DETERMINATION OF DIETARY FIBRE FERMENTATION BY THE HUMAN GUT MICROBIOTA USING AN *IN VITRO* BATCH MODEL

BY

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ABSTRACT

Dietary fibre (DF), which is not digested by mammalian enzymes, constitutes a critical substrate for bacterial fermentation in the gastrointestinal tract, resulting in short-chain fatty acids (SCFAs) production. However, the ability of the human gut microbiome to ferment specific DF structures is highly individualized, and little is known about how inter-individual differences in DF fermentation influence the health effects of DF. The primary objective of my research was to optimize a batch *in vitro* fermentation model and apply the model to human trials to characterize the importance of structural differences of DFs on the production of SCFAs by the human gut microbiota.

For optimization of a batch *in vitro* model, two fecal inoculum concentrations (5% and 2% w/v) and three growth media (Medium 1, Medium 2 and Medium 3) were tested. Medium 1 was a medium similar to what has been used in the literature, while Medium 2 and 3 used the same recipe but concentrations of peptone, yeast extract and Tween 80 were reduced to 20% and 10%, respectively. Fecal samples from three healthy individuals were used for *in vitro* fermentation (14 hours) with resistant starch type 4 (RS4) as a carbon source or no added carbohydrate (control). SCFAs and branched-chain fatty acids (BCFAs) were determined using gas chromatography (GC) and the growth of *Escherichia coli* (*E. coli*) during fermentation was quantified by plating. The concentration of fecal inoculum had no effect on the absolute amount of SCFAs and BCFAs produced and growth of *E. coli* during the fermentation. The concentration of total SCFAs relative to control increased by 2.16-fold ($p \leq 0.05$) while the concentration of total BCFAs relative to control decreased by 16-fold ($p \leq 0.05$) in Medium 3 as compared to Medium 1. In addition, there was a 4.54-log ($p \leq 0.05$) reduction in the growth of *E. coli* in Medium 3 as compared to its growth in Medium 1. These findings provide evidence that the growth medium with the concentration of

peptone and yeast extract reduced to 10% of their concentration commonly used in the literature resulted in an improved ability to detect SCFAs production from DF fermentation, reduced production of total BCFAs, and in an almost complete prevention in the growth of *E. coli.*

The second goal of the thesis was to determine the ability of the batch *IVFF* model to assess the capacity of fecal microbial communities of different individuals to ferment chemically distinct DFs to SCFAs. The isolated DFs arabinoxylan (AX), acacia gum, pectin, RS4, and microcrystalline cellulose (MCC) were subjected to an *in vitro* digestion mimicking conditions in the small intestine and *in vitro* fecal fermentations as described above. Significant differences were found between SCFAs produced by the different DFs with a high degree of variability between the subjects confirming that significant inter-individual differences occur. The third goal was to determine the impact of a six-week supplementation of a high daily dose of AX on the capacity of the gut microbiota of overweight individuals to ferment the DF into SCFAs. Healthy overweight subjects (n=31) consumed AX or MCC (control), either 25g/d or 35g/d for women and men respectively, for six weeks. Fecal samples were collected at baseline, week 1, and week 6. SCFAs were quantified directly in the fecal samples and using the *in vitro* fermentation method. There was a significant increase in the molar proportion of propionate in the fecal sample at week 6 as compared to baseline ($p \leq 0.05$) in the AX arm. An effect of AX was not detected using *in vitro* fermentations. Thus, the batch *IVFF* approach was less sensitive to study the response of gut microbiota to AX supplementation, especially in terms of propionate production.

Together, the findings in this dissertation indicate that the batch *IVFF* model was successfully optimized, which could be used to study the fermentation of chemically distinct DFs by fecal microbiota. The batch *IVFF* model developed here was already successfully used in one published

study (Jin et al., 2019) and is used in an ongoing study in the Walter lab, demonstrating that it provides a platform for future nutrition studies to assess individualized host responses to DF fermentation to eventually personalize the use of DF based on individual microbiome function.

Preface

This thesis is an original work by Nami Baskota to fulfill the requirements for an MSc degree. The results presented in this thesis are part of the Alberta FYBER (Feed Your gut Bacteria morE fibeR) study led by the University of Alberta under the leadership of Dr. Jens Walter. The DFs used in the study were provided by MGP Ingredients, Agrigum International Limited, Agrifiber Solutions LLC and Blanver. Edward Deehan, a PhD student at the University of Alberta and Janis Baarda, a study coordinator at the University of Alberta contributed to the recruitment of the participants and scheduled the participants' visits. Dr. Mingliang Jin, a visiting professor, Ngyuen Khoi Ngyuen, a master's student at the University of Alberta and Edward Deehan contributed to the study design of the batch *IVFF* and data analysis. This research project received ethics approval from the University of Alberta Health Research Ethics Board Biomedical Panel (Pro00050274). The FYBER study is registered under ClinicalTrials.gov with Identifier: NCT02322112.

The batch *in vitro* fermentation model optimized during the execution of the experiments as described in Chapter 2 of this thesis was used in a hepatology study published on March 2019 in the journal Liver International entitled "*Fecal microbiota from patients with cirrhosis has a low capacity to ferment non-digestible carbohydrates into short-chain fatty acids*".

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List of Abbreviations

AX	Arabinoxylan
AG	Acacia gum
BCFAs	Branched-chain fatty acids
BL	Baseline
BMI	Body Mass Index
CV	Coefficient of variation
DFs	Dietary DFs
E. coli	*Escherichia coli*
GC	Gas Chromatography
IVFF	*In Vitro* fecal fermentation
MACs	Microbiota-accessible carbohydrates
MCC	Micro-crystalline cellulose
PE	Pectin
RS	Resistant starch
RS2	Resistant starch type 2
RS3	Resistant starch type 3
RS4	Resistant starch type 4
SCFAs	Short-chain fatty acids
W1	Week 1
W6	Week 6

Chapter1. Background

1.1 Health benefits of dietary DF

It is widely recognized that high consumption of dietary DF (DF) lowers the incidence of chronic disease (Institute of Medicine, 2001). Epidemiological studies have consistently reported an inverse relationship between DF intake and the risk of developing cardiovascular and coronary heart diseases (Ajani et al., 2004; Pereira et al., 2004; Threapleton et al., 2013), cancer (Kim & Je, 2016; Murphy et al., 2012), type 2 diabetes (Yao et al., 2014) and obesity (Du et al., 2010; Liu et al., 2003). In contrast to epidemiological studies, human intervention trials produce conflicting results regarding the association of increased DF intake and reduction of disease biomarkers. For instance, in a five-week controlled study, daily consumption of 5g of oat barley significantly reduced postprandial glucose and insulin responses, while daily consumption of either 5g or 10g of barley beta-glucan did not benefit these markers (Biörklund et al., 2005). Similarly, in a randomized crossover intervention trial, neither a high DF (30g/day) diet nor a diet supplemented with 30g/day of isolated DF resulted in significant reductions of C-reactive protein (CRP), a marker of systemic inflammation (King et al., 2007). Additionally, conflicting results are not limited to health biomarkers but also include nutritional parameters, as a systematic review of DF intervention studies assessing appetite, acute and long-term energy intake and body weight reported relatively minor benefits of DF supplementation (Wanders et al., 2011). The inconsistencies between the epidemiological studies and human clinical trials might stem from the wide variability in the physicochemical properties of the DF (Fåk et al., 2015). High degrees of inter-individual differences in response to the DF intervention may also contribute to those

inconsistencies (Korpela et al., 2014). Both factors need to be addressed in order to elucidate the actual health benefits of DF.

1.2 Gut microbiota, fermentation of DF, and production of short-chain fatty acids

1.2.1 Introduction to DF fermentation by the gut microbiota

The gut microbiota consists of a dense ecosystem that is dominated by bacteria but also includes fungi, viruses, and archaea (Gibson et al., 2004). These organisms have been shown to play an essential role in normal digestive function, immune development, brain development and pathogenic defense (Bengmark, 2012). They can be seen as a "digestive partner" or an "additional organ" that has co-evolved with the host, allowing the host to benefit from food materials, like DF, that would otherwise not to be digested (Tuohy et al., 2012).

DF in the colon represents the most important fuel for the gut microbiota (Flint et al., 2012). The different types and amounts of DF that reach the large intestine depend on daily intake and type of food. The major components of DF that reach the gut microbiota are plant cell-wall polysaccharides, oligosaccharides, and resistant starches (RS) (Flint et al., 2008).

In addition to the fermentation of DF, the gut microbiota also participate in the fermentation of proteins (Williams et al., 2017). In contrast to short-chain fatty acids (SCFAs), branched-chain fatty acids (BCFAs) are produced when proteins pass through the small intestine unabsorbed and the branched-chain amino acids valine, isoleucine, and leucine are then fermented to iso-butyric acid, 2-methylbutyric acid, and iso-valeric acid, respectively (Brøbech & Rye Clausen, 1996; David et al., 2014; Macfarlane & Macfarlane, 2012). While SCFAs have been studied in detail and are considered beneficial, BCFAs are not well characterized and some researcher's consider them deleterious to health (Blakeney et al., 2018).

1.2.2 Effects of physicochemical properties of DF on fermentation

Understanding the physicochemical properties of a DF is key to understanding its physiological effects. These properties include its *solubility, viscosity, water-holding capacity, binding ability, bulking ability, and fermentability* (Guillon & Champ, 2000). The properties that relate to the gut microbiota and its fermentation ability will be discussed briefly.

The *solubility* of a DF is dependent on its chemical structure and its interactions with water molecules. In terms of behavior of the DF in the gastrointestinal tract, solubility is considered to be a useful predictor of fermentability by the gut microbiota (Williams et al., 2017). For example, linear DFs (such as cellulose and hemicellulose), which acquire crystalline conformations in solution resulting in a significantly reduced solubility in water, are considered unfermentable (Deehan et al., 2017). In contrast, branched DFs, which are water soluble, are less likely to form ordered structures in water (Capuano, 2017) and are mostly fermentable. However, one common exception is RS, which is not soluble in water but is highly fermentable (Deehan et al., 2017).

Viscosity, the gel-forming capacity of the DF (related to its ability to absorb water and form a gelatinous mass), is associated with its molecular weight, and is positively correlated with its solubility (Capuano, 2017). Viscosity is one of the most important gut microbiota-independent properties of DF that is believed to confer DF's associated health benefits (Jenkins et al., 2000; Würsch & Pi-Sunyer, 1997).

In terms of modulating the gut microbiota, the fermentability of DF is of the most relevance, (Deehan et al., 2017) and is, therefore, the focus of my research. Fermentation can be defined as the chemical breakdown of a substance by bacteria, yeasts, or other microorganisms. Bacterial fermentation of DF predominately results in SCFAs production, such as acetic, propionic, and

butyric acids, and a range of other carboxylic acids such as lactic acid (Tan et al., 2014). These end products of bacterial fermentation of DF are generally found to be beneficial for health (Besten et al., 2013).

1.2.3 Bacterial pathways for the production of SCFAs

The microbial conversion of DF to monosaccharides involves a number of reactions mediated by the enzyme repertoire in specific gut microbiota (Koh et al., 2016). The majority of the organisms in the colon are fermentative species that generate energy through substrate-level phosphorylation reactions. An equation describing the overall carbohydrate fermentation in the gut has been outlined based on the molar ratio for acetate, propionate, and butyrate of around 60:20:20. The equation is as follows:

$59C_6H_2O_6 + 38H_2O \rightarrow 60$acetate $+22$propionate $+ 18$butyrate $+96CO_2 + 256H^+$(Cummings & Macfarlane, 1997).

Metagenomic approaches have facilitated characterization of the bacteria responsible for the production of SCFAs. SCFAs are saturated aliphatic organic acids that consist of one to six carbons, being acetate (C2), propionate (C3), and butyrate (C4) the most abundant (Cook & Sellin, 2001). Acetate production pathways are widely distributed among bacterial groups, whereas pathways for propionate and butyrate production are highly conserved and are substrate-specific (Morrison & Preston, 2016; Reichardt et al., 2014). Their specific pathways and main producers are discussed below.

Acetate is formed by the hydrolysis of acetyl-CoA or from CO_2 via the Wood-Ljungdahl pathway, in which CO_2 is reduced to CO and converted with a methyl group and CoASH to acetyl-CoA (Besten et al., 2013; Duncan et al., 2006). Most of the enteric bacteria, for example,

Akkermansia muciniphila, *Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp., and *Ruminococcus* spp. have been shown to synthesize acetate from hydrogen, carbon dioxide, or formic acid (Petra et al., 2009)

The *succinate p*athway is the major route for the formation of propionate, which is found mainly in Bacteroidetes and in the Negativicutes class of Firmicutes (Louis & Flint, 2016; Reichardt et al., 2014). Propionate can also be synthesized from acrylate with lactate as a precursor through the acrylate pathway (Hetzel et al., 2003), occurring in the species of *Lachnospiraceae* and *Coprococcus catus* (Reichardt et al., 2014), and via the propanediol pathway in which deoxy sugars, such as fucose and rhamnose, are the substrates (Koh et al., 2016; Louis & Flint, 2016; Scott et al., 2006).

Butyrate is produced from the combination of two molecules of acetyl-CoA, followed by stepwise reduction to butyryl-CoA (Louis & Flint, 2016). Two different pathways are then involved for the final step in the formation of butyrate from butyryl-CoA, which proceeds either via the butyryl-CoA: acetate CoA-transferase or via phosphotransbutyrylase and butyrate kinase (Louis & Flint, 2009). Butyrate-producing species are found interspersed with butyrate non-producing species in two predominant families of human colonic Firmicutes: *Ruminococcaceae* and *Lachnospiraceae* (Louis et al., 2004), with the most abundant species present in human microbiota producing butyrate being *Faecalibacterium prausnitzii* (Louis & Flint, 2016).

1.2.4 Physiological benefits of SCFA

The SCFAs acetate, propionate, and butyrate have been shown to have health benefits. Acetate serves as an energy source for the liver and peripheral tissues. It also acts as a signaling molecule in metabolic pathways of gluconeogenesis and lipogenesis (Zambell et al., 2003). Animal studies

have suggested that acetate can help reduce hepatic cholesterol synthesis (Cheng & Lai, 2000). Propionate primarily serves as a precursor for gluconeogenesis. Additionally, propionate has been shown to decrease glucose-induced insulin secretion in isolated pancreatic islet cells of rats (Ximenes et al., 2007). Among the SCFAs, butyrate has been investigated the most extensively. Butyrate is the primary energy source for colonocytes and protects against inflammation; it plays an important role in the regulation of apoptosis and cellular proliferation, resulting in a lower risk of colorectal cancer (Koh et al., 2016), and has also been proposed to ameliorate mucosal inflammation (Canani et al., 2011).

These benefits are in contrast to the by-products of protein fermentation, which have been implicated to increase the risks of colorectal cancer, ulcerative colitis and other severe bowel disorders (Song et al., 2015). If there is a constant supply of DF and therefore, production of SCFAs, the detrimental effects of the metabolites generated by the fermentation of protein can be significantly reduced (Macfarlane & Macfarlane, 2012; Williams et al., 2017).

1.3 Factors influencing the production of SCFA in the gut

1.3.1 Effect of discrete chemical structure of DF on the production of SCFA

In an attempt to understand the interaction of DF structure and human colonic bacteria, Hamaker and Tuncil postulated DF as a vast collection of discrete structures providing a competitive advantage to gut microbes (Hamaker & Tuncil, 2014). Discrete structure of DFs are proposed to be carbohydrate structures that might be either highly complex due to sugar composition and linkage types (e.g., certain cereal arabinoxylans) or simple but different in size (e.g., long chain and short chain inulin) that would favor bacteria at the strain level in the competitive colon environment (Flint et al., 2012; Hamaker & Tuncil, 2014). The extent to which

different DFs are fermented by the gut microbiota is therefore structure-dependent, and relies on the metabolic capabilities of a subject's microbiome (Deehan et al., 2017).

Accordingly, both *in vitro* and *in vivo* studies have been conducted to identify the DFs capable of modulating the gut microbiota composition by promoting certain beneficial species, while maximizing the production of SCFAs (Benjamin et al., 2011; Damaskos & Kolios, 2008; Davis et al., 2010; Hughes et al., 2008; Martínez et al., 2010; Theuwissen et al., 2009; Yang et al., 2013). For instance, specific linkage features of AX were found to be associated with the rate of *in vitro* fermentation by fecal microbiota. The less branched structure of maize bran (as compared to rice and wheat bran) allowed for efficient utilization by the gut microbes to degrade the xylose chain and produce more SCFAs (Rumpagaporn et al., 2015). Likewise, a greater percentage of RS in whole grain wheat (as compared to bran) resulted in higher production of acetate and butyrate, and decreased propionate during *in vitro* fermentation (Hernot et al., 2008). These results highlight how compositional and structural properties of DFs play critical roles in regulating fermentation (Brahma et al., 2017). Furthermore, structurally distinct types of DF are associated with notable differences in their metabolic response (Ingerslev et al., 2014) and their effect on the gut microbiota. However, the composition and ability of the human microbiome to ferment a specific DF is highly individualized, and little is known of how the differences in individualized responses influence DF utilization in the gut.

1.3.2 Effect of individualized compositional response of the microbiome to DF

Humans host a very complex bacterial community composed of at least 100 trillion microbial cells (Whitman et al., 1998). There is large inter-individual variability in the microbiota composition which arises partly from host-genotype and environment but mainly from dietary

intake (Clemente et al., 2012). These diverse bacterial populations express distinct pools of carbohydrate-active enzymes and therefore, have markedly variable functional potentials (Flint et al., 2012), resulting in the same DF fermented differently between individuals. Inter-individual variation in the colonic microbiota composition hence influences responses to dietary manipulation (Flint et al., 2012). For example, fecal microbiota profiles tended to group by individual more than by the diet in obese male volunteers who were fed controlled diets differing in the type and content of DF for three weeks. On the other hand, dietary shifts, especially increased intake of RS, resulted in marked changes in the relative abundance of certain dominant phylotypes (Walker et al., 2010). Similarly, supplementation with galacto-oligosaccharides or inulin was shown to increase the relative abundance of bifidobacteria on average, but the responses varied widely between the individuals, with certain participants not responding to the treatment (Davis et al., 2011; Ramirez-Farias et al., 2008).

As such, the concept of a keystone species was reported in a study in 2012 where *Ruminococcus bromii* was shown to have superior ability to degrade RS when compared with other highly abundant species of amylolytic bacteria found in the human colon (Ze et al., 2012). Moreover, its representation in the fecal microbiota was increased in most volunteers when consuming diets containing RS2, RS3, or other types of RS. *R. ruminococci* was detected in the fecal samples of 12 out of 14 obese male volunteers examined in an intervention study by Walker and colleagues in 2011. Interestingly, the two exceptions were also the only two people to have residual unfermented starch in their fecal sample. Those two fecal samples were used in a follow-up study, which showed that introducing *R. bromii* restored the degradation of RS3 *in vitro* (Walker et al., 2010).

Considering the individual response, the concept of microbiota-accessible carbohydrates (MACs) has been introduced, which refers to carbohydrates that are not digested in the small intestine and are then metabolically available to the gut microbiota. MACs can be divided into dietary MACs and host-derived MACs, such as mucosal glycans (Sonnenburg & Sonnenburg, 2014). For a DF to be considered a MAC, the individual's gut microbiota must have the enzymatic capacity to utilize it (Deehan et al., 2017; Ze et al., 2012). For example, cellulose does not qualify as MAC because the capacity of human gut microbiota to ferment it is extremely low, though it would qualify as a MAC for other species that are hindgut and foregut fermenters (Chassard et al., 2010). Thus, the concept of MACs is especially applicable to personalized nutrition approaches given the importance of individualized microbiome response in their capacity to utilize certain DFs that reach the gut (Deehan et al., 2017).

1.3.3 Effect of pH in determining DF fermentation in the gut

In the neutral conditions of the large intestine, variations in pH resulting from the production of SCFAs have a major influence on the microbial community and their metabolic activity (Duncan et al., 2009; Walker et al., 2005). The pH of the lumen of the proximal colon *in vivo* is reported somewhat lower (5.5-6.5) than that of the distal colon (6.5-7.0), mainly as a result of the higher fermentation rate in the proximal colon (Duncan et al., 2009; Cummings and Macfarlane, 1991). An *in vitro* study found that a change of one unit in pH caused a drastic shift in the dominant groups of bacteria and the proportion of the produced SCFAs (Walker et al., 2005). Similarly, another study showed variable responses to pH between different phylogenetic groups. *Bacteroides* spp. demonstrated a sophisticated system for the utilization of soluble polysaccharides which allowed them to compete very effectively for substrates at a neutral pH. Suppression of the

Bacteroides spp. at pH 5.5 allowed populations of *Roseburia* spp. and *Eubacterium rectale*, which are butyrate-producing species to exploit the available substrates and increase dramatically (Duncan et al., 2009). The role of pH to predict the effects of different substrates on colonic fermentation has also been demonstrated in a recent study that investigated the impact of 15 different non-digestible carbohydrates upon microbiota composition on anaerobic batch cultures set at a pH of 5.5 or 6.5, simulating conditions in the healthy proximal and distal colon. The *in vitro* fermentation of non-digestible carbohydrates resulted in higher acetate and propionate levels at a pH of 6.5, and higher butyrate levels at a pH of 5.5. Interestingly, the butyrogenic effect was seen for inulin and oligofructose but not for RS at pH 6.5 (Reichardt et al., 2017).

Decreasing the pH is considered to be an important mechanism of action of DF *in vivo*, as lower pH is believed to prevent the growth of pH-sensitive pathogenic bacteria (Topping & Clifton, 2001). However, the drop in pH in *in vitro* batch culture studies assessing the fermentation of DF may differ from that occurring *in vivo* where absorption and turnover remove fermentation products (Walker et al., 2005).

1.4 Approaches to quantify SCFA in the gut

SCFAs are increasingly recognized as signaling molecules that mediate the interaction between diet, gut microbiota, and host (Besten et al., 2013). In humans, evidence for the beneficial physiological effects of SCFAs is more scarce due to the lack of reliable information on *in vivo* production, inaccessibility of the production sites, and rapid absorption by the colonocytes (Boets et al., 2015). The technical and ethical limitations of studying the production of SCFAs *in vivo* have driven the development of several *in vitro* techniques.

1.4.1 Direct measurement of SCFA in feces

Measurement of SCFAs directly in fecal samples is one of the most frequently used approaches to study fermentation of DF owing to its convenience (Filippo et al., 2017; Wu et al., 2016; Zhao et al., 2018). However, since more than 95% of the produced SCFAs are absorbed by the gut, measurements of these metabolites in fecal samples do not truly represent the fermentation of different DFs (Macfarlane & Macfarlane, 2011). For example, in one recent study, quantification of SCFAs in fecal samples revealed no differences between vegans and omnivores, despite the typical higher consumption of DF in vegan diets (Wu et al., 2016). Therefore, the value of this technique has major limitations and its use depends on the research question that is being investigated.

1.4.2 In vitro gut fermentation model

In vitro gut fermentation models make ideal systems for investigating the fermentation of DF and protein in the gut. Most importantly, inter-individual variations in the gut microbiota combined with individualized responses to the same diet intervention can be studied on a technological platform that can simulate the spatial, temporal and environmental features that microbes experience within the gut environment (Macfarlane & Macfarlane, 2007). Depending on the *in vitro* model, single or multiple vessels are inoculated with fecal samples and kept under anaerobic conditions, using a specific temperature, pH, and growth medium that mimics the colonic environment (Payne et al., 2012).

There are several different versions of *in vitro* gut fermentation models that vary in design and complexity. However, the most recent and advanced attempt at simulating the human gut is a model inclusive of the host digestive functions *in vitro* coupled with multistage continuous

fermentation known as SHIME (simulator of the human intestinal microbial ecosystem) (Payne et al., 2012). Even though the SHIME model integrates the entire gastrointestinal tract and, most importantly, inter-individual variability can be studied (Wiele et al., 2015), it is a sophisticated and expensive procedure to determine the fermentation of DF.

1.4.3 Batch fermentation model

Batch fermentation models are closed systems, sealed bottles or reactors, which allow the growth of pure and mixed bacterial suspensions in carefully selected media without further addition of nutrients (Payne et al., 2012). Batch cultures are helpful for determining SCFA profiles generated by the metabolism of DF by fecal microbes. Many studies have analyzed the impact of different DFs and how they affect the production of SCFAs using *in vitro* batch models (Carlson et al., 2016; Noack et al., 2013; Rumpagaporn et al., 2015; Yang et al., 2013). However, batch fermentation models are limited to short-term studies, as changes in substrate availability, pH, and redox potential result in the selection of non-representative microbial populations, which could lead to the distortion of the fermentation profile (Macfarlane & Macfarlane, 2007). Indeed, in an *in vitro* study by Yang and colleagues in 2013, there was a 37% increase in abundance in the family of Enterobacteriaceae after batch *in vitro* fermentation of RS (Yang et al., 2013), while this family usually constitutes only a small fraction (approximately 0.1%) of the gut microbiota (Eckburg et al., 2005; Winter et al., 2013).

While each of the *in vitro* models have their own strengths and limitations, their effectiveness depends on the objective of the research. The choice of the model requires a compromise between technical complexity, biological significance, and cost (Payne et al., 2012). Since batch *in vitro* models are relatively simple, inexpensive as compared to other *in vitro* models, and can be

effectively controlled they facilitate the rapid testing of a wide variety of substrates or fecal samples at high throughput (Payne et al., 2012).

1.5 Study objective and specific aims

Studies have indicated that the benefits of increased DFs in host health are mediated by a small number of bacteria with the genetic capacity of producing SCFAs in the colon (Keenan et al., 2015; Zhao et al., 2018). Because, SCFAs contribute to the health effects of DFs, their inclusion as an outcome variable in human trials might provide insight into the molecular mechanisms by which DFs work. However, the ability of the human gut microbiome to ferment specific DF structures is highly individualized, and little is known about how inter-individual differences in DF fermentation in individuals influence the health effects of DF. Therefore, the primary objective of my research was to optimize a batch *in vitro* fermentation model and apply the model to human trials to characterize the importance of structural differences of DFs on the production of SCFAs by the human gut microbiota. In order to achieve this objective, the following specific aims were targeted:

Specific aims

1. To optimize a batch *in vitro* fecal fermentation (*IVFF)* model that simulates the bacterial fermentation observed in the human colon.

2. To determine the ability of the batch *IVFF* model to assess the capacity of fecal microbial communities of different individuals to ferment chemically distinct DFs to SCFAs.

3. To determine the impact of a six-week supplementation of high daily dose of AX on the capacity of the gut microbiota of overweight individuals to ferment the DF into SCFAs.

Chapter 2. Optimization of a batch *IVFF* system that simulates bacterial fermentation in the colon

2.1 Introduction

When compared with *in vivo* methods, the *in vitro* colonic fermentation model is inexpensive and experiments can be conducted in a shorter period of time (Pham & Mohajeri, 2018). *In vitro* models enable the cultivation of human gut microbiota derived from fecal samples under simulated physiological conditions. The simplest model is the batch fermentation model, which is the most frequently used model to test the ability of fecal microbial communities to metabolize different substrates (Venema & Abbeele, 2013).

Batch *in vitro* fermentation systems are typically comprised of a fecal slurry derived from either a single fecal sample or a mixture of samples, a buffer, a growth media, and a substrate of interest. The substrate is inoculated with the fecal slurry and is incubated in an anaerobic environment for a defined period of time (Coles et al., 2005). When optimizing a batch *IVFF* model, the main objective is to have an *in vitro* method that is as representative as possible of the *in vivo* events. The *IVFF* model must provide a suitable environment that supports the fitness of the bacterial species present in the inoculum. For this reason, factors such as substrate, inoculum concentration, growth media, buffer, incubation time and output measured are important variables to consider (Coles et al., 2005).

2.1.1 Substrate

The microbiota in the colon ferment the substrate available to them after upper gastrointestinal digestion and absorption. Therefore, prior to the *IVFF*, the provided substrates should likewise

undergo an *in vitro* digestion (Coles et al., 2005). Furthermore, the particle size of the substrate should also be considered. For example, small/fine particle sizes or bran fractions have been shown to increase SCFA production as compared with large/coarse particles such as aleurone and whole bran during *IVFF* (Stewart & Slavin, 2009).

2.1.2 Inoculum

As the gut is comprised of different regions with distinct environmental conditions and microbial profiles, it would be ideal for the model simulating the proximal colon to be inoculated with the biological specimen of that region (Pham & Mohajeri, 2018). *In vitro* procedures often use feces as inoculum, because of (1) ease of collection, (2) the collection can be repeated from the same individual if needed, and (3) freshly collected feces typically provides a good source of all major groups of bacteria present in the colon (Pastorelli et al., 2014). However, fresh feces may not be always available. Instead, frozen fecal aliquots can provide inocula for more than one fermentation experiment, thereby potentially supplying repeatable and reproducible results over several months. Freezing of the fecal samples for 44 weeks at -80˚C did not affect the composition of the microbiota in terms of viable cells, indicating that frozen samples contained the microbial diversity of the fresh sample. In addition, the fresh and frozen microbiotas showed similar SCFA production over at least 150 hours of fermentation (Rose et al., 2010). Hence, using frozen fecal inoculum for *in vitro* fermentation of a substrate enhances the versatility of the study (Pastorelli et al., 2014).

Fecal samples are commonly obtained from healthy donors who have not taken antibiotics for at least three months prior to the study and are consuming their regular diet (Hughes et al., 2008; Sanz et al., 2005; Yang et al., 2013). The batch fermentation chambers are usually inoculated with

a liquid fecal suspension from a single subject in order to study the individuality of the microbiome or are inoculated with pooled stool samples from several subjects in order to limit the inter-individual variability and provide a standardized microbiota that can be used for different fermentations. However, an important consideration is that pooling of fecal samples might disturb the cross-feeding and trophic chains established in each individual donor resulting in different outcomes (Pham & Mohajeri, 2018).

2.1.3 Growth media

The growth medium is a solution containing a number of components including protein (peptone, yeast extract, casitone), micronutrients (salts, vitamins, minerals), and buffering agents (Rymer et al., 2005). Carbohydrates are limited as they could interfere with the fermentation of the substrate. A batch fermentation of the fecal inoculum in a defined fermentation medium facilitates the adaptation of the fecal microbiota to the experimental conditions (Aguirre & Venema, 2016). Unfortunately, there are considerable variations in the composition of the growth media used for *in vitro* studies reported in the literature, thus the effects of growth medium on fermentation outcomes are still inconclusive.

2.1.4 Buffer

In vivo, SCFAs are rapidly absorbed across the colon cell wall, thereby preventing the colonic contents from becoming too acidic. However, within batch *IVFF*s, the pH of the batch cultures cannot be controlled with precision as batch *IVFF*s are operated in a closed vessel, making it impossible to remove the metabolites produced during the fermentation. As such, a buffer is required in the fermentation vessel to maintain the pH for mimicking the environmental conditions.

Carbonate or phosphate buffers are commonly used in the media for this purpose (Coles et al., 2005).

2.1.5 Incubation time

Incubation time plays an important role in the fermentation process of the gut microbiota (Pham & Mohajeri, 2018). An *in vitro* study using a three stage continuous model showed that reducing the incubation time to 20 hours led to increased acetate and decreased butyrate production as compared to 60 hours incubation time (Child et al., 2006). Important bacterial groups, such as *Ruminococcus* spp., and butyrate-producing *Roseburia intestinalis* were absent in the system at 20 hours retention time, suggesting that their growth was not rapid enough to remain in the ecosystem (Child et al., 2006).

*IVFF*s are commonly conducted over 12 to 24 hours with multiple sampling time points. Studies with rumen samples have found the numbers of all microorganisms (total bacteria, protozoa, methanogens, fungi) declined sharply between 24 to 72 hours after incubation (Soto et al., 2013), which is likely due to the accumulation of the metabolites and the exhaustion of substrates (Yáñez-Ruiz et al., 2016).

2.1.6 Output Measurements

Analyzing the produced metabolites or substrate disappearance indicate the fermentation of substrate during *IVFF*. Substrate disappearance can be measured by losses of dry matter, gross energy or loss of specific components such as non-starch polysaccharide, RS, protein, carbohydrate or other fermentable substrates. Measuring SCFA production, being the primary indicator of the fermentation, is our choice of output assessment and falls into the category of measuring production of metabolites (Coles et al., 2005).

2.2 Study objective

The conditions used for *IVFF* systems reported in the literature differ to some degree, yet few explanations are provided as to why the researchers selected specific *in vitro* conditions. This is particularly true for the concentration of fecal inoculum and composition of growth media. The fecal inoculum concentration and the composition of peptone and yeast extract in the growth media used by different researchers vary considerably, which means that the results from different studies cannot be easily compared. Additionally, weak control of the growth of *E. coli* is another major limitation of the batch *IVFF* model, with previous studies reporting over-representation of *E. coli* after batch *IVFF* of DFs (Yang et al., 2013). Therefore, the main objective of this study was to optimize a batch *IVFF* model so as to simulate the bacterial fermentation in the colon. To achieve this objective, the following specific aims were developed.

1) Optimize a batch *in vitro* model that maximizes SCFA production, minimizes BCFA production, and avoid growth of *E. coli* during fermentation of DF.

2) To determine the effects of reducing concentration of fecal inoculum and peptone and yeast extract in the growth media on *in vitro* production of SCFAs, BCFAs, and *E. coli* growth.

3) To determine the repeatability of the optimized batch *IVFF* model.

2.3 Materials and methods

2.3.1 Subjects

Fecal sample donors (2 female and 1 male) were healthy overweight and class-I obese individuals between the ages of 20 and 33 whose BMI (body mass index) ranged from 25.0 to 33.3 kg/m^2. These three participants were chosen from the Alberta FYBER (Feed Your gut Bacteria morE fibeR) study who were recruited from the Edmonton area using campus-wide flyers, mailings

to specific Listservs, local events, and word of mouth. Inclusion criteria included (i) no known gastrointestinal disorders or surgeries; (ii) no history of diabetes mellitus; (iii) no chronic use of anti-hypertensive, lipid-lowering, anti-diabetic, analgesic, or laxative medications; (iv) no use of antibiotics in the three months prior to the study; (v) no use of probiotic, prebiotic or herbal supplements; (vi) non vegetarian (vii) no smoking; (viii) alcohol intake ≤ 7drinks/week; (ix) < 3 hours of vigorous exercise per week. All procedures involving human subjects were approved by the Health Research Ethics Board of the University of Alberta (Approval Number: Pro00050274). Written informed consent was obtained from the study subjects prior to enrollment into the study.

2.3.2 Fecal sample collection and processing

Participants were given stool collection kits consisting of a stool specimen container, an air-tight bag (Fisher, Canada), and a GasPak^{TM} EZ Anaerobe Sachet (BD, Canada) to generate an anaerobic environment within the container, and then delivered to the laboratory within 4 hours of defecation. Immediately upon receipt in the laboratory, the stool samples were processed in an anaerobic chamber (Bactron^{TM}, Shel Lab, Oregon, USA) with anaerobic environment (5% H_2, 5% $C0_2$, and 90% N_2). Each fecal sample was aliquoted and stored at -80˚C to serve as inoculums for later *in vitro* fermentation.

2.3.3 Growth media

The growth media (Medium 1) contained, per liter, 1.6 g of peptone, 1 g of yeast extract, 3.2 g of $NaHCO_3$, 3.6 g of NaCl, 1.6 g of K_2HPO_4, 0.32 g of L-cysteine HCl, 1.6 ml of Tween 80, 0.36 g of $CaCl_2.6H_20$, 0.4 g of $MgSO_4.7H_2O$, 0.01 g of hemin, 0.05 g of bile salts and 1 mg of vitamin K (Walker et al., 2005; Yang et al., 2013). In Medium 2, the concentration of peptone, yeast extract and Tween 80 were reduced to 20% of that in Medium 1, while in Medium 3 they were reduced

to 10% of that in Medium 1. Tween 80 is a solubilizing agent that acts as a surfactant and increases the solubility of one agent in another (Nielsen et al., 2016). All the other components in Medium 2 and 3 remained in the same amounts as in Medium 1.

2.3.4 DF substrate and in vitro digestion of DF

For the optimization trials, powdered RS4 provided by MGP Ingredients Inc. (Atchison, Kankas) was used as the DF substrate. The RS4 consists of phosphorylated cross-linked wheat starch containing >85% DF.

To remove the digestible components of DF, which would normally be absorbed by the host, the substrate was subjected to *in vitro* digestion. Simulated upper gastrointestinal tract digestion was carried out *in vitro*, according to Yang and colleagues (Yang et al., 2013) with some modifications including the addition of an "oral digestion phase" (Figure 2.1). Briefly, 25 g of DF was suspended in 300 ml of phosphate buffer (pH 6.9). After temperature equilibration at 37°C for 15 minutes, the oral digestion phase was carried out by adding 2.25 ml of 20 mg/ml human salivary α-amylase (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 1mM $CaCl_2$. The mixture was shaken at 150 rpm in a shaking water bath at 37°C for 15 minutes. The digestion in the stomach was replicated by adjusting the pH of the solution to 2.5 ± 0.1 with the addition of 1M HCl. Subsequently, 10% w/v of pepsin diluted in 50 mM of HCl was added to the acidified DF solution and shaken at 150 rpm at 37°C for 30 minutes. To emulate small intestinal digestion, 1M of $NaHCO_3$ was added to increase the pH of the solution to 6.9 ± 0.1. Afterwards, 50 ml of 12.5% w/v pancreatin diluted in sodium maleate buffer was added to the DF solution along with 2 ml of amyloglucosidase, followed by shaking at 150 rpm at 37°C for three hours. The sample was then precipitated with 6.5 L of 95% ethanol. Following the precipitation, the suspension was dialyzed

(50-1000 Da cutoff) against distilled water for two days. After dialysis, the samples were vacuum evaporated to remove the excess water and freeze-dried. The freeze-dried samples were then stored at -80°C.

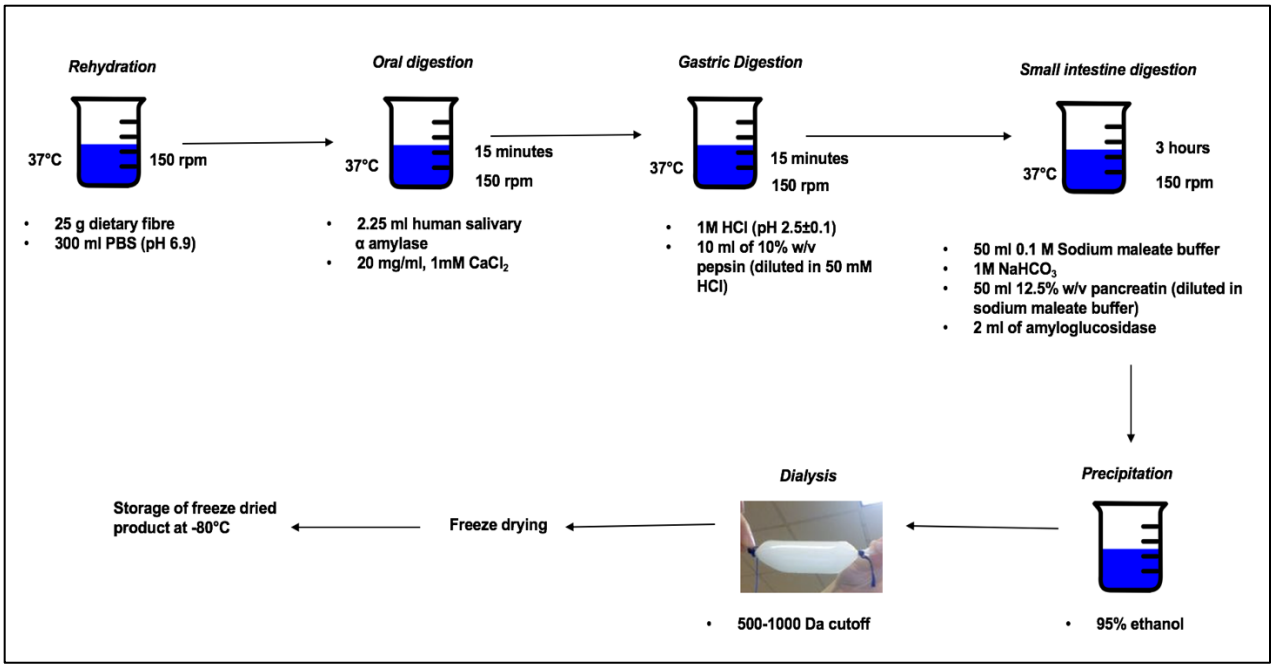

Figure 2.1. *In vitro* digestion of DF. *In vitro* model mimicking digestion of DF in the human upper gastrointestinal tract; DF, dietary fibre; Da, Dalton; PBS, phosphate buffered saline; rpm, rotation per minute.

2.3.5 IVFF of pre-digested DFs

The batch *IVFF* method used was carried out as previously described by Yang and colleagues (Yang et al., 2013) with a slight modification being the exclusion of the overnight hydration of the substrate. In short, 1 g of DF (freeze-dried) was dissolved in 25 ml of sterile growth medium to make a final concentration of 4%. Fecal samples were placed in an anaerobic chamber (GENEQinc, BACTRON^{TM}, USA) under the following environmental conditions: 5% $C0_2$, 5% H_2, and 90% N_2. The DF solution and the growth medium were allowed to reduce under the

anaerobic conditions for two hours. The reduced DF solution was then aliquoted into fermentation tubes (2.5 ml per tube). Concurrently, 0.1 g of the fecal samples were scooped into 50 ml conical tubes and growth medium was added to each tube to make a final concentration of 2% and 5%. Subsequently, individual fermentation tubes containing the DF solution were inoculated with 2.5 ml of the fecal solution (total incubation volume: 5 ml), capped, placed at a 45° angle, and incubated at 37°C with shaking. A sample containing only growth media (without substrate) and 2% and 5% of fecal slurry was included as a control. The solution was then fermented for 14 hours with continuous shaking at 125 rpm at 37˚C. Two tubes, each containing 1 ml of the fermentation slurry, were aliquoted for the quantification of SCFAs and the enumeration of *E. coli*. Sampling was performed at 14 hours, and the fermented materials were stored at -80°C until analysis by gas chromatography (GC).

2.3.6 Quantification of SCFAs and BCFAs

One milliliter of the aliquoted samples was removed from the anaerobic chamber and centrifuged at 20,000 xg for 20 minutes until a clear supernatant was obtained. In a 1.8 ml GC vial, 0.8 ml of clear supernatant was then combined with 0.2 ml of 25% phosphoric acid and 0.2 ml of internal standard, mixed well and stored at -80˚C until analysis of SCFAs and BCFAs by GC. The internal standard was prepared by adding 20 ml of 25% phosphoric acid, approximately 20 ml distilled water and 300 µl of isocaproic acid (4 methyl-valeric acid). The volume was then brought up to 100 ml with water and mixed well.

SCFAs and BCFAs were quantified at the Chromatography Core Facility at the University of Alberta (Edmonton, AB, Canada). An aliquot of 0.2 ml of the sample was injected into a Bruker SCION 456 gas chromatograph. SCFAs were separated on a Stabilwax-DA column (30-m × 0.53-

mm × 0.5-µm, Restek, Bellefonte, Pa) with a flame ionization detector, and quantified by calculating response factors for each SCFA relative to 4-methyl valeric acid using injections of pure standards. The total SCFAs were determined as the sum of acetate, propionate, and butyrate. Total BCFAs were determined as the sum of iso-butyrate and iso-valerate. SCFAs and BCFAs production was measured in µmol/ml of fermentation media and presented as the mean value with standard deviation. In order to investigate the concentration of SCFAs and BCFAs produced by the fermentation of substrate (RS4), the data were expressed as the ratio of SCFAs and BCFAs in substrate to the control.

2.3.7 Enumeration of E. coli from the fermented sample

The calculation of *E. coli* cell number in the samples pre-and post-fermentation was performed by plating. MacConkey agar (prepared according to manufacturer's instructions) was used as the selective medium. MacConkey agar not only selects for Gram-negative organisms by inhibiting Gram-positive bacteria, but also differentiates the Gram-negative organisms by lactose fermentation. Lactose fermenting bacteria such as *E.coli* produce an acid-by-product causing the medium to turn pink (Wanger et al., 2017), enabling the identification of the bacterium.

Briefly, 9 ml of autoclaved 0.9% saline was added in eppendorf tubes. Ten-fold serial dilutions (1:10) were carried out by adding 1 ml of the sample in each tube. An aliquot of 0.1 ml of each dilution was then transferred and streaked onto MacConkey agar plates. Plates were then incubated anaerobically at 37˚C for 24 hours. Pink-stained colonies were counted and recorded to determine colony-forming units per milliliter (CFU/ml) for each sample.

E. coli data were log- transformed and presented as fold change determined as the ratio of the number of *E. coli* colonies (CFU/ml) present at the end of the fermentation (14 hours) to the number of colonies (CFU/ml) at the start of fermentation (0 hour).

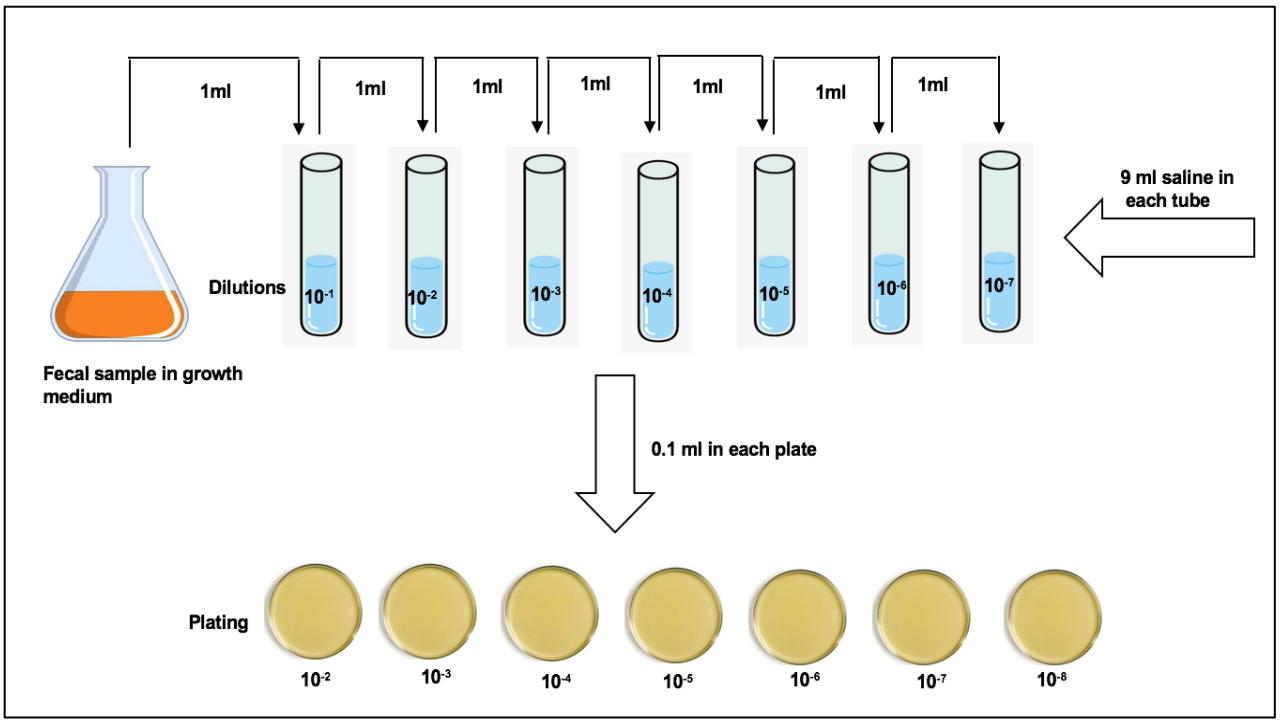

Figure 2.2 Enumeration of *E. coli* by plate count method. Colony forming units/ml (CFU/ml) was calculated by number of colonies on plate * reciprocal of dilution factor; *E. coli, Escherichia coli*.

2.3.8 Evaluation of the repeatability of batch IVFF model in producing SCFAs

Additional experiments were carried out to investigate the repeatability of the optimized batch *IVFF* model. Fecal samples were obtained from fifteen healthy individuals who were not undergoing any intervention. Fecal sample donors (10 females and 5 males) were healthy overweight and class-I obese individuals between the ages of 24 and 50 whose BMI ranged from 25.9 to 34.7 kg/m^2.They were recruited from the campus area by word of mouth who have had (i) no known gastrointestinal disorders or surgeries; (ii) no history of diabetes mellitus; (iii) no chronic

use of anti-hypertensive, lipid-lowering, anti-diabetic, analgesic, or laxative medications; (iv) no use of antibiotics in the three months prior to the study; (v) no use of probiotic, prebiotic or herbal supplements (vi) non vegetarian; (vii) no smoking; (viii) alcohol intake ≤ 7 drinks/week; (ix) < 3 hours of vigorous exercise per week. All procedures involving human subjects were approved by the Health Research Ethics Board of the University of Alberta (Approval Number: Pro00050274). Written informed consent was obtained from the study subjects prior to enrollment into the study.

Participants were given stool collection kits consisting of a stool specimen container, an air-tight bag (Fisher, Canada), and a GasPak^{TM} EZ Anaerobe Sachet (BD, Canada) to generate an anaerobic environment within the container, and then delivered to the laboratory within 4 hours of defecation as described in section 2.3.2 of the thesis. Each fecal sample was aliquoted into two equal samples. The replicates of each individual sample were then used to ferment pectin (PE) for 14 hours. PE derived from citrus peel was obtained from Cargill, Inc. (Wayzata, MN, USA). SCFAs in the fermented samples were then quantified by GC performed at the Chromatography Core Facility at the University of Alberta as described in section 2.3.6.

2.3.9 Statistical Analysis

All statistical tests were performed using GraphPad Prism version 6.0 (GraphPad Software, La Joya, CA, USA). Conventional statistical guidelines recommend using non-parametric test for analyzing data from studies with small sample sizes (Altman et al., 1983). Since the number of participants in our study were three, a non-parametric Wilcoxon signed-rank test was performed to compare absolute concentration, ratios of SCFAs, BCFAs and fold change in *E. coli* between 2% and 5% fecal inoculum concentration. Similarly, a non-parametric Friedman test was used to compare absolute concentration, ratios of SCFAs, BCFAs and fold change in *E. coli* among Media

1, Media 2, and Media 3. P values of ≤ 0.05 were deemed as statistically significant. In addition, coefficient of variation (CV) in the SCFA production was calculated using the formula: CV = (standard deviation/ mean) *100%.

2.4 Results

2.4.1 In vitro production of SCFAs and BCFAs at 5% and 2% fecal inoculum concentration in Medium 1

As shown in Figure 2.3, both RS4 and control were fermented to acetate, propionate, butyrate, and BCFAs (iso-butyrate and iso-valerate). However, there was no difference between the absolute amount of SCFAs and BCFAs produced between 5% and 2% fecal inoculum concentration in both RS4 and control ($p \geq 0.05$) (Figure 2.3 A, B, C, D & E).

Considering the fermentation in the control, we assessed the concentration of SCFAs and BCFAs produced during the fermentation of RS4 relative to control. There was no difference between the relative concentration of acetate, propionate, butyrate, total SCFA and total BCFA in RS4 to control between 5% and 2% fecal inoculum concentrations ($p \geq 0.05$) (Figure 2.3 F & G).

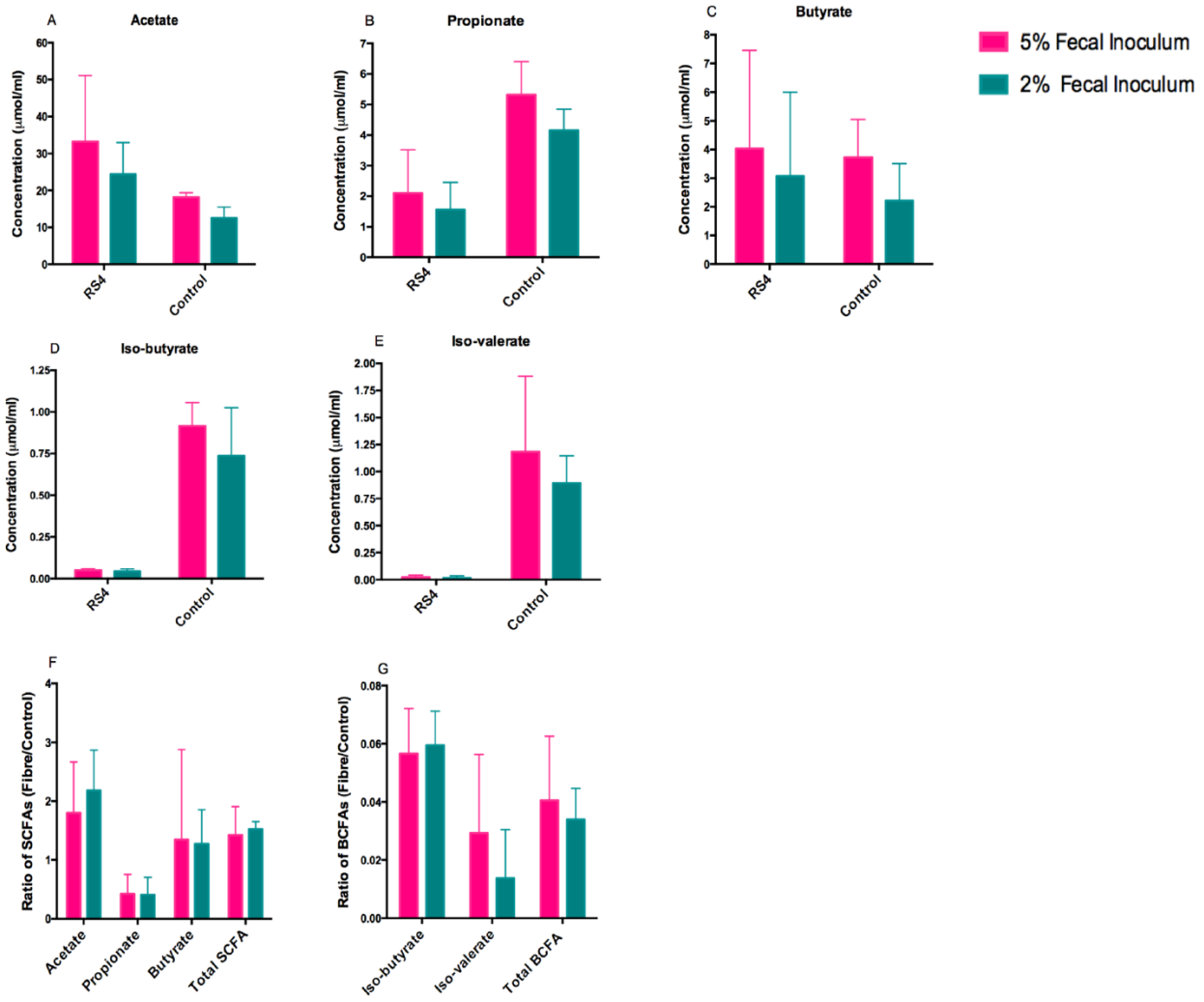

Figure 2.3. Effects of fecal inoculum concentration on the production of SCFAs and BCFAs during *IVFF* of RS4. Concentration (µmol/ml) of (A) acetate, (B) propionate, (C) butyrate, (D) iso-butyrate, (E) iso-valerate, (F) ratio of SCFAs (DF/ Control), (G) ratio of BCFAs (DF/ Control); reported as mean ± SD. Data was analyzed using Wilcoxon-signed rank test to assess the differences between absolute concentrations of SCFAs (A, B, C), BCFAs (D, E) and the ratio of SCFAs and BCFAs (F,G) between 2% and 5% fecal inoculum concentration. BCFAs, branched-chain fatty acids; Control, no added carbohydrate; *IVFF*, *in vitro* fecal fermentation; Medium 1, growth medium similar to what has been used in the literature; RS4, resistant starch type 4; SCFAs, short-chain fatty acids; n = 3.

2.4.2 Effect of fecal inoculum concentration on the growth of E. coli

Figure 2.4 shows the growth of *E. coli* at two different fecal inoculum concentrations with RS4 as a carbon source and no added carbohydrate (control) after 14 hours of *IVFF* in Medium 1. There was no difference in the growth of *E. coli* between 2% and 5% fecal inoculum concentration in both conditions, i.e. with RS4 and in control.

As no statistical differences in relative production of SCFAs and BCFAs and growth of *E. coli* were found between 5% and 2% fecal inoculum, we decided to use 2% fecal inoculum concentration for our future *in vitro* experiments.

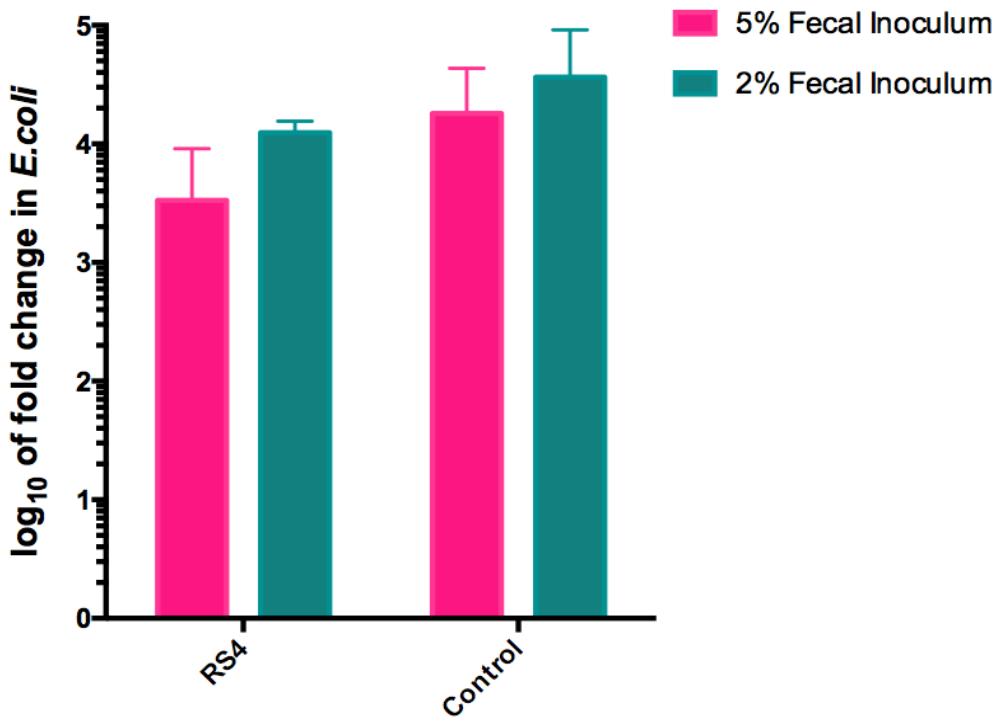

Figure 2.4. Effects of fecal inoculum concentration on the growth of *E. coli* during *IVFF* of RS4. Growth of *E. coli* at 2% and 5% fecal inoculum after *in vitro* fermentation with RS4 and no added carbohydrate (control) in Medium 1. The fold change in *E. coli* was determined as the ratio of the number of *E. coli* colonies (CFU/ml) present at the end of the fermentation (14 hours) to the number of colonies (CFU/ml) at the start of fermentation (0 hour). Values displayed are mean ± SD and were log transformed prior to the statistical analysis. Data was analyzed using Wilcoxon-signed rank tests to assess the differences in *E. coli* growth between 2% and 5% fecal inoculum concentration in RS4 and in control. CFU/ml, colony forming units/ml; *E. coli, Escherichia coli*; *IVFF, in vitro* fecal fermentation; Medium 1, growth medium similar to what has been used in the literature; RS4, resistant starch type 4; n = 3.

2.4.3 In vitro production of SCFAs and BCFAs at Media 1, 2 and 3 in 2% fecal inoculum concentration

As shown in the figure below, both RS4 and control were fermented to acetate, propionate, butyrate and BCFAs (iso-butyrate and iso-valerate) in Media 1, 2 and 3. However, there was no difference between the absolute amount of SCFAs and BCFAs produced among Medium 1, 2 and 3 ($p \geq 0.05$) (Figure 2.5 A, B, C, D & E).

Compared to the relative production of acetate, propionate, and total SCFA versus control in Medium 1, the concentration of acetate increased by 2.47-fold, the concentration of propionate increased by 2.42-fold, and the concentration of total SCFA increased by 2.16-fold in Medium 3 ($p \leq 0.05$) (Figure 2.5 F). Interestingly, the fermentation of RS4 resulted in a relative decrease of total BCFA in Medium 3 ($p \leq 0.05$) by 16-fold as compared to Medium 1 (Figure 2.5 G).

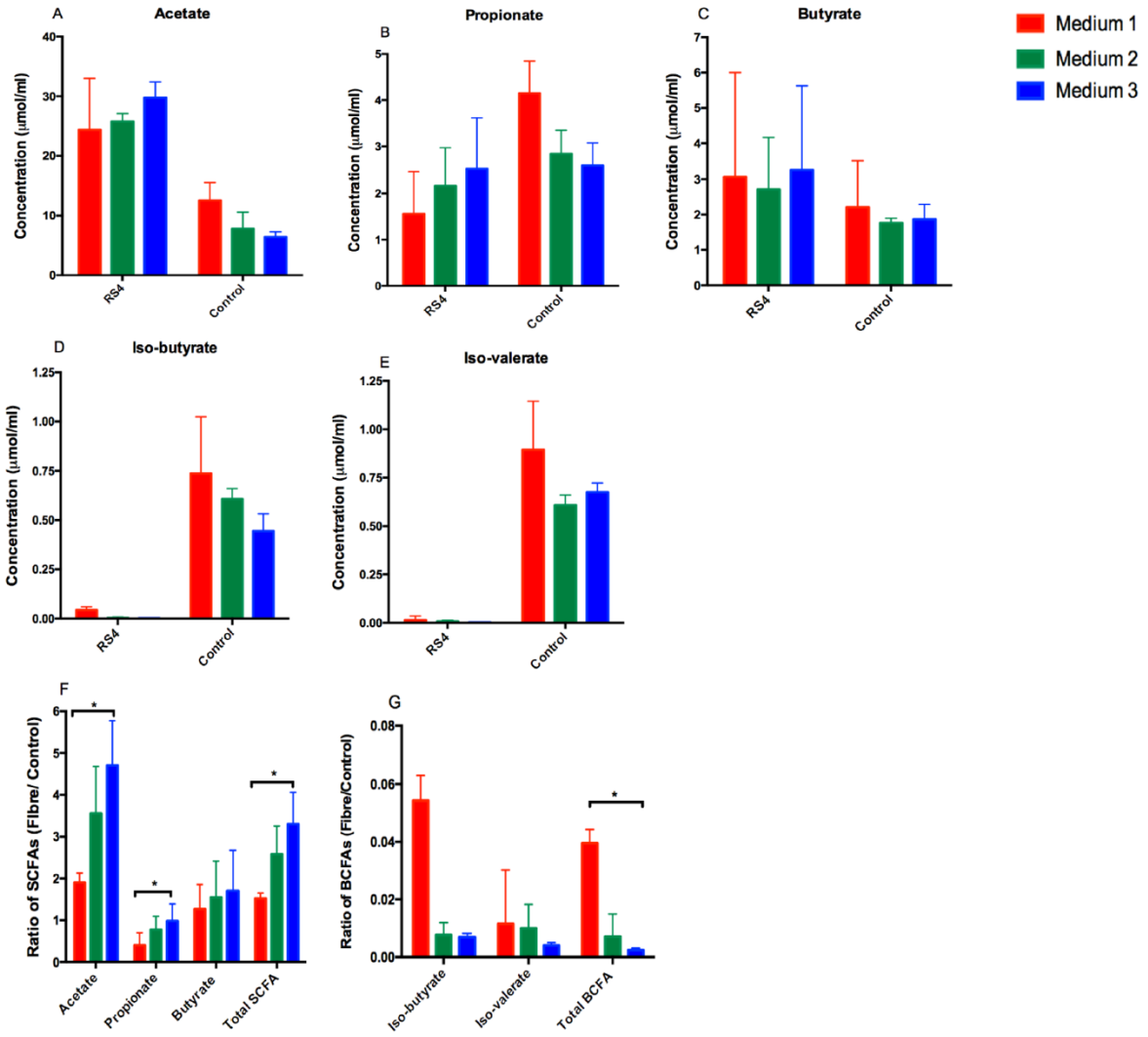

Figure 2.5. Effects of reduced concentration of peptone and yeast extract in the growth media on the production of SCFAs and BCFAs during *IVFF* of RS4. Concentration (µmol/ml) of (A) acetate, (B) propionate, (C) butyrate, (D) iso-butyrate, (E) iso-valerate, (F) ratio of SCFAs (DF/Control), (G) ratio of BCFAs (DF/Control); reported as mean ± SD. Data analyzed using Friedman test to assess the differences between absolute concentrations of SCFAs (A, B, C), BCFAs (D, E) and the ratio of SCFAs and BCFAs (F, G) among Media 1, 2 and 3; * $p \leq 0.05$. BCFAs, branched-chain fatty acids; Control, no added carbohydrate; *IVFF, in vitro* fecal fermentation; Medium 1, growth medium similar to what has been used in the literature; Medium 2, growth medium with the concentration of peptone and yeast extract 20% of Medium 1; Medium 3,

growth medium with the concentration of peptone and yeast extract 10% of Medium 1; RS4, resistant starch type 4; SCFAs, short-chain fatty acids; n = 3.

2.4.4 Effect of reducing the concentration of peptone and yeast extract in the growth medium on the growth of E. coli

Figure 2.6 shows the growth of *E. coli* in three different growth media (Medium 1, Medium 2, and Medium 3) at 2% fecal inoculum concentration in the presence of RS4 and without added carbohydrate (control) after 14 hours of *in vitro* fermentation. In the presence of substrate (RS4), Medium 3 had 4.54-log reduction ($p \leq 0.05$) in the growth of *E. coli* as compared to its growth in Medium 1. Similarly, in the absence of substrate (control) for fermentation, there was a 4.09-log reduction ($p \leq 0.05$) in the growth of *E. coli* in Medium 3 as compared to its growth in Medium 1.

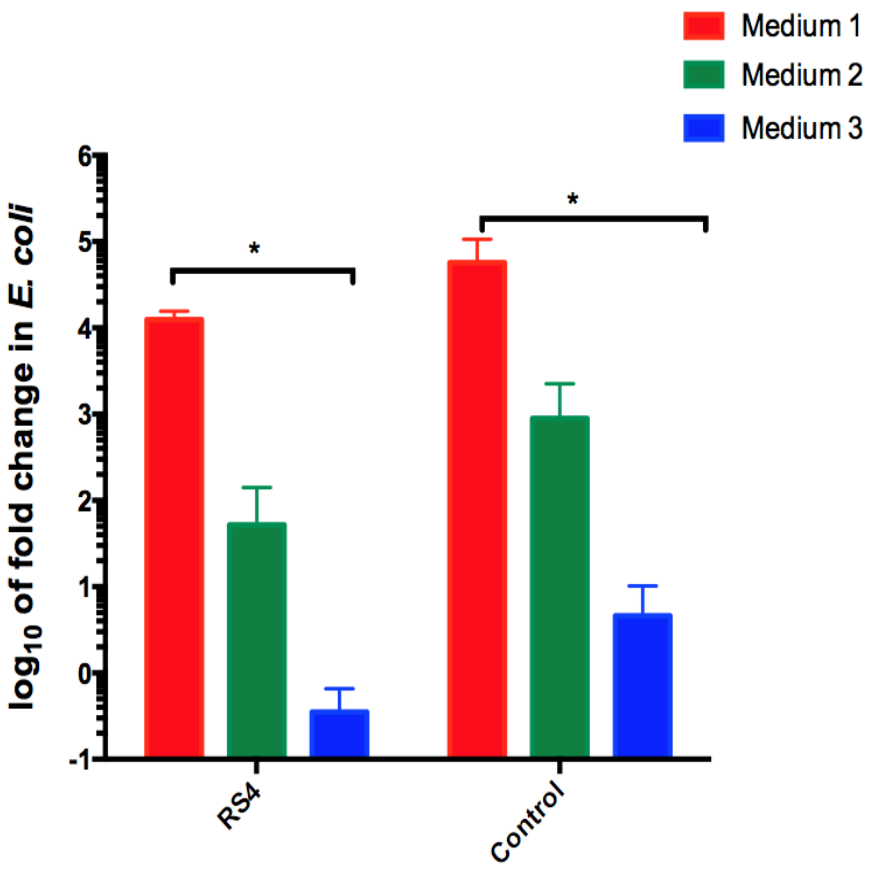

Figure 2.6. Effects of reduced concentration of peptone and yeast extract in the growth media on the growth of *E. coli* during *IVFF* of RS4. Growth of *E. coli* in Medium 1, Medium 2 and Medium 3 at 2% fecal inoculum concentration after *in vitro* fermentation with RS4 and no added carbohydrate (control). The fold change in *E. coli* was determined as the ratio of the number of *E. coli* colonies (CFU/ml) present at the end of the fermentation (14 hours) to the number of colonies (CFU/ml) at the start of fermentation (0 hour). Values displayed are mean ± SD and were log transformed prior to the statistical analysis. Data was analyzed using Friedman test to assess the differences in *E. coli* growth among Medium 1, Medium 2, and Medium 3; * $p \leq 0.05$. CFU/ml, colony forming units/ml; *E. coli, Escherichia coli*; *IVFF, in vitro* fecal fermentation; Medium 1, growth medium similar to what has been used in the literature; Medium 2, growth

medium with the concentration of peptone and yeast extract 20% of Medium 1; Medium 3, growth medium with the concentration of peptone and yeast extract 10% of Medium 1; RS4, resistant starch type 4; n = 3.

2.4.5 Repeatability of the SCFAs quantified via batch IVFF model (technical replicates)

The output of the repeatability experiment is shown in Table 2.1. The average of coefficients of variation (CV) in terms of absolute amount of SCFAs between 15 participants with their respective replicates were less than 5% for acetate and propionate and less than 8% for butyrate.

Table 2.1. Descriptive statistics for SCFAs results from technical replicates obtained after *in vitro* fermentation of pectin

Coefficient of variation (%) of technical replicates			
	Types of fatty acids		
Sample ID	Acetate(%)	Propionate(%)	Butyrate(%)
S1	3.16	1.81	7.71
S2	4.53	2.14	1.67
S3	6.69	13.19	14.60
S4	2.96	5.17	1.16
S5	1.46	2.05	2.30
S6	3.92	15.31	12.12
S7	1.61	2.27	2.92
S8	13.20	6.46	6.56
S9	3.06	1.37	12.85
S10	2.32	0.81	9.72
S11	1.56	1.50	13.40
S12	8.95	2.82	8.48
S13	0.29	0.55	6.18
S14	10.13	2.38	11.82
S15	3.61	5.59	0.70
Average of CV(%)	4.44	4.31	7.06

Each value for acetate, propionate, and butyrate represents coefficients of variation (CV) between the sample and its replicate sample. CV was calculated using the formula, CV = (SD/Mean) * 100%; SD = standard deviation; n = 15.

2.5 Discussion

Acetate, propionate, and butyrate are representative end products of colonic fermentation and their production is influenced by the microbes present and substrates utilized (Carlson et al., 2015). The most accessible and non-invasive means of obtaining a population of microorganisms for *in vitro* studies is from a fecal sample, which establishes the range and viability of the microbial species for the fermentation (Macfarlane & Smith, 1998). In the current study we demonstrated that the concentration of fecal inoculum has limited/unobvious effect on the *in vitro* production of SCFAs, BCFAs and growth of *E. coli*. However, the role of peptone and yeast extract in the growth media influenced the *in vitro* production of SCFAs, BCFAs and growth of *E. coli*.

Peptone and yeast extract are widely used in growth media as the source of amino acids for the fecal microbiota. *In vitro* batch studies using human fecal samples have shown that SCFAs and BCFAs are the end products formed during the degradation of protein where approximately 30% of the protein breakdown was converted to SCFAs (Macfarlane et at., 1992). In our study, we demonstrated that reducing the concentration of peptone and yeast extract in the growth media resulted in a significant increase in the relative production of total SCFA as compared to the relative amount of SCFA in the growth medium commonly used in the literature, when RS4 was fermented. This indicates that the quantified SCFAs are directly related to the substrate fermentation instead of products of fermented proteins present in the medium. Furthermore, the relative production of total BCFAs was decreased significantly in Medium 3 as compared to Medium 1, which was a desired outcome for the optimization of the *IVFF* model. BCFAs are reliable markers of proteolytic fermentation as they are produced exclusively through the fermentation of branched-chain amino acids (Diether & Willing, 2019). Limiting amino acid

sources creates an unfavorable environment for the growth and activity of BCFA-producing bacteria, which studies have shown could range from 0.6% (iso-valerate) to 40% (iso-butyrate) of total peptide and amino acid fermenting populations (Macfarlane & Smith, 1998).

In addition, we demonstrated the suppression in the growth of *E. coli* in Medium 3 as compared to Medium 1. Amino acids are building blocks for microbial protein. They serve as a source of energy via fermentation and are important for microbial growth (Diether & Willing, 2019). Aromatic amino acid metabolism reactions are thought to be primarily performed by *Enterobacter* and *Escherichia* spp (Sridharan et al., 2014). An *in vitro* study found that at low concentrations of phosphate and tryptophan, the specific growth rate of *E. coli* is directly proportional to the concentration of the available nutrients (Shehata & Marr, 1971), which could be another explanation as to why *E. coli* growth was reduced in the media with minimum amino acid concentration. After optimization of the batch *IVFF* model, we also investigated if the model is repeatable. We used pectin as the substrate for fermentation and demonstrated that the average of coefficient of variation of SCFAs for 15 samples and their replicates were less than 8%, indicating that the model is highly repeatable.

In our study, we did not optimize the fermentation time which could also have an effect on the amount of SCFAs produced. Future studies should study the impact of longer fermentation time on SCFAs production with multiple sampling points, as longer fermentation time such as 24 hours likely corresponds with the transit through the proximal colon (Metcalf et al., 1987). In conclusion, a batch *IVFF* model was optimized based on the relative amount of SCFAs and total BCFA and the growth of *E. coli* during fermentation of DF. The growth media with the concentration of peptone and yeast extract reduced to 10% of their concentration in the growth media commonly used in the literature resulted in the largest relative production of total SCFA, the lowest relative

production of total BCFA, and in an almost complete prevention in the growth of *E. coli*. This optimized batch *IVFF* model provides a simple alternative for characterizing the metabolic function of the gut microbiota to ferment DF in individuals.

Chapter 3. Application of the batch *IVFF* model to assess the capacity of the fecal microbiota to ferment chemically distinct DFs to SCFAs.

3.1 Introduction

DF and the gut microbiota interact positively to influence host physiology and metabolism (Makki et al., 2018). These effects depend in part on the type of DF consumed and the extent of DF degradation and fermentation by the gut microbiota (Bliss et al., 2013). As DF types differ vastly in their chemical structures, their utilization by the gut microbes can vary substantially because the bacteria have different metabolic abilities to cleave the linkages of complex structures yielding simple sugars (Tuncil et al., 2017). Functional differences exist between chemically distinct types of DFs in terms of how they impact gut microbiota composition and subsequent SCFA production. It has been reported that the monomeric composition, type of linkages between the monomers, solubility, molecular weight and degree of methylation of the DFs affect the rates and patterns of its fermentation (Hughes et al., 2008; Rose et al., 2007).

Even though structurally distinct types of DFs are associated with notable differences in their metabolic response (Ingerslev et al., 2014), the individualized nature of the microbiome determine whether a particular DF can be metabolized (Ze et al., 2012). The individualized response to a DF largely depends on the presence of keystone species capable of utilizing the specific DF (Deehan et al., 2017). The inter-individual variability of the gut microbiota largely influences the metabolism of dietary constituents and the magnitude and flux of metabolites to which the host is exposed (Lampe et al., 2013). However, limited information is available on how inter-individual differences in DF fermentation influences the health effects of DFs. In our previous study, a batch *IVFF* model was optimised, which now gives us an opportunity to further evaluate the fermentation

capacity of fecal microbial community. Therefore, the primary objective of this study was to determine the ability of the batch *IVFF* model to assess the capacity of fecal microbial communities of different individuals to ferment chemically distinct DFs to SCFAs.

3.2 Materials and method

3.2.1 Subjects

Fecal donors (83 females and 41 males) were healthy overweight and class-I obese between the ages of 19 and 50 whose BMI ranged from 25.0 to 34.9 kg/m^2. The participants were chosen from the Alberta FYBER (Feed Your gut Bacteria morE fibeR) study who were recruited from the Edmonton area using campus-wide flyers, mailings to specific Listservs, local events, and word of mouth. Inclusion criteria included (i) no known gastrointestinal disorders or surgeries; (ii) no history of diabetes mellitus; (iii) no chronic use of anti-hypertensive, lipid-lowering, anti-diabetic, analgesic, or laxative medications; (iv) no use of antibiotics in the three months prior to the study; (v) no use of probiotic, prebiotic or herbal supplements; (vi) non vegetarian; (vii) no smoking; (viii) alcohol intake ≤ 7drinks/week; (ix) < 3 hours of vigorous exercise per week. All procedures involving human subjects were approved by the Health Research Ethics Board of the University of Alberta (Approval Number: Pro00050274). Written informed consent was obtained from all study subjects prior to enrollment into the study.

3.2.2 Fecal sample collection and processing

Participants were given stool collection kits consisting of a stool specimen container, an air-tight bag (Fisher, Canada), and a GasPak^{TM} EZ Anaerobe Sachet (BD, Canada) to generate an anaerobic environment within the container, and then delivered to the laboratory within 4 hours of defecation. Immediately upon receipt in the laboratory, the stool samples were processed in an

anaerobic chamber (Bactron^{TM}, Shel Lab, Oregon, USA) with anaerobic environment (5% H_2, 5% $C0_2$, and 90% N_2). Each fecal sample was aliquoted and stored at -80˚C to serve as inoculums for later *in vitro* fermentation.

3.2.3 DF Substrates

AX derived from corn bran was provided by Agrifiber Solutions LLC (Mundelein, IL, USA). PE derived from citrus peel was obtained from Cargill, Inc. (Wayzata, MN, USA). RS4 (phosphorylated, cross-linked wheat starch) was provided by MGP Ingredients, Inc. (Atchison, Kansas, USA). AG derived from acacia tree exudate and largely arabinogalactan was provided by Agrigum International Limited (The Broadway, Old, Amersham). MCC derived from wood cellulose deprived of hemicellulose and amorphous regions was provided by Blanver (Boca Raton, FL, USA). All the DFs were subjected to *in vitro* digestion as described in Chapter 2 on section 2.3.4, prior to the *IVFF* in order to remove the digestible components that would normally be absorbed by the host. MCC was used as a control as it largely resists fermentation in the colon (Flint et al., 2014). The digested DF samples were vacuum evaporated to remove the excess water and freeze-dried. The freeze-dried samples were then stored at -80°C.

3.2.4 IVFF of the pre-digested DFs

IVFF of freeze-dried AX with fecal inoculum from the study participants was carried out according to the methods described by Yang and colleagues (Yang et al., 2013) with the modifications as described previously in Chapter 2 on section 2.3.5 of the thesis.

3.2.5 SCFA analysis

After *in vitro* fermentations, the produced SCFAs were quantified at the Chromatography Core Facility of the University of Alberta as described previously in Chapter 2 on section 2.3.6. The

total SCFAs were determined as the sum of acetate, propionate, and butyrate. SCFA production was measured in µmol/ml of fermentation media and presented as the mean value with standard deviation for each substrate, and as molar proportion (%) estimated by dividing the amount of a specific SCFA by the total amount of SCFA for the same subject and multiplied by 100.

3.2.6 Statistical Analysis

All statistical tests were performed using GraphPad Prism version 6.0 (GraphPad Software, La Joya, CA, USA). The D'Agostino & Pearson omnibus normality test was used to assess if the data had a normal distribution. Since the data did not follow a normal distribution, non-parametric Kruskal-Wallis test was used to identify differences in SCFA production among DFs. P values of ≤ 0.05 were deemed as statistically significant. Additionally, CV in the SCFA production was calculated using the formula: CV = (standard deviation/ mean) *100%.

3.3 Results

3.3.1 Effect of DF source on the amount of SCFA production

The fermentation of AX, PE, RS4 and AG produced SCFAs consisting primarily of acetate, propionate, and butyrate. Significant increase in the concentration of total SCFA as compared to the control (MCC) were observed for all DFs (Figure 3.1 D). The fermentation of AX resulted in a significant increase in propionate concentration as compared to PE ($p \leq 0.01$), RS4 ($p \leq 0.0001$), and MCC ($p \leq 0.01$) (Figure 3.1 B).

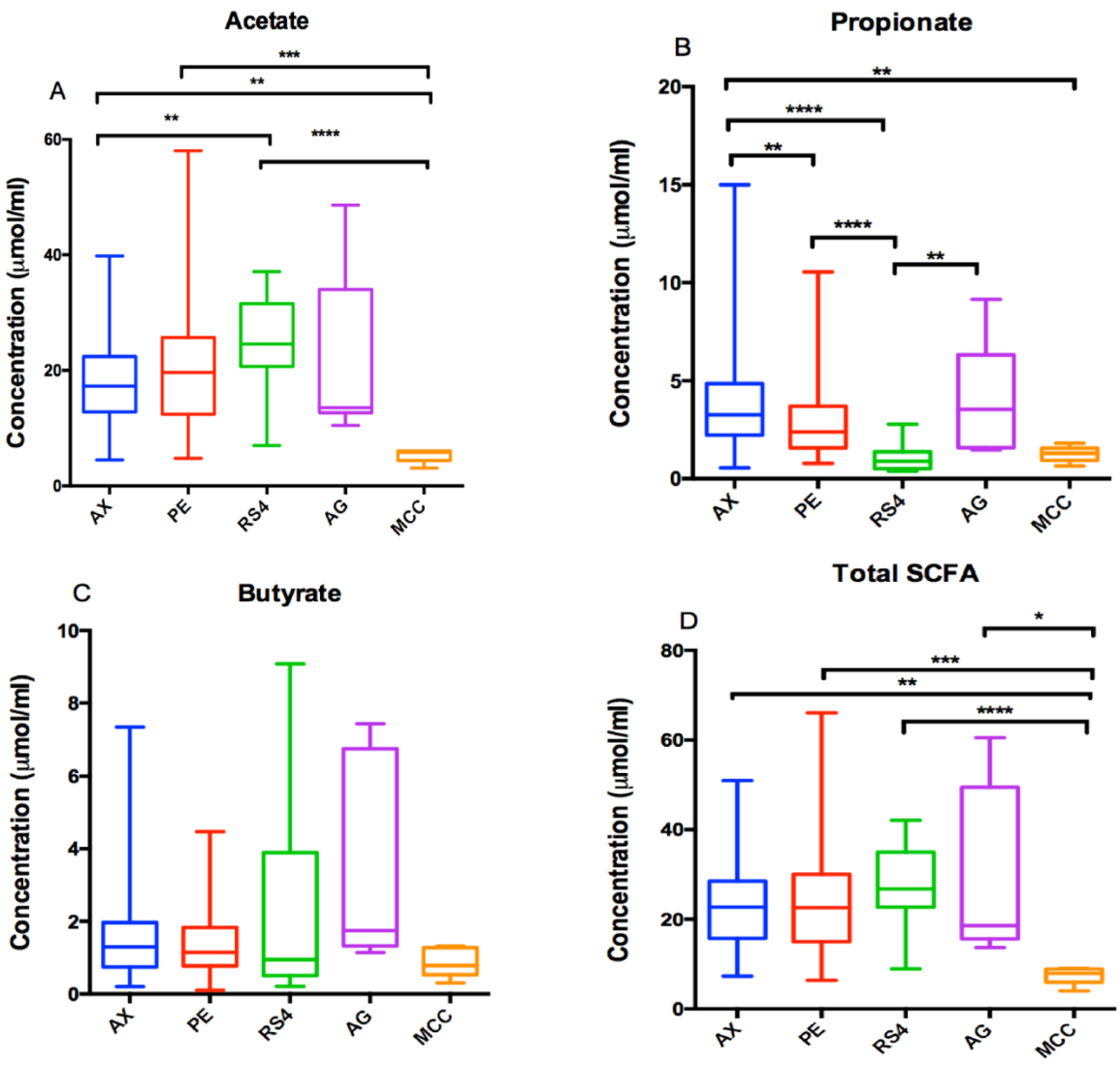

Figure 3.1. DF type determines the amount of SCFA production. *In vitro* production of (A) acetate, (B) propionate (C) butyrate, and (D) total SCFA obtained from the fermentation of different types of DFs, reported as mean ± SD. Data was analyzed using Kruskal-Wallis test to assess the differences in SCFAs production between different DFs; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. AX, arabinoxylan; AG, acacia gum; MCC, micro-crystalline cellulose; PE, pectin; RS4, resistant starch type 4; SCFA, short-chain fatty acid; n=92 for AX, n = 92 for PE, n=20 for RS4, n= 6 for AG, and n=6 for MCC.

3.3.2 Effect of DF source on the proportion of SCFA production

Calculating molar proportions provides values that are physiologically more relevant than the absolute values, as the absolute amount of SCFAs are affected by host factors such as absorption in the epithelial cells and gastrointestinal transit, among others (Cummings et al., 1987). In terms of molar proportions, the *in vitro* fermentation of RS4 resulted in a significantly higher molar proportion of acetate as compared to AX ($p \leq 0.0001$), AG ($p \leq 0.01$), and MCC ($p \leq 0.0001$), but a significantly lower proportion of propionate as compared to AX ($p \leq 0.0001$), PE ($p \leq 0.0001$), AG ($p \leq 0.01$), and MCC ($p \leq 0.0001$) (Figure 3.2 A & B). Interestingly, the *in vitro* fermentation of AG resulted in a significant increase in molar proportion of butyrate as compared to RS4 ($p \leq 0.05$), PE ($p \leq 0.05$) and AX ($p \leq 0.05$) (Figure 3.2 C).

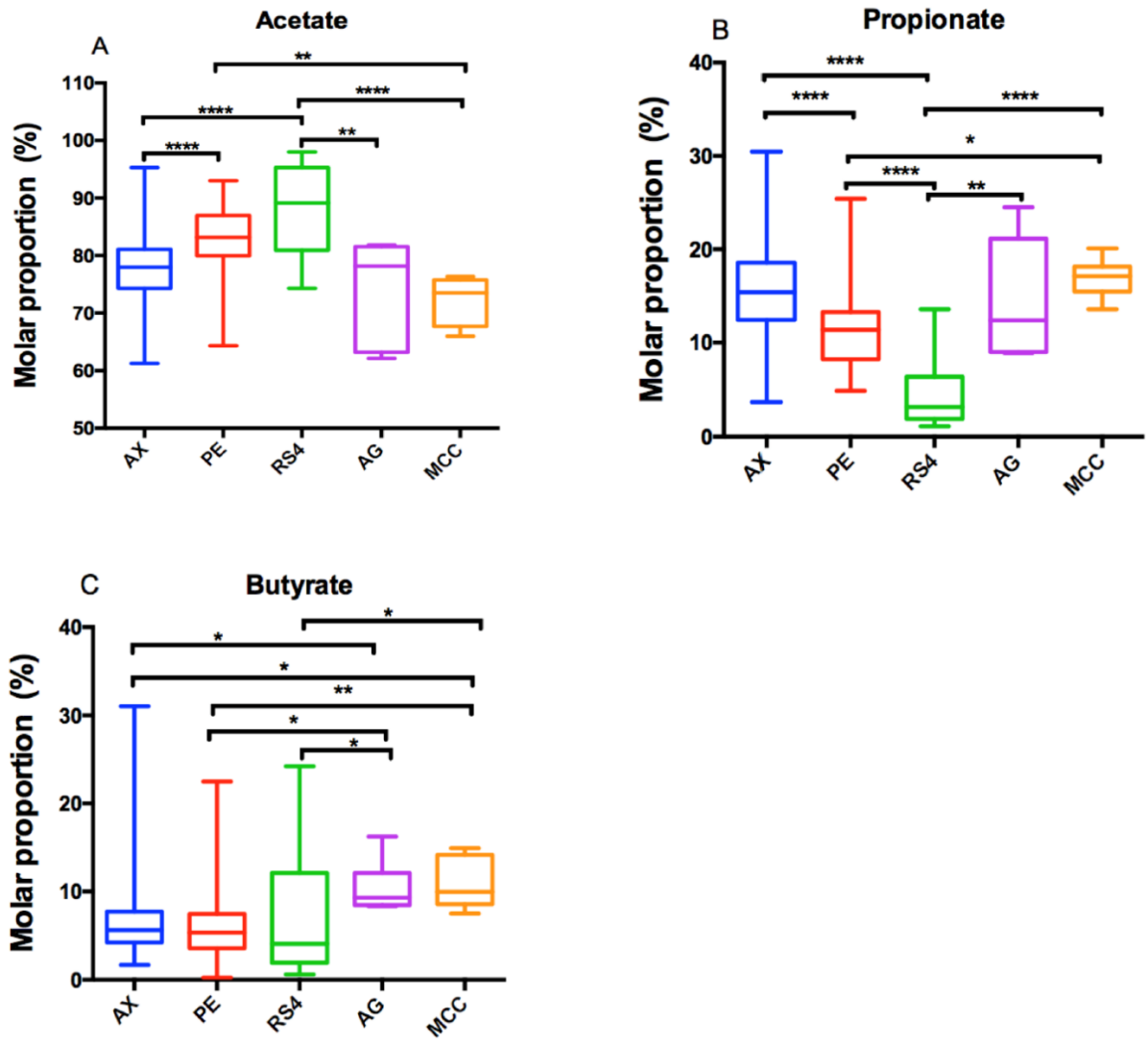

Figure 3.2. DF type determines the molar proportion (%) of SCFA production. *In vitro* molar proportion of (A) acetate, (B) propionate, and (C) butyrate obtained from the fermentation of different types of DFs, reported as mean ± SD. Molar proportion (%) was calculated by dividing the amount of a specific SCFA by the total amount of SCFA for the same subject and multiplied by 100. Data was analyzed using Kruskal-Wallis test to assess the differences in proportion of SCFAs between different DFs; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. AX, arabinoxylan; AG, acacia gum; MCC, micro-crystalline cellulose; PE, pectin; RS4, resistant starch type 4; SCFA, short-chain fatty acid; n=92 for AX, n = 92 for PE, n=20 for RS4, n= 6 for AG, and n=6 for MCC.

3.3.3 The inter-individual variation in SCFA production for different DFs

As shown in the Table 3.1, there exists substantial inter-individual variation in absolute amounts of SCFAs for each type of DF. In particular, the amount of propionate and butyrate varied by more than 60% for AX, PE, RS4, and AG. Similarly, RS4 had the highest CV (120.34%) for butyrate production among all other DFs studies and AG had highest CV for acetate (74.95%) production.

Table 3.1 Mean, standard deviation and coefficient of variation of SCFAs obtained by the *IVFF* of different types of DF

In vitro SCFAs with different types of DFs					
	AX	PE	RS4	AG	MCC
Acetate					
Mean (µmol/ml)	18.06	20.38	24.89	21.46	5.29
SD (µmol/ml)	6.87	9.93	7.51	16.09	1.29
CV (%)	38.03	48.72	30.17	74.97	24.38
Propionate					
Mean (µmol/ml)	3.88	2.82	1.10	4.12	1.26
SD (µmol/ml)	2.47	1.79	0.69	3.01	0.41
CV (%)	63.65	63.47	62.72	73.03	32.53
Butyrate					
Mean (µmol/ml)	1.47	1.34	2.19	3.33	0.84
SD (µmol/ml)	1.01	0.86	2.63	2.85	0.39
CV (%)	68.70	64.17	120.09	85.58	46.42

Data are presented as mean, SD and CV for acetate, propionate, and butyrate. CV was calculated using the formula, CV = (SD/Mean) * 100%. AX, arabinoxylan; AG, acacia gum; CV, coefficient of variation; DFs; dietary fibres; MCC, micro-crystalline cellulose; PE, pectin; RS4, resistant starch type 4; SD, standard

deviation; SCFAs, short-chain fatty acids; n=92 for AX, n = 92 for PE, n=20 for RS4, n= 6 for AG, and n=6 for MCC.

3.4 Discussion

Previous studies have shown that variations in the chemical structures of the DFs, such as linkage type or degree of polymerization, affect their utilization by the gut microbiota and the production of SCFAs (Fehlbaum et al., 2018; Glitsø et al., 2000). In agreement, the present study demonstrated that each of the specific DFs was associated with distinct profiles of SCFA production which can be attributed to the differences in their chemical integrity. For example, AX produced the highest concentration of propionate as compared to RS4, PE, and MCC. Researchers have shown that the structural configuration of corn bran AX allows fermentation by divergent groups of bacteria such as the xylan-degrading Bacteroides producing mainly acetate and propionate (Chassard et al., 2007; Rumpagaporn et al., 2016). The complexity of structure of AX varies greatly by source, with corn bran AX having highly branched and very complex structures. Corn bran AX, owing to their complex structure, are more difficult to hydrolyze into oligosaccharides (Kale et al., 2018) and slow fermentation rates have been reported for corn AX with terminal branched xyloses (Rumpagaporn et al., 2015).

Not only the chemical complexity of the DF, but also the structural characteristics have been associated with its fermentation ability. For instance, AG is a polysaccharide with high molecular weight, which has been linked to its fermentation in the distal colon (Marzorati et al., 2015). A study in which AGs with four molecular masses (15, 20, 400 and 1100 KDa) were fermented using human fecal inoculum found that the 400 KDa mass of AG resulted in the highest amount of butyrate production after 24 hours of fermentation (Stewart & Slavin, 2006). In our study, the

fermentation of AG resulted in a significantly higher proportion of butyrate as compared to AX, PE, and RS4. The butyrate producing ability of AG has been associated with the abundance of Roseburia/ E. rectale group of bacteria in the fecal samples (Ohashi et al., 2012). In contrast, the reduction in the proportion of butyrate in AX as compared to AG may be in part related to the inability of the *Roseburia intestinalis*, a key xylan degrading butyrate producing Firmicute to grow on the corn bran AX (Leth et al., 2018).

Another key point of this study was the high degree of inter-individual variation in regard to the fermentation of DFs. For instance, the coefficient of variation was greater than 60% for propionate and butyrate production in AX, PE, RS4 and AG. This indicates that the fecal microbiota of some of individuals were able to ferment the isolated DFs into propionate and butyrate more effectively than others. Moreover, a CV of 120.09% for butyrate production in RS4 was observed. Such profound inter-individual variability during *in vitro* fecal fermentation of DF demonstrates that it may be possible to stratify individuals into categories that respond to each DF. Specific dietary changes could have highly variable effects in different people owing to the individualized nature of their gut microbiota. For example, a study showed that *R. bromii* bloomed in the presence of RS in most of the 14 obese male participants (12 out of 14 individuals) , but lacked a response in 2 other individuals, which may be attributed to the absence of such taxa in those people (Walker et al., 2010). In a follow up study, two fecal samples that had residual unfermented starch restored degradation of RS3 after the addition of *R. bromii* (Walker et al., 2010). In our study, the microbiota of some individuals may already have the capacity to degrade RS4 efficiently as compared to their counterparts, while there might be some non-responders with microbiota inhibiting the fermentation. Such individualized response of the microbiota to DF

fermentation could open avenues for personalized nutrition approaches. Indeed, a recent study integrated an individual's microbiota in order to accurately predict glucose response to a range of foods, suggesting that personalized diets may successfully modify elevated postprandial blood glucose and its metabolic consequences (Zeevi et al., 2015).

Small sample size in RS4, AG, and MCC groups is the limitation of our study. As our study is progressing, with more enrollment of participants, we can have more powerful analysis in the future studies. In conclusion, the optimized batch *IVFF* model could be successfully used to study the fermentation of chemically distinct DFs into SCFAs. DFs differed in their ability to induce different SCFAs, reflecting the chemical structure of each fibre (Chassard et al., 2007; Marzorati et al., 2015; Rumpagaporn et al., 2016; Stewart & Slavin, 2006). In addition, extensive inter-individual variation in response to the fermentation of DFs were observed. Thus, the batch *IVFF* model provides a platform for future nutrition studies to assess individualized host response to DF fermentation and to eventually personalize the use of DF based on individual microbiome function.

Chapter 4. Effect of arabinoxylan supplementation for six weeks on short-chain fatty acid production in healthy overweight individuals

4.1 Introduction

Observational studies have shown an association between increased intake of whole grain and cereal derived DFs with reduced cases of chronic diseases, such as cardiovascular disease, type 2 diabetes, and obesity (Koh et al., 2004; Mellen et al., 2008; Ye et al., 2012). Emerging evidence indicates that the benefits of increased DFs on host health is mediated by a small number of bacteria with the genetic capacity of producing SCFAs in the colon (Keenan et al., 2015; Zhao et al., 2018).

One approach for stimulating the production of SCFA in the gut is to supplement the diet with DFs that can be metabolized by the gut microbes in the colon (Baxter et al., 2019). Diet-driven changes in microbial diversity have been shown to cause variations in SCFA production (Morrison & Preston, 2016; O'Keefe et al., 2015). Short-term consumption of diets composed of entirely animal or plant products altered microbial community structure and metabolic activity within 24 hours (David et al., 2014). However, supplementation of whole grain for six weeks in healthy subjects caused a decrease in the concentration of fecal SCFAs at week six of intervention compared to the baseline (Vanegas et al., 2017). Similarly, in another randomized study, consumption of diet enriched with AX and RS2 for 4 weeks by individuals did not influence the production of SCFAs. The difference in SCFA post intervention was only observed as a result of the declining SCFAs during the western style diet (Hald et al., 2016).

Such inconsistent findings from human dietary intervention trials may arise from extensive inter-individual differences in the ability of gut microbiota to metabolize the consumed DF into

SCFAs. However, our knowledge of how differences in DF fermentation among individuals influences the production of SCFAs is scarce. We hypothesized in this study that the production of SCFAs is enhanced after six weeks as compared to week one of DF feeding trial because of the ability of gut microbiota to adapt to the DF. To test this hypothesis, we evaluated the SCFAs concentrations produced via *IVFF* of AX and directly in the fecal sample to assess the impact of AX intervention on the metabolic function of the gut microbiota in overweight and obese men and women.

4.2 Materials and methods

4.2.1 Study participants

Overweight and class-I obese (BMI 25.0 to 34.9 kg/m^2), otherwise healthy males and pre-menopausal, non-pregnant or lactating females, aged 19 to 50 years were recruited from the Edmonton area using campus-wide flyers, mailings to specific Listservs, local events, and word of mouth. In total 31 subjects (21 females and 10 males) took part in this study. A total of 15 subjects were included in the AX arm while 16 subjects were assigned to the MCC arm (Figure 4.1). The inclusion criteria were: i) no history of gastrointestinal disorders or surgeries, ii) no history of diabetes mellitus; iii) no use of anti-hypertensive, lipid-lowering, anti-diabetic, anti-inflammatory (i.e. corticosteroids, or chronic nonsteroidal anti-inflammatory drugs), or laxative medications; (iv) no antibiotic treatment in the last three months prior to the start of the study; v) no use of probiotic, prebiotic, or herbal supplements; vi) no intolerance to corn; vii) non vegetarian; viii) no smoking, and ix) alcohol intake ≤ 7 drinks/week, x) < 3 hours of vigorous exercise per week.

4.2.2 Study design

This randomized, controlled, single-blind, parallel two-arm, six-week dietary intervention study (ClinicalTrials.gov Registration Number: NCT02322112) was conducted in accordance with the principles of the Declaration of Helsinki at the University of Alberta Human Nutrition Research Unit in Edmonton, Alberta, Canada between September 2015 and October 2016. All procedures involving human subjects were approved by the Health Research Ethics Board of the University of Alberta (Approval Number: Pro00050274). Written informed consents were obtained from all study subjects prior to enrollment into the study.

Study subjects were required to attend five clinic visits (Figure 4.1). During a 2-week screening/baseline period, potential subjects were pre-screened by telephone for initial eligibility and then attended a screening visit (visit 1) to confirm their eligibility and receive the study materials (including fecal sample collection supplies) to be completed prior to the baseline visit (visit 2). During the baseline visit, eligible subjects were enrolled, stratified based on sex, and then randomly assigned to either the AX or MCC treatment arms.

Thirty-one subjects completed the study. The participants were instructed to consume their corresponding DF daily for six-weeks at a DF dose of 25 g for females and 35 g for males, provided strictly as either AX or MCC. Half daily doses were provided for the first two days of treatment (12.5 g and 17.5 g for females and males, respectively), while complete doses were provided for the remainder of the intervention. Fecal samples were collected at baseline (visit 2), week 1 (visit 3) and week 6 (visit 5).

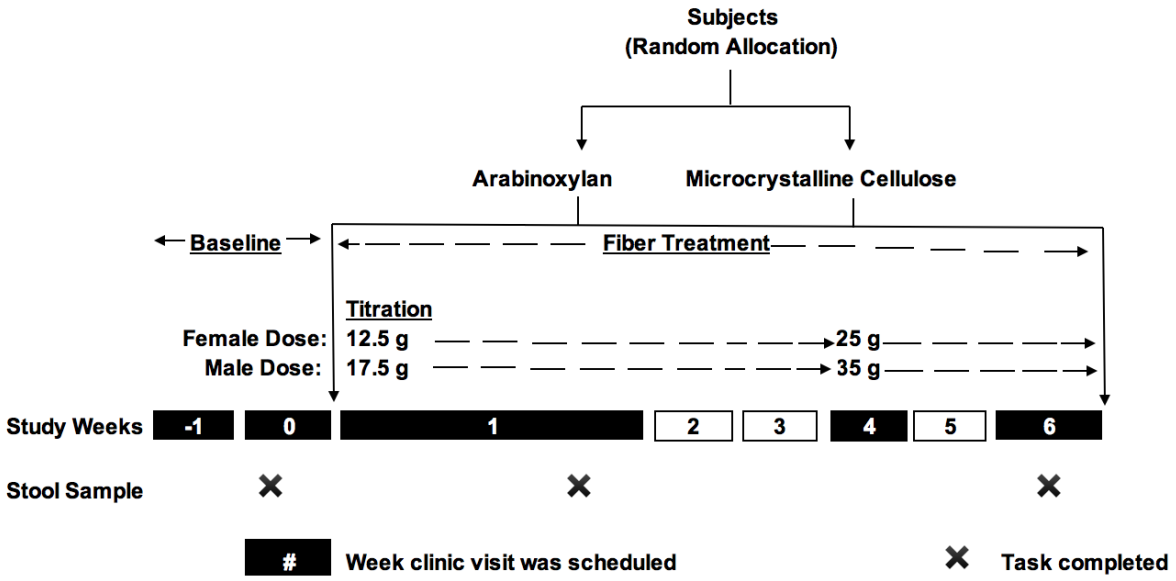

Figure 4.1. Study design of the human dietary intervention trial. The shaded study week block indicates a scheduled clinic visit. The '**X**' indicates that the stool sample was collected during the study week.

4.2.3 DF treatments

AX derived from corn bran was provided by Agrifiber Solutions LLC (Mundelein, IL, USA). MCC derived from wood cellulose and deprived of hemicellulose and amorphous regions was provided by Blanver (Boca Raton, FL, USA). The MCC was selected as the control treatment due to its excessive resistance to microbial fermentation (Flint et al., 2008). Both DF treatments were administered as powdered supplements that the subjects then incorporated into their preferred food and beverages. Subjects were blinded to which DF treatment they were receiving, and their weekly doses were provided in sealed opaque bags that contained individually packaged, ready-to-use daily sachets providing the specified dose of DF (i.e. 25g/d or 35g/d).

4.2.4 Fecal sample collection and processing

Three stool samples from each individual (delivered within four hours of defecation) were collected at baseline, week 1, and week 6. Stool samples were collected with stool collection kits as previously described in Chapter 2 on section 2.3.2 of the thesis. The fecal samples were then aliquoted in an anaerobic chamber with atmosphere conditions of 5% H_2, 5% $C0_2$, and 90% N_2, and subsequently stored at -80°C as described earlier (Chapter 2). One four-fold dilution aliquot of each sample diluted in 5% phosphoric acid solution was stored at -80°C for direct SCFA analysis.

4.2.5 IVFF of the pre-digested AX

IVFF of freeze-dried AX with fecal inoculum from the study participants was carried out according to the methods described by Yang and colleagues (Yang et al., 2013) with the modifications previously described in Chapter 2 on section 2.3.5. After 14 hours of *IVFF*, 1 ml of the aliquoted sample was removed from the anaerobic chamber and centrifuged at 20,000 x g for 20 minutes until a clear supernatant was obtained.

4.2.6 Analysis of fecal samples for SCFAs

For quantification of SCFAs directly from fecal samples, aliquots stored in 5% phosphoric acid were thawed at room temperature. Once thawed, they were centrifuged, and in a GC vial (1.8 ml), 1 ml of clear supernatant was added to 0.2 ml of internal standard, mixed well and stored at -80˚C until analysis. For the quantification of SCFAs from the fermented samples, 0.8 ml of the supernatant was mixed with 0.2 ml of 25% phosphoric acid and 0.2 ml of internal standard, mixed well, and stored at -80˚C until analysis. The internal standard was prepared by adding 20 ml of 25% phosphoric acid, 20 ml of distilled water and 300 µl of isocaproic acid.

SCFAs were quantified by GC at the Chromatography Core Facility at the University of Alberta (Edmonton, AB, Canada) as described earlier on section 2.3.6 in Chapter 2. Total SCFAs were determined as the sum of acetate, propionate, and butyrate. SCFAs production was measured in μmol/ml of fermentation media and presented as the mean value with standard deviation and as molar proportion (%) estimated by dividing the amount of a specific SCFA by the total amount of SCFA for the same subject and multiplied by 100.

4.2.7 Statistical Analysis

All statistical tests were performed using GraphPad Prism version 6.0 (GraphPad Software, La Joya, CA, USA). The D'Agostino & Pearson omnibus normality test was used to assess if the data had a normal distribution. Since the data did not follow a normal distribution, a non-parametric Friedman test was used to compare SCFAs (absolute concentration and molar proportion) between baseline, week 1 and week 6. Correlations between the shifts (ΔW6- W1) in the absolute amount of SCFAs *in vitro* and in fecal samples was computed with Spearman's correlation test. P values ≤ 0.05 were deemed as statistically significant.

4.4 Results

4.4.1 Effect of AX consumption for six weeks on the absolute concentration of SCFAs after in vitro fermentation and in fecal samples

The consumption of AX for six weeks did not change the *in vitro* production of SCFAs concentration between the baseline, week 1 and week 6 (Figure 4.2 A, B & C). Similarly, fecal SCFAs did not change in the MCC arm (Figure 4.2 G, H & I). Intriguingly, after one week of AX supplementation, a significant increase in fecal propionate was observed ($p \leq 0.05$), an effect not maintained after six weeks (Figure 4.2 E).

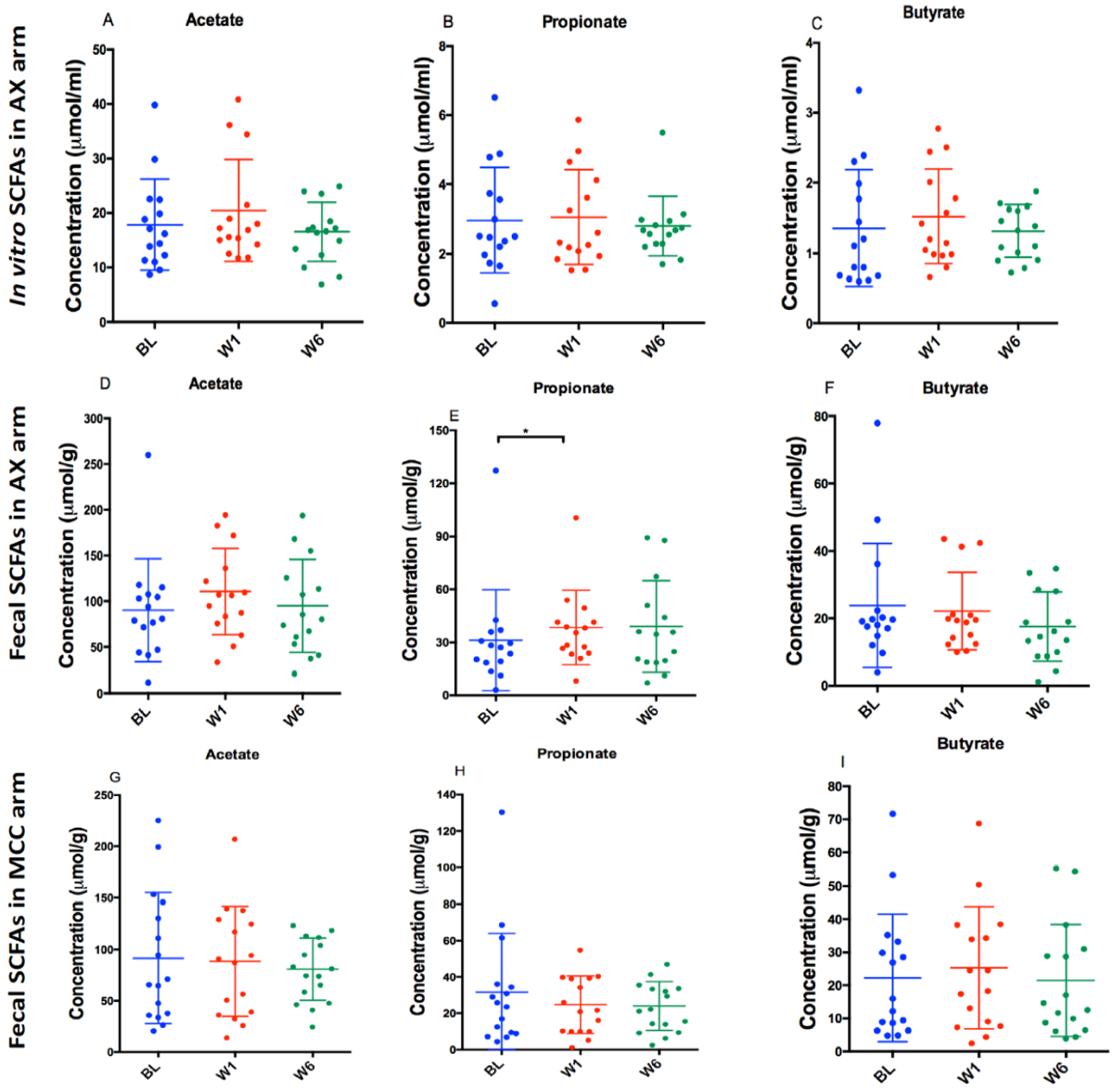

Figure 4.2 Comparison of SCFAs in fecal samples and after *in vitro* fermentation of AX. Absolute concentration (µmol/ml) of (A) acetate, (B) propionate, and (C) butyrate in AX arm after *in vitro* fermentation; concentration of fecal (µmol/g) (D) acetate, (E) propionate, and (F) butyrate in AX arm; concentration of fecal (µmol/g) (G) acetate, (H) propionate, and (I) butyrate in MCC arm, reported as mean ± SD. Friedman's test was applied to detect the effect of treatment on each type of SCFAs in between the

time points; * $p \leq 0.05$. AX, arabinoxylan; BL, baseline; MCC, microcrystalline cellulose; SCFAs, short-chain fatty acids; W1, week 1; W6, week 6; n = 15 in AX arm, n = 16 in MCC arm.

4.4.2 Effect of AX consumption for six weeks on the molar proportion of SCFAs after in vitro fermentation and in fecal samples

The *in vitro* molar proportion of SCFAs remain unchanged during the AX intervention (Figure 4.3 A, B & C). Similarly, no changes were observed for the molar proportion of SCFAs at week 6 as compared to the baseline for MCC (Figure 4.3 G, H & I). However, the consumption of AX increased the molar proportion of fecal propionate at week 6 as compared to the baseline ($p \leq 0.05$), in AX arm (Figure 4.3 E).

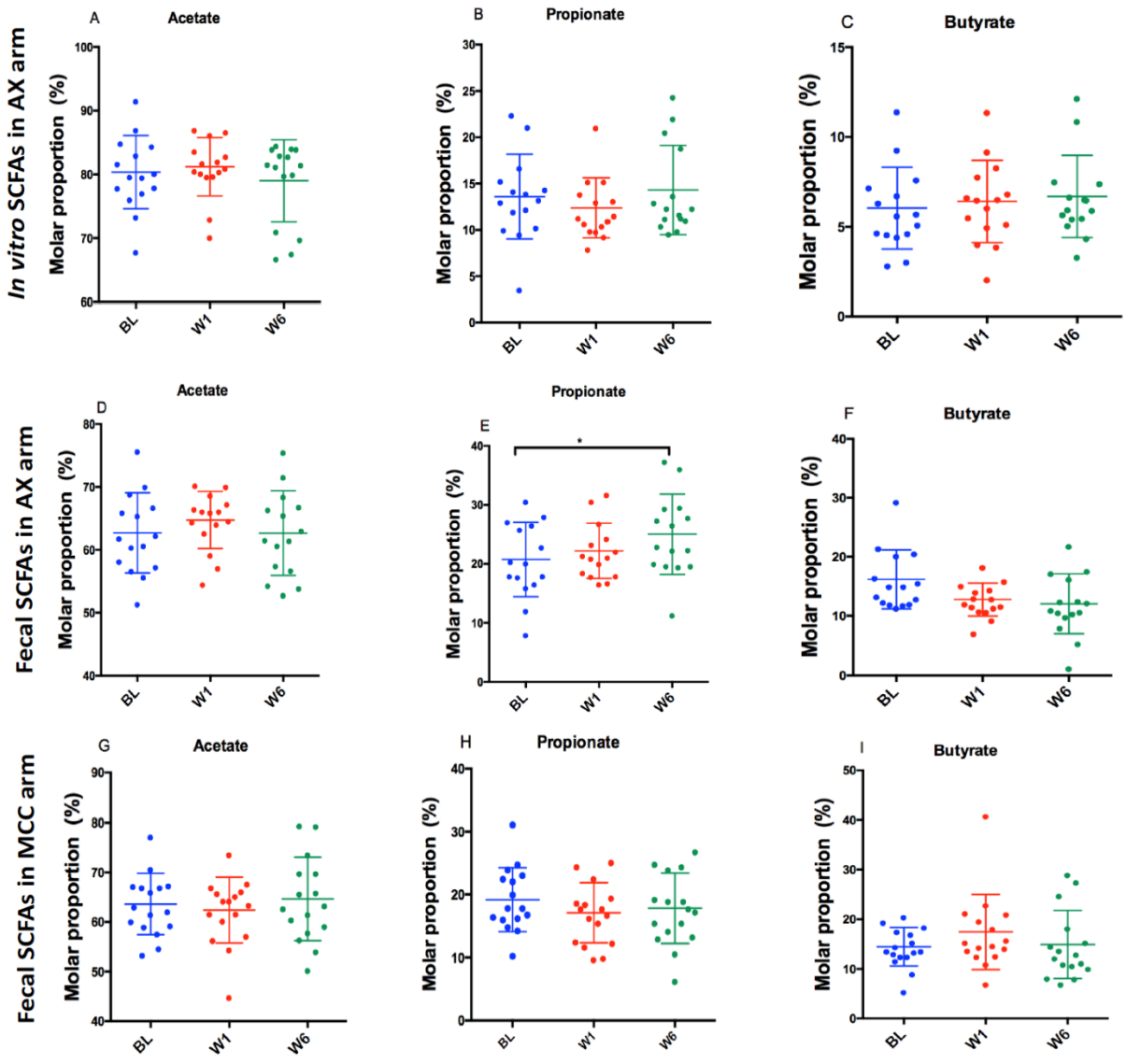

Figure 4.3 Comparisons of the proportions of SCFAs in fecal samples and after *in vitro* fermentation of AX. Molar proportion of (A) acetate, (B) propionate, and (C) butyrate in AX arm after *in vitro* fermentation; molar proportion of fecal (D) acetate, (E) propionate, (F) butyrate in AX arm; molar proportion of fecal (G) acetate, (H) propionate, and (I) butyrate in MCC arm. Molar proportion (%) was calculated by dividing the amount of a specific SCFA by the total amount of SCFA for the same subject and multiplied by 100. Friedman test was applied to detect the effect of treatment on the proportion of each type of SCFA in between the time points; * $p \leq 0.05$. AX, arabinoxylan; BL, baseline; MCC,

microcrystalline cellulose; SCFAs, short-chain fatty acids; W1, week 1; W6, week 6; n = 15 in AX arm, n = 16 in MCC arm.

4.4.3 Inter-individual response of the gut microbiota to AX supplementation

Our findings show inter-individual variation in the concentration of SCFAs production during six weeks of intervention. Based on the direction of change from week 1 to week 6 (i.e. positive or negative), subjects were grouped into positive shift group, which exhibited a higher output in SCFAs at week six (Δ W6-W1>0), and negative shift group, which exhibited a decrease in SCFAs at week six (ΔW6-W1<0) for both *in vitro* and in fecal sample in the AX arm (Figure 4.4).

Interestingly, 14 subjects out of 15 (93.33%) had similar response (either positive shift or negative shift) for propionate after *in vitro* fermentation of AX and in fecal sample, while 12 out of 15 subjects (80%) in butyrate and 9 out of 15 subjects (60%) in acetate showed similar response after *in vitro* fermentation of AX and in fecal sample.

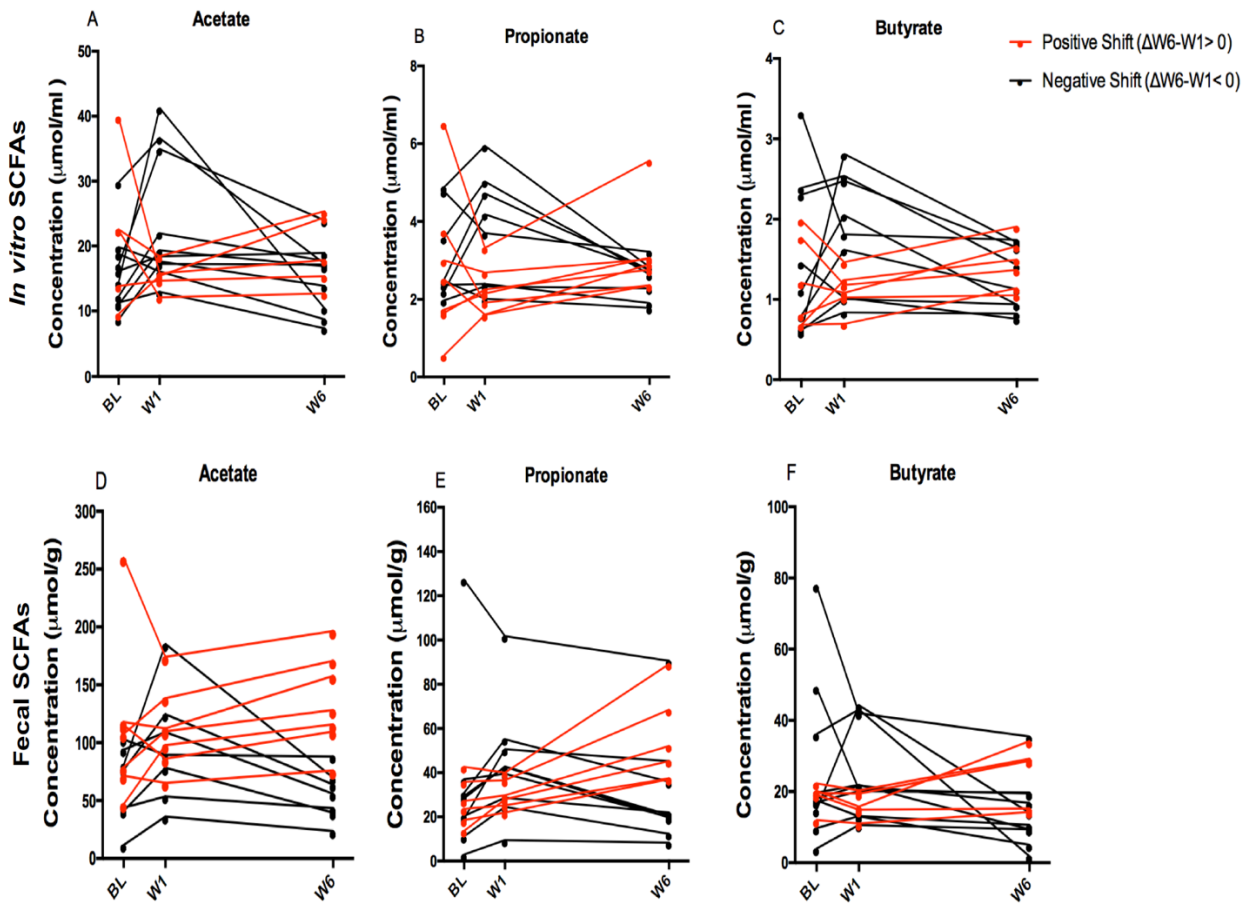

Figure 4.4. Individualized response of the gut microbiota to AX supplementation. Temporal SCFAs response of participants based on positive shift (ΔW6-W1>0, red) and negative shift (ΔW6-W1<0, black) for *in vitro* (A) acetate, (B) propionate, and (C) butyrate and for fecal (D) acetate, (E) propionate, and (F) butyrate in the AX arm. BL, baseline; SCFAs, short-chain fatty acids; (ΔW6-W1), absolute change from week 1 to week 6; W1, week 1; W6, week 6; n = 15.

4.4.4 Relationship between in vitro production of SCFAs and fecal SCFAs

We then determined the associations between *in vitro* production of SCFAs and fecal SCFAs in the AX arm. Significant positive correlation was obtained for the shifts in the concentration of butyrate from week one to week 6 (ΔW6-W1, p = 0.003) between *in vitro* and fecal sample, but

not for the shifts (ΔW6-W1) in the concentration of acetate (p = 0.06) and propionate (p = 0.11) in between the two approaches (Figure 4.5).

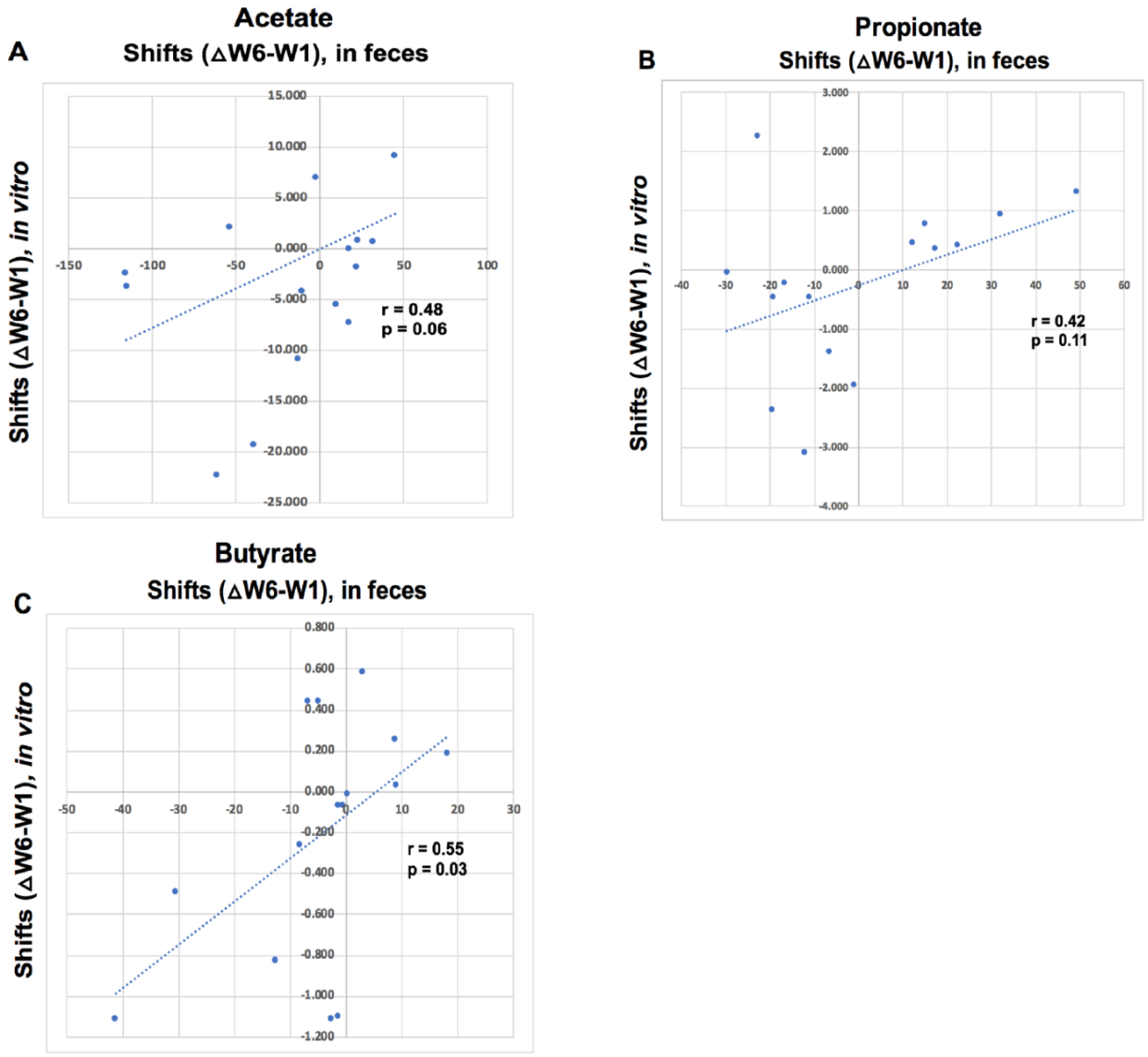

Figure 4.5. Correlations between the shifts in SCFAs concentration (ΔW6-W1) *in vitro* and in fecal sample in the AX arm. Spearman's correlation analysis was performed between the shifts (ΔW6-W1) in concentration for (A) acetate, (B) propionate, and (C) butyrate in fecal sample and *in vitro*. The Spearman

correlations (r) and p values are shown in the graphs. SCFAs, short-chain fatty acids; (ΔW6-W1), absolute change from week 1 to week 6; W1, week 1; W6, week 6; n = 15.

4.5 Discussion

In the present study, we characterized the impact of high daily dose of AX supplementation for one week and six weeks on the production of SCFAs in healthy overweight and obese individuals through determination of SCFAs in fecal samples and parallel *in vitro* fermentation assays. There was an increase in the absolute concentration of fecal propionate after week one of intervention, which was not maintained at six weeks. Considering that the absolute concentrations of fecal SCFAs are affected by multiple factors, including their almost complete absorption by gut epithelial cells, we additionally assessed the molar proportions between acetate, propionate, and butyrate relative to total SCFA concentrations, which has been previously shown to vary little across colonic regions (Cummings et al., 1987). Intriguingly, this analysis revealed that six weeks of AX supplementation significantly increased the molar proportion of fecal propionate.

However, findings from the *in vitro* study did not support our hypothesis. The ability of the microbiome to metabolize the consumed DF to SCFAs was not enhanced at six weeks of AX administration as compared to week one, but the responses varied widely between individuals. Individuals could be categorized into two groups, positive shift and negative shift group. This allowed us to reduce the variability and identify inter-individual variation in each group. From our results, we can conclude that each individual is different in terms of SCFA production. Therefore, we could not observe an overall significant response to the AX treatment. The variable response to the SCFA production in the individuals could be better explained by the microbiome data and

studies are underway to assess the effect of increased AX consumption on the gut microbiota composition of the participants, in this clinical trial.

We acknowledge the failure of the batch *in vitro* model to replicate with the SCFAs results obtained directly from the fecal samples. There were no significant correlations between the shifts in propionate concentration at week six from week 1(ΔW6-W1) after *in vitro* fermentation with AX and in fecal samples of the participants consuming AX. It is important to note that both the *in vitro* approach and measurement of SCFAs directly in the fecal sample have their own limitations. The absence of control of pH is one of the major limitations of the batch *IVFF* model. Rapid depletion of substrate during batch *IVFF* results in the accumulation of microbial metabolites such as SCFAs which reduces pH and in turn prevent further microbial activity resulting in distorted fermentation profiles (Payne et al., 2012). Previous studies have revealed a dramatic effect of a one-unit shift in pH upon microbial community composition and SCFA production ratios. Particularly, a mildly acidic pH (5.5) stimulated butyrate production and the population of butyrate producing species such as *Roseburia spp*. and *F. prausnitzii*. Propionate formation on the other hand was maximized at pH 6.5, correlating with greatly increased populations of Bacteroides-related bacteria (Walker et al., 2005). Therefore, it is of great importance to consider that although the system includes buffers, the batch *IVFF* model does not mimic the changes in pH that occur *in vivo*, nor does it include absorption of the metabolites as the computer-controlled continuous model does (Aura et al., 2006). The batch *IVFF* model mimics only the cecal conversion of dietary constituents and reveals the metabolite formation during fermentation. Nevertheless, the main strength of the *in vitro* fermentation model is that one can follow the in situ production of SCFAs upon treatment, whereas direct measurement of fecal SCFA concentrations are only a proxy for

colonic fermentation and mainly the result of absorption of SCFAs in the intestine, or lack thereof (Poeker et al., 2018). Therefore, a continuous *in vitro* model could be a better alternative to study the shifts in propionate production in future intervention studies.

4.6 Conclusion

Supplementation of high daily dose of AX for six weeks increased the molar proportion of fecal propionate. However, the *in vitro* results indicate that there was no evidence of the microbiome to adapt to the AX administration for six weeks. Thus, the batch *IVFF* approach might not be a good strategy to study the response of the gut microbiota to DF supplementation, especially in terms of propionate production.

Chapter 5. Conclusions and Implications of Research

5.1 Conclusions

We found no significant differences in the concentration of SCFAs and BCFAs produced and growth of *E. coli* between 2% and 5% fecal inoculum concentration during *IVFF* of RS4. However, significant differences in the relative amount of total SCFA, total BCFA and in the growth of *E. coli* was observed in Medium 3 as compared to Medium 1. The findings indicate that growth medium with the concentration of peptone and yeast extract reduced to 10% of their concentration in the medium commonly used in the literature resulted in an improved ability to detect SCFAs production from DF fermentation, reduced production of total BCFAs (which indicate fermentation of amino acids), and in an almost complete prevention in the growth of *E. coli*.

This optimized batch model has been successfully used to study the ability of the gut microbiota of patients suffering from liver cirrhosis to ferment DFs into SCFAs (Jin et al., 2019) which demonstrated that stool samples from cirrhotic patients had reduced capacity to produce SCFAs from *in vitro* fermentation AX, RS4, and PE, with butyrate being the most abnormal. This highlights the feasibility of the batch *IVFF* model for future studies. The batch model has also been used in a cross-sectional study (unpublished) which shows that *in vitro* production of propionate with AX and PE is significantly associated with high Prevotella to Bacteroides ratio. This finding is interesting as recent research have highlighted the importance of high Prevotella to Bacteroides ratio on weight loss on high DF diet (Hjorth et al., 2019).

However, the batch *IVFF* approach failed to show any shifts in SCFA production, in an intervention study that administered AX to human subject. This was contradictory to the shifts in SCFA especially in terms of propionate observed in the fecal sample. For this reason, the *in vitro*

SCFAs data was removed from the ongoing intervention study and only fecal SCFAs were considered for further analysis.

In conclusion, the batch model was successfully optimized which could determine the ability of fecal microbiota to ferment chemically distinct DFs to SCFAs in healthy individuals. In addition, it was able to assess the impact of cirrhotic dysbiosis on the production of SCFAs. As dysbiosis is often characterized by a reduced bacterial diversity and shifts in community structure and composition, the compositional abnormalities are associated with a reduced capacity to produce SCFAs from different substrates (Bajaj et al., 2014; Jin et al., 2019). The batch model therefore seems to be able to study more pronounced differences in fermentation, such as those between highly dysbiotic and healthy microbiota.

5.2 Implications of Research

The study provided optimization of a batch model for characterizing the metabolic function of the fecal microbiota in individuals. The second important implication of our study derives from the ability of the batch *IVFF* model to assess the individuality of the fecal microbial communities to ferment chemically distinct DFs into SCFAs. Such individualized nature of the microbiome can impact whether a specific type of DF can be metabolized (Ze et al., 2012) and on the concentration and profiles of SCFAs produced (Chen et al., 2017). Given the individualized nature of the gut microbiota, there is vast potential to personalize nutrition strategies based on the ability of the microbiota to ferment specific DFs. The batch *IVFF* model optimized in our study provides a platform for such future nutrition studies to assess the individualized host response to DF fermentation and personalize the use of DF based on individual microbiome function.

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