FABRICATION OF MODEL PLANT CELL WALL MATERIALS TO PROBE GUT MICROBIOTA USE OF DIETARY FIBER

by

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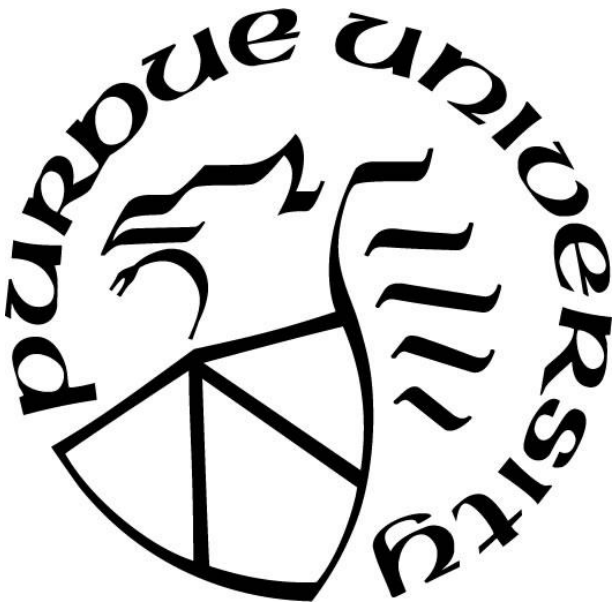

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To my mother and my sister, the bravest women I know

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Hayat kısa, kuşlar uçuyor!

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ABSTRACT

The cell wall provides a complex and rigid structure to the plant for support, protection from environmental factors, and transport. It is mainly composed of polysaccharides, proteins, and lignin. Arabinoxylan (AX), pectin (P), and cellulose (C) are the main components of cereal cell walls and are particularly concentrated in the bran portion of the grain. Cereal arabinoxylans create networks in plant cell walls in which other cell wall polysaccharides are imbedded forming complex matrices. These networks give an insolubility profile to plant cell wall. A previous study in our lab showed that soluble crosslinked arabinoxylan with relatively high residual ferulic acid from corn bran provided advantageous *in vitro* human fecal fermentation products and promoted butyrogenic gut bacteria. In the present work, arabinoxylan was isolated from corn bran with a mild sodium hydroxide concentration to keep most of its ferulic acid content. Highly ferulated corn bran arabinoxylan was crosslinked to create an insoluble network to mimic the cereal grain cell wall matrices. Firstly, arabinoxylan film (Cax-F), pectin film (P-F), the film produced by embedding pectin into arabinoxylan networks (CaxP-F), and cellulose embedding arabinoxylan matrices (CaxC-F), and embedding the mixture of cellulose and pectin into arabinoxylan networks (CaxCP-F) were fabricated into simulated plant cell wall materials. Water solubility of films in terms of monosaccharide content was examined and revealed that Cax-F was insoluble, and P-F was partially insoluble, and nanosized pectin and cellulose were partially entrapped inside the crosslinked-arabinoxylan matrices. In a further study, these films were used in an *in vitro* human fecal fermentation assay to understand how gut microbiota access and utilize the different simulated plant cell walls to highlight the role of each plant cell wall component during colonic fermentation. *In vitro* fecal samples, obtained from three healthy donors were used to ferment the films (Cax-F, P-F, CaxP-F, CaxC-F, and CaxCP-F) and controls (free form of cell wall components -Cax, P and C). The fabricated films that were compositionally similar to cell walls were fermented more slowly than the free polysaccharides (Cax and P). Besides, CaxP-F produced the highest short chain fatty acids (SCFA) amount among the films after 24 hour *in vitro* fecal fermentation. Regarding specific SCFA, butyrate molar ratio of all films was significantly higher than the free, soluble Cax and P. 16S rRNA gene sequencing explained the differences of the butyrate proportion derived from specific butyrogenic bacteria. Particularly, some bacteria, especially in a butyrogenic genera from Clostridium cluster XIVa, were increased in arabinoxylan films forms compared to the native free arabinoxylan

polysaccharide. However, no changes were observed between P and P-F in terms of both end products (SCFA) and microbiota compositions. Moreover, CaxP-F promoted the butyrogenic bacteria in fecal samples compared with pectin alone, arabinoxylan alone, and the arabinoxylan film. Differences in matrix insolubility of the film, which was high for the covalently linked arabinoxylan films, but low for the non-covalent ionic-linked pectin film, appears to play an important role in targeting Clostridial bacterial groups. Overall, the cell wall-like films were useful to understand which bacteria degrade them related to their physical form and location of the fiber polymers. This study showed how fabricated model plant cell wall films influence specificity and competitiveness of some gut bacteria and suggest that fabricated materials using natural fibers might be used for targeted support of certain gut bacteria and bacterial groups.

CHAPTER 1. LITERATURE REVIEW

1.1 Dietary Fibers and Human Health

Dietary fibers cannot be digested by humans in the small intestine but are fermented by resident bacteria in the large intestine (Sims & Monro, 2013). In an official way, dietary fibers are defined by the American Association of Cereal Chemist as "the edible part of plants and analogous carbohydrates that is resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the human large intestine. Dietary fibers include polysaccharides, oligosaccharides, lignin and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, blood cholesterol attenuation, and/or blood glucose attenuation" (DeVries, 2001). They are often further classified into soluble and insoluble fibers, though a more useful gut functional classification would be "fermentable" dietary fibers, which include nearly all soluble and to different degrees most insoluble fibers and provide nutrients to the gut microbes, and "unfermentable" fibers, which have a function of holding water and improving laxation (increase the bulking and softening of stools) (Gemen et al., 2011). Many of the common soluble dietary fibers (e.g. inulin, cereal β-glucans) are fast fermenting in the colon by gut microbiota (Jonathan et al., 2012), though this is dependent on the complexity of their chemical structures (Rumpagaporn et al., 2015). The main end-products of gut bacterial fermentation are gases and short chain fatty acids (SCFAs, i.e. acetate, propionate, butyrate) The majority of SCFAs are absorbed into the body where they are either provide energy for colonic epithelial cells or are transported to the liver where they are converted to energy (Wu et al., 2018). Some SCFAs, particularly acetate and propionate can be found in circulating plasma and appear to have other functions in the body. In addition, SCFAs decrease the pH in the colon promoting beneficial microorganisms and inhibit the growth of pathogenic microorganisms in the gut (Buttriss & Stokes, 2008).

The human colon contains 38 trillions bacteria per gram of wet content with hundreds of species and perhaps thousands of strains, and these microorganisms have a symbiotic relationship with the host (Sender, Fuchs, & Milo, 2016). The structure of the microbial community has a significant effect on human health. Imbalance in microbiota composition and ecology is related to diseases such as type 2 diabetes (Karlsson, Tremaroli, Nielsen, & Bäckhed, 2013; Kendall et al., 2010), inflammatory bowel disease (Rasmussen & Hamaker,

2017)and colorectal cancer (Kendall et al., 2010). Shifting of host microbiota to balanced community using a dietary fiber approach has good merit, but the intricacies of how to do it remain unclear. It largely depends on the types of dietary fiber utilized by different bacteria and bacterial groups (Hamaker & Tuncil, 2014; Jonathan et al., 2012), particle size (Tuncil et al., 2018), and molecular properties as molecular weight and monosaccharide composition (Gemen et al., 2011).

Most fermentable fiber such as inulin, fructo-oligosaccharides, and pectin are immediately fermented in cecum and proximal colon, and thus very low amounts of fiber substrate reach the distal region of the colon (Cummings & Macfarlane, 1991). Perhaps related to this, most colonic diseases appear in the distal colon due to the accumulation of toxic metabolites from protein fermentation such as ammonia (Macfarlane, Gibson, & Cummings, 1992). Moreover, fast fermentation of fibers causes high gas production which lead to bloating and abdominal discomfort. As a result, the ideal dietary fiber is proposed by Rose & Hamaker (2011) as: "(1) having low gas production to avoid undesirable bloating, (2) producing high amount of SCFA to optimize colon environment to inhibit pathogens, (3) being fermented gradually to reach all regions of the colon region to avoid production of undesired compounds (ammonia, phenols, etc.), (4) increasing the beneficial bacteria to maintain a healthy colonic environment. Therefore, the optimal fibers are preferred for slow and steady fermentation."

According to the Food and Nutrition Board of the Institute of Medicine (IoM), the recommendations of dietary fiber intake are 25 g for women and 38 g for men in healthy adults per day (IoM, 2002). Most people do not consume the daily recommended fiber amount. The inadequate fiber intake increases the risk of colonic disease such as inflammatory bowel disease and colon cancer (Zhang & Hamaker, 2010). Increases in the daily intake of dietary fibers help to control body weight, regulate diabetes, decrease the risk of heart disease, and prevent colorectal cancer (Kendall, Esfahani, & Jenkins, 2010). The close relationship between dietary fibers and human health is illustrated in Figure 1.1.

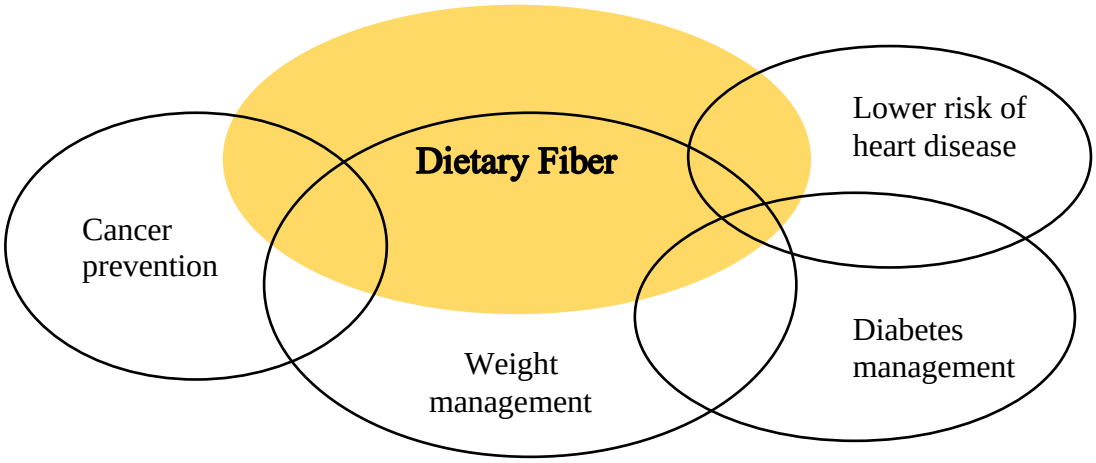

Figure 3 1 The connection between dietary fiber and health benefits (adapted from Kendall et al., 2010)

Many, if not most, dietary fibers derive from plant cell walls of cereals, tubers, legumes, and fruits and vegetables that include cellulose, pectin, heteroxylan (or arabinoxylan), xyloglucans, β-glucans, and lignin (Williams et al., 2017). From work in our laboratory, it has been shown that physical structure is important to supplying nutrients to important and beneficial bacteria in the gut, and in this thesis research simulated plant cell wall structures have been made to better understand their role in gut health.

1.2 Plant Cell Wall

The plant cell wall is a complex structure that provides rigidity and durability to the plant. It is composed of crosslinked polysaccharide matrices, proteins, and lignin. The crosslinking of cell wall components impacts on biodegradability of the plant (Ralph, Grabber, & Hatfield, 1995). Over the years, many models have been proposed to explain structural features of plant cell walls (Carpita, 2002; Carpita & Gibeaut, 1993; Fry, 2001; Harris & Smith, 2006; Keegstra, Talmadge, Bauer, & Albersheim, 1973). Carpita (2002) reported that cellulose fibrils are embedded into crosslinked non-cellulosic polysaccharide networks. These polymers are linked through the covalent, ionic and/or hydrogen bond assembling a complex matrix framework (Heredia, Jimnez, & Guilln, 1995).

The plant cell wall is morphologically characterized in two groups; the primary cell wall and the secondary cell wall. The primary wall of cells expands as the cell grows and the secondary plant wall occurs afterwards and involved laying down of structural polysaccharides such as heteroxylans and cellulose and they can additionally be lignified (Burton & Fincher,

2014). Primary wall thickness is around 0.1 μm, while the secondary wall ranges from 10 to 20 μm (Fry, 2001).

Plant cell walls vary according to the their composition, structure, and architecture. Carpita (1996) mentioned that the walls of growing corn coleoptile that are rigid pieces of plant tissue is composed of around 55% hemicellulose, 25% cellulose, and only 10% pectin. Saulnier, Marot, Chanliaud, & Thibault (1995) found that corn bran cell wall consists of around 68% hemicelluloses that contain arabinose, xylose, galactose, and glucuronic acid and about 22% cellulose. Other cell wall components are aromatic substances and structural proteins.

Many studies show that the specific genes are responsible for synthesis of plant cell wall polysaccharides and assembly of these polymers (Doblin, Kurek, Jacob-Wilk, & Delmer, 2002; Pauly et al., 2013). Despite limited information about the how specific polymers integrate and construct plant cell wall, this subject has been investigated. Mikkelsen et al, (2015) worked on simulated assemblage of the plant cell wall structure with cellulose produce by *Gluconacetobacter xylinus.* In this study, arabinoxylan and β-glucan was interacted with a cellulose network for constructing composites (Mikkelsen et al., 2015). In addition, Ying et al. (2013) produced a model plant cell wall from arabinoxylan and β-D-glucans film. Their hypothesis was to understand how polymer structure impacts hydration and mechanical properties of the cell walls (Ying et al., 2013).

1.3 Structure of Corn Bran and Its Cell Walls

Corn bran has thick cell walls and mainly originates from the testa, pericarp, aleurone layer, and some residual endosperm tissue in the outer layers of the corn kernel (Saulnier et al., 1995). The composite plant material consists of various polysaccharide components, e.g. primarily hemicellulose, cellulose, and phenolic acid (Chanliaud, Saulnier, & Thibault, 1995; Rose, Inglett, & Liu, 2010). The hemicellulose fraction is composed of heteroxylans, also termed arabinoxylans, which have mostly xylose and arabinose units, but also contain minor amounts of galactose, glucuronic acid, and mannose (Table 1.1) (Bonnin et al., 2002; Chanliaud et al., 1997; Rose et al., 2010; Saulnier, 1999).

Table 1. 1 Composition (g/kg dry matter of corn bran). Adapted from Rose et al. (2010).

Constituent	Corn Bran
Total dietary fiber	732-860
Arabinose	128-178
Xylose	217-243
Mannose	3
Galactose	44-51
Glucose*	182-248
Uronic Acid	39-42
Lignin	7-10
Ferulic acid	28-31
Diferulic acid	6.8-32
Protein**	50-115
Ash	6-10

*non-starch glucose
**Nitrogen × 6.25.

Corn bran cell wall also consists of arabinoxylan and cellulose. The potential pattern of corn bran cell wall structure is illustrated in Figure 1.2 by Saulnier and Thibault (1999). Phenolic acids, mostly ferulic acid, play an important role in connecting of arabinoxylans in corn bran cell wall. As described by Saulnier and Thilbaut (1999), the arabinoxylans are crosslinked through ferulic acids, and microfibrils of cellulose are imbedded into the diferulic-linked networks. Dimers and trimers of ferulic acids provide bridges between arabinoxylan chains. It is not only the diferulic bridge forms that cause insoluble arabinoxylan matrices in the cell wall, but also arabinoxylan matrices and protein linkages provide the cell structure a strong network (Saulnier et al., 1995). According to the Carpita & Gibeaut (1993)., there are covalent, ionic, and hydrogen bonds among the cell wall components However, hydrogen bonds between microfibrils of cellulose and arabinoxylan are not detected in corn bran cell wall (Saulnier et al., 1995).

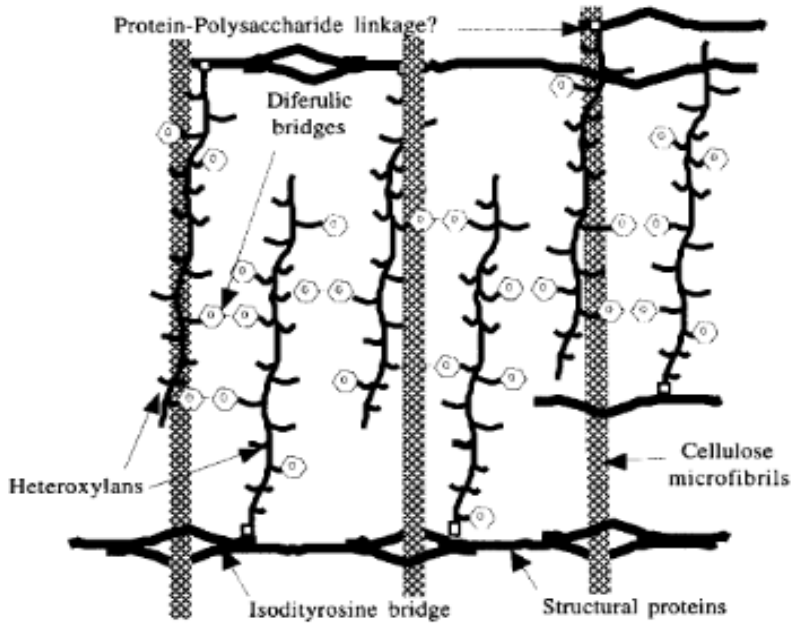

Figure 3 2 Schematic representation of model corn bran cell walls. From (Saulnier, 1999)

1.4 Arabinoxylan

1.4.1 Structure of Arabinoxylan

Arabinoxylan, which is classified in the general group of hemicelluloses, is the major component of cereal cell walls such as in corn and wheat. Arabinoxylan consists of a linear backbone of linked β-(1,4)-xylan residues with α-L-arabinofuranose residues linked as side-chains through α-(1→3) and/or α(1→ 2) linkages (Izydorczyk & Biliaderi, 1995) (Figure 1.3). The use of arabinose to xylose ratio is commonly employed to compare the arabinoxylan structures from different sources (B. Williams et al., 2017). Ferulic acids are linked to the arabinose residues and crosslinked to provide a rigid cell wall structure on its natural form (Saulnier et al., 1995).

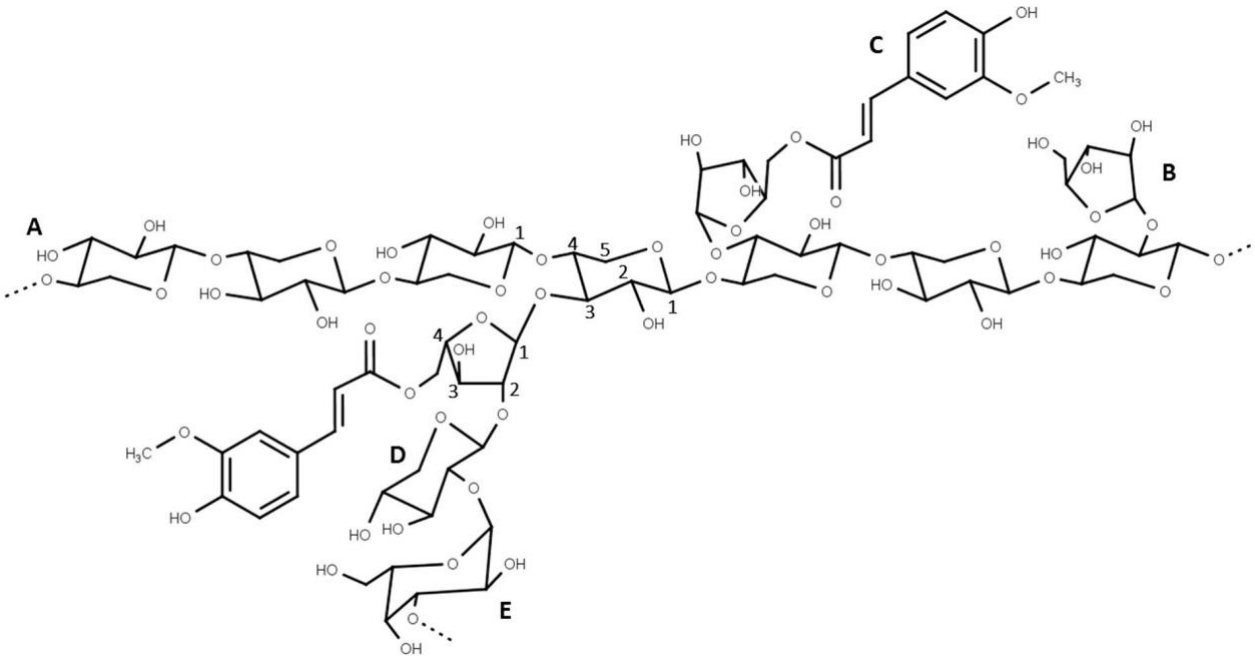

Figure 3 3 General structural features of corn bran arabinoxylan; A: Xylan backbone; B: Arabinosyl group; C: Feruloyl group; D: Xylosyl group; E: Galactosyl group. From Bento-Silva, Carlota Vaz Patto, Do, & Bronze (2018)

1.4.2 Alkaline Extraction of Arabinoxylans from Corn Bran

As described before, corn bran is mainly composed of arabinoxylans that contain not only various monosaccharides such as xylose, arabinose, galactose and glucuronic acid, but also phenolic compounds such as ferulic acids which are esterified to arabinose units. It is known that the interconnection of arabinoxylans is due to the presence of ferulic acids and their dimer and trimer crosslinking. This structural network among cell wall components cause the insolubility of arabinoxylan in aqueous solutions. Arabinoxylan can be extracted with enzymatic and alkali treatments from the corn bran cell wall. Alkaline treatment is applied on corn bran to de-esterfy the crosslinked ferulic acids and to release soluble arabinoxylan. Many studies demonstrate that alkaline extraction of arabinoxylan from different types of cereal bran remove the diferulic bridges (Bergmans et al., 1996; Chanliaud et al., 1995; Doner & Hicks, 1997a; Izydorczyk & Biliaderi, 1995; Maes & Delcour, 2001; Rose, Patterson, & Hamaker, 2010a; Rumpagaporn et al., 2012).

A variety of alkali reagents are used to extract arabinoxylan from cereals including sodium hydroxide (Rose, Patterson, et al., 2010; Zhang, Wang &Zhou, 2008), potassium hydroxide (Chanliaud et al., 1995), calcium hydroxide (Ogawa, Takeuchi, & Nakamura, 2005), and barium hydroxide (Gruppen, Hamer, & Voragen, 1991). It is noteworthy that the type of solvent impacts the final yield of arabinoxylan (Chanliaud et al., 1995). Hydroxy ions of alkali

solutions break down the ester bonds between arabinose residues and ferulic acids (Malgorzata, Courtin, & Delcour, 2003). The role of reagent cation ions has not being speculated yet (Chanliaud et al., 1995).

1.4.3 Ferulic Acid and Oxidative Gelation of Arabinoxylan

Phenolic compounds, mainly ferulic acids, are present in corn bran in amounts around 3.1-4 % of its dry weight (Rose, Inglett, et al., 2010; Saulnier, 1999; Saulniet, Chanliaud, & Thibault, 1995; Vitaglione, Napolitano, & Fogliano,2008; Zhao & Moghadasian, 2008). The amount of ferulic acid in corn bran is significantly higher than in other cereals (Vitaglione, Napolitano, & Fogliano, 2008).

Ferulic acid is available in corn bran as a free form that is esterified on arabinose residues, but not crosslinked, and their dimer and trimer forms that are in crosslinks. The total free ferulic acid is about the 1-6% of the total ferulic acid content (Martínez-López et al., 2013; Zhao & Moghadasian, 2008). Saulnier and Thibault (1999) calculated that, each arabinoxylan polymer esterifies with 75 ferulic acid units and 45 are in the non-crosslinked form. After alkali treatment, there is an increased amount of free ferulic acids. Kale, Hamaker, & Campanella showed that the alkali treatment condition (concentration of alkali and time of mixing corn bran with solution) affects the final content of esterified ferulic acid residues (2013). If the time and concentration of alkali solution are increased, the bound ferulic acid content is decreased (Kale et al., 2013).

It is known that soluble ferulated arabinoxylan can be gelled by covalent crosslinking of ferulic acid residues with treatments using chemical (ferric chloride, ammonium persulphate) or enzymatic (peroxidase/ H_2O_2, laccase/O2) oxidizing agents (Carvajal-Millan et al., 2005; Hoseney, Chem, & 1981;Izydorczyk, Biliaderis, & Bushuk, 1990; Geissmann & Neukom, 1973). Laccase is an oxidative agent and naturally present in many plants (Johannes & Majcherczyk, 2000). It was initially used as a free radicals-generating agent for crosslinking free ferulic acids by Figueroa-Espinoza et al. (1998). Recent studies show that laccase is a good crosslinking agent to gellify arabinoxylans (Berlanga-Reyes et al., 2011; Carvajal-Millanet al., 2005; Kale et al., 2013; Xiaowei Zhang et al., 2019).

Non-crosslinked ferulic acids estferified on arabinose residues are covalently crosslinked after oxidization with laccase and become diferulic acids and triferulic acids (Figure 1.4) (Carvajal-Millan et al., 2005; Vansteenkiste et al., 2004; Xiaowei Zhang et al., 2019). Kale et al. showed that degree of crosslinking of ferulic acids is associated with how much these polymers can participate in the gelling mechanism (2013). The role of ferulic acid

esterfication on arabinose units the key factor in making a strong gel. The diferulic bridges develop a network among arabinoxylans, and these interactions are responsible for the degree of gel viscosity (Kale et al., 2013). Also, the networks formed involving diferulic bridges give an opportunity to produce arabinoxylan-based films that has been investigated for food packaging. (Anderson & Simsek, 2019; Ebeaufort & Espre, 2002).

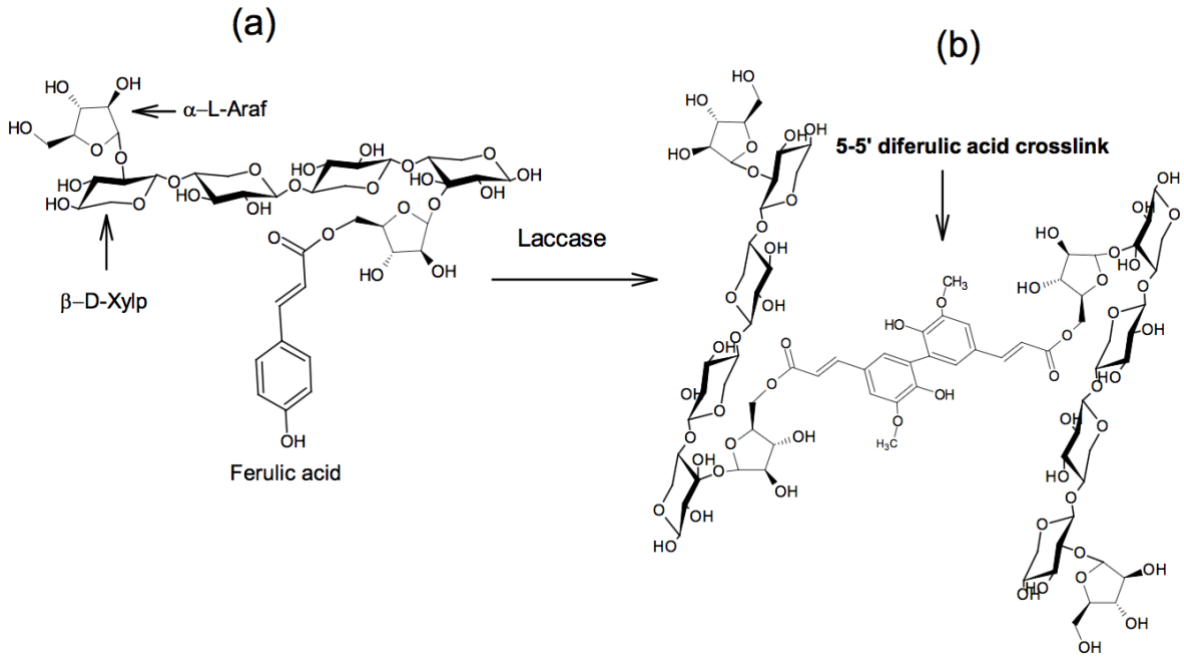

Figure 3 4 Schematic representation of the covalent cross-linking of ferulated arabinoxylans. Ferulated arabinoxylans **(a)** in solution and **(b)** in gel. Covalent cross-linking through a 5,5 diferuloyl moiety in the gel is presented as an example (From Martínez-López et al., 2013).

1.5 Cellulose

Cellulose is the most abundant polymer in earth and an important part of the plant cell wall (Klemm, Heublein, Fink, & Bohn, 2005). It is formed by consecutive D-glucose units linked linearly through β-1,4 linkages (Figure 1.4). Cellulose, crosslinked hemicellulose, and penetrated pectin create the rigidity and strength of the plant cell wall (Grundy et al., 2016). This strength and rigidity make the cell wall resistant to mechanical and chemical damage. Cellulose is insoluble in water in its natural form, but can be solubilized by either chemical modification or high shear with alkaline and hydrogen peroxide treatments (Rose & Hamaker, 2011).

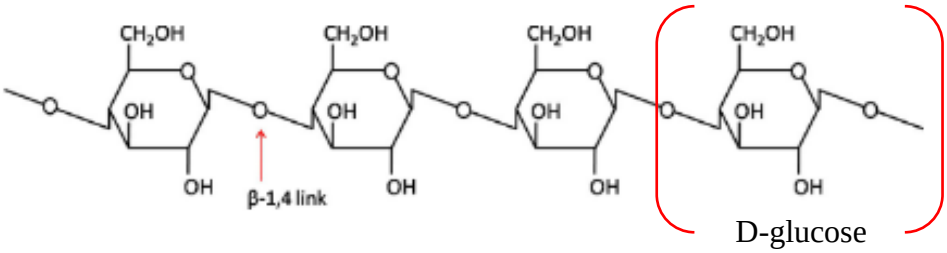

Figure 3 5 Chemical structure of cellulose.

1.6 Pectin

1.6.1 Structure of Pectin

Pectin is perhaps the most structurally complex polysaccharide found in primary cell walls. The pectin structure can be classified in three groups; (1) rhamnogalacturonan I that is composed by rhamnose and galacturonic acid at the backbone structure, (2) homogalacturonan backbone that is composed of (1–4) linked α-D-galacturonic acid residues, and (3) complex multi-sugar branches forming rhamnogalacturonan II (Figure 1.6). (Mohnen, 2008). Homogalacturonan has approximately 100 sequential galacturonic acid residues (Thibault et al., 1993), and has a simple structure compared to the other pectic polysaccharides rhamnogalacturonan I and II (Nakamura et al., 2002). The rhamnogalacturonan I backbone is composed of (1-4) linked α -D-galacturonic acid and (1-2) linked α -L-Rhamnose and may have a variety of branched oligosaccharides linked at rhamnose residues depending on the cell type (Yapo et al., 2007). D-galactosyluronic acid, L-rhamnosyl, D-galactosyl, L-arabinosyl, and small amounts of L-fucosyl residues can be found linked in rhamnogalacturonan I backbone (McNeil et al., 1984).

Rhamnogalacturonan II is the most complex part of the pectin structure. Different types of neutral sugars (primarily arabinose, xylose, galactose, fucose) are attached to a homogalacturonan backbone that has at least 8 α-D-galacturonic acid units (Mohnen, 2008). Vegetables and fruits contain comparably high amounts of pectin (Thakur et al., 1997). Most commercially available pectin is extracted from apple pomace and citrus peels. Pectin also gives the firmness to the cell wall structure because it is linked with cellulose (Carpita & Gibeaut, 1993).

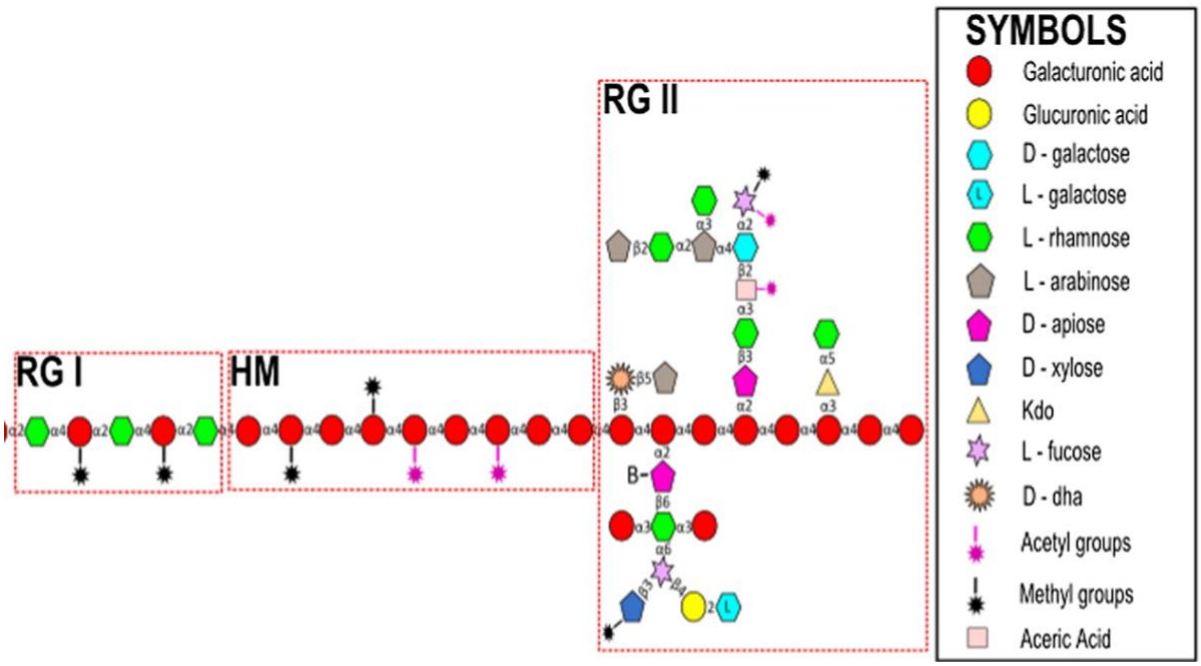

Figure 3 6 Chemical structure of pectin (From Hamaker & Tuncil 2014).

1.6.2 Gelling Mechanism

The homogalacturonan backbone is usually partially esterified by methoxyl groups and this degree of esterification (DE) is categorized from 0 to 100%. Based on degree of esterification, pectin is classified as high methoxy pectin (DE > 50%) or low methoxy pectin (DE < 50%).

The gelation potential of pectin depends of the degree of esterification, and high-methoxyl pectin and low-methoxyl pectin have different mechanisms for gelation (Raj, 2012). High-methoxyl pectin can be form gel in the presence of soluble solids such as sucrose and at low pH (2.5–3.5) (Lara-Espinoza et al., 1991; Raj, 2012). Low-methoxyl pectin gelation occurs via different intermolecular interactions. For gelation of low-methoxyl pectin, the number of carboxyl groups and calcium concentration play an important role (Axelos & Thibault, 1991). Its gelation mechanism is known as the ‘egg-box’ model (Figure 1.7). In this model, galacturonic acid monomers are intermolecularly crosslinked through electrostatic and ionic bonding in the presence of calcium ions (Axelos & Thibault, 1991). The strength of the gel depends on the stability of the electrostatic bond between carboxyl groups and calcium (Axelos & Thibault, 1991; Lootens et al., 2003; Raj, 2012).

Pectin is widely used in the food industry for thickening (Brejnholt, 2009), gelling (Fu & Rao, 2001), and as a prebiotic (Rose & Hamaker, 2011; Tuncil et al., 2017; Williams et al., 2017). In addition, there is a popular trend to develop environmentally friendly packaging

based on biodegradable polymers, and accordingly low methoxy pectin has been used experimentally to cast films for this purpose (Chaichi et al., 2019a; Spatafora et. al, 2019).

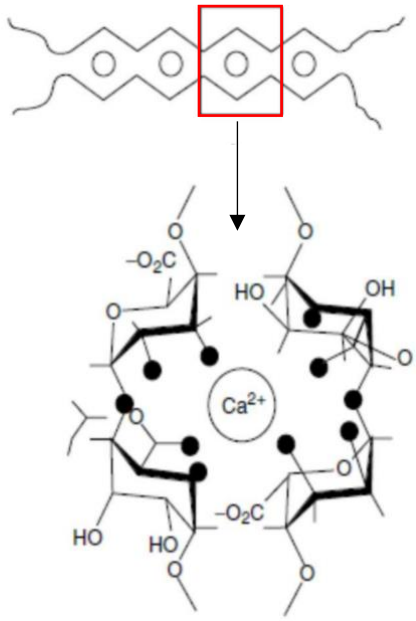

Figure 3 7 Schematic representation of low methoxyl pectin gelation mechanism as call “the egg box” model. From Axelos & Thibault (1991).

1.7 Fermentation Profile of Plant Cell Wall Components

Plant cell wall components are resistant to digestion in the small intestine, but can be partially or totally fermented by the microbiota residing in the large intestine (Sims & Monro, 2013). Most dietary fibers are components of the plant cell wall. There are many classification systems for dietary fibers, but they can be commonly classified into two groups based on their solubility in water. There is a general view that water-soluble fibers are more fermentable than insoluble fibers (Maathuis et al., 2009). However, there is a study showing that water-insoluble dietary fibers are fermentable in the large intestine (Williams et al., 2000).

Arabinoxylans are classified as water-soluble or water-insoluble, depending on their structure (e.g. branching and crosslinking degree). Arabinoxylan has been assumed as a prebiotic, because it cannot be digested by enzymatic hydrolysis in the small intestine while being fermented by gut microbiota (Ciudad-Mulero et al., 2019; Mendis & Simsek, 2014; Mendis et al., 2016). The fermentation of arabinoxylan provides beneficial end products such as short chain fatty acids, especially acetate, propionate, and butyrate, that are important to host health (Rumpagaporn et al., 2016, 2015). In addition, it was shown to promote the *Bacteroides*,

Coprococcus, and *Faecalibacterium spp.* using *in vitro* human fecal batch fermentations (Chen et al., 2017; Yang et al, 2013).

Cellulose cannot be dissolved in water and is only partially fermented by bacteria in the large intestine (Lattimer et al., 2010). However, cellulose plays a significant role during colonic transit, making stools bulky and decreasing bowel transit time (Dodevska et al., 2013). This helps to improve colonic health and possibly decrease colon cancer risk (Ciudad-Mulero et al., 2019). Although *in vitro* fecal fermentation performance shows a limited fermentation of cellulose, *Bacteroides* spp., *Ruminococcus* spp., and *Enterococcus* spp. were shown to be increased on its ferments (Jonathan et al., 2012).

Pectin is completely soluble in water and highly fermentable in the large intestine and it is a potential prebiotic, because it has many health benefits such as beneficial fermentation profiles in the gut (Gómez et al., 2015). *In vitro* fecal fermentation studies show that fermentation of pectin yielded particularly high amounts of acetate and butyrate (Ferreira-Lazarte et al., 2018; Gómez et al., 2015; Tuncil et al., 2017). Moreover, fermentation of pectin stimulated the growth of *Lachnospira*, *Clostridium*, and *Sutterella* (Yang et al., 2013).

1.8 Objectives

The main goal of this research was to develop simulated plant cell wall-like thin films to determine *in vitro* human fecal fermentation profiles of these films for the purpose of better understanding the role of composition and structure of the plant cell wall on the colonic microbiota and their fermentation metabolites. Specific objectives of this project were categorized as follows:

1. Construct different types of composite films containing the plant polysaccharides arabinoxylan, pectin, and cellulose, and to devise a method to make insoluble arabinoxylan film (Cax-F) and partially insoluble pectin film (P-F), and then arabinoxylan with embedded nanosized pectin (CaxP-F), arabinoxylan with embedded nano-fibrillated cellulose (CaxC-F), and a combination of arabinoxylan with embedded pectin and cellulose (CaxCP-F).
2. Analyze the physical properties of these films regarding the thickness of the films and their solubility in water and relative component dissolution.
3. Determine the fermentation outputs (SCFAs and microbiota analysis) of these model materials, compare with the control substrates that are plant polysaccharide in native

soluble forms; arabinoxylan (Cax), pectin (P), and cellulose (C), to understand how polymer structure in plant cell walls affect the the gut microbiota and fermentation metabolites during *in vitro* human fecal fermentation.

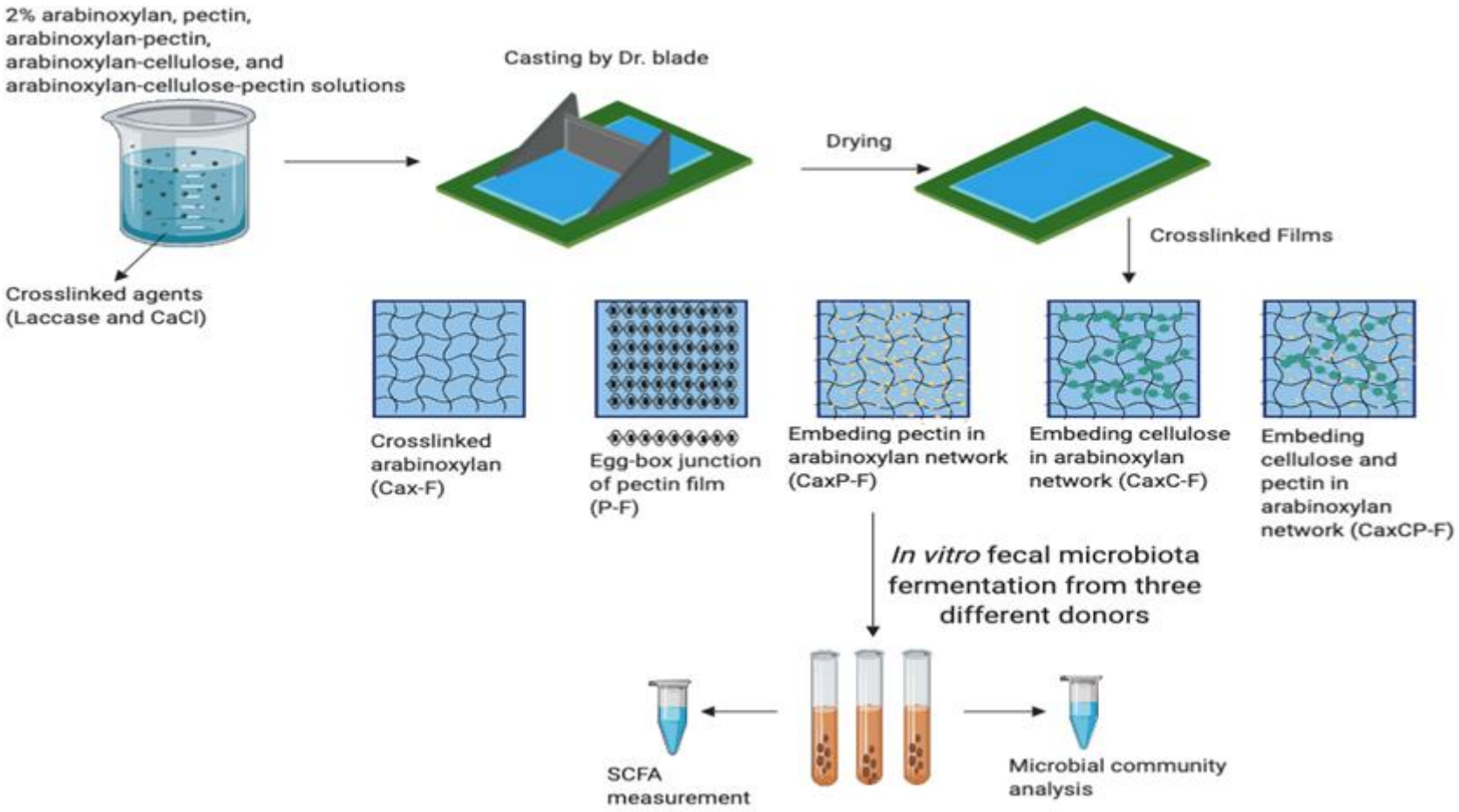

Figure 3 8 Diagram of films preparation and experimental design.

CHAPTER 2. DEVELOPMENT OF PLANT POLYSACCHARIDE-BASED THIN FILMS

2.1 Abstract

Arabinoxylan (AX), pectin (P), and cellulose (C) are the main components of cereal cell walls and are particularly concentrated in the bran portion of the grain. Cereal arabinoxylans create networks in plant cell walls in which other cell wall polysaccharides are embedded, forming complex matrices. In this study, corn arabinoxylan, which had been solubilized and isolated, was covalently linked and used to form an insoluble network in a fabricated film form. Likewise, pectin was formed to a calcium-crosslinked film, and also was made into nanoscale particles for incorporation into the arabinoxylan films. Nanofibrillated cellulose was obtained and also incorporated into the films. Therefore, films were formed as arabinoxylan film (Cax-F), pectin film (P-F), arabinoxylan film with embedded nanopectin (CaxP-F) or nanofibrillated cellulose (CaxC-F), and arabinoxylan film with embedded pectin and (CaxCP-F) and used as model plant cell wall study how access and utilization by the human gut microbiota. This study focused on how the polymer network can be made for this purpose. The solubility of Cax-F was only 6.6%, which is significantly lower than the pectin film at 75.1%, and was found adequate to construct the laminar structure that mimics the plant cell wall. The pectin film was mostly soluble (~75%), but the nanosized pectin placed inside the insoluble arabinoxylan film did not escape from the film despite being soluble in water. The addition of insoluble polymers into the cell wall matrix model increased its thickness, with CaxC-F exhibiting a much higher thickness than the other films.

2.2 Introduction

The cell wall provides a complex and rigid structure to the plant for support, protection from environmental factors, and transport. It is mainly composed of polysaccharides, proteins, and lignin. Recent studies have revealed the biosynthetic genes responsible for the plant cell wall (Doblin et al., 2002; Pauly et al., 2013).Regarding structure, Talbott & Ray (1992), initially considered that cellulose microfibrils were covered by loosely bounded polysaccharides in a ‘multicoat’ model. However , Cosgrove (2002) proposed that non-cellulosic matrix polymers and proteins create matrices with covalent crosslinking and cellulose is bonded to the network by hydrogen bonding.

In addition, phenolic acids are found in cell walls most bound to polysaccharides and are important to crosslink polymer chains to create the cell wall matrix networks (Saulnier et al., 1995). For example, Saulnier and Thilbaut showed that some of the ferulic acids that are esterified on arabinose monomer branches of arabinoxylan are crosslinked and suggested that the microfibril cellulose is embedded into this matrix in corn bran cell walls (1999). The insolubility of corn arabinoxylan in the cell wall is due to the high crosslinking rate of ferulic acids through diferulic bridges.

Many studies have shown that arabinoxylan can be isolated from the cereal cell walls by alkali treatment (Bergmans et al., 1996; Doner & Hicks, 1997b; Maes & Delcour, 2001; Rose, Patterson, & Hamaker, 2010b; Rumpagaporn et al., 2015; Saulnier et al., 1995). Alkali treatment de-esterfies ferulic acids bound to arabinose branches and their dimers and trimers to release the soluble arabinoxylan. Depending on the strength of the alkali used, crosslinks can be removed, while leaving different amounts of single ferulate residues bound to the polymer. Soluble arabinoxylan containing some amount of such ferulate residues can be used to form gels by covalent crosslinking with chemical and enzymatic agents (Carvajal-Millan et al., 2005; Izydorczyk et al., 1990)Thus, the gelling capacity of arabinoxylan depends on the alkali treatment. According to Kale et al. (2013), alkali treatment conditions including time and concentration of alkali reagents affect the crosslinking and gelling potential of arabinoxylan. The residual ferulic acid content determines the amount of crosslinking that can occur and thereby affects the strength of resulting gels. Severe extraction conditions such as treating with 1.5 M NaOH for 24 h can causes too much reduction in ferulate residues on arabinoxylans, resulting in poor crosslinking property, while treatment with 0.25 M NaOH for 0.5 h increased ferulate content causing increased crosslinking.

Pectin also forms a gel in the presence of cationic crosslinking agents. The strength of the gel depends on the amount of carboxyl groups on pectin and the crosslinking agent used. The interaction between galacturonic acid and calcium is commonly used to form gels by electrostatic interaction (Axelos & Thibault, 1991; Raj, 2012).

The objective of this research was to create thin films of different cereal cell wall compositions using solubilized arabinoxylan and pectin. Also, nanosized pectin or nanofibrillated cellulose and a combination of the pectin and cellulose were entrapped inside the covalently-bound insoluble arabinoxylan film matrix. Once casted, the mechanical properties of these films [arabinoxylan film (Cax-F) , pectin film (P-F), arabinoxylan-pectin film (CaxP-F), arabinoxylan-cellulose (CaxC-F), and arabinoxylan-cellulose-pectin (CaxCP-

F)] were determined to further understand the relationship between composition and structural properties. Scanning electron microscopy (SEM) was used to visualize the structure of these thin films. Solubility analysis of the films was done with monosaccharide composition for the soluble and insoluble fractions to determine polymers that were soluble and insoluble.

2.3 Material and Methods

2.3.1 Materials

Dry milled corn bran was gifted from Agricor, Ltd. (Marion, IN). A commercial sample of low methoxy pectin from citrus peel was purchased from Sigma Chemical Company (St. Louis, MO). Nanofibrillated cellulose was gifted from Cellulose Lab, Ltd (Canada). Thermostable α-amylase, laccase from *Trametes versicolor*, ferulic acid, 3,4,5-trimethoxy-cinnamic acid (TMCA), and methanolic-HCl, were obtained from Sigma Chemical Co. (St. Louis, MO). Hexane, ethanol, sodium hydroxide pellets and concentrated hydrochloric acid were obtained from Fisher Scientific Chemicals (Pittsburgh, PA). Tri-Sil and acetonitrile were obtained from Fisher Scientific (Thermo Fisher Scientific, Suwanee, GA).

2.3.2 Arabinoxylan Extraction from Corn Bran by Alkali Treatment

Arabinoxylan was extracted by using methodology described by Kale, Hamaker, & Campanella (2013) with some modifications. Corn bran was defatted with two volume of hexane (bran: hexane, 1:10 [w/v]) and stirred for 45 min. The slurry was filtered, and the residue dried in a hot air oven at 45 °C. One-hundred gram of the defatted bran was suspended in 900 ml of water and pH was adjusted to 7.0 using 1M NaOH. The mixture was boiled with constant stirring for 5 min to gelatinize the starch and then cooled to 90 °C. Thermostable α-amylase (4 mL) was added. The de-starching reaction was continued for 1 h at 90 °C – 95 °C. The slurry was centrifuged at 10,000 *g* for 20 min. The liquid part was discarded, and the residue was washed twice with purified water, and dried in a hot air oven at 45 °C to collect de-starched bran (DSB). DSB (50 g) was suspended in 500 mL of 0.25 M sodium hydroxide solution. After stirring for 30 min at room temperature, the suspension pH was adjusted to 4-5 using 1M HCl. The slurry was centrifuged at 10,000 *g* for 10 min. The residue was discarded, and the volume of supernatant was measured. Four volumes of absolute ethanol were added to the supernatant to precipitate arabinoxylan. The precipitate was siphoned off and dried in a hot air oven at 45 °C. The AX was re-dissolved in water and freeze-dried.

2.3.3 Preparation of Nanosized Pectin

Nanosized pectin powders were prepared by mechanical milling. Pectin (5 g) was milled using Thinky Mixer (ARE-310, Thinky Inc.) with 100 g of yttria-stabilized zirconia (YSZ) spherical grinding balls (2 mm in diameter) at 2400 rpm for 1 h. Dynamic light scattering (DLS) was used to analyze particle size of pectin using an ALV-CGS3 light scattering goniometer (ALV, Langen) with an HeNe laser (wavelength = 632.8 nm) at a 90 degree scattering angle.

2.3.4 Film Preparation

2% (w/v) arabinoxylan, pectin, arabinoxylan-pectin, arabinoxylan-cellulose, and arabinoxylan-cellulose-pectin solutions were prepared by mixing the pure polymers in purified water for 30 min in 2400 rpm with a Thinky Mixer (ARE-310, Thinky Inc.) in purified water that is a planetary centrifugal vacuum mixer. The percentages of fiber used to cast films are shown in Table 2.1. Laccase (1.675 nkat/mg AX) (Kale et al., 2013) and calcium chloride ($CaCl_2$) (1 % w/w pectin) were added for Cax and pectin, respectively, as crosslinking agents.

The solutions were cast on a glass platform by using a motorized drawdown coater equipped with a commercial 7.62 cm wide casting blade. The casting films were 17.78 cm in length and 7.62 cm width and 762 μm thickness.

2.3.5. Scanning Electron Microscopy of Films

A NOVA nanoSEM scanning electron microscope from FEI (Oreg., U.S.A.) was used to take the scanning electron micrograph (SEM) images. The beam was intensified at 5 kV in order to obtain good images. All the films were attached to the surface of a carbon tape and coated with a thin layer of palladium before SEM imaging.

2.4 Results and Discussion

2.4.1 Film Formation and Morphology

Arabinoxylans were extracted from corn bran with mild alkali extraction condition (0.25 M NaOH) in order to retain relatively high ferulic acid content during isolation for re-crosslinking during the film forming step (Kale et al., 2013). Laccase was used as a crosslinking enzyme of arabinose-bound ferulic acids to make di- and triferulate crosslinks among arabinoxylans from alkaline-extracted corn bran. The diferulic and triferulic bridges between

arabinoxylans create strong networks to form an insoluble gel. It is important to note that the ferulic acid content on arabinoxylan determining factor related to gelling capacity (Kale et al., 2013). Only arabinoxylan with sufficiently high ferulic acid content formed a film structure. Below a critical content of ferulic acid, arabinoxylan could not form a strong film by treating with laccase. Alkali-treated corn arabinoxylan efficiently formed a gel by oxidative gelation with laccase during the casting of the solution on a glass surface as a film. Pectin was milled to a particle size until 150-250 nm. Particle size was made small to entrap the nanosized pectin into the crosslinked arabinoxylan matrices, and nanofibrillated cellulose was obtained for the same purpose. Arabinoxylan, pectin, arabinoxylan-pectin, and arabinoxylan-cellulose, and arabinoxylan-cellulose-pectin mixtures (Cax-F, P-F, CaxP-F, CaxC-F, and CaxCP-F, respectively) were used to form composite films. A homogenous appearance was obtained after drying (Figure 2.1). Visually, P-F was more colorless and transparent than Cax-F. However, the transparency of the arabinoxylan film was further decreased with the addition of pectin and cellulose, with the CaxC-F, and CaxCP-F less transparent and opaquer than the CAX-F.

Scanning electron micrographs showed that the films had a compact and smooth structure (Figure 2.2a-e). Ying et al. reported that there were not any micropores or phase separation in arabinoxylan film (2015). Furthermore, micrographs revealed that Cax-F, P-F, and CaxCP-F were less rough and more homogenous than CaxP-F and CaxC-F. CaxP-F, and CaxC-F composite films, which had more aggregated nonhomogeneous structures. These were possibly due to the aggregation of nanofibrillated cellulose and calcium precipitation of the pectin in CaxC-F and CaxP-F, respectively. Cellulose cannot be dissolved in water due to its high molecular weight and crystalline structure (Strachan, 1938). Thus, the dispersion of cellulose in solution causes aggregation after drying (Chaichi, Badii, Mohammadi, & Hashemi, 2019b). Pectin polymers interact with calcium ions (Ca^{+}), which is the crosslinking agent. Xin at al. reported that calcium ion-mediated aggregation and precipitation causes white spots on surface of polysaccharide-based films (Xin et al., 2014).

2.4.2 Thickness of Films

Corn bran cell walls are thick with composition of polysaccharides such as arabinoxylan and cellulose (Saulnier et al., 1995). According to Fry (2001), the thickness of the primary cell wall of plants is about 0.1 μm, and secondary cell walls of seeds are usually 10–20 μm thick. The primary cell wall is laid down during the enlarging of the cell, and secondary cell wall is accumulated on the primary wall after cell expansion has stopped (Burton & Fincher, 2014). This thick secondary cell wall is found in the bran of cereal kernels. In this

study, the thickness of films was designed to be similar and model the cereal bran cell wall. Although initial thickness of the films (~750 μm) were the same, the final thicknesses, after drying, were different (Table 2.2). Cax-F thickness was the lowest between 15 to 17 μm, while the largest was the CaxC-F that was 21-25 μm thick. The addition of nanofibrillated cellulose or nanosized pectin or both in to the CAX based film-forming solution led to an increase in the thickness of films, but pectin did not contribute to the increasing of film thickness as much as cellulose. Moreover, all five films' thickness were measured at ten different point, Table 2.2 shows that the commercial casting blade helped to obtain a uniform film thickness.

2.4.3 Solubility of Films and Monosaccharide Composition of Soluble and Insoluble Portions

One of the objectives in making the model cell wall was to make the matrix material (i.e. arabinoxylan) insoluble with the possibility of keeping an included soluble pectin in a non-extractable state. This would create a gradient of accessibility of fiber prebiotics to the gut microbiota. In these experiments, solubility of the constituents was examined by simple solubility testing and monosaccharide analysis. Monosaccharide composition of released polysaccharides showed how much and which polysaccharides are more prone to escape the films. The solubility and monosaccharide composition of soluble and insoluble fractions for Cax-F, P-F, CaxP-F, CaxC-F and CaxCP-F are shown in Figure 2.3.

The average total solubility of Cax-F was only 6.6% showing it to be an essentially insoluble film. The soluble fraction of CAX-F was mainly composed of glucose (~60%), and xylose and arabinose were in lower amounts summing to 35% of the total extractable carbohydrate (Figure 2.3.a). The high glucose content, but in the minor soluble fraction, may have been from a minor amount of residual starch in the corn bran that was extracted with alkali. Ferulic acid residues remained on the solubilized arabinoxylan, as had been determined by Kale et al. (2013), and was used to covalently link CAX via ester bonds upon oxidation with laccase (Izydorczyk & Biliaderis, 1995). The ferulic acid content is the crucial part of the oxidative gelation to obtain strong gel and intensive ferulic acid crosslinking. (Carvajal-Millan, Guilbert, et al., 2005; Kale et al., 2013; Martínez-López et al., 2013). Therefore, the dense crosslinking of ferulic acids created complex networks and these matrices kept the film stable in water. Insoluble Cax-F had an unusually high amount of xylose (~65%) and low amount of arabinose (20%) compared to a typical ~0.5 ratio of these sugars in Cax.

Expectedly, the pectin-based film presented the highest water solubility (~75%). Galacturonic acid compositions were characteristically high for both the soluble and insoluble

fractions (Figure 2.3.b). The pectin gelation mechanism depends on the interaction of galacturonic acids and Ca^{+} ions, and this link was not strong enough to keep P-F insoluble and stable in water. Still, ~25% of the P-F remained insoluble. Many studies report that pectin films are soluble in water (Sartori, Feltre, do Amaral Sobral, Lopes da Cunha, & Menegalli, 2018; Sucheta, Rai, Chaturvedi, & Yadav, 2019).

CaxC-F had higher insoluble composition (~90%) than CaxP-F (~84%), thus the solubility of films was affected by whether the included fiber was cellulose or pectin. Still, a portion of the pectin was retained in the insoluble arabinoxylan film as evidenced by the galacturonic acid in its composition. When all the fiber constituents were included as CaxCP-F, solubility was ~16%, which was more soluble than CaxC-F and less than CaxP-F. The monosaccharide compositions of soluble fraction of CaxC-F and CaxP-F showed that mainly glucose and galacturonic acid escaped from the films. In addition, the amount of arabinose in soluble fraction was different for CaxC-F, CaxP-F and CaxCP-F. Perhaps pectin might interrupt more the crosslinking of arabinoxylan compared to cellulose. Overall, the results show that the arabinoxylan-based films had sufficient intermolecular crosslinking to entrap the pectin, cellulose, and the mixture of pectin-cellulose polymers in the complex matrix network.

2.5 Conclusions

Polysaccharide-based thin films were cast in this study to create insoluble plant cell wall-like matrices with arabinoxylan, pectin, and cellulose for study of the fermentation properties of the human gut microbiota. The molecular network was formed using crosslinked arabinoxylan, and nanosized pectin and cellulose. Pectin itself was shown not to create a strong network that would resist solubilization in water, yet there was still a significant fraction of pectin in the fabricated film that remained unextractable. Corn arabinoxylan was formed to create crosslinked matrices and its film form was mainly insoluble in water. As a result of the strong intermolecular crosslinking of the arabinoxylan in the fabricated films, nanosized pectin, nanofibrillated cellulose, and nanosized pectin-nanofibrillated cellulose were entrapped in the arabinoxylan matrices. Solubility analysis with monosaccharide compositional analysis confirmed that these molecules were still inside the arabinoxylan network after washing with water, and though much of the nanosized pectin was lost a significant portion remained.

Although the initial concentration of solutions and casting thickness were the same for the different combinations of polymers, the final thicknesses of different films were different. These differences were attributed to the addition of polymers in the network structure that

increased the film thickness. The results suggested that the thickness of the films was dependent on the initial condition of polymers (soluble or insoluble) that are entrapped inside the matrices.

In conclusion, in this work the polysaccharide-based thin films were successfully designed and cast to be used as a model of the secondary plant cell wall. In further study, these thin films were used in an *in vitro* human fecal fermentation assay to understand the fermentation profiles of plant cell wall components

Table 2.1 Materials used for casting the films.

Material No	Materials	Abbreviation	Source	% Incorporation
1	Arabinoxylan	Cax	corn arabinoxylan	100% AX
2	Pectin	P	pectin	100% P
3	Arabinoxylan + Pectin	CaxP	corn arabinoxylan and nanosized pectin	75% AX+ 25% P
4	Arabinoxylan + Cellulose	CaxC	corn arabinoxylan and nanofibrillated cellulose	75% AX+ 25% C
5	Arabinoxylan + Cellulose + Pectin	CaxCP	corn arabinoxylan, nanofibrillated and cellulose nanosized pectin	65%AX+25%C+ 10% P

Table 2.2 Thickness of films after drying.

Film Samples	Thickness (μm)
Cax-F	16.67 ± 1.05b
P-F	17.77 ± 0.69b
CaxP-F	19.23 ± 0.81ab
CaxC-F	22.85 ± 1.99a
CaxCP-F	21.21 ± 2.10ab

Values are mean ± S.E. and different letters indicate the significant difference ($p \leq 0.05$).

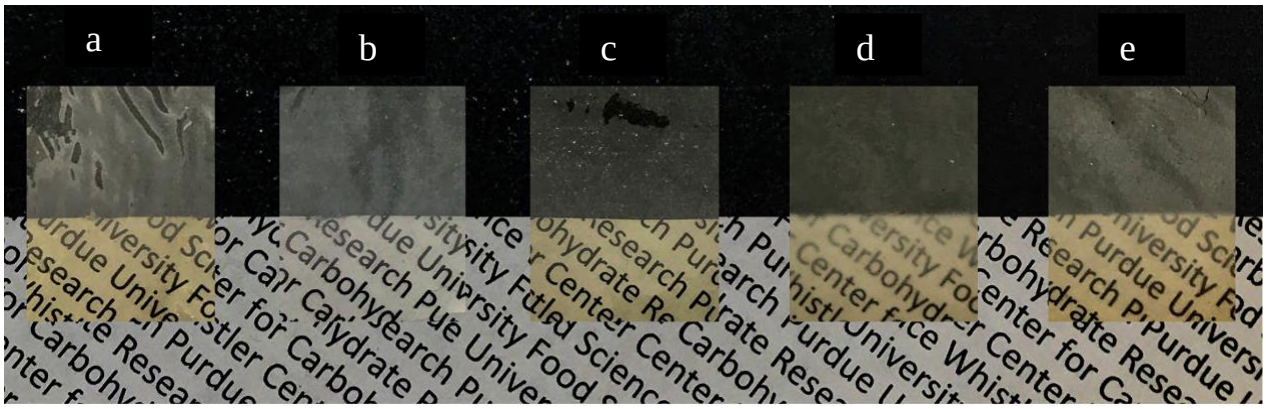

Figure 2.1 Visual appearance of films **(a)** Cax-F **(b)** P-F **(c)** CaxP-F **(d)** CaxC-F **(e)** CaxCP-F.

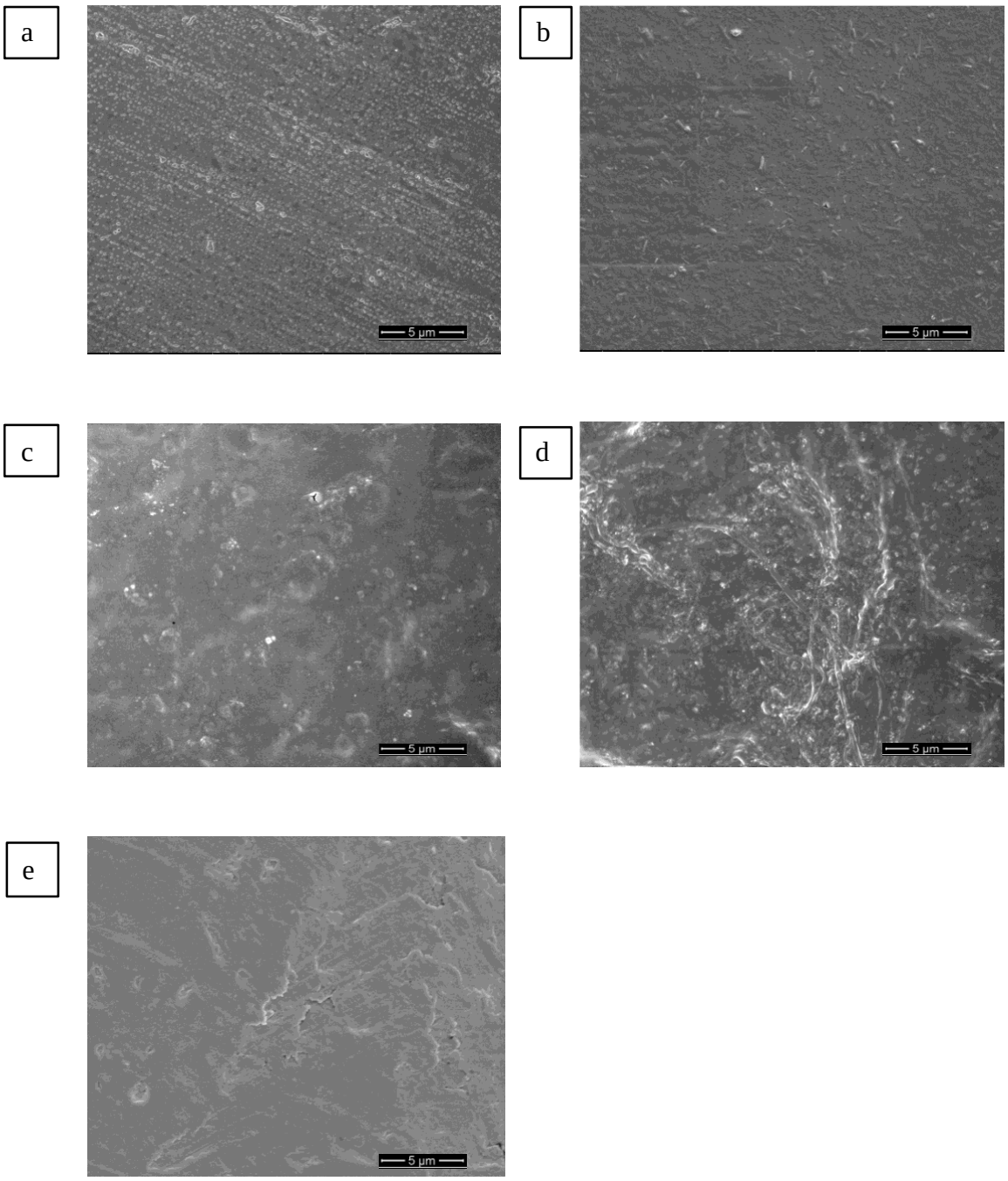

Figure 2.2 SEM images of films surface of (a) Cax-F (b) P-F (c) CaxP-F (d) CaxC-F (e) CaxCP-F. (Scale bar is 5 µm)

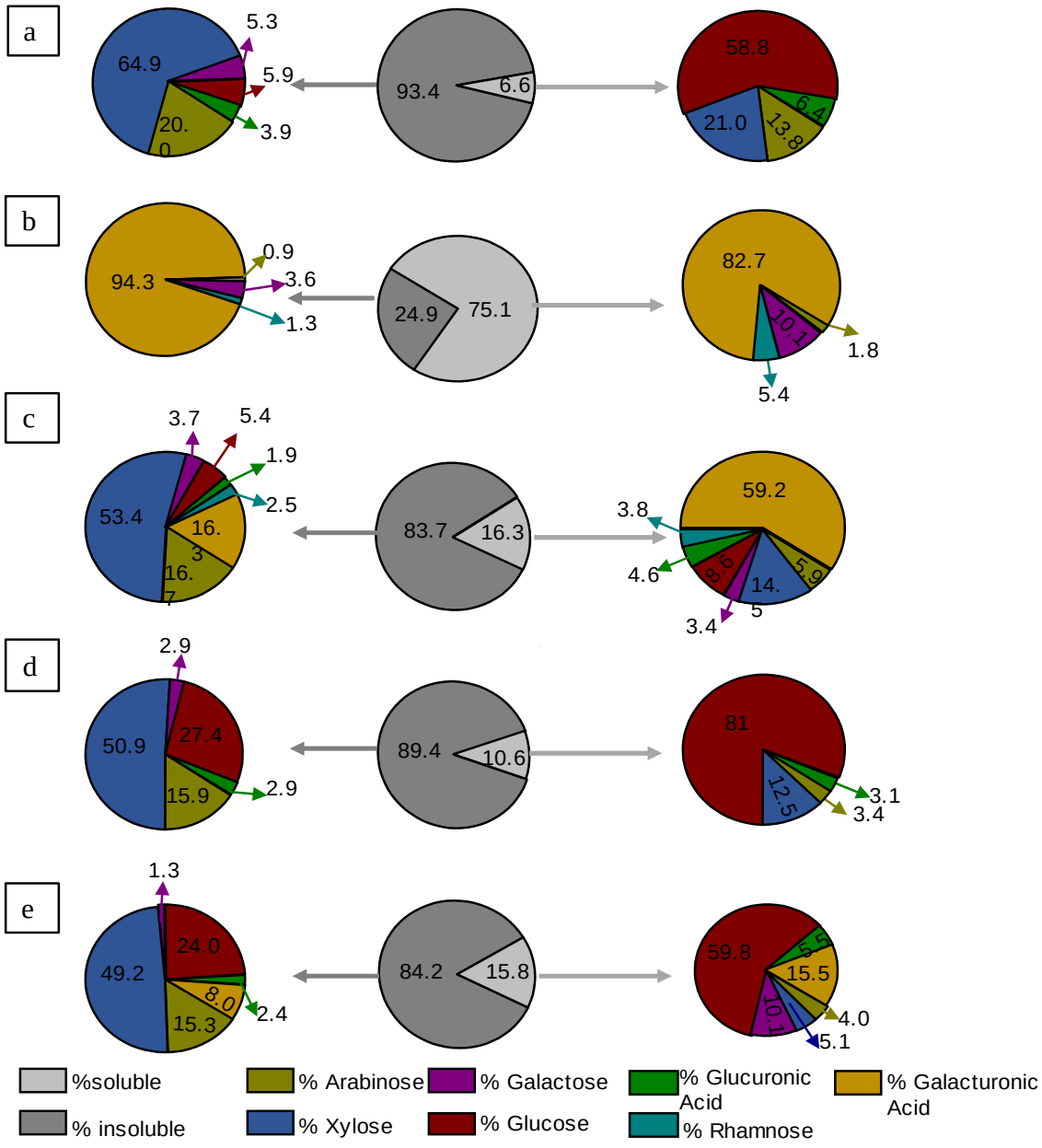

Figure 2.3 The solubility analysis of films. The pie chart in the middle represent the percentage of the films that was soluble and insoluble. Monosaccharide composition of the insoluble fraction is in the left column, and the soluble fraction in the right column. (a) Cax-F, (b) P-F (c) CaxP-F (d) CaxC-F (e) CaxCP-F.

CHAPTER 3. CONSTRUCTED PLANT CELL WALL-LIKE FILMS INCREASE BUTYRATE PERCENTAGES AND TARGET SPECIFIC COLONIC BACTERIA DURING *IN VITRO* FECAL FERMENTATION

3.1 Abstract

Plant cell wall has a complex chemical structure, so it is hard to comprehend the role that all of its components play during colonic fermentation. Using three donors, *in vitro* fecal fermentation of films modeling simple and complex plant cell wall matrixes (ferulic acid crosslinked arabinoxylan film, pectin film, arabinoxylan-pectin film, arabinoxylan-cellulose film, and arabinoxylan-pectin-cellulose) were compared with pure polysaccharides (soluble arabinoxylan and pectin, and insoluble cellulose). The fermentation rate was similar in all plant cell wall-like films, but lower than the native arabinoxylan and pectin. These are indicative that the film forms provide an additional challenge for microbial fermentation and thus are slower fermented than free fermentable polymers. The films made by pectin embedded into the arabinoxylan matrix produced the highest total short chain fatty acid production among the films. Also, all arabinoxylan films led to a significantly higher percentage of butyrate production than arabinoxylan or pectin alone, showing that cell wall-like forms are more selective towards butyrogenic bacteria than the isolated components of the film. The results suggest that the physical form of plant cell wall polysaccharides was significant to determine the end products and microbiota compositions after *in vitro* fecal fermentation. 16S rRNA sequencing revealed that rapidly fermentable pectin embedding inside the matrices of the crosslinked arabinoxylan film differently affected the microbial ecology in fecal samples compared to the arabinoxylan film alone or pectin alone. Heatmap analysis and the relative abundance of specific bacteria showed that in all three donors some taxa were specifically increased in arabinoxylan films forms if compared to the native free arabinoxylan polysaccharide. On the other hand, no specific bacterial shifts were observed between pectin and pectin film. Differences in matrix insolubility of the film, which was high for the covalently linked arabinoxylan films, but low for the non-covalent ionic-linked pectin films, may play an important role targeting Clostridial bacterial groups. Overall, the results suggest that the physical form of the plant cell wall is important in determining the end product of fermentation (SCFA) and shifting bacterial composition in gut towards butyrogenic Clostridia. This work also showed that polysaccharide-based thin films can be used as a model to show how the gradient of accessibility to plant polysaccharides changes the fermentation profile.

3.2 Introduction

The human gut contains around $3.8 \times \cdot 10^{13}$ microorganisms with about a thousand of microbial species (Sender et al., 2016). Generally, the most common bacteria in the colonic microbiota community are Firmicutes (~65%), followed by Bacteroidetes (~25%), Actinobacteria (~5%), and Proteobacteria (~8%) (Ley, Peterson, & Gordon, 2006). The human gut microbiota is important to human health, particularly because the colonic microbiota produce short chain fatty acids (SCFAs) that provide an energy for epithelial cells (Wang et al., 2019) and inhibit pathogenic microorganisms in the gut (Buttriss & Stokes, 2008). Imbalances in the colonic microbiota composition have shown to produce long-term negative health effects such as inflammatory bowel disease and colon cancer (G. Zhang & Hamaker, 2010). Thus, it is important to keep a healthy microbiota composition in the gut. Dietary fibers, which are mainly found in plant cell walls, are an important part of the diet and are known to be able to maintain and/or modify the colonic microbial composition (Buttriss & Stokes, 2008).

The plant cell wall is composed of cellulose microfibrils embedded in a non-cellulosic matrix. Cellulose exists in microfibril forms in plant cell wall and consists of linear chains of β (1,4)-linked glucose units linked through hydrogen bonding. Arabinoxylan is the main polysaccharide in cereal cell walls and consists of β (1,4)-linked xylose residues substituted with α-L-arabinofuranose residues (Izydorczyk & Biliaderi, 1995). Pectin is another complex polysaccharide and present in plant cell walls. Pectin structure contains α (1,4)-linked D-galacturonic acid and rhamnogalacturonan backbones that may be further branched by a variety of lateral side chains (Nakamura et al., 2002).

The fermentability of plant cell walls in the gut is noticeably different between dietary fibers from distinct plant species, varieties, and tissues (Flint, Scott, Duncan, Louis, & Forano, 2012). A number of studies have investigated the *in vitro* fecal fermentation profiles of cell wall polysaccharides such as arabinoxylan (Chen et al., 2017; Rumpagaporn et al., 2016, 2015; Yang et al., 2013), pectin (Bang et al., 2018; Ferreira-Lazarte et al., 2018; Jonathan et al., 2012), and cellulose (Jonathan et al., 2012). In addition, Mikkelsen, Gidley, & Williams (2011) studied fermentation profiles of cellulose composites that are composed of bacterial cellulose incorporated with non-cellulosic polymers (arabinoxylan, xyloglucan, and mixed-linked glucans) as a model of plant cell walls and compared those with their natural free forms (arabinoxylan, xyloglucan, and mixed glucan) by using pig fecal inocula. The results showed that the natural forms were rapidly fermented compared to the cellulose composites and that butyrate ratio significantly increased in cellulose composites (Mikkelsen et al., 2011). This

SCFA molar ratio could be due to the colonization and fermentation by different bacteria. The physical structure of dietary fiber can affect the gut microbial community and understanding this relationship is one of the factors necessary to understand how to make predictable shifts in gut microbes.

In this study, an insoluble arabinoxylan-based thin film were fabricated, and pectin, cellulose, or their mixture were embedded into the film to create models of the plant cell wall structures. *In vitro* fecal fermentation profiles of these films [crosslinked arabinoxylan film (Cax-F), crosslinked arabinoxylan and pectin film (CaxP-F), crosslinked arabinoxylan and cellulose film (CaxC-F), crosslinked arabinoxylan, pectin, and cellulose film (CaxCP-F), and pectin film (P-F)) were compared with their purified forms (Cax, P, and C). The aim of this study was to understand the impact of the complex structure of model cell wall-like films on changes in human fecal microbiota populations and fermentation metabolic outcomes (SCFA). The cell wall-like materials were useful to understand which bacteria degrade them related to their physical form and location of substrates in the plant cell wall. We hypothesized that incorporation of polysaccharides into insoluble crosslinked arabinoxylan matrices give a competitive advantage for specific butyrogenic bacteria.

3.3 Material and Methods

3.3.1 Preparation of Model Dietary Fibers

The different types of plant polysaccharides used in this study included soluble polymers (Cax, P), films (Cax-F, P-F, CaxP-F,CaxC-F and CaxCP-F), and insoluble polymers (C). Arabinoxylan and model films were produced as described in Chapter 2. The model films were cut and sieved to a size range of 0.5-1 mm. Fructooligosaccharides from chicory and pectin from citrus peel were purchased from Sigma Chemical Co. (St. Louis, MO), and nanofibrillated cellulose sourced from Cellulose Lab, Ltd (Canada). Table 3.1 shows the summary of the dietary fibers and model films used in this study.

3.3.2 *In-Vitro* Fecal Fermentation of Films

In vitro fecal fermentation was performed according to the methodology of Lebet et al. (1998) with some modifications (Rose, Patterson, et al., 2010a). Substrates (30 mg) were weighed into test tubes for each time point (0, 6, 12, 24 h) and then all tubes were transferred to an anaerobic chamber. All substrates for each time point were prepared in triplicates. Carbon-phosphate buffer was prepared and sterilized by autoclaving at 121 °C for 20 min.

After autoclaving, 0.25 g/L of cysteine hydrochloride was added, and carbon dioxide was used to remove oxygen by bubbling. The buffer was also directly transferred into the anaerobic chamber at least one night before using.

In the next day, 4 ml of carbonate-phosphate buffer was added to each tube already containing substrates. Feces were collected from 3 healthy donors consuming their routine diets and who had not taken antibiotics for at least 3 months. The collected fecal samples were sealed in plastic tubes and kept on ice and placed into the anaerobic chamber and used within 2 h of collection. The feces were combined with three times the volume of carbonate-phosphate buffer and filtered with 4 layers of cheese cloth. Filtered fecal slurry (1ml) was inoculated with the substrate in each tube. The tubes were sealed with rubber stoppers and incubated at 37 °C in a water bath with gently shaking.

At the planned time points, the tubes were removed from the water bath and gas production was measured using a graduated syringe with a needle, which was inserted through the rubber stopper. After recording gas production, the tubes were opened and the slurries were collected for DNA extraction (1 ml) and SCFA analysis (1 ml). Samples were stored at -80 °C until further analysis. The pH was measured on the residual samples.

3.3.3 Quantification of Short Chain Fatty Acids

Internal standard mixture for SCFA analysis was prepared from 157.5 µl of 4-supplied methylvaleric acid, 1.47 ml of 85% phosphoric acid, 39 mg of copper sulfate pentahydrate and the final volume was adjusted to 25 ml with purified water. Butyric acid, acetic acid, and propionic acid were used as external standards. The samples from the -80 °C freezer were defrosted at room temperature and centrifuged at 13,000 rpm for 10 min. Aliquots (400 µl) from the supernatant of the samples were mixed with 100 µl of internal standard. Four µl of this mixture were injected onto a gas chromatograph (GC-FID 7890A, Agilent Technologies, Inc., Santa Clara, CA) equipped with a fused silica capillary column (NukonTM, Supelco No: 40369-03A, Bellefonte, PA) under the following conditions: injector temperature, 230 ^{O}C; detector temperature 230 ^{O}C; initial oven temperature, 100 ^{O}C; temperature program, 8 ^{O}C /min to 200 ^{O}C with a hold for 3 min at final temperature; carrier gas, helium at 0.75 ml/min. Quantification of short chain fatty acids was calculated from the peaks areas of the acids relative to the internal standard.

3.3.4 Microbiota Analysis

Frozen fecal samples at 0 and 24 h were thawed and centrifuged at 13,000 rpm for 10 min for DNA extraction. The supernatants were discarded, and the pellets were homogenized in phosphate buffer. Then, the samples were transferred into the lysing matrix E tube from FastDNA SPIN® kit for feces (PC: 116570200) (MP Biomedical, Santa Ana, USA). DNA was extracted according to the manufacturer's instructions. The concentration of extracted DNA was evaluated using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technology). Extracted DNA samples were then stored at −20 ^{O}C until further sequencing.

Sequencing was performed using the Illumina MiSeq platform at the DNA Services Facility at the University of Illinois, Chicago. DNA was amplified using with PCR strategy with primers 515F and 806R (Caporaso et al., 2012). The V4 variable region of bacterial and archaeal small subunit (SSU) ribosomal RNA (rRNA) gene was amplified for each sample. Sequencing was performed on an Illumina MiSeq system. Illumina-generated sequencing data were analyzed using QIIME software and R statistical software was used to carry out principal components analysis (PCA) (Caporaso et al., 2010).

3.4 Results and Discussion

Negative control (blank), positive control (FOS), soluble polymers (Cax and P), and insoluble polymers (C) and their film assemblies (Cax-F, P-F, CaxP-F, CaxC-F, and CaxCP-F) were used as a model system to understand the fermentation characteristics of the plant cell matrix and its components (Table 3.1).

3.4.1 Gas Production and pH Changes During *In Vitro* Fermentation

Gas production was indicative of relative fiber fermentability, as well as microbiota depletion of the substrates during the anaerobic incubation. For example, a previous study showed that poorly fermented fibers had low gas production, while highly fermentable fibers produced high gas (Wood, Arrigoni, Shea Miller, & Amadò, 2002). Figure 3.1 shows the amount of gas production of substrates in three donors with all substrates during *in vitro* fecal fermentation.

Gas production of fermented dietary fibers were similarly ranked among the three donors, but differences were also apparent. For each donor, pectin and pectin film were rapidly and nearly completely fermented in the first 6 h. Soluble Cax was the next most fermentable test fiber with lower initial fermentation rate, but well fermented by 24 h. The crosslinked Cax

film reduced fermentation compared to soluble Cax by about 25-40% at 24 h, depending on the donor. Addition of pectin into the arabinoxylan film matrix made the film more fermentable than the arabinoxylan film alone. Cellulose itself was poorly fermentable and similar to the blank, though Donor 2 microbiota was able to ferment cellulose better than others. Cellulose incorporation into the simulated cell wall-like matrix also somewhat reduced fermentation in all donors. Overall, Donor 3 microbiota produced the highest amount of gas for the soluble polysaccharides and that was equal to the fast-fermenting FOS control (at 6 h fermentation, though then decreased to 24 h). FOS was somewhat less fermentable than soluble Cax and P for Donor 1 and even less so for Donor 2. The insoluble matrix film fibers were slightly higher in gas production in Donor 3 compared to the others,

As expected, soluble samples which were Cax, FOS, P and P-F (which was about 75% soluble), produced high levels of gas, and Cax had higher gas production than Cax-F in all donors. Williams, Mikkelsen, le Paih, & Gidley evaluated the fermentability of soluble arabinoxylan in comparison to insoluble arabinoxylan and showed that the soluble fraction had more gas production than insoluble arabinoxylan (2011). Adding the highly fermentable pectin inside the crosslinked arabinoxylan film further increased gas production, while the addition of cellulose to the films reduced fermentation. However, adding both pectin and cellulose in arabinoxylan films (CaxCP-F) did not have an effect in gas production if compared with only pectin in the arabinoxylan film (CaxP-F).

The pH changes of each donor and substrate during *in vitro* fecal fermentation are shown in Figure 3.2. During the anaerobic incubation, the pH of the media decreased due to the production of SCFAs during the *in vitro* fecal fermentation. The pH value of the initial inocula for the three donors were approximately the same (7.14 ± 0.04, 7.13 ± 0.04, and 7.11 ±0.05, respectively). They then responded with different pH drop ranges related to fermentation of the fibers. Shifts in pH were more most notable during the initial 6 h fermentations. As expected from the gas production results, the pH of the fecal slurries incubated with P and P-F decreased more than in other substrates due to the rapid and high fermentation. pH drops for Cax were significantly higher than that observed for Cax-F. For Cax-F, CaxP-F, CaxC-F, and CaxCP-F, the pH drops were moderate. In addition, similar to the blank, cellulose had little pH shift except for Donor 3 in which cellulose was more fermented.

3.4.2 Short Chain Fatty Acid Production During *In Vitro* Fermentation

Short chain fatty acids, mainly acetate, butyrate and propionate, are significant end products of dietary fiber fermentation by colonic microbiota. SCFAs have a number of beneficial effects on host health. They reduce the colonic pH and this helps to inhibit pathogenic microorganisms and to promote beneficial bacteria in colon (Buttriss & Stokes, 2008). Acetate and propionate reach the liver and butyrate is mainly used as an energy source for coloncytes (Tan et al., 2014).The chemical characteristic and physical features of dietary fibers are significant to determine fermentation rate, SCFA profile, and bacterial shifts (Gidley, 2013). Our laboratory group has further shown that physical, as well as chemical, form is a key feature that dictates such shifts, with insoluble and soluble fermentable matrices favoring butyrogenic Clostridial groups (Cantu Jungles et al., 2018; Zhang et al., 2019). To test the effect of physical positioning of plant cell wall-based fibers in soluble, insoluble film, and film-entrapped forms on SCFAs production, *in vitro* fermentation products using substrates (Cax, P, C, Cax-F, P-F, CaxC-F, CaxP-F and CaxCP-F) were quantified regarding SCFAs contents at different time points (0, 6, 12, and 24 h) for the three different donors. It is known that not only the type of dietary fiber, but also the fecal donor diet affect *in vitro* fermentation profiles (Brahma et al., 2017).

A significant boost in acetate production was observed for all substrates, except cellulose, as a result of microbial fermentation in the three donors (Figure 3.3). Acetate is the main SCFA produced by fiber fermentation. It is known to be an energy sources for the host (Flint et al., 2012) and certain Firmicutes (*Eubacterium hallii* and *Anaerostipes spp*.) can convert acetate into butyrate (Duncan, Louis, & Flint, 2004). The results show that the sharpest acetate increases of all substrates occurred during the first 6 h of fermentation in three donors, then followed by a leveling out of profiles from 6 to 24 h for most of the substrates for Donor 1 and a continual slight increase in acetate for Donors 2 and 3, P and P-F led to the production of highest amount of acetate in all donors. Other studies showed that pectic polymers produced high amounts of acetate (Ferreira-Lazarte et al., 2018; Moro Cantu-Jungles et al., 2019; Yang et al., 2013). In addition, Cax generated significantly more acetate than Cax-F at all incubation times for Donors 1 and 3. Likewise, soluble arabinoxylan produced a high amount of acetate during *in vitro* fecal fermentation (Rose, Patterson, et al., 2010a; Rumpagaporn et al., 2015; Yang et al., 2013). The differences in acetate production observed between Cax-F and Cax was due to the crosslinking of arabinoxylan that led to a slower fermentation profile and affected acetate production. Adding pectin inside the arabinoxylan-based film (CaxP-F) significantly

increased the amount of acetate production for Donors 1 and 3 compared with arabinoxylan film (Cax-F). However, adding cellulose did not significantly change the amount of acetate in Donors 2 and 3. Interestingly, there were significant differences in acetate for all substrates at 24 h fermentation for Donor 3.

The amount of propionate production is shown in Figure 3.4. Soluble Cax had the highest level of propionate generation at every time point and in all donors. Propionate is typically produced in high levels from microbiota fermentation of xylans, and particularly from the gram (-) *Prevotella* and *Bacteroides spp*., such as arabinoxylan of Cax (Hu, Nie, Li, & Xie, 2013). Accordingly, arabinoxylan fermentation produced high propionate, and relatively compared with FOS (Rose, Patterson, et al., 2010a; Rumpagaporn et al., 2015). Yang et al. (2013) also studied fecal fermentation of six different dietary fibers showing that arabinoxylan and guar gum had highest propionate concentrations at the end of the fermentation. The insoluble Cax-F produced significantly lower amounts of propionate in all donors due to lower accessibility caused by diferulate crosslinks. Moreover, results at 24 h fermentation indicate that adding soluble polymer (P) within the film (CaxP-F) did not change the propionate production compared to Cax-F in all donors. However, adding cellulose into the arabinoxylan film significantly reduced propionate production for Donors 2 and 3.

The butyrate production in all samples and donors is shown in Figure 3.5. The amount of butyrate produced from *in vitro* fermentation of original substrates and their film forms was higher for Donor 3 in all time points than Donors 1 and 2. FOS, which is known to be a butyrogenic substrate (Chen et al.,2017; Jonathan et al., 2012), generated significantly higher butyrate for Donor 2 and 3 in all time points, whereas Donor 1 produced more butyrate with fermentation of P, P-F and Cax. Jonathan et al. (2012) determined the fermentation profiles of 12 different fibers. They pointed that cellulose and pectin increased butyrate production (Jonathan et al., 2012). In addition, in all donors P-F produced butyrate more gradually than the native pectin, but by the end point (24 h), butyrate amounts between pectin film and the free polymer were not different. Thus P-F possess a slower fermentation profile despite pectin being a rapidly fermented fiber. Notably, Cax-F and CaxP-F resulted in significantly higher butyrate amounts than Cax at 24 h for Donor 3. Butyrate amounts for CaxP-F, CaxC-F and CaxCP-F were not significantly higher than Cax-F for Donors 2 and 3.

Total SCFA is the sum of acetate, propionate and butyrate. The total SCFA production from *in vitro* fecal fermentation of different fibers were highest for Donor 3 (Figure 3.6). As for acetate, the highest total SCFA production was observed for P and P-F in all donors and at all time points. This was an expected result as pectin is a rapidly fermented cell wall component

by microbiota. At the final time point (24 h), Cax produced more total SCFA compared to Cax-F in all three donors. Salvador et al. (1993) mentioned that SCFA profiles during *in vitro* fermentation depend on the chemical and physical form of glycosidic linkages and molecular complexity of dietary fibers. In the present study, crosslinking of ferulic acids turned arabinoxylan into a more complex structure and this complexity of the arabinoxylan structures affected fermentation rate. In addition, Williams et al., who using soluble and insoluble arabinoxylan for *in vitro* pig fecal fermentation, suggested that the physical form of arabinoxylan ('solubility' and 'insolubility') significantly impacted the generation of SCFAs (2011). The results showed that insoluble arabinoxylan produced significantly lower concentration of SCFA (B. A. Williams et al., 2000). Among the arabinoxylan-based films, CaxP-F produced highest amount of total SCFA in all donors, presumably due to the addition of rapidly fermentable pectin within the insoluble arabinoxylan film matrix. Interestingly, the percentage of pectin in films, which was 25% in CaxP-F and 10% in CaxCP-F had significant impact on the total SCFA results in all donors. To summarize, incorporating pectin into the arabinoxylan-based thin film enhanced the fermentation rate and SCFA profiles of the films. For cellulose, all SCFA values were as low or lower than the blank in all donors (Figure 3.6). Many studies based on *in vitro* fermentation models using pig or human feces show that cellulose is a poorly fermented fiber, because of its crystalline structure (Jonathan et al., 2012; Mikkelsen et al., 2011). Moreover, although FOS is known as a fast fermenting fiber (Chen et al., 2017; Jonathan et al., 2012; Kaur, Rose, Rumpagaporn, Patterson, & Hamaker, 2011), it produced lower total SCFAs than P,P-F, and Cax in all time points and donors, related to its lower generation of acetate.

Individual SCFA amounts can also be put on a percentage of total SCFAs basis, or molar ratio, which gives a better view of which types of bacteria (acetogenic, propiogenic, butyrogenic) are promoted by fiber substrates. A typical proportion of acetate, propionate and butyrate in the colon is 65:25:15 of the individual SCFAs, respectively (Tan et al., 2014). However, Hamer et al. (2008) noted that the proportions depend on various factors affecting fecal fermentation such as fiber type, microbiota compositions of host and fermentation site. In the current study, there were proportionally more acetate than the above noted ratio and lesser amounts of propionate and butyrate for all donors and substrates (Figure 3.7). Butyrate levels were significantly higher for the arabinoxylan-based thin films (Cax-F, P-F, CaxC-F, CaxP-F and CaxCP-F), than for the isolated soluble components of Cax and P in all donors despite the overall low concentration when then were incorporated into the films. X. Zhang et al., (2019) showed that during *in vitro* fecal fermentation, butyrate proportion was significantly

higher in crosslinked corn arabinoxylan with comparably high ferulic acid content than crosslinked corn arabinoxylan having comparably low ferulic acid content. They also claimed that this was due to Clostrodial butyrogenic bacteria being somewhat favored by matrices versus the free soluble polysaccharides (Xiaowei Zhang, Chen, Lim, Xie, et al., 2019). In addition, (Leitch, Walker, Duncan, Holtrop, & Flint, 2007) showed that Clostridium Cluster XIVa preferentially colonized insoluble substrates during *in vitro* fecal fermentation. Conversely, we observed that the butyrate proportion for P-F was not significantly different than P for the three donors. This may be because pectin in P-F is not covalently crosslinked, but there is only a light ionic bonding between galacturonic acid units. Unlike pectin, Cax-F had significantly lower proportion of propionate compared with Cax. Adding pectin polymers into the crosslinked arabinoxylan matrix (CaxP-F) decreased both butyrate and propionate proportions in total SCFAs compared with Cax-F for all donors. However, proportion of propionate remained stable when pectin and cellulose were both added to the arabinoxylan film (CaxC-F), while butyrate production decreased for Donors 1 and 3. There was also a trend in decreasing propionate and butyrate for the CaxCP-F substrate for Donors 1 and 3. Butyrate proportion increased when both pectin and cellulose were incorporated into the arabinoxylan film for Donor 2, while propionate level decreased. All in all, the arabinoxylan film forms resulted in higher molar ratios of butyrate production in all donors compared with soluble and free Cax and P.

3.4.3 The Fecal Microbiota Community Structure Over 24 h *In vitro* Fermentation of Polysaccharide-based Thin Films

The polysaccharide-based thin films (Cax-F, P-F, CaxP-F, CaxC-F, and CaxCP-F) and their original forms (Cax, P, and C), as well as positive (FOS) and negative (Blank) controls, were analyzed by *in vitro* fecal fermentation as previously described. Genomic DNA was extracted from fecal samples before (0 h) and after (24 h) incubation with different fiber substrates to understand microbial community changes during *in vitro* fecal fermentation.

3.4.3.1 Effects of *In Vitro* Fecal Fermentation of Polysaccharide-based Thin Films on α-Diversity of Microbial Communities

α-Diversity measures the diversity within a single sample and it is identified not only by the number of species in a community (richness), but also the relative abundance of the species (evenness) in a community (Lozupone & Knight, 2008). In this study, α-diversity was described by the number of species observed and the inverse Simpson (invSimpson), Chao1, Shannon, and Simpson evenness indices for Donors 1, 2, and 3. Number of species and

Chao1are indicators of specie richness. Shannon, Simpson and invSimpson indicate both richness and evenness indices, but Shannon mostly reflects richness of species while Simpson and invSimpson are sensitive to species evenness (Kim et al., 2017). The donor results are treated separately below as there were some differences found in community response to the different chemical and physical forms of the fibers.

The change in α-diversity of the communities for Donor 1 is presented in Figure 3.8. Community richness (Number of species and Chao1 indices) did not significantly change between substrates compared to the initial inocula. However, the other α-diversity metrics (Shannon, Simpson and invSimpson indices) significantly decreased for fiber treatments after 24 h incubation. For the Shannon index, Cax resulted in lower diversity than Cax-F although there is no significant alteration between Cax-F, CaxP-F, CaxC-F, and CaxCP-F (Figure 3.8.d). The same trend was observed among substrates in Simpson and invSimpson indexes. This might be because Cax is a structurally a simple fermentable polysaccharide, but Cax-F has a more complex physical structure due to the crosslinking of arabinoxylan polymers. The simple structure caused a more selective growth throughout the *in vitro* fermentation, while the complex structure of films preserved the evenness and richness of microbial communities. Therefore, we hypothesized that the higher α-diversity (Shannon, Simpson and invSimpson indices) values of films are due to the slower fermentation profiles by fecal microbial communities, if compared to the initial fecal inoculum over the same time period.

α-Diversity of the fecal microbiota communities in Donor 2 is shown in Figure 3.9. There was a significant decrease in Number of species observed and Chao1 values for cellulose (C). The fecal microbiota diversity of Donor 2 was dramatically reduced according to Shannon, Simpson, and invSimpson indices. Solubility of fibers also affected the α-diversity (Figure 3.9 d); for example, Cax-F and P-F, which were mainly insoluble in water, had significantly higher Shannon values than Cax, and P. As shown in Figure 3.9b-e, embedding pectin inside the arabinoxylan matrix (CaxP-F) resulted in significantly higher diversity than in all other substrates. Apparently, a higher diversity of polymers constituting the film supported a greater α-diversity than all other film forms in Donor 2. Interestingly, there were no significant differences in Shannon Index of Cax-F and the initial fecal inoculum (Figure 3.9d), while a significant reduction in Simpson and invSimpson indexes was observed (Figure 3.9 b and e). This indicates that while the richness of species was reduced, Cax-F was able to maintain a similar species evenness to that of the initial inocula. Thus, in Donor 2, it may be that the combination of highly fermentable fibers and complex structure in Cax-F led to a minimization of α-diversity change in microbial communities.

Analysis of the change in α-diversity of microbial communities in Donor 3 after *in vitro* fecal fermentation is demonstrated in Figure 3.10. There was no significant effect on samples on richness of species that were shown in Number of species observed and Chao1 indices. Donor 3 fecal microbiota showed significant differences in Simpson and invSimpson indexes when fermented with Cax, P, and polysaccharide-based thin films (Cax-F, P-F, CaxP-F, CaxC-F, and CaxCP-F). As for Donor 1, although no changes in Number of species and Chao1 indices were observed when treated with Cax and Cax-F, the other α-diversity metrics (Shannon, Simpson and inverse Simpson) increased significantly for Cax-F. As described before, the more complex physical structure of Cax-F may have supported the growth of a larger number of species, thus increasing α-diversity compared to the simpler arabinoxylan polysaccharide (Cax).

3.4.3.2 Effects of *In Vitro* Fecal Fermentation of Polysaccharide-based Thin Films and Native Polymers on β-Diversity of Microbial Communities

β-Diversity measurement is used to evaluate the diversity of two or more communities and to indicate how a microbial communities differ between each other (Lozupone & Knight, 2008). β-Diversity was measured between ferments of polysaccharide-based films (Cax-F, P-F, CaxP-F, CaxC-F, and CaxCP-F), their original forms (Cax, P and C), positive control (FOS), negative control (blank), and initial inocula for the three donors. The Bray-Curtis dissimilarity test based on the relative abundances of OTUs at 97% similarity level was used to reveal similarities between the initial, and 24 h time point for blank and separation was observed samples of each donor and among all donors (Figure 3.11). No significant differences were observed among the samples at 24 h in all donors (ANOVA, $p < 0.001$) (Figure 3.11 a-c). The PCoA analysis showed that the substrates had a noticeable lack of effect on the β-diversity.

3.4.3.3 Changes in Microbiota Composition After 24 h *In Vitro* Fecal Fermentation of Films and Native Polymers

Shifts in microbiota were observed after 24 h of *in vitro* fecal fermentation of polysaccharides-based thin films, their original forms, FOS and the blank which not contain any fibers. Heat maps at the genus level revealed that the structure of gut microbiota was altered after fermentation of films compared to their original forms through the *in vitro* fecal fermentation as demonstrated in Figures 3.12, 3.15, and 3.18 for Donors 1,2 and 3, respectively. Heatmaps showed differences between microbial communities after 24 h fermentation of cell wall-like films (Cax-F, CaxP-F, CaxC-F and CaxCP-F), their native polymers (Cax, P, C) and

positive (FOS) and negative controls (initial and blank). At the genus level, *Bacteroides* was dominant in all three host's gut microbiotas.

After fermentation, shifts in certain microbial taxa were observed for Donor 1 and relative abundance of specific taxa are shown in Figure 3.13. *Alistipes* was significantly higher in all ferments compared to the initial inoculum. A previous study showed that consumption of plant-based diet resulted in increased abundance of bile-tolerant microorganisms like *Alistipes* (David et al. 2014). In addition, a significant increase in *Anaerostipes,* which is a member of the *Clostridium* cluster XIVa, was observed in all arabinoxylan film forms compared to the free Cax. However, no significant differences were observed between the native pectic polymer and its film form. This taxa mostly fermented D-glucose, while weakly fermented *D*-arabinose and *L*-xylose (Phuong Nam Bui, de Vos, Plugge, & Thi Phuong Nam Bui, 2014). The major end products produced by *Anaerostipes* are lactate and butyrate (Schwiertz et al., 2002), and was likely one of the reasons why the molar ratio of butyrate was significantly higher in film forms than Cax for Donor 1 (Figure 3.7). Similar to *Anaerostipes*, *Eubacterium halii* was significantly higher in all arabinoxylan films (Cax-F, CaxP-F CaxC-F and CaxCP-F) than in arabinoxylan native polymer (Cax), while no differences were observed between pectin film (P-F) or its native polymer (P). *Eubacterium halii* has been reported as a high-butyrate producer (Yu et al., 2016). The significant difference between Cax and film forms of Cax was that the physical form of arabinoxylans played a major role in selecting specific microbial community members for growing on the substrates during fermentation.

The microbiota composition after 24 h fermentation was also compared at the genus level for Donor 2 (Figure 3.15). It is interesting to note that a *Bifidobacterium* population was higher in CaxP-F compared to Cax, Cax-F, P-F and P alone after 24 h fermentation. In addition, the relative abundance of *Anaerostipes* was significantly higher in all arabinoxylan films (Cax-F, CaxP-F, CaxC-F and CaxCP-F) than on the arabinoxylan native polymer (Cax). On the other hand, native pectic polymer (P) and pectin film (P-F) did not present significant differences in *Anaerostipes* aboundance. This is the same trend observed and previously discussed for *Anaerostipes* in Donor 1. Similar to *Anaerostipes, Parabacteroides* and *Unasigned Lachnospiraceae* were in higher abundance in all arabinoxylan film forms compared to the native arabinoxylan, but no differences were observed for the pectin native polymer or its film form. Another interesting aspect in Donor 2 was that *Anaerostipes* and Unassigned *Lachnospiraceae* were significantly higher in the arabinoxylan film with entrapped pectin (CaxP-F), than in the native pectin (P), native arabinoxylan (Cax) or the arabinoxylan film

(Cax-F). This indicates that both the film form and its composition play a role in selecting for the growth of these bacteria. Moreover, the complex structure of CaxP-C was more selective to the specific bacteria of *Anaerostipes* and Unassigned
Lachnospiraceae than other thin films or their native forms in Donor 2. Besides that, the growth of *Parabacteroides* was selectively promoted by fermentation of all the thin films compared to Cax. Members of *Parabacteroides* mainly produce acetic and succinic acids during the fermentation of substrates (Sakamoto & Benno, 2006).

The relative abundance of *Bacteroides, Alistipes, Anaerostipes*, and *Ruminococcaceae* in Donor 3 is shown in Figure 3.19. In general, all arabinoxylan films Cax-F yielded larger abundance of these microbial taxa than the native polymer (Cax) after 24 h fermentation. For pectin, however, no differences were observed between the native polymer (P) and its film form (P-F). *Ruminococcaceae* is known to be promoted by degradation of plant cell wall polysaccharides, especially cellulose (Flint et al., 2012). Therefore, higher abundance of these taxa after 24 h fermentation could be related to both the initial fecal inocula and the physical matrix form of films.

Linear discriminant analysis (LDA) can be used to explain the degree of consistent difference in relative abundance between the groups that are compared with each other and illustrated as a histograms (Segata et al., 2011). These LDA histograms demonstrate which communities among all those detected revealed considerable differences between the groups (Segata et al., 2011). LDA analysis was applied to identify the key bacterial phylotypes responsible for the difference between Cax and Cax-F, P and P-F, and among the films in Figure 3.14, 3.17, 3.120 for Donors 1, 2, and 3, respectively. The results revealed that the selective consumption of gut microbiota played an important role to degrade specific substrates during *in vitro* fermentation. For example, in Donor 1, *Alistipes*, which was abundant in Cax-F as previously discussed, was identified as a the dominant phylotype in Cax-F in the LDA histogram compared with Cax, highlighting differences between arabinoxylan in film and native forms (Figure 3.14a). These bacteria were also discriminative for CaxCP-F compared other films. In Donor 2, *Akkermansia* was a discriminator for Cax-F compared with Cax, and among the films, it was a differentiator for CaxCP-F (Figure 3.17a,c). Yassour et al. (2016) reported that the increase in *Akkermansia* might be related with the decreasing of the risk of type 2 diabetes and obesity. Linear discriminant analysis (LDA) effect size also indicated that the genera *Lachnospiraceea* was a differentiator for the P-F compared with P in Donor 3 (Figure 3.20). Bang et al. (2018) noted that *Lachnospiraceea* family was increased when

incubated with pectin. Based on LDA analysis, it appears that both the physical condition (soluble or insoluble) and monosaccharide composition of fibers were significant factors to promote changes in the microbiota composition. The results also showed that the colonic microbiota composition after fiber treatments was dependent on the initial microbiota community of each donor.

Overall, we have showed that the plant cell wall-like films promoted different species of bacteria than that of the native polysaccharide constituents. For example, *Anaerostipes* was higher in all arabinoxylan films than in the native arabinoxylan and this was consistent in all donors. Furthermore, there were instances where pectin or cellulose embedded in the insoluble arabinoxylan further differentiated bacterial response. Apparently, some bacteria have a competitive edge in utilizing fibers when they are hidden inside insoluble arabinoxylan matrices. Flint et al. reported that nutritionally specialized bacteria are critical to degrade complex substrates such as plant cell wall (2012). We propose that the complex structure of polysaccharide-based thin films could promote more specific bacterial groups shifts during *in vitro* fecal fermentation than simple isolated polymers.

3.5 Conclusion

In vitro fecal fermentation of insoluble crosslinked arabinoxylan matrices resulted in a fermentation rate slower compared with free and soluble arabinoxylan. Embedding pectin, cellulose, and the mixture of pectin and cellulose affected the total short chain fatty acid production. Total amount of short chain fatty acids of CaxP-F was significantly higher compared to Cax-F, CaxC-F and CaxCP-F. These results suggest that the presence of pectin embedded in arabinoxylan matrices (CaxP-F) modulate the microbial community in fecal inocula. Interestingly, the proportion of butyrate was significantly higher for the cell wall-like thin films (Cax-F, CaxP-F, CaxC-F, and CaxCP-F), compared with Cax at 24 h fermentation.

This study also showed that the initial microbiota composition impacted fiber fermentability and microbiota composition during *in vitro* fermentation of films and their initial counterparts. For example, Donor 3 produced a higher amount of total short chain fatty acids when compared to other donors. Similarly, shifts in microbial composition after 24 h fermentation was dependent on the donor initial microbiota. In addition, 16S rRNA sequencing was used to identify which bacterial groups were promoted by these films. This study supported

that each donor, with its own unique microbiota composition, responded differently to *in vitro* fecal fermentation of dietary fibers.

The complex structure of plant cell wall-like thin films revealed differences at the bacterial genus level. Some groups were specifically increased in all arabinoxylan film forms compared to the native free arabinoxylan. On the other hand, no specific bacterial shifts were observed between pectin and pectin film. Differences in matrix strength, which is high for the covalently linked arabinoxylan films, but low for the non-covalent ionic linked pectin films, may play an important role targeting specific bacterial groups. For example, a consistent increase in all three donors was observed for *Anaerostipes spp.*, a butyrogenic genera from Clostridium cluster XIVa, when fermented with plant polysaccharide-based thin films, but not its isolated polysaccharides counterparts. Overall, the assembled cell wall-like films were useful to understand which bacteria degrade them related to their physical form and location. This study showed that how fabricated films with cell wall materials show specificity and competitiveness of some gut bacteria and suggest that fabricated materials using natural fibers might be used for targeted support of certain gut bacteria and bacterial groups.

Table 3.1 Substrates used for fermentation.

substrate no	substrate	abbreviation	source	% incorporation
1	Fructo-oligosaccharide	FOS	chicory	na[a]
2	Arabinoxylan	Cax	corn bran	na
3	Pectin	P	citrus peel	na
4	Cellulose	C	nanofibrillated cellulose	na
5	Arabinoxylan film	Cax-F	corn arabinoxylan	100% AX
6	Pectin film	P-F	pectin	100% P
7	Arabinoxylan+ pectin film	CaxP-F	corn arabinoxylan and nanosized pectin	75% AX+ 25% P
8	Arabinoxylan+ cellulose film	CaxC-F	corn arabinoxylan and nanofibrillated cellulose	75% AX+ 25% C
9	Arabinoxylan+ cellulose + pectin	CaxCP-F	corn arabinoxylan, nanofibrillated and cellulose nanosized pectin	65%AX+25%C+10% P

[a]na, not applicable

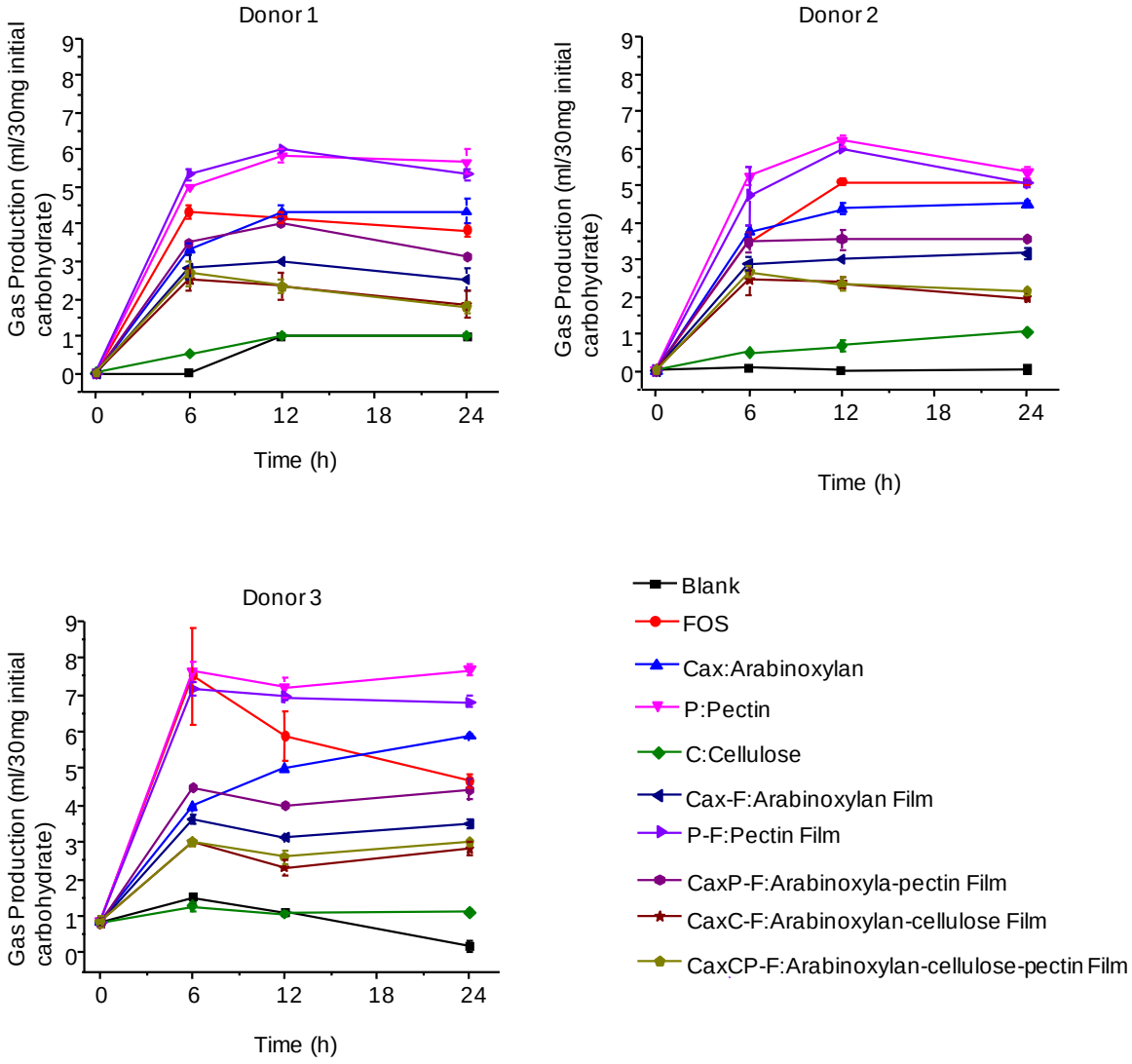

Figure 3.1 Total gas production for three donors during *in vitro* fecal fermentation of films and their free forms. Error bars show the standard error of the mean of three replicates of substrates.

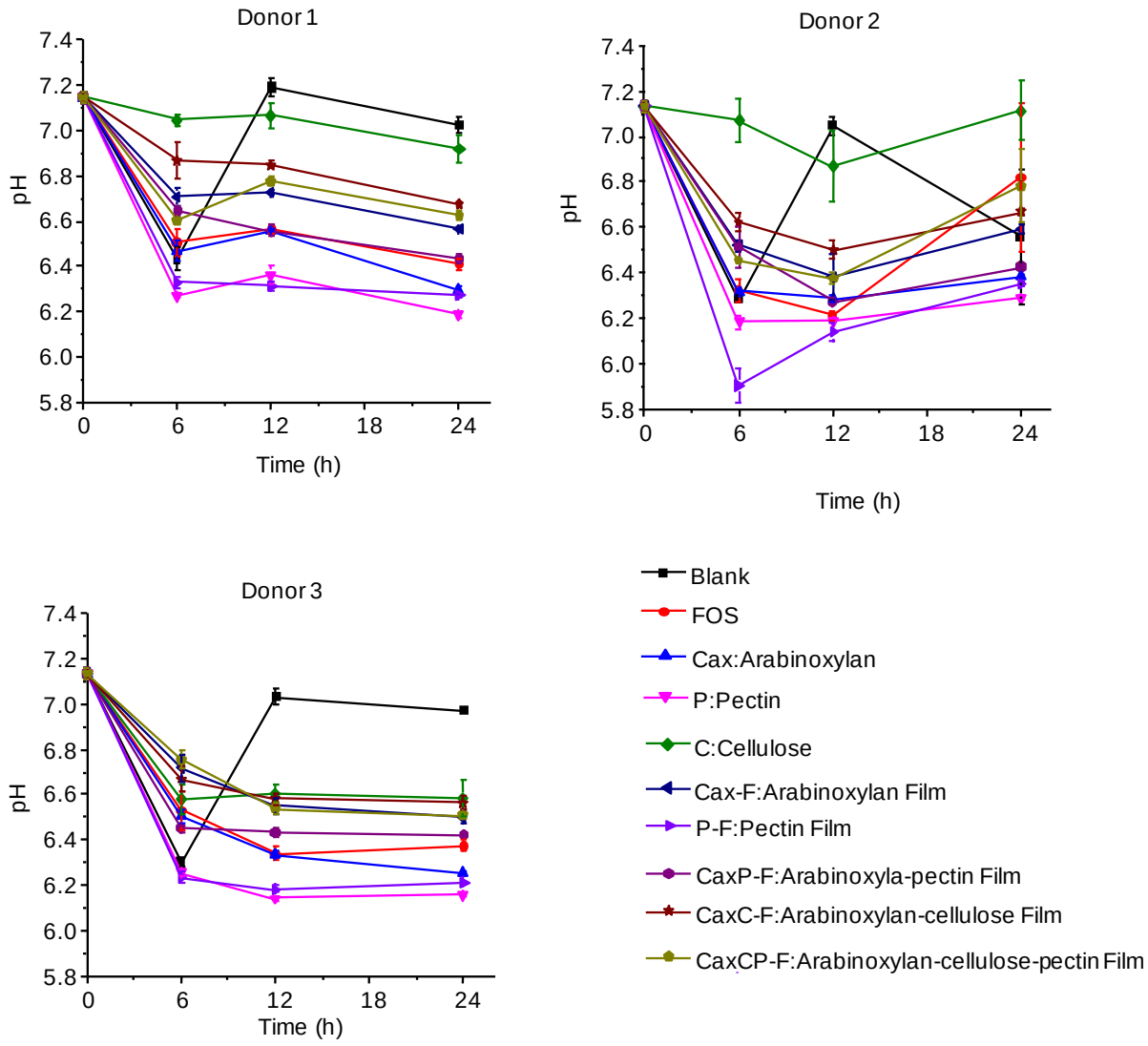

Figure 3. 2 pH shifts during *in vitro* fecal fermentation of films and their free forms. Error bars show the standard error of the mean of three replicates of substrates.

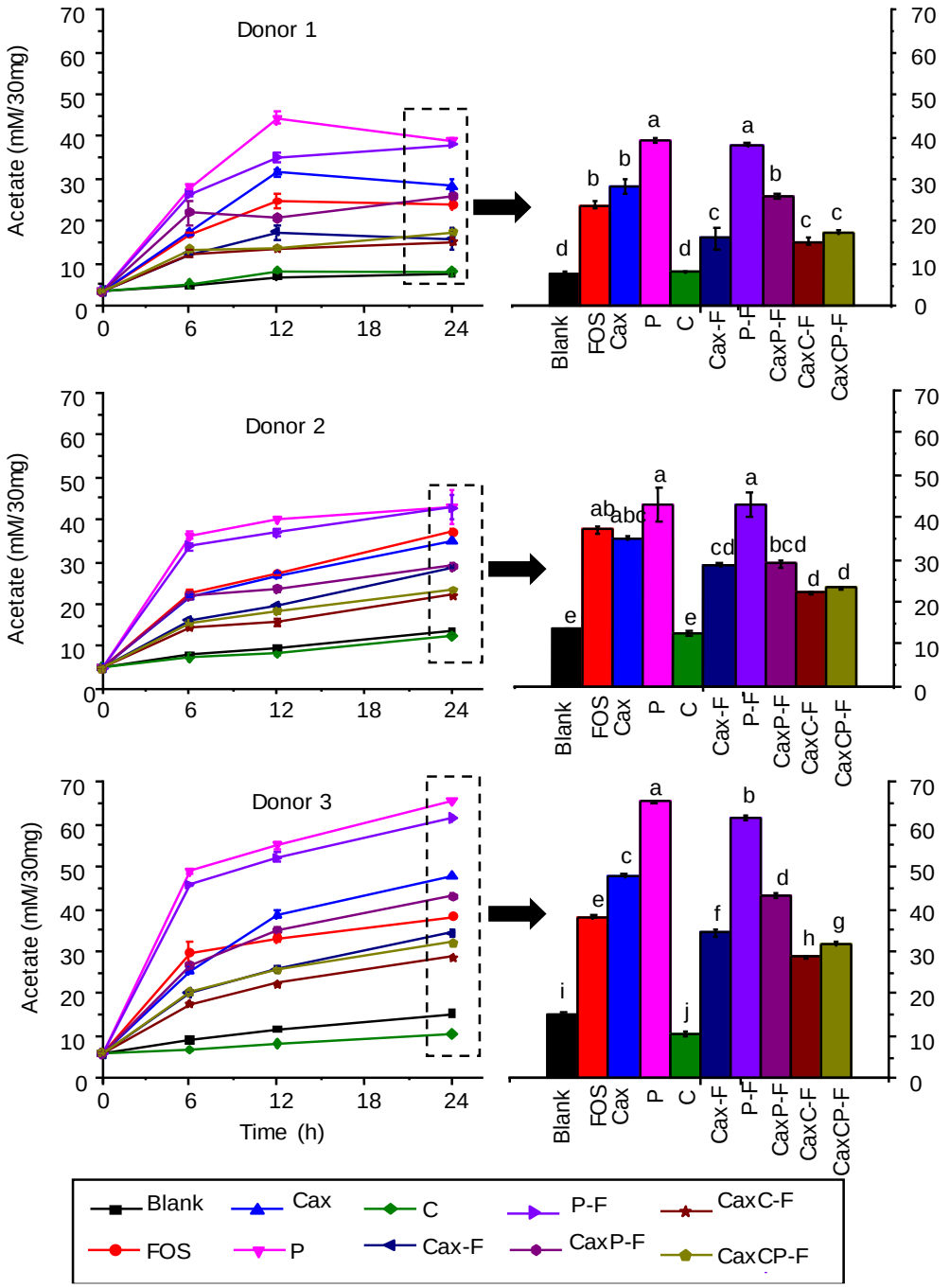

Figure 3. 3 The acetate productions during *in vitro* human fecal fermentation. FOS (fructooligosaccharides) was a positive control. The blank was a negative control that not icluding any substrates. Error bars represent the standard error of the mean of three separate replicates shows with error bars. Different letters indicate significant differences in SCFAs among treatments at the same time point (Tukey's multiple comparisons test, $\alpha = 0.05$).

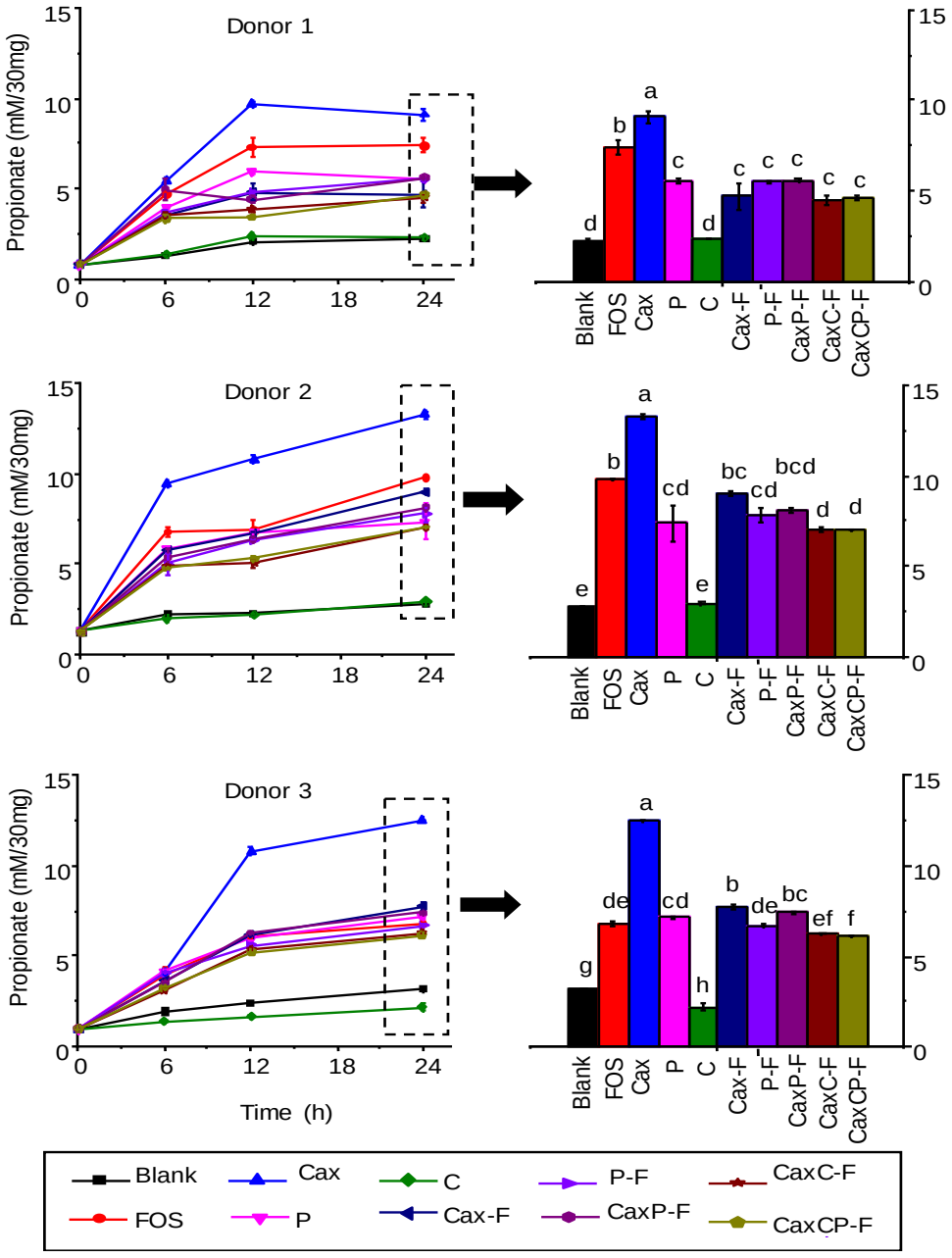

Figure 3. 4 The propionate productions during *in vitro* human fecal fermentation. FOS (fructooligosaccharides) was a positive control. The blank was a negative control that not icluding any substrates. Error bars represent the standard error of the mean of three separate replicates shows with error bars. Different letters indicate significant differences in SCFAs among treatments at the same time point (Tukey's multiple comparisons test, $\alpha = 0.05$).

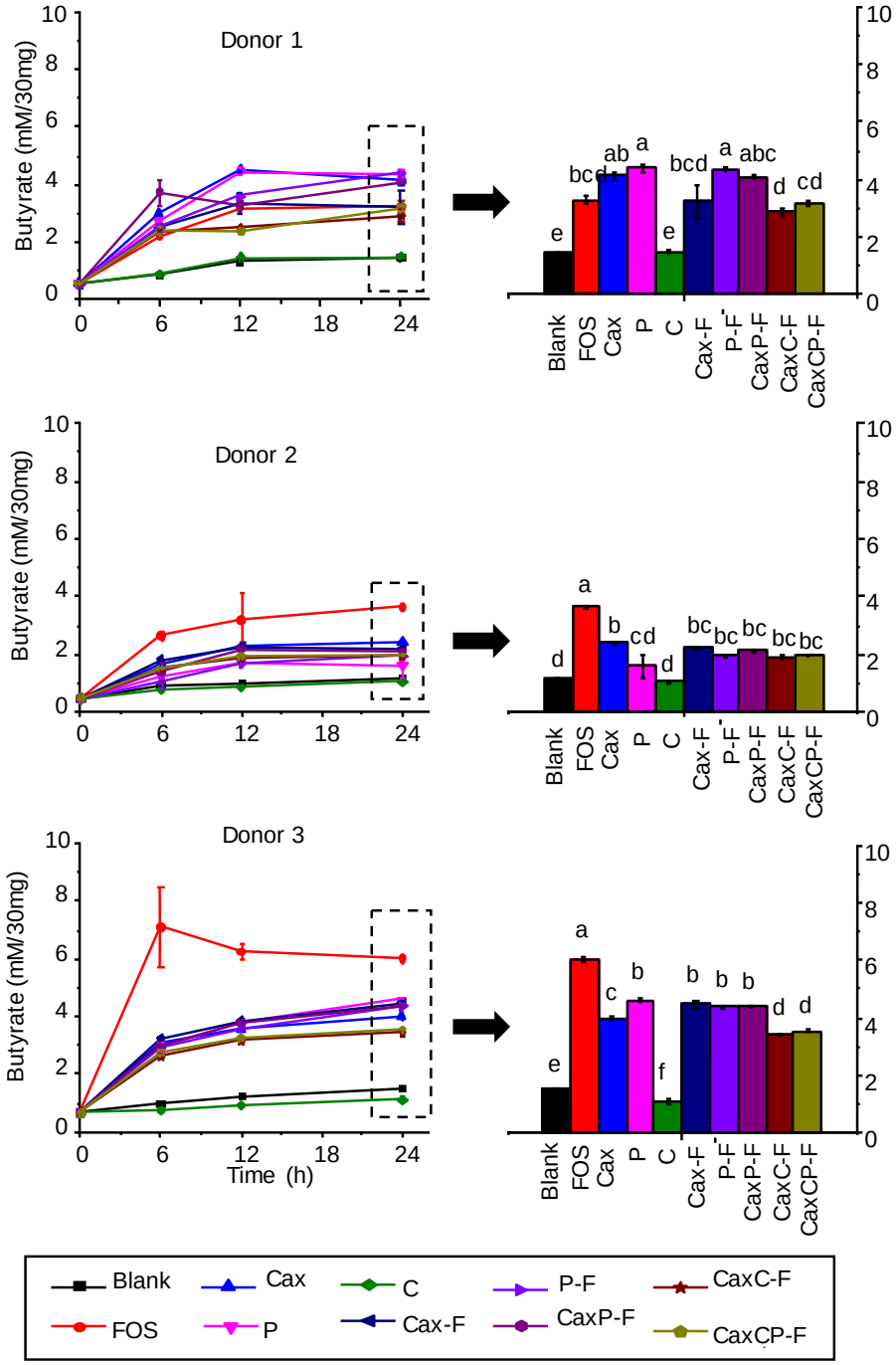

Figure 3. 5 The butyrate productions during *in vitro* human fecal fermentation. FOS (fructooligosaccharides) was a positive control. The blank was a negative control that not icluding any substrates. Error bars represent the standard error of the mean of three separate replicates shows with error bars. Different letters indicate significant differences in SCFAs among treatments at the same time point (Tukey's multiple comparisons test, $\alpha = 0.05$).

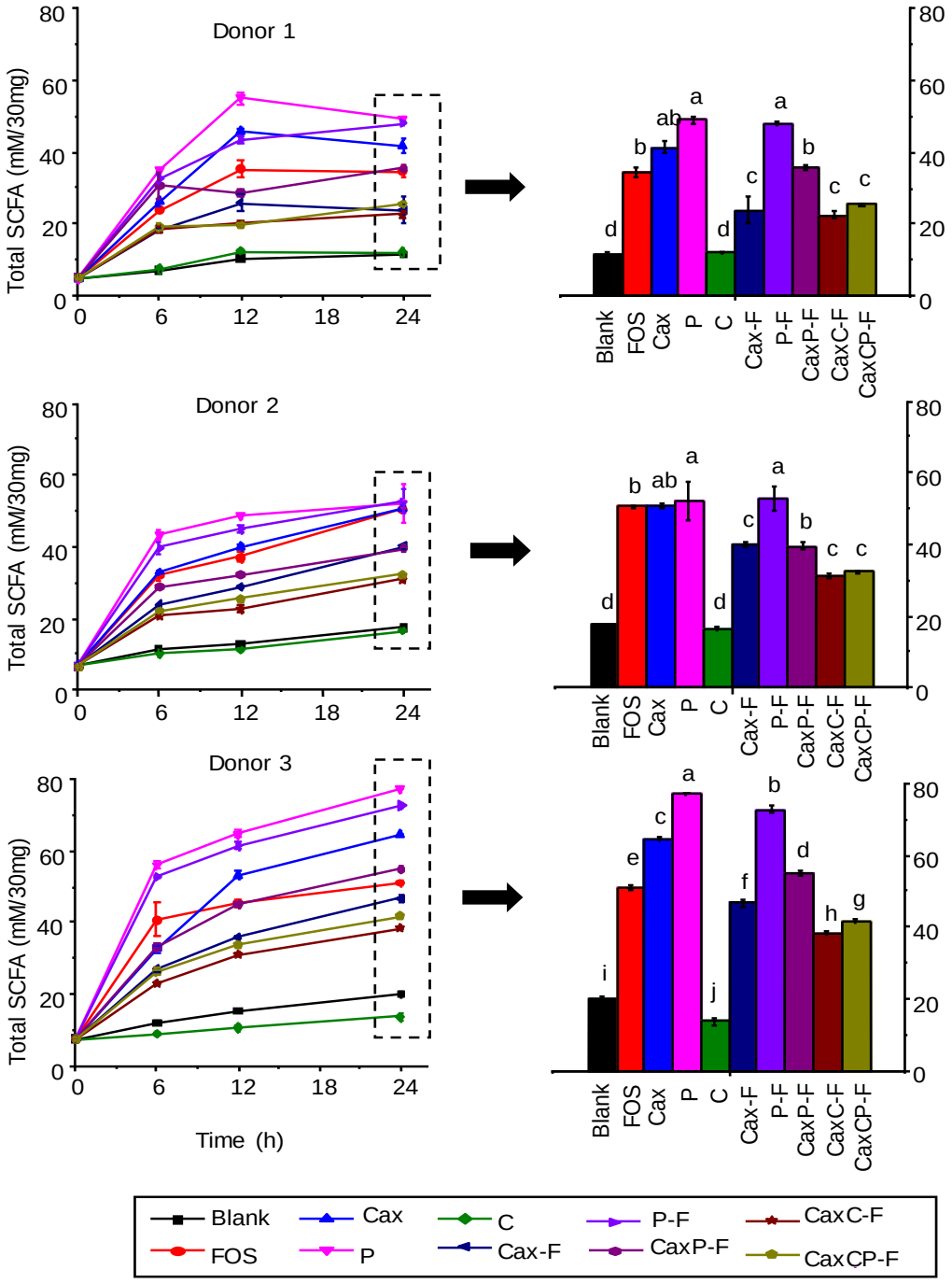

Figure 3. 6 Total short chain fatty acid (SCFA) productions during *in vitro* human fecal fermentation. FOS (fructooligosaccharides) was a positive control. The blank was a negative control that not icluding any substrates. Error bars represent the standard error of the mean of three separate replicates shows with error bars. Different letters indicate significant differences in SCFAs among treatments at the same time point (Tukey's multiple comparisons test, $\alpha = 0.05$).

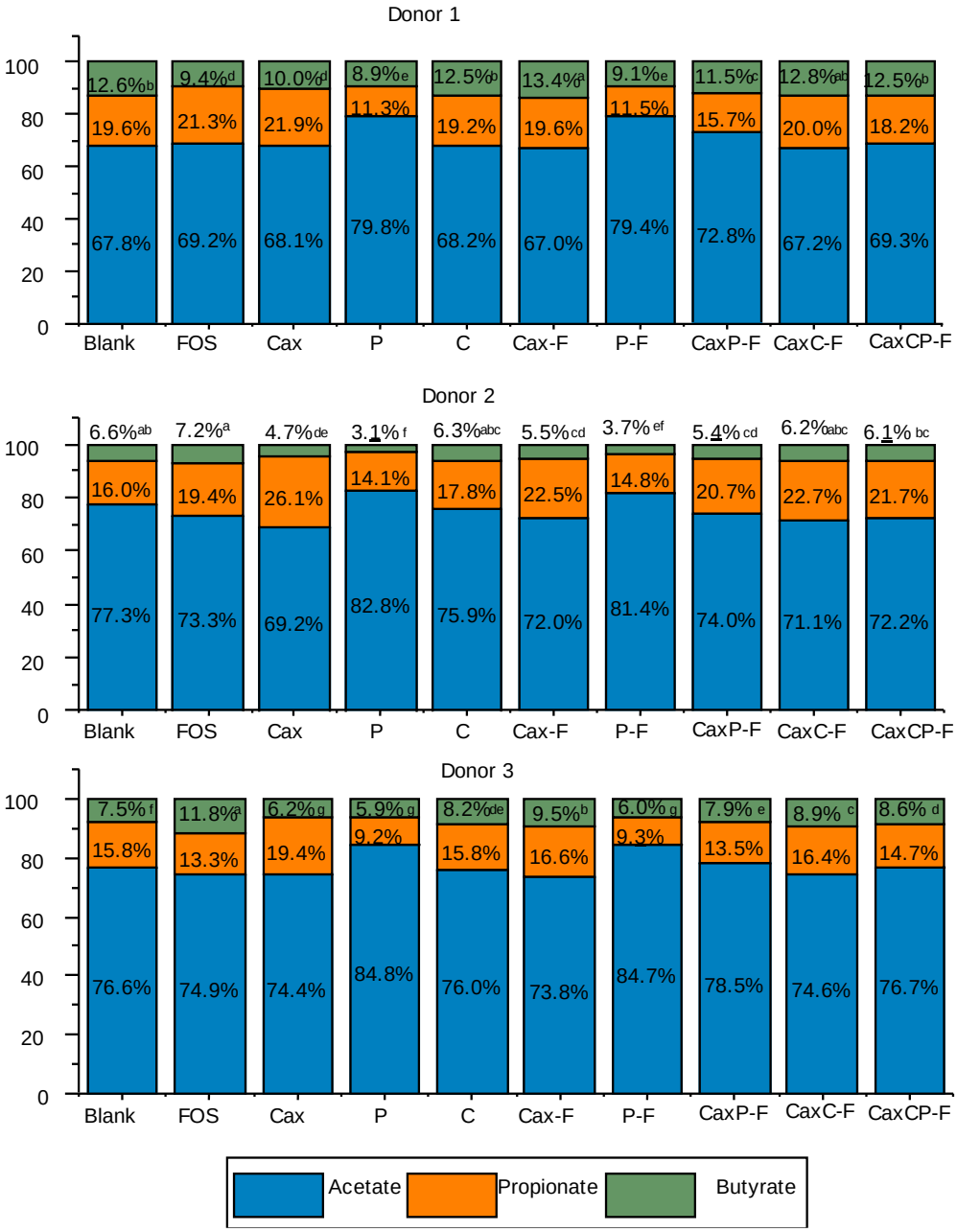

Figure 3. 7 Short chain fatt acids percentage ratio generated by Blank, FOS = Fructooligosaccharides, CAX = Arabinoxylan, P=Pectin, C=Cellulose, Cax-F= Arabinoxylan film, P-F=Pectin film, CaxP-F= Arabinoxylan-pectin film, CaxC-F=Arabinoxylan-cellulose film, CaxCP-F=Arabinoxylan-cellulose-pectin film. after 24h in vitro fecal fermentation in three donors.

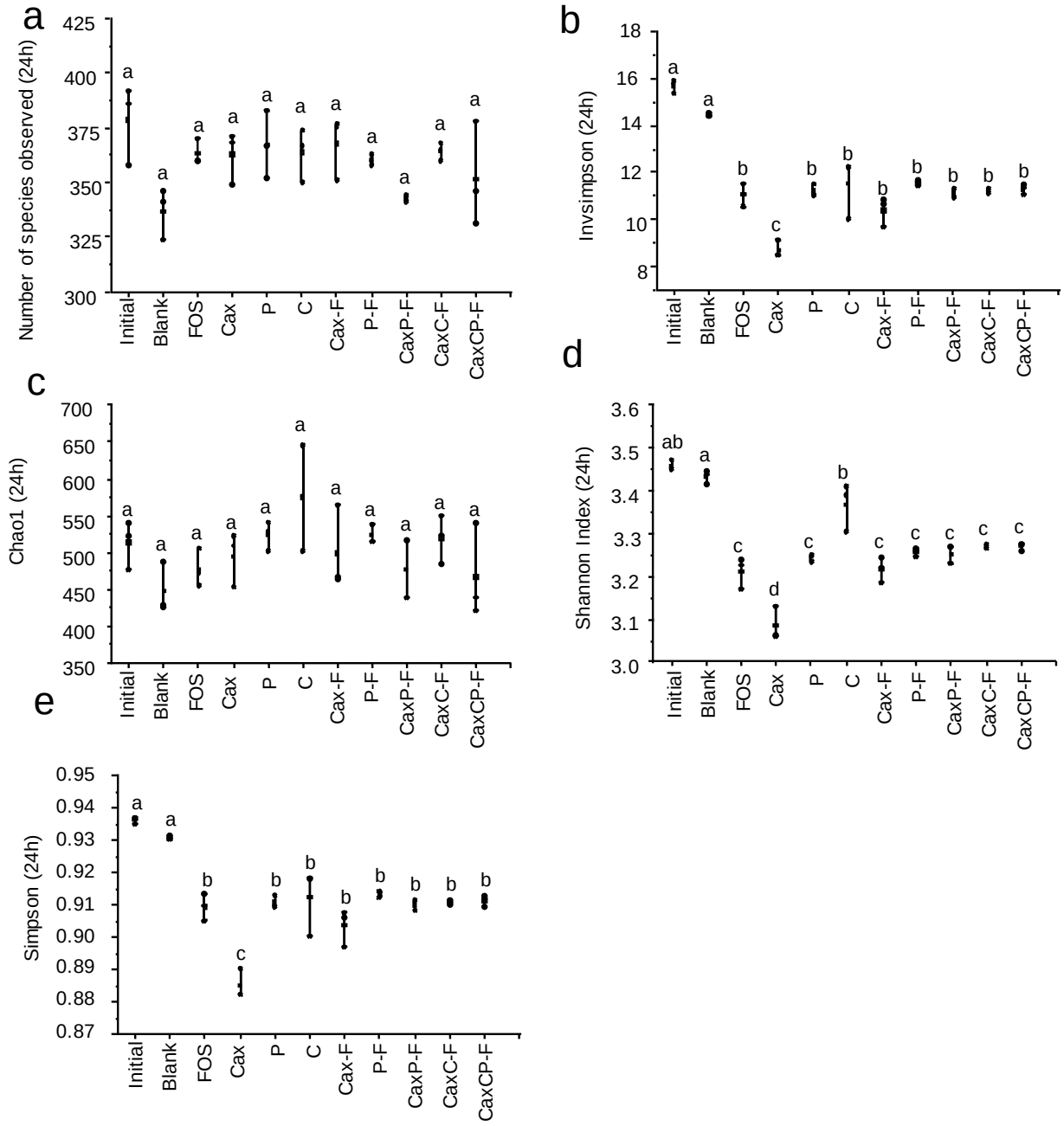

Figure 3. 8 Changes in α-diversity for donor 1 fecal microbiota communities after 24 h *in vitro* fecal fermentation of films and their free forms (a) number of species observed, (b) the inverse Simpson index (invSimpson), (c) Chao1, (d) Shannon index, and (e) Simpson evenness index. Different letters indicate significant differences between substrates at the same time point ($\alpha = 0.05$).

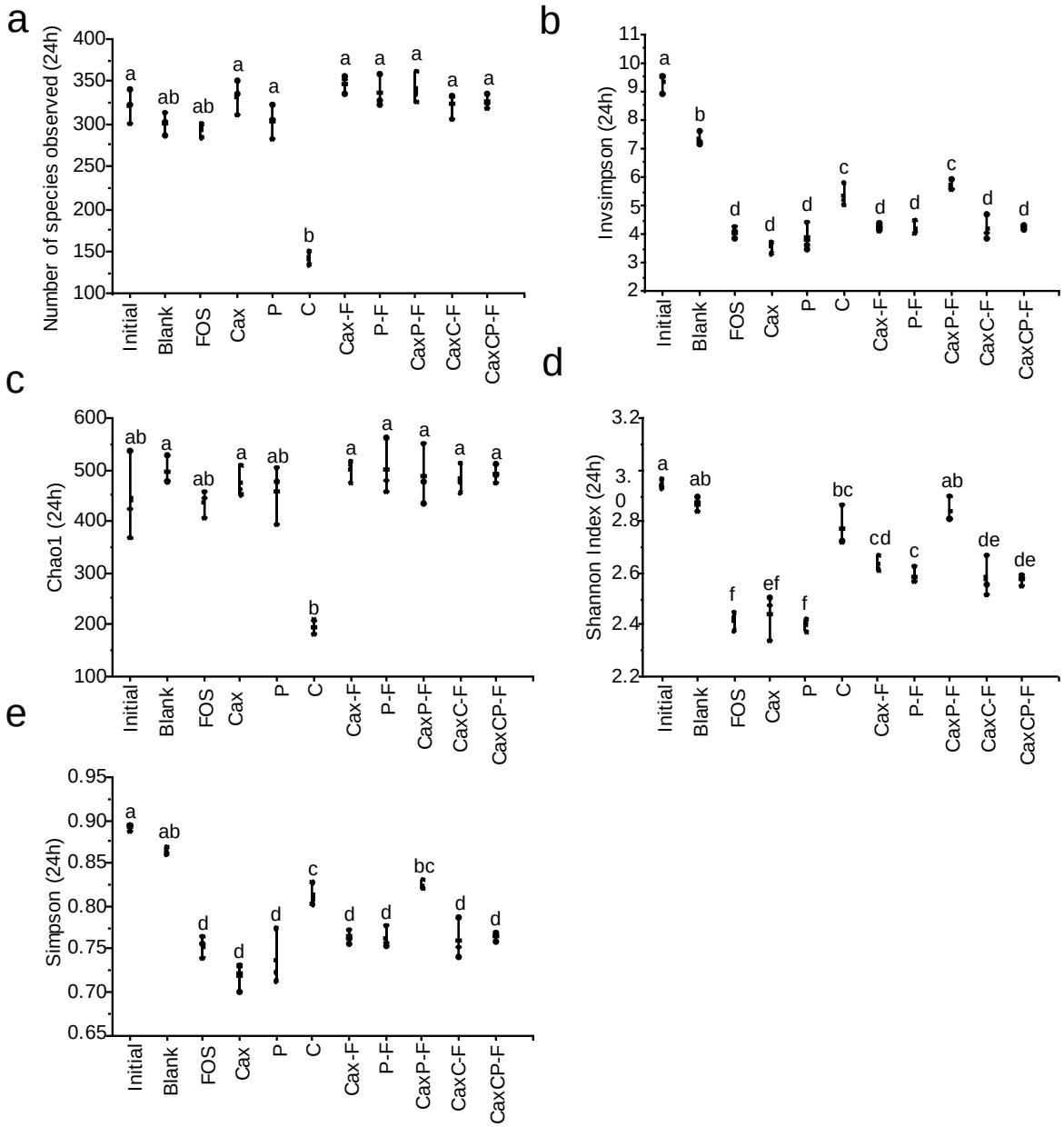

Figure 3. 9 Changes in α-diversity for donor 2 fecal microbiota communities after 24 h *in vitro* fecal fermentation of films and their free forms (a) number of species observed, (b) the inverse Simpson index (invSimpson), (c) Chao1, (d) Shannon index, and (e) Simpson evenness index. Different letters indicate significant differences between substrates at the same time point ($\alpha = 0.05$).

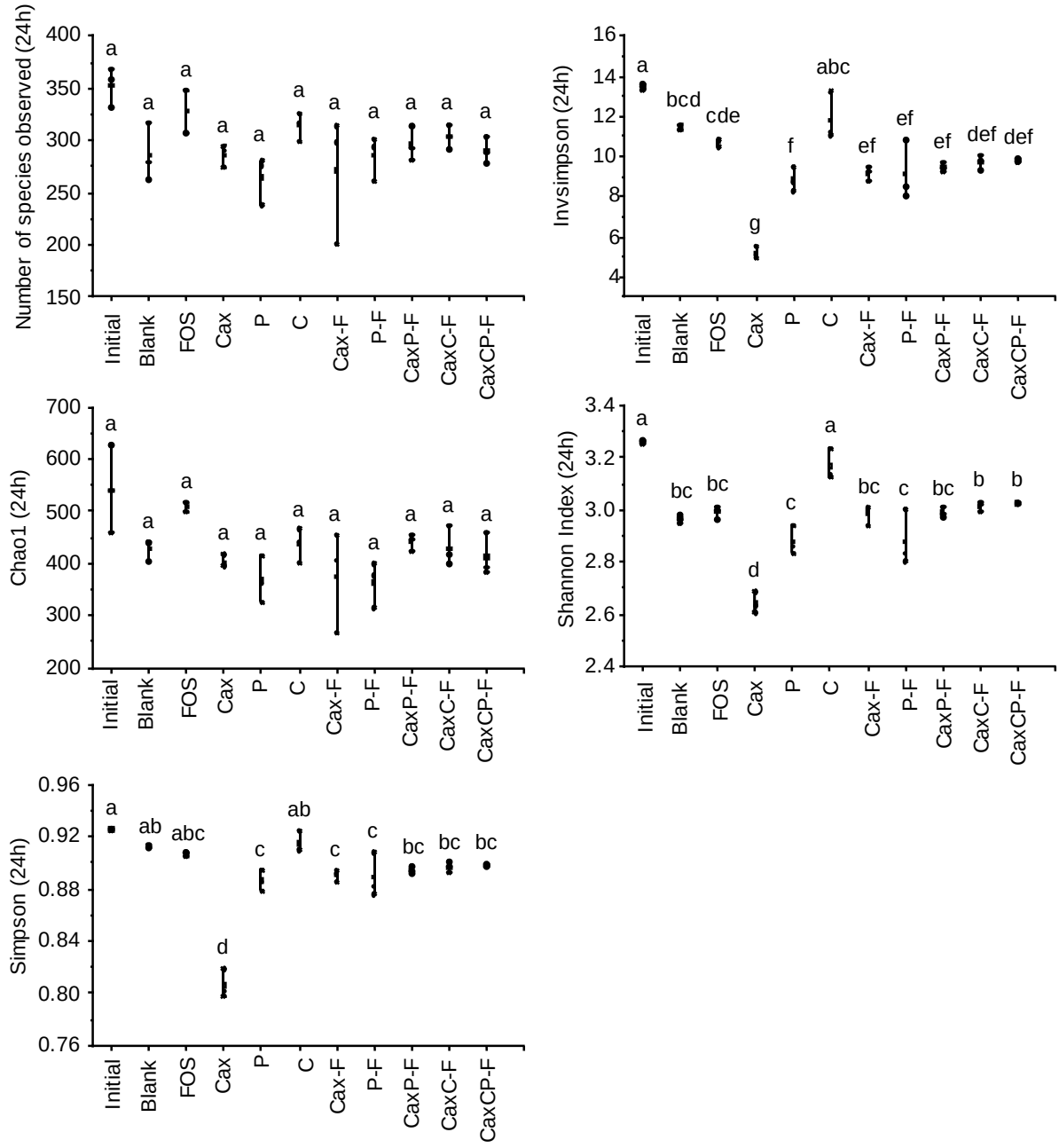

Figure 3. 10 Changes in α-diversity for donor 3 fecal microbiota communities after 24 h *in vitro* fecal fermentation of films and their free forms (a) number of species observed, (b) the inverse Simpson index (invSimpson), (c) Chao1, (d) Shannon index, and (e) Simpson evenness index. Different letters indicate significant differences between substrates at the same time point (α = 0.05).

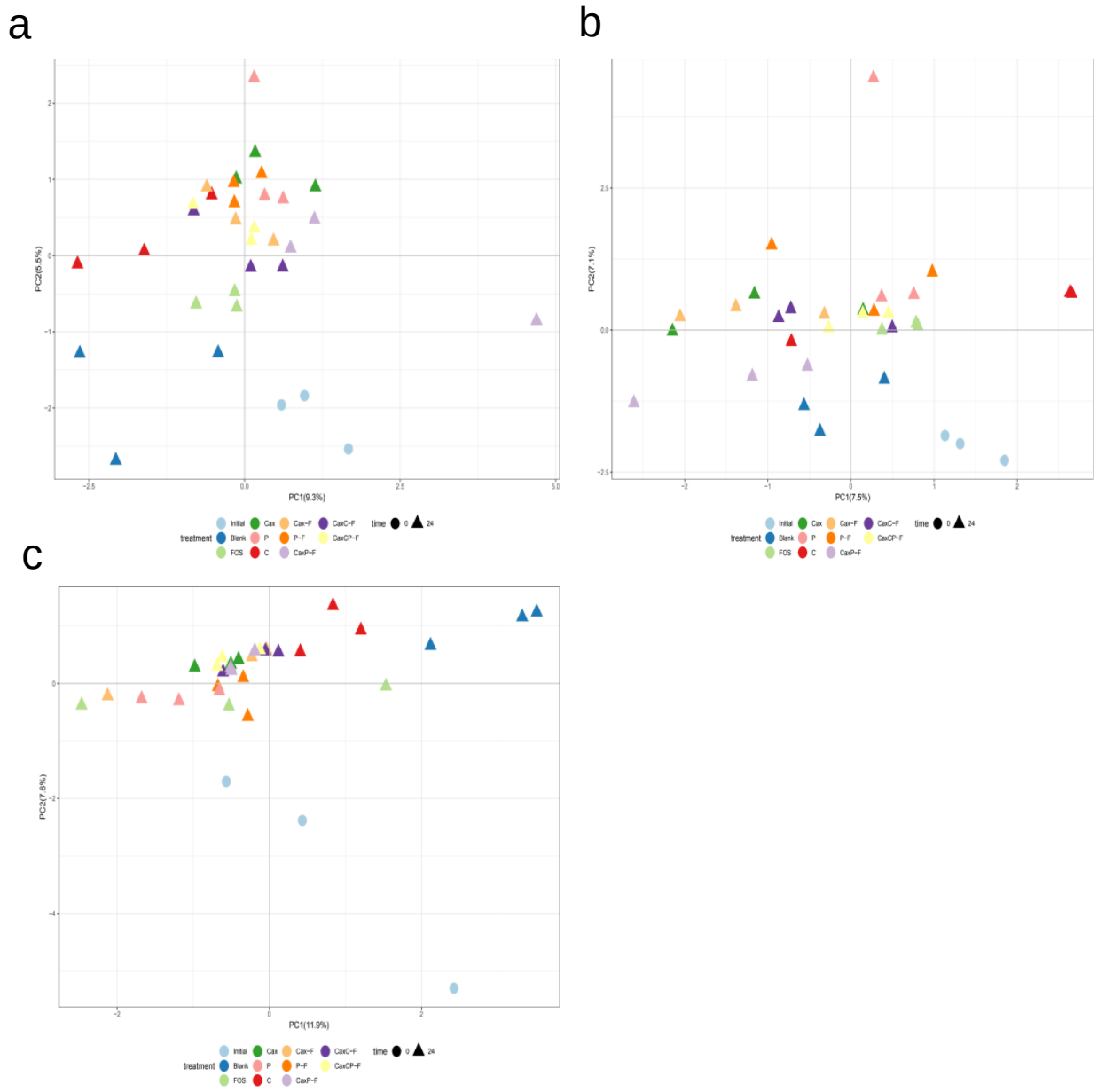

Figure 3. 11 Principal component analysis of community structures of **(a)** comparison of substrates based on donor 1, **(b)** comparison of fibers based on donor 2, **(c)** comparison of substrates based on donor 3 as determined by 16S rRNA gene amplicon sequencing. Bray−Curtis dissimilarity of fecal microbiota was based on the relative abundances of OTUs at a 97% identity level after in vitro fermentation.

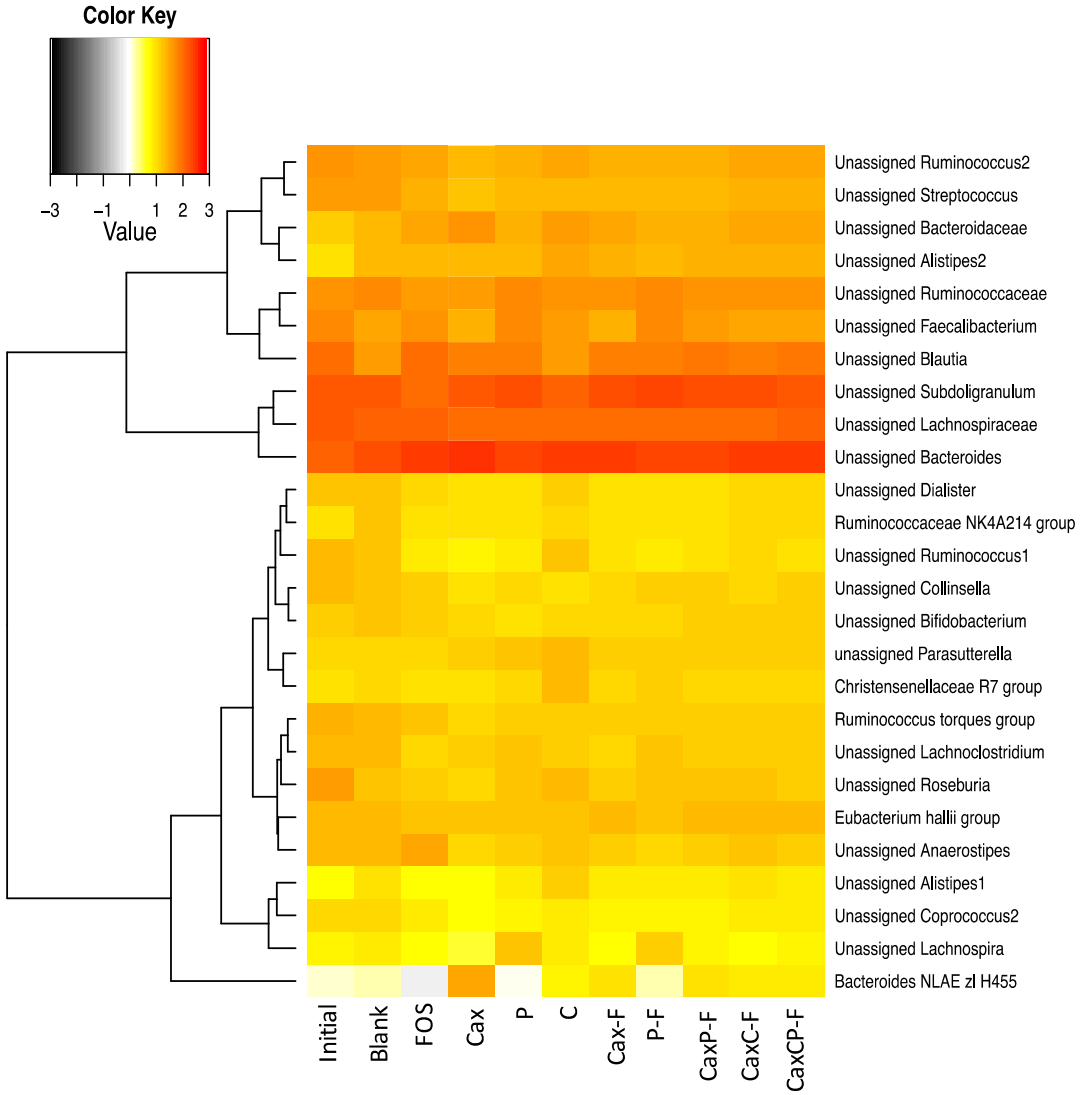

Figure 3. 12 Heatmap of relative abundances of microbial community after *in vitro* human fecal fermentation at 24 h for donor 1. Each column represents the mean value of three replicates for each sample. The color bar showing black to red indicate the relative content of bacterial microbiota composition.

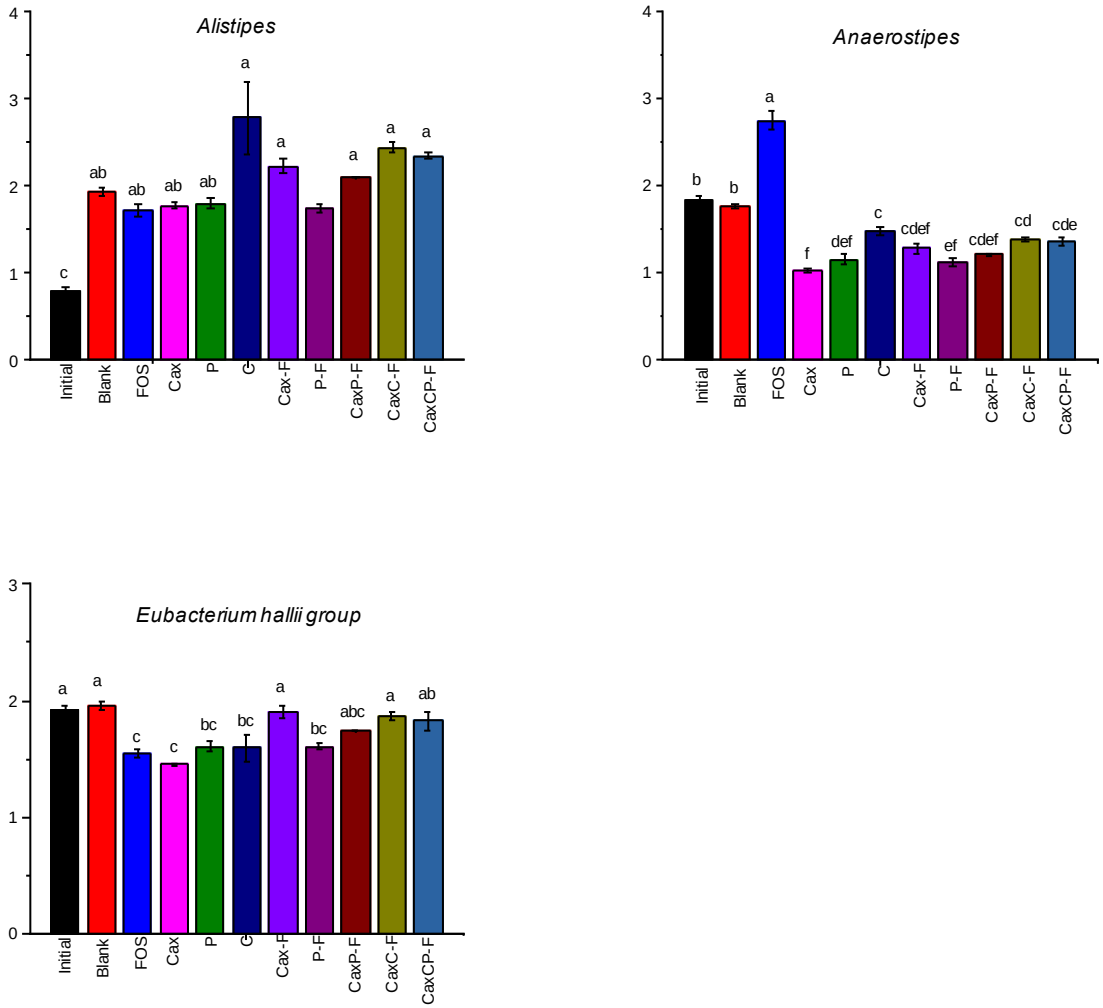

Figure 3. 13 Relative abundances (%) based on OTUs in each substrates for donor 1. Error bars represent the standard error of the mean of three separate replicates. Different letters indicate significant differences among the substrates (Tukey's multiple comparisons test, α = 0.05).

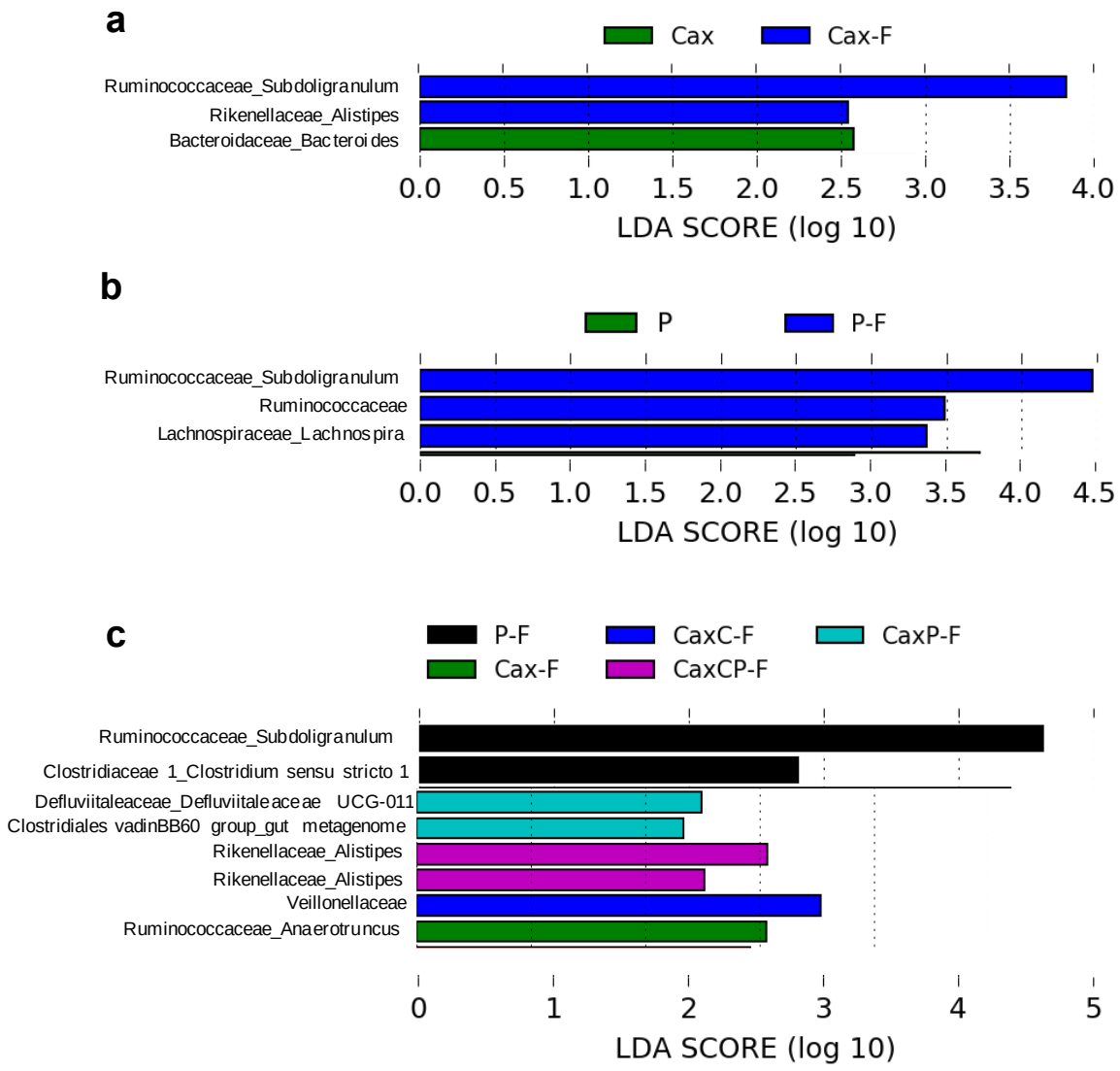

Figure 3. 14 Linear discriminant analysis of taxa for donor 1. Initial and blank communities are also included in the analysis and the default score is 2.0. Taxa with LDA scores **(a)** in Cax and Cax-F, **(b)** P and P-F, **(c)** among the films; linear discriminants of initial and blank conditions not shown.

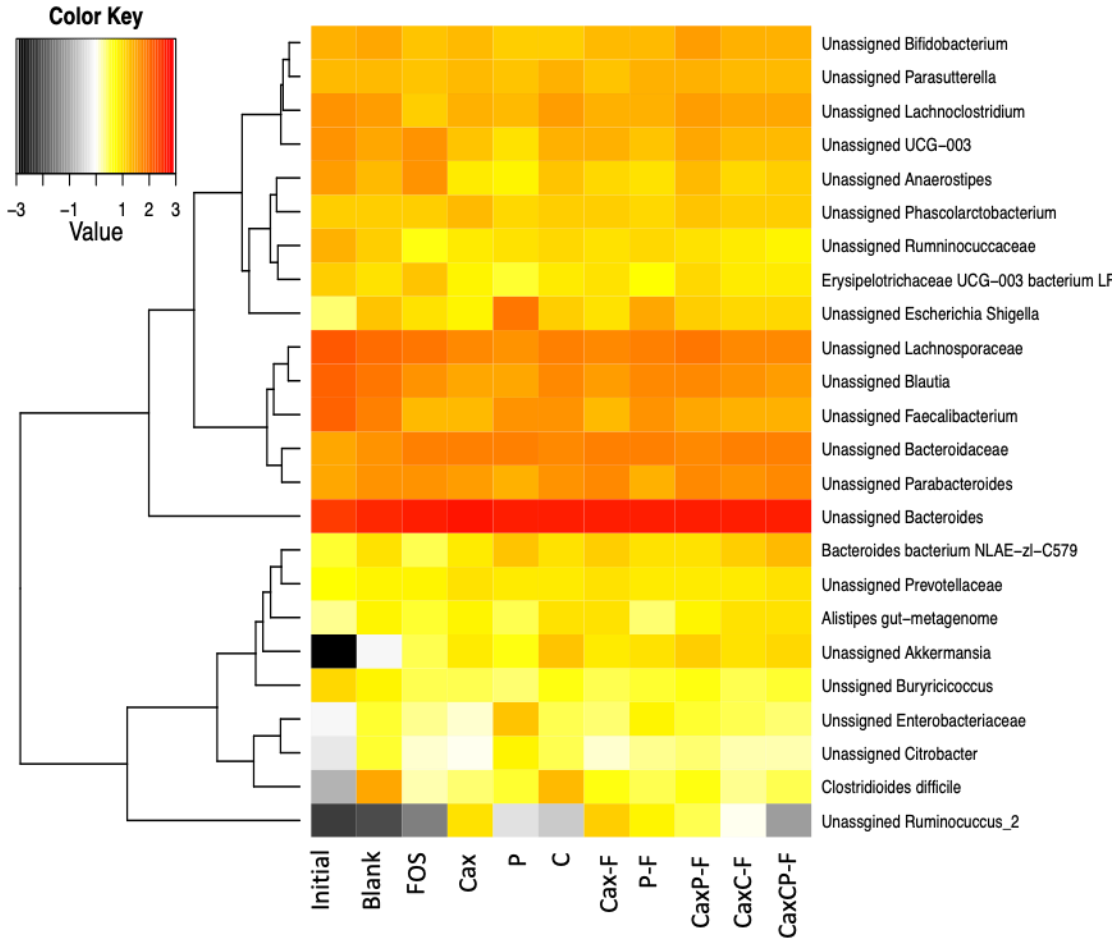

Figure 3. 15 Heatmap of relative abundances of microbial community after *in vitro* human fecal fermentation at 24 h for donor 2. Each column represents the mean value of three replicates for each sample. The color bar showing black to red indicate the relative content of bacterial microbiota

composition.

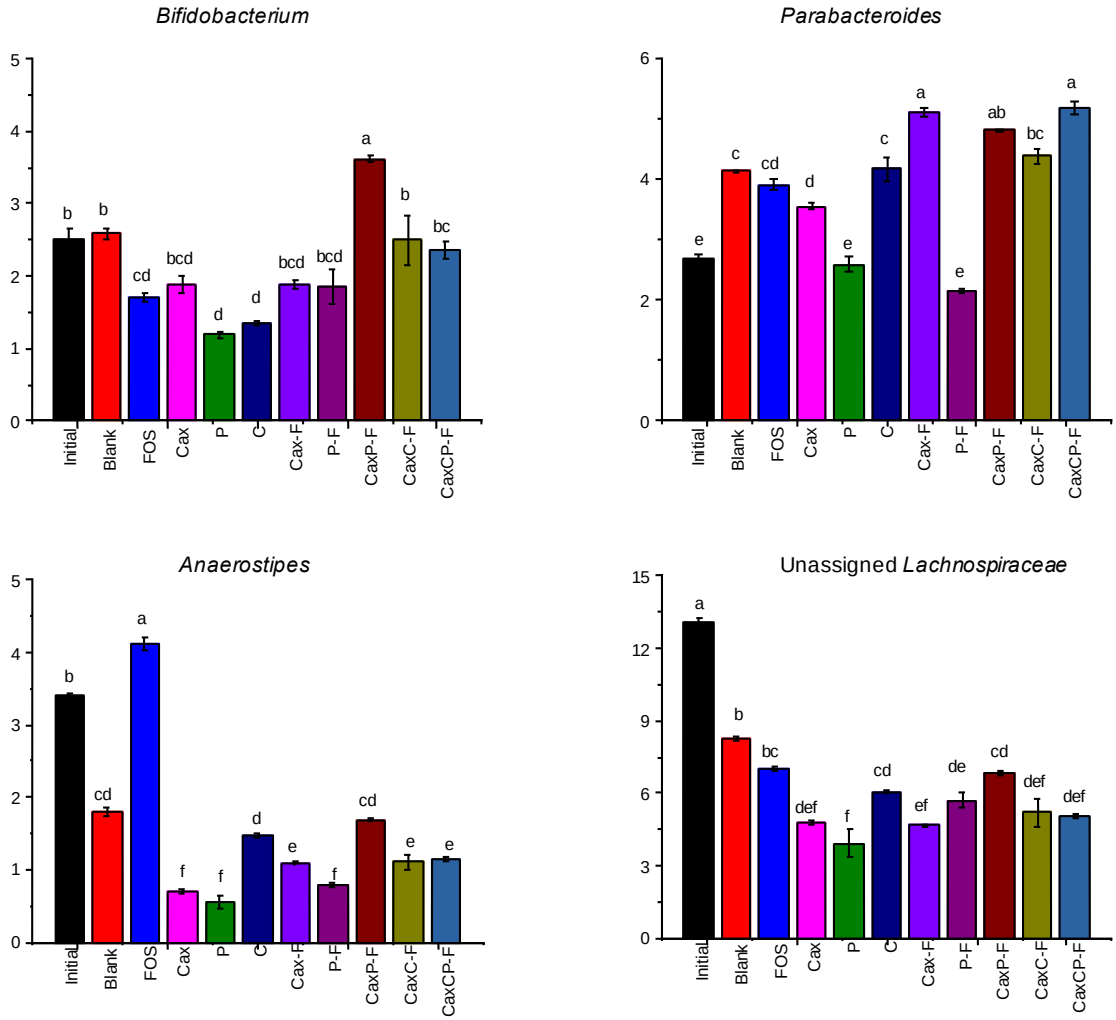

Figure 3. 16 Relative abundances (%) based on OTUs in each substrates for donor 2. Error bars represent the standard error of the mean of three separate replicates. Different letters indicate significant differences among the substrates (Tukey's multiple comparisons test, α = 0.05).

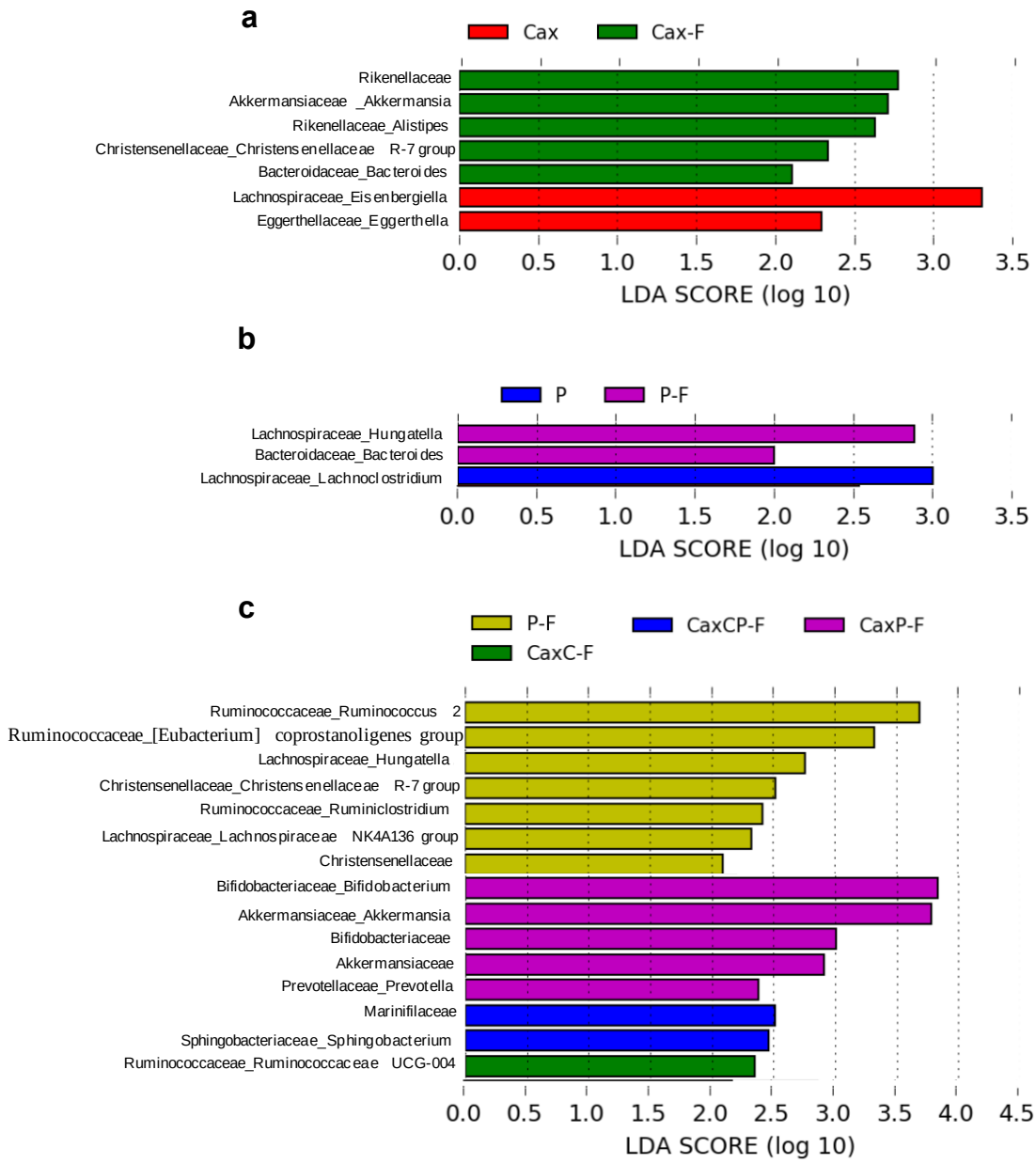

Figure 3. 17 Linear discriminant analysis of taxa for donor 2. Initial and blank communities are also included in the analysis and the default score is 2.0. Taxa with LDA scores **(a)** in Cax and Cax-F, **(b)** P and P-F, **(c)** among the films; linear discriminants of initial and blank conditions not shown.

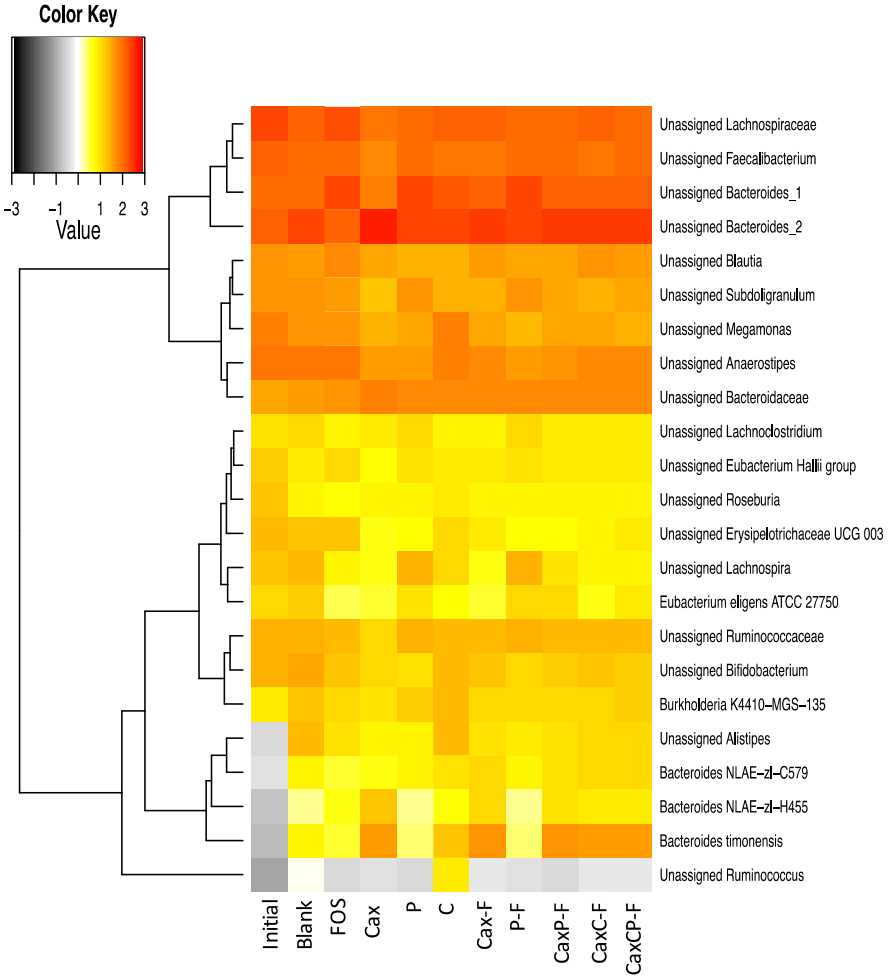

Figure 3. 18 Heatmap of relative abundances of microbial community after *in vitro* human fecal fermentation at 24 h for donor 3. Each column represents the mean value of three replicates for each sample. The color bar showing black to red indicate the relative content of bacterial microbiota composition.

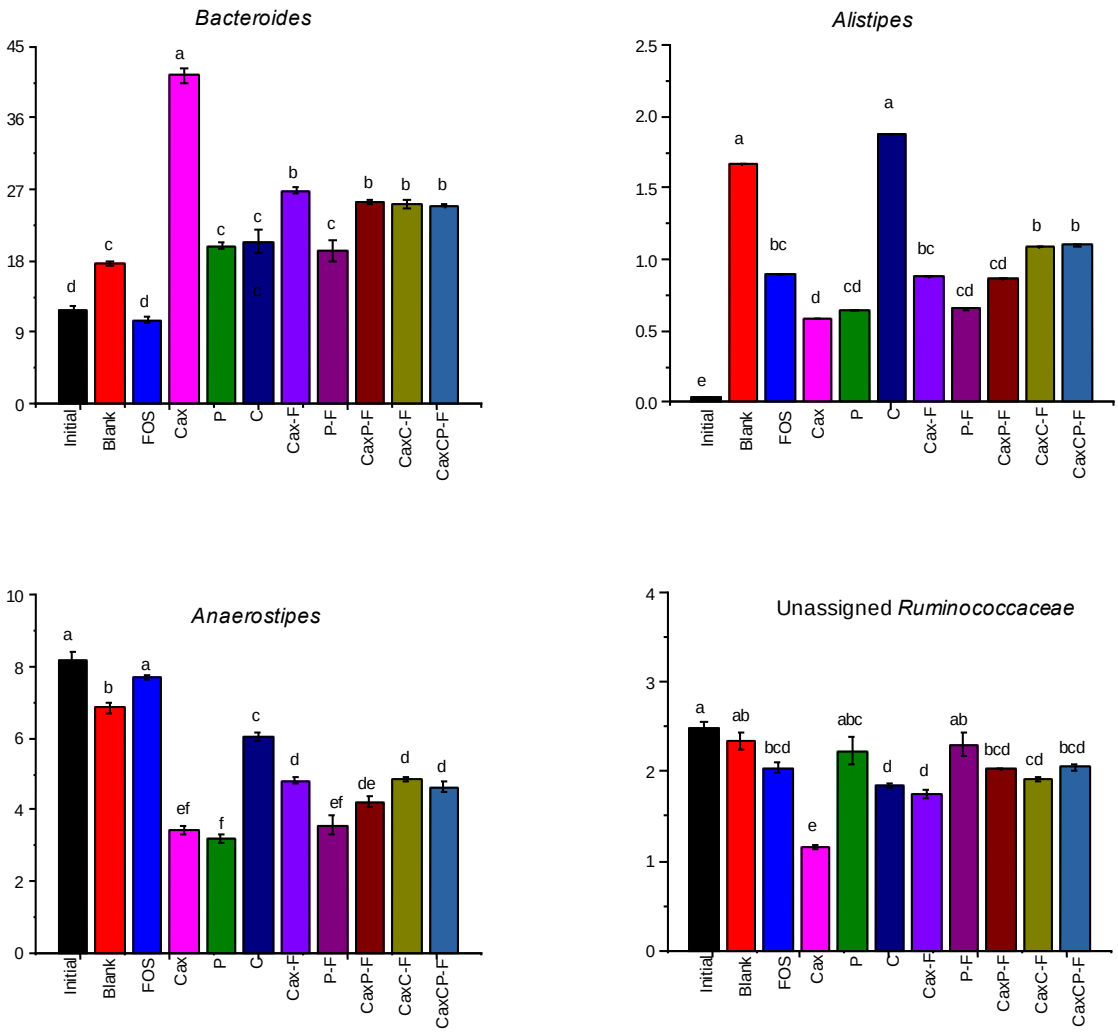

Figure 3. 19 Relative abundances (%) based on OTUs in each substrates for donor 3. Error bars represent the standard error of the mean of three separate replicates. Different letters indicate significant differences among the substrates (Tukey's multiple comparisons test, α = 0.05).

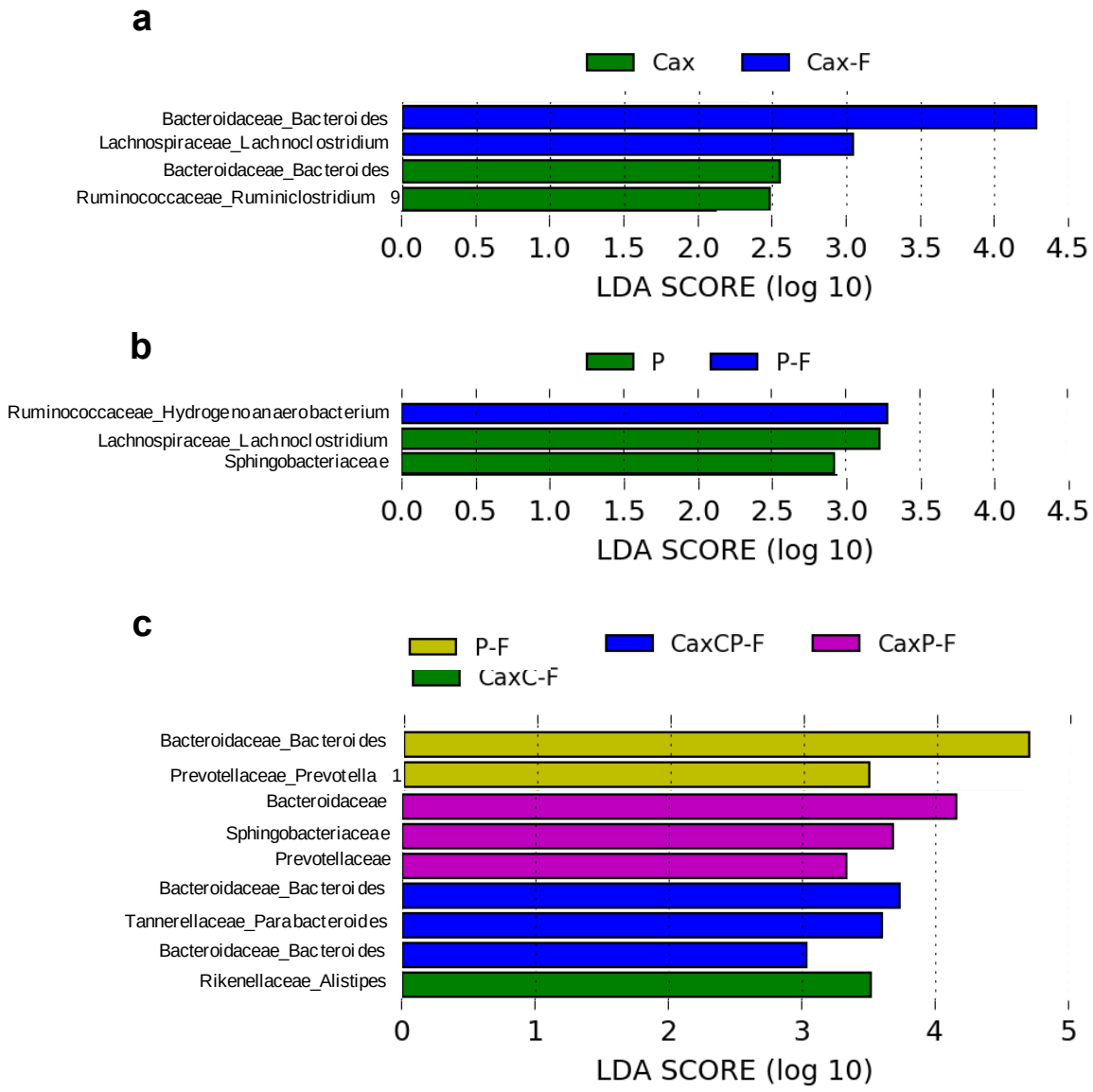

Figure 3. 20 Linear discriminant analysis of taxa for donor 3. Initial and blank communities are also included in the analysis and the default score is 2.0. Taxa with LDA scores **(a)** in Cax and Cax-F, **(b)** P and P-F, **(c)** among the films; linear discriminants of initial and blank conditions not shown.

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