXOS fractions, but there are

greater differences between the O-3 linked AXOS fractions XA^3XX , XA^3A^3XX , XA^3XA^3XX , $XA^3XA^{2+3}XX$.

	Control AX (nC*min)	RNAi GT61 AX (nC*min)
Xylose	4.68 (± 0.09)	3.64 (± 0.17)
Xyl ₂	1.38 (± 0.02)	0.80 (± 0.01)
XA^3XX	2.90 (± 0.02)	0.92 (± 0.03)
$XA^{2+3}XX$	1.90 (± 0.01)	1.65 (± 0.03)
XA^3A^3XX	1.13 (± 0.03)	0.34 (± 0.006)
XA^3XA^3XX	0.18 (± 0.02)	0.086 (± 0.15)
$XA^3A^{2+3}XX$	0.32 (± 0.03)	0.32 (± 0.02)
$XA^3XA^{2+3}XX$	0.70 (± 0.04)	0.31 (± 0.03)

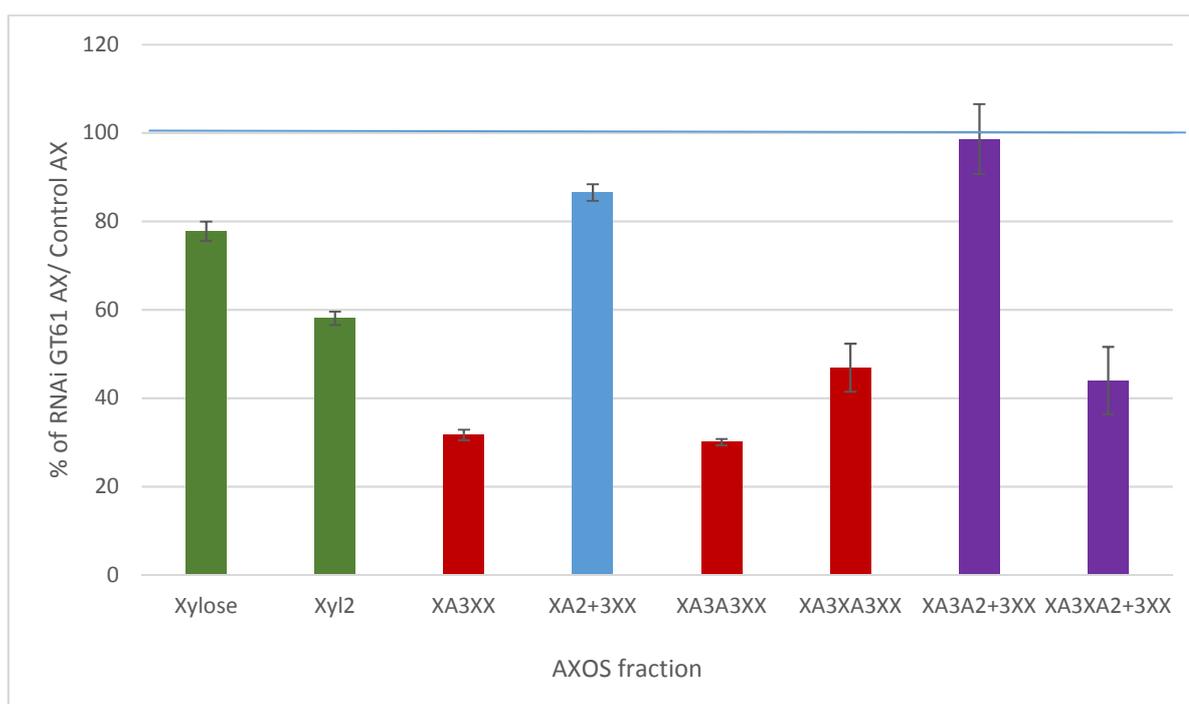


Figure 4.3 Analysis of xylan structure in endosperm samples from homozygous TaXAT1 RNAi transgenic wheat. Oligosaccharide abundance (HPAEC peak area) from RNAi GT61 AX samples relative to corresponding azygous control after xylanase digest (PRO-E0062). N=3. Columns for AX oligosaccharides are coloured according to substitution with Araf: unsubstituted (green), monosubstituted only (red), di-substituted only (blue), and mono- and di-substituted (purple). Abundances of (1,3) ;(1,4)- β -glucan oligosaccharides [Glucose3 (G3) and Glucose4 (G4)] as result of simultaneous lichenase digest show no overall change.

4.4.32.2. PACE of RNAi GT61 AX and azygous control

The samples that were analysed by HPAEC were also labelled with ANTS and subjected to PACE (Fig 4.6) Band thickness denotes abundance of AXOS fraction and Figure 4.4 clearly shows that the AX isolated from the RNAi GT61 wheat line has less O-3 linked XA^3XX , XA^3A^3XX and XA^3XA^3XX and less mixed linked $XA^{2+3}XX$, $XA^3A^{2+3}XX$ and $XA^3XA^{2+3}XX$ than the azygous control AX which supports the data from HPAEC analysis in Figure 4.3 and table 4.2.

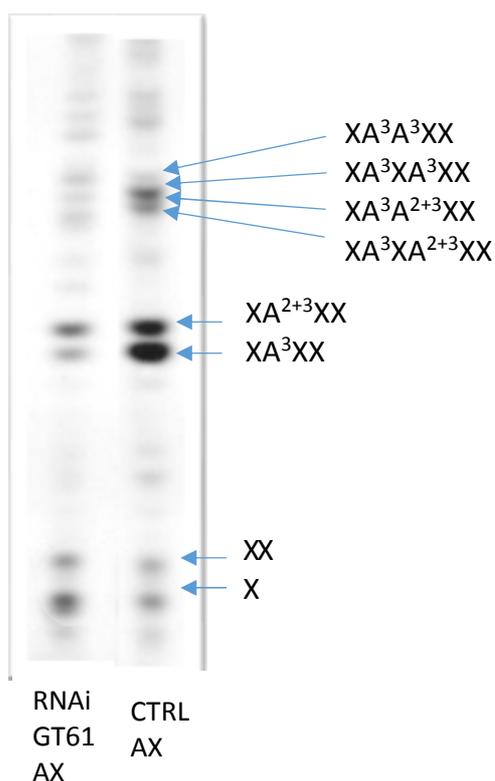


Figure 4.4: Image of PACE showing AX from RNAi GT61 (GT61, left) and an azygous control line (CTRL, right). AX samples were derivatised with ANTS and separated in a polyacrylamide gel by size, where smaller oligosaccharides travel further down the gel. A darker band represent a larger number of AXOS fractions. GT61 has less linked $XA^{2+3}XX$, $XA^3A^{2+3}XX$ and $XA^3XA^{2+3}XX$ and less O-3 linked XA^3XX , XA^3A^3XX and XA^3XA^3XX , than an azygous control AX but more X and XX.

4.4.33. Analysis of prebiotic activity of AX isolated from RNAi GT61 compared to control AX (isolated from azygous control) by quantification of SCFA and changes in bacterial populations

Table 4.3 shows the SCFA and lactate concentrations generated in the *in vitro* colonic fermentation system and Table 4.4 shows the bacterial populations. Fermentation of RNAi GT61 AX with reductions in mono-substituted O-3 linked arabinose substitutions resulted in

changes to the microbial composition and SCFA concentrations *in vitro* in comparison with fermentation of azygous control AX.

The largest contributor to total SCFA in both AX samples is acetate, which increases significantly ($p > 0.05$) with fermentation of the azygous control AX compared to the negative control from 8h. The concentration of acetate from fermentation of AX isolated from RNAi GT61 did not increase significantly compared to the negative control at any of the time points measured, indicating a reduction in prebiotic activity from the altered structure. The concomitant increase in *Bifidobacterium* populations may be the reason for the increase in acetate seen with the azygous control. *Bifidobacterium* populations do not increase significantly compared to the negative control with fermentation of the RNAi GT61 AX and therefore may explain why there is no significant ($p > 0.05$) increase in acetate concentrations.

Only the *Lactobacillus* bacterial group was increased significantly ($p > 0.05$) compared to the negative control with RNAi GT61 AX, whilst fermentation of the azygous control AX caused significant increases of total bacterial populations and increases in *Bifidobacterium* and *Clostridium coccooides/ Eubacterium rectales* groups. Fermentation of the AX isolated from the azygous control caused an increase in prebiotic associated bifidobacteria from 7.1% total bacteria at 0h to 35.5% after 24h, and whilst the other main prebiotic associated bacteria, *Lactobacillus*, populations dropped in all samples after 0h, fermentation of the RNAi GT61 AX caused a recovery of this from 7.5% at 8h to 23.9% at 24h, significant ($p > 0.05$) compared to the negative control and greater than that seen with FOS. Azygous control AX appears to select more for *Bifidobacterium spp.* whilst RNAi GT61 AX appears to select more for *Lactobacillus spp.* This is in contrast with Hughes et al. (2007) where commercial low MM AX was shown to increase *Lactobacillus* populations, however, in the Hughes study the AX was from a commercial source and differences in the AX structures may have caused differences in the prebiotic activity.

Formic acid levels increased significantly compared to the negative control for both the RNAi GT61 and control AX samples after 8 hours (Table 4.3). Formate production peaked with both AX samples at 24 hours. The concentration of formate from fermentation of FOS reaches much greater concentrations than is seen with either AX sample. Thus, FOS appears to be a more favoured substrate for production of formate than AX. This was unexpected and in contrast to other data (chapter 3 and 5) however, the isolated AX used in these experiments was not from a commercial source and thus may account for these differences. Relatively little is known about the role of formate in the gut. Formate is an electron donor which, along with H₂, reduces CO₂ to methane in methanogenesis and appears to be elevated in inflammatory conditions

(Vanderhaeghen, et al., 2015; Bereswill et al., 2011) therefore formate increases may not be beneficial to the host and increases in formate should not be considered as a prebiotic effect.

Lactate levels begin high and decrease to almost zero after 24 hours' fermentation with both AX samples. Lactate is often a transient metabolite and is utilised for other metabolic processes including the production of SCFA in cross-feeding mechanisms by some colonic bacteria. Duncan et al. (2004) state that lactate is normally detected only at low concentrations (<5 mM) in faeces from healthy individuals. It is not known which bacterial species are responsible for lactate utilisation, however, four bacterial species related to *Eubacterium hallii* have been implicated. The large number of *Lactobacillus* bacteria present at 0h for all samples. may explain the high initial levels of lactate.

Propionate concentration increased in all vessels, regardless of carbohydrate presence or modification. No significant differences were observed between either AX substrate or FOS and the negative control. Lactate is a precursor for propionate production so it is likely that lactate levels decrease due to utilisation for the formation of propionate.

Fermentation of FOS showed increased production of butyrate after 24 hours, compared to the negative control but this was not the case for either of the AX fractions tested, where there were no differences in butyrate production compared to the negative control.

Both the SCFA and bacterial population data agree that the azygous control AX shows greater prebiotic effects thorough stimulation of beneficial bacterial group bifidobacteria and resultant increase in acetate production.

Table 4.3: SCFA and lactate concentration in batch cultures at 0, 4, 8 and 24 hours' fermentation comparing no substrate, FOS, azygous control AX and RNAi GT61 AX. Negative control is no added carbohydrate and positive control is FOS. One-way ANVOA was applied to the data to test (F-test) the main effect of treatment. SEM is shown in italics Significant interaction between treatments and negative control are denoted * ($p < 0.05$) and shown in bold.

	Time (h)	Lactic acid (μM)	Formic Acid (μM)	Acetic Acid (μM)	Propionic acid (μM)	Butyric acid (μM)	Total (μM)
No treatment	0	11.02 (3.68)	0.00 (0.00)	4.21 (1.22)	4.36 (1.11)	2.22 (0.50)	19.15 (3.15)
	8	5.66 (0.44)	0.62 (0.27)	9.05 (2.40)	8.77 (1.42)	1.53 (0.36)	19.07 (3.97)
	24	6.45 (0.16)	3.71 (0.93)	9.84 (2.71)	9.33 (1.85)	1.20 (0.22)	26.57 (1.97)
FOS	0	24.05 (3.26)	0.11 (0.06)	4.93 (3.71)	5.20 (1.23)	1.54 (0.19)	39.90 (7.54)
	8	48.50* (6.97)	21.10* (4.99)	31.81* (5.44)	4.57 (0.94)	2.96 (0.87)	131.01* (14.50)
	24	65.44* (12.91)	29.57* (4.55)	75.06* (10.78)	11.76 (6.01)	10.48* (3.29)	207.60* (35.54)
RNAi GT61 AX	0	14.85 (2.32)	0.00 (0.00)	3.47 (0.96)	2.31 (0.42)	1.50 (0.08)	21.71 (2.38)
	8	6.80 (1.22)	4.94* (1.17)	14.45 (8.32)	12.12 (3.49)	0.93 (0.40)	36.60 (12.54)
	24	3.12 (1.18)	9.83* (3.09)	23.81 (9.42)	12.89 (3.23)	1.27 (0.63)	46.45 (13.17)
Azygous Control AX	0	16.39 (3.37)	0.00 (0.00)	4.93 (1.01)	5.05 (0.60)	1.54 (0.20)	26.55 (3.04)
	8	10.19 (2.86)	6.63* (2.30)	21.12* (4.46)	8.09 (0.48)	1.76 (0.11)	47.13* (4.99)
	24	2.56 (1.31)	15.90* (4.17)	27.64* (4.85)	9.76 (2.77)	1.29 (0.12)	51.07 (12.25)

Table 4.4: Bacterial enumeration (mean log₁₀ bacterial numbers / mL) of samples taken from static batch cultures after 0, 4, 8 and 24 hours' fermentation comparing no substrate, FOS, azygous control AX and RNAi GT61 AX. Negative control is no added carbohydrate and positive control is FOS. Values are mean log₁₀ bacterial numbers/mL found using flow FISH. One-way AVONA was applied to the data to test (F-test) the main interaction between treatments. Values in brackets are SEM. Significant interaction between treatments and negative control are denoted * (p< 0.05) and shown in bold.

	Time (h)	<i>Bifidobacterium</i> genus	<i>Lactobacillus</i> Enterococcus group	Bacteroides- Prevotella group	<i>Clostridium</i> <i>coccoides</i> / <i>Eubacterium</i> rectales groups	Roseburia	<i>Atopobium</i> cluster	<i>Clostridium</i> cluster IX	<i>Fusobacterium</i> prausnitzii group	Desulfovibrionales	<i>Clostridium</i> -cluster I and II	Total
No treatment	0	8.53 (0.33)	8.76 (0.13)	7.95 (0.12)	9.11 (0.04)	8.63 (0.11)	7.60 (0.16)	8.20 (0.09)	9.07 (0.04)	8.61 (0.06)	7.75 (0.15)	9.70 (0.03)
	8	8.58 (0.32)	7.65 (0.03)	8.55 (0.03)	9.01 (0.08)	8.25 (0.13)	7.70 (0.09)	8.70 (0.06)	8.90 (0.02)	8.54 (0.10)	8.20 (0.12)	9.67 (0.07)
	24	8.76 (0.3)	7.90 (0.18)	8.45 (0.13)	8.92 (0.07)	7.74 (0.20)	7.85 (0.10)	8.76 (0.08)	8.54 (0.06)	8.12 (0.12)	7.93 (0.12)	9.58 (0.10)
FOS	0	8.69 (0.61)	9.63 (0.19)	8.16 (0.21)	9.16 (0.14)	8.29 (0.24)	7.57 (0.12)	8.26 (0.18)	8.89 (0.04)	8.37 (0.14)	7.73 (0.22)	9.79 (0.26)
	8	9.98* (0.22)	7.85 (0.08)	8.82 (0.09)	9.39 (0.23)	8.37 (0.29)	7.95 (0.33)	8.86 (0.11)	9.11 (0.12)	8.44 (0.20)	8.27 (0.24)	10.30* (0.14)
	24	9.63 (0.29)	8.34* (0.18)	8.87 (0.11)	9.24 (0.31)	8.40 (0.42)	8.12 (0.28)	8.90 (0.16)	8.78 (0.21)	8.44 (0.31)	8.08 (0.20)	10.13* (0.16)
RNAi GT61 AX	0	8.38 (0.46)	9.31 (0.29)	7.79 (0.20)	8.99 (0.10)	8.46 (0.04)	7.49 (0.20)	7.99 (0.15)	8.84 (0.05)	8.48 (0.08)	7.83 (0.29)	9.58 (0.11)
	8	9.04 (0.17)	7.61 (0.20)	8.65 (0.09)	9.05 (0.10)	8.53 (0.14)	8.01 (0.22)	8.73 (0.16)	9.03 (0.13)	8.69 (0.17)	8.30 (0.20)	9.86 (0.01)
	24	9.31 (0.16)	8.58* (0.17)	9.03^b (0.04)	8.99 (0.17)	8.36 (0.29)	7.76 (0.15)	8.94 (0.09)	8.91 (0.32)	8.20 (0.14)	7.79 (0.14)	9.90 (0.13)
Azygous control AX	0	8.52 (0.41)	9.84 (0.14)	8.11 (0.17)	9.13 (0.01)	8.65 (0.06)	7.88 (0.05)	8.28 (0.12)	8.89 (0.09)	8.58 (0.03)	8.00 (0.16)	9.67 (0.07)
	8	9.23* (0.19)	7.92 (0.20)	8.84 (0.23)	9.09 (0.38)	8.32 (0.52)	7.65 (0.37)	8.93 (0.24)	9.00 (0.19)	8.61 (0.19)	7.81 (0.40)	9.94 (0.24)
	24	9.84* (0.09)	7.67 (0.22)	8.55 (0.44)	9.57* (0.18)	8.42 (0.40)	8.45 (0.53)	8.63 (0.44)	9.06* (0.17)	8.26 (0.20)	8.11 (0.42)	10.29* (0.05)

4.5. Results of *in vitro* gastric and duodenal digest of AX

In vitro digestion was used to test if non-specific changes in arabinosylation affect prebiotic activity. It was theorised that gastric digestion would reduce the A:X ratio, i.e., similar to the use of RNAi GT61 AX but with random removal of arabinose depending on the 3D structure of the AX polysaccharide and exposure of bonds to the low pH. RNAi GT61 AX has less arabinose specifically at the O-3 linked position, which may cause structural differences between the AX polysaccharides. It is hypothesized that AX exposed to the acid environment of a simulated gastric digestion would remove arabinose in a much more random fashion.

4.5.1. Monosaccharide analysis of AX before and after *in vitro* gastric and duodenal digestion.

Monosaccharide analysis of AX before and after *in vitro* gastric and duodenal digestion is shown in Figure 4.5. After *in vitro* digestion, there is a small but significant ($p>0.95$) decrease in arabinose in the AX sample, reducing the A:X ratio from 0.77 to 0.74. There is no reduction in xylose. Comparison of Figures 4.1 and 4.5 show that although *in vitro* digestion reduces the A:X ratio, however it remains much greater than that of RNAi GT61 AX

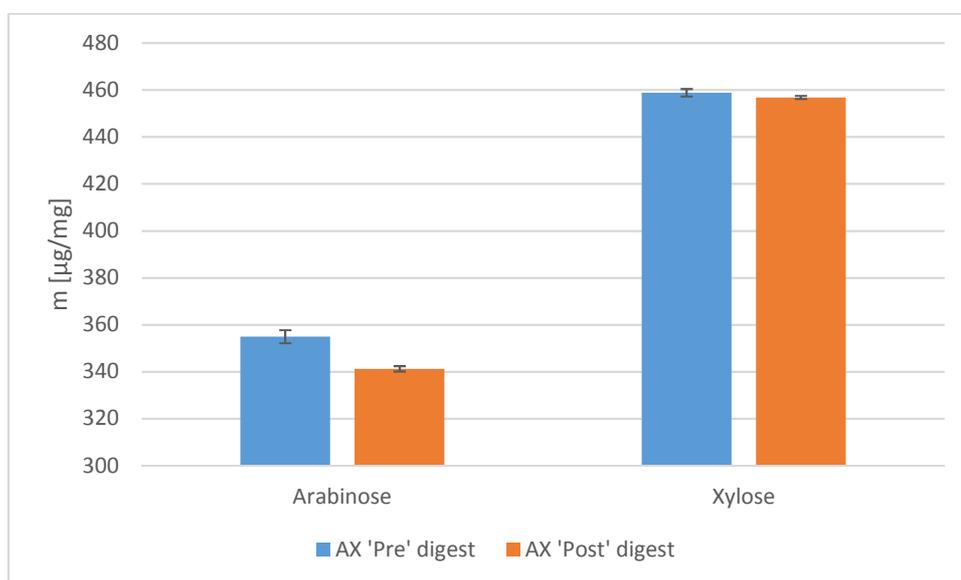


Figure 4.5: Monosaccharide analysis of Medium MM AX before and after *in vitro* gastric and duodenal digestion. Analysis of monosaccharides using a Carbowax PA20 column. (N=3) Error bars show standard deviation.

4.5.2. HPAEC of AX before (pre) and after simulated gastric and duodenal digestion (post)

HPAEC was used to determine changes in the structure of the AX by quantifying AXOS fragments before and after simulated gastric and duodenal digestion (Figure 4.6).

In vitro digestion of AX resulted in changes to the structure. Smaller AXOS fragments, xylose and xylobiose, increased by 9.7% and 4% respectively, whilst the larger more highly arabinosylated AXOS fragments, XA3XX, XA2+3XX, XA3A3XX and XA3XA3XX, decreased by 2.8%, 7.3%, 1.6% and 0.9% respectively. It is likely that the low pH in the gastric digestion caused de-arabinosylation of the larger AXOS fragment, as shown in Zhang et al. (2003), making the xylan backbone more accessible to the endoxylase used in HPAEC analysis. This is supported by the data in Figure 4.6, showing a decrease in arabinose after digestion.

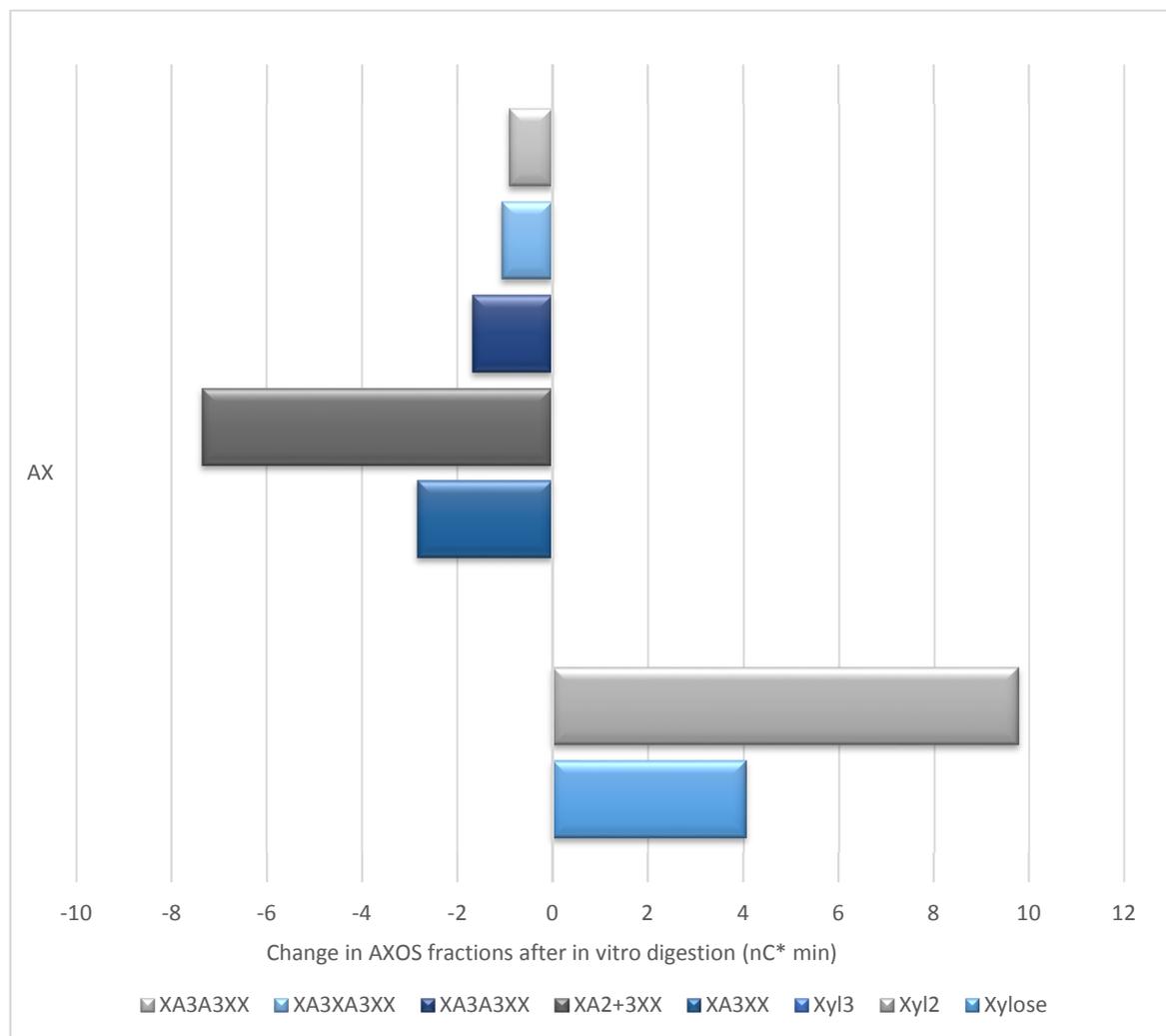


Figure 4.6: Change in AXOS shown by HPAEC analysis of AX before and after *in vitro* gastric digestion. Oligosaccharide abundance (HPAEC peak area) of AX, digested with endoxylase (Xylanase PRO-E0062 Prozomix) prior to running on HPAEC. Different patterns of AXOS are shown (as % positive or negative change pre-and post-gastric digestion). Values shown are average where n=3.

4.5.3. HP-SEC-MALS of AX before and after simulated digestion.

AX was separated by size exclusion chromatography before and after the *in vitro* gastric and duodenal digestion, to determine if digestion affects the size or viscosity of the polysaccharides (shown in Table 4.5). There was a reduction in MM and intrinsic viscosity after *in vitro* digestion, indicating that the gastric and duodenal digestion conditions do alter AX, most likely via hydrolysis of arabinofuranose by the low pH encountered in the simulated gastric phase, as stated in 4.5.2.

Table 4.5: SEC-MALS of AX before and after simulated gastric and duodenal digestion. MM and intrinsic viscosity values from Wyatt Astra software. One hundred μL was injected onto an OHPak SB 802.5 HQ column. The eluent was 0.1M NaNO_3 with 0.02% NaN_3 with a flow rate of 0.5mL/min⁻¹.

	MM (kDa)		Intrinsic viscosity (mL/g)	
	Pre-digest	Post digest	Pre-digest	Post digest
AX	323.9	177	288.632	180.121

4.5.4. Analysis of prebiotic activity of AX following *in vitro* gastric and duodenal digestion compared with untreated AX by quantification of SCFA and changes in bacterial populations

Table 4.6 shows the short chain fatty acid concentrations generated by *in vitro* colonic fermentation of AX fractions and Table 4.7 shows the bacterial populations.

Table 4.6 shows that acetate increases significantly in both AX samples compared to the negative control ($p > 0.95$), however the control AX (not exposed to prior gastric/duodenal digestion) showed a significant ($p > 0.95$) increase at 8h whilst the gastrically digested AX took 24h to show a significant difference. Almost twice as much acetic acid was produced from fermentation of untreated control AX at 8h, (29.76mM compared to 12.46mM) but by 24h the difference between the two AX fractions was no longer apparent. The rate of fermentation therefore appears to be greatest in the control AX sample and this may relate to the loss of arabinose observed in the gastric/duodenal-treated AX sample, resulting in more unsubstituted xylose residues making the pre-treated sample less soluble and therefore less fermentable as it has been hypothesised that decreased solubility results in decreased fermentability (Roland et al., 1995). The significant increase in acetate in all samples compared to the negative control may be related to an increase in *Bifidobacterium spp.*, which are known producers of acetate. Increases were observed in *Bifidobacterium* species after 24 hours for both AX samples compared to the negative control, although there were no differences between the two AX samples (i.e. pre-treatment with gastric/duodenal digest or no digestion).

The slower rate of acetate increasing in concentration from fermentation of the gastrically digested AX compared to the control AX may suggest that gastrically digested AX is slower to ferment, however, after 4h all bacterial populations showed an increased population with the *in vitro* digested AX as substrate compared to the untreated control AX (by 8h there was no longer any difference). It is possible that acetate was slower to accumulate due to increased utilization for bacterial cross feeding to generate other SCFAs as bacterial numbers were greater, particularly in two groups *Roseburia* and *Faecalibacterium prausnitzii*, of which, there were significant ($p > 0.95$) increases compared to the negative control with respective increases of 26% and 9.5%, with fermentation of the *in vitro* digested AX. These increases were not seen with untreated AX. These bacterial groups contain species which have been found to utilise acetate to produce butyrate (Duncan et al., 2002), which may explain why the increase in acetate is slower with the digested AX, however there is no concomitant increase in butyrate to support this theory.

The concentration of formate was significantly lower for the *in vitro* digested AX after 24 hours, even compared with the negative control. This was not the case for the untreated control AX. Formate has not been linked with any health benefits in humans but is used in cross feeding to generate acetate so reductions in formate are potentially beneficial. Propionate production did not differ significantly from the negative control for either AX samples. As lactate is a precursor to propionate it is of note that the lactate concentration decreased from the start of fermentation

for both digested and untreated AX. Lactate did, however, increase after 24hour fermentation in the FOS (positive control) treatment, which was unexpected as lactate is not usually recorded at high concentrations (18.71 mM) in healthy colonic conditions. The concentration of butyrate was not significantly different from the negative control for any sample, suggesting no effect of treatment on butyrate production, despite increases in butyrate producing bacterial groups with the *in vitro* digested AX.

Both AX samples caused a similar, significant ($p > 0.05$) increase in the beneficial *Clostridium coccooides*-*Eubacterium rectale* group after 24h supporting the theory that AX has prebiotic activity but showing that this bacterial group shows no distinction between the two samples.

These data show that *in vitro* gastric and duodenal digestion seems to increase beneficial bacterial groups *Roseburia* and *F. prau*. Which is contrary to SCFA data, as butyrate increases significantly compared to the negative control with untreated AX but not digested AX.

It could be concluded that structural changes including random de-arabinosylation of AX *in vitro* gastric and duodenal digestion affects prebiotic activity, possibly reducing speed of fermentation. The data from Table (4.3) and (4.4) show that RNAi GT61 AX (which has a loss of specific arabinose side chains) causes changes in prebiotic activity compared to its control as well. Although it is unclear whether a specific loss of O-3 linked arabinose side has a different effect than from random loss of arabinose side chains through acid hydrolysis, it has been shown that changes to arabinosylation appear to affect the prebiotic activity of AX.

The RNAi GT61 AX and its azygous control were extracted from wheat flour at Rothamsted Research, rather than being commercially bought and had a A:X ratio of 0.52 and 0.54 respectively. This is much lower than the A:X ratio found after *in vitro* digestion of medium MM AX of 0.73. A higher A:X ratio has been shown to decrease the degree of fermentability (Pollet et al., 2012), however, substitution was shown to be secondary to dp in rat studies where AX with A:X ratios of 0.69 and 0.27 affected the measured intestinal characteristics similarly (Van Craeyveld et al., 2008).

Different bacteria have different preferences for XOS or AXOS (Pastell et al., 2009), for example *Bifidobacterium adolescentis* possesses AX arabinofuranohydrolase-D3, an enzyme for releasing arabinose residues from double substituted xylose (van den Broek et al., 2005; Van Laere, Beldman, & Voragen, 1997). This bacterium is an example of bacteria which may be more able to break down *in vitro* digested AX, than RNAi GT61 AX, due to the presence of

double substitutions which survived digestion (as the A:X ratio was reduced only from 0.84 to 0.81 indicating that a lot of arabinose substitutions remain).

Therefore, the reduced A:X ratio found after *in vitro* gastric and duodenal digestion may have aided prebiotic activity by improving the degree of fermentability and causing a greater increase in beneficial bacterial populations, or the increase in bacterial populations may have been due to other changes in the AX structure like the reduction in MM and therefore solubility. The latter is more likely because the lower A:X ratio in RNAi GT61 AX did not also cause an increase in bacterial populations, which would be expected if substitution was the cause of increased prebiotic activity.

Table 4.6: SCFA production from faecal bacteria (mM per 1mL batch culture fluid) after incubation with either no substrate, FOS, untreated AX or AX that had undergone simulated gastric and duodenal digestion. AX is commercially available medium MM AX (Megazyme, Bray, UK). One-way ANOVA and F-test were used to compare treatments to negative control for each bacterial group. Values are means with SEM shown in italics. Significant interaction between treatments and negative control are denoted * ($p < 0.05$) and shown in bold.

	Time (h)	Lactic acid (μ M)	Formic Acid (μ M)	Acetic Acid (μ M)	Propionic acid (μ M)	Butyric acid (μ M)	Total (μ M)						
No treatment	0	25.53 (6.39)	2.70 (0.40)	2.08 (1.29)	4.14 (0.43)	4.06 (0.50)	38.51 (1.34)						
	4	17.81 (9.24)	4.39 (1.87)	1.92 (1.57)	5.15 (1.30)	8.15 (1.83)	37.42 (1.76)						
	8	4.78 (2.17)	5.19 (2.12)	4.80 (1.96)	2.43 (1.56)	5.58 (2.61)	22.78 (0.20)						
	24	2.66 (0.85)	9.55 (4.20)	10.10 (4.16)	5.06 (4.15)	3.64 (1.50)	31.01 (0.85)						
FOS	0	21.07 (8.25)	1.13 (0.92)	10.14 (9.38)	3.01 (1.09)	8.50 (1.84)	46.84 (2.31)						
	4	19.92 (7.91)	6.06 (1.78)	9.20 (2.52)	7.27 (3.16)	6.55 (1.04)	49.00* (1.40)						
	8	6.34 (2.98)	4.24 (1.84)	20.49* (3.18)	6.05 (2.97)	10.04 (4.52)	47.15* (0.49)						
	24	18.71* (5.08)	0.00* (0.01)	20.18 (3.86)	15.86 (6.59)	4.09 (0.61)	58.84* (1.47)						
AX	0	21.95 (6.21)	2.66 (1.10)	3.91 (3.20)	6.41 (1.78)	4.62 (0.60)	39.56 (1.16)						
	4	8.83 (0.51)	3.87 (1.19)	8.40 (3.58)	7.01 (3.09)	10.03 (4.31)	38.13* (0.83)						
	8	6.30 (3.23)	1.76 (1.44)	29.76* (9.47)	17.37 (12.33)	12.22* (1.37)	67.42* (2.60)						
	24	5.87 (3.92)	2.98 (2.43)	36.07* (12.11)	12.53 (5.07)	10.44* (3.68)	67.86* (1.98)						
Gastric digest AX	0	17.15 (3.56)	4.40 (2.68)	5.88 (3.40)	3.14 (0.28)	6.68 (1.63)	37.26 (0.70)						
	4	9.90 (3.12)	3.73 (0.92)	3.70 (1.53)	1.90 (0.54)	4.40 (1.24)	23.63 (0.51)						
	8	6.36 (3.13)	1.89 (0.83)	12.46 (4.31)	1.37 (2.44)	4.93 (2.15)	27.01 (0.66)						
	24	5.86 (4.46)	1.01* (0.82)	35.57* (0.70)	15.95 (8.74)	4.26 (0.64)	62.65* (1.84)						

Table 4.7: Bacterial enumeration (mean log₁₀ bacterial numbers / mL) of samples taken from static batch cultures after 0, 4, 8 and 24 hours' fermentation comparing no substrate, FOS, untreated AX or AX that had undergone simulated gastric and duodenal digestion. One-way ANOVA and F-test were used to compare treatments to negative control for each bacterial group. Values are means with SEM shown in brackets. Significant interaction between treatments and negative control are denoted * (p< 0.05) and shown in bold.

Time (h)	<i>Bifidobacterium</i> genus	<i>Lactobacillus</i> Enterococcus group	<i>Bacteroides-Prevotella</i> group	<i>Clostridium</i> <i>coccoides-Eubacterium</i> rectale group	<i>Roseburia</i>	<i>Atopobium</i> cluster	<i>Clostridium</i> cluster IX	<i>Faecalibacterium</i> <i>prausnitzii</i> group	<i>Desulfovibrionales</i>	<i>Clostridium</i> -cluster I and II	Total	
Negative	0	7.8 (0.16)	7.41 (0.19)	7.75 (0.24)	8.68 (0.22)	7.97 (0.23)	7.50 (0.52)	7.88 (0.27)	8.14 (0.16)	8.19 (0.31)	7.44 (0.22)	9.17 (0.21)
	4	7.7 (0.25)	7.32 (0.36)	7.81 (0.36)	8.37 (0.42)	7.29 (0.29)	7.50 (0.36)	7.99 (0.37)	8.67 (0.22)	8.06 (0.25)	7.70 (0.25)	9.20 (0.24)
	8	7.38 (0.45)	6.87 (0.26)	7.3(0.51)	8.23 (0.36)	6.80 (0.36)	7.27 (0.29)	7.49 (0.53)	8.08 (0.34)	7.76 (0.19)	7.04 (0.33)	8.85 (0.28)
	24	7.19 (0.60)	6.41 (0.33)	6.97 (0.55)	7.59 (0.76)	6.23 (0.33)	6.94 (0.59)	6.85 (0.62)	7.32 (0.75)	7.19 (0.58)	6.46 (0.41)	8.26 (0.62)
FOS	0	7.77 (0.25)	7.04 (0.40)	7.55 (0.40)	8.55 (0.24)	7.67 (0.51)	6.76 (0.41)	7.54 (0.41)	8.33 (0.19)	7.75 (0.32)	7.10 (0.40)	8.99 (0.26)
	4	8.32 (0.46)	6.76 (0.50)	7.73 (0.41)	8.37 (0.29)	7.21 (0.63)	7.44 (0.73)	7.85 (0.40)	8.17 (0.34)	7.06 (0.84)	6.95 (0.62)	9.03 (0.39)
	8	8.66* (0.61)	6.23 (0.96)	6.97 (0.15)	8.32 (0.32)	6.60 (0.54)	7.35 (0.59)	7.26 (0.56)	7.83 (0.29)	7.28 (0.57)	6.61 (0.77)	9.04 (0.47)
	24	9.02* (0.17)	5.87(0.67)	5.46 (0.45)	8.82 (0.19)	5.34 (0.33)	6.23 (0.39)	5.76 (0.33)	6.85 (0.37)	5.68 (0.15)	5.93 (0.82)	9.30 (0.16)
Untreated AX	0	7.21 (0.30)	7.47 (0.36)	7.22 (0.31)	8.18 (0.30)	7.62 (0.30)	6.61 (0.30)	7.32 (0.30)	7.85 (0.31)	7.49 (0.30)	6.96 (0.30)	8.65 (0.30)
	4	7.79 (0.70)	6.41 (0.64)	7.56 (0.65)	7.69 (0.50)	6.91 (0.92)	6.78 (0.71)	7.64 (0.65)	7.89 (0.53)	7.26 (0.67)	6.74 (0.62)	8.61 (0.61)
	8	8.54 (0.52)	6.7 (0.62)	6.29 (0.94)	8.17 (0.36)	6.79 (0.72)	6.95 (0.85)	7.35 (0.90)	7.78 (0.63)	7.30 (0.66)	6.44 (0.79)	8.99 (0.48)
	24	8.84* (0.23)	6.72 (0.66)	8.10 (0.86)	9.11* (0.16)	6.48 (0.67)	7.20 (0.71)	8.29 (0.47)	8.29 (0.28)	7.94 (0.56)	7.03 (0.67)	9.67* (0.21)
Gastric Digest AX	0	7.4 (0.12)	7.3 (0.25)	7.45 (0.09)	8.34 (0.14)	7.62 (0.08)	6.89 (0.0.7)	7.48 (0.10)	8.11 (0.15)	7.44 (0.13)	7.26 (0.06)	8.79 (0.12)
	4	8.28 (0.40)	6.94 (0.06)	7.68 (0.09)	8.42 (0.20)	7.36 (0.09)	7.54 (0.51)	7.64 (0.07)	8.37 (0.17)	7.96 (0.12)	7.29 (0.12)	9.13 (0.18)
	8	8.26 (0.36)	6.92 (0.50)	7.48 (0.24)	8.15 (0.45)	6.49 (0.98)	7.74 (0.09)	7.60 (0.26)	8.22 (0.30)	7.51 (0.32)	6.64 (0.49)	9.13 (0.19)
	24	8.58 (0.25)	7.2 (0.47)	8.04 (0.17)	9.10* (0.14)	8.14* (0.23)	7.81 (0.09)	8.04 (0.13)	9.08* (0.15)	8.12 (0.36)	7.22 (0.32)	9.65* (0.10)

4.6 . Conclusions

It was thought that structure plays a large role in the prebiotic activity of AX. Hughes et al (2007) demonstrated an effect of AX molecular size on prebiotic activity and the current study indicates that the arabinosylation pattern may also play a role in determining the level of prebiotic activity.

AX from transgenic wheat line RNAi GT61 had a lower A:X ratio resulting from reduced arabinose substitution specifically at the O-3 position, thereby reducing its solubility. Fermentation of this AX showed alterations and possible reduction to prebiotic properties compared to fermentation of AX from the control, azygous wheat line.

This study showed that *Bifidobacterium* species fermented AX isolated from white flour of the RNAi GT61 line to a lesser extent than AX isolated from the control line. Supporting previous studies which showed that XOS were poor substrates for bifidobacteria when compared to other carbohydrates (Hopkins et al., 1998; Crittenden et al., 2002; van den Broek et al., 2005; Van Laere, Beldman, & Voragen, 1997). Therefore, bifidobacteria would proliferate less when fermenting a less substituted AX because of reliance on other gut microbiota with xylanase activity for the release of xylose sugars.

It was shown that *in vitro* gastric and duodenal digestion also affected the structure of AX, reducing arabinose substitution in a more random pattern than the RNAi GT61 AX, reducing MM and solubility, which also appeared to affect the prebiotic activity of AX. Fingerprinting using HPAEC showed an increase in smaller AXOS fractions after simulated gastric and duodenal digestion and a decrease in larger AXOS fractions due to these structural changes.

Fermentation of digested AX increased two different butyrate producing bacterial groups that are thought to be beneficial to colonic health (Riviere et al., 2016), *Roseburia* and *F. prau*, suggesting that gastric and duodenal digestion does influence subsequent bacterial fermentation and that AX exposed to simulated gastric and duodenal conditions was a better substrate for bacterial fermentation, however the level of the SCFA butyrate was increased only with untreated AX, indicating that there may be other factors involved in prebiotic activity.

As the SCFA data has indicated that gastrically digested AX results in a lower A:X ratio and therefore, lower solubility and lower rate of fermentation. It may be possible that a longer fermentation period would be required for an increase in bacterial numbers to be observed.

It can be concluded that the structure of AX can be altered by gastric digestion, and this appears to influence the microbial composition in a positive way. Not only was arabinose lost but also the size of the AX chains appear to be reduced, and there a decrease in intrinsic viscosity was also observed. Specific reduction in the arabinose substitution pattern of AX (demonstrated with RNAi GT61 AX) can also affect the prebiotic activity, however this reduced prebiotic activity. Structure therefore clearly plays a role in influencing bacterial populations. Arabinosylation shows importance in modulation of prebiotic effects, as observed from the different effects of non-specific removal of arabinose units (simulated gastric digest), which appears to increase prebiotic activity compared to specific removal of arabinose units at the 3 position (RNAi GT61), which may reduce prebiotic activity.

Table 4.8: Summary of effects of RNAi GT61 AX compared to azygous control AX and prior *in vitro* gastric and duodenal digestion

Effect of GT61 AX	Effect of gastric digestion on AX
<ul style="list-style-type: none"> • Reduction in A: X ratio of AX from specific removal of arabinose • Reduction in concentration of acetate compared to azygous control AX • Decrease in total bacteria and bifidobacteria compared to non-transgenic AX • Possible reduction in prebiotic activity 	<ul style="list-style-type: none"> • Random reduction in A: X ratio of AX • Reduction in MM and viscosity • Slower initial production of acetate • Decrease in formate concentration • Increase in <i>Roseburia spp.</i> and <i>F.prau</i> bacteria compared to untreated AX • Possible increase in prebiotic activity

4.7. References

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5. *In vitro* fermentation of commercial AX fractions of different Molecular Mass after *in vitro* gastric and duodenal digestion

5.1. Abstract

Three soluble arabinoxylan (AX) fractions from wheat flour with average molecular mass (MM) of 56.9, 323.9 and 374.2 kDa and one insoluble AX fraction were purchased from Megazyme. These were subjected to *in vitro* gastric and duodenal digestion as described by Minekus et al., (2014) prior to addition to *in vitro* fermentation. The following changes in AX structure were observed in all samples after *in vitro* digestion: an increase in smaller AXOS, a decrease in MM and intrinsic viscosity and A:X ratio and a reduction in bound ferulic acid. Changes in microbiota were monitored using fluorescence *in-situ* hybridisation (FISH) to quantify bacteria and HPLC to quantify levels of short chain fatty acid (SCFA) and lactic acid. Differences in prebiotic activity were observed between AX samples of different MM after changes in structure caused by *in vitro* digestion. Significant increases in SCFA production compared to the negative control were observed in all AX samples. The AX fraction of 323.9 kDa gave the greatest increases in SCFA production, while the insoluble AX fraction showed the smallest increases in SCFA production. Changes in different bacterial populations were observed depending on the MM of the AX sample, however none were observed with the insoluble fraction.

5.2. Introduction

AX has previously been shown to have prebiotic properties (Vardakou et al., 2008, Grootaert et al., 2009, Neyrinck et al., 2011). A study by Hughes et al. (2007) indicated that commercial AX fractions of low MM were most effective in *in vitro* fermentation systems, producing the highest levels of SCFA and selectively stimulating the largest increase in beneficial colonic bacteria, bifidobacteria and lactobacilli. However, the study by Hughes et al, 2007 did not use AX fractions that had been exposed to *in vitro* gastric and duodenal digestion conditions prior to addition to the *in vitro* colonic fermentation system. It was shown that gastric digestion could generate AX with lower levels of arabinose substitution, and also increase overall prebiotic activity (as discussed in chapter 4), it was also hypothesised that exposure of AX to low gastric pH may release bound phenolic acids, including ferulic acid which links AX chains together at the O-5 position of the arabinose units (Zhang et al., 2003) and may also affect the rate at which the AX is fermented, hence affecting prebiotic activity.

It is thought that low MM polysaccharides are more rapidly and more selectively fermented by the beneficial bacteria, bifidobacteria and lactobacilli, than higher MM polysaccharides. This may be because low molecular mass substrates have more non-reducing ends per unit mass, which favours a more rapid attack by the exo-acting enzymes produced by bacteria in the gut (Gibson, 2004). It is likely that *in vitro* digestion lowers MM, therefore making polysaccharides more fermentable. AX degradation by bifidobacteria involves the consumption of arabinose but not always the consumption of xylose that makes up the backbone. The majority of bifidobacteria can only utilize xylan of length up to xylotetraose (Riviere et al., 2014). This could explain why lower MM AX was previously shown to promote the growth of bifidobacteria (Hughes et al., 2007). For complete utilization of AX, it is likely that bifidobacteria require co-operation with β -endoxyranase producing bacteria such as Roseburia and *Bacteroides* species.

Therefore, as a continuation of the work described in chapter 4 on *in vitro* gastric digestion of AX, we therefore determined whether these digestive pre-treatments would affect the results of *in vitro* AX fermentation compared to the previous study published by Hughes et al. (2007). Commercially available AX fractions with different average MMs were subjected to *in vitro* gastric and duodenal digestion prior to addition to the *in vitro* colonic fermentation system. Polysaccharides were characterised before and after simulated gastric/duodenal digests to determine if any structural changes had occurred before *in vitro* fermentation.

5.3. Materials and methods

5.3.1. Carbohydrates

All AX samples and FOS were purchased from Megazyme (Bray,co. Wicklow, Ireland).

5.3.2. Characterisation of AX

5.3.2.1. Monosaccharide composition:

Commercial AX fractions were characterised using monosaccharide analysis as described in 2.5.

5.3.2.2. Structural analysis of carbohydrates

AX fractions after *in-vitro* gastric/duodenal digestion were analysed by HPAEC and PACE following specific endoxylanase treatment, to determine effects of the simulated gastric/duodenal environment on AX structure. Methods are as described in 2.3 and 2.4 respectively.

5.3.3. Analysis of phenolic acid content

Analysis of phenolic acid content was done as described in 2.8.

5.3.4. *In vitro* gastric digestion of AX

In vitro gastric and duodenal digestion was carried out on all AX samples prior to fermentation as described in 2.9.

5.3.5. *In vitro* fermentation of AX

In vitro fermentation was performed as described in 2.10. AX (500mg) was compared to FOS (500mg) and a negative control in triplicate using faecal samples obtained from three healthy donors (n=3). Polysaccharide was added to basal media at 1% (w/v). Samples were inoculated

with 10% faecal slurry, as before. Samples were taken at 0h, 4, 6h, 24h and 48h. The additional time-point at 48h was added as it was hypothesized that the insoluble AX fraction might take longer to ferment.

5.3.6. Enumeration of bacteria by flow-FISH

Bacteria were enumerated from samples taken at 0h, 8h and 24h using flow-FISH as detailed in 2.12.

5.3.7. SCFA analysis

SCFA and lactate were quantified using HPLC as detailed in 2.11.

5.3.8. Statistical analysis

The Genstat (2015, 18th edition, © VSN International Ltd, Hemel Hempstead, UK) statistical package was used for all analysis. ANOVA data was supplied by SJ Powers (Statistical department, Rothamsted Research) and applied as detailed in 2.13.

5.4. Results

5.4.1. Monosaccharide Analysis of commercial AX samples of different MM following *in vitro* digestion.

Monosaccharide analysis of AX provides information about the average degree of arabinose substitution (A/X ratio) as well as the purity of the fractions. The A: X ratios of water-extractable wheat endosperm AX varies, Maes and Delcour (2002) found a range from 0.31 to 0.85.

Monosaccharide analysis of different commercial MM AX fractions from wheat using HPAEC showed that similar proportions of arabinose and xylose were present in each of the three soluble fractions(w/w) with very similar and very high A:X ratios ranging between 0.81 and 0.84 (Table 5.1) and a larger ratio of 0.93 for the insoluble AX. Figure 5.1 shows the A:X ratio before and after *in vitro* gastric and duodenal digestion. The A:X ratio for all samples decreased after digestion.

Glucose and galactose are present in the high MM AX (5.6% and 0.58% of total monosaccharides) and the insoluble AX samples (1.96% and 1.35% of total monosaccharides) before digestion. These monosaccharides are reduced or removed after dialysis in the *in vitro* digestion, in the high MM AX to 1.92% and 0% for glucose and galactose respectively and 0% for both for the insoluble AX.

The correction factor to account for arabinose not associated with xylose found by Loosveld et al. (1997) was not applied in this case as commercial AX has already been purified.

Table 5.1: Monosaccharide composition of commercial different MM AX pre-and post-*in vitro* gastric and duodenal digestion showing A:X ratio using a CarboPac PA20 column. Values are average m [$\mu\text{g}/\text{mg}$] where N=3 and standard deviation is shown in brackets.

			Arabinose	Xylose	Galactose	Glucose	A:X ratio
Low MM AX	Pre		391.5 (± 6.34)	465.0 (± 3.7)	0	0	0.84
	Post		359.1 (± 15.18)	463.9 (± 8.85)	0	0	0.77
Med MM AX	Pre		384.9 (± 2.77)	458.8 (± 12.25)	0	0	0.84
	Post		371.3 (± 1.15)	456.8 (± 0.67)	0	0	0.81
High MM AX	Pre		340.6 (± 11.5)	422.6 (± 12.25)	4.7 (± 4.71)	46.7 (± 1.39)	0.81
	Post		340.4 (± 6.76)	445.2 (± 25.65)	0	15.4 (± 14.55)	0.76
Insoluble AX	Pre		390.2 (± 1.04)	418.9 (± 27.50)	11.4 (± 6.42)	16.4 (± 7.15)	0.93
	Post		342.9 (± 28.79)	419.25 (± 26.61)	0	0	0.81

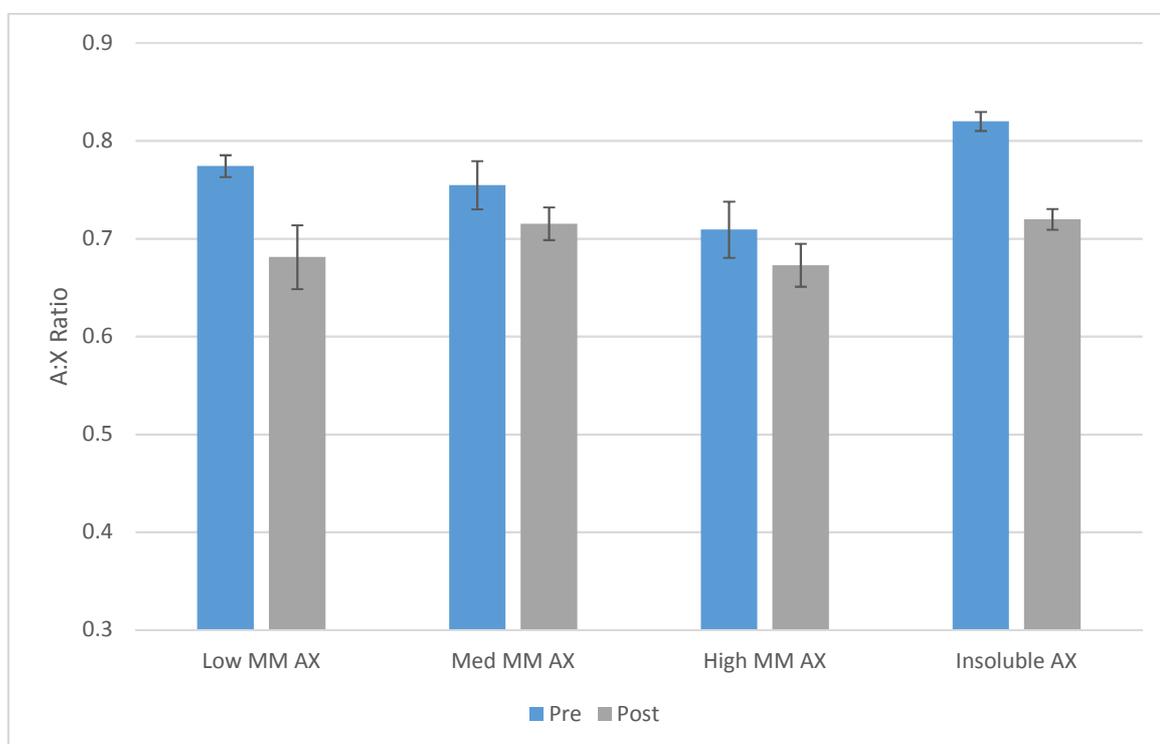


Figure 5.1: A:X ratio of AX samples pre-and post-*in vitro* gastric and duodenal digestion. Monosaccharides were resolved using a CarboPac PA20 column.

5.4.2. Structural analysis of AX by HPAEC before and after *in vitro* gastric and duodenal digestion

Following endoxylase digestion and HPAEC the peak areas for the identified AXOS (as seen in Figure 5.2) were compared for the various AXOS constituents before and after *in vitro* gastric and duodenal digestion. Figure 5.3 shows the relative % change in different AXOS fractions after *in vitro* gastric and duodenal digestion.

After *in vitro* gastric and duodenal digestion, there are a greater number of smaller AXOS fractions xylose and xylobiose (Xyl₂) in all samples and more xylotriose in the low and high MM AX samples. Subsequently the larger AXOS (XA³XX, XA²⁺³XX, XA³A³XX) decrease in all samples after digestion. The amount of the largest AXOS (XA³XA²⁺³XX) reduces very slightly after digestion, much less than the XA³XX, XA²⁺³XX and XA³A³XX fractions, indicating that this AXOS is less affected by the digestion than other AXOS, possibly due to its high level of substitution.

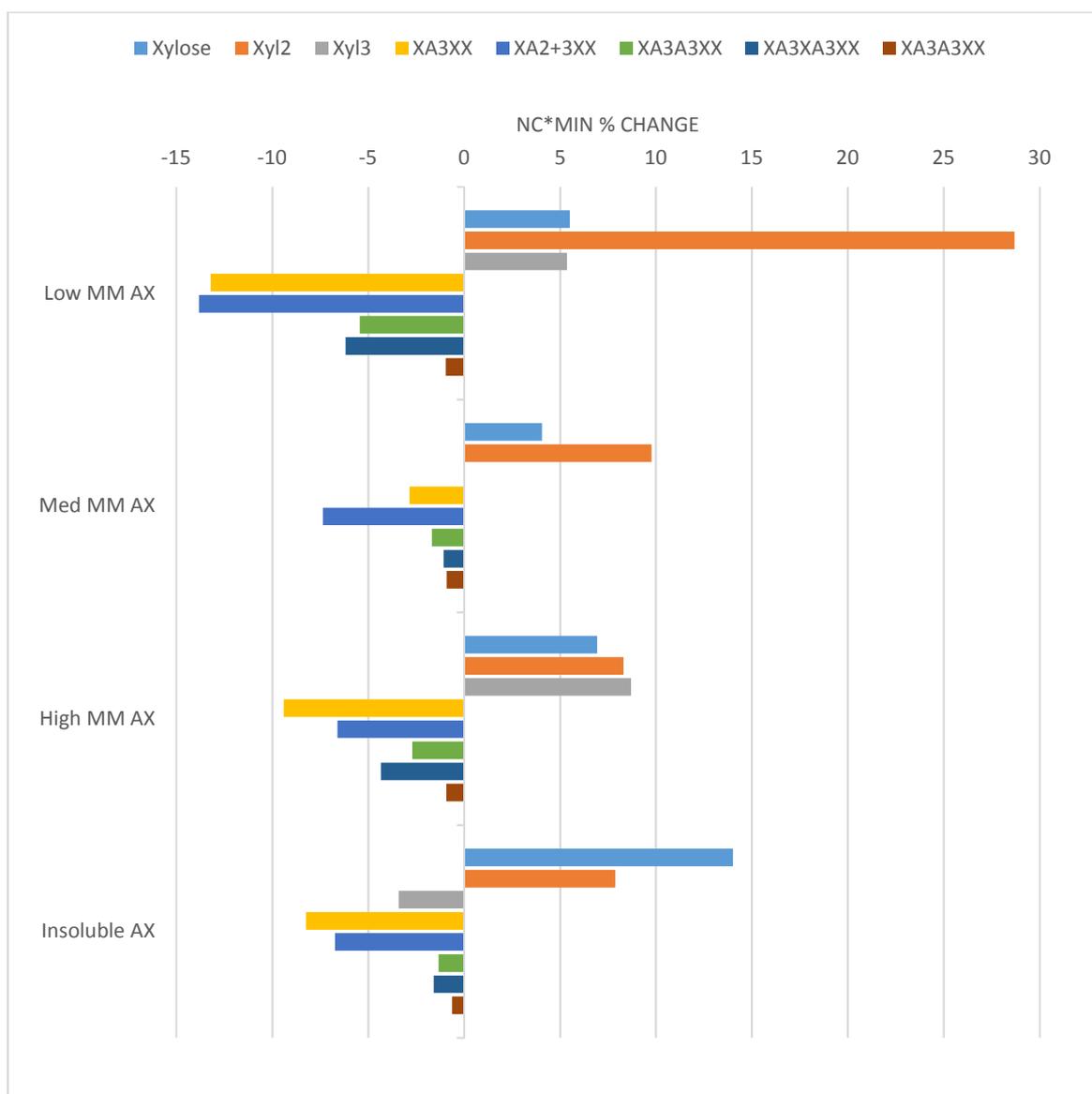


Figure 5.2: Changes in AXOS patterns in commercial AX samples (Megazyme co. Wicklow, Ireland) of different MM after *in vitro* gastric and duodenal digestion. Oligosaccharide abundance (HPAEC peak area). All Samples have undergone endoxylase digestion (Xylanase PRO-E0062 Prozomix) before analysis. Products of digestion were analysed by HPAEC. Values are average where (N=3).

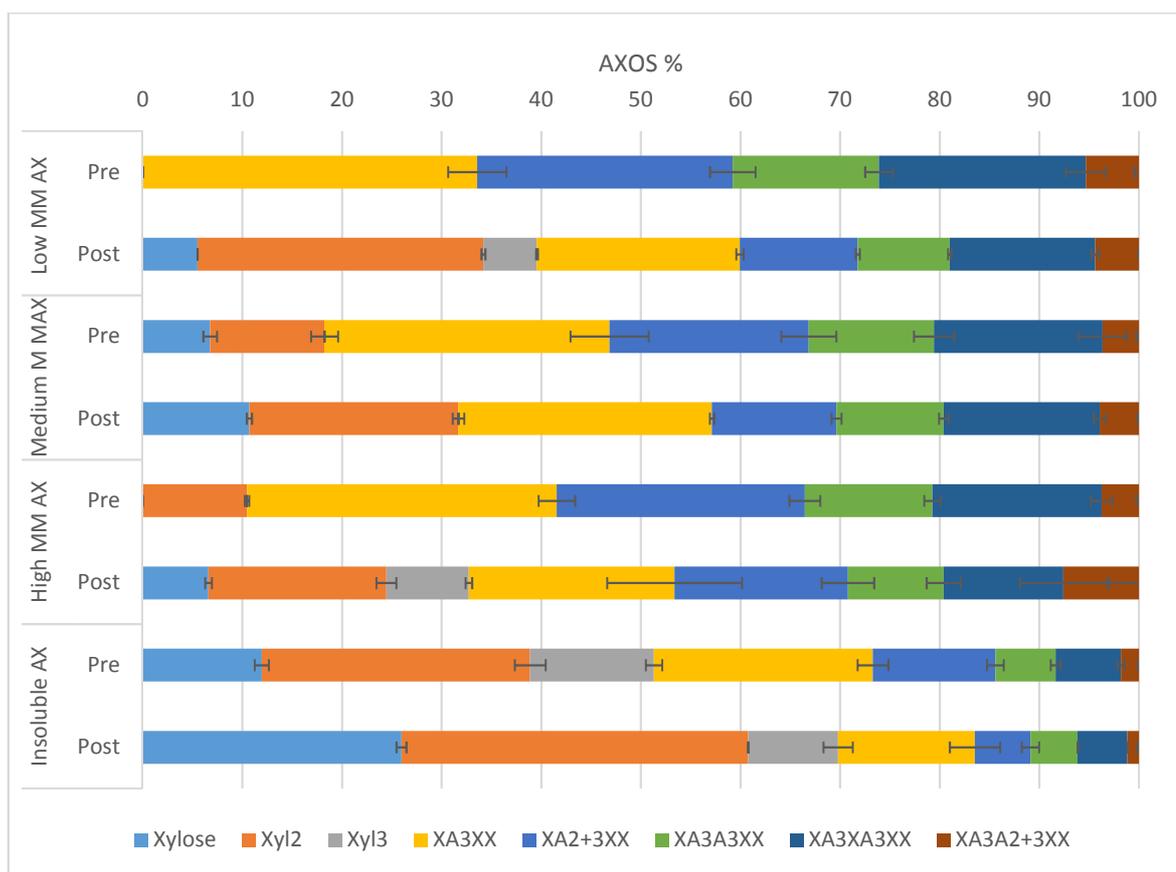


Figure 5.3: AXOS fragments, expressed as percent of total AXOS ‘pre’ and ‘post’ *in vitro* gastric and duodenal digestion. Oligosaccharide abundance (HPAEC peak area) of AXOS. All Samples have undergone endoxylase digestion (Xylanase PRO-E0062 Prozomix) before analysis. Products of digestion were analysed by HPAEC. (N=3) Error bars are SD. AXOS fractions X, XX, XXX, XA³XX, XA²⁺³XX and XA³A³XX, XA²⁺³XX, XA³A³XX are shown depending on quantity.

5.4.3. Structural analysis of different MM AX samples before and after simulated gastric and duodenal digestion by HP-SEC-MALS

Commercial AX samples were analysed by HP-SEC-MALS before and after *in vitro* gastric and duodenal digestion to find out whether the MM is affected by exposure to gastric/duodenal digestion conditions.

Samples of low MM AX, medium MM AX and high MM AX were separated by size exclusion chromatography before and after the *in vitro* gastric and duodenal digestion, to determine if digestion affects the size or viscosity of the polysaccharides (shown in Table 5.2 and Figure 5.4). HP-SEC-MALS analysis showed these fractions to have average MM of 56.9kDa, 326.9kDa,

and 374.2kDa respectively prior to digestion and 46.3kDa, 177 kDa and 236kDa post digestion. AXs with larger dp are accepted as having greater cross-linking capacity and a resulting capability to entrap and hold water, which in turn increases viscosity (Izydorczyk and Biliaderis, 1992). This is observed in Table 5.2 where the larger the MM of the AX, the higher the intrinsic viscosity. The intrinsic viscosity of all fractions also decreases after *in vitro* digestion. HP-SEC-MALS of insoluble AX was not possible under the aqueous conditions used.

All AX fractions showed reductions in MM and intrinsic viscosity after *in vitro* digestion, indicating that the gastric and duodenal digestion conditions do alter the AX fractions. It has been suggested that a positive correlation existed between solubility and fermentability (Roland et al. 1995) therefore an increase in solubility as observed in Table 5.2 and Figure 5.4 may increase fermentability.

There was a greater reduction in size of the high and medium MM AX compared to the low MM AX. This would fit with the hypothesis that the low pH environment acts to remove arabinose units, as observed in figs 5.2 and 5.3. The result of this may also be to release phenolic acids, that cross-link AX chains together via the arabinose unit. This was explored next (chapter 5.4.4).

Figure 5.4 shows the elution time of the AX samples, when using HP-SEC, larger solutes are eluted faster due to fewer interactions with the column. The high MM AX is eluted first, and the low MM AX is eluted last both before and after *in vitro* digestion. The elution time for the high MM AX appears to increase after *in vitro* digestion, due to its decrease in MM. There is no discernible difference in the elution time of the low MM AX which is likely because there was a smaller change in MM after *in vitro* digestion.

Variation in the MM of the high and medium MM AX samples appears to decrease after *in vitro* digestion (shown in Figure 5.4 by the reduced gradient of the line representing MM over time), suggesting the AX polysaccharides became more uniform.

Table 5.2: HP-SEC-MALS of different MM AX samples before and after simulated gastric and duodenal digestion. MM and intrinsic viscosity were obtained by HP-SEC-MALS. One hundred μL was injected onto an OHpak SB 802.5 HQ column. The eluent was 0.1M NaNO_3 with 0.02% NaN_3 with a flow rate of $0.5\text{mL}/\text{min}^{-1}$. Insoluble AX could not be run under the aqueous conditions. Lines at the bottom of each image show the signal strength.

	MM (kDa)		Intrinsic viscosity (mL/g)	
	Pre-digest	Post digest	Pre-digest	Post digest
Low MM AX	56.9 (± 0.1)	46.3 (± 0.3)	116.9	94.8
Medium MM AX	326.9 (± 0.3)	177 (± 0.3)	288.6	180.1
High MM AX	374.2 (± 0.3)	236.1 (± 0.2)	352.6	240.3

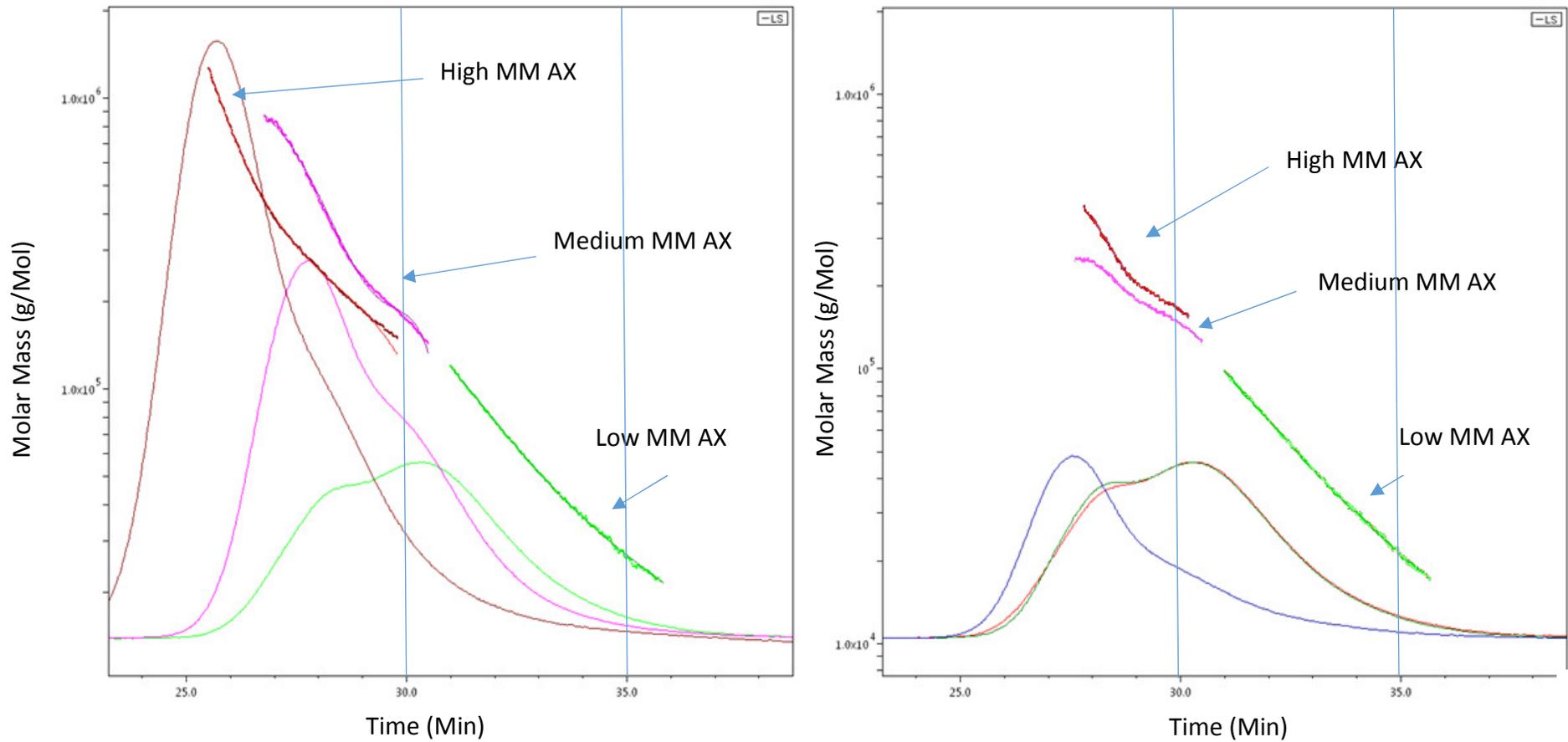


Figure 5.4.: HP-SEC-MALS of average Molar Mass of different MM AX samples before (left) and after (right) *in vitro* gastric and duodenal digestion on an OHpak SB 802.5 HQ column. Low MM AX is labelled in green, Medium MM AX is labelled in pink and High MM AX is labelled in red. Lines at the bottom of each image show the signal strength.

5.4.4. Phenolic acid Analysis of Different MM AX samples using HPLC

Commercial soluble AX fractions with low, medium and high MM and an insoluble fraction were analysed for phenolic acid content as it was hypothesised that acid hydrolysis removed bound phenolic acids. Comparison of phenolic acid content before and after *in vitro* digestion was analysed by HPLC (detailed in 2.8.2).

The major phenolic acid in wheat endosperm is trans-ferulic (tFA) which accounts for about 90% phenolic acids found in wheat flour) (Fulcher, 1982) and is present in free, conjugated and bound forms, the bound form being much more abundant than the free and conjugated (Sosulski et al., 1982). This was observed in the current study, tFA was the only phenolic acid present on commercial AX samples and is shown in Table 5.3.

The low and high MM AX initially had low levels of free and conjugated tFA (26.35-35.69 µg/mg) and lower levels of bound tFA (3.82-4.13 µg/mg). A much higher amount of tFA, including both bound and free and conjugated was present in the insoluble fraction. This is as expected as AX polymers can be cross-linked by their phenolic acid side chains, reducing their solubility (Saulnier et al., 2007). The high level of bound tFA would imply that the insoluble AX fraction is highly crosslinked. No tFA was detected on the medium MM AX but this is to be expected as the commercial soluble fractions had all been prepared from a high molecular mass stock preparation. The low MM AX was prepared from the high MM AX using partial enzyme digest and the medium MM AX prepared from the high MM AX using partial acid hydrolysis (Pitkanen et al., 2009) therefore phenolic acids would already have been removed by acid hydrolysis.

Free and conjugated tFA remain at similar levels after *in vitro* digestion (post) for the low and high MM AX, whilst increasing for the insoluble AX, whilst bound tFA fell to zero in all samples, suggesting that bound tFA was released by the low pH during gastric digestion. The little change observed in the low and high MM AX are likely due to the small amount of bound tFA present initially.

A decrease in bound phenolics due to a low pH in the stomach could contribute to the increase in fermentability of the AX. Bound phenolic acids cause cross linking of AX chains, increasing aggregation and this could also impede access of microbial digestive enzymes to the specific site required for enzyme action. Removal of bound tFA would also cause greater bioavailability of tFA in the gut, which has been linked to health benefits. A dose of 10mg/kg has been shown to reduce the risk of high-fat diet associated obesity in mouse models via modulation of

enzymatic, hormonal and inflammatory responses (De Melo et al., 2017). Thus it would seem that gastric digestion could improve the ability for AX to be fermented and increases potential absorption of tFA.

Table 5.3: Ferulic acid analysis of commercial different MM AX samples before and after *in vitro* gastric and duodenal digestion. Values are average where N=3, values in brackets are SD.

	Free and conjugated tFA ($\mu\text{g}/\text{mg}$)		Bound tFA ($\mu\text{g}/\text{mg}$)		Total ($\mu\text{g}/\text{mg}$)	
	pre	post	pre	post	Pre	Post
Low MM AX	35.69 (± 0.74)	37.03 (± 5.02)	3.82 (± 0.51)	0	39.51 (± 1.25)	37.03 (± 5.02)
Medium MM AX	0	0	0	0	0	0
High MM AX	26.35 (± 1.51)	22.95 (± 5.57)	4.13 (± 2.44)	0	30.48 (± 3.95)	22.95 (± 5.57)
Insoluble AX	212.21 (± 8.91)	722.20 (± 38.18)	469.66 (± 26.83)	0	681.87 (± 35.74)	722.20 (± 38.12)

5.4.5. HPLC quantification of SCFA production from *in vitro* fermentation of different MM AX fractions after *in vitro* gastric and duodenal digestion

Short chain fatty acid concentrations are shown in Table 5.4, and Table 5.5 shows the bacterial populations after the *in vitro* digested AX samples were fermented by colonic bacteria in static batch cultures for 0, 4, 6, 24 and 48 hours. Flow-FISH probes were used based on the earlier study by Hughes et al. (2007) to which this study compares. A longer fermentation time of 48h

was included in this study because in previous experiments (chapters 3 and 4) the highest concentrations of SCFA was observed at the final time point of 24h, therefore it was uncertain if the SCFA concentration could increase further with increased time and hypothesised that some AX samples may take longer than 24h to ferment.

It is shown in Table 5.4 and 5.5 that fermentation of AX can continue until 48h as the number of total bacteria continued to increase until 48h for the low MM AX and the total SCFA concentration also peaked at 48h. Total bacterial numbers declined after 24h fermentation of medium MM, high MM and insoluble AX (and the positive control, FOS) samples. Total SCFA production peaked at 48h for the low and medium MM AX and the insoluble AX. Interestingly, the concentration of total SCFAs also peaks at 48h with fermentation of FOS, which is thought to be rapidly fermented due to its low dp (Stewart et al., 2008). However, this late peak in SCFA is due to increases in propionate and butyrate (rather than acetate) which are secondary SCFAs formed through utilization of other metabolites through cross-feeding. Acetate concentration decreases after 24h in FOS, high MM AX and insoluble AX, indicating that the acetate may be being used in cross feeding. The lack of increases in acetate concentration for these samples indicates that fermentation has ceased. The concentration of acetate continues to increase with the low and medium MM AXs indicating that these samples were still being fermented after 24h.

Persistence in the colon is an important issue for prebiotics. It was thought that a greater MM would favour prolonged fermentation in the colon. This was not observed in this study as the low MM AX appeared to be fermented for longer than the medium, high MM AX or the insoluble AX. The low MM AX was thought to be fermented the fastest as it has the smallest dp and more non-reducing ends in the xylose backbone w/w than the larger fractions, which are more available for hydrolysis by bacterial enzymes. It is possible that the low MM AX was more *fully* fermented than the other AX samples, due to these structural attributes and therefore it was fermented for longer.

The total number of bacteria decreased after 24h of fermentation of the insoluble AX, indicating that fermentation rate had reduced and that bacterial populations would continue to decrease with increased time. The insoluble AX therefore did not appear to show the ability to persist further in the colon and be fermented in more distal regions than the soluble AX samples. A higher A:X ratio has been shown to decrease the degree of fermentability (Pollet et al., 2012) so it is possible that the insoluble AX was not fully fermented due to its complex structure. The insoluble AX fraction showed a significant ($p>0.95$) increase in SFCA production compared to the negative control after 24h fermentation, but this occurred slower than with the medium and

high MM AX samples, indicating that the insolubility of the AX structure makes it more difficult for bacterial enzymes to access the AX structure to hydrolyse it.

Bifidobacterium populations increased significantly ($p > 0.05$) compared to the negative control after 24h fermentation of the low mm AX (this was sustained until 48h) and high MM AX after 24h (not sustained until 48h). It is thought that bifidobacteria prefer lower molecular mass AXOS fractions (Hughes et al., 2007; Ho et al., 2017), however in this study bifidobacteria appear to prefer the high MM AX, reaching $7.34 \log_{10}$ bacteria/mL after 48 hours' fermentation compared $6.63 \log_{10}$ bacteria/mL. when fermenting the low MM AX. This is reflected in the concentration of acetate (as *Bifidobacterium* species are known producers of acetate (Fukuda et al., 2011; Bindels et al., 2015)) at 48h, for the high MM AX acetate concentration is higher, at 20.33 mM compared to 16.52mM for the low MM AX. The longer fermentation time could provide enough time for the higher MM AX to be degraded, however as early as the 6-hour time point the number of bifidobacteria is greater after fermenting the high MM AX than the low and medium MM AX.

The low MM AX appeared to be fermented more slowly than the medium MM or the high MM AX as it took up to 48 hours to cause a significant change in bacterial populations. The High MM AX increased *Bifidobacterium* populations after 24h compared to 48 hours for the low MM AX. The medium MM AX increased *Clostridium coccooides* / *Eubacterium rectales* after 24 hours compared to 48 hours for the low MM AX. This was contrary to expectations. After gastric digestion, the low MM AX was shown to have an A:X ratio higher than the medium or high MM AX, this may inhibit fermentation by blocking access to catalytic enzymes. Another hypothesis is that the low MM AX was more *fully* fermented due to the greater number of non-reducing ends per unit mass, which are more easily hydrolysed by the exo-acting enzymes produced by bacteria in the gut (Gibson, 2004).

The *Clostridium coccooides* / *Eubacterium rectales* groups increased with the medium MM AX after 24h (sustained until 48h) and the low MM AX after 48h. The AX did appear to result in selective fermentation by bacterial populations, for example, the *Clostridium histolyticum* subgroup, which can be pathogenic in humans, decreased for all AX samples.

Acetate levels increased steadily and peaked at 48hours for all samples. Significant increases in acetate concentration compared to the negative control ($p > 0.05$) were found for low MM AX from 24h-48h, for medium MM AX from 24h-48h, high MM AX for 6h-48h and insoluble AX at 48h. Increases were highest for acetate in the medium MM AX sample with increases of 3.03-fold compared to the negative control after 24 hours, the low, high and insoluble AX samples

gave increases in acetate of 1.73, 2.5 and 2.05-fold respectively. The increases in acetate occurred without significant increases in bifidobacteria in the medium MM AX but could be due to increased metabolism or from other bacterial groups, not measured by the FISH probes used in this study.

Significant increases in butyrate ($p < 0.05$) compared to the negative control were found for all AX samples. (The low MM AX from 4-48h, for medium MM AX from 4-48h, for high MM AX from 6-48h, insoluble AX from 6-48h and FOS at 48h). Butyric acid production was highest with the medium MM AX where there was a 10-fold increase. There were also increases in butyric acid of 5.29-fold for low MM AX, 6.52-fold for high MM AX and 7.52-fold for insoluble AX.

AX appears to stimulate butyrate production more than FOS, which may result from bacterial groups not measured in this study (e.g. *Roseburia* spp.) as the butyrate producing group *Clostridium coccooides/ Eubacterium rectales* that were measured in this study were increased with fermentation of FOS as well as with low and medium MM AX.

All AX samples showed reductions in numbers of *C. histolyticum* at 48h fermentation, although this was not statistically significant ($p > 0.05$). *C. histolyticum* preferentially ferments proteins potentially producing enterotoxins which cause a range of pathologies, from food poisoning to gas gangrene (Collins et al., 1994; Paredes-Sabja and Sarker, 2009). In the previous study (Hughes et al., 2007) all AX samples caused increases in this bacterium so prior *in vitro* gastric and duodenal digestion could cause AX to no longer stimulate this bacterium.

Although *Bacteroides* species are often numerically dominant in healthy adults, a selective increase in their colonic activity is not considered to be beneficial as they are often identified as the causative agent of diarrhoea. The *Bacteroides* group includes potentially pathogenic species such as *Bacteroides fragilis* (Macfarlane, Cummings and Allison, 1986) which produce high levels of the enzyme β -glucuronidase, that catalyses hydrolysis of β -D-glucuronic acid from polysaccharides and is associated with production of toxic metabolites (McBain and Macfarlane, 1998). *Bacteroides* bacteria did not increase significantly with any AX samples or the positive control, showing the selectivity of the AX substrates regardless of structure. Again, in the previous study (Hughes et al., 2007), *Bacteroides* species increase with all AX samples, so the changes resulting from the gastric/ duodenal environment appear to have changed the selectivity of AX.

No significant ($p < 0.05$) differences from the negative control were observed for formic acid and only one increase in lactic acid with the medium MM AX after 4h. This reflects the results

found by Hughes et al. (2007) for lactate (lactate levels increase from 0h-4h in this study but after 4h they reduce to less than 5mM due to cross feeding). It was previously found that propionate levels had increased by 5h in all AX samples, whereas in this study, propionate only increased after 24h for the medium and high MM AX and the insoluble AX and 48h for the low MM AX. The structural changes caused by prior *in vitro* digestion appear to have affected the fermentation profile and resulted in slowed and reduced propionate production. Formate was not measured in the previous study and so cannot be compared. Formic acid is a transient metabolite which peaked at 6 hours in all samples apart from the insoluble AX, which peaked at 4 hours.

It is unclear why AX caused a significant ($p > 0.05$) increase in butyrate in this study this was not observed in our previous studies (Chapter 3 and 4) where the same medium MM AX substrate was used. The same faecal donors could not be used in all the fermentation studies and differences in diet and lifestyle could add confounding factors into these studies. Individual differences in microbiota and reactions to fermentation of polysaccharides could be responsible for the large deviation between donors in this study.

Table 5.4: SCFA and lactate concentration in batch cultures at 0, 4, 8 and 24 hours' fermentation comparing no substrate, FOS, and AX with different molecular masses that had previously undergone in-vitro gastric and duodenal digestion. Negative control is no added carbohydrate and positive control is FOS. One way ANVOA was applied to the data to test (F-test) the main effect of treatment. SEM is shown in italics. Significant interaction between treatments and negative control are denoted * ($p < 0.05$).

	Time	Lactate (μM)	Formate (μM)	Acetate (μM)	Propionate (μM)	Butyrate (μM)	Total (μM)
Negative control	0	5.10 (0.09)	1.73 (0.04)	6.91 (1.39)	4.12 (2.01)	2.18 (0.18)	20.32 (5.77)
	4	8.67 (2.33)	2.25 (0.39)	7.34 (1.61)	5.37 (3.53)	1.97 (0.22)	25.60 (6.99)
	6	5.9 (0.19)	3.33 (0.40)	6.57 (1.74)	2.07 (0.12)	1.88 (0.23)	19.76 (3.55)
	24	5.27 (0.35)	1.96 (0.19)	8.28 (1.04)	2.03 (0.14)	1.24 (0.23)	18.79 (1.74)
	48	4.45 (0.02)	1.73 (0.08)	5.99 (0.82)	2.05 (0.11)	0.89 (0.22)	15.11 (1.40)
FOS (positive control)	0	4.72 (0.06)	1.7 (0.04)	1.57 (0.14)	3.71 (1.81)	1.91 (0.34)	13.60 (3.59)
	4	7.05 (2.37)	3.17 (1.22)	10.62 (6.00)	2.39 (0.50)	2.11 (0.73)	25.36 (15.64)
	6	6.36 (1.67)	5.46 (1.17)	17.31* (8.38)	4.04 (1.92)	1.89 (0.91)	35.06 (22.31)
	24	5.34 (0.98)	2.48 (0.54)	25.88* (4.63)	7.49 (5.01)	1.99 (0.98)	43.84* (15.76)
	48	4.47 (0.06)	1.72 (0.07)	25.29* (6.62)	15.94* (4.52)	3.7* (1.89)	51.14* (21.87)
Low MM AX	0	5.94 (0.04)	2.13 (0.02)	11.63 (2.89)	2.06 (0.15)	2.55 (0.42)	24.30 (6.64)
	4	10.76 (0.08)	2.16 (0.21)	9.2 (1.26)	1.99 (0.08)	5.05* (0.44)	25.82 (8.89)
	6	6.46 (0.82)	3.72 (0.96)	10.44 (5.44)	4.25 (2.28)	6.27* (0.36)	26.14 (11.20)
	24	4.51 (0.80)	2.24 (0.44)	14.03* (4.02)	5.51 (3.43)	6.56* (0.49)	27.19 (20.95)
	48	4.65 (0.07)	1.88 (0.19)	16.52* (7.59)	6.08* (2.62)	3.73* (2.93)	30.86 (18.72)
Medium MM AX	0	5.15 (0.26)	1.92 (0.16)	4.69 (1.20)	3.57 (1.66)	2.69 (0.18)	18.02 (4.11)
	4	14.85 (1.94)	3.31 (0.58)	8.72 (1.85)	2.12 (0.10)	4.97* (0.3)	31.63 (12.64)
	6	6.22 (0.39)	5.00 (0.67)	13.73 (4.78)	2.97 (1.05)	8.98* (0.28)	29.91 (14.07)
	24	7.2 (0.06)	2.06 (0.31)	25.09* (2.72)	7.74* (1.60)	12.61* (0.22)	44.69* (7.68)
	48	5.39 (0.31)	2.03 (0.22)	27.55* (6.26)	11.3* (3.16)	5.9* (3.86)	48.83* (15.69)
High MM AX	0	5.25 (0.35)	1.7 (0.05)	6.5 (0.35)	3.86 (1.86)	2.55 (0.16)	19.86 (3.37)
	4	7.14 (4.62)	3.21 (0.35)	12.51 (0.85)	6.37 (4.24)	3.41 (0.64)	30.97 (7.20)
	6	5.87 (0.11)	4.96 (0.14)	17.44* (1.5)	2.16 (0.22)	7.2* (0.02)	31.97 (3.51)
	24	5.67 (2.13)	2.37 (0.35)	20.7* (0.87)	7.57* (2.34)	8.08* (0.82)	38.71* (1.56)
	48	4.78 (1.16)	1.64 (0.02)	20.33* (2.86)	8.4* (2.00)	2.33 (0.76)	37.49* (9.23)
Insoluble AX	0	5.03 (0.34)	1.85 (0.09)	6.54 (1.24)	3.22 (1.05)	2.4 (0.24)	19.04 (1.47)
	4	7.86 (0.65)	3.52 (0.56)	7.92 (0.56)	2.1 (0.04)	4.11 (0.48)	25.51 (4.47)
	6	4.71 (1.06)	3.28 (0.30)	11.12 (2.43)	3.87 (0.96)	3.89 (0.28)	25.21 (3.79)
	24	4.87 (1.42)	1.59 (0.00)	17.05* (2.99)	7.05* (1.59)	9.33* (0.29)	32.90* (5.16)
	48	4.46 (0.48)	1.62 (0.03)	16.71* (2.28)	7.89* (0.57)	9.49* (0.58)	34.84* (4.65)

Table 5.5: Bacterial enumeration (mean log₁₀ bacterial numbers / mL) of samples taken from static batch cultures after 0, 4, 8 and 24 hours' fermentation comparing no substrate, FOS, and AX with different molecular masses that had previously undergone *in vitro* gastric and duodenal digestion, using flow FISH. SEM are shown in italics. Negative control is no added carbohydrate and positive control is FOS. One way ANVOA was applied to the data to test (F-test) the main effect of treatment. SEM is shown in italics. Significant interaction between treatments and negative control are denoted * (p< 0.05).

	Time (h)	<i>Bifidobacterium</i> genus	<i>Lactobaccillus</i> <i>Enterococcus</i> group	<i>Bacteroides-</i> <i>Prevotella</i> group	<i>Clostridium</i> <i>coccoides</i> / <i>Eubacterium</i> <i>rectales</i> groups	<i>Clostridium-</i> <i>histolyticum</i> sub group	Total
Negative control	0	6.14 (0.51)	5.76 (0.58)	5.52 (0.47)	6.04 (0.45)	6.04 (0.45)	6.85 (0.47)
	4	6.14 (0.33)	5.70 (0.23)	5.45 (0.46)	6.01 (0.29)	4.41 (0.69)	6.99 (0.04)
	6	6.60 (0.31)	6.20 (0.12)	6.37 (0.18)	6.60 (0.30)	5.83 (0.26)	7.50 (0.08)
	24	5.90 (0.26)	5.56 (0.36)	5.60 (0.47)	5.85 (0.28)	5.67 (0.31)	6.73 (0.35)
	48	6.09 (0.34)	5.78 (0.22)	6.30 (0.41)	6.09 (0.36)	5.96 (0.27)	7.36 (0.63)
FOS (positive control)	0	6.26 (0.26)	5.86 (0.44)	5.81 (0.29)	6.44 (0.28)	6.44 (0.28)	7.35 (0.19)
	4	7.19 (0.73)	6.04 (0.46)	6.41 (0.44)	7.06 (0.64)	5.28 (0.13)	7.70 (0.66)
	6	6.98 (0.61)	5.82 (0.11)	6.67 (0.29)	6.80 (0.50)	5.98 (0.08)	7.82 (0.33)
	24	7.45* (0.12)	6.73 (0.39)	6.90 (0.64)	7.46* (0.28)	6.17 (0.90)	8.28* (0.30)
	48	7.26* (0.34)	6.25 (0.57)	6.82 (0.60)	7.33* (0.27)	6.27 (0.72)	8.04 (0.32)
Low MM AX	0	6.25 (0.25)	5.48 (0.31)	5.92 (0.36)	6.44 (0.42)	6.44 (0.42)	7.28 (0.35)
	4	7.45 (0.59)	6.12 (0.68)	6.73 (0.67)	7.42 (0.71)	5.69 (0.66)	7.88 (0.53)
	6	6.65 (0.44)	5.74 (0.48)	6.26 (0.48)	5.98 (0.27)	5.74 (0.37)	7.20 (0.39)
	24	6.79 (0.93)	6.17 (0.90)	6.48 (0.96)	6.61 (1.01)	6.16 (0.81)	6.86 (0.77)
	48	6.63* (0.64)	5.86 (0.44)	6.37 (0.57)	6.67* (0.60)	6.04 (0.44)	7.15 (0.61)

	Time (h)	<i>Bifidobacterium</i> genus	<i>Lactobacillus</i> <i>Enterococcus</i> group	<i>Bacteroides-</i> <i>Prevotella</i> group	<i>Clostridium</i> <i>coccoides</i> / <i>Eubacterium</i> <i>rectales</i> groups	<i>Clostridium-</i> <i>histolyticum</i> sub group	Total
Medium MM AX	0	6.33 (0.18)	5.74 (0.25)	5.86 (0.31)	6.68 (0.33)	6.68 (0.33)	7.42 (0.31)
	4	6.64 (0.37)	5.54 (0.25)	6.06 (0.29)	6.73 (0.39)	5.37 (0.31)	7.27 (0.32)
	6	6.91 (0.30)	6.13 (0.30)	6.94 (0.25)	6.97 (0.26)	5.94 (0.34)	7.72 (0.12)
	24	6.80 (0.53)	5.65 (0.38)	7.62 (0.33)	6.69* (0.59)	5.79 (0.05)	8.26* (0.09)
	48	6.48 (0.71)	5.88 (0.68)	6.32 (0.80)	6.70* (0.75)	5.98 (0.67)	7.27 (0.74)
High MM AX	0	6.50 (0.20)	5.78 (0.48)	6.19 (0.23)	6.69 (0.35)	6.69 (0.35)	7.51 (0.29)
	4	7.14 (0.37)	5.62 (0.17)	6.58 (0.24)	6.87 (0.44)	5.51 (0.29)	7.53 (0.36)
	6	7.31 (0.36)	6.00 (0.14)	7.02 (0.22)	6.99 (0.24)	5.93 (0.07)	7.75 (0.29)
	24	6.94* (0.26)	5.79 (0.09)	6.96 (0.48)	6.82* (0.63)	6.11 (0.22)	7.74 (0.25)
	48	7.34* (0.47)	5.19 (0.14)	6.07 (0.17)	7.12* (0.41)	5.76 (0.06)	7.63 (0.43)
Insoluble AX	0	6.01 (0.330)	5.47 (0.17)	5.75 (0.23)	6.07 (0.43)	6.07 (0.43)	6.86 (0.25)
	4	6.80 (0.32)	5.86 (0.41)	6.27 (0.38)	6.78 (0.46)	5.80 (0.42)	7.38 (0.39)
	6	6.71 (0.36)	6.10 (0.40)	6.62 (0.31)	6.81 (0.44)	6.01 (0.40)	7.45 (0.38)
	24	6.55 (0.37)	5.79 (0.06)	6.14 (0.03)	6.52 (0.47)	5.82 (0.09)	7.31 (0.29)
	48	5.92 (0.52)	4.49 (0.61)	4.93 (0.78)	5.81 (0.56)	4.45 (1.01)	6.27 (0.56)

5.3. Conclusions

After *in vitro* gastric and duodenal digestion, different MM AX samples were reduced in arabinosylation, this combined with the removal of all bound phenolic acids through hydrolysis reduced the MM and the intrinsic viscosity of each of the samples due to a reduction in diferulate bridges formed between AX chains. Though digestion affected the structure, making them more similar in MM, phenolic content and viscosity, there were still differences between the samples and these differences were still sufficient to cause differences in prebiotic activity between the AX samples.

Bifidobacterium populations were increased by fermentation of low and high MM AX and *Clostridium coccooides/ Eubacterium rectales* group populations were increased by fermentation of low and medium MM AX. However, the greatest levels of SCFA, namely acetate and butyrate were produced from fermentation of medium MM AX (at 24h) so it is difficult to conclude which fraction provided the greatest prebiotic activity. Total bacterial populations were significantly increased only by fermentation of the medium MM AX despite no statistically significant increase in the bacterial groups measured by FLOW FISH. This is likely due to increases in bacterial populations that were not measured in this study and would explain the increased production of SCFA. This is in contrast to the data from a previous study by Hughes et al. (2007), who found that the lowest MM AX had in the greatest prebiotic activity. However, it has been shown that *in vitro* gastric and duodenal digestion affects the structure of AX and therefore alters prebiotic activity.

The insoluble AX sample took longest to ferment, as was expected. It did not start causing increases of SCFA until 24h fermentation whereas the other samples began after just 4h. As changes took so much longer to occur, it is possible that a longer time point still would have allowed changes in microbial composition to be observed. This is something that could be looked into in the future as insoluble AX would be expected to persist longer in the colon *in vivo*.

The release of bound phenolic acids makes them more bioavailable, creating beneficial effects for the host. Therefore, tFA could be an important component of AX in terms of its ability to modulate prebiotic activity and a future study comparing the prebiotic effects of AX with and without ferulic acid would be of interest to elucidate if absence of ferulic acid resulting from the *in vitro* gastric and duodenal digestion is responsible for changes in prebiotic activity.

Table 5.6 shows conclusions from this study, with comparison to the previous study which was done without gastric and duodenal digestion prior to fermentation to see if prior exposure to a gastric environment affected the prebiotic activity of different MM AX fractions. The insoluble AX was omitted as it was not used in the previous study.

Table 5.6 Conclusions compared with the previous study by Hughes et al. (2007)

Low MM AX after <i>in vitro</i> digestion	Medium MM AX after <i>in vitro</i> digestion	High MM AX after <i>in vitro</i> digestion
<p>Increase in smaller AXOS and decrease in larger AXOS after <i>in vitro</i> digestion</p> <p>Reduction in viscosity</p> <p>Removal of bound ferulic acid</p> <p>Increase in acetate from 4-48h, as observed in Hughes et al. (2007)</p> <p>Increase in butyrate from 4-48h, as observed in Hughes et al. (2007)</p> <p>Increase in bifidobacteria after 48h, observed sooner in Hughes et al. (2007)</p> <p>Increase in <i>Clostridium coccooides/ Eubacterium rectales</i> group after 48h, observed sooner in Hughes et al. (2007)</p>	<p>Increase in smaller AXOS and decrease in larger AXOS after <i>in vitro</i> digestion</p> <p>Reduction in viscosity</p> <p>Removal of bound ferulic acid</p> <p>Increase in acetate from 4-48h, as observed in Hughes et al. (2007)</p> <p>Increase in butyrate from 4-48h, as observed in Hughes et al. (2007)</p> <p>Increase in total bacteria after 48h</p> <p>Increase in <i>Clostridium coccooides/ Eubacterium rectales</i> group after 24h, observed sooner in Hughes et al. (2007)</p>	<p>Increase in smaller AXOS and decrease in larger AXOS after <i>in vitro</i> digestion</p> <p>Reduction in viscosity</p> <p>Removal of bound ferulic acid</p> <p>Increase in acetate from 4-24h, as observed in Hughes et al. (2007)</p> <p>Increase in butyrate from 4-48h, as observed in Hughes et al. (2007)</p> <p>Increase in bifidobacteria after 24h, observed sooner in Hughes et al. (2007)</p>

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6. General Discussion

6.1. General conclusions

This thesis explored the relationship between wheat dietary fibre (DF) and prebiotic effects, focussing on how the structure of arabinoxylan (AX) and how different combinations of types of DF affect prebiotic activity. Wheat DFs are highly abundant and already used in food ingredients. Therefore, further knowledge as to their effects as prebiotics could be exploited to design changes in their composition which could be incorporated into food products to create healthier foods.

Chapter three focussed on arabinogalactan-peptide (AGP), which comprises a complex glycan attached to a 15-residue peptide via O-glycosylation to hydroxyproline residues. The structure has been determined but no studies had been carried out on physiological effects of AGP as a food ingredient, therefore it had not been tested for prebiotic activity. In this study the prebiotic activity of AGP was compared with AX. AGP was shown to have prebiotic activity similar to that of AX and the prebiotic activities of both DFs were enhanced by providing AGP and AX together in *in vitro* experiments. This study showed that different types of DF could have the ability to enhance the effect of available prebiotics through synergy.

Chapter three also determined the effects of combinations of dietary fibre components on prebiotic activity, focussing on AX and β -glucan. AX and β -glucan were fermented in different ratios to determine whether there was an enhanced effect of the two DFs on prebiotic activity. It was shown that a ratio of AX: β -glucan (3:1) had the greatest prebiotic activity, performing better than AX alone. This is the ratio that is found in wheat flour so it was postulated that colonic bacteria have adapted to this ratio of DF in staple foods made from wheat flour and hence are able to efficiently ferment these substrates when provided together. This study supported the idea that different types of DF could have the ability to enhance the effect of available prebiotics through synergy and so further research in to this could yield more effective prebiotic formulas.

Chapter 4 focussed on structural changes to AX and how they affect prebiotic activity, using two approaches. Firstly, AX was isolated from a transgenic wheat line with altered expression of a glycosyl transferase enzyme responsible for addition of the O-3 linked arabinofuranose. The resultant AX had an altered A:X ratio and reduced arabinosylation. The rate and concentration

of SCFAs produced during fermentation, mainly acetate, were not as high as with the control AX, and the populations of total bacteria and *Bifidobacterium* were increased only in the control but not the RNAi GT61 AX. It was concluded that the RNAi GT61 AX had reduced prebiotic activity related to specific reduction in O-3 linked arabinose. It was shown in chapter 4 that the reduction of O-3 linked arabinose substitution caused by an altered GT enzyme in RNAi GT61 AX can reduce prebiotic effects of AX. This is contrary to some studies which claim that a reduction in arabinose substitution has no effect on prebiotic activity (Van Craeyveld et al., 2008) or even increased prebiotic activity (Pollett et al., 2012) however, the current study, uniquely, was able to use AX with a highly defined arabinosylation pattern, which may explain the contrasting results.

The effect of structure on prebiotic activity was also studied using *in vitro* gastric and duodenal digestion, to simulate effects on AX structure prior to fermentation in the colon. It was shown that prior *in vitro* gastric and duodenal digestion causes a reduction in A:X ratio of AX, a total removal of bound ferulic acid and reductions in MM and intrinsic viscosity. The digested AX had a lower initial rate of acetate generation compared to the untreated control AX, however, the digested AX caused modulation of the bacterial communities which the untreated control AX did not. The digested AX caused increases in beneficial groups *Bifidobacterium*, *F. prau* and *Roseburia*, the latter two use acetate to produce butyrate and it was therefore concluded that the initial low acetate concentration observed with the pre-digested substrate was due to utilization for cross feeding to produce other SCFA.

The random removal of arabinose units and reduction in arabinose substitution occurring during gastric digestion appears to enhance prebiotic activity. However, during the *in vitro* digestion, other aspects of AX structure were also affected as well as a reduction in arabinosylation, notably a reduction in dp and a removal of bound phenolic acids. These effects could also have contributed to the change in prebiotic activity, producing similar to the data obtained by Van Craeyveld et al. (2008), who postulated that changes in substitution were not as important as changes in dp.

From the present study, it appears that the specific removal of O-3 linked arabinose can negatively affect prebiotic activity. However, the random removal of arabinose and changes in size do not. Further work is required to explore this further. The exposure of the AX isolated from the transgenic wheat line might be a first step determine the basis for this effect.

Chapter 5 showed that although *in vitro* gastric and duodenal digestion of different MM AX samples resulted in greater similarity in structure, they still retained differences in prebiotic

activity. Fermentation of the low and medium MM AX resulted in the greatest increases in SCFA production and beneficial modulation of bacterial composition. The insoluble AX was very slow to ferment, probably due to its high degree of substitution and insolubility. Prior digestion modulated the prebiotic activity of the AX by increasing time taken to exert a prebiotic effect compared to a previous study (Hughes et al., 2007).

6.2. Future work

Wheat flour DFs show potential to be prebiotics but further work is required to provide a greater understanding of their potential. As with all model systems, *in vitro* fermentation systems are not a fool-proof way of measuring prebiotic potential. Increases in a limited number of bacterial genera in faecal slurries *in vitro* cannot confirm a prebiotic effect because faecal slurries have been proven to be more representative of more distal regions of the colon, whilst more proximal regions have a more saccharolytic profile (Macfarlane et al. 1992, 1998). The continuous, three-stage gut models, which replicate different the three regions of the colon, attempt to overcome this and further work should be carried out using first, continuous gut model and then human trials (Gibson et al., 2004).

- A further fermentation study on insoluble AX is required to determine its persistence in the colon. A three-stage fermentation model is available at the University of Reading which replicates the three stages of the colon. Persistence of insoluble AX would be demonstrated by increases in beneficial bacteria and SCFA productions in the most distal section. Greater persistence of AX into the distal regions should help to prevent the production of toxic metabolites from fermentation of proteins which occurs when carbohydrate sources are exhausted.
- This work has focussed mainly on how the structure of AX affects prebiotic activity, however β -glucan is also a significant DF in wheat flour although more abundant (and soluble) in other cereals including oats and barley. However, as wheat is more common in the diet the amount of wheat β -glucan consumed is probably much greater than that of barley or oat β -glucan. A study comparing the structure and prebiotic effects of wheat β -glucan and barley β -glucan would determine if the source of DF is important. There is conflicting evidence in this field currently.
- A study comparing AX with and without phenolic acids would demonstrate whether phenolic acids affect prebiotic activity. Based on a study by Grabber et al. (2008), who found that reductions in ferulate cross-linking of arabinoxylans to lignin enhanced the fermentation of hemicellulose. It can be hypothesised that increases in phenolic acids would increase fermentation times by creating cross-links in the arabinoxylan chains.

- Further studies should be carried out with wheat AGP to determine if it has prebiotic effects *in vivo* and at what dosage, including a dietary intervention study on humans. Development of methods to upscale AGP extraction would be required as the current method yields only ~200mg/200g WF.
- Prior exposure of potential prebiotics including AGP to a simulated gastric/duodenal environment to obtain a more realistic idea of their prebiotic potential.

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