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Resistant starch and sodium butyrate reduce body fat in rodents

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RESISTANT STARCH AND SODIUM BUTYRATE REDUCE BODY FAT IN RODENTS

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Abstract

Introduction: Obesity levels in the United States have significantly increased in the last forty years. Lifestyle and pharmacological treatments have been largely ineffective in treating obesity for most people. Both Resistant Starch (RS) and Dietary Sodium Butyrate (SB) are bioactivties which have shown the ability to decrease body fat levels of rodents without increasing physical activity or decreasing energy intake. Glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) are gut hormones that may be involved in increased energy expenditure at a cellular level with dietary RS and SB. **Objective:** To discern if SB and RS both work through the increase of plasma GLP-1 and PYY. Also to see if a combination of RS and SB would lead to an increased or even an additive effect on the reduction of body fat levels in rodents. **Methods:** 60 Sprague Dawley rats were fed isocaloric diets of either control, SB, RS or a combination of RS and SB for 60 days. Measurements included food intake, body weight, abdominal fat, plasma PYY and GLP-1, and gastrointestinal tract weights. **Results:** There was no difference in caloric consumptions between any groups. According to factorial results, SB and RS both lowered abdominal fat. While the combination of RS and SB showed the lowest levels of abdominal body fat levels by t tests compared to control, there was not an additive effect of SB and RS. GLP-1 and PYY levels were not increased in the SB fed group. **Conclusions:** SB effects on body fat reduction are not associated with increased plasma GLP-1 and PYY levels as found in RS fed rodents. The combination of SB and RS have a greater effect on body fat than either alone, but the lack of an additive effect suggests a saturation level in a cellular mechanism by which both RS and SB may increase energy expenditure.

Chapter 1

Introduction

Obesity is a growing disease state in western cultures. The incidence of obesity has risen in the United States from 13.4% of the population to 30.9% since 1960 (Bravata et al 2003). Obesity has a high correlation to high mortality disease states such as cardiovascular disease, hypertension, type 2 diabetes, cancer and stroke (Bravata et al 2003). Abdominal obesity in particular is strongly linked to cardiovascular disease and stroke and is considered a major risk factor for both. The obesity disease state is attributed primarily to an imbalance in energy metabolism which leads to an excessive accumulation of body fat by an individual. When an individual consumes more kilocalories than they use in biological metabolic functions the remaining energy is then transformed into fatty acids and stored in adiopocytes leading to an increase in body fat levels. This increase in body fat is only of medical concern once it has grown past levels which have been linked to increase incidences of disease states. Obesity is defined through multiple methods concerning body weight, height and fat levels (Hubbard 2000). The most commonly used method is the Body Mass Index (BMI) method, which defines obesity through the measurement of both weight and height. Using the BMI method obesity is defined as having a score of 30 or larger on the BMI scale (Hubbard 2000).

Due to the well-documented theory of energy metabolism and its effects on weight gain and loss in humans, many "lifestyle" methods of treating obesity have been used. These treatments involve either increasing energy expenditure in the patient or decreasing energy consumption or both. Unfortunately to date these methods have been largely unsuccessful in effectively eliminating obesity from most of the patients which undergo treatments and tend to have high dropout rates (Gao et al 2006, Klem et al 1997). This is most likely due to physical and mental discomforts of hunger and physical exertion which patients undergo when applying these

treatments (Cefalu et al 2008). One exception to these findings is gastric by-pass surgery, in which patients undergo a major surgery which severely reduces the size of the stomach and eliminates a portion of the small intestines from involvement in digestion (Gao et al 2006). This surgery leads to a severe reduction of food and kilocalorie intake by the patients because severe biological reactions (vomiting, dumping syndrome) occur when patients consume moderate to large volumes of food after recovery from surgery (Gao et al 2006). This reduced food intake state leads to sustained large reductions of both body weight and body fat levels in patients (Gao et al 2006). Recent studies have given evidence that some food boiactives can reduce body fat levels in rodents without an increase in physical activity or a decrease in caloric consumption (Zhou et al 2007). Specifically, both dietary sodium butyrate (SB) and dietary resistant starch (RS) have been shown to decrease body fat levels without an increase in energy expenditure or a decrease in energy intake (Zhou et al 2007).

Resistant Starch is starch which resists digestion in the small intestines and reaches the large intestines intact (Keenan et al 2006). Once in the large intestines, resistant starch is then metabolized and fermented by the intestinal microflora. This fermentation process seems to be an intergal part of the mechanism by which RS reduces body fat levels in rodents. Recent studies have found that when RS is fed to rodents and fermentation does not occur, body fat levels do not change compared to controls. The byproducts of RS metabolism in the large intestine are the increase of short chain fatty acids (SCFA). One very important SCFA created is butyrate. This SCFA provides energy for the clonocytes of the large intestine.

Resistant starch intake in rodents has also been found to consistently increase the gut hormones glucagon like peptide 1 (GLP-1) and peptide YY (PYY) (Keenan et al 2006). Primary cell

culture of cecal cells from control rats demonstrated production of PYY and GLP-1 in response to butyrate in the culture media. This finding suggests that butyrate produced by RS fermentation in the large intestines may be a primary method of GLP-1 and PYY production caused by RS intake. These hormones may be the mechanism by which RS starch increases fat oxidation without increases in physical activity. One recent study found that when C57L/6J (wild type) mice were fed a RS diet they had reduced body fat levels when compared to control mice (see figure 1). When the same treatment was given to GLP-1 receptor knockout mice the body fat, reductions were no longer observed. This study gives strong evidence that the action of the GLP-1 hormone plays an integral part in the mechanism of body fat reduction found in RS fed animals. This action may be direct upon peripheral tissues; muscle, liver and adipose (Ding et al); or by signaling through the brain by involving an increase in proopiomelnocortin (POMC) (Shen et al. 2009).

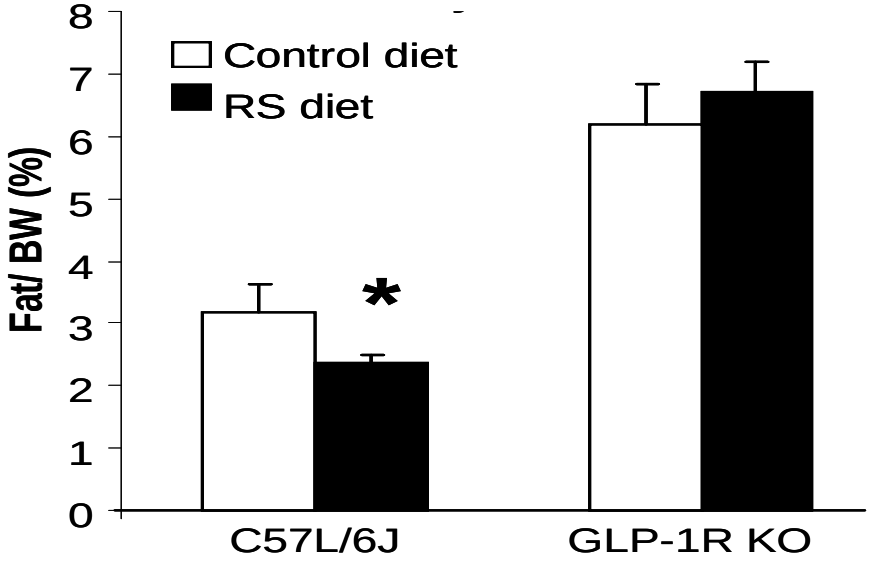

Figure 1 - GLP-1 Receptor Knock Out Mice Body Fat

Dietary sodium butyrate is a four carbon short chain fatty acid found primarily in dairy products such as cheese. Dietary sodium butyrate in absorbed in the small intestines and does not reach the large intestines and therefore there is no increase in butyrate levels found in the large intestines of sodium butyrate fed animals compared to control groups. In a recent study, Gau et al in 2009 found that mice fed dietary sodium butyrate showed decreases in body weight and body fat levels compared to control groups. These decreases in bodyweight and body fat were associated with increases in fat oxidation, energy expenditure, muscle tissue and food intake, but they were not associated with increases in physical activity. As with RS, dietary sodium butyrate has been shown to decrease body fat levels without a decrease in food intake or an increase in physical activity.

The similarities in physical changes found in animals fed either RS or SB has led to the question of whether they share a similar metabolic increase of energy expenditure and fat oxidation. At this juncture we have strong evidence linking the RS fermentation and increase gut hormone status found by RS supplementation to decreased body fat levels found in RS fed rodents. Additionally, in vitro evidence that butyrate fed cecal cells increase the output of the gut hormones GLP-1 and PYY, and that butyrate levels are increased in the large intestines of RS fed animals when fermentation is present. This leads us to the hypothesis that RS fermentation increases butyrate production in the lumen of the large intestines, which in turn increases the gut hormone levels of GLP-1 and PYY which are primary drivers of the increased fat oxidization and energy expenditure mechanism found in RS fed rodents. While dietary sodium butyrate is absorbed in the small intestines before reaching the large intestines it is possible that dietary

sodium butyrate circulates in the systemic blood and is taken up by L cells in the digestive tract and is also increasing the gut hormones GLP-1 and PYY. If both RS and SB are reducing body fat levels in rodents through the activation of GLP-1 and PYY then it may be possible through a combination of both in the diet to have an additive increase in body fat reductions leading to further decreased levels of body fat without decreases in food intake or increases in physical activity.

Chapter 2

Review of the Literature

Obesity. Obesity is defined as having a body mass index of >30, has increased in the United States from 13.4% to 30.9% since 1960.(Bravata et al 2003) There is an estimated 325,000 deaths each year that are attributed to obesity. These deaths account for direct health care cost of $39-52 billon dollars a year, which is 3%-5% of the annual health care cost (Bravata et al 2003). This increase in obesity is seen across population groups in all states within the United States of America. Increases have been shown in male, female, child, adolescent, in all races and across all education levels. Through the years of 1991 to 1998 the populations with the highest increases in obesity rates were, 18- to 29-year-olds (7.1% to 12.1%), those with some college education (10.6% to 17.8%), and those of Hispanic ethnicity (11.6% to 20.8%) (Mokdad et al 1999).

The growing prevalence and lack of effective treatments has caused obesity to become a major health concern to western societies and has been named a public health priority (Mokdad et al 1999). This concern is amplified by obesity's links to major morbidity disease states such as cardiovascular disease, hypertension, diabetes and cancer (Mokdad et al 1999).

The only long term effective treatment for obesity to date has been bariatric surgery (Sjostrom et al 2007). At this time bariatric surgery is only available for the severely obese population and has significant cost and risk for the patient undergoing the procedure both during and post operation. Pharmaceutical drugs and lifestyle treatments (Hill et al 2008) have also been unsuccessful to date in the treatment of the majority of the obese population. Lifestyle treatments usually require either a reduction of energy intake, increases in energy expenditure or both simultaneously (Klem et al 1997). Such methods of behavior change require strong mental and/or physical input from patients and have usually been found to be too difficult for most patients to maintain in the long term.

Resistant Starch. Starch is a naturally abundant nutrient, a type of carbohydrate, $(C_6H_{10}O_5)_n$, found chiefly in the seeds, fruits, tubers, roots, and stem pith of plants, notably in corn, potatoes, wheat, and rice. Starch is the polysaccharide storage form of glucose by plants (Nugent 2005). Most starches are hydrolyzed by the enzymes alpha-amylases, glucoamylase, and sucrose-isomaltase in the small intestines to create free glucose to meet immediate energy needs or for storage as glycogen or fatty acids to support future energy needs (Nugent 2005). Resistant starch however, is not hydrolized by enzymes in the small intestine and proceeds to the large intestine where it is fermented by colonic microflora. The major products of fermentation are short chain fatty acids (SCFAs). While many fatty acids are produced from the fermentation of RS, the primary resulting SCFAs are butyrate, acetate, and propionate. Other byproducts of RS fermentation are carbon dioxide, methane, hydrogen and organic acids (Nugent 2005).

Resistant starch intake is likely to be inversely proportional to obesity rates. Intake of RS has declined in diets with the introduction of modern processing techniques. Resistant starch is found primarily in bananas, whole grains, legumes and cooked then cooled potatoes. Medieval European diets contained 50-100 grams/day/person of resistant starch (Dyssler 2006). People in modern day developing countries average 30-40 g/day/person and developed countries average 3-8 g/day.

Four types of Resistant Starch exist: RS1, RS2, RS3, and RS4. RS1 is physically inaccessible resistant starch, such as that found in seeds or legumes and unprocessed whole grains. The starch form is inaccessible due to the protective nature of the amyloblasts within the plant which retards enzymatic degradation. RS2 is resistant starch that occurs in its natural granular form, such as uncooked potato, green banana flour and high amylose corn. The granular form is tightly packed in a radial pattern which protects the starch from enzymatic activity. RS3 is a resistant

starch that is formed when starch-containing foods are cooked and cooled such as in bread, cornflakes and cooked-and-chilled potatoes or retrograded high amylose corn. It also happens to be the most resistant form of RS by digestive enzymes. RS4 are Resistant Starches that have been chemically modified by the formation of chemical bonds other than the alpha-D-(1-4) and alpha-D-(1-6) linkages found in common starch granules (Jacobasch et al 1999). The change in chemical bonds increases starch resistance to enzymatic digestion. This type of resistant starch can have a wide variety of structures that are not found in nature.

Resistant Starch has been labeled as a functional food, due to the health benefits that have been found by the consumption of RS in the diet. Unlike normal forms of starch, RS resists digestion in the small intestine. This reduction in starch digestion caused by RS induces a lower glycemic response, lower postprandial insulinemia and possible increased satiety effects (Jenkins et al 1987, Raben et al 1994). As previously stated, 30% to 70% of RS is fermented by microbial flora in the large intestines, which leads to increased SCFA production (Bjorck et al 1987, Goni et al 1996). The fermentation process of RS leads to multiple intestine health benefits including: increasing crypt cell production rate, decreasing large intestinal cell atrophy, increasing fecal weight and output, reducing fecal and cecal pH (Vidrine 2009 unpublished), decreasing ammonia levels, improvement in immune response during inflammatory bowel disease states, increased levels of beneficial bacteria and decreased levels of pathological bacteria in the large intestines (Sjostrom et al 2007).

Resistant starch may play a role in the reduction of body fat levels. Low intakes of RS have been shown to have a satiety effect (cite human studies and animal studies if available), larger doses (>25%) have shown similar or increased dietary consumption to control groups. This may be due to a decreased sensitivity to PYY, a known regulator of satiety, which has been shown to

reduce food intake. Multiple studies have confirmed increased levels of PYY with RS intake, but not reduced food intake with the elevated PYY levels. The elevation of PYY levels found associated with RS diets may cause a resistance to the effects of PYY on food intake. (Reidelberger et al Reidelberger 2008)

Although food and nutrient absorption is not reduced in RS supplemented diets, RS diets have been found to inhibit fat gain (Vidrine 2009 unpublished). Rodent studies have shown increased energy expenditure and food intake with RS supplementation, where the experimental rodents showed lower body fat levels and increased fat oxidation when compared to control diets (Zhou et al 2009).

It has been theorized that the reduced kcal energy of RS compared to starch could be the mechanism for reduced body weight and fat in previous studies. However, this was not found in recent work (Vidrine 2009 unpublished) where energy intake was controlled and RS groups showed lower body fat levels than control groups. An alternate theory suggesting that the SCFA fermentation products of RS may be responsible for the reduction of body fat levels in RS supplemented diet. The fermentation of RS in the cecum creates SCFAs, particularly sodium butyrate. Fermented sodium butyrate is associated with increased GLP-1 transcription which may induce mitochondrial function in liver, muscle and adipose cells and effectively increase energy expenditure and reduce body fat stores (Ding et al 2006). The effects of RS fermented SCFAs on body fat are of great interest regarding obesity treatment and weight management.

Sodium Butyrate. Sodium butyrate (SB) is the salt form of butyric acid. Butyric acid is a fatty acid occurring in the form of esters in animal fats and plant oils. It is a member of the fatty acid sub-group called short chain fatty acids (SCFA). In the body SB is a lipid product from B-oxidation of long chain fatty acids. Dietary SB is absorbed by the upper GI tract in the body.

Sodium butyrate may also be produced as a byproduct of starches which bypass digestion in the small intestines and are then fermented by micro flora in the large intestines. (Pryde et al 2002, Roy et al 2007)

Supplementation of sodium butyrate in mouse diets showed an anti-obesity effect (Gao et al 2009). Mice treated with a sodium butyrate supplemented diet gained less body weight and less body fat while retaining more muscle mass than the control diet mice. This occurred even though food intake in the experimental group was increased above the control diet.

The same group found that energy expenditure and fatty acid oxidation was increased in the sodium butyrate diet, although physical activity was not increased. This suggests that sodium butyrate regulates a molecular mechanism which increases resting metabolic rate and promotes the oxidation of fatty acids. This theory is supported by a study our lab performed which used indirect calorimetry to monitor energy expenditure (Zhou et al. 2009). In this study mice fed an SB supplemented diet did not become obese even though food intake was increased and fat absorption was not reduced. This group found that both oxygen consumption and carbon dioxide production were increased SB supplementation.

Sodium butyrate affects the mitochondrial function of cells throughout the body. Sodium Butyrate induces peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha) which increases the metabolic activity of mitochondria and effectively increases energy expenditure. This process may be dependent on the balance between histone deacetylase (HDAC), a known suppressor or PGC-1alpha transcription and SIRTUIN 1, a NAD dependent histone deacetylase (SIRT1) and AMP-activated protein kinase (AMPK), both of which have been shown in enhance PGC-1 alpha activation in the cell.

Sodium butyrate has been shown to have other health benefits aside from reducing body fat. SB is a preferred fuel source by colonocytes even when glucose is available (MacFarlane et al 2003). The production of SCFAs has been shown in repeated studies to reduce inflammation, increase blood flow to the colon, lower luminal pH, regulate the coloncytes' gene expression, induce apoptosis (Mentschel 2003) and improve favorable microbial growth while decreasing pathogenic microbial growth in the large intestines.

Glucagon Like Peptide – 1. Glucagon like peptide - 1 (GLP-1) is a hormone which is expressed by the glucagon gene from the pancreas and also the endocrine cells of the intestinal mucosa. GLP-1 functions as an incretin hormone by enhancing insulin secretion stimulated by oral ingestion of nutrients. GLP-1 relationship with insulin makes it a prime candidate in the treatment of insulin resistant disease states such as type 2 diabetes. GLP-1 is also believed to play a role as an "ileal brake mechanism" which when activated by the presence of nutrients in the ileal lumen serves to inhibit gastric motility and secretion. GLP-1 intolerance effects were shown in a study which found the "mice, homozygous for a targeted deletion of the GLP-1 gene, gained weight at a normally but were glucose intolerant." These findings point to the importance of GLP-1 on insulin's effects on insulin secretion and the GLP-1's possible function in the treatment of the hyperglycemia of non-insulin-dependent diabetes mellitus (NIDDM).

Previous research has shown that while the majority of GLP-1 is secreted by the ileum and pancreas, changes in the amount of GLP-1 secreted by the L-cells of the large intestines is enhanced by fermentation of dietary fibers by the microflora found in the large intestines. Our lab has confirmed that GLP-1 levels which are created by the fermentation of dietary fibers in the cecum are the only changes in GLP-1 levels which differ from control fed rodents (Keenan et al 2006). Our lab has also found that fermentation is necessary for the effects of Resistant Starch

(RS) to reduce epididymal body fat levels to occur. Furthermore, our lab has published recent evidence of GLP-1 importance in the function of RS to reduce epididymal body fat levels. In our previous research, C57K/6J "wild type" mice and GLP-1 knockout mice were treated with a RS diet (Zhou 2009 unpublished). In this study, unlike our previous findings, there were no differences in epedidymal body fat levels between either group. This suggests that GLP-1 plays an integral role in the effects of RS to affect epididymal body fat levels.

Resistant starch (RS) induces GLP-1 expression (Zhou 2009 unpublished). This may be dependent on the fermentation products of RS which include sodium butyrate (SB). Sodium butyrate induces GLP-1 transcription through HDAC inhibition in the L-cells of the large intestines. GLP-1 was found in recent published work to increase cAMP levels in hepatic cells (Ding et al 2005). While there is no evidence currently, which reports GLP-1 as a regulator of PGC-1 α, a transcriptional coactivator which is known to increase energy expenditure in the cell (Lin 2006); it is well known that cAMP induces PGC -1α transcription through the PKA-CREB pathway. It may be that GLP-1 effects on epididymal body fat reduction are due to increases in cellular expenditure caused by increases of PGC -1α which are induced by increase cAMP levels caused by increased GLP-1 expression by the L-cells of the cecum.

Peptide YY. Peptide YY (PYY) is a polypeptide gut hormone produced by the L-cells of the gastrointestinal tract(le Rou et al 2006) PYY is present throughout the intestinal tract with its highest concentrations coming from the distal segments (Young et al2006)PYY is synthesized and released from specialized endocrine cells (L cells) that are found primarily in the distal gastrointestinal tract (Batterham et al. 2003). Levels of PYY increase within 15 minutes after feeding. These levels peak at 60 minutes and may remain elevated for up to 6 hours (Batterham et al. 2003). PYY plasma levels are increased postprandial in proportion with meal size for both

overweight and lean subjects, but the initial PYY levels and level of PYY increase is proportionately smaller in overweight individuals even when overweight individuals consume higher caloric intakes (le Rou et al 2006). Elevated fasting levels of PYY have been shown in gastrointestinal diseases associated with loss of appetite (Batterham et al. 2003). Furthermore, PYY infusion reduces food intake in subjects of normal weight, and repeated administration to rodents reduces weight gain (le Rou et al 2006). More recent research has described obesity as a PYY deficiency (Young et al 2006). These findings suggest that PYY may be a useful treatment for reducing energy intake and possibly combating obesity (Young et al 2006).

Resistant starch increases PYY levels in rodents (Zhou et al 2008). Interestingly, energy intake is unaffected by this increase (see figure 2) in PYY levels (Zhou et al 2008). It may be that because PYY levels are elevated continuously over a 24 hour period, unlike the relatively rapid rise and fall postprandial, that a reduction of PYY sensitivity by cells negates PYY effects on huger levels and food consumption. This is supported by the research of Reidelberger et al. (2008) demonstrating increased food intake with repeated injections of PYY (Reidelberger et al 2008).

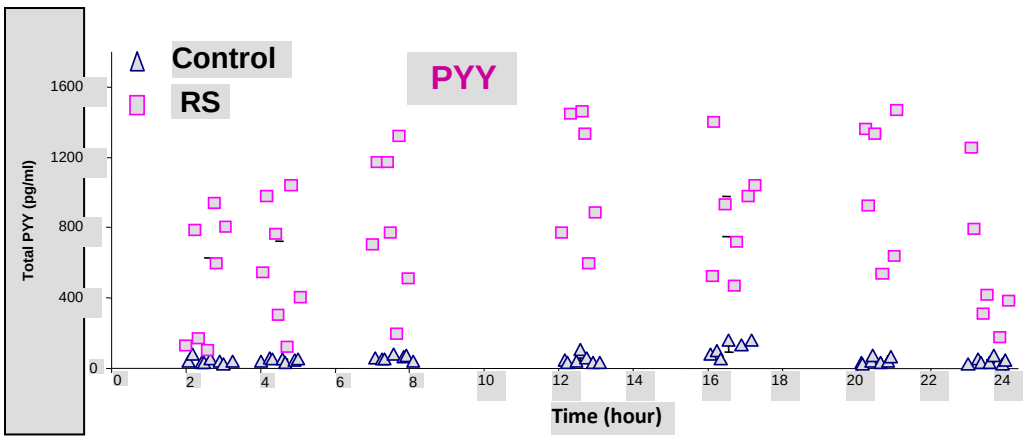

Figure 2 - PYY levels of Control and RS fed Rodents over a 24 hour period

Chapter 3

Methods and Results

Introduction. Obesity has been associated with high mortality disease states such as cardiovascular disease, hypertension, diabetes and stroke. Over the last forty years the obesity rate in the U. S. has increased from 13.4% to 30.9% (Bravata et al 2003)Difficulties of lifestyle treatments have led to the consideration of novel methods of obesity treatment. Resistant starch (RS), a fermentable dietary fiber found in carbohydrate foods such as raw potatoes (Murphy et al 2008), and dietary sodium butyrate (B), a short chain fatty acid found in dairy products (Pryde et al 2002), have both shown in previous research the ability to increase energy expenditure without increasing physical activity (Keenan et al 2006,). RS and SB in these studies also decreased body fat levels without decreasing energy intake (Keenan et al 2006).

Resistant starch's effects on reducing body fat levels have been associated with the fermentation products created by microbial digestion of RS, one of which is butyrate. In vitro evidence found that L-cells incubated with butyrate in the media increased production of the gut hormones GLP-1 and PYY (Zhou et al 2008). These hormones are associated with both the increased fermentation levels and decreased body fat levels associated with RS consumption (Keenan et al 2006). A GLP-1 knockout mouse study found that GLP-1 is necessary for RS effects on body fat levels to be present (Zhou 2009 Unpublished). In the previous study with dietary sodium butyrate, PYY and GLP-1 were not measured (Gao et al 2009). It is possible that dietary sodium butyrate could also increase PYY and GLP-1 by a different mechanism than for RS. Brubaker and Anini (2003) review the direct and indirect mechanisms for GLP-1 release by L endocrine cells. The indirect effect employs the nervous system that is stimulated by the presence of nutrients in the small intestine. The direct effect occurs for those substances that reach the later parts of the small intestine and large intestine and come into contact with L endocrine cells. Dietary sodium butyrate may increase PYY and GLP-1 through the indirect mechanism or

possibly by reaching the L endocrine cells in the systemic blood after absorption from the stomach and small intestine. The current study was done to 1) determine if dietary sodium butyrate is also associated with increased levels of GLP-1 and PYY, 2).ascertain if sodium butyrate and RS in combination in the diet are more effective than either compound alone.

Materials and Methods. All animals were housed in a humidity and temperature controlled (22.2°C, 65–67% humidity) on a 12:12-h light-dark cycle with free access to food and water. Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were stratified by weight, growth potential and age and were individually housed in suspended wire-bottom cages with numbered paper plates under the food jar in order to account for spillage. The powdered diet was placed in metal food jars with a plastic food cover by the experimenter, and secured by a wire spring that attached to the wire mesh front of the cage. All animals were fed semipurified powder diets prepared in our laboratory. The control (C), RS, B and BR diets were based on the AIN-93G diet formula for laboratory rodents (Reeves 1993), and all diets were isocaloric (diet table figure 3). Water was available via a water nozzle at the rear of the cage that released water when it was pushed on. Animal protocols were approved by the Louisiana State University Animal Care and Use Committee.

The purpose of this study was to determine the effects on body composition change through the combination of RS and B in diets. Sixty adult male Sprague-Dawley rats were divided into four dietary treatment groups (n = 15 each group) and fed the C, RS, B or BR combination diet for 12 weeks. Weight and food intake (by measurement of food jar weight and spillage for each rat) were taken twice a week for the duration of the study, with a final weight taken on the animals just prior to kill.

7% Fat / AIN 93-M			0% RS + 0g Butyrate			28% RS + 0g Butyrate			28% RS + 32g Butyrate			0% RS + 32g Butyrate		
	kJ/g	kcal/gm	grams	kJ	kcal	grams	kJ	kcal	grams	kJ	kcal	grams	kJ	kcal
AMYLOPECTIN	14.65	3.50	539.9	7912.0	1889.7	139.9	2050.2	489.7	139.9	2050.2	489.7	539.9	7912.0	1889.7
HI MAIZE	11.72	2.80				500.0	5861.8	1400.0	500.0	5861.8	1400.0			
SUCROSE	16.75	4.00	100.0	1674.8	400.0	100.0	1674.8	400.0	100.0	1674.8	400.0	100.0	1674.8	400.0
CASEIN	14.99	3.58	140.0	2098.5	501.2	140.0	2098.5	501.2	140.0	2098.5	501.2	140.0	2098.5	501.2
CELLULOSE			100.0									100.0		
CORN OIL	37.01	8.84	72.0	2664.9	636.5	72.0	2664.9	636.5	40.0	1480.5	353.6	40.0	1480.5	353.6
BUTYRATE	37.68	9.00							32.0	1205.9	288.0	32.0	1205.9	288.0
MINERAL MIX	3.68	0.88	35.0	129.0	30.8	35.0	129.0	30.8	35.0	129.0	30.8	35.0	129.0	30.8
VITAMIN MIX	16.20	3.87	10.0	162.0	38.7	10.0	162.0	38.7	10.0	162.0	38.7	10.0	162.0	38.7
CHOLINE CLORIDE			1.3			1.3			1.3			1.3		
L - CYSTINE	16.75	4.00	1.8	30.1	7.2	1.8	30.1	7.2	1.8	30.1	7.2	1.8	30.1	7.2
			1000	**14671**	**3.5**	**1000**	**14671**	**3.5**	**1000**	**14693**	**3.5**	**1000**	**14693**	**3.5**
			g/kg	**kJ**	**Kcal**	**g/kg**	**kJ**	**Kcal**	**g/kg**	**kJ**	**Kcal**	**g/kg**	**kJ**	**Kcal**
			0% RS + 0g Butyrate			28% RS + 0g Butyrate			28% RS + 32g Butyrate			0% RS + 32g Butyrate		
				3504	kcal/kg		3504	kcal/kg		3509	kcal/kg		3509	kcal/kg
				3.50	kcal/g		3.50	kcal/g		3.51	kcal/g		3.51	kcal/g
PROTEIN			**14%**	**2129**	**KJ**	**14%**	**2129**	**KJ**	**14%**	**2129**	**KJ**	**14%**	**2129**	**KJ**
				15	**%**		**15**	**%**		**14**	**%**		**14**	**%**
TOTAL CARBOHYDRATES			**68%**	**9878**	**KJ**	**56%**	**9878**	**KJ**	**56%**	**9878**	**KJ**	**68%**	**9878**	**KJ**
				67	**%**		**67**	**%**		**67**	**%**		**67**	**%**
FAT			**7%**	**2665**	**KJ**	**7%**	**2665**	**KJ**	**7%**	**2686**	**KJ**	**7%**	**2686**	**KJ**
				18	**%**		**18**	**%**		**18**	**%**		**18**	**%**
RS			**0%**	**0**	**KJ**	**28%**	**3517**	**KJ**	**28%**	**3517**	**KJ**	**0%**	**0**	**KJ**
			Kcal	**0**	**%**	**Kcal**	**24**	**%**	**Kcal**	**24**	**%**	**Kcal**	**0**	**%**
Total Fiber			**10%**			**30%**			**30%**			**10%**		

Figure 3 - Diet Table

After 12 weeks, rats from each group were initially exsanguinated by removal of blood via heart puncture, then killed rapidly by heart removal, disemboweled and then had abdominal fat pads (epididymal, retroperitoneal, and perirenal) and cecal contents removed. Animals were killed in groups of five from each treatment in order to keep physiological conditions similar to increase the accuracy of findings. The full GI tract, full cecum, empty cecum, and each individual fat pad were weighed. A reduced pH level in the cecal contents is an indicator of fermentation, so 0.5 grams of cecal contents were diluted with 4.5 ml of water for extraction of short chain fatty acids and measured the pH levels.

Values are means ± SE. A 2 x 2 factorial design analyzed by SAS statistical software was used to examine the effects of resistant starch and sodium butyrate on food intake, body weight, disemboweled body weight, full GI tract weight, body fat, full and empty cecal weights, cecal

pH, GLP-1 and PYY concentrations among groups. **Statistical results will be reported as main effects of RS and B and the interaction between RS and B.** A post hoc comparison of the RS and B groups individually to the BR combination group was also performed for total abdominal fat levels.

Results. Food intake did not statistically differ among the groups. Since all diets were made isocaloric to each other the results indicate no difference in energy intake between groups (R: p=0.0611; B: p=0.1041; BR: p=0.2603). (See figure 4)

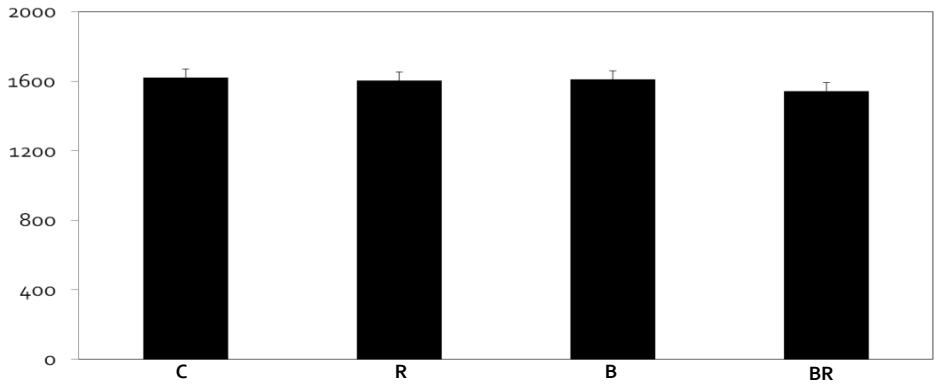

Figure 4 - Food Intake

Cecal contents and empty cecum weights were increased in R fed animals (both $p<0.0001$), and there was no B effect for either of these dependent variables ($p=0.195$ and $p=0.910$, respectively). The pH levels of the cecal contents were decreased in groups with R in the diet ($p<0.0001$) but there was no effect of B ($p=0.788$). The interaction of B with R reduced ($p=0.04$) cecal content levels compared to R alone but there was not an interaction effect on empty cecum weights ($p=0.3$) or pH levels ($p=0.4$). (See figure 5, 6, and 7)

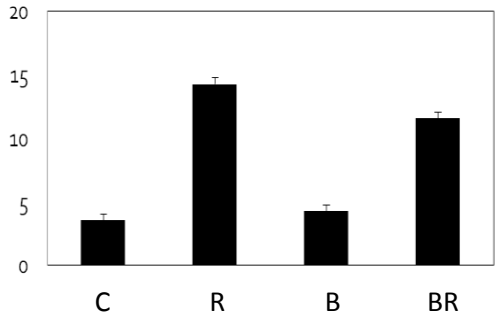

Figure 5 – Cecal Contents

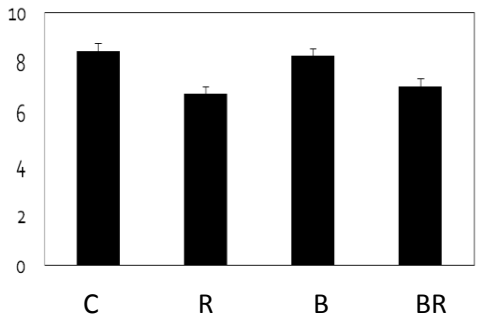

Figure 6 - pH

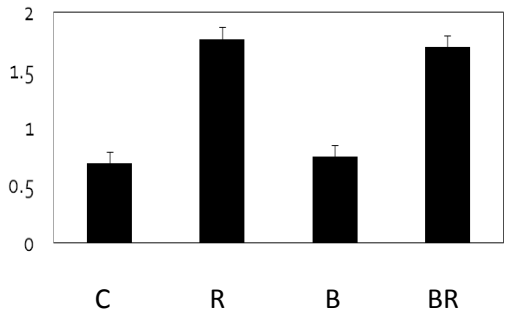

Figure 7 – Empty Cecum

RS in the diet was significantly associated with increased butyrate levels in the cecum (p=0.002) but there was not an effect of B (p=0.99) and there was no interaction between R and B (p=0.68). These findings were also seen in concentration levels of butyrate levels in the cecum, (R: p=<0.0001; B: p=0.58; BR: p=0.54) (See figure 8, 9)

Total PYY and GLP-1 plasma levels were increased by RS (PYY: p<0.0001 and GLP-1: p<0.0001), but B reduced PYY and had no effect on GLP-1 (PYY: p<0.007 and GLP-1: p=0.14). There was a significant interaction between RS and B on PYY levels (p=0.004) and GLP-1 levels (p=0.0167) as the presence of B in the diet was associated with reduced levels of both PYY and GLP-1. (See Figure 10, 11)

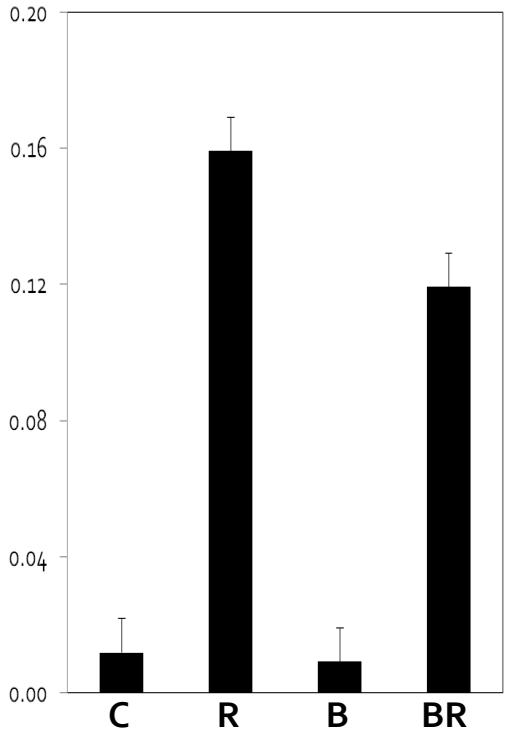

Figure 8 – mMoles Butyrate in cecum

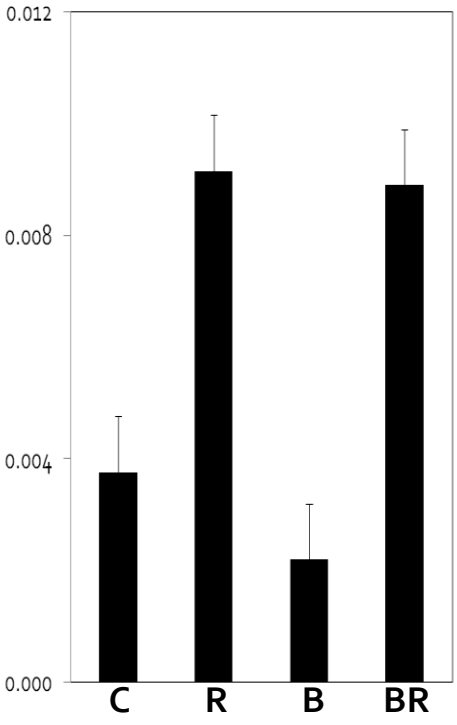

Figure 9 – mMoles Butryate/g in cecum

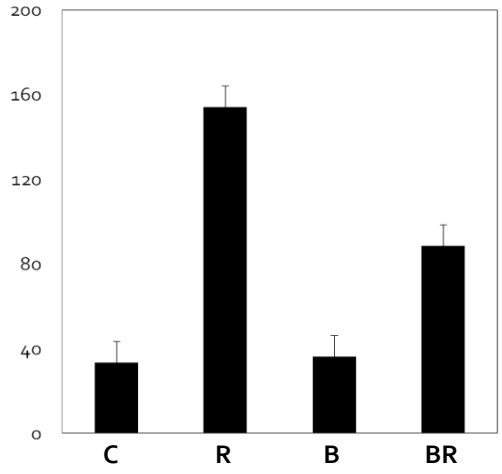

Figure 10 – Total PYY

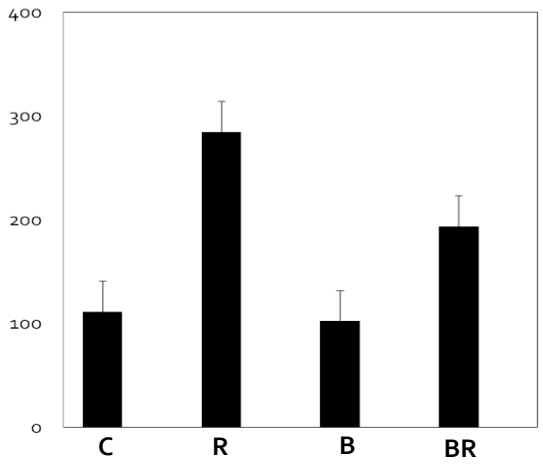

Figure 11 – Total GLP-1

Both R and B had reduced epididymal fat levels and epidiymal fat pads as a percent of disemboweled body weight (R; epidiymal: $p<0.0004$; percent: $p<0.008$; and B; epididymal: $p<0.0007$; percent: $p<0.0002$), but there was no interaction between the two independent variables (BR; epididymal: $p=0.6$; percent: $p=0.6$) (See figure 12, 13). The combination group BR had the lowest levels of body fat for both epididymal and percent epididymal fat and a *post hoc* comparison of the R group and the B group individually to the BR group showed the BR group had lower epididymal and percent epididymal fat than the R group (epididymal: $p<0.04$; percent: $p<0.002$) and the B group (epididymal: $p<0.002$; percent: $p<0.03$).

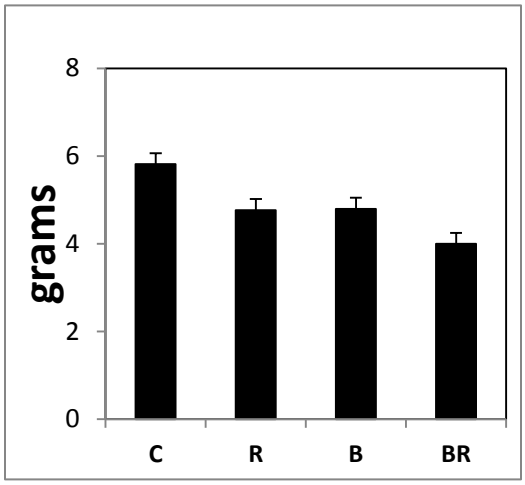

Figure 12 – Epididymal Fat

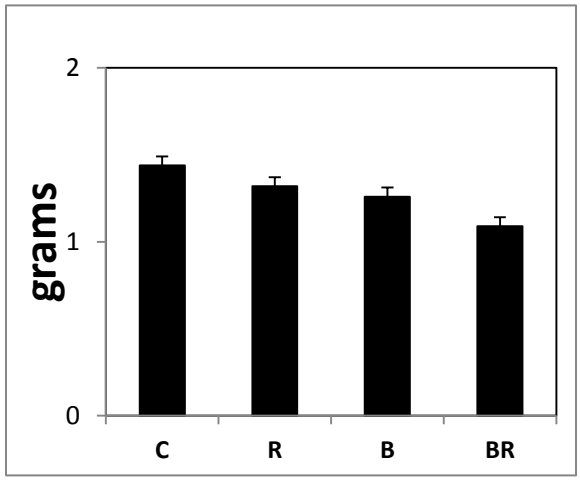

Figure 13 – Percent Epidymal Fat

Discussion. The overall aim of the current study was to determine if the combination of dietary butyrate and dietary resistant starch would be more effective than either compound individually in reducing body fat. A secondary aim was to determine if reduced body fat in rats fed butyrate also had increased plasma levels of PYY and GLP-1.

In line with the hypothesis, the BR group had lower epididymal and percent epididymal fat levels compared to both the RS and B groups. This meant that the combination is better than the individual compounds in combating obesity. However, the effect did not reach the level of a

significant interaction between the two independent variables, which would have indicated additive or synergistic effects. The lack of an interaction suggests that there may be a similar cellular mechanism and that the high levels of dietary butyrate (3.2% wt of diet) and dietary RS (30% wt of diet) are near to saturating the mechanism. PGC1-α is a transcriptional coactivator which increases lipid utilization by cells and was found to be elevated liver and muscle tissues in a sodium butyrate study (Gao et al 2009). This has not been measured yet for this study, but it may turn out that both B and RS may increase this transcriptional coactivator as the common link between the activities of the two compounds. It is important to remember that in the current study there are two sources of butyrate. One is the dietary butyrate fed to the B and BR rat treatment groups and this butyrate should be absorbed in the stomach and small intestine. The other is the butyrate produced in the large intestine as a product of fermentation of RS in the R and BR groups. In a previous study, butyrate was found to increase proglucagon (gene for GLP-1) RNA production in primary culture of cecal cells (presumably containing L-endocrine cells) in vitro. Another study found that GLP-1 was able to increase hepatocyte cAMP production (Ding et al 2005). While there is no current link to GLP-1 and PGC-1 α, cAMP is a known regulator of PGC-1 α (Lin et al 2005). It is possible that the increased production of butyrate by RS fermentation leads to increases of GLP-1, which may increase cAMP levels and concurrently increase PGC-1 α in liver and muscle tissues. Future research will investigate the possible convergence of RS and B in the cell by means of PGC-1α.

As expected, plasma GLP-1 and PYY levels were increased by feeding RS. However, the hypothesis that dietary B could increase these two gut peptide hormones was demonstrated not to be valid. The possible reasons for this are 1) the dietary butyrate that reaches the stomach and small intestine does not indirectly affect production of PYY and GLP-1 by L endocrine cells in

the later part of the small intestine and large intestine through some kind of neural loop or 2) dietary butyrate absorbed into the systemic blood does not stimulate L endocrine cell from the systemic surface. Since RS effects on body fat reduction are dependent on GLP-1 increases and these increases were not found by dietary B supplementation, B does not act through increases in the gut hormones GLP-1 or PYY as RS does. Reduction in both PYY and GLP-1 levels in the combination BR group may suggest speculation of a possible feedback loop to limit the levels of GLP-1 and PYY output produced by RS fermentation and body fat reduction.

As expected RS also significantly increased fermentation levels in rats as evidenced by the increased cecal contents and empty cecum weights, as well as reduced pH levels of the cecal contents. These effects were not found in the sodium butyrate group and demonstrated the expected result that an increase in fermentation does not occur with dietary sodium butyrate. The level of fermentation by the combination of RS and B fed rats was statistically (significant interaction effects) reduced compared to the RS fed group, again suggesting a speculation that dietary butyrate B when in combination may have a blocking effect on RS fermentation. The combination group's fermentation levels were still larger than control and B fed rats, which suggests that BR blocking effects on RS fermentation is incomplete; therefore significant fermentation by RS in the cecum was possible with BR supplementation.

Increases in total butyrate and butyrate concentrations found in the cecum of RS fed diets confirmed previous findings that fermentation of RS lead to increases in the short chain fatty acid butyrate. Butyrate levels were not increased in the cecum of the group fed butyrate, indicating that the ingested dietary sodium butyrate does not reach the cecum and must be taken up earlier in the stomach and small intestine.

Food intake did not differ between any groups in the study and since the diets were isocaloric this indicates that there were no energy intake differences between groups. These results imply that lower body fat levels in the B, R and BR groups in the study were the result of differences in energy expenditure. In previous studies, Zhou et al. (2009) and Gao et al. (2009) demonstrated increased energy expenditure and fat burning in mice fed RS or butyrate, respectively.

There are inherent issues with using RS or B as fat loss food chemicals in diets for humans. At higher levels, such as the levels used in this study, RS may promote gastrointestinal discomfort. Inclusion of relative levels of RS in human dietary intakes may lead to levels of discomfort that cause cessation of intake of high RS food sources. Butyrate has a particularly pungent odor, which may make it difficult to incorporate into food sources while keeping flavor and taste of the food source intact or acceptable to the food consumer. Given the previously mentioned results of a combination of RS and SB effectively lowering body fat levels lower then either one separately, it may be possible to use lower dosages of RS and SB and still reach similar body fat reduction levels. A reduce of both RS and SB may also also alevate any possible discomforts associated with higher intakes. Future research will focus on this aspect of research.

In conclusion, we have confirmed RS increased fermentation levels, increased cecal butyrate levels, increased plasma GLP-1 and PYY levels, while B did not increase any of these dependent variables. These findings provided evidence that dietary sodium butyrate is not functioning through the same initial mechanism by which dietary resistant starch in reducing body fat levels of rodents. The combination of resistant starch and dietary sodium butyrate reduced epididymal fat better than either one alone, but not additively. This may indicate a convergent mechanism at the mitochondrial level. It's possible the transcriptional coactivator PGC-1α is a component in the convergent mechanism and a speculated saturation point for RS and B. Future research using

PYY and GLP-1 knockout mice should be used to further demonstrate butyrate does not act through PYY and GLP-1. Further measures of PGC-1α levels of B and RS fed rodents may demonstrate a convergent mechanism of cellular respiration. Future research will also focus on finding optimal levels of RS and BR in the diet, which would decrease body fat levels while reducing the difficulties of ingesting high levels of RS or B in the diet.

Chapter 4

Conclusion

In the real world, replacement of regular starch in foods with starch containing resistant starch results in a decrease in energy density of the food. Any non-fermentable fiber can dilute the energy density of a food or diet. The goal of our research with resistant starch is to determine any metabolic effects beyond dilution of energy density of the experimental rodent diets. This is effectively done by using control diets with equal energy densities. Thus, any effects on food intake or energy expenditure can be attributed to metabolism of the resistant starch rather than an effect of reduced energy density of the food or diet.

In food related research, unknown caloric intake differences between groups can lead to major differences in dependent variables, which could cause erroneous conclusions. By housing each animal in individual cages and measuring food intake and spillage separately for each animal, the study format reduces the likelihood of inaccurate conclusions due to variations in energy consumption. Food intake results in the sodium butyrate and resistant starch study demonstrated that there was no difference in food intake for all isocaloric diets (C, B, R, and BR) used in the study. Therefore, there was no difference in caloric intake among groups. Given that food weight and caloric intake did not statistically differ among groups other independent variables rather than caloric intake differences can be considered as causes affecting dependent variable differences among groups.

Several dependent factor results confirmed some of the expected metabolic pathways of both dietary resistant starch and dietary sodium butyrate. One hypothesis was that resistant starch acted to reduce body fat, at least initially, via a fermentation mechanism in the cecum of the large intestine in rodents. This mechanism is in a sense, indirect, as products of fermentation of resistant starch rather than the resistant starch would be the effectors in reducing body fat.

However, sodium butyrate should be absorbed in the upper gut and not reach the large intestine. This would mean that the effect of sodium butyrate on reducing body fat as reported by Gao et al. (2009) would be that it is acting through the systemic circulation as sodium butyrate and would be more of a direct mechanism with butyrate itself acting on peripheral tissues and causing reduced body fat. Additionally, the B group diet containing sodium butyrate without resistant starch should not have any greater fermentation than the C group without both sodium butyrate and resistant starch. This hypothesis was confirmed with the two groups fed RS, R and BR, as evidenced by increased cecal contents, empty cecal weights, and a decrease in cecal contents pH levels. Fermentation was not increased in the group fed sodium butyrate, B ,compared to the other groups. There was also no interaction between RS and SB on cecal contents pH and empty cecal weights. However, there was some reduction of the cecal contents of the BR group compared to the R group, as demonstrated by a significant interaction between SB and RS ($p<0.04$). These cecal content levels were still much larger for the BR group compared to the C and B groups which did not have RS in the diet as demonstrated by the significant factorial effect of the combination of the R and BR groups compared to the C and B groups.

As in previous research findings (Keenan et al, 2006), RS fed groups, R and BR, had increased levels of butyrate in the cecum, while the group fed SB, B, did not show increases in cecum butyrate levels compared to control. Dietary SB also did not increase the cecal butyrate levels beyond that of feeding RS alone without SB as demonstrated in the BR group. These findings confirm that RS is reaching the cecum and being fermented into SCFA's, leading to an increase in butyrate levels, while dietary SB is being absorbed in the small intestinal tract and not reaching the cecum.

Another hypothesis tested was that SB may increase GLP-1 and PYY production by L cells as it could reach the L cells via systemic blood. As expected based on previous studies (Keenan et al. 2006; Zhou et al. 2008; Shen et al. 2009), rats fed diets containing resistant starch, R and BR, had increased gut hormones GLP-1 and PYY levels above control. Based on in vitro cell culture of cecal cells with butyrate in the culture media (Zhou et al 2006), it appears that butyrate produced by fermentation of RS may enter L cells from the lumen of the cecum and result in production of GLP-1 and PYY. Unexpectedly, these gut hormone levels were not increased in the B group compared to C group. . Also the presence of SB in the diet resulted in significantly reduced the PYY levels ($p<0.0037$) in the BR group and approached significance in reducing GLP-1 levels in the BR group ($p=0.20$). At this time, the reason for this interaction or near interaction is unknown.

Previous studies have found reductions in body fat levels without reduction of caloric intake individually with either dietary sodium butyrate (Gao et al. 2009) or with resistant starch (Keenan et al 2006, Shen et al. 2009, Zhou et al 2009). A further hypothesis was that the combination of RS and SB in the diet would have a greater effect in reducing body fat levels. Abdominal fat was reduced by both RS ($p<.0004$) and SB ($p<.0007$) as determined by factorial analysis. A *post hoc* individual mean comparison of BR to B or R demonstrated that the combination diet with both SB and RS was more effective in reducing body fat than the RS ($p<.04$) or the SB diets ($p<.03$) individually. However, the combination diet did not reduce body fat to a level to demonstrate an additive or synergistic effect using 2 x 2 factorial statistical examination.

In summary, it was confirmed that: 1) the mechanism of action for RS involves fermentation, 2) RS fermentation results in increased SCFA levels, specifically butyrate levels that may be the active compound for production of GLP-1 and PYY in the cecum, 3) RS fermentation leads to increases in the gut hormones GLP-1 and PYY, 4) fermentation of RS leads to reduced body fat levels compared to control without increases energy intake reductions, 5) dietary SB does not appear to reach the cecum to act on L endocrine cells, 6) the diet with SB alone as the treatment did not increase the gut hormones GLP-1 and PYY compared to diets with RS and addition of SB to a diet with RS did not increase the gut hormones to a greater extent than with RS alone in the diet, 7) diets with SB reduce body fat levels without reductions in energy intake, 8) SB combined in the diet with RS reduces the weight of the cecal contents to some extent but has no effect on empty cecal weights, pH of cecal contents or cecal butyrate levels, 9) when SB is combined in the diet with RS it limits the levels of PYY and may limit the levels of GLP-1 output that are associated with RS consumption, and 10) combination of SB with RS in the diet results in a greater reduction of body fat, but there was not enough reduction to be considered an additive effect.

The above findings imply that while there may still be a convergent mechanism in which both RS and SB increase energy expenditure and fat oxidation it is not through the gut hormones GLP-1 and PYY. Reduction of body fat with dietary RS is associated with increases in GLP-1 and PYY, but reductions with dietary SB are not associated with increases in these hormones. While this study gives evidence of at least an initial divergent pathway, the statistical result of no additively in effect on reduction in body fat when used in combination may indicate a convergent mechanism, possibly at the mitochondrial level.

In the previously mentioned SB study Gao et al (2009) found that mice fed SB had increases in mRNA and protein levels of peroxisome proliferator activated receptor gamma coactivator 1-alpha (PCG1-alpha), a transcriptional coactivator found in the cell. It is known that when PGC1-alpha is increased, mitochondrial proliferation and respiration increases. These increases lead to increased energy expenditure and beta oxidation by the cell. The mentioned study found that PCG1-alpha levels were increased in both BAT and muscle tissue. Increased PGC-1 alpha levels is the current proposed mechanism to body fat reduction caused by SB intake in rodents.

GLP-1 was found in one study to regulate the levels of cAMP in heptacytes (Ding et al. 2009) and cAMP is a known regulator of PGC-1 alpha. It may be possible that GLP-1 can increase cellular levels of PGC-1 alpha through increases in cAMP. Increases in PGC-1 alpha could be the mechanism by which RS decreases body fat levels in rodents. This may be a point of convergence by which both RS and SB work to increase energy expenditure and beta oxidation in the cell. Furthermore, this convergence point may have a saturation level that can be reached through either SB, RS or a combination of both. This would explain why the body fat levels of the combination diet were lower than either SB or RS alone but not low enough to be considered an additive effect.

Both SB (odor) and RS (discomfort with high amounts of fermentation) have current issues that must be solved in order to create dosages for both that are tolerable for human intake. If a convergence mechanism is confirmed in future research it could lead to profound effects on the usage of both SB and RS in the treatment of obesity. If the combination of SB and RS in the diet leads to similar body fat reductions of each compound individually without the need for reduced caloric or food intake it may be possible to use lower doses of both food bioactives to reach these

body fat reduction levels at dosages which are comfortable for human intake. Another possibility is that the dose for SB and RS may be lower for humans based on metabolic body size. Using the combination of SB and RS in addition to adjustments for metabolic body size may result in effective dosages in human subjects.

Results from the current research study have identified future research needed to advance the field. Future research using GLP-1 and PYY knockout mice is needed to mechanistically confirm that SB is not working through increases in these gut hormone levels. At this point from the results of the current study dietary SB is not associated with increases in GLP-1 and PYY. Also future research is needed to learn if reduced intakes of SB or RS in combination are able to create the same body fat reductions as the individual high dosages of SB or RS.

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Vita

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