In Vitro Analysis of Short Chain Fatty Acids and Human Fecal Microbiota Stimulated by Pectin Sources

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In Vitro Analysis of Short Chain Fatty Acids and Human Fecal Microbiota Stimulated by Pectin Sources
In Vitro Analysis of Short Chain Fatty Acids and Human Fecal Microbiota Stimulated by Pectin Sources

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Food Science

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ABSTRACT

As interest increases for colonic health, various studies have been conducted to investigate functional ingredients for host health and prevention of chronic disease in humans such as colon cancer and Type 2 diabetes. In particular dietary fiber has been reported to have beneficial effects on colonic health. More recently, dietary fiber has been categorized based on its characteristics and numerous studies have been conducted to determine correlations with chronic diseases. Pectin is a naturally occurring biopolymer that has been used in various applications for purposes of pharmaceutical and biotechnology. Pectin is a soluble dietary fiber and can be entirely fermented by gut microbiota. There has been a growing interest in the health effects of pectin. In the food industries, pectin is used as a gelling and thickening agent. Pectins from different sources might have different characteristics such as the molecular size, DM (Degree of Methylation) and structural characteristics. For the current study, we hypothesize that pectins from different sources could have different fermentability properties and beneficially affect colonic health. The objectives of this research were to determine the effects of pectin sources on SCFAs and BCFAs production and to investigate the changes in the community of the fecal microbiota. We selected 3 different pectins (HMP: high methoxy pectin from citrus pectin, SBP: sugar beet pectin, SOY: soybean pectin), based on the fact that citrus pectin and sugar beet pectins in particular are widely used throughout the world. From our results, pectin samples stimulated production of total SCFAs. Also composition of human fecal microbiota was modulated by pectin samples. Therefore, pectin samples can influence fecal culture composition and might help maintain colorectal health.
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CHAPTER 1

General Introduction

To date, colonic health has been continuously brought up to maintain overall health and reduce the dangers of various diseases related with changes in nutrition and lifestyle. The human colon is closely related with bacterial fermentation activities. The bacteria in the human colon obtain energy for growth by fermenting carbohydrates in the colonic lumen, mainly polysaccharides of plant cell wall (dietary fiber, DF). Currently, there is not a widely accepted definition of DF, but the original chemical definition of fiber, termed “crude fiber”, was based on resistance to hydrolysis by acid and alkali (Theander and others 1994; Van Soest 1963). DF is classified into two different fibers: soluble and insoluble. DF has been received attention due to its various health related functions. Numerous studies have designed and conducted to establish fiber’s beneficial effects such as prevention of certain cancers (Dahm and others 2010; Hébert and others 2012; Limburg and others 2011; Park and others 2009), diabetes (Wannamethee and others 2009; Hopping and others 2010; Post and others 2012) and obesity (Kendall and others 2010; Lattimer and Haub 2010; Papathanasopoulos and Camilleri 2010). In particular, pectin, one of the soluble fibers, has been suggested to possibly having more beneficial properties for colonic health, because it can be entirely fermented in colon by gut microbiota. Isolated pectin from dietary fiber might exhibit potential beneficial effects on human colonic health, compared to crude dietary fiber. However, most of studies have been conducted only to examine relations between intake of dietary fiber and disease. Thus, the goal of current study is to compare in vitro fermentability among different pectin sources. The hypothesis in the current study is that pectin can stimulate total the fermentation products produced by the gut microbiota (Short Chain Fatty Acids, SCFAs) and influence composition of gut microbiota. The objectives are to
measure production of SCFAs and BCFAs (Branched Chain Fatty Acids) during *in vitro* fermentation (30 hrs) and to investigate changes of composition of gut microbiota during fermentation.
1. References


CHAPTER 2

Literature Review

1. Potential Uses of Pectin for Colonic Health

1.1. Fiber and Pectin

Dietary Fiber (DF) has a long history and its definition has been revised many times. Currently, definitions of DF typically include the fiber components; non-starch polysaccharides (NSP) and resistant oligosaccharides (RO), lignin, substances related with the NSP and lignin complex in plants, and other analogous carbohydrates, such as resistant starch (RS) and dextrin (Tungland and Meyer 2002).

Dietary fiber is categorized into two types: soluble and insoluble fiber. Pectin (pectic polysaccharides) is one of the represented soluble fibers (Anderson and others 1990). Much of the interest in the effects of pectin has centered on its selective bioavailability or solubility. Numerous studies have been conducted to seek out the possibility of pectin as a functional ingredient (Liu and others 2001; Haskell and others 1992; Cerda 1988).

In 1825, Henri Braconnot, who was an expert for the extraction of plants in France, discovered pectin. He gave a name for the heteropolysaccharide with pectic acid, which exhibited gelling properties (Willats and others 2006). The plant cell wall is a complex macromolecular structure that protects the cell, and elicits important characteristics to plants survival. The primary wall is characterized with less cellulose and more pectin compared to secondary walls. Also, the primary cell wall is thought to have roles for wall structural integrity, cell adhesion, and signal transduction (Caffall and Mohnen 2009).

Pectins are a family of complex polysaccharides, which are commonly found in plant primary walls. Plant cell walls are major contribution to both the form and properties of plant
structures (Leclere and others 2013). As the primary role of plant wall component, pectin contributes mechanical strength of plants in order to provide a barrier from the external environment and to retain an extracellular water phase.

1.2. Structure of Pectin

Pectin is known to include various fragments of linear and highly branched regions. The pectin is comprised of rich GalA-containing polysaccharides that are abundant in the plant cell wall (Sriamornsak 2003). Galacturonic acid is made up of approximately 70% of pectin, and all the pectic polysaccharides include galacturonic acid linked at the O-1 and the O-4 position. The structural categories of pectin include homogalacturonan (HG), xylogalacturonana (XGA), apiogalacturonana (AGA), rhamnogalacturonan I (RG-I), and rhamnogalacturonan 2 (RG-II) (Mohnen 2008).

Among pectic polysaccharides, the most abundant is homogalacturonan (HG), which is a linear chain of α-1,4-linked galaturonic acid that comprises approximately 65% of pectin. HG has been reported that it consists of approximately 70 to 100 GalA residues (Thibault and others 1993). While HG displays a simple structure, the other pectic polysaccharides: XGA, AGA, RG-I, RG-II are considerably more complex in structure compared to HG. It is generally reported that the pectin polysaccharides are covalently cross-linked, because in order to break down the pectic polysaccharides harsh chemical treatments or enzyme treatment are needed to isolate HG, RG-I, and RG-II.

The backbone of HG referred to as the smooth region is covalently linked to RG-I and RG-II, XGA, has also been reported to be covalently cross-linked to HG. The structure of RG-I possess a repeating backbone [-α-D-GalpA-1,2-α-L-Rhap-1,4-]n. As the sidechains of RG-I,
three types of galactan polysaccharides have been isolated from RG-I: galactan, and Type-I and Type- II arabinogalactan (AG). The RG-I sidechains: arabinans and galactans have shown considerable variation in structure from source to source (Ponder and Richards 1997).

The most structurally complex pectic polysaccharides, RG-II, includes up to approximately 10% pectin. RG-II is comprised of an HG backbone of at least 8 of the 1,4-linked α-D-GalA residues with side branches consisting of 12 different types of sugars and over 20 different linkages. Because of this complexity, structural analysis of RG-II of pectic polysaccharides has been regarded as difficult task (O'Neill and others 2004). The chemical structure of pectic polysaccharides isolated from different sources displays different chemical properties: solubility, gelling formation and potential effects on health: lipid lowering effects, inhibition of growth of cancer cell, boosting effects of immune cells (Caffall and Mohnen 2009).

1.3. Pharmaceutical Uses of Pectin

High intake of fruits and vegetables has been recommended for a long time, as there are growing concerns about unhealthy life and bad dietary behavior (Boeing and others 2012). One of dietary fibers, pectin has been interested as functional food ingredient linked to beneficial effects on health (Maxwell and others 2012). This property allows pectin to be used as an important ingredient in pharmaceutical products and food industries (Moore and others 1998). To date, numerous studies have demonstrated beneficial effects of pectin for human health by using for delivery drug or intake of pectin (Ahrabi and others 2000; Ashford and others 1994; Wakerly and others 1996) (Figure 1). For drug ingredient delivery, the most unique property of pectin is gelling ability. Pectin has been suggested to exhibit hydrogen bonding and hydrophilic interactions the primary forces in the aggregation of pectin structure (Sawayama and others...
1988). The possibility of controlling drug delivery with oral administration is very limited as it depends on the unavailability of digestion in the gastrointestinal tract (Sriamornsak 2011). Pectin has the problem of inconsistency in its reproducibility, degradability and mechanical properties between final product and these differences might result in poor reproducibility for delivery properties (Sriamornsak 2011). Therefore further studies are needed to determine the efficient ways to produce pectin-based delivery systems with high reproducibility.

1.4. Pectin Sources and Extraction

Pectin is extracted by adding mineral acid at approximately pH 2. The extract is subsequently concentrated in vacuum evaporation and the pectin precipitated by adding 70% of ethanol or isopropanol. The initial pectin treated with dilute acid leads to low-esterified pectin (Yeoh and others 2008).

The pectins from different sources do not correspond with the same gelling ability due to variations of structure and its constituents. In order to characterize pectic polysaccharides, the degree of esterification (DE) of the uronide carboxyl groups with methyl alcohol. Initially, pectin is formed in a highly esterified form referred to as high methoxy pectin (HMP), but after undergoing processing procedures, such as acid hydrolysis and enzymic degradation, pectin can exhibit a lower DE becoming low methoxy pectin (LMP) (Fishman 1988). In general, tissue pectins range from 60 to 90% DM (Rolin and De Vries 1990), and there can be a wide range of DE dependent on species, tissue, and maturity (Van Buren and Walter 1991). Based on this characteristic, pectin is used to make gels in aqueous media containing sugar and acid (Fu and Rao 2001). Historically, peel material originating from citrus products such as orange, lemon, and lime and apple pomace is the most popular pectin source for gelling and stabilizing abilities.
(Figuerola and others 2005). Apple pomace contains 10 to 15% of pectin and by-products of citrus contain about 20 to 30% (May 1990).

More recently, numerous studies have been conducted with different or modified pectin sources for health effects. Interesting alternative pectin sources include by-products from food industries such as sugar beet pulp (Williams and others 2005; Phatak and others 1988; Norsker and others 2000), soy hull (Crandall and McCain 2006; Monsoor and Proctor 2001; Lam and others 2007), tomato peels (Sharma and others 1997), and mango peels (Berardini and others 2005; Koubala and others 2008).

1.5. Pectin and Health Effects

Many of the recommendations for modification to the western human diet, characterized as high fat and low dietary fiber intakes, have been suggested from a variety of studies (Carter and others 2010; Crowe and others 2011; Jenkins and others 2011), focused on possible health benefits of increased fiber intake. The pectin content of fruits and vegetables that are mainly consumed in U.S. is presented in Table 2. It appears that the intake of fruits and vegetables represents a beneficial way in which to increase the intake of pectin naturally. While pectin for pharmaceutical uses are closely related to its gelling properties, the health effects of pectin resulted in selective solubility as one of the dietary fibers (Onumpai and others 2011; Parkar and others 2010).

Numerous studies have investigated pectin and its biological effect for human health. Pectin has been reported to possess anti-oxidant activity (Candrawinata and others 2012), anti-inflammatory effects (Popov and others 2012; Markov and others 2011), lowering cholesterol and serum levels (Brouns and others 2011), and anti-cancer activities (Bergman and others 2010;
Zong and others 2012; Leclere and others 2013). Still, mechanisms and efficacy of pectin are unclear, thus, further studies are needed to determine its availability as a functional food ingredient. Also, most studies have been conducted with crude pectin preparations containing different structural domains. Since pectin displays such a complex structure, to date it has been regarded as nearly impossible to determine specific health related activities with defined molecular structures. However, as identification techniques developed, isolated pectic polysaccharides have demonstrated beneficial effects from various studies. In one of these studies, Yamada’s group (2009) reported that the complex side chains of pectin from medicinal herbs have anti-cancer activities and can enhance the function of immune cells (Yamada and others 2009). These results imply that advances in isolation and separation techniques are required to allow pectic polysaccharides have more positive effects on human health.

2. Changes of Metabolites and its Effects

2.1. Fermentation and its Metabolites

The large intestinal microbiota in humans is highly complex and consists of hundreds of bacterial species (or phylotypes) (Scott and others 2012). The composition of the gut microbiota is strongly influenced by various factors such as host genetics (Zhang and others 2009), immunological factors (Cerf-Bensussan and Gaboriau-Routhiau 2010), antibiotic usages (Clemente and others 2012), gastrointestinal disease (Sekirov and others 2010), and also dietary effects (Scott and others 2012).

Dietary intervention might have a significant impact on the gut microbiota both directly and indirectly on its environment, accompanied with gut transit time and pH (Scott and others 2012). Three main macronutrients: carbohydrates, proteins and fats have been reported to
influence the composition of the microbiota (Hang and others 2012). In the gut environment, fermentation of these three macronutrients can produce several metabolites, e.g. SCFAs, gases. Carbohydrates that can be degraded by microbiota in the colon include resistant starch, non-starch polysaccharides (NSP) and oligosaccharides (Laparra and Sanz 2010). Since the host does have different microbiota that are capable of degradation of carbohydrates, even the same dietary intervention with different hosts might have different alterations to the composition of microbiota (Nicholson and others 2012).

Fermentation of carbohydrates (saccharolytic by bacteria) results in the production of SCFAs comprised of acetate, propionate, and butyrate normally with ratio of 3:1:1 (Macfarlane and Gibson 1997). SCFAs have been reported to act in unique roles in colonic health (will be discussed later in this chapter). During fermentation with carbohydrates, the release and transformation of phytochemicals that have potent anti-inflammatory and antioxidant properties are occurring (Hugenholtz and others 2013).

Contrary to fermentation with carbohydrate, fermentation with dietary fats and proteins in the colon can result in the production of unhealthy metabolites such as nitrosamine and branched chain fatty acids (BCFAs) (Mu and Høy 2004; Windey and others 2012). The main pathway of amino acid fermentation in the human colon is deamination, contributing to the production of SCFAs and ammonia (Smith and Macfarlane 1997). Approximately 30% of protein substrate is converted to the major SCFAs and the BCFAs: *iso*-butyrate, 2-methylbutyrate and *iso*-valerate (Windey and others 2012). These BCFAs are mainly formed from the branched-chain amino acids: valine, isoleucine and leucine (Macfarlane and Macfarlane 2003). Also, BCFAs are commonly used as fecal markers for protein fermentation (will be discussed later in this chapter) (Valerio and others 2011).
Dietary fat is primarily absorbed in the small intestine. A few studies have been conducted on the effect of dietary fat on gut microbiota (Mu and Høy 2004). Brinkworth and others (2009) reported that a low fat diet, compared to a high fat diet significantly lowered concentrations of SCFAs in fecal matter, as well as the number of strains of bifidobacteria (Brinkworth and others 2009). However, from this study, low fat diet was formulated by higher content of carbohydrates in order to make balance with energy expenditure of a high fat diet. Increased concentration of SCFAs with a low fat diet might not only be associated with low fat content, since carbohydrates are also a major energy source for gut microbiota to metabolize SCFAs during fermentation.

Carbohydrates can also be used for fermentation by gut microbiota, indicating a connection between composition of microbiota and production of SCFAs (Cani and Delzenne 2009). It is hard to conclude that an increased fat diet will be crucial for causing changes of gut microbiota and production of SCFAs in human colon. However, from animal model studies, evidence has been generated that microbiota of mice fed a HF diet have a different gut microbial compositions (Daniel and others 2013; de La Serre and others 2010; An and others 2011). Consumption of HF diet resulted in a significant decrease in numbers of Roseburia species, which were restored by polysaccharide supplementation (Neyrinck and others 2011). These results indicated that fermentation with dietary fat might have deleterious impacts on colonic health. This review focuses on the impact of dietary carbohydrates on metabolites by gut microbiota and explores some of the possible consequences for human health.

3. SCFAs and Colonic Health

3.1. Production and Absorption of SCFAs
Dietary fiber and other dietary components target the colon and affect its environment. Fermentation involves various reactions and metabolic processes, producing energy for microbial growth and maintenance or other metabolites for the host (Tremaroli and Bäckhed 2012). The chief end products of colonic fermentation are SCFAs, some gases (CO$_2$, CH$_4$, and H$_2$), and heat (Macfarlane 1995). SCFAs are organic fatty acids with 1 to 6 carbon atoms and the principal metabolites that result from bacterial fermentation with polysaccharide, oligosaccharides, and protein sources in the colon (Rombeau and others 1990) (Figure 2). SCFAs: acetic acid, propionic acid, and butyrate are the key end products of colonic fermentation and these SCFAs have been associated with certain health effects such as reduced risk of cancer (Scheppach and others 1995) and some diseases, including irritable bowel syndrome (Mortensen and others 1987), inflammatory bowel disease (Tedelind and others 2007), and cardiovascular disease (Theuwissen and Mensink 2008; Anderson and others 1999).

The productions of SCFAs are affected by a number of conditions, including the substrate source (Titgemeyer and others 1991), gut transit time (El Oufir and others 1996), health status of host (Schwiertz and others 2010), and the type and number of microbiota in the colon (Pharmaceutiques 1995). The production of SCFAs can be changed by intervention of dietary sources. Non-digestible dietary source: dietary fibers are known as the substrates for fermentation by gut microbiota in anaerobic conditions, as they can be degraded or hydrolyzed in upper gastrointestinal tract. From previous studies, polysaccharides play the pivotal role in the production of SCFAs in colon (Manning and Gibson 2004).

Absorption of SCFAs occurs in an efficient manner because SCFAs are readily absorbed in the colon and only 5 to10% of SCFAs being excreted in the feces (Ruppin and others 1980). There are two possible hypotheses related to this absorption; a) diffusion of protonated SCFAs
and 2) anion exchange. Concentrations of SCFAs are higher in the proximal colon than in the distal colon (Cook and Sellin 1998). SCFAs uptake is associated with the transport of water and it seems to be higher in the distal colon than in proximal sites (Macfarlane and Macfarlane 2003).

3.2. Functions of SCFAs

The concept of SCFAs being a luminal nutrient source for the colonic mucosal cells was reported by Rodediger (Roediger 1980). SCFAs can modulate colonic and intracellular pH, cell volume, and other functions associated with cell proliferation (Roediger 1980) and differentiation (Blottiere and others 2003), as well as gene expression (Drozdowski and others 2002). Decreased pH with metabolized SCFAs indirectly influences the composition of the colonic microbiota, and also an increase in SCFA concentration can decrease the solubility of bile acids (Wong and others 2006). Other functions of SCFAs are related to the energy source for the host and gut microbiota (Hijova and Chmelarova 2007).

Numerous studies have been conducted to determine the health promoting functions of colonic fermentation with SCFAs production. Acetic acid is the principal SCFA in the colon. Since it readily absorbed and transported through the liver, lesser amounts are found in the colon (Cummings and Branch 1986). Acetate is known as the primary substrate for cholesterol synthesis (Hara and others 1999). Also, the presence of acetyl-CoA synthetase in the cytosol of adipose and mammary glands enables the use of acetic acid for lipogenesis once it enters the systemic circulation (Hijova and Chmelarova 2007). For clinical uses in humans, acetic acid has been used as an indirect marker to monitor colonic events, because it is the dominant SCFA. However, compared to other SCFAs, there have been no studies to show that acetic acid has
beneficial effects on human health. In the host, it might be absorbed and utilized by peripheral tissue. Duncan and others (2002) reported butyrate-producing strains isolated from human feces might utilize acetate to produce butyrate during in vitro fermentation (Duncan and others 2002a).

Propionic acid occurs naturally in dairy products such as yogurt and cheese, but only small amounts with milk (Fernandez-Garcia and McGregor 1994; Langsrud and Reinbold 1974). This is caused by bacterial fermentation. Since it has anti-fungal (Lind and others 2005) and anti-bacterial effects (Schnürer and Magnusson 2005), propionic acid has been used as a preservative in food industries (Ricke 2003). The propionic acid concentration in the human colon was reported to be 20 mmol/kg and to rely on the balance between production and absorption (Cummings and others 1987). Moreover, approximately 90% of propionic acid is metabolized by the liver and the rest is transported into the peripheral blood (Al-Lahham and others 2010a). High quantities of propionic acid can be found in specific situations, such as the propionic academia which is an autosomal recessive disorder of amino acid and odd-chain fatty acid metabolism (Childs and others 1961) and in gingival inflammation (Al-Lahham and others 2010a).

Dietary fiber intake is the primary substrate for propionic acid production and it has been connected with a reduction in low-grade inflammation (Al-Lahham and others 2010a). Wajner and others (1999) reported that the proliferation of human and animal lymphocytes is inhibited by propionic acid treatment, also another study conducted by Curi and others (1993) came to the same conclusion.

The evidence has been established that propionic acid could be a potential therapeutic agent for insulin sensitivity (Shinkai and others 1999) (Figure 3). Also, propionic acid increases GLUT-4 in primary human adipose explant in a dose-dependent manner (Hong and others 2005).
There are only limited studies that have been conducted for obesity-related studies with propionic acid. However, positive evidence exists that there is a systematic relevance between propionic acid and insulin sensitivity in the adipose compartment (Al-Lahham and others 2010a).

While only two studies have investigated absorbed propionic acid results on satiety and reduced food intake in humans (Ruijorschop and others 2008; Liljeberg and others 1995), some animal studies have revealed interesting perspectives about relations with satiety (Baile 1971; Anil and Forbes 1980; Bradford and Allen 2007). Anil and Forbes (1980) reported that receptors of liver show sensitivity to propionic acid (Anil and Forbes 1980). Also, previous studies showed that propionic acid increases production of the satiety hormone: leptin by mouse and ruminant adipose tissue (Al-Lahham and others 2010b; Xiong and others 2004).

Acetic acid and propionic acid are thought to be important in intestinal physiology; however, those SCFAs are less likely to have significant effects on human health (Sossai 2012). On the other hand, numerous studies related to SCFAs production have focused on butyrate production, because butyrate has been reported to play a major role in regulation of cell proliferation and differentiations (Canani and others 2011).

In human studies, the effects of butyrate can be divided into intestinal and extraintestinal (Figure 4). The intestinal ecosystem consists of epithelium, immune system cells, enteric neurons, microbiota, and prebiotics. Butyric acid can be the beneficial substance for gastroenterological therapies such as inflammatory bowel disease (Chauhan and others 2013) and colorectal cancer (Pouillart 1998) as well as metabolic disease such as obesity (Hotamisligil 2008).

Also, there is increasing evidence that butyrate plays a key role in colonic health from animals (Catuogno and others 2011; Brännning and Nyman 2011; Catuogno and others 2013) and
human model studies (van Zanten and others 2012; Van Immerseel and others 2010; Kokke and others 2011).

Butyrate improves the absorptive and anti-secretive capabilities of the intestinal mucosa (Sossai 2012). From various studies, it has been regarded as the one of the most important SCFA in colonocyte metabolism (Astbury and Corfe 2012). In particular, butyrate is metabolized by the colonocyte approximately in the range of 70 to 90% (Fung and others 2012). Apart from its well-known roles as preventing and apoptosis of neoplastic colonocytes (Wollowski and others 2001), butyrate has been reported to alleviate inflammation in colitis (Butzner and others 1996). Also, anti-inflammatory effects have been documented in normal colonocytes (Scheppach and others 1992).

More recently, butyrate enema has been administered to ulcerative colitis (UC) patients with favorable results from studies (Breuer and others 1997). This beneficial effect for UC patients were shown not only when administered as an enema, but also by high fermentable fiber or butyrate-producing bacteria supplementation (Videla and others 2001; Kanauchi and others 1999). Machlels and others (2013) reported that UC patients have a reduction in Roseburia hominis and Faecalibacterium prausnitzii, both of which are butyrate-producing bacteria of the Firmicutes phylum.

The main pathway of protein fermentation in the human colon is deamination, leading to the production of mostly BCFAs, SCFA, ammonia, iso-butyrate, 2-methylbutyrate, iso-valerate, hydrogen sulfide, poly-amines, indolic, phenolic compounds and N-nitroso compounds (Jacobs and others 2009). Branched chain fatty acids (BCFAs) are mostly saturated fatty acid (SFA) with one or more methyl branches on the carbon (Long and Short 1963). In contrast to SCFAs, the absorption and metabolism of BCFAs have only been minimally investigated (Cummings and
Although only a few studies have been conducted to determine the role of BCFAs in colonic health, there is evidence that they may interfere with ion movements through the colonic epithelium and be related to diarrhea regulation (Blachier and others 2007). The potential effects and mechanisms of BCFAs in colon have thus far been insufficiently explored.

Some studies have been conducted to investigate its negative effect on colonic health, using animal and human models. From a previous animal study, Propst and others (2003) reported that total BCFAs concentration was lower in dogs fed FOS (Propst and others 2003). In addition, microbiota from inflammatory bowel disease patients produced more BCFAs (25% greater) than the microbiota from healthy subjects and the concentration of total BCFAs (iso-butyrate and iso-valate) was increased after 16 hrs of fermentation to approximately 5 mmol, which was the average value of all subjects (Van Nuenen and others 2004). However, they also found that production of SCFAs by gut microbiota of IBD (Inflammatory Bowel Disease) patients is higher than healthy patients. Since only one study has been conducted on the production of BCFAs and SCFAs (n=14), it is hard to conclude that production of BCFAs and SCFAs are related to IBD. In contrast to butyrate, BCFAs do not appear to have measurable stimulatory effects on the proliferation of colonocytes in vitro, because of their cytotoxicity and limited intracellular availability (Siavoshian and others 1997; Waldecker and others 2008).

3.3. Interrelationships SCFAs and Gut Microbiota

The human large intestine can be described as complex microbial ecosystem. It is thought that at least 50 genera of bacteria reside in the colon (Rolfe 1984; Moore and Holdeman 1974; Finegold and others 1974). The bacteria present have fluctuating activities in response to substrate availability, redox potential, pH, O₂ tension and distribution in the colon (Cummings
and others 2001). The mechanism(s) associated with the contributions of individual anaerobic species to colonic fermentation and to the nutrition and health of the host remain unclear.

From a colonic health perspective, the gut microbiota play an important role in enhanced immunity through secretory and excretory substances and the competitive inhibition of pathogens (Breuer and others 1997). Dietary intervention can have an influence on the composition of the microbiota in colon. In order to beneficially influence gut health, the importance of selecting the right dietary source has been brought up from a previous study (Costabile and others 2008).

During bacterial fermentation, DF can be fermented to produce SCFAs: acetate, propionate, and butyrate. SCFAs are fermented by gut microbiota and are important on colonic health. In order to determine correlations between SCFAs and gut microbiota, various studies have been conducted (Macfarlane and Macfarlane 2011; Kleessen and others 2001; Cheng and Lai 2000; Beards and others 2010). The human gut flora is dominated by Bacteroides and Firmicutes (Barile and Rastall 2013).

It has been reported that Bacteroides are propionate producers through the succinate pathway (Louis and Flint 2009). These results were confirmed recently with another study (Yang and others 2013). They reported that the proportion of Bacteroidetes exhibited a positive correlation with propionate/SCFA ratio. Moreover, similar correlations were observed for Bacteroidia (class), Bacteroidales (order), Bacteroidaceae (family), Bacteroides (genus), Bacteroides uniformis and Parabacteroides distasonis (species) (Louis and Flint 2009).

One of the previous studies reported that microbiota can produce butyrate by Eubacterium hallii and Anaerostipes caccae (Duncan and others 2002b). Butyrate has received interest because of its preventive effect of colon cancer and various studies were conducted to
determine interrelationships between production of butyrate and changes of gut microbiota (Morrison and others 2006; Pryde and others 2002; Duncan and others 2002b). The most dominant microbiota that produce butyrate are Ruminococcaceae, Faecalibacterium, Eubacterium, and Clostridium that displayed positive correlations with butyrate/SCFA ratio using in vivo studies with rat models (Louis and Flint 2009; Benus and others 2010). However, the relationship of these bacteria and butyrate production are currently being characterized utilizing 16S rRNA sequencing (Vital and others 2013; Louis and others 2010).

Fermentable DF changes the gut microbial populations by providing substrates for bacterial fermentation. In particular, fructooligosaccharides and galactooligosaccharides increase the fecal population of Bifidobacteria and Lactobacillus in infants (Haarman and Knol 2006; Ramirez-Farias and others 2009), while inulin increases the Bifidobacteria population in adults (Tahri and others 1996). The fermentation patterns and the ratio of SCFAs produced relies on the DF type (Raninen and others 2011). These results indicated that relative production rates of these SCFAs provide an important connection between gut microbiota and colonic health.

At present, numerous studies have examined in vitro fermentation responses from gut microbiota. However, it is hard to identify every microorganism within that microbiota because of the considerable complexity of the overall microbial community. Also, each human subject potentially has a different composition of human fecal bacteria (Duncan and others 2008). In order to make it clear, efficient ways to identify the changes of microbiota during fermentation of samples should be elucidated. The objectives of current study are to measure metabolites: SCFAs and BCFAs and also to explore alterations in the composition of gut microbiota during in vitro fermentation (30 hrs).
4. References


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Figure 1. Pharmaceutical application of pectin, adopted from Sriamornsak study (2011).
Figure 2. The molecular structure of the SCFAs

Butyric acid

Propionic acid

Acetic acid
**Figure 3.** Physiological roles and potential applications of propionic acid, adopted from Al-Lahman’s study (2010)
Figure 4. The effects of butyric acid in human, adopted from (Sossai 2012)
CHAPTER 3

*In Vitro* Analysis of Short Chain Fatty Acids and Human Fecal Microbiota Stimulated by Pectin Sources

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1. Abstract

High Methoxy Pectin (HMP), Sugar Beet Pectin (SBP), Soy Pectin (SOY), and Fructooligosaccharide (FOS, as a positive control) were used to determine fermentation properties considering applicability as functional foods, particularly related to colon health. Certain beneficial effects of carbohydrates in humans can be postulated as being due to changes to microorganisms and metabolites: short-chain fatty acids (SCFAs) and branched chain fatty acids (BCFAs) produced during anaerobic fermentation in the colon. The average degree of polymerization (DP) of HMP, SBP, and SOY was 492, 3729, and 1510, respectively. Degree of methylation of each pectin sample was 76.0% (HMP), 21.2% (SBP), and 22.8% (SOY). Total SCFAs in SOY showed the highest value compared to other samples, in particular the highest concentration occurring with propionic acid (p<0.05). While fermentation with FOS showed higher butyrate production, the total SCFAs with SOY, HMP, and SBP were significantly higher than FOS over 30 h (p < 0.05). From the denaturing gradient gel electrophoresis (DGGE) analysis, changes of microbiota related to human colon health were found. In conclusion, pectin samples stimulated production of total SCFAs and the composition of human fecal microbiota was modulated. Therefore, pectin samples can alter fecal culture composition and may help maintain colorectal health.

2. Introduction

Colonic health has been continuously reported to maintain overall health and reduce the dangers of various diseases related to changes in nutrition and lifestyle (Slattery and others 1999). The human colon is closely involved with bacterial fermentation activities (Cummings
and Macfarlane 1991). The bacteria in the human colon obtain energy for growth by fermenting carbohydrates in the colonic lumen, especially polysaccharides of plant cell walls (dietary fiber) and some starch (Cani and Delzenne 2009). As concerns related to colonic health have become greater, functional ingredients coming from natural sources have received attention. For example, dietary fiber, pectin substances, prebiotics, probiotics, and other dietary components that are capable of influencing the colon and its environment are widely used for various purposes and increase SCFAs production (Hotchkiss and others 2003).

One of the efficient biomarkers for colonic health, SCFAs have been used as efficient indicators due to their characteristics: sensitivity and ease of analysis. Furthermore, SCFAs have been known to have an important role related to colon health (Scheppach and others 1987). They are subsequently transferred to the bloodstream after absorption by the intestinal mucosa (Mortensen and Clausen 1996). SCFAs are metabolites of the anaerobic bacterial fermentation of unabsorbed carbohydrates in the human intestine (Cani and Delzenne 2009). A number of general actions excreted by SCFAs promote large bowel function (Topping and Clifton 2001; Bornet and others 2002; Topping 2007). Furthermore, previous studies reported that SCFAs produced by anaerobic bacteria beneficially associate with inflammatory disease (Kurita-Ochiai and others 1995; Vinolo and others 2009). In order to determine SCFAs produced by microbiota, in vitro fermentation analyses have been used to evaluate the fermentability of substrates within the gastrointestinal tracts of human (Blümmel and others 1997).
Dietary fiber is divided into two different fibers: insoluble and soluble fibers. Insoluble fibers (e.g., lignins, cellulose and some hemicellulose), which are limited to fermentation by colonic microbiota, play a pivotal role in fecal bulking and may carry with them fermentable carbohydrate substrates, including starches and sugars (Jenkins and others 1998). Soluble fibers (e.g., pectins, gums, mucilages, and some hemicelluloses) are fermented mainly by colonic microbiota (Cummings 1981). Pectin plays an important role in food processing as a food additive and as a source of DF. It is also used as ingredients in pharmaceutical preventions such as coronary heart disease, colon cancer, hypercholesterolemia (Reiser 1987; Cerda 1988; Silalahi 2002; Salman and others 2008; Nangia-Makker and others 2002). Pectins from different sources exhibit different characteristics due to their unique structure. Pectin is defined as a heteropolysaccharide containing mostly galacturonic acid (GalA) residues. While there is a certain amount of neutral sugar present as a side chain, the homogalacturonan region of pectin consists of galacturonic acid (GalA) joined in chains by α-(1→4) glycosidic linkages (Voragen and others 2009). The GalA moieties are partly esterified by methanol. The degree of methylation (DM) is defined as the number of moles of methanol per 100 mol GalA. Physiological (fermentability by microbiota) and functional properties (gelation or binding of metal ions) of pectin are different, relying on its structural parameters such as molecular weight, DE, and distribution of free and methoxylated carboxyl groups within the galacturonan chains (Dongowski and others 2002). Prebiotics are a selectively fermented ingredient that allows specific changes in composition and activity of the gastrointestinal microbiota to confer benefits to the host’s well being and health (Gibson and others 2004). It is clear that fructooligosaccharide (FOS) and oligosaccharides stimulate *Bifidobacterium* and *Lactobacillus* which are known as beneficial microbiota and are common targets for dietary intervention (Kajiwara and others 2002; Manning and Gibson 2004).
Soluble DF has been investigated for its fermentability in previous studies and well established as a functional food source. Since pectin can be entirely fermented by gut microbiota, thus it can be expected that pectin might be a better source for fermentation by gut microbiota. However, few studies have been conducted with purified pectin to investigate its health beneficial effects. Hence, the objective of this research was to determine the effects of pectin sources on changes SCFA and BCFA production, also changes in the community profiles of the fecal microbiota were tracked using polymerase chain reaction coupled denaturing gradient gel electrophoresis (PCR-DGGE) with 16S rRNA gene-targeted universal primer sets.

3. Materials and Methods

3.1. Chemicals and Media

High purity SCFAs and BCFAs were used to make standard solutions. All chemicals: acetic acid (99.9%), propionic acid (99.6%), butyric acid (99.8%), iso-valeric acid (99%), n-valeric acid (99%), 4-methyl valeric acid (internal standard), acetone (> 99.5%, GC grade), metaphosphoric acid, alchol oxidase (from *Pichia pastoris*), Tris (Trizma grade), D- (+) galacturonic acid, sodium chloride, sulfamic acid, formaldehyde, 3-methyl-2-benzothiazolinone hydrazine (MBTH), ammonium iron sulfate dodecahydrate, Sulfuric acid, 3,5 dimethylphenol (DMP) and copper sulfate were purchased from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO). To construct a standard curve, a standard solution containing the three SCFAs and two BCFAs were prepared by mixing 100 μL of 4-methyl valeric acid (50 mM) of the internal standard and diluting with acetone.

Fecal samples were cultured in brain heart infusion (BHI broth). In order to make BHI media, BHI broth (7.4 g) and rezasurin (0.2 mg) were mixed with purified water (200 mL) and
heated until right before boiling, and then cooled down with ice. After mixing with sodium bicarbonate (0.8 g) and cysteine sulfide (50 mg), media were sterilized and stored at room temperature (Zheng and others 2003). During the making of BHI media, nitrogen gas was flushed into tubes continuously while making BHI media. All water used was purified using a Milli-Q reagent water system (Millipore, Molsheim, France).

3.2. Characterization of Pectin

Galacturonic acid was released after acid hydrolysis of pectin samples. Total galacturonic was determined using a modified DMP method (Scott 1979) with a maximum absorbance of 450 nm. A modified AO/MBTH method (Anthon and Barrett 2004) was used to determine the degree of methylation of pectin samples. Each pectin sample (1 mg) was dissolved into a mixture that contained 700 μL of 20 mM Tris-HCl buffer (pH 7.5), 100μL of MBTH (dissolved in water at 3 mg/mL) and 100 μL of 0.5M NaCl solution. The mixture reacted with alcohol oxidase (1 unit) at 30°C for 30 min for full digestion. A 200 μL aliquot of acidic iron solution was added after digestion. After 30 min, absorbance was determined using a spectrophotometer (DU 520, Beckman Coulter, Brea, Calif., U.S.A.) at 620 nm. The molecular weight distribution of the pectins was determined by gel-permeation chromatography on a series Shodex OHpak columns (KB-802, and KB-804) with HPLC system (Waters, Miliford, MA) that consisted of a 515 HPLC pump and 2410 refractive index detector.

3.3. Stool Samples from Participants
The study was reviewed and approved by the Institutional Review Board (IRB) at the University of Arkansas (Fayetteville, AR). Prospective volunteers were selected by using a dietary and health questionnaire. Informed consent forms were obtained from each participant before the sample collection.

Fecal samples were collected from 4 male participants (23 to 28 years of age) who had been on a routine diet and not taken any antibiotics or medicine for 6 months prior to the study. All nutrition data of every subject was processed with Nutritionist Pro (Version 4.4.0, Axxya system). Each fresh fecal sample was collected in in Commode Specimen Collection System (Fisher Scientific, Waltham, MA) and inoculated into media under anaerobic conditions.

An in vitro incubation was conducted to evaluate the fermentability of four substrates by human fecal bacteria. The examined substrates included FOS (Fructooligosaccharides, Herbstreith & Fox), HMP (High Methoxy Pectin, TIC), SBP (Sugar Beet Pectin, Herbstreith & Fox), and SOY (Soy Pectin). Soy pectin was prepared according to Crandall and McCain (2000). The physical form of these samples was fine powder. The incubation was prepared from fecal material by adding 22 mL of BHI media, 2 g of feces, and 0.5 g of each sample. Blank tubes were used for each sample at each time point. The mixture was homogenized by vortexing right before incubation (37 °C). Four milliliters were taken using a sterile syringe from the tube immediately for time 0 and stored at -20 °C. Subsequent aliquots were obtained at 6, 12, 24, and 30 h and stored at -20 °C until analysis.

3.4. SCFAs Analysis

For SCFA analysis, each aliquot was defrosted in room temperature and centrifuged at 3500 x g for 15 min. After centrifugation, 0.5 mL supernatant was pipetted into the tube and
mixed with 100 μL of a mixture containing 50 mM of 4-methyl-valeric acid, 5% metaphosphoric acid and copper sulfate (1.56 mg/mL). Each tube was centrifuged at 11,000 x g for 10 min after reacting with the mixture for 10 min and stored at -40 °C until analysis. The sample injection (1 μL) was performed in the split mode (30:1). Hydrogen was used as the carrier gas. The concentration of produced fatty acids was calculated by subtracting SCFA produced in blank tube (without substrate). The oven temperature was increased by 4 °C /min from 100 °C (2 min) to 120 °C (1 min), then at 3 °C /min until 150 °C was reached. The data was recorded and processed using the integrated Shimadzu database.

3.5. DNA Extraction from Fecal Samples

Bacterial DNA was extracted using QIAamp DNA stool kit (QIAGEN, Valencia, CA, Cat. No.51504) with some modification. An aliquot (250 mg) of fecal sample was lysed by vortexing using Garnet beads (MO BIO, Carlsbad, CA). Fecal tubes were heated for 5 min at 70 °C and mixed for 15s using vortex. The lysed sample was centrifuged at 3,000 x g for 1 min and transferred to bead beating tube (MO BIO). The sample was further lysed by vortexing for 10 min and incubated at 95 °C for 6 min. After incubation, the sample was centrifuged at 16,100 x g for 1 min. Eluted DNA was further processed for PCR procedures.

3.6. DNA Amplification

All DNA amplification and DGGE procedures were performed as previously described with modification (Hanning and Ricke 2011). A 233 bp portion of the 16s rRNA gene was amplified by PCR using primers of DGGE-F (5- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCCTAC GGG AGG CAG CAG -3) and DGGE- R (5- ATT ACC GCG GCT GCT GG -3) (Integrated DNA Technologies, Coralville, IA) (Muyzer and others
A 50 ng of template DNA, 0.4 µM of primers and PCR grade water were mixed with Jump Start Ready Mix (Sigma Chemical Co., St Louis, MO) to total of 25 µL for each reaction. The amplification was performed by touchdown PCR program: initial denaturation at 95 °C for 2 min, then 17 cycles of 1) denaturation at 94 °C for 1 min; 2) annealing at 67 °C for 45 s decreasing by -0.5 °C per cycle to a touchdown temperature of 59 °C and 3) annealing at 72 °C for 2 min. The reaction was followed with 12 cycles of 4) denaturation at 94 °C for 1 min; 5) annealing at 58°C for 45 s with a final elongation step at 72 °C for 7 min. The amplification was confirmed by 0.8% agarose gel electrophoresis with ultraviolet transillumination using the Quantity One software (Bio-Rad Laboratories, Richmond, CA).

3.7. Gel Electrophoresis

The 8% polyacrylamide gels (acrylamide:bisacrylamide = 37:1) consisted of a 35 to 60% of urea-deionized formamide gradient; the 100% denaturing acrylamide was composed of 7 M urea and 40% deionized formamide. The equal concentration of PCR product (2 µg) was mixed with loading buffer [0.05% bromophenol blue (w/v); 0.05% xylene cyanol (w/v); and 70% glycerol (v/v)] and the mixed samples were loaded in each well. Electrophoresis was carried out in a D-Code Universal Mutation Detection System (Bio-Rad Laboratories) in TAE buffer (1 mM EDTA, 20 mM acetic acid, 40 mM Tris) at 59°C for 17 h at 60V. The gels were stained with SYBR Green (1:50,000 dilution, Cambrex Bioscience, Walkersville, MD) in TAE for 40 min and the result was observed with ultraviolet trans illumination using Quantity One software (Bio-Rad Laboratories) for image acquisition.

3.8. DNA Extraction and Sequence Analysis
Bands of interest were excised from the DGGE gel for sequencing analysis. Excised gel fragments were disrupted, transferred in 300 µL of TE buffer (10 mM Tris–HCl; 1 mM EDTA, pH 8.0) and incubated at 65°C for 15 min to elute the DNA into the buffer. The contents were transferred to a Spin-X® centrifuge tube (Corning, Corning, NY) and centrifuged at 16,100 g for 5 min to purify DNA from acrylamide gel. The filtrate was mixed with 133 µl of 7.5M NH4OAc, 900 µL of EtOH and 3 µL of glycogen (20 mg ml-1), vortexed and incubated at -80°C for 1 h. The samples were then centrifuged at 16,100 g for 15 min and the pellet was washed twice in cold 70% EtOH. The extracted DNA was sequenced in the DNA Resource Center at the University of Arkansas (Fayetteville, AR) using an ABI 3100 capillary analyzing system (Applied Biosystems, Foster City, CA) and the sequences were compared with the GenBank database using the Blast algorithm.

For showing phylogenetic analysis, UPGMA’s method was used for the analysis using Bio-rad quantity One (software ver. 4.6.7.).

3.9. Statistical Analysis

Statistical analysis was carried out by statistical program: SAS 9.3 (SAS Inst., Cary, N.C., U.S.A.), using either (a) one-way ANOVA when comparing three or more data sets or (b) a t test when comparing two data sets. All data are presented as mean ± S.E.M (Standard Error of Mean). Differences were deemed significant when p < 0.05.

4. Result and Discussion

4.1. Subjects
Prior to experimentation, every subject answered the questionnaire in order to check their disease status and their eating behavior. The questionnaire summary of subjects (n=4) is shown in Table 1. The BMI (Body Mass Index) in Kg $m^{-2}$ of subject 4 was within the normal range (18.5 - 24.9), in contrast, subject 1 through 3 were overweight (25.0 to 29.9). The average daily calories were also determined. All subjects consumed less than 2000 calories/day. The U.S. Department of Agriculture recommends men consume 2,000 to 2,600 calories if they are sedentary, 2,200 to 2,800 calories if they’re moderately active. All of subjects showed they had consumed less than 2000 kcal for 8 days before this experiment.

Evidence has been firmly established that consuming recommended dietary fiber (25 g/day) exerts a beneficial influence on human colonic health and provides many health benefits such as preventing coronary heart disease (Pereira and others 2004), stroke, hypertension (Wallström and others 2012), diabetes (Pereira and others 2004) and obesity (Howarth and others 2001). The questionnaire determined the dietary fiber intake of each subject. Subject 1 and 3 consumed close to 30g daily for 8 days before the onset of the experiment, while subject 2 and 4 (18.9 g, 17.7 g respectively) consumed less than the recommended daily intake of dietary fiber. Long-term dietary fiber intake is associated with the number and composition of gut microbiota as well as reducing gut apoptosis (Wu and others 2011; Nofrarías and others 2007). It is presumed that the individuals consuming high dietary fiber (subject 1 and 3) might have greater numbers of gut microbiota and furthermore, it can be expected that the gut microbiota from subject 1 and 3 might have higher availability of dietary fiber than the individuals who consumed the lower level of dietary fiber (subject 2 and 4). Lesser availability of dietary fiber might be one of the fundamental reasons for lower production of SCFAs.
4.2. Characteristic of Samples

The average degree of polymerization (DP) of pectin samples was determined using an HPLC system; SOY-1510, SBP-3729, HMP-492 (Fig.1). Also, the degree of pectin methylation (DM, %) of each sample was determined as 22.78% (SOY), 21.16% (SBP), and 75.96% (HMP). With the exception of HMP, SOY and SBP were determined as low-methoxy pectins (DM<50%). As shown in Fig. 1, the HPLC profile of SBP and SOY exhibited a similar pattern, while the HPLC profile of HMP responded differently.

Intrinsic factors such as DP, DM, and chain length are key parameters affecting the behavior of pectins (Thakur and others 1997). Also, the pectin source and type impact health response including such characteristics as a cholesterol lowering effect (Brown and others 1999). Pectin sources (high DM and high MW) exerted higher cholesterol-lowering properties than low DM and low MW (Brouns and others 2011). Moreover, Patricia and others (1982) reported that fecal wet and dry weights were higher on HMP, while transit time and fecal water were similar (Judd and Truswell 1982). This result also might be connected with the fact that availability of LMP by gut microbiota is higher than HMP. To date, most pectin studies have been focused on chemical properties such as gel or film properties. There are only few studies that have compared bioavailability or health effects between pectin with different chemical characteristics (DP and DM). However, with even limited evidence from previous studies, differences with DP and DM of pectin samples might be assumed that they have different fermentability by human gut microbiota and bioavailability for human health.

4.3. Fatty Acid Analysis
Dietary carbohydrates escaping digestive absorption in the small intestine undergo fermentation in the colon and stimulate short chain fatty acids (SCFAs) production (Cani and Delzenne 2009). Previous studies showed that the presence of carbohydrates in the colon and their fermentation might alter the colonic physiology (Kaur and others 2011; Shen and others 2011). Pectin is one of the soluble DFs and its potential roles for human health have been discussed in previous studies. However, most of these studies were conducted with low-methoxy pectin and high-methoxy pectin and investigated their physical properties (gelation or pH stability) (Evageliou and others 2000; Yoo and others 2006). The purposes of this study were to evaluate SCFAs production with different pectin sources and examine their specific characteristics regarding human gut microbiota.

From previous studies, production of SCFAs can be stimulated with dietary fiber. Titgemeyer (1991) reported that fermentation with citrus and apple pectin increased the SCFAs more than fiber sources from sugar beet, soy and oat. To date, there have been no studies to determine SCFA production with different pectin sources. Also, soy fiber showed the highest total SCFAs production among fiber samples.

Total SCFAs production of subjects with samples is shown in Figure 2. These values are calculated for the sum of SCFAs: acetate, propionate, and butyrate. Each subject exhibits a different pattern of SCFAs production. From the beginning of fermentation (0 hr), considerable concentration levels of SCFAs and BCFAs were detected. These values were thought to be excreted SCFAs from subjects, since all of the subjects were not fasted before the experiment. Compared to SCFAs produced without samples, the concentration of SCFAs produced by fermentation with pectin samples increased after 6 to 12 hrs incubation and reached their highest concentration. In particular, subject 2 showed a different increased pattern of total SCFA. With
the exception of subject 2, gut microbiota from other subjects produced significantly higher total SCFAs with HMP, SBP, and SOY during 6 hrs. After 12 hrs, while microbiota from subject 1 and 4 showed the highest total SCFAs with SOY, microbiota from subject 3 showed that SBP was the most efficient source for producing SCFAs with in vitro fermentation. At the end point (after 30 hrs), fermentation with SOY showed significantly higher production of SCFAs among samples. SBP also generated higher production of SCFAs with microbiota from Subject 2, 3, and 4. HMP fermented with microbiota coming from subject 2 and 4 produced higher production of SCFAs compared to the blank and FOS. Pectin samples (HMP, SBP, and SOY) might stimulate metabolism of gut microbiota to produce more total SCFAs, compared to fermentation with FOS and fermentation without substrates.

Figure 3 shows acetate production by gut microbiota from each subject. For acetic acid production, all pectin samples (HMP, SBP, and SOY) showed higher acetate production than fermentation without substrate. However, fermentation with FOS showed lower acetate production and produced less acetate than the blank (without substrate) at the end point (30 hrs). This pattern was observed in subject 1, 3, and 4, except subject 2 during 30 hrs. Even subject 2 yielded higher acetate production than blank, after 24 hrs, fermentation with FOS decreased dramatically and it was not significantly different with fermentation without substrate at the end point (30 hrs). These results indicate that all pectin samples stimulate acetate production by gut microbiota in vitro. FOS might have inhibition effects on acetate production by human gut microbiota.

Acetate, the principal SCFA in the colon, is readily absorbed and transported to the liver, and therefore less metabolized in the colon (Cook and Sellin 1998). In addition, acetate can be used as a source for butyrate. Diez-Gonzalez and others (1999) reported two different
mechanisms (acetate utilization and lactate fermentation) and enzymes (butyryl CoA:acetate CoA transferase, butyrate kinase) that are important factors for production of butyrate in the gut (Diez-Gonzalez and others 1999). Also, *Coprococcus sp.*, *Roseburia sp.*, *R. intestinals*, and *Facalibacterium prausnitzii* are known as bacteria that can convert butyrate, using acetate (Duncan and others 2002b). To date, no data has been reported that acetate has positive effects related to human health. However, acetate is often used to monitor colonic events because it is the primary SCFA. Also, increasing the concentration of acetate resulted in lowering pH; this reaction might be related to the beneficial influence on the composition of gut microbiota and preventing the proliferation of harmful species and growth of pathogenic bacteria.

The production of propionic acid during *in vitro* fermentation (30 hrs) is illustrated in Figure 3. Fermentation with SOY demonstrated significantly higher propionate production than other pectin samples (HMP and SBP) and FOS during 30 hrs.

Propionate has been shown to lower glucose-induced insulin secretion in isolated pancreatic islet cells of rats (Ximenes and others 2007). Thus, high production of propionate may be responsible for the observed lipid lowering effect as being one of the possible mechanisms of action. It is also reported that propionate has anti-proliferative effect towards colon cancer (Scheppach and others 1995; Jan and others 2002) and is related to weight control and feeding behavior (Oba and Allen 2003; Ruijschop and others 2008; Zhou and others 2008). Fermentation with SOY might have stimulating effects with propionate production.

Butyrate is the preferred energy source of colonocytes; approximately 70 to 90% of butyrate is metabolized by the colonocytes. The mechanisms of action of butyrate in relation to colon cancer are not clearly defined. However, various studies have found that butyrate has a positive effect in the prevention of colon cancer (Hague and others 1995; Scheppach and others...
reported that butyrate is formed from two molecules of acetyl coenzyme A that yields acetoacetyl-CoA, which is converted into butyryl-CoA. Consequently, butyryl-CoA might yield butyrate through butyrate kinase by some butyrate-producing strains such as *Butyrivibrio fibrisolvens* or via butyryl-CoA: acetate-CoA transferase.

From Figure 5, compared to other SCFAs, concentration of butyrate increased at later time point than other SCFAs. According to Duncan’s study (Duncan and others 2002a), butyrate can be converted, as gut microbiota consumes acetate. Thus, current result implies production of SCFAs also might be affected by other SCFAs and SCFAs can be changed interdependently in anaerobic fermentation. For butyrate production, fermentation with pectin samples by subject 1 and 4 show higher butyrate production, compared to FOS. However, fermentation with subject 2 and 3 show FOS is most efficient source for producing of butyrate during anaerobic fermentation (30hrs).

There were significant differences with production of BCFAs: *iso*-valeric acids and n-vdaleric acids. BCFAs produced during 30 hrs incubaton are shown in Figure 6. The two types of fermentation are carried out in the gut: saccharolytic (with carbohydrates) and proteolytic (with proteins) (Kolida and Gibson 2007). Branched chain fatty acids (BCFAs) are composed of 15 to 17 carbon atoms, and may also be stimulated by dietary intervention. *Iso*-valate is metabolized by fermentation of leucine. Metabolites of proteolytic fermentation, such as certain phenolic compounds, amines, ammonia and BCFAs showed negative effects on liver and overall health (MacFarlane 1995). However, in comparison with SCFAs, the absorption and metabolism of BCFAs has been investigated only minimally. Some studies have been investigated for their negative effects on health, using animal and human models.
In a previous animal study, Propst and others (2003) reported that total BCFA concentration was lower in dogs fed FOS in an experiment. In addition, microbiota from inflammatory bowel disease patients produced more BCFAs (25% higher) than microbiota from healthy subjects, concentration of total BCFAs (iso-butyrate and iso-valate) were increased after 16 hrs of fermentation (approximately 5 mmol, which were average value of all subjects) (Van Nuenen and others 2004). However, they also found that production of SCFAs by gut microbiota of IBD (Inflammatory Bowel Disease) patients is higher than healthy patients. Since only the current study has investigated the production of BCFAs and SCFAs (n=14), it is hard to conclude that production of BCFAs and SCFAs are related to IBD. While SCFAs can be used beneficially for human health, BCFAs might have deleterious effects on the human colon. From the current experiment’s results, fermentation with all of pectin samples showed significantly lower total BCFAs production, compared to blank (Fig. 6). This result implies that HMP, SBP and SOY might inhibit metabolism of gut microbiota to metabolize the BCFAs that are regarded as negative factors on human and animal health.

4.4. DGGE Experiment

The human intestinal microbiota has been known to contain complicated colonies composed of at least several hundred different species of bacteria with approximately $10^{11}$ to $10^{12}$ cells per gram of feces (Carman and others 1992; Ley and others 2006). For this reason, although intestinal microbiota has been thought to be closely related to human health, direct analysis of the intestinal microbiota is intrinsically difficult with conventional experiments (Ricke and Pillai 1999).
Based on DGGE results, bands showing different intensity were excised from the polyacrylamide gel and extracted DNA to explore specific bacteria. Numerous bands were excised and identified by sequencing, however most bands failed to yield identifiable sequence and were classified as uncultured bacterium. Extracted DNA showed that specific bacteria were based on BLAST algorithm. Banding patterns of each subject, excised bands number and identifications are shown in Figure 7 and table 2.

In this study, all subjects have very diverse gut microbiota. Even though most of the sequences of bands were assigned to uncultured bacterium or were not identified, Table 3 shows the dominant strains indicated by the banding patterns.

Metabolites (SCFAs, BCFAs and some gases), potential growth factors in fecal material, and dietary habit are important factors that affect the composition of gut microbiota (Gao and others 2009). Firmicutes species, Faecalibacterium species, Bacteroides species that have been regarded dominant human fecal bacterium were detected in subjects 1 to 4. The band of Firmicutes species was enriched after exposure to dietary FOS, SBP and HMP from subject 1 as well as FOS with subject 3. While Firmicutes species were detected from subject 1 and 3, the band of Firmicutes species was not detected in subjects 2 and 4. Also, DGGE bands for fecalibacterium species were detected in subjects 1, 3, and 4. The band identified as fecalibacterium was present in all samples and the blank from subjects 1, 3, and 4 except of SOY. This result indicates that growth of fecalibacterium might be inhibited by fermentation with soy pectins. From the current study, Bacteroides was present in subject 2 and 3. These three strains: Firmicutes species, Faecalibacterium species, Bacteroides species were reported to be related to the production of SCFAs and host health. According to Shen’s study, Bacteroides numbers may correlate with the increase in propionate production (Shen and others 2011). Also,
the number of dominant fecal bacterium is changed by disease status or body weight (Martín and others 2013; Fujimoto and others 2013; Jia and others 2010). Andreas and others (2010) reported that the most abundant bacterial groups in fecal samples of lean and obese subjects are *Firmicutes* species and *Bacteroidetes* species. The ratio of *Firmicutes* to *Bacteroidetes* altered in favor of the *Bacteroidetes* in overweight and obese subjects. Despite not knowing exactly the quantity of *Bacteroides* from this result, the number of *Bacteroides* might be affected depending on the fermentable sources, particularly pectins.

Interestingly, specific bacteria were also detected from each subject. *Lactobacillus rumis* is one of the dominant *Lactobacillus* species that have been known as probiotics. There are numerous studies in humans that provide evidence about the beneficial effects of probiotics. From the subject 1 results, the band for *L. rumis* was found to be most intense with Soy pectin sample. Based on this result, soy pectin might have a positive effect on the growth of *L. rumis*, exhibiting positive effects on human colonic health, such as inhibition of pathogenic microorganisms and relief of lactose maldigestion symptoms (Saxelin and others 2005). The band of *Bifidobacterium* species was also found in subject 3 and 4. Especially, the band of *Bifidobacterium* was enriched in fermentation with SBP or without substrates. This result is likely that HMP, SOY and FOS are not be related for growth of *Bifidobacterium* species, based on the results of the current study. *Pseudobutyrivibrio* species were also detected in subject 4. This strain has been reported that it is closely related to butyric acid production (Mrazek and others 2006). The bands of *Pseudobutyrivibrio* species in subject 4 were enriched with fermentation of all pectin samples and FOS, except Blank. From analysis of SCFAs, all pectin samples showed higher butyrate production, compared to blank. This result might be thought that
pectins and FOS stimulate growth of *Pseudobutyribrio* species and subsequently metabolize more butyrate production.

In order to compare composition of microbiota fermented with different samples by each subject, analysis with the phylogenetic trees was shown and illustrated the correlation between samples and subjects (Figure 8). The phylogenetic trees of samples generated by the UPGMA algorithm exhibited 5 different groups in each treatment. Also, subject 4 can be categorized as a normal weight person, while other subjects are considered overweight persons. From phylogenetic analysis with bands from samples fermented without substrates it appears that subjects 4 and subject 2 are clustered with 70% of relatedness. Also, subject 4 was clustered with subject 3 (65%) and subject 1 (63%), respectively. This result indicates that subject 4 and subject 2 have similar pattern of microbiota, than the other subjects. This pattern was also detected with FOS and SBP samples. However, samples fermented with SOY and HMP exhibited different patterns compared to the blank. In particular, the relatedness was decreased by fermentation with the respective samples. Even if same source was utilized for fermentation, the composition of microbiota became more diverse, due to the difference of initial microbiota composition of subjects.

This result implies that composition of microbiota can be altered by substrates, but ecology of microbiota did not change significantly during 30 hrs of fermentation, because each subject had a different composition of microbiota at the beginning.

The same fecal microbiota from the subject were fermented in different batches with different samples, and each batch has a different composition of microbiota. From the questionnaires that were conducted before the experiment, subject 4 is within normal range of BMI (from 18.5 to 25), while other subjects are within the overweight range of BMI (from 25 to
The relationship between bodyweight and composition of microbiota has been recently investigated in several studies (Schwiertz and others 2010; Turnbaugh and others 2006; de La Serre and others 2010; Cani and Delzenne 2009). There is a large amount of evidence to suggest that the metabolic activities of the gut microbiota produce energy from ingested dietary substances and store these calories in the host’s adipose tissue. Moreover, the gut bacterial flora of obese mice and humans consist of lower number of Bacteroides, on the other hand more Firmicutes were detected than their lean counterparts. These results suggest that differences in caloric extraction of ingested food substances may be due to the composition of the gut microbiota.

The distal tract contains the most abundant and diverse communities of microbiota and they have roles to interact with the human host and systemic effects (nutritional and metabolic). In order to determine the composition of gut microbiota with intervention of energy sources, appropriate experimental design should be conducted, considering its complexity and requirement to be cultivated. An in vitro fermentation has been widely used to cultivate human gut microbiota in combination with PCR-based DGGE for qualitative analysis and Fluorescent in Situ Hybridization (FISH) for quantitative analysis. The FISH technique has been used in the field of microbial ecology to identify microbiota, and enumerate the predominant bacterial groups (e.g. Bifidobacteria, Eubacteria, Clostridia, Atopobia, Bacteroides, Lactobacillus, and Escherichia coli) (Bezirtzoglou and others 2011; Schwiertz and others 2010; Roger and McCartney 2010).

The DGGE experiment investigated the changes in microbiota during in vitro fermentation. Even though no relationships between specific species and pectin samples were determined due to the limitation of the DGGE experiment, the data demonstrated that
fermentation with pectin samples showed different fermentation patterns. We can make a conclusion that certain pectin samples might modify microbiota during *in vitro* fermentation. Further study could validate this with quantification to determine the specific microbiota related to SCFA production.

5. Conclusions

All pectin samples showed higher total SCFAs production, compared to FOS (used as positive control). In particular SOY pectin shows that it has stimulating effects on propionic acid and butyric acid that are known for beneficial effects on human colonic health. Also, all pectins inhibit the production of BCFAs that might have deleterious effects on human colonic health. Furthermore, from the DGGE results, fermentation by human fecal microbiota with pectin samples might modulate profiles of microbiota. Instead of intervention of sample, the initial composition of microbiota is a major factor that can make differences between subjects. In sum, pectins coming from different sources have different characteristics to produce metabolites and to affect the composition of gut microbiota. Pectin might have beneficial effects by stimulation of SCFAs.
6. References


Table 1. Summary of subjects (n=4)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>BMI (KG *m^2)</th>
<th>Average Daily calories (Kcal)</th>
<th>Dietary fiber intakes (g / a day)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>27.8</td>
<td>1899</td>
<td>29.6</td>
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<tr>
<td>2</td>
<td>28</td>
<td>26.2</td>
<td>1713</td>
<td>18.9</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>28.0</td>
<td>1912</td>
<td>30.5</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>19.2</td>
<td>1898</td>
<td>17.7</td>
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<tr>
<td>Band No.</td>
<td>Bacteria (Genbank Accession No.)</td>
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<tr>
<td>---------</td>
<td>----------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A,B</td>
<td>Uncultured bacterium</td>
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<td></td>
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<tr>
<td>1C</td>
<td><em>Lactobacillus ruminis</em></td>
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<tr>
<td>1D</td>
<td><em>Faecalibacterium</em> species</td>
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</tr>
<tr>
<td>1E</td>
<td>Uncultured <em>Catenibacterium</em> species</td>
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<td></td>
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<tr>
<td>1F</td>
<td><em>Prevotella bergepis</em></td>
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<tr>
<td>1G</td>
<td>Uncultured <em>Firmicutes bacterium</em></td>
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<tr>
<td>2B</td>
<td><em>Bacteroides</em> species</td>
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</tr>
<tr>
<td>2C</td>
<td><em>Alistipes</em> species</td>
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<tr>
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<td><em>Eubacterium eligens</em></td>
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<td>2E</td>
<td>Uncultured <em>Ruminococcaceae bacterium</em></td>
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<tr>
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<td><em>Thermaerobacter marianensis</em></td>
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<td><em>Bacteroides</em> species</td>
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<td>3B</td>
<td><em>Catonella</em> species</td>
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<tr>
<td>3D</td>
<td><em>Faecalibacterium praunitzii</em></td>
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<tr>
<td>3E</td>
<td>Uncultured <em>Firmicutes bacterium</em></td>
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<tr>
<td>3F</td>
<td>Uncultured <em>Clostridiales bacterium</em></td>
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<tr>
<td>3G</td>
<td>Uncultured <em>Bifidobacterium</em></td>
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<tr>
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<td>4A</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4B</td>
<td>Uncultured bacterium</td>
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<td></td>
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<tr>
<td>4C</td>
<td><em>Pseudobutyriivibrio</em> species</td>
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<tr>
<td>4D</td>
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<tr>
<td>4E</td>
<td>Uncultured <em>Bifidobacterium</em></td>
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</tr>
<tr>
<td>4F</td>
<td><em>Faecalibacterium praunitzii</em></td>
<td></td>
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</tr>
<tr>
<td>4G</td>
<td><em>Coprococcus</em> species</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4H</td>
<td>Uncultured <em>Barnesiella</em> species</td>
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</table>
Figure 1. Chromatogram of pectins and FOS, using RI detector

*HMP = High Methoxy Pectin; SBP = Sugar Beet Pectin; FOS = Fructooligosaccharides; SOY = Soy pectin
Figure 2. The concentration of total SCFAs with subject 1 through 4 during 30hrs incubation

Subject 1

Subject 2

Subject 3

Subject 4

*Bars indicate standard error of the mean, different letters indicate that there are significant differences between samples (p<0.05)
Figure 3. Acetate production with subject 1 through 4 during 30hrs incubation

*Different letter indicates significant difference within same time, BL=Blank; SOY=Soy pectin; SBP=Sugar beet pectin; FOS=Fructooligosaccharides; HMP=High Methoxy Pectin
Figure 4. Propionate production with subject 1 through 4 during 30hrs incubation

*aDifferent letter indicates significant difference within same time, BL=Blank; SOY=Soy pectin; SBP=Sugar beet pectin; FOS=Fructooligosaccharides; HMP=High Methoxy Pectin
Figure 5. Butyrate production with subject 1 through 4 during 30 hrs incubation

*Different letter indicates significant difference within same time, BL=Blank; SOY=Soy pectin; SBP=Sugar beet pectin; FOS=Fructooligosaccharides; HMP=High Methoxy Pectin.*
Figure 6. Total BCFAs production with subject 1 through 4 during 30hrs incubation

*Each value is sum of iso-valate and n-valate, different letter indicates significant difference within same time.
Figure 7. Denaturing gradient gel electrophoresis banding patterns of 16S rRNA gene fragments generated from bacterial DNA isolated from fecal batch after 30 hrs incubation.
Figure 8. Phylogenetic tree based on DGGE results

*BL=Blank; SOY=Soy pectin; SBP=Sugar beet pectin; FOS=Fructooligosaccharides; HMP=High Methoxy Pectin.
7. Appendix

7.1. Authorship statement for chapter 3

Byungjiek Min is the first author of the paper and has written at least 51% of the paper among coauthors in chapter 3: title is “In Vitro Analysis of Short Chain Fatty acids and Human Fecal Microbiota Stimulated by Pectin Sources”

Major Advisor: Dr. Sun-Ok Lee

Date: December 11th, 2013
CHAPTER 4

Overall Conclusion

Dietary fiber (DF) has been regarded as a functional ingredient for colonic health. DF can be categorized according to its solubility. Unlike insoluble fiber, soluble fiber can be entirely digested by gut microbiota. We should consider not only correlations between soluble fiber (especially pectin) intake and colonic health, but also which pectin is suitable for fermentation by gut microbiota. Pectin can be extracted from plants, fruits, and some byproducts from food industries. Pectin has been used as a thickening agent for food ingredients, and more recently, it has received interests as an ingredient of functional food. Pectins came from various sources having different structures and this difference permits unique properties for fermentation. However, few studies have been conducted to compare pectins, thus more studies are required to determine differences among different pectin sources. We examined three different pectin samples using 4 subjects in order to determine its fermentability in vitro. All pectin samples showed higher total SCFAs production, compared to FOS (used as positive control) from all subjects. In particular, SOY pectin shows that it has stimulating effects on propionic acid and butyric acid that are known for beneficial effects on human colonic health. Also, all pectins inhibited the production of BCFAs that might have deleterious effects on human colonic health. Furthermore, from the DGGE results, fermentation by human fecal microbiota with pectin samples might modulate profiles of microbiota. Instead of intervention of sample, initial composition of microbiota is a major factor that can cause differences among subjects.

Pectin samples may have a positive effect for colon health by stimulating production of SCFAs and modulating microbiota composition. We might utilize pectin sources to apply functional ingredient for human colon health.