Evaluating the use of resistant starch as a beneficial dietary fiber and its effect on physiological response of glucose, insulin, and fermentation

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Evaluating the use of resistant starch as a beneficial dietary fiber and its effect on physiological response of glucose, insulin, and fermentation

by

Esther Haugabrooks

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

DOCTOR OF PHILOSOPHY

Major: Toxicology

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Iowa State University
Ames, Iowa
2013

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DEDICATION

To Don and Mini for always believing.
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ABSTRACT

Resistant starches (RS) are complex partly indigestible carbohydrates that have shown promise in improving digestive health, lowering postprandial blood glucose (PPG) and insulin (PPI) concentrations among other beneficial health effects. There are four types of RS and a fifth type is emerging; although there is very little experimental data on the physiological responses of RS type V. Three experiments were constructed to test the overarching hypothesis that RS (type II and V) can favorably impact human physiological responses of PPG, PPI, and microbial fermentation in the colon with minimal gastrointestinal distress. Two of the three experiments were randomized blind crossover human feeding studies. Study one, restricted to PPG response, was conducted using conventional and novel crossbred corn starches (type II) fed to 11 participants in pudding product. Study two investigated the effects of stearic acid-modified RS and its parent high amylose starch in crackers on PPG, PPI, and microbial fermentation in 30 participants. The third experiment was an in vitro batch-fermentation of human fecal microbiota from lean and obese individuals. Short chain fatty acids (SCFA) fermentation patterns and shifts in pH were assessed with RS type V, type IV, and type II starch residues (SR) inoculated in brain heart infusion broth, brain heart infusion broth without dextrose, and a basal nutrient growth medium. Overall in vivo results supported the use of RS type II and type V to lower PPG, RS type II to lower PPI, and for type II and V to be more fermentable than type II, based on breath hydrogen production at 8 h after RS intake. Both types of RS were very well tolerated by participants based on gastrointestinal symptom scores after RS-containing meals. In vitro analysis of human fecal batch anaerobic fermentations showed no differences in SCFA production by SR of RS types V and II or body mass index (BMI) of fecal donors. More investigation on the fermentability of RS type V is needed, but so far results support the role of both types of starches in lower PPG with favorable implications for people with diabetes and metabolic syndrome.
CHAPTER 1. GENERAL INTRODUCTION

Research Introduction

It would seem this is the era of resistant starch (RS). Since the discovery of RS in 1982, there have been numerous publications on the effects and application of RS (Abell et al., 2008; Asp et al., 1996; Bassaganya-Riera et al., 2011; Fässler et al., 2006a; Silvester et al., 1995; Storey et al., 2007; Topping et al., 2003; Willis et al., 2009). Research studies utilizing RS cover a wide scope of interest including digestion rates and bioavailability (Cummings et al., 1996; Fässler et al., 2006b; Granfeldt et al., 1993; Hoebler et al., 1999; Holm and Björck, 1992); product development and sensory evaluation\(^1\) (Aigster et al., 2011; Baixauli et al., 2008; Maziarz et al., 2013); incorporation or efficacy of RS as a functional ingredient (Fuentes-Zaragoza et al., 2010); metabolic responses and health benefits (Brouns et al., 2007; Heijnen et al., 1995; Higgins et al., 2004; Johnston et al., 2010); and other aspects. Test species in these studies have included rats (Bauer-Marinovic et al., 2006; Perrin et al., 2001), pigs (Giuberti et al., 2013), and humans (Worthley et al., 2009); in additions, observations have been made in vivo and in vitro when assessing certain attributes such as physiological responses of glucose, insulin, and fermentation (Al-Tamimi et al., 2010; García-Rodríguez et al., 2012; Li et al., 2010; van Munster et al., 1994a; Zhou et al., 2013). Furthermore, scientific evaluation of the benefits of RS has also generated commercial interest.

The ‘RS movement’ came around the advent of efforts to increase dietary fiber and has become the new diet craze. However, the use of RS as a dietary fiber is not its only application.

\(^{1}\) Defined as “a scientific discipline used to evoke, measure, analyze, and interpret reactions to the characteristics of food and materials as they are perceived by the senses of sight, smell, taste, touch, and hearing” by IFT’s Sensory Evaluation Division. (from http://sfs.wsu.edu/sensory/ and Moskowitz et al., 2012)
Within the past couple of decades, the potato diet emerged in which the objective was to lose weight by eating copious amounts of potatoes. Though scientific communities have not rendered enough evidence to strongly support the use of RS in weight loss, the underlying premise was that potatoes contain RS that facilitate weight loss. Another use of RS is as a prebiotic. Arguably, the chief nutritional hot topics of the day include prebiotics and probiotics. Thus, in the area of prebiotic research, RS is of major interest for its potential as an effective prebiotic.

With much ado about RS in the scientific community, a central question pertains: how effective is the use of RS? Implications such as RS providing benefit toward inhibiting carcinogenesis, inflammatory bowel diseases, and metabolic disorders are at issue. For example, due to the slow digestive rates of RS suggestions have been made that RS will lower postprandial glucose and be of benefit to people with diabetes. There is evidence to support pros and cons as to the efficacy of RS to benefit human health. RS has been observed to be effective in lowering postprandial glucose response (Achour et al., 1997; Granfeldt et al., 1995; Raben et al., 1994) and RS has been observed not to be effective in lowering postprandial glucose response (Jenkins et al., 1998; Noakes et al., 1996). Because the majority of this type of research is done in healthy individuals or animal models, there is not enough evidence to support the benefit to people with diabetes, thus, the question of efficacy remains unresolved.

Similar to the focus of this dissertation research introduction, throughout this dissertation careful attention has been given to human studies for this is the main focus of the dissertation. Furthermore, humans tend to be the main interest in many RS physiological response studies. The dissertation’s review of the literature has aimed to establish the growing interest in RS as a dietary fiber and as a functional ingredient in connection to human health. Undoubtedly, the platform for RS is expanding along with our knowledge of gut microbiota and human host
interactions. RS can be used as a substrate to influence events of digestion in addition to the capacity of colonic microbiota for carbohydrate fermentation. However, the role of gut microbiota and gastrointestinal status has been implicated in far more aspects of human health than fermentation.

The goal of this dissertation research is to evaluate in vivo and in vitro effects of RS and how it pertains to human health. The forms of RS that will be used are RS$_2$ and RS$_5$. If there was a postulation on which form of RS is used the most in human studies, it would probably be RS$_2$. But without a doubt, RS$_2$ is used in human studies more frequently than RS$_5$. The types of RS will be covered in the literature review. The objectives of the dissertation research is to investigate the role of RS in glucose, insulin, and fermentation responses in vivo; assess in vitro fermentation patterns of the degradation of RS; and address if different types of RS influence physiological responses. Moreover, through this dissertation literature review and research, the author hopes to provoke discussion on meticulously defining parameters and conditions under which the use of RS is effective.

Dissertation organization

The organization of the dissertation is comprised as follows:

- Review of RS literature largely from a human health perspective
- Three papers for journal submission, authors, and author contributions
  1) The Effects Of Naturally Occurring Resistant Starch In Maize On Postprandial Glycemic Response In Healthy Humans
Esther M. Haugabrooks – drafting of manuscript, data acquisition, analysis, and interpretation

Hyun Jung Kim – data analysis

Linda Pollak – production of study material

Suzanne Hendrich – revision of content

2) Influence Of Crackers Containing Resistant Starch V On Postprandial Glucose, Insulin, And Fermentation Response In Healthy Humans

Esther M. Haugabrooks - drafting of manuscript, data acquisition, analysis, and interpretation

Yongfeng Ai – production of study material (RS5)

Jay-lin Jane – study conception, production of study material (RS5)

Suzanne Hendrich – revision of content

3) In Vitro Screening Of Lipid Modified Resistant Starches And Media Comparison On Short Chain Fatty Acid Production In Batch Fermentation

Esther M. Haugabrooks - drafting of manuscript, data acquisition, analysis, and interpretation, study conception and design

Yongfeng Ai – production of study material (RS5)

Jay-lin Jane – production of study material (RS5)

Suzanne Hendrich – study conception and design, revision of content
A. Resistant Starch

Worldwide, carbohydrates are the main staple in the human diet. Sources of carbohydrates come from a myriad of foods and beverages that act largely as a supply of fuel to energize the body. Components of biological systems like the brain, blood, and nervous use carbohydrates in their simplest form as a principal source of energy. Additionally, the use of this vital nutrient provides an array of health benefits when certain types of carbohydrates are consumed and potential protection from diseases (Asp et al. 1996; Fuentes-Zaragoza et al. 2010; Nugent 2005).

Investigation into identifying and classifying types of carbohydrates, especially those that convey added benefit other than sources of energy, has become multitudinous. Broad classification of carbohydrates ranges from basic simple sugars to complex carbohydrates while encompassing type of sugar, number of sugar units, and type of bond for further specification. Simple sugars, or monosaccharaides, are one sugar unit. Two or more sugars linked by glycosidic bonds are termed di-, oligo-, or poly- saccharides. Starch and glycogen are examples of polysaccharides. Furthermore, polysaccharides can also be categorized as complex carbohydrate and simple carbohydrates can be monosaccharaides and disaccharides. Some complex carbohydrates are classified as dietary fiber, which are the leading carbohydrate currently under great investigation.

An estimated 50% or more of a typical Western diet is carbohydrates, making it the most common and abundant nutrient. A concerning fact when considering carbohydrates that are rapidly digested, such as simple carbohydrates, pose a risk of deteriorating health issues such as diabetes mellitus and coronary heart disease (Liu, 2002), although dietary fiber, a subset of
carbohydrates has been widely accepted for the ability to improve aspects of health. Recommendations have been given for daily intake of carbohydrates and dietary fiber; however, over the past couple decades carbohydrate consumption is increasing while dietary fiber consumption is decreasing (Gross et al., 2004). Thus, nutritional advances are being made to increase overall dietary fiber consumptions and in unsuspecting carbohydrate-rich foods.

Perhaps the most interesting carbohydrate currently being investigated for that role is resistant starch (RS) because of its physiological benefit as a dietary fiber. Though the discovery of resistant starches among dietary components is fairly recent, resistant starch is rapidly becoming a hot topic among functional foods as the scientific knowledge accumulates on structure, uses, function, and biological effects.

A.1 General structure and classification

Starch and cellulose are examples of complex carbohydrates found in plants. Both are storage polysaccharides composed of monomeric glucose, which makes starch and cellulose chemically the same. Furthermore, they both can be classified as dietary fiber, though in the case of cellulose that is strictly the case: whereas, starches are only considered dietary fibers if they are digestion resistant. However, these two compounds are not identical. The biggest distinction between starch and cellulose is the glycosidic linkages. Cellulose contains β- (1-4)-glucosidic bonds that link monomeric glucose units, while starch uses starch α- (1-4) glucosidic bonds. Depending on the polymer composition, starch also contains α- (1-6) glucosidic bonds.

The main structural composition of starches is made up of two polymers: amylose and amylopectin. Amylose is mainly a linear polymer with α- (1-4) bonds, though some branching can occur. Amylopectin is a highly branched polymer with α- (1-4) and α- (1-6) bonds.
Amylopectin is a larger molecule and is typically the more abundant polymer in starches (Buléon et al., 1998). The ratio of amylose to amylopectin has a strong influence on the rate of digestion.

In humans, enzymatic digestion of starches begins in the mouth and ends in the small intestine. However, some starches were found to resist enzymatic digestion. Not long after this discovery (Englyst et al., 1982), the term resistant starch (RS) was coined. RS was described as the sum or fraction of starch that is not absorbed in the small intestine of healthy humans. This fraction of starch was not hydrolyzed in vitro after 120 minutes of incubation with α-amylase and pullulanase (Berry, 1986; Englyst et al., 1982, 1999). Classifications of starches then began to be categorized by the degree of digestion or its crystalline structure, which also gives insight to how the starch will be digested.

The most simplistic way to classify all starches is under the umbrella of three general categories: rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). Because of the straightforward nomenclature, this classification is commonly used to describe the rate of digestion in starches particularly regarding postprandial glucose response (see section D.2). Simple carbohydrates are examples of RDS, which tend to be refined carbohydrates. Starches with slower digestion rates such as SDS and RS can be examples of dietary fibers.

A different method to classify starches is through crystalline structure. Using x-ray diffraction patterns starches can be categorized into types A, B, C, and sometimes V. Type A and type B are somewhat similar in structure because both have starch granules with structural polymers packed in a double helical formation. Type B differs from type A due to water molecules dispersed inside helices, whereas type A has densely packed polymers with typically less water molecules than type B. Type A is commonly found in cereals and type B is found
raw potatoes (Sajilata et al., 2006). Pure amylose has also been used to understand type A and type B starches (see figure 1), which have been shown to crystalize in both forms (Wu and Sarko, 1978a, 1978b). Type C is a combination of type A and B, while type V is an x-ray diffraction pattern observed in swollen starch granules.

![Figure 1. Type A and B starch granular structure. Double helix formation and packing of type A amylose on the left and type B amylose to the right (Wu and Sarko, 1978b).](image)

Though Wu and Sarko (1978a,b) showed crystallinity in amylose, type A, B, and C starches are also typed from the crystallinity of amylopectin, which has been attributed with the crystalline nature of starch granules. Amylopectin has branched chains. Short branched chains are more abundant in type A, longer branched chains are abundant in B, and reiteratively type C has a mix of both long and short branched chains (Jane, 2006). Type A is more susceptible to enzyme hydrolysis, which in part could be attributable to shorter double helices in comparison to type B; therefore, this makes type A more digestible and a common type seen in RDS and SDS than type B, whilst type B is found more in RS (Jane et al., 1997). This method of x-ray diffraction classification, though relevant, is infrequently used especially in biological in vivo studies.
Typing of starches by x-ray diffraction patterns favors more to the physicochemical nature of starch, which gives insight to physiological responses through in vitro structural behavior or visual characterization of starch. For example, a x-ray pattern of a bread rich in amylose exhibited a B-type pattern in vitro and showed an in vivo trend of a lower postprandial peak (Hoebler et al., 1999), supporting B type starches having lower digestibility. However, as previously stated, this type of classification is rarely used to describe starches used in in vivo carbohydrate studies. Broader classification terms of starches like RDS, SDS, and RS are commonly used in biological studies where the terms are applicable. RS is further subdivided into four main categories or types (Eerlingen et al., 1993; Englyst et al., 1992):

**Type I** (RS$_1$) is physically inaccessible to digestive amylolytic enzymes for degradation due to entrapment in a non-digestible matrix; found in starchy foods that are not fractionated or refined.

Examples: whole grains, coarsely ground cereals/partly-milled grains, legumes, seeds

**Type II** (RS$_2$) refers to native resistant starch granule crystallinity with high amylose content; primary structure and conformation of starch granules results in its natural resistance to degradation.

Examples: green banana, crude potato, and raw starch granules

**Type III** (RS$_3$) comprises retrograded starches, which is a recrystallization or re-association of the starch molecules that can form a gel.

Examples: bread (stale), cooked and cooled potatoes, cornflakes, pasta salad
Type IV (RS₄) is chemically modified starch with a high amount of chemical modifications normally through crosslinking (etherized, esterified, or cross-bonded - distarch phosphate ester); synthetic and not found in nature.

Examples: chemically modified starches used as additives, such as Fibersym® RW (MGP Ingredients, Inc., Atchison, KS)

Classifying RS by RS₁-RS₄ is perhaps the most common way RS is described in literature. There is an emerging classification of RS proposed unlike RS type I –IV. This new class of RS is classified as RS type V or RS₅. RS₅ are starch granules that have been modified with lipids forming a starch-lipid complex (Hasjim et al., 2010; Jane et al., 2009), which can be found in native starch granules or processed starch. Differentiation of RS types has become essential in biological research studies because differences in physiological effects of RS have been found between RS types. As a whole, RS is known for its benefit but when addressing particular advancements in health and food productions, specific detail should be given on type and dose of RS.

A.2 Methods of Analysis for Resistant Starch Content

Measurement of RS is quantified as a portion of total starch, mathematically represented as the difference between total starch from the rapidly or slowly digested starch (equation 1). Where TS is total starch, RDS is rapidly digested starch, and SDS is slowly digested starch.

Equation 1: RS% = TS – (RDS + SDS)
The first method developed for the analysis resistant starch seemed to be serendipitous (Englyst et al., 1982). In reporting a method to measure non-starch polysaccharides, Englyst and colleagues recognized starch “made resistant” to pancreatic α-amylase and pullulanase hydrolysis during incubation. Nearly a decade after their discovery of resistant starch, Englyst and others developed a method specifically for the measurement of RDS, SDS, and RS (Englyst et al., 1992).

At first, Englyst’s methods were accepted, but scrutinized for employing non-physiological parameters such as a 100°C incubation. Subsequently, investigation of other techniques or modifications to Englyst’s original methods have been made to produce analytical in vitro RS measurements that reflect in vivo conditions in human digestion: modifications involve added ethanol or industrial methylated spirits precipitation, protein removal, elimination of acetone washing and drying, increased sample size, removal of pullulanase digestions, and increased incubation pH (Berry, 1986; Björck et al., 1986; Champ, 1992; Goni et al., 1996; McCleary et al., 2011). Various analytical methods for measurements of dietary fiber have also been used to measure RS; however, McCleary and Monaghan developed a method exclusively to measure RS (McCleary and Monaghan, 2002) that has become a widely accepted method of RS analysis. Though this method still employs incubation temperatures (50°C) normally not encountered under healthy conditions in vivo, it is currently viewed as an extremely reliable method to predict in vivo conditions of RS digestion.

At present, the two popular methods of analysis of RS are both accepted by AACC International, formerly the American Association of Cereal Chemists (AACC1 or AACC), and Association of Analytical Communities International (AOAC). Lee and company (1992) optimized a method for Total, Soluble, and Insoluble Dietary Fiber in Foods that became official
method AACC 32-07.01-AOAC 991.43; and McCleary and Monaghan Resistant Starch assay became official method AACC 32-40.01-AOAC 2002.02 (Lee et al., 1992; McCleary and Monaghan, 2002). Both employ enzymatic digestions, but AOAC 991.43 is a gravimetric test and AOAC 2002.02 is a colorimetric test (see table 1 for method comparison). AOAC 991.43 is a non-specific method for all dietary fiber and as such has been criticized as an analytical method to measure RS. Some report AOAC 991.43 to overestimate RS content, while in some food preparations it is thought to underestimate RS content in comparison to AOAC 2002.02 (Sanz et al., 2010).

A.3 Resistant Starches as Functional foods or Functional Ingredients

The term “functional food” or “functional ingredient” is quickly gaining popularity. Functional foods fundamentally have been defined as natural and/or modified components with physiological value beyond basic nutrition, which aid in the improvement of human health (Henry, 2010). Functional ingredients can be food additives used to fortify foods or nutritional components naturally present in food. These definitions are loosely accepted because in the US the terms have no legal definition. Notwithstanding that in the US, functional foods are reported to be purchased for health issues such as weight loss, cholesterol reduction, and digestive health (Arvanitoyannis and Van Houwelingen-Koukaliaroglou, 2005). Functional foods span a wide range of foods and beverages. Examples of functional foods or ingredients are salmon, green vegetables, cereals, breads, garlic, onion, vitamins, calcium, and yogurt. RS is also a functional ingredient (Fuentes-Zaragoza et al., 2010) that is naturally present or incorporated into products to make functional foods.
Commercial companies have developed functional foods with dietary fiber, but more specifically products are being formulated with RS or RS as an additive (Aigster et al., 2011). Many research studies have successfully used RS as a functional ingredient to evaluate physiological efficacy and health benefits such as lower cholesterol or improved lipid profile, modulation of glycemic and insulin response, and improved bowel health (Fuentes-Zaragoza et al., 2010); although the term ‘functional ingredient’ might not always be used.

Efforts to enrich low-fiber foods with dietary fiber have encountered setbacks due to sensory issues. Breads, muffins, and crackers are foods commonly fortified with whole grain
dietary fiber. However, sensory evaluation of appearance/color, taste, and feel of high fiber fortified foods has been somewhat unfavorable. For example, fiber-fortified bread has been summarized to have a dark color, reduced loaf volume, poor mouthfeel, and flavor masking properties (Sajilata et al., 2006). Breads formulated to be functional foods, can replace part of rapidly digestible flour with RS to improve sensory concerns. Sanz and associates (2009) replaced 15% of wheat flour with four different types of RS (2 type RS₂, 2 type RS₃) for final RS g (%) of muffin product to be between 8.3g (1.55% RS) and 12.5 g (1.76) as compared to the wheat control 0.65g (0.031% RS) (Sanz et al., 2009).

The appearance of RS can range from a coarse to fine powder that is white in color. RS has a bland taste, which makes it desirable as a food additive. Other desirable traits that are present in types of RS are small particle size, low water-holding capacity, viscosity, solubility, gel formation, light texture, and high gelatinization temperatures. One or more of these traits allow for successful incorporation of RS into a food product while still maintaining a fairly palatable food product, although other sensory attributes such as grittiness, chewiness, and cohesiveness have been affected by the addition of RS to bread products (Baixauli et al., 2008). Baixauli and associates (2008) noticed an increase in grittiness, sweetness, and an overall increase in moisture as RS % increased, while springiness, chewiness, and cohesiveness decreased as RS% increased.

Because of desirable physicochemical characteristics of RS, it improves sensory properties in baked goods compared to control baked goods (Maziarz et al., 2013), and in some cases, addition of RS can improve sensory properties above other dietary fibers. This allows RS to be used to increase dietary fiber in foods not typically viewed to be good sources of dietary fiber, as in the case of Maziarz et al (2013) who also tested the use of RS in a meat entrée,
chicken curry, for sensory evaluation. However, RS is more commonly incorporated into baked goods to modify texture and act as a crisping agent (Sajilata et al., 2006). With the increase of RS and its use as a functional ingredient, understanding physicochemical dynamics of RS when incorporated into food products is not only good for the improvement of human health but also has monetary importance for the food industry.

A.4 Bioavailability

The concept of bioavailability is important in the fields of toxicology and nutrition. In toxicology, bioavailability focuses on the form of toxin and its effects to target tissues through various routes of exposure. Oral exposure is the predominate route in nutrition; although in intravenous is an employed route of exposure by comparison. After ingestion, nutrients that are broken down through digestive enzymes can be absorbed from the GI tract to become available to target tissues. Overall, bioavailability in human health fields deals with how much of the compound of interest or its metabolites appear in systemic circulation and/or target tissues.

Ingestion of nutrients in food does not guarantee complete absorbance of nutrients for bodily utilization, fuel, or storage. Generally, only a portion of total nutrients ingested from foods become bioavailable. Some nutrients have more availability than others, while in whole food interactions can influence the rate or extent of bioavailability. Other factors that can hinder nutritional bioavailability are the form of nutrient and enzymatic activity.

In humans, the digestion of starch starts on a small scale immediately in the mouth with salivary α –amylase. As the food bolus moves through the digestive tract, pancreatic α -amylases (1,4-α -D-glucan glucanohydrolase, EC 3.2.1.1) continues to break down polysaccharides of starch to oligosaccharides and dextrins for release into the lumen of the small
intestine. The small intestine is the last location for native enzymatic digestion of food. Here in
the brush border region of the small intestine, glucose and other sugars are hydrolyzed by
glucoamylase, α–glucosidase, maltase-glucoamylase, and sucrose-isomaltase for absorption and
release into the bloodstream. The undigested portion passes from the small to the large intestine
where it becomes available to resident microbes for enzymatic fermentation. The colon is where
RS has the most effect because this is the site of fermentation and where fermentation products,
such as short-chain fatty acids (SCFA) and gases (carbon dioxide, methane and hydrogen) are
formed.

Bioavailability of RS to lower intestinal microbiota for fermentation can be considered a
desirable outcome with health implications. However, the bioavailability of glucose from any
carbohydrate can be both desirable and undesirable. Another classification of carbohydrates
with respect to glucose bioavailability is glycemic\(^2\) vs. non-glycemic carbohydrates. Glycemic
carbohydrates are free sugars, maltodextrins, and simple starches while non-glycemic
carbohydrates are non-starch polysaccharides (NSP), resistant short-chain carbohydrates, sugar
alcohols, and resistant starches (Englyst and Englyst, 2005).

Glycemic carbohydrates are bioavailable in the small intestine where glucose can be
absorbed into the bloodstream. Resistant starch, on the other hand as a non-glycemic
carbohydrate provides limited glucose bioavailability.

This classification of carbohydrates as glycemic and non-glycemic is infrequently used,
although quantifying how much sugar becomes available after carbohydrate digestion is the key
question in many carbohydrate human feeding studies (Hoebler et al., 1999; Holm and Björck,
1992) and animal studies (Granfeldt et al., 1993; Hildebrandt and Marlett, 1991). Availability of

\(^2\) The term glycemic refers to glucose present in the blood; the usage of glycemic carbohydrates
refers carbohydrates that rapidly release glucose into the blood in the same manner as RDS.
RS for fermentation is also a key research question, to such a degree that significant research effort has gone into producing starches modified to reduce glycemic bioavailability and enhance fermentation bioavailability.

A.5 Interactions of RS with dietary components

Previously brief mention has been made of factors that affect bioavailability or digestion of starch, one of which is dietary interactions. Food components can interact with starch to alter in vivo bioavailability or alter the formation of RS in preparation (Escarpa et al., 1997). Sajilata and associates (2006) reviewed in vitro interactions of starch and other dietary compounds. To summarize: protein, calcium and potassium ions, soluble sugars (glucose, sucrose, lactose, maltose, ribose), and defatting lowered RS formation; other dietary fibers such as cellulose and lignin had minimal effect on RS formation; polyphenols, phytic acid, lectins and tannic acid acted as enzyme inhibitors decreasing RS digestion; and amylose-lipid complexes formed during cooking and/or processing reduced RS formation or contradictory were forms of RS (Sajilata et al., 2006).

In addition, Sajilata’s review also cited an in vitro starch digestibility study that observed enzyme inhibition of trypsin and pancreatic α-amylase activity on insoluble indigestible residues from black beans, green beans, carrots, and rice bran. Though the use of the term ‘indigestible residue’ was poorly defined, the inhibitory effect on amylase of black bean residues was attributed to tannin content (Moron et al., 1989). Another amylose inhibitor is the bacterial-derived oligosaccharide, acarbose, which restricts starch digestion in the small intestine (Wolin et al., 1999).
The lipid starch relationship is a notable interaction because starch-lipid complexes have been used to make novel types of RS (Jane et al., 2009). Interaction of fat and starch in vivo lends inconsistent results. As implied by Sajilata and company (2006), amylose-lipid complexes can lower or increase RS. However, overall fat content whether complexed with starch granules or present in the food matrix can lower postprandial glucose response. Hasjim et al (2010) studied a complexed lipid with a high amylose starch in a human (20 males) feeding study and found a decrease in postprandial glucose and insulin response. A starch interaction study was conducted where 9 women were fed 75 g glucose equivalent of in the form of pasta with 0 g (low fat; LF), 15 g (medium fat; MF), or 40g (high fat; HF) of sunflower oil to measure glucose and insulin response among other variable over a 7-hour period (Normand et al., 2001). Postprandial glucose AUC was initially decreased in HF and MF diet within the first 3 hours although no significant difference was observed in total glucose AUC between meals. Insulin and insulin:glucose AUC was found to be highest in HF diet. These results lead to the conclusion that there is an important need for further investigation on the mechanism in which fat modifies carbohydrate metabolism.

**B. Beneficial health effects**

As a dietary fiber, RS has been implicated in the improvement of digestive health as well as aiding in other positive biological effects. Research has associated RS with beneficial health effects such as increased mineral uptake (such as calcium and magnesium in rats and apparent retention of iron and zinc in humans), increased fermentation and other gastrointestinal events such as fecal bulking, modulated gut microbiota towards beneficial bacterial species, increased
lipid oxidation and satiety for possibly improved weight loss and/or weight maintenance, lowered thermogenesis, and decreased blood glucose and insulin response (see Table 2).

Table 2. Potential health effects of RS

<table>
<thead>
<tr>
<th>Gut health</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prebiotic and synbiotic</td>
<td>Topping et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Brown et al. 1998</td>
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<tr>
<td>Fermentation</td>
<td></td>
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<tr>
<td>Total SCFA production</td>
<td>Cummings et al. 1996</td>
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<tr>
<td>Butyrate production</td>
<td>van Munster et al. 1994</td>
</tr>
<tr>
<td>Breath $\text{H}_2$</td>
<td>Muir et al. 1994</td>
</tr>
<tr>
<td>Fecal bulking</td>
<td>Li et al. 2010</td>
</tr>
<tr>
<td>Modulate gut microbes</td>
<td>Jenkins et al. 1998</td>
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<tr>
<td></td>
<td>Abell et al. 2008</td>
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<tr>
<td></td>
<td>Martínez et al. 2010</td>
</tr>
<tr>
<td>Improves</td>
<td></td>
</tr>
<tr>
<td>Glucose and insulin response</td>
<td>Achour et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Hasjim et al. 2010</td>
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<tr>
<td>Lipid oxidization/fat accumulation</td>
<td>Higgins et al. 2004</td>
</tr>
<tr>
<td>Satiety and reduced energy intake</td>
<td>Willis et al. 2009</td>
</tr>
<tr>
<td>Thermogenesis</td>
<td>Heijnen et al. 1995</td>
</tr>
<tr>
<td>Mineral absorption</td>
<td>Behall et al. 2002</td>
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<tr>
<td></td>
<td>Younes et al. 2001</td>
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<tr>
<td>Protective</td>
<td></td>
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<tr>
<td>Colon/rectal cancer</td>
<td>Cassidy et al. 1994</td>
</tr>
<tr>
<td></td>
<td>Topping et al. 2003</td>
</tr>
<tr>
<td>Cardiovascular diseases (inhibitor) or Improves cardiovascular health</td>
<td>Martínez-Flores et al. 2004</td>
</tr>
<tr>
<td>Inflammatory bowel diseases</td>
<td>Bassaganya-Riera et al. 2011</td>
</tr>
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</table>
B.1 Resistant starch as a dietary fiber

On average, Americans are consuming less dietary fiber than the recommended 25-38 g per day. In order to meet the dietary fiber recommendations, the 2010 Dietary Guidelines for Americans suggested increasing consumption of foods with naturally occurring fiber that can be found in legumes, vegetables, fruits, whole grains (U.S. Department of Agriculture and U.S. Department of Health and Services Human, 2010). The benefits of increased dietary fiber consumption have been substantiated by various scientific publications as well as approved health claims by the U.S Food Drug Administration (FDA). The FDA health claim outlines the benefits for the consumption of dietary fiber in fruit, vegetables, and grains for the reduction of some types of cancer (§ 101.76). Scientific publications provide evidence that increased dietary fiber consumption assists in hypertension and cancer prevention, constipation, diverticulitis, and reducing risk of cardiovascular disease, diabetes, and other metabolic syndromes (American Dietetic Association, 2008; Carlson et al., 2011; Keenan et al., 2002; Wolk et al., 1999).

Sources of dietary fibers are numerous, but refining of grain flours has depleted dietary fiber content of some food products. In efforts to increase nutritious qualities, foods are being enriched with dietary fibers such as fructans, resistant maltodextrins, polydextrose, and RS. In general, RS conveys many of the same health effects as the broader class of dietary fibers and possibly for some outcomes with more efficacy than dietary fiber. The health benefits of RS have been outlined in table 2. However, all known benefits of RS as a dietary fiber are still being investigated as researchers continue to analyze this fermentable fiber in various forms and sources. Studies of the range of health effects of RS have not always generated consistent results
(Nugent 2005), but such studies have generated enough evidence of benefit to warrant further investigation.

Resistant starch has been compared with other dietary fibers, such as wheat bran, for well-established health endpoints. Jenkins et al. (1998) compared four treatments for their effects on colonic function (fecal bulking, fecal SCFA production), glycemic control, and serum lipid metabolism. Twenty-four healthy human participants were randomly assigned a low-fiber control, high-fiber wheat bran, RS2 or RS3 supplemented foods for two weeks, with a two-week wash out period between each treatment. Parameters such as serum lipids, urea, and creatinine, breath hydrogen and methane, and glycemic index had no significant differences between treatments. RS supplement products produced a greater satiety score than the low-fiber control, while the high-fiber supplement satiety score was not statistically different from any treatment. Overall, the wheat bran supplement significantly increased fecal bulking more than any other treatment; however, RS treatments also increased fecal mass more than the low-fiber control. Fecal butyrate was found to be the highest after RS3 treatment among all the treatments. The study concluded RS could be used to gain potential benefits of improve colonic health by increasing fecal bulking and SCFA production. This study also passively addresses the concept that all dietary fibers are not physiologically equal and some dietary fibers may have advantages over others.

B.2 Resistant starch as a prebiotic

Good gastrointestinal health seems to depend, at least in part, on a healthy composition of gut microbiota. Functional dietary components, such as probiotics and prebiotics, can be added to foods to improve gastrointestinal health. Probiotics are live microbes incorporated into food
products or administered in pill or other supplemented form that when ingested in adequate amounts can provide a benefit to the host (e.g., *Lactobacillus acidophilus*, a common probiotic found in yogurt). Prebiotics have been defined as ‘nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species resident in the colon in attempt to improve host health’ (Gibson and Roberfroid, 1995). Simply, prebiotics are substrates that promote the growth of beneficial microbes, whereas probiotics are the viable beneficial microbes and synbiotics are a combination of prebiotics and probiotics resulting in a synergistic interaction between the two.

Examples of prebiotics are hemicellulose, pectins, gums, inulin, lactulose, fructooligosaccharides (FOS), and other non-digestible oligosaccharides. Resistant starches can be classified as prebiotics, but the question still remains can all types of RS prebiotics. A strong case has been made for the use of RS as a prebiotic that considers three fundamental facts:

1) The GI tract is colonized by $10^{13-14}$ organism (Hao and Lee, 2004). The large intestine particularly has the largest population of microbes in the GI tract with about $10^{11-12}$ CFU/ml (Pandeya et al., 2012).

2) The definition of RS is the sum of starch and starch degradation products not absorbed in the healthy small intestine and therefore passed to the large intestine.

3) As previously defined, prebiotics are substrates that promote microbial growth and activity but are non-digestible implying that prebiotic target organisms within the large bowel alone.

Considering these facts, inferences from experimental research in human and animal models have been made for the use of RS as a prebiotic and moreover as components of synbiotics (Topping et al., 2003). The degree of microbial utilization of RS as a prebiotic might vary by
type of RS and type of autochthonous\textsuperscript{3} organisms of host. Efficacy of different types of prebiotics such as lactulose, lactitol, inulin, soybean oligosaccharides, FOS, galacto-oligosaccharides (GOS), and isomalto-, gluco-, and xylo-oligosaccharides have been evaluated (Cani et al., 2009; Gibson and Fuller, 2000); however, RS is not as common a prebiotic as other prebiotics like FOS and GOS. However, RS has been used as an effective prebiotic and is being established in scientific literature as a prebiotic.

Prebiotics are used to increase the growth of beneficial bacterial, such as \textit{Bifidobacterium} and \textit{Lactobacillus}, but more over to enhance the growth and activity of any beneficially bacterial in the gastrointestinal tract. The efficacy of RS has been reviewed and shown to promote the growth of \textit{Bifidobacterium} and \textit{Lactobacillus}; elevate colonic butyrate concentrations; act as a synergist with other oligosaccharides; reduce intestinal pathogens (Conway, 2001). In a long-term in vivo randomized study, 96 inbred rats were fed a low-fiber control diet (CD), starch free wheat bran (WB), resistant starch (RS3), or FOS diet to evaluate fermentation and induction of induction of aberrant crypt foci (ACF). After a 44-day diet stabilization period 36 rats were injected with azoxymethane (AOM) and ACF was evaluated 30 days later (Perrin et al., 2001). Perrin and associates (2001) found that RS3 and FOS diets induced the production of large amounts of butyrate and rats on those diets had a significant reduction in ACF formation (p=0.022 and p=0.043 respectively) when compared with CD. This study compared the prebiotic effects of RS and FOS and found them comparable in producing potential anti-carcinogenic metabolites (i.e., butyrate).

\textsuperscript{3} Used in this context for microbiota considered indigenous, resident, or permanent members that colonizes [parts of] the gastrointestinal (GI) tract in contrast to allochthonous organisms which are transient members “passing through” and only colonize GI tract under abnormal or contrived conditions as described by Savage (1977) and Wu and Gordon (2003)
In a randomized, double-blind, placebo-controlled, crossover human trial, RS was found to have stronger effects on induction of microbial shifts as a synbiotic than as a prebiotic or when compared with probiotic *Bifidobacterium lactis* alone (Worthley et al., 2009). The study was conducted as a 4-week intervention of 25 g high amylose maize starch (~12.5 g RS), 5 g *B. lactis* (LAFTI B94 at $10^9$ colony-forming units/g), or both for a total of 16 weeks (n=17). When participants were on prebiotic intervention, they were given a placebo probiotic and given a placebo prebiotic when on the probiotic intervention. Overall, any beneficial outcomes in microbiota of the gastrointestinal tract due to RS, builds stronger evidence of RS as an effective prebiotic.

**B.3 Postprandial glucose and insulin**

Discussions and recommended strategies on controlling glucose and insulin responses through diet are commonly associated with metabolic syndromes such as diabetes mellitus. Extensive study has been conducted implicating RS as a dietary agent that can be used to control postprandial glucose and insulin. Furthermore, because RS has shown to reduce glycemic response by impaired glucose bioavailability, it can also lower the insulin surge. Postprandial glucose and insulin response after a carbohydrate dietary intervention are conventionally studied within a 2 to 8 hour period. Likewise with experimental feeding of RS, however, RS feeding studies can have prolonged study periods to assess if RS has a second meal effect\(^4\) or other overlapping interactions.

\(^4\) A physiological phenomenon in which a carry over effect, normal of decreased glucose is observed at the second meal in conjunction to the prior meal. However, the term can also be used when the effect of decreased insulin is observed,
The topic of RS on postprandial glucose and insulin is rather controversial. In response to RS some studies have observed significant decrease in glucose and insulin, other have observed no change due to treatment, while a few studies have observed an increase glucose or insulin response. Although it is generally thought that RS lowers postprandial response, careful attention should be given to experimental conditions. Due to the breadth of this research area and our specific research interest in humans, further discussion on RS and postprandial glucose and insulin have been narrowed to glucose and insulin response in humans (see section D.2).

B.4 Satiety and Obesity

In United States of America and other developed countries, the rate of obesity has risen to historic and epidemic proportions. People who are overweight and obese increase their risk for metabolic diseases and other adverse health effects (Ma et al., 2013). Maintaining healthy weights or reducing body fat composition within a body mass index (BMI) of 18-25 is a health goal for some. Through diet and exercise, BMI can be controlled. With strict regard to diet and obesity, appetite control is essential. Feeling satiated plays an enormous role in appetite control. If RS can affect satiety, it can exert a beneficial role in weight regulation. In turn, weight regulation influenced by diet may play a role in lower gut microbial population and gastrointestinal functions.

While RS does share a lot of the same properties of dietary fiber, the mechanism of weight management through the use of RS is unclear. Some of the proposed ways dietary fiber can aid in weight management is by slowing gastric emptying promoting longer feelings of fullness, while lowering food energy density (Slavin, 2005). The mechanisms for effects of RS on satiety and weight management have not been well established.
One can assume all dietary fiber is not equal, but what effect might different fibers have on satiety? This question was addressed in a human study (7 men, 13 women) in which investigators evaluated effects of different fibers on satiety after a single meal, quantified by AUC taken from 100 mm visual analog scale (VAS) measuring hunger, satisfaction, fullness, and prospective food intake at 15, 30, 45, 60, 120, and 180 minutes after muffin meal (Willis et al., 2009). Four types of high-fiber muffins (corn bran, barley β-glucan + oat fiber, RS, and polydextrose) were tested against a low-fiber muffin. In this study, RS and corn bran muffins reduced AUC for desire of food intake (p = 0.009 and p = 0.025 respectively) and were overall more satiating than the other types of muffins tested.

A human study providing 48 g of type II RS to 20 healthy adult males investigated ad libitum energy intake after test meals (Bodinham et al., 2010). The 48 g of RS was divided into 2 portions incorporated in mousse products and eaten as part of a meal, one at breakfast and one at lunch. The same meals were given for the placebo treatment except the mousse products were supplemented with 32 g RDS. At dinner, participants were given an ad libitum meal and energy intake was calculated. Other parameters were measured but with respect to energy intake, Bodinham et al. (2010) observed after the RS treatment energy intake of 5241 ± 313 kJ was lower when compared to placebo at 5606 ± 345 kJ (p = 0.033). These results infer that increased RS consumption might suppress the urge to overeat facilitating weight manage or reduce weight gain.

There is not sufficient evidence that RS promotes weight loss, especially in humans. However, there are implications that RS could decrease long-term fat accumulation by significantly increasing lipid oxidation (Higgins et al., 2004). In this study, Higgins and company spiked bread with 50 µCi [1-14C]-triolein (glycerol tri[1-14C] oleate) suspended in
olive oil and added it to each test meal containing a total of either 0%, 5.4%, or 10.7% RS. Through indirect calorimetry, they measured fat oxidation by conversion of $[^{14}\text{C}]-\text{triolein}$ to $^{14}\text{CO}_2$. Postprandial oxidation of radiolabeled triolein was 23% greater with RS meal at 5.4% resistance than 0% resistance ($p = 0.0062$). Unexpectedly, fat oxidation at the 5.4% RS dose was also higher than for a 10.7% RS dose ($p < 0.0001$) but researchers could not explain the non-linear dose response. Higgins and others did not report fluctuations in weight of the 12 healthy participants, BMI 24.7±2.4, nor was it part of the experimental design provided that each participant was fed a RS diet for only one day. Fat storage, however, was assessed by gluteal biopsies and appeared lower for 5.4% RS meal, but was not statistically significant.

In a parallel arm design, two overweight groups were supplemented in their normal diets with 24 g of either resistant starch (RS) or control starch (CS) for 21 days (Park et al., 2004). Group 1 ($n=13$, initial BMI of 27.9 ± 0.5) received CS and group 2 ($n=12$, initial BMI of 26.6 ± 0.7) received RS. After 21 days, there was no significant change in BMI. This study directly assessed weight fluctuations among other variables due to ingestion of RS, however the experimental design to assess RS impacts on BMI might have been flawed. For a parallel design, it is possible the cohort was too small to observed significant weight reduction and could have benefited from a larger cohort.

### B.5 Gastrointestinal Health

Hippocrates is credited for the sweeping generalization ‘all disease begins in the gut’. Though this statement may not always be true, more and more correlations between overall health and gastrointestinal health are being made. While Hippocrates established a link between
the gut and disease, gut health is now linked with the health of other periphery symptoms (e.g., cardiac, immune, and neural).

At the most basic level of gut health, proper bowel function is vital for waste elimination but also for adequate nutrition absorption. Conditions like constipation and diarrhea can interfere with proper bowel function. As isolated incidences, they are only moderately harmful and can be corrected. However, other conditions like Crohn’s disease and cancers of the lower gut are harder to manage and can be lethal. Yet, the use of RS for all these conditions might be advantageous.

Mammalian cells contribute largely to digestion in the gastrointestinal (GI) tract but other key contributors are resident microbes. The microbe facilitated fermentative property of RS is key, and because of this aspect, it is hypothesized that the main target organ of RS is the lower gut. Regional diseases and ailments like colorectal cancer, inflammatory bowel diseases (IBD), diverticulitis, and constipation are conditions for which RS putatively will protect against or improve GI health. The fermentative properties of RS in the lower gut can act as a weak laxative, which is suggested to aid in constipation and diverticular disease (Topping et al., 2003).

An inverse association was found between starch, non-starch polysaccharides (NSP), and RS and incidence of colorectal cancer in a correlative study of 12 countries (Cassidy et al., 1994). Significant inverse association between ingestion of RS and incidence of colon, rectal, and large bowel cancer were not obtained even after adjusting for fat and protein interactions. However, associations were found for a combination of NSP and RS.

Better transit time, fecal bulking, and frequency of bowel movements are attributes of dietary fiber. Bowel health can be improved as well as absorption of nutrients, such as microminerals zinc and iron. Though bioavailability of glucose in RS is lower than rapidly
digestible and slowly digestible starches, bioavailability of micronutrients such as mineral absorption seems to be improved with RS. Increased absorption of mineral after ingestion of RS has been observed more consistently in animal models than in humans. However, there is evidence that RS will increase zinc mineral uptake in humans (Behall et al., 2002).

Behall and associates (2002) in a long-term crossover human study fed 10 healthy and 14 hyperinsulinemic men 5 food products formulated with standard (70% amylopectin, 30% amylose; AP) or high amylose (70% amylose, 30% amylopectin; AM) cornstarch. Food products bread, muffins, cookies, corn flakes and cheese puffs RS content ranged from 3.3-12.7 g/day for AM and 0.4-1.0 g/day for AP food products were test products as part of controlled meals. The duration of each diet intervention was 14 week, at 2 weeks participants collected all urine and feces and mineral content was measured by flame atomic absorption spectrophotometry. In this study, though Behall and associates did observe an apparent mineral uptake in zinc and iron on the AM diet. In addition they observed after AM diets fecal starch excretion and transit time was greater though fecal weights did not differ by diets. Little differences form study variables were observed between the two types of study participants, but the healthy controls had greater transit time compared to hyperinsulinemic. This is one of the few studies, by comparison, that reports an increase in transit time on a RS diet.

Overall RS supplementation can assist in: normalizing bowel function and improving a host of ailments such as diarrhea (Raghupathy et al., 2006), antibiotic-associated diarrhea, traveler's diarrhea, gastroenteritis and colitis, reducing irritable bowel problems /IBD, prevention/protection from colon or colorectal cancer, boosting immune function, infant health
and nutrition, prebiotic promoted growth of beneficial microorganisms such as *Bifidobacterium* and culture protagonist\(^5\), and modulating gut microbes.

### B.6 Lower intestinal microbiota and fermentation

The gastrointestinal tract is colonized with microbiota from birth. Microbial communities and populations become more dense and diverse as the GI tract progresses from the stomach to the rectum. Maintaining human health for many centuries has been related to GI health, which could be accomplished through a simplistic perspective of good nutrition and avoiding toxins or harmful organisms. Now, it is further understood that maintaining health should also be considered from the perspective of symbiotic relationships (Nicholson et al., 2005; Pandeya et al., 2012; Rooks and Garrett, 2011; Xu and Gordon, 2003). Symbiotic relationships between a host and another organism can be parasitic, commensalistic, or mutualistic. Microbes, especially bacteria, as a whole have rather a poor public reputation as valuable health agents, however, emerging public perception is accepting that not only are the vast majority of bacteria harmless in relative amounts but rather ingestion of live bacteria (probiotics) might be propitious to health.

Lower intestinal microbiota are fed by the indigestible part of the diet that host cells were unable to break down. The predominate anaerobic bacterial genera of the lower gut are *Bifidobacterium, Ruminococcus, Clostridium, Bacteroides*, and *Eubacterium* (Moore and Holdeman, 1974). *Bifidobacterium* contribute to substrate production of lactate and succinate, which can then be converted to butyrate and propionate, two of the three principal gut microbial SCFA fermentation products. The production of butyrate, a chief metabolically active

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\(^5\) Used to enhance the survival of probiotic cultures
fermentation product, comes mainly from bacterial classes Clostridia and Fusobacteria. The microbial utilization of food that host cannot metabolize would appear to be a commensal relationship. However, the perception of a commensal relationship is slowly being revamped because microbes and microbial activity can be to the benefit or detriment of host. Xu and Gibson (2003) captured the gravity of the host-microbiota relationship with a review article entitled ‘Honor thy symbionts’ in which they emphasized contributions towards understanding the human microbiota and beneficial symbiotic host–bacterial relationships through genome-based techniques. The complexity of the gut microbiome and an inclusive range of its function would profit from even greater insight, specifically concerning biological mechanism (such as how intestinal microbiota affect intestinal and immune homeostasis; elucidating microbiota-related host tolerance; understanding intestinal uptake of polysaccharide; and others discussed in Xu et al, 2013) and microbial species associations in gastrointestinal health and disease. Although a great deal of work in this area has already begun a full grasp of gut microbiota is still in its infancy.

B.6.1 Overview of Gut Microbiota and implications for health and disease

The subject of gut microbiota is an extensive area that surpasses digestive health and affects many different biological systems. Because of the number of microbes in comparison to host eukaryotic cells and their specialized function, gut microbiota may loosely be termed an organ. As an exogenous organ, they work to influence host systems (e.g., digestive, cardiovascular, immune, integumentary, and nervous) through fermentation, energy salvage, production of micronutrients, epithelial regulation, and microbial competition. Fermentation is an important specialized function of gut microbiota, but understanding microbial communities
has also become a main interest. Gut microbiota fingerprints or distinct changes in gut microbiota have been implicated in:

- Increased energy harvesting and obesity (Ley et al., 2005; Turnbaugh et al., 2006) due to shift in Bacteroidetes and Firmicutes ratios
- Diabetes type II (Cani et al., 2008) reduced *Lactobacillus spp.*, *Bifidobacterium spp.*, and *Bacteroides-Prevotella spp.*
- GI inflammation (Lupp et al., 2007) reduces total colonic bacteria, increase in *Enterococcus faecalis* and *Enterobacteriaceae*
- Inflammatory bowel diseases (Frank et al., 2007) depletion of bacterial groups in Firmicutes, Bacteroidetes, *Lachnospiraceae*, and *Bacteroidales*; enrichment of *Actinobacteria, Bacilli, Alphaproteobacteria, Betaproteobacteria Gammaproteobacteria*, and smaller group of Firmicutes
- Colitis, colon and colorectal cancer (Uronis et al., 2009) potentially attenuated in presence of *Bacteroides vulgatus*

The exact role of microbiota in the progression of diseases is still being clarified. What is known is that gut microbiota have dichotomous effects with the potential, for instance, to exert both pro- and/or anti-inflammatory responses and be protective against and/or associated with gastric cancers or other GI cancers. In the case of carcinogenesis, the link between gut microbiota and GI cancers has been associated but no strong mechanisms of causation, especially for cancers of the lower gut, has been proposed. Mechanistic hypotheses of how certain gut microbiota initiate cancer are diverse with one general postulation that the host immune system has not fully
recognized the symbionts and induce host-signaling pathways that effect cancer growth and development.

### B.6.2 Effects of Microbial fermentation products

The indigestible portion of starch is fermented in the large intestine, which results in the production of products like carbon dioxide, methane, hydrogen, organic acids (e.g., lactic acid) and short chain fatty acids (SCFA). Fermentation by-products from protein metabolism are also a likely event; however, it occurs at a lesser rate in comparison to carbohydrate fermentation (Macfarlane and Macfarlane, 2011). Furthermore, favored fermentation end products for protein metabolism are not the same as for carbohydrate fermentation. A more detailed description of carbohydrate fermentation products in response to RS in human studies will be discussed in a subsequent section.

The principal products of carbohydrate fermentation are SCFA. Breath gases are products of overall carbohydrate fermentation but are not strongly correlated with any beneficial health effects. Breath gases have been used as diagnostic tools for carbohydrate malabsorption and cancer but are more widely used as indicators of fermentation. In the case of hydrogen, it is an indicator for fermentation while playing a role in microbial redox reactions. Naturally SCFA are also used as indicators of microbial fermentation because the majority of physiological production of SCFA is from gut microbes.

Attention to adverse effects due to carbohydrate fermentation is more obscure and less reported in comparison to advantageous health effects of fermentation and its implications. Furthermore, toxic effects are more commonly referenced in concurrence with protein fermentation rather than carbohydrate fermentation. MacFarlane and MacFarlane (2012)
reviewed physiological effects indicative of improved health from acetate, propionate, and butyrate in various studies (see Table 3). These are the main SCFA produced and recognized for their role in maintaining health.

Table 3. Physiological effects of principal SCFA in colonic fermentation

<table>
<thead>
<tr>
<th></th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune system</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Carcinogenesis</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Colonic function</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Adipogenesis</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol metabolism and lipogenesis</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Satiety</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Neurological effects</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Oxidative stress</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Barrier (epithelial) function</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Visceral perception</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Insulin sensitivity</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

Summarized adaption from MacFarlane and MacFarlane, 2012 review of in vivo animal and human and in vitro studies, improved lipogenesis from Hosseini et al., 2011.

The MacFarlane and MacFarlane (2012) review of physiological effects of SCFA acetate, propionate, and butyrate resulted in categories that highlight a range of detailed effects (categories seen in table above). Out of eleven categories immune responses, carcinogenesis, and colonic function were the three physiological categories that were affected by each of the principal SCFA. Though specific physiological responses in the three categories were not the same, similarities did arise. In the immune system lipopolysaccharide (LPS) stimulated tumor necrosis factor (TNFα) release was decreased by acetate but inhibited by propionate and butyrate
suppressed production of TNFα. For carcinogenesis propionate and butyrate induce apoptosis in colonic cancer cells. MacFarlane and MacFarlane (2012) did not review any article in which acetate was shown to influence apoptosis, although they did highlight a study in which acetate administered intravenous to cancer patients increased polyclonal antibody secretion in the blood and natural killer cell activity. Lastly for colonic function, like the other categories SCFA effects were not the same. In a dose-dependent manner, propionate increased but acetate decreased rat smooth muscle contractions in vitro, whereas in large bowel of rats, butyrate concentration decreased smooth muscle contractions. Overall, the MacFarlane and MacFarlane (2012) review was fairly extensive and invaluable to understanding the impact of principal SCFA to health.

B.6.3 Gut microbial interactions with RS as substrate

The human microbiome in the lower gut contains approximately 500 or more microbial species, mostly anaerobes, in the magnitude of $10^{11}$ - $10^{13}$. The human intestinal tract is colonized in large part by facultative anaerobic microbiota, such as members of the family Enterobacteriaceae (Finegold et al., 1978). The genus Bacteroides belonging to the Bacteroidaceae family are also predominant microorganisms in normal feces. Gut microbiota obtain carbon sources from the host diet and in the case of the colon, fermentable fibers are their food. Which raises the research question: how and what microbiota can be modulated by diet or more specifically can diet preferentially influence microbiota for targeted health effects?

There is evidence that microbiota can be modulated by dietary RS. Intricacies of the human gut microbiota were observed in human flora-associated (HFA) rats colonized with fecal microbiota from either UK or Italian donors (Silvi et al., 1999). In both UK and Italian HFA rats, 4 out of 7 main bacterial groups were affect by RS for 4 weeks in which bacterial counts for
Lactobacilli and Bifidobacteria increased while Enterobacteria counts decreased. As for Staphlococci, the 4 bacterial groups that were affected in both UK and Italian HFA rats, UK HFA had an increase in bacterial counts while Italian HFA rats had a decrease. Other bacterial difference between HFA rat groups were observed, Italian HFA rats also had no change in total aerobes whereas UK rats did. Also shifts in 6 out of the 7 bacterial groups were observed in UK HFA rats while only 4 out of 7 significant shifts in bacterial groups was observed in Italian HFA rats.

In humans, colonic microbial changes in response to RS diets have also been observed. Two studies with human fecal specimens contributed after RS feeding found bacteria related to Ruminococcus bromii increased on RS diets when compared to NSP and other diets (Abell et al., 2008; Walker et al., 2011). In a double-blind crossover design, 10 (5 female, 5 male) healthy participants consumed crackers containing either RS$_2$, RS$_4$, or control starch for 3 weeks with a 2 week washout (Martínez et al., 2010) human fecal bacterial shifts were comparable to that found by Silvi and associates (1999) in HFA rats. Silvi and associates (1999) noticed increases in Bifidobacterium of the Phylum Actinobacteria and Bacteroides from the phylum Bacteroidetes. With human fecal microbiota 16S rRNA multiplex sequencing, Martínez and associates (2010) showed both types of RS induced significant microbial shifts even on a species-level. At the phylum-level RS$_2$ and RS$_4$ show different effects on bacterial community structure, where Bacteroidetes and Actinobacteria had the highest proportions after consumption of RS$_4$. From their study Martínez and associates inferred that specific microbial populations could be targeted with RS functional foods.
C. In vitro Fermentation for metabolic analysis of RS

Assessing fermentation using in vitro systems is valuable for understanding metabolites and microbial communities in a well-controlled environment. Research conducted using in vitro methods lead to the discovery of RS and its classification via digestion rates. Afterward, in vitro fermentation studies of RS led to the elucidation of fermentation mechanisms and pathways, although substantial investigation is still ongoing. In vitro fermentation models are also useful for understanding chemical events occurring during starch degradation and SCFA production by tracing carbon conversion.

C.1. Defining In vitro Fermentation Systems

Methods for fermentation in vitro studies fall under one of two motifs: batch or continuous fermentation. Following the ideology that a system is either 1) contained with a set mixture of microbes, nutrients, and substrates for a short period of time or 2) a continuous system with replenishment of nutrients, substrate, or other environmental inputs (e.g., sustained pH).

Batch fermentation typically spans from 24 to 96 hours, while continuous fermentations have been recorded to go as long as 8 weeks. Both fermentation systems are used to quantify microbial shifts, microbial growth, pH changes, fermentation patterns, and RS degradation. Fermentations can be done with pure cultures, a mix of strains, or with crude environmental samples such as feces. Fecal matter is used to make inocula with media and other nutrients. In some cases, inocula from ileal digest is used although it requires a more effort for researchers to acquire than fecal specimens. When addressing research questions pertaining to bacterial species and bacterial pathways, pure cultures or co-cultures can be used. When addressing research
questions about bacterial communities and fermentation patterns, ileal or fecal inoculum are used. For outcomes of in vitro RS fermentation research, see section C.4 (In vitro RS fermentation studies).

Similarly, batch and continuous in vitro systems can also be used to simulate digestion as well. When the digestibility of RS$_2$ and RS$_3$ was compared using batch and dynamic in vitro system (TIM-1), Fässler et al. (2006) found similar amounts of RS$_3$ escaping digestion in both models but RS$_2$ produced more indigestible starch fraction for batch digestions than dynamic TIM-1 digestion ($P < 0.05$). Interestingly, RS$_2$ digestion in TIM-1, which is a sophisticated compartmentalized system created to simulate conditions in human stomach, duodenum, jejunum, and ileum was not significantly different from RS$_2$ digestion using a Megazyme kit, which is a much simpler in vitro digestion method (Fässler et al., 2006b).

C.2 Media Selection

In order to do fecal incubations, it would seem to be beneficial to mimic the gut environment as closely as possible; however, simulating the gut environment is very complex. A major challenge for a survey of the complete microbiome is how to stimulate the growth of microbes without creating a selective milieu.

Media can be solid or liquid (broth). Either form can be synthetic in which the exact chemical composition is known, or non-synthetic in which the amounts but not the exact composition of major constituents is known. Selection of appropriate media for research purposes requires much consideration, expertise, and above all should not be trivialized. In the book Bailey and Scott’s Diagnostic Microbiology, Finegold et al. (1978) suggest several general guidelines for selecting a good culture medium: consistent quality control, adequate
concentrations of essential nutrients and salt, adequate amount of water, appropriate pH for required metabolic function, lack of inhibitory substances of interest, and sterile environment. Other factors worth considering for maintaining a successful culture are temperature and oxygen requirements.

Carbohydrates, such as glucose, are often included in media, which is known to enhance the growth of microorganisms in plate (solid) or broth cultures. Finegold et al (1978) state when selecting a standard medium, a glucose free medium tends to give more consistent and reliable results because of the elimination of enhanced growth due to small amounts of carbohydrate. Also, they describe that the fermentation of glucose can alter the pH and be harmful to acid-sensitive organisms.

After the appropriate broth is chosen the method chosen to sterilize is also critical. Media is commonly sterilized through autoclaving, however certain media due to particular constituents cannot withstand high temperature. After choosing a media, especially if it has been tailored to specific needs of an experiment, proper sterilization is necessary. Finegold et al (1978) suggest sterilization methods that include autoclaving (moist heat), flowing steam, inspissation, filtration, and chemical methods.

Often when investigating intestinal microbiota, even when comparing media, selective media is used to target specific bacterial genera (Mundy et al., 1995; Vael et al., 2011). In some media comparison studies, liquid and solid media have been evaluated in addition to different anaerobic culture methods to analysis clinical specimens (Rosenblatt et al., 1973). However, media comparisons with total fecal populations have been done. When 10 different media were compared to evaluate floral enumeration in mice feces some proved to be more selective for specific bacteria although each media was chosen for comparisons were considered to be similar.
to each other (Nelson and George, 1995). Nelson and George (1995) found all media to be comparable for enumeration of total anaerobes. However, when identified colonies in media was compared, Mannitol salt agar was more selective than Baird-Parker agar for staphylococci; Brucella blood agar + vancomycin and kanamycin (VK) agar was more selective than Wilkins-Chalgren blood agar + nalidixic acid and vancomycin (NAV) agar for obligate anaerobic Gram negative bacilli; Rogosa agar more selective than LBS agar for Gram positive rods; and KF (Kenner Fecal) Streptococcus agar was found to be most appropriate for fecal enterococci and streptococci.

Similarly an earlier study compared “non” selective media for anaerobic fecal bacteria in mice, some media proved to be more selective than others (Itoh and Mitsuoka, 1985). These studies both suggest that choosing a non-selective media is still limited to what organisms it can actually grow. This notion is well established considering culture methods have never been able to grow all bacteria present in biological specimens.

Very few articles compare media for use of total microbiota in healthy human fecal samples. Notwithstanding, Hartemink and Rombouts (1999) compared total anaerobes in human fecal samples by evaluating plate counts. They were able to rank nine different media. In the end they reported no significant difference between the non-chosen selective media. Though they also evaluated selective media for the detection of Bifidobateria and Lactobacilli. Plate counts, colony morphology, and colony size were used to determine if the none-selective media performed the same (Hartemink and Rombouts, 1999). Though the actual diversity of colonies grown via each media is unknown.
C.3. Preparation of fecal samples for microbial analysis

Fecal samples are used to evaluate microbial community and metabolites in the intestinal tract. Waiting for feces to pass and taking an effluent sample or biopsy are the methods by which fecal samples are obtained for analysis. Biopsies are far more invasive than fecal elimination though and fecal samples from biopsies are a “cleaner” sample since these generally contain very few epithelial cells. During elimination, food solidifies in the large intestines and moves through the colon picking up resident cells (i.e., sloughed off intestinal epithelial cells and microbes). Indubitably, the easiest way to sample the gut microbial population is through fecal passing. Assessing microbial metabolic activity through fecal samples though not completely accurate might be the best type of sample or sample preparation for representation of the large intestinal environment. Currently, no other specimen or method of specimen collection producing more accurate results has been defined.

Genomic methods are used to analyze microbial composition. However, analysis of microbial metabolites and microbial activity in fecal samples are commonly evaluated through fecal incubations. Fecal incubations can also be used to evaluate microbial shifts in controlled environments, generally followed by molecular methods. Regardless of what method of analysis is chosen, the way in which samples are handled and prepared is critical to appropriately interpreting results. Factors that will influence analysis and results of fecal microbial activity are collection time, form of substrate, type of incubations (batch or continuous), media selection, inocula/substrate ratio, and anaerobic conditions (Li, 2010a).

The time interval between fecal excretion and analysis or start of incubations is sometimes not reported. Some articles will report working with freshly prepared samples without citing a specific time frame (hours). This might introduce significant variability in results of in
vitro fecal incubations making comparison between studies difficult. The underlying targets when conducting fecal incubations are the microbiota; whether there is a direct measurement of microbiota through molecular fingerprinting or indirect measurements through production of SCFA’s. Roesch et al. (2009) investigated if time affects fecal microbial communities when they compared bacterial communities from human donors immediately after defecation from time points 0h, 12, 24, 48, and 72 hours. All time points except for 0h, which was immediately frozen, were permitted to sit at room temperature before frozen at -80 C. Samples were kept frozen until DNA was isolated. Through 16S rRNA gene amplification and 454 pyrosequencing a gradual change in bacterial diversity over time was observed, reporting the least change, 3.06%, at 12 hours (Roesch et al., 2009).

In the Roesch et al. (2009) study, the most diversity change was 10.14% at 72 hours. The maximal time period over which a fecal sample is considered viable is 72 hrs. In light of their results, an important research question is raised: is ~10% change too high of a background to accurately interpret results? This question is also applicable for fecal incubations where samples cannot be frozen immediately. If samples cannot be processed immediately (within 0-1 hr), no more than 12 hours should be permitted before microbial diversity shifts become different from the original samples.

Depending on the method of analysis, samples must be handled differently. For microbial activity assays such as measuring SCFA, viable samples are needed. Therefore, freezing and thawing samples or prolonged exposure of samples to aerobic conditions would produce questionable estimates. Thus, timing around sample collection if important for assessing viable microbial activity. For molecular methods, immediate anaerobic conditions are not as crucial but immediate freezing of the samples is preferred.
C.4 In vitro RS fermentation Studies

In vitro fermentation models are also useful in assessing fermentability of RS and understanding chemical events occurred during starch degradation and SCFA production by indirectly or directly tracing carbon conversion. For an overview of RS metabolism and microbial fermentation end products, see figure 2 in Section D.3.

Many of the functions of the most metabolically active SCFA, butyrate, has been evaluated through in vitro studies. An in vitro study showed that butyrate had synergistic effects by improving probiotic viability in conjunction with RS (Brown et al., 1998). Another in vitro study also showed synergistic effects in the production of butyrate and propionate when incubating RS with one or two bacterial strains in human fecal extracts (Zhao and Zhu, 2013). In addition, in vitro studies have been used to postulate mechanistically the fermentation of RS in vivo and fermentation by-products. Duncan and associates (2004) suggested in an in vitro study that a cross-feeding between Bifidobacterium adolescentis and species related to Eubacterium hallii and Anaerostipes caccae isolated from human feces may be the contributing reason to a butyrogenic effect to dietary substrates like RS (Duncan et al., 2004).

Measuring SCFA production from in vitro fermentation is a good way to screen starches or predict in vivo fermentation response. Pyrodextrinized starches from potato, lentil and cocoyam made resistant due to acid treatment were fermented anaerobically using human fecal inocula for 24 h (Laurentin and Edwards, 2004). The native starches were compared with pyrodextrinized starches from the same sources. Net total SCFA production for each RS source was significantly higher when compared with native starch. For all RS sources, acetate production decreased while propionate production increased. Laurentin and Edwards (2004) found that pyrodextrinized RS was fermentable in vitro by the colonic bacteria of healthy adults,
indicating pyrodextrinised starches may be fermentable in vivo but the mechanism of increased SCFA production by these RS forms remains to be elucidated.

During in vitro fermentation substrates can be added to assess fermentation or substrates can be administrated in vivo. Langkilde and associates (2002) provided 30 g of RS$_2$ in raw banana flour (RBF) or cooked green banana flour (CBF) mixed into yogurt to 10 ileostomy volunteers in addition to their ordinary diet for a period of four days. After each of the two periods, ileal effluents were collected from 6 of the patients and incubated for a total of 24h with samples analyzed at 0, 4, 8, and 24 hours. The study found lower pH and percent molar propionate ratios but higher total SCFA concentrations and percent molar ratios for butyrate for RBF when compared to CBF (Langkilde et al., 2002). The results suggest that fermentation after in vivo digestion of RS fermentation continues in vitro. This study was complicated by the use of yogurt, which contains probiotics, as the food vehicle for the prebiotic RS. Investigators did not address additive effects of yogurt and RS on fermentation and SCFA production.

D. Human Studies with RS

D. 1 Methodology of Human Feeding Studies

To further the knowledge of human health, data have been used from in vitro and animal studies. Where applicable, inferences and extrapolations can be made from these experimental findings to humans; however, inferring in vitro results to humans can lead to misapplied interpretations. In addition, though in vivo studies are more dynamic than in vitro studies, extrapolations of in vivo studies across species can also pose problems. Notwithstanding that
both in vivo and in vitro methods are invaluable, understanding appropriate conditions in which to use each method is necessary.

Data obtained from in vivo studies of species of interest are a more direct approach for measuring and understanding physical phenomena where ethically applicable and financially feasible. Human nutritional in vivo studies are a common tool, designed to be innocuous and easily reproducible. When humans are the species of interest, human feeding studies tend to be a more powerful tool in nutritional research than animal studies since intra-species extrapolation generates less confounding variables than interspecies extrapolations.

However, human studies come with their own set of drawbacks. When answering research questions, controlled human feeding trials can be a useful tool if sources of variation are properly controlled. Designing experiments to reduce plausible sources of variation is critical. The subsequent three sections (D1.1-D1.3) provided general information addressing some of the basic issues to consider when conducting experimental human feeding studies: careful screening of participants, careful selection of food treatments, and sound experimental setup.

**D.1.1 Experimental Setup**

Planning human studies starts with a sound experimental design and should be the first order of business after a research question is generated. The experimental set up should be well thought out and employ appropriate methods. Ways to reduce sources of variability should strategically be incorporated into experimental setup.

Possible sources of variability are: human (researchers and participants/experimental unit) and experimental error, food formulations (varying concentrations, different cooking parameters or days), time, and non-compliance. Variation will always exist and some sources of
variation cannot be controlled, however, streamlining experiments for what can be controlled (e.g., consistent time points and collection methods) helps to reduce high variation.

The design of an experiment can also be constructed to reduce unwanted variation slightly harder to control. For instance, if there are systematic characteristics that are of no interest to the researcher, one could ‘block’ for those factors to increase accuracy of characteristics or factors of interest. Blocking, randomization, and nesting are design techniques that help reduce unwanted variation. Popular design terms to be familiar with when evaluating or constructing human studies are as follows:

- **Block**
- **Case series**
- **Case study**
- **Clinical**
- **Cohort study**
- **Controlled trials**
- **Crossover**
- **Intervention**
- **Latin-square**
- **Nested**
- **Observational**
- **Parallel or parallel-arm**
- **Prospective**
- **Randomized**
- **Retrospective**
- **Stratified**

* can be an example of an intervention study

Experimental designs are normally a combination of above terms. For example, randomized studies can be crossover and parallel. Other example designs are case-controlled study, nested case control, or a controlled observational cohort study.

Use of a control, or placebo, is common in human studies. However, it is well known in human studies investigating a physiological response can sometime be affected by a psychological adjustment, which is termed placebo effect. Because of the placebo effect, human studies can be blinded or double blinded to avoid this phenomenon and reduce bias. Terms like run in and washout are part of experimental design, and setup that should also be carefully considered to reduce interactions or carry over effect.
Randomization of participants and treatments is important to remove unintentional bias. In experimental designs such as crossovers, balance of randomized treatment order among participants also limits treatment interactions/carryover effect. When a balanced randomization is not conducted in crossover designs, it is impossible to partition effects of time from effects of test products. For example, a crossover study conducted in Japan stratified participants into two groups; on the first experimental day group one (n=10) was fed test product of bread containing 6g RS per two slices and group two (n=10) was fed placebo (Yamada et al., 2005). After a two-week washout, treatments were switched, group one was fed placebo and group two was fed test product. Though initially this experimental setup might seemed balanced and assurance was given by researchers that the two test days were same, there is no certainty that after analysis with paired t-test observed differences in experimental variables of blood and insulin profiles and characterization of participants vitals (blood pressure, pulse rate, body weight, BMI) was solely from treatment differences. This study could have benefited from randomization of treatments and participants, which was not described in study outline.

D.1.2 Selection and Design of RS Food Treatments

Controlled human feeding studies are designed around punctiliously crafted food products. Careful thought should be given to dose, dietary interactions, physicochemical properties, and food preparation to insure a reliable vehicle for dietary components of interest. Using high precision research foods allows for 1) the biological response of interest to be more accurately estimated, 2) a shorter experimental period (as short as 1 day) to establish biological response, 3) the use of a smaller cohort (5-25 participants), and 4) a maximal physiological response due to diet (Most et al., 2003). Factors such as minimizing experimental period can
remove confounding variables that can occur over long periods of time (e.g., weight change). Feeding studies can also be designed in which weight change is specifically investigated as a desirable variable to be altered due to treatment, although typically weight is maintained throughout short-term human feeding study.

To address specific research question(s), the appropriate selection and preparation of RS must be employed especially when the aim is to increase the RS content of foods. Thus food product formulation and preparation is critical. Not only are food products the vehicle of delivery for test material but also formulation and preparation can affect test material. It is known that different types of RS can elicit different response to the same variable, such as glucose response (Haub et al., 2010), which could be due to physicochemical nature of specific starch types. Furthermore, the source of RS (e.g. beans, bananas, corn, or potatoes) can also have different physicochemical properties that can influence physiological responses such as digestion and glucose and insulin responses. Thus, the first step in product formulation should be selection of what type of RS is to be used and in what products can it be incorporated. Some types of RS are water-soluble that can easily be incorporated into beverages, while others are not readily dissolved in water and different test products must be used.

Once the type of starch and ideal food product has been chosen, formulation and preparation can begin with careful consideration avoiding potential confounding variables. Ideally, studies with multiple treatments should have a food formulation with minimal to no variation due to food components other than the test material. Not only should food formulations be congruent with other test treatments in experiment, but preferably simple and palatable. When researchers strictly consider food formulations without regard to palatability, participant compliance and moreover retention can drastically decrease.
RS stability can be altered by food preparation with different food matrices and cooking conditions. High temperature heating and cooling of high moisture starch (such as potatoes) can form RS3 due to retrogradation after gelatinization and subsequently lower glycemic response (Najjar et al., 2004); however, in other starches gelatinization can occur during boiling or cooking inducing a more digestible starch. Boiling and pressure cooking have been reported to increase RS while roasting, extrusion cooking, frying, and drum drying decreased RS when food processing methods were compared (Parchure and Kulkarni, 1997).

After test product is completed even storage and post-handling (heating and freezing) of final food product can alter RS content. Long-term storage can not only affect sensory outcomes, but also make foods stale. Whether a food product is stale or not stale can alter RS content, where stale starches are higher in RS content than fresh starches (Ahmed et al., 2000).

D.1.3 Screening of Participants

Though inter-human variability is a natural source of variation, it can potentially be too high occluding evidence of significant results. Ways to address and control for this inevitable variation is through proper screening criteria and obtaining the desired level of statistical power.

Parameters that naturally vary among individuals like genetics and environmental conditions may also increase experimental variability. Though most researchers try to streamline parameters by tailoring recruitment requirements (weight, diet, energy expenditure, disease, etc.), influential variations that are harder to streamline, such as metabolic differences, can still exist. For instance, McOrist et al (2011) found different entry fecal butyrate levels varied among 46 healthy adults. Change in fecal butyrate concentration after RS test meals subsequently varied, though in general they observed that RS consumption increased butyrate. Some inter-individual
butyrate variation was attributed to BMI (low butyrate entry levels corresponded with lower BMI) and nutrient intake of protein, starch, carbohydrate, fiber, and fat in normal diets.

Variation is certain even in the simplest experiment. The goal in dynamic experiments such as human studies is to reduce as many sources of variation to glean a better understanding of what is being tested. Differences in experimental conditions and experimental design can alter the results of a study, which can make comparisons between studies difficult, but even in human studies when experimental designs and setup are kept the same, great variability can exist. Moreover, keeping participants in the study until completion is important to maintain balance of design. Coercion of participants of any sort (ex. excessive monetary reimbursement) is not ethical or allowed, therefore strategic methods to increase retention and compliance can be employed in the experimental setup (examples: palatable food treatment). The human palate is largely variable; therefore, screening participants via questionnaires for palatable of test products is of great value for the purposes of study retention and compliance. Though this practice has been employed before commencement of human feeding studies, author did not find a published example of a human research study where they reported prescreening of test food product.

D.1.4 Dissertation methodology employed

Three research experiments were included the dissertation research, two human in vivo studies and one in vitro study. Both human studies were conducted as randomized blind crossover design. This design was chosen so each participant could act as their own control instead of having a control group as seen in parallel designs and some intervention studies. In addition, one study was had 15 participants and benefited from crossover design due to a smaller cohort. For both studies careful consideration was given to test food product. Simple recipes
were used (i.e., pudding and crackers) in which the same recipe was used for all products only differing by test starches and in some cases addition of water. However, despite consideration to food products, in the first human feeding study, three participants dropped out after the first experimental day two expressing inability to finish test meal. By the second human study, rating of palatability for test product was implemented and only participants who rated test product above a 6 on a scale of 1-10 (1 meant participant liked the product ‘not at all’ and 10 meant they liked the product ‘very much’) were entered into the study. Resulting in one participant who dropped the study for unspecified reason.

D.2 Glycemic and Insulin Response of RS

The main dietary source of blood glucose comes from carbohydrates (e.g., starch), which according to the Department of Agriculture’s 2010 Dietary Guidelines for Americans, should be 45 to 65 percent of daily calories. Therefore, deductively starches are a major contributor to the glycemic response and thus are categorized by their digestibility with respect to glycemic response: rapidly digestible starch (RDS), slowly digestible starch (SDS) or low-digestible carbohydrates (LDC), and resistant starch (RS) - see table 4.

Table 4. Categorization of carbohydrates by digestion rates.

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<thead>
<tr>
<th>In vitro digestion time</th>
<th>Rapidly Digestible Starch (RDS)</th>
<th>Slowly Digestible Starch (SDS)</th>
<th>Resistant Starch (RS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within 20 min</td>
<td>20-120 min</td>
<td>&gt;120 min</td>
</tr>
<tr>
<td>Location of digestion</td>
<td>Mouth and small intestine</td>
<td>Small intestine</td>
<td>Escapes small intestine, main action in colon</td>
</tr>
<tr>
<td>Example</td>
<td>Freshly cooked foods, pregelatinized starch</td>
<td>Uncooked cereal starches maize, waxy maize, barley, wheat, and rice --- change</td>
<td>Cooked and cooled potatoes, bread, cornflakes</td>
</tr>
</tbody>
</table>

Adapted from Englyst et al. 1992; Lehmann and Robin 2007
RDS is not known for physiological benefits other than when quick release of glucose is needed, such as in cases of hypoglycemia. Otherwise, large doses of rapidly available glucose can have drawbacks, especially for prolonged periods of time. Diets with low glycemic foods have established their benefit in reducing the risk of developing diabetes (Salmerón et al., 1997a, 1997b) and cardiovascular disease (Liu et al., 2000) in long-term perspective cohort studies. Such observations have led to the sustained investigation of foods and dietary components with lower glycemic index.

SDS and RS are being investigated for physiological benefits akin to dietary fiber. Though SDS and RS are dietary fibers, each with specific physiochemical properties that respond differently to enzymatic hydrolysis of glucose into the bloodstream. With respect to RS digestion and absorption of glucose the source, structure, preparation, and concentration of starches are important variables to consider.

Ratios of amylose and amylopectin influence digestive resistance and thus alter glycemic response. Depending on the amylose:amylopectin structure, hydrolysis to monomeric glucose in digestion can be enzymatically hindered. Starches with higher amylose content have a higher digestion resistant than starches high in amylopectin. This is the induction for the ideology that RS lowers postprandial glucose as well as insulimic response, which has been shown in select human studies.

Behall et al. (1988) fed 25 people (12 women, 13 men) 2 cracker meals in a crossover design. The two meals were comprised of either a cracker with 70% amylose and 30% amylopectin or 30% amylose and 70% amylopectin. The cracker recipe was the same for each type of cracker only varying by the different type of starch. Crackers were administered to give a dose of 1-gram carbohydrate and 0.33gram fat/kg body weight. The goal was to observe if
different chemical structure of starch due to different amylopectin and amylose ratios had an effect on glycemic responses. Insulin was also measured. Six blood samples over a period of 180 minutes were used for evaluation. The sum insulin response above fasting measurements was significantly different between test meals but the same was not true for glucose measurements above fasting. However, the high amylose cracker obtained a lower glucose peak at 30 min and lower insulin responses at 30 and 60 minutes when compared with the high amylopectin cracker (Behall et al., 1988). When glucose response was summed, there was no difference between treatments. This study noticed a stable glucose response and lower insulin response after a high amylose meal. Being one of the earlier RS human feeding studies they surmised that these results suggested a benefit to carbohydrate sensitive or diabetic individuals.

Bodinham et al. (2010) conducted a study where they incorporated RS supplements into a flavored mousse fed to 20 healthy males. The study was an acute randomized, single-blind crossover design where the subjects participated in two separate test days scheduled a week apart. The supplements were either a RS product comprised of 60% RS and 40% RDS or a placebo comprised of 100% RDS. The supplemented mousse was administered to the subjects with a standardized breakfast and lunch. This study also tested satiety by separately consuming an ad libitum pasta dinner at the end of the dietary intervention until subjects felt full. The results from this study were similar to Behall et al (1988) with respect to glucose and insulin response. They observed no change overall in postprandial glucose concentrations between treatments, but a significantly lower insulin response was observed with the RS supplemented meal when compared to the placebo-supplemented meal (Bodinham et al., 2010).

The Bodinham et al. (2010) study differed from the Behall et al (1988) study because instead of one test meal per day, Bodinham et al. (2010)served two test meals per day.
Bodinham et al. also supplemented standard meals with their test product, instead of only the test product being served as a meal. Generally, it is believed RS lowers postprandial glucose as well as insulinemic response; however, overall both studies reported no change in glucose response. Nonetheless, other human feeding studies have reported a lower glucose coupled with a lower insulin response (Hoebler et al., 1999; Raben et al., 1994).

There is a wealth of publications on the effects of RS on glycemic and insulin responses. These studies assess glucose and insulin response with food products composed mainly of starch and/or where portions of food constituents have been substituted with RS (Hoebler et al., 1999). Observed differences in glucose or insulin response after ingestion of RS have led to conflicting reports. Many researchers have reported a decrease in postprandial glycemic or insulin response while others have reported no observed difference (see table 5(a-d)). Major differences have been found in glucose response when consumption of a RS meal was only compared with control. For example the use of raw potato starch against pregelatinized potato starch (Raben et al., 1994) and a RS bread against a white bread (Hasjim et al., 2010) expressed noticeable differences between treatment and control.

The use of RS for lowering blood glucose has promising implications for people with diabetes since, for this subpopulation, controlling blood glucose concentrations is a beneficial trait. However, in comparison to healthy individuals little research is available for people with diabetes (Table 5(a) and (b)) probably in part due to ethical issues since no true control can be administered.
Table 5(a). Human studies evaluating glucose and insulin response to RS feeding in healthy participants

<table>
<thead>
<tr>
<th>Participants Characterization</th>
<th>Type, RS Dose</th>
<th>Treatments/ Products</th>
<th>Design and Duration</th>
<th>Main Effect of RS</th>
<th>Comments</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HEALTHY</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>n= 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6M, 4F</td>
<td>Type II*, 19.5g</td>
<td>Trt1. RS supp meal</td>
<td>1 day</td>
<td>Glucose AUC</td>
<td>Trt 1 and trt 2 differed in energy 2385kJ and 2282kJ respectively; and CHO 61g and 54.5g respectively. Though small, could account some for unsuspecting increase in glucose from trt1 the RS supp. meal, though research assume potato starch was more digestible than presumed.</td>
<td>Marchini et al. 1998</td>
</tr>
<tr>
<td>Age: 19-27</td>
<td></td>
<td>Trt2. Non-RS supp. meal</td>
<td>Total visits: 2 within 15 days</td>
<td>NS for insulin AUC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI: 22.4±0.8</td>
<td></td>
<td>Meals low in fiber and RS, 30 gram raw potato starch (19.5g RS and 6.5g DS) supp. to trt1</td>
<td>DESIGN: randomized crossover*</td>
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<tr>
<td>n= 8</td>
<td>Type II, 16.5g</td>
<td>Trt 1: white bread, 1.3g RS</td>
<td>1 day with ~4day washout period</td>
<td>glucose and insulin trend with 16.5g RS when compared to other treatments</td>
<td>Trt 3 lowered glucose concentration though not all TP reached a significant level. Trt 3 at TP 90 and 1034 from trt 1 (p&lt;0.05). Insulin also showed a lower curve however trt3 produced sig. lower insulin response from trt1 TP 90- 200 min. and was sig. lower than trt2 at 170min.</td>
<td>Hoebl er et al. 1999</td>
</tr>
<tr>
<td>6M, 2F</td>
<td></td>
<td>Trt2: wheat spaghetti, 2.5g RS</td>
<td>Total visits: 3</td>
<td>glycemic and insulnemic indexes of RS bread was different from white bread reference index (p&lt;0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age: 21-28</td>
<td></td>
<td>Trt3: Supp. bread, 16.5g RS</td>
<td>DESIGN: crossover*</td>
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<tr>
<td>Weight: 55-70kg</td>
<td></td>
<td>RS only added to trt3 was high amylose maize starch</td>
<td></td>
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<tr>
<td>n= 7</td>
<td>Type II and Type III, 40g of starch</td>
<td>Trt1: RDS, GI 98</td>
<td>1 day, 48 hour washout period</td>
<td>GI 48 and 22 from 98, 43± 15%, 44±16%, and 82±23% respectively after 2hr.</td>
<td>All treatments were corn starches. Researchers used $^{13}$C marker naturally enriched in corn to asses plasma glucose and ultimate oxidization of glucose to $^{13}$CO$_2$ during digestion. Subjects were given a list of $^{13}$C foods not to ingest 2days before test day</td>
<td>Vonk et al. 2000</td>
</tr>
<tr>
<td>Gender: NR</td>
<td></td>
<td>Trt2: RS2, GI 22</td>
<td>DESIGN: parallel group</td>
<td>No evaluation of insulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age: 20-26</td>
<td></td>
<td>Trt3: RS3, GI 48</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BMI: NR</td>
<td></td>
<td>40 g dissolved in low fat milk and non caloric flavors and sweeteners added for palatability</td>
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</tbody>
</table>
Table 5(a). Continued

<table>
<thead>
<tr>
<th>Participants Characterization</th>
<th>Type, RS Dose</th>
<th>Treatments/Products</th>
<th>Design and Duration</th>
<th>Main Effect of RS</th>
<th>Comments</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>n= 30</td>
<td>Type IV, 25g</td>
<td>Trt1: glucose</td>
<td>25 g of either trt was incorporated into test beverage</td>
<td>↓ glucose at TP 15 and 30 min (p&lt;0.05)</td>
<td>Trt 2 is an octenyl succinic anhydride (OSA) modified starch. Each test beverage was made to a viscosity &lt; 5 mPa*s. Diets were controlled 3 days leading into test day.</td>
<td>Wolf et al. 2001</td>
</tr>
<tr>
<td>Age: 20-74</td>
<td>Trt2: OSA</td>
<td>25 g of either trt was incorporated into test beverage</td>
<td>1 day, washout 5-14 days</td>
<td>↓ mean glucose peak reduced by 19% (p&lt;0.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI: 24.1 ± 0.6</td>
<td></td>
<td>DESIGN: double blind randomized crossover</td>
<td>NO evaluation of insulin</td>
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<tr>
<td>12M, 18F</td>
<td></td>
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<tr>
<td>n= 25</td>
<td>Type II, 2-13.4g RS</td>
<td>Trt1: glucose sol.</td>
<td>1 day</td>
<td>↓ glucose AUC for all trt were lower than glucose (p&lt;0.0001), trt 5 and 6 lowest AUC</td>
<td>Trt 2-6 were given as test corn starches in bread, trt1 was not a bread. Subjects consumed 1g CHO/kg body weight based on the mean of weights 2 days before MTT. Men and women were paired for age and BMI, but there were no sig. gender differences in glucose or insulin response.</td>
<td>Behall and Hallfrisch 2002</td>
</tr>
<tr>
<td>13M, 12F</td>
<td>Trt2: 30%, 2g RS</td>
<td>Trt3: 40%, 3.8g RS</td>
<td>Total visits: 6</td>
<td>↓ insulin AUC for trt 5 with 11.5g RS and 6 with 13.4 RS than all MTT (p&lt;0.0001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age: 23-53</td>
<td>Trt4: 50%, 8.2g RS</td>
<td>Trt5: 60%, 11.5g RS</td>
<td>DESIGN: Latin square</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI: 21.3 - 32.8</td>
<td>Trt6: 70%, 13.4g RS</td>
<td>Bread products with varying amounts (by %) of amylose</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Trt1: glucose
- Trt2: OSA
- Trt3: 40%, 3.8g RS
- Trt4: 50%, 8.2g RS
- Trt5: 60%, 11.5g RS
- Trt6: 70%, 13.4g RS
- Trt1 was not a bread.
<table>
<thead>
<tr>
<th>Participants Characterization</th>
<th>Type, RS Dose</th>
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</tr>
</thead>
</table>
| n= 10                         | Type II, 60g | *Trt 1.* RS: 60g and 40g RDS=100g  
*Trt 2.* Placebo: 40g RDS  
Given basal meals with Trt 1 divided into 4 15g doses in jelly, or trt 2 of 4 jelly products with 0g RS over 24hrs | 24hrs: diets implemented for 24hrs next morning MTT  
DESIGN: single-blind, crossover (acute) | ↓ Insulin sensitivity (p=0.03)  
↑ Hepatic insulin clearance (p=0.02)  
↑ postprandial glucose (p=0.04) and insulin (p=0.04) | Identical background ready-to-eat diets where used to evaluate the effects of inulin solely on the addition of RS to diet. Measurements taken after RS ingestion and evaluated with MTT. Also measured plasma C-peptide, GLP-1, NEFAs, breathe H₂  
Other effects: fermentation byproducts SCFA and H₂ were slightly elevated with RS though NS | Robertson et al. 2003 |
| n= 12                         | Type II; 2.5, 5, and 10g | Trt 1. Meal 0% RS  
Trt 2. Meal 2.7% RS  
Trt 3. Meal 5.4% RS  
Trt 4. Meal 10% RS  
isocaloric meals adjusted to be 30% individual daily energy needs. | 1 d, 4 w washout period  
DESIGN: crossover* | NS in postprandial glucose or insulin response for AUC or at specific tp | Mainly a lipid oxidation experiment. Important to note glucose and insulin response was measured in response to a mixed meal. Gluteal fat biopsies and breath samples were collected. Also measured blood TAG, FFA, RQ, and urine N content. Significant differences were found in RQ | Higgins et al. 2004 |
Table 5(a). Continued

<table>
<thead>
<tr>
<th>Participants Characterization</th>
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<th>Treatments/Products</th>
<th>Design and Duration</th>
<th>Main Effect of RS</th>
<th>Comments</th>
<th>Study</th>
</tr>
</thead>
</table>
| n= 10                         | Type II, 30g/d | **Trt 1.** RS: 30 RS and 20 RDS=50g  
**Trt 2.** Placebo: 20g RDS  
Supp. prepackaged sachets into normal diet | 4 w over 12 w period  
DESIGN: single-blind, crossover dietary intervention | ↑ insulin sensitivity with clamp (p=0.03) and meal tolerance (p=0.5)  
↑ mean glucose clearance (p= 0.01)  
↓ postprandial insulin (p=0.02) | Hyperinsulinemic-euglycemic clamp used to maintain glucose plasma at 5 mmol/L (2 visits) and arteriovenous MTT to assess metabolism of adipose tissue and skeletal muscles (2 visits) also measured NEFAs, C-peptide, and ghrelin Minor effects: found 1.1 kg increase in lean body mass (p=0.003) | Robertson et al. 2005 |
| **n= 12** (healthy lean)  
7 M, 5 F  
Age: NR  
BMI: NR | Type III, ~30g | **Trt1:** glucose control  
**Trt2:** tapioca-based retrograded maltodextrin 59.7% RS  
50g of CHO dissolved in water | 1 day  
DESIGN: randomized single-blind crossover study | ↓ glucose AUC (p < 0.05) 58.52 ± 7.33% compared to trt1  
↓ insulin AUC (p < 0.01) 24.81 ± 10.12% compared to trt 1 | Blood samples via an intravenous catheter were collected from participants after a 10 hour overnight fast | Brouns et al 2007 STUDY 2 |
| n= 24  
11 M, 13 F  
Age: 22–59  
BMI: 23.2 ± 0.6 | Type IV, RS dose NR but 7.2g DF for DSF (Group 2, trt 1)  
**Group 1 (n=10)**  
Trt 1: CHO constituent product (0g DF)  
Trt 2: control glucose --- 50g of trt dissolved in 400 ml water  
**Group 2 (n = 14)**  
Trt 1: DSF (T-Diet PlusÔ Diabet NP)  
Trt 2: control product --- 400 ml of each trt | 1 day, 2 days total, 1 week washout  
DESIGN: randomized parallel arm* | ↓ glycaemic index and glycaemic load both trt  
NS glucose AUC  
↓ insulin AUC after CHO constituent as well asafter the new DSF (p < 0.001) when compared to controls | The new DSF consisted of slowly digestible CHO mainly RS type IV. No comparison of between treatments in different groups. | García-Rodríguez et al. 2012 STUDY 1 |
Table 5(b). Human studies evaluating glucose and insulin response to RS feeding in people with diabetes

<table>
<thead>
<tr>
<th>Participants Characterization</th>
<th>Type, RS Dose</th>
<th>Treatments/ Products</th>
<th>Duration</th>
<th>Main Effect of RS</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DIAGNOSED TYPE II DIABETIC</strong></td>
<td></td>
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</tr>
<tr>
<td>n = 90 - see comments</td>
<td>Type NR, 6.51 g RS</td>
<td>Trt 1: cooked refined rice (control) Trt 2: test rice with corn derived RS</td>
<td>4-week 2-week run-in</td>
<td>↓ fasting insulin ↓ insulin resistance ↓ postprandial insulin at tp 30 min ↓ postprandial glucose (P= 0.010)</td>
<td>Participants (n=90) had either impaired fasting glucose (IFG) or glucose tolerance (IGT) or newly diagnosed diabetes. Diabetes was assessed as fasting plasma glucose ≥126 mg/dL, prediabetes was defined as having IFG levels of 100-125 mg/dL, and IGT was described as after 2-hr in oral glucose tolerance test having 140-199 mg/dLglucose. Before and after 4-wk intervention, subjects received a standard meal tolerance test after an overnight fast</td>
</tr>
<tr>
<td>Control group (n=44)</td>
<td>26 M, 18 F</td>
<td>Age: 49.4 ± 1.74 BMI: 24.5 ± 0.37</td>
<td>Rice trts were pre-packaged as 210g serving to be consumed for one meal a day on top of normal diets</td>
<td>DESIGN: randomized double-blind, placebo-controlled parallel arm</td>
<td></td>
</tr>
<tr>
<td>RS group (n=41)</td>
<td>21 M, 20 F</td>
<td>Age: 51.7 ± 2.03 BMI: 25.0 ± 0.49</td>
<td></td>
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</tr>
<tr>
<td>n= 10</td>
<td>4 M, 6 F</td>
<td>Age: 37–64 BMI: 35.5 ± 3.6</td>
<td>Type IV, RS dose NR but 3.04 - 8g DF</td>
<td>Trt 1: new DSF T-Diet Plus® Diabet NP (8g DF) Trt 2: DSF Glucerna® SR (3.04g DF) Trt 3: Novasource® Diabet (6g DF) --- 400 mL of each trt</td>
<td>↓ glucose AUC was lower after new DSF, trt 1, (P = 0.037) compared with the other trts. NS insulin AUC among trt</td>
</tr>
<tr>
<td>Garcia-Rodríguez et al. 2012 STUDY 2</td>
<td></td>
<td></td>
<td>1 day, 3 days total, 1 week washout DESIGN: crossover*</td>
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<tr>
<td>Kwak et al. 2012</td>
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</tbody>
</table>
Table 5(c). Human studies evaluating glucose and insulin response to RS feeding in overweight participants.

<table>
<thead>
<tr>
<th>Participants Characterization</th>
<th>Type, RS Dose</th>
<th>Treatments/Products</th>
<th>Duration</th>
<th>Main Effect of RS</th>
<th>Comments</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overweight hypertriglyceridemic n= 29</td>
<td>Type II*, 17g for W, 25g for M only in trt1</td>
<td>Trt1: high-amylose Trt2: oat bran Trt3: low-amylose Test products (muffins, bread, breakfast cereal, and pasta) were given as supplements to background diet</td>
<td>12 wks, 3wks/trt no washout DESIGN: randomized crossover</td>
<td>↓ postprandial glucose after trt1 (RS) when compared to trt 3 ↓ postprandial plasma insulin, total NS fasting insulin and glucose for all trt</td>
<td>Diets had 55%&lt; CHO and 30%&gt; fat. Background diets were self-selected within researchers constraints and treatments were given as test products. Other effects: RS, trt 1, had no effect on plasma lipids but increase fecal butyrate. Trt 1 and 2 increased stool frequency and lowered pH</td>
<td>Noakes et al. 1996</td>
</tr>
<tr>
<td>Healthy overweight female n= 25</td>
<td>Type III*, 24g/d</td>
<td>Trt1. Resistant Corn starch group, RS Trt2. Regular corn starch group, CS Supp. into regular diet</td>
<td>21 days DESIGN: double-blind parallel</td>
<td>↓ fasting glucose (p&lt;0.05) NS fasting insulin</td>
<td>Fasting baseline measurements were taken before the 21 day and fasting measurements were taken after. Change in fasting glucose was observed but not in insulin. Other effects: lowered cholesterol (total and LDL)</td>
<td>Park et al. 2004</td>
</tr>
</tbody>
</table>
Table 5(c). Continued

<table>
<thead>
<tr>
<th>Participants Characterization</th>
<th>Type, RS Dose</th>
<th>Treatments/Products</th>
<th>Duration</th>
<th>Main Effect of RS</th>
<th>Comments</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (NW) and overweight (OW) women n=20 F</td>
<td>Type II, 0.71, 2.57, or 5.06 g RS/100 g muffin</td>
<td>Trt 1: glucose control Trt 2: 0.26 β: 0.71 RS Trt 3: 0.26 β: 2.57 RS Trt 4: 0.26 β: 5.06 RS Trt 5: 0.68 β: 0.71 RS Trt 6: 0.68 β: 2.57 RS Trt 7: 0.68 β: 5.06 RS Trt 8: 2.3 β: 0.71 RS Trt 9: 2.3 β: 2.57 RS Trt 10: 2.3 β: 5.06 RS</td>
<td>1 test day, total 10 visits</td>
<td>➧ overall AUC for insulin trt 10, high β-glucan and high RS DESIGN: Latin Square</td>
<td>Investigating interactions with three levels of resistant starch and three levels of β-glucan. Ten subjects in each group. OW paired for age with NW as a control. Subjects consumed 1g CHO/kg body weight. Standard equilibration diets were given for 2 days before and test day, but between meals/trt normal diet was consumed. OW had plasma insulin concentrations higher and were marginally more insulin resistant than NW but maintained similar plasma glucose concentrations.</td>
<td>Behall et al. 2006</td>
</tr>
<tr>
<td>NW (n=10) Age: 43.4 BMI: 22.0</td>
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<tr>
<td>OW (n=10) Age: 43.3 BMI: 30.4</td>
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<td></td>
</tr>
<tr>
<td>Overweight, n=33 11 M, 22 F Age: (18-69y) 49.5 ±1.6 y BMI: 30.6 ± 0.5</td>
<td>Type II, 2 levels of ~60% RS</td>
<td>Trt1: 0g/d Amioca Trt2: 15g/d Hi-maize 260 Trt3: 30g/d Hi-maize 260 trts individually packaged in ready-to-use sachets to be mixed with cold or room-temperature beverages or foods 2 times per day</td>
<td>4 wk, 3wk washout DESIGN: double blind randomized crossover</td>
<td>➧ insulin sensitivity for men in both levels of RS (trt2 and 3) when compared to control (trt1) NS change between RS levels in men and women NS insulin sensitivity in women among treatments</td>
<td>Participants’ waist circumference ≥ 89 cm for F or ≥ 102.0 cm for M to increase insulin-resistant probability, but individuals with BMI ≥ 35 were excluded. Mean fasting glucose was 5.29 ± 0.08 mmol/L, which is within normal range. Insulin sensitivity was measured using insulin-modified i.v. glucose tolerance test. There was a treatment and sex interaction (P = 0.033) but no proposed mechanism.</td>
<td>Maki et al. 2012</td>
</tr>
</tbody>
</table>
Table 5(d). Human studies evaluating glucose and insulin response to RS feeding in healthy participant

<table>
<thead>
<tr>
<th>Study</th>
<th>Participants Characterization</th>
<th>Type, RS Dose</th>
<th>Treatments/ Products</th>
<th>Duration</th>
<th>Main Effect of RS</th>
<th>Comments</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTHER</td>
<td>insulin resistance (IR) and normoinsulinemic (NI), n=9 M</td>
<td>Type III, RS (g) NR but 3.8 g dietary fiber for trt 1 and 2</td>
<td>Trt 1: hot potato (HP) at 83.6 ± 2.0°F Trt 2: cooled potato (CP) at 26.0 ± 0.6°F Trt 3: white bread</td>
<td>1 day, total 3 days with 1-5 week washout DESIGN: randomized crossover *</td>
<td>↓ glucose in trt2 the cooled potato NS insulin</td>
<td>Insulin sensitivity was calculated based on fasting insulin:glucose concentration. No diabetics were entered into the study. To avoid second-meal effect no legumes in the meal preceding overnight fast.</td>
<td>Najjar et al. 2004</td>
</tr>
<tr>
<td></td>
<td>4 IR, 5 NI Age: 20.9 ± 0.7 BMI: 30.6 ± 3.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borderline (untreated) diabetes mellitus, 20</td>
<td>Type III, 6g</td>
<td>Trt 1. bread Supp. with tapioca RS Trt 2. Placebo control bread 140g/2 slices/trt</td>
<td>1 day, 2 week washout total visits: 2 DESIGN: double blind, single-ingestion crossover</td>
<td>NS in glucose AUC ↓ Insulin AUC in all subjects (p=0.05) ↓ glucose concentration in borderline group 1hr after ingestion of trt1</td>
<td>Participants screened for fasting blood glucose of 100-140 mg/dl then stratified into borderline group (glucose≥111mg/dl) or normal (glucose ≤ 110mg/dl). Stated to be crossover but could have benefitted from being a randomized crossover</td>
<td>Yamada et al. 2005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9M, 11F Age: 50.5±7.5 y BMI NR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin resistant 12 M, 8 F</td>
<td>Type II, 40 g/day RS</td>
<td>Trt 1: Hi-Maize 260 (RS- group 1) Trt 2: Amioca (placebo- group2) starch in ready-to-use sachets</td>
<td>12 weeks DESIGN: randomized, single-blind, placebo-controlled, parallel intervention</td>
<td>↑ insulin sensitivity (p = 0.023) NS fasting insulin sensitivity</td>
<td>Insulin resistant qualified as fasting plasma insulin value &gt; 60 pmol/L in healthy participants. For intervention 2 sachets/day incorporated into participants diet, 2-test days before and after 12 weeks for measurements. Total of 4 test days. Insulin sensitivity was measured by euglycaemic–hyperinsulinaemic clamp.</td>
<td>Johnston et al. 2010</td>
<td></td>
</tr>
<tr>
<td>Group 1 (n=10) Age: 45.2 ±3.55 BMI: 31.3 ± 1.70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2 (n=10) Age: 50.1 ± 4.05 BMI: 30.4 ± 1.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5(a-d). Continued

Abbreviation used in Table 5:
BMI or iBMI: (initial-i) Body Mass Index, measured as kg/m²
CHO- carbohydrate
DSF – diabetes-specific formula
F- Female
FFA – free fatty acids
GI- Glycemic Index
M- Male
MTT- Meal Tolerance Test
NEFA- Non-esterified fatty acids
NR – not reported
NS- Not Significant
Supp. – supplemented
RDS – Rapidly Digestible Starch
RQ- Respiratory quotient
RS- Resistant Starch
SCFA- Short chain fatty acids
sol.- solution
TAG – triacylglyceride
tp- time point
trt – treatment
wk - week

--- Age and BMI were reported as mean (± SE were applicable) not unless written as a range.
* - Not reported but assessed to be as indicated by description of procedures
a – 40g of total starch grams of RS was NR only GI
D.3 Fermentation Studies

Currently, considerable public attention is given to dietary fiber and colon health, but perhaps the most underrated aspect of colon health in public perception is resident microbiota and their fermentative ability. Gut microbiota play a very active role in host interactions, one of which is fermentation. Fermentation of the gastrointestinal tract is the sum of metabolic processes and reactions from the decomposition of organic substances by anaerobic microbiota. RS, like other dietary fibers, can be fermented in the colon by microbiota. The portion of RS that is not absorbed in the small intestine enters the large intestine where it is utilized as a substrate by colonic bacteria resulting in the release of fermentation by-products carbon dioxide, methane, hydrogen, energy, organic acids (e.g. lactic acid) and SCFA.

Anaerobic metabolism of starch does induce physiological changes, which can offer benefit to the host. Once glucose from starches enters the glycolytic pathway, it is broken down to pyruvate, from which ultimately all fermentation products can be produced (Figure 2). In humans, the large bowel is the main site of fermentation and SCFA production, specifically the large proximal bowel where fermentation and absorption of fermentation products is the greatest.

Fermentation, as a whole, can increases colonic blood flow (Scheppach, 1994), reduce fecal/colon luminal pH (Ahmed et al., 2000) and secondary bile acids, improve colon epithelial cell function, stimulate fluid and electrolyte absorption, and has implications in colon cancer protection (Topping and Clifton, 2001). Other implicated physiological benefits of fermentation from dietary fibers are decreased growth/binding of pathogenic bacteria, increased mineral absorption, and sources of energy for the colon epithelium. Thus, fermentation not only plays a large role in colon health but also in colonic function (e.g., nutrient absorption) and plausibly as an aid in the immune system’s first line of defense not only by decreasing pathogenic bacteria
adhesion but also by eliciting anti-inflammatory effects (Meijer et al., 2010). Moreover, specific fermentation by-products can elicit different physiological responses, which can aid in improving human health (refer to table 3).

(Figure 2). Fermentation schematic of major carbohydrate fermentation by-products. Dotted arrows are not direct production of metabolite but do contribute to overall production, like in the case of lactate to butyrate.
A carbohydrate-rich diet has been shown to increase fermentation, although gender differences are thought to decrease fermentation due to menstruation. There have been fermentation studies, which have excluded women. One of which (van Munster et al., 1994b) explained that fermentation and starch absorption was influenced by menstruation, citing an article that observed the effects of the menstrual cycle on GI changes in starch malabsorption, stool bulking, stool mucinase, and beta-glucuronidase activities when on a low-fiber diet were investigated (McBurney, 1991). There are GI differences of women versus men, but fermentation studies have been conducted with both genders in which no gender differences were reported. Conversely, fermentation studies have reported significant gender differences in response to dietary fibers and RS (McOrist et al., 2011).

Fermentation studies in humans are practical and can provided constructive information towards the improvement of colon health especially for people suffering from GI disorders. The study of microbial fermentation by-products of RS in human studies, though useful, does not always lend clear or predictable results. However, as for postprandial glucose and insulin response, there are notable trends or hypothesis driven generalizations that RS increases fermentation. Human fermentation has provided varied results as to the efficacy of RS (see table 6), although there is evidence for the use of RS as a fermentable fiber, results are not consistent.

D.3.1 Quantification of Fermentation

The most common variables used for fermentation quantification are breath gases and SCFA production. SCFA are readily absorbed and metabolized in the liver and muscle tissues to produce energy. What is not absorbed or utilized is excreted in the feces. Gases produced in fermentation, on the other hand, have to be absorbed in the colon before pulmonary excretion.
The two variables, breath gases and SCFA production, can be used to survey fermentation activity but is not always a direct measurement of gut fermentation. Sample analysis to measure fermentation by-products in the colon can be taken from blood, breath, and/or feces. Use of excreted samples are the more common methods for human in vivo models, yet ileostomy effluent and intestinal intubations are also in vivo methods used in fermentation studies though the latter methods require more care due to their evasive nature by comparison. Sampling from feces is not invasive and can be used in vitro and in vivo models and are considered the ‘gold standard’ for overall digestion and fermentation studies (Salminen et al., 1998).

Gas chromatography (GC) is the most common techniques for analysis of SCFA. Quantification of SCFA by GC can be done with free fatty acids; however, due to the volatile nature of acetate and other SCFA, normally derivatization or esterification techniques are employed. Blood samples have been measured by automated head space GC methods with esterified SCFA (Achour et al., 1997). SCFA quantification in blood samples is a useful method but not as widely used as quantification of SCFA in fecal samples since concentrations of SCFA in the periphery are low and are believed to be difficult to measure accurately because an increase in SCFA production might not be proportionally detected in peripheral circulation.
Table 6. Summary of in vivo human studies examining effects of RS on change in colonic fermentation

<table>
<thead>
<tr>
<th>Reference</th>
<th>RS Dose</th>
<th>Type</th>
<th>No. trt</th>
<th>Duration</th>
<th>N</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>Total</th>
<th>H₂</th>
<th>CH₄</th>
<th>CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achour et al. 1997</td>
<td>50 g starch</td>
<td>RS₂</td>
<td>2</td>
<td>Acute</td>
<td>8</td>
<td>+, Blood</td>
<td>☒</td>
<td>☒</td>
<td>☒, blood</td>
<td>+*</td>
<td>+ (5)</td>
<td>+*</td>
</tr>
<tr>
<td>Muir et al. 1994</td>
<td>59.1g ¥</td>
<td>RS₂, RS₁</td>
<td>2</td>
<td>Acute</td>
<td>8</td>
<td>+*, Blood</td>
<td>*</td>
<td>*</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
</tr>
<tr>
<td>Vonk et al. 2000</td>
<td>40g starch</td>
<td>RS₂, RS₃</td>
<td>3</td>
<td>Acute</td>
<td>7</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
</tr>
<tr>
<td>van Munster et al. 1994</td>
<td>28g</td>
<td>RS₂</td>
<td>2</td>
<td>1 w</td>
<td>19</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
</tr>
<tr>
<td>Ahmed et al. 2000</td>
<td>50 g CHO</td>
<td>RS₁</td>
<td>2</td>
<td>8 d</td>
<td>14</td>
<td>+</td>
<td>NS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>☒</td>
<td>☒</td>
</tr>
<tr>
<td>Cummings et al. 1996</td>
<td>Various</td>
<td>RS₂, RS₃</td>
<td>7</td>
<td>15 d</td>
<td>12</td>
<td>+¹</td>
<td>+¹</td>
<td>+⁴</td>
<td>+⁴</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
</tr>
<tr>
<td>Phillips et al. 1995</td>
<td>26-50g ¥</td>
<td>RS₂, RS₁</td>
<td>2</td>
<td>3 w</td>
<td>11</td>
<td>+</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
</tr>
<tr>
<td>McOrist et al. 2011</td>
<td>22g</td>
<td>RS₂</td>
<td>2</td>
<td>4 w</td>
<td>46</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
</tr>
</tbody>
</table>

Acute, 2 < test days, 1 RS treatment meal
+ (green), increase p<0.05 unless otherwise specified
- (pink), decrease p<0.05 unless otherwise specified
* (blue), no effect
☒, not measured/not reported
NS, non-significant trend
*, p < 0.01
¹, highest or lowest in some forms of RS
¥, highest of two RS doses

Note: Studies Muir et al. 1994, Ahmed et al. 2000, Phillips et al. 1995, and McOrist et al. 2011, did not clearly define what type of RS was used, therefore, type information used in table was interpreted from description of meals in text of respective article.
D.3.2 Short Chain Fatty Acids (SCFA)

SCFA are anionic organic fatty acids with a chain length 1-6 carbon long. The production of SCFA by gut fermentation of RS has already been reviewed to be beneficial to health. Fermentation of RS produces SCFA that are active metabolites utilized by the colonic epithelium and enter the portal circulation. If fermentation of dietary fibers such as RS is sufficient, SCFA produced in the large intestine will increase epithelial and portal concentrations. Thus, SCFA can be used as biochemical markers of gut microbiota fermentation.

The most abundant SCFA of microbial fermentation in the human large bowel in order of proportion are acetate, propionate, and butyrate, though the order of metabolic significance is reversed (butyrate>propionate>acetate). However, all of these SCFA are of interest in maintaining human health and have an implicated role in gut health. Valerate (C5) and hexanoate (C6) are also observed but at lower concentrations and furthermore with little interest to carbohydrate fermentation health effects. Branched SCFA are predominantly proteolytic bacteria fermentation products of proteins and amino acids.

With substrates like RS and other fermentable dietary fiber substrates, saccharolytic bacteria produce SCFA. These fermentable fibers are targeted for their putative ability to increase SCFA; but, in some human studies, no change in SCFA in response to RS has been observed (Heijnen et al., 1998; Hylla et al., 1998). However, generally RS on some level increases SCFA production. With an increase in SCFA production, a decrease in fecal pH from colon effluent has also been observed (Ahmed et al., 2000). Overall, SCFA are useful in colonic health as nutrients or regulators of proliferation, differentiation, and gene expression for the colonic epithelium; and modulators of colonic pH (Cook and Sellin, 1998).
Acetate is the most abundant SCFA in the gut. It is formed through the reduction of CO\textsubscript{2} by acetogenic bacteria, its production is also described as oxidative decarboxylation of pyruvate (Cummings, 1981). Initial substrates for the production of acetate can either come from hepatic or peripheral glucose or fatty acid metabolism. Once formed, acetate is readily absorbed and taken up by colonic epithelium to eventually enter peripheral circulation where it can be metabolized by peripheral tissues. As the liver absorbs acetate as it appears in portal blood from the intestines, it can then be used in hepatic de novo lipogenesis via acetyl-coA synthetase/fatty acid synthase and hepatic cholesterol synthesis (Bergman, 1990).

Colonic fermentation provides microbial production of acetate allowing the body to utilize indigestible carbohydrates for energy. Most of acetate from the gut is taken up by the liver, which makes it less likely to be metabolized in the colon than by other tissues (e.g., muscle). Acetate, however, is utilized by colonic bacteria, mainly for the production of butyrate as shown in bacterial culture and human microbiota fermentation (Duncan et al., 2002). Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, and Lentisphaerae are some of the human gut phyla that contribute to acetate as a fermentation end product (Jacobs et al., 2009) as reviewed from 16S rRNA methods in the human gut microbiota.

The mechanistic study of the production and utilization of acetate in gut is conducted mostly in vitro. Measurements of concentrations of acetate with other SCFA and fermentation products as a function of fermentation are conducted in human studies. Acetate concentrations in plasma after an RS intervention were found to be greater in women than in men. This study examined 11 men and 22 women who were overweight or obese in a crossover intervention with 4-week test periods on either control (0 g/d RS2), 15 g/d RS2, or 30 g/d RS2 (Maki et al., 2012).
Plasma acetate had a treatment effect ($P = 0.0007$), but no treatment and gender interaction. Acetate concentration of $4.4 \pm 0.1 \ \mu\text{mol/L}$ was greater in the highest RS level (30-g/d RS2) than in the control ($P = 0.047$). Gender differences in RS fermentation were attributed to generally different gut transit time between genders influencing the rate of fermentation events, although gut transit time in this study was not a direct measurement. There was no change in any other SCFA. This study is of interest because generally butyrate is the SCFA in vivo that is reported to change due to RS treatments.

**D.3.2.b Propionate**

The formation of propionate is by two main pathways: the dicarboxylic acid pathway and the acrylate pathway (Cummings, 1981). Like acetate, the liver also takes up propionate. But unlike acetate, it is implicated for its effect on glucose as well as lipid metabolism and thereby attributed with the reduction blood lipid profile and insulin resistance.

The role of propionate in carbohydrate metabolism is species specific and its effect on glucose metabolism across and within species seems to vary. An example of species variation is in ruminants propionate is thought to be a glucose precursor but not in hindgut fermenters. Though more species differences exist, within species (like in humans) effects of propionate also differ.

Propionate, like acetate, tends not to be proportionally affected by percent in total SCFA production due RS consumption. Contrarily, RS consumption is thought to induce higher propionate and butyrate production, but research studies have shown the spread of propionate concentration to have all-inclusive results. A few human studies have shown specific increase of fecal propionate, like in a study when 12 healthy participants were feed different forms of RS
and found RS₃ increased propionate concentrations over RS₂ (Cummings et al., 1996). Generally, RS diets have increased total SCFA production but one study showed that not only was there no effect on total fecal SCFA concentration but over a 4 week period in 12 healthy participants propionate concentration after a high RS treatment decreased when compared to low RS treatment; 10.8 ± 0.6 and 14.6 ± 1.3 µmol/g wet weight respectively (Hylla et al., 1998). Also, in vitro studies have shown that overweight and obese individuals have higher amounts of propionate (Schwiertz et al., 2010), though there is little human in vivo evidence that propionate changes due to RS treatment when considering metabolic factors such as weight.

In a study where propionate improved postprandial glucose response, it was in carbohydrate products supplemented with propionate and not from gut fermented propionate (Todesco et al., 1991). Here propionate appeared to have an inhibitory effect on amylolytic activity. More effort can be spent expanding the knowledge of propionate from RS fermentation in humans and specifically in overweight and/or obese individuals.

\[D.3.2.c \text{ Butyrate}\]

Butyrate seems to be the SCFA of interest when considering colonic health. Acetate feeds into the production of butyrate when it is reduced to acetoacetate. However, lactate is also a major contributor of butyrate (Bourriaud et al., 2005). Butyrate is a preferred substrate for colonocytes (Schwiertz et al., 2002) providing a major source of energy. In addition, butyrate is also attributed with playing a role in maintaining a normal colonocyte phenotype and population.

The main SCFA acetate, propionate and butyrate are all taken up by the colonic mucosa, but butyrate is preferentially transported by colonocytes (Ritzhaupt et al., 1998). It is estimated
that the majority of butyrate (95%) produced by microbes is transported across the epithelium and utilized, making butyrate hard to detect in portal blood. Common ways to examine butyrate production are through fecal samples, colon cell lines, or luminal effluent mostly likely because it is believed butyrate exerts its main effects in the lower gut.

Butyrate production can be sensitive to some forms of RS consumption (Jenkins et al., 1998), and when compared with a low fiber diet RS might be the dietary component most influential on butyrate production. In a 3-week dietary trial of 19 healthy participant consuming 45g of RS approximately 32% resistant or 20g natural fiber low in RS showed an increase in butyrate (van Munster et al., 1994b). This study also showed an association between high fecal butyrate and lower colonocyte proliferation. Overall, RS is thought to increase butyrate production and which can bear beneficial colonic health effects such as inflammation inhibition by influence on NF-kB expression and activity (Segain et al., 2000) and potentiating protection at various stages of colorectal cancer by suppressing growth of tumor cells and enhancing differentiation (Scheppach et al., 1995).

Starch as a substrate has lead to increased butyrate fermentation over other substrates (Weaver et al., 1992), and RS specifically has shown to increase fecal butyrate production over other dietary fibers (Noakes et al., 1996). Though inter-individual and gender differences to SCFA concentrations, particularly butyrate, has been reported (McOrist et al., 2011). McOrist and associates (2011) conducted a randomized crossover study were 46 participants (16 males, 30 female) were feed 25 g of NSP or 25 g of NSP plus 22 g of RS per day for 4 weeks. Entry fecal butyrate concentrations varied from 3.5–32.6 mmol/kg where 27% of inter-individual variability was explained by BMI. From butyrate entry levels, participants were ranked into quintiles. Participants in the quintile with low butyrate entry levels tended to have an increase
butyrate in response to RS treatment, but those with high entry levels often decreased in butyrate concentration. Total SCFA concentrations overall did increase due to treatment with RS and gender differences were observed. Men had higher SCFA concentration than women for acetate, propionate, and total SCFA, which is plausible since it is believed men have greater fermentation than women. What was interesting in this study was there were no gender differences in only butyrate concentrations ($p=0.08$).

Butyrate is oxidized in the colonic mucosa, which can lead to production in CO$_2$. Compromised populations, such as people with inflammatory bowel disease (ulcerative colitis), have been shown have lower levels of butyrate oxidation in the lower gut than population with healthy digestive tract (Chapman et al., 1994). Due to the symptomatology of IBD, it is postulated that lower levels of butyrate exist in this group. Thus, esterified starches have been studied to supplement SCFA of interest in food engineered to be delivered to the colon. In a randomized crossover trial 16 participants (4 men, 12 women) in good health supplemented their diets with a treatment of low RS with 20 or 40 g of high amylose corn starch (HAMS) or butyrylated HAMS, HAMS20, HAMS40, HAMSB20, or HAMSB40 respectively, each for 2 weeks (Clarke et al., 2011). Fecal samples were collected for 4 days out of a two-week period (day 2, 3, 12, and 13). Esterified butyrate concentrations on days 12 and 30 and free fecal butyrate were highest for HAMSB40. The esterified starches, HAMSB40 and HAMSB20 excreted more (g/100g feces) than non-esterified starches, HAMS20 and HAMS40; which leads to the inferences that the HASB starches are not bioavailable and would lead to more lower gut microbial fermentation. Microbial analysis showed an increase in abundance of *Parabacteroides distasonis* after HAMSB40 on days 12 and 13, concluding that this bacteria probably facilitated butyrate production.
D.3.3 Breath Gases

Intestinal gases $N_2$, $O_2$, $CO_2$, $H_2$, and $CH_4$ come from ingestion, diffusion from bloodstream, or colonic bacterial activity. Gases from colonic bacterial fermentation ($H_2$, $CO_2$, and $CH_4$) can diffuse into the blood and be excreted in the breath, though only $H_2$ and $CH_4$ are exclusively from bacterial fermentation. This premise, through the measurement of breath samples, has been used to estimate gastrointestinal conditions and as a non-invasive gastroenterological diagnostic tool.

D3.3.a Hydrogen

Breath hydrogen is an indicator of intestinal fermentation. When $H_2$ is produced, it is rapidly absorbed into the bloodstream and excreted by the lungs where it can be measured. Formate, a product of anaerobic metabolism, can be converted into carbon dioxide and hydrogen. In turn, $H_2$ can either be converted into methane if appropriate bacteria are present or excreted.

In 1970 Levitt and Donaldson wrote a popular article “Use of respiratory hydrogen ($H_2$) excretion to detect carbohydrate malabsorption” in the Journal of Laboratory and Clinical Medicine. From this article and other works (Bond and Levitt, 1972), excretion of $H_2$ was linked to the malabsorption of carbohydrates. Carbohydrate malabsorption, where a carbohydrate cannot be absorbed in the small intestines, is usually seen in people with abnormal GI functions. When breath hydrogen and carbohydrate malabsorption studies were being conducted in the early 1970s, it was before the discovery of what is known as resistant starches today. Strictly speaking, since RS is not fully digested in the small intestine it is malabsorbed. However, the incomplete digestion of RS is not considered carbohydrate malabsorption though the ingestion of RS can also cause an increase in breath hydrogen just like carbohydrate malabsorption (Bond...
and Levitt, 1972; Born, 2007). Though unlike in carbohydrate malabsorption, fermentative properties of RS does not have to be associated with GI disorders. In addition, RS is suspected to preferentially increase the concentration of colonic bacteria involved in fermentation.

Ingestion of RS has shown signs of increased fermentation by increased breath $H_2$. When 8 subjects were fed a 3 meal diet of high and low RS (type I and II) there was a comparative increase in breath hydrogen production $34.1 \pm 4.7$ and $23.9 \pm 3.9$ ppm ($P < 0.001$) respectively (Muir et al., 1994). Likewise, after 16 hrs test period, a genetically modified rice for enriched RS was found to increase (p<0.05) peak breath $H_2$ at $38.9 \pm 17.6$ ppm over its wild type $10.5 \pm 3.7$ ppm (Li et al., 2010). There is also evidence of no change to breath hydrogen in response to RS or other fermentable fibers (Jenkins et al., 1998).

There are drawbacks to measuring $H_2$ as a metabolite of fermentation. Although $H_2$ can be excreted in the lungs it can also be used in other colonic bacterial pathways such as sulphate reduction, acetogenesis, and methanogenesis. Consumption of $H_2$ is essential for the formation of byproducts such as methane. Thus, though fermentation of RS represented as hydrogen production might be occurring, it is utilized before it can be excreted in the breath. Bacterial use of $H_2$ is one factor that influence excretion of $H_2$ but colonic environment plays a role in $H_2$ production. Gas production is directly proportional to colonic pH, as fecal pH is lowered so is the production of $H_2$. Notwithstanding since there is evidence that $H_2$ is influenced in response to RS it is a good indicator to survey fermentation.

### D.3.3.b Methane

The production of methane is dependent on whether the host has resident colonic methanogenic microbiota. Methanogens consume large quantities of hydrogen to produce methane. Euryarchaeota and Firmicutes are examples of human gut microbial phyla that
produce methane as fermentation end products (Jacobs et al., 2009). Some people do not have the appropriate microbiota to produce methane and considering the production of methane has not been shown to be a function of eukaryotic cells, measurements and inferences of methane production are only applicable for a sub-population of humans.

Methane is normally a breath gas that rarely reaches significance when assessing the effect of RS, in diets even when divided into producers and non-producers (Hallfrisch and Behall, 1999). Although studies have shown an increase in methane fermentation (van Munster et al., 1994b). It is estimated that 30% of the adult population are methane producers (Gasbarrini et al., 2009), but experimental data stratified for production of methane have varied. An in vitro study quantified 23 out of 30 of human fecal samples to produced methane as the primary method of hydrogen consumption (Gibson et al., 1990). Though the research question of Gibson and associates (1990) was to identify the main method of hydrogen disposal, it also showed that 77% of their sampled size had methane-producing bacteria. Other human feeding studies have found 58% (11 of 19) (van Munster et al., 1994b), and 63% (5 of 8) (Achour et al., 1997) of participants to be methane producers. A more stable estimation of methane producers is 30-60% of adult population on westernized diets though in other countries where a carbohydrate rich diet is more prevalent, higher incidents upwards of 70- 85% of methane producers can be observed (Cummings, 1981; Segal et al., 1988).

The criteria for categorizing CH$_4$ producer are also inconsistent. When pulmonary methane excretion rate was measured in 22 adults, participants with methane > 1 ppm were considered producers while all participants < 1 ppm where non-producers (Bond et al., 1971), but this same study is accredited elsewhere as defining methane producers as having methane concentrations >2 ppm higher than ambient air. Other studies have cited cut off of corrected
fasting methane to be as high as 10ppm to be considered as a methane producer (Hallfrisch and Behall, 1999).

Methane production can also decrease overall fermentation. Substantial reduction in SCFA production has been observed in methane producers when compared with non-methane producers, where producers were defined as ≥ 5 µmol methane in 24 hours and non-producers <0.3 µmol methane in 24 hours (Weaver et al., 1992). This study used methane measurements from feces and not breath samples and the definition of producers verse non-producers was based off of gram per feces per unit time instead of conventional (breath) ppm for fermentation gases. To date, no studies could be found with long-term measurements of methane in only methane producers. An increased sample size with only methane producers might strengthen evidence and correlations between methane production and RS consumption.

D.3.3.c Carbon dioxide

Like breath hydrogen, carbon dioxide (CO₂) can be absorbed in the gut to be excreted by the lungs. In the human body, CO₂ can be produced from a number of reactions. Formate is produced from anaerobic metabolism and can be converted into carbon dioxide and hydrogen. In the small intestine carbonic anhydrase will rapidly convert hydrogen ions and bicarbonate to CO₂. It can also be oxidized from butyrate in the colon. However, when considering lower gut fermentation, carbon dioxide is the lesser reported breath gas.

Studying CO₂ as a gut bacterial fermentation product from carbohydrates has been commonly evaluated though the use of labeled carbon. Corn has a natural carbon isotope, ^13C. Oxidation of this ^13C to CO₂ generates a stable labeled fermentation by-product, which allows the quantification of the production of CO₂ strictly from a corn RS source, since CO₂ is a product of
the citric acid cycle fed by all glycolysis. Excretion of $^{13}$CO$_2$, when measured in response to naturally $^{13}$C- enriched corn starches, was found to be higher after a corn derived RS (retrograded) meal than after a digestible corn starch meal fed to humans (Achour et al., 1997), indicative of higher fermentative ability of RS than RDS. Although CO$_2$ is used as an indicator of bacterial fermentation; no reports of change due to RS consumption can be found if not assed by $^{13}$CO$_2$. Furthermore, CO$_2$ is a ubiquitous metabolic product in humans and may not be a strong measurement for bacterial fermentation.

D.3.4 RS Comparisons in fermentation Studies

Resistant starches have been compared to other dietary fibers for its health benefits. RS might be more useful for lower gut fermentation than some other dietary fibers. However, research by Jenkins et al. (1998) did not support this hypothesis when they compared four dietary fibers for their effects on colonic function (fecal bulking, fecal SCFA production), glycemic control, and serum lipid metabolism. Healthy human participants were randomly assigned low-fiber control (LF), high-fiber wheat bran, RS$_2$ or RS$_3$ enriched foods for two weeks, with a two-week wash out period between treatments. Out of all the treatments, fecal butyrate was found to increase 45±17% after RS$_2$ and 66± 26% after RS$_3$ treatment ($p=0.015$ and $p=0.02$ respectively) when compared to LF, whereas wheat bran increased 48± 21% ($p=0.030$) when compared to LF. Yet these results still supports that some types of RS might improve colonic health by increasing SCFA production particularly butyrate.

Similarly, another study was conducted comparing diets containing no starch (SF), wheat starch fully digested in the small intestine (R+SDS), wheat bran (Bran-NSP), potato RS (Potato-RS2), banana RS (Banana-RS2), processed wheat starch (Wheat-RS3) and processed maize
starch (Maize- RS3) in 12 healthy adults over a 15 day period (Cummings et al., 1996). Two
diets, Potato-RS2 and R + SDS, showed the highest total SCFA concentrations excreted in feces.
Lower fecal acetate molar ratios were observed for diets with RS3 and lower propionate molar
ratios were observed for diets with RS2. Differences in fecal acetate concentrations reached
significance (p<0.05) when RS3 diets were compared to bran-NSP and banana-RS2 diets and for
propionate when RS2 diets were compared to R + SDS and wheat-RS3 diets. Changes in fecal
butyrate were only observed in potato-RS2 diet, which was found to be significantly greater than
R+SDS, bran-NSP and wheat-RS3 diets. To summarize results of this study, RS2 shows promise
in increasing total SCFA production and increasing butyrate production over RS3; while RS2 and
RS3 might be used lower propionate and acetate concentrations respectively.

Evidence of comparative fermentation studies point to different types of starches
generating different fermentation profiles. Not all RS increases butyrate production. Some
studies have found additive effects when dietary fibers were mixed. Muir and associates (1994)
found that a combination of wheat bran and RS reduced transit time and fecal pH, but increased
fecal output and excretion of SCFA. As for glycemic response there seems to be no observable
trend as to which dietary fibers preforms best.

D.4 Gastrointestinal Symptoms

The health and gastrointestinal benefits of dietary fibers and RS have previously been
discussed (see section B.1). But as for all substances there is an upper and lower tolerance.
Therefore, at some dose we know RS will cause adverse GI effects. Effective and recommended
doses have been made for RS by different researchers to improve various physiological
endpoints; however, defining an adverse dose has been challenging. Adverse GI effects of low-
digestible carbohydrates are influenced by characterization of carbohydrate (e.g., type, degree of polymerization), dose, and human host interaction and variability (Grabitske and Slavin, 2009); which makes blanket recommendations for tolerance of RS hard to quantify.

With the growing interest in the use of RS there is yet little research on possible adverse effects, which is not alarming because dietary fibers in general seem to pose little risk of adverse effects. According the Dietary Reference Intake by the Institute of Medicine, no tolerable upper intake level (UL) has been set for dietary fibers since consumption of dietary fibers are normally self-limiting.

Furthermore, adverse GI symptoms are normally not primary end points in RS or dietary fiber studies. There are some RS feeding studies that have reported GI symptoms, although RS dose and type along with observed GI symptoms are underreported. Gastrointestinal symptoms such as bloating, abdominal discomfort and diarrhea have been anecdotally associated with an increase in dietary fiber. However, the dose of dietary fiber loosely correlated with adverse GI symptoms has not been well defined. As increased consumption of dietary fiber can have minor adverse side effects, one can postulate that theoretically the increased consumption of RS will do the same.

RS as well as other forms of dietary fiber can have a laxative effect and increase the ease and/or frequency of laxation (Heijnen et al., 1996; Noakes et al., 1996). Increased or excess intakes may cause acid reflux and heartburn, diarrhea, flatulence, bloating, and/or abdominal discomfort (See table 7). Such GI symptom though normally transient can negatively alter the perceptions of consumers that may cause conscious reductions in the amount of RS/dietary fiber foods consumed.
Table 7. Tolerance terms used to assess symptoms of RS consumption. Adapted from Livesey 2007

<table>
<thead>
<tr>
<th>Acute reflux</th>
<th>Flatulence</th>
<th>Rumbling in stomach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belching (eructation)</td>
<td>Gripe</td>
<td>Stomachache</td>
</tr>
<tr>
<td>Borborygmi – rumbling in intestines</td>
<td>Heartburn</td>
<td>Toilet visits</td>
</tr>
<tr>
<td>Burping</td>
<td>Laxation</td>
<td>Undergarment staining</td>
</tr>
<tr>
<td>Colic</td>
<td>Loss of appetite</td>
<td>Vomiting</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>Meteorism</td>
<td>Watery feces</td>
</tr>
<tr>
<td>Distension</td>
<td>Nausea</td>
<td></td>
</tr>
<tr>
<td>Fecal incontinence</td>
<td>Rumbling in gut</td>
<td></td>
</tr>
</tbody>
</table>

Gastrointestinal symptoms and discomforts, such as flatulence, bloating, and abdominal pain are unpleasant but normally not harmful. Likewise flatulence, burping and stomach rumbling are possibly embarrassing but not harmful. Notwithstanding, GI distress such as diarrhea can become problematic. Excessive diarrhea can lead to dehydration and loss of fluids, sodium, chloride, and potassium, which can lead to serious health issues for children, elderly, and immune-compromised people. Besides GI symptoms and discomforts, other serious side affects can occur with dietary fiber consumption: interfere with absorption of nutrients, inhibit efficacy of medication, and GI blockages normally occurring with supplemented dietary fibers.

Gastrointestinal symptoms can occur for a number of reasons; however, adverse GI symptoms are a concern with any increases in fiber that putatively intensify with dose. Conversely, minimal GI symptoms were observed by symptoms survey in a human study where four dietary fibers, one of which was resistant starch, were used to increase fiber consumption by 12g a day for 2 weeks (Stewart et al., 2010). Stewart and associates (2010) supplemented dietary fibers to increase consumption of fiber above what was naturally present in participants’ habitual diets. A daily 12g increase of dietary fiber was well tolerated even though plausibly any increase in fiber could cause GI distress, since dietary fiber tolerance is individually variable. Specifically for RS, no adverse effects were reported by bowel habit diaries in an acute study where 20 males were given 80g of a 60% resistant Hi-Maize® 260 product (48g of type II RS) in a day.
(Bodinham et al., 2010). The dose of 48g would be considered a high dose of dietary fiber since recommended values of dietary fiber are 25-38 g per day.

In the human feeding studies conducted as part of this dissertation, moderate doses of RS were fed as a one-meal replacement in normal diet and few GI symptoms were reported. Most human feeding studies of RS also report minimal or no adverse effects to RS even at high does of 60 g (Karalus et al., 2012; Klosterbuer et al., 2013; Li et al., 2010; Storey et al., 2007; Tomlin and Read, 1990; Wolf et al., 2001). Therefore, there is sufficient evidence to support shifting the percentage of RDS to SDS or RS might prove to be advantages to lowering postprandial glucose response and improving health with little to no adverse gastrointestinal events.

E. References


CHAPTER 3. THE EFFECTS OF NATURALLY OCCURRING RESISTANT STARCH IN MAIZE ON POSTPRANDIAL GLYCEMIC RESPONSE IN HEALTHY HUMANS

A paper to be submitted to *Nutrition Research*

Esther M. Haugabrooks, Hyun Jung Kim, Linda Pollak, Suzanne Hendrich

**List of Abbreviations**
AUC: Area Under the Curve  
AR: Argentine Normal yellow dent corn starch  
DF: Dietary Fiber  
Guat: Guatamala high-amylose corn starch  
Guat/AR: Hybrid cornstarch from Guat and AR

**ABSTRACT**

Resistant starches (RS) are complex partly indigestible carbohydrates that have shown promise in improving digestive health and glycemic response. The use of RS is gaining popularity in commercial products, generating interest in practical agronomic production for variable RS sources. In the present study, cornstarch derived from conventional maize (AR), high RS maize (Guat), and maize crossbred to enhance yield of high RS maize (AR/Guat) were used to evaluate the change in blood glucose compared with standard commercial cornstarch (CCS). A four-week randomized blinded crossover study was conducted using AR, Guat, AR/Guat, and CCS in a pudding fed to 11 participants (6 men and 5 women). Blood samples were collected -15, 0, 15, 30, 45, 60, 75, 90, 120, 180, and 240 min after ingesting the pudding starches. There was no significant treatment effect on plasma glucose Area Under the Curve (AUC). Guat, the treatment with the highest digestion resistance at 26.2% of total starch, showed the lowest change from baseline with a 30 min postprandial glucose peak of 6.87 ± 0.27 mmol/L, indicating that Guat had a lower glycemic increase after a RS meal at 30 min, significantly different from Guat/AR (7.48 ± 0.26, p= 0.03) and AR (7.57 ± 0.26, p=0.01).
Participants reported no adverse gastrointestinal effects in response to resistant starch meals. Lower glucose responses with minimal to no adverse gastrointestinal effects supports the role of RS in novel corn lines potentially improving postprandial blood glucose with promise to aiding in preventing diabetic complications.

Key Words: resistant starch, glycemic response, humans, dietary fiber, crossover

INTRODUCTION

Various scientific publications have substantiated the health benefits and implications of increased dietary fiber (DF) consumption to aid in hypertension, risk of cardiovascular disease, and metabolic syndrome lowering risk of diabetes mellitus (Carlson et al., 2011; Keenan et al., 2002; Marlett et al., 2002; Wolk et al., 1999). In addition, the benefits of DF have been supported by approved U.S Food and Drug Administration (FDA) health claims outlining the consumption of DF in fruit, vegetables, and grains for the reduction of some types of cancer (21 CFR 101.76). Resistant starch (RS), as a source of DF, has some of the same health benefits as other DFs and can be used to aid in improvement of digestive health and further favorable biological effects (Jenkins et al., 1998; Nugent, 2005). Research has associated RS with increased mineral uptake of zinc in humans (Behall et al., 2002), modulation of gut microbiota presumably for enhanced fermentation (Abell et al., 2008; Walker et al., 2011), colorectal cancer protection (Cassidy et al., 1994), and improved blood glucose and insulin response (Behall and Hallfrisch, 2002).

RS is defined as the portion of starch that undergoes incomplete digestion in the small intestine (Asp, 1992) and is largely fermentable in the lower gut (Cummings and Englyst, 1991;
Cummings et al., 1996). Due to low glucose bioavailability of RS in the small intestines, the
glycemic load for RS containing foods is lower than rapidly digestible (RDS) or slowly
digestible (SDS) starches (Englyst et al., 1996, 1999), as the names (SDS and RDS) imply.
Maintaining stable blood glucose concentrations has positive implications especially for the
improvement of metabolic syndrome where controlling blood glucose within normal
concentrations is ideal. There is emerging evidence that the use of RS in the diet beneficially
modulates blood glucose, insulin and C-peptide; improves blood lipid profile; reduces oxidative
stress and energy to aid in management of diabetes and obesity (Bodinham et al., 2010; García-
Rodríguez et al., 2012; Kwak et al., 2012; Park et al., 2004). For people with diabetes and
people with high risk factors for diabetes mellitus, like obesity, controlling blood glucose
through diet is advantageous. Increasing RS concentration in food products fed to healthy
individuals has shown to reduce blood glucose area under the curve (AUC) by over half (57%)
when compared to a control (Granfeldt et al., 1995). Although RS meals have been shown to
significantly decrease postprandial glucose when compared to a control (Raben et al., 1994),
contrary results were reported with RS (type 2 and 3) had no effect on glucose response when
compared to a low-fiber control (Jenkins et al., 1998). However, overall replacing portions of a
rapidly digestible starch with slowly digestible or digestion resistant starch generally reduces
glucose concentrations (Achour et al., 1997; Behall and Hallfrisch, 2002; Behall et al., 2006;
Vonk et al., 2000) people with diabetes mellitus (García-Rodríguez et al., 2012) and even in
borderline diabetics (Yamada et al., 2005).

On average Americans are consuming less DF than the recommended 25-38 grams per
day. Though hard to truly estimate, based on combined data from the US Department of Health,
Human Services and National Health and Nutrition Examination Surveys (NHANES), and
literature-based RS database, Americans are consuming approximately 3 to 8 grams of RS per person per day (Murphy et al., 2008). In order to meet the DF recommendations, the 2010 Dietary Guidelines for Americans suggested increasing consumption of foods with naturally occurring fiber (U.S. Department of Agriculture and U.S. Department of Health and Services Human, 2010). There are many naturally occurring sources of RS, such as bananas, potatoes, certain legumes, whole grains, and corn, although RS content can be altered by chemical (Al-Tamimi et al., 2010) and genetic modification (Li et al., 2010). Providing natural sources of RS that have not been chemically or genetically modified might be a consumer conscious approach to increasing recommended DF consumption while promoting lower postprandial glycemic response for subpopulations of interest.

Producing RS sources or products with physiologically relevant doses is a concept of considerable scientific interest. When formulating a RS product, various food preparation conditions (e.g., cooking, baking) can cause alteration of RS concentrations (Eggum et al., 1993; Siljestriim and Asp, 1985). However, subsequent to any product preparation, RS concentrations also vary by source. Several commercial products are available which use RS maize as a DF substitute. Maize is an abundant, popular, and variable source of RS since its amylose content can be genetically manipulated. Although genetic modification has produced viable RS, which can be classified as type IV, manipulation of amylose content can be done through conventional crossbreeding of maize. Investigation to produce novel and stable corn line with increased amylose is still ongoing.

In this study we used resistant starches from a novel crossbred corn lines in a pudding-like product to evaluate their influence on blood glucose response. We hypothesized that starches from parent corn lines and starches from corn conventional crossbred to vary in RS concentration
will provide naturally occurring resistant starches that lower postprandial blood glucose response when compared to commercially available a corn starch with low RS content.

**MATERIALS AND METHODS**

*Participants and Study Design*

Healthy non-smoking adults between 18-45 years of age were recruited from Iowa State University and surrounding community. Recruited volunteers were assessed by a health questionnaire for inclusion. Participants were selected based on the following inclusion criteria: not taking any drugs or medications, no use of antibiotics within the last six months, non-smokers, and not allergic to corn. Individuals with diabetes (type I or II) and/or gastrointestinal diseases were excluded.

Out of 49 volunteers who screened for the study, 15 eligible participants were entered, and 11 (6 men, 5 women) completed. Characteristics for the 11 participants were 25 ± 4.3 years of age, 67.8 ± 4.11 height (in), 162.4 ± 31.5 pounds, and 24.8 ± 3.6 BMI. The experiment was conducted in a randomized blinded crossover design. Participants were scheduled for a total of four days one week apart to consume the pudding product. After a 12-hour overnight fast, participants consumed one of four lemon-flavored puddings made from corn starch. Thereafter, finger pricks for blood glucose samples were obtained at time points 0, 15, 30, 45, 60, 75, 90, 120, 180, and 240 minutes. Gastrointestinal symptoms where assessed 24-hours after consuming pudding product by a gastrointestinal questionnaire. The Institutional Review Board of Iowa State University approved the study design and recruitment procedures. Informed consent was obtained from all participants prior to entry into the study.
Assessment of Gastrointestinal Distress

Participants were asked to fill out a gastrointestinal questionnaire for 24 hours after ingestion of test pudding describing individual events of gastrointestinal distress and rating overall intensity of gastrointestinal symptoms. Overall occurrences of gastrointestinal bloating, gastrointestinal pain, and diarrhea were rated on a 0-10 scale where 0 = none; 1 to 5 = mild; 6 to 7 = moderate; 8 to 9 = severe, 10 = worst possible. Individual events of gastrointestinal distress (such as discomfort, feeling bloated, gas, pain, burping, flatulence, diarrhea, or loose stools) experienced over the 24-hour period were recorded by symptom time of onset and cessation, and level of discomfort. Level of discomfort was expressed numerically 1 = lowest, 10 = most severe possible; or number of flatulence where applicable.

Corn lines and corn starches

Three corn starches were tested against a commercially available cornstarch for postprandial glucose response. Of the three starches of interest, two were parent lines, Normal yellow dent corn (AR) and high-amylose corn (Guat), and the third conventionally crossbred line Guat/AR high amylose hybrid (Guat/AR). Guat was an exotic corn accession from Guatemala with pedigree Guat209:S13/Oh43ae/H99ae-1-2-1-B-B-02-B. AR pedigree AR011050:S01:1081-1-2-B was an exotic non-mutant breeding corn line from Argentina. Guat/AR pedigree 209/S13 OH43aeH99ae had an amylose extender recessive gene expressed in the presence of both alleles. Guat and AR were planted in 200 rows, Guat/AR was planted in 267 rows. Corn was grown in 2009 at Iowa State University Agronomy Farms. The plants were self-pollinated, hand harvested, force-air dried at room temperature, and mechanically shelled. The seeds were bulked, ground into meal, and found to be negative for the presence of mycotoxins.
(deoxynivalenol and related trichothecenes, aflatoxin B1, fumonisin B1) by the North Dakota State University Veterinary Diagnostic laboratory. Bulk seeds were processed via wet milling at Iowa State University for corn starch. The resulting three corn starches were tested against Argo corn starch (ACH Food Companies Inc. Cordova, TN), a commercially available corn starch (CCS) that contains very little RS.

*Mycotoxin analysis of corn lines*

Corn meal from each of the corn lines showed no detectable contamination for any mycotoxin (deoxynivalenol and related trichothecenes, aflatoxin B1, fumonisin B1), tests performed at Veterinary Diagnostic laboratory, North Dakota State University. Therefore, starches derived from wet milling of corn meal would contain no detectable levels of mycotoxins.

*Test Meal: Pudding recipe*

The corn starches were mixed in three parts water. Splenda® Sucralose micronized powder (200 mg/serving, Tate & Lyle Sucralose, Inc. Decatur, IL, USA) was added for a sweet taste and lemon extract (5g/serving, McCormick Corporation, Sparks MD) was added for flavor. Under continual stirring the puddings were made by bringing the corn starch and water mixture to a medium boil. Yellow food coloring (McCormick Corporation, Sparks MD) was added for aesthetics (1-5 drops were added to achieve pale yellow coloring) and puddings were portioned and allowed to cool at room temperature before stored at 4 °C until consumption (less than 48 hours after preparation). Each pudding portion contained 50 g of cornstarch.
Analysis: Resistant Starch, Moisture, and Blood Glucose

The determination of resistant starch content in puddings and in raw starch samples was conducted by AOAC method 2002.02 using Megazyme Kit (Megazyme International Ireland Limited, Bray Business Park, Bray, Co. Wicklow, Ireland). RS concentrations were determined from wet weight. Moisture content was measured gravimetrically for each sample by drying pudding samples overnight at 110°C.

Blood samples were collected by finger pricks in heparinzed capillary tubes (Fisher Scientific Inc. Hampton, NH). To obtain plasma, whole blood samples were centrifuged at 3000 rpm for 7 minutes. All plasma was frozen at – 80°C until analysis. Plasma glucose concentrations were analyzed using a Biochemistry Analyzer 2700 Select (YSI Incorporated, Yellow Springs, OH). An Area Under the Curve for plasma glucose concentration was calculated using the trapezoidal rule, omitting negative measurements.

Statistical Analysis

Data were analyzed using SAS 9.2 (SAS Institute, Cary, N.C., USA). AUC data were analyzed using an ANOVA linear model with treatment and week as fixed effects. Random effect terms were used for participant difference to consider dependency among observations from the same participants and variability between participants. Analysis of glucose response treatment differences by time point followed the same model as AUC data, except time point 0 was included as a baseline in the fixed effect in which the difference from baseline was analyzed. Data was pooled for male and female. Statistical significance was set at \( p < 0.05 \).
RESULTS

Resistant Starch Content

Corn starches used to make test meals were Guat, Guat/AR, AR, and CCS. The RS content of the raw starch was 28.8, 11.7, 3.1, and 0.6 percent, respectively (Table 1). Crossbreeding of Guat and AR corn lines resulted in the production of a cornstarch, Guat/AR, with an intermediate resistance. Therefore, the parent line Guat was the test starch with the highest RS content, followed by Guat/AR, AR, and CCS. Moisture content for all puddings was between 85-90 percent. Resistant starch analysis of pudding products provided similar characterization of RS content as did the raw starch except for AR and CCS values were closer in pudding products than raw starches. Guat, Guat/AR, AR, and CCS had 13.1, 5.3, 2.4, and 2.7 grams of RS per serving size, respectively.

Blood Glucose Response

Although Guat had the lowest AUC of all starches, there was no significant difference in AUC between pudding types. Total AUC values were 194.38 ± 30.51, 211.08 ± 30.51, 159.55 ± 30.41, 183.61 ± 32.43 for Guat/AR, Guat, AR, and CCS, respectively (Fig 1). General trends of glucose response due to treatment; however, can been observed at the time point level. Glucose concentrations in plasma reached maximum between 30-45 minutes after ingestion of pudding for all treatments. Plasma samples from individuals that consumed Guat pudding had the lowest glucose values at 30 minutes, but the change from baseline for Guat was only significantly different from Guat/AR (7.48 ± 0.26, p= 0.03), AR (7.57 ± 0.26, p=0.01). Despite their difference in RS content (Table 1) and difference in glucose concentration at 30 min, Guat difference from baseline was the same as CCS. Mean AUC values across treatments were not
significant though trends can be observed. Inter-individual variability for time points of all treatments was evident, specifically during elevation of postprandial peak.

*Gastrointestinal symptoms*

No gastrointestinal symptoms differences were observed due to resistant starch puddings. Overall, few gastrointestinal symptoms were reported. Throughout the duration of four weeks 5 events of bloating, 2 events of pain, and 1 event of diarrhea were reported for all treatments. No symptoms were reported with intensity above 7 (moderate score could be reported as 6 or 7).

**DISCUSSION**

We expected to see a lower postprandial peak from puddings formulated from starches with the higher RS contents. Although a trend was observed with Guat at postprandial peak, total glucose AUC did not reach significance. However, this outcome is not uncommon. Glycemic responses to a RS meal have shown divergent results. In a glucose tolerance test where 25 healthy participants were fed cornstarch in a bread product with 30, 40, 50, 60, or 70% amylose (2, 3.8, 8.2, 11.5, and 13.4 g of RS respectively), a lower AUC in glucose response was observed when participants were fed 60 or 70% amylose thus estimating the recommended amounts of RS (presumably type II) to lower blood glucose response to be between 11.5 and 13.4 g (Behall and Hallfrisch, 2002). Another study used 5, 15, and 25g of type III RS in cereal bars and beverages and observed no difference between RS doses in postprandial blood glucose AUC of 22 healthy participants (Kendall et al., 2010). Likewise, other studies have investigated consumption of RS and glucose response resulting in variable outcomes, which have raised the question- what is a biologically relevant type and dose of RS?
In our study 13.1 g of RS in the Guat pudding was fed to participants, which encompasses the range that Behall and associates recommended for lower glucose AUC. Yet, overall we found no significant reduction in postprandial glucose AUC, although, an apparent reduction in postprandial peak from consumption of Guat was evident. Differences have been found in glucose response after consumption of one RS meal compared only to a control. The following studies fed healthy humans 50g/treatment/person of raw potato starch and pregelatinized potato starch in a syrup to 10 males (Raben et al., 1994); retrograded cornstarch and pregelatinized cornstarch in a porridge-like gel to 8 participants (Achour et al., 1997); RS bread against a white bread to 20 males (Hasjim et al., 2010) in order to evaluate the change in glucose response from the control.

Glycemic response studies ideally generate an AUC from 5 or more time points and where overall AUC has failed to provide evidence of a difference, investigation of specific time points has given insightful results. Numerous studies have seen no difference in treatments with AUC, however, describe notable differences when comparing treatments across time points (Behall et al., 1988; Kendall et al., 2010; Yamada et al., 2005). A postprandial glucose peak is typically observed between 30-45 minutes. This time frame is a good indication of the amount of glucose absorbed from the small intestine and released into the blood stream. During this time frame bioavailability of glucose from meal due to treatment might be easier to observe. As in our study, significant difference between treatments have been observed during this peak when AUC failed to show evidence of a differences (Behall et al., 1988).

Glucose concentration will begin to return to baseline values between 90 and 240 minutes. By 240 minutes, glucose concentrations should return to concentrations (3.9 - 5.6 mmol/L) similar to fasting baseline measurements. The quick return of glucose after a meal is
presumed as healthy for prevention of type II diabetes in which is implicated to lower the risk of cardiovascular disease. For people who suffer from type II diabetes, maintaining glucose concentrations below 7.8 mmol/L after meals is advantageous. Thus, starches that show promise of lower glycemic response at 30-45 and 90-240 minutes are ideal.

When accounting for the change from baseline after 30 minutes and despite their differences in RS content, the rise in postprandial plasma glucose concentration as a result of consuming the Guat pudding was comparable to that of CCS though different from Guat/AR and AR (Fig 1). Although no explanation can be given for baseline differences since participants were entered into the study based on fasting glucose \( \leq 100 \text{ mg/dL} \), in addition to overnight fasting before each experimental day.

Crossbred corn lines to improve amylose content have been shown to resist enzymatic digestion in vitro (Li et al., 2008) making them plausible candidates to use in the reduction of glucose response. However, composition of starches, crystallinity and RS classification (Type I-IV), food product, matrix, and processing are all critical components to consider in digestion of starches. For instance, in the present study, raw starches showed different RS content for each starch (Table 1). When cooked, AR and CCS had similar RS content per gram even though they were derived from different corn lines (CCS. Argo #10 Dent) and processed on a different scale. The process of pudding preparation altered RS contents from raw starches resulting in the same RS content in AR and CCS puddings.

Shifting the percentage of rapidly digestible starch to slowly digestible or resistant starch might prove to be advantageous to people with diabetes and obesity. An increase in dietary fiber anecdotally can be associated with gastrointestinal distress, although very few studies report strong evidence of gastrointestinal distress with increased RS consumption. In studies in which
an increase of 35-60g/day of RS increased gastrointestinal distress (Hylla et al., 1998; Muir et al., 1994; Phillips et al., 1995), reports were only a mild to moderate. Mild to moderate gastrointestinal symptoms have been observed at lower doses (12g of RS) (Stewart et al., 2010), but a larger number of studies in which gastrointestinal events were reported report minimal to no evidence of adverse responses due to RS consumption (Karalus et al., 2012; Klosterbuer et al., 2013; Li et al., 2010; van Munster et al., 1994b; Storey et al., 2007; Tomlin and Read, 1990). Overall, gastrointestinal symptoms are easy to assess though tricky to define meaningful application since most measurements of gastrointestinal distress (e.g., bloating, pain, increased flatulence, stool consistency) through questionnaires, bowel diaries, or interviews are subjective.

In this study gastrointestinal distress was self-reported. Participants were encouraged to be detailed and record symptoms as they occurred, because this was an acute feeding study in which total food intake of the day was likely to be greater than food intake from one treatment meal, symptoms of gastrointestinal distress could be from other sources. In present study, prior diets and subsequent meals after puddings were not controlled, although other researchers have controlled the diet of participants via interventions of planned diets (Noakes et al., 1996); however, those tend to be for long-term studies with multiple variables and might not be necessary for acute feeding studies to assessing postprandial glucose response and gastrointestinal stress from RS meal.

Overall, participants reported no adverse gastrointestinal effects due to resistant starch meals. In our study, participants were fed at most 13 g of RS per serving size. Minimal gastrointestinal symptoms were also reported in a human study in which four DFs, one of which was resistant starch, were used to increase fiber consumption by 12 g a day for 2 weeks (Stewart et al., 2010). No adverse effects were found in an acute study in which 20 males ingested 48 g of
RS in a day (Bodinham et al., 2010). Our study is similar to Bodinham et al. (2010) with respect to duration; therefore, the acute feeding of 13 g RS in a day showing a lack of adverse gastrointestinal effects is not surprising.

Lower glucose responses with minimal to no adverse gastrointestinal effects supports the role of resistant starches in potentially improving postprandial blood glucose and maintaining health. This research showed the pudding with the highest resistance starch played a biological role in reducing peak blood glucose concentrations compared to two of the more digestion resistant treatments. However, differences between starch sources of 8-10g of RS, with the greatest content fed of 13 g in a meal, did not result in significant differences in glucose AUC. A more palatable product might facilitate volunteer participation since in present study a quarter of original participants did not complete the pudding study.

ACKNOWLEDGMENTS

The Iowa State University Plant Sciences Institute supported this study and special thanks to Ignacio Alvarez-Castro and Dason Kurkiewicz for statistical consulting.

REFERENCES


## FIGURES AND TABLES

Table 1. RS percentages of total starch, and grams of RS/serving

<table>
<thead>
<tr>
<th>Starches</th>
<th>%RS Pudding</th>
<th>%RS Raw starch</th>
<th>RS/serving size (g)</th>
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<tbody>
<tr>
<td>Guat</td>
<td>26.2 ± 0.31</td>
<td>28.8 ± 0.57</td>
<td>13.1</td>
</tr>
<tr>
<td>Guat/AR</td>
<td>10.5 ± 0.42</td>
<td>11.7 ± 1.61</td>
<td>5.3</td>
</tr>
<tr>
<td>AR</td>
<td>4.8 ± 0.31</td>
<td>3.1 ± 2.09</td>
<td>2.4</td>
</tr>
<tr>
<td>CCS</td>
<td>5.4 ± 0.30</td>
<td>0.6 ± 0.03</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Percentage of resistant starch was determined from wet weight of total starch content for each pudding treatment. Estimate of grams RS/serving size calculated from 50g of raw starch used in pudding preparations. Means ± SE for RS percentage of total starch of pudding treatments across all weeks compared to mean ± SE of raw starches.
Fig 1. Plasma glucose response curves after consumption of pudding treatment expressing the variability at each time point as mean ± SE, n=11.
### APPENDIX

Table 1. Plasma glucose mean ± SE mmol/L by time points.

<table>
<thead>
<tr>
<th>minutes</th>
<th>Guat/AR</th>
<th>AR</th>
<th>Guat</th>
<th>CCS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-15</td>
<td>4.45 ± 0.28</td>
<td>4.44 ± 0.29</td>
<td>4.61 ± 0.29</td>
<td>4.61 ± 0.31</td>
<td>0.92</td>
</tr>
<tr>
<td>0</td>
<td>4.65 ± 0.26</td>
<td>4.59 ± 0.26</td>
<td>4.59 ± 0.27</td>
<td>4.73 ± 0.28</td>
<td>0.96</td>
</tr>
<tr>
<td>15</td>
<td>5.72 ± 0.26</td>
<td>5.72 ± 0.26</td>
<td>5.57 ± 0.27</td>
<td>5.92 ± 0.28</td>
<td>0.69</td>
</tr>
<tr>
<td>30</td>
<td>7.48 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.57 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.87 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.41 ± 0.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.05</td>
</tr>
<tr>
<td>45</td>
<td>7.07 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.39 ± 0.26&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.43 ± 0.27&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>6.92 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
</tr>
<tr>
<td>60</td>
<td>6.07 ± 0.26</td>
<td>6.53 ± 0.26</td>
<td>5.84 ± 0.27</td>
<td>6.30 ± 0.28</td>
<td>0.08</td>
</tr>
<tr>
<td>75</td>
<td>5.41 ± 0.26</td>
<td>5.68 ± 0.26</td>
<td>5.48 ± 0.27</td>
<td>5.76 ± 0.28</td>
<td>0.57</td>
</tr>
<tr>
<td>90</td>
<td>5.11 ± 0.27</td>
<td>5.18 ± 0.26</td>
<td>5.18 ± 0.27</td>
<td>5.17 ± 0.28</td>
<td>0.99</td>
</tr>
<tr>
<td>120</td>
<td>4.94 ± 0.26</td>
<td>4.86 ± 0.26</td>
<td>4.95 ± 0.27</td>
<td>5.01 ± 0.28</td>
<td>0.96</td>
</tr>
<tr>
<td>180</td>
<td>4.92 ± 0.26</td>
<td>4.47 ± 0.26</td>
<td>4.76 ± 0.27</td>
<td>4.82 ± 0.28</td>
<td>0.41</td>
</tr>
<tr>
<td>240</td>
<td>4.49 ± 0.27</td>
<td>4.37 ± 0.26</td>
<td>4.54 ± 0.267</td>
<td>4.68 ± 0.28</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Means sharing the same superscript within a row are not significantly different. P values are a main time point effect and superscripts within rows are treatment within time point comparisons. Time point -15 is baseline measurements before pudding consumption; time point 0 (zero) was glucose measurements taken immediately after.

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Table 1.1. Least Square differences of pudding treatments by time point

<table>
<thead>
<tr>
<th>Differences of LSM</th>
<th>30 minutes</th>
<th>45 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guat/AR vs 2</td>
<td>0.70</td>
<td>0.48</td>
</tr>
<tr>
<td>Guat/AR vs Guat</td>
<td>0.03</td>
<td>0.19</td>
</tr>
<tr>
<td>Guat/AR vs CS</td>
<td>0.54</td>
<td>0.93</td>
</tr>
<tr>
<td>AR vs Guat</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>AR vs CS</td>
<td>0.35</td>
<td>0.46</td>
</tr>
<tr>
<td>AR vs CS</td>
<td>0.15</td>
<td>0.26</td>
</tr>
<tr>
<td>Main treatment effect</td>
<td>0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>
ABSTRACT

Stearic acid-modified resistant starch was compared with its parent high amylose starch for physiological effects on healthy humans. In a randomized blinded crossover study, after a 10-h fast, 30 participants ate crackers providing 50 g carbohydrate approximately 6% (control starch crackers, CS), 24% (high amylose crackers, HA) and 58% (stearic acid-high amylose crackers, RS5) resistant starch as measured by AOAC method 991.43. Cracker types were given solely as a test meal once a week over a period of 3 weeks. Glucose, insulin, and fermentation responses were evaluated. Area under the curve (AUC) for blood glucose, was not different between HA and RS5; both produced lesser AUC responses than CS (p < 0.001). Reduced insulin AUC was found only in HA when compared to CS (p < 0.01). In vitro digestion of crackers was also evaluated by AOAC method 2002.02 showing different RS content than 991.43 method. In vivo postprandial glucose (PPG) response corresponded with AOAC 2002.02 in vitro digestion for each starch. Fermentation, assessed by breath hydrogen, was greater after ingestion of RS5 than after eating HA or CS (p < 0.05). Fermentation assessed by short chain fatty acid (SCFA) production was not different between cracker types. Gastrointestinal symptoms assessed from a questionnaire were minimal and not statistically different between cracker types. This study indicates that further study of health effects of the fermentability of RS5 should be feasible in humans.
INTRODUCTION

The portion of starch not digested in the small intestine is passed to the colon where fermentation can ensue is termed resistant starch (RS). These types of starches (I-V) have been implicated for reduction of glucose and insulinemic response (Behall and Hallfrisch, 2002; Brouns et al., 2007; Noakes et al., 1996; Wolf et al., 2001) and for their fermentative ability (Ahmed et al., 2000; Cummings et al., 1996; McOrist et al., 2011). Fermentation by colonic microbiota which has been associated with a bevy of beneficial physiological responses (Fuentes-Zaragoza et al., 2010; Nugent, 2005; Topping et al., 2003) mainly through the production of acetate (C2), propionate (C3), and butyrate (C4); however, valerate (C5) and caproate (C6) can also be produced. In addition, consumption of RS has been implicated for potential amelioration in gastrointestinal ailments (Higgins and Brown, 2013) and metabolic syndrome (Johnston et al., 2010). Though accumulated evidence for the use dietary RS for the modulation of human health is not always consistent, unequivocally RS has been used as a dietary fiber.

RS starches have evolved from mere scientific intrigue to incorporation into commercial products. Interest in RS as a dietary fiber has become so prodigious new starches are being modified and tested to quantify efficacy to influence physiological responses such as postprandial glucose (PPG), insulin (PPI), and fermentation. RS types I-V are commonly cited in literature. However, a novel type of RS is emerging, typed RSV, which has been modified by lipid starch interactions. RSV has shown evidence to improve PPG and PPI responses (Hasjim et al., 2010) with far-reaching implications for management of diabetes mellitus and obesity. The
goal of this study was to evaluate the effect of RSV and RSII incorporated into a practical food on glucose, insulin, and fermentation response with humans in vivo.

MATERIALS AND METHODS

Participants

Healthy participants, assessed by health questionnaire, were recruited from Iowa State University and surrounding community. Participants were chosen based on inclusion criteria of not taking any drugs, medications, or antibiotics within the last six months, non-smokers, not allergic to corn, no gluten sensitivity, and within 18-45 years of age. Exclusion criteria were volunteers with diabetes (type I or II), gastrointestinal diseases, fasting plasma glucose concentration ≥ 100 mg/dL, body mass index (BMI) > 30, and pregnant. While entered in the study, use of oral contraceptives was permissible.

A total of 58 volunteers were screened for the study, 30 participants who met all inclusion and exclusion criteria were entered into the study. In total, 29 participants completed the study (14 female, 15 male). Participant characteristics for male and female participants were 25 ± 5.4 (19-42) years of age, 23 ± 3.5 (17-29) BMI, 89 ± 6.2 (79-98) mg/dL fasting glucose. Where gender differences were present, males and females were represented separately in results. Therefore, participant characteristics by gender are as follows: females 24 ± 3.6 (19-30) years of age, 23 ± 3.5 (17-29) BMI, 90 ± 5.9 (81-98) mg/dL fasting glucose; males 26 ± 6.6 (19-42) years of age, 24 ± 3.6 (18-29) BMI, 88 ± 6.9 (79-97) mg/dL fasting glucose. Participant characteristics are reported as mean ± SD (range). Ethnicities of participants were 12 White, 9 Asian/Asian American, 5 African/African American, 3 Other, and 1 Latino/Hispanic.
The Institutional Review Board at the Iowa State University approved the study design and recruitment procedures. Oral and writing explanations for the design and purpose of the study was given to each participant prior to entry into the study. The experimental design and possible gastrointestinal discomforts were explained to the participants before written informed consent was obtained. All participants who entered the study signed an informed consent before first experimental day.

**Cracker preparation**

Three test starches were used to formulate experimental crackers: 1) commercial corn starch, product name Argo (ACH Food Companies Inc. Cordova, TN), obtained from a local grocery store; 2) a native high-amylose maize starch, commercial product name AmyloGel 03003, courtesy of Cargill (Hammond, IN); 3) a stearic acid lipid-modified corn starch developed at Iowa State University. Crackers prepared from these starches are termed CC, HA, and RS5, respectively.

Crackers were formulated to have 50 grams of starch per serving. For RS5, 10 % of the volume was attributed to stearic acid complex; therefore, 55 g of raw starch was used per serving to account for total starch differences. For all of other ingredients, each test cracker contained 8 g gluten, 7 g vegetable shortening, 1 g whey, 1 g salt, and 0.7 g baking powder per serving size. Varying amounts of water per cracker type was added to knead ingredients together. Due to different water binding capacity, 33 ml, 45 ml, and 68ml per serving size was used to obtain workable doughs for CC for HA, for RS5 crackers, respectively. CC, HA, and RS5 crackers were baked at 400 °C until light to golden brown which was approximately 9, 12, and 17 minutes, respectively. The crackers were prepared each week and frozen until consumption.
Total serving size of crackers/day was served in a sealed bag at breakfast with a glass of water. Participants were required to consume the entire bag within 10 minutes.

**Experimental Design**

Study was conducted as a randomized blind crossover design. The duration of the study was 3 weeks with one-week washouts between test crackers. Each participant was randomly assigned to 1 of 6 treatment sequences to avoid treatment carryover interactions. Before consuming test crackers as a breakfast meal, participants were asked to undergo at least a 10-hour overnight fast. Participants were asked to stay at Nutrition and Wellness Center (NWRC) for an 8-hour experimental day. Upon arrival, participants were given a bag of crackers designed to be similar in appearance for their first meal of the day. The study design intended for each participant to receive all three treatments at the end of the three-week period; however, eight participants during the second week of the study were accidently fed the wrong treatment. Therefore, 8 out of 29 participants that completed the study were not randomized and treatment order was corrected where applicable. Of the eight participants, three had already been fed the same treatment previously.

Before consuming cracker products, baseline breath and blood samples were obtained. In total 4 breath samples and 10 blood samples collected at time points 0 (baseline), 2, 4, 8 hours and -15 (baseline), 0, 15, 30, 45, 60, 75, 90, 120, and 240 minutes, respectively. Participants were given a gastrointestinal symptoms questionnaire in which they were asked to list all symptoms and rate gastrointestinal distress symptoms bloating, pain, and diarrhea on a scale of 0 (none) to 10 (most severe) over the 24-hour period after the consumption of cracker meal.
Participants were served lunch four hours into their eight-hour experimental day. A standardized lunch of turkey or cheese sandwich (supplied by local Jimmy Johns, nutritional facts at https://www.jimmyjohns.com/menu/#/), 16 potato chips (Pringles, Kellogg Company, Battle Creek, MI), 3 small cookies (Oreo, Nabisco, East Hanover, NJ) was served immediately after their 240-minute blood time point. All fecal samples were collected from cracker consumption until 72 h after.

**Chemical Analysis**

**Physiological variables**

Blood from finger pricks were centrifuged to retrieve plasma. Plasma was analyzed with YSI Model 2700 Select Biochemistry Analyzer (YSI Inc., Yellow springs, OH) for glucose concentration and Ultrasensitive Insulin ELISA 80-INSHUU-E01.1 kits (APLCO diagnostics, Salem NH) were used to measure plasma insulin.

Breath samples were collected with Quintron EasySampler Collection Device and Vacuum tubes (QuinTron Instrument Company, Inc, Milwaukee, WI) and analyzed less than two hours after collection by the Quintron Gas Analyzer (MicroLyzer Model SC). The Quintron Gas Analyzer was calibrated every hour with a linearity check using QuinGas™-3 (QT07031-G – 100 ppm H₂, 50 ppm CH₄ and 5 % CO₂) error established to be no greater than ±0.05 % absolute or ± 2 parts per million (ppm) for calibration gas. Participants were divided into non-producers and producers by quantification of methane productions, producers were defined as > 7 ppm of methane at one or more time points.
Fecal and SCFA analysis

Fecal weight, frequency, and pH were evaluated. Fecal frequency was determined by number of bowel movements obtained in 72 hours after cracker consumption. Fecal weight of the total sample was determined on wet weight basis by weighing the sample in the collection bag and subtracting the weight of an average collection bag. Samples were homogenized by kneading the fecal sample in the collection bag, and then a subsample was obtained for a 1:3 dilution of fecal sample to sterile water. Diluted fecal mixtures were centrifuged, and supernatant was frozen at 80°C until SCFA analysis. Remaining supernatant was analyzed to determine fecal pH.

Supernatant from fecal dilutions were subjected to silyl derivatization before GC analysis on Agilent 7890A GC system model (Agilent Technologies, Inc, USA). Silylation outlined as follows: 100 µL of 2-ethylbutyric acid (internal standard) added to 1 ml supernatant, protonated with concentrated hydrochloric acid, silylated with N-(tert-butyldimethylsilyl)-N-methyltrifluoro- acetamide (MTBSTFA), SCFA extracted with diethyl ether. Derivatives incubated for 20 min at 80°C stood at room temperature for 24 hours. After derivatization was competed, samples were injected on a HP-5MSI column (30 m x 0.25 mm x 0.25 µm, Agilent Technologies). Helium was used as a carrier gas flow rate 1 mL/min, with split mode (5:1) 1µL injection volume. Injector (inlet) temperature was 250°C and MS detector temperatures were and 230°C and 150 for source and quad, respectively. Ion source was EI with voltage at 70 eV. The column temperature was held at 40°C for 4 minutes the programeed to rise to 240 °C at 25 °C/min, then to 320 °C at 30 °C/min. SCFA C2-C6 and isomers of C4 and C5 were present in detection. However, due to recognized physiological relevance of SCFA C2-C4 only acetate, propionate, and butyrate concentration are reported. Likewise, where SCFA total is reported, it
is only for the 3 SCFA of interest. The ratio of peak area of a SCFA to internal standard (2-ethyl butyric acid) versus the SCFA concentration was used as a standard calibration curve to estimate the concentration of SCFA in in vitro fecal fermentations.

*Other parameters*

Resistant starch content of finely ground crackers was measured using method AOAC 991.43 and AOAC 2002 using Megazyme kit (Megazyme International Ireland Limited). Gastrointestinal symptoms were also assessed using a gastrointestinal questionnaire. Participants were asked to list any gastrointestinal distress up until 24 hours after ingestion of test crackers where they would record the time, duration, and severity of individual symptoms. Estimated intensity of overall occurrences of gastrointestinal bloating, gastrointestinal pain, and diarrhea was scored on a 0 -10 scale where 0 = none; 1 to 5 = mild; 6 to 7 = moderate; 8 to 9 = severe, 10 = worst possible.

*Statistical Analysis*

All variables were analyzed with linear models in SAS 9.2 (SAS Institute, Cary, N.C., USA). An ANOVA linear model was used for resistant starch content with starch treatment as a fixed effect. Postprandial glucose and insulin was analyzed using mixed model procedures by time points and AUC. Main effects evaluated were cracker treatments, time point, gender, week, and interactions between main effects. Breath gases (H₂ and CO₂) were analyzed with a mixed linear model with treatment, time point, gender, and treatment gender interactions as main effects. Breath gas methane was analyzed using a generalized linear mixed model (GLMM) with Poisson distribution. SCFA
was analyzed with a mixed linear model fixed effects gender, treatment, gender treatment interaction, and methane on response variables acetate, propionate, butyrate and SCFA total with subject as a random effect. Gastrointestinal symptoms used a mixed model with treatment as the main effect. Significance level was set at $p < 0.05$.

**RESULTS**

*Resistant starch content*

Two methods of analysis were used to estimate RS content. For AOAC 991.43 Insoluble Fiber method RS content for crackers and raw starch was significantly different from each other ($p < 0.001$). Megazyme analysis (AOAC 200.12) of raw starch and crackers showed that CS was different from RS5 and HA; however, RS5 and HA were not different from each other. The cooking processes seemed to reduce RS content of RS5 and HA almost by half but had little effect on CS RS content. The difference in RS content by method analysis estimates the average intake of RS differently (Table 1). Moisture content for all cracker treatments was measure to be $9.03 \pm 2.09 \%$ (mean + SD) but not accounted for in RS calculations.

*Postprandial Glucose and Insulin*

For all participants, there was strong evidence that PPG AUC was lower for HA and RS5 when compared to CS ($p<0.001$). PPG peak reached maximum at 15 minutes for CS and HA but reached maximum peak at 30 minutes for RS5. By time point PPG with CS was higher than with HA and RS5 from 0-45 minutes. CS for PPI AUC was only different from HA ($p<0.01$) and there was borderline evidence HA and RS5 were different from each other ($p=0.0529$). RS5 was not different from CS. Gender differences were found in AUC for PPG and PPI (Figure 2). For
every cracker type females had higher PPG and PPI than males. There was evidence in PPI AUC of treatment and gender interaction in males (p=0.0394) but not females, however the opposite was found in PPG AUC where treatment gender interaction was observed for females (p=0.0107) but not in males.

By time point PPG for CS was significantly different from HA and RS5 from 0-45 minutes (Figure 2.). There were no gender differences when PPG was analyzed by time point, however gender differences were found when PPI was analyzed by time point (p = 0.035). PPI analyzed by time point for RS5 showed an oscillating trend in concentration after 45 minutes. RS5 trend differed from CS and HA which both showed a slightly more stable glucose response with a postprandial peak at 15 min and subsequent decline (Figure 2). Similar to PPG, PPI time point differences were found between 0-45 minutes; however, results were not as consistent. CS was different from HA and RS5 at 0 (p<0.01), 15 (p<0.001), and 30 minutes (p<0.05). At 45 min, HA was different CS and RS5, but HA was only different from RS5 at 75 min and CS at 90 min (p<0.05). Due to the complexity of PPI time point response by gender, gender differences were only discussed in PPI AUC.

**Breath Samples for gases H\textsubscript{2}, CO\textsubscript{2}, CH\textsubscript{4}**

Fermentation, assessed by breath hydrogen, was greater after ingestion of RS5 than after eating HA or CS. For all participants, there was no evidence of treatment differences until 4 and 8 hours after ingestion of crackers, where H\textsubscript{2} was highest in RS5 at 10.22 ± 2.06 and 11.25 ± 2.08 ppm respectively (Figure 3). Gender differences were observed in breath hydrogen production, in which females produces more than males (p=0.0485). However, there was no change in CO\textsubscript{2} by treatment or gender.
Participants were divided into two groups: non-producers and producers. Producers were defined as participants with breath methane measurement $\geq 7$ ppm at any time point. With the methane cut-off, 15 participants were found to be producers. When methane was analyzed for all participants (producers and non-producers) there was still evidence of treatment differences and gender treatment interactions. Overall, methane production for all participants was the same to the results for just methane producers alone for both genders except with lower means.

Time point differences were analyzed among producers and non-producers. However, because there was no gender difference at the time point level, results are expressed for as one for both genders (Figure 4). Overall significant results for producers and all participants were the same except for at 2 hours where in all participants there was no difference between treatments whereas in producers, RS5 was significantly lower than HA ($p=0.0295$) but not CS. For methane producers at 0 hours (baseline) RS5 was again significantly lower than HA but not significantly different from CS. At 4 hours HA was significantly higher from all other treatments, but no significant difference among treatments was observed at 8 hours.

**SCFA**

No change in the production of SCFA acetate, propionate, and butyrate due to cracker treatments was observed for all three days. However, gender differences and treatment and gender interaction was observed. Figure 5 represents total production of SCFA profiles for Day 1 and Day 2 grouped by treatment and gender, however, there were no significant treatment differences for SCFA production. For total SCFA production, there was a gender effect ($p=0.0358$) only in day 2 in which the mean total SCFA production for all treatments was $149.20 \pm 14.14$ µmoles/g in males and $113.96 \pm 15.61$ µmoles/g for females. There was a gender main
effect for acetate on day two (p = 0.0386) and propionate on day one (p = 0.0204), in which males produced more fecal SCFA than females but no main effect gender difference for butyrate on either day. Treatment and gender interaction were evident only in propionate (p = 0.0369) and butyrate (p = 0.0191) on day two. HA produced more butyrate and propionate in males on day two than in females.

**Gastrointestinal Symptoms and Fecal Parameters**

There was no evidence of difference for bowel movement fecal frequency, fecal weight or fecal pH across treatments. Reports of gastrointestinal symptoms were minimal. Summed gastrointestinal symptoms were not statistically different between cracker types. The sum of all gastrointestinal symptoms indicated with a positive mean that there was gastrointestinal events were reported but at a low rate and severity (Table 2). However investigation of each symptom showed RS5 crackers had more events of bloating reported than HA and CS (p < 0.01). For all gastrointestinal events, most severity scores were 0. There was a higher frequency of severity score 0 for gastrointestinal symptoms pain and diarrhea than for bloating. Pain and diarrhea gastrointestinal symptoms were not different among all treatment and over three weeks 15 events were reported for pain and 4 events were reported for diarrhea.

**DISCUSSION**

RS content in RS5 and HA crackers were substantially different via AOAC 991.43 but similar when assessed with AOAC 2002.12. The difference in AOAC methods to measure RS content in starches and crackers was not particularly astonishing. Discrepancies between the two methods and RS content have been cited. Furthermore, AOAC 2002.12 resembles in vivo
digestion of resistant starch (McCleary and Monaghan, 2002) as shown in present study in which glucose AUC was not different for RS5 and HA. However, what was particularly interesting was for HA and RS5 in 2002.12 method (Megazyme) RS content in crackers was reduced more than half when compared to respective raw starches, while in 991.43 method RS content in crackers and respective raw starches were comparable. Cooking and preparation conditions can influence RS content (Parchure and Kulkarni, 1997) but cracker preparations employed low moisture, reducing significant gelatinization or even retrogradation. Though this reduction in RS content from raw starch to cracker product was noticed in 991.43 it was marginal compared to 2002.12, which was reduced by half form starch to final cracker product. Although writers propose no explanation for varied RS content, the results support the use of AOAC 2002.12 method as reliable predictor of in vivo glucose digestion.

*Postprandial Glucose and insulin*

Various human feeding studies using RS in a food product have reported the reduction of PPG either by AUC (Behall and Hallfrisch, 2002; Li et al., 2010; Marchini et al., 1998; Najjar et al., 2004; Raben et al., 1994) or by time point (Anderson et al., 2010; Brighenti et al., 2006; Robertson et al., 2003), while other studies have shown no change PPG after consumption of a RS meal (Bodinham et al., 2010; Higgins et al., 2004). This introduces the idea that some RS dose and/or types might not significantly modulate PPG. We used two types of RS in present study HA (type II) and RS5 (type V) at approximate 8-9 grams RS per serving size, when analyzed by Megazyme. We observed a reduction in PPG AUC for both types of RS when compared to conventional starch. Hasjim and associates (2010) fed a type V RS in a bread product to 20 males and also found a reduction in PPG. Studies in which RS did not reduce
overall PPG, evaluation by time points also provided insightful results when considering if RS can lower glucose surge or reduce PPG faster after 90 minutes than other carbohydrates. Although we observed an overall reduction in PPG following consumption of HA or RS5 by AUC, there were also significant differences at given time points. Consumption of HA or RS5 significantly reduced the postprandial glycaemia at 0-45 minutes compared to CS. However, plasma PPG after 90 minutes was the same for all treatments. There was a gender difference observed in glucose AUC as evidenced by a higher PPG for males than females, but this did not interfere with significance observed in the main effect for treatment and the overall trend for genders was similar to pooled glucose AUC (CS > HA=RS5). Dietary fat can lower glycemic response, as observed in a second meal effect (Ercan et al., 1994), and lipid-starch complexes will also drastically reduce PPG when compared to a control (Hasjim et al., 2010). Consumption of RS5, which was produced by modifying raw starch with stearic acid, produced PPG only marginally lower with no statistical significance than its parent starch HA. Therefore, we surmise the 10 percent fat content of RS5 had a negligible effect on overall PPG response. Although dietary fat can decrease PPG, inversely, it can increase PPI even in people with diabetes mellitus (Gannon et al., 1993). However, findings from Hasjim and associates (2010) found that lipid-starch complex starch incorporated into a bread product reduced PPI when compared to conventional bread. We did not find RS5 to be different from any of the other crackers with respect to PPI. HA (type II) produced a PPI significantly lower than CS but PPI after the consumption of HA was not different from RS5. Thus, our evidence does not support a change in PPI due to lipid-starch complex. Gender differences were observed for PPI. Insulin AUC for females was also greater than males. These data supports gender differences in glucose metabolism that have been reported between men and women (Basu et al., 2006). Insulin
sensitivity was observed after RS meals in men but not in women (Maki et al., 2012) which is congruent with our observation that women had elevated PPG and PPI compared to men. Our difference found to increased PPG in women could be due to a set dose of RS was given to each participant and with males being larger than females RSg/kg could be higher; however, when tolerance meals were fed to men and women by to kcal/kg, women still had higher PPG than men (Basu et al., 2006).

**Fermentation, breath gases, and SCFA**

Fermentation was measured by breath hydrogen (H$_2$) and methane (CH$_4$) and SCFA (acetate, propionate, and butyrate). Consumption of RS5 produced more hydrogen from 4-8hrs than CS and HA. HA was not different in fermentation from CS, based on utilization of the breath hydrogen after cracker consumption. Other human studies have shown an increase in breath hydrogen due to RS meals from type II – IV (Hylla et al., 1998; Li et al., 2010; Muir et al., 1994; Robertson et al., 2003). What is notable is that HA a type II RS when assessing breath hydrogen was not fermentable, but RS5 type IV was fermentable over time.

HA seemed to be the more fermentable fiber among methane producers, consistently producing more methane than RS5 and at time point 4 h producing more methane than CS. This pattern was also the same when all participants were included for methane production (time point data not shown) except that at 2 hrs there were no treatment differences and HA was only marginally higher (p=0.0513) than RS5 in methane production.

SCFA production has been implicated in its role to help in colon cancer prevention and possibly amelioration; however, a clinical study with 24 colon adenoma patients had no change in SCFA production from fecal samples collected 48 after a 4 week intervention (Grubben et al.,
RS starch fermentation studies in healthy humans have found increase in total SCFA production (Ahmed et al., 2000; McOrist et al., 2011), while others found no difference (Phillips et al., 1995). Overall, RS types II-IV are generally fermentable fibers, however, to our knowledge there are no human feeding studies that have investigated lower gut fermentation using type IV RS. No difference in cracker and SCFA production was found in present study for individual SCFA (acetate, propionate, or butyrate) or total SCFA. Gender differences, however, were found. The main effect for SCFA production was higher in males than females. Fermentation gender differences have been reported in other studies (McOrist et al., 2011; Pitt et al., 1980).

No marker was used to estimate gut transit time, therefore complicating the estimate of when fermentation of cracker products actually took place. Furthermore, bowel movements for each participant were different. Some participants showed signs of constipation with no bowel movements over the 3 days after cracker consumption. To assess the cracker fermentation without aggressive sampling or use of medical devices, there was no true way of standardization of fecal samples other than through time. Thus a sum of SCFA production by time (i.e., days) was the best estimate of SCFA production among all participants.

**Gastrointestinal symptoms and fecal parameters**

We found no difference for fecal frequency, fecal weight, or pH across treatments. Increase in fecal bulking/weight (Cummings et al., 1996; Jenkins et al., 1998; Phillips et al., 1995), increased bowel movement frequency (Heijnen et al., 1996; Noakes et al., 1996), and decreased pH (Birkett et al., 1996; McOrist et al., 2011) have been observed after consumption of RS, as RS is a dietary fiber. McOrist and associates (2011) observed a change in fecal pH after
RS treatment although for males a decrease in pH was observed when RS treatment was compared to entry fecal samples and in women was lower than another dietary fiber (NSP). Unlike McOrist, we did not obtain entry fecal samples before treatments and found no fecal pH treatment difference, similar to other research groups (Grubben et al., 2001). HA and RS5 were the dietary fiber cracker and overall were well tolerated with minimal reports of gastrointestinal symptoms (bloating pain, and diarrhea). The sum of all gastrointestinal symptoms indicated no difference in treatment for symptoms. However RS5 crackers had more events of bloating reported than HA and CS (p<0.01) although reported events were mild on the severity scale.

Chocolate crisp bars formulated with a wheat RS increased bloating when compared to the control (Karalus et al., 2012). Gastrointestinal distress has been reported after consumption of high RS meals (Muir et al., 1994), however our finding are similar to other acute RS feeding studies, were research groups found RS treatments to be well tolerated (Heacock et al., 2004; Stewart et al., 2010; Wolf et al., 2001).

**Conclusion**

Two types of RS when compared to a control starch expressed different responses by gender and to fermentation as measured by breath gases. Total SCFA production was not affected by treatment but overall production was different among genders. Fermentation assessed by breath H showed RS5 to be more fermentable, but HA was more fermentable when assessed by breath methane. Overall RS5 and HA proved to be to lower postprandial glucose, but HA seemed to be a viable dietary agent to lower postprandial glucose response over RS5. Both forms of RS were well tolerated although RS5 may mild induce bloating. This study supports the use of RS to lower postprandial glucose to aid in the diabetes and metabolic
syndrome. Further research is necessary to understanding mechanisms to fermentation patterns between RS types; however, this study supports the use of RS5 and HA as fermentable fibers.

ACKNOWLEDGEMENTS

Research support received from AFRI Proj. no. 2009-65503-05798. The author would like to thanks Ann Perera, Zhihong Song, and the W.M. Keck Metabolomics Research Laboratory at Iowa State University for use of the facility and their insightful assistance. Thanks to the Nutrition and Wellness Center of Iowa State University for providing a comfortable facility during experimental days, and lastly special thanks to the dedicated helpers and participants of this study.

REFERENCES


FIGURES AND TABLES

<table>
<thead>
<tr>
<th>HOURS</th>
<th>Night before</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fasted</td>
<td>-10</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>2. Arrive at NWRC</td>
<td></td>
<td>6</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>3. Blood Samples</td>
<td></td>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>4. Breath Samples</td>
<td></td>
<td></td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>5. Lunch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Leave NWRC</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7. Questionnaires</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>8. Fecal Samples</td>
<td></td>
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</tr>
</tbody>
</table>

Figure 1. Time line of research week as explained to each participant. 1) Overnight fast required. 2) Upon arrival baseline (BL) samples will be taken then crackers eaten. 3) Total of 10 blood samples were collected 4) Total of 4 breath samples were taken. 5) A standardized lunch provided. 6) Allowed to leave research facility, Nutrition and Wellness Research Center (NWRC). 7) After 24hrs questionnaires for food consumption and gastrointestinal symptoms were completed. 8) Fecal samples were collected for 72 hours after consumption of cracker.
### Table 1. Method Comparison

<table>
<thead>
<tr>
<th></th>
<th>RS Content (% of total starch)</th>
<th>Average RS intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AOAC 991.43</td>
<td>Megazyme</td>
</tr>
<tr>
<td><strong>Raw Starch</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>1.1 ± 0.2(^a)</td>
<td>1.5 ± 0.2(^a)</td>
</tr>
<tr>
<td>HA</td>
<td>28.4 ± 2.3(^b)</td>
<td>36.7 ± 1.2(^b)</td>
</tr>
<tr>
<td>RS5</td>
<td>58.0 ± 0.6(^c)</td>
<td>32.8 ± 1.7(^b)</td>
</tr>
<tr>
<td><strong>Crackers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>5.8 ± 1.2(^a)</td>
<td>0.3 ± 0.0(^a)</td>
</tr>
<tr>
<td>HA</td>
<td>24.3 ± 1.0(^b)</td>
<td>17.5 ± 0.2(^b)</td>
</tr>
<tr>
<td>RS5</td>
<td>57.8 ± 2.3(^c)</td>
<td>16.4 ± 0.7(^b)</td>
</tr>
</tbody>
</table>

Mean ± SE for RS content with the same superscript within a column with in type (i.e., raw starch or crackers) are not significantly different from each other. Estimates for average RS intake was not expressed for raw starch since participants consumed cracker products. Average RS intake was estimated from 50g starch/serving.
Figure 2. A and C. Glucose and insulin response mean and SE by time for all participants (n=29), CS was significantly different from HA and RS5 at time point 0, 15, 30, and 45 for glucose response. ** p < 0.0001 * p < 0.05.  Insulin response by time point for all participants expressed treatment time point interactions between at 0-45 minutes. B and D. AUC mean and SE for postprandial glucose and postprandial insulin was expressed by gender. For total glucose AUC CS was significantly higher than HA and RS5 (p=0.0010). Total insulin AUC CS was significantly higher than HA (p=0.0043) but RS5 insulin AUC was the same for CS and HA.
Figure 3. Breath hydrogen fermentation (ppm) represented as mean and SE (n=29). RS5 was significantly higher at 4 (* p< 0.05) and 8 hours (** p<0.01) from CS and HA.
Figure 4. Methane production by time point for male and female producers mean and SE (n=15) showed treatment differences 0, 2, and 4h but none at 8h. Letters within time point (h) that are the same are not significantly different from each other. Notably HA was significantly higher than RS5 and CS at 4 h.
Figure 5. Total fecal SCFA profiles for each treatment by gender and days. A) From fecal samples received on day 1 of cracker treatment. B) From fecal samples received on day 2, 24h after ingestion of cracker treatment.
Table 2. Reported gastrointestinal symptoms and averaged severity.

<table>
<thead>
<tr>
<th></th>
<th>Bloating</th>
<th>All symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Events</td>
<td>Overall mean</td>
</tr>
<tr>
<td></td>
<td>Reported</td>
<td>Median</td>
</tr>
<tr>
<td>CS</td>
<td>6</td>
<td>2.5</td>
</tr>
<tr>
<td>HA</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>RS5</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>p = 0.0245</td>
<td></td>
</tr>
</tbody>
</table>

For all participants, bloating was the only gastrointestinal symptom that showed to have a difference among treatments. Superscripts that are different within each column are significantly different in overall mean ± SE from each other (p<0.01). All symptoms were averaged (bloating, diarrhea, and pain) and reported as overall mean ± SE.
APPENDIX

Record any events of gastrointestinal distress (such as discomfort, feeling bloated, gas, pain, burping, flatulence, diarrhea or loose stools) that you experience over the next 24 h.

<table>
<thead>
<tr>
<th>Time of onset H: min am/pm</th>
<th>Time of cessation H: min am/pm</th>
<th>Symptom</th>
<th>Level of discomfort (1 = lowest, 10 = most severe possible); number of gas passages (if applicable)</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
</tbody>
</table>

Overall, circle the rating of your level of gastrointestinal symptoms over the 24 h after eating the test product:

Gastrointestinal bloating
(Estimated intensity 0 = None; 1 to 5 = Mild; 6 to 7 = Moderate; 8 to 9 = Severe, 10 = Worst possible)

\[
\begin{array}{cccccccc}
0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 \\
\end{array}
\]

Gastrointestinal pain
(Estimated intensity 0 = No pain; 1 to 5 = Mild pain; 6 to 7 = Moderate pain; 8 to 9 = Severe pain, 10 = Worst pain possible)

\[
\begin{array}{cccccccc}
0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 \\
\end{array}
\]

Diarrhea
(Estimated severity 0 = None; 1 to 5 = Mild; 6 to 7 = Moderate; 8 to 9 = Severe, 10 = Worst possible)

\[
\begin{array}{cccccccc}
0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 \\
\end{array}
\]

Figure 1. Gastrointestinal symptoms questionnaire
Table 1. Nutritional facts from Jimmy Johns website of turkey and cheese sandwiches (https://www.jimmyjohns.com/menu/#/).

<table>
<thead>
<tr>
<th>Nutrition Info</th>
<th>Slim 4 (Turkey)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amount per serving</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Serving size</strong></td>
<td>201 g</td>
</tr>
<tr>
<td><strong>Calories</strong></td>
<td>410</td>
</tr>
<tr>
<td><strong>Total Fat</strong></td>
<td>3 g</td>
</tr>
<tr>
<td>Saturated Fat</td>
<td>0 g</td>
</tr>
<tr>
<td><strong>Cholesterol</strong></td>
<td>30 mg</td>
</tr>
<tr>
<td><strong>Sodium</strong></td>
<td>1070 mg</td>
</tr>
<tr>
<td><strong>Total Carbohydrate</strong></td>
<td>65 g</td>
</tr>
<tr>
<td>Dietary Fiber</td>
<td>0 g</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td>27 g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nutrition Info</th>
<th>Slim 6 (Cheese)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amount per serving</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Serving size</strong></td>
<td>194 g</td>
</tr>
<tr>
<td><strong>Calories</strong></td>
<td>550</td>
</tr>
<tr>
<td><strong>Total Fat</strong></td>
<td>18.5 g</td>
</tr>
<tr>
<td>Saturated Fat</td>
<td>9 g</td>
</tr>
<tr>
<td><strong>Cholesterol</strong></td>
<td>40 mg</td>
</tr>
<tr>
<td><strong>Sodium</strong></td>
<td>990 mg</td>
</tr>
<tr>
<td><strong>Total Carbohydrate</strong></td>
<td>66 g</td>
</tr>
<tr>
<td>Dietary Fiber</td>
<td>0 g</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td>29 g</td>
</tr>
</tbody>
</table>
Table 2. Mean ± SE μmole/g of fecal SCFA production by gender across treatments, stratified by days.

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th></th>
<th></th>
<th>Female</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate</td>
<td>Propionate</td>
<td>Butyrate</td>
<td>Acetate</td>
<td>Propionate</td>
<td>Butyrate</td>
</tr>
<tr>
<td>CS</td>
<td>132.74 ± 20.66</td>
<td>13.02 ± 2.83</td>
<td>2.85 ± 3.06</td>
<td>104.03 ± 25.82</td>
<td>6.40 ± 3.53</td>
<td>7.23 ± 3.85</td>
</tr>
<tr>
<td>HA</td>
<td>131.38 ± 19.23</td>
<td>13.30 ± 2.68</td>
<td>13.62 ± 2.87</td>
<td>88.21 ± 19.15</td>
<td>7.53 ± 2.68</td>
<td>9.32 ± 2.88</td>
</tr>
<tr>
<td>RS5</td>
<td>108.90 ± 19.39</td>
<td>10.32 ± 2.72</td>
<td>11.13 ± 2.91</td>
<td>100.55 ± 20.64</td>
<td>8.02 ± 2.87</td>
<td>10.75 ± 3.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th></th>
<th></th>
<th>Female</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate</td>
<td>Propionate</td>
<td>Butyrate</td>
<td>Acetate</td>
<td>Propionate</td>
<td>Butyrate</td>
</tr>
<tr>
<td>CS</td>
<td>244.92 ± 63.42</td>
<td>26.46 ± 7.30</td>
<td>26.54 ± 10.09</td>
<td>122.99 ± 77.73</td>
<td>7.58 ± 8.94</td>
<td>10.31 ± 12.36</td>
</tr>
<tr>
<td>HA</td>
<td>341.59 ± 73.58</td>
<td>29.82 ± 8.46</td>
<td>50.76 ± 11.69</td>
<td>157.03 ± 77.85</td>
<td>10.79 ± 8.96</td>
<td>13.52 ± 12.38</td>
</tr>
<tr>
<td>RS5</td>
<td>198.10 ± 73.93</td>
<td>14.69 ± 8.51</td>
<td>19.53 ± 11.76</td>
<td>285.95 ± 78.21</td>
<td>28.17 ± 9.00</td>
<td>44.76 ± 12.44</td>
</tr>
</tbody>
</table>
CHAPTER 5. IN VITRO SCREENING OF LIPID-MODIFIED RESISTANT STARCHES AND MEDIA COMPARISON ON SHORT CHAIN FATTY ACID PRODUCTION IN BATCH FERMENTATION

A paper to be submitted to *Journal of Food Science*

Esther Haugabrooks, Yongfeng Ai, Jay-lin Jane, Suzanne Hendrich

**Abstract**

Carbohydrate fermentation is a valuable function in human health. Fermentable dietary fibers, such as resistant starch, have been heavily used to influence fermentation events of the large intestine to advance gastrointestinal health. In vitro comparative studies have been conducted to estimate and screen potential efficacy of fermentable fibers in vivo. Starch residues (SR), obtained by in vitro digestion of two novel lipid complexed starches and their parent starch, were used as substrates for anaerobic in vitro batch fecal fermentation. Fecal slurries were incubated in brain heart infusion (BHI), brain heart infusion without dextrose (BHI-D), or a basal nutrient growth (BNG) medium for 24 hrs. Short chain fatty production (SCFA) and pH were evaluated at the end of incubation period for changes due to SR, media, or body mass index (BMI) of fecal donors. SCFA production was unaffected by SR treatment; however, media differences were found. BNG had the lowest SCFA production when compared to BHI and BHI-D. All treatments significantly lowered fecal inocula from baseline pH however, RS5 and HA in all 3 media caused the lowest pH. Although no differences were found for SR fermentation between BMI groups, further investigation on in vitro SR fermentation is warranted. Ultimately, fermentation comparison should be conducted between in vitro and in vivo conditions to establish effective in vitro fermentation methods for resistant starch screening.
Introduction

With the discovery of resistant starch (RS) and the advent of the dietary fiber movement, growing awareness has been given to evaluating the effects of human gut fermentation and gut microbiota. This has introduced opportunities not only for greater understanding of public health application but also for comparative screening for suitable fermentable conditions and substrates.

Short chain fatty acids (SCFA) are microbial fermentation products that have been implicated in gastrointestinal functions and beneficial biological roles such as reducing the risk of colon cancer, gastrointestinal disorders, and cardiovascular disease (Cook and Sellin, 1998; Hosseini et al., 2011; Scheppach, 1994; Scheppach et al., 1995; Suzuki et al., 2008; Vinolo et al., 2011). In vitro fermentation by human gut microbiota has increased SCFA production with soygerm powder rich in phytoestrogenic isoflavones (Boever et al., 2000); agave fructans (Gomez et al., 2010); and various other dietary fibers (Barry et al., 1995) as substrates. Digestion of resistant dietary fibers, such as resistant starches, has been recommended for their fermentative capability (Topping and Clifton, 2001) and are used as substrates to improve overall gastrointestinal health such as lesser transit time, increase fecal output, lower fecal pH, and higher fecal SCFA (Muir et al., 2004; Phillips et al., 1995).

Resistant starch (RS) is defined as the ingested portion of starch indigestible in the small intestine (Asp, 1992). In vitro techniques, batch and dynamic models, have been used to screen resistant fibers to predict their fermentative capabilities in vivo (Fässler et al., 2006a). However, in vitro screening can be influenced by starting material (i.e., entry fecal specimen) or by in vitro conditions and preparation (i.e., media used for the assay). Different methods and media selections, mineral salts, mineral salts with tryptone, complex yeast media, have been
documented for use in fermentation studies (Edwards et al., 1996), while in resistant starch fermentation studies both media and buffers have been used. With the array of different fermentation methods and preparation of fecal inocula, no particular method has been outlined as the best for in vitro fermentation studies. A standardized method would facilitate research that might rapidly predict in vivo effects by accurately stimulating lower gut conditions in vitro.

Carbohydrates as a whole are fermentable by lower gut microbiota. Considering the positive association of carbohydrate fermentation and beneficial health effects, slower carbohydrate fermentation is a desirable trait. Glucose is fermented but much more quickly than in the form of dietary fibers. Media with glucose as a source of energy can enhance growth and fermentation of some microbiota. Thus, for in vitro fermentation studies in which RS is evaluated as substrate, to what extent should glucose be limited in media? Furthermore, the use of enriched media can be criticized for simulating conditions unrealistic to the gastrointestinal environment.

Media is consequently a feasible influence on SCFA production. For fecal in vitro fermentation incubations the inherent nature of the specimen may also influence SCFA production. There is evidence from human fecal evaluation that fermentation patterns are different among body type lean, overweight, and obese (Schwiertz et al., 2010). SCFA production and overall carbohydrate fermentation is attributed to resident gut microbiota. Due to different microbial communities that can exist across body type (Turnbaugh et al., 2006) different fermentation patterns can be observed generally with more fermentation observed in overweight or obese individuals (Schwiertz et al., 2010). Although the mechanism is unclear, the phenomenon is slightly paradoxical since increased fermentation is generally perceived as a beneficial towards human health while obesity increases the risk of health problems.
Fermentation studies have compared substrates in a predigested form in vitro (Sayar et al., 2007; Zhou et al., 2013); differences between body mass index (BMI) groupings for SCFA production (Payne et al., 2011; Schwertz et al., 2002); and media selection (Kim et al., 2011; Nelson and George, 1995). However, RS fermentation studies comparing non-selective media are few. Research has compared fermentable fibers among which have been RS types in vivo and in vitro. RS types I-IV are fairly well studied, but fermentative capacity of RS type V is not known. This study aims to investigate the effect of 3 starch residues (SR) form in vitro digestion on human fecal inocula from lean and obese humans through the use of 3 media. We hypothesize body type (BMI) and SR will influence the in vitro production of SCFA from gut microbial committees in fecal samples with little difference in fermentation across different media types.

Materials and Methods

Preparation of Starch residues

Three starch residues (SR) were prepared from test starches HA7, stearic acid complexed (RS5) and octenyl succinic starch (OS). HA7 is a native high-amylose maize starch, commercial product name AmyloGel 03003, courtesy of Cargill (Hammond, IN). RS5 and OS are lipid-modified starches developed at Iowa State University by Jay-lin Jane’s laboratory. RS5 and OS were prepared at pilot-plant scale from starting material high-amylose cornstarch. Starting material for RS5 was suspended in distilled water (10%, w/w) and heated at 80°C in a stem-jacketed kettle with vigorous agitation. After 1 hour it was cooled to 55°C, pullulanase (1.25%, dry starch basis, dsb) was added to suspension and incubated with agitation at 55-60°C for 24h in a hot-water-jacketed stainless steel tank (Model 70-gallon JOVC, Viatec™, Belding, MI). Stearic acid (10%, dsb) and solution heated to 80°C for 1 hour to allow lipid complex to form.
Starch suspension was stored at 4°C for 48h RS5 was recovered by centrifugation. Pasted was dried at 50°C in convection oven and ground to a powder. Starting material for OS was suspended in distilled water to solid content of 35% (w/w, dsb), pH adjusted to 8.0 with 3% (w/w) NaOH. Maintaining the pH at 8.0 ± 0.1 and temperature at 33 ± 1°C, octenyl succinic anhydride (10% of dry starch weight, w/w, dsb) was gradually added. After stabilization of pH the reaction was terminated with 1.0M HCl to pH of 6.5. Starch suspension was centrifuged and washed twice with distilled water and 100% ethanol. Starch was dried in an oven at 35°C and ground to a powder. To obtain starch residues all test starches were digested with AOAC 991.43 method to simulate human digestion of cooked starch.

**Participant Characteristics**

Participants (5 female, 1 male) were screened to be within 18-65 years of age, no gastrointestinal disease, not on any drugs or medication, no use of antibiotics within past 6 month. Inclusion criteria based on BMI and divided into two groups: Group 1, lean (BMI<25) and Group 2, overweight (BMI> 30). Six participants considered healthy were selected for the study three in each group. Participants summarize daily eating habits via a health and diet questionnaire. Specific participants characteristics and eating habits are found in Table 1. The Institutional Review Board of Iowa State University approved the study. Each participant reviewed and signed informed consent prior to donation of fecal specimen.

**Fecal Collection**

Whole stools were collected from six participants. Participants were instructed to a metabolic restroom at ISU where they would leave a fecal specimen in a commode specimen
collection system (Sage Products, Inc Crystal Lake IL) placed in the refrigerator at 4°C, and call a specific research telephone line. Once participants placed a call to the research telephone line, fecal samples were immediately collected and transferred to Bactron I anaerobic chamber (Sheldon Manufacturing, Inc., Cornelius, OR) to be processed. Fecal samples were transferred to anaerobic conditions in a one hour or less upon receipt. All processing of samples were done in anaerobic chamber including transfer of samples into media and application of SR treatments in crimper top test tubes.

Batch fermentation/In vitro incubations

Three media were selected: brain heart infusion (BHI), brain heart infusion without dextrose (BHI-D), and a basal nutrient growth media (BNG). BHI and BHI-D (Alpha biosciences, Baltimore, MD) were prepared according to manufacture’s directions. Prior to autoclaving BHI and BHI-D, 50 ml/L of 8 % (w/v) NaHCO₃, 20 ml/L of 1.25 % (w/v) cysteine hydrochloride, and 1mL of 0.01% (w/v) resazurin were added. BNG was prepared with 2 g/L peptone water, 2 g/L yeast extract, 0.1 g/L NaCl, 0.04 g/L KH₂PO₄, 0.04 g/L K₂HPO₄, 0.01 g/L MgSO₄ · 7H₂O, 0.01 g/L CaCl₂ · 6H₂O, 2 g/L NaHCO₃, 2 ml/L Tween 80, 0.05 g/L hemin, 10 µl/L vitamin K, 0.5 g/L L-cysteine hydrochloride, 0.5 g/L bile salts. Prior to autoclaving BNG medium was adjusted to pH 6 and 4 ml 0.025% (w/v) resazurin was added. Each prepared media was placed in a 2L aspirator bottled adapted for media sampling by Milton Allison, ISU Affiliate Professor. All media were gassed with CO₂ using sterile needles prior to autoclaving and post autoclaving during cooling. When cooled media was transferred and stored in anaerobic chamber to equilibrate to anaerobic chamber gases for at least a day before use.
Crimper top test tubes with 100mg of SR were hydrated in 2mL of test media for 24 hours then flash frozen before stored at -80 °C until use. Upon receipt of fecal sample, SR hydrates were thawed to temperature. Fecal inocula were prepared immediately by diluting fecal specimens (1/10 w/v) in BHI, BHI-D, and BNG. Inoculum was stirred and filtered through 4 layers of cheesecloth to remove particles. After fecal processing, 8mL of inoculum with corresponding media was added to SR hydrate, stoppered with butyl stoppers and crimped. Treatments levels were 4 starches (SR-OS, SR-RS5, SR-HA7, and control with no SR) and 3 media. Therefore, each participant’s specimen received a combination of 12 treatments. Baseline measurements were taken for each media. Crimped test tubes were incubated for 24 hr at 37°C with gentle agitation. Upon completion of batch incubation, inocula were centrifuged and aliquots of supernatant were frozen at -80 °C for SCFA analysis. Remaining supernatants was measured for change in pH

Chemical analysis of SCFA
Supernatants from inoculum were subjected to silyl derivatization before GC analysis on Agilent 7890A GC system model (Agilent Technologies, Inc, USA). Silylation outlined as follows: 100 µL of 2-ethylbutyric acid (internal standard) added to 1ml supernatant, protonated with concentrated hydrochloric acid, silylated with N-(tert-butyldimethylsilyl)-N-methyltrifluoro- acetamide (MTBSTFA), SCFA extracted with diethyl ether. Derivatives incubated for 20 min at 80°C the stood at room temperature for 24 hours. After derivatization was competed, samples were injected on a HP-5MSI column (30 m x 0.25 mm x 0.25 µm, Agilent Technologies). Helium was used as a carrier gas flow rate 1mL/min, with split mode (5:1) 1µL injection volume. Injector (inlet) temperature was 250°C and MS detector
temperatures were and 230 °C and 150 for source and quad, respectively. Ion source was EI with voltage at 70 eV. The column temperature was held at 40°C for 4 minutes the programed to rise to 240 °C at 25 °C/min, then to 320 °C at 30 °C/min. SCFA C2-C6 (as in acetate, propionate, butyrate, valerate and caproate) and isomers of C4 and C5 (isobutyrate and isovalerate) were present in detection. However, due to known physiological relevance of C2-C4 only acetate, propionate, and butyrate concentration are reported. Likewise, where SCFA total is reported it is only for the 3 SCFA of interest. The ratio of peak area of a SCFA to internal standard (2-ethyl butyric acid) versus the SCFA concentration was used as a standard calibration curve to estimate the concentration of flavonoids in in vitro fecal fermentations.

Statistical Analysis

All data, SCFA and pH analysis, were run in duplicate. Means were analyzed using SAS v. 9.2 (SAS Institute, Cary, NC) conducting analyses of variance (ANOVA) with a mixed linear model. Mixed linear model for pH was defined where fixed effects were media, treatment, media treatment interaction and participants (BMI); with random terms participant (BMI) and participant media interaction. Mixed linear model for SCFA was defined using BMI, baseline measurements, media, treatment, and treatment media interaction as fixed effects; and subject and subject media interaction as random terms. Statistical differences were evaluated using least significant difference (LSD) with significance level was set at $p < 0.05$. 
Results

SCFA production in fecal inocula

There was no difference in SCFA production by treatment. Differences were found in SCFA production when media was compared (Table 2). For total SCFA production BNG was the lowest and BHI with and without glucose performed the same. This was also the trend for each individual SCFA (acetate, propionate, and butyrate). There was no significant difference in media for butyrate production in fecal incubations. However, butyrate was lower in BNG than BHI and BHI-D and almost differed from butyrate concentrations in BHI-D (p=0.0562) although not significantly. BNG was significantly lowest in acetate production and lower from BHI-D in propionate production. All concentrations for SCFA were analyzed as an increase from baseline. Therefore, overall the use of BNG for in vitro fermentation did not elevate microbial production of SCFA in 24h incubations compared to BHI and BHI-D.

Change in pH

Treatment differences were observed in pH. Comparably, all media performed the same with respect to treatment differences in pH response (Figure 2). As anticipated, pH decreased from baseline in all treatments (p<0.01) for all media. The control (C) had the least change in pH compared to all other treatments. Likewise, OS was different from all other treatments. RS5 and HA7 had the lowest change from baseline when compared with OS and C. RS5 and HA7 did not differ from each other in effects on media pH. SR had an effect on pH, but change in pH did affect total SCFA production (Figure 1).

BMI
This study found no evidence to support a difference in SCFA fermentation due to BMI or SR. Groups analyzed by weight were lean versus obese assessed by BMI. When analyzing weight as a variable there was no difference in pH or SCFA production by media or SR treatment. Therefore, results are represented as a sum of all participants within both weight categories.

Discussion

As a dietary fiber, RS has been heavily investigated for its fermentability, among other physiological effects. Comparison of what type of RS has the greatest fermentative capacity has also been conducted. In the present study we compared three different types of RS: stearic acid, RS5 in which we term RS type V, OS a type IV, and HA a type II RS. RS underwent in vitro digestion to simulate the portion of the starch residue (SR) that would be encountered by lower intestinal microbiota for fermentation. This study also aimed to compare SR, body weights, and media selection to see what variables had an effect on pH and SCFA production. Results will be discussed in the order of SR, media, and body weight comparisons.

The digestion resistant portion of RS can be fermentable. Fecal SCFA production has increased in vivo on a high RS diet in comparison to a low RS diet (Ahmed et al., 2000). Increased SCFA production to RS treatments can also be observed in vitro from ilea effluent (Silvester et al., 1995). In vitro studies utilize in vitro digestion methods to obtain SR, which are closer simulation to what lower gut microbes would encounter in vivo. Overall no difference in SCFA production was found between SR treatments. We did find that pH decline more in RS5 and HA7 when compared to control. Fermentation of RS produces an acidic environment, which is expressed with the decline in pH, was observe from all SR treatments. However, RS5 and OS
were the lipid-starch complexed residues and were found to perform differently with respect to reduction of pH. Substrates used in RS fermentation studies are often residues from RS II or RS III. Little is known about the fermentability of RS type V or lipid-starch complex fermentation.

Although anaerobic bacteria were targeted for fermentation, media selection of BHI, BHI-D, and BNG in present study was chosen for non-selective properties. BHI and BHI-D are commercially available media and BNG has been used in other anaerobic fecal studies (Fooks and Gibson, 2003; Gomez et al., 2010). An early media comparative study used selective and none-selective media to recover anaerobic bacteria in human clinical specimens from anaerobic infection and found certain selective media (laked blood-agar plate containing vancomycin and kanamycin and neomycin Brucella blood-agar plate) enhanced the appearance of some anaerobic bacteria easier isolation in specimens with heavy growth of facultative organisms; however, selective media overall decreased recovery of most anaerobic bacteria especially gram-positive rods (Rosenblatt et al., 1973).

In a media comparison study for enumeration and isolation of anaerobic fecal bacteria of mice found using a plate-in-bottle method six non-selective media, one of which was BHI, colony counts and percent recoveries was higher among habitat-stimulating media which contained low concentration of organic matter than enriched media (i.e., BHI) (Itoh and Mitsuoka, 1985). In addition, Itoh and Mitsuoka (1985) found that BHI and a media described by Wensinck & Ruselervan Embden (W&E agar) were more selective than other media excluding the growth of bacterial group Peptococcaccae in BHI and suppressing the growth Eubacteria in W&E agar. The use of BHI has also been compared in media comparison study to evaluate culture conditions to maintain metabolically active and diverse population of human bacteria in feces over an 18-24 hour bath incubation (Kim et al., 2011). Kim and associates
(2011) found that between BHI, a low-carbohydrate medium (LCM), and a high-carbohydrate medium (HCM) that LCM performed best in maintaining a diverse metabolically active population and would be suitable for in vitro studies using human intestinal microbiota.

In the present study we used BHI and two media we considered low carbohydrate, BHI-D and BNG. We supplied each media with carbohydrate source of SR, one from RS type II and two from RS type II and also evaluated the SR fermentation against a blank control. Considering main effects of media we found that BHI and BHI-D performed the same while BNG was consistently lower for individual SCFA and total SCFA production. Molar ratios for each media were 146:13:17, 170:14:19, and 34:8:12 for BHI, BHI-D, and BNG respectively. We expected to see a slight elevation in SCFA production from BHI due to the carbohydrate source when compared to its counterpart depleted of dextrose. However, we found no statistical difference between the two enriched media BHI and BHI-D.

Bacteria that contribute to fermentation in the colon are often obligate anaerobes that do not thrive in the presence of oxygen. Obtaining fecal samples in oxygen-depleted environments for in vitro incubations are a challenge, and where mentioned methods have been poorly described. It can be concluded that fermentation contributions from strict anaerobic bacterial species including extremely oxygen-sensitive organisms cannot be accessed in vitro, which parallels the current understand in microbiology that only a fraction of gut microbiota is culturable. At most, with quick and anaerobic fecal processing times, viable anaerobes that remain can be surmised to exert the main effects related to treatment differences.

We found no difference in SCFA production due to weight at entry level or after 24 h batch fermentation incubations. Li and associates (2010) compared in 15 lean to 9 overweight and 6 obese individuals in an in vitro RS fermentation. Similar to our study they found no significant
differences in SCFA production between weight groups. This study was conducted over 3 weeks in a semi-continuous fermentation system using BHI as media and also showed no change in SCFA production due to various SR (RS type II) treatments (Li, 2010b). Thus we do not believe longer incubation periods would have provided evidence to weight and SCFA fermentation differences, even though various RS in vitro studies utilize 24-hour incubation periods have produced varied results in SCFA production (Fässler et al., 2007; Laurentin and Edwards, 2004).

There is evidence that in vitro anaerobic fermentation in fecal samples from overweight or obese individuals tend to be greater than in lean individuals (Schwiertz et al., 2010). Schwiertz and associates (2010) evaluated intestinal microbiota and fecal SCFA concentration of lean (n=30, 18.5-24.9 BMI), overweight (n=35, 24-30 BMI), and obese (n=33, BMI>30) individuals. They found total SCFA was significantly higher for overweight (98.7 ± 33.9 mmol/l) and obese (103.9 ± 34.3 mmol/l) when compared to lean individuals, furthermore propionate concentrations were elevated over lean individuals. The mechanism for this phenomenon is unclear. Furthermore, it does not bridge the discrepancy that if overweight and obese individuals tend to have increased fermentation, which have been postulated to lead to beneficial effects, why are they the subpopulation at higher risk for gastrointestinal complications such as colorectal cancer (Ma et al., 2013)?

Eating habits of participants were similar, except for whole grain consumption. Lean individuals consumed more whole grains servings/day than individuals with higher BMI, although individuals with a higher BMI seemed to consume more servings of fruit per day. Similar background diets could be an explanation to no difference in fermentation at entry level or after fecal incubation with SR. Although Schwiertz and associates (2010) found SCFA
differences by weight, fermentation was assessed from participants on a background western diet, independent of strict diet normalization or fecal incubation with carbohydrate substrates.

Currently a wide variety of methods are used for in vitro fermentation studies with no method clearly described as the best. Statements for biologically relevant substrates are being made when even in vivo results for suitable fermentable substrates are mixed. More comparative studies should be conducted but more over between in vitro screening leading into in vivo experiments. Due to the fragile nature of the amount of human microbiota that can be cultured, it is hard to say which media or batch fermentation method best represent the complex environment of the gastrointestinal tract. However batch fermentations can be an easy and invaluable tool of in vitro results parallel in vivo findings. Little is known about the fermentative capacity of RS type V, but per our in vitro results, we found no difference between type V, IV, or type II RS. Dose response and in vivo comparisons would add to our understanding and knowledge of this new type of resistant starch.

Acknowledgments

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REFERENCES


Figures and Tables

Table 1. Participant characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>27 ± 3 (24-29)</td>
<td>27 ± 5 (22-32)</td>
</tr>
<tr>
<td>BMI</td>
<td>23.3 ± 1.5 (22-25)</td>
<td>34.9 ± 0.9 (34-36)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>166 ± 3.9 (162-171)</td>
<td>169 ± 6.4 (162-175)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>64.9 ± 5.1 (58-69)</td>
<td>98.1 ± 7.4 (89-104)</td>
</tr>
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</table>

Eating habits by serving size

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat (~3oz)</td>
<td>≥ 2.7</td>
<td>≥ 2</td>
</tr>
<tr>
<td>Fruit (~1/2 cup or one piece)</td>
<td>2</td>
<td>≥ 2.7</td>
</tr>
<tr>
<td>Vegetables (~1/2 cup)</td>
<td>3.5</td>
<td>3.3</td>
</tr>
<tr>
<td>Whole grains (~1/2 cup, 1 slice of bread)</td>
<td>4.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Dairy products (~1 cup)</td>
<td>1.3</td>
<td>1</td>
</tr>
</tbody>
</table>

Characteristic of age, BMI, height, and weight are reported as mean ± SD (range). Eating habits were reported by means. Each participant reported daily serving sizes either from a range of 0-2 for meat, 0-4 for fruit, 1-5 for vegetables, 0, 2, 4, 6 or >6 for grains, and 0-3 or >3 for dairy products. If one or more participant reported servings sizes ‘>2’, ‘>4’, ‘>5’, ‘>6’, and ‘>3’ for meat, fruit, vegetable, whole grains, and dairy products respectively the succeeding whole number was used to calculate the mean and expressed as ≥ mean. (See Appendix C for health and diet questionnaire)
Figure 1. Total SCFA production and relation to pH by treatment within medium. Left y-axis is SCFA acid concentration (mmole/L). Right y-axis is pH scale. BHI- brain heart infusion medium, BHI-D – brain heart infusion medium without dextrose, BNG- basal nutrient growth medium.
Table 2. Media comparison of SCFA fermentation for all treatments

<table>
<thead>
<tr>
<th></th>
<th>BHI</th>
<th>BHI-D</th>
<th>BNG</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>145.72 ± 32.7149&lt;sup&gt;a&lt;/sup&gt;</td>
<td>170.39 ± 33.0553&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.0361 ± 32.9499&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0100</td>
</tr>
<tr>
<td>Propionate</td>
<td>12.5332 ± 1.8095&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>13.9238 ± 1.7818&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.6328 ± 1.8066&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0695</td>
</tr>
<tr>
<td>Butyrate</td>
<td>17.1515 ± 3.4951</td>
<td>18.7966 ± 3.2701</td>
<td>11.3514 ± 3.3032</td>
<td>0.1339</td>
</tr>
<tr>
<td>Total</td>
<td>178.02 ± 38.8286&lt;sup&gt;a&lt;/sup&gt;</td>
<td>203.32 ± 39.2102&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.2008 ± 38.8974&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0100</td>
</tr>
</tbody>
</table>

Values represented as means ± SE in mmoles/L. Means with the same superscripts within row are not significantly different. P value represents the main effect of media, whereas superscripts are differences of least square means (p < 0.05).
Figure 2. Change in pH from baseline. Measured from fecal inocula of three SR and a blank control (C).
APPENDIX

Health and diet questionnaire:

| Age: _____ (must be 18 years of age or older) | Date of birth: ____________ mo/day/year |

Ethnicity (circle as many as appropriate):

- African or African American
- Asian or Asian American
- American Indian
- White
- Latino/Hispanic
- Native Hawaiian or Other Pacific Islander

| Body weight ______________ | Height ______________ |

Do you currently have any disease or illness? yes no
specify:

Are you taking any drugs? yes no
specify:

Have you taken antibiotics in the past 6 months? yes no

How many servings of meat (~3 oz. portion) do you consume in a typical day? 0 1 2 >2

How many servings of fruits (~1/2 cup or one piece portion) do you consume in a typical day? 0 1 2 3 4 >4

How many servings of vegetables (~1/2 cup portion) do you consume in a typical day? 0 1 2 3 4 5 >5

How many servings of whole grains (~1/2 cup, 1 slice of bread) do you consume in a typical day? 0 2 4 6 >6

How many servings of dairy products (~1 cup portion) do you consume in a typical day? 0 1 2 3 >3

Figure 1. Health and diet questionnaire used to assess estimated daily food intake and if participant was eligible to be entered into the study.
CHAPTER 6. SUMMARY AND CONCLUSIONS

Digestion of resistant starches (RS) is a topic of great interest in science with a large number of implications for health and observable outcomes. However, there are four different forms of RS that have the potential to respond differently to physiological responses, and this dissertation research examines human physiological responses to a new less common type of RS. Lipid-starch or lipid-amylose complexes are being proposed to be different from other types of RS and worthy to be classified in their own category, RS type V. The other types of RS are type I that are physically inaccessible starches not fractionated and/or refined, type II are starches with native resistant granules normally amylose rich, type 3 are retrogradated starches, and type IV are chemically modified starches. Research findings have built a framework that dietary fiber fermentation in the lower gut is beneficial to gastrointestinal health and may be helpful in prevention or amelioration of gastrointestinal diseases. RS can be used as a fermentable fiber. Furthermore, in metabolic disease and diabetes it is necessary to manage glucose and insulin responses: RS can be used to lower glucose and insulin response. These are the physiological responses we targeted through the use of dietary RS. The overarching hypothesis of this dissertation is that RS (type II and V) can favorably impact human physiological responses of PPG, PPI, and lower gut microbial fermentation with minimal gastrointestinal distress. We tested this hypothesis through three research studies.

Study One (1) entitled ‘Post-Prandial Affects Of Naturally Occurring Resistant Starch In Maize On Glycemic Response In Healthy Humans’

Objectives:
• Evaluate postprandial glucose response from RS content in corn starches derived from
  exotic corn lines or a conventionally crossbred line derived from these lines when
  compared to control corn starch
• Make simple food product (pudding) to deliver test starches
• Assess gastrointestinal symptoms associated with pudding treatments

Study Two (2) entitled ‘Influence Of Crackers Containing Resistant Starch Type V On
Postprandial Glucose, Insulin, And Fermentation Response In Healthy Humans’

Objectives:
• Evaluate postprandial glucose response and insulin response from high amyllose (type II
  RS) and stearic acid complexed RS (type V)
• Make simple palatable food product (cracker) to deliver test starches
• Evaluate fermentation via breath gases (H₂, CH₄, CO₂) and fecal short chain fatty acid
  (SCFA) production.
• Record fecal parameters (frequency, weight, pH)
• Assess gastrointestinal symptoms associated with cracker treatments

Study Three (3) entitled ‘In Vitro Screening of Lipid Modified Resistant Starches And Media
Comparison On Short Chain Fatty Acid Production In Batch Fermentation’

Objectives:
• Prepare starch resides (SR) from high amyllose starch (type II RS), stearic acid complexed
  starch (type V RS), and octenyl succinate (type IV) for 24 hour in vitro batch
  fermentation comparison
• Investigate if SCFA profiles are different between lean and obese individuals and if different type of RS have an effect on SCFA production
• Compare the effects of media selections (brain heart infusion with and without dextrose and a basal nutrient growth) on SCFA production

Postprandial glucose and insulin response and fermentation are some of the physiological responses that have also been targeted by other research groups with varied results. The research submitted in this dissertation also found varied results. Study 1 and 2 investigated glucose response. In Study 1, total glucose AUC was unaffected by RS types when compared to a cornstarch control or other low RS corn starches. However, there was a postprandial decrease from the cornstarch in participants ingesting pudding with the highest RS content. Study 2 showed that RS type 2 and RS type V were effective in lowering glucose AUC when compared to a control cornstarch.

In Study 2, it was very evident that both forms of RS lowered glucose AUC. However, we also evaluated postprandial insulin response in Study two and noticed differences in RS type; HA significantly lowered insulin AUC when compared to the control starch but RS5 did not. RS content measured by AOAC 2002.12 did not differ between the two high RS crackers. HA was the parent starch complexed with stearic acid to obtain RS5. Therefore, the only difference between these starches was the lipid content, which may have contributed to the lack of significant change in insulin AUC when compared to control even though RS5 produced a lesser glucose AUC than CS. However, when insulin AUC was compared between RS5 and HA no difference was observed. Overall both forms of RS did lower glucose response and further
investigation is needed to understand lipid-starch complexes on insulin and fermentation response in vivo.

The use of RS type V in Study 2 and 3 was intended to advance what is known about physiological responses and practical application for the use of this new type of RS. Overall the experimental designs compared type V to well-established RS type II and type IV (only in Study 3) to evaluate in vivo or in vitro fermentation as well as in vivo glucose and insulin response in humans. In Study 2 fermentation differences were found between RS type II and type V. Breath hydrogen measurements after RS5 were significantly higher than all other treatments between 4 and 8 hours. In breath methane production we found HA to generate greater response than other starches, especially at 4 hours. But we found no fermentation differences between starches from analysis of fecal SCFA production. In study 3 we only evaluated fermentation by SCFA production and also found no difference between type II, IV, and V when dose and RS content was the same.

Study 1 and 2 assessed how well RS was tolerated. No treatment differences were observed for adverse gastrointestinal effects in study 1. Study 2 showed more events of bloating for RS5 than the other treatments, but these effects were very mild. Therefore these studies supported overall gastrointestinal tolerance of RS was good.

The central question still remains, how effective is the consumption of RS in improving human health? Cumulative literature supports the use of RS for improving human health. Furthermore, RS is already being manufactured and commercially distributed as additives and in food products, which supports the need for development of new RS. The research presented in this dissertation evaluated the efficacy of new resistant starches from crossbreeding exotic corn lines or lipid modification on glucose and insulin response, colonic fermentation, and
gastrointestinal tolerance. Study one used crossbred exotic corn lines with varied RS content. The objective was to test the starches for their efficacy in lowering postprandial glucose. Reduced postprandial glucose effects were observed in a parent line; however, crossbreeding did not improve RS content. The results from Study 1 did not support the use of the specific crossbred line (Guat/AR) for lowering glucose, however, suggests that crossbreeding to obtain starches with approximate RS content > 25% (similar to the parent line, Guat, with lowest postprandial peak) could produce new and effective starches. Results of this dissertation indicate that RS5, from raw starches with ~ 33% resistance, seems efficacious for glucose and fermentation responses. The author supports RS5 as a strong candidate for further study in protecting the lower intestine from diseases through colonic fermentation, and in benefitting individuals with the need to control their blood glucose.