Assessing prebiotic effects of resistant starch on modulating gut microbiota with an in vivo animal model and an in vitro semi-continuous fermentation model

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Assessing prebiotic effects of resistant starch on modulating gut microbiota with an in vivo animal model and an in vitro semi-continuous fermentation model

by

Li Li

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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Major: Genetics

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ABSTRACT

Resistant starch (RS) is the starch that escapes human digestion system and will be delivered to the lower gut for fermentation by gut microbiota. Five types of resistant starch have been described, including 1) physically amylase-inaccessible type 1 RS, 2) native high amylose type 2 RS, 3) retrograded type 3 RS, 4) chemically modified type 4 RS, and 5) fatty acid complexed type 5 RS. RS has been shown to lower postprandial glycemic index (including plasma glucose and insulin levels), indicating effects of RS in reducing risk of type II diabetes and possible obesity as well. In addition, RS fermentation in the lower gut has been shown to maintain colon health and prevent colon carcinogenesis, mainly through production of SCFA. Physiological significances of SCFA, especially butyrate, include providing an energy source for colonocytes, maintaining the colonic epithelia cell layer, stimulating basal crypt cell proliferation, and inducing apoptosis of hyper-proliferative cancer cells. Furthermore, RS has also been considered as a prebiotic to modulate gut microbiota favoring RS degradation and SCFA production. The long-term objective of our study is to understand the underlying mechanisms of RS in improving human health from two correlated aspects, modulating gut microbiota and producing SCFA. We hypothesized that when using an in vivo animal model and an in vitro fermentation model, resistant starch consumption would shift gut microbiota pattern towards favoring RS fermentation, especially SCFA production.

In respect to the physiological significance of resistant starch implicated in improving colon health, we hypothesized that HA7 (high amylose starch VII, type 2 RS), and HA7-SA (palmitic acid complexed HA7, type 5 RS) would modulate colonic microbiota into different patterns. The bacterial pattern shifts can explain the differential efficacy of two RS suppressing colon carcinogenesis. Previous studies showed that pre-neoplasm, aberrant crypt foci (ACF) induced by carcinogen Azoxymethane (AOM) were only decreased by 8-wk feeding of HA7 (21 ± 9) in male Fisher 344 rats, but not by HA7-SA (38 ± 18), when compared to highly digested control starch (48 ± 14). We used PCR-DGGE (Denaturing Gradient Gel Electrophoresis) to analyze colonic microbiota with bacteria universal 16S rRNA gene primers. Our results showed two RSs induced significant shifts of colonic microbiota compared to control digestible starch-fed rats. Moreover, differential bacterial
patterns were observed between two RSs such as the specific enrichment of putative *Bifidobacterium pseudolongum* by HA7-SA, but not by HA7. More importantly, a significant correlation was observed between gut microbiota patterns and ACF numbers developed in AOM treated animals. Further analysis of starch fermentation-associated bacteria groups showed similar shifts of *Clostridium* cluster IV by two RSs with increased putative *Ruminococcus bromii*. By contrast, the bacterial pattern of *Clostridium* cluster XIVa showed correlation with ACF number. Specific enrichment of putative *Ruminococcus obeum* R. sp. SRI/5 was observed in HA7-fed animals, whereas specific decrease of *Bacteroides* sp. ASF519 or *Parabacteroides goldsteinii* (T) by HA7-SA was observed in the *Bacteroides fragilis* group. Our results suggested that gut microbiota patterns modulated by RS were related to differential efficacy of HA7 and HA7-SA in decreasing colonic carcinogenesis.

We established an *in vitro* semi-continuous anaerobic incubation model to compare fermentability, i.e. SCFA production, of four high amylose starches: HAV, HAVI, HAVII and GEMS-067 from four maize lines with different genetic background and amylose contents (55%, 65%, 70% and 70% respectively). This was done to evaluate prebiotic effects of resistant starch on modulating gut microbiota hypothetically towards favoring RS fermentation. The digested starch residues (SR), obtained from *in vitro* digestion with the AOAC 991.43 method, were incubated with fecal microbiota to simulate human digestion of cooked starchy food. In our study a total of 32 individuals were recruited: 17 lean, 9 overweight and 6 obese individuals. The study was conducted in two phases 5 months apart. Each phase consisted of a 3-wk incubation period with a frequency of changing BHI (Brain Heart Infusion) medium and SR substrates every 3.5 d. We observed significantly decreased pH, increased gas production, increased butyrate and total SCFA concentration in incubations with the four SR compared to blank BHI medium control starting at wk 1. There was no difference between SR. Molar proportions of butyrate was increased by SR with decreased acetate proportion, both of which achieved stability starting at wk 2. Additionally, propionate concentration was only increased by SRV at the end of the 3-wk incubation compared to BHI medium, but not by other SR. Large inter-individual variation was observed in the proportional increase of butyrate by SR compared to blank BHI medium control. No significant difference was found between lean and overweight individuals from
fermentation indicators measured. We concluded that a stable long-term semi-continuous *in vitro* fermentation model was established to simulate carbohydrate fermentation in human lower gut. We also showed significant increase of butyrate production by RS fermentation with human fecal microbiota.

The modulation of microbiota by amylase digestion residues of HAV, VI and VII in our semi-continuous fermentation model was further analyzed with PCR-DGGE by examining total bacterial pattern and that of RS fermentation associated bacteria groups, including *Clostridium* cluster IV, XIVa and *Bacteroides fragilis* group. We hypothesized that bacterial patterns obtained after 3 wk fermentation will be selected by three SR incubation and lean/overweight microbiota as well. Our results showed that total bacterial patterns were shifted by three SR incubation with fecal microbiota from 30 donors at the end of 3-wk fermentation compared to control BHI medium. Moreover, bacterial pattern in SRV fermentation samples differed from that of SRVI and SRVII, which shared a certain degree of similarity. However, bacterial pattern of *Clostridium* cluster IV and XIVa in SRVI fermentation samples differed from SRV and SRVII, which shared similar patterns, whereas no shifts were observed by any SR in *Bacteroides fragilis* group bacterial pattern compared to blank BHI medium control. Most important, we observed the putative *Ruminococcus bromii* was specifically selected during SR incubation by microbiota from lean individuals, but not by microbiota from overweight and obese individuals. We concluded that our *in vitro* semi-continuous fermentation model can be used to assess prebiotic effects of RS by simulating long-term RS consumption in human. We also showed that *Ruminococcus bromii*, belonging to *Clostridium* cluster IV, was selectively enriched by SR and microbiota of lean individuals.

In summary, our studies with both *in vivo* animal model and *in vitro* fermentation model supported previous recognition of resistant starch acting as a prebiotic to modulate gut microbiota, especially on *Clostridium* clusters IV and XIVa. *Ruminococcus bromii* was specifically induced in rat model fed RS as well as *in vitro* fermentation model using lean microbiota. Moreover, different RS may have different fermentation outcomes. Our findings provided solid evidence to answer the fundamental question of how RS exerted effects on
shifting bacterial pattern. In addition, we also showed that the physiological significance of RS might be affected by physical-chemical properties of starch and pre-existing microbiota as well.
CHAPTER 1. GENERAL INTRODUCTION

Introduction
Resistant starch (RS) is the starch that escapes human small intestine digestion. It can be delivered to the lower gut for further microbial anaerobic fermentation. Five types of RS have been classified. Type I RS are referred to those physically inaccessible starches, such as partially milled grains and seeds. Type II RS are native starches with highly packed structure inside starch granules, such as potato starch, bean starch, and high amylose cornstarch. Type III RS are retrograded starches, which undergo heating and cooling procedures, creating a crystalline structure resistant to amylase action. Type IV RS are chemically modified starches with etherized, esterized, or cross bonded sugars to decrease digestibility. Type V RS are fatty acid modified starches that have recently been proposed.

Recognition of RS can be traced back to the 1980s with their potential in improving health and preventing chronic diseases. A single consumption of a RS meal has been shown to decrease glycemic index, i.e. blood glucose and insulin levels, as well as regulate satiety and satiety hormones levels, indicating the role of RS in preventing and improving type II diabetes and obesity. Long term consumption of RS has been shown to decrease colon carcinogenesis and improve inflammatory damages caused by colitis. Human feeding trials, animal models, and in vitro fermentation models have been extensively used to discover physiological significances of resistant starch and to understand underlying mechanisms.

Fermentation of RS by lower gut microbiota is one of the most important aspects of RS to maintaining colon health. Increased fecal bulking, decreasing colonic pH, increased short chain fatty acid (SCFA), as well as gas production are major fermentation outcomes observed during starch anaerobic fermentation. Acetate, propionate, and butyrate account for 90-95% of the SCFA produced by RS. These major SCFA, especially butyrate, have attracted attention in the past decades because of a body of in vitro evidence showing multi-function of butyrate in maintaining colon health. Butyrate is a primary energy source for colonocytes. SCFA can stimulate the proliferation of enterocytes in the basal crypt. SCFA can also enhance colonic epithelial barrier function. More importantly, butyrate is a potent inducer of
apoptosis when applied to hyper-proliferative cell lines in vitro. Inhibition of histone deacetylase (HDACs) has been considered as one of the molecular mechanisms by which butyrate regulates gene expression. Regulation of specific cellular events through G protein-coupled receptors GPR41 and 43 are surfacing, shedding new light on the involvement of SCFA in adipogenesis and anti-inflammation.

Giving the significant luminal effects of RS and SCFA, understanding prebiotic effects of RS on modulating gut microbiota and on metabolic outcomes is thus very important. In addition, with the advent of 16S rRNA deep sequencing techniques, gut microbiota has been related to chronic diseases such as colitis and obesity. Distinct fecal microbiota patterns have been observed in patients with ulcerative colitis disease from that of healthy human subject. When compared to lean individuals with BMI of 18.5-24.9, obese individuals (BMI ≥ 30) have higher fecal Firmicutes/Bacteroidetes ratios.

These findings led us to focus on how gut microbiota contributed to the effects of RS in decreasing colon carcinogenesis with an in vivo animal model. We also took an alternative strategy using an in vitro semi-fermentation model to investigate how human fecal microbiota adapts to long-term RS anaerobic incubation. Shifts of genetic biodiversity of gut microbiota by RS were monitored with molecular fingerprinting analysis by PCR-DGGE (denaturing gradient gel electrophoresis). Metabolic or functional activity of microbiota in SCFA production was examined with bio-analytical GC (gas chromatograph). Our studies would be one of the initial steps to understand prebiotic effects of RS in modulating gut microbiota. The ultimate goal is to lay groundwork for future dietary intervention with resistant starch or similar prebiotic compounds to improve human host health.

**Dissertation organization**

This dissertation contains a literature review focusing mainly on recent research progress on resistant starch from two aspects: 1) prebiotic effects of resistant starch on modulating gut microbiota, and 2) the physiological significance of resistant starch and its fermentation products, i.e. short chain fatty acid (SCFA) in preventing colon cancer. All three papers “Gut
microbial profile is related to the efficacy of resistant starch in decreasing aberrant crypt foci in Fisher 344 rats”, “Increased butyrate production by long term in vitro fermentation of digested high amylose cornstarch residues with human fecal microbiota” and “Ruminococcus bromii related organisms was selective enriched by high amylose starch digested residues in lean microbiota with a semi-continuous in vitro fermentation model” will be submitted to The Journal of Nutrition.
CHAPTER 2. LITERATURE REVIEW

1. Resistant starch

1.1 Starch structure and digestion

Starch is a polysaccharide with glucose units joined by glycoside bonds, which is naturally found in plants as the major carbohydrate source in most human diets. There are two types of molecules that are commonly found in starch with varied proportions, amylose and amylpectin, which differ in the glycosidic linkage and branch structure. Amylose is a linear polymer with glucose linked by \( \alpha - (1, 4) \) glycosidic bonds, with little or none branching. Whereas amylpectin usually has a glucose backbone, and that is highly branched by \( \alpha-(1, 6) \) glycosidic bonds every 25-30 glucose molecules (BNF 1990). Amylose usually displays helical structure with six glucose molecules per turn. The hydrophilic hydroxyl groups are exposed outward, creating a hydrophobic environment inside where fatty acids or iodine can be bound to (Takeda et al. 1989). Amylopectin, on the other hand, is usually clustered randomly or regularly, and linked by long chains extending to two or more clusters (Hizukuri 1986).

As food is digested in humans, starch hydrolysis starts by salivary amylase, followed by pancreatic alpha-amylase to degrade the polymer into maltose and glucose in the small intestine for absorption. Various inhibitors of human amylase are found in the human diet, including tannins, polyphenols, and certain legume proteins, which might affect starch digestion, as summarized in Bennick (2002). In addition, the physical-chemical properties of starch itself can also affect the digestion, such as the starch granule size, shape, and structure, which may vary with its resource plants. The granule of normal corn starch in the food is round or polygonal shaped. The potato starch granule is large ellipsoid or spherical, whereas cereal starch granules are small and polyhedric (Frazier 1997). Moreover, the starch structure, largely determined by the ratio of amylose and amylpectin, also affects the digestion. Waxy starch is mainly composed of amylpectin and does not have amylose, which is highly
digestible in human. Normal corn starch is composed of around 25% amylose and 75% amylopectin. High amylose starch (HAS), known as amylomaize usually contain more than 50% amylose. Increased proportion of amylose in starch can increase digestion resistance (Jiang et al. 2010), probably due to the interchain association of amylose resulting in the crystallization structure interfering with amylase activity. Undigested starch fractions are delivered to the lower gut for bacteria anaerobic fermentation.

1.2 Resistant starch

Resistant starch (RS) is defined as “the starch or the portion of starch that escapes digestion in the small intestine of healthy individuals” (N-G Asp 1992). The digestibility of RS can be evaluated in vitro by simulating digestion events happening in human upper gut, including acidic gastric hydrolysis and saliva or pancreatic amylase digestion in small intestine. Standard analytic methods for RS have not been established yet, due to the varied efficacy of amylase and the quantification method of starch residues. The AOAC 991.43 method (AOAC 2003) for total dietary fiber was considered more relevant to cooked starchy food in human diet because of the thermal stable α-amylase hydrolysis step at ~100 °C for 30 min (Li et al. 2008), whereas other RS quantification methods, such as AOAC 2002.02 (AOAC 2005) and Englyst’s method (Englyst et al. 1992) are widely used to analyze raw starch with 37 °C amylase digestion.

RS was classified into four types: type I RS represent those starches whose structures are physically inaccessible to amylase, such as the partially milled grains and seeds; type II RS represent those native starches with highly packed structure inside the granules, such as potato starch, bean starch, and high amylose cornstarch (HAS); type III RS represent those retrograded starches with altered structure by heating and cooling cycle; and type IV RS represent those chemically modified starches with etherisation, esterisation or cross bonded with chemicals to decrease digestibility (Englyst et al. 1990; Englyst et al. 1992). Recently, a type V RS has been proposed as starch-lipid complex (Jane et al. 2009). Type II and type III RS are widely used in studying the physiological significance of RS due to their commercial availability and common application in food processing.
The physiological significance of RS fermentation in the lower gut, especially their fermentation products, short chain fatty acid (SCFA), may maintain colon health and improve colon disease symptoms. Additionally, long term consumption of RS may modulate microbial population in the lower gut, indicating it effects as prebiotics. In the current thesis, these two aspects of RS were studied with an animal model in vivo and fermentation model in vitro.

2. RS and gut microbes

2.1 Gut microbes and human health

The involvement of resistant starch and gut microbiota in maintaining human health are summarized in Figure 1. We will first take the development of obesity as an example to show the cutting-edge research progress in the role of gut microbes involved in maintaining human health and contributing to disease. A further detailed discussion of gut microbiota and their metabolic products, i.e. short chain fatty acids in colon carcinogenesis will be included in the end of the dissertation.

2.1.1 Gut microbes

The distal gut (including cecum, colon and rectum) is most densely populated part of the gut with $10^{12}$-$10^{14}$ bacteria, archaea and yeast cells per gram feces (Whitman et al. 1998). Bacteria comprise about 40-55% of solid stool in people having typical Western diets (Stephen et al. 1980). According to 13 335 full length 16S rRNA sequences obtained with PCR amplification and cloning, 9 phyla were detected with variable dominance in the human large intestine, including Actinobacteria, Bacteriodetes (also known as Cytophaga-Flavobacterium-Bacteroides), Cyanobacteria, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, VadinBE97 and Verrucomicrobia in fecal and mucosal tissue samples from 3 healthy adult human (Eckburg et al. 2005; Ley et al. 2006). Two major methanogenic archaea species have been identified with the more prominent Methanobrevibacter smithii and less dominant Methanosphaera stadtmanae (Eckburg et al. 2005; Dridi et al. 2009). The concept of phylotype, or operational taxonomic unit (OTU), which is determined by using
certain percent of minimum similarity as the threshold for any pair of sequences, was proposed by Eckburg et al. (2005) and accepted by most studies later on. Bacteriodetes and Firmicutes are the two dominant divisions accounting for almost 95% phylotypes (with the cut-off value defined as 80% similarity). On the lower taxonomic levels, more than 50 genera (with the cutoff defined as more than 95% similarity) and over 400 species (with the cutoff defined as more than 97% similarity) have been identified in human gut and more than 7,000 strains were obtained with unique sequences (Eckburg et al. 2005).

Usually, the whole microbial population in a healthy subject can be described as mutualistic to the host with the functions identified so far (Backhed et al. 2005). Gut microbes are involved in the energy extraction, xenobiotic/drug metabolism, postnatal angiogenesis of villus network, and maturation/regulation of gut associated immune system (GALT). Among those, the most important function of gut microbes is the energy salvation, which otherwise will be lost through excretion. The regional specific distribution of bacteria along the gut reflects the metabolic adaptability achieved after long evolution. The upper gut is inhabited with sparse bacteria to avoid competition with host for digestible mono-saccharides. Whereas, abundant bacteria reside in the lower gut are efficient in fermenting endogenous and exogenous carbohydrate and protein. Studies showed that conventionally raised (CV) Wistar male rats metabolized 80% of their dietary Kcal intake (148 kcal/kg per day), as shown by measuring calories excreted in the feces (Wostmann et al. 1983). Whereas, with similar Kcal intake (143 kcal/kg per day), germ free (GF) animals devoid of gut microbes metabolized 71.9%. These results suggested gut microbes consumed about 10% energy of host dietary intake. It was estimated that gut microbial fermentation of glucose could potentially provide 60% ATP, in the form of short chain fatty acid (SCFA) for colonocytes utilization. The remaining 40% can be used by bacteria for growth or otherwise wasted as methane and hydrogen (Bergman 1990). Moreover, the inoculation of GF C57BL/6 mice with microbes from CV mice resulted in 60% increase of the body fat, increased mono-saccharides absorption from gut lumen, induced de novo lipogenesis, and reduced food intake within 14-d inoculation (Backhed et al. 2004). These results suggested that gut microbes have the potential to maintain colon health by providing energy needed for the host health but also
may increase the risk of obesity by increasing body fat. Therefore, as an essential “organ” to the host, the gut microbiota has become one of the research hotspot for the past decade considering its double-edge roles as the mediator during disease-prevention and disease-promotion.

2.1.2 Role of gut microbes in obesity

Obesity is recently known as one of the gut microbe-associated diseases. It was described here as an example showing how gut microbes contributed to human health and disease development. The energy imbalance caused by the energy extraction capacity of gut microbes was credited as one of the reasons leading to obesity. Ley et al. (2006) reported higher Firmicutes and lower Bacteroidetes in fecal microbiota in 12 obese people from United States, according to 16S rRNA sequencing results. In the same study, feeding fat-restricted or carbohydrate restricted low-calorie diet for a year progressively increased the abundance of Bacteroidetes, but decreased the proportion of Firmicutes in these obese subjects along with significant weight loss. As a consequence, the higher energy extraction efficiency of Firmicutes from polysaccharides was blamed as one reason of obese development.

Further, Turnbaugh et al. (2009) compared the taxonomic structure of fecal microbes from 154 individuals in US with obese or lean body mass index (BMI), and consistently found that obese people contained less Bacteroidetes compared to lean people. Actinobacteria was higher in obese people instead of previously reported Firmicutes. Additionally, the majority of obesity related genes were from Actinobacteria (75%) and Firmicutes (25%) according to genome shot-gun sequencing and gene function prediction of gut microbiota. They concluded that the functional difference between gut microbiota might be important for understanding the development of obesity.
Figure 1 Resistant starch, gut microbiota and human health
However, Zhang et al. (2009) reported that hydrogen-producing Prevotellaceae, a subgroup of Bacteroidetes and hydrogen-utilizing methanogenic archaea (*Methanobrevibacter smithii*) were particularly enriched in obese subjects (n = 3 each) compared to lean people. However, in this study, the distribution of fecal Firmicutes and Bacteroidetes was comparable between two groups. They indicated that the efficiency of hydrogen consumption as a result of polysaccharides fermentation might be important in the energy extraction in obesity development.

More intriguingly, Schwiertz et al. (2009) reported that significantly higher proportion of *Bacteroides* (45% for overweight and 51% for obese individuals) but less Firmicutes (47.7% for overweight and 51.0% for obese individuals) was observed in fecal microbiota of overweight (n = 35) and obese people (n = 33) compared to lean people (n = 30) (22.9% for Bacteroidetes and 73.1% for Firmicutes) from Germany using quantitative real time PCR with bacteria group specific primers. Moreover, fecal SCFA level, especially propionate, measured by gas chromatography, was significantly higher in overweight and obese subjects (18.3 ± 7.9 and 19.3 ± 8.7 mmol/L) than lean subjects (13.6 ± 5.2 mmol/L), as well as higher total SCFA level. Therefore they concluded that metabolic effects of the gut microbiome, i.e. SCFA production, were more important in assessing the contribution of gut microbiota in human obesity development.

The discrepancy in the genetic diversity of gut microbiota from these human studies might be due to the geographic difference of subjects, the difference in the number of subjects and detection limits of the techniques as well, such as comparing 16S rRNA deep sequencing versus quantitative PCR. However, metabolic activities, of gut microbiota such as SCFA and hydrogen transfer, are proposed as one mechanism for obesity development due to increased energy generation by carbohydrate fermentation, which might increase the risk of energy unbalance.

The role of gut microbiota in obesity development was further investigated with gnotobiotic animal studies. When animals were fed polysaccharide-rich diet, PicoLab chow diet (Purina)
*ad libitum*, the microbiota of genetically induced obese C57BL/6J mice (ob/ob) showed 50% reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes compared to lean counterparts ob/-/+ mice and wild type mice (Ley et al. 2005). In addition, obese C57BL/6J mice induced by a high fat/high sugar western diet harbored a significant increased of bacteria of Mollicutes class, belonging to phylum Firmicutes. And the genomic analysis of one of the most dominant bacteria, *Eubacterium dolichum* belonging to Firmicutes phylum and Mollicutes class, in obese mice indicated that it was very competitive in the utilization of simple sugars to produce butyrate (Turnbaugh et al. 2008). Moreover, transplantation of cecal microbiota from obese mice (genetic-induced or high fat diet-induced) significantly increased body fat in GF animals compared to those colonized with lean microbiota (Turnbaugh et al. 2006; Turnbaugh et al. 2008). Co-colonization of *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii* increased hydrogen consumption and cecal SCFA in the NMRI inbred mice, as well as body fat (Samuel et al. 2006).

The molecular mechanism of gut microbes facilitating obesity has also been investigated with conventionally raised animals compared with germ free animals, the latter of which was shown to be resistant to diet induced obesity due to lack of gut microbes. Backhed et al. (2007) addressed the mechanism of how germ free animals were resistant to high fat diet-induced obesity. They reported that GF C57BL/6J mice had higher level of phosphorylated AMP-activated protein kinase (AMPK), which was related to the decreased fat oxidation in skeleton muscles, compared to CV mice. In addition, there was an increased body fat in adipose tissues in response to high fat diet in knock out GF animals lacking fasting-induced adipose factor (Fiaf), which is a lipoprotein lipase inhibitor that is normally suppressed by gut microbiota. Their results indicated gut microbes are involved in regulating host body fat distribution and contributed to obesity development.

Given the dual roles of gut microbes in disease-promotion and disease-prevention as revealed by the obesity studies, the identification of beneficial microbial population is a vital area of research that focuses on understanding the other gut microbes-associated disease, such as colitis and colon cancer. In addition, the metabolic outcomes of gut microbes are associated
with dietary habits that influence substrates availability. Dietary manipulation of gut microbes towards healthy balanced profile is thus considered as an alternative for clinical treatment and dietary prevention. Prebiotics, for example, are referred to as a group of food ingredients including carbohydrates, peptide, protein and lipids, all of which are not digestible by human digestive enzymes, but fermentable by gut microbiota (Gibson et al. 1995). One of the major classification criteria for prebiotics is their ability to stimulate growth or activities of beneficial bacteria to human luminal or systemic health. Our study mainly focuses on these aspects by investigating the modulation of microbial population by RS and exploring its metabolic effects on the colon carcinogenesis.

2.2 Analysis of gut microbiota

Many early studies identified the amylolytic bacteria with screening of amylase activity of fecal isolates on the agar plate or liquid medium supplemented with starch. However, single isolates screen may overlook the bacteria involved in cross-feeding, in which the products released from one bacteria species could be utilized as substrates by other bacteria. In addition to human or animal in vivo feeding, continuous in vitro fermentation system provides a better platform to identify bacteria involved in RS fermentation, which will be discussed later. In addition, molecular methods are more commonly used with advantages of increased sensitivity and culture-independence. The 16S ribosomal RNA gene is usually used as the taxonomic marker since it is universally present with highly conserved sequence in the bacteria population. Based on the alignment of 16S rRNA genes sequences, the evolitional relationship can be identified and their metabolic capacity and characteristics can be predicted as well. For example, Collins et al. (1994) examined the full length 16S rRNA gene sequences from more than 114 Clostridium related bacteria obtained from the EMBL Data Library and Ribosomal Database Project (RDP). They reclassified the genera of Clostridium, Fusobacterium and Eubacterium into 19 Clostridial clusters. It is now known that the majority of members of Clostridium cluster IV and XIVa, belonging to phylum Firmicutes, were amylolytic and butyrogenic bacteria identified in human feces as summarized in the review of Pryde et al. (2002)). These results suggested these bacteria are highly involved in
resistant starch fermentation, which may be used as biomarkers identifying fermentation capacity of human individuals.

2.2.1 DGGE analysis

Various fingerprint analyses of the microbial population have been used to investigate the microbial population of samples collected from in vivo and in vitro studies. DNA fingerprint analysis, or known as DNA profiling, was referred to those techniques used to characterize and identify individual DNA pattern as biomarker. PCR (Polymerase chain reaction) is commonly coupled with fingerprint analysis to increase the sensitivity detecting low abundance bacteria species in the sample. Advantages of DNA fingerprint analysis are fast and high resolution, which can be used to easily compare the difference between groups by visualization on gels. Taking DGGE (Denaturing Gradient Gel Electrophoresis) as an example, it was first applied by Muyzer et al. (1993) to analyze the bacterial communities using 16S rRNA primer for the amplification of the variable 3 (V3) region. The separation of PCR fragments on the DGGE gel is based on the difference of the denaturing energy required for opening or melting the PCR fragments with similar size (bp). It is known that cytosine / guanine base-pairing (GC) is generally stronger than adenosine / thymine base-pairing (AT). Therefore, during the migration of PCR products on the polyacrylamide gel with gradient generated by denaturing reagents, mainly formamide and urea, the double strand structure will be “melted” or denatured at the weaker AT regions, resulting in much slower migration. Other PCR fragments with more GC content may continue to migrate on the gels till their denaturing gradient. The pattern of bands representing the bacteria community is generated and visualized with a following staining procedure.

Theoretically, an individual band on the DGGE represents a unique sequence of a particular bacteria strain or species. But it has been shown by DNA sequencing that single band may represent several sequences due to very similar GC content or very closely related species (Sekiguchi et al. 2001). In addition, artificial bands caused by heteroduplex (mismatch DNA double helix) formation during PCR denaturing-annealing cycle may also interfere with the band identification on the gels when analyzing complex DNA templates (Ferris et al. 1997).
On the other hand, the same bacteria may have multiple copies of 16S rRNA which might differ in their sequences. This was reflected on the DGGE gels as separated bands for *Bifidobacterium adolescentis* strain E-981074 (Satokari et al. 2001). To avoid this problem, it may be necessary to optimize or narrow down the gradient of the DGGE gel to separate similar bands. Additionally, species-specific primers are used to reduce the complexity of the DGGE gel and enhance the separation of those bacteria belonging to the same genus such as *Bifidobacterium* (Satokari et al. 2001), Lactobacilli (Walter et al. 2001), or same group such as Clostridial cluster IV (Shen et al. 2006), Clostridial cluster XIVa (Maukonen et al. 2006), *Bacteroides fragilis* (Pang et al. 2005) for investigating the modulation of bacteria population caused by RS fermentation.

DGGE is a relatively sensitive fingerprinting method. It has been commonly accepted that species with the density of more than 0.1-1% in the population could be detected by DGGE (Muyzer et al. 1993) using ethidium bromide (EB) staining. The application of silver staining instead enhances the sensitivity of gel visualization with 1 pg DNA/mm² band cross-section (Bassam et al. 1991). Neufeld et al. (2005) described using fluorophore (Cy5)-labelled PCR primers to even improve the sensitivity to load 10ng DNA per lane and detect 0.1 ng DNA with the decreased background noise.

Usually, the intensity of the band can reflect some information of the abundance of bacteria species in the population. However, amplification bias during PCR of multiple DNA templates may exist, as summarized in a review of Kanagawa et al. (2003). For example, GC-rich DNA templates in the priming sites were preferably amplified over AT-rich fragments, probably due to higher binding energy of GC than AT. In contrast, higher GC content in the DNA template may lead to PCR bias producing lower detection, probably due to incomplete DNA denaturing, as shown in a study using 16S rRNA universal primers to detect *Bifidobacterium* species (Suau et al. 1999). Additionally, increased PCR cycles will lead to a strong bias of final PCR products towards equal ratio, regardless of the initial ratio of multiple DNA templates, probably due to re-annealing of PCR products. Limiting the
PCR cycle, optimizing primer specificity, and more efficient replication of PCR are other common strategies to reduce the bias caused by PCR amplification.

It was shown that the template DNA/RNA used in PCR-DGGE analysis may affect the analysis of diversity of bacterial populations. According to Tannock et al. (2004)), cDNA-, obtained with RT-PCR (reverse transcription-PCR) of bacteria RNA, DGGE or named as RNA–DGGE, was markedly different from DNA-DGGE with the Dice’s similarity of only 58.5% when examining bacteria population from human fecal samples. And DNA-DGGE showed no shifts of bacterial pattern by consumption of oligosaccharides in human, whereas increased *Bifidobacterium adolescentis* and *Clostridium aerofaciens* were detected by RNA/cDNA-DGGE. Furthermore, when analyzing the bacteria population pattern in rats fed different carbohydrates, Licht et al. (2006) also reported that RNA/ or DNA-DGGE showed different pattern and *Bacteroides sp.* and *Rikenella sp.* were only detected by RNA-DGGE. It is difficult to explain what caused the discrepancy between these results obtained from DNA and RNA-DGGE, although it was hypothesized that RNA-DGGE might be a better indicator for live bacteria population, and PCR bias caused by different ratio of bacteria DNA templates in genomic DNA or reversed transcribed cDNA might further cause differential DGGE patterns. Further studies with other molecular methods with higher detection sensitivity and specificity, such as gut microbiota sequencing are needed to provide experimental evidence answer the fundamental question of whether the discrepancy is an artefact caused by technical limitation of PCR-DGGE or is a long time overlooked aspects investigating genetic composition of gut microbiota.

The interpretation of DGGE was developed and used with various purposes. In most cases, a database of band patterns based on the presence and absence of the band at its migration position and/or their intensity is collected with the assistance of a computer program. Clustering analysis is a common strategy to explain the similarity/dissimilarly among samples from different treatment or temporal changes. Unweighted pair group method with arithmetic average (UPGAMA) is recommended by some DGGE analyzing program, such as Quantity One (Bio-Rad Laboratories, Hercules, CA), which is based on the presence and
absence of the band only. The dendrographic tree is usually obtained showing similarity index.

However, multivariate ordination methods have been more widely used currently in DGGE analysis. By transforming the complicated data set and presenting on a two or three dimensional graph, ordination analysis could simplify the data and highlight the key factors of the variation observed. Commonly used ordination methods include non-metric multidimensional scaling (nMDs), principle component analysis (PCA) and canonical correspondence analysis (CCA). Non-metric multidimensional scaling (nMDs), which is used in our study, based on the similarity matrix obtained from cluster analysis to explain the variation among individuals within group by calculating the dissimilarity distance. The variation among groups by connecting consecutive points could be showed on a two dimensional graph. Principle component analysis (PCA), is based on the generation of new variables representing the highest distribution of samples, as used by Licht et al. (2006) to reveal distinctive bacterial patterns obtained in rat feeding with five types of carbohydrate diets, including corn starch, inulin oligosaccharides, sucrose and potato starch. Canonical correspondence analysis (CCA) has been used to correlate other environmental variables with the DGGE pattern. In the study of Tian et al. (2009), the bacterioplankton diversity in water samples from different location and different time were assessed by PCR-DGGE, and CCA analysis showed that DGGE pattern was significantly related to dissolved oxygen, total nitrogen and total phosphorus in water samples.

In summary, given the advantage of DGGE as a fast, low cost and culture-independent technique, it is widely used to monitor the shifts of bacteria population from large number of samples.

2.2.2 Genomics of gut microbiota

Even though DGGE analysis is one of the effective methods to characterize the bacterial population, the drawbacks limiting its application include the challenging statistical analysis and other technological difficulties such as PCR bias and the limitation of band resolution,
which make quantification difficult. Other methods, such as real time PCR and FISH (fluorescence in situ-hybridization), are also usually used to further confirm results in the studies. The recently advent of pyrosequencing is a more advanced technique with the advantages of rapidity and high output as described by Ronaghi et al. (1998). Pyrosequencing is based on the theory of “sequencing by synthesis”, in which sequencing of a single strand DNA was conducted by synthesizing its complementary strand. The successful synthesis of one base pair will release ATP, which will be utilized to generate light and further captured and analyzed by computer program. Although only short DNA reads (300-500 bp) are obtained by pyrosequencing, it can be used in large scale sequencing. It is fast and low cost compared to traditional Sanger sequencing. Its application in characterization of human gut microbiome provides the possibility to further understand many gut microbes related health issues such as obesity development (Eckburg et al. 2005).

Recently, a high-density 16S rRNA gene-target DNA microarray was developed to monitor the changes in bacterial populations. In the study of Brodie et al. (2006), a Phylochip was designed to detect bacteria population shifts with soil samples and the probes were designed based on 16S rRNA gene sequences > 600 bp in the database Greengenes (www.greengenes.lbl.gov, release March 2002). Palmer et al. (2006) described a DNA microarray to monitor changes with human colon biopsies samples and the probes were designed based on the full length 16S rRNA genes available in the database (Ribosomal Database Project II (RDP), release in 2001). Very recently, Rajilic-Stojanovic et al. (2009) described another microarray called HIT-Chip (Human-intestinal tract chip) with probes designed based on two most variable regions (V1, 87.51% and V6, 86.74%), as indicated by percentage of distinct sequences detected in the database of http://www.arb-silva.de (release December 2006). However, due to high variability of gut microbes within individual and among individuals, the efficacy of probes covering the bacteria population studied needs to be validated.
2.2.3 Transcriptomics and Proteomics of gut microbiota

In addition to identifying the changes of genetic diversity of gut microbes, gene expression profile at RNA or protein level is important for the functional analysis, especially for the comparison of whole microbial gene expression pattern between individuals with different phenotype, such as lean versus obese, methane versus non-methane producer and etc. The large-scale analysis of RNA or protein, particularly their expression level, structures and functions, was thus known as transcriptomics and proteomics. It might be challenging due to so many unidentified ORFs (Open reading frame) as potential genes with unknown function in the whole gut microbial genome. However, progress is reported by He et al. (2007) who have developed a functional gene array, called Geochip, to detect the functional activity of more than 10 000 genes involved in 150 gene groups of carbon, nitrogen, sulfur and phosphorus cycling. It is anticipated that similar RNA microarray for bacteria carbohydrate fermentation and SCFA production could be developed in the near future.

2.2.4 Metabolomics of gut microbiota

One of the needs in this field is to correlate the microbial profile or their metabolites with particular host phenotype. It is known that the inter-individual variability of drug metabolism and drug efficacy was due metabolic activity of gut microbes, and maybe caused by those minor members in the population. The analytic method currently available is one major limitation to identify the unknown metabolites. And our understanding of the metabolites profiles of gut contents is very limited, let alone the correlation of the metabolite profile with the bacteria population changes. Li et al. (2008) analyzed the fecal and urinary samples from seven Chinese people with 1H NMR spectroscopy and fecal bacteria population with DGGE and cloning library sequencing. With the correlation of the NMR pattern and the microbial pattern using PCA and co-regression analysis, they confirmed many well known bacteria related metabolites such as phenylacetylglutamine, 4-cresol sulfate, and 4-hydroxyphenylacetate. They also found out that Bacteroidetes thetaiotaomicron was correlated to 3-aminoisobutyrate, which was also gender correlated. In addition, Faecalibacterium prausnitzii was correlated to the presence of 8 urinary metabolites
including dimethylamine, taurine, lactate, glycine, 2-hydroxyisobutyrate, glycolate, 3,5-hydroxylbenzoate, and 3-aminoisobutyrate. It is anticipated that the effect of RS on gut microbes could be further clarified at genomic, transcriptional and metabolic levels, which is important for the further manipulation of gut micro-system with dietary intervention.

2.3 RS as substrates for gut microbes

Gut microbes are estimated to be exposed to 60-80 g carbohydrates and 3-9 g protein coming from undigested diet and endogenous host cells as summarized in the review of Topping et al. (2001). The daily intake of RS, as one of the major carbohydrate substrates, was estimated to be 8-40 g depending on the dietary habitation and geographic factors. Others potential carbohydrates reaching the colon in a adult human are non-starch polysaccharides (NSP) with 8-18 g/d, oligosaccharides with 2-8 g/d, simple sugars with 2-10 g/d. The end fermentation products of RS are gases (methane, hydrogen, carbon dioxide) and short chain fatty acid (acetate, propionate, butyrate and valerate), as shown by Figure 2. Other intermediate products such as organic acids (lactate, succinate and formate), branch short chain fatty acid (BCFA) (isobutyrate and isovalerate), and alcohols (methanol and ethanol) are also produced, although with much smaller amount.

Carbohydrate degradation is a cooperative process in the lower gut, which could be generalized as (Figure 2): 1) the degradation of polymer structure into sugar monomer, 2) the glycolysis and the production of SCFA or other organic acids, and 3) the hydrogen consumption during methanogenesis, sulphate reduction and acetate production. The amylolytic bacteria and butyrogenic bacteria are the major focuses in determining fermentation outcome of RS in the lower gut.
Figure 2 Starch degradation and short chain fatty acid (SCFA) production
2.3.1 Amylolytic bacteria

The amylase mediated starch break-down includes α-amylase for α-1, 4 linkage, type I pullulanase for α-1,6 linkage, and amylopullulanases for both 1, 4 and 1, 6 linkage (Erra-Pujada et al. 1999). Members from three major phyla, Firmicutes, Bacteroidetes and Actinobacterium, which account for almost 95% of total population in the gut, have been implicated to be involved in starch fermentation. Macfarlane et al. (1986) used peptone-yeast (PY) agar plate to screen soluble starch-hydrolyzing strains by clear zone formed around colonies. They reported that based on 120 amylolytic colonies randomly selected from fecal samples of 6 human subjects, 58% were identified as Bifidobacterium spp. with lactate and acetate as major products. About 18% of starch-hydrolyzing fecal isolates were identified as Bacteroides spp. with acetate and propionate as major products, and another 10% of amylolytic isolates were identified as Fusobacterium and Butyribrio with butyrate as major product.

However, the picture of amylolytic bacteria might be different when using other types of starch with the differences in structure or composition. Wang et al. (1999) screened 38 human colonic bacteria strains by measuring the clear zone on PY medium agar plates supplemented with autoclaved starch granules to mimic food preparation. They reported that although Bifidobacterium spp., Bacteroides spp., Fusobacterium spp. and strains from Eubacterium, Clostridium, Streptococcus, and Propionibacterium all had amylase activity to utilize high amylopectin and soluble starch, only Bifidobacterium spp. and Clostridium butyricum could efficiently utilize high amylose starch granules.

The binding to starch granules might be important for bacteria degradation in the colon over constant bowel movement. Currently, only two structures have been clarified in gut bacteria which might be related to starch binding: the cellulosome structure in Ruminococcus flavefaciens for insoluble substrates and outer membrane protein (OMP) complex in Bacteroidetes thetaiotaomicron for soluble substrates.
The cellulosome complex was first identified in rumen isolate *Ruminococcus flavefaciens*, which is also the commensal bacteria in human gut (Flint et al. 2008). Most components in the complex are encoded by *Sca* cluster, including scaffolding proteins ScaA-ScaB-ScaE and various glycoside hydrolases, polysaccharide lyases and esterases. The anchor of these enzymes to the cell member through dockerin-cohesin recognition between enzymes and structural scaffolding proteins could enhance the efficiency of polymers degradation. The solubilized products such as oligosaccharides and polymers could be further utilized by other amylolytic bacteria as cross-feeding substrates. It is unknown whether other Ruminococcus species in have similar complex, but *Ruminococcus bromii*, which has been reported to be enriched by RS feeding both *in vivo* and *in vitro* studies, has been shown to be evolutionarily close-related to *R. flavefaciens*.

OMP complex in *Bacteroidetes thetaiotaomicron* is related to solubilized starch utilization with its *sus* (starch-utilization-structure) gene clusters. Seven structural genes (*susA* to *susG*) encode a outer-membrane protein (OMPs) complex (*susC, -D, -E, -F, and -G*) involved in starch binding and *susA* and *B* encode periplasmic amylase for starch hydrolysis. SusC and G have been shown to break-down the larger polymers and transport the smaller molecules with the size ranging from maltose or (G2) to maltoheptaose (G7) into periplasm for further degradation (Reeves et al. 1997).

The starch binding ability in other gram positive bacteria might be mediated by cell associated alpha-amylase or other surface protein. Ramsay et al. (2006) identified the amylase (Mw = 140-210 KDa) in *Butyrivibrio fibrisolvens Roseburia inulinviorans* and *Roseburia intestinalis*, which were known as butyrate producing bacteria. They also reported that the alpha amylase activity is cell-associated by comparing the amylase activity of supernatant and whole-cell fraction resuspended in sodium phosphate buffer. In addition, Crittenden et al. (2001) used co-sedimentation assay to examine the affinity of 19 Bifidobacterium strains with the high amylose starch *in vitro*. They found out that two amylolytic strains, *Bifidobacterium pseudolongum* (ATCC 25526) and *Bifidobacterium adolescentis* (VTT E-001561), had the highest affinity and specificity for α-1,4-linked
glucose sugars. The starch binding could be inhibited by pancreatin and low pH (< 3), indicating that the cell surface proteins were involved in the starch attachment. The strong attachment by *Bifidobacterium spp.* to starch granules might partly explain its enrichment by RS feeding studies.

### 2.3.2 Butyrogenic bacteria

With the physiological significance of butyrate identified so far, which will be further discussed later in the thesis, the identification of butyrogenic bacteria is one of the major focuses in the field. The early work of isolation of butyrogenic bacteria was described in Barcenilla et al. (2000). In the study, fecal samples from three individuals: one infant, one adult omnivore and one adult vegetarian were taken at the interval of one year. Microbes from fresh fecal suspension was grown on nutrient rich M2 medium supplemented with glucose, soluble starch and cellobiose (M2GSC), and butyrate producing level higher than 2 mM in the M2GSC broth was used as criteria for butyrate producing bacteria. 74 isolates out of total 313 (23%) showed the ability to produce butyrate. Further analysis of the full length 16S rRNA sequences showed that 80% of butyrate-producing isolates fell within the XIVa cluster with the most abundant group (42%) related to *Eubacterium rectale*, *Eubacterium ramulus*, and *Roseburia cecicola*. So far, most members from cluster XIVa (or *Roseburia/coccoides/E. rectale* group) and cluster IV (*Clostridium leptum* group) (family *Lachnospiraceae* and *Ruminococcaceae* respectively) are frequently detected in human fecal microbiota as summarized in Pryde et al. (2002) and adapted in (Table 1).
<table>
<thead>
<tr>
<th>Cluster XIVa</th>
<th>Butyrate producing</th>
<th>Non-butyrate producing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eubacterium sp. L2-7</td>
<td>Eub. haii</td>
<td>Eu. eligen s</td>
</tr>
<tr>
<td>Anaerostipes caccae L1-92</td>
<td>Eu. hadru m</td>
<td>Clostridium aminovalericum</td>
</tr>
<tr>
<td>Butyrivibrio crossotus</td>
<td>B. fibrisolvens NCDO 2223</td>
<td>Cl. indicolis</td>
</tr>
<tr>
<td>Coprococcus sp. L2-50</td>
<td>Co.eu tacuts</td>
<td>Cl. sphenoide s</td>
</tr>
<tr>
<td>Co.sp. A2-175</td>
<td>Co.catus</td>
<td>Cl. clostridium forms</td>
</tr>
<tr>
<td>Cl. symbiosum</td>
<td>Cl.symbiosum</td>
<td>Ruminococcus obeum</td>
</tr>
<tr>
<td>Eu.cellulosolves</td>
<td>Eu.cellulosolves</td>
<td>Eu. hansenii</td>
</tr>
<tr>
<td>Eu.saburreum</td>
<td></td>
<td>Ru. product us</td>
</tr>
<tr>
<td>Ru.hansenii</td>
<td>Eu. ventrius</td>
<td>Ru. amnisenii</td>
</tr>
<tr>
<td>Ru.productus</td>
<td>Eu. oxidoreduces</td>
<td>Ru. pr. productus</td>
</tr>
<tr>
<td>Eu.sp. A2-231</td>
<td>Eu.rectale</td>
<td>Eu.uniforme</td>
</tr>
<tr>
<td>Ru.sp. A2-183</td>
<td>Roseburia eecioia</td>
<td>Ru.gnavuses</td>
</tr>
<tr>
<td>Eu.sp. L1-86</td>
<td>Ru.intesinalis L1-82</td>
<td>Ru.torques</td>
</tr>
<tr>
<td>Eu.rectale</td>
<td>Eu.ramulus</td>
<td>Cl. cutortum</td>
</tr>
<tr>
<td>Eu.sp. A2-194</td>
<td></td>
<td>Eu.controltum</td>
</tr>
</tbody>
</table>

*Table 1* Butyrate producing and non-butyrate producing bacteria in *Clostridium* clusters identified in human gut microbiota (Adapted from Pryde et al. (2002))
**Table 1** Butyrate producing and non-butyrate producing bacteria in *Clostridium* clusters identified in human gut microbiota (Adapted from Pryde et al. (2002)) (continued)

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Butyrate producing</th>
<th>Non-Butyrate producing</th>
</tr>
</thead>
<tbody>
<tr>
<td>XVIII</td>
<td></td>
<td><em>Cl.ramosum</em></td>
</tr>
<tr>
<td>XVI</td>
<td></td>
<td><em>Cl.spiroforme</em></td>
</tr>
<tr>
<td>Xlb</td>
<td></td>
<td><em>Cl.glycolicum</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cl.difficile</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cl.bifermentans</em></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td><em>Cl.limosum</em></td>
</tr>
<tr>
<td>I</td>
<td></td>
<td><em>Cl.beijenrincii</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cl.butyricum</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cl.tertium</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Eu.moniforme</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cl.aurantibutyricum</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cl.acetobutyricum</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cl.tyrobutyrisum</em></td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td><em>Faecalibacterium</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>F.prausnitzii</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>F.prausnitzii</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cl.sporophylochaetes</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Eu.sp. A2 = 207</em></td>
</tr>
<tr>
<td>XV</td>
<td></td>
<td><em>Ru.bromii</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cl.leptum</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Eu.siraeum</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ru.albus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ru.flavefaciens</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Eu.desmolans</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Eu.barkeri</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Eu.limosum</em></td>
</tr>
</tbody>
</table>
Two mechanisms for butyrate production have been implied as summarized in the review of Louis et al. (2007) with butyrate kinase and butyryl CoA : acetate CoA transferase. After hydrolysis of glucose through EMP (Embden-Meyerhof-Parnas) pathway, pyruvate is considered as the major precursor with further conversion into acetyl-CoA, lactate and succinate by gut microbes. Acetyl-CoA, for example, could be either used for butyrate synthesis by butyrate kinase (Zhu et al. 2005) or converted into acetate with reciprocal transition. Nevertheless, acetate could be utilized by butyryl-CoA : acetate-CoA transferase as a CoA acceptor in the second route of butyrate synthesis in bacteria such as in *Roseburia intestinalis* (Duncan et al. 2002). A third route has been implicated recently by Belenguer et al. (2006). They co-cultured lactate producing bacteria *Bifidobacterium adolescentis* with starch utilizing bacteria *Eubacterium hallii* and *Anaerostipes caccae* in the yeast extract-casitone-fatty acid (YCFA) medium supplemented with soluble potato starch. By tracing the (1-13C) labeled acetate or (U-13C) labeled lactate, they found out that butyrate could be converted directly from lactic acid without exogenous acetate involved. Although acetyl CoA was involved as a intermediate, it was unknown whether the butyryl CoA : acetate CoA transferase was involved.

The distribution of butyrate kinase gene (*buk*) and butyryl-CoA : acetate CoA transferase genes (*ptb*) in gut bacteria were largely unknown until the study of Louis et al. (2004). They obtained 38 butyrate producing isolates from 4 subjects and the presence of two genes with PCR. They found out that only 4 out of 38 butyrate-producing strains contained butyrate kinase genes, whereas the butyryl-CoA : acetate CoA transferase gene was detected in all the isolated bacteria. This might be consistent with what Barcenilla et al. (2000) reported that 50% of butyrate-producing isolates showed significant correlation between the butyrate production and acetate disappearance ($r^2 = 0.6$) with the rate of 1 mol acetate consumed along with 2 mol butyrate production. Further in Louis et al. (2010), they collected fecal samples from 10 healthy human subjects and obtained 1 718 sequences with degenerate primers to amplify butyryl-CoA : acetate CoA transferase genes. They identified 32 OTUs as butyryl-CoA : acetate CoA transferase related genes (with cut off value as more than 98% similarity) in the human gut, and four of them, from *Eubacterium rectale, Roseburia faecis,*
*Eubacterium hallii* and an unnamed cultured species SS2/1, were highly abundant in the human gut. Beyond the results, the study is more significant in its approach of using certain functional gene to analyze the changes induced by diet intervention or treatment, as shown in the same study that *Faecalibacterium prausnitzii* was significantly increased ($P = 0.019$) after ingestion of inulin (5 g, twice per day) for 21 d compared to their normal diet without inulin supplement. Compared to 16S rRNA gene profiling, the analysis of key genes in a certain metabolic process of gut microbiota is more specific and important for understanding the function of gut microbes.

In addition to varying abundance of the bacteria or functional genes in the gut, the butyrate producing ability may vary among bacteria species. Louis et al. (2004) showed the varied activity of both butyrate kinase and butyryl-CoA : acetate CoA transferase among strains and species of butyrate producing isolates. However, many questions are left unresolved yet. For example, with metagenomic analysis butyrate kinase related genes were found to be one of the most enriched Clusters of Orthologous Groups (COGs) in fecal bacteria from two human subjects, whereas the butyryl-CoA : acetate CoA transferase has not been implicated as an enriched COGs (Gill et al. 2006). COG is a taxonomy protein families (usually more than three orthologs) based on both characterized proteins or predicted proteins from completed genome sequenced. The database of COGs can be used to annotate function of new gene sequence. It is unknown whether the methods and variability among individuals might cause the discrepancy. In addition, butyrate producing regulating gene haven’t been determined to date. Moreover, although some *Bacteroides spp.* and *Clostridium spp.* were known to produce propionate its production pathway and especially the regulation of its production in the bacteria by other SCFA are largely unknown. The detailed functional analysis of SCFA producing activity is anticipated with transcriptomics in the near future.

### 2.3.3 Methanogenic archaea

Gas production is another well known fermentation outcome, especially the production of methane, which can be considered as the final end product consuming hydrogen and carbon dioxide. The distribution of methanogenic archaea in the population is one of the examples of inter-individual variability. It was estimated that the proportion of methane producer in the
population varied from 24% in Oriental population to 48% in Caucasian population by measuring breath hydrogen with lactulose intake (Pitt et al. 1980) Methanogens have been suggested to be related to irritable bowel syndrome (IBS). For example, Rana et al. (2009) reported that the proportion of methane producers in healthy Indian subjects were significantly higher than Indian patients with IBS by measuring breath methane level after 10 g lactulose consumption (34.6% (88 out of 254) versus 14.5% (50 out of 354), \( P < 0.001 \)).

The existence of methanogenic archaea might affect the fermentation capacity of individuals. For example, Abell et al. (2006) reported that the abundance of methanogenic archaea was negatively related to the fecal butyrate concentration (\( R = -0.729, P < 0.05, n = 8 \)) but not with other SCFA, with PCR-DGGE analysis of methanogens with methanogen specific primers coupled with and real time PCR for the quantification analysis. Although genome sequences of two methanogens are available, their physiological significance is still largely unknown. This might be similar to the situation of other microbes in the gut, especially in the context of a microbial population with dynamic interaction (competition and cross-feeding). Further studies are needed to identify the role that methanogenic archaea play in RS fermentation.

In summary, as discussed above, the utilization of RS is cooperative and redundant in the gut. The identification of bacteria related to RS fermentation is important in the predication of fermentation outcome of RS. Therefore, recent studies have been focused on two interrelated aspects: one is to identify the bacteria related to RS fermentation, and the other is to characterize the metabolic outcomes of RS fermentation, which are crucial for understanding the underlying mechanism of RS in disease prevention.

### 2.4 RS as a prebiotic

“Prebiotic” was described by Gibson et al. (1995) as “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health”. Correspondingly, these beneficial bacteria are considered as probiotic, which was defined as “the live microorganisms which when administered in adequate amounts confer a health benefit on the
host”. A range of non-digestible food carbohydrates are categorized into prebiotics, including lactulose, inulin, non-digestible oligosaccharides, such as fructo-oligosaccharides and mannan-oligosaccharides (MOS) and non-starch polysaccharides.

As the major substrate for gut microbes, RS has been shown to modulate the bacterial pattern by enriching amylolytic and SCFA producing bacteria (Sharp et al. 2000; Leitch et al. 2007; Abell et al. 2008; Kovatcheva-Datchary et al. 2009). In addition, the fermentation products, mainly short chain fatty acid (SCFA) have been implicated to be involved in many physiological processes to promote human health. Therefore, RS now is considered as a prebiotic with the properties to modulate bacteria population to increase SCFA production.

2.4.1 RS and SCFA production

2.4.1.1 Human studies

The common fermentation outcomes of RS can be summarized as increasing fecal bulking, decrease fecal pH, increase fecal SCFA production, decreasing fecal secondary bile acids levels (Phillips et al. 1995; Hylla et al. 1998; Jenkins et al. 1998). The production of SCFA is most studied with its physiological significance to improve colon health.

In the study of Jenkins et al. (1998), 12 women and 12 men were fed low fiber diet with or without the supplementation of 30 g RS2 (high amylose starch) or RS3 (retrograded high amylose starch) daily for 2 wk, followed by a 2-wk washout intervals. They reported that butyrate concentration in the fecal water was increased by RS (mean of RS2 and RS3) (22.7 ± 1.7 mmol/L) compared to low fiber diet (19.2 ± 2.4 mol/L, P = 0.006). Similar results were reported by other studies. For examples, human feeding trial showed that 3-wk feeding of high RS diet (39 g/d) significantly increased total daily fecal output (by an average of 42%) , lowered fecal pH (by 0.6 unit) and increased fecal acetate (by 38%) and butyrate concentration (by 26%) compared to low RS diet (5 g/d) (Phillips et al. 1995).

However, discrepancies have been reported. In the study of Hylla et al. (1998), high RS starch diet amylomaize (55.2 ± 3.5 g RS/d) or low RS starch diet (7.7 ± 0.3 g RS/d) was given daily to 12 human subjects for 4 wk. They reported that fecal propionate was
significantly decreased by RS feeding (HRS: 10.8 ± 0.6 versus LRS: 14.6 ± 1.3 mmol/g wet wt, \( P < 0.05 \)). No difference in other SCFA concentration was observed between two diets. Similar results were reported by Heijnen et al. (1998), in which 1-wk consumption of uncooked high amylose starch (RS2) and retrograded high amylose starch (RS3) daily (32g/d) did not change SCFA concentration compared to glucose feeding (\( n = 23 \)), although it was argued that feeding period of 1 wk might not be sufficient for the adaptation to an RS diet.

In addition, the profile of individual SCFA might differ with varied RS type or its plant source. Cummings et al. (1996) reported that, compared to a starch free (SF) diet, total fecal SCFA was only increased by consumption of type2 RS from potato (17-30 g/d) for 15 d (SF: 80.2 mmol/kg versus potato RS2: 99.7 mmol/kg), but not by RS from banana (type2 RS), retrograded wheat and maize (type3 RS). In addition, butyrate proportion was only increased by potato starch (SF: 15.2% versus potato RS2: 18.4%), and propionate proportion was only increased by retrograded wheat starch (SF: 16.8% versus wheat RS3: 20%). RS from retrograded wheat and maize starch decreased acetate proportion (SF: 55.1% versus wheat RS3: 52.1% and maize RS3: 51.6%). Even though, daily fecal output of SCFA (total and individual) were increased by all RS feeding, since the daily fecal output was increased 30% to 49% compared to SF diet.

Due to the discrepancies in observed SCFAs in the cecum, colonic content and fecal content in \textit{in vivo} studies of RS, it might raise the question as to whether fecal and colonic contents could reflect the uptake and utilization of SCFA in colonocytes. Although higher fecal SCFA was accepted as the indicator of higher colonic production of SCFA, Vogt et al. (2003) found out that it might not be the case and showed fecal acetate level might be an indicator of its lack of absorption, rather than production. In their study, 10 healthy subjects were recruited and rectal infusions were repeated 10 times in each subject at 3 d intervals. Subjects were given high fiber foods before 12 h fasting, followed by emptying colon by infusion 500 mL distilled water and collecting baseline samples as well. The rectal infusions contained 300 mL single SCFA or combined SCFA at the concentration of acetate (60 mmol/L), propionate (20 mmol/L) and butyrate (20 mmol/L) were given to subjects and retained for 30 min. Then void samples were taken. The absorption of SCFA was calculated as the absolute difference
of SCFA detected before and after the rectal infusion. Fecal samples were collected at least one week after the infusion protocols. They found out that the fecal acetate concentration was inversely correlated to the acetate absorption calculated during rectal infusion of 3 SCFA ($r = -0.834, P <0.005$). Although further studies are needed to validate the conclusion, the results lead us to rethink the analytic methods of measuring SCFA absorbed by colonocytes, rather than how much SCFA is left or is excreted into feces.

Gas chromatography analysis is one of the most common techniques for quantification analysis of SCFA with its high sensitivity and resolution capacity. The extraction methods of SCFA for GC analysis include organic solvents extraction using acids, ether, ethanol, or chloroform, steam distillation and vacuum distillation to clean up the samples. The efficiency and the recovery rate of these methods may affect SCFA detection. Timm et al. (Timm et al. 2008) compared the two published SCFA extraction methods with aqueous (AQ) and diethyl ether (DE) in their ability to detect fecal SCFA level. They found out that AQ methods gave higher acetate ($P = 0.0001$) concentration, whereas DE gave higher propionate ($P = 0.0147$) and butyrate ($P = 0.0002$) concentration when the same samples were used ($n = 10$). Further studies are needed to compare the effects of extraction methods on the SCFA measurement.

Even with the methodological challenges, most human studies showed RS consumption increased SCFA production, as shown either by the fecal concentration or total fecal output, although variability exists in the individual SCFA profile depending on the type of RS or its origin.

### 2.4.1.2 Animal studies

A body of studies has shown that RS increased SCFA level in cecum, colon and feces in animals fed RS diet as described in (Cassand et al. 1997; Le Leu et al. 2002; Le Leu et al. 2007; Liu et al. 2008; Le Leu et al. 2009). The commonly used RSs include type 2 RS (such as raw potato starch and high amylose starch (HAS) and type 3 RS (retrograde HAS or gelatinized RS) and the dose of RS commonly used is 10% and 20% in the diet (wt/wt). Heat moisture-treated RS was another strategy to increase RS content. However discrepancies still exist due to varied type of RS used.
Similar to human studies, although total SCFA were shown to be increased by RS feeding, individual SCFA profile may be different depending on the type of RS used in the study. In the study of Ferguson et al. (2000), three types of RS were used: Hi-maize starch (HAS), α-amylase-treated HAS and potato starch, and in the concentration of 35% (wt/wt) in the diet for male Wistar rats (six-wk-old). After 4-wk feeding, they reported that the concentration of total SCFA and acetate were increased by all 3 RS compared to control diet with normal corn starch in the cecum, midcolon and rectum, although HAS showed highest concentration. However, varied profiles of propionate and butyrate were observed: butyrate was decreased by HAS feeding throughout the colon (e.g. 0.88 mmol/g wet wt compared to 1.89 mmol/g wet wt in control diet in cecum), but increased by the other two RS diets. Propionate was decreased in cecum by potato starch feeding and was not detectable in colon and rectum, but it was unchanged by other two RS in cecum and increased in midcolon and rectum. Even though, with the significant increase of cecum weight, the total SCFA (µmol/cecum) was increased by all 3 RS feeding with up to 10 folds difference.

In addition, it was thought that increased RS dose or RS content might increase the SCFA production by RS feeding. However, increased RS content in the diet didn’t increase SCFA level proportionally. In the study of Le Leu et al. (2003), 10%, 20% and 30% HAS was fed to 5-wk-old male Sprague-Dawley (SD) rats for 4 wk. Only acetate was increased proportionally by three RS doses. The total SCFA and butyrate concentration in cecum were increased to the same extent among three RS dose (e.g. butyrate: 10.1 ± 1.1 µmol/g, 10.0 ± 1.1 µmol/g, 10.3 ± 1.4 µmol/g compared to control 7.4 ± 0.5 µmol/g). Propionate was decreased by 30% HAS feeding. Similar patterns of propionate and butyrate concentration were observed in the feces.

On the other hand, heat moisture treatment (HMT), which is carried out by heating starch at 100–125 °C under 100% relative humidity, is one strategy to increase RS content. Jacobasch et al. (2006) reported that heat moisture treated Novelose 330 (HMT-N) for 25 d, which is a retrograde HAS, significantly increased butyrate concentration in the cecal and proximal colon compared to control diet in male Shoe-Wistar rats (no age provided), and in the distal colon and feces compared to both untreated Novelose330 and control. In addition,
there was no effect of starch diet on propionate concentration in cecal and proximal colon, but significantly higher propionate was observed in the distal colon and feces of animals fed two RS diets. However, HMT did not increase SCFA production in the study of Kishida et al. (2001), in which gelatinized normal cornstarch (G-CS), HACS, gelatinized HAS (G-HACS) and heat moisture-treated HACS (HMCS) were fed to male Wistar rats (6-wk-old) in the dose of 40% in the diet (wt/wt) for 21 d. RS content in HACS, G-HACS and HMCS was 19.3, 2.4 and 64.5% respectively. There was no difference in cecal acetate and propionate concentration. However, butyrate level was significantly decreased by HACS (17.3 ± 4.4 µmol/L) and HMCS (12.8 ± 2.6 µmmol/L) compared to CS (45.5 ± 2.5 µmol/L) and G-HACS (45.2 ± 9.4 µmol/L). Instead, succinic acid was significantly increased by HACS (79.6 ± 25.7 µmol/L) and HMCS (82.5 ± 15.7 µmol/L) feeding compared to G-CS (14.2 ± 4.5 µmol/L) and G-HACS (25.9 ± 13.2 µmol/L). Cecum pH was significantly decreased by all three RS, but in the order of G-HACS (6.7 ± 0.1), HACS (5.4 ± 0.1) and HMCS (5.7 ± 0.1) compared to G-CS (7.2 ± 0.2). Similar results were reported by Satio et al. (2001), in which the RS content was increased from 31.8% in HAS to 67.3% in HMT-HAS. After 10-13 d feeding of starch diet (10% wt/wt diet), total SCFA concentration in the cecum was increased by both HAS and HMT-HAS compared to control corn starch feeding group in male Wistar rats (4-wk-old). However, individual SCFA concentration in the cecum was only increased by HAS feeding, but not by HMT-HAS feeding. It is not fully understood what determined the varied SCFA profile by different type of RS, although gut microbes might be the major reason with their varied fermentation capacity to utilize RS as substrates. This needs further exploration. In addition, the physiological significance of these RS needs to be further tested on its effect to improve colonic function or prevent colon cancer risk.

2.4.1.3 *In vitro* studies

Most *in vitro* studies showed that RS or starch favors butyrate production, compared to other fermentable substrates available in the human diet. Macfarlane et al. (1993) reported that the molar proportion of butyrate produced by starch was the highest (acetate: propionate: butyrate 50:22:29) compared to pectin (84:14:2), arabinogalactan (50:42:8), xylan (82:15:3) in a pH controlled batch fermentation model for 50 h (no other details available).
Similarly, Wang et al. (2004) collected the ileal effluent of pigs fed animal proteins only or animal protein with potato starch (as RS), sugar beet pulp (SBP), or wheat bran (Nanthakumar et al. 2005) respectively and incubated with the feces collecting from the same pigs. The mineral salts medium was used in the batch fermentation and the SCFA production at the end of 48 h were compared among four treatments. They reported that both RS and SBP increased butyrate concentration compared to non-carbohydrate feeding group (4.2 and 4.4 versus 3.2 mmol, $P < 0.05$) in 10 ml fluid volume, but only RS significantly increased molar ratio of butyrate (acetate: propionate: butyrate = 54:22:16) and only WB increased the molar ratio of propionate (acetate: propionate: butyrate = 55:28:10).

One of the advantages of in vitro incubation system is in tracing SCFA formation, as shown by Miller et al. (1996). In the study, $1^{-14}$C labeled glucose, $3, 4^{-14}$C labeled glucose, or $^{14}$CO$_2$ was added to human fecal inocula ($n = 2$), and they found out that Wood-Ljungdahl pathway as one of the major acetate producing pathway and Embden-Meyerhof-Parnas (EMP) pathway of glucose metabolism. Propionate was shown to be produced from CO2 fixation pathways in Propionibacteria and butyrate was formed by acetyl-S coenzyme A pathway. In addition, when cabbage cellulose was used as one of the substrate for fermentation, the acetate was formed partially from a Bifidobacterium pathway in the fecal inocula from one subject, indicating that Bifidobacterium was involved in the digestion of cellulose. Another very interesting study is using in vitro fermentation model analyzing the effect of acarbose, which is an amylase inhibitor in human intestine to increase starch delivered to colon, on the fermentation capacity of gut microbes (Wolin et al. 1999). In the study, 40 subjects were recruited for an acarbose-placebo cross-over trial, in which acarbose were given as tablet (100 mg daily) for 4 months, followed by a 3 or 4 months resting period without any tablet intake before starting the next treatment. With anaerobic incubation of ($1^{-13}$C) glucose and circulating $^{12}$CO$_2$, which were measured with NMR (nuclear magnetic resonance spectrometry) and GC, with fecal inocula collected from these subjects, molar ratios of acetate, propionate and butyrate were 57:20:23 for placebo individuals, and 57:13:30 for acarbose treated individuals, indicating butyrate production ability was increased by acarbose treatment and propionate ability was decreased.
In addition, *in vitro* fermentation has been used to study the adaptation of subjects to the long term RS feeding. For example, Chrisl et al. (1997) took fecal samples from 10 subjects who ingested 7.0 to 8.3 or 50.7 to 59.7 g/d of resistant starch (Hylon VII) for 4 wk, and incubated with starch *in vitro* for up to 48 h with 10 g/L RS or digestible starch. They reported that two out of ten subjects didn’t completely ferment RS, and showed lower butyrate production compared to the other 8 subjects with higher fermentation capacity, indicating great inter-individual variability in the human subjects responding to RS feeding. (Abstract available only).

In summary, based on the results of human and animal feeding studies discussed above, RS has been shown to increase SCFA, especially butyrate production, the extent of which may be affected by preparation methods of RS, physical-chemical properties of RS and etc. Additionally, *in vitro* fermentation models are also useful in assessing fermentability of RS and understanding chemical events occurred during starch degradation and SCFA production by tracing carbon conversion.

### 2.4.2 Methodology of *in vitro* fermentation system

Currently, simple culture is still the major method used to characterize RS fermentability with the advantages of rapidity, low cost and easy control in a standard laboratory, compared to expensive *in vivo* human feeding studies. The fermentation systems used currently include simple batch fermentation and dynamic fermentation.

#### 2.4.2.1 Batch fermentation system

Batch fermentation refers to simple incubation of substrates with bacteria mixture (usually fecal inoculums) for a certain period of time. Usually, the starch disappearance, ammonia and SCFA production, pH changes and gases production within 24-48 h incubation are monitored as indicator for the fermentation outcomes. Some batch fermentation was extended to 72 or 96 h with the purpose of full fermentation of carbohydrate substrates. Batch fermentation system is widely used in characterizing the degradation rate of RS and time course changes in the metabolites pattern. For example, Macfarlane et al. (1986) used pH controlled batch
fermentation system showed that during the first 6 h incubation of freeze-dried, boiled potato starch, lactate (14.6 mM) was produced in the similar amount as butyrate (11.6 mM) and propionate (14.6 mM), but was undetectable at 24 h. Other three SCFA production showed continuous increasing till 12 h and kept at plateaus till 96 h later.

2.4.2.2 Dynamic fermentation system

The simplest dynamic system is similar to batch incubation but with controlled fluid volume by constantly feeding and draining medium and substrates, thus also known as continuous fermentation. It has better controlled condition than batch fermentation for longer incubation, but still needs special experimental equipment. More complicated dynamic fermentation system was described as three stage-system described by Macfarlane et al. (1998). In the system, three vessels (V1, 2 and 3) are controlled with different pH condition to simulate the proximal colon (pH = 5.5), transverse colon (pH = 6.2) and distal colon (pH = 6.8). Another example is Dutch TIM2 (TNO gastro-Intestinal Model-2) as described by Minekus et al. (1999). Similar multi-compartmental systems are included to simulate the large intestinal conditions but more controlled by computer. The advantage of these complicated dynamic systems is that they are more similar to large intestine conditions in vivo, which are characterized by regional differences in the substrate availability and pH condition. However, more expensive experimental equipment and special training are required for routine laboratories. Only limited number of fermentations could be tested at each time due to the availability of the equipment. Therefore, in most cases, the three-stage system or TIM2 described above were used to characterize the bacteria population by particular substrate with long-term incubation.

Only one study so far has been conducted by Fassler et al. (2006) to compare the batch system and dynamic system (TIM2) so far. They reported that with batch fermentation system, the blank samples (ileal delivery medium only) after 8 hr fermentation showed similar SCFA ratios (66:11:18) to that reported in the colon of sudden death victims (57:22:20). In addition, with batch system, the accumulation of SCFA was rapid in the first 8 h, and maintain plateau till the end of 24 h incubation, whereas with TIM2 system, the accumulation of SCFA was progressively increased along with 72 h incubation, indicating different dynamics of substrate degradation. However, it seems to be inappropriate to
compare the two systems since in batch system, substrate was added at once and in dynamic system, substrate was fed continuously. The author concluded that the batch model was more reproducible and appropriate for screening compounds and TIM2 model was better for the simulation of human colon behavior in vivo.

2.4.2.3 Factors affecting the efficacy of in vitro fermentation system

In addition to the influence of in vitro system themselves, other parameters are needed to be considered in order to simulate the in vivo conditions, including substrates preparation, inoculums/substrates concentration, medium components, incubation condition, and the fluid surface/volume ratio.

2.4.2.3.1 Starch substrate

The pre-digestion of starch substrates is crucial in simulating in vivo conditions, which has been overlooked in many early in vitro studies. In reality, the starch residue is the substrate for the gut microbes. Additionally, the cooking procedure has been shown to influence the RS content by comparing (Parchure et al. 1997). They reported boiling and pressure cooking increased RS content, whereas roasting, extrusion cooking, frying and drying decreased RS content. Therefore, the application of raw starch in vitro will not accurately reflect the fermentable outcomes, as well as in the animal feeding study in vivo. Currently, the pre-digestion of RS or other dietary fiber has been included before in vitro fermentation as shown by Sayar et al. (2007), in which β-glucan (oat flours) was heated in the boiling water bath, followed by salivary amylase digestion (pH = 6.9, 37 °C), pepsin digestion at acidic stomach condition (pH = 2.0, 37 °C), and pancreatin digestion at small intestine condition (pH = 6.9, 37 °C). For RS, AOAC 991.43 is one of the standard protocols to measure the resistant starch content, and it could be utilized for the preparation of RS starch residue for in vitro fermentation. Another alternative for fermentation substrates, but more complicated, is the ileal digesta taken from animals or humans as shown by Langkilde et al. (2002). In the study, ileostomy subjects with previous proctocolectomy for ulcerative colitis were recruited (n = 10). Ileostomy bags were changed every 2 h during 24 h, and ileostomy effluents were pooled and analyzed. They also reported that less starch residues was recovered from ileal
digesta of the subjects given cooked banana flours (6.3 g residue/30 g raw flours) compared to that of subjects given raw banana flours (15 g residue/30 g raw flours). Therefore, choosing an appropriate substrate is crucial in assessing physiological significance of RS with an aim to simulate human consumption of cooked resistant starchy food in vivo.

2.4.2.3.2 Inocula/substrate ratio

The ratio of inoculum to substrate may also influence the fermentation outcome. In the study of Stevenson et al. (1997), with the increase of substrate amount (2.5 g/liter, 5 g/liter) incubated with an aliquot of rat cecal inoculum, the SCFA production was increased proportionally at the end of 24 h incubation, but decreased with 10g/liter substrates. However, there is no commonly established standard for the inoculum/substrate used. In addition, since the digested residue should be used in the in vitro fermentation system, the determination of the amount of substrates and inoculums added is more challenging.

2.4.2.3.3 Anaerobic condition

A strict anaerobic condition is required for studying the metabolic effects of RS and identifying the bacteria degrading RS as well. The continuous CO₂ flow is applied during the handling of the fecal samples, and anaerobic jar and anaerobic chamber are commonly used for anaerobic incubation and sample handling. For example, with the anaerobic chamber used in our study, the sample could be transferred through an air lock, from which the oxygen was vacuuming out by two cycles filling in with N₂, and one cycle filling in with Anaerobic Mixture Gas (AMG: 10% H₂, 5% CO₂ and 90% N₂). The medium or substrates could be kept in the oxygen free chamber and trace oxygen will be absorbed by the palladium catalyst. Oxygen consuming reagent (such as cysteine sulfate) and oxygen sensitive indicator (such as resazurin) are also usually present in the culture medium to help to guarantee anaerobic conditions as well.

2.4.2.3.4 Medium Selection

The medium chosen in the starch fermentation studies varied depending on the experimental purpose. Some studies used nutrient-rich medium including both abundant peptide and sugar
sources (Sayar et al. 2007), whereas other studies used mineral-salts only (Wang et al. 2004). Sharp et al. (2000) used basic peptone-yeast medium supplemented with salts and vitamins to exclude other carbon source to identify degradation bacteria. However, the profile of ileal digesta delivered to the lower gut might be more complicated due to the various plant-derived polysaccharides and undigested protein sources, let alone the host-derived carbon and nitrogen sources available. Unfortunately, there is no systematic comparison of the influence of nutrient rich or nutrient limited media on the fermentation outcome of RS. Although one study showed that nitrogen limited condition significantly affected bacteria counting and SCFA pattern with a continuous one-stage fermentor (Walker et al. 2005). Two peptide (composed by casein hydrolysate and peptone) concentrations were used: peptide limited condition (0.1%) and normal peptide condition (0.6%), indicated by comparing ammonia level produced in vivo. After 150 h incubation with a mixture of carbohydrate (including potato starch, xylan, pectin, amylopectin and arabinogalactan) at control pH condition (pH = 5.5), they found that low peptide supply significantly decreased the butyrate (13.8 mM) production compared to the presence of 0.6% peptide in the medium (24.9 mM), whereas propionate level showed the opposite profile with up to 5 folds increase by 0.1% peptide. Additionally, total bacterial numbers were also significantly decreased by low peptide supply as indicated by FISH. Therefore, the medium might be a critical factor for the validation of in vitro fermentation results, and the reproducibility among laboratories as well.

2.4.2.3.5 Other factors

Other factors may also affect the substrates fermentation outcomes. Stevenson et al. (1997) designed the experiments investigating the influence fluid surface area/fluid volume ratio (SA/V). They reported that the bigger fluid surface area might enable the increased gas exchanges with liquid phase and accelerate fermentation process, as indicated by the results that more SCFA was produced with 1:4 SA/V ratio compared to 1:1 SA/V ratio. In the same study, they also reported that shaking or stationary condition didn’t affect the fermentation outcome of SCFA production at the end of 24 h incubation, although in most fermentation studies, shaking or stirring are recommended especially with those insoluble substrates particles.
In summary, the major drawback of in vitro fermentation is, as for other in vitro systems, the limitation in simulating the interaction between host and gut microbes. In addition, the variable experimental condition adopted by each laboratory makes it difficult to compare results from in vitro studies and thus the reproducibility among laboratories. Although the continuous incubation system is proposed to be more precise to mimic colonic events, the complexity and high expense make it less helpful in terms of investigating the inter-individual variability, which might interfere with the prediction of the physiological outcomes of particular compounds or substrates. However, both batch and dynamic fermentation systems provide the opportunity to screen substrates before application to human or animal feeding studies, and further validation of their efficiency is anticipated in the near future with in vivo studies.

The in vitro fermentation system we developed is a combination of batch and continuous incubation with frequently replenished medium and substrates after a certain period of time based on the gut transit time previously measured in our lab. Although the replenishment amount of the medium was arbitrary determined in our study, the stability of system was reached by constant pH, SCFA and gas production, as well as the bacteria population monitored with PCR-DGGE. We would like to meet the needs of comparing the fermentation capacity of individual bacterial population originated from human subjects with different phenotypes (lean versus obese), as well as the fermentability of different resistant starch residues obtained by pre-digestion with AOAC 991.43 methods.

2.4.3 RS as a prebiotic to modulate microbial population

The early establishment of RS as a prebiotic was based on its ability to enrich probiotics, mainly LAB, including Bifidobacterium spp. and Lactobacillus spp., or help them to survive in the lower gut. In the study of Brown et al. (1997), Large White pigs were fed low amylose starch and high amylose starch supplemented with a freeze-dried Bifidobacterium longum (CSCC 1941) in a cross-over design with 7 day feeding period followed by 1-wk wash-out, during which antibiotics were given and diet is starch-free. They reported that HAS can significantly increase the content (log₁₀ cfu/g wet wt) of B. longum in feces on an agar plate compared to low amylose starch (8.91 versus 8.12, \( P < 0.01 \)) in the groups fed the strain,
whereas no *B. longum* was detected in the groups without its supplementation (with the detection limit of 4 cfu/g). In addition, fecal butyrate and propionate concentration were increased by high amylose starch feeding, but not affected by supplementation of *B. longum*. Similar results have been reported by Wang et al. (1999) in which a *Bifidobacterium spp.* Lafti™ 8B strain, which was isolated from a healthy human, was used. 3 groups of Balb/c mice were fed normal diet without starch for 3 months. One group was changed to a high amylose starch diet 24 h prior to bacteria supplementation. Two other groups were kept on a normal diet, but orally administered HAS with bacteria or glucose with bacteria in the drinking water. They reported that the recovery rate of the strain in the feces within 10 h was significantly higher in the two groups with prior or simultaneous HAS feeding compared to glucose group (7.44 ± 0.28, 7.40 ± 0.20 versus 7.14 ± 0.08). Lasrado et al. (2008) reported their preliminary results of the human feeding studies with 4 RS (1 g/day) for 14 d in 20 human adults consumed. The study was a cross-over design with a 21-day washout period before next treatment started. With *Bifidobacterium* specific primers and PCR-DGGE, they found that half of the subjects showed changes of *Bifidobacterium* pattern by RS feeding. (No further details were given in the study).

With further analysis, it was known that RS could modulate more than LAB. Theoretically, bacteria enriched by RS feeding reflect their involvement in RS fermentation, including those amylolytic and butyrogenic bacteria. Unfortunately, most studies available so far are not thorough, mostly due to the limitation of the technologies. Abell et al. (2008) has investigated the effect of RS feeding on fecal microbial population and SCFA level by a randomized crossover intervention study. In the study, they recruited 46 healthy human subjects and gave high RS diet (30% HiMaize starch wt/wt), daily for 4 wk. They found out that *Ruminococcus bromii* was significantly increased by RS feeding compared to regular diet with low RS or detected by molecular analysis (PCR-DGGE and Quantitative Real-time PCR). Other SCFA production related bacteria, such as *Faecalibacterium prausnitizii*, *Eubacterium rectale* and *Bacteroides thetaiotaomicron*, were also increased along with the increased butyrate and acetate level by 22.1% and 9.5% respectively (P < 0.001).
Kovatcheve-Datchary et al. (2009) used an in vitro fermentation model (TIM2) to simulate colonic events of RS fermentation and monitored the shifts of bacterial pattern, coupled with 16S rRNA-based based stable isotope probing (SIP) and terminal-restriction fragment length polymorphism (T-RFLP) with AluI and MspI as restriction endonuclease. The inoculum was prepared with a mixture of feces from seven human subjects and incubated with (U-^{13}C) labeled starch and unlabeled starch for 8 h. They reported that Ruminococcus bromii, Prevotella spp., Eubacterium rectale, and Bifidobacterium adolescentis were directly involved in starch degradation as shown by its abundance in high intensity fraction (^{13}C) after 8 h incubation. However, Ruminococcus bromii was implicated as the major starch degrader, as shown by increased labeled (T-RFLP) profiles (labeled starch) compared to unlabeled profiles (unlabeled starch), whereas Prevotella spp., Bifidobacterium adolescentis and Eubacterium rectale were involved in SCFA production, as shown by their similar abundance between labeled and unlabeled T-RFs.

However, many factors may affect the prebiotics effect of RS, including the type of RS preparation, the RS amount used in the system and the original bacteria population in the individual.

The type of RS seems to influence the modulation effects even the starch is from the same origin. Kleessen et al. (1997) compared the effects of native potato starch (type 2 RS) and retrograded potato starch (type 3 RS) feeding on the shifts of bacteria population in male Wistar rats. Although both RS given at 10% (wt/wt diet) increased cecal Bifidobacterium counting compared to control starch (highly digestible waxy corn starch), only retrograded starch significantly increased cecal Lactobacillus, Streptococcus and Enterobacteria counting. Further analysis of Lactobacillus species with FISH showed that Lactobacillus cellobiosus was enriched with the highest abundance in animals fed retrograded starch.

The retrogradation procedure may also affect bacteria profiles, probably due to different crystalline structure produced. For example, Lesmes et al. (2008) evaluated the fermentation outcome of two high amylase starch polymorphisms generated by different cooling procedure after autoclave during retrogradation. A polymorphism (RS A) was obtained with retrograding at 95 °C for 24 h and type B polymorphism (RS B) was obtained with
retrogradation at 40 °C for 24 h. They showed that only RSB significantly increased the proportion of *Bifidobacterium spp.* and *Atopobium spp.* in the vessel simulating proximal colon compared to that of native HAS and RS A during 11 d continuous incubation using pH controlled three-stage fermentation system.

In addition, Sharp et al. (2000) investigate the effect of dilution rate of RS on the bacteria population and SCFA production in a three-stage continuous fermentation system. Dilution rate is defined as the ratio of medium flow rate to the culture fluid volume. Since the culture fluid volume is usually controlled to be consistent, low dilution rate could be considered as slow bowel movement, which may be used to select slow-growing amylolytic bacteria. Two flow rates were used, D = 0.03/h and D = 0.3/h for continuous incubation for 240 h and bacteria were counted under microscope and 16S rRNA dot hybridization. They reported that *Bacteroides spp.*, *Bifidobacterium spp.* and *Clostridium spp.* were predominant bacteria enriched at both flow rates but with different pattern. For *Bacteroides spp.*, low dilution system has more diversity with *B. ovatus, B. uniformis, B. thetaiotaomicron,* and *B. caccae* compared to high dilution system with only *B. uniformis* and *B. thetaiotaomicron*. Similarly for *Bifidobacterium* spp., *B. angulatum, B. adolescentis, B.sp.strain1, B.sp.strain2* were enriched in low dilution system, but only *B. angulatum* in high dilution one. In contrast, for *Clostridium* spp., low dilution rate enriched *C. sporogenes,* and *C. sordellii* only, whereas *C. sporogenes, C. sordellii, C. perfringens, C. butyricum* were enriched in high dilution system. In addition, *Eubacterium limosum, Peptostreptococcus micros* and *Lactobacillus acidophilus* were specifically enriched high dilution rate system.

The modulation of bacteria profile by RS might be also influenced by the bacterial pattern originally found in the host (Silvi et al. 1999). Fisher 344 germ free rats (both male and female) were transplanted with fecal mixture from three Italian subjects or three UK subjects, who were consuming normal omnivorous diets. The original bacteria population were compared and significantly difference were observed with one order higher Lactobacillus and Bifidobacterium in Italian subjects, but lower total aerobes than UK subjects. The human-flora associated animals (HFA) were fed 30% (wt/wt diet) CrystaLean starch (retrograded high amylose starch) for 4 wk. They reported RS significantly increased counting of cecal
lactic acid producing bacteria, including Lactobacilli, Bifidobacterium and Enterobacteria in both two HFA animals compared to sucrose fed animals. However, significantly diet-donor effects were observed, indicating the animals responded differently to the RS feeding. For example, RS feeding increased the total aerobic count only in UK-HFA rats, which was unchanged in Italian-HFA rats. In contrast, total anaerobes were shown the opposite pattern. In addition, reduction of Bacteroides and increase of Streptococci were observed in UK-HFA rats, which were unchanged in Italian-HFA rats. Staphylococci was increased in UK-HFA rats but decreased in Italian-HFA rats. However, the difference response of bacteria profile didn’t affect the metabolic outcome of RS. Increased cecal butyrate proportion and decreased propionate portion were observed in both UK-HFA and Italian-HFA animals compared to sucrose-fed counterparts. Similarly consistent changes with decreased ammonia level and increased β-glucosidase activity were observed in two groups of rat fed RS also.

Intake of other nutrients might also affect the prebiotic activity of RS. In the study reported by Senevirathne et al. (2009), C57Bl/6J mice were fed high fat diet (41% dietary energy), moderate fat diet (28% dietary energy) and low fat diet (18% diet energy) supplemented with RS for 10 wk. They reported that although the total bacteria population was not affected by fat diet, Bifidobacterium /Lactobacillus and Clostridia spp. were only enriched in animals fed low and moderate fat diets, but not in high fat diets. (Abstract available only).

According to the implication of those studies discussed above, the prebiotic properties of RS to modulate gut microbes were established. The structure or the composition of RS will determine the microbial profiles, and probably the metabolic outcomes and the physiological effects of RS in disease prevention.

3. Physiological significance of SCFA

3.1 Distribution and absorption of SCFA

The reaction of SCFA production from glucose can be summarized as 59 glucose + 38 H₂O $\rightarrow$ 60 acetate + 22 propionate + 18 butyrate. The daily production of SCFA has been estimated to be in the range of 400 mmol in human (Topping et al. 2001). SCFA
concentration decreased along the lower gut with the horizontal diffusion of SCFA to the host tissue, together the decreased bacteria/substrate density. Cummings et al. (1987) measured the colonic SCFA concentration within 4 h from the subjects who were victims of sudden death (n = 6). In the order of ascending colon (n = 6), traverse colon (n = 5), descending colon (n = 4) and sigmoid/rectum (n = 5), they reported that SCFA concentration (in the unit of mmol/kg content) was 63.4 (6.8), 57.9 (5.4), 43.5 (11.1), 50.1 (16.2) for acetate, 26.7 (4.0), 23.1 (2.8), 14.2 (3.1), 19.5 (6.7) for propionate, and 24.5 (4.2), 24.4 (2.2), 14.7 (0.4), 17.9 (5.6) for butyrate. In line with the SCFA level, the pH is lowest in proximal (pH = 5.7 ± 0.2), and increases towards rectum as pH = 5.7 ± 0.2 in the proximal colon, 6.2 ± 0.1 in the traverse colon, 6.6 ± 0.1 in the descending colon and 6.3 ± 0.2 in the distal/rectum.

SCFA exist in the intestinal lumen with two forms: free/protonated (HA) and ionized (A⁻) SCFA. Free SCFA, non-ionized SCFA, is readily absorbed by simple diffusion with their lipophilicity. It is a major route for SCFA transferring into the cells. Ionized SCFA are transported by SCFA/HCO₃⁻ or SCFA/Na⁺ exchange system to cross in and out of the cell.

Two SCFA transporters have been identified so far. Hadjiagapiou et al. (2000) first identified MCT1 as the major butyrate transporters of five monocarboxylate transporters in Caco-2 cells. Knocking downing MCT1 expression with the transient transfection of the antisense gene inhibited the butyrate uptake. In addition, they also reported that butyrate uptake was pH-dependent and dose dependent with a saturate plateau. Two-fold or seven-fold increase of butyrate uptake was observed when pH dropped to 6.5 or 5.5 respectively. Another SCFA transporter in human is named as sodium-coupled monocarboxylate transporter (SMCT), (previously known as SLC5A8) (Miyauchi et al. 2004). SMCT has no sequence similarity with MCT1. The affinity of SMCT1 to SCFA is in the order of butyrate (81 ± 17 µM > propionate (127 ± 14 µM) > acetate (2.46 ± 0.89 mM). This particular transporter has been shown to be silenced by methylation in aberrant crypt foci (ACF) and human colon cells, indicating the abnormal uptake of SCFA or butyrate in the colonocytes might be related to colon carcinogenesis (Li et al. 2003).

After being absorbed, the SCFA would be either utilized by colonocytes (especially butyrate) or enter the circulation and reach liver (especially acetate and propionate). Human portal
SCFA was estimated to be 98-143 µM for acetate, 3.8-5.4 µM for propionate and 0.5-3.3 µM for butyrate (Wolever et al. 1996; Wolever et al. 1997). Therefore, due to the availability of three SCFA, butyrate may be more involved in luminal events and acetate and propionate may be more involved in the portal effects during circulation to other tissues.

3.2 Luminal effects of SCFA

3.2.1 Energy source

The oxidation of SCFA provides 60-70% energy needs for the colonocytes and up to 10% of daily caloric intake for host. The preference of utilization of three SCFA has been shown to be butyrate>propionate>acetate, which were all more than glucose utilization by isolated rats colonocytes as shown by Clausen et al.(1995). In the study, increased CO₂ production from oxidation of ¹⁴C labelled SCFA was observed compared to glucose in colonocytes isolated from human.

Butyrate is metabolized in the mammalian cells through beta-oxidation pathway with HMG-CoA cycle (Topping et al. 2001). Impaired butyrate oxidation has been considered as one pathological phenomena related to colitis. In the study of Ahmad et al. (2000), the colonocytes isolated from colitic mice (induced by dextran sulphate sodium (DSS) after 7 d) were incubated with ¹⁴C labeled butyrate or glucose, they reported that the butyrate oxidation was significantly reduced in DSS colitis as indicated by the decreased production of ¹⁴CO₂, whereas the utilization of glucose was higher compared to control.

All three SCFA can stimulate proliferation of the colonocytes in the basal crypt, which is a physiological proliferation zone to maintain rapid turn-over of the epithelial layer (Scheppach et al. 1992). The potency of SCFA were different when they were applied to the cecal mucosa biopsy in vitro, in which 10 mmol/L butyrate had 89% increased DNA labelling index, compared to 70% with 25 mmol/L propionate and only 31% with 60 mmol/L acetate. Both the tropic effects and pro-proliferation effects of SCFA support the rapid turn-over of epithelia layer to replace the damaged cells.
3.2.2 Regulating epithelia permeability

The integrity of epithelial layer is important for the defence against penetration of bacteria and influx of toxic substance and molecules. SCFA, especially butyrate and propionate have been shown to increase transepithelial electrical resistance (TEER) in a dose dependent manner (Mariadason et al. 1997). 2 mM butyrate could increase the TEER by 299 ± 69%, for which 16 mM propionate was needed. 32 mM acetate was still less effective than 2mM butyrate. Peng et al. (2009) further reported that the mechanism of butyrate enhancing epithelia barrier was related to the activation of AMP-activated protein kinase (AMPK) to facilitate the assembly of tight junction protein such as protein ZO-1 and occludin.

However, Peng et al. (2007) reported that a high concentration of butyrate (8 mM) significantly decreased the TEER at 48 h compared to medium control, and at 96hr, the TEER was decreased to 23 ± 18 Ω cm² compared to a control of 200 ± 21 Ω cm². Further analysis of cell viability showed that 5 mM and 8 mM butyrate significantly decreased the cell viability to more than 50%, as revealed by increasing floating cells. DNA fragmentation experiments further showed that high doses of butyrate induce apoptosis and impair epithelia cell integrity. Similar results were reported by Mariadason et al. (1997) that acute exposure of 10 mM butyrate increased the paracellular permeability of isolated distal colon mucosa from rats, but not 1 or 5 mM butyrate. Therefore, the regulation of epithelia permeability is dose-determined: low concentration of SCFA can increase tight junction and high concentration of SCFA can impair epithelia layer and induce cell apoptosis.

3.2.3 Anti-inflammation

The identification of SCFA receptors enhanced our understanding of the anti-inflammatory effects in immune cells. Two orphan G protein coupled receptors (GPRs), GPR41 and GPR43 was identified as SCFA receptors with fishing strategy applied in yeast and CHO-K1 cell line by two separate groups (Brown et al. 2003; Le Poul et al. 2003). The activation of these receptors is SCFA concentration-dependent and the one carboxylic group at the end of aliphatic chain is required. GPR43 has equal affinity for all three SCFA, whereas GPR41 selectively binds to SCFA in the order of propionate > butyrate > acetate. The expression of
these two receptors has been detected in the colon epithelia, adipose, immune cells, and skeleton muscle cells. However, GPR41 is preferentially distributed in adipose tissue, whereas GPR43 is highly expressed in immune cells (particularly monocytes and neutrophils).

It was proposed that anti-inflammatory effects of SCFA were mediated by GPR43 (Maslowski et al. 2009). In the study, they showed that 200mM acetate in the drinking water reduced colitis symptoms induced by DSS in wild type mice, as indicated by the increase in colon length and decrease inflammatory mediator myeloperoxidase (MPO) and TNFα. However, in the GPR43 K/O mice, the same dose of acetate could not improve colitis. In addition, Cox et al. (2009) reported that the GPR43 was highly expressed on isolated human monocytes donated by 10 subjects. SCFAs induced prostaglandin E2 (PGE2) production in monocytes at the concentration of 20 mmol/L acetate, 20 mmol/L propionate, and 2 mmol/L butyrate. In the presence of LPS as a pro-inflammatory stimulus, the induction of PGE2 was enhanced synergistically through the inhibition of COX1, which was a known pro-inflammatory enzyme. Furthermore, they showed that SCFA could significantly suppress LPS induced IL10 level, which is one of the cytokines upregulated in colitis. In addition, other colitis related protein including MCP-1, IFN-γ and TNF-α could also be suppressed by SCFA in the presence of LPS.

The anti-inflammatory effect of SCFA in colon cells was probably through the suppression of nuclear factor (NFkB) in colon cancer cells. NF-kB is a transcription factor which can regulate the expression of pro-inflammatory cytokines and inflammatory enzymes (such as inducible NO synthase and cyclo-oxygenase-2). In the study of Inan et al. (2000), 4mM butyrate significantly decreased NF-kB and its downstream gene expression in HT29 cells, and propionate was also effective in reducing NF-kB but with less degree. In addition, Wachtershauser et al. (2000) reported that 2 mM butyrate increased the expression of peroxisome proliferator-activator-activated receptor (PPAR) in Caco-2 cells, but not by 1 mM butyrate or 2 mM propionate and valerate. Expression of PPAR, which is a nuclear receptor which has been shown to inhibit the expression of pro-inflammatory cytokines and chemokines, was significant lower in confined colonic epithelia cells of ulcerative colitis
patients than that of healthy controls and Crohn’s disease (CD) patients (Dubuquoy et al. 2003).

In summary, SCFA has anti-inflammatory effects mediated by G protein coupled receptors (GPR41 and GPR43). The downstream molecular pathways of SCFA are involved with downregulating NF-kB and upregulating PPAR to suppress pro-inflammatory cytokines and chemokines.

3.2.4 Induction of apoptosis

The inhibition of cell proliferation and induction of apoptosis of cancer cells are considered as the major effects of SCFA in reducing colon cancer risk by a body of in vitro studies. Varied efficacy of the three SCFA has also been observed. Hague et al. (1995) showed that extent of apoptosis induced by in Caco-2 cells was similar when 2 mM butyrate, 5 mM propionate and 10 mM valerate were applied to the medium, and other SCFA didn’t have similar effects even when 20 mM acetate was applied. However, Singh et al. (1997) reported that the effect of butyrate on cell proliferation was influenced by the presence or absence of other energy alternatives such as glucose and pyruvate. In the study, adenoma (S/RG/C2) cell line and carcinoma HT29 cell line were cultured in the standard growth medium (DMEM) with or without 25 mM glucose and 1mM sodium pyruvate. The effects of 4-day incubation with 0-10 mM sodium butyrate was evaluated by counting the attached cells and floating cells in the culture flask, the latter of which were considered as apoptotic cells. They reported that with the glucose-rich condition, butyrate showed inhibitory effect on cell growth and induced apoptosis (0.5 mM for both two cells), whereas with the glucose-depleted condition, low concentration of butyrate increased cell growth as shown by increased attached cell percentage (0.5 mM for S/RG/C2 and 0.5 and 2 mM for HT29). Therefore, the butyrate induced apoptosis effect is also dose-determined and influenced by the presence of other energy alternatives.

In addition to the evidence from in vitro studies, some in vivo animal feeding studies showed correlation of SCFA level with apoptosis index. In the study of Le Leu et al. (2002) six groups of SD rats were fed non-fiber diet, 5% cellulose or 5% wheat bran diet
supplemented with or without 6.95% Hi-Maize fed male SD rats (n = 12) for 4 wk, followed by single i. p. injection of carcinogen (azoxymethane) AOM (10mg/kg b.w.). The colon was collected 6 hr after treatment to measure the apoptosis and proliferation response to the acute dose of carcinogen, including crypt column height, number of apoptotic cells per crypt column and number of PCNA labeled cells per crypt column. They reported that the apoptotic index (%) in the distal colon was significantly correlated to fecal total SCFA (25.2 ± 9.5 µmol/g in RS, r = 0.5, P < 0.001), acetate (17 ± 7 µmol/g in RS, r = 0.43, P < 0.001), propionate (4.3 ± 1.9 µmol/g in RS, r = 0.56, P < 0.001) and butyrate (3.2 ± 1.9 µmol/g in RS, r = 0.41, P < 0.001), but no correlation was found with cecum SCFA concentration (total and individual SCFA). Similarly, in the study of Le Leu et al. (2003) 10%, 20% and 30% HAS was fed to SD rats (n = 16 each) 3 wk prior to AOM ip injection (10 mg/kg bw). A correlation of fecal SCFA (total and individual SCFA) with apoptosis index 6h after AOM injection was reported but to a lesser degree (r = 0.21-0.28, P < 0.05).

The molecular mechanism of butyrate inducing apoptosis is largely unknown. Although it has long been reported that butyrate could inhibit histone deacetylase (HDACs), and the treatment with 5mM sodium butyrate for 21 h could result in hyperacetylation, especially on H3 and H4, by tracing the $^{13}$C or $^1$H labelled acetate incorporation in histone fraction of Hela cells (Boffa et al. 1981). HDACs is considered as a global regulating mechanism which may affect 10% mammalian gene expression (Davie 2003) Other specific mechanisms have been reported. Mandal et al. (2001) showed that the apoptosis by butyrate in human DiFi and FET colon cells was signaled by JNK (c-Jun N-terminal kinase). JNK belongs to mitogen-activated protein kinase family (MAPK), and is known to mediate apoptosis by phosphorylating c-Jun, which could bind to activation protein1 (AP1) as a transcription dimer to activate caspase and the downstream apoptosis induced by apoptotic protein BAX. In addition, Ruemmele et al. (2003) proposed that butyrate-inducing apoptotic pathway in the Caco-2 cells was involved with mitochondria. They proposed the model that with butyrate stimulation, the increase of pro-apoptotic protein BAK and decrease of anti-apoptotic protein BCL1 could induce the transportation of cytochrome-C from mitochondria to cytosol resulting caspase-9 and caspase-3 induced apoptosis.
3.2.5 Satiety hormones

Gut secreted satiety hormones including glucagon-like peptide-1 (GLP-1), total peptide YY (PYY) have been shown to be induced by SCFA. In the study of Zhou et al. (2006) cecum and colonic epithelia cells from Sprague-Dawley male rats were collected and incubated with a gradient of butyrate concentration. They found out that butyrate could increase the mRNA expression of PYY at the concentration of 20 nM, 0.5 µM, 50 µM and 1 mM, and 20 mM butyrate inhibited PYY expression. Further in Zhou et al. (2008), they reported that low pH (6.0) increased proglucagon gene expression in STC-1 cells compared to neutral pH (7.5) after 24 h incubation. In addition, with the physiological concentration of SCFA detected in the rats fed RS for 5 wk, the addition of three SCFA could significantly increase the gene expression in a dose-dependent manner, with acetate at 3-30 mM, propionate at 0.1-1 mM, and butyrate 0.016-0.4 mM. And three SCFA also significantly increase the activity of PYY promoter in HEK-293 cells with a dose- and time-response manner, and the potency was in the order of butyrate > propionate > acetate. The expression of PYY was shown to be related to epithelial receptor GPR41 as shown by Samuel et al. (2008). In their study the inoculation of B. thetaiotamicron and M. smithii did not increase the epidermal fat in GPR41 K/O gnotobiatic mice as wild type gnotobiatic mice usually did, although with similar food intake. Further analysis showed that the serum PYY level in gnotobiatic K/O mice was significantly lower than that of gnotobiatic wild type mice, indicating that lower energy extraction might be related to the reduced expression of PYY. However, the inoculation increased the cecal and fecal acetate and propionate level to an extent higher in K/O animals than that of wild type gnotobiatic mice, indicating that the induction of PYY by SCFA might be blocked by GPR41 deficiency.

In summary, SCFA are important energy sources, can regulate epithelial permeability, contribute to anti-inflammation process, induce apoptosis to reduce colon cancer, and also induce satiety hormones for the management of obesity. Practically, SCFAs function in a dose-dependent manner, so the concentration of SCFA applied in experiments should be adjusted when considering the above effects. In addition, most evidence of SCFA effect is
based on the *in vitro* models, and thus more studies including *in vivo* animal models and/or primary epithelial cells are require to further elucidate the luminal effects of SCFA.

### 3.3 Portal effects

The target tissues for circulated SCFA include adipose tissue, and immune cells and liver. Acetate (2 mM) and butyrate (2 mM) have been reported as substrates for fatty acid synthesis as shown by tracing $^{13}$C labelled substrates incubated with isolated rat hepatocytes (Nishina et al. 1990). However, with the presence of 1 mM propionate, a 90% decreased of fatty acid synthesis from acetate was observed in the hepatocytes. The results were similar to what reported by Demigne et al. (1995) who showed 0.6 mM propionate effectively inhibited fatty acid synthesis, and cholesterol synthesis although to a less extent in isolated rat hepatocytes.

Propionate is also the most potent ligand for GPR43, as shown by the lowest EC50 of 2.1 μM among C2-C6 SCFA (Xiong et al. 2004). In addition, it was also reported that acute administration of 500μM sodium propionate increased plasma leptin concentration by 80% after 7 h gavage in mice. Since leptin is a hormone produced from adipose tissue that suppresses food intake, the study indicated that SCFA might be involved in regulating food intake and possibly obesity. However, Hong et al. (2005) reported that showed that acetate and propionate (0.1 μM) induced the differentiation of 3T3-L1 cells, which is an inducible cell line with adipocyte-like phenotype, indicating SCFA might get involved in the development of adipose tissue, known as adipogenesis, through the increase of GPR43 expression. Based on these pieces of information, it is still unknown how portal propionate or acetate regulates the function of adipose tissue.

Overall, SCFA is absorbed either through the simple diffusion along the lower gut into the cells, or less likely through SCFA transporters MCT1 and SMCT. Once absorbed, SCFA is distributed directly to luminal colon cells or with the systemic circulation to target tissues. For those SCFA accumulated in luminal colon cells, they are important energy sources, may regulate epithelial permeability, or contribute to anti-inflammation process. It has also been well documented that SCFA can induce apoptosis to reduce colon cancer and/or induce satiety hormones for the management of obesity. On the other hand, for those SCFA
distributed into target tissues through the portal effects, they may affect the local physiological pathways such as those in adipose tissues and hepatocytes.

4. RS, gut microbes and colon cancer

Giving the physiological significance of SCFA identified so far, RS was implicated to improve colon health and reduce the risk of colon related disease. Our studies are mainly focusing on the protective effect of RS against colon cancer in the context of gut microbes and their metabolic products, i.e. SCFA. We are trying to correlate the shifts of gut microbial population to the preneoplasm incidence using rat model, and hoping our finding could contribute the understanding of physiological effects of RS with the long-term goal to identify probiotics for cancer prevention and treatment.

4.1 RS and colon cancer

4.1.1 Epidemiological studies

Epidemiological studies have shown that dietary habits were significantly related to the risk of colon cancer besides the inherited genetic factors. Generally speaking, fresh fruits, vegetables, dietary fiber, calcium, vitamin D, and antioxidants could improve colon function, whereas red meat and saturated animal fat increase the risk of colon cancer, as summarized in the review of Bingham (1996). One of the most fundamental epidemiological studies was carried out by Cassidy et al. (1994), with the international comparison analysis on the dietary intake and the incidence of large bowel cancer (including colon and rectum cancer). They collected and reanalyzed the dietary intake of protein, fat, NSP and starch from 11 countries (28 groups) based on publications since 1975 and follow-up studies as well. The RS intake was calculated by a conservative estimation of 5% of total starch. They found out that starch intake (73–371 g/day) was significantly inversely related to the incidence of colon cancer ($r = -0.76$ to $-0.84$ for men or women or both). Moreover, protein intake (66–106 g/day) was positively related to colon cancer risk in men only ($r = 0.67$) and fat consumption (44–118 g/day) was positively related to colon cancer in female only ($r = 0.69$). Additionally, there was no significant correlation between NSP intake and colon cancer rates. According to this
study, starch or RS was first considered as one of the most important dietary factors reducing colon cancer risks.

4.1.2 Animal studies

4.1.2.1 Animal study protocols

Rodent models especially rats were used to study the disease prevention effects of RS. Carcinogenesis is usually induced by the carcinogen, dimethylhydrazine (DMH) or its metabolite azoxymethane (AOM), the latter of which is more commonly used with its enhanced potency and chemical stability over its parent compound (Druckrey 1970). Commonly measured endpoints in the animal studies include: the incidence of preneoplasm formed with short-term RS feeding (including aberrant crypt foci (ACF) and mucin depleted foci (MDF), and the incidence of cancer with long-term feeding or high dose/frequency of carcinogen. It has been suggested that MDF, as a subgroup of ACF, were better predictors of carcinogenesis with higher dysplastic score (4.95 ± 1.60) than ACF (3.61 ± 1.61), as shown by Cademi et al. (2003). Formation of DNA adducts, cell proliferation index, induction of apoptosis are also measured as response to acute dose of carcinogen. Metabolic effects of RS including fecal/colonic/cecal pH, fecal SCFA, bile acids, activity of bacterial enzyme are also measured commonly to understand the underlying mechanism.

Colon carcinogenesis includes three stages: the initiation phase with DNA mutation induced by carcinogen, the promotion phase with preneoplasms formation and proliferation from normal crypts, and the progression phase with neoplasm and cancer formation. Three major experimental protocols have been taken with the investigation of the protective effects of RS against carcinogenesis. One is the immediate response of colonocytes to acute carcinogen injection in the rats fed a period time of RS, which is usually used to investigate the apoptosis induced by SCFA in vivo. In the other two protocols, the formation of pre-neoplasm or neoplasm is measured, but different carcinogen protocols are applied: 1) applying carcinogen during RS feeding, with which RS may affect all three carcinogenic phases including initiation, and 2) applying carcinogen ahead of RS feeding, with which RS only affects post-initiation phase.
Unfortunately, big discrepancies exist with the studies finished so far, which might be explained by carcinogen protocol (dose, injection timing and frequency, period), starch protocol (type, dose and preparation) and even animal protocol (strains, genetic background and ages).

4.1.2.2 Effects of RS against initiation phase or post-initiation phase

Conflicting results have been reported on the protective effects of RS against colon cancer, especially when it was applied before the carcinogen injection. In the study of Young et al. (1996), three groups of male Sprague-Dawley (SD) rats (n = 30-35) were fed low fiber diet only, or supplemented with 20% raw potato starch, or supplemented with both 10% wheat bran and 20% raw potato starch for wk, followed by dimethylhydrazine (DMH) (20 mg/kg bw) injection once a week for 10 wk and a continuous feeding for another 20 wk. They reported that the tumor incidence was increased by RS feeding compared to control diet (88% versus 74%, P <0.05), as well as the number of tumors per animal (2.0 ± 0.27 versus 1.26 ± 0.25, P < 0.05) and the tumor size (86 ± 40 versus 191 ± 40, P = 0.01). The addition of wheat bran in the RS diet suppressed the tumor incidence and other measurement to the extent similar to that of control diet. No SCFA measurement has been performed in that study though. Similarly, in the study of Sakamoto et al. (1996), the digested residue of high amylose starch (HAS) with pancreatin (including amylase, lipase and trypsin) in vitro were used as RS sources and added to the basal diet in the concentration of 3% or 10% RS (wt/wt diet). After 7-wk feeding of experimental diets, the DMH (20 mg/kg bw) was given to SD rats (n = 13-15 each group) once a week for 20 wk. They showed that RS did not change the incidence of tumors compared to basal diet. There was a trend of increased incidence of colon cancer in both distal and proximal colon, although not significantly different. However, the cecum and distal butyrate level was increased by two doses of RS. Similar results were reported by Maziere et al. (1998), in which 25% retrograded HAS were added to the diets and fed to SD rats 4 wk before DMH injection (50 mg/kg bw) twice per week for one week. After another continuous 20-wk feeding, they reported that although all HAS diets significantly decreased cecal pH, no changes of SCFA was observed, and no effects of HAS on the incidence of ACF was observed.
However, in the study of Le Leu et al. (2007), three groups (n = 30) of male SD rats were fed control diets (no fiber and RS), 10% (wt/wt diet) high amylose starch (HAS) or 20% HAS diet for 4 wk, followed by AOM (15 mg/kg bw) injection twice (once a week) and continuous cultivation for another 25 wk. They reported that RS were effective to reduce colon carcinogenesis and the two RS doses were similar in decreasing the incidence of cancer (27% and 27% versus 57%, *P* < 0.01) and the number of cancer developed (0.33 ± 0.11 and 0.30 ± 0.10 versus 0.63 ± 0.11, *P* < 0.05).

In addition, in the study of Le Leu et al. (2007), four groups of male SD rats were fed basal diet with digestible starch, 10% HAS replacing digestible starch, 15% potato protein (PP) replacing casein, or both HAS and PP (n = 10 each) for 4 wk, followed by twice S.C. injection of AOM (15 mg/kg bw) once a week and a continuous cultivation for another 10 wk. They reported that the incidence of adenocarcinomas was significantly reduced by the addition of RS in the diet regardless of PP (27% by RS and 23% by RS+PP versus 50% by control and 40% by PP, *P* = 0.038).

In those studies when RS was applied after the carcinogen injection, the effect of reducing cancer risk by RS was relatively consistent. In the study of Thorup et al. (1995) four groups of Wistar rats (n = 16 each) were fed basal diet, sucrose diet, cornstarch and potato starch after consecutively injection of AOM (15 mg/kg bw) twice (once a week). They found out that potato starch group showed significantly lower ACF number, especially fewer larger ACF, but not in the sucrose and cornstarch fed group (only abstract is available). Similarly, in the study of Cassand et al. (1997), one week after the DMH (50 mg/kg bw) injection, the experimental diets with RS (25% wt/wt diet) were given to male SD rats (n = 8 each) for 12 wk. The ACF number was decreased by RS feeding compared to control diets but not significantly different (288 ± 166 in RS versus 364 ± 155 in control). But small ACF number (with less than 3 AC per foci) was significantly decreased by RS addition (2.4 ± 1.1 in RS versus 2.5 ± 0.7 in control, *P* < 0.05). Fecal butyrate content was also increased by RS feeding. Again, in the study of Bauer-Marinovic et al. (2006), two groups of SD rats were injected with DMH twice (once a week) followed by feeding of standard diet (n = 12) with or without 10% retrograde high amylase starch (Novelose 330) (n = 8) for 20 wk. They reported
that no tumors were developed in the RS group, and 6 out of 12 rats fed standard diet developed tumor (number of tumor: $1.2 \pm 0.4$). However, no difference in luminal SCFA and butyrate concentration was observed due to big variability in the animals.

Due to the conflicting results reported, Liu et al. (2008) investigated the protective effects of RS during the different phases of carcinogen. In the study, eight groups of Wistar rats fed no RS diet, low dose (10% wt/wt diet, medium dose (20% wt/wt diet) and high dose (30% wt/wt diet) RS diet ($n = 12$). The RS they used was raw potato starch and basal diet was AIN-76. Half of the groups were fed starch diet 3 wk prior AOM (15 mg/kg b.w.,) intraperitoneal injection and the other half groups were given AOM 3 wk before feeding with starch diet. AOM was given twice with once a week. After another 13 wk, the animals were sacrificed and they reported that ACF number was significantly increased by prior 20% and 30% RS feeding with a dose response also (distal colon, for example, $48 \pm 3$ for 30% RS versus $43 \pm 5$ for 20% RS versus $31 \pm 4$ for 10% RS and $31 \pm 3$ for control). In contrast, ACF was significantly decreased by post all three RS doses (distal colon, for example, $20 \pm 2$ for 30% RS versus $24 \pm 2$ for 20% RS versus $26 \pm 4$ for 10% RS versus $30 \pm 3$ for control). With the further analysis of SCFA concentration in cecum, they reported that the carcinogen application protocol didn’t affect the SCFA level. 20% and 30% RS significantly increase acetate and propionate level ($\mu$mol/g) in a dose pattern, whereas butyrate was only increased by 30% RS feeding. In the feces, acetate and butyrate were increased by 20% and 30% RS in a dose response, whereas propionate was only increased by 20% RS feeding. No correlation between SCFA levels with ACF was observed. Similarly, 20% and 30% RS induced similar increase of acetate pattern in the cecum, distal colon and fecal content. However, butyrate was increased by both RS doses in distal colon, but only increased by 20% HAS in the cecum, proximal colon and feces.

They tried to explain the opposite effects of RS on carcinogenesis with the hypothesis that the AOM might be metabolized by the bacteria population modified by prior RS feeding, resulting in increased absorption of carcinogen. Ferguson et al. (2003) reported that the plasma level of metabolites of carcinogen IQ (2-Amino-3-methylimidazo [4,5-flquinoline) was only found in RS fed animals but showed excretion in feces compared to animals fed
wheat bran, which was known to dilute the carcinogen by increasing fecal bulking. The plasma level enhanced level of carcinogen might involve in the increase risk of cancer, which is originated from stem cell in the base crypt and nurtured by blood, but not by luminal content. This was consistent with the study of Young et al. (1996) showing that wheat bran could suppress the potentiating effect of RS in tumorigenesis. However, further validation of the hypothesis is needed.

Another interesting observation from the animals studies mentioned above is that in both studies of Young et al. (1996) and Sakamoto et al. (1996), the carcinogen application was repeated once a week until the end of study. It might be somewhat similar to the situation in genetically mutated animals that spontaneously develop colon cancer, and RS has not been shown to be effective either. For example, Pierre et al. (1997) reported that 6-wk RS feeding did not suppress the spontaneous tumor formation in Min mice, the heterozygous Apc knock out C57B/J mice, which was consistent with the results of Williamson et al. (1999) with another Apc mutated C57B/J mice, Apc1638N, fed high amylase starch for up to five month, indicating that RS or SCFA induced apoptosis may not sufficient to fix the colon cancer caused by genetic mutation.

Even though, the question is still unresolved on the conflicting results mentioned above. The varied types of RS used make it even harder to compare the results available. It is anticipated a systematic study would be designed to evaluate the influence of carcinogen dose, starch dose and etc.

4.1.3 In vitro studies

The combination of in vitro fermentation and in vitro cancer cell model has been described only recently by Fassler et al. (2007). In the study, the pre-digested RS residues were applied to both batch fermentation and three-stage pH-controlled dynamic fermentation system. The supernatants of 24h fermentation products were applied to Caco-2 cells and a combination of three SCFA standards was used as control with 8.5 mM acetate, 1.0mM propionate and 2.3 mM butyrate. The fermentation products from dynamic system had high cytotoxicity on Caco-2 cells, even at the similar concentration of SCFA with batch fermentation products
and SCFA standards. Therefore, they only measured the effects of batch fermentation products on Caco-2 cells. With the comet assay, they reported a 9-30% decrease of DNA damages induced by H₂O₂ was observed after Caco-2 cells were incubated with RS fermentation products for 24 h. In addition, a 22% increase of transepithelial electrical resistance (TEER) of Caco-2 cell monolayer was observed after 24 and 48 h treatment. However, the conditions in this study were not well controlled. For example, the fecal inocula for batch system were collected from three non-methanogenic human subjects and prepared with carbonate-phosphate buffer, whereas the inocula for dynamic model were the frozen products from prior anaerobic incubation of fresh feces from 5 subjects with NaCl, cysteine and ileal delivery medium for 16 h. This still provides a piece of evidence that RS fermentation products are involved in the maintenance of epithelial layer and protection against genotoxicity.

4.1.4 Mechanism of RS against colon cancer

Giving the physiological significance of SCFA, the correlation of SCFA with the incidence of neoplasm and preneoplasm in animal feeding studies has been explored in some of the studies discussed above. Le Leu et al. (2007) reported the negative correlation of the incidence of cancer with butyrate (r = -0.37, P = 0.03) and acetate level (r = -0.36, P = 0.04) in distal colon content from SD rats fed high amylose starch (10% and 20% wt/wt diet).

However, the SCFA might not be the only mediator for the protective effects conferred by RS, since Bauer-Marinovic et al. (2006) reported that RS reduced colon cancer risk, with no SCFA changes. In addition, Liu et al.(2008) reported that even though SCFA level was increased by RS feeding. The incidence of preneoplasms was increased by RS feeding when carcinogen AOM was applied after RS diet. Evidence from epidemiology studies and human studies for SCFA mediating reducing cancer risk is still lacking. For example, Vernia et al. (1989) (cited from Topping et al. (2001), since no full text is available) reported that fecal acetate was significantly lower in cancer patients (59.7 mM) than in those with polyps (79.6 mM) or in controls (89.2 mM). However, butyrate concentrations were not significantly different between those with cancer (12.5 mM) and the other two groups (20.9 and 19.3 mM).
Therefore, other metabolic effects of RS were also considered to contribute to RS-mediated prevention against colon cancer. In addition to the production of SCFA, the decreased pH, decreased secondary bile acids and beta-glucuronidase activity have all been reported in RS feeding in human and in animal study (Hylla et al. 1998) It was thought the acidic environment was epidemiological related to lower risk of colon cancer In the study of Malhotra et al. (1982), lower fecal pH (6.5) was observed in North Indian population with low risk, compared to high risk South Indian population with fecal pH = 7.8 (n = 60 each). Low pH has been shown to inhibit pathogen growth, such as E. coli and Salmonella, whose growth will be inhibited at pH = 5 (Cherriton et al. 1991). Additionally, the decreased pH could modulate the microbial metabolic activity, such as 7-alpha dehydroxidase, beta-glucuronidase and b-glucosidase, which all involved in the production of cancer promoter. Reddy et al. (1978) reported that the concentration of fecal secondary bile acids and β-glucuronidase activity were higher in the population in the New York city area with high colon cancer risk compared to the population in rural Kuopio of Finland with low cancer risk. In addition, in the study of Bayerdorffer et al. (1995), unconjugated DCA (deoxycholate) was reported to be 2.8 folds higher in the plasma of patients (n = 10) with colon cancer than that of control (0.89 versus 0.32 µmol/L, P < 0.0025). DCA was produced from hepatic primary bile acids by 7-alpha dehydroxilase of Eubacterium spp. and Clostridium leptum, which are commensal bacteria in the gut (Stellwag et al. 1979; Hylemon et al. 1991). The genotoxicity of DCA was mediated by the oxidative damage induced by ROS and RNS (reactive oxygen/nitrogen species), as summarized in the review of Bernstein et al. (2005). RS feeding could decrease the secondary bile acids level and reduce the risk of the DNA damage as shown by Vanmunster et al. (1994). In their study, the consumption of 45 g native amylomaize starch (Hylon-VII) significantly increased the secretion of primary bile acids and decreased soluble secondary bile acids (DCA) by 50% (P < 0.01). Although fecal SCFA concentration was increased by 35%, there was no difference in the butyrate level. Further analysis showed that the cytotoxicity of fecal aqueous on colon cancer cell line was significantly decreased compared to the samples from non-RS subjects (P = 0.007). In addition, applying fecal aqueous on the rectal biopsies significantly decreased colonic mucosa proliferation index (5.4 versus 6.7, P = 0.05). The decrease of fecal secondary bile
acids level was by. Therefore, the modification of bile acids excretion by RS might be one mechanism to prevent the incidence of colon cancer via the decreased pH.

In addition, high beta-glucuronidase activity was also claimed as one indicator for colon cancer as reported by Kim et al. (2001). In their study, fecal beta-glucuronidase activity was reported to be 1.7-fold higher in patients (n = 13) with colon cancer than that of healthy control subjects, and 12.1 times higher when bacteria was sonicated. In addition, high risk diet (high fat and sucrose) has been shown to induce 3-fold higher beta-glucuronidase activity than that of low risk diet (starch, fiber and calcium) as shown by Hambly et al. (1997), along with the higher ammonia level in the feces of rats. Beta-glucuronidase can release the glucuronides from conjugated potential toxins and increase the active carcinogens in the lower gut, which will enter the entero-hepatic recirculation. Probiotic *Bifidobacterium longum* has been shown to decrease the AOM-induced ACF number in male Fisher 344 rats with 5-wk feeding of 1.5% or 3% lyophilized cultures of bacteria prior to AOM injection followed by 8 months continuing feeding, along with the reduced fecal beta-glucuronidase activity (Kulkarni et al. 1994) (Abstract available only). In addition, Arimochi et al. (1999) have shown a significant correlation ($r = 0.978, P < 0.05$) between the fecal $\beta$-glucuronidase activity and ACF number induced by AOM in rats. However, Dabek et al. (2008) screened 40 human fecal strains with $\beta$-glucuronidase gene ($gus$) specific primer and reported it was mainly found in the Clostridial clusters XIVa and IV, which are two major clusters known for RS degradation and butyrate production. It is unknown whether there is regulation within bacteria between the SCFA synthesis and $\beta$-glucuronidase gene expression, but the theme of the double-edged role of gut microbes determining disease or health emerges again.

4.2 Gut microbes and colon cancer

The role of gut microbes in colon tumorigenesis is double-edged as it is in obesity, which can be seen from its promotion by some commensal bacteria and its prevention by probiotics.
4.2.1 Promotion of colon cancer

The common strategies to identify colon disease related gut microbes are 1) the forward comparison of gut microbial profile between healthy and colon/colitis patients, and 2) the reverse identification of particular bacteria which promote or induce the development of cancer or colitis.

Gut microbial patterns related to colon cancer has been shown by Moore et al. (1995). In the study, they compared the fecal bacteria population from colon cancer patients, Japanese-Hawaiians (n = 15), North American Caucasians (n = 17), rural native Japanese (n = 22), and rural native Africans (n = 16). The four populations were chosen due to their variable cancer risk in the described order from highest to lowest. With 5,350 isolated obtained from traditional cultivation methods, they reported that fifteen bacteria were significantly higher in the population with high risk of colon cancer, including Bacteroides vulgatus (P = 0.001), Eubacterium rectale (P = 0.0013), Ruminococcus torques (P = 0.0018), Streptococcus hansenii (P = 0.009), Bifidobacterium longum (P = 0.010), Ruminococcus albus (P = 0.018), Peptostreptococcus productus (P = 0.018), Bacteroides stercoris (P = 0.018), Bifidobacterium angulatum (P = 0.023), Eubacterium eligens (P = 0.024), Eubacterium eligens (P = 0.027), Ruminococcus gnavus (P = 0.028), Fusobacterium prausnitzii (P = 0.036), Eubacterium cylindroides (P = 0.037), and Eubacterium rectale (P = 0.042). In contrast, five bacteria were significantly higher in the population of low risk of colon cancer, including Lactobacillus S06 (P = 0.0043), Fusobacterium AB (P = 0.0055), Eubacterium aerofaciens (P = 0.0066), Eubacterium BN 37 (P = 0.032), Eubacterium aerofaciens (P = 0.034) and Peptostreptococcus DZ2 (P = 0.048). In addition, they also reported the correlation of total counting of Bacteorides and Bifidobacterium were positively related to the increased risk of colon cancer. In addition, Horie et al. (1999) studied the effects of intestinal bacteria on the incidence of neoplasm induced by carcinogen DMH (1,2-dimethylhydrazine) with comparing germ free IQI/Jic mice with gnotobiotic mice mono-inoculated with bacteria species interested. They reported that the incidence of adenoma observed in gnotobiotic mice were 68% by Mitsuokella multiacida inoculation, 68% by
Clostridium butyricum, 63% by Bifidobacterium longum, (50%) by Clostridium paraputrificum and 50% by Escherichia coli (50%) (Only abstract is available).

Oxygen intermediates (ROI) has been implicated to be related to the incidence of colon cancer as carcinogen initiator or promoter to damage cell membrane and cause DNA mutation (Huycke et al. 2004). H₂S is the only one available in the gut to diffuse into cells.

The role of sulphate reducing bacteria (SRB), has been implicated to act as cancer initiator or promoter, For examples, Balish et al. (2002) reported that the inoculation of one of the commensal SRB, Enterococcus faecalis, to GF IL10 knockout mice (KO) induced the incidence of colitis (colon and rectum) and rectal tumor after 20-27 wk of colonization. Another known commensal SRB, Desulfovibrio desulfuricans, has been implicated to be related to bowel disease by Gibson et al. (1991).

In addition to ROIs, bacteria surface protein may also induced colon cancer, such as Streptococcus bovis (Ellmerich et al. 2000). In the study, rats were injected IP with AOM (15 mg/kg) twice with once a week, followed by 15 d cultivation. Then either S. bovis or its cell wall protein was given to the animals by gavage twice per week for another 5 wk. They found out that the ACF were significantly increased in the animals given S. bovis or proteins compared to AOM injected only group (36 ± 2 and 38 ± 3 versus 20 ± 1.4, \( P < 0.05 \)), and only groups with bacteria cell wall proteins showed incidence of adenomas (3 out 6 animals).

Other more pathogenic bacteria such as Helicobacter hepaticus also has the similar promoter effects of colon cancer as shown by Nagamine et al. (2008) using gnotobiotic BALB/c-IL10 deficient mice, which were inherent with colonic inflammation. In their study, infection of Helicobacter hepaticus prior to AOM indjection synergistically increased tumor compared to animals treated with AOM alone.

Although the correlation of gut microbes with the incidence of colon cancer hasn’t been established yet, some metabolic products of gut microbes were known to act as promoters for the carcinogenesis. Correspondingly, the activities of bacterial \( \beta \)-glycosidases, \( \beta \)-glucuronidases, and alcohol dehydrogenases were implicated with the generation of those promoting metabolites. However, it is still too early to make the conclusion of the role of
these bacteria in the colon cancer, especially when the evaluation was not in the context of commensal bacteria population.

### 4.2.2 Prevention of colon cancer by RS and probiotics

The evidence of gut microbes against colon cancer mainly comes from the application of probiotics and synbiotics. As commonly accepted probiotics, lactic acid bacteria (LAB), have been shown to reduce the risk of colon cancer. Early in 1980s, Goldin and Gorbach (1980) designed a study with male Fisher 344 rats fed beef diet simulating Western diet, which was known to increase the risk of colon cancer. With the supplementation of live *Lactobacillus acidophilus* culture in the diet, the incidence of colon cancer was reduced from 77% in control group to 40% at the end of 20 wk. Similarly, the addition of 2% lyophilized culture of *Bifidobacterium longum* to the basal AIN76A diet has also been shown to inhibit the incidence and the multiplicity of colonic tumor induced by AOM in Fisher 344 rats at the end of 20-wk feeding (Orrhage et al. 1994). It was proposed that LAB might include binding and degrading carcinogens, producing anti-tumorigenic compounds, enhancing immune response and etc, as summarized by Hirayama et al. (2000).

With the physiological significance of butyrate, butyrate producing bacteria have also been implicated as effective in reducing colon cancer. *Butyribrio fibrisolvens*, for example, one low abundance human intestinal bacteria, has been studied by Ohkaware et al. (2009). In the study, Jcl:ICR mice (n = 10 each) were orally administrated live *B. fibrisolvens* (strain MDT-1) once per week, live bacteria (10⁹ cfu/dose) 3 times per week, or cell homogenates of bacteria 3 times per week for 4 wk. The DMH (0.75 mg/day) was given with stomach tube twice with the frequency of once a week for the first two week. They found out that ACF number was significantly decreased by *B. fibrisolvens* administration in a dose response compared to saline group (24 ± 4.2 versus 17.9 ± 3.0 versus 9.4 ± 1.3, P < 0.05), but with no effects of cell homogenate (19.7 ± 2.7). In addition, the β-glucuronidase activity in the cecal content was only decreased by the inoculation of *B. fibrisolvens* 3 times per week compared to control (34.1 ± 3.1 versus 26.7 ± 4.1 µmol/ (h·g wet contents).
Due to the survivability of probiotics in the gut, the synbiotic application of prebiotics and probiotics has been expected to be a more efficient dietary intervention to manipulate gut microbial population with the aim to improve human health and prevent disease development. For example, *Clostridium butyricum* (strain CBM588), for example, is one known butyrate and acetate producing bacteria, has been shown to enhance the effect of high amylose starch in reducing ACF number in rats (Nakanishi et al. 2003). However, although cecal butyrate was increased by *C. butyricum* administration, no effect was observed in decrease ACF. On the other hand, butyrate didn’t changed by the HAS and synbiotic feeding, but intestinal acetate and propionate level were increased. In addition, β-glucuronidase activity was also decreased significantly in the two groups of rats fed HAS. (Only abstract available).

Similarly effects have been reported by Leu et al. (2010) reported with the synbiotic application of RS and *Bifidobacterium lactis*, which was shown to degrade RS *in vitro*. In the study, 1% (wt/wt diet) *B. lactis* in form of lyophilized culture (1X10^{11} cfu/g) was given with or without two type of RS: native or retrograded HiMaize starch (10% wt/wt diet) for 4 wk before carcinogen azoxymethane (AOM) injection once weekly for 2 wk. After another 26 wk, they found out that synbiotic application of bacteria with RS significantly decreased the neoplasm number (18.3%, *P* < 0.001) compared to control diet (53.3%) but not by either single application (50%, *P* = 0.08 for *B. lactis* and 33.3%, *P* = 0.07 for pooled of two RS). In addition, the *B. lactis* alone didn’t change the SCFA production, but the HAS addition increased three SCFA level in the distal colon compared to control (9.0 ± 5.2 versus 4.1 ± 2.1, *P* < 0.001), but not different from HAS feeding alone (9.8 ± 5.9).

In addition, the synbiotic application of RS and *Bifidobacterium lactis* has been performed in a double-blind, placebo-controlled cross-over human study (Worthley et al. 2009). In the study, 20 healthy human subjects were recruited and given 25 g HAS (Hi-maize 958) in the form of sachet and 5g B.lactis (strain LAFTI B94, 10^9 cfu/g) in the form of capsule per day as supplement to their usual diet. 4-wk feeding was followed by 4-wk washout intervals between treatments. Only the incidence of MINT2 methylation in rectal mucosa was significantly decreased by synbiotic application (*P* = 0.04). MINT2 is one of those type C DNA methylation markers, which might be important in early colon carcinogenesis with
further epigenetic events. In addition, although they didn’t find difference of fecal SCFA level, serum cytokine level, crypt proliferation index and colon cell height by dietary intervention, they reported correlation of total fecal SCFA concentration with the crypt height before treatment ($r = -0.56$, $P = 0.038$). Moreover, with DGGE, they reported the significantly difference of fecal bacteria profile between the synbiotic group and HAS alone ($P = 0.032$) or $B. lactis$ ($P = 0.001$) alone, although no difference were found between the two treatments ($P = 0.49$). The major difference was due to bands with the sequences related to Lachnospiraceae, particularly clone MS146A1_G07 detected 61% in the synbiotic group compared to 50% in HAMS group, and 32% in $B. lactis$ group.

Dietary intervention is one promising strategy to tune the gut microbes into a healthy-status against the disease and even overcome the genetic disease predisposition. It is unknown whether other bacteria related to RS fermentation, such as Ruminococcus bromii could have similar effects to reduce the risk of colon cancer, which is anticipated for the application in the near future. Although the application of synbiotics has been shown promising, there is still a long way to go for the identification of probiotics related to RS and fully understand the properties of bacteria considering the safety issues.

In summary, although it was inconclusive in the prevention effect of RS against colon cancer, due to the varied experimental protocols and RSs used in the studies, RS, as one of the prebiotics to increase SCFA production, to modulate gut microbial profiles and their metabolic activity, could be one promising candidate to reduce colon cancer risk. It is anticipated that the underlying mechanisms could be identified in the near future with genomics, transcriptomics and metabolomics studies.

5. Literature Cited


CHAPTER 3. GUT MICROBIAL PROFILE IS RELATED TO THE EFFICACY OF RESISTANT STARCH IN DECREASING ABERRANT CRYPT FOCI (ACF) IN FISHER 344 RATS

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Abstract

The study was designed to understand how gut microbiota contribute to effects of resistant starch (RS) in preventing colon carcinogenesis. Compared to the raw normal cornstarch (control), raw high amylose cornstarch VII (HA7, type 2 RS) feeding significantly lowered the number of aberrant crypt foci (ACF) induced by azoxymethane (AOM) in Fisher 344 rats, but a stearic acid-complexed HA7 cornstarch (HA7-SA, type 5 RS) did not. We hypothesized that the shift of gut microbiota induced by RS feeding was correlated with its differential efficacy in decreasing ACF. PCR-DGGE (Denaturing Gradient Gel Electrophoresis) analysis of microbes from rat colon contents was used to assess patterns of total bacteria, *Clostridium* clusters IV and XIVa, and *Bacteroides fragilis* group. Our results showed that the two RSs induced differential shifts in colonic total bacteria and *Clostridium* cluster XIVa, which were also different from control diet. A putative *Ruminococcus bromii*-related organism belonging to *Clostridium* cluster IV was enriched by both two RSs feeding. Whereas, HA7-SA feeding significantly enriched a putative *Bifidobacterium pseudolongum* bacteria species, and HA7 feeding specifically enriched a *Ruminococcus spp.* (*R. obeum* or *R. sp. SR1/5*) belonging to *Clostridium* cluster XIVa. More importantly, ACF number was significantly correlated with DGGE DNA patterns of total bacteria and *Clostridium* cluster XIVa. We concluded that two
types of RS had different prebiotic effects on modulating colonic microbiota, which were related to their effectiveness in inhibiting colon carcinogenesis. *Clostridium* cluster XIVa, especially a *R. spp* (*R. obeum* or *R. sp. SR1/5*), putatively stimulated by HA7, may be important in mediating the suppression of colon carcinogenesis by RS.

**Introduction**

Resistant starch (RS) is defined as the portion of starch that escapes digestion in the small intestine of healthy individuals (1). Four types of RS were classified: type 1 RS are physically inaccessible starches; type 2 RS are native B- and some C-polymorphic starches with highly packed structure inside the granules; type 3 RS are retrograded amylose; and type 4 RS are chemically modified starches through etherization, esterification, and crosslinking to decrease digestibility (2, 3).

Epidemiological studies suggested that higher RS consumption was related to the lower risk of large bowel cancer (4). Animal studies have provided evidence showing a decrease in the incidence of cancer induced by carcinogen azoxymethane (AOM) by 25-wk feeding of 10% and 20% (wt/wt diet) high amylose cornstarch (HAS) in male Sprague-Dawley (SD) rats (5). Similarly, in the study of Bauer-Marinovic et al. (6), SD rats fed 10% retrograded HAS (Novelose 330) feeding for 20 wk did not develop tumors induced by carcinogen dimethylhydrazine (DMH), whereas half animals in control group fed highly digestible waxy starch developed tumors. However, some contradictory results showed that RS feeding was not effective in prevention of colon carcinogenesis. In the study of Sakamoto et al. (7), the addition of pancreatin-digested residue of HAS to the basal diet (3% or 10%, wt/wt diet) did not change the incidence of tumors induced by DMH in SD rats, compared to the basal diet after 7-wk feeding. Moreover, one study showed that RS even potentiated the tumor development, as shown by the increase of tumor incidence, tumor size, and tumor frequency induced by DMH in SD rats fed diet containing 20% raw potato starch, compared to control diet without RS (8). Various aspects might affect the efficacy of RS in suppressing colon carcinogenesis. It has been demonstrated that the incidence of ACF was decreased by 13-wk HAS feeding in Wistar rats when carcinogen AOM was applied with prior 3-wk RS feeding,
but was increased when AOM applied before RS feeding (9). These results indicated that RS was effective against the post-initiation/progression phase of carcinogenesis, but not the initiation phase of carcinogenesis. Additionally, Zhao et al. (unpublished, 2010) showed that one type of RS, stearic acid-processed HAS, decreased ACF number in AOM-treated Fisher 344 rats when the diet was prepared with heating procedure to mimic human cooked starch food, but not by raw diet feeding with the same type of RS. These results indicated that the preparation of RS diet might affect the efficacy of RS inhibiting colon carcinogenesis, probably through the newly formed physicochemical structure of RS during heating processing.

RS is currently considered as a prebiotic to selectively stimulate the growth of colonic bacteria and/or activity beneficial to human host health (10). Major fermentation products, SCFA, have been linked to the maintenance of colon health and prevention of colon cancer. SCFA, especially butyrate, are primary energy sources for colonocytes (11). SCFA could also stimulate the proliferation of basal crypt colonocytes (12), enhance colonic epithelial barrier function (13), and more importantly, induce apoptosis of a body of hyper-proliferative cell lines in vitro (14, 15). RS fermentation-associated bacteria have been identified with both in vitro (16) and in vivo feeding studies (17) (18). *Bifidobacterium spp.*, *Bacteroides spp.*, and *Clostridium spp.* are major RS-associated bacteria and 80% of the members of *Clostridium* XIVa microbial cluster are butyrate producers (19). Therefore, a fundamental question is how gut microbiota will be modified during RS fermentation, which might be highly associated with the efficacy of RS inhibiting colon carcinogenesis.

Molecular methods based on 16S rRNA genes are widely used to study the biodiversity of gut microbial community, including fluorescence in situ hybridization (FISH), quantitative real time PCR (qRT-PCR), and PCR coupled with denaturing gradient gel electrophoresis (PCR-DGGE). Next generation sequencing developed in the past decade significantly enhanced our understanding of the diversity of human gut microbiome (20). Although large inter-individual variation has been reported, patterns of gut microbial populations have recently been correlated with human disease, such as obesity (21, 22) and inflammatory bowel disease (IBD) (23), (24). The role of gut microbes in colon carcinogenesis has long
been studied. Early studies identified 20 fecal bacteria species which were significantly associated positively or negatively related to colon cancer risks (25). Animals studies showed that inoculation of H₂S-producing bacteria *Enterococcus faecalis* induced colitis and rectal tumors after 20-27 wk of colonization in germ-free (GF) IL10 knock-out mice (26). On the other hand, orally administration of live *Butyrivibrio fibrisolvens*, which was a butyrate producing bacteria, to GF Jcl : ICR mice significantly decreased the preneoplasm formation induced by the carcinogen (27). However, the gut microbial patterns related to colon cancer are still largely unknown.

Recently, a new group of RS (type 5) was proposed for amylose-lipid complex (28). Human feeding trial with single meal showed that a type 5 RS made from complexing high amylose cornstarch VII (HA7) with palmitic acid, named as HA7-PA, significantly decreased postprandial glycemic and insulinemic responses compared to commercial wheat flour starch when they were served as bread for breakfast, suggesting the lower digestibility of HA7-PA by human digestion in vivo (29). In the study of Zhao et al. (unpublished, 2010), feeding of another type 5 raw RS, HA7 processed with stearic acid (HA7-SA), and raw HA7 induced similar fermentation outcomes of gut microbiota in Fisher 344 rats, as implicated by the same extent of the decrease of cecum pH and the increase of cecum weight compared to raw normal cornstarch (CS) feeding. However, only HA7 feeding significantly decreased ACF numbers induced by azoxymethane (AOM) detected in the distal colon, but not by HA7-SA, compared to raw normal CS.

In this study, we further assessed the prebiotic effects of RS on modulating gut microbiota in rats fed HA7 or HA7-FA diet conducted in the study of Zhao et al. (unpublished, 2010). We hypothesized that the shifts of gut microbiota induced by RS feeding was related to the differential efficacy of two RSs in the prevention of colon carcinogenesis. PCR-DGGE was used to monitor colonic bacterial pattern with universal primers of 16S rRNA gene and specific primers for *Clostridium* clusters IV and XIVa, and *Bacteroides fragilis* group. The formation of preneoplastic lesions, ACF induced by AOM was correlated with the bacterial pattern examined to elucidate the role of gut microbiota in preventing colon carcinogenesis.
Materials and Methods

Animals and diets. Details of the protocol for animal care, diet preparation and carcinogen treatment were reported in Zhao et al. (unpublished, 2010). Briefly, fifty-four male Fischer 344 (F344) rats (5-wk-old) (Charles Rivers laboratory, Wilmington, MA) were housed individually. Three types of raw cornstarches were used: normal cornstarch (CS) (Cargill Gel™ 03420; Cargill Inc., Minneapolis, MN), high-amylose cornstarch VII (a type 2 RS, HA7) (AmyloGel 03003; Cargill Inc., Minneapolis, MN), and HA7 processed with stearic acid (a type 5 RS, HA7-SA), kindly provided by Dr. Jay-lin Jane (Iowa State University). Starches were added to the standard animal diet AIN-93M (Harland Tekland, Madison, WI) to give a mixture of 50% (dry wt starch/wt diet). Animals were adapted for 2 wk with raw CS feeding before twice weekly injection of saline or carcinogen azoxymethane (AOM) (Midwest Research Institute, Kansas City, MO) (15 mg/kg b.w.) intraperitoneally, followed by switching to raw RS diets for another 8 wk.

Bacterial genomic DNA extraction. After 8-wk starch feeding, animals were euthanized with CO₂. Two rats did not have colon contents at the time when samples were collected. Hence, fresh colon contents from a total of 52 rats were obtained and kept frozen at -70 °C until analyzed. Bacterial genomic DNA from colonic contents of rats was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). The obtained DNA was quantified with NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE).

PCR. Primers used in the current study were listed in Table 1. Reversed primers were attached with a GC-clamp (GCCCCGCCCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGGACGCGCG) for DGGE analysis (30). PCR reactions were performed with 400 ng genomic DNA, 5 µL Taq buffer, 4 uL dNTP (0.25 mmol/L), 50 pmol of each primer, and 0.25 µL (5 units/µL) Hotstart Taq polymerase (TAKARA Bio Inc., Shiga, Japan) in a final 50 µL reaction volume. PCR was performed on a Thermal cycler (Bio-Rad Laboratories, Hercules, CA) and conditions were as follows: 1 cycle of denaturation at 94 °C for 5 min, followed by 21 cycles of denaturation at 95 °C for 15 sec, annealing for 30 sec and extension at 72 °C for 2 min, with the final extension at 72 °C for 10
All PCR products were analyzed by electrophoresis with 2% agarose gel to confirm the presence of amplicons before applying to DGGE analysis.

**DGGE.** Equal amounts of PCR products were applied to denaturing gradient gels as described by Muzyer et al. (30). DGGE marker III (Wako, Osaka, Japan) was used for the normalization between gels. Polyacrylamide (37:5:1 acrylamide/bisacrylamide) gels (16 cm × 16 cm) with a defined gradient of urea and formamide were casted with a gradient former (Thermo Fisher Scientific Inc., Pittsburgh, PA). 100% denaturing solution contains 7 mol/L urea and 40% (v/v) formamide. DGGE electrophoresis was performed in 1 × TAE (Tris-acetate-EDTA, pH 8.0) buffer at constant temperature of 60 °C for 14 h using the De-code system (Bio-Rad Laboratories, Hercules, CA). Gels were stained with 0.1% silver nitrate (AgNO₃) for 20 min and washed twice with distilled water for 30 sec, followed by soaking in the exposing solution containing NaOH (1.5% wt/v), NaBH₄ (0.01%, wt/v), and formaldehyde (0.4%, v/v) until bands were clear (31). DGGE gels were visualized with GS-800 Calibrated Imaging Densitometry (Bio-Rad Laboratories., Hercules, CA) and analyzed with “Quantity One” software.

**DGGE bands purification and sequencing.** DGGE bands of particular interest were excised and extracted with Wizard SC gel and PCR clean-up system (Promega, Madison, WI). A second round PCR was used to enrich DNA with same PCR conditions described above. Purified PCR fragments were cloned into sequencing vector and transformed to competent cells provided in TOPO-TA cloning kits (Invitrogen, Carlsbad, CA) according to the manufacture manual. Single colonies were cultured and plasmids were extracted with QIAamp Plasmid mini kit (Qiagen, Valencia, CA). Closely matched bacteria species were identified by aligning DNA sequences to GenBank DNA database and Ribosomal Release Project database.

**Statistical analysis.** Presence/absence of DNA bands and intensity were used for statistical analysis of DGGE gels. 2-dimensional non-metric multidimensional scaling (nMDs) analysis was performed with R package (R Foundation for Statistical Computing, Vienna, Austria) to determine the similarity/dissimilarity of DGGE patterns across individual animals. Partial
least square analysis (PLS) was performed with SAS 9.2.1 (SAS Institute Inc, Irvine, CA) to determine the relationship between DGGE pattern and ACF number.

Results

DGGE band pattern of total bacteria from rat colon contents. PCR-DGGE DNA band pattern shown in Fig. 1 represented colonic microbial profile of all 52 rats fed with three types of raw starch diets, with or without prior AOM intraperitoneal injection. The number of DNA bands detected in individual samples varied from 7 to 20 (with an average of 16), which is similar to that previously reported in male Fisher 344 rats (32). There was no significant difference in the average band numbers among six groups of animals (Table 2).

Non-metric multidimensional scaling (nMDs) analysis was used to show the similarity/dissimilarity of colonic microbial DGGE band patterns based on the presence/absence of DNA bands and intensity. Colonic microbial DNA band patterns from animals fed raw normal cornstarch (CS) diet were clustered away from patterns found in rats fed either RS diet, which were similar to each other (Fig. 2). In addition, a larger dissimilarity in the DGGE pattern was observed among individual animals within HA7 group than individuals within CS or HA7-SA-feeding group, as shown by the average dissimilarity distance among individuals in each group (Table 2).

Table 3 listed closely matched bacterial candidates corresponding to DNA bands with visual predominance. The average intensity of each band was compared across AOM-treated animals fed the three diets (n=29) to determine which putative microbial species were most strongly related to inhibition of carcinogenesis. The average intensity of Band 1, identified as Parabacteroides spp. (with 97% similarity to Bacteroides sp. ASF519 or Parabacteroides goldsteinii (T)), was lower in animals fed either raw RS diet compared with raw CS diet. The average intensity of Band 3, identified with 100% similarity to an Alistipes spp. (Alistipes finegoldii or Alistipes sp. DJF_B185), was increased by both RSs. In addition, Band 7, putatively Bifidobacterium pseudolongum (100% similarity), was consistently detected with higher intensity in animals fed HA7-SA than with the other two starches.
Correlation between DGGE band pattern of total colonic bacteria and the number of Aberrant Crypt Foci (ACF). Partial least square (PLS) analysis was used to determine if ACF numbers detected in the distal colon of AOM-treated animals fed three raw starch diets could be explained by DGGE band patterns of colonic microbial population. A criterion to exclude bands occurring in the colon content samples from less than 5 out of 29 individuals was applied, and hence a total of 26 bands were used in the PLS regression analysis, which accounted for 44.9% of the variability in DGGE DNA band patterns and 85.2% of the variability in ACF numbers. The coefficients in Fig. 3 showed the positive/negative association as well as the extent of the association of each band with ACF numbers. Three bands (U77, U75 and Band 7 (Bifidobacterium pseudolongum) (Fig. 1) showed the highest coefficients (0.25, 0.30, and 0.33 respectively), indicating their positive correlation with ACF numbers, whereas three other bands (U92, U30 and U63) showed the lowest coefficients (-0.31, -0.38 and -0.44 respectively), indicating their negative correlation with ACF numbers.

PCR-DGGE pattern of RS fermentation-associated bacteria groups. In order to further understand changes in bacterial groups known to be associated with RS fermentation and their relationship with ACF, a set of group-specific primers of 16S rRNA gene were applied to analyze colonic bacterial patterns of 29 AOM-treated animals.

Clostridium cluster IV. A total of 21 unique bands were observed in DGGE band patterns of rat colon contents with Clostridium cluster IV specific primers (Fig. 4A). The average DNA band numbers of colon content samples from individual animal were significantly decreased by both RS (Table 4). The nMDs analysis showed that individuals fed diets containing raw CS and HA7-SA showed distinct clusters from each other in the bacteria DGGE pattern (Fig. 5). One predominant band (Band8) was particular observed in most individuals fed either RS, which was most closely matched to Ruminococcus bromii (99% similarity) (Table 5). The intensity of this particular band was negatively correlated with ACF numbers detected in animals (r = -0.4152, P = 0.0251). Two DNA bands were detected in colon contents of rats fed raw CS (Band 11 and 12), but were less frequently seen in rats fed either RS. There was no correlation between Clostridium IV DGGE band pattern and the ACF number detected in AOM-treated animals according to the PLS analysis (data not shown).
Clostridium cluster XIVa. A total of 17 unique bands were obtained with specific primers for Clostridium cluster XIVa bacteria (Fig. 4B). The average number of DNA bands detected in colon contents from animals fed either raw RS diet was significantly decreased (Table 4). Three distinct clusters of DGGE patterns corresponding to different starch feeding treatment were observed with nMDs analysis (Fig. 5). One band (Band 13) was only detected in rats fed RSs, but not in animals fed CS, which was closely matched to uncultured Dorea sp. (96% similarity) (Table 6). Higher average intensity of Band17 was observed in animals fed raw HA7, which was negatively correlated with ACF numbers detected ($r = -0.5835$, $P = 0.0009$). Sequence analysis showed that it was closely matched to Ruminococcus sp. SRI/5 or Ruminococcus obeum (96% similarity).

With inclusion of bands detected in more than 3 individuals, we observed a significant correlation between the Clostridium XIVa DGGE pattern and ACF numbers detected in 29 AOM-treated rats according to PLS analysis, which accounted for 30.1% of the variability of DGGE band pattern and 55.5% of the variability of the observed ACF numbers. Among 13 bands used in the analysis, Band 17 (Ruminococcus sp. SRI/5 or R. obeum) showed the lowest coefficient (-0.16) in the model, indicating its strongest negative correlation with ACF numbers, whereas Band 15 (uncultured Lachnospiraceae or Clostridiales bacterium) and two other unidentified bands (U6 and U7 in Fig. 4B) showed the highest coefficients (0.13, 0.15, and 0.16 respectively) in the model.

Bacteroides fragilis group. A total of 7 unique bands were obtained from rat colon contents samples with Bacteroides fragilis group specific primers (Fig. 4C). Similar average band numbers were observed among the three different diet treatments (Table 4). A very consistent DGGE band pattern in the colon contents fed raw CS diet was observed, as shown by the smallest average dissimilarity distance (0.164). No distinct cluster was observed based on the starch diet according to the nMDs analysis (Fig. 5). Band 12, which had a close similarity (97%) to Bacteroides sp. ASF519 or Parabacteroides goldsteinii, was consistently decreased in rats fed HA7-SA diet compared to rats fed CS. PLS analysis did not reveal a significant correlation of band pattern of Bacteroides fragilis group with ACF number detected in AOM-treated animals (data not shown).
Discussion

In our study, we observed significant shifts of colonic bacterial species according to PCR/DGGE analysis in Fisher 344 rats after 8-wk feeding of two types of raw RS diets containing high amylose VII (HA7) or stearic acid-complex HA7 (HA7-SA). Additionally, the DGGE pattern of RS fermentation-associated bacteria group *Clostridium* cluster IV was shifted similarly by both RSs, whereas *Clostridium* cluster XIVa was shifted differentially by the two RSs. More importantly, DGGE band patterns of colonic total bacteria and *Clostridium* cluster XIVa were found to be significantly correlated with the number of pre-neoplasms, aberrant crypt foci (ACF), detected in the distal colon of animals treated with carcinogen azoxymethane (AOM).

Previous studies have shown that rats fed potato starch, inulin or oligofructose contained unique fecal bacterial pattern when analyzed with PCR-DGGE, which were different from that of animals fed control cornstarch (32). In our study, colonic bacteria composition in Fisher 344 rats was shifted by two raw RS diets feeding compared with raw normal cornstarch, as shown by nMDs cluster analysis of DGGE band pattern. Major shifts by two RS were observed visually on DGGE gels, as shown by the presence of Band 4 and Band 5, as well as the increased abundance of Band 3 (Table 2). These results are expected considering that a higher amount of undigested starch residues from two RS diets would be delivered to the lower gut, as predicated by the higher RS content measured in two RS diets (HA7: 20.2 ± 2.5 and HA7-SA: 30.7 ± 1.1% of dry feed basis), compared to CS diet (6.7 ± 1.2%) (Zhao et al., unpublished, 2010).

Although total colonic bacterial pattern did not reveal differences between the two RSs, some major bands showed specific response to HA7-FA by visual observation. For example, Band 7 (putatively *Bifidobacterium pseudolongum*) (Fig. 1) was consistently detected with high intensity in 16 out 17 rats fed HA7-SA. However, only 5 out of 17 animals fed HA7 showed comparable intensity of this band. *B. pseudolongum* has been shown to be the most predominant bacteria in the intestine of female Balb/C mice after 4-wk feeding of fructo-oligosaccharide with PCR-DGGE analysis (33). To our knowledge, this is the first report showing that *B. pseudolongum* was increased by RS feeding in rats. *In vitro* studies have
shown that *B. pseudolongum* has the highest affinity to HAS granules compared to 18 other *Bifidobacterium* isolates from human feces (34).

The differing bacteria profile between two RS indicated by the selective enrichment of *B. pseudolongum* in HA7-SA-feeding animals might be related to the chemical composition and physical structure of starches. The effect of starch crystalline structure on bacterial patterns has been studied *in vitro* with two polymorphs of retrograded HAS (35). In the study, type A and type B polymorphic retrograded starches were obtained by incubating autoclaved starches at 95 °C and 40 °C for 24 h, respectively. FISH analysis showed that type B polymorphic retrograded starch induced *Bifidobacterium spp.* after 11-d continuous incubation with human fecal microbes, whereas type A polymorph enriched *Atopobium spp.*. The type 5 RS, HA7-SA, used in our study was produced by treating HA7 starch with pullulanase, a debranching enzyme, followed by complexing with stearic acid at 80 °C, which may form different molecular structure and crystalline structure from those of HA7. The addition of stearic acid (C18:0) to the undigested starch complex, on the other hand, may also introduce new substrates for gut microbes, and its effect on gut microbial populations has not been studied. Further analysis of HA7-SA starch residues after mammalian digestion to determine the crystalline structure of the starch complex and the presence of stearic acid before the starch is exposed to microbes would give a better understanding of the mechanism of two types of RS in inducing different patterns of gut bacteria as observed in the current study.

We also observed that the bacterial pattern of animals fed the same diet tended to cluster together, independent of AOM treatment, as shown by the overlapping distribution of animals fed each starch diet on the nMDs plot (**Fig. 2**). This indicated that the type of starch in the diet, rather than the pre-cancer status with ACF or AOM treatment, is the major factor influencing the bacterial pattern shifts in our study. However, our technique might miss some important bacteria since DGGE has a detection limit of bacteria with > 1% abundance in the overall population (30). In addition, one particular band (Band 1), which was closely matched to *Parabacteroides spp.* (with 97% similarity to *Bacteroides sp. ASF519* or *Parabacteroides goldsteinii* (T), **Fig. 1**), showed decreased band intensity in rats fed both RS
that had also been exposed to AOM compared with rats given AOM and CS, whereas in animals not given AOM, there was no difference among the three starches in the band intensity of this putative bacterial species. It is unlikely that the carcinogen itself caused the difference between RS and CS since in our study all animals were fed raw CS diet during AOM injection and RS diets were introduced three days after AOM injection. The mechanism for two RSs to suppress this Parabacteroides spp. may be of some interest for further study, although this species was seemingly suppressed by both an RS (HA7) that suppressed ACF and by an RS that did not (HA7-SA).

It has been reported that the number of ACF was only decreased by HA7 feeding but not by HA-SA compared to highly digestible control starch (CS) (Zhao et al., unpublished, 2010). The correlation between colonic microbial pattern and the ACF number detected in AOM treated animals according to PLS analysis indicated shifts of gut microbial patterns might explain the different efficacy of these two types of RS in decreasing colon carcinogenesis. Unexpectedly, of those 26 bands, the intensity of Band 7 (a putative Bifidobacteria pseudolongum) showed the strongest positive correlation with ACF number (Fig. 3). Bifidobacterium spp. such as B. lactis and B. longum are the major recognized probiotic bacteria. Symbiotic administration of B. lactis and HiMaize significantly decreased incidence and multiplicity of colonic tumors by >50% compared to the control group, but marginally by RS alone and not by B. lactis (36). Similarly, 38-wk co-administration of B. pseudolongum biovar b and Neosugar significantly decreased ACF number induced by DMH in CF1 mice (37). Our observation of the ineffectiveness of B. pseudolongum to decrease ACF number in HA7-SA-feeding rats suggested that examining changes of single bacteria species might not be sufficient to understand the effects of RS due to the cooperation (such as cross-feeding) and competition among gut microbiota. Bifidobacterium spp. are known lactic acid-producing bacteria and also contribute to the butyrate production, as shown by co-culturing B. adolescentis with non-amylolytic but butyrogenic bacteria in starch supplemented medium (38, 39). Butyrate has been reported as the most potent SCFA to induce apoptosis of hyperproliferative and cancer cell lines in vitro, indicating its roles in preventing colon carcinogenesis (13). Therefore, we suspected that although the abundance of B. pseudolongum was increased in animals, no corresponding lactate-utilizing and butyrate-
producing bacteria were increased by HA7-SA, thus compromising the effect of this species in suppressing ACF incidence.

Using specific primers targeting known RS fermentation-related bacteria groups helped reveal the similarity and difference between two RSs in their influences on gut microbial population shifts. Both RSs significantly decreased diversity of the two Clostridium clusters as shown by a decrease in the average band numbers per individual compared with CS. Increased diversity of Clostridium cluster IV and XIVa has been observed in colon cancer patients (40). Physiological significance of the diversity within Clostridium cluster is still unknown, although it is possible that some Clostridium species in these clusters might have other undiscovered detrimental activity. For example, fecal beta-glucuronidase activity was reported to be 1.7-fold higher (or 12.1-fold higher with sonicated fecal specimens) in patients with colon cancer than in healthy control subjects (41). Intriguingly, PCR analysis on 40 dominant human fecal isolates showed that beta-glucuronidase gene (gus) was mainly detected in bacteria belonging to Clostridium clusters XIVa and IV (42).

One consistent shift by both RSs was the putative enrichment of Ruminococcus bromii (Band 9, Fig. 4A) belonging to Clostridium cluster IV group. This is consistent with the findings from one recent human feeding trial, in which a 4-wk feeding of HiMaize starch diet enriched the abundance of fecal R. bromii-related phylotypes compared to a low RS diet or regular diet (16). In vitro fermentation studies showed that R. bromii was selectively enriched by high amylose cornstarch (32) and it was a major starch degrader identified by tracing C\textsuperscript{13} labeled starch (43). We only observed a weak correlation between the band intensity and ACF numbers ($r = 0.4152$), which is not unexpected considering the major fermentation products of starch by R. bromii are acetate, formate and ethanol (44), but not butyrate, which is important in preventing colon carcinogenesis. Abell et al. (16) did not find significant correlation between increased fecal butyrate level by high RS consumption and increased R. bromii abundance.

Differential Clostridium XIVa DGGE pattern detected in animals fed raw HA7 from HA7-SA indicated that Clostridium XIVa was related to the difference in efficacy of the two RS in decreasing ACF number. Clostridium cluster XIVa, also known as
Roseburia/coccoides/Eubacterium rectale group, is a major bacterial cluster with butyrate producing capability. The varied efficiency of starch utilization and SCFA production by phylogenetically related bacteria in this cluster has been reported by Louis et al. (45), in which butyrate producing ability varied from 1.9 mM (Eubacterium ruminantium) to 24 mM (Roseburia intestinalis) with starch fermentation. Clostridium cluster XIVa pattern was correlated with efficacy of RS to suppress ACF as shown by the PLS regression model. Additionally, Band 17 (Ruminococcus obeum or Ruminococcus sp. SR1/5, 96% similarity) (Fig. 4B) has the lowest negative coefficient in the model and the band intensity was negatively correlated with ACF numbers (r = -0.5835, P = 0.0009). Interestingly, R. obeum has been reported to be saccharolytic with glucosidase activity to produce and acetate as the major fermentation products (46). Acetate has been known as a substrate for butyrate production by butyryl-CoA: acetate transferase in other Clostridium XIVa bacteria, such as Roseburia intestinalis or Clostridium IV bacteria such as Faecalibacterium prausnitzii, again suggesting the importance of cross-feeding in gut microbiota (47, 48).

Bacteroides thetaiotaomicron has been indicated to have the ability to degrade and utilize starch by its outer-membrane complex encoded by sus gene cluster (49). A 4-wk human feeding trial of HiMaize diet (22 g/d) also increased B. thetaiotaomicron in fecal samples as shown by PCR-DGGE analysis (16). However, we did not observe the enrichment of any bands in Bacteroides fragilis group in response to RS feeding. By contrast, rats fed HA7-SA and some rats fed HA7 showed decreased band intensity for Bacteroides sp. ASF519-related phylotype, which was consistent with our observation of colon microbial DNA bands using universal 16S rRNA primers. The suppression of Bacteroides spp. by RS may be related to the increased acidity of gut contents by RS fermentation. It has been reported that in the in vitro anaerobic fermentation culture of fecal microbiota with a mixed carbohydrate sources, Bacteroides levels was increased from 20% to 75% of total bacteria when pH shifts from 5.5 to 6.5 with FISH analysis (50). Further examination of three dominant fecal Bacteroides species individually (Bacteroides ovatus V975, B. thetaiotaomicron DSM 2079 and B. vulgatus DSM 1447) showed that they grew poorly at pH 5.5, but well at pH 6.5.

In conclusion, our study supported a previous study that Ruminococcus bromii-related organisms were highly involved in RS fermentation. We also first reported that
Bifidobacterium pseudolon was increased by RS5 feeding. In addition, similar shifts by both RS were observed in Clostridium cluster IV, whereas the two RSs differed in shifts of Clostridium cluster XIVa and Bacteroides fragilis group in colon microbiota. More importantly, Clostridium cluster XIVa pattern was related to ACF number, which is an indirect evidence of the importance of butyrate production in the prevention of colon cancer. These results clarify the ability of RS to modulate gut microbes, and suggest major beneficial or detrimental bacteria species that might be targets for manipulation, with the long-term goal to maintain colon health and decrease colon cancer risk.

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**Footnotes**

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2 Author disclosures: M-Y. Yum, Y. Zhao, J. Hasjim, D.F. Birt, J-L. Jane, P.M. Dixon, and S. Hendrich, no conflicts of interest.

6 Abbreviations used: ACF, aberrant crypt foci; AOM, azoxymethane; DMH, dimethylhydrazine; HA7, high-amylose cornstarch VII; HA7-PA, high amylose cornstarch VII processed with palmitic acid; HA7-SA, high amylose cornstarch VII processed with stearic acid; PCR-DGGE, PCR-denaturing gradient gel electrophoresis; RS, resistant starch; SCFA: short chain fatty acid.

* To whom correspondence should be addressed. Email: shendric@iastate.edu
Literature Cited


Table 1 Primers used for PCR-DGGE analysis.

<table>
<thead>
<tr>
<th>Target groups</th>
<th>Primers</th>
<th>Sequence (5’-3’) (^1)</th>
<th>PCR Annealing Temp. (°C)</th>
<th>DGGE gradient (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>357F</td>
<td>ATTACCGCGGCTGCTGG</td>
<td>65-55</td>
<td>20-80</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td>518R-GC</td>
<td>GC clamp-CCTACGGGAGGCGGAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroides fragilis group</td>
<td>Bfr-F</td>
<td>CTGAACCAGCCAAGTAGCG</td>
<td>52</td>
<td>20-70</td>
<td>(51)</td>
</tr>
<tr>
<td></td>
<td>Bfr-R-GC</td>
<td>GC clamp-CCGCAACTTCAACAAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium cluster XIVa (Clostridium coccoides-Eubacterium rectale group)</td>
<td>Cocc-F</td>
<td>AAATGACGGTGACCTGACAAA</td>
<td>54</td>
<td>38-60</td>
<td>(52)</td>
</tr>
<tr>
<td></td>
<td>Cocc-R-GC</td>
<td>GC clamp-TGGGATGTTTCTTGGCA</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Clostridium cluster IV (Clostridium leptum group)</td>
<td>sg-Clept-F</td>
<td>GCACAAGCAGTGAGT</td>
<td>50</td>
<td>35-55</td>
<td>(53)</td>
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<tr>
<td></td>
<td>sg-Clept-R-GC</td>
<td>GC clamp-TTCCTCGGTTTGTCAA</td>
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</table>

\(^1\) *GC-clamp: GCCCGCCGCCGCCGCCGGGGGCGGCGGCGGGGCGGGGCGGGG-CACGCGGCGG
Table 2  Sequence information of predominant bands indicated in DGGE gel (Fig. 1) of colonic total bacteria in AOM-treated Fisher 344 rats fed three raw starch diets.

<table>
<thead>
<tr>
<th>Band</th>
<th>Mean intensity</th>
<th>Closest relatives (sequence similarities)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>HA7</td>
</tr>
<tr>
<td>Band 1</td>
<td>ND</td>
<td>66.9 ± 23.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 5)</td>
</tr>
<tr>
<td>Band 2</td>
<td>ND</td>
<td>52.4 ± 22.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 4)</td>
</tr>
<tr>
<td>Band 3</td>
<td>176.7 ± 5.4</td>
<td>133.3 ± 35.6</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>139.4 ± 21.2</td>
<td>b</td>
</tr>
<tr>
<td>Band 4</td>
<td>106.1 ± 22.1</td>
<td>143.5 ± 21.2</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>139.4 ± 38.9</td>
<td>b</td>
</tr>
<tr>
<td>Band 5</td>
<td>129.2 ± 24.6</td>
<td>139.4 ± 38.9</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>139.4 ± 38.9</td>
<td>b</td>
</tr>
<tr>
<td>Band 6</td>
<td>123.9 ± 21.5</td>
<td>126.4 ± 21.5</td>
</tr>
<tr>
<td></td>
<td>(n = 9)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Band 7</td>
<td>93.4 ± 34.4</td>
<td>92.0 ± 70.1</td>
</tr>
<tr>
<td></td>
<td>(n = 9) a</td>
<td>(n = 5) ab</td>
</tr>
</tbody>
</table>

1 Values are band mean intensity ± SD. Values within a row followed by a same letter are not significantly different (P < 0.05). ND: not detected.
2 Sequence similarities were obtained with the alignment of PCR fragments excluding primers against NCBI database.
Table 3 Average band number detected on DGGE gels of colonic total bacteria (Fig. 1) and average within group distance of each treatment with nMDs analysis (Fig. 2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Band Number ¹</th>
<th>Average within group distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS + Saline (n = 8)</td>
<td>17 ± 4</td>
<td>0.372</td>
</tr>
<tr>
<td>CS + AOM (n = 10)</td>
<td>15 ± 3</td>
<td>0.397</td>
</tr>
<tr>
<td>HA7 + Saline (n = 8)</td>
<td>15 ± 5</td>
<td>0.538</td>
</tr>
<tr>
<td>HA7 + AOM (n = 9)</td>
<td>15 ± 4</td>
<td>0.523</td>
</tr>
<tr>
<td>HA7-SA + Saline (n = 7)</td>
<td>16 ± 1</td>
<td>0.442</td>
</tr>
<tr>
<td>HA7-SA + AOM (n = 10)</td>
<td>16 ± 2</td>
<td>0.395</td>
</tr>
</tbody>
</table>

¹ Values are mean ± SD
Table 4 Average band number detected on DGGE gels of *Clostridium* cluster IV, *Clostridium* cluster XIVa, and *Bacteroides fragilis* group (Fig. 4) and average within group distance of each treatment according to the nMDs plot (Fig. 5).

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>Clostridium</em> cluster IV</th>
<th><em>Clostridium</em> cluster XIVa</th>
<th><em>Bacteroides fragilis</em> group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Band Number 1</td>
<td>Average within group distance</td>
<td>Band Number 1</td>
</tr>
<tr>
<td>CS+AOM (n = 10)</td>
<td>10 ± 2 b</td>
<td>0.443</td>
<td>10 ± 2 b</td>
</tr>
<tr>
<td>HA7+AOM (n = 9)</td>
<td>4 ± 2 a</td>
<td>0.591</td>
<td>4 ± 2 a</td>
</tr>
<tr>
<td>HA7-SA+AOM (n = 10)</td>
<td>3 ± 2 a</td>
<td>0.52</td>
<td>5 ± 1 a</td>
</tr>
</tbody>
</table>

1 Values are mean ± SD. Values within a column followed by a same letter are not significantly different (P <0.05).
Table 5 Information of bands indicated in DGGE gels with specific primers targeting *Clostridium* cluster IV, *Clostridium* cluster XIVa, and *Bacteroides fragilis* group in AOM-treated Fisher 344 rats fed three raw starch diets.

<table>
<thead>
<tr>
<th>Target groups</th>
<th>Band</th>
<th>Mean intensity $^1$</th>
<th>Closest relatives</th>
<th>Similarities $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>HA7-SA</td>
<td>HA7</td>
<td>Phylum</td>
</tr>
<tr>
<td>Clostridium Cluster IV</td>
<td>Band 8</td>
<td>156.3 a</td>
<td>399.9 b</td>
<td>321.6 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 10)</td>
<td>(n = 8)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td></td>
<td>Band 9</td>
<td>95.9 a</td>
<td>181.1 b</td>
<td>82.1 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 3)</td>
<td>(n = 4)</td>
<td>(n = 3)</td>
</tr>
<tr>
<td></td>
<td>Band 10</td>
<td>11.4</td>
<td>42.3</td>
<td>24.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 9)</td>
<td>(n = 10)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td></td>
<td>Band 11</td>
<td>53.0</td>
<td>ND</td>
<td>40.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 10)</td>
<td></td>
<td>(n = 5)</td>
</tr>
<tr>
<td></td>
<td>Band 12</td>
<td>66.2</td>
<td>ND</td>
<td>7.8</td>
</tr>
</tbody>
</table>
Table 5 (continued) Information of bands indicated in DGGE gels with specific primers targeting *Clostridium* cluster IV, *Clostridium* cluster XIVa, and *Bacteroides fragilis* group in AOM-treated Fisher 344 rats fed three raw starch diets.

<table>
<thead>
<tr>
<th>Target group</th>
<th>Band</th>
<th>Mean intensity $^1$</th>
<th>Phylum</th>
<th>Family</th>
<th>Genus</th>
<th>Species (Accession No.)</th>
<th>Similarities $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>HA7-SA</td>
<td>HA7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 13</td>
<td>ND</td>
<td>83.9 (n = 10)</td>
<td>106.5 (n = 7)</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Dorea</td>
<td>Uncultured Dorea sp. (EU530244)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium</td>
<td>Band 14</td>
<td>41.7 a (n = 10)</td>
<td>207.9 b (n = 8)</td>
<td>173.8 b (n = 1)</td>
<td>Firmicutes</td>
<td>Incertae Sedis XIV</td>
<td>Blautia</td>
</tr>
<tr>
<td>Cluster XIVa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Band 15</td>
<td>119.7 b (n = 10)</td>
<td>42.1 a (n = 4)</td>
<td>26.8 a (n = 4)</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Band 16</td>
<td>116.9 b (n = 10)</td>
<td>169.5 b (n = 10)</td>
<td>34.8 a (n = 6)</td>
<td>Firmicutes</td>
<td>Unclassified</td>
<td>Clostridiales</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Band 17</td>
<td>65.8 a (n = 8)</td>
<td>156.9 b (n = 5)</td>
<td>217.8 b (n = 7)</td>
<td>Firmicutes</td>
<td>Ruminococcaceae</td>
<td>Ruminococcus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Band 18</td>
<td>72.5 (n = 10)</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
Table 5 (continued) Information of bands indicated in DGGE gels with specific primers targeting *Clostridium* cluster IV, *Clostridium* cluster XIVa, and *Bacteroides fragilis* group in AOM-treated Fisher 344 rats fed three raw starch diets.

<table>
<thead>
<tr>
<th>Target group</th>
<th>Band</th>
<th>Mean intensity</th>
<th>Closest relatives</th>
<th>Similarities</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacteroides fragilis</em> group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Band 19</td>
<td>248.4 b (n = 10)</td>
<td>105.8 a (n = 10)</td>
<td>235.6 b (n = 7)</td>
</tr>
<tr>
<td></td>
<td>Band 20</td>
<td>75.5 (n = 10)</td>
<td>119.1 (n = 10)</td>
<td>106.9 (n = 6)</td>
</tr>
<tr>
<td></td>
<td>Band 21</td>
<td>65.8 (n = 10)</td>
<td>100.1 (n = 10)</td>
<td>90.3 (n = 6)</td>
</tr>
</tbody>
</table>

1 Values are band mean intensity. Values within a row followed by a same letter are not significantly different (P < 0.05). ND: not detected. NA: not available.

2 Sequence similarities were obtained with the alignment of PCR fragments excluding primers against NCBI database.
Table 6  Intensity of six bands (Fig. 3) showing lowest (U63, U30 and U92) and highest coefficients (U77, U75 and Band 7) according to PLS analysis of DGGE total colonic bacteria in AOM-treated animals fed three raw starch diets $^1$.

<table>
<thead>
<tr>
<th></th>
<th>U63</th>
<th>U30</th>
<th>U92</th>
<th>U77</th>
<th>U75</th>
<th>Band 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>$73.6 \pm 7.0$ (n = 3)</td>
<td>117.1 (n = 1)</td>
<td>$83.9 \pm 33.0$ (n = 7)</td>
<td>$121.7 \pm 39.9$ (n = 9)</td>
<td>$93.4 \pm 34.4$ (n = 9)</td>
<td>38.1 (n = 1)</td>
</tr>
<tr>
<td>HA7-SA</td>
<td>$133.6 \pm 40.6$ (n = 8)</td>
<td>$110.4 \pm 40.6$ (n = 6)</td>
<td>$89.7 \pm 18.5$ (n = 4)</td>
<td>$117.8 \pm 14.0$ (n = 9)</td>
<td>$165.4 \pm 5.3$ (n = 10)</td>
<td>$39.8 \pm 24.3$ (n = 9)</td>
</tr>
<tr>
<td>HA7</td>
<td>$138.6 \pm 36.0$ (n = 6)</td>
<td>$140.7 \pm 43.1$ (n = 8)</td>
<td>$92.0 \pm 33.0$ (n = 7)</td>
<td>$88.5 \pm 38.6$ (n = 4)</td>
<td>$103.3 \pm 70.1$ (n = 5)</td>
<td>$24.4 \pm 9.2$ (n = 2)</td>
</tr>
</tbody>
</table>

$^1$ Values are mean ± SD.
**FIGURE 1** PCR-DGGE pattern of colonic total bacteria in Fisher 344 rats at the end of 8-wk raw starch diets feeding (n = 52).
FIGURE 2 The nMDs (Non-metric multidimensional scaling) analysis of PCR-DGGE band pattern of colonic total bacteria in Fisher 344 rats fed three raw starch diets (n = 52).
FIGURE 3 Centered and scaled regression coefficients of 26 bands for predicting ACF numbers with PLS analysis of PCR-DGGE pattern of total colonic bacteria in AOM-treated Fisher 344 rats fed three raw starch diets (n = 29).
**FIGURE 4** PCR-DGGE analysis of RS fermentation-associated bacteria groups: *Clostridium* cluster IV (A), *Clostridium* cluster XIVa (B), and *Bacteroides fragilis* group (C) in AOM-treated Fisher 344 rats fed three raw starch diets (n = 29). M: DGGE marker.
FIGURE 5 The nMDs analysis of PCR-DGGE band patterns of *Clostridium* cluster IV (A), *Clostridium* cluster XIVa (B), and *Bacteroides fragilis* group (C) in AOM-treated Fisher 344 rats fed three raw starch diets (n = 29).
CHAPTER 4. INCREASED BUTYRATE PRODUCTION BY LONG-TERM IN VITRO FERMENTATION OF DIGESTED HIGH AMYLOSE CORNSTARCH RESIDUES WITH HUMAN FECAL MICROBIOTA 1,2

A paper to be submitted to The Journal of Nutrition

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Abstract

An in vitro semi-continuous anaerobic incubation system was established to simulate lower gut fermentation and to determine the extent of difference in fermentability of digested resistant starch (RS) residues (SR) by human fecal microbiota according to BMI and across a large of individuals. Four high amylose cornstarches (HAS): HAV, VI, VII, and GEMS067, were pre-digested with AOAC 991.43 method to mimic human digestion of cooked starch. Thirty-two human subjects were recruited for two visits (Phase I and Phase II) 5 mo apart: 17 lean, 9 overweight, and 6 obese; 27 subjects participated in both phases of the study. SR was applied to a semi-continuous fermentation system for 3 wk with replenishment of substrates and Brain Heart Infusion (BHI) medium every 3.5 d to simulate colonic transit time. SR physicochemical analysis showed that SRV displayed the highest peak temperature, enthalpy changes and proportion of small molecules among four SR, which was consistent with its lowest digestibility. In both phases starting at wk 1, compared to blank medium control, SR incubation significantly decreased pH, increased gas production, increased butyrate, and total SCFA concentration. Increased molar proportions of butyrate and decreased acetate proportion, which were negatively correlated (r = -0.78 in Phase I and r = -0.87 in Phase II), were stabilized starting at wk 2.
SRV, but not the other SR, showed higher propionate concentration than blank at the end of 3-wk incubation in both phases. This effect might have been partly mediated by the lesser digestibility of SRV compared with the other SR. There were no significant differences in major SCFA concentrations between lean individuals and individuals with BMI ≥ 25, predicting that RSs would have similar effects regardless of body weight status. There were extensive interindividual variation in butyrate production ranging from 4 to 13 mmol/L for each SR, and variation within individual over time (5 mo) with 10 and 19 out of 27 subjects behaved differently between two phases in responding to SRV and SR VII, respectively. In conclusion, a stable in vitro semi-continuous fermentation model was established to simulate RS fermentation in the human lower gut. SR increased butyrate production, and one SR also increased propionate, which may mediate some health effects of the SR. Identifying subject characteristics including gut microbial populations associated with interindividual and intraindividual variations may be important in understanding health effects of RS.

Introduction

Resistant starch (RS) is defined as “starch or starch portion that escapes digestion in the small intestine of healthy individuals” (2). Physiological significances of RS have been associated with its lower digestibility by human host to reduce the risks of type 2 diabetes and obesity by improving glycemic index and regulation food intake. Human feeding trials showed that one single breakfast meal with RS decreased postprandial blood glucose, insulin, and satiety hormones levels, as well as increased sensation of fullness, compared to highly digestible starch control (3, 4). Fermentation of RS by microbiota colonized mainly in large intestine, on the other hand, increased fecal bulking, lowered fecal pH, and increased fecal short chain fatty acid (SCFA) production, as shown by long-term human feeding trials with high RS diets (5, 6).

Fermentation outcomes of RS, especially SCFA production, have been considered as major mechanisms of RS in improving colon health and preventing colon disease. SCFA are primary energy source for colonocytes (7), and have been implicated in maintaining
colon health through stimulating proliferation of basal colonocytes (8), decreasing paracellular permeability (9), inducing apoptosis of hyper-proliferative cancer cells (10, 11), and ameliorating inflammation injuries (12). Animals studies showed that 14-d RS feeding improved ceco-colonic inflammatory injuries and increased cecal butyrate amount in Sprague-Dawley (SD) rats with dextran sodium sulfate (DSS)-induced colitis, but not by fructo-oligosaccharides feeding (14). Long-term (25-wk) feeding with 10% or 20% RS significantly decreased the incidence of colon tumor induced by carcinogen azoxymethane (AOM) in SD rats (15). Moreover, the colon cancer incidence was negatively correlated with distal colonic pH, colonic acetate, and butyrate concentrations.

Fermentability of RS can be evaluated by measuring fecal SCFA \textit{in vivo} with long-term human feeding trials (5, 6, 16). However, it is difficult to obtain colonic samples from human for ethical, cost and procedural reasons. In addition, it is difficult to control dietary intake of other food components in long term human feeding trials, which may alter RS fermentation. Moreover, fecal SCFA profiles may differ among samples collected at different times even from the same individual (17). As an alternative, \textit{in vitro} incubation models, including batch and dynamic models, can be used to investigate fermentation of a single substrate with advantages of reduced cost, simple procedures, and easily controlled conditions. Short-term (24-72 h) batch fermentation models are widely used to compare fermentabilities of various carbohydrates by measuring fermentation indicators including pH, gas, and SCFA production (18). Dynamic fermentation systems, on the other hand, are characterized by the long-term continuous feeding of substrates at a certain dilution rate, and usually in pH controlled vessels/reactors to simulate physiological conditions of each part of the gastrointestinal tract. Dynamic fermentation models are more suitable to study adaption of gut microbes to substrates, but special equipment has been used in such models, as shown by the development of SHIME (simulator of the human intestinal microbial ecosystem) (19), TIM2 (TNO \textit{in vivo} model of human colon) (20), and the three-stage system (21) to study carbohydrate fermentation-associated bacteria.
Considerable variation of gut microbiota has been well recognized with the application of 16S rRNA-based phylogenetic analysis, and no consistent single phylotype was detected in 154 human subjects (22). Recently, a higher ratio of fecal Firmicutes to Bacteroidetes was detected in obese individuals than in lean individuals (23). In addition, the gut microbiome in obese individuals was enriched with genes from Actinobacterium (75%) and Firmicutes (25%) when compared with that in lean individuals (22). These results suggested that microbiota in obese individuals may have higher energy extraction capacity from RS since Bifidobacterium and Clostridium spp., which belong to phyla Actinobacterium and Firmicutes respectively, were predominant amylolytic and butyrogenic bacteria identified with in vitro fermentation of RS (24). The gut microbiome in obese mice with genetic deficiency of leptin were enriched with pathways involved in starch/sucrose fermentation compared to wild type lean animals (25).

The work reported here aimed to establish a long term semi-continuous incubation model to simulate lower gut fermentation in vitro, which can be used as an alternative strategy to in vivo human feeding trial to investigate the adaptation to RS across numerous individuals and to compare effects of RSs and other dietary fibers. We hypothesized that different SCFA profiles would be observed with a 3-wk incubation of digested cooked starch residues from four different high amylose cornstarches with human fecal microbiota. We also hypothesize that overweight/obese individuals would show greater fermentation capacity than lean individuals during 3-wk adaptation to SR.

Materials and Methods

Subjects. Lean, overweight and obese subjects (n = 32) were recruited with the following inclusion criteria: aged 18–45 years old, no use of antibiotics within past 6 mo, without gastrointestinal diseases. Two study phases (two visits) were included with 5 mo apart. In Phase I (first visit), 15 subjects were with lean body mass index (BMI) (in kg/m²) (18.5-24.9), 9 were overweight (25.0-29.9) and 6 were obese (≥ 30.0). In Phase II (second visit), two lean and one obese subjects dropped the study and two new lean subjects were recruited. Thus a total of 29 subjects participated in Phase II and 27 subjects participated in both phases. Typical food habits (including about 70 food items and their portion size)
were self-reported by subjects with online Block Brief Food Frequency Questionnaire 2000 (FFQ) (www.nutritionquest.com) (Berkeley, CA). The study was approved by the Institutional Review Board of Iowa State University (IRB 07-322). Subjects were informed and signed consent forms were obtained before the study.

_Preparation of SR._ Four maize lines of high amylose starch (HAS), HAV \((H_{99ae})\), HAVI (F1 hybrid of \(Pa91ae\) and \(AR16035:S02-615-1-B-B///GUAT209:S13//OH43ae/H99ae\)), HAVII (F1 hybrid of \(AR16035:S02-615-1-B-B///GUAT209:S13//OH43ae/H99ae\) and \(DKXL370:N11a20-31-1-B-B-SIB///GUAT209:S13//OH43ae/H99ae\)) and GEMS067 (\(GUAT209:S13//OH43ae/H99ae\)) provided by Dr. Mark Campbell (Truman State University, Kirksville, MO) were used in the study. HAV, HAVI and HAVII, estimated with 55%, 65% and 70% amylose respectively were used in Phase I. In Phase II, GEMS067 with an estimated of 70% amylose was selected instead of HAVI due to the similar SCFA pattern between HAVI and HAS VII observed in Phase I.

To simulate human digestion of cooked starch, total dietary fiber method (AOAC 991.43) with slight modification was used to prepare starch residues (SR) for large scale. Briefly, starches were digested with a thermostable \(\alpha\)-amylase from _Bacillus licheniformis_ in 2-morpholinoethanesulfonic acid (MES) \((pH = 8.2)\) buffer at boiling water temperature for 30 min. Followed by protease digestion at 60 °C for 30 min, amyloglucosidase from _Aspergillus Niger_ was used for another 30 min digestion at 60 °C after adjusting pH to 4.4-4.6. Starch residues (pellets) were collected in 50 mL tubes (Fisher Scientific, Pittsburgh, PA) by centrifuging at 8000×g for 10 min. Pellets were further washed with distilled water once, 100% ethanol twice, 78% ethanol twice and 100% acetone once at room temperature before they were dried in 37 °C oven for 48 h). Grounded starch residues (SR) in powder with a coffee blender were kept in desiccators at room temperature before use.

_Physicochemical properties analysis of SR._ Total starch and resistant starch content were analyzed with resistant starch assay kit (Megazyme International Ireland Ltd., Co. Wicklow, Ireland) by AACC method 32-40 and AOAC method 2002.02 respectively. The digestibility of SR at 20 min, 2 h, and 24 h was determined using the Englyst method.
Thermal properties of SR, including onset (To), peak and conclusion (Tc) gelatinization temperatures and enthalpy change (ΔH) were determined using a differential scanning calorimeter (DSC) (DSC-7, Perkin-Elmer, Norwalk, CT) according to Jiang et al. (27). Molecular-weight distribution of SR were analyzed with high-performance size-exclusion chromatograph (HPSEC) equipped with a SB-803 analytical column (Showa Denko K.K., Tokyo, Japan).

Semi-continuous in vitro fermentation of SR. Fresh feces were collected with commode specimen collection container (Fisher Scientific, Pittsburgh, PA) from each individual. All experimental procedures were conducted in anaerobic environment. Fecal inocula were prepared immediately by diluting feces (1/10 w/v) with Brain Heart Infusion medium (Difco Laboratories, Detroit, MI) under the circulation of CO₂ (28). Homogenous fecal slurry was obtained by filtering through four-layer cheesecloth and transferred into BactronI anaerobic chamber (Sheldon Manufacturing, Inc., Cornelius, OR). Two milliliters of inocula were added to 50 mL serum bottles containing 100 mg substrates (SR) pre-hydrating in 8 mL BHI medium overnight at room temperature. Anaerobic fermentation of SR was carried out in serum bottles sealed by aluminum caps with PTFE/silicone septa (Supelco Inc., Bellefonte, PA) at 37 °C in a shaking ambient chamber. Samples were prepared in duplicates, and BHI medium without any substrate was used as a blank control. Medium and substrate were replenished every 3.5 d by discarding 6 mL fermentation mixture to simulate food transit of filling and emptying in the gut. Total gas production was measured as the overpressure in fermentation vials at wk 0.5, 1, 1.5, 2, 2.5, and 3 with a digital manometer (Fisher Scientific, Pittsburgh, PA). Fermentation was stopped by adding 0.1 mL saturated HgCl₂, and pH was measured thereafter at wk 0, 1, 2, and 3. The fermentation mixture was centrifuged and the supernatant used for further SCFA analysis. All samples were stored at -80 °C before analysis.

SCFA measurement. Organic acids: acetate, propionate, butyrate, valerate, isobutyrate and isovalerate were analyzed according to the method described in Sayar et al. (29). Briefly, 1 mL supernatant was acidified by 0.5 mL hydrochloric acid and SCFA was
extracted with 2 mL diethyl ether. 200 µL supernatant was silylated with 20 µL N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) at 80 °C for 20 min. After standing for 24 h at room temperature, 1 uL silylated derivatives were injected to Gas Chromatograph (HP6890) (Hewlett Packard, Roseville, CA) equipped with a flame ionization detector (FID) and a SPB5 capillary column (30 m ×0.25 mm i.d.× 1 µm df, Supelco, Bellefonte, PA). Helium was used as a carrier gas. The initial oven temperature was held at 70 °C for 4 min, and was increased at a rate of 7 °C/min to 160 °C and retained for 5 min. The injector temperature was 200 °C, and the detector temperature was 220 °C. SCFA concentrations were calculated using the peak area ratio of the analyte to the internal standard (ethyl butyric acid), based on the standard curve with a good linear correlation between the peak area ratio and the corresponding concentration showing r values > 0.99 for all SCFA.

Statistical analysis. All statistical analysis was conducted with SAS version 9.1 (SAS Institute, Cary, NC). Food component was analyzed separately for Phase I and II with Student’s t test. The effects of treatment and time point on SCFA, pH and gas production data were analyzed by MIXED procedure as a Randomized Complete Block Design (RCBD). Significance level of 0.05 was used. The effect of treatment and BMI category (lean, overweight and obese) on SCFA was analyzed by MIXED procedure as a split-plot design. Bonferroni’s method was applied to account for the multiple comparisons made between treatments. Pearson correlation coefficient was used to measure linear relationships between pH, gas, and SCFA production. A paired t-test was conducted to analyze the consistency of data between two phases in 27 subjects who participated both phases.

Results

Typical food intake. In two study phases, subjects in BMI ≥ 25 group consistently self-reported lower daily intake of total fat, vitamin A and beta-carotene and fats/oils/sweets (servings) compared to lean subjects (Table 1). Food and nutrients differences between two BMI groups in either Phase I and Phase II were also listed. There was no significant difference in BMI or daily intake or any other food or nutrients between the two visits in
27 subjects who participated in Phase I and Phase II as analyzed with paired t-test (data not shown).

**Physicochemical properties of SR.** Yields of HAVI digested starch residues (40.7%), calculated as percentage of residues obtained from starch flours treated with AOAC 991.43 method, were similar to HAVII (43.1%), which were higher than that of HAV (17.3%) but lower than GEMS-067 (53.0%). Further quantification of resistant starch content in SR showed that the undigested component of SR GEMS-067 (37%) was the lowest, followed by SRVI (39%) and SRVII (39%), and SRV had the highest undigested residues with 47% (Table 2). Consistently, time course enzymatic hydrolysis at 37 °C showed that SRV had the lower digestibility with 41.3%, compared to other three SR with 55-61% after 24 h digestion.

Thermal properties analyzed with a differential scanning calorimeter (DSC) showed SRGEMS-067 had the lowest gelatinization conclusion temperature (145 °C) compared to other SR with 161-164 °C. In addition, the highest enthalpy change was observed in SRV (14.8 J/g), followed by SRVI (10.8 J/g), SRVII (9.7 J/g), and SR GEMS-067 (8.1 J/g). No significant differences in the onset and peak temperature were observed among SR (data not shown).

The molecular-weight distributions of SR were analyzed with high performance size-exclusion chromatographs (Fig. 1). Two major peaks (Peak 1 and Peak 2) were observed in chromatograms corresponding to the highest molecular weight (Mw) standard (~ DP 2472), and a range of low Mw standard with DP 36-146. SR-GEMS0067 had the highest intensity of Peak 1, followed by similar intensity in SRVI and SRVII, and SRV has the lowest intensity. By contrast, SRV contained the highest intensity of Peak 2 compared to other three SR, which were not different from each other.

*Changes of pH.* Changes of pH in the fermentation mixture during 3-wk incubation were shown in Fig. 2A. In both phases, pH in the blank (BHI medium fermentation mixture) was kept stable at 7.3-7.5 during the 3-wk incubation. All SR incubation significantly decreased pH to 6.2-6.4 at wk 1 and kept stable as fermentation proceeded. There were
no significant differences in pH among SR, although a less degree of pH drop was observed in SRV compared to other SR in both phases.

*Gas production.* Overpressure of gas in the headspace of fermentation vials was measured at every 3.5 d (Fig. 2B). In both phases, similar pattern was observed in all treatments, with the highest gas pressure measured at 0.5 wk ($P < 0.05$, compared to that of other time points). Starting at wk 1, gas production maintained stable until the end of the 3-wk incubation, with significantly increased by SR (25 mL) compared to BHI (12 mL) in any week. There were no significant differences in total gas production among SR. Moderate negative correlation between pH and gas production was found in Phase I ($r = -0.67$, $P < 0.0001$) and Phase II ($r = -0.76$, $P < 0.0001$).

*SCFA production.* Major SCFA (i.e. acetate, propionate and butyrate) and minor SCFA (i.e. isobutyrate, isovalerate and valerate) were measured (Fig. 3). In all treatments, the three major SCFA accounted for 89-92% of total SCFA produced during *in vitro* fermentation. Acetate was the most abundant SCFA detected in the fermentation mixture, followed by butyrate, and propionate (Table 3).

In both two phases, increased butyrate (Fig. 3C) and total SCFA (Fig. 3G) concentrations were consistently observed by SR incubation during 3-wk incubation. There were no significant differences among SR. The molar proportion of butyrate in blank was kept stable with 18-20% in Phase I and 17-22% in phase II as fermentation proceeded, which was increased by SR to 30-31% in Phase I and 28-30% in Phase II at wk 1 (Table 3). Starting at wk 2, butyrate proportion of SRV increased to 35-36%, whereas higher butyrate proportion was observed with SRVII, SRVI and GEMS-067 with 39-40%. However, such differences among SR were not statistically significant.

Molar proportion of acetate in blank treatment was consistent across three weeks in Phase I (59-60%) and Phase II (56-58%). Although acetate concentrations at wk 2 and 3 were only decreased by SR in Phase I (Fig. 3A), molar proportion of acetate at wk 1 was consistently decreased to 48-51% in both Phase I and II. Starting at wk 2, acetate
proportion in SR further decreased to 41-44% at wk 2 and 43-45% at wk 3 for both phases.

Compared to blank without substrate in BHI medium, propionate concentration was increased by SRV fermentation consistently at the end of incubation in both phases, but showed different dynamics (Fig. 3B). In Phase I, SRV increased propionate concentration, compared to SRVI and blank starting at wk 1 till the end of incubation, whereas in Phase II, propionate level formed from SRV, VII, and GEMS-067 increased at wk 1, but only SRV increased propionate at wk 3.

Minor SCFA accounted for 8-11% of total SCFA produced. Compared to blank and SRV, isobutyrate concentration was decreased by SRVI and SRVII in Phase I, and by SRVII and GEMS-067 in Phase II (Fig. 3F). On the contrary, valerate concentration was consistently increased by all SR starting at wk 2 compared to blank in both phases (Fig. 3D). At the end of incubation (wk 3) in both phases, isovalerate production was only increased by SRV compared to blank BHI medium (Fig. 3E).

**Fermentation capacity between lean group and overweight/obese group.** There were no significant differences in pH and total gas production between fecal samples from lean individuals and overweight/obese individuals in the two study phases (data not shown). At the end of incubation in Phase I, there was a trend of lower SCFA concentration in fecal incubations of overweight/obese individuals by all treatments compared to lean individuals, but it was not statistically significant (adjusted \( P = 1.000 \) for blank, 0.9528 for SRV, 0.0181 for SRVI, and 0.1124 for SRVII, an adjusted \( P \) value < 0.0021 was considered significant) (Fig. 4). This trend was not noticed in Phase II. Further comparison of fermentation indicators across lean, overweight, and obese groups revealed a significantly lower butyrate production in SRVII treatment with fecal microbiota from obese individuals (\( n = 6 \)) compared to lean individuals (\( n = 15 \)) at the end of incubation in Phase I (data not shown). No other significant differences were observed across BMI groups in either phase.
**Interindividual and intraindividual variations.** To further explore the interindividual and intraindividual variation in the fermentation capacity over 5 mo, we examined butyrate production by fecal microbiota from 27 individuals who participated in both Phase I and Phase II. As shown in Fig. 5, a larger variation was observed in the butyrate concentration with SR incubation among 27 subjects compared to blank regardless of the BMI categorization. One subject with the highest BMI (= 53.56) showed minimal to no change in butyrate production compared to blank in both phases.

The intraindividual variation, or the stability of fecal microbiota from these 27 donors in response to 3-wk SR adaptation were analyzed based on the percentage of butyrate production increased by SRV and VII compared to blank, which can be considered as the situation in which subjects consuming low RS in their diet (Table 4). Paired t-test showed a similar average increase of butyrate concentration by SRV (92 ± 54% and 92 ± 53%) and SRVII (107 ± 62% and 102 ± 63%) in Phase I and II respectively. We further categorized 27 donors into three groups as low responders with percentage of increased butyrate production less than 100%, moderate responders with the percentage increased between 100-200%, and high responders with more than 200%. Compared between Phase I and II, 11 individuals behaved similarly as low responders to SRV, and the other 6 behaved similarly as moderate responders in the percentage of butyrate increased by SR compared to blank. Therefore, a total of 17 individuals (out of 27) maintained their fermentation capacities on SRV over 5 mo with our in vitro semi-continuous system. For SRVII, there were only 6 low responders and 2 moderate responders (a total 8 out of 27) who behaved similarly between two phases in the percentage of butyrate increased after 3-wk SR incubation compared to blank. On the other hand, in Phase I, 8 individuals were consistently grouped as low responders to both SR V and VII, and 3 individuals were moderate responders (a total of 11 out of 27). In Phase II, 15 individual were consistently grouped as low responders and 7 were consistent moderate responders to both SR V and VII (a total of 22 out 27).

**Discussion**
Justification of the semi-continuous fermentation model. The major aim of current study is to establish a long term in vitro semi-continuous fermentation model with frequent replenishment of substrates and medium to simulate adaptation of gut microbes to undigested dietary carbohydrates across numerous individuals. Stability of our model was demonstrated by constant pH, gas production, key SCFA production, i.e. butyrate, starting at wk 1, and molecular proportion of SCFA starting at wk 2. Further investigation of bacterial pattern with PCR-DGGE (Denaturing Gradient Gel Electrophoresis) analysis, which will be discussed in a separate paper, showing that dominant bacteria were preserved as fermentation proceeded starting at wk 2. We concluded that it took at least two weeks for microbiota to fully adapt to SR and reached function stability with our model, but that inter-individual variability was significant.

Conditions chosen in our model were determined following the decision to simulate in vivo human gut fermentation events. First of all, since it is crucial to use appropriate substrates as they enter into colon, we used AOAC 991.43 method to pre-digest RS to obtain SR. This method was considered more relevant to the digestion of cooked starchy food in human diet with its thermal stable α-amylase hydrolysis step at ~100 °C for 30 min (30), whereas other RS quantification methods, such as AOAC 2002.02 and Englyst’s method are widely used to analyze raw starch with amylase digestion at 37 °C. Studies have shown that fermentation rates of SR prepared from alpha-amylase digestion of retrograded or retrograded boiled high amylose starch were slower than digested SR of raw starch during 72 h batch incubation, indicating different fermentation bacteria enzymatic activities and kinetics might be involved (31), which possibility affected long-term in vitro fermentation outcomes.

The interval between replenishments of SR was used to simulate the retention time of substrates exposed to gut microbes in the colon. The 3.5 d (around 84 h) chosen in our study was within the range reported by previous studies of colonic transit time in healthy subjects with 62 to 93 h (32), 17.5 h in diarrhea patients, and 118 h in constipation patients (33). It would be interesting to examine the effects of altering replenishment frequency on fermentation outcomes to simulate individual condition with varied colonic
transit time, which might affect nutrition absorption as shown by the association of rapid gut transit time with greater bioavailability of genistein in female subjects (28). In addition, the dose of SR we used (100 mg SR/2 mL fecal inocula prepared with 1/10 dilution of feces) is a standard dose used in most batch fermentation studies (29, 34), with which substrates might be fully depleted at the end of incubation. It would be interesting to compare effects of different amounts of SR on the adaptation outcomes to simulate individual food habits with varied RS intake. Nevertheless, validation of our in vitro fermentation model with human or animal feeding trials would be the first priority.

**Fermentability of SR.** The second aim of our study is to assess the fermentability of SR obtained from four HAS with different amylose content during 3-wk adaptation in vitro. Four SR behaved similarly in the major fermentation indicators, as shown by the similar extent of pH drop (~ 0.6-1 unit) and gas production increase (~ one-fold), which were similar to previous studies using same batch incubation protocols fermenting oat flours (35). More importantly, around 100% increase in butyrate production and 20% increase in total SCFA concentration were also observed starting at wk 1 compared to blank and maintained as fermentation proceeded. Four SR incubation also significantly increased molar proportion of butyrate but decreased acetate proportion starting at wk 2 in both phases, which were inversely correlated (r = -0.78, P < 0.001 in Phase I and r = -0.87, P < 0.001 in Phase II). Interestingly, SRV showed a trend of less fermentable in all of these indicators than other SR, but they were not statistically significant different.

After recalculation of molar proportions from Table 3 by only considering three major SCFA, molar ratios of acetate: propionate: butyrate were 66:12:22 for blank, 49:15:37 for SRV, and 47:13:40 for SRVI, SRVII and SR-GEMS-067. Previous short-term fermentation studies (24-72 h) showed a broad range of molar ratios of acetate: propionate: butyrate as (38-66): (12-26): (22-36) using raw starches from varied origin as substrates (36), and (60-69): (8-28) : (10-22) using starch residues with varied pre-digested methods (31, 37). The higher butyrate/acetate ratio we observed with SR incubation could be explained as the result of adaptation of microbiota favoring SR utilization. However, it has to be mentioned that comparison of results obtained in
different in vitro fermentation studies is difficult due to different protocols used, including type of substrates, culture medium, fecal inoculums preparation and in vitro models applied (38).

However, differential patterns were observed in propionate and isobutyrate production across four SR. For examples, SRV incubation produced a higher propionate concentration than blank at the end of 3-wk incubation in Phase I and II, but not compared with other SR. Isobutyrate concentration, on the other hand, was not affected by SRV, but decreased by SRVI, SRVII, and SRGEMS incubation. Further analysis of physical properties showed SRV contained more undigested starch than other SR (Table 2), which could be explained by its higher enthalpy change required for converting crystalline SRV to amorphous structure during amylase digestion. Moreover, higher proportion of small molecules was specifically observed in SRV (Fig. 1), which was also associated with its degraded granule structure observed under light microscope, compared to that of other SR, although they were ground under the same condition (data not shown). Even though, the differences between SRVII and GEMS-067 in their physicochemical properties, such as enthalpy change, RS content and 24-h digestibility, were not consistent with their similarities in SCFA profiles in Phase II. Therefore, the physicochemical properties of SR examined in the current study may not be sufficient to explain or predict the fermentation outcome. Further studies on bacteria population shifts will be helpful in understanding the specificity of SRV fermentation.

Fermentation capacities between fecal microbiota from lean and overweight/obese subjects. Although it was hypothesized that gut microbiota in obese individuals had higher energy-extracting capacity with higher ratio of Firmicutes/Bacteroidetes compared to lean individuals (23), we did not observe such difference in fermentation indicators measured between lean and overweight/obese BMI groups. This might be explained by the long-term adaptation to SR incubation, which led to the comparable fermentation outcomes observed. It is also possible that there was no difference in pre-existing fermentation capacity of fecal microbiota between two groups, as indicated by similar carbohydrate and dietary fiber intake, which are major food components associated with
RS degradation bacteria. We will not emphasize the information obtained from questionnaire since it was self-reported by subjects and other parameters such as genetic background, physical activity, fitness and recognition of food control by subjects were not taken into account.

The role of higher energy extraction capacity of gut microbiota in obese development is still not conclusive considering the importance of dietary factors recognized. Studies indicated that gut microbiota in obese C57BL/6J mice induced by Western diet preferred simple sugar rather than complex carbohydrates, as shown by the enrichment of glycolysis, fructose and mannose metabolic pathway and the depletion of starch and sucrose pathway in the microbiome compared to animals fed carbohydrate-restricted diets (39). If so, due to the deficiency in starch-utilization bacteria in obese people consuming typical Western diets, RS application might be less effective or require a longer period of adaptation to reach the comparable SCFA production to that in lean individuals. This might explain the limited butyrate production in SR incubation with fecal microbiota from one subject with the highest BMI (53.4). Further studies focusing on more obese subjects may help compare the adaptation efficiency of SR by lean and obese gut microbiota.

Although carbohydrate restriction may help to reduce the risk of obesity, it might also increase the risk of colon cancer by decreasing SCFA, especially butyrate concentration. A recent study demonstrated that the restricted carbohydrate intake (including starch and non-starch polysaccharides) decreased fecal SCFA concentration in obese people after 4-wk feeding, as well as bacteria counts of *Bifidobacterium spp.*, Clostridium clusters IV and XIVa (40). Hence, RS application might also be a better strategy than carbohydrate restriction to reduce the risk of both obesity and colon cancer in overweight/obese individuals, by reducing glucose released in small intestine and increasing butyrate production in large intestine.

*Inter-individual variability and stability in the fermentation efficiency during SR adaptation.* Exploration of inter-individual variation in the adaptation efficiency of gut microbiota is important for the future dietary intervention of RS to modulate gut
microbes and improve their fermentation capacity. In our study, large inter-individual variation in the response of bacteria to SR incubation was observed as shown by the increased percentage of butyrate production compared to blank. The variation in the adaption efficiency to RS feeding has been addressed in human feeding trials. With 4-wk feeding of RS diet in 24 subjects, the increase of fecal butyrate concentration varied from -100-200% with type 2 RS diet compared to low RS diet, and -50-400% with type 3 RS (6). Additionally, in the study of Christl et al. (41), 2 out of 10 subjects showed limited adaptation to RS supplementation for 4 wk, as shown by their slower and incomplete breaking-down of the same starch \textit{in vitro} by the adapted fecal microbiota.

Previous studies have shown that fecal bacteria PCR-DGGE pattern of 8 individuals were stable at the genera level over a period of up to 12 wk (42). However, much is unknown about the stability at species or strain level, which may be important in determining the function activity of gut microbiota. In our study, a third of individuals (a total 10 out of 27) shifted their response to SRV adaptation between two study phases in the percentage of butyrate increased compared to blank, and a two-third (a total 19 out of 27) shifted with SRVII incubation. Shifts of metabolic activities of gut microbiota during a certain period of time has been reported in the study of Zheng et al. (28). They examined the stability of degradation capacity of isoflavone compounds, including daidzein and genistein, by human fecal inocula collected 5 mo apart using a 24 h \textit{in vitro} incubation system. They reported that 39 out of 66 individuals switched daidzein disappearance phenotypes across low, moderate and high groups, and 27 out of 66 switched genistein disappearance phenotypes. We suspected that environmental climate changes in Iowa may lead to dietary habit shifts and thus the fermentation capacity of the subjects, since two study phases covered seasons of winter and summer. Characterization of pre-existing fecal bacteria pattern before and after SR incubation will help understanding interindividudal and intraindividual variations observed in the current study.

In conclusion, our studies established a stable semi-continuous \textit{in vitro} incubation model to demonstrate the increase of butyrate production by SR fermentation with human fecal microbiota. We observed comparable fermentation capacity on SR between lean and
overweight/obese BMI groups after 3-wk incubation. Further studies on the microbial pattern during SR incubation will help understanding the relationship between microbiota and their metabolic function.

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**Footnotes**

1 Supported by Iowa State University Plant Science Institute.


7 Abbreviations used: BMI, body mass index; HAS, high amylose starch; RS, resistant starch; SCFA, short chain fatty acid; SR, starch residues.

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**Literature Cited**


Table 1 Average daily intake of nutrients/foods showing difference between two BMI groups in Phase I and Phase II (5 mo apart) \(^1\).

<table>
<thead>
<tr>
<th>Nutrient/Food</th>
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<th>Phase II</th>
<th>P value</th>
<th>Nutrient/Food</th>
<th>Phase I</th>
<th>Phase II</th>
<th>P value</th>
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</thead>
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<td>BMI ≥ 25 (n=15)</td>
<td>P value</td>
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<td>18 &lt;BMI ≤ 24.9 (n=15)</td>
<td>BMI ≥ 25 (n=14)</td>
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<td>22.4</td>
<td>32.3</td>
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<td>22.3</td>
<td>32.0</td>
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\(^1\) *P* <0.05, \(^{**}\) *P* <0.01, \(^{***}\) *P* <0.001, NS, not significant. \(^2\) USF, unsaturated fat.
Table 2 Physicochemical properties of SR V, VI, VII, and GEMS-067.

<table>
<thead>
<tr>
<th>SR</th>
<th>Resistant starch (%)</th>
<th>Digestibility at 24 h (%)</th>
<th>Tp (°C)</th>
<th>Tc (°C)</th>
<th>Enthalpy ΔH (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRV</td>
<td>46.5 ± 0.7 c</td>
<td>41.3 ± 0.1 a</td>
<td>123.3 ± 0.3 b</td>
<td>163.5 ± 0.0 b</td>
<td>14.8 ± 0.0 d</td>
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<tr>
<td>SRVI</td>
<td>39.4 ± 0.8 b</td>
<td>55.7 ± 0.1 b</td>
<td>119.9 ± 0.5 a</td>
<td>161.3 ± 1.9 b</td>
<td>10.8 ± 0.3 c</td>
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<tr>
<td>SRVII</td>
<td>39.1 ± 0.5 b</td>
<td>55.3 ± 1.0 b</td>
<td>120.2 ± 0.0 a</td>
<td>161.3 ± 1.8 b</td>
<td>9.7 ± 0.5 b</td>
</tr>
<tr>
<td>SRGEMS-067</td>
<td>39.6 ± 0.4 a</td>
<td>60.7 ± 0.7 c</td>
<td>119.2 ± 0.2 a</td>
<td>145.5 ± 0.7 a</td>
<td>8.1 ± 0.1 a</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM of two replicates. Values within a column followed by a same letter are not significantly different (P <0.05).
2 RS content of the SR was determined using AACC method 32-40 and AOAC method 2002.02.
3 Enzyme digestibility of the SR was determined using the Englyst method (27).
4 Tp: Peak temperature of the SR.
5 Tc: Conclusion gelatinization temperature of the SR.
**Table 3** Molar proportions of acetate, propionate, butyrate and valerate during 3-wk *in vitro* fermentation of SR V, VI, VII, and GEMS-067.  

<table>
<thead>
<tr>
<th>Week</th>
<th>SRs</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Propionate</th>
<th>Valerate</th>
<th>SRs</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Propionate</th>
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<td>59 b</td>
<td>18 a</td>
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<td>SRVII</td>
<td>51 c</td>
<td>28 b</td>
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<td>SRVI</td>
<td>50 c</td>
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<td>GEMS</td>
<td>48 c</td>
<td>30 b</td>
<td>13 b</td>
<td>3 c</td>
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<tr>
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<td>56 b</td>
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<tr>
<td>2</td>
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<td>10 a</td>
<td>2 b</td>
<td>GEMS</td>
<td>41 a</td>
<td>39 c</td>
<td>10 a</td>
<td>3 c</td>
</tr>
<tr>
<td>3</td>
<td>Blank</td>
<td>59 b</td>
<td>20 a</td>
<td>11 ab</td>
<td>1 a</td>
<td>Blank</td>
<td>56 b</td>
<td>22 a</td>
<td>12 ab</td>
<td>1 ab</td>
</tr>
<tr>
<td>3</td>
<td>SRV</td>
<td>44 a</td>
<td>33 bc</td>
<td>13 b</td>
<td>2 b</td>
<td>SRV</td>
<td>45 ac</td>
<td>33 bc</td>
<td>12 ab</td>
<td>3 bc</td>
</tr>
<tr>
<td>3</td>
<td>SRVII</td>
<td>45 a</td>
<td>36 c</td>
<td>12 ab</td>
<td>2 b</td>
<td>SRVII</td>
<td>43 a</td>
<td>36 c</td>
<td>11 ab</td>
<td>3 bc</td>
</tr>
<tr>
<td>3</td>
<td>SRVI</td>
<td>44 a</td>
<td>37 c</td>
<td>11 ab</td>
<td>2 b</td>
<td>GEMS</td>
<td>43 a</td>
<td>36 c</td>
<td>12 ab</td>
<td>3 c</td>
</tr>
</tbody>
</table>

1 Values are means of observations (with 2 replicates). Values within a column followed by a same letter are not significantly different ($P < 0.05$).
Table 4 Low, moderate, and high responder groups of individuals in the increased percentage of butyrate concentration by SR V and VII incubation compared to blank in 27 subjects who participated in Phase I and Phase II. 

<table>
<thead>
<tr>
<th>Phase</th>
<th>Group</th>
<th>SRV</th>
<th>SRVII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>Increase (%)</td>
<td>n Increase (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low (&lt;100%)</td>
<td>14 (11²) (8³)</td>
<td>51 ± 30</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>12 (6²) (3³)</td>
<td>129 ± 25</td>
</tr>
<tr>
<td></td>
<td>High (&gt;200%)</td>
<td>1</td>
<td>225</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>27 (17²) (11³)</td>
<td>92 ± 54</td>
</tr>
</tbody>
</table>

¹ Values are mean ± SD.

² Number of individuals in the parenthesis responded similarly between phase I and II to the same SR.

³ Number of individuals in the parenthesis responded similarly to SR V and VII in the same phase.
FIGURE 1 High performance size-exclusion chromatograms of digested starch residues (SR) obtained from high amylose V, VI, VII and GEMS-067 cornstarches treated with AOAC991.43 method. The linear line is a standard curve with the degree of polymerization (DP) 3 (maltotriose), DP 6 (maltohexose), DP 10 (maltodextrin, α-1,4), DP 15 (maltodextrin α-1,4) and DP 36, 73, 146, 292, 617, 1148 and 2472 (pullulan).
**FIGURE 2** Changes of pH (A) and total gas production (B) during 3-wk *in vitro* fermentation of SR V, VI, VII, and GEMS-067 by human fecal microbiota in Phase I and Phase II (5 mo apart). Values are means of observations (with 2 replicates) with standard errors represented by vertical bars. Common letters indicate treatments within each week and treatments across each week are not significantly different ($P > 0.05$).
(D) Valerate

(E) Isovalerate

(F) Isobutyrate
FIGURE 3 Concentrations of acetate (A), propionate (B), butyrate (C), valerate (D), isobutyrate (E), isovalerate (F) and total SCFA (G) during 3-wk *in vitro* fermentation with SR by human fecal microbiota in Phase I and Phase II (5 mo apart). Values are means of observations (with 2 replicates) with standard errors represented by vertical bars. Common letters indicate treatments within each week and treatments across each week are not significantly different (*P* > 0.05).
FIGURE 4 SCFA productions at the end of 3-wk *in vitro* fermentation of SR residues by fecal microbiota of lean BMI group (18.5 ≤ BMI < 25) and the group with BMI ≥ 25. Values are means of observations (with 2 replicates) with standard errors represented by vertical bars.
FIGURE 5 Individual responses in butyrate concentration to 3-wk incubation with BHI medium and SR V, VI, VII, and GEMS-067 from fecal microbiota of 27 subjects who participated in Phase I and Phase II.
CHAPTER 5. RUMINOCOCCUS BROMII-RELATED ORGANIMS WERE SELECTVIELY ENRICHED BY HIGH AMYLOSE CORNSTARCH DIGESTED RESIDUES IN LEAN MICROBIOTA WITH A SEMI-CONTINUOUS FERMENTATION MODEL ¹

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Abstract

With an in vitro semi-continuous incubation model to simulate lower gut fermentation, we investigated prebiotic effects of digested resistant starch residues (SR) from three high amylose starches (HAS). 30 human subjects were recruited: 15 were lean (18.5 ≤ BMI ≤24.9), 10 were overweight (25 ≤ BMI ≤ 29.9) and 5 were obese (BMI ≥ 30). Three HAS, HAV, HAVI, and HAVII, with estimated 55%, 65% and 70% amylose, were pre-digested with AOAC 991.43 method. Starch residues were applied to our anaerobic human fecal incubation model for 3 wk with the replenishment of SR and Brain Heart Infusion (1) medium every half wk to simulate colonic retention of substrates. We observed a stable anaerobic fermentation of SR starting at wk 2 as shown by the analysis of bacterial pattern measured with PCR-DGGE (Denaturing Gradient Gel Electrophoresis). Compared to blank BHI control, three SR significantly shifted DGGE patterns of total bacteria, Clostridium clusters IV and cluster XIVa at the end of 3-wk incubation, but not Bacteroides fragilis group. However, SRV induced different fermentation bacteria DGGE pattern from that of SRVI and VII, whereas SRVI induced different Clostridium cluster IV and XIVa bacterial pattern from those of SRV and SRVII. Additionally, one DGGE band, which was putative Ruminococcus bromii-related organism belonging to Clostridium cluster IV, was detected with higher
frequency after 3-wk SR incubation with fecal microbiota from lean individuals than with fecal microbiota from overweight and obese individuals. However, fecal bacteria DGGE pattern of Clostridium cluster IV was similar between lean and overweight/obese individuals before incubation, suggesting the selective response of microbiota from lean individuals to SR incubation. Our results suggested that combination effects of SR and pre-existing microbiota were important in predicting the fermentation bacterial pattern after long-term RS incubation.

Introduction

Resistant starch (RS) is defined as the “starch or starch portion that escapes digestion in the small intestine of healthy individuals” (2). Physiological significance of RS is mainly associated with RS fermentation products by gut microbes, i.e. short chain fatty acid (SCFA), especially butyrate. Butyrate is a primary energy source for colonocytes compared to glucose when applied to human isolated colonocytes (3). SCFA could also stimulate the proliferation of basal enterocytes of mice mucosa biopsy in vitro, which is the proliferation zone of the epithelial layer (4). SCFA also enhance the colonic epithelial barrier as shown by decreasing paracellular permeability of Caco-2 cells in vitro (5). More importantly, butyrate is the most potent inducer of apoptosis when applied to hyper-proliferative cell lines in vitro (6, 7).

RS fermentation-associated amylolytic bacteria have been identified mainly with traditional culture methods. For examples, amylolytic bacteria have been identified at genus level as Bifidobacterium spp. (58%), Bacteroides spp. (18%) and Fusobacterium and Butyribibrio spp. (10%) by selecting starch-hydrolyzing colonies when culturing human fecal bacteria (8). In addition, 38 strains of Bifidobacteria spp., Bacteroides spp. and Clostridium spp. isolated from human feces could utilize soluble gelatinized amylopectin, whereas only Bifidobacterium spp. and Clostridium Butyricum could degrade insoluble gelatinized amylose (9). With the development of molecular methods, major butyrogenic bacteria were identified to belong to Clostridium clusters IV and XIVa in human fecal microbiota using 16S rRNA taxonomy analysis (10). Additionally, detection of butyryl-CoA: acetate CoA transferase gene (ptb), which is a key gene in microbial butyrate-producing pathway,
revealed that there were 32 homologues of \textit{ptb} in human fecal microbiota (11). Four of these homologues identified from \textit{Clostridia} \textit{spp.}, \textit{Eubacterium} \textit{rectale}, \textit{Roseburia} \textit{faecis}, \textit{Eubacterium} \textit{hallii}, and an unnamed cultured species SS2/1 were highly abundant in the human gut.

Instead of targeting particular metabolic activities, monitoring overall changes of gut microbiota with fingerprinting methods is another strategy to identify RS fermentation-related species or bacterial pattern in human and animal feeding studies. For examples, PCR-DGGE analysis (Denaturing Gradient Gel Electrophoresis) of fecal bacteria showed that the abundance of \textit{Clostridales} \textit{spp.}, \textit{Mollicutes} \textit{spp.} and \textit{Bacteroides} \textit{spp.} were increased in individuals fed a supplementation of a high RS diet for 4 wk (12). qRT-PCR (quantitative real-time PCR) further showed \textit{Ruminococcus} \textit{bromii}-related phylotypes were significantly increased in their proportion of total bacterial community by high RS diet feeding compared to normal diet. The recent emerging next generation sequencing techniques, such as pyrosequencing and illumina-based sequencing, provide a platform for understanding biodiversity of gut microbiota at an even deeper level. However, these sequencing methods have not been applied to investigate the shifts of gut microbiota induced by RS feeding.

RS is currently considered to be a prebiotic that modulates gut microbiota favoring starch degradation and SCFA production (13). However, it is difficult to study gut microbiota during RS adaptation in human due to the influence of other dietary components, which may cause greater differences between individuals than the difference caused by dietary intervention. Animal model may be useful in the aspect of diet control, but the substantial difference between human and animal gut microbiota at genus and species level (14) may make it difficult to extrapolate results from animal feeding study. Gnotobiotic animals transplanted with human microbiota pose problems in terms of gastrointestinal tract structure and behavior of animals (e.g., coprophagy), as well as being very costly. \textit{In vitro} anaerobic fermentation models, especially continuous ones, are therefore a cost-saving alternative for analyzing prebiotic effects of a single carbohydrate source on gut microbiota under a better-controlled condition in standard laboratories.
Coupling of molecular methods and *in vitro* fermentation models has been applied to simulate lower gut fermentation in humans to understand various aspects of prebiotic effect of RS. For example, physicochemical properties, such as crystalline structure, could affect long-term adaptation outcome of gut microbiota to RS, as shown by a recent study using two polymorphs of retrograded high amylose starch (HAS) obtained by incubating autoclaved starch at 95 °C (polymorph A) or 40 °C (polymorph B) for 24 h. They found out that after 11-d continuous incubation in a pH-controlled three-stage fermentation system, polymorph B starch enriched the growth of *Bifidobacterium* spp., whereas polymorph A starch stimulated the growth of *Atopobium* spp. (15). Additionally, large variability in host pre-existing gut microbiota makes it hard to predict individual response to RS. It was shown that the fecal bacterial pattern of a vegetarian donor differed from three omnivorous donors without the detection of *Bifidobacterium* spp., although similar percentages of *Clostridium* spp. were observed among four individuals (18). After 24-h continuous incubation with HAS, about 95% of bacterial clones identified from that vegetarian donor were from *Clostridium* cluster IV and XIVa, whereas in one omnivorous donor, *Bifidobacteria* spp. accounted for 70% clones isolated.

We have established an *in vitro* semi-continuous anaerobic incubation model to simulate the fermentation outcome of digested cooked high amylose starch residues (SR) (Li et al., unpublished, 2010). With that model, we showed that butyrate production was increased by three SR, SRV, VI and VII, similarly during 3-wk incubation compared to blank medium control without any substrate. However, SRV showed higher propionate production compared to blank and SRVI. Additionally, there were no significant differences in SCFA production between fermentation samples with fecal microbiota from 15 lean individuals and 15 individuals with BMI ≥ 25.0 (9 overweight and 6 obese).

In the current study, we further investigated prebiotic effects of RS after 3-wk adaptation of fecal microbiota to the digestion residues of three types of high amylose starches, SRV, SRVI and SRVII. We hypothesized that microbial pattern shifted by SRV differed from that of VI and VII, which could explain its different SCFA pattern. In addition, we hypothesized
that fecal microbiota from lean and overweight/obese donors would respond differently to SR, although they reached comparable SCFA production at the end of incubation.

**Materials and Methods**

*Subjects and in vitro fermentation.* Detailed information of subjects, preparation of starch residues and anaerobic incubation were described in Li et al. (unpublished, 2010). The study was approved by Institutional Review Board of Iowa State University (IRB 07-322). 30 healthy participants: 15 lean, 9 overweight and 6 obese were recruited and informed consent was obtained before the study began. Three HAS with different amylose content, HAV (55%), VI (65%) and VII (70%) were digested with AOAC 991.43 method to simulate human digestion of cooked starch. Fecal inocula were prepared from fresh fecal samples of each individual and applied to fermentation vials containing each SR. Brain Heart Infusion (BHI #954) medium (Difco Laboratories, Detroit, MI) was used as a blank control. All procedures were conducted in anaerobic conditions with the circulation of CO₂ or in Bactron I anaerobic chamber (Sheldon Manufacturing, Inc., Cornelius, OR). Medium and substrate were replenished every 3.5 d by discarding 6 mL fermentation mixture to simulate colonic retention of digesta. Bacterial cultures at wk 0, 1, 2, and 3 were collected by centrifuging and stored at -80 °C until analyzed.

*Genomic DNA extraction and PCR.* Bacteria genomic DNA was extracted with a QIAamp DNA stool mini kit (Qiagen, Valencia, CA). DNA was quantified with Nano-Drop ND-100UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Reverse primers used in the study were attached with a GC-clamp: 5’ CGCCCGCAGCCGAGCAGGGCCGGGCCCAGCGGTCGGTCCGGGGCCGGCCGGCCGGGGG 3’ for DGGE analysis (16) . Universal bacterial 16S rRNA gene primers (forward primer: 5’ CCAGCAGCCGAGCTATGATTTGTAAT 3’ and reverse primer: 5’ GC-clamp-TACGGGAGGCAGCAG 3’) were used to amplify 230 bp fragment of 16S rRNA variable 3 (V3) region for total bacterial population analysis (16) . *Clostridium* cluster IV (*Clostridium leptum* subgroup) specific primers (sg-Clept-F: 5’ GCACAAGCAGTGGAGT 3’ and sg-Clept-R3: 5’ GC-clamp-TTCCTCCGTTTTGTCGA 3’ were used to amplify ~ 240 bp fragment of 16S rRNA gene (17). *Clostridium* cluster XIVa (*Clostridium coccoides–Eubacterium rectale* group)
specific primers (Ccoc-F: 5’ AAATGACGGTGACCTGACTAA 3’ and Ccoc-R: 5’ GCTTGTGAGCTTTACATTCTTGCGAA 3’) were used to amplify ~ 450 bp fragments of 16S rRNA gene (18). Bacteroides fragilis group specific primers (Bfr-F 5’ CTGAACCAGGCAAAGTAGCG 3’ and Bfr-R: 5’ GC-clamp-CCGCAAACTTTTCACAATGACTTA 3’) were used to amplify ~ 230 bp fragments of 16S rRNA gene (19). All primers with PAGE (Preparative Purification by Gel Electrophoresis) purification were purchased from Sigma-Aldrich (St Louis, MO).

PCR reactions were performed with 200 ng genomic DNA, 5 µL Taq buffer, 0.25 mmol/L each dNTP, 50 pmol of each primer, and 0.25 µL (5 units/µL) Hotstart Taq polymerase (TaKaRa Bio Inc. Japan) in a 50 µL volume. PCR procedures were as follows: 1 cycle of denaturing at 94 °C for 5 min, followed by 21 cycles of denaturing at 95 °C for 15 sec, annealing for 30 sec and extension at 72 °C for 2 min, with the final extension at 72 °C for 10 min with Bio-Rad Thermal cycler (Bio-Rad Laboratories, Hercules, CA ). For universal primers for total bacteria, touch-down PCR procedure was used with annealing temperature decreased at the rate of 1 °C every two cycles from 62 °C to 52 °C. Annealing temperature was 50 °C for Clostridium cluster IV, 54 °C for Clostridium cluster XIVa, and 52 °C for Bacteroides fragilis group. All PCR products were analyzed by electrophoresis with 2% agarose gel before applying to the DGGE analysis to confirm the presence of amplicons.

DGGE. 2 µL PCR products were applied to DGGE analysis (16). DGGE marker III (Wako, Osaka, Japan) was used to allow normalization of band retention distances between gels. Polyacrylamide (37:5:1 acrylamide/bisacrylamide) gel (16 cm × 16 cm) with a defined gradient of denaturing reagents (urea and formamide) were casted with a Hoefer SG50 gradient former (Thermo Fisher Scientific, Pittsburgh, PA). A Gelbond PAG film (Lonza Rockland Inc. Rockland, Maine) was used to support the gel during electrophoresis and staining procedures. 100% denaturing solution contained 7 mol/L urea and 40% (v/v) formamide. DGGE gel with 20-80% gradient was used for total bacteria analysis, 35-55% for Clostridium cluster IV, 38-60% for Clostridium cluster XIVa, and 20-70% for Bacteroides fragilis group. DGGE electrophoresis was performed at a constant temperature of 60 °C.
using the D-code system (BioRad Laboratories, Hercules, CA) for 16 h for total bacteria, *Clostridium* cluster XIVa, and *Bacteroides fragilis* group, and 14 h for *Clostridium* cluster IV.

Silver staining was used for DGGE gels according to Liu et al. (20) with slight modifications. Gels were soaked in 0.1% AgCl₂ solution for 30 min, followed by the exposing solution (1.5% (wt/v) NaOH, 0.01% (wt/v) NaBH₄, and 0.4% (v/v) formaldehyde) until bands were clear. Gels were visualized with GS-800 Calibrated Imaging Densitometry (Biorad Laboratories., Hercules, CA). Migration distance and band intensity were analyzed with “Quantity One” software (Biorad Laboratories., Hercules, CA).

**Band purification and sequencing.** Bands of interest were excised from DGGE gels. DNA was eluted with Milli-Q water at 4 °C overnight. Following a brief spin, supernatant was used as template for PCR amplification to enrich DNA fragments according to the procedures above. PCR products were purified with Wizard Plus SV Minipreps DNA purification system (Promega Co., Madison, WI) and cloned to the PCR2.1 vector following manual instruction of TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Colonies were picked and cultured with LB medium overnight at 37 °C in a shaking chamber at 220 rpm. Plasmids was extracted with QIAGEN plasmid mini kit (Qiagen, Valencia, CA) and sent to DNA sequencing facility of Iowa State University. DNA sequences within primers excluding GC-clamp were matched to NCBI GenBank DNA database and Ribosomal Release Project database 9.1.

**Statistical analysis.** WPGAMA (weighted pair group method using arithmetic averages) (also known as average linkage), was used to cluster lanes based on bacterial pattern and construct dendrograms. DGGE gels across treatments were aligned based on retention distance of markers, followed by visual inspection for verification. Band intensity was logarithm (base 10) transformed and used to generalize liner probability model considering treatment and BMI effects, as well as their interaction: $y_{ijkl} = b_i + t_{xj} + w_k + btx_{ij} + bw_{ik} + wtx_{jk} + \epsilon_{ijkl}, (y_{ijkl})$. ($y_{ijkl}$: logarithm of band intensity +1, $b_i$: expected band intensity, $t_{xj}$: expected effect of treatment, $w_k$: effect of BMI, $\epsilon_{ijkl}$: residual error). Non-significant two-way or three-way interaction was not considered in the model. Significant bands identified were further used for Principle Component Analysis (PCA) and Linear Discriminant Analysis (LDA #624) to
assess selection effects of SR and lean/overweight/obese microbiota on fermentation bacterial DGGE patterns with Hotelling’s T test. Band numbers were reported as mean ± SD unless specified.

Results

Specificity and stability of bacterial pattern during 3-wk adaptation to SR incubation. To evaluate the specificity and stability of fermentation bacteria during 3-wk adaptation to SR in our continuous fermentation model, PCR-DGGE analysis was performed on fermentation samples collected at wk 1, 2, and 3 of each treatment from four donors randomly selected (Fig. 1). Dendrogram trees were constructed by WPGAMA (weighted pair group method using arithmetic averages), which was suggested by “Quantity One” manual as one of the cluster methods providing the most plausible results and least affected by outliers.

Two types of selective response to SR/blank treatment could be generalized by comparing fermentation bacterial pattern of these four donors. In Donor 1 and 3, fermentation bacterial pattern formed two distinct clusters with one including SRV and blank control, and the other one including SRVI and SRVII. Correspondingly, Band 1 (Donor 1) and Band 2 (Donor 3) were two dominant bands selectively enriched by SRVI and SRVII, but not by SRV and blank. By contrast, in Donor 2 and 4, fermentation bacterial pattern for three SR were clustered away from blank BHI control. Accordingly, Band 3 (Donor 2) and Band 4 (Donor 4) were similarly enriched by three SR across 3 wk, but not in blank. DNA sequence of Band 3 was closely matched to *Eubacterium rectale* (100% similarity, Table 1), which has been recognized as a butyrate-producing bacterium belonging to *Clostridium* cluster XIVa.

The stability of fermentation system during 3-wk incubation could be examined by comparing the fermentation bacterial pattern across each time point. Cluster analysis demonstrated that in three donors (Donor 1, 3, and 4), fermentation bacterial pattern in blank at wk 2 and 3 were clustered together with high Dice coefficients (0.57-0.80). For SR treatment, in Donor 1, 2 and 3, although there were exceptions, fermentation bacterial pattern in SR at wk 2 and 3 were similar to each other, especially for SRVII. In accordance, the enrichment of Band 5 (Donor 3) and the disappearance of Band 6 (Donor 1) were observed
after wk 1 in SR treatment, suggesting that at least two weeks were required for microbiota to achieve stability in our fermentation model. Interestingly, in Donor 4, fermentation bacterial pattern were clustered by wk and three SR induced similar profile at each time point, indicating the similarity of three SR in the dynamic adaption of fecal microbiota from this donor.

**PCR-DGGE analysis of fermentation total bacteria.** At the end of 3-wk incubation, total fermentation bacterial pattern of 30 donors were analyzed by PCR-DGGE with universal 16S rRNA primers for V3 region (**Fig. 2**). Compared to blank and SRV, both of which had an average DNA band number of 17 ± 4, SRVI incubation significantly increased band number to 20 ± 4, and SR VII decreased the band number to 14 ± 4. The band number observed were similar to what reported previously with 20 ± 3 (21) and in the range of 7-26 bands (12) detected in fecal samples of healthy individuals with PCR-DGGE. Band 7 and 8 were the two dominant bands observed in blank control as shown by DGGE analysis of fermentation bacteria (**Fig. 2**). The detection frequency of was 28/30 for Band 7 and 25/30 for Band 8. DNA sequence of Band 8 was closely matched to an uncultured bacterial clone belonging to the Firmicutes phylum (99% similarity, **Table 1**). SR incubation induced shifts in the fermentation bacterial pattern, but to varied extent among three SR. For example, SRVI and VII reduced the incidence of Band 8 to 10/30 and Band 7 to 14-15/30. A moderate effect of SRV on these two bands was observed with the detection frequencies of 21/30 for Band 7 and 22/30 for Band 8. On the contrary, a couple of bands with similar migration distances in the region between bands 3 and 4 of the marker were enriched by SRVI and VII, but to a less extent by SRV, compared to blank control, which lacked major bands.

**PCR-DGGE analysis of RS fermentation-associated bacteria.** RS fermentation-associated bacterial patterns of *Clostridium* cluster IV, *Clostridium* cluster XIVa and *Bacteroides fragilis* group were analyzed with specific primers for PCR-DGGE at the end of 3-wk SR incubation.
*Clostridium cluster XIVa.* At the end of 3-wk incubation, three SR significantly increased the average DNA band number (5 ± 2) compared to blank control (3 ± 1), as shown in the DGGE profiles of fermentation bacteria in *Clostridium* cluster XIVa (Fig. 3). There was one major band (Band 9) (*Clostridium symbiosum,* 99% similarity) (Table 1) detected in almost all samples in blank (29/30), but its detection frequency was decreased by SRV (16/30), SRVI (23/30) and SRVII (18/30). Instead, Band 10, which was not detected in blank, was observed with the frequency of 12/30 in SRV, 17/30 in SRVI and 14/30 in SRVII. Similarly, the incidence of Band 11, which was only detected in 1 individual sample of blank, was increased by SRV (12/30), SRVI (20/30) and SRVII (13/30).

*Bacteroides fragilis group.* At the end of 3-wk incubation Fermentation bacteria DGGE pattern of *Bacteroides fragilis* group was similar between SR and blank control (Fig. 4). The average band number was similar across four treatments. Band 12 and 13 were the two major bands detected in more than 25 individual samples in each treatment. DNA sequence of Band 12 was closely matched to *Bacteroidaceae bacterium Smarlab,* and Band 13 was closely matched to *Bacteroides thetaiotaomicron* (Table 1).

*Clostridium cluster IV.* DGGE profiles obtained with *Clostridium* cluster IV specific primers were shown in Fig. 5. There was no significant difference in the average band number across treatments (5 ± 2 in blank, 6 ± 2 in SRV, 5 ± 2 in SR VI, and 7 ± 2 in SRVII). Major shifts caused by SR incubation were observed by the detection frequency of two dominant bands, Band 14 and 15 as highlighted in Fig. 5. The detection frequency of Band 14 was high in blank (28/30), and was significantly decreased by SR incubation (14/30 in SRV, 12/30 in SRVI and SRVII). By contrast, the detection frequency of Band 15 (*Ruminococcus bromii,* 99% similarity, Table 1) was 10/30 in blank, and was increased by SR incubation with 14/30 in SRV and 19/30 in both SRVI and SRVII. Interestingly, Band 15 was detected more often in fermentation samples with fecal microbiota from lean donors than from overweight/obese donors: 7 versus 3 in blank, 10 versus 4 in SRV, 14 versus 5 in SRVI and 13 versus 6 in SRVII. Additionally, the incidence of Band 16 was only increased by SRVI (20/30) compared to blank (12/30), but not by other SR (10/30 for SRV and 11/30 for SRVII) suggesting a possible selective response of this putative bacteria species to SRVI.
Selection of SR and fecal microbiota from lean and overweight individuals on DGGE patterns of Clostridium cluster IV. The difference in the incidence of Band 15 between fermentation samples from lean and overweight/obese donors led us further explore the selection effects of SR treatment and BMI on shifting bacterial pattern of Clostridium cluster IV after 3-wk semi-continuous incubation. 11 bands on Clostridium cluster IV DGGE gels were identified in the linear model (multiple $R^2 = 0.3862$, adjusted $R^2 = 0.3595$, $P < 0.05$) (Table 2), which showed the significant effects of SR treatment and BMI on bacterial pattern. These bands were further used in Principle Component Analysis (PCA) and Linear Discriminant Analysis (LDA).

PCA analysis showed that the first two components accounted for the 18.2% variability in the Clostridium cluster IV bacterial pattern across four treatments (Fig. 6). Band 14 and Band 15 were the two bands showing the lowest and the highest coefficient with -0.593 and 0.508 respectively in the first principle component, which revealed the significant difference in the bacteria DGGE pattern of Clostridium cluster IV between fermentation samples with fecal microbiota from lean and overweight/obese donors. On the other hand, three DNA bands (Band 16, U8 and U23) had the highest coefficients with 0.462, 0.461 and 0.487 respectively in the second principle component, which revealed a smaller inter-individual variability of Clostridium IV DGGE pattern within the treatment of SRV or SRVII than that within the treatment of SRVI or blank control. Moreover, there were significant differences in the DGGE bacterial pattern across the four treatments (Fig. 9), with an exception in that between SRV and SRVII with $P > 0.05$. LDA analysis further confirmed the substantial effects of four bands (Band 14, 15 and 16) on the selection of fermentation bacteria DGGE pattern of Clostridium cluster IV by SR and microbiota from lean donors (Fig. 8). The detection frequency and band intensity of five major DNA bands recognized in the PCA and LDA analysis (Band 14, Band 15, Band 16, U8 and U23) were showed in Fig. 7. Most noticeably, the average band intensity of Band 15 in the fermentation samples with fecal microbiota from lean donors was 5-fold higher in SRV, 3-fold higher in SRVI and 4-fold higher in SRVII than that from overweight/obese donors.
Pre-incubation fecal bacteria DGGE pattern of Clostridium cluster IV. Fig. 10 showed fecal bacteria DGGE pattern of Clostridium cluster IV before incubation in 30 donors. An average band number was 8 ± 2 per lane. WPGAMA analysis did not reveal distinct clusters associated with BMI grouping (lean, overweight or obese). In contrast, a relatively consistent pattern was observed between lean, overweight and obese donors, as shown by three major bands (Band 17, 18 and 19) highlighted. The detection frequency was 29/30 for Band 17, 27/30 for Band 18 and 29/30 for Band 19.

Discussion

With an aim to investigate prebiotic effects of RS, we established a long-term in vitro semi-continuous fermentation system to simulate the adaptation process of gut microbes to digested starch residues (SR). PCR-DGGE analysis with bacterial group-specific primers, we demonstrated that Clostridium cluster IV and Cluster XIVa were shifted by 3-wk incubation with SR compared to blank control. Additionally, a higher frequency of putative Ruminococcus bromii was observed by SR incubation with fecal inocula from lean donors (10-14/15) than from overweight and obese donors (4-6/15).

Assessing the stability of bacterial pattern is essential in validating an in vitro fermentation model to simulate the in vivo situation of gut microbiota. Short-term fermentation with 24 or 48 h may not be sufficient for bacterial populations to fully adapt to substrates and establish a stabilized micro-ecosystem. In our study, we used a relatively simple batch fermentation model coupled with frequent replenishment of pre-digested SR every 3.5 d (84 h) to simulate retention of gut microbiota exposed to substrates. Our results of fermentation mixtures from four donors indicated that at least two weeks were required for microbiota to adapt to SR/blank as shown by the consistency of total bacterial pattern observed at wk 2 and 3 with PCR-DGGE analysis (Fig. 1). Measurement of functional indicators, i.e. major SCFA concentration, gas production and pH showed that they reached stability starting at wk 1, but it took two weeks for molar proportions of major SCFA to reach a stable status (Li et al., unpublished, 2010), which was consistent with PCR-DGGE results observed in the current study. Our findings were similar to the previous studies using a five-stage continuous reactor fermentation system called SHIME (simulator of the human intestinal microbial ecosystem)
with the same purpose to examine the time needed to form a stabilized bacteria community in response to a mixture of carbohydrate sources commonly found in human diet, including pectin, potato starch, xylan, arabinogalactan and glucose (26). Based on the quantification analysis with PCR-DGGE on total bacteria, *Lactobacillus* spp. and *Bacteroides/Prevotella* spp. pattern, they estimated that at least two weeks were needed for bacteria community stability and three weeks for functional stability (SCFA production).

As expected, we observed some evident shifts of fermentation bacterial pattern after 3-wk SR incubation compared to blank control, such as total bacteria, *Clostridium* clusters IV and XIVa. Interestingly, the response to blank/BHI medium was relatively consistent across 30 donors, as shown by the higher detection frequency of Band 7 and 8 in total bacteria (Fig. 2). Band 9 (*Clostridium symbiosum*, 99% similarity) in *Clostridium* *Clostridium* cluster XIVa (Fig. 3), and Band 14 in *Clostridium* cluster IV (Fig. 5). The BHI medium containing 0.2% dextrose used in the current study is a general nutrient rich medium for anaerobic bacteria culture. We thought it was appropriate as a control to simulate a common situation *in vivo* when gut microbiota exposed to a low amount of carbohydrates escaping small intestinal absorption. SR incubation either decreased the detection frequency of these bands or enriched other dominant bands, such as Band 10 and 11 in *Clostridium* cluster XIVa (Fig. 3) and Band 15 in *Clostridium* cluster IV (Fig. 5) to change the biodiversity of fermentation microbiota. These results were in line with the general recognition of the prebiotic effect of RS with *in vivo* animal feeding studies (27).

In contrast to *Clostridium* clusters, limited shifts of *Bacteroides* *fragilis* group were observed by SR incubation compared to blank BHI control (Fig. 4), suggesting *Bacteroides* spp. were less responsive to SR incubation in our system. For example, Band 13 (putative *B. thetaiotaomicron*) was consistently detected across most individuals in each treatment. The role of *Bacteroides* spp. in starch fermentation is still inconclusive. On one hand, one dominant *Bacteroides* spp. in human gut, *B. thetaiotaomicron*, has been known as a starch-utilizing bacterium based on the basic functional/metabolic analysis (24). Identification and characterization of starch utilization complex encoded by *sus* gene clusters in *B. thetaiotaomicron* further supported its role in starch fermentation (25). However, on the other
hand, *B. thetaiotaomicron* could only utilize soluble amylopectin, as measured by a clearing zone on an agar plate containing amylopectin, but not insoluble amylase (8). Additionally, the abundance of *B. thetaiotaomicron* was not affected during the continuous incubation with insoluble HAS (Hylon II) (26). Therefore, it is possible that members of *Bacteroides fragilis* group, including *B. thetaiotaomicron*, are efficient in utilizing soluble starch polymers and glucose/dextrose either in the BHI medium or released by other microbial action, rather than insoluble polysaccharides substrates, such as SR used in our study.

The fermentation outcome of RS, including SCFA production and bacterial pattern shifts, is mainly determined by physicochemical properties of RS and host pre-existing gut microbiota as well. However, there was only one study available showing that two polymorphs of retrograded RS with distinct crystalline structures induced different fermentation bacterial pattern using a three-stage continuous culture system *in vitro* (15). In the current study, we have observed selective effects of three SR on fermentation bacterial pattern at the end of 3-wk incubation. For examples, the total bacterial pattern obtained by SR V incubation was different from SRVI and SRVII as shown by the detection frequency of Band 7 and 8 ([Fig. 2](#)), whereas SRVI differed from SRV and SRVII in bacterial patterns of *Clostridium* clusters IV ([Fig. 5](#) and [Fig. 9](#)) and XIVa ([Fig. 4](#)). We suspected that the digestibility, determined by the crystalline structure of SR, may account for the different total bacterial pattern observed, as shown by a higher proportion of small molecules and higher enthalpy changes for SRV than SRVI and VII (Li et al., unpublished, 2010). The SR used in the current study was obtained by predigesting three types of HAS, which had varied genetic background and amylase content, with AOAC 991.43 method, which includes retrogradation-like procedures by incubating RS with thermal-stable amylase at boiling temperature (100 °C) for 30 min, followed by protease and amyloglucosidase digestion at 60 °C for a total of 1 h. The difference in the total bacterial pattern induced by the lower digestibility of SRV may explain its higher propionate production compared with the blank BHI control and SRVI. However, the similar bacterial patterns in *Clostridium* clusters IV and XIVa between SRV and SRVII could not be explained by their different digestibility and crystalline structure. Therefore, the combination effects of RS and gut microbiota might be essential in predicting the fermentation outcome of three SR after 3-wk incubation.
Recent studies have suggested that compared to lean individuals, microbiota of obese individuals had a higher energy harvesting capacity from starch/carbohydrates with the higher proportion of Firmicutes/Bacteroidetes (28), since the former of which contained major starch-utilizing bacteria, such as Clostridium clusters IV and XIVa (10). However, we observed comparable energy extraction capacity, i.e., SCFA production between fermentation samples with fecal microbiota from lean and overweight/obese donors during 3-wk SR adaption (Li et al., unpublished, 2010). Intriguingly, there was no particular bacteria species or DNA band in two Clostridium clusters showing increased incidence or band intensity in fermentation samples of overweight and obese donors. On the contrary, the detection frequency and band intensity of Band 15, which was closely matched to Ruminococcus bromii, belonging to Clostridium cluster IV, was significantly increased by three SR in fermentation samples of lean donors, but not in those of overweight/obese donors (Fig. 5), and not by blank BHI control either. More intriguingly, analysis of pre-existing fecal bacteria DGGE pattern of Clostridium cluster IV was similar between lean and overweight/obese donors as shown by the cluster analysis (Fig. 10). These results suggested the specific selection for R. bromii by the combination effects of SR and fecal microbiota from lean donors after 3-wk adaptation in the current study.

The comparable functional/metabolic activities, i.e. SCFA production suggested that other obesity-associated bacteria might thrive in fermentation microbiota of overweight and obese donors to compensate such a low abundance of R. bromii. For example, it has been shown that 75% of the microbiome in obese individuals were from bacteria belonging to Actinobacteria phylum, most of which were phylogenically related to B. adolescentis and B. longum (31). Bifidobacteria spp. were previously identified as major amylolytic bacteria in human gut and B. adolescentis and B. breves were enriched during HAS incubation in vitro (26), probably by their abilities to attach to insoluble starch granules (30). Bifidobacteria spp. has been suggested to contribute to butyrate production through cross-feeding by utilizing glucose with the “bifidus shunt” (i.e., fructose phosphate shunt) to provide lactate and acetate for butyrate-producing bacteria as substrates (32). In addition to the cross-feeding effects of Bifidobacteria spp., inter-species hydrogen transfer has also been suggested to be associated with higher energy extraction capacity of gut microbiota of obese individuals. Studies
showed that H$_2$-producing bacteria *Prevotellaceae*, a family in *Bacteroidetes* phylum, and H$_2$-consuming archaea *Methanobacteriales*, mainly *Methanobrevibacter smithii* were highly enriched in obese individuals (34). Since SCFA and gas are major fermentation products of RS, these bacteria might be possible candidates increased during SR incubation with fecal microbiota from overweight/obese donors contributing to the comparable butyrate and gas production. Further investigation may help to understand the adaptation outcome of these bacteria or archaea species in response to RS in the bacteria community from obese donors.

The role of *Ruminococcus bromii* in RS fermentation has been recognized recently with evidences from both *in vitro* and *in vivo* studies. Selective enrichment of *R. bromii* was observed by the incubation of HAS with fecal inocula from 4 donors in a continuous three-stage fermentation model, but not by wheat bran or mucin (26). This finding also suggested the ability of *R. bromii* specific utilization of insoluble starch granules. Further studies using 16S rRNA-SIP (stable isotope probing) to trace the utilization of U-$^{13}$C labeled potato starch by fecal microbiota showed that *R. bromii* was a primary starch degrader in a continuous fermentation model TIM2 (TNO *in vivo* model of human colon) (27). Recently, an *in vivo* human feeding trial with a high RS diet (22g/d) significantly increased the proportion of *R. bromii*-related phylotypes in total fecal bacteria (6.6-7.9%) compared to control diet feeding (4.4%) (12). Consistently, animal feeding studies conducted in our lab showed the band intensity of *R. bromii* on DGGE gels of *Clostridium* cluster IV was significantly increased by both two RS diets feeding (HAS and stearic acid-complexed HAS) for 8 wk in Fisher 344 rats (Li et al. unpublished, 2010). The identification of *R. bromii* in the current study not only further confirmed its role in RS fermentation, but also validated the efficiency of our model as a cost-saving alternative in simulating *in vivo* fermentation to study gut microbiota *in vitro*.

In conclusion, with a stable *in vitro* semi-continuous fermentation model, our results demonstrated prebiotic effects of RS, especially on the bacteria species distribution in *Clostridium* cluster IV and XIVa as analyzed by group-specific primers with PCR-DGGE. Additionally, the fermentation bacteria DGGE pattern was different across three SR in total bacteria, *Clostridium* clusters IV and XIVa, suggesting the combination effects of physiochemical prosperities of RS and host pre-existing microbiota. More importantly, the
selection for *Ruminococcus bromii* was observed by the combination effects of SR and microbiota from lean donors.

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**Footnotes**

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6. Abbreviations used: BMI, body mass index; HAS, high amylose starch; PCR-DGGE, PCR-Denaturing Gradient Gel Electrophoresis; RS, resistant starch; SCFA, short chain fatty acid; SR, starch residue; WPGAMA, weighted pair

**Literature Cited**


Table 1 Sequence similarity of excised DNA bands identified from DGGE gels in our study.

<table>
<thead>
<tr>
<th>Band</th>
<th>Phylum</th>
<th>Family</th>
<th>Genus</th>
<th>Closest match (Accession No.)</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band1</td>
<td>Bacteroidetes</td>
<td>Prevotellaceae</td>
<td>Prevotella</td>
<td>Uncultured bacteriira Prevotella copri DSM 18205 (AB064923)</td>
<td>100%</td>
</tr>
<tr>
<td>Band2</td>
<td>Firmicutes</td>
<td>Rumniococcaceae</td>
<td>Oscillibacter</td>
<td>Uncultured bacterium clone</td>
<td>98%</td>
</tr>
<tr>
<td>Band3</td>
<td>Firmicutes</td>
<td>Eubacteriaceae</td>
<td>Eubacterium</td>
<td>Eubacterium rectale</td>
<td>100%</td>
</tr>
<tr>
<td>Band4</td>
<td>Firmicutes</td>
<td>Porphyromonadaceae</td>
<td>Porphyromonas</td>
<td>Porphyromonas somerae (AY968205)</td>
<td>100%</td>
</tr>
<tr>
<td>Band5</td>
<td>Bacteroidetes</td>
<td>Porphyromonadaceae</td>
<td>Barnesiella</td>
<td>Barnesiella sp.EBA4-14 (EF608208) Barnesiella intestinihominis (AB370251)</td>
<td>99%</td>
</tr>
<tr>
<td>Band8</td>
<td>Firmicutes</td>
<td>Unclassified Clostridia</td>
<td>NA</td>
<td>Unncultured bacterium colone</td>
<td>99%</td>
</tr>
<tr>
<td>Band15-1</td>
<td>Firmicutes</td>
<td>Ruminococcaceae</td>
<td>Ruminococcus</td>
<td>Ruminococcus bromii (FP929051)</td>
<td>99%</td>
</tr>
<tr>
<td>Band15-2</td>
<td>Firmicutes</td>
<td>Ruminococcaceae</td>
<td>Ruminococcus</td>
<td>Ruminococcus bromii (FP929051)</td>
<td>99%</td>
</tr>
<tr>
<td>Band15-3</td>
<td>Firmicutes</td>
<td>Ruminococcaceae</td>
<td>Ruminococcus</td>
<td>Ruminococcus bromii (FP929051)</td>
<td>99%</td>
</tr>
<tr>
<td>Band9</td>
<td>Firmicutes</td>
<td>Clostridiaceae</td>
<td>Clostridium</td>
<td>Clostridium symbiosum (EF025909)</td>
<td>99%</td>
</tr>
</tbody>
</table>
Table 2 Type II variance table of the linear model generalized based on *Clostridium* cluster IV bacteria DGGE pattern.

<table>
<thead>
<tr>
<th></th>
<th>Sum Sq</th>
<th>Df</th>
<th>F-value</th>
<th>Pr(&gt;F)</th>
<th>Significant codes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band</td>
<td>270.04</td>
<td>25</td>
<td>27.3265</td>
<td>&lt;2.2e-16</td>
<td>***</td>
</tr>
<tr>
<td>Treatment</td>
<td>8.87</td>
<td>3</td>
<td>7.4777</td>
<td>5.516e-05</td>
<td>***</td>
</tr>
<tr>
<td>BMI</td>
<td>1.51</td>
<td>1</td>
<td>3.8085</td>
<td>0.05109</td>
<td>*</td>
</tr>
<tr>
<td>Band*treatment</td>
<td>112.77</td>
<td>72</td>
<td>3.9624</td>
<td>&lt;2.2e-16</td>
<td>***</td>
</tr>
<tr>
<td>Band*BMI</td>
<td>57.00</td>
<td>24</td>
<td>6.0080</td>
<td>&lt;2.2e-16</td>
<td>***</td>
</tr>
<tr>
<td>Residuals</td>
<td>1136.43</td>
<td>2875</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R squared = 0.3862. Adjusted R-squared = 0.3595 (p-value: <2.2e-16). *P <0.05, ** P <0.01, ***P <0.001
FIGURE 1 Total bacteria DGGE pattern of fermentation samples from Donor 1, 2, 3, and 4 during 3-wk incubation with SRV, VI and VII. Dendrogram trees were constructed with WPGAMA labeled with Dice coefficient. BK/Blank: blank BHI medium. M: DGGE marker III (Wako, Osaka, Japan). Pre: fecal microbiota before incubation.
FIGURE 2 Total bacteria DGGE pattern of fermentation samples from 30 donors at the end of 3-wk incubation with SRV, VI and VII. M: DGGE marker III (Wako, Osaka, Japan). B: band.
**FIGURE 3** *Clostridium* cluster XIVa bacteria DGGE pattern of fermentation samples from 30 donors at the end of 3-wk incubation with SRV, VI and VII. M: DGGE marker III (Wako, Osaka, Japan). B: band.
FIGURE 4 Bacteroides fragilis Bacteria group DGGE pattern of fermentation samples from 30 donors at the end of 3-wk incubation with SRV, VI and VII. M: DGGE marker III (Wako, Osaka, Japan. B: band.
FIGURE 5  *Clostridium* cluster IV bacterial DGGE pattern of fermentation samples from 30 donors at the end of 3-wk incubation with SRV, VI and VII. M: DGGE marker III (Wako, Osaka, Japan). B: band.
FIGURE 6 First two principle components in PCA analysis of *Clostridium* Cluster IV bacteria DGGE pattern of fermentation samples from 30 donors at the end of 3-wk incubation.
FIGURE 7 LDA analysis of *Clostridium* Cluster IV bacteria DGGE pattern of fermentation samples from 30 donors at the end of 3-wk incubation.
FIGURE 8 PCA analysis showed differences in the bacteria DGGE pattern of *Clostridium* cluster IV by SR fermentation with fecal microbiota from 30 donors.
FIGURE 9 Incidence and band intensity of five major bands (Band 14, 15, 16, U8, and U23) in *Clostridium* Cluster IV bacteria DGGE gels of fermentation samples from 30 donors at the end of 3-wk incubation.
FIGURE 10 Fecal *Clostridium* cluster IV bacteria DGGE pattern of 30 donors before incubation (wk 0). Dendrogram trees were constructed with WPGAMA methods and labeled with Dice coefficients. M: DGGE marker III (Wako, Osaka, Japan).
CHAPTER 6. GENERAL CONCLUSIONS

Assessing prebiotic effects of resistant starch (RS) during gut microbial fermentation is essential to understand physiological significance of resistant starch in improving human health and preventing chronic colonic diseases. The studies performed clearly showed with an \textit{in vivo} animal model and an \textit{in vitro} fermentation model how gut microbiota patterns and microbial metabolizing activities were affected by RS feeding. Our results provided evidence to key aspects for understanding the underlying mechanism of RS.

A new type of resistant starch (type5), fatty-acid modified high amylose starch VII (HA7-SA), was first examined with our \textit{in vivo} animal model by comparing it to its parent starch HA7. By PCR-DGGE analysis, we provided solid evidences showing that both RS significantly shifted colonic microbiota patterns in Fischer344 male rats after 8 wk feeding period. A similar shift of \textit{Clostridium} cluster IV was observed by HA7 and HA7-SA with the enrichment of \textit{Ruminococcus bromii} related organisms. Whereas, a specific enrichment of \textit{Bifidobacterium pseudolongum} related organisms was observed by HA7-SA. More importantly, modulation of microbiota by RS was significantly correlated with the differential efficacy of RS in preventing colonic carcinogenesis, i.e. decreasing the numbers of pre-neoplastic lesions, aberrant crypt foci (ACF). Putative bacteria \textit{Ruminococcus obeum}, belonging to \textit{Clostridium} cluster XIVa, was of particular interest with the specific enrichment by HA7 which significantly decreased ACF number but not by HA7-SA. These results suggest the importance of butyrate in RS prevention carcinogenesis because \textit{Clostridium} cluster XIVa contained major butyrate producing bacteria predominant in human gut microbiota.

We established an \textit{in vitro} semi-continuous anaerobic fermentation model to simulate long-term adaption to RS consumption by fecal microbiota from 32 human donors: 17 lean, 9 overweight, and 6 obese. In a two phase study we observed comparable fermentability with increased butyrate production by digested starch residues from the four resistant cornstarches, HAV, VI, VII and GEMS-67. These RS are all high amylose starch, (type II RS) having different genetic backgrounds and amylose content. However, an increased propionate
production was observed by one starch residue (SR) from HAV, SRV, but not by other 3 SR. Further investigation of fermentation bacteria with PCR-DGGE analysis showed the unique bacterial pattern by SRV incubation from SR VI and VII. More interestingly, *R. bromii* related organisms were selectively enriched with SR fermentation by fecal microbiota from lean individuals but not by that of overweight individuals. However, a comparable fermentation capacity with SCFA production was observed between lean and overweight individuals during 3-wk *in vitro* adaption.

As a conclusion, prebiotic effects of resistant starch were confirmed by our studies. *Bifidobacterium spp.* and *Clostridium spp.* are major RS fermentation associated bacteria identified in our studies. Our results also raised many questions for more consideration and future exploration. For example, we observed the large inter-individual variation of genetic diversity of microbiota in both animal studies and *in vitro* fermentation studies. This makes us wonder the efficacy of RS or any other gut-microbiota associated dietary intervention or drug application. More complicated, the nature of genetic diversity of gut microbiota creates functional redundancy. This makes us wonder whether we need to manipulate gut microbiota towards a defined pattern and moreover, if we can. Beyond RS, there are many other questions remaining to be answered, such as how many bacteria species or strains are required and sufficient to attain beneficial effects? Will it be possible to targeting specific bacteria species to introduce or deplete it in humans? Whether bacterial pattern can be sustained after dietary intervention stops? Are there other metabolites of gut microbiota playing a crucial but undiscovered role to improve human health? Other more practical concerns are what food products and appropriate dose is needed to be included in the diet without comprising individual food preference and habits but obtain equivalent beneficial effects. We anticipated that genomic, transcriptomic, and metabolomic analysis would be applied in the future to display comprehensive picture on the effects of prebiotic compounds including resistant starch on gut microbiota and human health.
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