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A high fat diet attenuates the fermentation effects of resistant starches and fructans

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A HIGH FAT DIET ATTENUATES THE FERMENTATION EFFECTS OF
RESISTANT STARCHES AND FRUCTANS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
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requirements for the degree of
Master of Science

in

The School of Human Ecology

By
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ABSTRACT

In our previous studies, ingestion of prebiotics in low fat diets resulted in decreased cecal pH from 8 to about 6 and increases in short chain fatty acids indicating robust fermentation. However, in some preliminary studies this effect on fermentation was not seen when rodents were obese and/or fed a high-fat diet. This comprehensive high-fat diet study was conducted to determine which sources and combinations of prebiotics would enhance fermentation despite a high dietary fat content. The effects of prebiotics in a high-fat diet (44% energy) on pH, cecal weights, abdominal fat, and body weight were studied in mature male C57Bl/6 mice fed one of 9 diets of similar energy (4.0 ± 0.2 kcal/g) for 12 weeks. A control (C) diet was compared to 4 prebiotics: Hi-Maize® RS2 (R), Novelose® RS3 (N), Nutraflora® fructooligosaccharide (F), BENEEO-Orafti HP gel® inulin (I), which were fed individually and combined (F+I, R+F, R+I, and R+N). Results were significant at $p < 0.05$. Fermentation, indicated by lower pH values, occurred with all F and I diets and combination diets. However, none of the groups had reduced abdominal fat compared to control as has been observed in previous studies with consumption of RS in low fat diets. All prebiotic diets had larger empty ceca, but only F and I had greater full ceca than the RS groups. It is proposed that diets with high concentrations of fat affect monogastric fermentation and microbial populations in a manner similar to ruminants. It is possible that the beneficial health effects of prebiotic ingestion may be most effective if consumed with a low-fat diet.

CHAPTER 1: INTRODUCTION

Significance of Research

The number of individuals in the United States that are overweight and obese, which is defined as a BMI of 25-29.9 or ≥ 30 , respectively, has risen dramatically over the past 35 years. According to Flegal *et al* (2010), in 2007-2008, the age-adjusted prevalence of obesity was 32.2% for men and 35.5% for women. The corresponding prevalence for overweight and obesity combined was estimated to be 72.3% for men and 64.1% for women. Obesity is associated with increased morbidity and mortality since it is a risk factor for many life-threatening, nutrition-related diseases and conditions such as: cardiovascular disease (Willett *et al*, 1995; Manson *et al*, 1990), hypertension (Montani *et al*, 2002), diabetes (Mokdad *et al*, 2004), and a variety of cancers (Calle and Thun, 2004). Therefore, finding effective means of preventing and treating overweight and obesity is of great importance to public health.

Dietary intervention has its limitations as most people are incapable of making the deliberate, lifelong dietary changes necessary for sustainable weight loss (Friedman, 2004). This is why attention should be paid to the development of new food additives and/or food products that improve the health of the consumer, and helps them to make positive lifestyle changes that do not require significant mental effort. Fiber has the potential to fill this role since its consumption is correlated with reduced food intake and improved weight status, ostensibly by promoting satiety by slowing gastric emptying (Heaton, 1973); by diluting the energy of the diet in which it is found (Rolls, 2000; Kim and Popkin, 2006); by stimulating the production of satiating gut hormones (Slavin, 2005); and by decreasing the absorption of fatty acids in the small intestine (Gades and Stern, 2003). In fact, populations that report higher fiber consumption also exhibit lower rates of obesity in both adults and children (Kimm, 1995). A handful of cross-

sectional, observational studies reveal inverse associations between fiber and body weight (Alfieri et al, 1995; Appleby et al, 1998) and between fiber and body fat (Nelson and Tucker, 1996). Optimally, people should increase the fiber content of their diet by increasing the consumption of fruits and vegetables, because these whole foods contain other compounds, such as phytochemicals, which are beneficial to human health and would be absent from purified fibers. However, some fiber is better than no fiber at all. Fiber can be added to preexisting foodstuffs with relative ease, which can be accomplished by the food industry, thereby eliminating the need for consumers to exert unnecessary effort to include it in his or her diet.

There are many different types of fiber. Resistant starches (RS) belong to a group of nondigestible fibers that resist, by varying degrees, digestion in the small intestine. By definition of being a starch, glucose molecules in the chains of RS are bonded together by α -bonds and are digestible by α -amylase. However, RS is usually not digested because of the matrix (RS1), granular structure (RS2 and RS3), or the addition of novel chemical bonds (RS4). Thus, they have a reduced caloric value compared to most starches. RS is fermented, or metabolized, for energy and growth by the resident microflora of the large intestine, resulting in increased bacterial biomass. Other byproducts of this reaction are short chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate (Topping and Clifton, 2001). These products reduce the pH of the gut, prevent the proliferation of harmful, pathogenic bacteria, and are a major source of energy for colonocytes, thereby contributing to overall gut health (Xu and Gordon, 2003). Also, the consumption of RS results in significantly higher concentrations of plasma peptide YY (PYY) and glucagon-like peptide -1 (GLP-1) (Keenan et al, 2006; Zhou et al, 2006; Zhou et al., 2008), which are satiety hormones released by the gut in response to nutrients. Both are candidates for anti-obesity treatment (Murphy, Dhillon and Bloom, 2006; Young, 2006) because

they reduce food intake and body weight when administered to humans and animals (Neary et al, 2005; Drucker, 2006).

Fructans are another type of fermentable fiber that occur naturally in a variety of plant species (Van Loo et al, 1995). These fibers are fructose polymers whose β bonds make them resistant to enzymatic digestion; ergo, they have a reduced caloric value, lead to fermentation and increased concentrations of SCFAs in the colon, and stimulate the growth of beneficial bacteria (Niness, 1999). Inulin and its hydrolysis product, oligofructose (a.k.a. fructooligosaccharides [FOS]), are two well-known members of this group. Due to its longer chain length, inulin is less soluble than oligofructose, allowing it to form microcrystals that imitate a fat-like mouthfeel in food products. Oligofructose has a sweet, satisfying flavor and has been used to replace sugar in many low-calorie and low-sugar foodstuffs.

While there is no single, overriding cause of obesity, the elevated fat and sugar content of modern Western diets is thought to be one of the main culprits, along with a sedentary lifestyle, increased food availability and portion size, and chronic stress (Hill and Peters, 1998). Fat contains more energy per gram than other nutrients, with high-fat (HF) foods having a higher energy density than low-fat (LF) foods. Since humans tend to consume a relatively constant weight of food, regardless of the fat content of the meal, diets that contain more HF foods have a greater energy density than those that do not. This, together with the increased palatability of HF foods, could lead to increased consumption and weight gain over time (Rolls, 2000; Drenowski, 1998; Rolls and Bell, 1999; Warwick et al, 2000).

Our lab is building a body of evidence supporting the idea that HF diets interfere with the process of fermentation (Badkoobeh et al, 2010; Goldsmith et al, 2010; Senevirathne et al, 2009), which could also help explain the effects of HF diets in increasing the incidence of obesity. In

previous studies, ingestion of RS in LF diets resulted in decreased cecal pH from 8 to about 6, indicating robust fermentation. However, in some preliminary studies, this effect of fermentation was not seen when rodents were fed a HF diet. The amount of RS reaching the cecum was greatly reduced with the HF diet. It appeared to have been digested during a potentially lengthened transit time through the GI tract. In a separate study, we noticed that the numbers of cecal bacteria that ferment RS were also reduced as well (Senevirathne et al, 2009). Moreover, no significant loss of body fat was observed. It would behoove the scientific community to investigate: (a) through what mechanism or mechanisms dietary fat might interfere with the fermentation of dietary fiber, and (b) does dietary fat affect the fermentation of all fermentable fibers equally regardless of theoretical digestibility (RS) or indigestibility (fructan).

Objective

The purpose of this study is to compare the effects of different types and combinations of fermentable fibers on fermentation and body fat loss. Therefore, the goal is to determine which types and/or combinations of prebiotics are better at enhancing fermentation than others in obese animals fed a HF diet.

Hypothesis

A HF diet will interfere with the fermentation of dietary RS, but not dietary fructans. This will be demonstrated by a decrease in percent total abdominal fat, a decrease in the pH of cecal contents, increased full and empty cecal weights, increased serum PYY and GLP-1 concentrations, and an increased disemboweled body weight (DBW) in subjects fed fructans, but not RS, in conjunction with a HF diet.

The reasoning behind this conclusion is that a HF diet increases transit time through the gastrointestinal (GI) tract, and this may give α -amylase more time to digest the resistant starch. If

this hypothesis is correct, then there should be less carbohydrate substrates available for fermentation in the colon (Saunders and Sillery, 1988). We expect dietary fructans to reach the cecum and be fermented regardless of the rate of intestinal transit, because fructans contain β bonds, which are not broken down by human digestive enzymes. Ergo, fructans should not be affected by the increase in transit time associated with the consumption of dietary fat.

Assumptions and Limitations

Although mice possess similar digestive physiology to that of humans, the results from this study do not apply directly to humans. Researchers that work with murine models function under the assumption that the blood and tissue samples collected from mice are representative of human subjects. Morphological and behavioral differences between mice and humans include, but are not limited to: coprophagia; possessing a much larger, more well-defined cecum; possessing a diffuse, glandular pancreas that consists of many white nodules embedded within the mesentery (Olds and Olds, 1979); and have a greater metabolic body size than humans, which necessitates higher doses of prebiotics than would be easily tolerated by humans. Nevertheless, mice and rats display similar phenotypical responses to bioactive dietary compounds such as fermentable fibers. Also, they enable us to take samples and measurements that would be too difficult in humans. Ergo, rodents are considered one of the best available models of human digestive mechanisms.

It is impossible to balance energy and total (fermentable and non-fermentable) fiber between diets simultaneously, since non-fermentable fiber is used to dilute the energy of the control diet to the same level as the prebiotic diets. The addition of Hi-Maize® to the diet as the source of RS2 adds energy to the diet at 2.8 kcal/g as well as total fiber as both fermentable and non-fermentable. The latter occurs at the levels of RS added to the rodent diets. On the other

hand, the purified cellulose used as the source of non-fermentable fiber in the control diet does not provide any energy to the diet. Therefore, the RS diet has greater total fiber than the control diet. This difference in total fiber content could be considered a confounding factor in this study. However, by balancing for non-fermentable fiber, instead of total fiber, we are able to discern which effects are occurring due to fermentation and independent of energy dilution.

CHAPTER 2: REVIEW OF LITERATURE

(Re)Defining Fiber

A renewed interest in the relationship between colonic function, fiber, and health emerged in the latter half of the twentieth century thanks to the “dietary fiber hypothesis” of Burkitt and Trowell (1975), which blamed many of the so-called “diseases of civilization” (i.e. cardiovascular disease [CVD], diabetes, obesity, constipation, diverticular disease, hemorrhoids, colon cancer, etc.) on the consumption of refined carbohydrates and/or the lack of fiber-rich foods in the diet. This theory was corroborated by several other prominent nutritionists of the time, including, but not limited to, T.L. Cleave, N.S. Painter, A.R.P. Walker, and D. Kritchevsky (Eastwood and Kritchevsky, 2005). This burgeoning interest in fiber and the beneficial effects of its consumption resulted in a veritable battle to characterize it. As a result, the definition of fiber has changed dramatically over the past half century, and has resulted in many slightly different and, occasionally, conflicting descriptions worldwide.

Originally, the term “dietary fiber” strictly meant those portion(s) of plants foods that were indigestible to human enzymes. These fibers were synonymous with “unavailable carbohydrates” (Southgate, 1969) and included structural plant polysaccharides (e.g. cellulose, hemicellulose, and pectin) and lignin, an associated polyphenol propane polymer. This was opposed to “available carbohydrates,” which consisted primarily of starches that could be digested and absorbed in the small intestine. These fibers were then subdivided into two main categories: soluble fiber, which dissolves in water, and insoluble fiber, which does not. The former gelatinizes during its journey through the intestine, thereby contributing bulk to stool, while the latter alleviates constipation and encourages laxation. Soluble fiber is also, in part, defined by its hypocholesterolemic effects, which are connected to its ability to bind bile salts.

This definition was inevitably found wanting, and has been the subject of much debate

and revision since then. This has been driven, at least in part, by the constant evolution of analytical methods: The invention of new methods of extraction and isolation resulted in the discovery of new fiber-like substances – that is, molecules that behave like traditional fiber inasmuch as they are not digestible and provide some benefit to human health – which necessitated an expansion in the definition of fiber. For example, since 1987, the United States Food and Drug Administration (FDA) has used a strictly analytical method to define fiber: Only those materials isolated by the enzymatic-gravimetric process known as AOAC method 985.29, which was developed by Prosky *et al* in 1985, are counted as dietary fiber on nutrition labels. In 1995, the World Health Organization (WHO) developed a similar definition, which includes AOAC method 985.29 and 991.43.

Most recently, in 2005, the Institute of Medicine expanded the definition of fiber to include purified gums and pectic substances, oligosaccharides such as inulin and fructooligosaccharides, and resistant starches, under a new heading labeled “functional fiber.” This new classification of fiber includes starch and non-starch polysaccharides (NSP) that may or may not be naturally occurring; have been isolated and extracted from preexisting food products; or were created by some other chemical or enzymatic means. The Institute of Medicine also suggested phasing out the terms “soluble” and “insoluble” in favor of others that provide a better description of a given fiber’s physiological properties, such as viscosity and fermentability.

The National Academy of Sciences recommends that men and women consume 38 and 25 grams of total fiber per day, respectively. However, the recommendation does not distinguish between dietary and functional fibers, or between fermentable and non-fermentable fibers. The effects of fermentable fibers on gut health have been a source of great interest in recent years due

to their numerous health benefits, especially in regards to weight management. These effects include, but are not limited to, increased satiety, reduced body weight and body fat, protection from colorectal cancer, and reduced postprandial glycemia and insulinemia (Slavin, 2005).

Fermentation and SCFAs

Our digestive system consists of not one, but two distinct processes. The first is digestion by the human body, which begins with maceration in the mouth and continues with the secretion of various enzymes and the absorption of breakdown products in the stomach and small intestine, respectively. When carbohydrates or other non-starch polysaccharides (NSP) bypass this first form of digestion, they may be fermented, or metabolized, by the resident bacteria of the large intestine for energy. These bacteria include over 400 different species and exist in a concentration of approximately 10^{11} organisms per gram of luminal contents (Savage, 1977). They possess the glycoside hydrolases and polysaccharide lyases necessary for breakdown of complex polysaccharides that are lacking in their human hosts' small intestines (Stevens and Hume, 2004).

The byproducts of this secondary digestion reaction by bacteria are short chain fatty acids (SCFAs), lactate, hydrogen gas, carbon dioxide, and methane in addition to bacterial biomass (Topping and Clifton, 2001). Acetate, propionate, and butyrate are the major SCFAs produced, but others, such as isobutyrate, isovalerate, and valerate, are produced in smaller amounts. SCFAs are important anions in the colonic lumen, because they increase colonic blood flow, stimulate sodium and fluid absorption, prevent the growth and development of aberrant cells, and have a proliferative effect on colonocytes, resulting in a thicker, healthier gut mucosa (Soergel, 1994; Scheppach, 1994). SCFA levels are highest in the proximal colon, where the concentration of nondigestible carbohydrates is the greatest and the rate of fermentation is the highest. The

lowest levels of SCFAs are found in the distal colon, since the majority of fermentable substrates in fecal matter are fermented and absorbed in the proximal and transverse colon. SCFAs generate approximately 1-2 kcal/g (Slavin et al, 2005) and are the primary energy source for colonocytes, with butyrate being the preferred fuel type. Butyrate has been thought to be particularly important for gut health, especially in the prevention of cancer. The mechanism is believed to involve regulating cell growth and differentiation and encouraging apoptosis when necessary. This has been determined in both *in vitro* (Whitehead et al, 1986) and *in vivo* (McIntyre et al, 1993) studies.

Many of the positive health effects attributed to increased levels of SCFAs and reduced intestinal pH are linked to: stimulation and/or improved maintenance of mucosal and gut-associated lymphoid tissue (GALT); increased growth and activity of beneficial microorganisms in the gut; and prevention of colonization and proliferation of potentially pathogenic bacteria (Anderson et al, 2009). Buddington, Buddington, and Sunvold (1999) showed that adult dogs consuming fermentable fibers had longer small intestines with a thicker mucosa and more surface area – resulting in a greater capacity for nutrient absorption – than those supplemented with non-fermentable fibers. In turn, Buddington and Weiher (1999) demonstrated that fermentable fibers are useful dietary management tools when recovering from diarrhea, especially when used in conjunction with oral rehydration/electrolyte solutions. Similar results, i.e. the occurrence of thicker, stronger mucosa, were documented by our lab in regards to the large intestine of rats fed RS (unpublished data). Neonatal pigs whose diets were supplemented with fermentable fibers showed reduced recovery time and improved symptoms following infection by *Salmonella typhimurium*, as measured by stool consistency and level of physical activity (Correa-Matos et al, 2003). Enteral formulas supplemented with fermentable or non-

fermentable fibers were also compared in infant pigs: the fermentable fibers were better at maintaining and improving migration of small intestinal lymphocytes and neutrophils following infection with the same pathogen (Milo et al, 2004). Similarly, supplementing total parenteral nutrition with SCFAs results in increased natural killer cell activity in rats (Pratt et al, 1996). Several other rat studies have shown that the inclusion of dietary fiber significantly decreases the rate of bacterial translocation across the intestinal mucosal barrier (Spaeth et al, 1990; Schley and Field, 2002).

Resistant Starch

Starch is a white, tasteless carbohydrate $(C_6H_{10}O_5)_n$ that occurs, primarily, in the seeds and tubers of plants. It provides plant life with an economical means of storing glucose in an insoluble and tightly packed manner (Imberty et al, 1991). Structurally, starch comes in two main forms: *amylose* – a linear, chainlike molecule consisting of $\alpha(1-4)$ bonds; and *amylopectin* – a larger, branched polymer that contains both $\alpha(1-4)$ and $\alpha(1-6)$ glycosidic linkages. Amylopectin is the most abundant polymer in starch, while the amount of amylose varies between 15-20%, depending on the plant species from which the starch comes.

Starch is largely digestible by α -amylase, glucoamylase, and sucrose-isomaltase in the small intestine (Nugent, 2005). However, RS is able to resist digestion by mammalian enzymes in the small intestine and pass into the large intestine, where it is fermented by colonic microflora. The fermentation of RS results in the same byproducts as the fermentation of fiber, with the exception of the ratios of SCFAs produced: RS generates more butyrate and less acetate than fiber (Nugent, 2005). RS was discovered in 1982 by Englyst and his colleagues when they figured out that certain processed foods, such as white bread and cooked and cooled potatoes, had greater NSP values than the equivalent raw products (Englyst, Wiggins, and Cummings,

1982). As it turned out, this excess “fiber” was really starch that failed to be hydrolyzed into glucose by digestive enzymes.

There are four major types of RS, which are defined based on the cause of resistance (Table 1) (Topping, Fukushima, and Bird, 2003). RS1 escapes digestion because it is in a physically protected form, i.e. embedded in a matrix that renders it inaccessible to digestive enzymes. This type of RS is found in unprocessed whole grains, seeds, and legumes. Actions that reduce particle size, such as mastication, milling, or grinding, generally reduce the amount of RS1 present in a given food. RS2 exists in a physically dense form known as a starch granule, which is composed solely of amylose molecules and can be tightly packed and folded into the aforementioned shape, making it harder for digestive enzymes to gain access to its terminal glucose units. It is commonly found in raw potatoes and unripe bananas. RS3, also known as retrograded starch, describes non-granular starch that becomes resistant only after it has been cooked and then cooled. This gelatinized starch is common in bread products and cooled potato salad. RS4 is resistant to digestion because its bonds have been chemically modified. That is, new chemical bonds other than the $\alpha(1-4)$ and $\alpha(1-6)$ linkages traditionally found in starch are added. Examples include etherized, esterified, and/or cross-linked starch molecules and fatty acid attachments.

Table 1. Description and potential food sources of different RS types

Type of RS	Description	Food Sources
RS1	Physically inaccessible starches; protected by food matrix	Whole or partially milled grain, rice, seeds, legumes, and pasta
RS2	Raw granular starches	Raw potatoes, green bananas, high amylose starches
RS3	Retrograded starches	Cooked and cooled potatoes, bread, and cornflakes
RS4	Chemically modified starches	Not naturally occurring; foods in which modified starches have been used

Concerning microbial fermentation, RS has a prebiotic effect in the large intestine and leads to increased SCFA production, which reduces cecal and fecal pH levels. Fermentation and the liberation of SCFAs are associated with increased proglucagon – the precursor for GLP-1 – and PYY gene expression (Keenan et al, 2006; Zhou et al. 2006,), especially in the cecum and proximal colon, where the bulk of fermentation occurs and its SCFA byproducts are located. Increases in plasma GLP-1 and PYY concentrations have also been documented (Keenan et al. 2006; Zhou et al., 2008; Shen et al, 2008). These outcomes could be beneficial in the control of metabolic syndrome, diabetes, and, potentially, obesity.

GLP-1 and PYY are anorexic/satiety hormones secreted by the gut in response to the presence of nutrients. PYY has been shown to reduce food intake and weight gain when infused in human subjects as well as animal models (Batterham et al, 2002; Batterham et al, 2003). Proglucagon is the precursor of several peptide hormones, including glucagon and GLP-1. Proglucagon is destined to become GLP-1 if it is secreted by the L cells of the small intestine. GLP-1 also reduces food intake and body weight in animals and humans (Neary, 2005; Drucker, 2006). These two hormones and others like them alter energy balance by sending signals from the gut to the brain, thereby changing brain neuropeptide expression (Wren and Bloom, 2007). Specifically, through activation of hypothalamic proopiomelanocortin (POMC) neurons, which are associated with increased energy expenditure and could be, in part, responsible for the body fat effect seen in RS-fed animals when compared to an energy-control (Keenan et al., 2006; Shen et al, 2009; Zhou et al., 2009). Both have been proposed as potential anti-obesity/diabetes drugs.

RS has a smaller net metabolizable energy value than digestible starch; approximately 2.68 to 3.06 kcal per gram of RS rather than 4.00 kcal per gram of regular starch (Tulley et al, 2009). Adding RS to a diet dilutes the energy density of the diet, resulting in reduced caloric

intake as well as reduced postprandial glycemia and insulinemia (Brennan, 2005). Also, when rodents were fed a diet that consisted of 25% RS2, they ended up with less body fat than rodents eating the same number of calories of an isocaloric diet, but no RS (Keenan et al, 2006; Shen et al, 2008; Zhou et al, 2009). This statistic does more than highlight the potential weight capabilities of RS replacement. It shows that the aforementioned reductions in adiposity go beyond simple energy dilution, because they are compared to an energy control, whose energy density has been diluted by the addition of cellulose. Other documented effects of RS replacement are greater full and empty cecal weights, lower pH value of cecal contents (which is beneficial for gut health), and increased plasma GLP-1 and PYY concentrations. All of which are associated with, and may be, the result of the fermentation of the RS that has reached the colon. However, these effects, as well as the reduction in percent total abdominal fat, are attenuated when the diet in question has a HF (20%) content as opposed to a LF (7%) content (Zhou et al, 2009) . Ostensibly, this occurs because RS is not reaching the cecum (unpublished data), potentially due to increased transit time resulting from the greater fat content of the diet (Saunders and Sillery, 1988). According to Kendall *et al* (2004), RS doses of 20-30 g/day are needed to observe physiological effects in humans. This level of consumption is 3-4 times higher than actual levels of RS consumption in the United States, which is estimated to be 3-8 g/day (Murphy et al, 2008).

Fructans

Fructan is the name given to members of a family of oligo- and polyfructoses composed primarily of $\beta(2-1)$ and/or $\beta(2-6)$ linkages between several to many fructose molecules, with or without a terminal glucose molecule. These bonds make fructans indigestible to mammalian enzymes and, therefore, fermentable. After starch, fructans are the most abundant non-structural

polysaccharide found in nature. Americans consume an approximately 1–4 g of inulin and oligofructose per day, while Europeans average 3–10 g/d (Van Loo et al, 1995). Intake varies according to geographic region and season as well as race and socioeconomic status (Moshfegh et al, 1999). One of the most common structural forms of fructans is the polymer inulin, which can be hydrolyzed into oligomers of fructooligosaccharides (FOS). Both contribute 1.5 kcal/g and are abundant in a variety of plants including, but not limited to, wheat, onions, Jerusalem artichokes, jicama, green beans, and leeks (Roberfoid, 2007). Studies have shown that the ingestion and subsequent fermentation of fructans provide many of the same health benefits as other fibers, such as aiding in colon function by increasing stool frequency and weight (Cani and Neyrinck et al, 2005; Roberfoid, 2005). For all of these reasons, fructans can be classified as a part of the dietary fiber complex (Roberfoid, 1993). However, since most commercially available inulin and oligofructose is either synthesized from precursors or extracted from fructan-containing plants, they can also be sorted into the functional fiber category.

The number of publications proclaiming the health benefits of inulin and oligofructose is impressive. Since fructans are not broken down by mammalian enzymes, they have no real influence on blood glucose or insulin levels after ingestion. This makes them useful ingredients in processed foods geared towards diabetics. The consumption of fructans has been linked to improved lipid metabolism in humans, resulting in reduced serum triglyceride and LDL cholesterol levels in hyperlipidemic individuals (Davidson and Maki, 1999). The effects of fructans on lipid metabolism have consequences on the development of atherosclerosis as well. ApoE-deficient mice fed inulin at 10% w/w developed significantly less aortic plaque than controls (Rault-Nania et al, 2006).

Both inulin and oligofructose are well-known as stimulators of the growth and metabolic

activity of a select number of beneficial bacterial species, including lactic acid-producing *Bifidobacterium spp* and *Lactobacillus spp* (Gibson and Roberfoid, 1995). Consumption of fructans is tantamount to feeding these valuable bacterial species; helping them to outcompete other, potentially pathogenic organisms such as certain *Clostridia spp*, *Candida albicans*, *Salmonella*, or *Listeria* (Buddington, Donahoo, and Buddington, 2002; Gibson and Roberfoid, 1995; Roberfoid, 2007; Wand and Gibson, 1993).

Like RS, fructans helps modulate food intake and satiety through the agency of its SCFA byproducts. The consumption of inulin-type fructans is associated with increased portal serum GLP-1 and colonic proglucagon gene expression, increased serum glucose-dependent insulinotropic polypeptide (GIP), and decreased serum ghrelin in both rats and mice (Cani et al, 2004; Cani et al, 2005). GLP-1 is of particular importance, since treatment with a GLP-1 receptor antagonist abolishes improvements in glucose tolerance and insulin sensitivity normally associated with the consumption of oligofructose (Cani et al, 2006).

High Fat Diets and Gut Microbiota

The obesity epidemic is the result of interplay between a myriad of biological, psychological, and environmental factors. Even so, much of the credit is given to the HF content of the modern Western diet, in part because fat has a greater energy density than either protein or carbohydrate (9 kcal/g versus 4 kcal/g), and is considered more palatable, which can easily lead to overconsumption of calories (Rolls, 2000; Drenowski, 1998; Rolls and Bell, 1999; Warwick et al, 2000). What's more, eating foods that contain large amounts of dietary fat is not the same thing as eating a HF diet. The consumption of dietary fat results in the inhibition of gastric emptying; stimulation and subsequent release of various satiety hormones, including PYY and GLP-1; and reduction of energy intake at successive meals. However, many animal and some

human studies have shown that chronic consumption of foods with high dietary fat content can alter the normal effects of fat on gastric emptying and intestinal transit as well as the secretion and action of GI peptides (Little, Horowitz and Fienle-Bisset, 2007). Chronically HF diets have also been known to affect the gut microbiota which, in turn, affects the fermentation of fiber and the health of the host (Cani et al, 2007; Senevirathne et al, 2009).

The microbiota constitutes a metabolic “organ” in so much as it performs functions that we have not had to evolve on our own. These functions include the ability to process otherwise indigestible components of our diet. The result is an increased capacity for energy harvest. Adult-germ free (GF) mice conventionalized with normal microbiota harvested from conventionally raised animals results in a 60% increase in body fat content despite decreased food intake (Bäckhed et al, 2004). It is not just the presence of the microbes in the gut that affects health and adiposity, but the composition of the bacterial population as well. According to Lay *et al* (2005), in healthy adults, 80% of known fecal microbiota can be classified into 3 dominant phyla: *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. In general, the *Firmicutes* to *Bacteroidetes* ratio is considered to be extremely relevant in human gut microbiota composition. The microbiota in obese subjects shows an elevated proportion of *Firmicutes* and a reduced population of *Bacteroides*. Conversely, a decreased *Firmicutes* to *Bacteroidetes* ratio has been directly related to weight loss (Ley et al, 2006). This ratio is not static. It is greatly affected by the composition of the host’s diet.

Our lab has had some difficulty preserving the fermentation of RS in rodents, especially mice, made obese by a HF diet. The stereotypical outcomes associated with RS consumption – i.e. increased cecal size, reduced cecal pH, and reduced adiposity – are lost in mice made obese on a HF diet (Zhou et al, 2009). Humans and animals made obese and/or diabetic through HF-

feeding also exhibit increased metabolic endotoxemia – as measured by increased plasma concentrations of lipopolysaccharide (LPS), an inflammatory compound and major component of the outer membrane of Gram (-) bacteria – in addition to adipose tissue inflammation and metabolic disorder (Amar et al, 2008; Cani et al, 2008). HF diets are also known to alter the Gram (-) to Gram (+) ratio, reducing the concentrations of important Gram (+) bacteria, such as *Bifidobacteria spp*, which help reduce endotoxemia and improve mucosal function (Cani et al, 2007); ostensibly, through its ability to ferment fiber and stimulate the host's immune system. As a doctoral student in our lab, Dr. Reshani Senevirathne determined that the number of colony forming units (CFU) of *Bifidobacterium spp.*, *Lactobacillus spp.*, and beneficial *Clostridia spp.* isolated from cecal contents of C57Bl/6J mice fed RS, were greatly reduced in a HF (41% total energy), but not low (18%) or medium fat diets (28%) (Senevirathne et al, 2009).

CHAPTER 3: MATERIALS AND METHODS

Animals, Housing, and Diets

One hundred male C57BL/6 mice (JAX[®] Mice and Services, Ben Harbor, Maine), aged 7-8 weeks, were housed in groups of 3 or 4 in shoebox cages in a climate-controlled environment ($22 \pm 2^{\circ}\text{C}$, 65-67% humidity) with a 12:12 h light-dark cycle illuminated at 7 AM. For three weeks prior to treatment, subjects were fed a high-fat, high-energy diet (D12331, Research Diets, New Brunswick, NJ) in order to induce weight gain, as well as to become acclimated to the powdered diet and new environment.

Next, the animals were stratified according to weight, and assigned to one of ten energy-balanced (4.0 ± 0.1 kcal/g) diets whose general percent compositions by weight (g/kg) are shown in Table 2. (Note that the study was analyzed as a completely randomized design because body weight is a dependent variable and randomization without regard to body weight would bias the analyses). A complete diet table can be found in Appendix C: Diet Table and Mixing Instructions.

Table 2. General percent compositions by weight (g/100 g) of experimental diets

	C (n=20)	R (n=10)	N (n=10)	F (n=10)	I (n=10)	F/I (n=10)	R/F (n=10)	R/I (n=10)	R/N (n=10)
Protein	14%	14%	14%	14%	14%	14%	14%	14%	14%
Carbs	43%	55%	55%	45%	45%	45%	55%	55%	55%
Fat	20%	20%	20%	20%	20%	20%	20%	20%	20%
RS	-	29%	29%	-	-	-	23%	23%	29%
Fructan	-	-	-	10%	10%	10%	10%	10%	10%

Originally, we planned on having two levels of FOS consumption: 10% by weight to match the fermentable fiber levels popular in the scientific literature, and 30% by weight to match the levels of RS that our lab uses for our RS diets. We also created two different control diets in order to ensure that we did not make an unfair comparison between diet groups containing two drastically different levels of fermentable fiber. Since RS is not as readily fermentable as fructans, more of it is required in order to achieve beneficial health effects. Unfortunately, we were unable to get the higher concentration of fructans into the diets: the mixture became discolored and started to clump in a very unappetizing manner. In the end, we determined that the two control diets were similar enough in general composition, nonfermentable fiber content, and energy density to be pooled together for analyses, resulting in one large control group (See Appendix B).

The combined control diets (C) were compared to diets containing four different prebiotics: *Hi-Maize*® (R), *Novelose*® (N), (National Starch Food Innovation, Bridgewater, NJ) *Nutraflora*® *fructooligo-saccharide* (F), (GTC Nutrition and Corn Products International, Westchester, IL) and *Orafti HP gel*® (I), (a gift from Dr. Kai Aryana, LSU, Dairy Science) which were fed individually and in certain combinations (F/I, R/F, R/I, and R/N). Water and assigned semipurified powder diet were available *ad libitum* throughout the experiment. The experimental protocol was approved by the Louisiana State University Institutional Animal Care and Use Committee (IACUC #06100), which can be found in full in Appendix A.

Methods and Measurements

Due to the large number of subjects, the sacrifice took place over a three-day period. At sacrifice, mice were weighed prior to exsanguination via cardiac puncture under isoflurane anesthesia. After all of the blood was withdrawn, the chest was opened and the heart was

removed to assure death prior to dissection. The blood was collected in EDTA tubes and centrifuged at 4000g for 20 minutes in order to extract serum, which was used to determine plasma PYY and total GLP-1 concentrations using a radioimmunoassay kit from Millipore (St. Louis, MO).

Epididymal, perirenal, and retroperitoneal fat pads from the abdominal cavity were dissected, and their combined weight was counted as total abdominal fat. Full and empty cecal weights were recorded, and the weight of the cecal contents was calculated as full cecal weight minus empty cecal weight. Also, disemboweled body weight (DBW) was determined by subtracting the weight of the full GI tract (rats not fasted prior to sacrifice), which included the stomach as well as the small and large intestines, from the weight of the animal from which it came.

The cecal contents were stored in separate 1.5mL microcentrifuge tubes and preserved in liquid nitrogen. Later the samples were transferred to a deep freeze (-80°C) for long term storage. The pH of the cecal contents was determined by using a combination electrode in thawed cecal contents that had been homogenized in distilled water at a ratio of 0.5 g sample to 5 ml water. Next, the samples were acidified with 1 ml of a 25% (w/w) solution of metaphosphoric acid, which contained 2g/l of 2-ethyl-butyric acid as an internal standard for the SCFA contents. The solids in the homogenates were separated by centrifugation, and the effluent was analyzed using gas-liquid chromatography.

Statistical Analysis

Data was analyzed using PASW® (formerly SPSS) Statistics GradPack 18. A one-way ANOVA followed by LSD *post hoc* mean comparison test was performed for all measurements. Results were considered significant at $p < 0.05$. Values are expressed as means \pm SE.

CHAPTER 4: RESULTS

All prebiotic groups had significantly greater full and empty cecal weights than the control group ($p < 0.000$). The R group consistently displayed the smallest increases among the treatment groups (Fig 1 and 2).

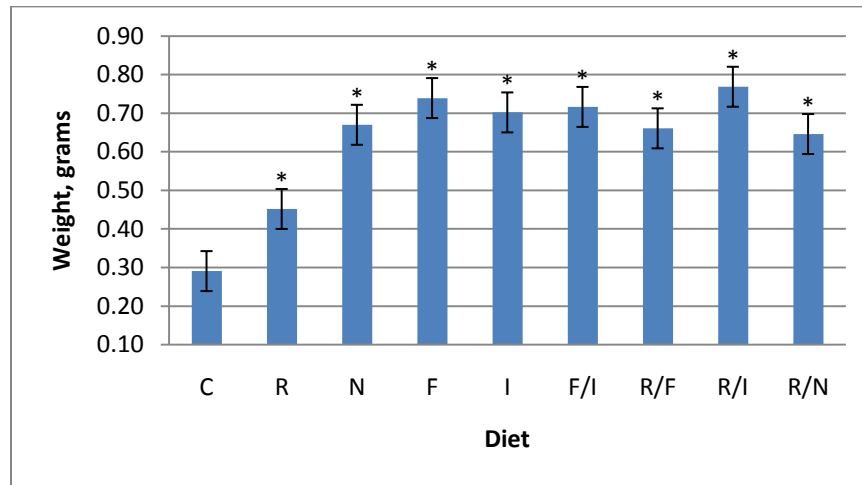


Fig 1. Full Cecal Weight.

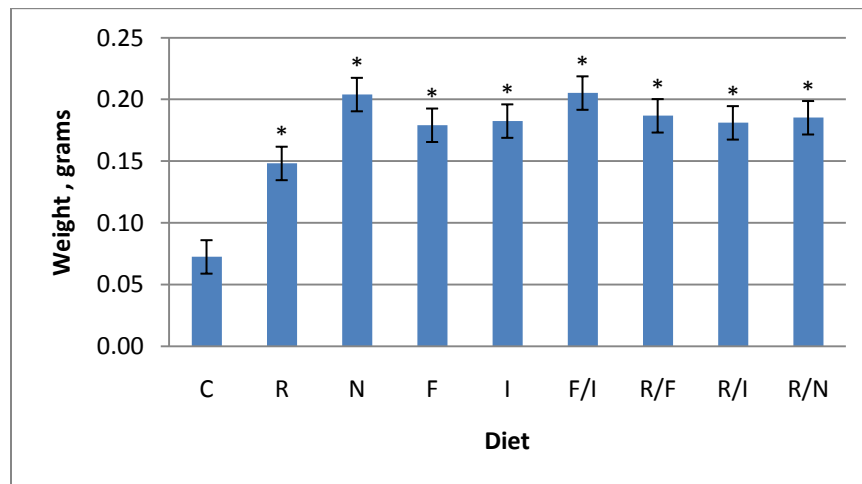


Fig 2. Empty Cecal Weight.

Our lab considers fermentation to have occurred when the pH value of the cecal contents is significantly reduced when compared to the control. This occurred with all fructan (F, I, and F/I) and combination (R/F, R/I, R/N) groups. The R and N groups individually failed to reduce the

pH of the cecal contents, despite the aforementioned increases in full and empty cecal weights. In fact, the N group exhibited a pH value that was numerically greater than the control (Fig 3).

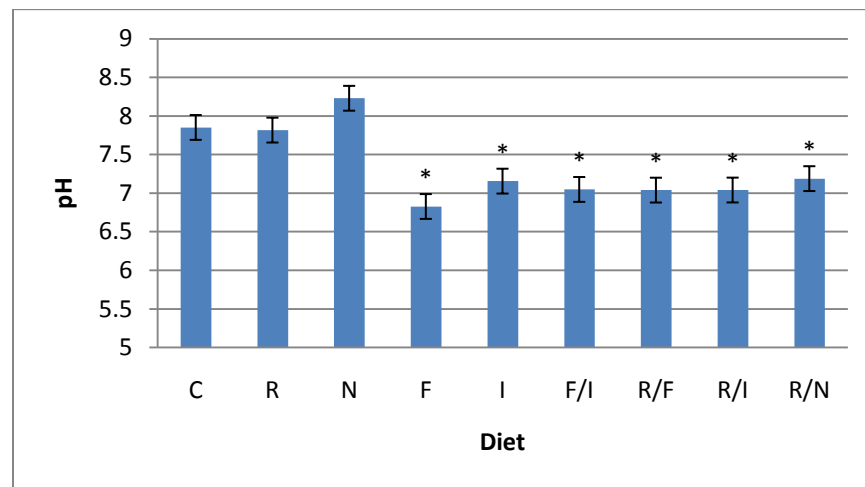


Fig 3. pH of Cecal Contents.

The concentration of SCFAs (acetate, propionate and butyrate) in the cecal contents (mM/g wet weight) varied greatly between groups, and depicted no correlated increases in significant decreases in pH levels. There was no significant difference between the C, R, N, and I groups. F, R/F, R/I, and R/N were significantly greater than both the C and R groups, and F/I was significantly greater than R, but not C (Fig 4 and Fig 5; a = significantly different from C; b =

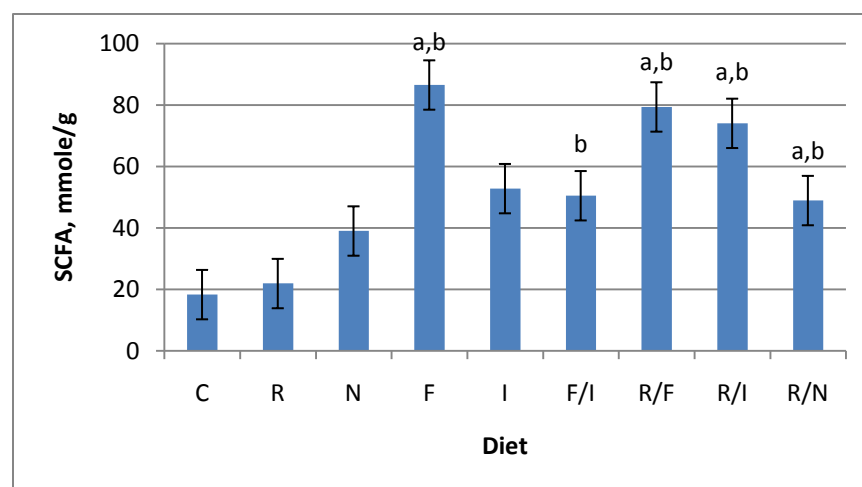


Fig 4. Total SCFA Concentration, mmole/g.

significantly different from R; a,b = significantly different from both C and R).

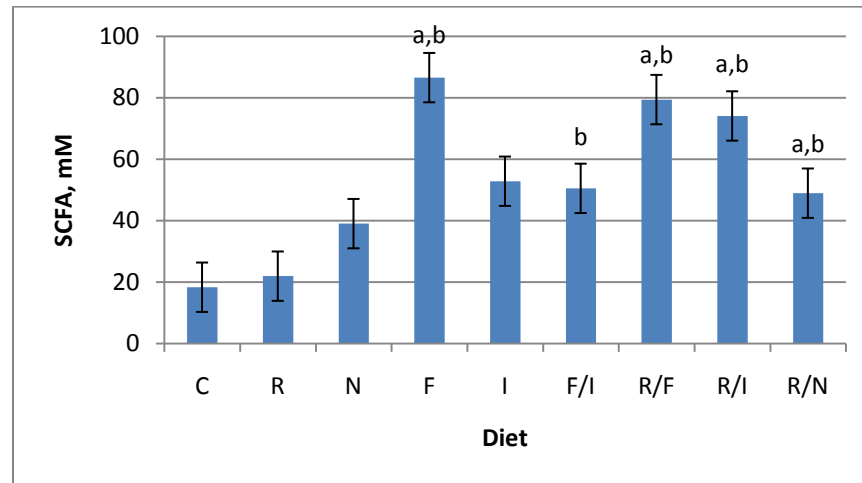


Fig 5. Total SCFA Concentration, mmole.

There were no significant differences in percent total abdominal fat (total abdominal fat/DBW) between the groups ($p>0.056$; Fig 6). An increase in DBW ($p<0.000$; Fig 7) was observed in subjects that consumed FOS, either alone or in combination with other prebiotics, but there was no concurrent increase in the DBW to fat ratio ($p>0.566$).

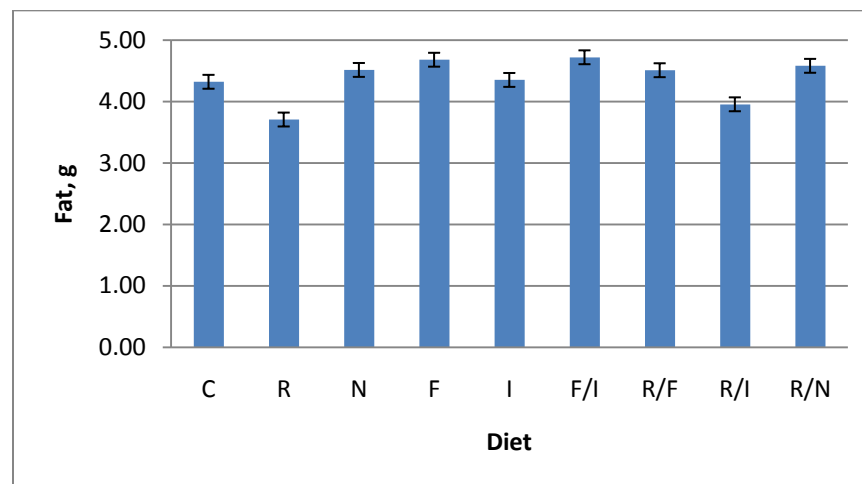


Fig 6. Abdominal Fat at Sacrifice.

We were unable to obtain data on the serum concentrations for PYY and GLP-1, because the antibody in our RIA kits was defective and we did not have enough serum sample to run the

assay a second time.

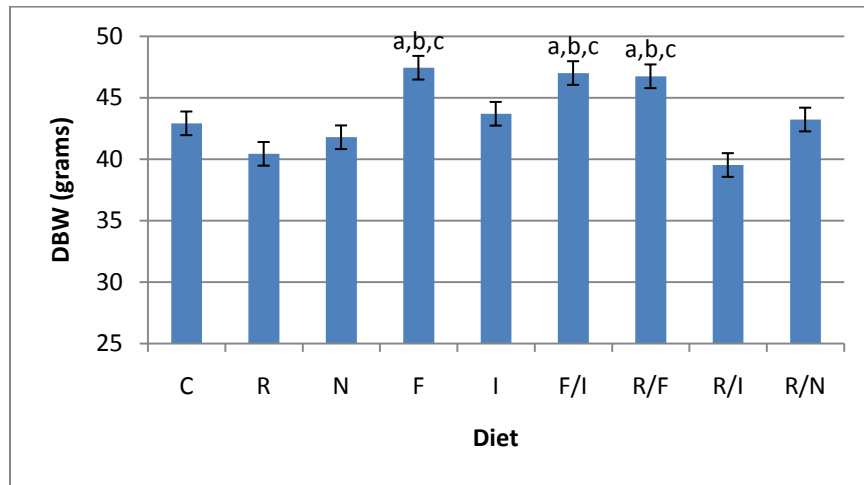


Fig 7. Disemboweled Body Weight.

CHAPTER 5: DISCUSSION

The purpose of this study was to determine which fermentable fibers, if any, would be fermented better by the intestinal microflora of mice fed a HF diet. We hoped that, by experimenting with different types and combinations of fermentable fibers, that we could (a) determine whether or not a HF diet interferes with the fermentation of all fermentable fibers equally; and (b) determine whether or not we could preserve the fermentation of RS and salvage the fibers' ability to reduce body fat on a HF diet. We assumed that the cause of the diminutive fermentation seen in animals on HF diets was due to RS not reaching the cecum (unpublished data), because the consumption of fat is known to slow intestinal transit (Saunders and Sillery, 1988). Presumably, digestive enzymes would have more time to degrade the RS, resulting in less RS making it to the cecum to be fermented. We also assumed that, because fructans were completely immune to digestion in the small intestine, that they would not be affected by changes in intestinal transit. Diets containing fructans, or a combination of fructans and RS, would result in greater fermentation than diets that just had RS, and as a result of this fermentation there would be a decrease in body fat. We may have been mistaken.

Body Fat and DBW

Unfortunately, we did not see an effect of any treatments on reducing body fat as has been observed in our studies with low fat diet consumption with RS (Keenan et al., 2006, Shen et al., 2009; Zhou et al. 2009). Differences in percent total abdominal fat levels (abdominal fat pads) at sacrifice were statistically not significant. There were promising, significant increases in the DBW of animals fed diets containing FOS, but there was no parallel decrease in the abdominal fat pads/DBW ratio. Therefore, we cannot be certain that those subjects predominantly gained lean muscle over fat since total body protein was not measured in this

study. Our lab normally uses DBW and the abdominal fat pads/DBW ratio, because we do not fast animals prior to sacrifice. Fasting would interfere with the observation of fermentation effects (unpublished data) and hormone production (Roy et al. 2003). Additionally, rodents fed RS diets have greater GI tract contents than control rodents due to fermentation (Keenan et al. 2006). Thus, DBW is used in order to prevent bias of the total abdominal fat/body weight ratio in favor of the RS fed rodents. An equal amount of total abdominal fat for RS and control would end up with a lower total abdominal fat/DBW ratio if body weights with the contents of the GI tract are used. However, the use of DBW may actually favor the control rodents, since the GI tract is a legitimate component of the animal's lean tissue. Future studies will use the DBW plus the empty GI tract weight in order to avoid that particular bias. The latter may be the superior factor for measurements of adiposity when studying animals consuming fermentable fibers. We expected to see a lack of favorable changes in adiposity with obese, RS-fed animals, because of previous results obtained from experiments on genetic models of obesity (Zhou et al, 2009). What we did not expect to see was the same outcome for animals fed inulin or FOS. This was surprising for a number of reasons: (1) With the exception of the R group, the prebiotic groups had increased both full and empty cecal weights compared to control, which is typically indicative of robust fermentation reactions; and (2) according to the scientific literature, we should have seen reductions in body fat in animals fed fructans with significant, positive changes in cecal weight. Collaborating scientists, Dr. Patrice Cani, Dr. Nathalie Delzenne, Dr. Catherine Daubioul, and Dr. Jacques Amar, have consistently documented body fat and body weight loss in various animal models (i.e. rats on normal and HF diets, obese and diabetic rats) supplemented with 10% inulin or oligofructose (Cani and Neyrinck et al, 2005; Cani and Daubioul et al, 2005; Daubioul et al, 2000; Daubioul et al, 2002). These changes in adiposity were often accompanied

by reduced postprandial serum triglyceride and cholesterol levels.

The results of Daubioul *et al* (2002) were particularly interesting, since fatty Zucker (fa/fa) rats are decent corollaries to our diet-induced obese mice as both are obese models. However, Zucker rats are a genetic model of obesity that may have a microflora that can robustly ferment fructans. Our diet-induced obese mice were lean before feeding a Western diet and may have had their ability to robustly ferment fructans reduced with the Western diet used to induce obesity in the run-up to the current study. Thus, feeding a HF diet prior to feeding a fermentable fiber may produce different effects than feeding the high fat in conjunction with the inclusion of a fermentable fiber in the diet. It is also possible that rats are better able to compensate for the effects of prior obesity in regards to fermentation. If that is the case, it might behoove our lab to consider using rats exclusively in future studies that include both HF diets and fermentable fibers. Another added benefit of using rats is directly related to their greater size. There is significantly more blood and tissue in rats compared to mice, both of which are easier to collect in the former compared to the latter. Also, there would be a decreased likelihood of missing or altering the statistical significance of data points by accident through human error at sacrifice.

The work of Daubioul *et al* (2002) was also the only one of the aforementioned studies that used animals that were already obese (fatty [fa/fa] Zucker rats) prior to supplementation with fructans, as was done for the current study. In the current study, it is also possible that the microbial composition of the animals' GI tract was significantly and negatively altered during the initial HF feeding referred to above and described in the Materials and Methods section. Without the proper concentration and/or composition of bacteria in the colon, the fructans and other fermentable fibers cannot be robustly broken down and thus cannot contribute to the body fat effect normally characteristic of its consumption. The Zucker rats may not have had similar

changes in the gut microflora as in the current study.

pH and SCFAs

The amount of fermentation in the cecum as determined by pH levels alone was possibly reduced as compared to a previous study (Keenan et al. 2006), which possibly explains why the animal subjects did not lose significant amounts of body fat. Significant decreases in pH levels were documented for all groups ingesting fructans individually and in combination with other fibers. The group fed the combination of RS2 and RS3, R/N, also had a significantly lower cecal content pH, but neither type of RS affected pH when fed individually. Additionally, the N group had increased full and empty cecal weights without a corresponding reduction in the pH of cecal contents. This result is an anomaly. It is possible that this type of RS is able to affect cecal size by some means other than fermentation, perhaps through a bulking effect caused by the simultaneous stretching and growth of the organ itself, but this is an unknown at this time.

As previously stated, the amount of fermentation was less than expected. In this study, most of the groups that had significant changes in pH had pH values between 7.0 and 7.5, but in a previous study we observed pH values between 6.5 and 7.0. Only the pH of cecal contents for the F group fit within the range of previously published data. These possibly moderate reductions in pH were accompanied by reduced production of combined acetate, propionate and butyrate concentrations overall. Once again, the R and N groups were ineffective at reducing the pH of the cecal contents compared to control but, groups that contained FOS or a combination of fermentable fibers in their diets had statistically significant lower pH of cecal contents compared to control. The amount of SCFAs measured for F, F/I, R/F, R/I, and R/N groups were greater than the C and R groups, which were statistically identical.

Gut Hormones

The lack of data regarding plasma concentrations of gut hormones is disappointing, to say the least. If I had to speculate about what the results would have been, I would expect either no significant changes or a slight trend towards increased plasma concentrations of PYY and GLP-1 in animals fed fructans. This would match the results we obtained for pH levels and total SCFA concentrations of the cecal contents.

The central GLP-1 receptor system in the brain decreases fat storage by direct modulation of lipid metabolism in lean, but not obese, animals. Diet-induced obese animals receiving an intracerebroventricular infusion of GLP-1 fail to decrease lipid stores in white adipose tissue. This suggests that obese animals become resistant to the actions of GLP-1 (Nogueiras et al, 2009). Diet-induced obese mice also develop hyperglycemia, hyperinsulinemia, hyperleptinemia, and develop leptin resistance. Leptin stimulates the secretion of GLP-1. Obese animals, including humans, develop concurrent resistances to both leptin and GLP-1, resulting in reduced plasma GLP-1 levels overall (Anini et al, 2003). It is possible that leptin-resistance developed in the mouse model used in this study. If this is true then fermentation could have been enough to stimulate fat oxidation and weight loss, but was undermined by the resulting leptin resistance. Obese animals do not appear to be resistant to the actions of PYY as they are with leptin and GLP-1. Obese humans are able to respond to the effects of PYY, but possess lower endogenous fasting and postprandial levels of PYY. It is possible that the reduced level of fermentation seen in obese animals contributes to the pathophysiology of obesity by reducing plasma PYY concentrations (Batterham et al, 2003).

Reflections

Reduced fermentation of RS, but not fructans, can be explained by the hypothesis present

in my introduction. An alternative hypothesis, which was partially explained and supported in the subsection “Body Fat and DBW”, could be that a HF diet reduces the concentration of beneficial, fermentative bacteria in the colon, in which case we would expect to see little or no fermentation of both types of fiber no matter what individual fibers or combination of fibers were used. The intestinal microflora could have been changed in one of two ways: during the initial high fat feeding, which made all of the mice overweight, or during the treatment, which included a high fat diet. Most likely, this deleterious change would have occurred prior to treatment, since HF-fed diabetic mice treated with oligofructose reduced body fat gain compared to a HF-fed control (Cani et al, 2006). This wouldn’t be possible if the microflora had changed during treatment. Based on Cani *et al* (2006), it would appear that the inclusion of fermentable fibers in a HF diet might prevent the negative changes to the intestinal microflora normally associated with fat-enriched diets.

Another possibility is that the fermentation of fructans was enough to induce changes in gut hormones and adiposity, but the HF diet interferes with the body fat effect somewhere distal to the gut. Likely sites of interference include; within the brain (Nogueiras et al, 2009), specifically, within the arcuate nucleus of the hypothalamus, where gut hormones bind and affect lipid metabolism directly; or at the level of gut hormones themselves (Batterham et al, 2003), which would be mediated by interactions with other hormones such as leptin produced in white adipose tissue.

A third alternative hypothesis is that the high energy density of the HF diets causes decreased consumption of the powered diets. That is, the animals are not consuming enough fiber because, volumetrically speaking, they don’t need to consume as much of the diet in order to meet their caloric needs. However, without accurate data on food intake, I can neither confirm

nor refute this hypothesis at this time.

Conclusion

The term "RS" refers to starch that is not digested into glucose in the small intestine like refined carbohydrates, such as starch in white bread and pasta, typically are digested. Because RS withstands digestion in the small intestine, it travels on to the large intestine, where it becomes food for the bacteria that reside there. This reaction is known as fermentation, and its byproducts include increased concentrations of SCFAs and the proliferation of beneficial bacteria. Both of which are thought to have beneficial effects on human health.

Our lab has difficulty documenting the fermentation of RS and the health benefits that normally accompany its consumption in animals that are obese and/or consuming a chronically HF diet. The purpose of this study was to determine whether or not this was true for all types and combinations of fibers. We thought that, due to their chemical nature, fructans would be capable of withstanding the increased transit time that accompanies the consumption of a HF diet. We expected that the consumption of fructans or a combination of fructans and RS would result in fermentation and body fat loss.

As it turns out, our hypothesis was only partially correct. Fermentation occurred with individual fructans and with all combination groups, but there was no reduction in percent total abdominal fat. The R and N groups were the least effective fermentable fibers out of all the prebiotic treatments when included in a HF diet. Conversely, the F group and, to a lesser extent, the I group, were the most fermentable fibers overall. Somehow, the consumption of Novelose® resulted in increased full and empty cecal weights without a corresponding reduction in cecal pH or increase total SCFA concentration. This is an anomaly and we have no plausible explanation for this result.

It is possible that a HF diet inhibits the growth of microbes that engage in fermentation, thereby reducing fermentation in the gut and contributing to weight gain. This would suggest that the health benefits of prebiotic ingestion may only be evident on a LF diet, or that prebiotics may need to be consumed separately from HF foods. It is also possible that, while the level of fermentation was seemingly reduced on the HF diet, it is still enough to elicit the characteristic effects of increased fat oxidation and weight loss through increased gut hormone production. If this is the case then the HF diet would have to act in a manner that is distal to the gut; potentially by blocking the effects of gut hormones within the brain.

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VITA

Felicia Robin Goldsmith was born in 1987 in Oceanside, New York. She is the youngest child and only daughter of Sherry and Elliot Goldsmith. She graduated from Oceanside High School in May of 2005. Three years later, Felicia completed her Bachelor of Science degree in cellular and molecular biology at Tulane University, New Orleans, Louisiana. She began graduate school at Louisiana State University Agricultural and Mechanical College in January of 2009. During her career as a graduate student, she worked as both a graduate teaching assistant and as a graduate research assistant. She also presented her thesis research as an oral presentation at Experimental Biology 2010 in Anaheim, California. Felicia plans to graduate with her master's in human ecology with a concentration in molecular nutrition. Upon completing her master's program, Felicia plans to continue her education at LSU by obtaining her doctorate in the same. Eventually, she would like to pursue a career as a professional academic and researcher.

APPENDIX: DIET TABLE AND MIXING INSTRUCTIONS

INGREDIENTS			C1		C2		R		N		F	
	<i>kJ/g</i>	<i>kcal/g</i>	<i>gram</i>	<i>kJ</i>	<i>gram</i>	<i>kJ</i>	<i>gram</i>	<i>kJ</i>	<i>gram</i>	<i>kJ</i>	<i>gram</i>	<i>kJ</i>
Amioca	14.65	3.50	386.90	5669.83	366.90	5376.74					296.90	4350.92
Hi-Maize	11.72	2.80					511.90	6001.31				
Novolose	11.72	2.80							511.90	6001.31		
Inulin / scFOS	6.28	1.50									100.00	628.05
Sucrose	16.75	4.00	100.00	1674.80	100.00	1674.80	100.00	1674.80	100.00	1674.80	100.00	1674.80
Casein	14.99	3.58	140.00	2098.52	140.00	2098.52	140.00	2098.52	140.00	2098.52	140.00	2098.52
Cellulose			125.00	0.00	145.00	0.00					115.00	0.00
Oil	35.38	8.45	100.00	3538.02	100.00	3538.02	100.00	3538.02	100.00	3538.02	100.00	3538.02
Lard	36.85	8.80	100.00	3684.56	100.00	3684.56	100.00	3684.56	100.00	3684.56	100.00	3684.56
Mineral Mix	3.68	0.88	35.00	128.96	35.00	128.96	35.00	128.96	35.00	128.96	35.00	128.96
Vitamin Mix	16.20	3.87	10.00	162.04	10.00	162.04	10.00	162.04	10.00	162.04	10.00	162.04
Choline Chloride			1.30	0.00	1.30	0.00	1.30	0.00	1.30	0.00	1.30	0.00
L-Cysteine	16.75	4.00	1.80	30.15	1.80	30.15	1.80	30.15	1.80	30.15	1.80	30.15
Total			1000.00	16986.87	1000.00	16693.78	1000.00	17318.35	1000.00	17318.35	1000.00	16296.01
Total (kcal/g)				4057.05		3987.05		4136.22		4136.22		3892.05
% diet				4.06		3.99		4.14		4.14		3.89

INGREDIENTS			I		F/I		R/F		R/I		R/N	
	<i>kJ/g</i>	<i>kcal/g</i>	<i>gram</i>	<i>kJ</i>	<i>gram</i>	<i>kJ</i>	<i>gram</i>	<i>kJ</i>	<i>gram</i>	<i>kJ</i>	<i>gram</i>	<i>kJ</i>
Amioca	14.65	3.50	296.90	4350.92	296.90	4350.92						
Hi-Maize	11.72	2.80					256.00	3001.24	411.90	4828.95	411.90	4828.95
Novolose	11.72	2.80					255.90	3000.07				
Inulin / scFOS	6.28	1.50	100.00	628.05	100.00	628.05			100.00	628.05	100.00	628.05
Sucrose	16.75	4.00	100.00	1674.80	100.00	1674.80	100.00	1674.80	100.00	1674.80	100.00	1674.80
Casein	14.99	3.58	140.00	2098.52	140.00	2098.52	140.00	2098.52	140.00	2098.52	140.00	2098.52
Cellulose			115.00	0.00	115.00	0.00						
Oil	35.38	8.45	100.00	3538.02	100.00	3538.02	100.00	3538.02	100.00	3538.02	100.00	3538.02
Lard	36.85	8.80	100.00	3684.56	100.00	3684.56	100.00	3684.56	100.00	3684.56	100.00	3684.56
Mineral Mix	3.68	0.88	35.00	128.96	35.00	128.96	35.00	128.96	35.00	128.96	35.00	128.96
Vitamin Mix	16.20	3.87	10.00	162.04	10.00	162.04	10.00	162.04	10.00	162.04	10.00	162.04
Choline Chloride			1.30	0.00	1.30	0.00	1.30	0.00	1.30	0.00	1.30	0.00
L-Cysteine	16.75	4.00	1.80	30.15	1.80	30.15	1.80	30.15	1.80	30.15	1.80	30.15
Total			1000.00	16296.01	1000.00	16296.01	1000.00	17318.35	1000.00	16774.04	1000.00	16774.04
Total (kcal/g)				3892.05		3892.05		4136.22		4006.22		4006.22
% diet				3.89		3.89		4.14		4.01		4.01

1. MACRO MIX

- a. Weight each ingredient individually on balance
- b. Add to larger mixing bowl in the following order:
 - i. Sucrose
 - ii. Casein
 - iii. Starch
 - iv. Fiber
- c. Stir evenly to distribute
- d. Place in Hobart MFG commercial mixer and process at low speed for 10 minutes
 - i. Scrape sides
 - ii. Repeat

2. MICRO MIX

- a. Weigh remaining dry ingredients individually on balance
- b. Add to smaller mixing bowl in the following order:
 - i. Mineral Mix (AIN-93G)
 - ii. Vitamin Mix
 - iii. Choline Chloride
 - iv. L-Cysteine
- c. Stir evenly to distribute
- d. Mix desired food coloring into 1 cup distilled water and add to dry ingredients
- e. Using handheld mix, process at low speed for 10 minutes

- f. Add micro mixture to macro mix in Hobart MFG commercial mixer and process at low speed for 10 minutes
 - i. Scrape sides
 - ii. Repeat

3. OIL MIX

- a. Weight each ingredient individually in small bowl on balance
 - i. Corn oil
 - ii. Lard
- b. Place ingredients into medium-sized bowl
- c. Using handheld mixer, process at low speed until creamy

4. FINAL MIXING

- a. Add oil mixture to center of dry mixture in Hobart MFG commercial mixer
- b. Process at low speed for 10 minutes
 - i. Scrape sides
 - ii. Repeat twice

5. STORAGE

- a. Place in gallon Ziplock bags
- b. Mark container in the following manner:

Study ID / Diet Name / Date / Initials
Batch # / Date / Initials
- c. Store in freezer