EFFECT OF EXTRACTION CONDITIONS AND OXIDATIVE CROSS-LINKING ON THE CHEMICAL STRUCTURE AND MICROBIAL FERMENTABILITY OF ARABINOXYLANS

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Dedication

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List of Abbreviations

AACC	**A**merican **A**ssociation of **C**ereal **C**hemists
AI	**A**dequate **I**ntake
AOAC	**A**ssociation of **O**fficial **A**nalytical **C**hemists
AX	**A**rabino**x**ylans
BM	**B**all **M**illed
BS	**B**uffer **S**alts
DFA	**D**i-**F**erulic **A**cid
DRI	**D**ietary **R**eference **I**ntake
EDTA	**E**thylene**d**iaminete**t**raacetic **A**cid
FA	**F**erulic **A**cid
GC-FID	**G**as **C**hromatography **F**lame **I**onization **D**etector
IOM	**I**nstitute **o**f **M**edicine
LDL	**L**ow **D**ensity **L**ipoproteins
M	**M**olarity
MeOH	**M**ethanol
N	**N**ormal
PTFE	**P**oly**t**etra**f**luoro**e**thylene
SCFA	**S**hort **C**hain **F**atty **A**cid
TFA	**T**rifluoroacetic **A**cid
UA	**U**ronic **A**cid
UL	**U**pper **L**imit
X	**C**ross-**L**inked

Chapter 1. Introduction

Dietary fiber, which consists of non-starch polysaccharides such as arabinoxylans (AX), cellulose, and other undigestable components intrinsic to plants such as lignin, is a very popular topic in today's food industry and in the health and nutrition sector. The consumption of dietary fiber has been linked to health benefits such as, but not limited to: lowering the risk of hypertension, coronary heart disease, diabetes, obesity, and gastrointestinal diseases (diverticulitis, constipation, hemorrhoids, and gastroesophageal reflux disease). The dominant constituents of cereal dietary fiber are AX and cellulose. The chemical structure of AX determines the overall physiological effects they impart on the human body. AX can be substituted with ferulic acid (FA) residues covalently linked through ester bonds to the O-5 position of the arabinose residue. Cross-linking AX (X AX) through coupling these FA residues forming ferulate dimers or higher oligomers allows for increased molecular weight and viscosity. Studies have demonstrated that fibers that stay viscous in the gastrointestinal tract can likely provide health benefits such as lowering cholesterol and postprandial blood glucose levels. Besides having an impact on viscosity, cross-linking may also affect AX fermentation in the large intestine. However, there are limited studies on comparing the fermentation of non cross-linked vs. cross-linked AX, extracted under different conditions, by human gut microorganisms. *In vitro* fermentation of cross-linked and non-cross linked AX can provide valuable information on how AX cross-linking through ferulates affect the extent of fermentation and the profile of fermentation products. Extraction and cross-linking of AX under different conditions and *in vitro* fermentation of these dietary fiber constituents is described in this thesis.

Chapter 2. Literature review and study objectives

2.1. Dietary fiber

Dietary fiber is among the most popular food constituents for health promotion and disease prevention. However, there is much controversy amongst food scientists, nutritionists, and medical experts on the exact definition of 'dietary fiber.'

2.1.1. Definition of dietary fiber

The definition of 'dietary fiber' has been debated for years. Hipsley first coined the term 'dietary fiber' in 1953 as non-digestible constituents of foods (Hipsley 1953). Later, in 1972, Trowell adjusted the term and suggested a definition for dietary fiber including potential physiological benefits to consumption of dietary fiber (Trowell 1972). Since Trowell's adjustment there have been multiple debates on the 'dietary fiber' definition mainly covering three key areas: components of plant materials that are in fact considered dietary fiber, analytical methods used to analyze dietary fiber contents, and the physiological benefits that 'dietary fiber' imparts on an individual.

Currently, there are four widely discussed definitions of 'dietary fiber,' with the Codex Alimentarius definition becoming legally binding in the future.

The Association of Official Analytical Chemists (AOAC) came to a consensus on their definition of 'dietary fiber' in 1981 looking more at analytical aspects of dietary fiber. AOAC defined dietary fiber as, "consists of the remnants of edible plant cells, polysaccharides, lignin, and associated substances resistant to digestion by alimentary enzymes of humans," and developed methodologies to analyze dietary fiber according to said definition (Jones et al. 2006). They aimed to develop methods of analysis to simulate the human digestive system and enable all laboratories to be able to analyze foods for dietary fiber components (Prosky and DeVries 1992). The AOAC currently has various methods that are used for capturing the components described by the working definition as 'dietary fiber'. These methods include among others: total dietary fiber "Official

Method of Analysis 985.29", insoluble dietary fiber "Official Method 991.42", and soluble dietary fiber "Official Method 993.19 (Jones and al. 2006)." AOAC methods for dietary fiber analysis have been adopted and used by many countries including the United States.

The American Association of Cereal Chemists (AACC) also came to a consensus on their definition of 'dietary fiber'which describes dietary fiber constituents as well as physiological benefits associated with the consumption of fiber. According to AACC, "dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation (Jones 2000)." This definition builds off the AOAC definition while incorporating physiological benefits. In addition, the AACC built their definition in a way that allows for future research and discoveries to add in very easily.

The third discussed definition of dietary fiber comes from the Institute of Medicine (IOM) during the development of new dietary reference intakes (DRI) for fiber. The IOM wanted isolated fiber components to be considered as different entities with their own proven physiological benefits and set their definition as following (Medicine 2005):

- Dietary fiber consists of non-digestible carbohydrates and lignin that are intrinsic and intact in plants.
- Functional fiber consists of isolated, non-digestible carbohydrates that have beneficial physiological effects in humans.
- Total fiber is the sum of dietary fiber and functional fiber.

The IOM's definition divides fiber into two categories. The first category is dietary fiber and defined as "carbohydrates and lignin intrinsic and intact in plants but not digested or absorbed in the small intestine," (Medicine 2005). The other category is functional fiber and defined as "isolated, non-digestible, carbohydrates possessing beneficial

physiological benefits in humans" (Medicine 2005). IOM's rationale for creating two categories of dietary fiber is due to the potential that intact dietary fiber may possess different benefits than artificial or isolated functional fiber components. IOM's definition allows for the incorporation of fibers into foods with the ability to declare either functional fiber or dietary fiber as long as the physiological benefits have been established (Jones 2000).

The Codex Alimentarius Commission defines dietary fiber as, "carbohydrate polymers with ten or more monomeric units[b], which are not hydrolyzed by the endogenous enzymes in the small intestine of humans and belong to the following categories:

- Edible carbohydrate polymers naturally occurring in the food as consumed,
- 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,
- 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.

The footnotes in the Codex Alimentarious Commission definition were modified in 2010 and read as follows:

- a. When derived from a plant origin, dietary fiber 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 fiber. However, such compounds are not included in the definition of dietary fiber if extracted and re-introduced into a food.
- b. Decision on whether to include carbohydrates of 3 to 9 monomeric units should be left up to national authorities.

Also, according to the Codex definition dietary fiber generally exhibits one or more of the following properties:

- Decrease intestinal transit time and increase stools bulk
- Fermentable by colonic microflora
- Reduce blood total and/or low density lipoproteins (LDL) cholesterol levels
- Reduce post-prandial blood glucose and/or insulin levels.

While different organizations still use slightly different definitions for dietary fiber overall consensus on the broad based definition of dietary fiber still stands universally as, "plant or analogous carbohydrates that are resistant to digestion and absorption in the small intestine with complete or partial fermentation in the large intestine."

The specific physiological effects described for dietary fiber have led to discussions and studies on the required amount of dietary fiber to consume in order to potentially see beneficial health effects.

2.1.2 Dietary fiber recommendations

A table extracted from "Dietary Reference Intakes: Macronutrients" shows the DRI Committee's stance on dietary fiber (**Table 22, Appendix C**). Generally speaking, the AI's for dietary fiber are ~14 grams of dietary fiber per 1000 kcal, which is equivalent to 25 g/day for women and 38 g/day for men, with the exception of the elderly. An overall recommendation for children over the age of 2 is to consume 19-25 g/day of dietary fiber. However, few Americans meet the above recommendations (Medicine 2005). In fact, the average fiber intake for the United States is at 13 g/day for women and 18 g/day for men, which is roughly half of the recommended values (Variyam 2003). Individuals can meet half of their daily requirement of dietary fiber by regular consumption of whole grain cereals due to the dietary fiber found in the cell walls of cereal grains (Ayano, Sanada et al. 1993).

Currently, there is no tolerable upper limit (UL) set for dietary fiber, as the DRI committee believed excess consumption of fiber was unlikely. However, recently dietary fiber has become quite trendy and can be found in many different food products, whether

inherent or added, causing some to speculate whether ULs should be set for dietary fiber consumption.

Without the physiological effects of dietary fiber there would be limited interest of researchers, consumers, regulators, and manufacturers in fiber. For example, the recommended DRI's for dietary fiber are set in place in order for individuals to potentially benefit from their physiological effects.

2.1.3. Physiological effects of dietary fiber

Dietary fiber has been linked to improving health and preventing diseases through mechanisms such as reducing glycemic index, cholesterol absorption (Marlett 1997), and improving intestinal health due to fermentation activity by gut microbiota (Burton-Freeman 2000). Fibers isolated from different sources have not been shown to induce all the positive effects but have been found to be responsible for one or more of them.

2.1.3.1. Glycemic index

Diabetes, particularly type II diabetes, is an area of increasing concern due to the large number of current cases (>300 million worldwide) and the expected steep increase of cases over the next few decades. Research has indicated increased dietary fiber consumption decreases the risk of diabetes. Determining the blood glucose levels for several hours after ingestion gives good indication of how dietary fiber and high fiber foods may delay glucose absorption and thus blunt postprandial blood glucose levels. One of the most common ways to analyze this effect is to determine the glycemic index, which measures the area under the blood glucose curve (concentration vs. time) following ingestion of food and comparing it to the area under the blood glucose curve following ingestion of a standard dose of glucose or white bread (Jenkins, Wolever, et al. 1981). Many fibers and fiber components, including cross-linked AX, have been shown to reduce the glycemic index and flatten glucose response curves (Vogel et al. 2012). Especially viscous dietary fiber components help reduce the rate of digestion and

absorption of carbohydrates and could be part of a low glycemic index diet, therefore potentially decreasing the incidence of diabetes.

2.1.3.2. Cholesterol absorption

Coronary artery disease is a major cause of death in the United States with cholesterol being a major risk factor (Rosner, Polk 1983). Reductions in total and LDL cholesterol through dietary and pharmacologic actions help decrease the risk of coronary problems. Some controlled clinical trials indicate high soluble fiber diets decrease total and LDL cholesterol (Brown, Rosner, et al. 1999). The exact mechanisms and also which type of fiber lowers blood cholesterol are still undefined. It was shown that some fibers, especially viscous fibers, bind bile acids or cholesterol during the intraluminal formation of micelles (Anderson, Tietyen-Clark 1986). Bile acids, which are not recycled by the enterohepatic circulation but which are excreted with the feces, need to be synthesized de novo in the human body, starting from cholesterol. Other proposed mechanisms included inhibition of hepatic fatty acid synthesis by products of fermentation (Nishina, Freedland 1990); fibers with high viscosity causing slowed absorption of macronutrients (Schneeman, 1987); and increased satiety leading to overall decreased energy intake (Blundell, Lawton, et al. 1987). The formulation of fiber rich foods result in diets that are lower in saturated, *trans*-unsaturated fats, and cholesterol and higher in protective nutrients such as unsaturated fatty acids, minerals, folate, and antioxidants (Brown, Rosner, et al. 1999).

2.1.3.3. Colonic fermentation and microbiota

Dietary fiber fermentation by the gut microbiota produces gas and short chain fatty acids (SCFAs) improving the potential for the growth of specific bacteria that enhance the intestinal health of individuals. SCFAs are organic fatty acids with 1 to 6 carbons and are the principal acids which arise from bacterial fermentation of polysaccharides, oligosaccharides, proteins, peptides, and glycoproteins in the colon (Wong, de Souza et al. 2006). Fermentation of fiber to produce SCFAs provides energy to the host, 1.5-2.5 kcal/g (Livesey 1990). SCFA production is dependent on the environment of the gut,

species and amount of microflora present in an individual, as well as substrate source and transit time through the gastrointestinal tract. Multiple SCFAs and other acids are produced through fermentation including lactate, isobutyrate, valerate, and isovalerate, but the three most abundant and important SCFAs produced are acetate, propionate, and butyrate with a ratio of roughly 60:25:10 (acetate: propionate: butyrate), depending on the fiber source and other factors (Cummings, Pomare et al. 1987). The availability of SCFAs through the entire colon, not just the proximal colon, is most sought after due to the fact that at least 50 to 60% of large intestinal cancer occurs in the distal colon (Cummings 1997). Production of SCFA in the distal colon could be useful in the prevention of distal inflammatory bowel disease or colon cancer where fermentation metabolites, specifically butyrate, have been indicated to combat inflamed or proliferated colonic mucosal epithelium (Yu, Waby et al. 2010). Therefore, fibers with slow, extended fermentation patterns are most desirable due to the low initial gas production, SCFA production throughout the entire colon, and beneficial microbiota changes that occur throughout the colon.

SCFAs act as nutrients for the colonic epithelium, modulate many variables/functions associated with ion transport, regulate proliferation, differentiation, and gene expression, influence composition of colon microflora, decrease solubility of bile acids, and increase mineral absorption (indirectly) (Cook and Sellin 1998). The three dominant SCFAs (acetate, propionate, and butyrate) are all absorbed in the body at similar rates but act in different regions of the body yielding different potential benefits. The two major regions of the body where these SCFAs act include: (1) ceco-colonic epithelium cells which use butyrate as their main energy substrate; (2) liver cells which utilize residual butyrate and especially propionate for gluconeogenesis with 50-70% acetate being utilized by the liver (Robberfroid 2005). Specific information on the three dominant SCFAs is given below.

Acetate: Acetate is usually the predominant SCFA formed in the colon. It is easily absorbed by the body and transported directly to the liver. Research on acetate is largely focused on cholesterol synthesis as acetate-CoA is the primary substrate for this.

Propionate: There are two main pathways that yield propionate: (1) formation of succinate from the fixation of CO_2 ("Dicarboxylic acid pathway"); (2) from lactate and acrylate ("acrylate pathway") (Cummings 1981). Most of the current knowledge about propionate is from ruminant studies whereas human metabolism is less understood meaning propionate may have other effects within the human body.

Butyrate: Butyrate plays a pivotal role in regulation of cell proliferation and differentiation as well as fuel for colonic epithelial cells (Wong, de Souza et al. 2006). Butyrate is considered the most important SCFA for colonocyte metabolism as 70% to 90% is metabolized by the colonocytes (Cook and Sellin 1998). Butyrate has been studied in many ways, all showing that it is a preferred substrate for energy and that it, depending on the experimental conditions, helps promote cell proliferation (Wong, de Souza et al. 2006).

In addition to the production of SCFAs via fermentation of dietary fiber there is also production of gas.

Gas production: The three main gases of interest are carbon dioxide, hydrogen, and methane. Carbon dioxide is the gas of least interest as it is commonly found in breath. Hydrogen and methane are produced exclusively through bacterial fermentation of different fibers in the body (Cloarec, Bornet et al. 1990). *In vitro* studies showed that different fermentable substrates produce different levels of gas. The fermentation of corn bran AX produces significantly less gas if compared to inulin and β-glucans suggesting the potential benefits of low flatulence, bloating, and abdominal pain (Kaur, Rose et al. 2011).

Hydrogen: Bacteria of the genus *Clostridium* are mostly responsible for the production of hydrogen gas (Chinda, Nakaji et al. 2004). It has been speculated that ~27% of individuals do not produce hydrogen while the remainder produce it as a result of the consumption of dietary fibers (Cloarec, Bornet et al. 1990).

Methane: *Methanobrevibacter smithii* and other methanogenic bacteria are responsible for the production of methane through usage of hydrogen and carbon dioxide (Gibson and Wang 1994). Studies have found that only 30-38% of Western populations expel air that contains detectable levels of methane gas due to individuals having different gut microbiotas (Miller and Wolin 1985). African populations, 80-91%, have been shown to produce methane, which may indicate methane as a preventative factor for colon health, along with other important factors, due to the fact that this population has an extremely low rate of colon cancer (Segal, Walker et al. 1988).

Gas tolerance: High levels of gas in the body can cause discomfort in individuals through flatulence, abdominal pain, and bloating. High fiber diets affect individuals differently and are dependent not only on the individual but also the amount and type of fiber consumed.

Different studies have shown that abdominal discomfort is not correlated with volume of gas expelled nor infusion rate of gas and that the specific site of gas infusion has an effect on intensity of abdominal pain through gas infusion experiments (Serra, Azpiroz et al. 1998; Serra, Azpiroz et al. 2001). A typical individual retains 100 to 200 mL of gas in the intestinal tract (Levitt 1971). Individuals with 400 mL of gas complained of higher discomfort which could mean that retention of gas in individuals could be a good indicator of discomfort, with physical activity increasing gas transit and lowering overall discomfort (Serra, Azpiroz et al. 1998). When looking at the site of gas infusion the small intestine can tolerate lower levels of gas compared to the colon (Harder, Serra et al. 2003). Overall, the culprit for abdominal discomfort seems to be linked with the ability of individuals to pass gas not necessarily the volume of formed gas, which is promising news for dietary fiber consumption.

The formation of SCFAs and the production of gas are often investigated in *in vitro* fermentation studies. Due to the dynamic nature of the human body it is hard to study the effects of fiber within humans' digestive systems and colonic studies are invasive and expensive.

In-vitro fermentation: *In-vitro* fermentation with human colonic microbiota is a noninvasive, time efficient means to estimate fiber fermentability (Stewart, Savarino et al. 2009). *In vitro* fermentations do not perfectly model the *in vivo* situation but it has been proven through multiple studies that batch fermentation degrades non-starch polysaccharides comparably to that of the human colon. The method chosen for fermentation is based on the study design as well as available resources.

In vitro fermentations are classified as either static or dynamic. Static involves placing the fermentable samples into a vessel with inoculum and sealing the vessel for a designated period of time. Dynamic methods, for example, take four separate glass vessels with flexible tubing, and a temperature of 37°C is maintained by passing water through the layers and creating peristaltic waves (Minekus, Smeets-Peters et al. 1999). The dynamic system uses nitrogen to create an anaerobic environment and computer additions of alkali to maintain a pH of 5.8. The system is colonized by microflora at a level of 10^{10} CFU/mL (colony forming unit). Dynamic state allows for dialysis where the SCFA can diffuse preventing accumulation of SCFAs, which can alter the microbial microflora. The dialysis portion of dynamic methods is a key advantage over the static method and gives more accurate results overall.

Fassler and co-workers did a study comparing dynamic and static model systems. They found that dynamic models have less total SCFA production for resistant starch 2 and resistant starch 3 while static models show similar SCFA production between the two (Fassler, Arrigoni et al. 2006). In addition, SCFA ratios formed by fermentation of resistant starch 3 were similar between static (31:48:20) and dynamic (35:49:16) methods, but the SCFA ratios formed by fermentation of resistant starch 2 was different between the two. Previous studies have shown that dynamic models are closely aligned with the *in vivo* situation but the overall system is more complicated, not consistent, and difficult to compare samples between different runs or experiments (Fassler, Arrigoni, et al. 2006; Barry, Hoebler, et al. 1995; Minekus, Smeets-Peters, et al. 1999).

Another feature of fermentation studies to consider is the vessel chosen to execute the study. Hall and co-workers, studied on three different types of vessels used to ferment substrates in *in-vitro* fermentations. The three vessels chosen included a 125 mL Erlenmeyer flask with continuous gas infusion, a serum vial with or without gas release, and a 50 mL polyethylene centrifuge tube (Hall and Mertens 2008). Results showed that sealed vessels showed no differences, but vessels with gas release or continuous gas infusion had lower fermentability (Hall and Mertens 2008). This showed that vessels with gas parameters had an influence on the fermentation study and end products formed (Hall and Mertens 2008). Concluding remarks of the research stated that vessel type and gas dynamics needed to be highlighted when comparing results from *in vitro* fermentation studies. Another variable to consider in fermentation studies is the effect shaking has on the vessels with substrate and inoculum. Stevenson and co-workers, did a study on pectin, psyllium, and corn starch which showed no differences attributed to shaking of vessels while substrate concentrations were held constant (Stevenson, Buchanana et al. 1997).

Inoculum used for *in vitro* fermentations is also a much debated topic as microflora differs greatly between individuals (McOrist, Abell et al. 2008). There are different strategies that can be employed to help control the *in vitro* fermentation inoculum. Subjects are excluded if they have used antibiotics 3 months prior to the study because antibiotics can significantly affect the intestinal microflora of individuals. Subjects are questioned about their eating patterns and excluded if they follow restrictive or specialty diets as well as if they have gastrointestinal diseases or other abnormalities that exclude them from the 'healthy population.' The last attempt to reduce variability in the study is to pool fecal inoculum from three different subjects and use this 'pooled' inoculum for the fermentation study to help eliminate individual influence. Multiple donors, at least three, should be used to improve the accuracy of the *in vitro* estimates of colonic production (Mc Burney and Thompson 1989). Variation attributable to donors decreased with increasing incubation time (up to 24 h) showing that a 24 h incubation time seems to be most appropriate for comparing the fermentation variables (Mc Burney and Thompson 1989).

There are many variables, including dynamic or batch models, vessel type, type of substrate, and inoculum, that affect the fermentation profiles of substrates run through *in vitro* fermentations. Most researchers use static *in vitro* fermentation set-ups as they use fewer resources than dynamic *in vitro* fermentation set-ups. Despite the *in vitro* set-up chosen, all methodologies still look at similar components of interest including, but not limited to: SCFAs, gas production, pH, phenolic metabolites, and microbial organisms.

The fermentability of various dietary fiber components and thus the formation of fermentation products such as SCFAs and phenolic metabolites strongly depend on the structures of the fiber components, which are more closely described in the following section.

2.1.4. Dietary fiber composition

Dietary fiber polysaccharides and oligosaccharides originate in plant cell walls or as storage carbohydrates so the amount and type of dietary fiber available is dependent on factors including type of plant, plant origin, and developmental stage of the plant. Plant cell walls are composed of polysaccharides including cellulose, hemicelluloses, and pectin's. Structural proteins and other non-carbohydrate polymers such as lignin and suberin, depend on factors mentioned above. In the following, the hemicelluloses especially the AX that dominate the hemicellulose fraction in monocotyledonous plants are described in more detail.

2.1.4.1. Arabinoxylans

AX are the dominant hemicelluloses of most cereal grains in particular wheat, rye, and corn. AX have not only been found in all major cereal grains (wheat, barley, oats, rye, rice, sorghum, maize, and millet), but also in psyllium, pangola grass, bamboo shoots, rye grass, flaxseed cake, banana peels, etc. The contents of total AX (%), water soluble AX (%), and arabinose to xylose ratios (A:X) are dependent on genus, species, as well as genetic and environmental factors (**Table 23, Appendix C**). AX were first identified in wheat flour in 1927 (Hoffman and Gorter 1927) and have been of interest to cereal

chemists ever since due to their technological importance and their potential physiological effects. AX offer, depending on their chemical structure, nutritional benefits of both soluble and insoluble fiber. Additionally, due to cereal AX phenolic moieties within their structure they also exhibit antioxidant properties (Katapodis, Vardakou et al. 2003).

Structures of AX differ dependent on sources. For example, AX from sorghum, millet, rice, and maize bran are more complex than those of other cereal grains due to the side branches containing, additionally to arabinose, xylose, galactose, α-D-glucuronic acid, and 4-O-methyl-α-D-glucuronic acid (Shibuya 1984). The AX backbone is a linear chair composed of (1,4)-β-D-xylopyranosyl (Xyl*p*) units. The side chains of AX are mostly composed of α-L-arabinofuranosyl residues (Ara*f*) attached to the linear backbone at the O-2, O-3, or both O-2,3 positions (**Figure 1**) (Izydorczyk and Dexter 2008). Most arabinofuranosyl units are monomeric but studies have shown oligomeric side chains of two or more arabinosyl residues linked via 1,2-, 1,3-, and 1,5- linkages (Izydorczyk and Biliaderis 1995).

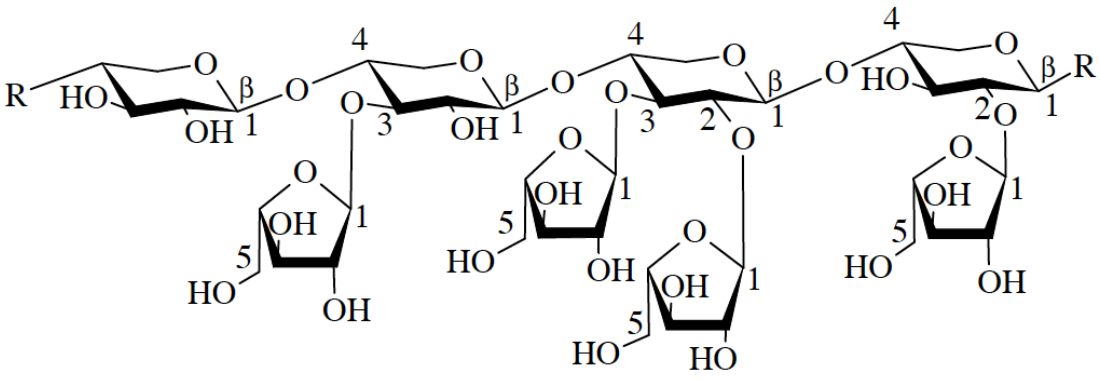

Figure 1. Molecular structure of arabinoxylans and potential side chains

The variable degree of branching results in different A:X ratios for AX from different sources. The ratio of A:X typically ranges from 0.3 to 1.1, with a few exceptions(Izydorczyk and Biliaderis 1995). The A:X ratio gives insight into the degree of branching but not the structure. Xylose units can be unsubstituted, monosubstituted,

and doubly substituted and it is also of interest whether side chains occur clustered or random. AX are mainly heterogeneous with a variety of different patterns and levels of substitution. However, sometimes AX can be found showing nonrandom distribution of Ara*f* units on the backbone of AX presenting more of a homogenous structure (Gruppen, Kormelink et al. 1993).

Biosynthesis: The biosynthesis of AX is very complex and dependent on many intracellular activities making AX usually heterogeneous and polydispersed. Research on AX biosynthesis is ongoing meaning there are constant additions and changes to the predicted synthesis mechanisms. The biosynthesis of AX occurs in the cell either in the golgi apparatus or the endoplasmic reticulum(Delmer and Stone 1988). AX are secondary gene products due to the fact that they are produced from synthases and glycosyl transferases (Bacic, Harris et al. 1988). AX polymerization has three main steps: chain initiation, elongation, and termination. UDP-D-Xyl*p* and UDP-L-Ara*f* donate monosaccharide's to produce AX (Fry 2000). First, the sugar is donated to a protein or lipid primer and the chain elongates by adding onto the non-reducing end. Xylose residues are continually added to the non-reducing end through β-(1,4)-linkages, growing the polymer chain. Research by Brett and Waldron supported the idea of simultaneous inclusion of arabinose onto the xylan backbone making the branching points of AX (Brett and Waldron 1996). Feruloyl groups are attached to AX through transfer of acyl groups concurrent with the polysaccharide polymerization process (Fry, Willis et al. 2000). The last step of AX polymerization is chain termination which is the least researched. There are speculations of how chain termination occurs but nothing directly supports them.

Physiochemical properties: Conformation of AX is most heavily influenced by the one hydrogen bond that holds the adjacent xylose residues together as this bond allows for intramolecular hydrogen bonding to occur, which can twist into threefold strands that are flexible in nature (Atkins 1992). The amount and distribution of arabinose units on the xylan backbone determine the solubility and interaction with other polysaccharides (Biliaderis and Izydorczyk 2007). The unsubstituted β-(1,4)-linked xylan backbone allows the structure to form intermolecular hydrogen bonds which reduces AX solubility.

If the xylan backbone is substituted hydrogen bonding is reduced making the AX partially soluble in water (Atkins 1992).

AX conformation and structure allow them to form viscosity in aqueous solutions. The viscosity of AX solutions is largely dependent on concentration, molecular size, and shear rate of when measurements are taken (Biliaderis and Izydorczyk 2007). AX aqueous solutions can form macrostructures due to chain aggregation and entanglements resulting in gel like behaviors for some solutions (Warrand 2005). Viscosity and solution characteristics of AX are important properties of the AX contributing to their physiological effects. The arabinose side chains of AX can be further substituted with hydroxycinnamic acids.

2.1.4.2. Hydroxycinnamic acids

Hydroxycinnamic acids are phenolic compounds formed in the phenylpropanoid pathway of plants. AX from cereal grains contain specific hydroxycinnamic acids (mostly FA but also *p*-coumaric acid) within their structure via esterification to the O-5 position of Ara*f* (Smith and Hartley 1983).

Ferulic acid: FA (**Figure 2**) is found in the cell walls of different plant species including graminaceous plants such as rice, wheat, oats, and corn (Jung and Himmelsbach 1989). FA concentrations are dependent on the grain source as well as the grain layer. Dervilly-Pinel and co-workers compared FA substitution of AX from the endosperm of wheat, barley, rye, and triticale reporting a range of 18 to 60 FA residues per 10,000 xylose units (Dervilly-Pinel, Thibault et al. 2001). AX in the outer layers of the grain (aleurone and pericarp) are more heavily substituted with FA than endosperm AX.

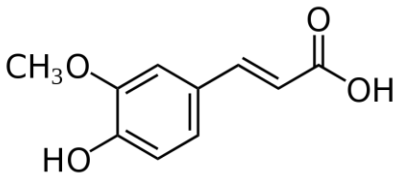

Figure 2. Chemical structure of ferulic acid

In the phenylpropanoid pathway FA is predominantly formed by the action of the O-methyltransferase converting caffeoyl-CoA into feruloyl-CoA (Pacheco-Palencia, Mertens-Talcott, et al. 2008). Then, FA is incorporated into the cell wall by acylating the O-5 position of the AX (Ralph, Helm et al. 1992). However, most likely 1-O-feruloyl-glucose is transferred to the AX and not the free acid.

Hydroxycinnamic acids in cereal grains are important due to their antioxidant properties (Krupnikova, Dranik, et al., 1971); more important is their ability to cross-link AX, increasing rigidity of plant cell walls, as well as increasing the viscosity of AX as dietary fiber constituents.

2.1.4.3. Hydroxycinnamic acid oligomers

FA derivatives can serve as cross-links between polysaccharides or between polysaccharides and lignin altering the physiochemical properties of the cell wall. Dimerization of ferulate-polysaccharide esters through free radical coupling or through a photochemical mechanism forming dehydrodiferulates or cyclobutane dimers, respectively, greatly affects the physiochemical properties of AX . Radical dimerization occurs via electron-delocalized phenoxy radicals with resonance structures having the single electron position in 4-O, C-5, or C-8 of ferulic acid. Therefore, at least five diferulate regioisomers coupled via 8-5-, 8-O-4-, 5-5-, 8-8-, and 4-O-5-linkages are possible(Biliaderis and Izydorczyk 2007). Isolation and identification of diferuloylated saccharide fragments showed that polysaccharide cross-linking via dehydrodiferulates occurs in the plants. For example, the isolation of 5-5-diferuloyl saccharides (Saulnier, Crepeau et al. 1999) and diarabinosyl esters of 8-O-4-dehydrodiferulate (Allerdings,

Ralph et al. 2005) demonstrated that AX can be cross-linked via these diferulic acid (DFA) regioisomers. Isolation and identification of FA trimers, e.g. 5-5/8-O-4, 8-O-4/8-O-4, and 8-8/8-O-4 triferulic acids, and FA tetramers showed that higher FA oligomers also play a role in cross-linking cell wall polysaccharides (Funk, Ralph, et al. 2004). The degree of AX cross-linking can be roughly determined by analyzing DFA to xylose ratios (Bunzel, Ralph et al. 2001).

Treatment of AX solutions with free radical generating agents, such as laccase, can cause the formation of hydrogels (Carvajal-Millan, Landillon et al. 2005). Hydrogel formation is due to covalent cross-linking via FA oligomers and subsequent formation of a three dimensional AX network incorporating water. Initial FA contents of the AX but also the molecular weight and the structure of the AX are important factors determining gel setting and gel hardness (Izydorczyk and Biliaderis 1995).

2.1.5. Interest of dietary fiber to industry

Dietary fiber received increased attention after the release of the 2005 Dietary Guidelines for Americans and MyPyramid, which recommend individuals to consume more fiber-rich foods, such as whole grains, fruits, and vegetables. The U.S. food fiber industry earned revenue of $495.2 million in 2011 and is estimated to keep increasing (Frost, Sullivan 2011). As interest in dietary fiber continues there will be economic incentives for development and marketing of more fiber ingredients and fiber products.

AX function as both technologically and nutritionally important ingredients to the food industry. Due to the nutritional benefits associated with AX and dietary fiber there is a large push to incorporate them into finished goods for consumers.

2.1.5.1. Effects of arabinoylans on breadmaking

The addition of AX as functional ingredients to breads has been very contradictive, mainly due to the different properties of added AX. AX solubility greatly influences its overall effect on the end product due to the interaction of the AX with other components

in the bread matrix. For example, if water soluble AX are added they compete with other flour constituents for water and as a result dough consistency is increased. Thus, addition of water soluble AX to breads has shown favorable benefits such as increased dough consistency, viscosity, dough structure, higher loaf volume, and improved crumb structure, once conditions are optimized (Biliaderis, Izydorczyk et al. 1995).

Counterparts, water insoluble AX typically show negative effects on breadmaking unless used with specific endoxylanases which can reverse actions (Courtin and Declour 2002). The negative effects of water insoluble AX in bread making are lower extensibility, lower loaf volume, and coarser crumbs. In addition, AX potentially play a role in bread staling through interactions with amylopectin crystallization in gels along with the final water content (Gudmudson, Eliasson et al. 1991).

2.2 Study objectives

Objectives of this research

The aim of this thesis project was to obtain corn bran AX of varying structure by using different extraction conditions, oxidatively cross-link them, and determine the microbial fermentability of both the non-cross-linked and cross-linked samples.

To reach this goal the following objectives were defined:

- Determine the influence of NaOH concentration used for AX extraction on structural characteristics of extracted AX.
- Determine the influence of a post extraction procedure, ball milling, on the structural characteristics of extracted AX.
- Cross-link differently extracted AX by using laccase and oxygen.
- Determine the effects of ferulate cross-linking on fermentability in an *in vitro* fermentation experiment and analyze fermentation end products (pH, gas volume, and SCFAs).
- Determine the release/formation of phenolic metabolites by gut microorganisms as well as the percentage of original FA being metabolized by gut microbiota.

Chapter 3. Materials and methods

3.1 Materials and methods

3.1.1. Chemicals, solvents, and instruments

A list of instruments as well as chemicals used can be found in **Appendices A and B.** If not otherwise specified, chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), Fischer Scientific (Pittsburgh, PA, USA), Becton, Dickinson, & Company (Sparks, MD, USA), Cambrex BioScience Walkersville, Inc. (Walkersville, MD, USA), Ricca Chemical Company (Arlington, TX, USA), or Oxyrase Incorporated (Mansfield, Ohio, USA). All chemicals were reagent grade or better.

3.1.2 Preparation and isolation of arabinoxylans from corn bran

AX were isolated from corn bran because it has high AX contents and also contains the highest FA concentrations among cereals. Corn bran was obtained for this study from Cargill, Inc. (Indianapolis, IN, USA). Bran was milled to a size of <0.5 mm and defatted for 2 h using acetone (bran/acetone ratio, 20 g/75 mL) at 60 °C. The procedure was repeated twice and acetone was discarded after each extraction. Defatted bran was dried overnight in a hood and then placed in a vacuum oven for 3 days at 40 °C.

Sodium phosphate buffer (0.08 M, pH6) was prepared by adding a, monosodium phosphate solution (8.00 g/L) at 88.9% to a disodium phosphate solution (11.87 g/L) at 11.1%. Defatted bran (500 g) was suspended in the buffer (2.6 L) and α-amylase (Termamyl®, 15 mL) was added. The suspension was heated for 1 h in a boiling water

bath with constant shaking. Destarched corn bran was collected by filtration (filter paper, pore size 20-25 µm) and washed with hot water (75 °C, 40 mL), 95% (v/v) ethanol (40 mL), and acetone (40 mL) to remove solubilized starch mono-/oligosaccharides as well as lipophilic compounds and to remove water. Destarched bran was dried in a hood overnight and then in a vacuum oven for 3 days at 40 °C.

Destarched bran (150 g) was suspended in aqueous NaOH solutions of varying concentrations (3 L for each variable; 0.1 M, 0.5 M, and 1.0 M) and stirred for 4 h. The pH of the solution was adjusted to 4.0 with 6 M HCl and the residue was removed by filtration (filter paper, pore size 20-25 µm). Solubilized arabinoxylans (1 L) were precipitated by adding ethanol (1680 mL, final concentration 65% (v/v)). Precipitated AX were separated by filtration (filter paper, pores size 20-25 µm) and washed with 80% (v/v) ethanol (400 mL) and acetone (400 mL). AX (and various co-extracted compounds) were suspended in acetone and treated 12 min in an ultrasonic bath, filtered, and dried in a hood (overnight) and a vacuum oven (4 days at 40 °C). AX samples were placed in screw cap glass jars and placed in a desiccator at room temperature.

3.1.3 Post extraction treatments

Half of the AX, extracted by using 1.0 M NaOH, were pulverized using a ball mill. AX were placed into a grinding jar with stainless steel balls. The planetary ball mill is a cylindrical device that rotates around a horizontal axis and uses stainless steel balls to pulverize the material in the container to a fine powder by impact forces. Ball milling (BM) does not only reduce the particle size of lumped AX but can also break covalent

linkages thus potentially reducing the molecular weight of the AX. Once pulverized the BM AX were placed into a sealed jar and stored in a desiccator at room temperature.

3.1.4 Preparation of arabinoxylan gels through oxidative cross-linking

Cross-linked AX samples were prepared by dissolving the dried AX (5 g of each NaOH variable of interest (0.1 M, 0.5 M, and 1.0 M)) in a citric acid/phosphate buffer (0.05 M, pH 5.5, 125 mL). The beaker was placed in a heated water bath (40° C) and, after the temperature of the buffer was adjusted, laccase (from *Trametes versicolor*; Fluka, Steinheim, Germany) (12.5 mL, 0.04 U/µL citric acid/phosphate buffer) was added. The gel was allowed to set for 2 h (unagitated). Gelled AX were freeze-dried for 5 days and pulverized by using mortar and pestle. The freeze-dried powder contained 5 g of AX (and co-extracted compounds) and 3.3 g buffer salts (BS). The amount of BS present in cross-linked AX was calculated and also determined by freeze-drying the exact amount of citric acid/phosphate buffer added to each batch followed by a gravimetric determination of the salts.

3.1.5 Chemical characterization of non-cross-linked and cross-linked arabinoxylans

The extracted non-cross-linked and cross-linked AX were analyzed for the following properties: moisture, ash, protein, total carbohydrate content, neutral sugar content and composition, uronic acid (UA) content, and hydroxycinnamic acids.

3.1.5.1 Moisture content analysis of non-cross-linked and cross-linked arabinoxylans

The Karl Fischer method (adapted from the Food Analysis Laboratory Manual, 2003) was used to determine the moisture contents of both non-cross-linked and cross-linked AX samples in triplicate.

AX samples, non cross-linked and cross-linked (30-50 mg), were weighed into Kimax® tubes with polytetrafluoroethylene (PTFE) caps. Methanol (MeOH, 25 mL) was added to the samples. Triplicate blanks of MeOH in extraction vials were also prepared. The vials were all capped immediately after addition of MeOH and additionally sealed with parafilm. Vials containing samples and blanks were shaken on a shaker at low speed (100 rpm) overnight (18 h) at room temperature. The vials were taken off the shaker and held at room temperature for at least 1 h before they were titrated by using an automated Karl-Fischer titration apparatus.

3.1.5.2. Total ash determination of non-cross-linked and cross-linked arabinoxylans

A number of dry ashing methods have been officially recognized for the determination of the ash content of various foods (AOAC Official Methods of Analysis). Total ash determination for AX samples was adapted from the AOAC Official Method 923.03 (AOAC Official Methods of Analysis, 1990).

Crucibles were heated over low flame for a few minutes and marking ink was applied to the bottom of the crucibles while still warm for identification purposes. Crucibles were left to dry. Crucibles were carefully submerged in aqua regia solution (3 parts concentrated HCl (375 mL), 1 part concentrated HNO_3 (125 mL), 4 parts deionized water

(500 mL)), and soaked for several hours (preferably overnight). Crucibles were removed from the aqua regia solution, rinsed with reverse osmosis water, and dried in an atmospheric oven at 100 °C for approximately 1 h. Dry crucibles were ignited in a muffle furnace to dull redness, cooled to room temperature, and placed in a desiccator until use.

Samples were analyzed in triplicate with two blank crucibles included. After weighing the crucibles 100 mg of AX samples were weighed into crucibles. The samples were carefully carbonized over a Bunsen burner. The crucibles were placed in a muffle furnace at 550 °C for at least 12 h or overnight. If the color of the samples did not turn to a light gray after 12 h a small volume of H_2O_2 (30 % v/v) was added to the cool crucibles and they were put back into the furnace for 1-2 h.

After ashing was completed, the crucibles were transferred to a desiccator in the same order they were placed in the muffle furnace. Once the crucibles and contents were cooled to room temperature they were removed from the desiccator and weighed.

3.1.5.3. Determination of total protein content of non-cross-linked and cross-linked arabinoxylans

The Dumas method, a combustion method, is an official method of AOAC International to determine the total protein content of samples (Fox and McSweeney, 2003; AOAC Official Methods of Analysis). Total protein determination for AX samples was adapted from the AOAC Official Method 990.03. In this study, the TruSpec® N instrument was used for total protein analysis.

"System check" as well as "Whole O_2" and "Whole He" leak checks were performed to ensure equipment was operating properly. System was purged with high purity ethylenediaminetetraacetic acid (EDTA) (~0.15 g) twice. Blank capsules were analyzed until readings stabilized and then readings logged in to calibrate the TruSpec® N. Drift corrects were executed with EDTA (~0.1 g) until readings stabilized, with standard deviation <0.03. Stable readings were used to properly set the standard curve. Gelatin Method 1 was chosen and total nitrogen determined by the TruSpec® N was multiplied by 6.38 to get total protein content. AX samples, non-cross-linked and cross-linked (30-40 mg), were weighed into gel capsules and backfilled with cellulose to reach total weight of 100 mg. Samples were analyzed in duplicate with gelatin capsules.

3.1.5.4. Determination of total carbohydrate content of non-cross-linked and cross-linked arabinoxylans using phenol-sulfuric acid assay

Total carbohydrate contents were determined using the phenol-sulfuric acid method (Dubois, Gilles et al. 1956).

A standard curve for this spectrophotometric assay was recorded using xylose because this monosaccharide is usually the most dominant AX constituent. Each standard solution was subjected to the procedure described below for the samples, in duplicate, reading the absorbance at 480 nm. Standard concentrations ranging from 10-210 μg/ml were tested. A typical standard curve is shown in (**Figure 3, Appendix D**).

AX samples, non-cross-linked and cross-linked (30-40 mg), were added to a test tube. For the blank 50 μL deionized H_2O was added to a test tube. Phenol reagent (5% w/v in

H_2O, 500 μL) was added to samples and vortexed immediately to ensure adequate mixing. Next, concentrated sulfuric acid (2.5 mL) was added rapidly to each tube using an automatic dispenser. Tubes were vortexed again, 2-4 times, and left at room temperature until cool. Absorbance was read at 480 nm. Analysis was done in duplicate.

3.1.5.5. Analysis of the neutral sugar composition of non-cross-linked and cross-linked arabinoxylans

The neutral carbohydrate composition of the AX samples was analyzed using a modified method published by Blakeney and co-workers (Blakeney, Harris et al. 1983).

Polysaccharide hydrolysis (acidic hydrolysis to determine hemicellulosic carbohydrates): AX samples (30 mg), non cross-linked and cross-linked, were weighed into medium (15 mL) Kimax® tubes with PTFE caps. Addition of one small glass bead to each Kimax tube ensured that all material was mixed well during careful vortexing following the addition of 3 mL of 2 M H_2SO_4. Capped tubes were heated for 60 min in a boiling water bath, while being carefully mixed at 20, 40, and 60 min time points. After 1, h samples were cooled down to room temperature. Ammonium hydroxide (25%, 1.2 mL) was added to reach an alkaline pH of 8-9. If necessary, additional ammonium hydroxide was added to adjust the pH. The solution was then filtered into a 10 mL volumetric flask. The tubes were rinsed with distilled water and the washes were used to bring the volume up to 10 mL.

Reduction and acetylation of monosaccharides, extraction of alditol acetates: An aliquot of the filtered solution (100 μL) was transferred to a 15 mL Kimax® tube and freshly prepared 2% sodium borohydride in DMSO (100 μL per sample) was added.

Capped tubes were placed in a 60 °C water bath for 60 min and shaken at constant speed. After 1 h, samples were cooled down to room temperature and 80% (v/v) acetic acid solution (100 µL) containing the internal standards inositol and erythritol (0.8 mg/mL) was added. Acetylation was performed by adding 2 mL acetic anhydride and 200 µL of the catalyst 1-methylimidazole. After 10 min at room temperature, the samples were cooled in an ice bath and 5 mL water was added. The acetylated alditol acetates were extracted into 2 mL chloroform. The chloroform phase was washed twice with water and the aqueous phases were discarded. Capped tubes were placed in an -80 °C freezer overnight to freeze-out residual water. The alditol acetates were analyzed using gas chromatography with flame ionization detection (GC-FID) on a DB-225 capillary column (30 m x 0.25 mm i.d., film thickness 0.15 µm). Column temperature was maintained at 180 °C for 5 min; increased to 186 °C (1 °C/min); increased to 210 °C (4 °C/min) and held for 8 min; increased to 220 °C (10 °C/min) and held for 2 min. Helium was used as the carrier gas (3 mL/min). Quantification was performed by using the internal standard method. Correction factors for the analyzed monosaccharides against inositol were determined by treating a standard mixture containing rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose as described before. A standard stock solution containing the analyzed monosaccharides was prepared and a 100 µL aliquot of the standard solution was added to a test tube and dried under a stream of nitrogen after re-dissolving in 100 µL of 0.1 M ammonia solution. The standard was run through the protocol as described above (reduction, acetylation, and extraction of alditol acetates). The correction factors used for quantification purposes are displayed in (**Table 25, Appendix D)**. A standard chromatogram is shown in (**Figure 8, Appendix E).**

3.1.5.6. Determination of uronic acid contents of non-cross-linked and cross-linked arabinoxylans

UA contents were determined in duplicate using an adapted colorimetric assay method as described by Ahmed & Labavitch (Ahmed and Labavitch 1977).

AX samples (75-100 mg), non cross-linked and cross-linked, were weighed into a medium (15 mL) Kimax® tube. Sulfuric acid (5 mL, 12 M) was added to Kimax® tubes and immediately vortexed. The samples were placed in an ice bath for 30 min, and then kept at room temperature for 2 h. During hydrolysis, samples were vortexed every 10 min. Distilled water (9.75 mL) was carefully added to each tube, vortexed, and then immediately centrifuged. An aliquot of the supernatant (1 mL) was transferred into a 10 mL volumetric flask and the flask was filled with deionized H_2O for a 1:10 dilution. Diluted solution (600 μL) was added to each of two Pyrex tubes. Samples were cooled in an ice bath and 3.6 mL of a 0.0125 M sodium tetraborate solution in 18 M sulfuric acid were added. The tubes were vortexed 3 times, incubated for 5 min in a 100 °C water bath, and cooled down to room temperature. To one of the two tubes *m*-hydroxy diphenyl solution (60 μL 0.15% (w/v) in 0.5% (w/v) NaOH solution) was added. NaOH solution (60 μL, 0.5%) was added to the other tube, serving as a blank. Calibration was performed using a standard curve prepared from using standard solutions of galacturonic acid dissolved in distilled water (**Figure 4, Appendix D**).

3.1.5.7. Analysis of select monomeric hydroxycinnamic acids

AX samples (30 mg), non cross-linked and cross-linked, were weighed into a small PTFE-lined screw-capped Kimax® tubes, nitrogen purged, and NaOH solution (1.5 mL, 2M) was added. The headspace was purged with nitrogen and the samples were capped immediately. Tubes were lightly vortexed to ensure complete wetting of the samples and prevent the formation of clumps, and placed in darkness for 18 h with periodic vortexing. Following hydrolysis samples were acidified to a pH <2.0 using ca. 1 mL HCl (37% solution).

Liberated hydroxycinnamic acids were extracted into 2 mL diethyl ether followed by three additional extractions, using 1 mL of diethyl ether. Diethyl ether fractions were pooled in 5 mL glass conical tubes and dried under a stream of nitrogen.

Residues were reconstituted in MeOH/H_2O (50/50 (v/v), 150 µL) for RP-HPLC analysis. Diluted samples were analyzed for *cis*-ferulic acid, *trans*-ferulic acid, and *trans*-*p*-coumaric acid using a photo diode array detector monitoring the effluent at 280 and 325 nm and a Luna phenyl-hexyl column (250 x 4.6 mm i.d., 5 µm particle size). Solvents used for gradient elution at 45°C were 1 mM trifluoroacetic acid (TFA) (A), MeOH/1 mM TFA 90/10 (v/v) (B) and acetonitrile/1 mM TFA 90/10 (v/v) (C). The gradient program used was adapted from a published method (Dobberstein and Bunzel 2010): 87% A and 13% C for 10 min; changed over 10 min to 77% A, 3% B, 20% C; over 5 min to 70% A, 5% B, 25% C; over 5 min to 25% A, 25% B, 50% C; and completed with a 10 min equilibration step for the starting conditions. Quantification was done using

correction factors of the individual monomers (**Table 26, Appendix D**) against the internal standard. A standard chromatogram is shown in (**Figure 11, Appendix E**).

3.1.5.8. Analysis of ferulic acid dehydrodimers

Alkaline hydrolysis and extraction were performed as described for hydroxycinnamic acid monomers. For dehydrodiferulic analysis a different internal standard (5-5(methylated)-dehydrodiferulic acid in MeOH/H_2O (50/50, v/v), 15µg) was added before acidification. The reconstituted samples were analyzed using RP-HPLC using a Luna phenyl-hexyl column (250 x 4.6 mm i.d., 5 µm particle size), and monitoring the effluent at 280 nm. Solvents used for gradient elution at 45°C were 1 mM TFA (A), acetonitrile/1 mM TFA 90/10 (v/v) (B) and MeOH/1 mM TFA 90/10 (v/v) (C). The gradient program used was adapted from a published method (Dobberstein and Bunzel 2010): 85% A and 15% C for initial; changed over 15 min to 82% A and 18% C; over 5 min to 80% A, 20% B, 5% C; over 5 min to 72% A, 25% B, 3% C; over 5 minutes to 70% A, 25% B, 5% C; over 10 minutes to 65% A, 30% B, 5% C; held 5 minutes at 80% A, 20% B, 5% C and completed with10 minutes at 55% A, 40% B, 5% C. Chromatograms were monitored at 280 and 325 nm and FA dehydrodimers were quantified using correction factors of the individual dimers (**Table 27, Appendix D)** against the internal standard. A standard chromatogram is shown in (**Figure 12, Appendix E**).

3.1.6 Fermentation studies

The non-cross-linked and cross-linked AX which were isolated and characterized as described in the previous sections were used for *in vitro* fermentation studies. The eight

different AX used were AX extracted with 0.1 M NaOH, non-cross-linked; 0.5 M NaOH, non-cross-linked; 1.0 M NaOH, non-cross-linked; 1.0 M NaOH, non-cross-linked, ball-milled; 0.1 M NaOH, cross-linked; 0.5 M NaOH, cross-linked; 1.0 M NaOH, cross-linked; and 1.0 M NaOH, cross-linked, ball-milled. The *in vitro* fermentation set up followed a protocol from McBurney and co-workers (McBurney, Thompson et al. 1989). The "no added fiber samples" served as a control for all tested parameters (SCFA, H_2 and CH_4, pH, and phenolic acid metabolites). All AX were fermented in triplicate.

All 100 mL serum bottles, total of 108 bottles, were heated at 100° C in an atmospheric oven for 20 min. Bottles were removed from the oven, cooled at room temperature, labeled with an appropriate AX treatment number and fermentation time point, and capped with rubber stoppers to avoid contamination before the start of the fermentation study. Different amounts of cross-linked (0.8 g) and non-cross-linked (0.5 g) AX were used in the fermentation studies because the cross-linked samples contained buffer salts from AX cross-linking (**Table 28, Appendix D**).

A total volume of 5 L of fermentation medium was prepared in a 6 L flask (**Table 29, Appendix D)**. The fermentation medium was autoclaved for 20 min at liquid setting. The AX were weighed into the serum bottles and hydrated 12 h prior to the fermentation with 40 mL of sterile trypticase peptone fermentation medium at 4 °C. All bottles were then heated to 37 °C in a shaking water bath 2 h before inoculation with fecal inoculum, which was prepared as described below.

Three subjects who consume a typical western diet and have not taken antibiotics within the last 6 months were recruited for this fermentation study. They completed consent forms and dietary records as well as anaerobically collected a fecal sample to provide for the study. Collection of the fecal samples followed rigid protocol instructions (**D-1, Appendix D)**. Fecal inoculum was prepared from the fecal samples as described by McBurney and Thompson (Mc Burney and Thompson 1989). Phosphate buffer solution (1000 mL) was prepared in a volumetric flask by dissolving: sodium dihydrogen phosphate (2.401 g), sodium chloride (0.809 g) in 1000 mL deionized water. Reducing solution (200 mL) was prepared in a volumetric flask by mixing: 1.25 g cysteine hydrochloride, 8 mL 1N NaOH, 1.25 g sodium sulfide nonhydrate, and 190 mL deionized water. Equal amounts of fecal samples (total 126.05 grams of feces) from all three individuals were combined in a blender and homogenized until desired consistency was achieved. Phosphate buffer solution (756.3 mL) was added to the feces (ratio of 6:1) after the homogenization step (Goering and VanSoest 1970). Mixing was continued and 2 parts reducing solution were combined with 15 parts fecal slurry (Goering and VanSoest 1970). Each bottle was filled with 10 mL of fecal inoculum and 0.8 mL of an oxygen scavenger Oxyrase were added. The bottles were flushed with carbon dioxide and immediately sealed with a rubber stopper. Bottles were placed in 37 °C shaking water baths and shook gently until time point removal (4, 8, 12, 24 h). At given time points bottles were removed from the water bath and gas volume was determined by puncturing rubber cap with a syringe and allowing the gas to fill it. Overpressure in the flask forced the gas to fill the syringe, the gas was transferred to sample holding bags until hydrogen analysis was conducted on the Quintron machine, as described below. The pH of each

bottle was measured using an Orion model 350 PerpHect Log R meter. Copper sulfate (1 mL, 200 g/L) was added to each bottle to stop the fermentation. Two different aliquots were taken from each bottle, one 1 mL aliquot for SCFA analysis, and one 10 mL aliquot for phenolic acid metabolite analysis. The aliquots were promptly stored at -80 °C until analysis.

3.1.6.1 Determination of the pH in the fermentation media

Orion model 350 PerpHect Log R meter (**Appendix A)** was calibrated for the pH range 4-7. If samples had a pH>7 or <4 the pH meter was re-calibrated to meet the specified pH range for the sample set. Inoculum samples from each bottle were poured into small beakers and the pH was determined.

3.1.6.2 Gas analysis

Gas analysis was done with a calibrated Quintron Instrument, QTL0055 Rev C (**Appendix A)**. Concentrations of hydrogen and methane were determined by injecting 20 mL of gas from the sample holding bags into the Quintron Model DP Microanalyzer. All samples were diluted to a 1 to 29 dilution, taking 1 mL of gas from the sample bag and combining it with 29 mL of atmospheric air for analysis. Gas amounts were quantified using specific equations (**D-2, Appendix D)**.

3.1.6.3 Analysis of short chain fatty acids

SCFA extraction was done according to Schneider and co-workers (Schneider, Girard-Pipau et al. 2006). Aliquot samples were thawed at room temperature and 1 mL of the inoculum sample was diluted with 1.6 mL distilled water in a 10 mL centrifuge tube. Ether (300 mL) was pre-mixed with 2-ethylbutyric acid (300 μL, internal standard) and 2 mL of this ether/internal standard solution plus 0.4 mL H_2SO_4 (50%) sulfuric acid were added to the centrifuge tubes. The tubes were vortexed for 20 sec to extract the SCFA into the ether, with cap slightly ajar to prevent pressure build up, placed in an ortibal shaker for 45 min, and then centrifuged at 3000 rpm for 5 min at room temperature. The organic supernatant was removed and transferred to a 5 mL glass test tube and calcium chloride (~20 mg) was added to remove any residual water. The solution was filtered through a FisherBrand 13 mm nylon filter with pore size 0.2 μm into gas chromatography vials.

GC-FID was used to analyze SCFA concentrations. Samples were analyzed using a HP 5890 series gas chromatograph with a Stabilwax-DA and fused silica column (30 m x 0.53 mm i.d., 1 μm film thickness) (**Appendix A)**. Detector and inlet temperatures were 200 °C, the injection volume was 0.5 μL. A temperature gradient was used: start temperature 90 °C, held for 2 min, increase of 60 °C per minute to 120 °C, held for 10 min. Flow rates were 33 mL/min, 32 mL/min, and 40 mL/min for helium (carrier gas), hydrogen, and air. Peaks were identified by comparing retention times to those of standard compounds (acetic acid, propionic acid, butyric acid, and ethyl butyrate). HP Chem Station was the software used to integrate the chromatograms. Quantification of SCFAs was performed by using the internal standard method (ethyl butyrate as internal

standard) (**Table 30, Appendix D**). Calibration curves are shown in (**Figures 5, 6, & 7, Appendix D)**.

3.1.6.4 Phenolic metabolite analysis

The aliquot of the fecal inoculum (10 mL) was centrifuged and 1 mL of the supernatant was removed and placed into a 12 mL screw cap tube. Internal standard solution σ-hydroxy cinnamic acid (10 μg/mL in MeOH/H_2O 50/50 (v/v), 40 μL), approximately 5 drops of concentrated hydrochloric acid, and 1.5 mL of ethyl acetate were added and the tube was vortexed for 1 min. The tube was centrifuged at 1500 g for 10 min and the organic supernatant was transferred to new screw cap tube. The extraction was repeated with 1.5 mL ethyl acetate and the organic phases were combined. $NaHCO_3$ solution (5% (w/v), 1.5 mL) was added to the pooled ethyl acetate extract and the tube was vortexed for 1 min. Following centrifugation at 1500 g for 10 min the lower aqueous phase, now containing the phenolic acid metabolites, was transferred to a new screw cap tube. The extraction of the phenolic acid metabolites into $NaHCO_3$ solution was repeated. Approximately 10 to 12 drops of concentrated hydrochloric acid were added to the combined aqeous $NaHCO_3$ layers to reach a pH of 1-2 and the protonated phenolic acid metabolites were extracted into 1.5 mL of ethyl acetate by vortexing for 1 min. After centrifugation at 1500 g for 10 min the supernatant was transferred to a new screw cap tube and the ethyl acetate extraction step was repeated. The pooled ethyl acetate was dried under a stream of nitrogen (35° C), 200 μL of 50% (v/v) methanol was added, and the mixture was ultrasonicated for 5 min. The solution was transferred into an HPLC vial

with insert and kept at -20 °C in a freezer until HPLC analysis. Analysis was done in triplicate.

Phenolic metabolite analysis was performed by RP-HPLC analysis using a photo diode array detector monitoring the effluent at 280 nm. A Luna phenyl-hexyl column (250 x 4.6 mm i.d., 5 µm particle size) was used for separation. Oven temperature was held at 45° C, the flow rate was 1 mL/min. Solvents used for the gradient elution were MeOH/1 mM TFA 90/10 (v/v) (A), acetonitrile/1 mM TFA 90/10 (v/v) (B), and 1 mM TFA (C). The gradient program used was: 10% A and 90% C for 30 min; changed over 10 min to 35% A and 65% C; over 5 min to 50% A and 50% C; over 10 min to 50% A, 25% B, 25% C; over 10 min to 13% B and 87% C; and completed with a 10 min equilibration step for the starting conditions.

The following compounds were included in the standard solution for the standard curve: *trans*-ferulic acid, *trans*-caffeic acid, *p*-hydroxycinamic acid, 3-(3,4 dihydroxyphenyl) propionic acid, 3-(4 hydroxyphenyl) propionic acid, 3-(3 hydroxyphenyl) propionic acid, 3-(4-hydroxy-3-methoxyphenyl) propionic acid, 3,4-dihydroxyphenyl acetic acid homovanillic acid, *o*-hydroxycinnamic acid. A standard chromatogram can be found in (**Figure 26, Appendix E)**. The correction factors were determined for phenolic metabolites and quantification using *o*-hydroxy cinnamic acid as internal standards (**Table 31, Appendix D**).

3.1.7 Statistical analysis

Statistical analyses for results were executed using SAS statistical software version 9.1 (SAS Institute, Cary, NC). Data analysis was completed using a general linear model procedure. The fixed effect of fiber was tested. Time was considered a random effect. Least square means were used to determine the statistical significance ($P<0.05$) among samples. All data was analyzed in duplicate or triplicate, test dependent.

Chapter 4. Results and discussion

AX, non starch polysaccharides, resist digestion in the upper gastrointestinal tract and act as fermentable carbon sources for bacteria in the lower gastrointestinal tract. However, little is understood about the actual fermentation process within the body (Hopkins, Englyst, et al. 2003). A previous *in vitro* study was done on the fermentation of cross-linked and non-cross-linked AX, modeling a human digestion system (Hopkins, Englyst, et al. 2003). This study aimed to investigate the breakdown of non-cross-linked and FA cross-linked AX by children's microbiota. Formation of fermentation products and changes in specific gut microorganisms were analyzed in this study having a large emphasis on microbial ecology. A major drawback of this study was that the non-cross-linked and cross-linked AX were only minimally characterized. For example, information about the degree of cross-linking was not given. Thus, critical information to interpret the data from the fermentation study was missing. The aim of our study deviated from this approach as we used well-described AX. Also, we used AX extracted by applying different conditions and studied the effect of different chemical structures on the microbial fermentation process.

The fate of AX in the digestive system is dependent on many different factors but the first to consider was AX composition. In our study, differently extracted as well as cross-linked AX preparations were characterized by the following parameters: moisture, ash, protein, total carbohydrate contents, neutral sugar composition, UA content, and FA content and composition (monomers and dimers).

4.1 Chemical characterization of the arabinoxylan preparations

4.1.1 Moisture contents of the arabinoxylan preparations

Moisture contents were determined by using Karl Fischer. The average moisture content of non-cross-linked samples (average 8.75 g/100 g) was larger in comparison to cross-linked samples (average 1.61 g/100 g) (**Table 1**). These differences were attributed to cross-linked samples being stored in desiccators. The extracted AX samples, being very hygroscopic after drying, easily absorb water from the surrounding environment unless placed in a controlled environment. All subsequent calculations of AX preparations were corrected for moisture.

Table 1. Moisture content of the arabinoxylan preparations[ijk]

	Average % ± SD
Sample	**Moisture %**
0.1 M AX	8.86±0.007[b]
0.5 M AX	7.65±0.021[d]
1.0 M AX	10.18±0.014[a]
1.0 M BM AX	8.25±0.007[c]
0.1 M X AX	1.22±0.006[g]
0.5 M X AX	2.12±0.021[e]
1.0 M X AX	2.05±0.014[f]
1.0 M BM X AX	1.01±0.016[h]

[i]standard deviations were calculated from 3 replicates
[j]values with different letters represent statistical difference ($P<0.05$)
[k]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

4.1.2 Ash contents of the arabinoxylan preparations

Ash contents were determined by using the AOAC Official Method 923.03. There was a large difference in ash contents between non-cross-linked (average 4.70 g/100 g) and cross-linked samples (average 6.89 g/100 g) (**Table 2**). Differences in ash content were due to the partial presence of BS from the cross-linking step. Samples were heated to

high temperatures in the ashing procedure where citric acid, an organic compound, was incinerated and phosphate and the cations were left within the ash, from the citric-phosphate buffer, contributing to a higher overall percentage of ash for the cross-linked samples.

BS from the citrate-phosphate buffer used for oxidative cross-linking largely contributed to the cross-linked AX preparations. The addition of BS and potential impurities was determined to be 39.50% and was used in all subsequent calculations for cross-linked samples, as well as *in vitro* fermentation calculations, ensuring all results reflected the same amount of AX for data comparison.

Table 2. Ash contents of the arabinoxylan preparations[ghij]

	Average % ± SD
Sample	**Ash %**
0.1 M AX	5.08±0.005[d]
0.5 M AX	4.92±0.004[e]
1.0 M AX	4.33±0.003[f]
1.0 M BM AX	4.39±0.006[f]
0.1 M X AX	7.11±0.008[a]
0.5 M X AX	6.75±0.002[c]
1.0 M X AX	6.82±0.006[bc]
1.0 M BM X AX	6.88±0.007[b]

[g]standard deviations were calculated from 3 replicates
[h]all data corrected for buffer salts and protein
[i]values with different letters represent statistical difference ($P<0.05$)
[j]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

4.1.3 Protein contents of the arabinoxylan preparations

Protein contents of AX preparations were determined using the Dumas method. A large difference was observed between the non-BM (average 5.34 g protein/100 g) and BM AX preparations (average 4.78 g protein/100 g) (**Table 3**). The 1.0 M BM preparations, both

non-cross-linked and cross-linked, showed the lowest percentage of protein implying that BM decreased the amount of protein detected in the samples. Lower amounts of protein in other BM samples such as flour have been reported from a previous study (D'appolonia and Gilles 1996). The study concluded that flour ground in a ball mill had alterations in protein structure as well as overall lower protein concentrations compared to samples that had not been BM (D'appolonia and Gilles 1996). All subsequent calculations of AX preparations were corrected for moisture, BS (just cross-linked samples), and protein.

Table 3. Protein contents of the arabinoxylan preparations[hijk]

	Average % ± SD
Sample	**Protein %**
0.1 M AX	5.40±0.007[b]
0.5 M AX	5.25±0.002[d]
1.0 M AX	5.74±0.009[a]
1.0 M BM AX	4.85±0.001[f]
0.1 M X AX	5.26±0.008[d]
0.5 M X AX	5.37±0.009[c]
1.0 M X AX	5.00±0.010[e]
1.0 M BM X AX	4.70±0.009[g]

[h]standard deviations were calculated from 3 replicates
[i]data corrected for moisture and buffer salts
[j]values with different letters represent statistical difference ($P<0.05$)
[k]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

4.1.4 Total carbohydrate contents and neutral sugar compositions of the arabinoxylan preparations

Total carbohydrates were determined using the phenol sulfuric acid assay. There were differences between AX preparations in total carbohydrate contents (**Table 4**). Although the carbohydrate contents of the non-cross-linked preparations seemed to be higher than the total carbohydrate contents for the corresponding cross-linked preparations, the

opposite is true for the AX extracted by using 1.0 M NaOH. The total carbohydrate contents are slightly different but data shows that correction for BS, moisture, and protein works to adjust the sample amounts for the fermentation studies.

Neutral sugar analysis using GC-FID after polysaccharide hydrolysis and reduction and acetylation of the liberated monosaccharides determined at the overall recovery rate (%) as well as the A:X ratio, which is a common reference point for arabinoxylans (**Table 4**). The average A:X ratio for the non-cross-linked and cross-linked preparations is between 0.61 and 0.77. These A:X ratios indicate a moderate level of branching and are similar to other results reported from corn bran AX (Singgh, Doner, Johnston, et al., 2000). There is a trend that non-cross-linked AX are slightly more branched (higher A/X ratio) than the cross-linked AX. This result was rather unexpected since the cross-linking process should, theoretically, not affect the carbohydrate portion of the carbohydrates. Why cross-linking decreases the AX branching cannot be explained here.

Due to monosaccharide degradation during the acid hydrolysis and due to incomplete hydrolysis the recovery rates (sum of monosaccharides compared to total carbohydrates) are always lower than 100%. Also, UA, which were not analyzed here, contribute to the total carbohydrate content. Recovery data show similarity between most preparations except 1.0 M BMAX and 0.5 M cross-linked AX. Previous studies have reported that recovery rates of 60%-70% are normal and considered acceptable for the analysis of the neutral sugar composition of polysaccharides.

Table 4. Carbohydrate analysis of arabinoxylan samples (total carbohydrate and neutral sugars)[ghij]

	Average % ± SD	Neutral Sugar Analysis	
	Total CHO %	**Arabinose: Xylose Ratio**	**Recovery %**
0.1 M AX	94.36±0.028[a]	0.69	67.76
0.5 M AX	93.39±0.141[c]	0.77	66.21
1.0 M AX	93.40±0.143[c]	0.74	67.22
1.0 M BM AX	90.83±0.021[d]	0.62	61.00
0.1 M X AX	90.93±0.071[d]	0.62	66.46
0.5 M X AX	89.92±0.153[e]	0.68	73.63
1.0 M X AX	94.19±0.071[b]	0.68	67.48
1.0 M BM X AX	88.86±0.063[f]	0.61	68.57

[g]standard deviations were calculated from 3 replicates
[h]all data corrected for buffer salts, moisture, and protein
[i]values with different letters represent statistical difference ($P<0.05$)
[j]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

4.1.5 Uronic acid contents of arabinoxylan preparations

UA contents were determined by using a colorimetric assay. As expected, there was no large difference in the UA concentrations between non-cross-linked (average 0.44 g/100 g) and cross-linked preparations (average 0.43 g/100 g) (**Table 5**). In addition, the contribution of UA to the AX structures is rather small and independent from the extraction conditions except that BM preparations, both non-cross-linked and cross-linked, were lower in UA than all other condition preparations.

Table 5. Uronic acid contents of the arabinoxylan preparations[fghi]

	Average % ± SD
Sample	**UA %**
0.1 M AX	0.47±0.007[ab]
0.5 M AX	0.43±0.001[c]
1.0 M AX	0.45±0.008[bc]
1.0 M BM AX	0.40±0.007[de]
0.1 M X AX	0.47±0.002[a]
0.5 M X AX	0.44±0.002[c]
1.0 M X AX	0.43±0.008[cd]

1.0 M BM X AX	0.39±0.002[e]

[f]standard deviations were calculated from 3 replicates
[g]data corrected for moisture, buffer salts, and protein
[h]values with different letters represent statistical difference ($P<0.05$)
[i]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

4.1.6 Contents and composition of ferulic acid monomers and dimers of the arabinoxylan preparations

Non-cross-linked and cross-linked preparations were analyzed for their monomeric and dimeric FA contents by using RP HPLC after alkaline hydrolysis and extraction of the liberated phenolic acids (**Table 6 & 7**). The results showed large differences in both FA monomer and dimer contents between non-cross-linked and cross-linked preparations. The concentration of total monomers (sum of *cis/trans*-FA) was larger for the non-cross-linked samples in comparison to the cross-linked samples. In addition, you can see for both non-cross linked and cross-linked arabinoxylan samples the higher the concentration of NaOH the lower the amount of total FA. These results were expected since stronger concentrations of base yield less FA since higher alkaline conditions cleave ester linkages.

Opposite trends were observed for the dimer contents of the non-cross-linked and cross-linked preparations (**Table 7**). Total dimer contents of all non-cross-linked preparations were lower than cross-linked preparations. Since all 8-5 coupled dimers have the same precursor in the plant, all 8-5 coupled dimers are grouped together in this section. The situation is less clear for the 8-8 dimers. Although the different 8-8 dimers may exist in the plant they are also grouped together in this study. Similar trends are seen for both contents of 8-8 DFAs and 5-5 DFA with non-cross-linked preparations having lower concentrations than cross-linked preparations. The 8-O-4 DFA contents of the cross-linked and non-cross-linked preparations show similar trends as the 8-8 and 5-5 DFAs

except for 0.1 M preparation having a higher concentration (0.10 µg/mg) than other non-cross-linked preparations (0.01-0.02 µg/mg).

The results shown here were as expected. Since FA monomers and dimers are predominantly ester-linked to AX, extraction procedures using more concentrated NaOH reduce the contents of FA monomers and dimers in the extracted AX preparations. Data suggests that the extractions with 0.1 M NaOH leave more esterified ferulate monomers and dimers behind then extractions with 1.0 M NaOH. As expected, monomeric FA contents decreased after oxidative cross-linking from an average of 2.32 µg/mg to 0.50 µg/mg while FA dimers were formed (contents increased from 0.43 µg/mg to 3.30 µg/mg.) Observed monomeric FA contents for non-cross-linked preparations were higher than reported by Carajal-Millan et al. for maize bran AX (0.34 µg/mg) whereas the DFA contents of the non-cross-linked AX measured here were lower than in Carajal-Millan's study (0.58 µg/mg) (Carvajal-Millan, Rascón-Chu et al. 2007). Carajal-Millan's study extracted AX using mild alkali conditions (0.5 M NaOH) using a procedure very similar to our study. The increase of DFA's induced by cross-linking was expected and has been reported by many other sources (Lapierre, Pollet et al. 2001; Carvajal-Millan, Rascón-Chu et al. 2007). Surprisingly, most of the newly formed dimers were 8-8 coupled DFA's (~67-88%). 8-5 (~3-9%), and 8-O-4 (~3-11%) coupled dimers, which usually dominate, were formed in much lower amounts. From these results we conclude that cross-linking of AX results in an extensive formation of dimers having a profound impact on the AX functionality.

Table 6. Monomeric ferulic acid contents of non-cross-linked and cross-linked preparations [μg/mg][ijkl]

Fiber	*trans*-FA	*cis*-FA	Total FA
0.1 M	2.68±0.008[a]	0.21±0.004[a]	2.88±0.006[a]
0.5 M	2.41±0.018[b]	0.23±0.011[a]	2.65±0.019[b]
1.0 M	2.34±0.003[c]	0.21±0.018[a]	2.55±0.009[c]
1.0 M BM	1.18±0.019[d]	0.04±0.013[bc]	1.21±0.016[d]
0.1 M X	0.64±0.021[e]	0.08±0.012[b]	0.71±0.012[e]
0.5 M X	0.5±0.044[f]	0.05±0.011[b]	0.53±0.025[f]
1.0 M X	0.41±0.045[g]	0.08±0.011[b]	0.51±0.026[f]
1.0 M BM X	0.24±0.101[h]	0.01±0.012[c]	0.26±0.052[g]

[i]standard deviations were calculated from 3 replicates
[j]all data corrected for buffer salts, moisture, and protein
[k]values with different letters represent statistical difference (P<0.05) within column
[l]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

Table 7. Contents of ferulic acid dimers of non-cross-linked and cross-linked preparations [μg/mg][hijk]

Fiber	8-8' DFA's	5-5' DFA	8-O-4' DFA	8-5' DFA	Total
0.1 M	0.60±0.011[d]	0.11±0.011[e]	0.12±0.011[b]	0.08±0.011[bc]	0.89±0.011[d]
0.5 M	0.32±0.011[e]	0.02±0.011[f]	0.01±0.011[c]	0.01±0.011[c]	0.28±0.011[e]
1.0 M	0.22±0.012[f]	0.02±0.012[f]	0.02±0.012[c]	0.01±0.012[c]	0.27±0.011[e]
1.0 M BM	0.16±0.012[g]	0.03±0.011[f]	0.03±0.011[c]	0.01±0.013[c]	0.20±0.011[e]
0.1 M X	2.95±0.011[a]	0.45±0.013[a]	0.19±0.011[a]	0.25±0.012[a]	3.86±0.014[a]
0.5 M X	2.79±0.013[b]	0.30±0.011[b]	0.13±0.011[ab]	0.13±0.011[b]	3.35±0.013[b]
1.0 M X	2.80±0.011[b]	0.24±0.013[c]	0.11±0.013[b]	0.09±0.011[bc]	3.24±0.013[b]
1.0 M BM X	2.22±0.019[c]	0.19±0.011[d]	0.14±0.013[ab]	0.10±0.012[bc]	2.63±0.101[c]

[h]standard deviations were calculated from 3 replicates
[i]data corrected for buffer salts, moisture, and protein
[j]values with different letters represent statistical difference (P<0.05) within columns
[k]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

Table 8. Diferulic acid composition[a]

	8-8' DFA's (%)	5-5' DFA (%)	8-O-4' DFA (%)	8-5' DFA (%)

0.1 M	67	12	11	9
0.5 M	88	6	3	3
1.0 M	84	8	4	4
1.0 M BM	71	14	10	5
0.1 M X	76	11	5	8
0.5 M X	83	9	4	4
1.0 M X	86	8	4	2
1.0 M BM X	84	7	6	3

[a]percentage of total diferulic acids

4.2 Microbial fermentation of the arabinoxylan preparations

Bacteria in the human digestive system use dietary carbohydrates as important energy sources. However, limited research has investigated how AX are fermented and whether or not cross-linking has an effect on the fermentation. Different but well defined AX preparations were obtained by using different extraction conditions and partial oxidative cross-linking of these AX. Chemical characterization of these preparations ensured that well characterized carbohydrates were used in the fermentation studies and that misinterpretation due to unknown substrates were minimized. In this study, the different AX preparations were fermented by fecal samples and the fermentation slurries were analyzed for pH, gas (methane and hydrogen), SCFAs, and phenolic metabolites at 4, 8, 12, and 24 h time points. Results from the *in vitro* fermentation are reliant on many different factors, which should be considered when analyzing data, such as the type of microflora present in the pooled slurry, the substrate used in the fermentation (in this case AX), and the time of fermentation which has passed all greatly influence the results as the body takes time for specific reactions to occur.

4.2.1 pH of the fermentation slurries

A decrease in pH in the course of time was observed for all preparations up to 8 h (**Table 9**). After 8 h the pH was more stable. No large differences were noticed between the 12 and 24 h time points. The decrease in pH was due to the production of SCFAs during the fermentation and is considered physiologically beneficial. A lower colonic pH can decrease the growth of pathogenic bacteria and increase the growth of beneficial bacteria (Wang and Gibson 1993) as well as increase the absorption of calcium and magnesium in the large intestine (Holloway, Moynihan et al. 2007). Increased pH values at 12 and 24 h time points for select preparations were not statistically valid. The comparison of the pH values of fermentation slurries containing non-cross-linked and cross-linked AX is difficult. As described earlier, the cross-linked AX preparations contain BS from cross-linking procedure, which affected the pH of the fermentation slurries. Thus higher pH values in the slurries containing cross-linked preparations may not be due to a reduced formation of SCFAs but due to the buffering capacity of the salts added with the AX preparations.

Table 9. pH of the fermentation slurries at different time points[ijk]

Hour	Ctrl	0.1 M	0.5 M	1.0 M	1.0 M BM	0.1 M X	0.5 M X	1.0 M X	1.0 M BM X
4	7.11±0.011^{a}	6.93±0.032^{c}	6.98±0.011^{b}	6.95±0.081^{c}	6.98±0.013^{b}	6.81±0.013^{e}	6.85±0.015^{d}	6.82±0.011^{e}	6.71±0.017^{f}
8	7.17±0.011^{a}	6.33±0.036^{f}	6.31±0.041^{g}	6.21±0.013^{h}	6.35±0.011^{e}	6.60±0.014^{b}	6.61±0.021^{b}	6.47±0.014^{d}	6.55±0.011^{c}
12	7.14±0.021^{a}	6.17±0.021^{f}	6.20±0.021^{e}	6.21±0.062^{e}	6.20±0.021^{e}	6.61±0.013^{b}	6.51±0.021^{cd}	6.49±0.013^{d}	6.52±0.014^{c}
24	7.15±0.021^{a}	6.21±0.031^{f}	6.15±0.011^{g}	6.13±0.022^{h}	6.23±0.013^{e}	6.53±0.012^{b}	6.51±0.011^{c}	6.48±0.012^{d}	6.50±0.013^{c}

[i]standard deviations were calculated from 3 replicates
[j]values with different letters represent statistical difference ($P<0.05$) within each line
[k]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

4.2.2 Gas production during fermentation of the arabinoxylan preparations

Gas volumes produced in the course of fermentations were determined by using the Quintron instrument. Total gas volume is an important parameter to measure because a large amount of gas in the gastrointestinal tract can lead to discomfort for many individuals. However, individuals react to gas in different ways due to their gastrointestinal tolerance (Serra, Azpiroz et al. 1998). An individual's tolerance to gas is dependent on its equilibrium between gas production and gas excretion as well as the exact location of the gas pressure in the system (Harder, Serra et al. 2003). The rate of fermentation is also a key factor in determining the tolerance of specific samples. Based on our results non-cross-linked preparations produced higher levels of gas (499 mL total) compared to the cross-linked preparations (257 mL total) (**Table 10**). Therefore, cross-linking AX could be beneficial as there is less gas production in the large intestine and less gas, flatulence, and discomfort for individuals. Less gas production during the fermentation of the cross-linked preparations could indicate that these AX are less fermented or more slowly fermented than their non-cross-linked counterparts as already indicated (but not demonstrated due to the addition of BS with the cross-linked preparations) by the higher pH values after 8 h of fermentation. Rates of fermentation are pretty consistent amongst groups (non-cross-linked and cross-linked) showing small fluctuations in gas volume throughout the four designated time points. The analysis of total gas production also produced values for individual gases present, hydrogen, and methane.

Table 10. Gas production in the course of arabinoxylan fermentation. Produced gas volumes are indicated in mL.[hij]

Hour	Ctrl	0.1 M	0.5 M	1.0 M	1.0 M BM	0.1 M X	0.5 M X	1.0 M X	1.0 M BM X
4	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
8	0[f]	20.50±0.011[b]	20.50±0.012[b]	25.00±0.013[a]	0[f]	3.50±0.017[e]	6.50±0.009[c]	0[f]	5.00±0.011[d]
12	0[g]	54.50±0.011[a]	51.50±0.012[b]	45.00±0.011[d]	43.00±0.011[d]	28.50±0.014[f]	30.50±0.013[e]	30.50±0.021[e]	29.50±0.023[ef]
24	0[f]	56.50±0.015[b]	57.50±0.019[b]	61.50±0.027[a]	58.00±0.031[b]	29.50±0.012[e]	36.50±0.042[c]	34.00±0.032[d]	30.50±0.011[e]

[h]standard deviations were calculated from 3 replicates
[i]values with different letters represent statistical difference ($P<0.05$) within each line
[j]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

Hydrogen gas is not normally found in the human body (Bjorneklett, Jensen 1982). The presence of hydrogen gas indicates the fermentation of undigestable carbohydrates in the colon (Bjorneklett and Jenssen 1982). Once hydrogen gas is produced in the body it is absorbed and transported through the blood stream, excreted through the lungs, anus, or converted to one of three compounds: acetate, methane, or hydrogen sulfide (Gibson, MacFarlane et al. 1993). The conversion of the latter is of concern because hydrogen sulfide is cell toxic and can harm the colonic epithelium ((Attene-Ramos, Wagner et al. 2006). Previous studies have found that a pH decrease from 7 to 6 can cause a reduction in hydrogen sulfide production of up to 50% (Gibson, Cummings et al. 1990). In this study, only methane and hydrogen gas were measured, but hydrogen sulfide could also be present. The highest production of hydrogen was found where the 1.0 M AX preparation was fermented for 24 h followed closely by non-cross-linked 0.1 M and 1.0 M AX preparations (**Table 11**). Hydrogen concentration in the fermentation vessels fermenting the 0.5 M and 1.0 M BM cross-linked AX preparations decreased over time due to the possible conversion of hydrogen to acetate, methane, or hydrogen sulfide as has been seen in previous studies (Lebet, Arrigoni et al. 1998). Why this decrease was seen for these preparations but not for the other preparations can not be explained. The high levels

of hydrogen for non-cross-linked preparations correlate with the higher production of total gas from non-cross-linked preparations by microbial fermentation.

Table 11. Concentration (in ppm) of hydrogen in the headspace of the fermentation vessels in the course of arabinoxylan fermentations[ijk]

Hour	Ctrl	0.1 M	0.5 M	1.0 M	1.0 M BM	0.1 M X	0.5 M X	1.0 M X	1.0 M BM X
4	0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
8	0^{h}	48 ± 0.011^{g}	104 ± 0.013^{a}	87 ± 0.041^{e}	90 ± 0.012^{d}	83 ± 0.041^{f}	93 ± 0.022^{c}	90 ± 0.015^{de}	96 ± 0.012^{b}
12	0^{g}	151 ± 0.011^{a}	99 ± 0.016^{d}	123 ± 0.033^{b}	61 ± 0.011^{f}	87 ± 0.016^{e}	87 ± 0.022^{e}	115 ± 0.022^{c}	117 ± 0.013^{c}
24	0^{g}	146 ± 0.018^{b}	80 ± 0.012^{f}	156 ± 0.042^{a}	142 ± 0.023^{b}	110 ± 0.013^{d}	101 ± 0.024^{e}	122 ± 0.016^{c}	81 ± 0.013^{f}

[i]standard deviations were calculated from 3 replicates
[j]values with different letters represent statistical difference ($P<0.05$) within each line
[k]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

Methane, CH_4, is not present in most individuals and is dependent on the presence of *Methanobrevibacter smithii* populations within the colon, microorganisms that produce methane (Weaver, Krause et al. 1986). Previous studies have shown that populations or cultures with high levels of *Methanobrevibacter smithii* are more likely to have lower rates of colon cancer suggesting that high levels of methane could hold a role in cancer prevention (Segal, Walker et al. 1988). Methane is also important as it is thought to decrease the production of hydrogen sulfide in the body through the consumption of hydrogen, which is used in the production of hydrogen sulfide. While relatively low levels of methane (4-16 ppm) were produced from both non-cross-linked and cross-linked preparations slightly higher concentrations of methane were produced from the cross-linked AX preparations (**Table 12**). These results are opposite to the hydrogen results. Methane levels of the control fecal slurries were constant at zero for all

preparations proving that methane was produced through the fermentation and not originally present in the fecal slurry.

Table 12. Concentration (in ppm) of methane in the headspace of the fermentation vessels in the course of arabinoxylan fermentations[ghi]

Hour	Ctrl	0.1 M	0.5 M	1.0 M	1.0 M BM	0.1 M X	0.5 M X	1.0 M X	1.0 M BM X
4	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
8	0^{d}	4 ± 0.011^{c}	7 ± 0.023^{b}	7 ± 0.031^{b}	11 ± 0.011^{a}	6 ± 0.012^{b}	6 ± 0.031^{b}	7 ± 0.015^{b}	6 ± 0.011^{b}
12	0^{f}	4 ± 0.013^{e}	10 ± 0.012^{d}	12 ± 0.022^{c}	12 ± 0.011^{bc}	13 ± 0.023^{bc}	13 ± 0.033^{b}	16 ± 0.016^{a}	14 ± 0.033^{b}
24	0^{e}	6 ± 0.012^{d}	11 ± 0.022^{c}	12 ± 0.022^{c}	20 ± 0.011^{b}	20 ± 0.014^{b}	20 ± 0.026^{b}	26 ± 0.011^{a}	20 ± 0.014^{b}

[g]standard deviations were calculated from 3 replicates
[h]values with different letters represent statistical difference ($P<0.05$) within each line
[i]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

4.2.3 Formation of short chain fatty acids during fermentation of the arabinoxylan preparations

Numerous health beneficial effects of SCFA production in the colon are described in the literature, including: increased apoptosis rate, reduced formation of aberrant crypt foci, and decreased tumor development (Vergheses, Rao et al. 2002; Bauer-Marinovic, Florian et al. 2006). In this study, a 24 h fermentation period was analyzed which is consistent with multiple other research studies. Some studies extended the fermentation to 48 h as the average colonic transit time for humans varies between 26 and 59 h (Saad, S.S.C et al. 2010).

No difference in SCFA production from cross-linked and non-cross-linked AX was observed for the first two time points of the fermentation study, 4 and 8 h **(Tables 14 & 15)**. However, at 12 and 24 h there was a difference between non-cross-linked and cross-

linked preparations with higher SCFA concentrations produced from the non-cross-linked preparations. Similar results were shown in an *in vitro* fermentation study done by Hopkins and co-workers (Hopkins, Englyst et al. 2003). The lower total SCFA and total gas volume production after 24 h may imply that cross-linked preparations are more slowly fermented than their non-cross-linked counterparts. The structural complexity of the cross-linked AX allows them to withstand fermentation reactions in the gut longer than non-cross-linked AX which are degraded more rapidly. Thus, our fermentation results support the hypothesis that cross-linking AX makes them less susceptible to fermentation. However, whether or not the residual non-fermented material is fermented after 24 h or whether this material is not fermented at all cannot be judged from this study. Ideally, the residual material will be fermented after 24 h allowing the interpretation that this material reaches the distal colon where it can be fermented by the microflora located in this part of the colon.

4.2.4 Short chain fatty acid profiles produced from the different arabinoxylan preparations

Comparing SCFA ratios (acetate: propionate: butyrate) at the end of the 24 h *in vitro* fermentation we observe butyrate production being the lowest and propionate production being highest, or equivalent to acetate production (**Table 13**).

The predominant formation of propionate and acetate is not unique to this study but was demonstrated in numerous *in-vitro* fermentation studies analyzing these fiber types before. Other studies align with other studies results showing corn bran AX produce higher concentrations of propionate. However, other observed SCFA ratios show acetate

as the leading SCFA whereas our results show propionate as the leading SCFA. Trends show that non-cross-linked preparations showed more similarity to the control preparation producing higher levels of propionate, whereas cross-linked preparations produced lower levels of propionate (**Tables 14 & 15**).

Table 13. Ratios of short chain fatty acids at 24 h time point[a]

Sample	SCFA Ratio
Control	2:4:1
0.1 M	2:3:1
0.5 M	2:4:1
1.0 M	2:4:1
1.0 M BM	2:4:1
0.1 M X	2:3:1
0.5 M X	2:3:1
1.0 M X	2:2:1
1.0 M BM X	2:3:1

[a] Ratios defined as (acetate: propionate: butyrate)

Table 14. Production of short chain fatty acids during the fermentation of non-cross-linked and cross-linked arabinoxylans (μmol/mL)[jklm]

	Acetate				Propionate				Buturate			
Sample	**4 h**	**8 h**	**12 h**	**24 h**	**4 h**	**8 h**	**12 h**	**24 h**	**4 h**	**8 h**	**12 h**	**24 h**
Control	0.66±0.031[i]	1.71±0.015[i]	3.61±0.081[g]	3.81±0.022[i]	0.97±0.091[h]	1.32±0.033[h]	5.74±0.021[h]	7.04±0.089[i]	0.67±0.011[f]	0.65±0.052[f]	1.53±0.043[h]	1.84±0.019[g]
0.1 M AX	1.37±0.071[h]	3.91±0.069[g]	6.86±0.088[b]	8.07±0.066[c]	3.58±0.012[b]	7.31±0.071[c]	13.21±0.037[a]	16.51±0.067[b]	0.91±0.012[c]	1.67±0.031[e]	3.07±0.025[b]	5.63±0.084[a]
0.5 M AX	1.65±0.015[g]	4.14±0.035[e]	6.63±0.063[c]	9.82±0.015[a]	2.71±0.025[g]	8.26±0.056[b]	12.92±0.042[b]	21.16±0.024[a]	0.75±0.021[c]	1.76±0.012[d]	2.25±0.014[g]	5.31±0.013[b]
1.0 M AX	1.79±0.005[f]	4.61±0.035[a]	6.74±0.055[c]	9.44±0.023[b]	3.55±0.003[c]	8.56±0.072[a]	11.45±0.063[d]	15.58±0.079[c]	0.85±0.014[d]	1.84±0.021[c]	2.72±0.015[e]	4.30±0.091[c]
1.0 M BM AX	1.88±0.003[e]	4.22±0.022[d]	7.08±0.025[a]	7.36±0.041[d]	3.69±0.031[a]	6.81±0.037[e]	12.04±0.043[c]	12.82±0.028[d]	0.86±0.005[d]	1.61±0.001[e]	3.18±0.031[a]	3.54±0.015[e]
0.1 M X AX	2.53±0.012[a]	4.05±0.011[f]	5.23±0.036[f]	5.59±0.011[g]	3.15±0.017[f]	6.63±0.005[f]	9.22±0.048[g]	9.55±0.013[h]	1.27±0.007[a]	2.11±0.019[b]	3.08±0.028[b]	3.56±0.105[e]
0.5 M X AX	2.27±0.021[d]	4.31±0.031[c]	5.83±0.045[d]	6.31±0.032[e]	3.17±0.031[f]	3.02±0.038[g]	10.15±0.045[e]	10.46±0.035[f]	1.13±0.009[b]	2.17±0.013[a]	3.01±0.015[c]	3.61±0.024[d]
1.0 M X AX	2.37±0.016[c]	3.66±0.034[h]	5.56±0.053[e]	4.62±0.048[h]	3.35±0.023[d]	7.19±0.025[d]	9.26±0.042[g]	11.88±0.061[e]	1.15±0.017[b]	2.12±0.008[b]	2.85±0.011[d]	3.52±0.012[e]
1.0 M X BM AX	2.51±0.007[b]	4.45±0.036[b]	5.74±0.031[d]	6.28±0.068[f]	3.32±0.018[e]	6.88±0.011[e]	9.46±0.075[f]	9.89±0.014[g]	1.28±0.022[a]	2.12±0.034[b]	2.68±0.008[f]	3.36±0.051[f]

[j]standard deviations were calculated from 3 replicates

[k]values with different letters represent statistical difference ($P<0.05$) within each column

[l]tested difference between samples at each specific time point (4, 8, 12, and 24 h)

[m]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

Table 15. Production of total short chain fatty acid during the fermentation of non-cross-linked and cross-linked arabinoxylans (μmol/mL)[jklm]

	Total			
Sample	**4 h**	**8 h**	**12 h**	**24 h**
Control	2.31±0.033[h]	3.69±0.074[g]	10.88±0.083[g]	12.70±0.085[i]
0.1 M AX	5.86±0.081[f]	12.89±0.022[d]	23.13±0.029[a]	30.21±0.084[b]
0.5 M AX	5.11±0.061[g]	14.16±0.014[b]	21.81±0.021[b]	36.29±0.021[a]
1.0 M AX	6.21±0.022[e]	15.02±1.028[a]	20.92±1.030[c]	29.32±1.079[c]
1.0 M BM AX	6.44±0.036[d]	12.63±0.065[f]	22.31±0.023[b]	23.73±0.061[d]
0.1 M X AX	6.95±0.031[b]	12.78±0.007[e]	17.62±0.003[d]	18.72±0.021[h]
0.5 M X AX	6.59±0.059[c]	13.49±0.082[c]	18.99±0.005[e]	20.39±0.088[e]
1.0 M X AX	6.88±0.045[b]	12.98±0.067[d]	17.69±0.097[f]	20.02±0.024[f]
1.0 M X BM AX	7.12±0.014[a]	13.46±0.035[c]	17.89±0.013[e]	19.54±0.025[g]

[j]standard deviations were calculated from 3 replicates
[k]values with different letters represent statistical difference ($P<0.05$) within each column
[l]tested difference between samples at each specific time point (4, 8, 12, and 24 h)
[m]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

4.2.5 Formation of microbial metabolites of ferulic acid during fermentation

Phenolic metabolites were determined by using RP-HPLC after extraction and clean up. Major phenolic metabolites and liberated phenolic compounds observed throughout the *in vitro* fermentation study included: caffeic acid, FA, 3-(3,4-dihydroxy phenyl) propionic acid, 3-(4-hydroxy-3-methoxyphenyl) propionic acid, and 3-(3-hydroxyphenyl) propionic acid. The formation pattern of individual metabolites are affected by many attributing factors but major factors to consider would be the source of the original starting material, corn bran, the extraction procedure of the AX, post-extraction treatments such as oxidative cross-linking, as well as the variation of the microbiota in the stool samples that contributed to the pooled fecal slurry. Phenolic metabolite concentrations were compared across time points in relation to the total phenolic metabolite formation as well as back to the original amount of FA present in the sample that was run through the fermentation study.

FA, one of the dominant hydroxycinnamic acids, was liberated from the AX during the fermentation study starting with high concentrations at 4 h, and then decreasing in concentration from 4 h to 24 h (**Table 16**). Due to the higher amounts of esterified FA, the slurries containing non-cross-linked preparations show higher FA concentrations after 4 h of fermentation than slurries containing cross-linked AX preparations. Liberation of FA from its ester linkage requires the action of microbial esterases. The concentrations of FA decrease in all preparations after 4 h. In several samples the concentrations of FA in the fermentation slurries after 8 h of fermentation are reduced to half compared to the 4 h fermentation slurries. and slower reduction rates from 4 h to 8 h which is likely due to

having a lower concentration of AX-bound FA which can be liberated by the microbial esterases. The reduction in FA over time is likely due to the microbial reduction or microbial demethylation of FA acid by gut microbiota which yield other metabolites such as caffeic acid and various phenyl propionic acids. Other research studies have demonstrated earlier that microbial reduction, demethylation, and dehydroxylation are the dominant reactions in the microbial metabolism of hydroxycinnamic acids (Russell, Scobbie et al. 2008).

Depending on whether liberated FA is first reduced or demethylated 3-(4-hydroxy-3-methoxyphenyl) propionic acid or caffeic acid are formed. During the *in vitro* fermentation microbial esterases demethylate FA to produce caffeic acid. Data suggest that FA is heavily demethylated between 4 and 8 h time points because caffeic acid concentrations are the highest for all AX preparations at 8 h (**Table 16**). After 8 h, caffeic acid concentrations start to decrease suggesting that caffeic acid is microbially reduced to form 3-(3,4-dehydroxyphenyl) propionic acid.

The concentration of the phenolic metabolite 3-(4-hydroxy-3-methoxyphenyl) propionic acid increases from 4 to 12 h time points (**Table 16**). At 12 h it peaks and starts to decrease in concentration until the 24 h time point. 3-(4-Hydroxy-3-methoxyphenyl) propionic acid is a product of FA being reduced by gut microorganisms. Because the concentration of 3-(4-hydroxy-3-methoxyphenyl) propionic acid starts to decrease after 12 h more of the metabolite is further transformed into, most likely, 3-(3,4-dihydroxy-phenyl) propionic acid after 12 h than is formed from FA.

The phenolic metabolite, 3-(3,4-dihydroxyphenyl) propionic acid, was present at higher concentration levels at the ending stages of the fermentation, 24 h. 3-(3,4-Dihydroxyphenyl) propionic acid can be formed through two pathways: 1) microbial demethylation of 3-(4-hydroxy-3-methoxyphenyl) propionic acid or 2) microbial reduction of caffeic acid (**Table 17**).

The least abundant phenolic metabolite observed was 3-(3-hydroxyphenyl) propionic acid. The low concentrations of 3-(3-hydroxyphenyl) propionic acid in all fermented preparations reflect that this compound is most likely the end product of the fermentation of FA. Although a decarboxylation of this compound was described in earlier studies, other studies could not confirm this (Braune, Bunzel et al, 2009). Low concentrations of 3-(3-hydoxyphenyl) propionic acid could be analyzed at later points of the fermentation study, 12 or 24 h, indicating a slow formation of this metabolite. 3-(3-Hydroxyphenyl) propionic acid is produced by microbial dehydroxylation of 3-(3,4-dehydroxyphenyl) propionic acid (**Table 17**).

Table 16. Individual phenolic metabolites found in non-cross-linked and cross-linked arabinoxylan samples during the *in vitro* fermentation (μM)[fghi]

	Caffeic Acid				Ferulic Acid				3-(4-Hydroxy-3-methoxyphenyl) Propionic Acid			
Sample	**4 h**	**8 h**	**12 h**	**24 h**	**4 h**	**8 h**	**12 h**	**24 h**	**4 h**	**8 h**	**12 h**	**24 h**
Faecal Control	0.91±0.033[g]	1.08±0.022[i]	1.11±0.079[i]	1.71±0.042[f]	6.14±0.028[i]	1.81±0.098[e]	1.02±0.057[i]	1.14±0.018[h]	0[g]	0[i]	0[i]	0[i]
0.1 M AX	6.65±0.005[a]	19.80±0.016[a]	8.51±0.017[a]	4.89±0.011[a]	78.61±0.012[a]	31.79±0.020[b]	20.94±0.042[a]	7.12±0.028[a]	2.12±0.041[a]	15.43±0.017[a]	31.52±0.034[a]	19.31±0.024[a]
0.5 M AX	3.12±0.076[b]	18.68±0.028[b]	8.19±0.038[b]	3.16±0.015[b]	76.12±0.037[b]	37.52±0.014[a]	18.22±0.058[b]	6.91±0.014[b]	1.98±0.088[b]	11.04±0.016[b]	15.59±0.021[d]	18.02±0.056[b]
1.0 M AX	2.03±0.073[e]	16.21±0.051[c]	7.89±0.051[c]	2.81±0.032[c]	49.97±0.054[c]	37.62±0.044[a]	18.15±0.061[c]	3.61±0.024[e]	1.79±0.021[c]	9.78±0.056[c]	26.66±0.045[b]	17.87±0.067[c]
1.0 M BM AX	2.32±0.040[c]	11.01±0.012[d]	5.21±0.061[d]	2.11±0.052[d]	46.68±0.021[d]	31.60±0.019[b]	8.27±0.043[h]	1.61±0.019[g]	1.50±0.018[d]	9.65±0.074[d]	13.45±0.047[e]	8.12±0.015[e]
0.1 M X AX	2.17±0.022[d]	10.01±0.071[e]	4.76±0.043[e]	1.81±0.061[e]	40.97±0.017[e]	22.14±0.064[c]	14.12±0.054[d]	5.39±0.064[d]	1.12±0.017[e]	5.38±0.056[e]	16.12±0.022[c]	12.21±0.019[d]
0.5 M X AX	1.38±0.024[f]	9.34±0.011[f]	4.61±0.052[f]	1.64±0.026[g]	38.45±0.010[f]	17.21±0.020[c]	13.27±0.039[e]	5.62±0.020[c]	1.09±0.019[f]	4.47±0.061[f]	9.70±0.032[f]	5.82±0.041[g]
1.0 M X AX	1.41±0.010[f]	9.21±0.020[g]	3.99±0.002[g]	1.39±0.056[h]	36.62±0.017[g]	22.01±0.031[c]	11.21±0.028[f]	2.92±0.061[f]	0[g]	3.71±0.077[g]	8.51±0.045[g]	5.96±0.099[f]
1.0 M BM X AX	0.78±0.031[h]	5.21±0.020[h]	2.12±0.023[h]	1.21±0.054[i]	14.44±0.053[h]	11.81±0.022[d]	8.65±0.087[g]	1.62±0.052[g]	0[g]	2.12±0.007[h]	7.16±0.003[h]	3.31±0.018[h]

[f]standard deviations were calculated from 3 replicates
[g]values with different letters represent statistical difference ($P<0.05$) within each column
[h]value comparison of individual phenolic metabolites between samples at each time point
[k]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

Table 17. Individual phenolic metabolites found in non-cross-linked and cross-linked arabinoxylan samples during the *in vitro* fermentation continued (μM)[fghi]

	3-(3.4-Dihydroxyphenyl) Propionic Acid				3-(3-Hydroxyphenyl) Propionic Acid			
Sample	**4 h**	**8 h**	**12 h**	**24 h**	**4 h**	**8 h**	**12 h**	**24 h**
Faecal Control	0[i]	0[h]	0[i]	0[i]	0[a]	0[b]	0[g]	0[e]
0.1 M AX	3.41±0.056[b]	5.56±0.043[a]	8.02±0.021[a]	27.41±0.017[a]	0[a]	0[b]	0.36±0.0002[a]	0.56±0.0001[a]
0.5 M AX	3.98±0.032[a]	5.54±0.051[a]	7.49±0.018[b]	16.49±0.043[b]	0[a]	0[b]	0.21±0.0001[d]	0.25±0.0005[c]
1.0 M AX	3.31±0.021[c]	4.49±0.017[b]	5.26±0.012[c]	13.79±0.066[d]	0[a]	0[b]	0.24±0.0001[c]	0.26±0.0005[bc]
1.0 M BM AX	2.16±0.051[e]	2.81±0.021[d]	3.12±0.043[g]	9.28±0.073[e]	0[a]	0[b]	0[g]	0.12±0.0003[d]
0.1 M X AX	2.19±0.012[d]	3.96±0.042[c]	4.41±0.064[e]	14.51±0.074[c]	0[a]	0.32±0.0003[a]	0.32±0.0001[b]	0.32±0.0003[b]
0.5 M X AX	2.14±0.021[f]	2.38±0.066[e]	4.81±0.065[d]	8.02±0.043[g]	0[a]	0[b]	0.18±0.0002[e]	0.24±0.0004[c]
1.0 M X AX	1.82±0.023[g]	1.90±0.086[f]	4.27±0.031[f]	8.12±0.021[f]	0[a]	0[b]	0.13±0.0001[f]	0.19±0.0004[c]
1.0 M BM X AX	0.81±0.082[h]	1.02±0.014[g]	1.29±0.071[h]	3.58±0.081[h]	0[a]	0[b]	0.19±0.0002[de]	0.11±0.0004[d]

[f]standard deviations were calculated from 3 replicates
[g]values with different letters represent statistical difference ($P<0.05$) within each column
[h]value comparison of individual phenolic metabolites between samples at each time point
[i]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

4.2.6 Metabolite formation depending on the arabinoxylan preparation:

Phenolics are suggested to have biological and nutritional benefits mainly through their antioxidant activity. Research shows that certain phenolic compounds can be metabolized by gut microorganisms in the digestive system through a series of enzymatic/chemical reactions thus altering their antioxidant and other potentially health-beneficial activities. In this study, total phenolic metabolites/compounds were calculated as the sum of caffeic acid, FA, and phenyl propionic acids as these were the main metabolites observed (**Table 18**). The highest concentration of total phenolic metabolites/compounds were detected after 4 and 8 h. However, this does not give any information about the total antioxidant activity since different phenolic compounds have different antioxidant activities. Also, it is not known how well these compounds are re-absorbed from the colon. Results show differences between cross-linked and non-cross-linked preparations in relationship to total phenolic metabolites/compounds mainly due to the initial amount of AX-bound FA.

Table 18. Total amount of phenolic metabolites within fermentation vessel from *in vitro* fermentation (μM)[ij]

Sample	4 h	8 h	12 h	24 h
Control	7.05±0.020[i]	2.89±0.074[f]	2.13±0.021[i]	2.85±0.012[h]
0.1 M AX	90.79±0.021[a]	72.58±0.078[a]	68.99±0.013[a]	58.73±0.036[a]
0.5 M AX	85.2±0.021[b]	72.7±0.090[a]	49.49±0.043[c]	45.57±0.028[b]
1.0 M AX	57.1±0.059[c]	68.41±0.091[a]	57.96±0.080[b]	38.08±0.072[c]
1.0 M BM AX	52.66±0.046[d]	55.07±0.010[b]	30.05±0.029[f]	21.12±0.065[e]
0.1 M X AX	46.45±0.018[e]	41.49±0.044[c]	39.41±0.017[d]	35.92±0.077[d]
0.5 M X AX	43.06±0.054[f]	33.40±0.077[d]	32.39±0.004[e]	21.34±0.070[e]
1.0 M X AX	39.85±0.088[g]	36.88±0.042[cd]	27.98±0.083[g]	19.39±0.080[f]
1.0 M BM X AX	16.03±0.047[h]	20.16±0.038[e]	19.22±0.029[h]	10.72±0.090[g]

[i]standard deviations were calculated from 3 replicates
[j]values with different letters represent statistical difference ($P<0.05$) within each column
[k]statistical significance obtained by running Tukey's Studentized Range (HSD) Test

The percentage of original FA recovered as phenolic metabolites can be determined by comparing the total phenolic metabolite concentration (μM) at each time point to the amount of ester-linked FA in the AX preparations used for the fermentation study. Results show that between 34.62% and 86.32% of the original ester-linked FA was recovered as phenolic metabolites (**Table 19**).

The fact that recovery rates only went up to 86.32% may be attributed to multiple factors such as: incomplete recovery of the metabolites, formation of unknown metabolites not included in the analytical procedure, FA still being bound to the polysaccharide.

Table 19. Percentage of original ferulic acid recovered as phenolic metabolites throughout *in vitro* fermentation[ab]

Sample	4 h %	8 h %	12 h %	24 h %
0.1 M AX	62.83	50.22	47.74	40.64
0.5 M AX	64.31	54.86	37.35	34.39
1.0 M AX	86.32	53.43	45.28	29.75
1.0 M BM AX	66.32	58.27	49.26	34.62
0.1 M X AX	81.77	73.04	69.38	63.23
0.5 M X AX	86.12	66.89	64.78	46.89
1.0 M X AX	80.56	72.54	63.59	44.06
1.0 M BM X AX	76.61	75.90	80.18	50.56

[a]percentages were calculated from 3 replicates
[b]individual percentages calculated from original ferulic acid amounts for each individual sample

4.3 Conclusions and future research avenues

In conclusion, AX were extracted from corn bran under four different extraction conditions, oxidatively cross-linked, fully characterized, and microbial fermentability of

both non-cross-linked and cross-linked samples determined through analysis of fermentation profiles. Characterization of AX preparations showed that cross-linking decreased the amount of AX-bound FA but increased the amount of FA dimers. In addition, higher concentrations of extraction conditions, molarity of NaOH, altered the characterization of AX giving yield to lower amounts of FA (both monomeric and dimeric) due to alkaline conditions cleaving the ester linkages. Fermentation profiles showed cross-linked samples have higher pH values, lower gas volumes, lower SCFA concentrations, and lower phenolic metabolite concentrations compared to non-cross-linked samples. Lower/delayed fermentability was seen with the cross-linked samples which could provide benefits such as decreased gas discomfort and higher production of SCFA in the distal colon.

Key topics to consider when moving forward on this area of research include analyzing the effects buffer salts have on the fermentation; looking at a fermentation beyond 24 h to determine whether or not residual non-fermented material would be fermented or whether it is not fermentable; and analyzing individual phenolic metabolites for antioxidant activity as well as how they are or are not absorbed by the colon.

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Chapter 5. References

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Appendices

Chapter 6. Appendices

6.1. Appendix A. List of instruments

1) pH meter:	Corning
	Corning, NY, USA
2) Orion pH meter	Orion Research, Inc.
	Beverly, MA, USA
3) Magnetic stirrer:	Chemglass
	Vineland, NJ, USA
4) Analytical balance:	Shimadzu
	Columbia, MD,USA
5) Balance:	H&C Weighing Systems™
	Columbia, MD, USA
6) Centrifuge:	BD Diagnostic Systems
	Franklin Lakes, NJ, USA
7) Incubator shaker:	New Brunswick Scientific Co.,Inc.
	Edison, NJ, USA
8) Shaker bath:	Pegasus Scientific Inc.
	Rockville, MD, USA
9) Sonicator:	All Spec Industries
	Wilmington, NC, USA
10) Vacuum oven:	Stifflers Surplus, INC.
	Chandler, AZ, USA
11) Rotovap:	Büchi Corporation
	New Castle, DE, USA

12) Muffle furnace	Thermolyn Company
	USA
13) Freeze dryer:	Labconco Corporation
	Kansas City, MO, USA
14) Freeze dryer:	SP Scientific
	Gardiner, NY, USA
15) HP Chem Station	Hewlett-Packard
	Palo Alto, CA, USA
16) HPLC System	Shimadzu
	Colombia, MD, USA
17) GC-FID system:	Thermo Scientific Inc.,
	Milan, Italy
18) GC System	Hewlett-Packard
	Palo Alto, CA, USA
19) Aquatest titrator:	Photovolt Co.,
	Minneapolis, MN., USA
20) TruSpec ®N:	Leco Corporation,
	St. Joseph, MI., USA
21) Pulverisette 6	Fritsch Company
	Goshen, NY, USA
22) Warring blender	Waring Company
	Calhoun, GA, USA
23) Colworth stomacher	Seward Company
	Port Saint Lucie, FL, USA

24) Spectrophotometer	GMI Inc. (Beckman) Ramsey, MN, USA
25) Quintron breathtracker	Quintron Instrument Company USA

6.2. Appendix B. List of chemicals

Name of chemical	Hazard symbol				Manufacturer details
	Health	**F**ire	**R**eactivity	Personal protection	
Acetic acid	3	2	0	H	Fisher Scientific, Pittsburgh, PA, USA
Acetic anhydride	3	2	0	-	Sigma Aldrich, St. Louis, MO, USA
Acetone	2	3	0	H	Fisher Scientific, Pittsburgh, PA, USA
Acetonitrile	2	3	0	H	Fisher Scientific, Pittsburgh, PA, USA
α-Amylase, Termamyl®	-	-	-	-	Novozymes Denmark
Ammonium bicarbonate	2	0	0	E	Sigma Aldrich, St. Louis, MO, USA
Ammonium hydroxide solution	3	0	0	-	Sigma Aldrich, St. Louis, MO, USA
Calcium chloride	2	0	0	E	Sigma Aldrich, St. Louis, MO, USA
p-Coumaric acid	2	0	0	1	Acros Organics, Fairlawn, NJ, USA
Citric acid	2	1	0	E	Sigma Aldrich, St. Louis, MO, USA
Cobalt chloride	2	0	0	E	Sigma Aldrich, St. Louis, MO, USA
Cysteine hydrochloride	2	0	0	E	Sigma Aldrich, St. Louis, MO, USA
Diethyl ether	2	2	0	H	Sigma Aldrich, St. Louis, MO, USA
Erythritol	1	1	0	E	Acros Organics, Fairlawn, NJ, USA
Ethanol	2	3	0	H	Decon Labs, Inc., King of Prussia, PA, USA
Ethyl acetate	2	3	0	G	Sigma Aldrich, St. Louis, MO, USA
Ferric chloride	3	0	0	-	Sigma Aldrich, St. Louis, MO, USA

Ferulic acid	2	1	0	E	MP Biomedicals Inc., Solon, OH, USA
Fucose	1	1	0	E	Acros Organics, Fairlawn, NJ, USA
Galactose	2	1	0	E	Sigma Aldrich, St. Louis, MO, USA
Galacturonic acid	1	1	0	E	Acros Organics, Fairlawn, NJ, USA
Glucose	0	0	0	-	Sigma Aldrich, St. Louis, MO, USA
Hydrochloric acid (37%)	3	0	1	-	Sigma Aldrich, St. Louis, MO, USA
Inositol	1	1	0	E	Fluka(Sigma Aldrich) St Louis, MO, USA
Laccase	2	1	0	E	Fluka(Sigma Aldrich) St Louis, MO, USA
m-Hydroxydiphenyl	1	1	0	-	MP Biomedicals Inc., Solon, OH, USA
Manganous chloride	2	0	0	E	Fischer Science, Rochester, NY, USA
Methanol	2	3	0	H	Sigma Aldrich, St. Louis, MO, USA
Magnesium sulfate	2	1	0	E	Sigma Aldrich, St. Louis, MO, USA
Mannose	1	1	0	E	Sigma Aldrich, St. Louis, MO, USA
n-Methylimidazole	3	2	0	-	MP Biomedicals Inc., Solon, OH, USA
Methyl sulfoxide	1	2	0	F	Acros Organics, Fairlawn, NJ, USA
Potassium phosphate monobasic	1	0	0	E	Sigma Aldrich, St. Louis, MO, USA
Resazurin	1	0	0	-	Sigma Aldrich, St. Louis, MO, USA
Rhamnose	1	1	0	E	Sigma Aldrich, St. Louis, MO, USA
Sinapic acid	2	0	0	-	Fluka, St. Louis, MO, USA
Sodium bicarbonate	1	0	0	E	Cambrix Bioscience, Walkersville, MD, USA
Sodium hydroxide	3	0	2	J	Fischer Scientific, Pittsburgh, PA, USA
Sodium phosphate dibasic	1	0	0	E	Sigma Aldrich, St. Louis, MO, USA
Sodium phosphate	1	0	0	E	Sigma Aldrich,

monobasic					St. Louis, MO, USA
Sodium tetraborate	2	0	0	-	Sigma Aldrich, St. Louis, MO, USA
Sodium sulfide nonahydrate	3	3	0	E	Science Lab, Houston, TX, USA
Sulfuric acid	3	1	2	-	EM Science, Gibbstown, NJ, USA
Trifluoroacetic acid	3	0	0	H	Alfa Aesar, Ward Hill, MA, USA
Trypticase peptone	0	0	0	-	Dickinson & Company, Sparks, MD, USA

6.3. Appendix C. Additional supporting material

Table 20. Dietary reference intakes (DRI) values for carbohydrates and total fiber[abc]

Nutrient	Function	Life Stage Group	Carbohydrates (g/d)	Total Fiber (g/d)	Selected Food Sources	Adverse effects of excessive consumption
Dietary Fiber	Improves laxation, reduces risk of coronary heart disease, and assists in maintaining normal blood glucose levels.	Infants 0-6 mo 7-12 mo Children 1-3 yr 4-8 yr Males 9-13 yr 14-18 yr 19-30 yr 31-50 yr 50-70 yr >70 yr Females 9-13 yr 14-18 r 19-30 yr 31-50 yr 50-70 yr >70 yr Pregnancy < 18 yr 19-30 yr 31-50 yr Lactation <18 yr 19-30 yr 31-50 yr	 60* 95* **130** **130** **130** **130** **130** **130** **130** **130** **130** **130** **130** **130** **130** **130** **175** **175** **175** **210** **210** **210**	 ND ND 19* 25* 31* 38* 38* 38* 30* 30* 26* 26* 25* 25* 21* 21* 28* 28* 28* 29* 29* 29*	Includes dietary fiber naturally present in grains (such as found in oats, wheat, or unmilled rice) and functional fiber synthesized or isolated from plants or animals and shown to be of benefit to health	Dietary fiber can have variable compositions and therefore it is difficult to link a specific source of fiber with a particular adverse effect, especially when phytate is also present in the natural fiber source. It is concluded that as part of an overall healthy diet, a high intake of dietary fiber will not produce deleterious effects in healthy individuals. While occasional adverse gastrointestinal symptoms are observed when consuming some isolated or synthetic fibers, serious chronic adverse effects have not been observed. Due to the bulky nature of fibers, excess consumption is likely to be self-limiting. Therefore, a UL was not set for individual functional fibers.

[a]This table was adopted from DRI report, see www.nap.edu.

[b]This table presents RDAs in **bold type**, AIs in ordinary type followed by an asterisk (*).

[c]ND=not determined

Table 21. Literature data on arabinoxylan contents and composition in cereals

Source	Total AX (%)	Water-Soluble AX (%)	Reference	A/X Ratio
Corn Bran	29.86	0.28	Hashimoto & Grossmann, 2003	
Whole Wheat	5.77	0.59	Hashimoto & Grossmann, 2003	
Whole Wheat	NA	0.38-0.83	Saulnier, Sado, et al., 2007	
Wheat Bran	19.38	0.88	Hashimoto & Grossmann, 2003	
Whole Barley	6.11	0.35	Hashimoto & Grossmann, 2003	
Whole Barley	3.4-4.1	NA	Izydorczyk, Biliaderis, et al., 1995, 2006	
Whole Barley	NA	0.4-0.88	Oscarsson, Parkkonen, et al., 1997	
Whole Rye	7.6	NA	Bengtsson & Aman, 1991	
Whole Rye	8-12.1	2.6-4.1	Hansen, Jones, et al. 1991	
Rye Bran	NA	1.7	Figueroa-Espinoza, Poulsen, et al., 2004	
Whole Oat	2.73	0.17	Hashimoto & Grossmann, 2003	
Oat Bran	3.5	0.33	Hashimoto & Grossmann, 2003	
Whole Rice	2.64	0.06	Hashimoto & Grossmann, 2003	
Rice Bran	4.84-5.11	0.35-0.77	Hashimoto & Grossmann, 2003	
Wheat Flour	2.35	0.51	Saulnier, Sado, et al., 2007	0.47
Wheat Outer Pericarp	44.1		Saulnier, Sado, et al., 2007	1.14 mg/g
Wheat Aleruone	20.8		Saulnier, Sado, et al., 2007	0.39 mg/g
Rice			Rao & Murariknisha, 2005	0.81
Barley Husks	32.5		Pitkanen, Tuomainen, et al., 2008	0.28
Barley Fiber	50		Pitkanen, Tuomainen, et al., 2008	0.51
Wheat Flour		0.44	Dervilly-Pinel, Rimsten, et al., 2001	0.63
Barley Flour		0.46	Dervilly-Pinel, Rimsten, et al., 2001	0.56
Rye Flour		2.1	Dervilly-Pinel, Rimsten, et al., 2001	0.54
Triticale Flour		0.55	Dervilly-Pinel, Rimsten, et al., 2001	0.69
Wheat Bran	1.01-2.81		Craeyveld, Swennen, et al., 2010	0.68-0.96
Whole Grain	67-121 g/kg	36 g/kg dry matter	Saini & Henry, 1989	0.54-0.59
Whole Grain	73 g/kg	22 g/kg	Vinkx, Delcour, et al., 1995	0.59
Whole Grain	90-110 g/kg	22-47 g/kg	Back Knudsen, Laerke, et al., 1997	0.49-0.65

Whole Grain	80-121 g/kg	26-40 g/kg	Hansen, Jones, et al. 2003	0.59-0.69
Whole Grain	90 g/kg	24 g/kg	Andersson, 2009	0.53
Wheat Flour		0.5	Hartmann, Piber, et al, 2005	0.83
Rye Flour		1.99	Hartmann, Piber, et al, 2005	0.59
Whole Rye		0.66	Delcour & Bleukx, 1999	0.63
Rye Outer Layer	32.92		Cyran & Anderson, 2005	0.52
Wheat Flour	0.68	0.84	Cleemput, Laere, et al., 1997	0.52
Wheat Bran		10.5	Beaugrand, Debeire, et al., 2004	0.16
Wheat Bran	42% dry matter		Pollet, Craeyveld, et al., 2012	0.61
Whole Rye	75 g/kg DM		Glitso & Knudsen 1998	0.67

6.4. Appendix D. Additional method details

Table 22. Xylose concentration levels for the calibration of the total carbohydrate analysis method

Solution concentration (µg/mL)	Addition of deionized water to 1 mL stock solution (1 mg xylose/ mL)
10	100
50	20
90	11
130	7.69
170	5.88
210	4.76

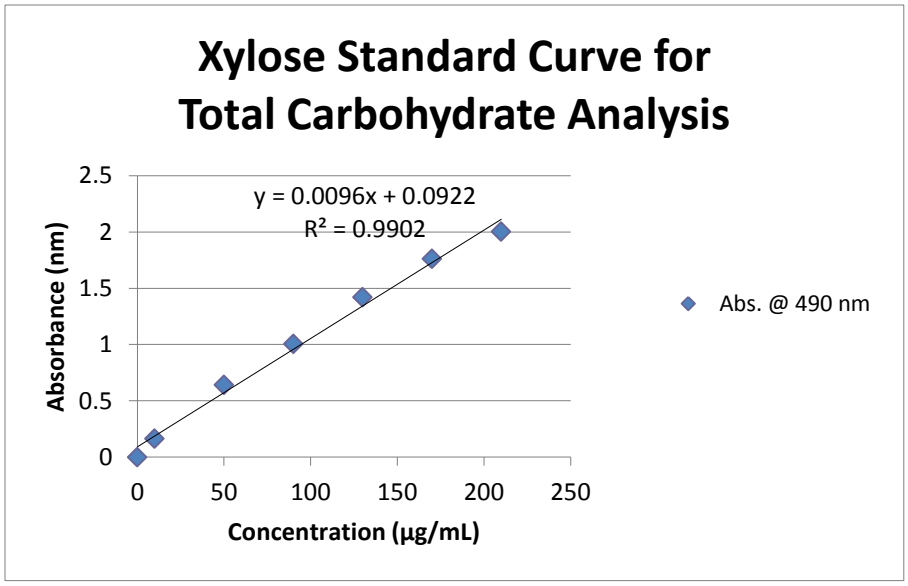

Figure 3. Calibration curve for xylose used for total carbohydrate quantification

Table 23. Correction factors for the analysis of monosaccharides in form of their alditolacetates against acetylated inositol

Monosaccharide	Calculated Correction Factor
Rhamnose	1.78
Arabinose	0.97
Xylose	0.91
Mannose	1.87
Galactose	1.20
Glucose	1.31

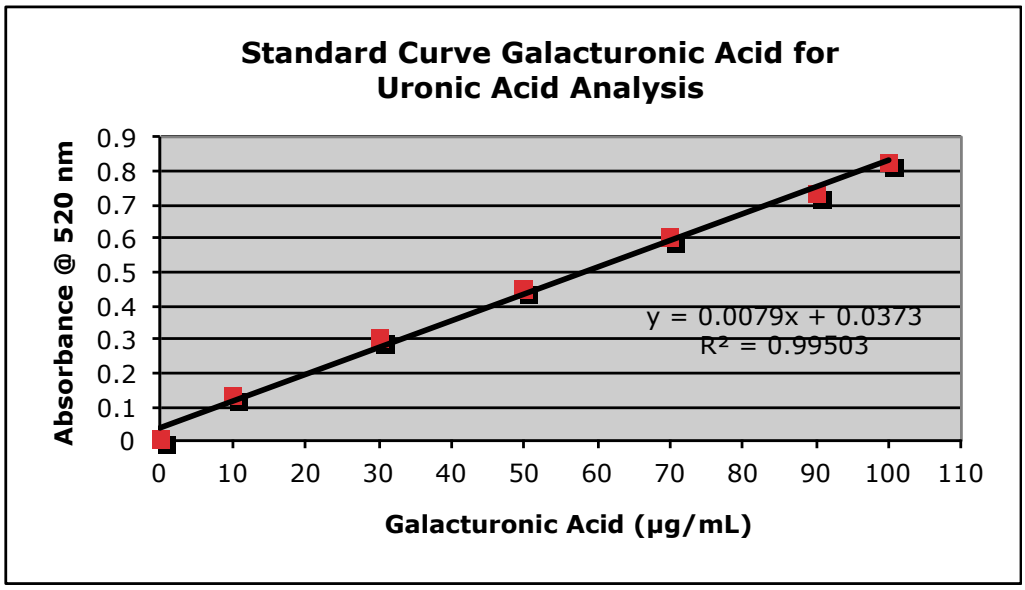

Figure 4. Calibration curve for galacturonic acid used for uronic acid quantification

Table 24. Correction factors used for quantification of monomeric hydroxycinnamic acids using caffeic acid as internal standard

Ferulate Monomer	Calculated Correction Factor
caffeic acid	1
trans-*p*-coumaric acid	0.44
trans-ferulic acid	0.75
cis-ferulic acid	1.11

Table 25. Correction factors for ferulate dimers against 5-5(methylated)-dehydrodiferulic acid as internal standard

Ferulate Dimer	Correction Factor
8-8(cyclic)-dehydrodiferulic acid	4.35
8-8(noncyclic)-dehydrodiferulic acid	1.94
5-5-dehydrodiferulic acid	1.50
8-O-4-dehydrodiferulic acid	0.84
8-5(cyclic)-dehydrodiferulic acid	1.51

5-5 methylated-dehydrodiferulic acid	1

Table 26. Arabinoxylan addition to vessels for *in vitro* fermentation

Cross-linked samples	Total carbohydrates (%)	Added to fermentation vessel (g)	Total carbohydrates in sample added (amount added * % total carbohydrates)
0.1 M X	54.14	0.83	0.45
0.5 M X	53.81	0.83	0.44
1.0 M X	53.72	0.83	0.44
1.0 M BM X	53.72	0.83	0.44
Non-cross-linked			
0.1 M	94.38	0.5	0.47
0.5 M	93.38	0.5	0.47
1.0 M	93.41	0.5	0.47
1.0 M BM	90.85	0.5	0.45

Table 27. Fermentation media formulation

Ingredients	Recipe for 1 L solution (g)	Recipe for 5 L solution (g)	Actual amount (g)
Trypticase peptone	2.49	12.45	12.4588
Ammonium bicarbonate	1.0	5.0	5.0134
Sodium bicarbonate	8.75	43.75	43.7591
Anhydrous sodium phosphate, dibasic	1.43	7.15	7.1540
Anhydrous potassium phosphate monobasic	1.55	7.75	7.7514
Magnesium sulfate	0.6	3.0	3.0125
Reaszurim	0.000096	0.00012	0.0016
Calcium chloride	0.1242976	0.621488	0.6264
Manganous chloride	0.07928172	0.3964086	0.3942
Cobalt chloride	0.0194758	0.0973792	0.1046

Ferric chloride	0.00648816	0.0324408	0.0381

Table 28. Correction factors against ethyl butyrate as internal standard used for short chain fatty acid quantification

	Correction factors		
Concentration	Acetate	Propionate	Butyrate
3 µmol	0.09	0.18	0.32
6 µmol	0.09	0.19	0.31
12 µmol	0.19	0.39	0.62
25 µmol	0.43	0.89	1.39
50 µmol	0.94	1.89	2.90

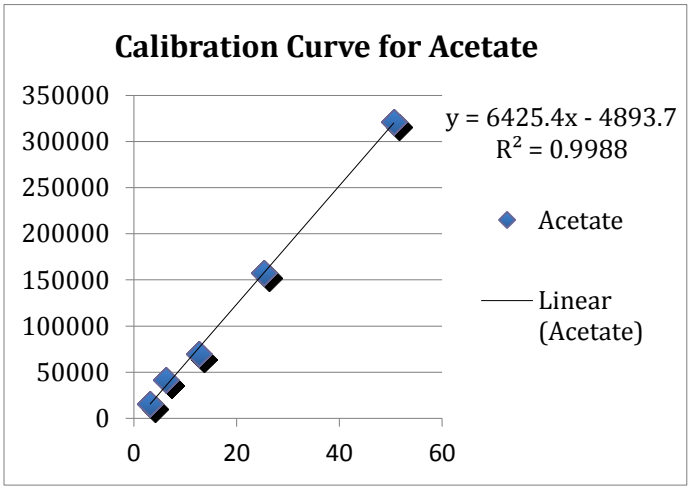

Figure 5. Calibration curve for acetate used for short chain fatty acid quantification

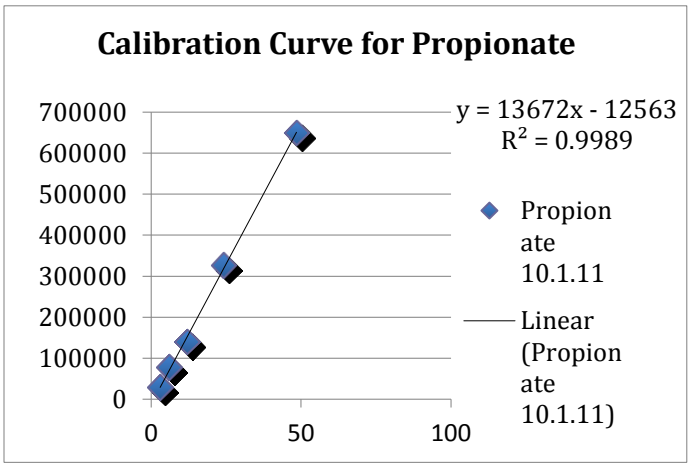

Figure 6. Calibration curve for propionate used for short chain fatty acid quantification

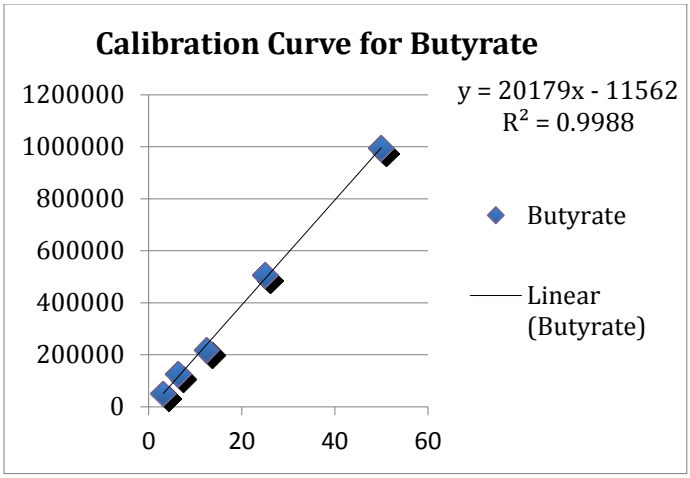

Figure 7. Calibration curve for butyrate used for short chain fatty acid quantification

Table 29. Correction factors used for the quantification of phenolic metabolites using *o*-hydroxy cinnamic acid as internal standard

Phenolic Metabolite	Calculated Correction Factor
3-(3,4-dihydroxyphenyl) propionic acid	0.81
trans-caffeic acid	3.29
p-hydroxy cinnamic acid	4.92
3-(4-hydroxy-3-methoxyphenyl) propionic acid	0.69
trans-ferulic acid	2.79
o-hydroxy-cinnamic acid	1

D-1. Detailed fecal collection instructions

Each subject was given the proper materials for fecal collection including: toilet device, white collection container, collection bag, sharpie, ice packs, anaerobic packet (inside silver pouch), 2 anaerobic clips (blue), anaerobic bag (clear non-ziploc), and cooler. Subjects were asked to collect their fecal matter either late the evening before the fermentation study or early the day of the fermentation study. To collect fecal matter they executed the following steps: placed toilet device under the toilet seat, lined the white collection container with the collection bag, put white collection container lined with collection bag into the toilet device. Subjects defecated making sure not to

incorporate any urine or toilet paper into the collection bag. Removed collection bag from white container and folded the unsealed bag over to place into the anaerobic bag. Tore open anaerobic packet, removed the pouch, placed into the anaerobic bag along with the collection bag. The pouch was not to come into direct contact with the sample. Excess air was removed from the anaerobic bag, anaerobic clips were snapped onto open end of anaerobic bag to seal it closed. Clips should be secure and immobile when done. Donors were prompted to immediately place storage bag into the cooler with frozen ice packs. Samples were then used for the fermentation study.

D-2. Gas analysis equations

Gas concentrations were quantitatively calculated using the following equations:

Original concentration calculated using the equation: $\mathbf{A(B^{\wedge C})}$

A= the diluted ppm (number shown on the Quintron Breath Tracker)

B= the amount of ambient air used to dilute (Ex: 1 mL aliquot/ 35 mL ambient air), or (1 mL aliquot/ 50 mL ambient air).

C= the number of times diluted (1^{st}, 2^{nd}, 3^{rd}…..)

6.5. Appendix E. Chromatograms

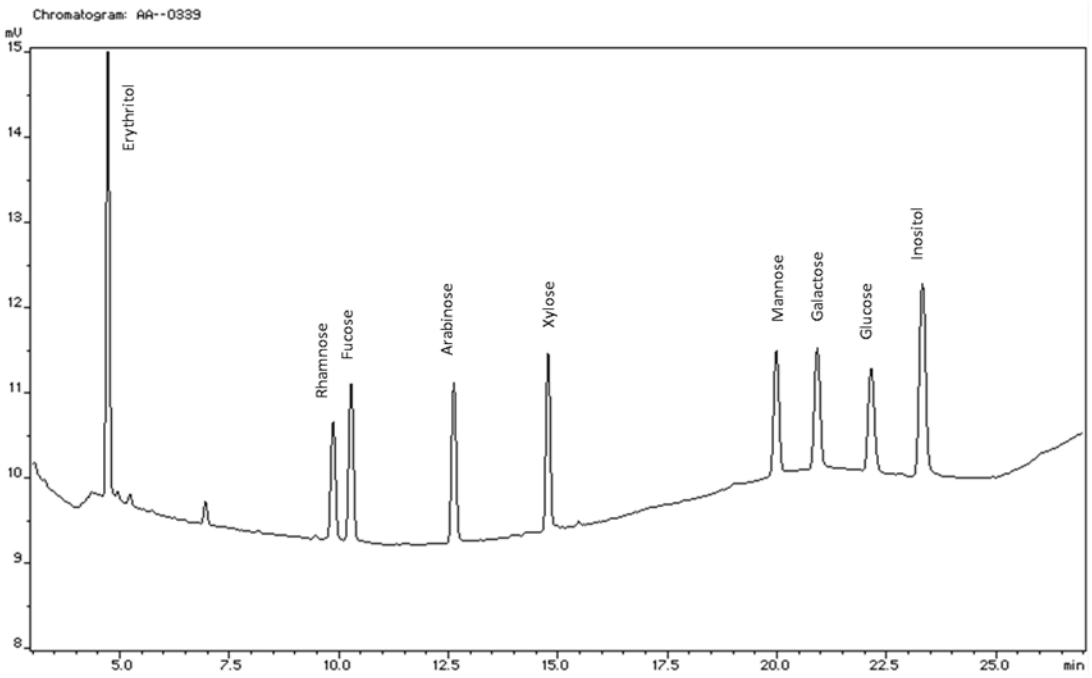

Figure 8: GC-FID chromatogram showing the analysis of standard monosaccharides in form of their aditol acetates

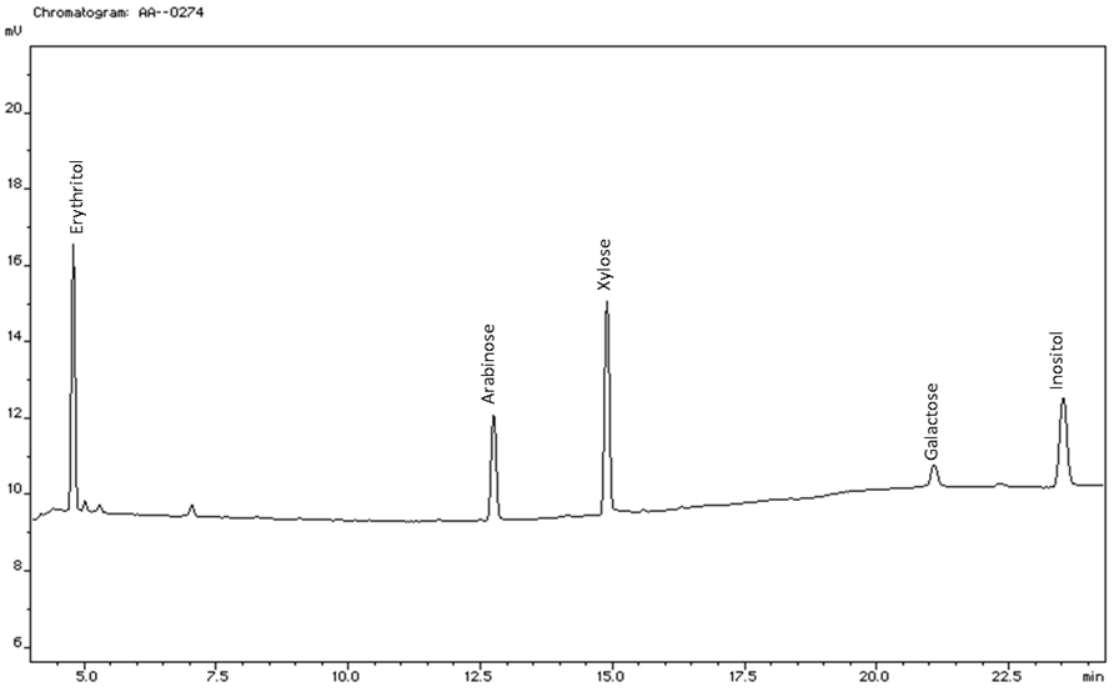

Figure 9: Characterization of the monosaccharide composition of non-cross-linked arabinoxylans extracted by using 0.5 M NaOH. Monosaccharides were analyzed in form of their alditol acetates.

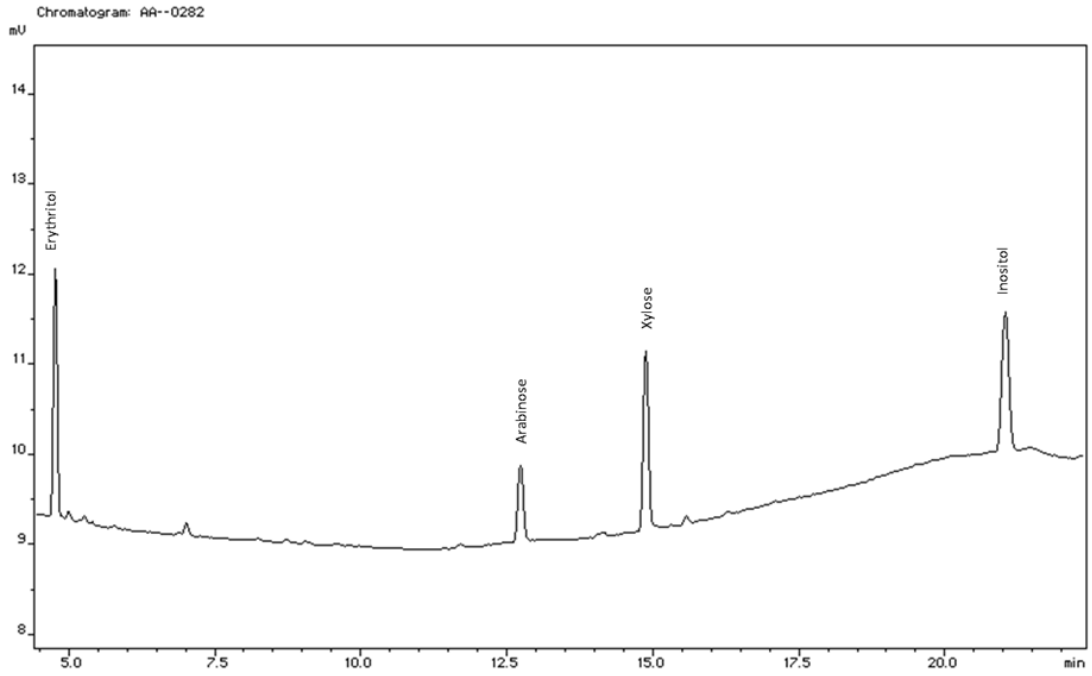

Figure 10: Characterization of the monosaccharide composition of cross-linked arabinoxylans extracted by using 0.5 M NaOH. Monosaccharide's were analyzed in form of their alditol acetates.

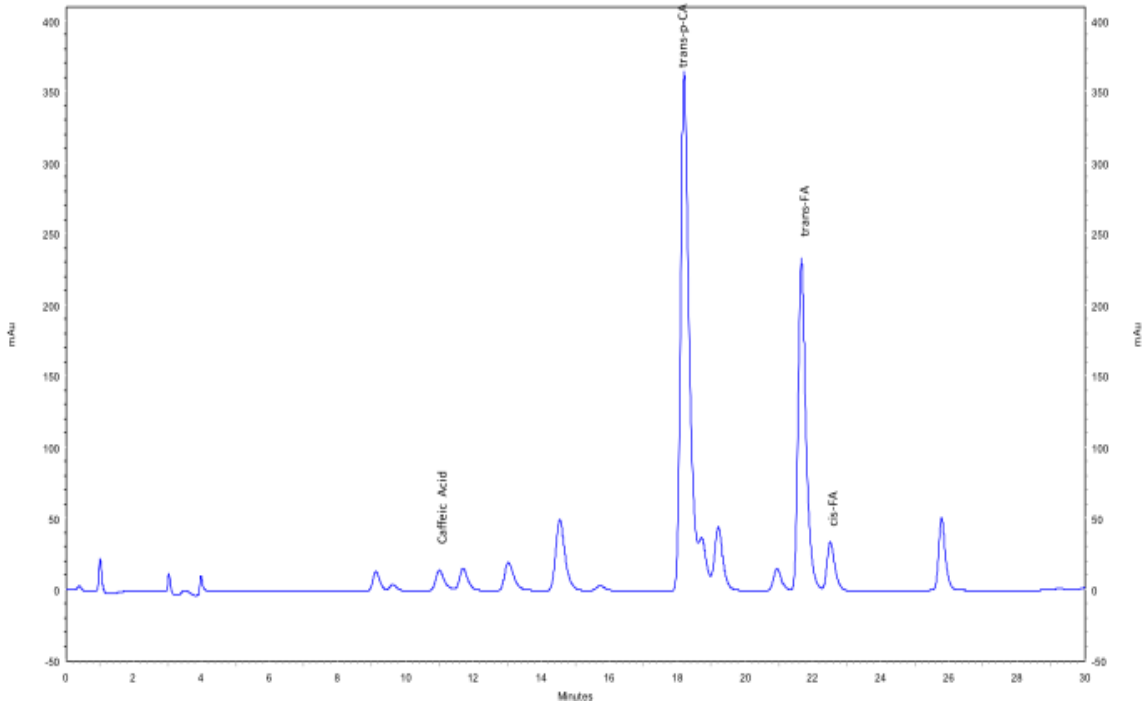

Figure 11: HPLC-UV standard chromatogram showing the analysis of monomeric hydroxycinnamic acids.

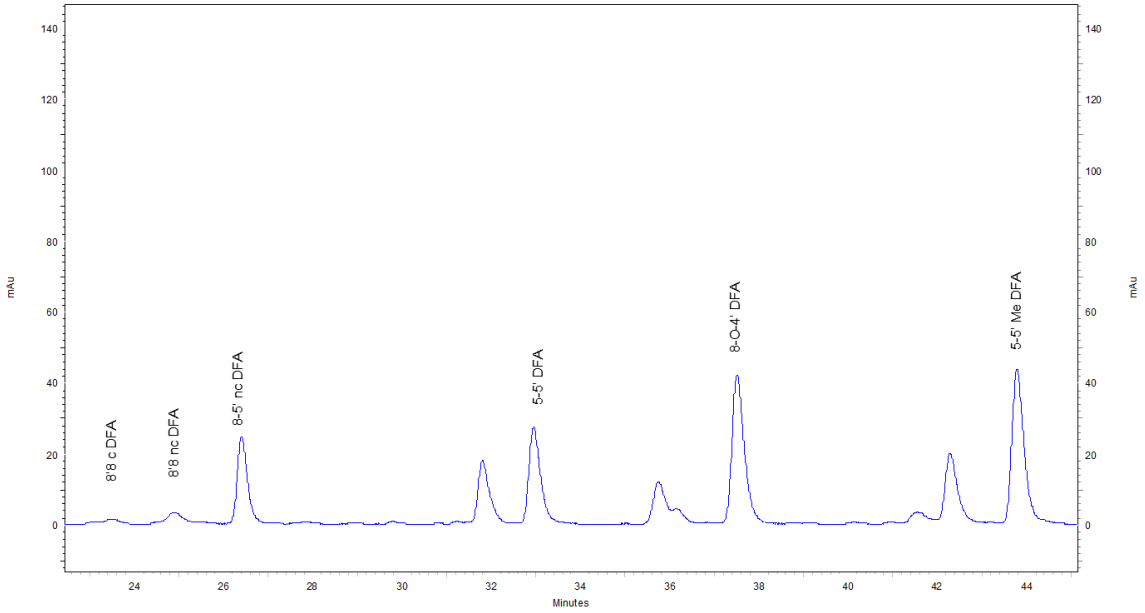

Figure 12: HPLC-UV standard chromatogram showing the analysis of ferulic acid dehydrodimers.

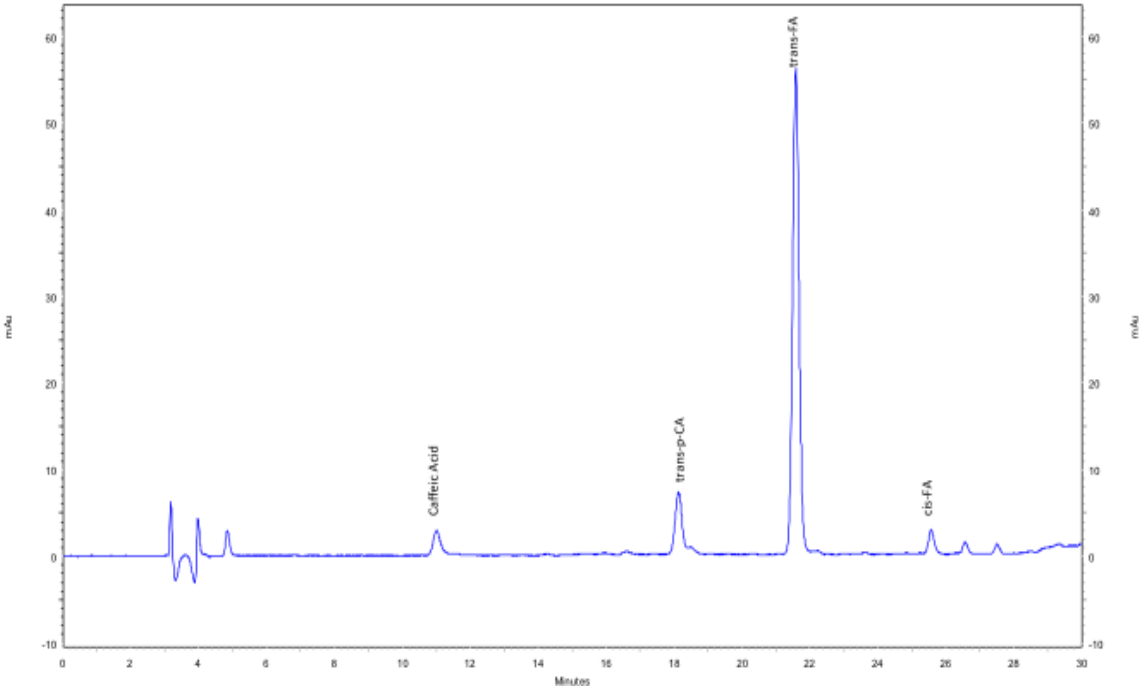

Figure 13: Ferulic acid monomers obtained from analysis of non-cross-linked arabinoxylans extracted by using 0.5 M NaOH.

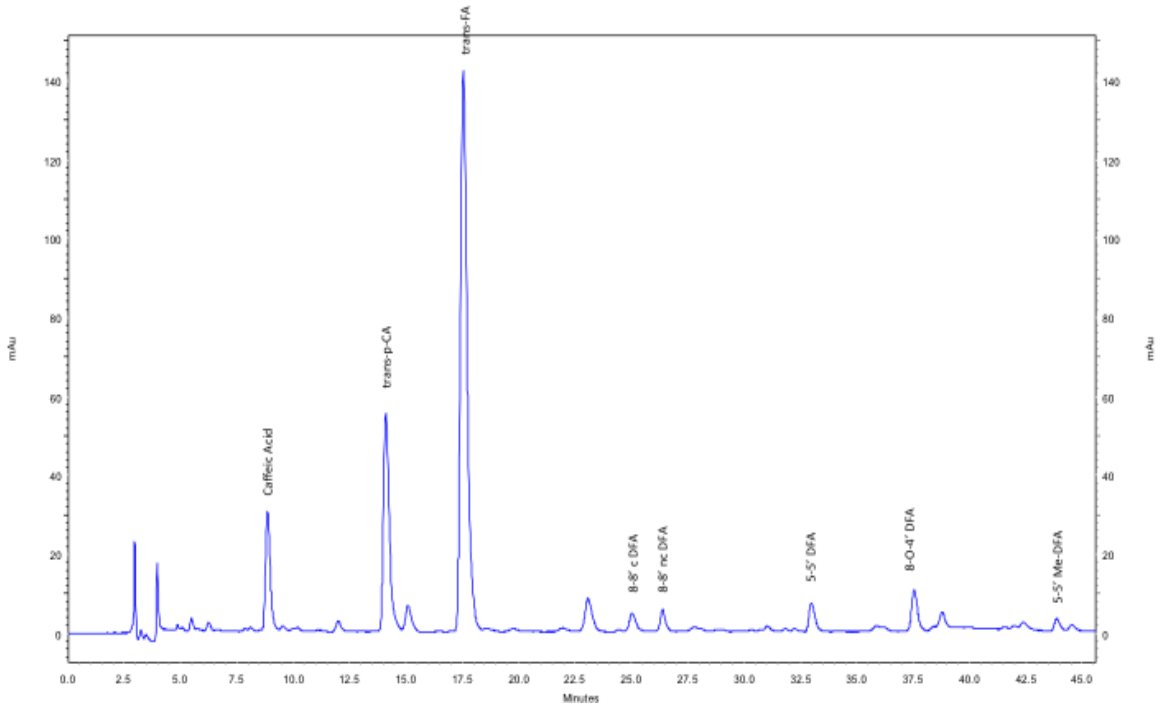

Figure 14: Ferulic acid dehydrodimers obtained from analysis of non-cross-linked arabinoxylans extracted by using 0.5 M NaOH.

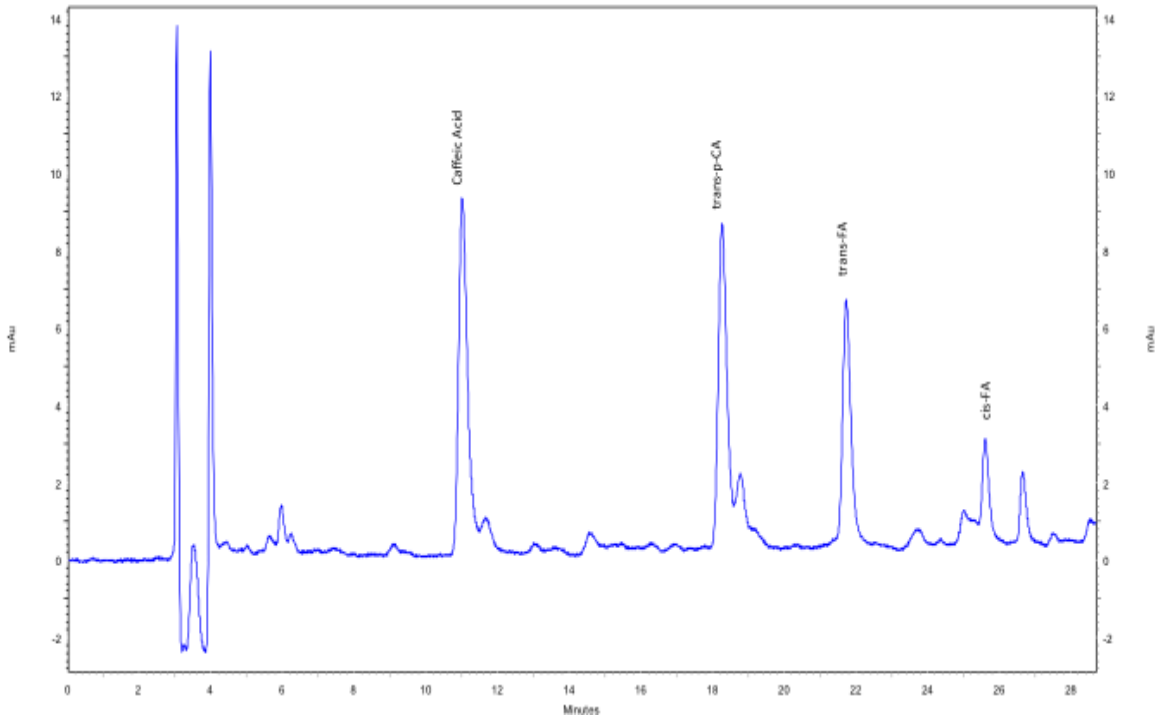

Figure 15: Ferulic acid monomers obtained from analysis of cross-linked arabinoxylans extracted by using 0.5 M NaOH.

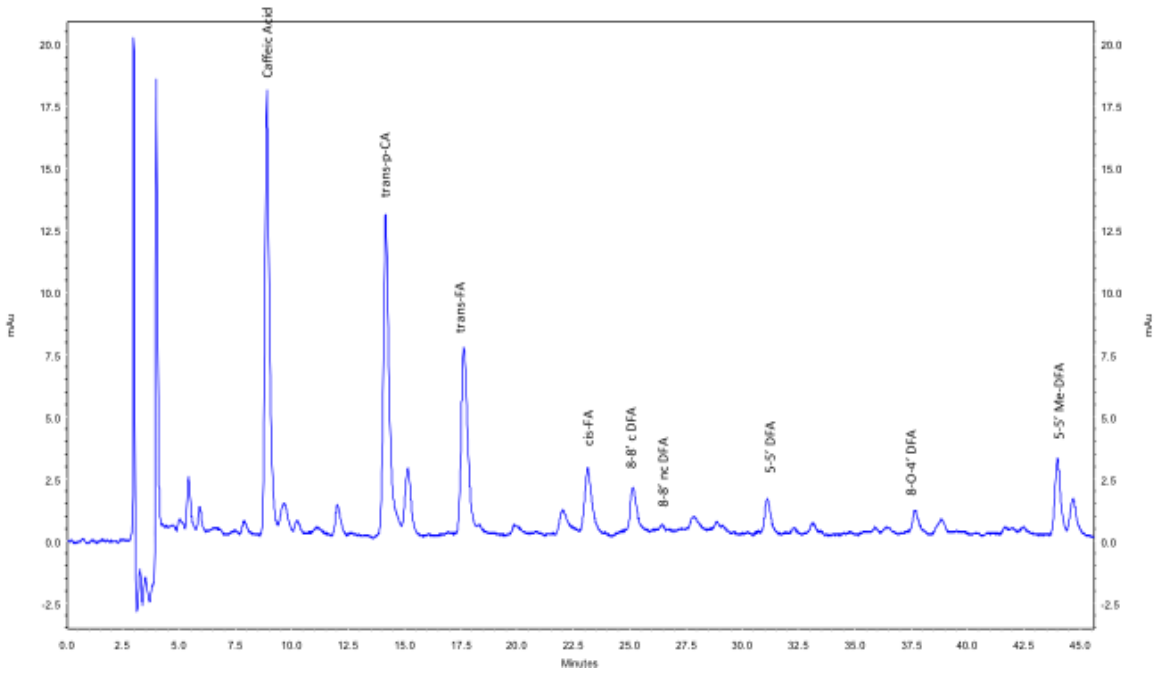

Figure 16: Ferulic acid dehydrodimers obtained from analysis of cross-linked arabinoxylans extracted by using 0.5 M NaOH.

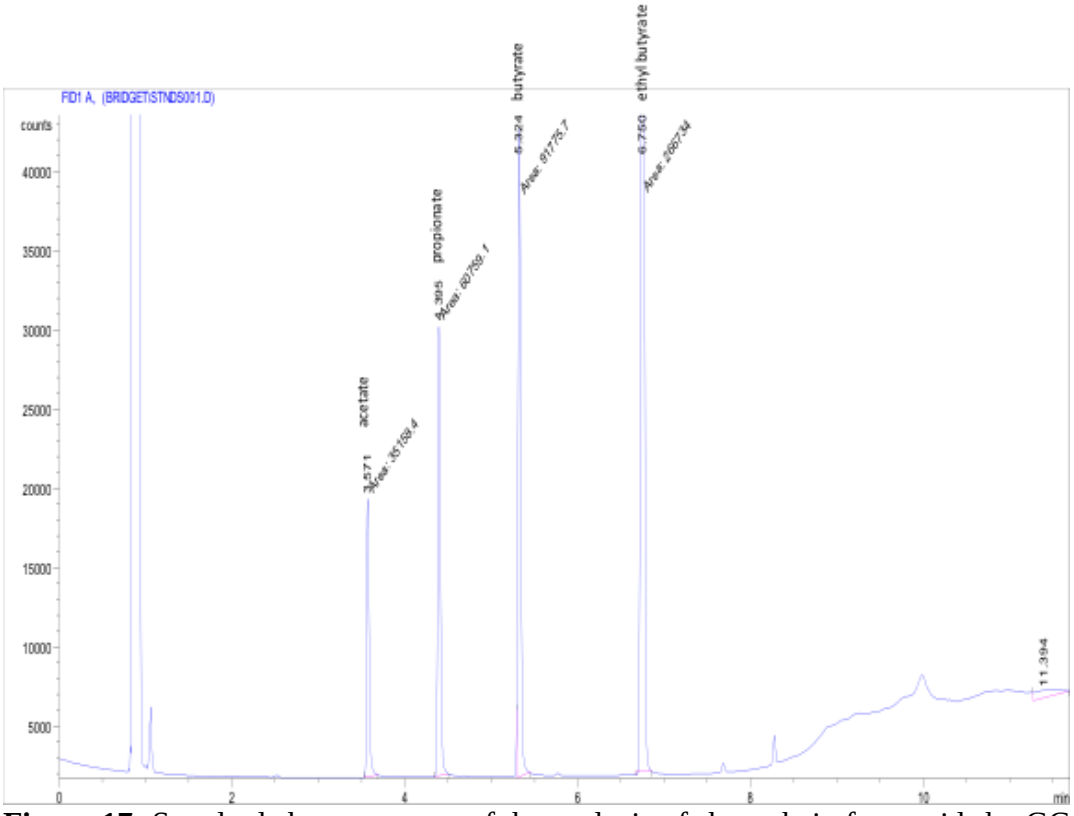

Figure 17: Standard chromatogram of the analysis of short chain fatty acids by GC-FID

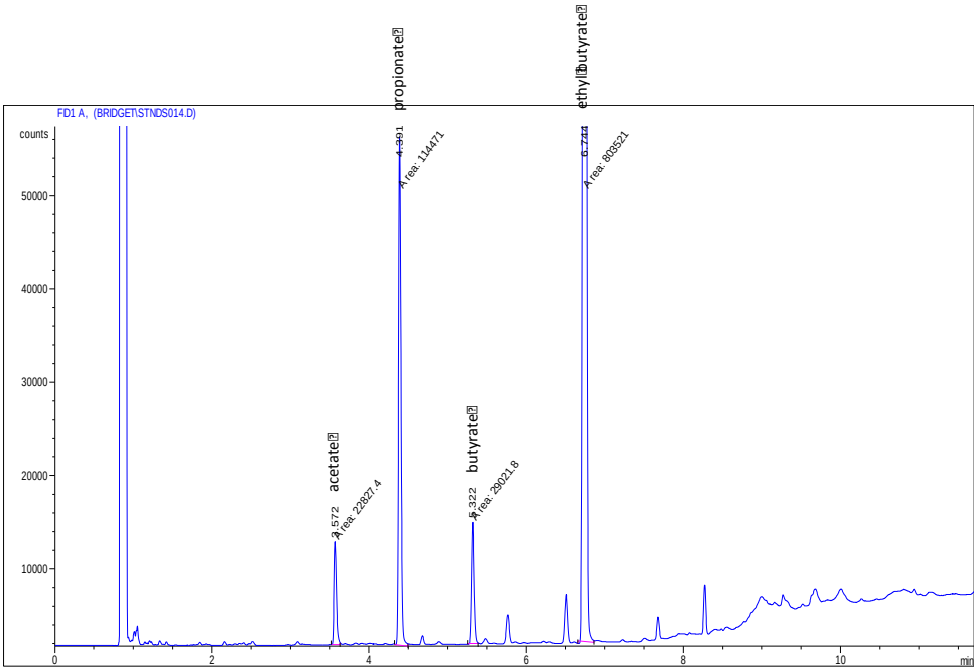

Figure 18: Analysis of the short chain fatty acids obtained from the fermentation (4h) of non-cross-linked arabinoxylans (extracted by using 0.5 M NaOH)

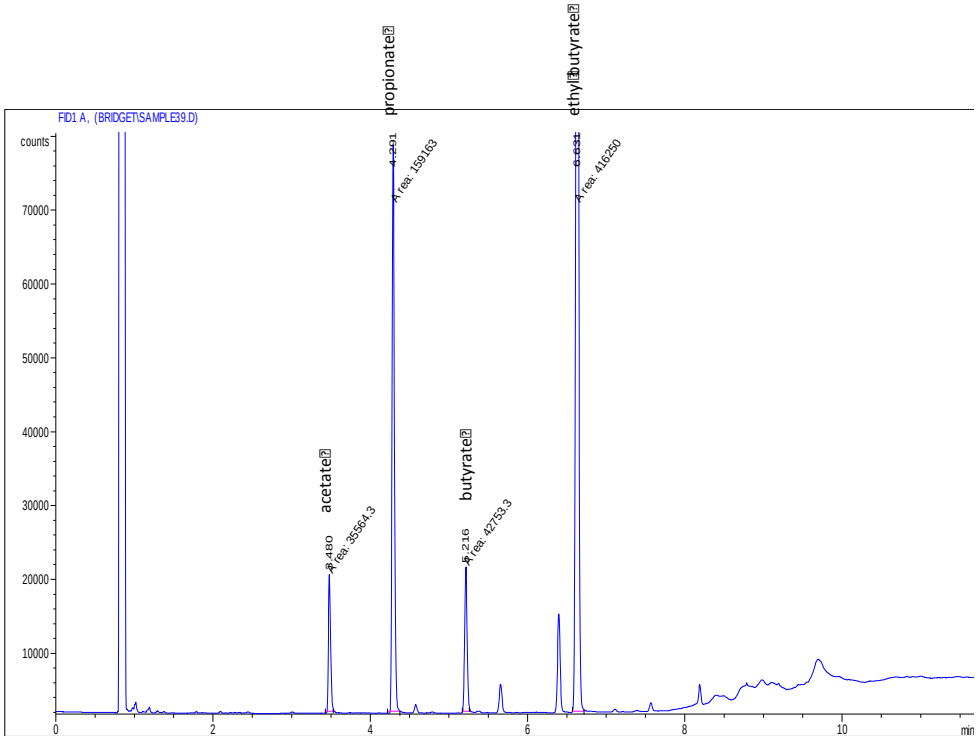

Figure 19: Analysis of the short chain fatty acids obtained from the fermentation (8 h) of non-cross-linked arabinoxylans (extracted by using 0.5 M NaOH)

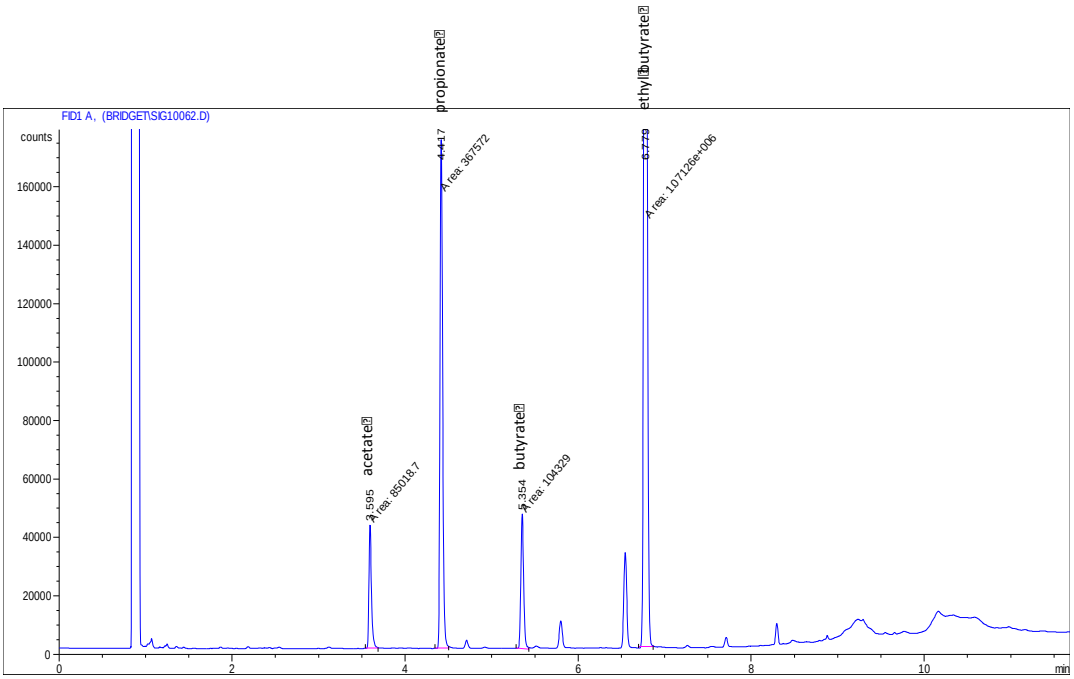

Figure 20: Analysis of the short chain fatty acids obtained from the fermentation (12 h) of non-cross-linked arabinoxylans (extracted by using 0.5 M NaOH)

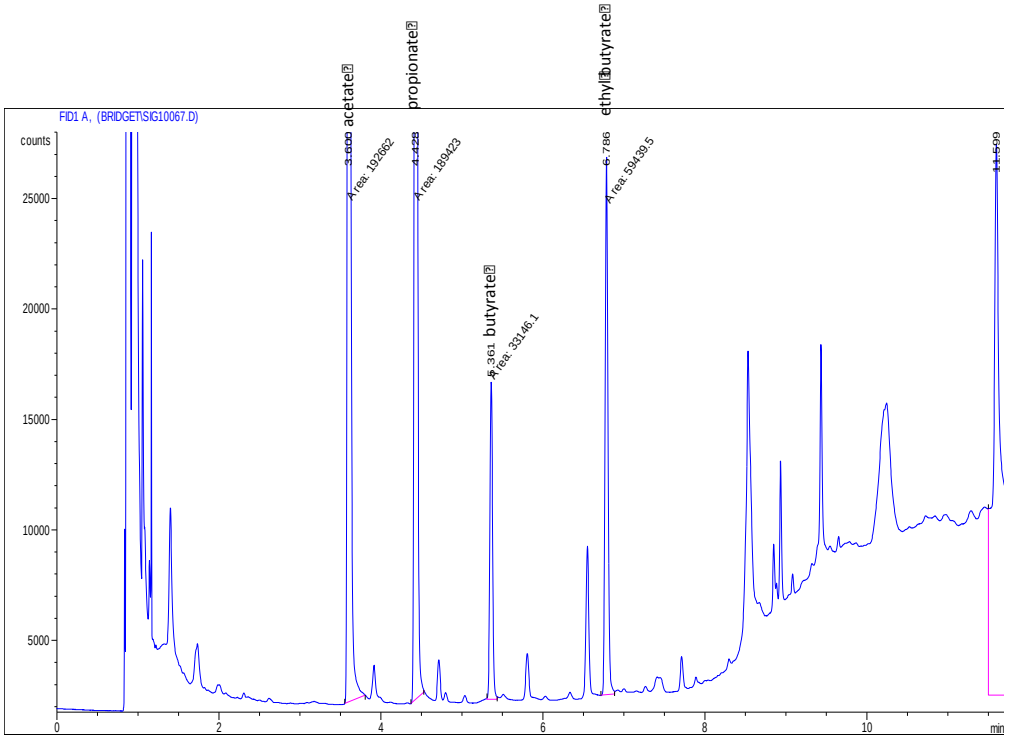

Figure 21: Analysis of the short chain fatty acids obtained from the fermentation (24 h) of non-cross-linked arabinoxylans (extracted by using 0.5 M NaOH)

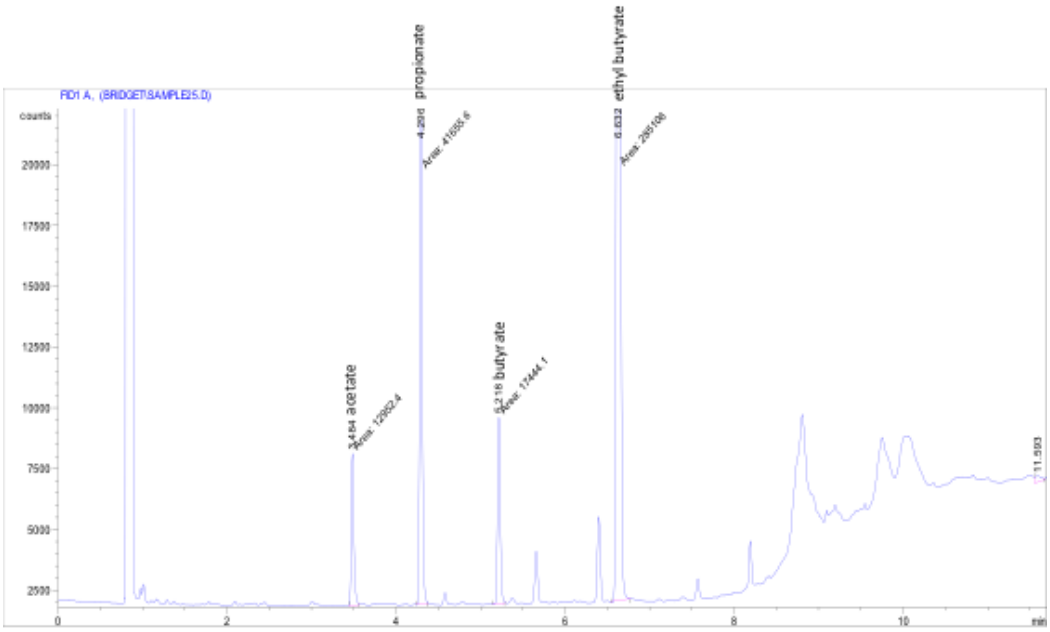

Figure 22: Analysis of the short chain fatty acids obtained from the fermentation (4 h) of cross-linked arabinoxylans (extracted by using 0.5 M NaOH)

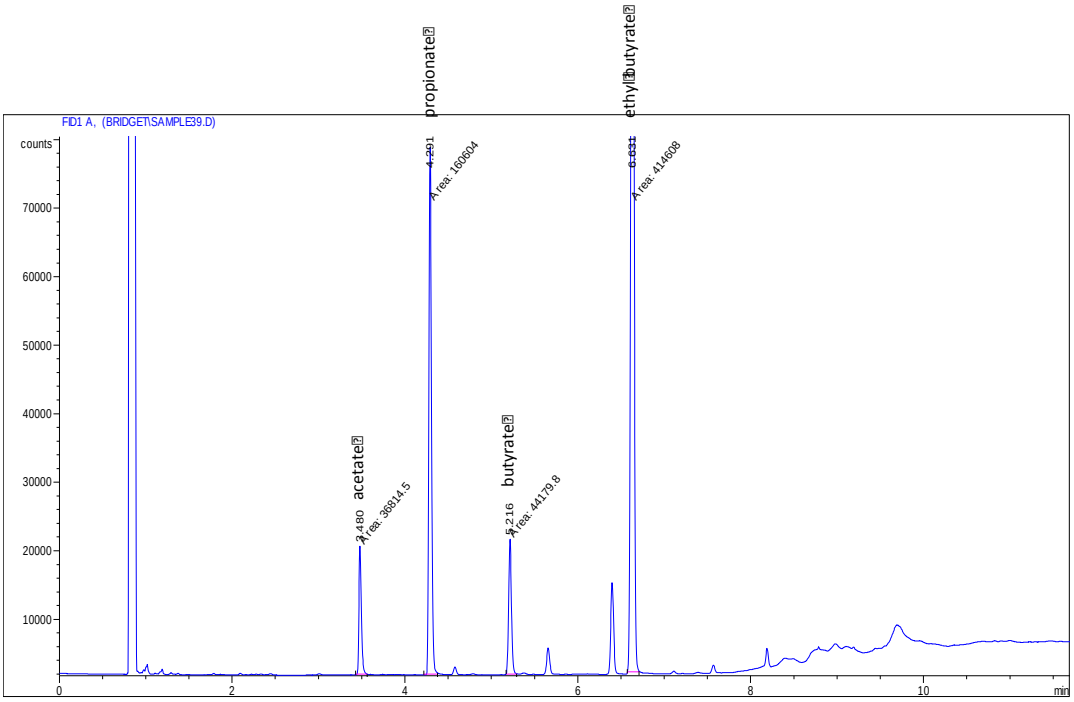

Figure 23: Analysis of the short chain fatty acids obtained from the fermentation (8 h) of cross-linked arabinoxylans (extracted by using 0.5 M NaOH)

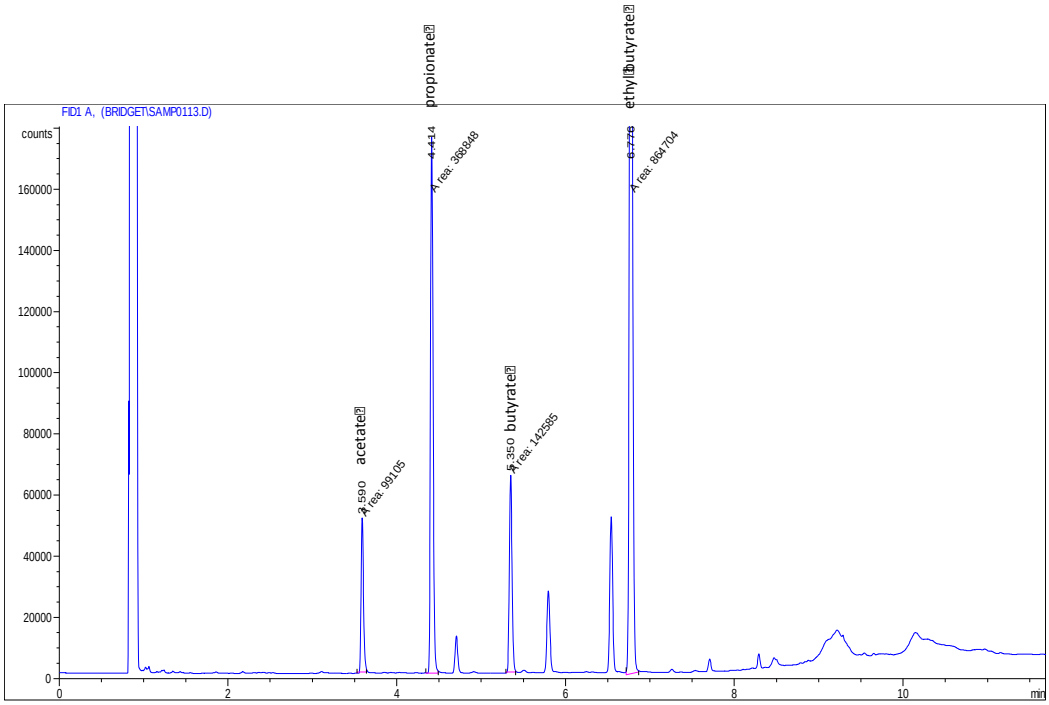

Figure 24: Analysis of the short chain fatty acids obtained from the fermentation (12 h) of cross-linked arabinoxylans (extracted by using 0.5 M NaOH)

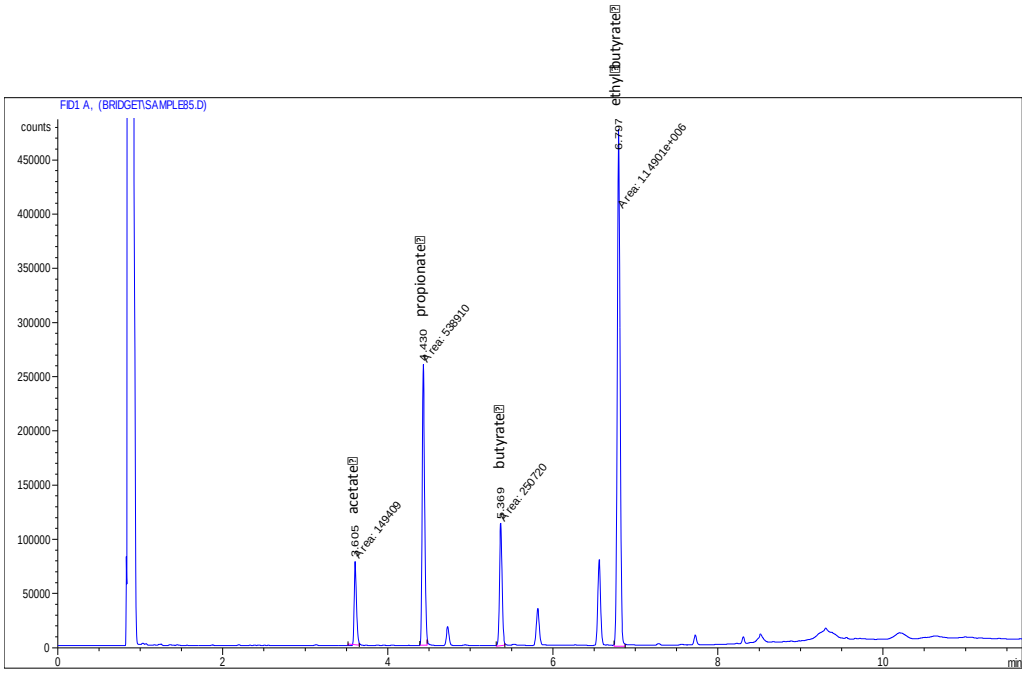

Figure 25: Analysis of the short chain fatty acids obtained from the fermentation (24 h) of cross-linked arabinoxylans (extracted by using 0.5 M NaOH)

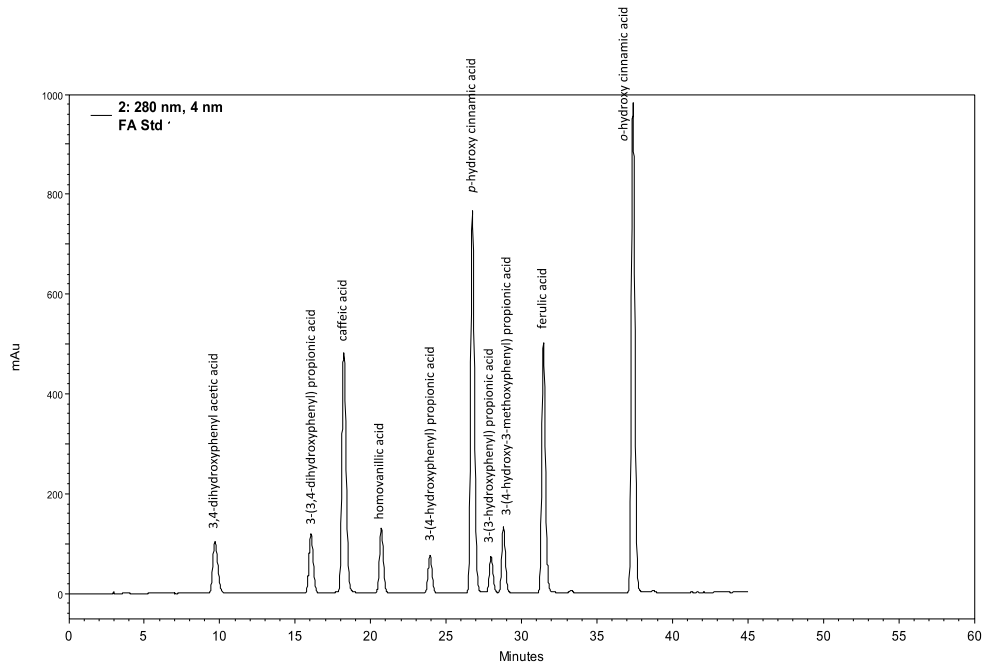

Figure 26: HPLC-UV standard chromatogram showing the analysis of phenolic metabolites (monitored at 280 nm)

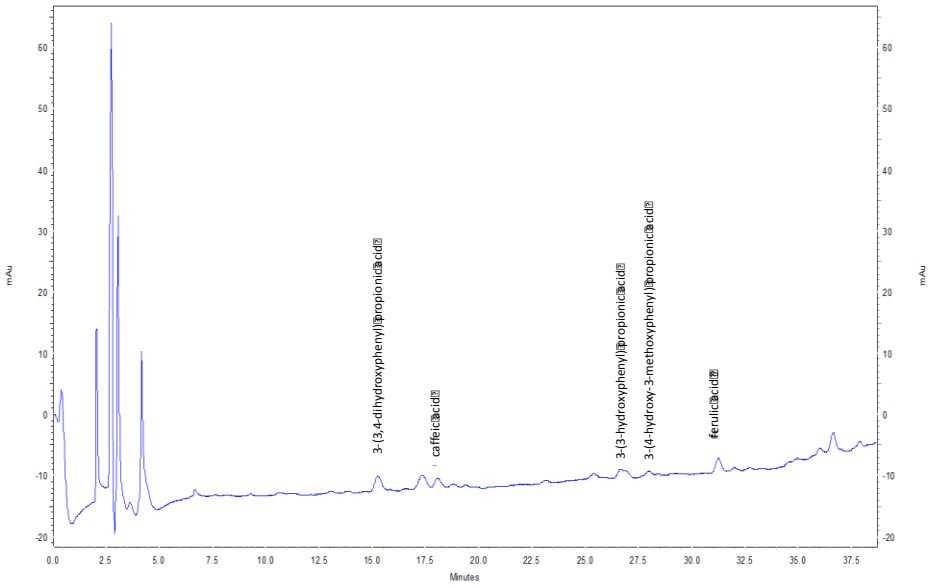

Figure 27: HPLC-UV chromatogram showing the analysis of phenolic metabolites obtained after a 4 h fermentation of non-cross-linked arabinoxylans (extracted using 0.5 M NaOH) (monitored at 280 nm)

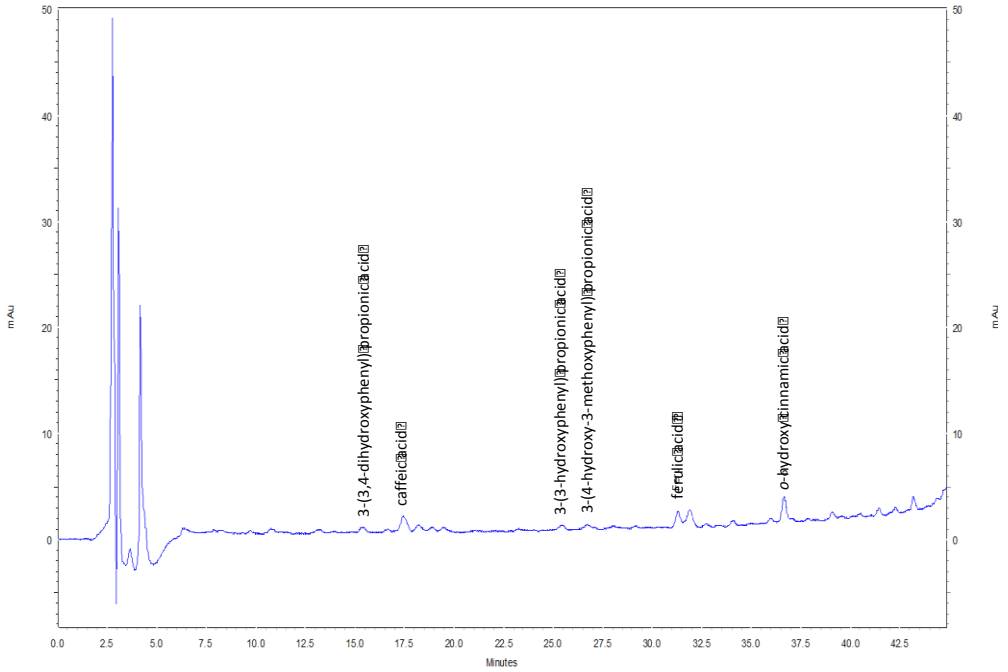

Figure 28: HPLC-UV chromatogram showing the analysis of phenolic metabolites obtained after a 4 h fermentation of cross-linked arabinoxylans (extracted by using 0.5 M NaOH) (monitored at 280 nm)

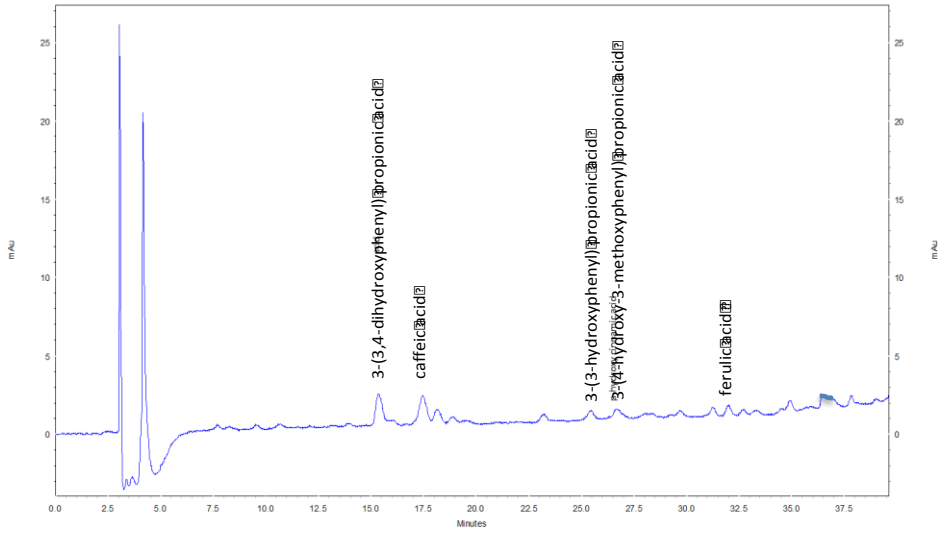

Figure 29: HPLC-UV chromatogram showing the analysis of phenolic metabolites obtained after a 8 h fermentation of non-cross-linked arabinoxylans (extracted by using 0.5 M NaOH) (monitored at 280 nm)

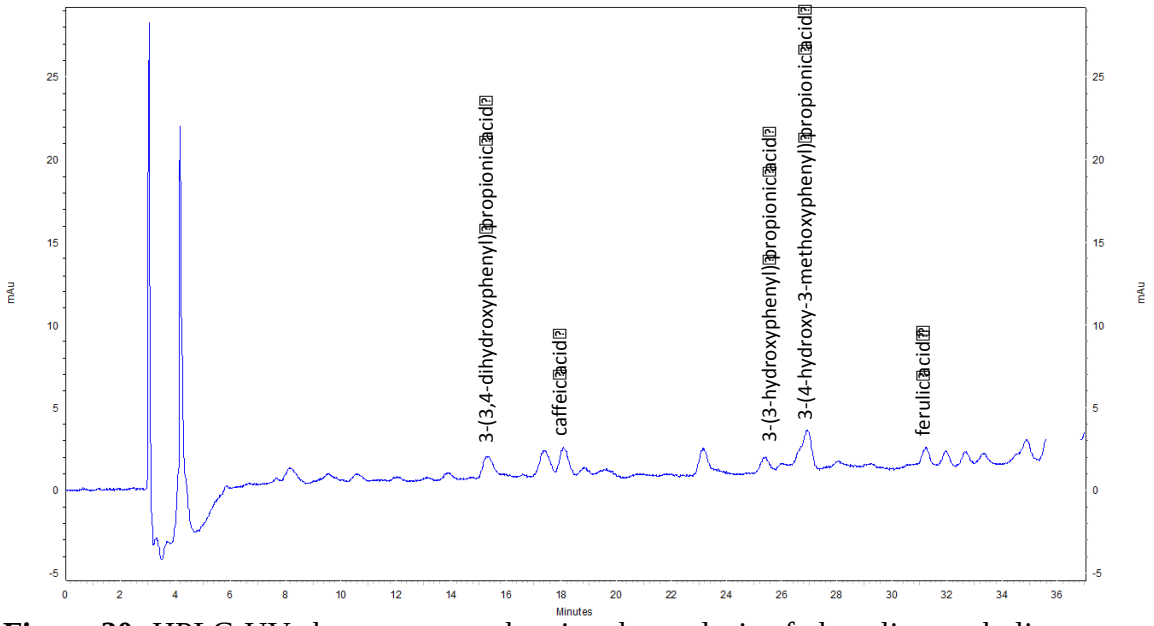

Figure 30: HPLC-UV chromatogram showing the analysis of phenolic metabolites obtained after a 8 h fermentation of cross-linked arabinoxylans (extracted by using 0.5 M NaOH) (monitored at 280 nm)

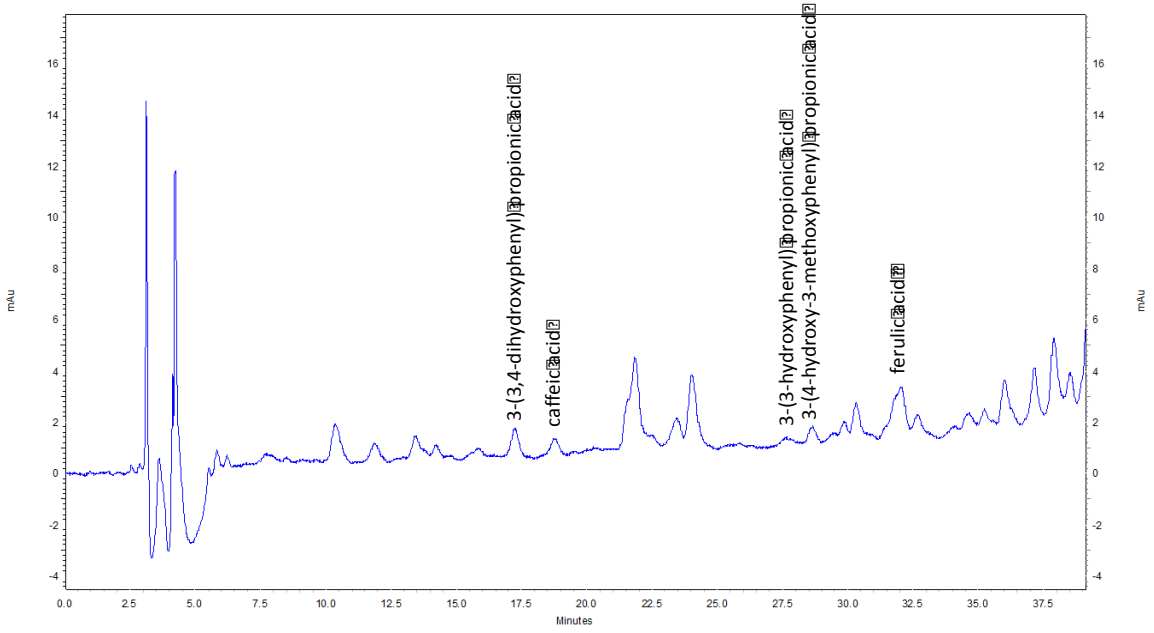

Figure 31: HPLC-UV chromatogram showing the analysis of phenolic metabolites obtained after a 12 h fermentation of non-cross-linked arabinoxylans (extracted by using 0.5 M NaOH) (monitored at 280 nm)

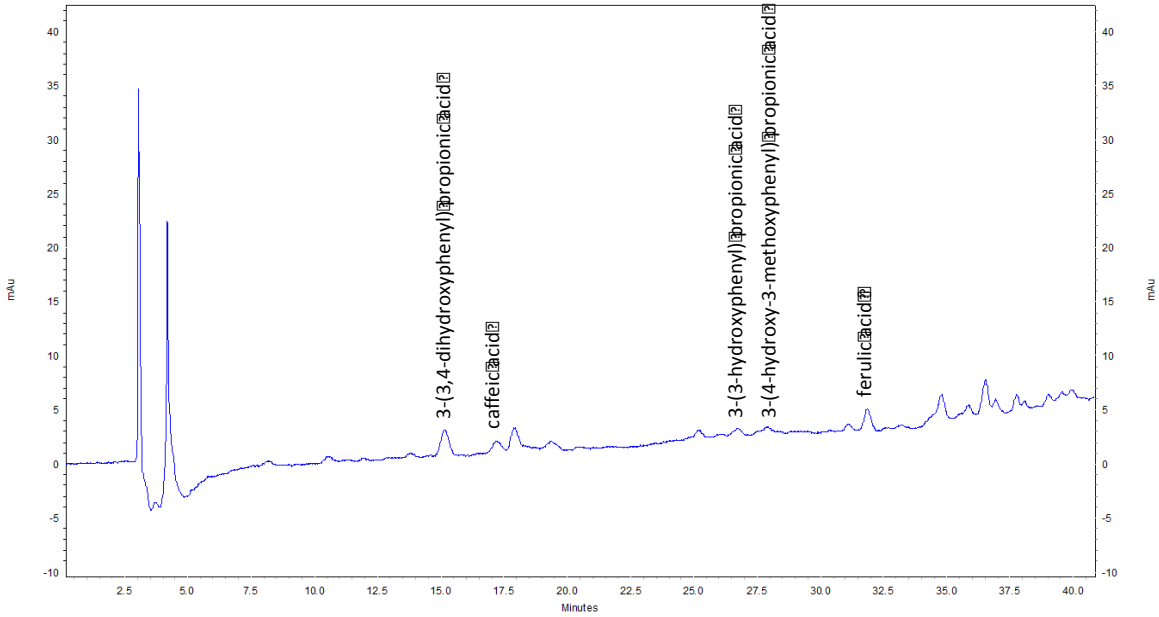

Figure 32: HPLC-UV chromatogram showing the analysis of phenolic metabolites obtained after a 12 h fermentation of cross-linked arabinoxylans (extracted by using 0.5 M NaOH) (monitored at 280 nm)

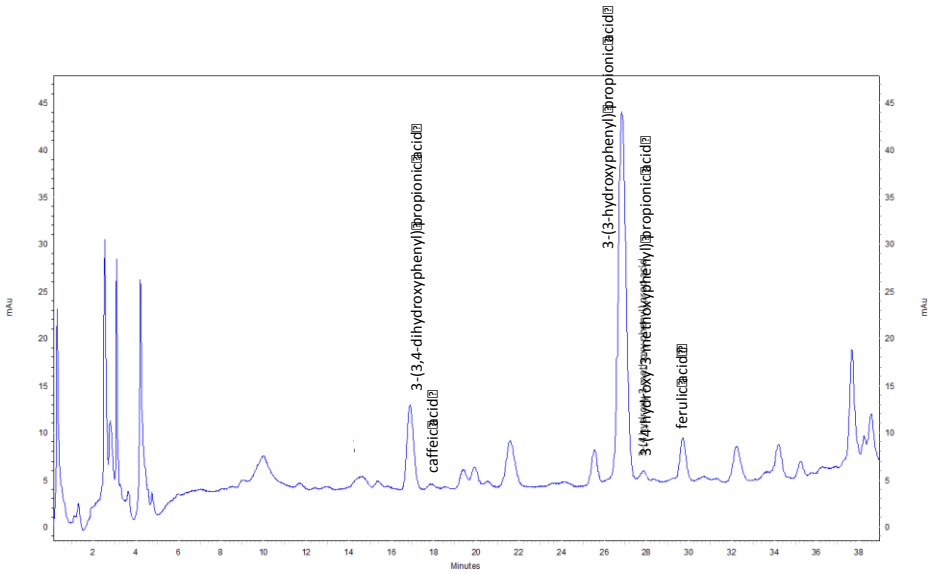

Figure 33: HPLC-UV chromatogram showing the analysis of phenolic metabolites obtained after a 24 h fermentation of non-cross-linked arabinoxylans (extracted by using 0.5 M NaOH) (monitored at 280 nm)

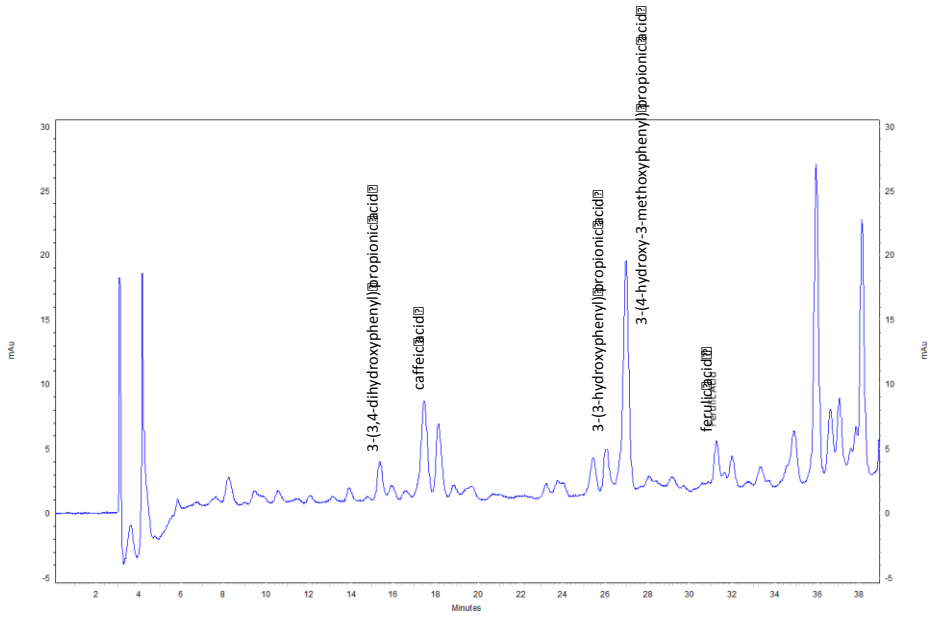

Figure 34: HPLC-UV chromatogram showing the analysis of phenolic metabolites obtained after a 24 h fermentation of cross-linked arabinoxylans (extracted by using 0.5 M NaOH) (monitored at 280 nm)