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The physiological effects of resistant starch on obesity and diabetes

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THE PHYSIOLOGICAL EFFECTS OF RESISTANT STARCH
ON OBESITY AND DIABETES

A Dissertation

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by
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This dissertation is dedicated to my family: to my big brother, Adam, for his gentle teasing and for showing me the importance of stepping outside of my comfort zone; to my mother, Sherry, for her eternal optimism and 24-hour mental and emotional support; and to my father, Elliot, who taught me to shoot for nothing less than the stars.

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ABBREVIATIONS

AC	Amioca® control
ADA	American Diabetes Association
AHA	American Heart Association
AT	Adipose tissue
AOAC	American Association of Analytical Chemists
BG	Blood glucose
BMI	Body mass index
BPA	Bisphenol-A
C2	Acetate
C3	Propionate
C4	Butyrate
cAMP	Cyclic adenosine monophosphate
CO2	Carbon dioxide
CDC	Center for Disease Control
CLAMS	Comprehensive lab animal monitoring system
DF	Dietary fiber
DIO	Diet-induced obesity
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DPP-4	Dipeptidyl peptidase-4
DWGC	Dent whole-grain resistant corn
EBW	Emboweled body weight

EE	Energy expenditure
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated protein kinase
FI	Food intake
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
HF	High-fat
HFD	High-fat diet
HM260	Hi-Maize® 260
HMWG	Hi-Maize® whole-grain resistant starch
HOMA-IR	Homeostasis model assessment of insulin resistance
IACUC	International animal care and use committee
IRS-1	Insulin receptor substrate-1
KO	Knock-out
kcal	Kilocalorie
LF	Low-fat
NMR	Nuclear magnetic resonance
NSP	Non-starch polysaccharides
NF-κB	Nuclear factor kappa beta
O2	Oxygen
PA	Physical activity
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
PYY	Peptide YY

qRT-PCR	Qualitative real-time polymerase chain reaction
REM	Rapid eye movement
RS	Resistant starch
SCFA	Short chain fatty acids
SE	Standard error
SG	Sitagliptin
STZ	Streptozotocin
TLR-4	Toll-like receptor-4
TNF-α	Tumor necrosis factor-alpha
US	Unites States
WHO	World Health Organization
WT	Wild-type
ZDF	Zucker diabetic fatty

ABSTRACT

Three studies were performed to determine the effects of RS on body weight and adiposity in HF DIO, diabetic C57BL/6J and GLP-1 receptor KO mice as well as genetically obese ZDF rats.

The first study was a dose-response experiment for HM260 (0, 15, or 28 g/100 g diet) against the anti-diabetes drug SG (Januvia®) (0 or 0.4 g/100 g diet) in HF DIO C57BL/6J (n=55) mice injected with STZ in order to assess synergy. The most effective combination was used in the second study, the purpose of which was to determine the mechanistic importance of GLP-1 in GLP-1R KO (n=25) in aforementioned synergy. HM260 and SG interact synergistically in HF diet to reduce adiposity at the 28% HM260 level, and SG appears to promote increased active GLP-1 when combined with 28% HM260. Combination treatment resulted in increased energy expenditure and attenuated weight gain in mice, and these activities are dependent on a functioning GLP-1 receptor. GLP-1 receptor may help regulate serum GLP-1 concentration by facilitating clearance.

For the third study, the fermentation response of ZDF rats was characterized using four diets differing in starch source/type: AC, HM260, DWGC, a novel HMWG, which contained 0, 25, 6.9, and 25% RS by weight, respectively. Empty cecum weight and short chain fatty acid concentrations were significantly increased for all fiber-containing groups compared to the non-fiber control. Animals fed the whole-grain RS had a 30% greater effect than non-whole-grain RS. However, no significant differences in body weight or percent body fat were found for any diet group. These results demonstrate a synergistic effect between whole-grain and RS, and provide evidence for greater potential health benefits with whole-grain varieties of RS. ZDF rats have a defective leptin receptor, and, thus, beneficial phenotypic changes observed in previous studies in rodents fed RS appear to require leptin signaling.

CHAPTER 1
INTRODUCTION

Significance of Research

Over the past several decades, Americans have become an exceedingly overweight and obese population. According to the CDC, approximately 35% of adults and close to 17% of children and adolescents (aged 2-19 years) in the US were obese between 2009 and 2010. This is a dramatic increase in prevalence from the 1980s and 1990s when only 15% of adults and 5% of children fit into the obese category. The problem of overweight and obesity – defined as having a BMI of 25-29.9 or ≥30, respectively – is not isolated to the US. According to the WHO, the worldwide obesity rate has nearly doubled since 1980 with 1 out of every 10 adults around the world considered obese in 2008. In 2010, 43 million preschool children were overweight or obese, a 60% increase since 1990 (de Onis et al. 2010). The prevalence of obesity-related diseases and conditions such as DM has also increased.

Type 2 DM is a chronic metabolic disorder characterized by altered glucose homeostasis. It is distinguished from other types of DM, such as type 1, gestational and maturity onset diabetes of the young, by hyperglycemia coupled with hyperinsulinemia: the body's tissues become less responsive to insulin over time, so greater concentrations of insulin are required to regulate BG. Eventually, the beta cells responsible for producing insulin fail and full-blown type 2 DM develops. From 1980-2010, the crude prevalence of diagnosed diabetes increased by 176%; that is, from 2.5% to 6.9% nationwide (CDC, 2014). Almost two million American adults were diagnosed with diabetes in 2010 alone (CDC, 2014). Moreover, statistics show that 60-90% of patients with type 2 DM are or were obese at some point in their lives (Stumyoll et al. 2005).

Finding an effective means of preventing and/or treating obesity and diabetes is of great importance to public health. The burden on national healthcare systems and personal finances is

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enormous! In 2008, the annual medical burden of obesity was estimated at $147 billion with medical costs for obese individuals approximately $1,400 higher than those for their normal weight counterparts (Finkelstein et al. 2006). The direct medical costs associated with diabetes in 2007 were $116 billion; medical costs for diabetics were, on average, 2.3 times higher than for non-diabetics (CDC, 2014).

While there is no single, overriding cause of obesity, much of the blame has been placed on the elevated fat content of modern "Westernized" diets. Dietary fat contributes to weight gain by increasing the energy density of the diet while also encouraging overconsumption (Rolls & Bell 1999; Rolls, 2000; Kim & Popkin 2006). However, any energy-yielding macronutrient, if consumed in excess of the body's needs, can result in weight gain. Carbohydrates are not blameless in the battle against the bulge; since the 1970s, the average total daily kcal intake has increased (by approximately 500 kcal per day), as has average total dietary fat intake, but percentage of kcals from dietary fat has not (Gross et al. 2004). In fact, according to Chanmugam *et al.* (2003) it has decreased! Nearly 80% of these excess kcals are derived from carbohydrates, especially refined and processed grain products such as corn syrups.

According to Gross *et al.* (2004), dietary carbohydrate consumption steadily decreased from 500 grams per day in 1909 to 374 grams per day in 1963, thanks mostly to reduced whole grain consumption. As a result, Americans experienced a 40% drop in the amount of DF consumed. Since 1963, dietary carbohydrate levels have rebounded to the original 500 grams per day level; however, DF consumption has not increased proportionately. Consumption of high-carb, low-fiber foodstuffs results in an intense, rapid rises in BG. This is believed to contribute to the diabetes epidemic by promoting weight gain and insulin resistance and then overtaxing the pancreas. The result is a vicious cycle of inflammation that exacerbates and confounds the

body's ability to regulate BG. These findings indicate an increase in refined grain consumption over time, and denote a substantial positive correlation between refined carbohydrate consumption and disease (Venn & Mann 2004; Aune et al. 2013).

Lack of DF also denies human commensal bacteria important substrates for metabolism and growth. The human body, especially the lower GI tract, is teeming with microorganisms. In fact, bacterial cells are believed to outnumber human somatic and germ cells by at least one order of magnitude (Pietzak, 2004). The greatest concentration of these microbes – approximately 10^{11} per gram of contents – resides in the colon and is believed to participate in the maintenance and proper functioning of several human metabolic processes (Savage, 1977). Among these occupations is the breakdown of indigestible plant fibers, also known as microbial fermentation (Hill & Peters 1998).

The major metabolic products of these fermentation reactions are SCFAs – mainly C2, C3, and C4 – as well as carbon dioxide and hydrogen gases (Topping & Clifton 2001). SCFAs, of which butyrate is the most well-studied (Schwiertz et al. 2002), reduce the pH levels in the intestine, and are readily absorbed and used as energy by colonocytes, which preferentially utilize C4, and other tissues such as the liver and muscle, which metabolize C2 and C3, respectively. It is estimated that up to 10% of human basal energy needs are provided by SCFAs (Hooper et al. 2002). SCFAs also play a role in many of their host's biological processes, including cell proliferation and differentiation, regulation of inflammatory response, gut hormone secretion, and regulation of AT stores (Mentschel & Claur 2003; Toden et al. 2007).

GLP-1, a hormone produced by the L endocrine cells of the terminal ileum and colon, in response to nutrient intake, is capable of crossing the blood brain barrier and affecting neuronal signaling in the brain. It has garnered a significant amount of scientific and clinical interest due

to its effect on satiety and FI as well as insulin sensitivity and BG in individuals with insulin resistance and/or full-blown DM (Neary et al. 2005; Drucker 2006; Janssen et al. 2012). In fact, it is so effective at improving glucose homeostasis that it precipitated the invention of several anti-diabetes medications. SG, a DPP-4 inhibitor, is one such drug. Manufactured by Merck & Co. under the brand name Januvia®, SG extends the half-life of circulating GLP-1 by reducing its rate of enzymatic degradation and extending the duration of its effects.

Treatment for both conditions, i.e. obesity and diabetes, includes a combination of diet and exercise. However, the effectiveness of such interventions has been questioned, not because they do not work, per se, but because people dislike both the effort and discomfort involved in dietary restriction and exercise. Most individuals appear incapable of making the deliberate, long-term lifestyle changes necessary for sustained weight loss and improved insulin sensitivity, as evidenced by consistent failure to lose sufficient weight and/or regaining much of the weight lost five years post-adherence to a structured weight loss program (Friedman, 2004). This is why heightened attention is being paid to new food additives and/or products that improve consumer health without significant, conscious mental effort or radical changes to one's diet and lifestyle.

DF is a very popular choice among food scientists and nutritionists alike, since increased consumption is correlated with reduced FI (by promoting satiety through mechanistic and hormonal pathways) (Heaton, 1973; Slavin, 2005) as well as improved weight status (by diluting the energy density of the diet in which it is found. After water, which increases food volume without adding any energy [i.e. 0 kcal per gram], fiber contributes the most food volume for the fewest kcal per unit volume [1.5-2.5 kcal per gram]) (Rolls & Bell 1999; Rolls 2000; Kim and Popkin 2006). This also affects fiber's role in BG metabolism (by virtue of its indigestibility and interference with the absorption of other dietary components within the small intestine) as well

as its prebiotic capacity (by acting as a substrate for the metabolism of several different species of commensal bacteria.) (Marlett et al. 2002; Anderson et al. 2009).

RS is a relatively new addition to the category of DF, but it has a great deal of clinical and industrial potential. It has reduced caloric value and glycemic index compared to most starches. It is also readily fermented, producing very high concentrations of SCFAs, especially C4, (Cummings et al. 2001) and increased bacterial biomass. Several animal models, e.g. rats (Keenan et al. 2006; Zhou et al. 2006; Zhou et al. 2008; Shen et al. 2009), mice (Zhou et al. 2009; Zhou et al. 2012), dogs (Massimino et al. 1998), and pigs (Regmi et al. 2011), respond to RS supplementation with increased circulating levels of GLP-1, which is correlated with decreased FI, body weight, and adiposity as well as improved glucose homeostasis. These changes have not appeared to be readily achievable in humans (Robertson et al. 2005; Johnston et al. 2010). However, a recent study by the Robertson lab found increased GLP-1 in human subjects fed RS (Bodinham et al. 2014).

Certain environmental factors may be interacting to blunt the effects of RS and other fermentable fibers in the diet. First and foremost, we no longer consume sufficient amounts of all types of DF, including RS. The US Department of Agriculture suggests that, regardless of age, the adequate intake for DF is 14 grams per 1000 kcal consumed. According to King *et al.*. (2012), average total DF intakes for Americans have hovered around 15 grams per day for the past 25 years. Since Americans do not consume sufficient amounts of DF overall they are not likely to meet the recommended intake values of RS either. US citizens consume an estimated range of 3-8 grams of RS per day (Murphy et al. 2008). Warshaw (2007) suggests consuming at least double that, i.e. 15-20 grams of RS per day, which is equivalent to the amount of RS in the

diets of people living in less developed countries. Their diets are stereotypically richer in whole-grain cereals, fruits, and vegetables.

Secondarily, macronutrient content of the diet can have a profound effect on colonic fermentation, although the exact mechanisms through which this occurs are not fully known at this time. Several studies conducted by and in conjunction with Dr. Michael Keenan's laboratory at Louisiana State University have reported difficulty preserving fermentation of HM260, a type of high-amylose corn starch, and its effects in conjunction with HF diets (Zhou et al. 2009; Senevirathne et al. 2009; Goldsmith et al. 2010; Charrier et al. 2013). That is, the stereotypical outcomes associated with fermentation – i.e. increased empty cecal weight, reduced cecal pH, and reduced body weight and adiposity – were not as robust as previously documented studies using low- or moderate-fat diets. This led us to hypothesize that the high dietary fat content altered the intestinal microbiota and/or gut hormone – namely, GLP-1 – production in such a way as to diminish fermentation of RS and prevent or reduce the intensity of commonly associated physiological outcomes of RS consumption.

The aforementioned potential of RS as a tool for controlling the obesity and diabetes epidemics warrants investigations into the mechanisms behind and means of overcoming subpar levels of fermentation. This dissertation is devoted to the scientific and clinical advancement of that goal.

Objectives

1. Determine if the full effect(s) of HM260 supplementation can be rescued on a HF diet through combinatory treatment with the anti-diabetes drug SG.

2. Determine the minimum effective dose of HM260 necessary to achieve synergy with SG.

3. Determine if the biological effects associated with the combination treatment are dependent on GLP-1 receptor signaling.

4. Determine the efficacy of HM260 versus its whole grain variety of RS in an insulin resistant rodent model of obesity.

5. Determine if leptin signaling is required for production of beneficial phenotypic effects associated with fermentation of RS.

Proposal

Study 1 - Investigating synergistic effects between RS and the drug SG

SG has been approved by the FDA for the treatment of type 2 diabetes in the US since 2006. As previously described, it works by inhibiting the actions of an enzyme called DPP-4, which degrades active GLP-1 in the circulation. GLP-1 functions as a satiety signal and increased secretion is generally associated with reduced FI and body weight in both animals and humans. GLP-1 is also an incretin hormone, meaning that it increases insulin secretion from beta cells and improves insulin response. While use of SG results in improved glucose control, it does not result in improvements in weight status and adiposity. This is in contrast to GLP-1 agonists such as extenatide, which do result in improved adiposity.

Our lab surmised that reduced degradation does not sufficiently stimulate the GLP-1 receptor system to cause weight loss. SG administration coupled with HM260 supplementation, which stimulates endogenous GLP-1 secretion, may succeed where both compounds failed: acting synergistically to augment GLP-1 production, resulting in weight loss in addition to improved insulin sensitivity on a HF diet. That is, SG may rescue the effect of HM260 that is attenuated with feeding of a HF diet, and HM260 may amplify the SG effects by increasing the endogenous synthesis of active GLP-1.

The first step in this investigation was a 2x3 factorial study in order to determine the optimal combination of SG and HM260 in the diet. "Optimal" is defined as that which results in the greatest increase in serum concentrations of GLP-1 active, greater insulin sensitivity, and greater reductions in body weight and/or body fat. The most effective combination was used in the next study, which investigated the importance of GLP-1 receptor signaling in the synergistic interactions between the two substances.

Animals were made obese through administration of a HF, high-energy diet then injected with a low dose of STZ in order to induce diabetes. Next, the animals were placed in one of six groups and placed on HF diets combined with one of two levels of SG (0 or 0.4% by weight) and one of three levels of HM260 (0, 15, and 28% by weight) for 10 weeks. After the appropriate amount of time had elapsed, animals were euthanized and blood, cecal contents, and abdominal fat pads were collected in order to determine the following endpoints: the concentration of insulin and GLP-1 in the plasma, the pH and concentration of SCFAs in the cecal contents, empty cecum weights, cecal content weight, and visceral fat mass.

Study 2 – Investigating importance of GLP-1 receptor system in RS and SG responses

From the previous study, we determined that the most effective drug and functional food combination was 28% HM260 coupled with 0.4% SG. The next step in our research was to investigate how this combination influences weight loss and STZ-induced diabetes in WT C57BL6 and age-matched GLP-1 receptor KO mice. The same timeline and endpoints of interest that were used in the previous study were also used here. Deviations from the previously described protocol include the type and number of levels of independent variables used for the study – study one utilized the presence or absence of SG and one of three levels of HM260 supplementation, while the second study utilized two animal strains and presence or absence of

the SG+RS combination – and the use of metabolism cages for indirect calorimetry measurements during the eighth week of treatment.

Study 3 – Comparing fermentation profiles of two different types of RS

The ZDF rat possesses a mutation in the leptin receptor that causes it to spontaneously develop obesity and insulin resistance between 7-10 weeks of age (Srinivasan & Ramarao 2007). Scientific literature characterizing the fermentation response of the animal model is sparse, almost nonexistent. Whether or not this animal model's genotype has altered the microbiota to make it unresponsive to treatment with RS is unknown, as well as if RS supplementation is sufficient to alter their gut microbial composition and induce weight loss. However, there could be fermentation of RS, but no phenotypic changes if leptin signaling is required.

In order to fill in this gap in the literature, and in order to test the efficacy of a new RS product on fermentation and phenotypic changes, we proposed the following study: A one-way ANOVA featuring pre-diabetic ZDF rats fed one of four diets varying in presence and type of RS for 12 weeks. These diets were named according to their identifying starch component: a control diet made with waxy corn starch; an RS-containing diet made with high-amylose maize starch; a whole-grain control diet containing dent corn flour; and a whole-grain RS diet made with a novel functional food product, whole-grain high-amylose flour. These diets were labeled AC, HM260, DWGC, and HMWG, respectively.

Upon euthanasia we collected blood, cecal contents, and abdominal fat pads to determine the following endpoints: serum concentrations of insulin and GLP-1, the pH and concentration of SCFAs in the cecal contents, empty cecum weights, weight of cecal contents, and visceral fat mass. We intended to characterize the ZDF gut microbiota down to the phyla and genus levels using NGS techniques in addition to targeted qRT-PCR to quantify the amount of butyrate-

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producing bacteria (i.e. *Lactobacillus*, *Bifidobacteria*, and Clostridial clusters IV and XIV a & b). However, we decided against the former after no phenotypic changes were documented in the ZDF rats. The latter has been delayed until culturing of representative bacterial species and extraction of DNA for improved standard curves is completed. Standard curves made from arbitrary DNA (pooled samples) were not optimal.

Typically, the standard curve is generated from a dilution series constructed from a "reference" sample. qRT-PCR is performed on both the experimental samples and reference standards. Relative values for target abundance in each experimental sample are extrapolated from the standard curve generated from the reference standard. While the absolute values calculated from the experimental samples are meaningless, the relative differences in nucleic acid abundance between the samples are accurate. The reliability of any relative qRT-PCR can be improved by (1) including an invariant endogenous control in the assay to correct for sample to sample variations in RT-PCT efficiency and errors in sample quantitation, and (2) developing the dilution series from known template concentrations, which is what our lab is currently doing.

Hypotheses

Study 1

1. A combination of RS, which increases endogenous GLP-1 production, and SG, which slows the rate of GLP-1 degradation in the bloodstream, will cause a significantly greater increase in circulating GLP-1 concentrations than either treatment alone under HF diet and type 2 DM conditions. This also will foster better glycemic control and lead to greater weight loss than either treatment alone.

2. The combination of 15% and 0.4% (by weight) for RS and SG, respectively, should be sufficient to induce biologically effective increases in circulating GLP-1 concentrations.

Study 2

3. If improvements in glucose homeostasis and adiposity levels are dependent upon GLP-1 activity, then the combination of RS+SG will fail to produce fat loss and/or improved insulin sensitivity in GLP-1 receptor KO mice.

Study 3

4. ZDF rats will respond to RS supplementation with adequate cecal fermentation as defined by reduced pH and increased SCFA concentrations within cecal contents, and greater empty cecum size. Groups supplemented with RS will exhibit reduced body weight and/or body fat and better BG control compared to non-supplemented groups. Animals fed HMWG will produce a more robust fermentation response and, thus, exhibit greater improvements in weight status and insulin sensitivity compared to those fed HM260, because the former is a source of two types of RS, while the latter contains one.

6. If leptin signaling is required for observed fermentation to be associated with beneficial phenotypic effects, then ZDF with a defective leptin receptor, will not show phenotypic changes even if they robustly ferment RS.

Assumptions and Limitations

Although mice and rats possess similar digestive physiology to that of humans and are a commonly used animal model for humans, the results from this study do not apply directly to humans. Researchers who work with rodent models function under the assumption that the blood and tissue samples collected from animal models are representative of human subjects. Morphological and behavioral differences between rodents and humans include, but are not limited to: Coprophagy, i.e. the consumption of feces, which could influence the composition of

gut bacteria through consistent re-inoculation; possessing a much larger, more well-defined cecum; having a greater metabolic body size than humans, which necessitates higher doses of prebiotics than would be easily tolerated by humans; and, in the case of rodents, possessing a diffuse, glandular pancreas that consists of many white nodules embedded within the mesentery (Olds & Olds, 1979). Nevertheless, mice and rats display similar phenotypic responses to bioactive dietary compounds such as fermentable fibers as humans. Also, they enable us to take samples and measurements that would be unethical and too difficult in humans. Ergo, after pigs, rodents are considered the best corollary of human digestive processes.

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 high-amylose starch adds energy to the diet at 2.8 kcal/g as well as total fiber, including both fermentable and non-fermentable (Tulley et al. 2009). The latter occurs at the levels of RS added to the rodent diets with using proof-of-concept dietary levels of greater than 25% of the weight of the diet. 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.

Similarly, feeding studies make it impossible to provide experimental diets that meet the exact macronutrient specifications of the provider. ZDF rats were developed in the mid-1970s when a mutation occurred in a colony of outbred Zucker rats in the laboratory of Dr. Walter Shaw at Eli Lilly Research Laboratories in Indianapolis, IN. Over the next few years, several

groups of diabetic animals were identified and re-derived, and subsequent inbreeding of selected pairs led to the establishment of the ZDF line. According to Charles River Laboratories International Inc., which maintains the line, these rats are ideal candidates for research involving obesity, altered glucose homeostasis, including type 2 DM, and hyperlipidemia. However, the company recommends that in order to effectively induce the diabetic phenotype, animals should be maintained on Purina #5008 and D12468 from Research Diets, Inc, for obese male and female rats, respectively. It is possible that, by (a) not using the exact diets specified by the manufacturers and (b) by replacing digestible starch with RS (regardless of type) alters the diet too much, making the manifestation of DM unreliable and, thereby, confounding our results relating to insulin sensitivity. However, by stratifying by HOMA-IR in addition to body weight, we compensate for any potential variation in response to non-standard diet.

Using STZ in order to induce hyperglycemia in our mouse model is closer in etiology to type 1, not type 2, DM. In spite of this, the extremely small doses used should have resulted in partial not total beta-cell knock out, which does fit the pathophysiology of type 2 DM. Also, STZ produces a one-time knock down of insulin production, and research shows that beta cells can be regenerated. Al-Hasani *et al.* (2013) conducted studies in transgenic mice that show how ductal cells in the pancreas can be mobilized and transformed into insulin-producing beta cells, and that this process can occur at any age. This means that the hyperglycemic effects of this treatment may not be permanent, that the mice in our control groups may have experienced improvements in glucose homeostasis even without RS treatment.

CHAPTER 2
LITERATURE REVIEW

Overweight and Obesity

The terms "overweight" and "obesity" refer to body weights that are greater than what is considered healthy for a given height. The most expedient method of determining healthy or unhealthy weight status is BMI. The BMI is calculated by dividing an individual's weight in kilograms by his or her height in meters squared (kg/m^2). A BMI between 25.0 and 29.9 is considered overweight, while a BMI greater than or equal to 30 qualifies as obese. There are different classes of obesity: 30.0-34.9, 35.0-39.9, and >40 being levels one, two, and three, respectively. Falling within the third class is also known as being "morbidly obese."

BMI is a convenient, albeit imperfect means of determining adiposity (Frankenfield et al. 2002; Romero-Corral et al. 2008). BMI does not differentiate between fat mass and lean mass, and because muscle has a greater density than AT, BMI may overestimate adiposity in individuals that are extremely muscular, such as professional athletes, and underestimate it in the sick and elderly. BMI does not take into account where the body stores fat either: Abdominal or visceral fat, which is located around the midsection (contributing to the so-called 'apple-shape'), confers a greater risk of morbidity and early mortality than peripheral fat, which is stored in the buttocks and thighs (also known as being pear-shaped) (Figure 1). Therefore, measuring waist circumference along with BMI is considered a better estimate of overweight- and obesity-related health risks than BMI alone (Han, 2006). A high-risk waist circumference is greater than 35 inches for women and greater than 40 inches for men. Waist-to-hip and waist-to-height ratios are also simple, popular alternatives for BMI. It is important to note, however, that at a BMI >35, abdominal adiposity is assumed.

Fig. 1 – Apple- versus pear-shaped body types.

Notwithstanding its limitations, BMI is a useful mathematical corollary for "fatness," especially at the population level, because it utilizes noninvasive, easily acquired demographics. Elevated BMI is a major risk factor for several non-communicable diseases such as hypertension (Montani et al. 2002), dyslipidemia (Datillo & Kris-Etherton 1992), cardiovascular disease (i.e. coronary artery disease and stroke) (Kenchaiah 2002), sleep disorders such as sleep apnea (Crummy et al. 2008), musculoskeletal disorders like osteoarthritis (Vincent et al. 2012), insulin resistance and diabetes (Chan et al. 1994; Colditz et al. 1995), as well as several types of cancer (Calle 2004). Childhood obesity contributes to respiratory distress and an increased risk of bone fracture due to increased stress placed on muscles and joints and, potentially, altered leptin signaling (Wang et al. 2012). It is also associated with greater chances of obesity, disability, and premature death in adulthood, often as a result of complications due to the aforementioned obesity-associated diseases and conditions (Hardy et al. 2004; Biro & Wien 2010).

The incidence of worldwide obesity has nearly doubled since 1980, which is why clinicians and scientists often refer to it as an obesity 'epidemic' (WHO, 2012). Although there

are many potential causes of overweight and obesity, the majority of cases are the result of energy imbalance. When an individual is in positive energy balance, i.e. obtains more energy from food than he or she expends through basal metabolism and PA, the excess energy is stored as AT. Modern humans living in developed countries engage in very little PA compared to those living in underdeveloped countries (Popkin, 1999; Giraldo et al. 2012), and especially compared to those of our hunter-gatherer ancestors (Leonard, 2010). Also, thanks to the invention of agriculture and subsequent improvements made during the Industrial Era, we no longer live through alternating periods of feast and famine. We live in a state of perpetual feasting on energy dense foodstuffs, made that way through the addition of dietary fat and/or highly refined carbohydrates. Over the last several decades, a significant increase in portion size as well as the caloric content of food products has been observed, augmenting consumption and contributing to obesity and its related conditions (Nielsen & Popkin 2003).

Additional environmental factors can further complicate energy balance and promote even greater weight gain. Since the 1960s, people have lost about 2 hours of sleep per night (Knutson et al. 2010). That is, from 8.5 hours to <7 hours. Several studies have shown that, as you sleep less, BMI goes up (Spiegel et al. 2009). Because obesity can lead to difficulties maintaining proper sleeping patterns (often due to difficulty breathing) this leads to the birth of a vicious cycle of positive reinforcement: the less sleep a person gets, the more likely he or she will put on weight, which leads to greater difficulty achieving rapid eye movement sleep, and so on and so forth. Also, humans are warm-blooded creatures and, as such, we expend a great deal of energy in order to keep our bodies at the optimum temperature. Advanced climate control technology, i.e. heaters and air conditioners, eliminates the need of modern individuals to burn so many calories. According to an observational study conducted by Bo *et al.* (2011) it was

discovered that individuals who preferred warmer indoor temperatures during the fall and winter months were twice as likely to become obese compared to people who kept their homes under 70 degrees Fahrenheit.

Increased exposure to industrial chemicals such as dyes, solvents, pesticides, perfumes, etc. is another potential culprit. Several studies have demonstrated a link between increased exposure to industrial chemicals (e.g. organochlorides) and excessive weight gain and/or abnormal fat metabolism. Rubin *et al.* (2001) found that perinatal exposure to BPA, a chemical compound found in canned food and plastic bottles, in Sprague-Dawley rats resulted in greater body weights at birth and into adulthood. High urinary BPA concentrations were significantly associated with obesity in a cross-sectional study of children and adolescents (Trasande et al. 2012). High urinary levels of BPA are also associated with an increased risk of obesity, diabetes, cardiovascular disease, and fertility issues in adults as well (Fenichel, 2013).

Typically, health professionals recommend a combination of diet and exercise to balance caloric intake with expenditure for the purpose of preventing or treating obesity and its comorbidities. However, the effectiveness of such interventions has been brought into question. Improved knowledge does not automatically equate to improved attitudes and behaviors: despite awareness of the problem – being overweight or obese – and understanding the steps that need to be taken in order to treat the problem – reduced calorie intake and increased energy expenditure through diet and exercise – the vast majority of people seem incapable of making the deliberate, long-term lifestyle changes necessary for sustained weight loss. Even when weight loss is achieved, the results are often difficult to sustain. Friedman *et al.* (2004) used National Health and Nutrition Examination Survey data to identify over 1300 Americans aged 20 to 84 who were, at their heaviest, classified as overweight or obese. They had since lost a substantial

amount of weight, defined as at least 10% of their maximum weight one year before they were surveyed. The researchers discovered that 30% to 35% of lost weight is regained in the first year, and most, if not all, of lost weight is regained by the fifth year. Understanding the barriers to achieving and maintaining weight loss is crucial for preventing relapse and for reducing morbidity and mortality rates associated with it. Developing means to overcome such barriers that do not involve (a) conscious effort and/or (b) significant investments of time or money to increase compliance and, thus, effectiveness are needed.

Diabetes Mellitus

DM describes a collection of diseases that affect how the body metabolizes BG, commonly referred to as blood sugar. Having diabetes, no matter what type, means that an individual possesses too much glucose in their blood. Some of the common signs are increased thirst, frequent urination, extreme hunger and fatigue, unexplained weight loss, blurred vision, frequent infections and slow-healing sores, as well as the presence of ketone bodies in the urine. These symptoms and their severity vary depending on how high one's BG level is and for how long. The longer an individual has diabetes – and the less controlled their BG is – the higher the risk of developing more debilitating and even life-threatening complications such as neuropathy (nerve damage), nephropathy (kidney damage), retinopathy (blindness), etc..

Potentially reversible diabetic conditions include pre-diabetes – when BG levels are higher than normal but not so high as to be classified as full-blown diabetes (i.e. fasted plasma BG 100-125 mg/dL on two separate occasions or <140 mg/dL after taking an oral glucose tolerance test) – and gestational diabetes, which occurs during pregnancy but usually resolves postpartum. However, the mother is at an increased risk of developing diabetes, mostly type 2, over the subsequent 5 to 20 years (Kim et al. 2002). Chronic forms of diabetes include type 1 and

type 2 DM. Type 1 DM is usually diagnosed in children and adolescents, and was previously known as juvenile diabetes, but has since been rechristened as insulin-dependent diabetes. According to the ADA, only about 5% of people with diabetes have this form of the disease. With type 1, the body does not produce insulin, the hormone needed to transport glucose into muscle and adipose cells so it can be broken down and used to produce energy, because the specialized cells that produce it have been destroyed by the immune system. Type 2 DM is the most common form of the disease – affecting over 90% of all diabetics worldwide – and is characterized by the improper utilization of insulin, also known as insulin resistance. At first, the pancreas makes extra insulin to make up for the defect but, over time, the organ isn't able to keep up and BG rises to diabetic levels (i.e. fasted plasma BG $\geq$126 mg/dL on two separate occasions).[1]

Of all the diseases and conditions that make up the metabolic syndrome, the link between obesity and diabetes is particularly strong. The incidence of DM, especially of type 2, is on the rise, correlating with the increased prevalence of obesity. According to the CDC, from 1980-2010, the crude prevalence of diagnosed diabetes increased by 176%. That is, from 2.5% to 6.9% nationwide (Tirosh et al. 2005). Almost two million adults were diagnosed with diabetes in 2010 alone. Significantly, between 80 and 90% of individuals diagnosed with type 2 DM are obese as well. While the exact mechanism linking obesity to diabetes is unknown, insulin resistance in both human and rodent models of obesity is closely related to the presence of inflammation in

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[1] There is another type of diabetes known as diabetes insipidus, but this rare disease has nothing to do with abnormal functioning or circulating levels of insulin. Instead, it is the result of the kidneys' inability to conserve water while filtering the blood. Diabetes insipidus can be caused by either a lack of antidiuretic hormone or inability of the kidneys to respond to this hormone, also called vasopressin, which regulates water conservation by said organ. It is not a component of metabolic syndrome.

AT (Wentworth et al. 2010; DeFuria 2013). Macrophages are white blood cells that respond to infection; made in bone marrow, and are secreted into the blood stream and then infiltrate tissues in order to seek out and destroy foreign particles. In obese animals, macrophages preferentially invade fat tissue, resulting in inflammation and the release of inflammatory cytokines (Kershaw & Flier 2004). Certain cytokines cause cells to become resistant to insulin, e.g. TNF-α, leading to diabetes and other hallmarks of metabolic syndrome. Potential instigators of this cascade include free fatty acids and their derivatives, oxidative stress, tissue hypoxia leading to adipocyte death, and endoplasmic reticulum stress, to name a few (Ye, 2011).

It is true that AT inflammation is pronounced among obese people, and that it contributes to the pathophysiology of type 2 DM through inhibition of the insulin signaling molecules like IRS-1 and PPARγ. More specifically, during the obese state, rapidly expanding AT causes elevated levels of free fatty acids in the bloodstream. The body responds by producing the pro-inflammatory cytokine TLR-4, which reduces the functionality of the aforementioned compounds and, eventually, leads to insulin resistance (Shi et al. 2006; Kim & Sears 2010). However, this does not mean that all inflammation is bad: the inflammatory response is a natural bodily process that, when properly applied, ensures the continued health and survival of the organism (Gao & Ye 2012). Ye and McGuinness (2013) suggest that chronic, runaway inflammation that occurs in the absence of increased energy metabolism and/or storage is the insidious variety that leads to insulin resistance and DM. That is, inflammation is a symptom of the problem, not the problem itself. This conclusion is supported by data from (a) Tang *et al.* (2010) who showed that mice that overexpressed NF-κB, a signaling molecule downstream of TNF-α, were resistant to diet-induced obesity despite no reductions in food intake; and (b) the

fact that anti-inflammatory, insulin-sensitizing drugs such as rosiglitazone and pioglitazone do not improve insulin sensitivity in animal models.

Like overweight and obesity, treatment for diabetes includes a combination of diet and exercise as well as regular BG monitoring and, depending upon the type and severity of the disease, insulin and/or other medications. Testing BG multiple times per day using a glucometer – ideally, before and after meals as well as before sleep and PA – in addition to measuring hemoglobin A1C every 3-4 months is considered standard. A1C is a better overall indicator of how well a diabetes treatment plan is working; it provides an "average" BG measurement as opposed to "snapshot" values obtained from a glucometer. An A1C level greater than or equal to 6.5% on two separate occasions indicates that a patient has DM, and the ADA recommends keeping A1C levels below 7%. While there is no such thing as a diabetes diet per se, it is a good idea to center on fruits, vegetables, and whole grains, i.e. foods that are high in nutrients and fiber and relatively low in fat and calories. Limiting consumption of animal products, which are high in saturated fat, as well as limiting consumption of refined carbohydrates, is also highly recommended.

Relatively few type 2 diabetics require insulin as a part of their treatment regimen; several other injectable and oral medications exist. Some stimulate the pancreas to produce and release more insulin (sulfonylureas and meglitinides), while others inhibit the production and release of glucagon from the liver (biguanides and thiazolidinediones), reducing insulin requirement. Alpha-glucosidase inhibitors block the action of enzymes that digest carbohydrate, impeding glucose uptake into the blood stream. Sodium-glucose linked transporter 2 (SGLT2) inhibitors prevent glucose reabsorption by the kidneys, causing excess glucose to be excreted in the urine. Thiazolidinediones sensitize various tissues to the actions of insulin (Table 1).

Table 1. Diabetes Drugs and Their Mode of Action. Generic Name (Brand Name).

Drug Class	Prescriptions	Mode of Action
Alpha-glucosidase inhibitors	Acarbose (Precose), Meglitol (Glyset), Voglibose (Voglib)	Limits carbohydrate digestion and subsequent absorption into the bloodstream
Biguanides	Metformin (Glucophage, Fortamet, Riomet),	Decreases amount of glucose released from the liver
Bile acid sequestrants	Colesevelam (Welchol)	Binds bile acids; unknown mechanism for improving BG levels
DPP-4 inhibitors	Sitagliptin (Januvia), Saxagliptin (Onglyza), Linagliptin (Tradjenta), Alogliptin (Nesina)	Inhibit enzyme responsible for breaking down the hormone GLP-1, which helps lower BG
Meglitinides	Regaplinide (Prandin), Nateglinide (Starlix)	Stimulates the pancreas to release more insulin
SGLT2 inhibitors	Canagliflozin (Invokana), Dapagliflozin (Farxiga)	Blocks reabsorption of glucose by kidneys, causing excess to be eliminated in the urine.
Sulfonylureas	Glyburide (Micronase, Glynase, DiaBeta), Glimepiride (Amaryl), Glipizide (Glucotrol)	Stimulates the pancreas to release more insulin
Thiazolidinediones	Rosiglitazone (Avandia), Pioglitazone (ACTOS)	Makes the body more sensitive to the effects of insulin

SG belongs to another class of diabetes medications called DPP-4 inhibitors. First marketed in the US by Merck & Co., LLC in 2006, it improves glucose metabolism in patients with type 2 DM by increasing the half-life of GLP-1. It does so by inhibiting the DPP-4 enzyme, which is responsible for the degradation of GLP-1 in the bloodstream. Augmenting GLP-1 activity through SG significantly improves insulin sensitivity and BG in both human and animal models. Body weight reductions have not been documented as they have been with GLP-1 agonists, which consistently reduce both BG and body weight.

High-Fat Diet

Dietary fat contributes to weight gain through both passive and active mechanisms. To our native hominid senses, dietary fat is extremely palatable, with a smooth texture and creamy mouthfeel. It contributes desirable characteristics to foods, including the crispiness of fried foods, the tenderness of baked goods, and the juiciness of meat products. These qualities contribute to active overconsumption through appetite stimulation. Dietary fat is also the most energy-dense macronutrient, containing more than twice the amount of energy per unit weight than either proteins or carbohydrates (i.e. nine versus four kcal per gram). Therefore, HF foods have a greater energy density than LF ones. Since humans tend to consume a relatively constant weight or volume of food, regardless of the energy content and/or nutrient composition of the meal, diets containing more HF food items result in greater energy consumption because they possess a greater energy density than those that do not (Drenowski, 1998; Rolls, 2000; Rolls & Bell 1999; Warwick et al. 2000). This phenomenon is not under conscious control, and is referred to as passive overconsumption.

HF diets do more than just provide excess energy: excessive consumption of dietary fat is linked to the increased production of pro-inflammatory molecules, which accelerates weight gain and contributes to the advancement of metabolic syndrome. HF diets accomplish this by increasing endotoxin levels in the intestinal lumen (Cani et al. 2007) as well as in the plasma by altering the gut microbiota composition and increasing its intestinal permeability through the induction of TLR-4 (Kim, Sears, et al. 2012; Kim, Gu, et al. 2012). Indeed, research has shown that commensal gut microbiota is highly susceptible to changes induced by HF dietary factors (Turnbaugh et al. 2009; Devkota et al. 2012) suggesting that diet-mediated changes in gut microbiota could be playing a role in inflammatory propagation (de La Serre et al. 2010)

The type of fat as well as the amount of fat consumed plays an important role in the pathophysiology of DM and metabolic syndrome. Studies have shown that rats given HF diets based on lard or soybean leads to obesity and inflamed AT (Wang et al. 2013). Furthermore, mice fed HF diets made with lard and olive oil – sources of saturated and omega-6 fatty acids, respectively – exhibit increased levels of plasma insulin, leptin, and resistin, which are characteristic of an inflammatory state (Catta-Preta et al. 2012). Also, animals fed the lard-based diet gained the most weight out of all HF-diet groups. Huang *et al.* (2013) observed mild inflammation in mice that consumed milk fat or lard based diets, whereas a diet rich in safflower oil, a concentrated source of omega-6 fatty acids, led to more powerful amounts of inflammation in mesenteric and gonadal fat pads, specifically.

Our lab has been collecting a substantial amount of evidence that HF diet-induced changes in the gut microbiota may interfere with the process of RS fermentation. We have previously demonstrated that the addition of a specific type of RS from Hi-Maize® corn starch to a LF diet was associated with increased fermentation and reduced abdominal body fat (Keenan et al. 2006). The reduced body fat was associated with (a) increased gene expression of pro-opiomelanocortinin, a complex polypeptide precursor critical for normal energy homeostasis, in the arcuate nucleus of the hypothalamus, the area of the brain known to regulate food intake and satiety (Shen et al. 2009); and (b) increased oxidation of fat as demonstrated by a decreased respiratory quotient, a ratio used in indirect calorimetry methods to determine whether carbohydrate or fat is the predominant type of fuel being used by the body (Zhou et al. 2009). Use of a LF diet on Goto-Kakizaki rats, a diabetic model (Shen et al. 2011) as well as a medium-fat diet in C57BL6 mice (Zhou et al. 2009) and LF diet in ovariectomized rats, which constitute an endocrine model of obesity (Keenan et al. 2013) exhibited reduced body fat with Hi-Maize®.

However, this body fat effect was not seen in several preliminary studies utilizing rodents fed HF diets (Badkoobeh et al. 2010; Goldsmith et al. 2010). The strength of the fermentation response was diminished as indicated by significantly higher cecal pH levels than expected, reduced levels of RS-fermenting cecal bacteria (Senevirathne et al. 2009), and no significant loss of body fat. Most recently, Charrier *et al.* (2013) investigated the effects of Hi-Maize® in HF diet-induced obese rat model plus tuna oil, a potent source of omega-3 fatty acids. Since fish oils have been reported to have an effect on inflammation and body fat accumulation completely opposite to other types of fat (Buettner et al. 2007), inclusion of fish oil in a HF diet may exhibit a protective effect on the gut microbiota and, thereby, rescue the effects of RS fermentation that are usually diminished on a HF diet. In this study, animals fed a HF were still capable of responding to Hi-Maize® 260 with reduced cecal pH, increased SCFA concentrations, and increased GLP-1 production, but the positive health implications were partially attenuated when compared to the LF RS groups. That is, the animals did not lose substantial amounts of body fat when compared to a LF+RS diet. No discernible differences were seen in either LF or HF diets supplemented with the fish oil. It is possible that the minimum effective dose for fish oil was not reached in this study, because fish oil content was not determined on a percent fat basis.

Dietary Fiber and Resistant Starch

Starch is a glucose polymer that is synthesized and stored within granules inside the seeds, fruits, and tubers of various plants. These granules contain two basic forms of starch: a linear, chainlike molecule consisting of α(1-4) glycosidic linkages known as amylose, and a larger, branched polymer with both α(1-4) and α(1-6) bonds called amylopectin (Imberty et al. 1991, Nugent, 2005). The ratio of amylose to amylopectin varies according to botanical origin, with so-called 'waxy' starch containing <15% amylose by weight while 'normal' and 'high'

amylose starches have 20-35% and >40% amylose, respectively (Tester et al. 2004). The botanical role of starch is to provide plants with a stable reserve of glucose for metabolism and/or germination. The digestibility of the starch is an important parameter in meeting this function.

In humans, starch digestion begins in the mouth with salivary α-amylase, which hydrolyzes the aforementioned α(1-4) glycosidic linkages, resulting in partial breakdown of both amylose and amylopectin into dextrins. There is no starch digestion in the stomach because the low pH value of its contents deactivates salivary α-amylase. Therefore, dextrins remain unchanged and, thus, unabsorbed until they pass into the small intestine, where they are acted upon by enzymes, e.g. pancreatic α-amylase, maltase, isomaltase, etc. (Nugent 2005). The term "available carbohydrate" is used to describe the sum of all CHOs that are digested and absorbed and, thus, made available for metabolism by the body. Some starches manage to resist digestion by endogenous enzymes, and pass through to the colon where they are broken down or fermented by the bacteria residing there. This starch fraction is called RS, and was formally defined by EURESTA in 1992 as "the total amount of starch and the products of starch degradation that resist digestion in the small intestine of healthy people" (Asp, 1992).

That RS is treated like a type of DF is a testament to the extremely complex nature of DF as a particular food fraction, which is fraught with analytical and taxonomical challenges. The original definition of DF was devised by Hipsley in 1953, and referred strictly to the indigestible components of plant cells walls, i.e. cellulose and hemicellulose along with the closely associated aromatic alcohol, lignin. Today, DF is now an umbrella term used to describe various types of starch and non-starch polysaccharides that are edible but resistant to enzymatic digestion

in the small intestine. These include those mentioned above, but are not limited to, naturally-occurring gums, pectins, mucilages as well as a handful of synthetic compounds.

Scientific and clinical interest in DF did not really take off until the 1970s: various papers examining the relationship between DF and human health were published (Cleave, 1973; Burkitt et al. 1974; Cleave, 1975). This culminated with the dissemination of Burkitt and Trowell's "DF hypothesis," which posited that the so-called "diseases of civilization," i.e. obesity, cardiovascular disease, diverticulitis, and colon cancer, were caused by the overconsumption of refined carbohydrates and/or lack of fiber-rich foods in the diet (Burkitt & Trowell 1975; Trowell, 1976). Further investigations into the chemical nature of DF, and the physiological mechanisms that governed its relationship to human health were conducted (Champ et al. 2003a; Champ et al. 2003b; Eastwood & Kritchevsky 2005), and new and improved methods of extraction and analysis were soon developed. These novel techniques added even greater nuance to the definition of DF, including distinctions between soluble and insoluble fibers (Southgate, 1969). As the names suggest, soluble fiber dissolves in water, but insoluble fiber does not. The former gelatinizes within the intestines, which contributes bulk to stool, and has a hypocholesterolemic effect (Lund 1984), while the latter helps ease constipation by encouraging laxation. At this time, novel substances that behaved similarly to fiber in the GI tract, but didn't meet the standard chemical and/or botanical definition thereof were also discovered, e.g. inulin, fructans, and RS.

Englyst *et al.* (1982) is credited with discovering and defining RS as the starch fraction that resisted enzymatic digestion *in vitro*. Rapidly digestible starch is completely converted into glucose within 20 minutes of exposure. Slowly digestible starch is also successfully hydrolyzed, but only after an additional 100 minutes of incubation. Starches that are not hydrolyzed by 120

minutes are considered RS. Depending on the reason(s) behind its characteristic resistance, RS can be further classified into five sub-groups (Topping et al. 2003) (Table 2).

Starch granules are surrounded by protein matrix and thick cell wall material. Whole or partially milled grains, seeds, and legumes retain these compounds, which hinder starch digestibility by 1) providing a physical barrier, preventing enzymes from reaching and hydrolyzing starch, and 2) preventing water penetration into the granule. Without adequate moisture, the granule fails to swell and burst during cooking and the starch remains contained. This is referred to as RS1. RS2 is isolated from high-amylose starch granules which, due to their aforementioned linear shape, are capable of tighter packing and folding, reducing the surface area available for hydrolysis. RS2 is commonly found in foods that have higher amylose:amylopectin ratios such as raw potatoes, green bananas, and certain types of corn, e.g. Ingredion's Hi-Maize® 260, Hi-Maize® whole grain corn flour, and Hylon VII® .

RS3 describes starch that becomes resistant after it has been cooked (gelatinization) and then cooled (retrogradation). That is, the starch has been cooked in water, causing its granules to burst and form a thick paste. Then, during prolonged cooling and/or storage, the starch molecules reorganize into a more crystalline structure, causing the paste to thicken and become resistant to digestion. Food sources of RS3 include cooled potato salad and bread products. RS4 is resistant to digestion because novel chemical bonds and/or functional groups have been introduced. Examples include cross-linked starches (Al-Tamimi et al. 2010), starch esters (Clarke et al.. 2007), starch ethers (Shimotoyodome et al. 2010), as well as pyrodextrins with glycosidic linkages other than the stereotypical α(1-4) and α(1-6) varieties (Ohkuma & Wakabayashi 2008; Haub et al. 2010). Hasjim *et al.* (2010) describe a novel type of RS made up of retrograded

debranched-starch and starch-lipid complexes dubbed RS5. Its resistance to enzymatic digestion is based on steric hindrance resulting from such complexes.

Table 2. Types of RS and their Sources in the Diet.

Type of RS	Reason for Resistance	Food Sources
RS1	Physically inaccessible to digestive enzymes; protected by carbohydrate food matrix	Whole or partially milled cereal grains, rice, seeds, legumes
RS2	Raw granular starches; possess high amylose to amylopectin ratio	Raw potatoes, green bananas, Hi-Maize® 260
RS3	Retrograded starches; starches that have been cooked (gelatinization), cooled, and granules allowed to reorganize	Cooked and cooled potato salad, bread products
RS4	Chemically modified starches	Not naturally occurring; foods in which modified starches with novel chemical bonds or chemical groups attached have been used
RS5	Chemically de-branched starch and amylose-lipid complexes	Steric hindrance at enzyme-substrate complex

Once again, it should be noted that the digestibility of a given starch sample is never due to a single factor as classification systems suggest; rather, the extrinsic factor with the greatest influence on digestibility is generally used to classify the starch. Starch digestibility can be influenced by non-starch components in the digest (e.g. lipid, protein, etc.), the structure of the starch itself, as well as the type and amount of processing prior to digestion (Annison & Topping 1994; Sharma et al. 2008). The most influential structural feature is the degree and type of crystallinity within the granule. Starch with long, linear chains has a greater tendency to form crystalline structures than starch with short, highly branched chains. Because the amylose component of starch is less branched than amylopectin, high-amylose starch tends to be more resistant to digestion than low-amylose starch.

The National Academy of Sciences recommends that adult men and women consume 38 and 25 grams of total fiber per day, respectively. According to the AHA, the average American consumes around 15 grams of DF per day. If we are not getting enough total fiber, then we likely are not getting enough RS, either. According to Kendall *et al.* (2004), RS doses of 20-30 g/day are needed to observe substantial physiological effects in humans. This level of consumption is 3-4 times higher than actual levels of RS consumption in the US, which are estimated to be between 3 grams and 8 grams per day (Murphy et al. 2008). These levels of consumption stand in sharp contrast to most of human history, and many places around the world. Estimated levels of consumption of RS in medieval Europe – before modern processing methods were invented – indicate daily consumption was between 50 and 100 grams. In developing / third-world countries, which lack that same modern infrastructure, citizens regularly consume between 30 and 40 grams RS per day (Birkett et al. 1997).

RS has been a part of the human diet for centuries as unrefined, unprocessed starch, even though we lacked the ability to identify and manipulate it until recently. Englyst *et al.* (1982) is credited with discovering and defining RS as the starch fraction that resisted enzymatic digestion *in vitro*.[2] Berry *et al.* (1986) modified Englyst's original procedure: most notably by eliminating the boiling step 100°C, which more closely mimics physiological conditions. Such harsh conditions destroyed RS1 and RS3, making complete and accurate quantification of RS impossible. As expected, Berry's changes resulted in greater amounts of RS being isolated from samples. These totals were subsequently confirmed through studies in healthy ileostomy patients (Englyst & Cummings 1985; Macfarlane & Englyst 1986; Englyst et al. 1987).

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[2] *In vivo* methods of measuring RS do exist, and include the breath hydrogen test and direct measurement of effluent from human ileostomy patients, but in vitro methods are generally preferred because they are less invasive and can be applied to a wider variety of creatures and substances.

In 1992, Englyst and his colleagues reported on their method for distinguishing starch fractions based on digestibility *in vitro*. Further modifications were also made to the Berry method at this time by Champ (1992), Faisant *et al.* (1995), and Åkerberg *et al*. (1998) in order to improve underestimation of RS. These changes included an increase in sample size from 10 mg to 100 mg, the addition of sodium azide to prevent microbial contamination as well as de-proteinization using pepsin (Champ 1992; Faisant et al. 1995). Muir and O'Dea (1992) even added a "chewing" step! These changes allowed all naturally-occurring forms of RS to be accounted for.

While both the modified Barry method and Englyst's technique are considered suitable methods for measuring RS *in vitro*, significant variations existed (e.g. in the types and concentrations of enzymes used, pH of the solution, temperature and length of incubation period(s), etc. and did not encourage inter-laboratory evaluation. McCleary *et al.* (2002) attempted to fix that creating a standardized analytical method with the help of the AOAC International, a non-profit organization that functions as an independent third party in the development and dissemination of reliable, standardized analytical methods for a variety of compounds. There are currently a number of Official Methods of Analysis ™ for measuring DF; some of which isolate RS and others which do not, but all of which attempt to remain abreast of major changes and trends in DF research.

Since 1987, the US Food and Drug Administration only counts materials isolated by Official Method™ 985.29, which was developed for quantification of DF on nutrition facts labels (Prosky et al. 1985). Extensions 991.42, 993.19 and equivalent methods 991.43, 992.16, 993.21, and 994.13 adequately quantify DF content as it was originally described by Burkitt and Trowell (McCleary et al. 2012), but do not distinguish RS and other NSPs within said contents.

AOAC 2002.2 is the basis for Megazyme International's RS assay, and includes an enzymatic digestion using pancreatic α-amylase and amyloglucosidase at 37°C for 16 hours followed by isolation and dissolution of the undigested components, which are then quantified as glucose (McCleary & Monaghan 2002).[3] AOAC 2009.01 quantitates the most complete range of DF elements by including fractionation and deionization procedures of other Official Methods™ (i.e. AOAC 991.42, 993.19, and 2001.3) along with the digestion conditions of AOAC 2002.02 (McCleary et al. 2012)

Gut Microbiota

DFs are not chemically identical nor do they share all of the same physiological properties, e.g. viscosity, water-holding capacity, solubility, and fermentability (Burton-Freeman, 2000; Howarth et al. 2001). The beneficial health effects of fermentable fibers have received a great deal of scientific and lay attention, especially in regard to weight management and diabetes. Augmenting the number of beneficial bacteria in the gut, known as the "prebiotic effect," is another positive effect of DF and, especially, RS consumption.

The body of an average healthy human adult is home to over 100 trillion bacteria (Turnbaugh et al. 2007), the majority of which reside in the large intestine (Savage, 1977). This community is dominated by anaerobic bacteria and includes up to 1000 different species whose collective genetic potential is around 100-times greater than the human host (Cani & Delzenne, 2009; Xu & Gordon, 2003). The gut microbiota performs many important duties above and beyond the digestion of otherwise unusable foodstuffs. Gut microbes produce essential nutrients,

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[3] Ingredion Inc. does not use this particular assay as they believe its enzymatic digestion step is too harsh, and leads to underestimation of RS content. They run a modified version of methods described by Englyst *et al.* (1996) instead.

e.g. the B-complex and K vitamins, educate the host's immune system, and protect the host from infection by outcompeting or killing potential pathogens (Turnbaugh et al. 2007).

The composition of the gut microbiota is not static: it is greatly affected by the composition of the host's diet. Prebiotics, as defined by Gibson and Roberfoid, are a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon (Gibson & Roberfoid, 1995). Compared with a probiotic, which introduces non-native bacteria directly into the GI tract, a prebiotic adjusts the composition of the resident microflora indirectly by providing substrate for specific beneficial endogenous microorganisms.

RS and NSPs such as inulin, fructo-, and galactooligosaccharides, are the most well-known and thoroughly studied prebiotics to date. All have a strong bifidogenic effect and, thus, have a number of health benefits associated with them. This includes displacement of injurious microbes, such as certain *Clostridia* or *Salmonella* spp., by *bifidobacteria*; the stimulation of gut-associated lymphoid tissue and subsequent strengthening of gut barrier function, which prevents colonization and infection by potential pathogens; and increased production of SCFAs, resulting in improved mineral absorption and, possibly, protection against colon cancer (Kolida & Gibson 2007). RS may exert its cancer-protective effects by modifying important biological consequences related to cancer development such as apoptosis or cell proliferation (Dronamraju et al. 2009; Leu et al. 2003). The synbiotic combination of RS and a single probiotic bifidobacteria, *B. lactis*, significantly protected azoxymethane-treated Sprague Dawley rats from developing coloerectal cancer.

Dysbiosis is the term used to describe an imbalance in the composition of an individual's microbiota, and has been implicated in the pathophysiology of a number of diseases and

conditions such as obesity, diabetes, and various inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis (Elson et al. 2005; Lupp et al. 2007; Sartor, 2008). Adult germ-free mice inoculated with bacteria harvested from the guts of obese animals have significantly greater increases in body fat content than animals colonized with bacteria from lean animals (Turnbaugh et al. 2006). Interestingly, these differences in adiposity are associated with dissimilarity in the relative abundance of *Bacteroidetes* and *Firmicutes*, which are two of the predominant phyla present in both human and mouse GI tracts. The microbiota of obese subjects is dominated by *Firmicutes* and a reduced population of *Bacteroidetes*. Conversely, a decrease in the *Firmicutes*:*Bacteroidetes* ratio has been found in response to weight loss (Ley et al. 2006).

As previously stated, the by-products of microbial fermentation of RS and other DFs are lactate, hydrogen gas, carbon dioxide, and methane in addition to SCFAs, the latter of which provide energy for both the microbiota and the human host (Topping & Clifton 2001). While the amount varies based on the amount and type of polysaccharide consumed as well as the exact composition of the microbiota, microbial fermentation is believed to account for around 10% of our daily caloric intake (Hooper et al. 2002). The main SCFAs produced are acetate, propionate, and butyrate.

Acetate is the most abundant SCFA formed in both *in vivo* and *in vitro* samples (Topping & Clifton 2001), and it is widely used throughout the body during the formation of ATP (Kolida & Gibson 2007). It is synthesized by acetogenic and methanogenic bacteria using the Wood–Ljungdahl pathway, also known as the reductive acetyl-CoA pathway, under the anaerobic conditions of the gut. This route requires two enzymes – carbon monoxide dehydrogenase and acetyl-CoA synthase – that produce acetyl CoA from CO_2 and CO using hydrogen as the electron donor. It can also be made from pyruvate using pyruvate dehydrogenase.

Propionate is primarily taken up by the liver via the portal vein and is involved in hepatic lipid metabolism. More specifically, it is converted to propionyl-CoA by propanoate-CoA ligase then into succinyl-CoA in three consecutive steps catalyzed by propionyl-CoA carboxylase, methylmalonyl-CoA epimerase, and methylmalonyl-CoA mutase. Succinyl-CoA enters the tricarboxylic acid cycle and is converted to oxaloacetate, the precursor of gluconeogenesis.

Approximately 95% of the butyrate produced in the colon is absorbed by the colonic mucosa, for which it is the preferred energy source. Butyrate is believed to be particularly important for gut health, especially in regard to cancer prevention (Whitehead et al. 1986; McIntyre et al. 1993; McOrist et al. 2008). Butyrate is produced from two molecules of acetyl-CoA, yielding acetoacetyl-CoA, which is further converted, finally, to butyryl CoA. This metabolite can be converted to butyrate via butyrate kinase or butyryl CoA:acetate CoA transferase. In general RS fermentation generates proportionally more butyrate and less acetate than other types of DF (Nugent 2005).

Production of SCFAs is also 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 saccharolytic fermentation occurs. Increases in plasma GLP-1 and PYY concentrations have also been documented in several studies (Keenan et al.. 2006; Zhou et al. 2008; Shen et al. 2008). Positive health outcomes associated with increased circulating GLP-1 concentrations could be beneficial in the control of diabetes and obesity. Most recently, research has demonstrated that butyrate and propionate stimulate intestinal gluconeogenesis (De Vadder et al. 2014). Butyrate activates intestinal gluconeogenesis via signaling through a cAMP-based mechanism while propionate serves as both a substrate and an activator for intestinal gluconeogenesis through a gut-brain neural circuit controlled by binding

to the propionate receptor, i.e. fatty acid receptor-3. Glucose from the intestinal gluconeogenesis enters the portal blood and is detected by a sensor and this signals the brain via the peripheral nervous system. Together, this promotes reduced on FI and improved glucose metabolism.

The existence of intestinal gluconeogenesis is a relatively recent discovery (Rajas et al.. 1999, Croset et al.. 2001, Mithieux et al.. 2004) and represents a mechanism by which the products of fermentation can produce beneficial health effects. Feeding of fructooligosaccharide, butyrate, or propionate improves insulin sensitivity and BG regulation in WT mice, but not in intestinal glucose 6-phosphatase KO mice. The glucose 6-phosphatase enzyme is necessary for the production of free glucose, which enters into portal circulation, via intestinal gluconeogenesis. Some humans fail to increase GLP-1 production after consumption of RS, but still exhibit improved insulin sensitivity and BG control (Robertson et al. 2005). Our research group (Keenan et al. 2011) has demonstrated variability in GLP-1 production in humans fed RS, and recently Robertson's group has observed increased GLP-1 production in her human subjects given RS (Bodinham et al. 2014). There is possible mechanism for non-responders, e.g. different gene variants of transcription factor 7-like 2 (also called T-cell-specific factor 4), involved in activation of the proglucagon; some of the best known gene variants are associated with type 2 DM (Jin et al. 2008). Thus, beneficial health effects associated with RS consumption are not fully dependent on GLP-1, and are also possible through stimulation of intestinal gluconeogenesis.

CHAPTER 3
NUTRIENT-DRUG INTERACTION STUDIES

Introduction

Obesity is a significant risk factor for many diseases and conditions, including type 2 DM, and the number of individuals who meet the criteria for diagnosis has reached pandemic proportions (Flegal et al. 2012; Kim & Popkin et al. 2012). Weight loss and glycemic control are cornerstones in obesity and diabetes treatments. Several studies suggest that diet and lifestyle interventions induce weight loss (5-10% baseline weight) and improve glucose control (Hamman et al. 2006; Garber 2012). However, lifestyle changes are often difficult to maintain and, as such, are not the most reliable means of treating and preventing diabetes (Friedman 2004; Wilding 2007). Clinicians prescribe various drugs, such as SG and metformin, in order to help control BG (Brown & Evans 2012).

The drug SG, made by Merck & Co., LLC, was first marketed in the US in 2006 (Kim et al. 2005). It improves glucose metabolism in type 2 DM patients by up-regulating the activity of an incretin hormone known as GLP-1, which is produced by L endocrine cells of both small and large intestines in response to nutrient intake (Brown & Evans 2012). SG increases GLP-1 half-life by inhibiting the enzyme DPP-4, which degrades GLP-1 in the bloodstream (Brown & Evans 2012). Augmenting GLP-1 activity through SG significantly improves insulin sensitivity and BG in both human and animal models, without reductions in body weight. In contrast, GLP-1 agonists consistently reduce both BG and body weight (Madsbad 2009; Gerich 2010).

RS is a type of DF that stimulates endogenous GLP-1 secretion through its fermentation into SCFAs by the intestinal microbiota (Englyst et al. 1987; Cummings et al. 1996). Supplementation with HM260, a type of resistant starch, has been shown to reduce body fat by diluting the energy density of the diet and by increasing β-oxidation of fatty acids, resulting in

reduced body fat in various models (Higgins et al. 2004; Keenan et al. 2006). Our lab has had some difficulty maintaining robust levels of fermentation in rodents fed diets that are high in dietary fat, resulting in reduced gut hormone levels and body fat loss (Charrier et al. 2013).

These observations led us to hypothesize that: (1) HM260 may enhance SG activity by stimulating endogenous GLP-1 production, augmenting serum GLP-1 concentrations and leading to reduced body weight and adiposity; and (2) SG may "rescue" the effects the HM260 supplementation on BG and body fat control despite HF diet content. That is, a combination of HM260 and SG may foster better glycemic control and lead to greater weight loss than either treatment alone under HF diet and/or type 2 DM conditions.

To test these possibilities, we conducted two cohort studies in a mouse model of type 2 diabetes. In the first study, various combinations of HM260 were tested in wild-type mice in order to identify an effective HM260 concentration for synergy. In the second study, the role of GLP-1 in said synergy was tested in GLP-1 receptor KO mice. We did not observe significant effects of HM260 alone on body weight or adiposity. However, the combination of 28% HM260 with 0.4% SG (28R/S) reduced body weight and fat gain, and lessened BG levels in the wild-type model. Those effects were abolished in GLP-1R KO mice. Fermentation of HM260 was attenuated on the HF diet and, therefore, the 15% HM260 did not enhance SG activity.

Research Design and Methods

Animal models – All study procedures were approved by the IACUC committee at the Pennington Biomedical Research Center. In the first study, fifty five (N=55), 7-week-old male C57BL/6 WT mice were purchased from the JAX® Laboratories (Bar Harbor, Maine), and housed individually in shoebox cages in a climate-controlled environment (21-22°C, 55% humidity) with a 12:12 hour light-dark cycle illuminated at 7 AM. The mice were fed a semi-

purified HF diet (42% energy) in order to induce weight gain. This pre-study diet also contained a small amount of HM260 (3% weight) in order to maintain proper fermentative microbial communities despite dietary fat content. Mice were given one injection of a low dose of STZ (i.p. at 40 mg/kg) during the third week on the HF diet in order to induce hyperglycemia by partial β-cell loss to establish the type 2 DM model.

Animals were stratified according to fasting BG (244 ± 4 mg/dL) and body composition (25-26% body fat) as determined by NMR spectroscopy, which was assessed at one and three weeks post-STZ injection, respectively. The mice were divided into six groups after a total of six weeks on pre-study HF diet (i.e. three weeks after STZ injection), and treated for ten weeks with isocaloric diets (4.16 ± 0.02 kcal/g) (n=9-10) containing HM260 and/or SG (0 or 0.4% WT of diet; 12.65 mg/kg/day). HM260 was tested at three dosages: 0, 15, and 28% weight of diet and labeled as C, 15R, and 28R, respectively. Prior to consumption, powdered diets were formed into bars by mixing with water followed by freeze drying overnight in the -20°C freezer. Fasted BG and body composition were assessed at the end of week 10 of treatment, prior to euthanasia.

In the second study, we repeated the experiment in male GLP-1 receptor KO mice with one combination of HM260 (28% weight of diet) and SG (0.4% weight of diet). The GLP-1 receptor KO mice were previously obtained from the original breeder, Dr. Drucker of the University of Toronto, Ontario, Canada. Fifty male KO mice and fifty male WT mice were generated through in-house breeding and used at 7-8 weeks of age. Hyperglycemia was induced in mice using HF diet + STZ injection as was done in the previous study. Baseline body weight (25 and 28 g ± 1 for WT and KO mice, respectively) and composition (25 and 35% ± 1 for WT and KO mice, respectively) were assessed at week 5 on HF diet, and was followed by stratification along with glycemic status (220 mg/dL ± 3) into one of four groups (n=12; N=48).

The mice were then fed either a control diet (C) or diet supplemented with HM260 and SG (R/S) for 11 weeks. Additionally, metabolic rate and food intake were assessed by OxyMax®/CLAMS for three days following a three-day acclimation period during experimental weeks 7 and 8. The chamber was maintained at the same temperature range and light-dark cycle as for the shoebox cages. Body composition and fasted BG were assessed at the end of week 9 and at the beginning of week 10 of treatment, respectively.

Blood glucose − Fasting BG was tested using AlphaTRAK glucometer with AlphaTRAK strips (Abbott Laboratories, Inc., Chicago, IL) for both studies. After overnight fasting, blood was drawn by either tail or submandibular bleeding using sterile 5 mm Goldenrod Animal Lancets (MEDIpoint Inc., Mineola, NY).

GLP-1, insulin, and leptin test − In the 1st study, plasma was used to determine the concentration of active GLP-1 using ALPCO Diagnostic's GLP-1 ELISA (Active 7-36) kit (cat # 43-GP1HU-E01, Salem, NH). An EMD Millipore Multiplex® MAP kit (cat# MMHMAG-44K St. Louis, MO) was used to assess the concentrations of various hormones, including active GLP-1 active, leptin and insulin in the 2nd study. The GLP-1 assay used in the first study required plasma, which was collected in tubes containing another DPP-4 inhibitor (Millipore) 30 minutes following re-feeding after an overnight fast. Fasting plasma was used for GLP-1 and other hormone tests in the second study.

Tissue collection − Mice were sacrificed via decapitation and blood samples were collected in EDTA tubes and centrifuged at 4000g for 20 minutes in order to extract plasma. 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. Cecal contents were collected in microcentrifuge tubes and their pH was determined.

Statistical Analysis – Data from the first and second studies were analyzed as 2x3 and 2x2 factorials, respectfully, using the MIXED procedure of SAS® Version 9.3 (SAS Institute, Inc., Car, N.C.). Exceptions include end-of-study data from the first study used to plan the second study, which were evaluated using student t-tests in Excel. The UNIVARIATE procedure was used in the MIXED procedure to assure equal variance, normal distribution, and to identify outliers. Any observations that were more than three standard deviations away from the mean were considered outliers. Four total data points were removed: 28R1 and 28R5 were removed from active GLP-1 and fasting BG measurements from the first study; KO11 and WTC9 from the second study's insulin and leptin measurements, respectively. WTR/S1 and WTR/S2 were removed from food intake data due to suspected equipment malfunction. Data were transformed by log if the normality assumption was not met. This was followed by F-protected least significant difference mean comparison tests in order to determine differences among dietary treatments. Results were considered significant at $p<0.05$ and expressed as means ± SE.

Results

An interaction effect (p=0.0198) was documented for empty cecum weight in the first study (Fig. 2). SG increased empty cecum weight, but only when consumed in conjunction with HM260, whereas HM260 was effective both alone and in combination with SG. No difference between 15 and 28% HM260 consumption was observed. Cecal content weights were similarly affected: HM260 (p<0.0001) and SG (p=0.0281) increased cecal content weight, albeit SG only worked at the 28% HM260 level (Fig. 3). Significant main effects for both RS (p=0.0001) and SG (p=0.0002) were documented for cecal pH, but numerical differences were not substantial i.e. less than half of a pH point (Fig. 4). This is considered a very modest reduction compared to

those documented in previous studies on low- and medium-fat diets, suggesting diminished rates

of HM260 fermentation with the feeding of the HF diet.
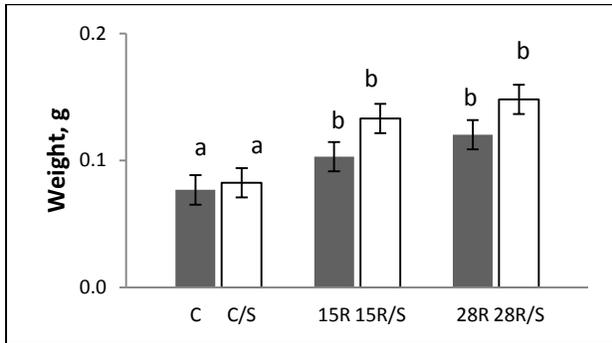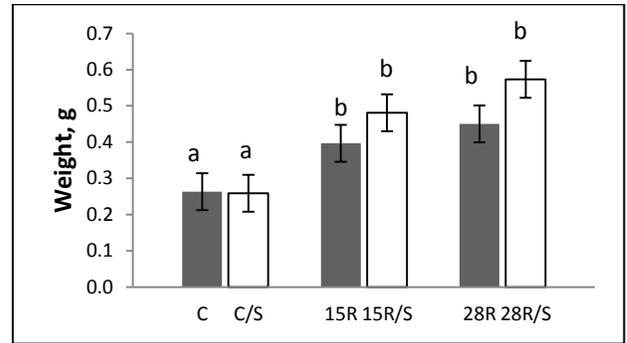

Fig 2. Empty cecum weights for
study 1.

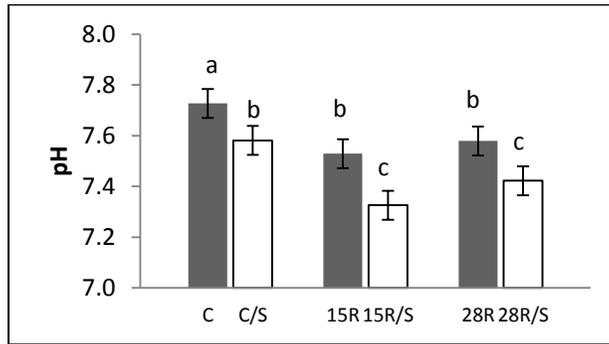Fig 3. Cecal content weights for
study 1.

Fig 4. Cecal pH for study 1.

In the second study, KO animals had greater empty cecum (Fig. 5) and cecal content

weights (Fig. 6) compared to WT (p<0.0001 and p=0.0014, respectively) – most likely as a result

of their greater overall size – and both strains responded to 28R/S treatment (p=0.0004 and

p<0.0001, respectively) with increased values compared to those fed control diets. As expected,

combination treatment (p<0.0001), but not strain (p=0.1268), reduced cecal pH values by

approximately half a pH point (Fig. 7).
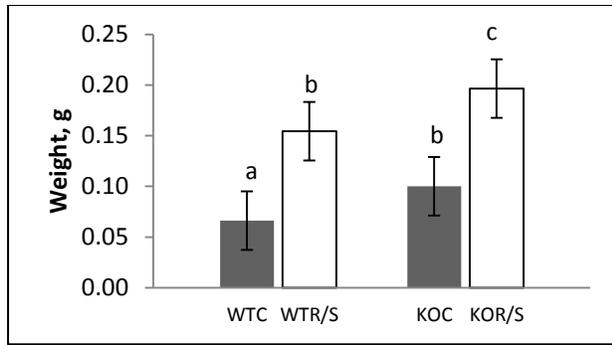
Fig 5. Empty cecum weights for study 2.
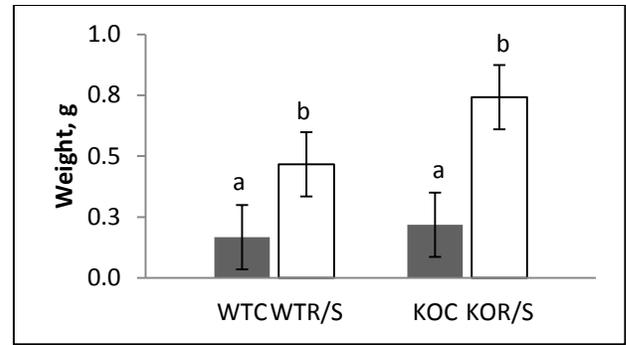
Fig 6. Cecal content weights for study 2.
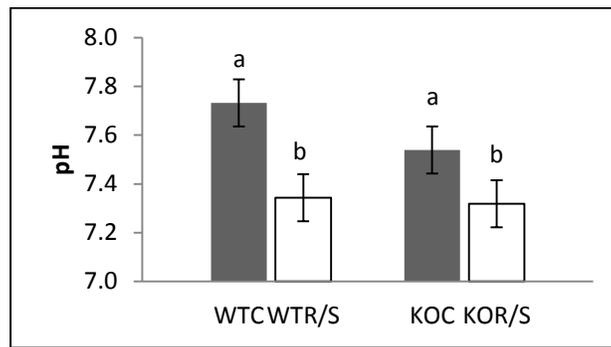
Fig 7. Cecal pH for study 2.

Active GLP-1 concentrations and fasted BG are the primary factors for evaluating treatment efficacy. In the first study, HM260 did not significantly stimulate serum GLP-1 production at either the 15 or 28% level (p=0.1772). An SG effect was documented (p=0.0010), with increased GLP-1 at the 0 (p=0.0227) and 28% (p=0.0256) HM260, but not 15% (p=0.1485) (Fig. 8). However, 28% HM260 had the highest numerical amount of active GLP-1 and as stated above, had a higher amount of active GLP-1 than SG alone. Since there was not a significant interaction (p=0.7747) between HM260 and SG, this difference appears to be isolated to the 28% HM260 and the HF diet may have impaired active GLP-1 production with 15% HM260. The data do not support a dose-dependent response of SG with HM260 in regard to GLP-1. Something else could have interfered with the effectiveness of SG treatment at the 15% level. It

may be that the HF diet reduced fermentation and GLP-1 production, even though GLP-1 production is usually stimulated by nutrient intake, especially dietary fat.
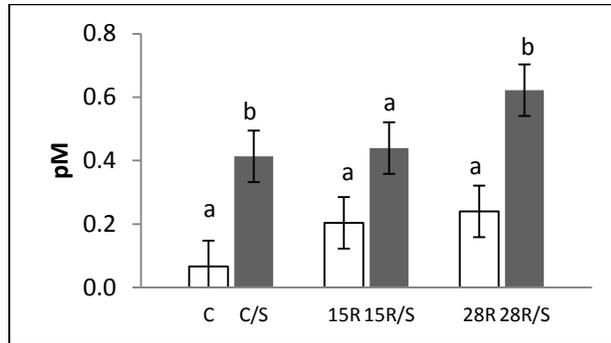

Fig 8. Serum GLP-1 active for study 1.

Despite SG-induced elevations in GLP-1 concentration, no significant changes in fasting BG were found in the first study (data not shown). These data are likely inaccurate due to human error: animals were not given new cages during the fasting period prior to testing; food was merely removed from the hoppers and cages per visual inspection. It is possible that small amounts of food at the bottom of the cage went undetected by researchers, allowing the mice to feed during the designated fasting period.

Since elevation of active GLP-1 concentration was seen in SG-treated mice in study 1, and in order to determine the role of GLP-1 on treatment outcomes, we performed a second study with GLP-1 receptor KO and WT mice. The test was conducted after 10 weeks of combined treatment with SG (0.4%) and HM260 (28%). Treatment amplified serum GLP-1 active concentration (strain*diet, p=0.0454) and reduced fasting BG (diet, p<0.001) in WT mice (Fig. 9). In KO mice, the GLP-1 elevation was almost double that of their WT counterparts (p=0.0013). However, the increased GLP-1 was not faithfully translated into a glucose reduction in KO mice. While KO animals were capable of responding to treatment with reduced BG levels, they exhibited consistently and significantly higher BG values than WT mice, regardless of the

doubled level of GLP-1. The data confirm that the GLP-1R is required for glucose control by SG-induced GLP-1. The data also suggest that a functioning GLP-1R is required for effective clearance of GLP-1.
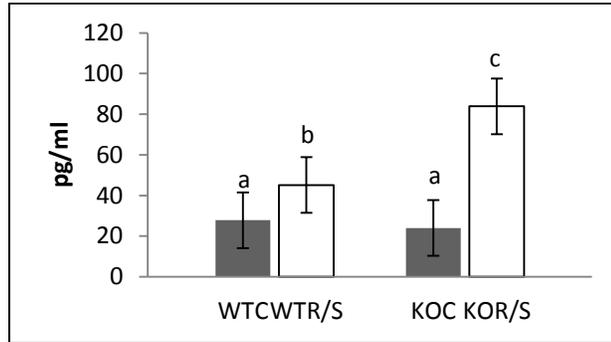

Fig 9. Serum GLP-1 active for study 2.

Fasting BG obtained during the second study in response to 28R/S treatment likely confirms human error in study 1 (Fig. 10). Animals fed the treatment diet had reduced fasting BG levels compared to animals of the same strain fed a control diet (p<0.0001). Similar results were discovered for strain (p<0.0001): KO animals had greater fasting BG levels compared to WT controls regardless of diet. However, neither dietary treatment nor strain had any significant influence under ad-libitum feeding conditions.
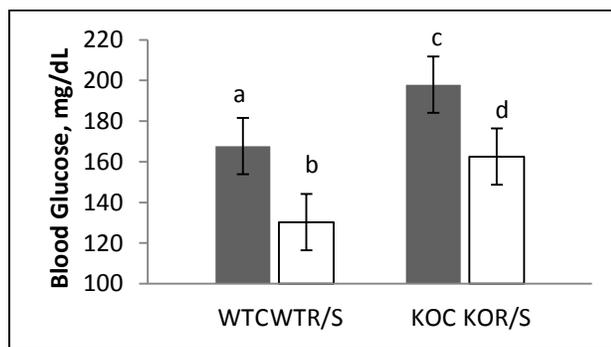
Fig 10. Fasted blood glucose for study 2.

Animals receiving SG treatment in the first study had significantly reduced body weights (p<0.0001) and adiposity – based on NMR (p<0.0001) and total abdominal fat pad weight

(p<0.0001) – compared to control animals (Fig. 11, 12, and 13, respectively). SG appeared to rescue the impeded 28% HM260 effect on body fat loss by HFD in study 1. A combination of SG and 28% HM260 resulted in the most substantial reduction in fat mass (p=0.0048, NMR and p=0.0111, excised abdominal respectively).
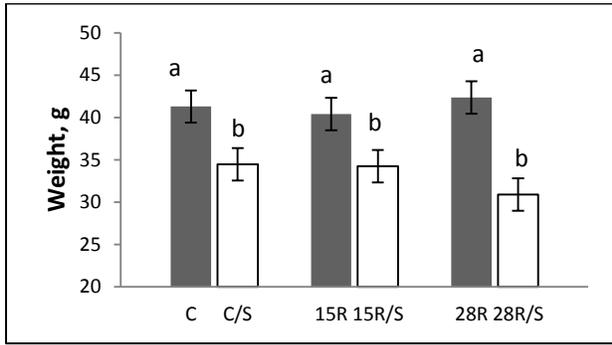

Fig 11. Body weight for study 1.

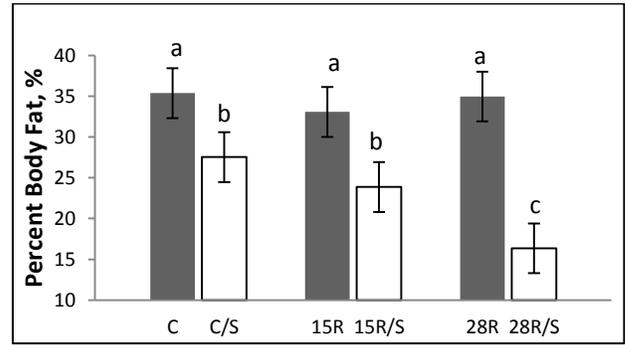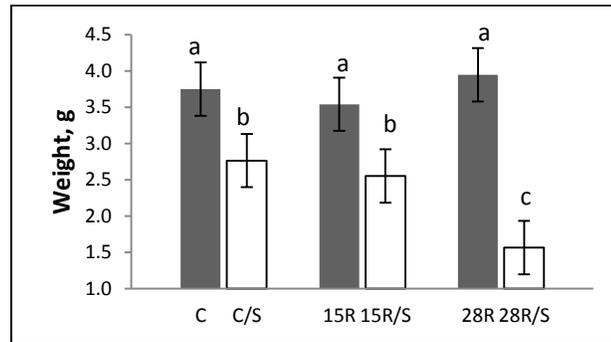Fig 12. Percent body fat for study 1.

Fig 13. Total abdominal fat for study 1.

Phenotypic differences in body weight and adiposity between KO and WT mice were observed both before and after treatment (data not shown). KO mice exhibited ~10% more body weight than WT mice before treatment (p=0.0003) (Fig. 14) due to ~50% more fat mass in the former (p<0.0001) (Fig. 15). Treatment attenuated weight gain in WT mice (p=0.0047), but failed in KO mice (p<0.0001). That is, KO mice gained more weight and had either diminished or no reductions in fat mass over the course of the study compared to controls according to NMR and total abdominal fat pad weights (Fig. 16), respectively.
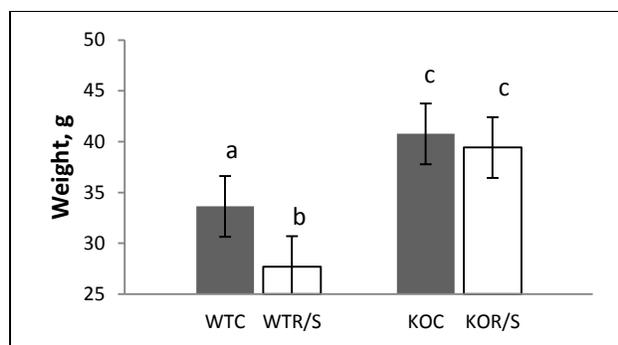
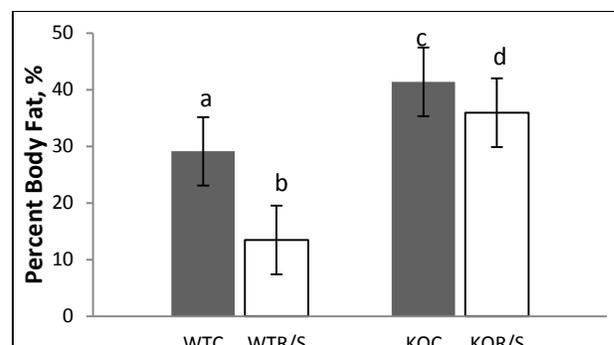Fig 14. Final body weight for study 2.

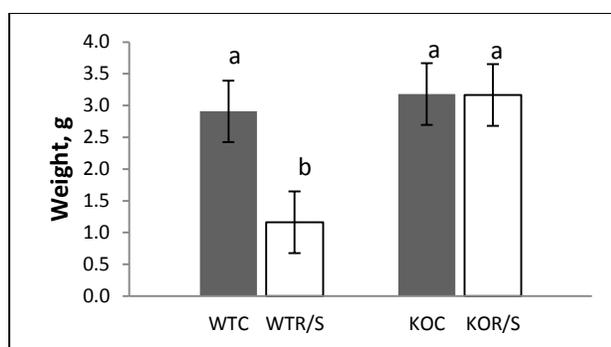Fig 15. Percent total body fat for study 2.

Fig 16. Total abdominal fat for study 2.

Collectively, the data suggest that SG together with HM260 has a significant activity in the prevention of diet-induced weight and fat gain in a mouse model of type 2 DM, and the effect is dependent on a functioning GLP-1R.

To understand GLP-1 activity in the regulation of energy metabolism, we monitored FI, PA, and EE via metabolism cages. Treatment significantly increased FI (p=0.0216) (Fig. 17) and EE in WT mice without altering PA (p=0.9065) (Fig. 19). Increased EE in response to treatment was indicated by increased oxygen consumption (O2) and carbon dioxide production (CO2) in WT mice, resulting in increased values for respiratory exchange ratio (RER) (p=0.0002) (Fig. 18, 20, and 21, respectively) . KO animals had reduced overall O2 and CO2 levels compared to controls, and their response to treatment was either non-existent (CO2) or less pronounced (O2).

Increased RER despite minimal exchange of gases can be explained by the severely diminished

PA and lack of increased FI accomplished by the KO strain (p<0.0001).
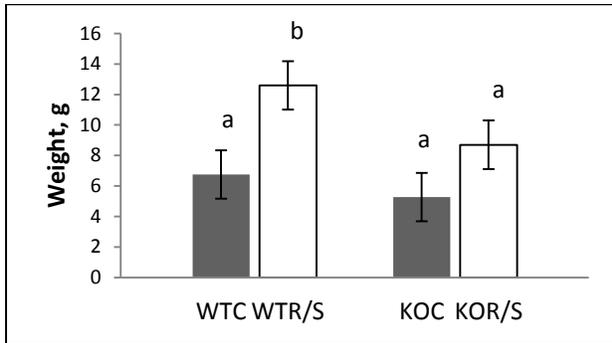

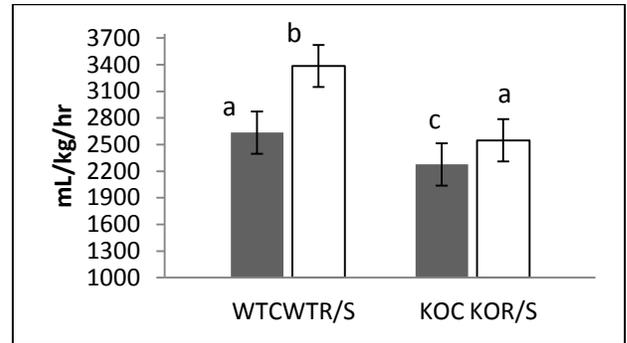Fig 17. Food intake for study 2.
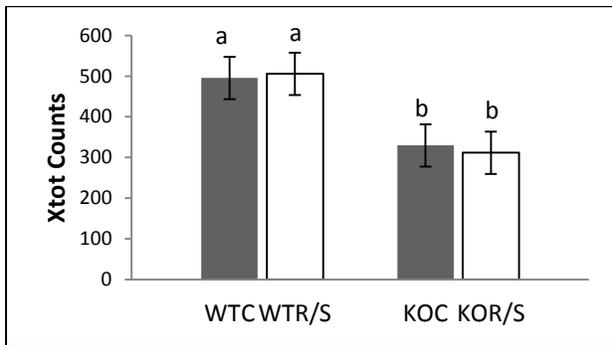

Fig 18. Oxygen consumption for study 2.

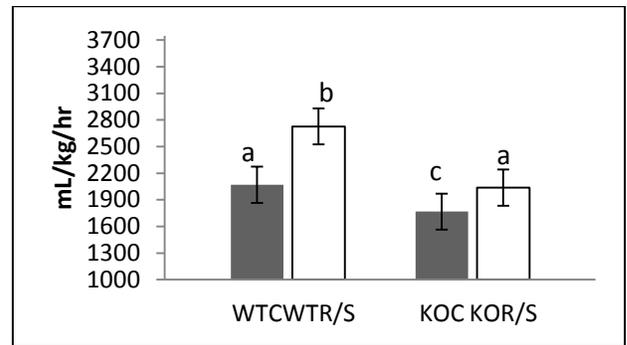Fig 19. Physical activity for study 2.

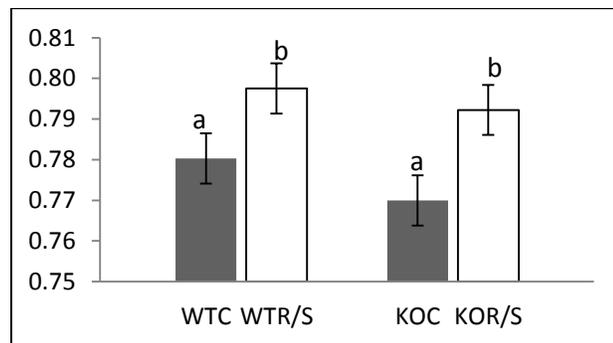Fig 20. Carbon dioxide production for study 2.

Fig 21. Respiratory exchange ratio for study 2.

The effect of enhanced EE may exceed that of increased FI as the weight gain was reduced by the treatment. That treatment failed to induce significant changes in FI and EE in KO mice suggests that the GLP-1R is required for the induction of EE in WT mice in response to the treatment. The extreme differences in PA in KO animals may undermine the beneficial effects of 28R/S treatment in that strain. An alternative possibility is that WT animals supplemented with treatment diet, due to their increased FI, consumed significantly more SG than KO animals, which contributed to that group's substantial weight loss.

Insulin and leptin levels were measured in order to determine relative sensitivities in the DIO diabetic mice used in the second study. The test was conducted at 10 weeks of treatment using fasting plasma. Diet (p=0.0346) and strain (p=0.0113) independently affected insulin levels, and an interaction effect was discovered for leptin (p=0.0003). That is, WT animals fed the 28R/S combination had significantly reduced insulin levels compared to those fed the control diet (p=0.0303), but KO animals were not responsive (Fig. 22). Leptin was significantly reduced in response to treatment for both strains, but the reduction in KO was diminished compared to WT mice (Fig. 23). The hormone data are consistent with the lower body weight and lower adiposity of treated mice as well as the higher relative adiposity of KO animals.
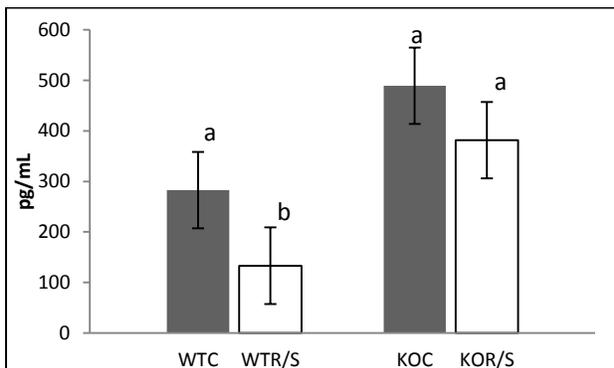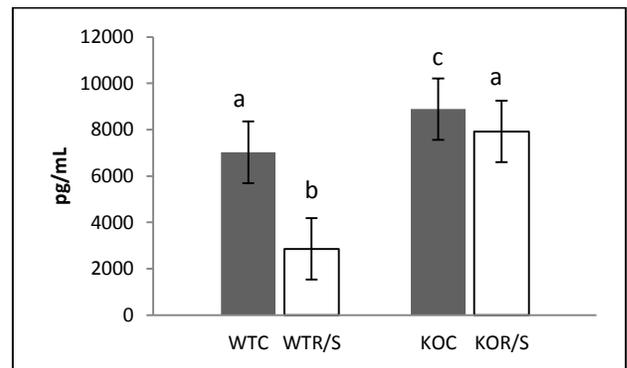
Fig 22. Plasma insulin levels.   Fig 23. Plasma leptin levels.

Discussion

In the first study, HM260 did not enhance SG-mediated induction of GLP-1, but it did result in greater reductions in total and abdominal body fat content. Partial synergy between HM260 and SG occurred at the 28% HM260 level only, causing our lab to choose 28R/S as the treatment diet for the second study using GLP-1R KO mice. The limited interaction between the two compounds is likely due to inhibition of HM260 fermentation by the HFD used in generation of the DIO model. HM260 was fermented, as indicated by increased empty cecum and cecal content weights, and by decreased cecal pH. However, changes in cecal pH were only about half a pH point. Moreover, measurements remained above 7.0, well within the normal pH range of the colonic lumen. Charrier *et al.* (2013) also documented diminished reductions in cecal pH, and partial attenuation of other stereotypical effects associated with HM260 consumption when consumed in conjunction with a HFD. Superior synergy, leading to more pronounced physiological effects, is expected in animals fed low- and medium-fat diets.

The SG effect on cecal pH was unexpected, and the mechanism(s) by which this reaction occurs is unknown. Poor gastrointestinal absorption and use by intestinal bacteria as a substrate for fermentation has never been reported, and is highly unlikely due to its documented 87% bioavailability (Herman et al. 2005). To our knowledge, no laboratory has specifically and intentionally investigated the effects of SG on colonic fermentation. We suspect this result is related to GLP-1's inhibitory effect on gastric emptying: slowing down intestinal transit may give bacteria more time to ferment dietary fibers before excretion. Interestingly, SG-induced reductions in cecal pH are not diminished by a HFD, albeit its effects are not as strong as unimpeded HM260 fermentation.

A multitude of studies have shown that DPP-4 inhibitors such as SG are body weight-neutral (Charbonnel et al. 2006; Raz et al. 2006; Rosenstock et al. 2006), so SG's ability to attenuate body weight and fat gain in the first study was also surprising. In our study, the weight loss might be a result of dietary supplementation as opposed to injection, which likely keeps plasma GLP-1 active concentrations consistently elevated thanks to more frequent consumption by mice and the long half-life of SG, i.e. between 8-14 hours (Herman et al. 2005). Higher plasma GLP-1 concentrations are associated with higher rates of EE and fat oxidation independent of age, sex, and body composition (Pannacciulli et al. 2006). In rats, GLP-1 injection (50 pmol-20 nmol, iv) elicited dose-dependent increases in the rate of EE as indicated by whole-body O2 consumption, heart rate of urethane-anesthetized rats, and core body temperature (Osaka et al. 2005). GLP-1R agonists, with or without concurrent administration of a DPP-4 inhibitor, consistently reduce body weight (Boschmann et al. 2009; Bradley et al. 2010; Garber 2011), and inactivation of the DPP-4 gene prevents DIO, insulin resistance, and type 2 DM in mice (Marguet et al. 2000; Conarello et al. 2003). Our laboratory hypothesized that the majority of the body weight and fat losses seen in association with augmented GLP-1 concentration occurred through activation of the GLP-1R. In support of this theory, anti-obesity effects of 28R/S treatment were lost in GLP-1R KO mice.

We observed that 28R/S treatment reduced fasting BG, insulin, and leptin levels in WT mice, suggesting improved sensitivities to both hormones. As expected, these effects were associated with lower body weights and decreased adiposity. Once again, the anti-diabetes and anti-obesity effects of 28R/S were lost in GLP-1R KO mice, suggesting the role of GLP-1R in the therapeutic activities of the combination treatment.

Our data from KO mice also indicate that GLP-1R may regulate serum GLP-1. The circulating GLP-1 concentration reflects a balance of secretion and degradation. The secretion by L-cells is induced by food intake. SG was reported to stimulate total GLP-1 secretion by directly activating cAMP and ERK1/2 signaling pathways in L-cells (Sangle et al. 2012), which is independent of DPP-4 inhibition. This activity is likely unrelated to the elevated GLP-1 in KO mice. The GLP-1 protein is degraded by DPP-4 in the deactivation process. It is not known if the GLP-1 receptor is required in the control of GLP-1 degradation. In this study, GLP-1R KO mice exhibited a 100% greater serum GLP-1 in response to SG treatment. The elevation is induced by SG as serum GLP-1 levels were identical between WT and KO mice in the untreated control groups. The data suggest that in a physiological condition, GLP-1R may accelerate GLP-1 degradation. The effect is impaired in the GLP-1R KO mice, which leads to the GLP-1 super reaction to SG.

In summary, we used GLP-1R KO mice to study potentially synergistic therapeutic activities of SG and HM260. We observed that a combination of SG with 28% HM260 was able to attenuate weight gain in addition to reducing fasting BG in type 2 DM mice. This combination enhanced EE in the mice, which may represent a mechanism of the anti-obesity effect commonly associated with HM260 and inconsistently with SG. The therapeutic effects are dependent on GLP-1R, which may regulate plasma GLP-1 as well. The mechanisms of GLP-1R actions remain to be established for the induction of EE and regulation of circulating GLP-1.

CHAPTER 4
WHOLE-GRAIN RESISTANT STARCH STUDY

Introduction

Dietary RS in the form of HM260, a type of high-amylose corn starch containing significant amounts of RS2, increases gut fermentation in several non-obese rodent models including C57BLK6 mice, Wistar rats, and Goto Kakazaki rats. Characteristic results of fermentation are increased empty cecum weight, increased cecal content weight, reduced cecal pH value (i.e. below 7.0), and increased concentrations of various SCFAs in the cecum. Reductions in body fat and body weight have also been documented. However, these results are inconsistent and/or not as robust in rodent models of obesity, including both genetic and diet-induced obese varieties.

Reid *et al.* (2010) makes several recommendations designed to minimize problems with experimental design that could obscure important biological differences between those that do (responders) and those that do not respond (non-responders) to probiotic treatment; these suggestions can also be applied to studies using prebiotics. Identifying which individuals or groups of individuals are likely to exhibit which physiological responses is important when attempting to prescribe the most effective treatment modality. In regard to RS, certain animal models appear to be non-responders to dietary RS. For example, Zhou *et al.* (2009) saw no reductions in abdominal fat for two different polygenic obese mouse models fed low-fat diets; these mice did not appear to ferment RS. Goldsmith *et al.* (2010) and Badkoobeh *et al.* (2010) failed to document abdominal fat reduction in mice made obese through HF-feeding prior to beginning RS supplementation.

Our laboratory is curious about these irregularities: what is responsible for these variations in fermentation? Is it something to do with a particular animal model's genetic make-

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up or a peculiarity of the particular type of resistant starch, e.g. RS1, RS2, RS3, etc. or a combination of the above? The ZDF rat possesses a mutation in the leptin receptor that causes it to develop obesity and insulin resistance between 7-10 weeks of age (Srinivasan & Ramarao 2007). Scientific literature characterizing the fermentation response of the animal model is sparse, if not nonexistent. Whether or not these animals are capable of fermenting, i.e. whether or not this animal model's genotype has altered the microbiota to make it unresponsive to treatment with RS is likewise unknown.

We hypothesize that obese ZDF rats will be capable of robustly fermenting RS demonstrated by increased cecal weights and increased SCFA and reduced cecal contents pH. However, beneficial phenotypic changes such as lower abdominal fat and increased insulin sensitivity that we have previously observed in combination with RS fermentation, may not occur if leptin signaling is required for the effects. ZDF rats have a defective leptin receptor and are used in this study as a knockout for leptin signaling. That is, feeding of RS to ZDF rats for enhancing fermentation and improving the gut microbiota (Tachon et al. 2012) may not override the animals' genetics.. We also hypothesize that more vigorous levels of fermentation will be seen in animals fed HMWG, which is a source of two types of RS, i.e. RS1 and RS2, compared to Hi-Maize RS, which only provides RS1. Additionally, the corn bran in the whole-grain corn flour has fructans, β-glucan, and arabinoxylans (Lu et al. 2000, Dodevska et al. 2013, Maki et al.. 2012).

Research Design and Methods

Animal Models – All study protocols were approved by the IACUC committee at the Louisiana State University Agricultural and Mechanical College. ZDF rats (n=48) arrived from Charles River Laboratories International Inc. at 4 weeks of age, and 47 rats remained in

quarantine for 1 week, where they were maintained on a standard chow diet.[4] Animals were housed in wire-mesh cages in a climate-controlled environment (21-22°C, 55% humidity) with a 12:12 hour light-dark cycle illuminated at 7 AM. Rats were given two weeks to acclimate to a powdered control diet before stratification and administration of experimental diets. Fasted blood glucose and insulin values were determined from blood collected through retro-orbital bleeding using a glucometer and Millipore's insulin kit, respectively. Measurements were used to calculate HOMA-IR, which equals fasting plasma glucose multiplied (mmol/L) by fasting plasma insulin (mU/L) and then divided by 2430, in order to properly stratify animals into four groups (n=11-12) based on body weight and diabetic status.

At 7 weeks of age, rats were stratified by HOMA-IR and body weight into one of four isocaloric (3.2 kcal/g) diets: AC (n=12), HM260 (n=12), DWGC (n=12), and HMWG (n=11). The signifying diet components, i.e. Amioca® starch, Hi-Maize® RS, dent corn flour, and WG RS flour, were analyzed by proximate analysis and modified Englyst assay (Englyst, 1996) for formulating diets for macronutrient content and RS amount of the carbohydrate fraction, respectively. The RS by weight of the final diets was: AC 0%, DWGC 6.9%, HM260 25%, and HMWG 25%. Body weight, food intake, and spillage measurements were taken twice per week for the 11-week study. Collections for HOMA-IR calculations were repeated at 8 weeks into the study.

Tissue Collection – At the end of the study period, non-fasted rats were euthanized via isoflurane inhalation. Blood was collected in 1.5 mL microcentrifuge tubes containing DPP-4 inhibitor and used to measure GLP-1 active with ALPCO's ELISA kit. The GI tract was removed from the base of esophagus to anus and weighed. Stomach, small intestine, and large

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[4] One rat died soon after arrival due to kidney-related health problems.

intestine were weighed full, divested of their contents, and washed then reweighed. Peritoneal, retroperitoneal, and epididymal fat pads were collected and weighed, and the sum of which is referred to as total abdominal fat weight. EBW was calculated as disemboweled body weight (final body weight minus weight of full GI tract) plus the weight of the empty GI tract. Percent abdominal fat was calculated as total abdominal fat divided by EBW, multiplied by 100. Cecal contents were saved in microcentrifuge tubes and frozen in liquid nitrogen. Samples were thawed and subjected to pH and SCFA analysis. pH was assayed using a Mettler Toledo SevenEasy pH meter (Model: S20), and SCFA concentrations using gas-liquid chromatography.

Statistical Analysis – Data were analyzed as a one-way ANOVA followed by LSD post-hoc mean comparison tests using the MIXED procedure of SAS® Version 9.3 (SAS Institute, Inc., Car, N.C.). The UNIVARIATE procedure was used in the MIXED procedure to assure equal variance, normal distribution, and to identify outliers. Data were transformed by log if the normality assumption was not met. Any observations that were more than three standard deviations away from the mean were considered outliers. Three outliers were discovered and subsequently removed for HOMA-IR: two for HM260 (1.29 and 7.20) and for one for HMWG (5.12). Results were considered significant at $p<0.05$. Values are expressed as means $\pm$ SE.
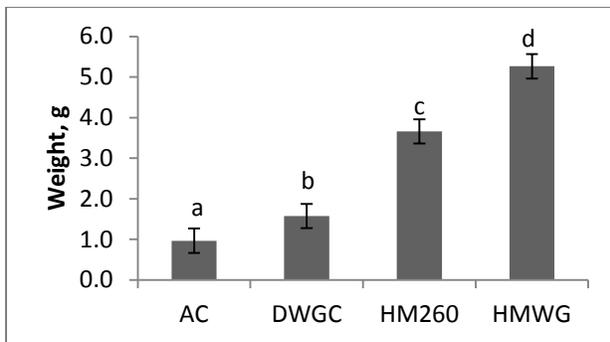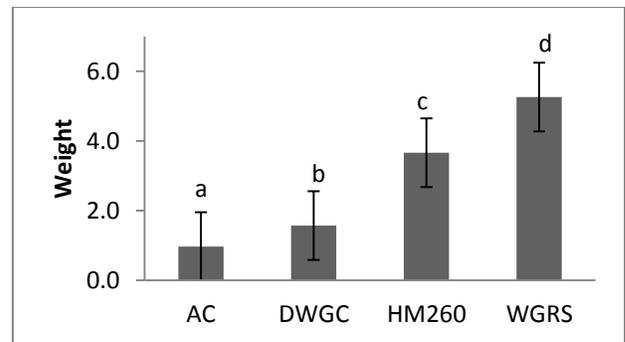
Results

Fig 24. Empty cecum weights.

Fig 25. Cecal content weights.

Empty cecum and cecal content weights (Fig. 24 and 25, respectively) were significantly different among all groups (p<0.0001), and increased with increased RS and whole-grain contents. This demonstrated that ZDF rats are capable of RS fermentation and that the presence of whole-grain (HMWG vs. HM260 and AC vs. DWGC) resulted in a more robust fermentation response than for treatments with no whole-grain. RS-containing groups (DWGC, HM260, and HMWG) had significantly reduced cecal pH values compared to the non-RS control (AC) (Fig 26). HMWG was significantly greater than DWGC, but not HM260.

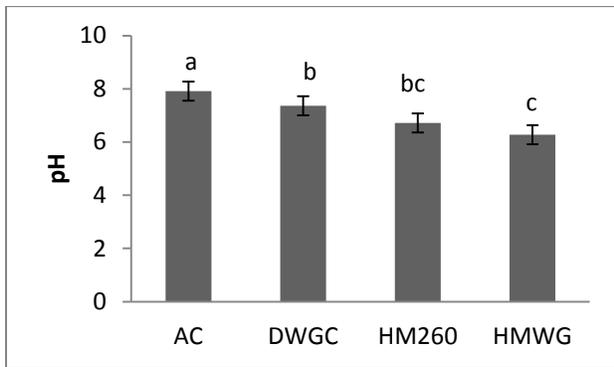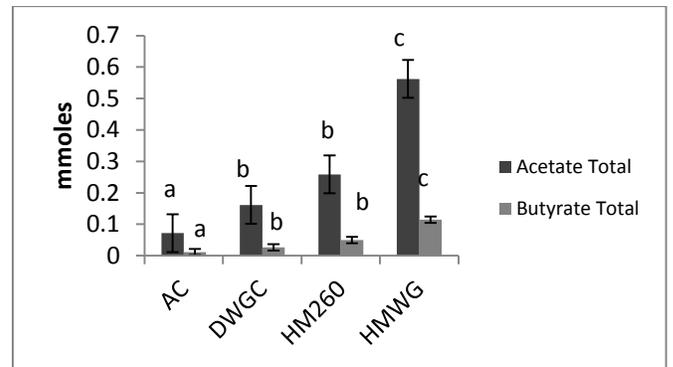Fig 26. Cecal pH values.

Fig 27. SCFA in cecal contents.

Differences in total SCFAs were documented for acetate and butyrate, but not propionate (Fig 27). For both acetate and butyrate, RS consumption resulted in significantly increased values compared to AC, with the HMWG group producing significantly more than DWGC or HM260.
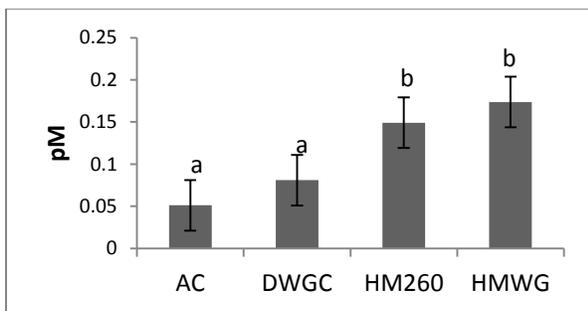
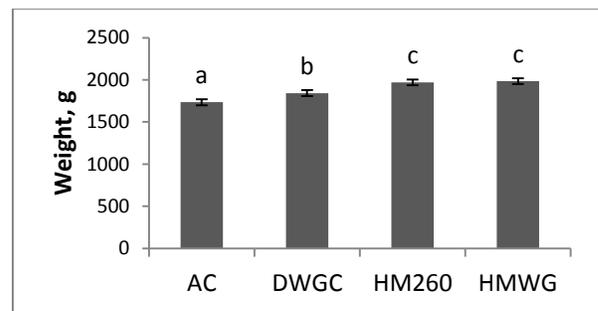Fig 28. GLP-1 active concentrations.

Fig 29. Total food intake values.
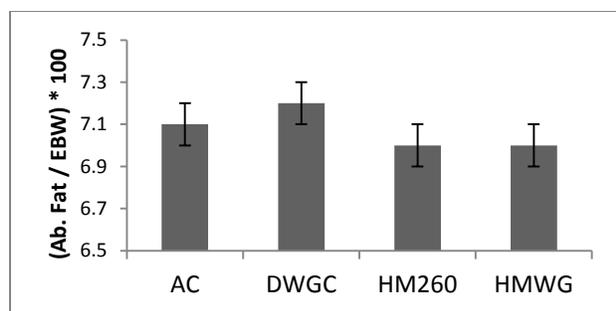
Fig 30. Percent abdominal fat.
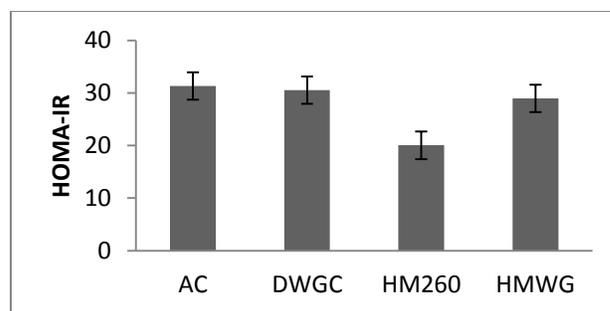
Fig 31. HOMA-IR values.

GLP-1 active was significantly greater in groups supplemented with the higher amounts of RS only (p<0.01), but HM260 and HMWG were not significantly different from one another (p=0.2048) (Fig. 28). All three RS-containing groups consumed more food compared to the non-RS control (p<0.03) (Fig 29). Animals supplemented with either HM260 or HMWG consumed significantly more than the WG-control group, DWGC (DWGC versus HM260 p=0.0138; DWGC versus HMWG p=0.0072), but not more than each other (p=0.7578). Despite differences in food intake and GLP-1 status, no differences in percent abdominal fat (p=0.4981) (Fig. 30) or HOMA-IR (p=0.08) were documented (Fig 31).

Discussion

Fermentation was demonstrated in groups whose diets were supplemented with carbohydrate sources containing dietary RS, with significantly more being documented in groups with diets containing WG, specifically. Groups whose dietary carbohydrate contained RS, regardless of type, responded with increased cecal content weights (p<0.0001), decreased pH of cecal contents (p<0.0001), increased empty cecum weights, increased SCFA produced, and increased GLP-1 active secreted. These results indicated that ZDF rats possess microbiota capable of fermenting dietary RS and other possible fermentable fibers in WG flours.

Animals exhibited a more consistent and, thus, more robust fermentation response to HMWG than the HM260 variety. There was a great deal of variation in fermentation response

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for HM260 compared to HMWG: 5 out of 12 HM260 rats appeared to not ferment the RS as indicated by cecal contents pH above 7 compared to 1 out of 11 rats for HMWG. This suggests that there are additional factors affecting fermentation capacity, which we are not controlling for in this study.

Despite robust levels of fermentation, no metabolic effects, i.e. no reductions in percent body fat or HOMA-IR, were statistically evident. This suggests that ZDF genotype, with its strong propensity towards obesity, insulin resistance, and type 2 DM, is more powerful than the fermentative effects produced by either source of RS, whole–grain or no whole-grain. The ZDF rat is a type of knockout for leptin signaling because it has a defective leptin receptor. The results from this study demonstrate that a functioning leptin receptor and leptin signaling is required to convert the robust fermentation effects into beneficial phenotypic changes. These beneficial changes include a healthier large intestine (Keenan et al. 2012), a healthier microbiota (Shen et al. 2011, Tachon et al. 2012; Keenan et al. 2013), and improved insulin sensitivity and pancreatic mass in a lean model of type 2 DM (Shen et al. 2011). Previous research results that indicated that a response to leptin was necessary for beneficial phenotypic changes to occur were the increased energy expenditure and increased fat burning (Zhou et al. 2009) and increased POMC gene expression in the arcuate nucleus of the hypothalamus (Shen et al. 2009).

Dent corn is stereotypical of corn products found in today's food supply, and possesses a high amylopectin:amylose ratio. Therefore, dent WG corn flour likely has some RS1 in its WG component plus a small amount of RS2 with the amylose in its starch component. Amioca® starch is produced from the isolated starch fraction of waxy corn flour. Recently, the Ingredion Company analyzed the RS content in the major dietary components in a later RS study that included the whole grain flour product from waxy corn,WG Amioca® flour, again using the

modified Englyst method (Englyst 1996). The percent of RS in the WG.Amioca® in the carbohydrate fraction (proximate analysis) was 13% compared to 14% for the whole-grain Dent corn. Since Amioca® starch is 100% amylopectin, the amount of RS in WGAmioca® is likely all RS1. HM260 starch contains only RS1 as it is a high-amylose isolated starch. The results, therefore, indicate that the presence of RS1 and other fermentable fibers in the WG flours (e.g. arabinoxylans) (Lu et al. 2000, Maki et al. 2012), in addition to RS2 in DWGC and HMWG, are responsible for the increased fermentation in the ZDF rats compared to non-WG counterparts, i.e. AC and HM260, respectively.

These results are further evidence that inclusion of WG products are likely to have increased health benefits compared to diets with no or low amounts of WG products. This would likely include improved gut health and health-positive phenotypic changes in individuals without strong genetic tendencies for chronic diseases. The current study demonstrated that the strong genetics toward obesity, insulin resistance, and type 2 DM likely driven predominately by a defective leptin receptor, could not be overcome despite a very robust fermentation produced by the HMWG product.

CHAPTER 5
CONCLUSION

While obesity does not produce the swift and highly visible devastation that tuberculosis, polio, and other communicable diseases do, its increasing prevalence and associated co-morbidities, i.e. metabolic syndrome, constitute a severe public health crisis. The defining characteristics of metabolic syndrome are obesity, dyslipidemia, hypertension, poor glycemic control, and several types of cancer (Galisteo, 2008). The pathophysiology of metabolic syndrome and the exact relationships between its components are obscured by its complex etiology, making effective, long-term treatment extraordinarily difficult. Recently, DFs have garnered significant attention due to their beneficial effects on body weight, food intake, glucose homeostasis, and insulin sensitivity (Marlett, 2002; Delzenne & Cani 2005). In fact, populations that report higher fiber consumption also exhibit lower rates of obesity in both adults and children (Kimm, 1995; Galisteo, 2008). Epidemiological studies reveal inverse associations between fiber and body weight (Alfieri et al., 1995; Appleby et al., 1998) as well as body fat (Nelson and Tucker, 1996). Reduced risk for cardiovascular disease, diabetes, and colon cancer have also been documented (Galisteo, 2008).

SCFAs, the end products of fermentation of DFs by the anaerobic intestinal microbiota, have been shown to exert multiple beneficial effects on mammalian energy metabolism and overall health. Colonic fermentation of the unique DF known as RS is associated with a number of positive health effects including: increased cecal SCFA concentrations leading to reduced cecal pH; increased circulating levels of GLP-1; reduced FI, body weight, and adiposity; and improved insulin sensitivity and glucose homeostasis. In addition, use of RS as a substrate for microbial metabolism alters the composition of the gut microbiota, supporting the growth of

beneficial bacteria, which may prevent colonization of the GI tract by potential pathogens and reduce the risk of inflammatory bowel diseases, among other things.

Previous studies have showed that the macronutrient content of the diet affects the strength of colonic fermentation and/or effectiveness of RS supplementation as a treatment for obesity- and diabetes-related health outcomes: That is, high levels of dietary fat reduce the intensity of RS fermentation in the colon and subsequent benefits to overall health. Our lab and others have described the influence that the amount as well as type of fat in the diet has on colonic fermentation and its associated outcomes, too. In addition, we discovered that certain genetic models of obesity and diabetes successfully ferment RS, but fail to exhibit any discernible, beneficial physiological response. Whether or not this is true for all types of RS or just HM260 WG and non-WG is unknown. Knowing if an individual is capable of responding to and deriving benefit from a particular functional food product like RS is important and necessary information when attempting to prescribe the most efficient treatment.

The studies included in this dissertation attempted to address these phenomena by determining: (a) whether or not RS fermentation effects could be rescued during concurrent administration of a HF diet; (b) the mechanistic importance of the GLP-1 hormone on RS fermentation effects; and (c) whether or not ZDF rats were capable of responding to RS supplementation, that also included utilization of a novel product consisting of whole-grain RS. Our hypotheses were that (1) RS plus the anti-diabetes drug SG on a HF diet would be sufficient to raise GLP-1 concentrations in the bloodstream to sufficiently induce body fat loss and improve insulin sensitivity; (2) intact GLP-1 receptor signaling was both necessary and sufficient to induce positive changes in percent body fat and insulin sensitivity; (3) the microbiota present within the ZDF rat would be capable of fermenting RS, but the lack of a functional leptin

receptor may prevent the production of a leaner, more insulin sensitive phenotype ; and (4) the whole-grain variety of RS would be more highly fermented than regular RS, because it is a source of both RS1 and RS2 and other fermentable fibers, while the latter is composed of RS2.

Fermentation of HM260 occurred on the HF diet but was diminished compared to results from previous studies using LF and medium-fat diets. This likely explains why we failed to see an HM260 effect for circulating GLP-1 concentrations and, subsequently, insulin sensitivity in study one. We were surprised by SG's effect on body weight and adiposity: DPP-4 inhibitors have not previously been associated with weight loss. But in the studies described here we saw significant reductions in body weight and adiposity with SG administration; effects that were enhanced by concomitant administration of HM260 at 28% of the weight of the diet. It was these results that encouraged us to pursue the second mechanistic study with GLP-1 receptor KO mice.

We observed that a combination of SG with 28% HM260 was able to raise GLP-1 concentrations in the blood stream, attenuate weight gain, and reduce fasting BG in a mouse model of type 2 DM. This combination enhanced EE in the mice, which may represent a part of the mechanism of the anti-obesity and anti-diabetes effect commonly, but inconsistently, associated with both HM260 and SG. The therapeutic effects are dependent on GLP-1 receptor; as GLP-1 receptor KO animals were capable of responding to the combinatory treatment with increased GLP-1 production, but they were incapable of manifesting the same beneficial health effects seen in the WT strain. Our data shed some light on the potential role of the GLP-1 receptor system in induction of EE and regulation of circulating GLP-1 levels.

The exploratory experiment into the fermentative capacity of the ZDF rat provided two important insights. First, that the influence the microbiota has on host phenotype is limited; host genotype trumps its microbial fermentation, at least in this particular animal model and with this

particular prebiotic compound. In particular, this animal model's genetic resistance to positive effects that are normally associated with fermentation of RS is likely dominated by it possession of a defective leptin receptor. Secondly, that a combination of multiple types of RS and, ostensibly, multiple types of DF overall, is more effective than a single one. This realization may warrant revisiting our HF diet experiments – this time with HMWG instead of HM260 – to see if (a) the fermentation response is still reduced on a HF diet and/or (b) whether or not the amount of fermentation is enough to generate synergy with SG. Additionally, it may be warranted to repeat the investigation of the SG and HM260 with a low- or moderate-fat diet. With a LF diet, lower dietary doses of HM260 may be effective in reducing body fat/weight and increasing GLP-1 active and synergistic effects of HM260 with SG would be observed.

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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, and completed her Bachelor's degree in cellular and molecular biology at Tulane University, New Orleans, Louisiana in 2008. She loved the Pelican State so much that she decided to attend graduate school at Louisiana State University Agricultural and Mechanical College, where she completed her Master's in human ecology with a concentration in molecular nutrition in 2010. Her thesis dealt with the effects of a high-fat diet on the fermentation of various dietary fibers in the rodent colon, a line of research that she continued to pursue for her Doctorate in the same. Felicia's work in dietary fibers and the intestinal microbiota has fueled a fascination with the prebiotic potential of human milk oligosaccharides, and the effect that maternal diet has on the quantity and quality of breast milk. After graduation, Felicia plans to accept a position as a post-doctoral researcher at the Foods for Health Institute located at the University of California, Davis.