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Health Effects of Dietary Fermentable Fiber (Resistant Starch)

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HEALTH EFFECTS OF DIETARY FERMENTABLE FIBER (RESISTANT STARCH)

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Dedicated to my beloved parents Ramiro Carvajal and Fanny Aldaz, my brother Marco Carvajal my grandmother Clara Vega, and my adored future husband Francisco Grau. Without their patience, support, and love, the completion of this work would not have been possible. And specially dedicated to the Lord, who put on my way the right people and opportunities. The Lord gave me strength and faith to continue this journey far away from my home country and without my family.

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TABLE OF CONTENTS

ABBREVIATIONS

ABF %: Abdominal fat percent

AI: Adequate Intake

ATCC: American Type Culture Collection

CDC: Centers for Disease Control and Prevention

CFU: Colony forming units

Ct: Cycles to threshold

CYC: Cyclophilin

DNA: Deoxyribonucleic acid

DRI: Dietary Reference Intake

ELISA: Enzyme linked immunosorbent assay

ECW: Empty cecum weight

F: Fat factor

FOS: Fructo-oligosaccharide

GCG: Proglucagon

GI: Gastro-intestinal

GLP-1: Glucagon-like peptide 1

GK: Goto-Kakizaki

G6Pase: Glucose-6-phosphatase

HAMRS: High amylose maize resistant starch

HOMA-IR: Homeostatic model assessment of insulin resistance

I: Injection factor

IACUC: Institutional Animal Care and Use Committee

IGN: Intestinal gluconeogenesis

IP: Intraperitoneal

PCase: Pyruvate carboxylase

PYY: Peptide YY

Real-time qPCR: Quantitative real-time polymerase chain reaction

R: Resistant starch factor

SCFAs: Short chain fatty acids

T2D: Type 2 Diabetes

W: Whole grain factor

ZDF: Zucker Diabetic Fatty

ABSTRACT

Resistant starch (RS) reaches the large intestine for fermentation and is considered a prebiotic. We wanted to determine if we could reduce fermentation for future mechanistic studies and how there is improved insulin sensitivity in human studies with or without increased incretin hormone glucagon-like peptide 1 (GLP-1). Two main studies were performed.

In the first main study, low potency antibiotics (Ampicillin 1g/L and Neomycin 0.5g/L) were added to the drinking water of rats to reduce fermentation of RS. Antibiotics were used either prior (first) to or during (second) feeding of RS. Results demonstrated that low potency antibiotics given prior to resistant starch feeding were not able to prevent fermentation in the cecum independently of water or cecal contents gavage (donor rats fed resistant starch). Low potency antibiotics given during resistant starch feeding were able to reduce fermentation.

The main purpose of second main study was to determine if resistant starch, as either an isolated starch or in a whole grain flour, increases gene expression of pyruvate carboxylase (PCase), and glucose-6-phosphatase (G6Pase). These two enzymes are involved in intestinal gluconeogenesis (IGN) which improves endogenous glucose control. Certain diets can trigger IGN. Goto-Kakizaki (GK) rats (first) and Sprague Dawley rats (second) were used to measure IGN gene expression in the fasted or fed state, respectively. GK rats were in four diet groups, two control diets (highly digestible isolated starch, CON; and low resistant starch whole grain flour, WG) and two high resistant starch diets (high amount of isolated resistant starch, RS; high amounts of resistant starch in a whole grain flour, WG+RS). Gene expression was measured in the fasted state with insulin injection used to model fed state. Sprague Dawley rats were fed the same diets but with moderate or high fat and gene expression measured in the fed state. High resistant starch in the diet increased IGN gene expression in the fed state, regardless of fat level. In the fasted state,

there were no significant increases of PCase or G6Pase even with insulin injection. Improvement of insulin sensitivity regardless of GLP-1 production in humans fed resistant starch may be a result of IGN.

CHAPTER 1: INTRODUCTION

1.1. Significance of Research

Obesity is considered a key risk factor in the development of hypertension, diabetes, coronary heart diseases, and colon cancer [1]. Obesity is caused as a consequence of an enriched fat diet and a sedentary lifestyle. The Centers for Disease Control and Prevention (CDC) stated that the average fat intake of American people was 33% of energy in 2010. The gut microbiota is now known as an environmental factor involved in body weight, energy homeostasis, nutrition, immunomodulation, behavior, and stress response [2, 3]. A high fat diet can lead to dysbiosis, which is the disruption of the normal balanced state of the gut microbiota [4]. Dysbiosis can lead to diseases and can be associated with pathogens or can be associated with the change of former symbionts into pathobionts [5] which release potential toxic products that play an important role in illnesses such as inflammatory bowel, chronic fatigue syndrome, obesity, cancer, colitis, and others [6, 7]. The obese microbiome may harvest more energy from the diet without compensation of increased energy expenditure [8]. Also, in obese people it has been observed that there is a decrease in phylum *Bacteriodetes* and an increase in phylum *Firmicutes* [2]. Cani in 2007, demonstrated that in mice fed a high fat diet, the variation of gut microbiota is associated with an increased intestinal permeability and an inflammatory endotoxemia [9]. High saturated fat diets can induce severe insulin resistance in skeletal muscle [10].

The gut microbiota is the collection of microbial populations that exists in the gastrointestinal tract including bacteria, archaea, viruses, and some unicellular eukaryotes. In humans it is estimated that there are 10^{14} microorganisms, and the colon has up to 10^{12} microorganisms/ml which is the highest density found in humans. The interaction of the gut microbiota with the host has influential consequences with metabolism and health. The majority

of microorganisms found in the colon belong to the phyla Firmicutes, Bacteroidetes, Actinobacteria, and Protobacteria.

Fermentable fiber is considered a prebiotic that stimulates the growth and/or activity of bacteria in the colon, and, thus, improve host health [11]. The Dietary Reference Intake (DRI) committee on fiber set the Adequate Intake (AI) for fiber as 35 g/day for men and 28 g/day for women. The Agricultural Research Service reported that the mean intake of dietary fiber is 12.1-13.8 g/day for women and 13.5-17.9 g/day for men. Both levels of intake are lower than recommended. Fermentable fiber is more bioactive than other non-fermentable fibers because during its fermentation gases and acids produced result in health benefits. Studies have provided evidence that resistant starch is a prebiotic, which stimulates the growth of beneficial bacteria in the colon. Our lab is interested in the fermentation of resistant starch, which is one of the many types of fermentable fiber. Its fermentation involves *Bacteriodes thetaiotaomicron*, *Bifidobacterium spp.*, and some *Lactobacillus spp* [12]. Bacteria, mostly in Clostridial clusters use the products of these bacteria, acetate and lactate, and produce butyrate [13]. Butyrate and other short chain fatty acids are associated with improved health [14].

It is important to increase the intake of fibers in the daily food consumption. A variety of fibers is important including consumption of fermentable fibers. Our lab is interested in the investigation of the benefits of consumption of resistant starch diets in dysbiosis and its role in obesity and diabetes. It is important to study the effects of resistant starch in rodent models of obesity and insulin resistance and diabetes as well as the interaction of resistant starch and high fat diets. In the future, more people can include the consumption of resistant starch in their daily diets and may improve their health.

1.2. Objectives

1. Determine if lower potency antibiotics can reduce fermentation for future mechanistic studies in order to avoid the use of very potent antibiotics such as vancomycin.

2. Determine if a resistant starch diet can improve the insulin sensitivity of Goto-Kakizaki (GK) rats.

3. Determine if feeding an isolated high resistant starch product or a high resistant starch whole grain flour would result in increased intestinal gluconeogenesis (IGN) gene expression in either the fasted or fed state.

4. Determine if a high fat diet affected IGN gene expression compared to a moderate fat diet.

CHAPTER 2: LITERATURE REVIEW

2.1. Resistant Starch

The starch properties depend on how organized are amylose and amylopectin molecules within the granule. Starches were classified by Englyst et al. (1992) by in vitro assay into three types: (1) rapidly digestible starch digested to glucose in less than 20 minutes, (2) slowly digestible starch digested between 20 and 120 minutes, and (3) resistant starch [15]. Any starch digested after 120 minutes is considered a resistant starch [16]. Resistant starch resists amylase digestion in the small intestine and passes directly to the large intestine where it is fermented [17]. Resistant starch is a non-viscous fiber and it can be classified into four groups depending on source and processing. Resistant starch 1 is found in whole grains because starch granules are inserted in indigestible plant matrix. Resistant starch 2 is found in raw potatoes, wheat, high-amylose maize, and others; its starch granules are native. Resistant starch 3 is any crystallized starch by cooking-cooling. Finally, resistant starch 4 is starch chemically modified by esterification, cross-linking, or trans-glycosylation [18]. There is a possible type 5 which is a mixture of starch with lipid moiety[19].

Resistant starch is considered a prebiotic because it is a non-digestible food ingredient that stimulates the growth and/or activity of certain bacteria in the colon and, thus, benefits host health [20]. Bacterial fermentation of resistant starch results in production of short chain fatty acids (SCFAs) mainly as acetate, propionate, and butyrate. *Bacteroides thetaiotaomicron*, *Bifidobacterium longum*, and some *Lactobacillus spp* attach to the surface of starch molecules and produce acetate, propionate, succinate, and lactate [21]. Lactate and acetate are used by bacteria in Clostridium cluster IV and Clostridium cluster XIV to produce butyrate [22, 23]. Acetate, propionate, and butyrate are involved in energy homeostasis and metabolism. Among the many functions some examples are: acetate is the most abundant SCFA and it is used for ATP formation,

propionate is involved in hepatic lipid metabolism, and butyrate is absorbed by the colonic mucosa and it's the preferred energy source [24].

Resistant starch has been attributed to promoting several beneficial health effects such as reduction of colon cancer risk [25], improvement of colon health [26], reduction of diabetes and reduction of body weight and fat [27], and others. Also, commercial resistant starch is available and can be used as a food ingredient for lowering the caloric value and improving, textural and organoleptic characteristics [28]. Therefore, the food industry has developed a special interest to use resistant starch as a food ingredient to replace rapidly digestible starch.

Our lab uses high amylose maize resistant starch 2 (HAMRS2) which contains ~60% amylose and ~40% amylopectin [16]. HAMRS2 is used for animal and human studies, and different results have been observed. Rodent studies demonstrated postprandial glycaemia reduction [29], increased GLP-1 and PYY peptide [29, 30], increased energy expenditure [27], reduction of body fat [31], increased pancreatic beta-cell density [32], and others. Human studies demonstrated that inclusion of resistant starch in the diet can significantly reduce insulin levels [18], improve peripheral insulin resistance [33], enhance postprandial insulin sensitivity [34], reduce food intake [35], and others. Americans consume approximately 4.9 grams per day of resistant starch [36]. However, more research is required in human subjects.

2.2. Gut Microbiota

The human body is populated by several bacteria, archaea, viruses, and unicellular eukaryotes. Microbiota, microflora, or normal flora is the collection of microorganisms that coexist peacefully within the host. The human microbiota is estimated to contain 10^{14} bacterial cells, and 70% of microbes in the human body live in the colon [37]. Gut microbiota colonization starts at birth and its composition depends on the way of delivery, vaginal vs. caesarean [38]. The gut

microbiota is dominated by two bacterial phyla: Bacteroides and Firmicutes, and in minor proportions: Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria [39]. The gut microbiota is important for the human host because it affects metabolism and immune functions [40]. Recent research has demonstrated that the gut microbiota plays a key role in diseases such as colon cancer, obesity, type 2 diabetes, and inflammatory bowel disease [41, 42].

Alterations of the gut microbiota composition are known as dysbiosis. Dysbiosis is the imbalance of gut microbiota and it is associated with diseases and conditions such as obesity, diabetes, Crohn's disease, and ulcerative colitis. The Western diet (rich in fat and sugar) and life style contribute to the development of dysbiosis [7]. Excess food intake, or the consumption of wrong types of foods can result in the production of intestinal toxins. The fermentation of these toxins can increase the growth of pathogens. Also, usage of antibiotics may cause significant alterations of normal gut microbiota [43]. Dysbiosis leads to gut permeability, causing an increase in the passage of lipopolysaccharide (LPS), a cell wall component of gram negative bacteria, into the portal and systemic circulations [44].

The gut microbiota composition is not static because it is greatly affected by the host's diet [20]. The gut microbiota of obese subjects is dominated by *Firmicutes* in greater proportions than *Bacteroidetes*; and opposite proportions are found in lean subjects [45]. Resistant starch affects colonic health, fecal bulk and SCFA metabolism and stimulates probiotic bacteria growth and activity [28]. Resistant starch is a source of nutrients for colonic bacteria, thus it is considered as a tool for the modulation of the gut microbiota [46]. Studies *in vitro* and using animal models had reported increases in bifidobacteria [47, 48] and *Bacteroides* [49]. Probiotic bacteria stimulated by fermentation of resistant starch prevent colonization of the gut and infection by pathogens.

2.3. Antibiotics

Antibiotics are usually used to treat infections and they are prescribed depending on the targeted pathogen, Gram positive or Gram negative. The usage of antibiotics could reshape the microbiota for short or long periods of time [50]. Cani et al. showed that mice fed a high fat diet that were treated with low potency antibiotics (neomycin and ampicillin) improved glucose tolerance and cured dysbiosis [51].

2.4. Insulin resistance

Insulin resistance occurs when body cells become resistant to the effect of insulin. In order to achieve proper effects of insulin, higher levels are demanded. Therefore, the pancreas tries to compensate by producing more insulin until it is not able to produce enough insulin and the pancreas suffers damage and type 2 diabetes develops. Insulin resistance is a risk factor for development of diabetes. High saturated fat diets induce severe insulin resistance. Diets containing HAMRS2 have demonstrated improvement in insulin resistance in partially diabetic mice [29], and also increased pancreatic mass [32] in Goto-Kakizaki rats, a lean model of type 2 diabetes. Also, humans fed diets containing HAMRS2 had demonstrated improvements in insulin sensitivity [33, 34, 52]. Robertson et al. observed improvement of peripheral but not hepatic insulin resistance by providing 40 g per day of HAMRS2 for 8 weeks in the diet of men and women with insulin resistance [33].

Diabetes mellitus is a chronic degenerative metabolic disease characterized by a high concentration of glucose in blood and insulin cannot clear it. Normal individuals have fasting glucose in a range of 80 to 100 mg/dl, pre-diabetic individuals have 101 to 125 mg/dl of fasting glucose, and diabetic individuals have more than 126 mg/dl of fasting glucose. Diabetic subjects commonly experience excessive thirst, frequent urination, hunger, fatigue, inexplicable weight gain or loss, and others. Diabetes has reached epidemic proportions due to an increasing sedentary

lifestyle, overweight, and obesity [53]. According to the National Institute of Diabetes & Digestive & Kidney Diseases, 35% of Americans have pre-diabetes and 11.3% of Americans older than 20 years old already have diabetes. Diabetes includes type 1 and type 2. Diabetes type 1 is prevalent in children and adolescents, but now it is known as insulin independent diabetes. Diabetes type 2 is characterized by inadequate utilization of insulin and it is the most common type of diabetes diagnosed in obese subjects.

2.5. Intestinal Gluconeogenesis

Glucogenic amino acids, and pyruvate, lactate, propionate, and glycerol are non-carbohydrate carbon substrates utilized for generation of glucose, and this metabolic pathway is called gluconeogenesis. Gluconeogenesis is necessary for long fasting periods or during intensive exercise in order to maintain the blood glucose levels necessary for metabolic demands of brain, muscle and red blood cells. The liver does 90% of the gluconeogenesis and the other 10% is produced by the kidney.

Nowadays, evidence of intestinal gluconeogenesis (IGN) has been observed. Recent research demonstrated that IGN has beneficial effects on glucose and energy homeostasis. De Vadder et al. observed that fermentation products of soluble fiber as propionate and butyrate activate IGN. IGN leads to signaling from the gut to the brain to reduce hepatic gluconeogenesis for better glycemic control and insulin sensitivity [54]. The intestine contributes approximately 20 to 25% of total endogenous glucose during fasting periods [55]. Key enzymes that are involved in IGN are glucose-6-phosphatase (G6Pase), pyruvate carboxylase (PCase), and phosphoenolpyruvate carboxykinase cytosolic (PEPCK-C). G6Pase and PEPCK-C gene expression has been shown in rat and human small intestine [56]. Gene expression of G6Pase and

PEPCK-C are controlled by insulin in small intestine. IGN could have anti-obesity, antidiabetic effects, and regulate food intake.

CHAPTER 3: MICROBIOTA REDUCTION WITH LOW POTENCY ANTIBIOTICS AND COMPARISON OF RECOVERY WITH PREBIOTIC (RESISTANT STARCH) AND PROBIOTIC (CECAL TRANSPLANT BY GAVAGE)

3.1. Introduction

The human microbiota is estimated to contain approximately 10^{14} bacterial cells [45]. The gastrointestinal tract is the largest organ in the human body and it is rich in molecules considered nutrients for microorganisms [37]. The large intestine alone contains over 70% of all microorganisms in the human body [45]. The gut microbiota plays a key role in host health [57], and it is well recognized as an environmental factor that affects body weight, energy homeostasis, nutrition, immunodulation, behavior, and stress response [2, 8, 58]. The human gut microbiota is dominated mainly by phyla Bacteroidetes and Firmicutes, and has minor proportions of Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria [39]. Lately, gut microbiota has been emphasized as a critical organ that plays an essential role in health and disease [39]. The disturbance of the ecological equilibrium of the gut is known as dysbiosis and it is linked with pathological processes [59]. Cani et al. demonstrated that the low potency antibiotics neomycin and ampicillin improved dysbiosis [51] in obese mice. Both are broad-spectrum antibiotics [60]. Additionally, antibiotics can modulate gut microbiota [61] by producing drastic short and long term alterations [50, 62].

The gut microbiota is not static and it is greatly affected by composition of the host's diet. Resistant starch is a fermentable fiber and is considered a prebiotic [63] because it is a substrate for specific beneficial endogenous microorganisms and it modifies the composition of the host gut microbiota [64]. The short chain fatty acids (SCFAs) production is stimulated by fermentation in large intestine [16]. Fermentation of resistant starch involves the attachment of *Bacteroides thetaiotaomicron* (Gram negative), *Bifidobacterium longum* (Gram positive), and some *Lactobacillus spp* (Gram positive) to the surface of starch molecules. *Bacteroides spp* fermentation

products are acetate, propionate and succinate; while products of *Bifidobacterium spp* and *Lactobacillus spp* are lactate and acetate [21]. Lactate and acetate are used by bacteria in Clostridium cluster IV and Clostridium cluster XIV (Gram positives) to produce butyrate [22, 23].

Rodents have been demonstrated to be good models to study alteration of microbial communities by administration of antibiotics and interactions between the gut microbiota and the host [51, 62]. The aim of this research was to determine if low potency antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L) added to drinking water is able to reduce bacteria and fermentation of resistant starch by using them either prior to or during feeding of a resistant starch diet. The overall future purpose of the current research is to be able to do further studies to determine other beneficial effects of dietary resistant starch besides fermentation and SCFAs production. The second purpose of this research was to determine if ampicillin and neomycin can reduce the gut microbiota in order to avoid the use of very potent antibiotics for treatment of dysbiosis and if resistant starch would be able to promote recovery of the gut microbiota after antibiotic treatment. In order to accomplish these objectives, we conducted two studies and tested the effect of antibiotic treatment on the presence of bacteria that ferment resistant starch and use the products; and measured fermentation markers such as pH, SCFAs, and empty cecum weight. In Study 1.1, we determined if reduction of the microbiota prior to feeding resistant starch would subsequently prevent fermentation of resistant starch. Surprisingly rats were able to robustly ferment resistant starch after the antibiotic treatment. This result led us to the study 1.2, where we determined if low potency antibiotics would be able to reduce fermentation when resistant starch is fed at the same time as the antibiotic treatment.

3.2. Research Design and Methods
Animals and diets

For both studies, protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University. Male Sprague Dawley rats were purchased from Harlan Laboratories Inc. (Indianapolis, IN) at 6 weeks old, maintained in quarantine for 1 week, and fed a standard chow diet. After quarantine, rats were stratified according to their body weight. Rats were housed individually in wire bottom cages at 21-22°C, 55% humidity, and 12:12 hour light-dark cycle. Rats were fed *ad libitum*, and had free access to water. During the studies the body weight, food intake, and food spillage were measured twice a week.

Study 1.1. Twenty nine (n=29) rats were used in this study in two phases (Fig. 3.1.). During the first phase, the rats were divided into two groups: resistant starch in the diet and no antibiotics (RS+NAB, n=10) and low dietary resistant starch with antibiotics (NRS+AB, n=19). The rats in RS+NAB group were fed an AIN-93M based diet (Table 3.1.) with high amylose maize resistant starch (HAMRS) corn starch diet for 4 weeks. When, they were euthanized the rats were divided into two subgroups (n=5), one for collecting cecal contents for pooling for a transplant, and the other subgroup had cecal contents collected individually for bacterial analyses by quantitative real-time polymerase chain reaction (real-time qPCR). Simultaneously, the NRS+AB group was fed the AIN-93M diet [65], prepared, pelleted, and irradiated before use by Dyets Company (Bethlehem, PA) and given the antibiotic treatment for 4 weeks (Ampicillin 1g/L and Neomycin 0.5g/L added to their drinking water). This group would have low amylose in the starch in the diet. The drinking water (before addition of antibiotics) and food cups for this group receiving antibiotics were autoclaved before use. After week four, five rats from this group were euthanized to individually collect cecal contents for bacterial analyses by real-time qPCR and the remaining

14 rats continued into the second phase. In the second phase, after antibiotic treatment, the remaining 14 rats from the NRS+AB group from phase 1 were divided into two subgroups (n= 7). The subgroups received either a control water gavage (RS+WtG) or a cecal contents gavage (RS+CG) from the cecal contents from RS+NAB in phase 1 of the study (RS donor rats), respectively. The volume for the gavages was 5 ml of 1:10 diluted cecal contents in saline solution. After the gavage procedure, rats were fed the RS diet used in phase 1 for 3.5 weeks.
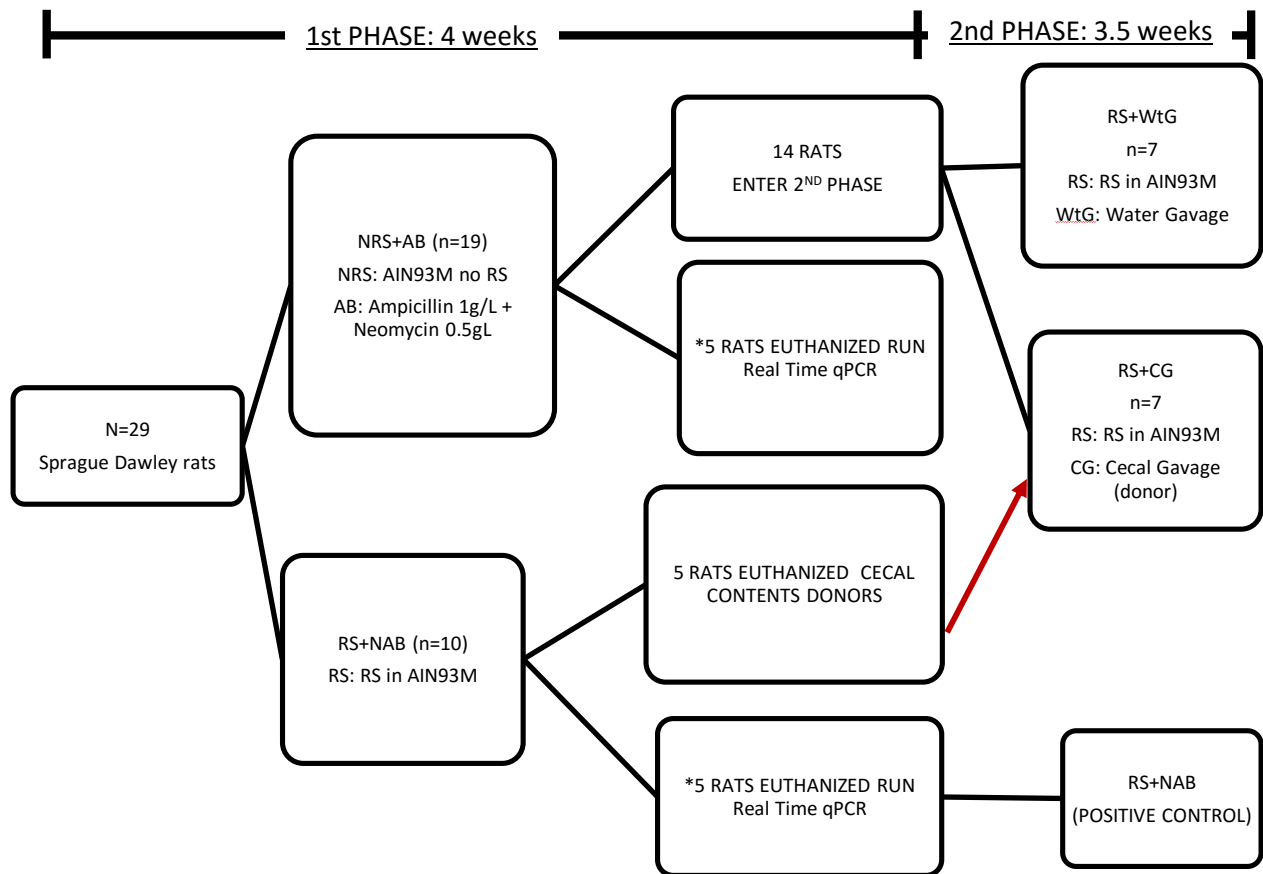
* Targeted bacteria: *Lactobacillus spp., Clostridial clusters XIV a & b, Bacteroides spp., Bifidobacterium spp.*, 16S universal

Figure 3.1. Experimental Design for Study 1.1. Groups included in Phase 1: NRS+AB = AIN93M no resistant starch diet + antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L added in their drinking water); and RS + NAB = AIN-93M based diet with high amylose maize resistant starch (HAMRS) corn starch + no antibiotic treatment. Groups included in Phase 2: RS+NAB = AIN-93M based diet with high amylose maize resistant starch (HAMRS) corn starch + no antibiotic treatment; RS+WtG = HAMRS corn starch + Water gavage; and RS+CG = HAMRS corn starch diet + Cecal gavage from donors in phase 1. RS+NAB: Group from Phase 1 considered as positive control for Phase 2 to compare with RS+WtG and RS+CG groups.

13

Table 3.1. Diets composition in Study 1.1.

Ingredients	NRS[1]		RS	
	Grams	kcal	Grams	kcal
Waxy corn starch[2]	620.69	2208.42	61.7	219.55
High-amylose corn starch[3,4]	0		619	1733.2
Sucrose	100	387	100	387
Casein[5]	140	501.2	135.1	483.66
Cellulose	50	0	0	0
Soy bean oil[6]	40	353.6	34.4	304.1
Mineral mix	35	29.4	35	29.4
Vitamin mix	10	38.7	10	38.7
Choline cloride	2.5	0	2.5	0
L-Cystine	1.8	7.2	1.8	7.2
Total	1000	3525.52	1000	3202.81

[1]Diets include: NRS= AIN-93M diet [65] and RS = AIN-93M based-with purified high amylose maize resistant starch (HAMRS) corn starch.
[2]AMIOCA® corn starch.
[3]HI-MAIZE® resistant corn starch.
[4]Waxy corn starch and high-amylose corn starch were gifts from Ingredion Incorporated (Bridgewater, NJ).
[5]Casein was reduced from AIN-93M amount (140 g/kg) for RS diet based on the protein in the HI-MAIZE® resistant corn starch as analyzed by proximate analysis by Medallion Labs for Ingredion Incorporated.
[6]Soy bean oil was decreased from AIN-93M amount (40 g/kg) for RS diet based on the fat in the HI-MAIZE® resistant corn starch as analyzed by proximate analysis by Medallion Labs for Ingredion Incorporated.

Study 1.2. Twenty-four rats were fed *ad libitum* for 4 weeks and had free access to water. The diets provided were (1) low fat and HAMRS corn starch (LFRS), (2) high fat and HAMRS corn starch (HFRS), and (3) low fat and amylopectin control corn starch with no resistant starch (LFNRS). The compositions of the diets used are listed in Table 3.2. Two groups of rats received an antibiotic treatment in the drinking water containing Ampicillin 1g/L and Neomycin 0.5g/L (AB=antibiotic and NAB=no antibiotic). The four groups (n=6) were LFRS+AB, HFRS+AB, LFNRS+NAB, and LFRS+NAB, where LFNRS+NAB was considered the negative control and LFRS+NAB was considered the positive control.

Table 3.2. Diets composition Study 1.2.

Ingredients	LFRS[1]		HFRS		LFNRS	
	Grams	kcal	Grams	kcal	Grams	kcal
Waxy corn starch[2]	138.35	489.34	0.00	0.00	536.80	1898.66
High-amylose corn starch[3,4]	524.00	1467.20	524.66	1469.05	0.00	0.00
Sucrose	100.00	387.00	100.00	387.00	100.00	387.00
Casein[5]	132.27	473.53	133.70	478.65	136.00	486.88
Cellulose	18.31	0.00	0.00	0.00	135.00	0.00
Soy bean oil[6]	38.87	343.61	93.44	826.01	44.00	388.96
Lard[7]	0.00	0.00	100.00	900.00	0.00	0.00
Mineral mix	35.00	29.40	35.00	29.40	35.00	29.40
Vitamin mix	10.00	38.70	10.00	38.70	10.00	38.70
Choline cloride	1.40	0.00	1.40	0.00	1.40	0.00
L-Cystine	1.80	7.20	1.80	7.20	1.80	7.20
Total	1000.00	3235.98	1000.00	4136.00	1000.00	3236.80

[1]Diets include: LFRS = low fat and purified high amylose maize resistant starch (HAMRS) corn starch diet; HFRS = high fat and HAMRS corn starch diet; LFNRS = low fat and amylopectin control corn starch with no resistant starch.

[2]AMIOCA® corn starch.

[3]HI-MAIZE® resistant corn starch.

[4]Waxy corn starch and high-amylose corn starch were gifts from Ingredion Incorporated (Bridgewater, NJ).

[5]Casein was reduced from AIN-93M amount (140 g/kg) for three diets based on the protein in the AMIOCA® corn starch and HI-MAIZE® resistant corn starch as analyzed by proximate analysis by Medallion Labs for Ingredion Incorporated.

[6]Soy bean oil was decreased from AIN-93M amount (40 g/kg) for RS diets based on the fat in the AMIOCA® corn starch and HI-MAIZE® resistant corn starch as analyzed by proximate analysis by Medallion Labs for Ingredion Incorporated.

[7]Lard was added to complete ~40% fat in conjunction with soy bean oil and the fat in the HI-MAIZE® resistant corn starch as analyzed by proximate analysis by Medallion Labs for Ingredion Incorporated for HFRS diet.

Procedures

In studies 1.1 and 1.2, rats were euthanized by cardiac puncture using inhalation of isoflurane soaked cotton balls as anesthesia in a bell jar. The gastrointestinal tract (GI) was removed from esophagus to anus, and then it was divided into stomach, small intestine, cecum and large intestine. The GI parts were weighed full and empty. Fat pads (peritoneal, retroperitoneal,

and epididymal) were weighed for calculation of total abdominal fat percentage per animal. Abdominal fat percent was calculated from abdominal cavity fat divided by body weight of the rats with the GI tract contents removed. Cecal contents were frozen in liquid nitrogen for further measurements of pH, short chain fatty acids (SCFAs), and targeted bacterial genera that ferment resistant starch by real-time qPCR. For Study 1.2, blood samples were collected by cardiac puncture for measurement of GLP-1 active with ELISA kit (ALPCO, NH) and one full cecum per group was kept for histology of cecum wall.

Cecal contents pH and SCFAs analysis

For studies 1.1 and 1.2, cecal contents were thawed and 0.5 g of each rat's wet sample was homogenized in 5 ml of distilled water, for pH measurements. Subsequently, each sample was acidified with 1 ml of a 25% (w/w) solution of metaphosphoric acid containing 2 g/L 2-ethyl-butiryc acid as an internal standard for SCFAs contents. Solids were separated by centrifugation and filtration. The supernatant was transferred to a GC autosampler vial. Concentrations of SCFAs were quantitatively determined by gas chromatography by a method described in previous publication from our laboratory [66].

Bacterial DNA extraction

Study 1.1. After cecal contents were thawed, 500 mg for each rat's sample were weighed and placed into a 2 ml Lysing Matrix E tube (MP Biomedicals, OH) containing 1.4 mm ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead; then 825 µl of sodium phosphate, and 275 µl of PLS solution from FastDNA® SPIN kit for Feces (MP Biomedicals, OH) were added. Immediately, the tubes with cecal contents, Lysing Matrix E, sodium phosphate, and PLS solution were vortexed for 15 seconds, then centrifuged at 11,400 rpm for 5 minutes and supernatant was decanted. Next, 978 µl of sodium phosphate buffer and 122 µl of MT buffer were added into the

tubes and vortexed briefly. Mixture was homogenized using the FastPrep®-24 Instruments (MP Biomedicals, OH) for 40 seconds at speed setting 6.0 m/s. The lysate was centrifuged at 11,400 rpm for 5 minutes, and DNA was extracted from supernatant by following FastDNA® SPIN kit for Feces protocol. Purified DNA was quantified using a NanoDrop® Spectrophotometer and diluted to 1 ng/ μl for storage at -80℃.

Study 1.2. After cecal contents were thawed, approximately 200 mg for each rat's sample were weighed and placed into a 2 ml screw-cap tube containing ~ 300 mg of 0.1 mm Zirconia Silica beads (BioSpec Products Inc, OK), then 100 μl of Lysis buffer prepared with 5 M NaCl, 1 M Tris-HCl (pH 8), 0.5 M EDTA (pH 8), distilled autoclaved water and Lysozyme (Thermo Scientific, IL) were added. Immediately, the tubes with cecal contents, Zirconia Silica beads, and Lysis buffer were vortexed and incubated for 30 minutes at 37℃. Next, 1 ml of InhibitEX buffer from QIAamp® Fast DNA Stool Mini kit (QIAGEN, CA) was added. Next, the mixture was homogenized two times using a FastPrep®-24 Instrument (MP Biomedicals, OH) for 60 seconds at speed setting 6.5 m/s. Subsequently, the suspension was heated in a heat-block at 95℃ and 250 rpm for 5 minutes, then vortexed for 15 seconds and centrifuged for 3 minutes at 14,000 rpm. Finally, DNA was extracted from supernatant by following QIAamp® Fast DNA Stool Mini kit protocol. Purified DNA was quantified using a NanoDrop® Spectrophotometer and diluted to 1 ng/μl for storage at -80℃. The method for DNA extraction was changed for this study due to a high absorbance at 230 nm wavelength in previous method used in study 1. The method for study 2 resulted in low absorbance at 230 nm wavelength and greater purity for the DNA extract.

Quantitative real-time PCR

In studies 1.1 and 1.2, ABI PRISM 7900 Sequence Detection System, and SDS 2.4 Software (Life Technologies, NY) were used to perform SYBR® Green method of real-time

qPCR. Targeted bacterial genera, primer sequences, annealing temperature and literature references are listed in Table 3.3 and 3.4 for studies 1.1 and 1.2, respectively. All reactions were performed using sterile MicroAmp® Optical 384-well Reaction Plates with Barcode and sealed with MicroAmp® Optical Adhesive Films (Life Technologies, CA). Each reaction was performed in triplicates and made of 5µl of 2X SYBR Green Master Mix (Life Technologies, CA), 0.5 µl of each primer at 10 µM (Integrated DNA Technologies, IA), 0.5 µl of 250 mg/ml bovine serum albumin (BSA), 0.5 µl of nuclease free water and 3 µl of DNA template in a 10 µl total volume.

Table 3.3. Real-time qPCR primers used to profile cecal contents samples for Study 1.1.

Targeted bacteria	Primer Sequence	Annealing Temp (℃)	Reference
Firmicutes			
Lactobacillus spp	F: TGG ATG CCT TGG CAC TAG GA R: AAA TCT CCG GAT CAA AGC TTA CTT AT	55	Haarman & Knol, 2006[67]
Clostridial cluster IV *Clostridium leptum* subgroup, includes *Faecalibacterium prausnutzii*	F: TTA CTG GGT GTA AAG GG R: TAG AGT GCT CTT GCG TA	55	Wise & Siragusa, 2007[68]
Clostridium cluster XIVa and XIVb *Clostridium coccoides – Eubacterium rectale* subgroup	F: AAA TGA CGG TAC CTG ACT AA R: CTT TGA GTT TCA TTC TTG CGA A	55	Matsuki et al., 2002[69]
Bacteroidetes Bacteroides group including *Prevotella* and *Porphyromonas*	F: GAA GGT CCC CCA CAT TG R: CAA TCG GAG TTC TTC GTG	55	Wise & Siragusa, 2007[68]
Actinobacteria *Bifidobacterium spp*	F: GGG TGG TAA TGC CGG ATG R: TAA GCC ATG GAC TTT CAC ACC	55	Bartosch et al., 2005[70]
Bacterial Domain 16S universal primers	F: GTG STG CAY GGY YGT CGT CA R: ACG TCR TCC MCN CCT TCC TC	55	Belenguer, et al., 2006[71]

Table 3.4. Real-time qPCR primers used to profile cecal contents samples for Study 12.

Targeted bacteria	Primer Sequence	Annealing Temp (°C)	Reference
Firmicutes			
Lactobacillus spp	F: TGG ATG CCT TGG CAC TAG GA R: AAA TCT CCG GAT CAA AGC TTA CTT AT	60	Haarman & Knol, 2006[67]
Clostridium cluster XIVa and XIVb *Clostridium coccoides – Eubacterium rectale* subgroup	F: AAA TGA CGG TAC CTG ACT AA R: CTT TGA GTT TCA TTC TTG CGA A	60	Matsuki et al., 2002[69]
Bacteroidetes			
Bacteroides group including *Prevotella* and *Porphyromonas*	F: GAA GGT CCC CCA CAT TG R: CAA TCG GAG TTC TTC GTG	60	Wise & Siragusa, 2007[68]
Actinobacteria			
Bifidobacterium spp	F: CTC CTG GAA ACG GGT GG R: GGT GTT CTT CCC GAT ATC TAC A	60	Matsuda et al., 2009[72]
Bacterial Domain			
16S universal primers	F: GTG STG CAY GGY YGT CGT CA R: ACG TCR TCC MCN CCT TCC TC	60	Belenguer, et al., 2006[71]

The primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and their specificity checked with the GenBank database by blast search of primers sequences in National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) on September 30, 2015. The cycling conditions were one cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, primer-specific annealing temperature (Table 3.2.) for 1 minute, 72°C for 40 seconds, and one cycle of 72°C for 30 seconds. After amplification, a dissociation step of 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds, was included. The results were

expressed as Ct values for targeted bacterial genera, where higher Ct equals lower apparent starting amounts of a bacterial genera DNA. For study 1.1, we stopped at the use of Ct only for analysis because we observed that feeding resistant starch subsequent to antibiotic treatment resulted in the same Ct values as rats fed resistant starch that were never treated with antibiotics. We viewed this as a pilot study for study 1.2.

Study 1.2. Several bacteria strains representing targeted genera were cultured in specific broths (conditions described in Table 3.5). Serial dilutions (1:10) starting with one milliliter of the cultured bacteria plus 9 milliliters of PBS were made. These dilutions of bacteria were spread on agar plates using 100 µl. Colony Forming Units (CFU) were determined for the serial dilutions and converted to log CFU. The specific bacteria cultured were: *Lactobacillus plantarum* strain ATCC 4163 (*Lactobacillus* genera)*, Clostridium coccoides* strain ATCC 29236 (*Clostridial cluster XIVa and b*, which includes *Clostridium coccoide, Eubacterium rectale* subgroup, *Lachnospiraceae,* and *Ruminococcus*)*, Bacteroides fragilis* strain ATCC 23745 (*Bacteroides* group, including *Prevotella* and *Porphyromonas* genera), *Bifidobacterium longum* strain ATCC 15708 (*Bifidobacteria* genera), and *Escherichia coli* strain ATCC 25922 (total bacteria using 16S universal primers). DNA was extracted from 1 ml of undiluted broth culture by the method described above for study 1.2, and dilutions of DNA (serial 1:4 dilutions) were interpolated with dilutions for log CFU. Standard curves were constructed as Ct versus log CFU (Table 3.6). The primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and their specificity checked as described above in study 1.1. The cycling conditions were one cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, primer-specific annealing temperature (Table 3.4.) for 1 minute, 78°C for 40 seconds, and after amplification a dissociation step was included as mentioned above. Treatment replicate log CFUs for each targeted bacteria were

determined using the equations for the lines obtained from standard curves.

Table 3.5. Culture conditions Study 1.2.

Bacteria strains	Media	Incubation Temp (℃)	Time (hours)
Firmicutes			
Lactobacillus plantarum	de *Man-Rogosa*-Sharpe Agar (Difco Laboratories, MI)[1]	30	48
Clostridium coccoides	reinforced Clostridial agar (Oxoid, UK)[1]	37	72-96
Bacteroidetes			
Bacteroides fragilis	Brewer Modified Thioglycollate medium (BD Diagnostic Systems, MD)[1]	37	72-96
Actinobacteria			
Bifidobacterium longum	Brewer Modified Thioglycollate medium (BD Diagnostic Systems, MD)[1]	37	72-96
Bacterial Domain 16S universal			
Escherichia coli	Luria Broth (Difco Laboratories, MI)[2]	37	24

[1]Grown in aerobic conditions.
[2]Grown in a chemically generated anaerobic system using an *anaerobis box* GasPak™ EZ (Mitsubishi Gas Chemical America Inc., NY).

Table 3.6. Standard curves Study 2.

Bacteria strains	Equation	Slope	R^2	Efficiency (%)
Firmicutes				
Lactobacillus plantarum	log CFU= -0.2947*(Ct)+14.694	-3.557	0.999	91.00
Clostridium coccoides	log CFU= -0.2395*(Ct)+12.102	-3.38	0.996	97.50
Bacteroidetes				
Bacteroides fragilis	log CFU= -0.2881*(Ct)+11.785	-3.38	0.990	97.60
Actinobacteria				
Bifidobacterium longum	log CFU= -0.2215*(Ct)+9.3945	-3.553	0.996	91.20
Bacterial Domain 16S universal *Escherichia coli*	log CFU= -0.2897*(Ct)+13.815	-3.45	0.999	95.00

Resistant Starch Assay

For study 1.2, 100±5 mg of thawed cecal contents were weighed for each rat sample. Resistant starch was measured following the protocol of the Resistant Starch Assay Kit (Megazyme Inc, IL).

Histology of cecum wall

For study 1.2., one cecum per group was not used for cecal contents analysis and was placed with contents into a jar with 100 ml of 10% formalin for 72 hours until each one acquired a firm texture. Next, approximately one third of each hardened cecum was cut off from the bottom away from the two openings (small intestine or rest of large intestine) and placed into histology cassettes. The histologist carefully removed the contents and cut cross-sectional slices so that the wall of the cecum could be visualized with Hematoxylin and Eosin stain. Pictures of slides were made by the Cell Biology & Bioimaging Core at Pennington Biomedical Research Center using a NanoZoomer-SQ Digital slide scanner (Hamamatsu, Japan) at 20X magnification. Images from tissue samples were analyzed using NanoZoomer Digital PathologyView2 Software. The heights of mucosal, submucosal, and muscularis layers were measured in three different locations per slide image and then averaged.

Statistical Analysis

Statistical differences were analyzed in the Statistical Analysis Software SAS® version 9.3. A one-way ANOVA analysis was executed and followed by F-protected least significant difference (LSD) post-hoc mean comparison tests using the MIXED procedure. In order to test equal variance, normal distribution, and to identify outliers an UNIVARIATE procedure was performed in the MIXED procedure. If normality assumption was not met, data were transformed to log10. A p value of < 0.05 was considered statistically significant. In Study 1.1, one outlier was

detected for Clostridium Cluster XIV (NRS+AB: 21.34) in phase 1, and in phase 2 Clostridium Cluster IV data was log 10 transformed for statistical analyses. In Study 1.2, two outliers were detected in GLP-1 active data (LFRS+AB: 2.99; LFRS+AB: 0.63), and propionate data were transformed to log 10 for statistical analyses. Data were presented in their original form and expressed as means ± standard error.

3.3. Results
Study 1.1.

Cecal contents pH, ECW, SCFAs, and ABF%

In phase 1 (Table 3.7.), as expected, the group fed resistant starch (RS+NAB) demonstrated greater fermentation than the group that received antibiotic treatment and no dietary resistant starch (NRS+AB); through lower pH of cecal contents (p<0.0002), higher production of propionate (p<0.0166), and lower abdominal fat percent (ABF %) (p<0.0213). Also, production of acetate and butyrate were not detectable in NRS+AB group. However, empty cecum weights (ECW) were not significantly different (p=0.0652) from one another.

In phase 2 (Table 3.7.), rats in the RS+NAB group from phase 1 were considered a positive control to compare with rats in the RS+WtG and RS+CG groups for pH and SCFAs production, but not for ECW or ABF% because rats in the RS+NAB group were younger (11 weeks old) than rats in RS+WtG and RS+CG groups (14.5 weeks old) at euthanasia. The positive control group pH was significantly lower than RS+WtG (p<0.0067) or RS+CG (p<0.0439) groups. However, RS+WtG and RS+CG groups produced acetate and butyrate to the same extent as the positive control group. Only propionate production of RS+CG group was significantly lower than the

positive control (p<0.0276) but not significantly different from RS+WtG (p=0.0817) group. Additionally, ECW and ABF% were not significantly different between RS+WtG and RS+CG.

Table 3.7. Cecal contents pH, ECW, SCFAs, and ABF% for study 1.1[1].

Variables	Phase 1[2]		Phase 2[3]		
	NRS+AB	RS+NAB	RS+NAB[4]	RS+WtG	RS+CG
pH	8.44±0.02a	6.17±0.18b	6.17±0.19b	6.94±0.16a	6.71±0.16a
ECW[5] (g)	0.67±0.11a	0.93±0.07a	-	1.33±0.15a	1.30±0.15a
Acetate (mmol)	UND[6]	8.61±1.35	8.61±1.35a	9.35±1.66a	7.86±1.56a
Propionate (mmol)	0.39±0.04b	1.66±0.32a	1.66±0.32a	1.10±0.19ab	0.93±0.19b
Butyrate (mmol)	UND	1.99±0.43	1.99±0.43a	3.24±0.54a	2.61±0.54a
ABF%[7]	1.88±0.14a	1.43±0.10b	-	1.79±0.10a	1.78±0.10a

[1]Data are presented in their original form and expressed as means ± standard error. P value< 0.05 was considered statistically significant and it is represented with different letters horizontally.

[2]Groups include in Phase 1: NRS+AB = AIN93M no resistant starch diet + antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L diluted in their drinking water); and RS + NAB = AIN-93M diet based high amylose maize resistant starch (HAMRS) corn starch + no antibiotic treatment.

[3]Groups include in Phase 2: RS+NAB = AIN-93M diet based high amylose maize resistant starch (HAMRS) corn starch + no antibiotic treatment; RS+WtG = HAMRS corn starch diet + Water gavage; and RS+CG = HAMRS corn starch diet + Cecal gavage from donors in phase 1.

[4]RS+NAB: Group from Phase 1 consider as positive control for Phase 2 to compare with RS+WtG and RS+CG groups for pH, acetate, propionate, and butyrate.

[5]ECW = Empty cecum weight.

[6]UND = undetectable.

[7]ABF% = Abdominal fat percent.

DNA amplification of the targeted bacteria in phase 1 and phase 2

In phase 1 (Fig. 3.2), the starting amounts of total bacteria appeared to be similar as amplified DNA extracts reached the threshold at similar cycles using 16S universal primers for the RS+NAB group and NRS+AB group (p= 0.1118, RS+NAB's Ct = NRS+AB's Ct). However, RS+NAB group appeared to have greater starting amounts of *Bididobacterium spp* (p<0.0001),

Clostridium cluster XIVa and b (p< 0.0001), and Lactobacillus (p<0.0007) than NRS+AB (RS+NAB's Ct < NRS+AB's Ct). Of the targeted genera, only *Bacteroides* for NRS+AB group had apparently greater starting amounts (as their amplified DNA extracts reached the threshold at a significantly lower cycle) than the RS+NAB group (p<0.012) (NRS+AB's Ct < RS+NAB's Ct). This result concurs with the result of propionate being the only SCFA produced for NRS+AB group, and propionate being one of the major fermentation products of *Bacteroides spp*.

Figure 3.2. Cycles to threshold (Ct) for targeted bacteria in phase 1 of Study 1.1. Higher Ct means slower amplification. Asterisk (*) next to bars indicates a statistically significant difference (p< 0.05). Data are presented in their original form and expressed as means ± standard error. Groups included in phase 1: AB+NRS = Amylopectin control corn starch with no resistant starch and antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L added in their drinking water); and RS+NAB = purified high amylose maize resistant starch (HAMRS) corn starch diet and no antibiotic treatment.

In phase 2 (Fig 3.3), there were no differences in the apparent starting amounts of DNA for total bacteria with 16S universal primers (p=0.3632), *Bifidobacterium spp* (p=0.6529), *Clostridium cluster XIVa and b* (p=0.3329), and *Lactobacillus spp* (p=0.3887) between the positive control group (RS+NAB), RS+WtG, and RS+CG (RS+NAB's Ct = RS+WtG's Ct= RS+CG's Ct). The positive control group had a significantly greater apparent starting amount for *Bacteroides spp*

than RS+CG group (p<0.0312) and RS+WtG (p<0.0364) (RS+NAB's Ct < RS+WtG's Ct; RS+NAB's Ct < RS+CG's Ct). However, the apparent starting amount of *Bacteroides spp* for RS+CG group and RS+WtG group (p=0.9322) were not significantly different (RS+WtG's Ct= RS+CG's Ct). These results demonstrated that regardless of the type of gavage (water or cecal contents from donor rats), the apparent amounts of all but one of the targeted genera of bacteria were the same. Also the groups with prior antibiotic treatment had reduced apparent amounts of *Bacteroides spp* compared to the positive control group never treated with antibiotics. This was a reversal from antibiotic treatment.
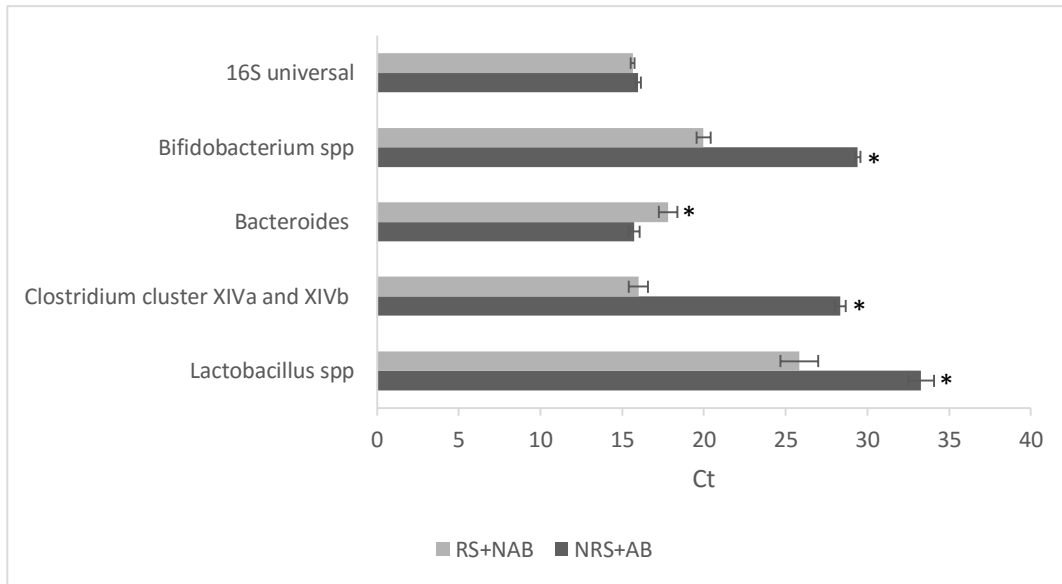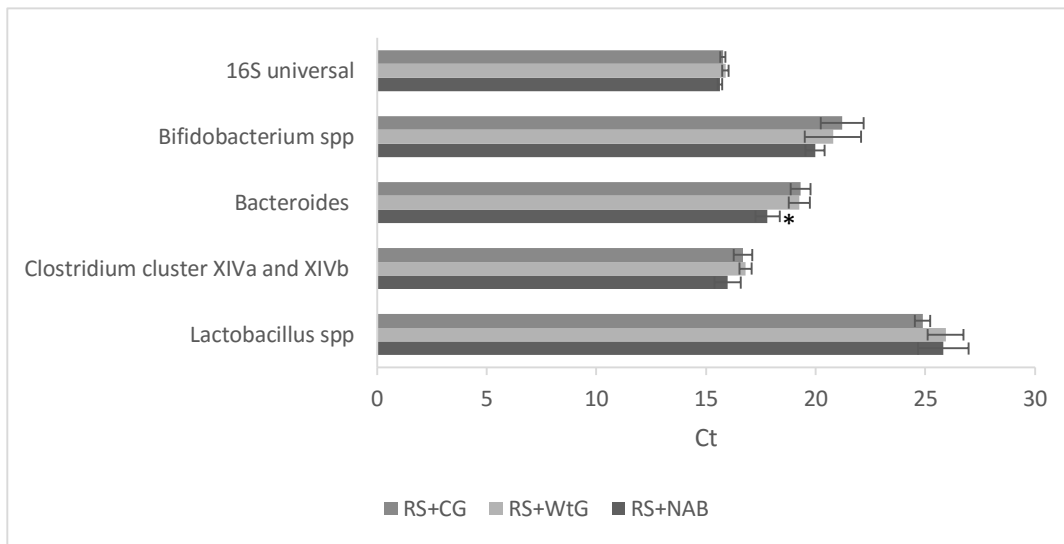

Figure 3.3. Cycles to threshold (Ct) for targeted bacteria in phase 2 of Study 1.1. Higher Ct means slower amplification. Asterisk (*) next to bars indicates a statistically significant difference (p< 0.05). Data are presented in their original form and expressed as means ± standard error. Groups included in Phase 2: RS+NAB = no antibiotic treatment + purified HAMRS corn starch diet; RS+WtG = HAMRS corn starch diet and water gavage; and RS+CG = Cecal gavage from donors fed resistant starch in phase 1 in HAMRS corn starch diet.

Study 1.2.

Cecal contents pH, ECW, SCFAs, and ABF%

Results (Table 3.8.) demonstrated that antibiotic treatment at the same time as feeding resistant starch was able to diminish the ability of the rats to ferment resistant starch. The

LFNRS+NAB group (negative control) did not have a significantly different pH of cecal contents compared to groups fed resistant starch and concurrently treated with antibiotics (LFRS+AB, p=0.3448 and HFRS+AB, p=0.9903). As also stated above in phase 1 in study 1.1, ECW was also not a reliable indicator of fermentation when rats are given antibiotic treatment. ECW of groups given antibiotic treatment, LFRS+AB (p<0.0001) and HFRS+AB (p<0.0005), were significantly greater than the negative control group (LFNRS+NAB); and ECW of LFRS+AB (p=0.1940) was not significantly different from the positive control group (LFRS+NAB) or from the HFRS+AB group (p=0.1632). However, the ECW for the HFRS+AB group was significantly lower than the positive control group (p<0.0124).

Acetate and butyrate short-chain fatty acid production was also reduced with antibiotic treatment (Table 3.8.). Acetate production for the LFRS+AB group was significantly lower than the positive control (p<0.0140), significantly greater than the negative control (p<0.0163), and not different from the HFRS+AB group (p=0.9639). Similar differences were observed for butyrate production except that the two groups treated with antibiotics had lower amounts than the negative control group (Table 3.8.). Thus, there was some increased production of acetate with the combination of resistant starch and antibiotics with both low and high fat diets, but the antibiotics reduced production compared to the positive control. Propionate production was similar for the two groups with low fat diets with resistant starch regardless of antibiotic treatment. The high fat diet with antibiotic treatment and resistant starch had less propionate production than the low fat group with antibiotic treatment and resistant starch, but had similar production as the positive control group.

The abdominal fat percent (Table 3.8.) was different according to dietary fat levels with the group with a high fat diet having the greatest ABF %. ABF % was significantly greater for the

HFRS+AB group compared to the LFRS+AB (p<0.045), and negative control group (p<0.0263) or positive control group (p<0.0169).

Table 3.8. Cecal contents pH, ECW, SCFAs, and ABF% for study 1.2[1].

Variables	LFRS+AB[2]	HFRS+AB	LFNRS+NAB[3]	LFRS+NAB[4]
pH	8.23±0.06a	8.39±0.07a	8.39±0.10a	6.05±0.18b
ECW[5] (g)	1.27±0.07ab	1.08±0.07b	0.51±0.04c	1.45±0.15a
Acetate (mmol)	0.151±0.024b	0.149±0.035bc	0.060±0.007c	0.469±0.080a
Propionate (mmol)	0.096±0.004a	0.074±0.006b	0.013±0.018c	0.087±0.023ab
Butyrate (mmol)	0.004±0.001c	0.004±0.002c	0.013±0.021b	0.074±0.007a
ABF%[6]	1.46±0.14b	1.81±0.05a	1.41±0.13b	1.38±0.12b

[1]Data are presented in their original form and expressed as means ± standard error. P value< 0.05 was considered statistically significant and it is represented with different letters horizontally.
[2]Groups include: LFRS+AB = low fat, purified high amylose maize resistant starch (HAMRS) corn starch diet, antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L added in their drinking water); HFRS+AB = high fat, HAMRS corn starch diet, antibiotic treatment; LFNRS+NAB = low fat, AIN-93M no resistant starch diet, no antibiotic treatment; and LFRS+NAB = low fat, HAMRS corn starch diet, no antibiotic treatment.
[3]LFNRS+NAB = negative control.
[4]LFRS+NAB = positive control.
[5]ECW = Empty cecum weight.
[6]ABF% = Abdominal fat percent.

Targeted bacteria

The results for standard curves of bacteria are listed in table 3.9. For *Bacteroides spp*, *Bifidobacterium spp*, and *Clostridium cluster XIVa* and *b* bacteria not all standard dilutions fell within the acceptable range for amplification of 90 (slope = -3.6) to 110% (slope = -3.0). However, some treatment replicates for rats treated with antibiotics for some genera fell below the lowest acceptable standard; and some treatment replicates from rats not treated with antibiotics fell above the highest acceptable standard. This was in line with our hypotheses for this study. To address this, the equations for the two lines, acceptable slope and inclusion of a standard that when included

gave a line with an efficiency below 90% (~80%), were used to determine the treatment replicate log CFU. The results for the samples were less than 1% different for the two equations and were considered acceptable for statistical analysis. Other options were deemed either technically not possible or not feasible. For example, greater dilutions of some treatment replicates from rats not treated with antibiotics resulted in too low amounts of DNA to promote amplification; and for extraction of some treatment replicates for rats treated with antibiotics much greater amounts of precious cecal contents would be required.

Table 3.9. Standard curves for study 1.2.

Bacteria strains	Equation	Slope	R^2	Efficiency (%)
Firmicutes				
Lactobacillus plantarum	log CFU= -0.2947*(Ct)+14.694	-3.557	0.999	91.00
Clostridium coccoides	log CFU= -0.2395*(Ct)+12.102	-3.38	0.996	97.50
Bacteroidetes				
Bacteroides fragilis	log CFU= -0.2881*(Ct)+11.785	-3.38	0.990	97.60
Actinobacteria				
Bifidobacterium longum	log CFU= -0.2215*(Ct)+9.3945	-3.553	0.996	91.20
Bacterial Domain				
16S universal	log CFU= -0.2897*(Ct)+13.815	-3.45	0.999	95.00
Escherichia coli				

Primers for *Lactobacillus spp.* and *Clostridium cluster XIVa* and *b* were initially designed for use with a Taqman® probe [67, 69], but the specificity of the probe did not allow for detection of amplification. Therefore, we successfully changed to use of the primers with SYBR green. The primers for *Lactobacillus spp.* resulted in 89% specificity and *Clostridium cluster XIVa* and *b* resulted in 60% specificity excluding chloroplasts and clones. Primers initially used for

Bifidobacterium spp.[70], exhibited two peaks in the melting curve at ~80°C that appeared to not be primer dimers based on the high temperature for melting. We changed to the primers from Matsuda et al[72]. Primers for *Bacteroides spp.* and *Bifidobacterium spp.* had 57% and 75%, specificity, respectively.

The results for targeted bacteria expressed in log CFU are shown in Table 3.10. The negative control group (LNRS+NAB) had the greatest log CFU of *Lactobacillus spp.* compared to LFRS+AB group (p<0.0333) or HFRS+AB group (p<0.0334). However, the negative control group was not significantly different from the positive control group (LFRS+NAB, p=0.1453). Also, the positive control group was not significantly different from LFRS+AB group (p=0.3512) or HFRS+AB group (p=0.2206). The log CFU of *Clostridium cluster XIVa and b* were not significantly different among the four groups. The negative control group had the lowest log CFU of *Bacteroides spp.* compared to the positive control group (p<0.0114), LFRS+AB group (p<0.0008) or HFRS+AB group (p<0.0463). However, the positive control group had similar log CFU of *Bacteroides spp.* as LFRS+AB group (p=0.2285) or HFRS+AB group (p=0.4949). The positive and negative control groups had similar (p=0.9416) log CFU of *Bifidobacterium spp.* and they both had greater log CFU compared to LFRS+AB group (LFRS+NAB, p=0.0056; LFNRS+NAB, p<0.0065) or HFRS+AB group (LFRS+NAB, p<0.0426; LFNRS+NAB, p<0.0487). Additionally, the LFRS+AB and the HFRS+AB groups had similar (p=0.4180) log CFU of *Bifidobacterium spp.* Finally, the 16S universal bacteria domain log CFU for the LFRS+AB group was greater than log CFU of the HFRS+AB group (p<0.0286) or the negative control group (p<0.0018), but not significantly different from the positive control group (p=0.0528). The negative control group had the lowest log CFU of 16S universal bacteria domain

compared to the LFRS+AB group, however it was not significantly different from the positive control group (p=0.1169) or the HFRS+AB group (p=0.1977).

Table 3.10. Bacteria genera in log CFU for study 1.2.

Bacterial Populations[2] (log CFU)	LFRS+AB	HFRS+AB	LFNRS+NAB[3]	LFRS+NAB[4]
Lactobacillus spp.	5.48±0.40b	5.20±0.49b	6.75±0.07a	6.04±0.39ab
Clostridium XIVa and b	6.36±0.34a	5.91±0.55a	6.74±0.12a	6.66±0.43a
Bacteroides spp.	5.00±0.23a	3.74±0.59a	2.35±0.56b	4.19±0.32a
Bifidobacterium spp.	2.62±0.27b	3.27±0.97b	4.95±0.54a	5.01±0.49a
16S universal	9.27±0.11a	8.83±0.14b	8.58±0.12b	8.88±0.14ab

[1]Data are presented in their original form and expressed as means ± standard error. P value< 0.05 was considered statistically significant and it is represented with different letters horizontally.
[2]Groups include: LFRS+AB = low fat, purified high amylose maize resistant starch (HAMRS) corn starch diet, and antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L added in their drinking water); HFRS+AB = high fat, HAMRS corn starch diet, and antibiotic treatment; LFNRS+NAB = low fat, amylopectin control corn starch with no resistant starch, and no antibiotic treatment; and LFRS+NAB = low fat, HAMRS corn starch diet, and no antibiotic treatment.
[3]LFNRS+NAB: Negative control group
[4]LFRS+NAB: Positive control group

GLP-1 active secretion in plasma

Previous research demonstrated that feeding resistant starch stimulates GLP-1 active secretion [30, 31]. GLP-1 active (Fig. 3.4.) measured in plasma in groups given antibiotic treatment, LFRS+AB (p<0.0121) and HFRS+AB (p<0.0076), was greater than the positive control group (LFRS+NAB). The negative control group was different from the other three groups (HFRS+AB, p<0.0001; LFRS+AB, p<0.0001; LFRS+NAB, p<0.0152).
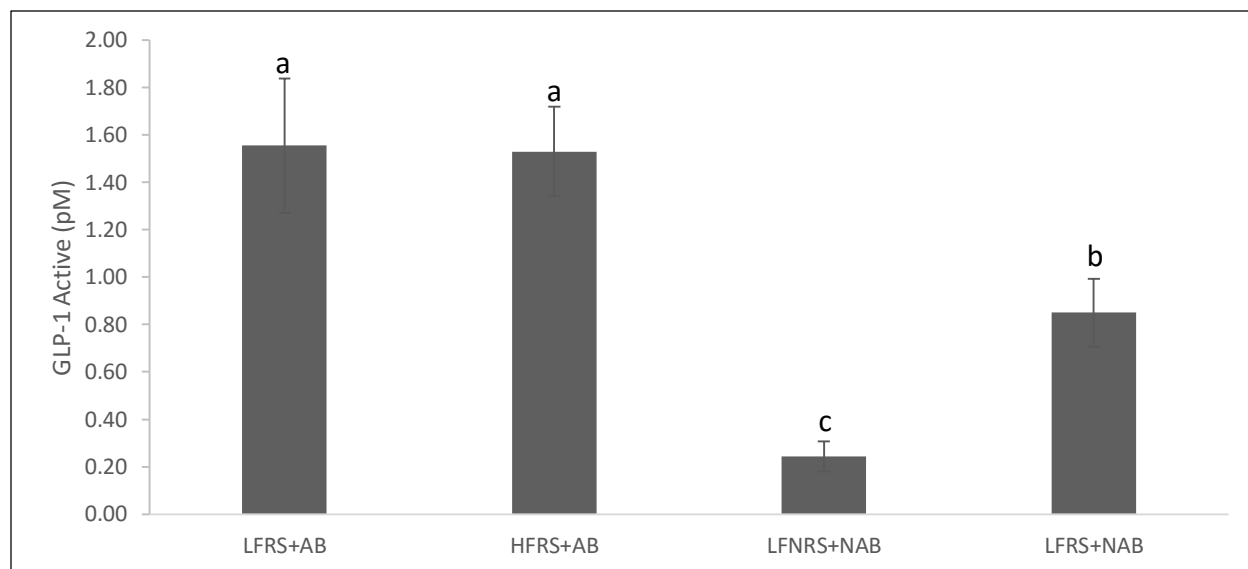
Figure 3.4. GLP-1 active for Study 1.2. Groups include: LFRS+AB = low fat, purified high amylose maize resistant starch (HAMRS) corn starch diet, and antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L added in their drinking water); HFRS+AB = high fat, HAMRS corn starch diet, and antibiotic treatment; LFNRS+NAB = low fat, amylopectin control corn starch with no resistant starch., and no antibiotic treatment; and LFRS+NAB = low fat, HAMRS corn starch diet, and no antibiotic treatment. Data are presented in their original form and expressed as means ± standard error. Different letters above columns indicate a statistically significant difference.

Resistant starch assay results

Results shown in Figure 3.5 demonstrated that antibiotic treatment completely eliminated the ability to ferment resistant starch in the cecum. Groups given antibiotic treatment, LFRS+AB ($p<0.0001$) and HFRS+AB ($p<0.0001$), had greater resistant starch in cecal contents than the positive control group (LFRS+NAB), which was about double the amount. This demonstrated that at the mechanistic, proof-of-concept dietary levels of resistant starch used in this study and previous studies about half of the resistant starch is not fermented. Resistant starch in cecal contents for groups given antibiotic treatment, LFRS+AB and HFRS+AB, were not significantly different from one another ($p<0.6897$). These results demonstrated that antibiotic treatment eliminated the ability to ferment resistant starch in the cecum of rats in both low and high fat diets.
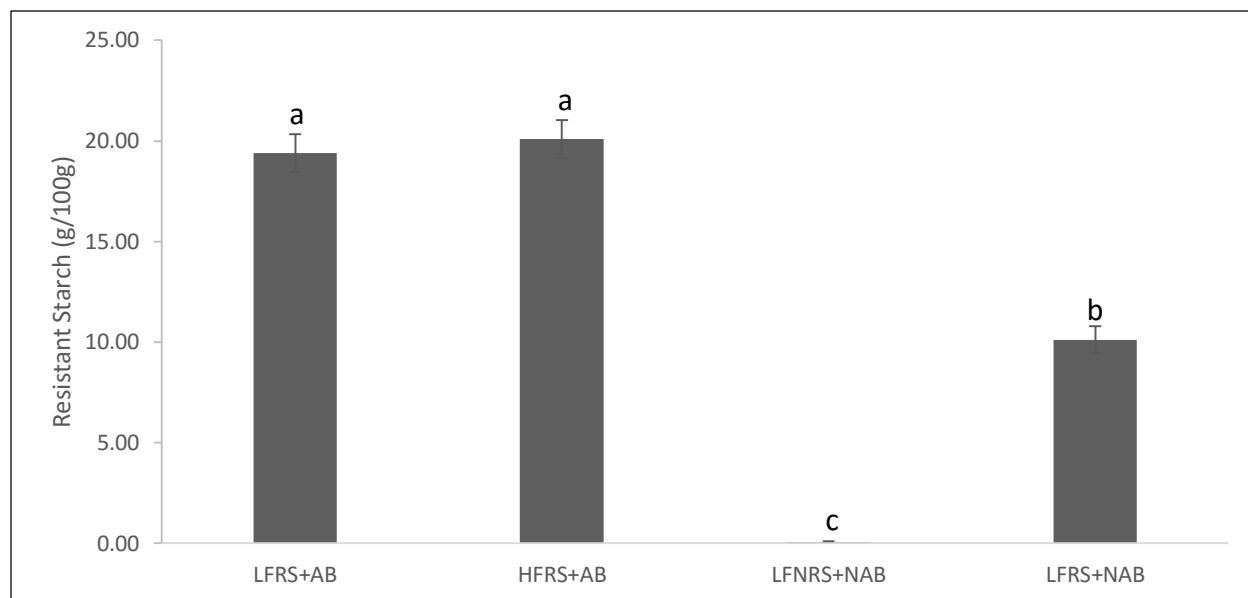
Figure 3.5. Resistant starch (g/100g) for Study 1.2. Groups include: LFRS+AB = low fat, purified high amylose maize resistant starch (HAMRS) corn starch diet, and antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L added in their drinking water); HFRS+AB = high fat, HAMRS corn starch diet, and antibiotic treatment; LFNRS+NAB = low fat, amylopectin control corn starch with no resistant starch., and no antibiotic treatment; and LFRS+NAB = low fat, HAMRS corn starch diet, and no antibiotic treatment. Data are presented in their original form and expressed as means ± standard error. Different letters above columns indicate a statistically significant difference.

Histology

In phase 1 of study 1.1 the ECW were not different, so one rat from each group was used in study 1.2 for histological examination of the cecal wall (Fig 3.6 and Table 3.10.). The cecal walls of the positive control (LFRS+NAB) and the two groups treated with antibiotics (HFRS+AB and LFRS+AB) were numerically greater than the cecal wall of the negative control (LFNRS+NAB). This gives some indication of why rats treated with antibiotics have increased weights for empty cecum weights.

A. LFRS+AB
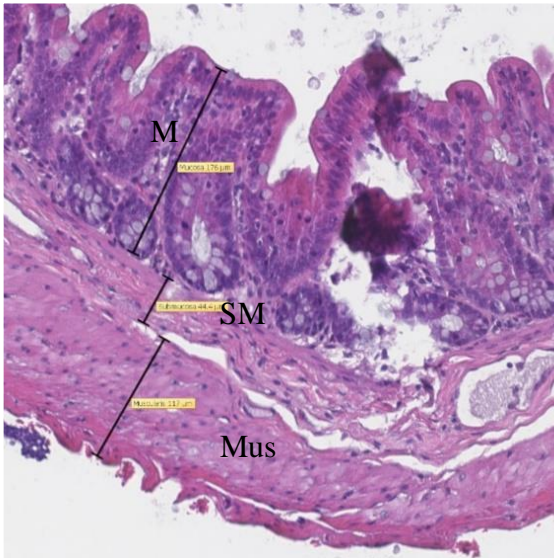
B. HFRS+AB
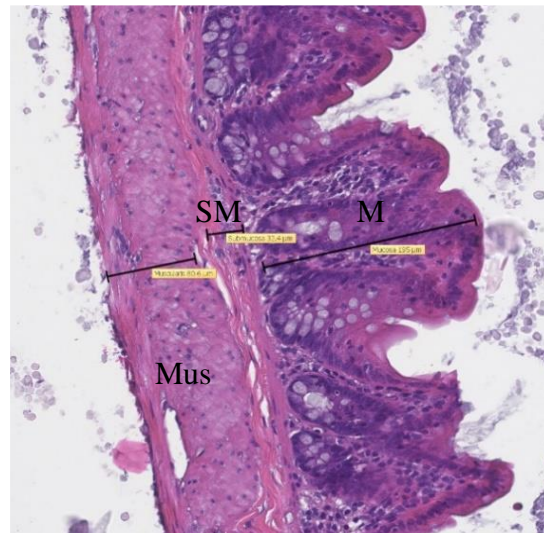
C. LFNRS+NAB
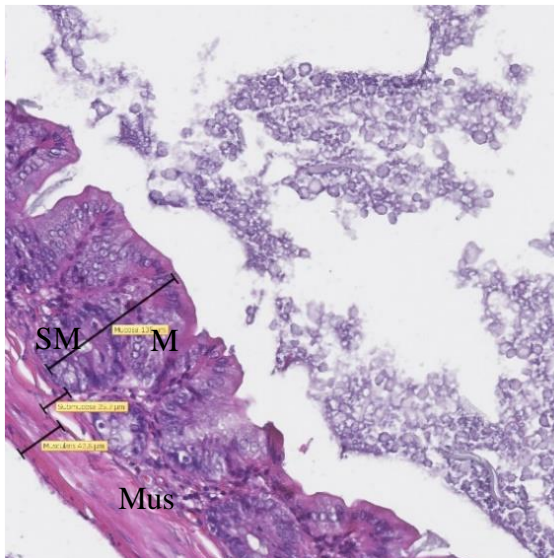
D. LFRS+NAB
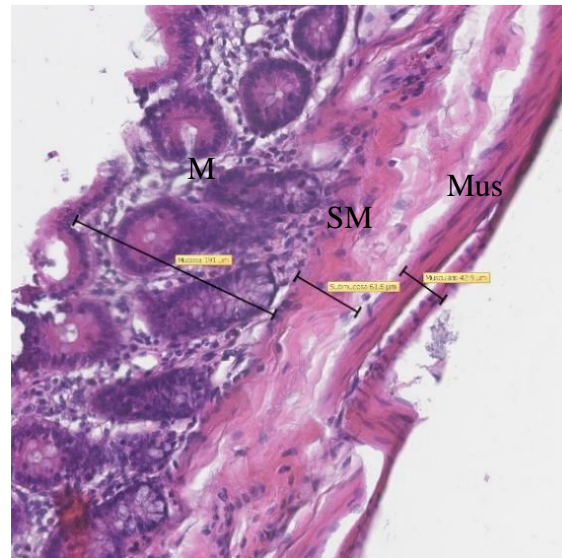
Figure 3.6. Histology of cecum wall at magnification 20X. Layers: mucosal (M), submucosal (SM), and muscularis (Mus). **A.** LFRS+AB = low fat, purified high amylose maize resistant starch (HAMRS) corn starch diet, and antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L added in their drinking water). **B.** HFRS+AB = high fat, HAMRS corn starch diet, and antibiotic treatment. **C.** LFNRS+NAB = low fat, amylopectin control corn starch with no resistant starch and no antibiotic treatment. **D.** LFRS+NAB = low fat, HAMRS corn starch diet, and no antibiotic treatment.

Table 3.10. Cecal wall measurements of mucosa, submucosa and muscularis layers for study 1.2.

Groups	LFRS+AB[1]	HFRS+AB	LFNRS+NAB[2]	LFRS+NAB[3]
Mucosa (μm)	171.741	192.381	180.430	180.630
Submucosa (μm)	20.031	42.808	26.015	63.491
Muscularis (μm)	95.283	91.668	58.593	76.069
Total (μm)	287.055	326.857	265.038	320.191

[1]Groups include: LFRS+AB = low fat, purified high amylose maize resistant starch (HAMRS) corn starch diet, and antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L diluted in their drinking water); HFRS+AB = high fat, HAMRS corn starch diet, and antibiotic treatment; LFNRS+NAB = low fat, amylopectin control corn starch with no resistant starch., and no antibiotic treatment; and LFRS+NAB = low fat, HAMRS corn starch diet, and no antibiotic treatment.
[2]LFNRS+NAB: Negative control group
[3]LFRS+NAB: Positive control group

3.4. Discussion

The results observed in this research demonstrated that antibiotic treatment with low potency antibiotics added to drinking water prior to resistant starch feeding was able to reduce bacteria, but not able to prevent subsequent fermentation of resistant starch. However, antibiotic treatment given during resistant starch feeding was able to completely reduce 100% of the fermentation of resistant starch as demonstrated by the resistant starch assay for groups given the antibiotic treatment (LFRS+AB and HFRS+AB). Previously we estimated, based on the metabolizable energy value for the HAMRS product, that the mechanistic, proof-of-concept amount of resistant starch used in our studies was 50% fermented. This was confirmed in study 1.2 as the antibiotic treatment resulted in ~two times the amount of cecal contents resistant starch. The reduction of fermentation was also reflected by cecal contents pH as groups treated with antibiotics had greater pH values than the positive control group and similar to the negative control group. The SCFA acetate is produced in much greater amounts compared to propionate and butyrate and appears to be the major reason for the lower cecal contents pH for the positive control group.

Antibiotic treatment resulted in a reduction of *Lactobacillus spp.* and *Bifidobacterium spp.* However, the combination of resistant starch and antibiotic treatment in study 1.2 with either low or high fat diets maintained similar amounts of *Bacteroides spp.* as the positive control group, LFRS+NAB . Additionally, use of primers predominantly for *Clostridium cluster XIVa and b* bacteria were not knocked down for groups treated by low potency antibiotics. Interestingly, the antibiotic treatment with the low fat diet was not different from the positive control group regarding total bacteria reflecting Bacteroides spp. (with *Prevotella spp.* and *Poryphromonas spp.*) and likely other bacteria not measured. Similarities in *Bacteroides spp.* were also reflected in similar production of propionate as *Bacteroides spp.* are major producers of propionate. Thus, the low potency antibiotics used in the current studies appear to cause a shift in bacterial populations, rather than a reduction of bacteria in general.

The results observed in study 1.2 for the targeted bacteria match the results observed for SCFAs production. *Bifidobacterium spp* and *Lactobacillus spp.* bacteria fermentation products are acetate and lactate. With low potency antibiotic treatment acetate production for both groups given antibiotics (LFRS+AB and HFRS+AB) was diminished compared to the positive control group (LFRS+NAB). Lactate was not measured in this study. In addition, *Clostridium cluster XIVa and b* use acetate and lactate to produce butyrate. In this case *Clostridium cluster XIVa and b* bacteria were not reduced by the low potency antibiotic treatment, but groups given antibiotics might not have adequate acetate and lactate to produce levels of butyrate with feeding of resistant starch. This resulted in low butyrate production for groups given antibiotics with levels even lower than the negative control group. However propionate production for the group fed resistant starch in a low fat diet and given antibiotics had the greatest numerical amounts that were statistically similar to those for the positive control group. The positive control group had propionate levels that fell

36

between the two groups treated with antibiotics and statistically similar with both groups. This reflects the results for *Bacteroides spp.* and means that the low potency antibiotics treatment allows these species to feed on the resistant starch granule as they normally do without antibiotics.

In phase 1 from study 1.1, we expected a greater ECW for RS+NAB group than NRS+AB group due to previous data from our lab demonstrating increased ECW for rats fed resistant starch [31, 66]. But ECW for the groups were not significantly different, even though one group was fed resistant starch. Also, in study 1.2 it was observed again that groups given antibiotic treatment had greater ECW than the negative control group (LFNRS+NAB) and similar to the positive control group (LFRS+NAB). These results are likely the consequence of antibiotic treatment as another study reported cecal dilation and increased osmotic activity of the cecal contents in rodents treated with antibiotics [73]. This was partially confirmed by histology examination with numerically greater cecal cell wall size for a rat from each of the two antibiotic treatment groups and a rat from the positive control group compared to the negative control group.

Previous research done in our lab demonstrated that high fat in the diet partially attenuates the ability to ferment resistant starch [66]. In study 1.2, we demonstrated that resistant starch in low or high fat diets was not fermented in the cecum of the large intestine in the presence of low potency antibiotics. For study 1.2 we had two alternative hypotheses. The first was that the antibiotics should reduce fermentation. The second was that these low potency antibiotics may actually improve the fermentation of resistant starch. Cani et al. demonstrated a decrease in gram negative bacteria and decreased inflammatory endotoxemia with these antibiotics [51]. However, in our study, fermentation was knocked out regardless of the level of fat in the diet.

Low potency antibiotics also stimulated increased production of GLP-1 in our study. Similarly, other studies had reported that antibiotics stimulate over-secretion of GLP-1 active from intestinal cells [74, 75]. In like manner, other researchers had reported that vancomycin and bacitracin improved insulin resistance because of augmentation of GLP-1 secretion even in rodents that had diet-induced obesity [76].

Limited histological examination in study 1.2 revealed some explanation for why antibiotic treatment might increase the ECW in rats. The muscularis layer may be increased with antibiotics and the submucosa layer decreased, but the overall height of the cecal wall appears to be somewhat greater than the negative control and less than the positive control. Other factors such as amount of water in the cecal wall, not measured in this study, may be responsible for the lack of difference for ECW between groups treated with antibiotics and the positive control group. Other studies have demonstrated similar results of antibiotics on cecum weight [73]. This effect of antibiotics on ECW means that this measure of fermentation cannot be used to document fermentation when antibiotics are used, but researchers can still rely on cecal contents pH and production of acetate and butyrate.

Finally, low potency antibiotics such as neomycin and ampicillin were able to reduce fermentation of resistant starch when they are given at the same time as the feeding of the resistant starch. This means that stronger potency antibiotics that come with possible greater risks of side effects are not necessary to reduce fermentation of resistant starch when they are given at the same time. It is also encouraging that feeding resistant starch without probiotic treatments appears to promote almost full recovery of the targeted bacteria after treatment with antibiotics as demonstrated by improvements in fermentation markers. However, treatment with more potent antibiotics may cause more damage to the microbiota and require probiotic treatment. Future

studies are warranted for expanding our knowledge on recovery of the microbiota after antibiotic treatments. One of our main objectives was to determine if we could reduce fermentation of resistant to be able to demonstrate effects of feeding resistant starch beyond the effects of fermentation. However, increased empty cecum weights and increased GLP-1 active are a concern for the future use of antibiotics for reduction of fermentation. Other methods such as bacteriophages with lytic peptides that kill specific bacteria may be more targeted and not have such broad effects as the use of antibiotics [77].

CHAPTER 4: GENE EXPRESSION FOR INTESTINAL GLUCONEOGENESIS ENZYMES IN THE FED AND FASTED STATE AFTER RESISTANT STARCH FEEDING

4.1. Introduction

Resistant starch is a fermentable fiber that lowers the glycemic index of the diet because it resists digestion in the small intestine [78]. It is fermented in the large intestine by bacteria that produce short chain fatty acids (SCFAs) mainly as acetate, propionate, and butyrate [16]. Previously, we showed that resistant starch can decrease body fat accumulation, increase fat oxidation, increase glucagon-like peptide 1 (GLP-1) cecal gene expression and plasma levels, up-regulate peptide YY (PYY) gene expression and plasma levels, and affect expression of other genes [29, 30, 79]. GLP-1 is an incretin that increases metabolic activity and improves insulin sensitivity [80]. Robertson et al. showed improved insulin sensitivity in humans with [81] and without increased GLP-1 compared to control subjects [18]. Some people do not produce as much GLP-1 as others due to a defective transcription factor [82]. Therefore, it is important to study how insulin sensitivity can be improved without increased GLP-1.

Animal studies consistently have shown increases of GLP-1 active and proglucagon gene expression in response to resistant starch feeding [29, 31, 83]. On the other hand, the majority of human studies have not shown greater concentrations of endogenous GLP-1 in response to resistant starch feeding [34, 52, 84]. These outcomes in human studies might be due to the resistant starch feeding time lasting only a few hours; which is different from animal studies where the consumption of resistant starch lasts for weeks. However, beneficial effects of feeding resistant starch have been observed in humans that include improvement in insulin sensitivity [34, 52], increased insulin secretion [85], and increased satiety [86]. Additionally, resistant starch has shown improved insulin sensitivity in the periphery (adipose and muscle) but not reduced hepatic glucose production in subjects at risk of developing type 2 diabetes [33].

Previously, the intestine has been described as a gluconeogenic organ due to its endogenous glucose production capacity [87]. Therefore, intestinal gluconeogenesis (IGN) might have the ability to decrease hunger and food intake [88]. It is proposed that certain diets such as protein-enriched [88-90], fiber-enriched [54], or certain procedures such as gastric bypass surgery [91] can trigger IGN. De Vadder et al. demonstrated IGN increased as a result of fermentation of fructo-oligosaccharide (FOS) to butyrate, and propionate. IGN (presence of glucose in the portal blood) leads to signaling from the gut to the brain to reduce hepatic gluconeogenesis for better glycemic control and insulin sensitivity [54]. Our objective was to determine if feeding resistant starch would result in increased IGN gene expression. To accomplish this objective we conducted two studies and tested gene expression for proglucagon (GCG), pyruvate carboxylase (PCase), and glucose-6-phosphatase (G6Pase). Proglucagon was used as a positive control by contrasting its gene expression with plasma levels of GLP-1 active found in both studies. PCase is a mitochondrial non-regulatory enzyme involved in gluconeogenesis that catalyzes the carboxylation of pyruvate to form oxaloacetate the first step of IGN in the mitochondria [92]. G6Pase is a key regulatory enzyme catalyzing the last step of gluconeogenesis to hydrolyze glucose-6-phosphate into glucose and P_i in the cytoplasm [93]. Much more is known about gluconeogenesis in the small intestine [93]. However, De Vadder et al. have reported increased gluconeogenesis in the large intestine in mice fed FOS. Our goal was to determine if there was increased gluconeogenesis in response to feeding resistant starch as either a pure starch or in whole grain flour. In GK rats we investigated the fasted state (saline injection) and modeled the fed state with injection of insulin. In study 2.1, fermentation indicators of resistant starch such as cecal contents pH and SCFAs production are reported in Goto-Kakizaki (GK) rats. In study 2.2, Sprague Dawley rats fed either moderate or high fat diets, as part of a larger study that will be reported later, were used in the fed state to

determine if there was increased gene expression for IGN. In both studies GCG, PCase and G6Pase gene expression were assessed.

4.2. Research Design and Methods
Animals and diets

For both studies, male rats were purchased at 6 weeks old and maintained in quarantine for 1 week. For the studies, rats were individually housed in wire bottom cages in a climate-controlled environment (21-22°C, 55% humidity) with a 12:12 hour light-dark cycle. Rats were fed *ad libitum* and had free access to water. Body weight, food intake and food spillage were measured twice a week.

Study 2.1. Protocol was approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee. Forty eight (n=48), GK rats were purchased from Charles River Laboratories International Inc. (Wilmington, MA). After quarantine, the GK rats were fasted the night before blood draw for ~ 12 hours after allowing access to food for three hours in the dark cycle (7-10 pm). Blood samples were obtained through retro-orbital bleeding, using inhalation of 5% isoflurane as anesthesia, for measuring glucose and insulin values using AlphaTRAK glucometer (Abbott Laboratories Inc., IL) and insulin kit (Millipore, MA), respectively. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated with the measurements obtained by the following formula:

$$HOMA - IR = \frac{fasting\ plasma\ glucose\ \left(\frac{mg}{dl}\right) \times plasma\ insulin\ (\frac{\mu U}{ml})}{2430}$$

Then 12 rats each were designated to one of the four isocaloric diets (3.23 kcal/g) based on their weight and HOMA-IR. The study lasted 12 weeks and the diets were designated by their

major ingredient: (1) Amylopectin control corn starch with no whole grain or resistant starch (CON), (2) isolated high amylose maize resistant starch (HAMRS) corn starch (RS), (3) whole grain waxy corn flour with low resistant starch (WG), and (4) whole grain HAMRS corn flour (WG+RS). The composition of the four diets used is listed in Table 4.1. Purified non-fermentable cellulose (Dyets, PA) was used to dilute the energy density of the control diets, non-whole grain and whole grain, to produce isocaloric control diets as high resistant starch diet ingredients have a lower metabolizable energy than amylopectin starch in isolated starch and waxy whole grain corn flour [17]. Before diets were formulated, the starch and whole grain ingredients were analyzed by proximate analysis and modified Englyst [94] assay to determine macronutrient and resistant starch content, respectively. Casein was reduced from AIN-93M amount (140 g/kg) based on the protein content in the major ingredient of each diet as analyzed by proximate analysis by Medallion Labs for Ingredion Incorporated. Also, corn oil was 10% higher than AIN-93M amount (40 g/kg) based on the fat content in whole grain HAMRS corn flour.

Table 4.1. Diets composition for Study 2.1.

Ingredients	CON[1]	RS	WG	WG+RS
	Grams	Grams	Grams	Grams
Waxy corn starch[2]	536.80	97.66	85.98	164.50
High-amylose corn starch[3]	0.00	576.00	0.00	0.00
Whole grain high-amylose corn flour[4]	0.00	0.00	0.00	572.00
Waxy whole grain flour[5]	0.00	0.00	550.00	0.00
Sucrose	100.00	100.00	100.00	100.00
Casein[6]	136.00	132.27	95.42	74.80
Cellulose	135.00	7.00	97.20	40.50
Corn Oil[7]	44.00	38.87	23.20	0.00
Mineral mix	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00
Choline chloride	1.40	1.40	1.40	1.40
L-Cystine	1.80	1.80	1.80	1.80

(Table 4.1. Con'd)

Ingredients	CON[1]	RS	WG	WG+RS
	Grams	Grams	Grams	Grams
Total	1000.00	1000.00	1000.00	1000.00
Total energy (kcal)	3.24	3.24	3.24	3.24
% RS[8]	1.00	26.47	6.10	26.79

[1]Groups include: CON= Amylopectin control corn starch with no whole grain or resistant starch, RS= isolated high amylose maize resistant starch (HAMRS) corn starch, WG= whole grain waxy corn flour with low resistant starch, and WG+RS = whole grain HAMRS corn flour.

[2]AMIOCA® corn starch

[3]HI-MAIZE® resistant corn starch

[4]HI-MAIZE® whole grain corn flour

[5]Waxy corn starch, high-amylose corn starch, whole grain high-amylose corn flour and waxy whole grain were all gifts from Ingredion Incorporated (Bridgewater, NJ).

[6]Casein was reduced from AIN-93M amount (140 g/kg) based on the protein in the AMIOCA® corn starch, HI-MAIZE® resistant corn starch, HI-MAIZE® whole grain corn flour, and waxy whole grain as analyzed by proximate analysis by Medallion Labs for Ingredion Incorporated.

[7]Corn oil was increased from AIN-93M amount (40 g/kg) to 44 g/kg based on the fat in the HI-MAIZE® whole grain corn flour, and waxy whole grain flour as analyzed by proximate analysis by Medallion Labs for Ingredion Incorporated. And corn oil was adjusted based on fat in the main ingredients.

[8]Resistant starch content of the four experimental starch ingredients determined by Ingredion Incorporated using the modified Englyst assay [94].

Study 2.2. Protocol was approved by the Louisiana State University Institutional Animal Care and Use Committee. Sprague Dawley rats (n=96) were purchased from Harlan Laboratories Inc. (Indianapolis, IN). After quarantine twelve rats were assigned into one of the four isocaloric diets for high fat content (4.18±0.02 kcal/g, 42.48%±0.17 of total energy) or into one of the four isocaloric diets for moderate fat content (3.76±0.002 kcal/g, 30.10%±0.04 of total energy) based on their body weight. The diets, within high fat or moderate fat content, were designated by the major ingredient the same as in study 2.1 as: (1) CON, (2) RS, (3) WG, and (4) WG+RS. The composition of the eight diets used is listed in Table 4.2.

Table 4.2. Diets composition for Study 2.2.

Ingredients	MODERATE FAT			
	CON[1]	RS	WG	WG+RS
	Grams	Grams	Grams	Grams
Waxy corn starch[2]	473.30	72.31	63.83	137.74
High-amylose corn starch[3]	0.00	524.00	0.00	0.00
Whole grain high-amylose corn flour[4]	0.00	0.00	0.00	520.00
Waxy whole grain flour[5]	0.00	0.00	500.00	0.00
Sucrose	100.00	100.00	100.00	100.00
Casein[6]	136.00	133.12	99.42	80.56
Cellulose	115.00	0.00	81.00	27.00
Corn Oil[7]	85.00	79.87	65.05	44.00
Lard	42.50	42.50	42.50	42.50
Mineral mix	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00
Choline cloride	1.40	1.40	1.40	1.40
L-Cystine	1.80	1.80	1.80	1.80
Total	1000.00	1000.00	1000.00	1000.00
Total energy (kcal)	3.76	3.75	3.76	3.76
% RS[8]	0.42	23.37	4.93	23.45

Ingredients	HIGH FAT			
	CON[1]	RS	WG	WG+RS
	Grams	Grams	Grams	Grams
Waxy corn starch[2]	405.80	0.00	0.00	77.85
High-amylose corn starch[3]	0.00	524.66	0.00	0.00
Whole grain high-amylose corn flour[4]	0.00	0.00	0.00	525.00
Waxy whole grain flour[5]	0.00	0.00	517.00	0.00
Sucrose	100.00	100.00	100.00	100.00
Casein[6]	136.75	133.70	98.74	80.58
Cellulose	110.00	0.00	56.91	10.00
Corn Oil[7]	99.25	93.44	79.15	58.37
Lard	100.00	100.00	100.00	100.00
Mineral mix	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00
Choline cloride	1.40	1.40	1.40	1.40
L-Cystine	1.80	1.80	1.80	1.80
Total	1000.00	1000.00	1000.00	1000.00
Total energy (kcal)	4.16	4.14	4.23	4.21

(Table 4.2. Con'd)

Ingredients	HIGH FAT			
	CON[1]	RS	WG	WG+RS
	Grams	Grams	Grams	Grams
% RS[8]	0.36	23.32	5.04	23.41

[1]Groups include: CON= Amylopectin control corn starch with no whole grain or resistant starch, RS= isolated high amylose maize resistant starch (HAMRS) corn starch, WG= whole grain waxy corn flour with low resistant starch, and WG+RS= whole grain HAMRS corn flour.
[2]AMIOCA® corn starch
[3]HI-MAIZE® resistant corn starch
[4]HI-MAIZE® whole grain corn flour
[5]Waxy corn starch, high-amylose corn starch, whole grain high-amylose corn flour and waxy whole grain flour were all gifts from Ingredion Incorporated (Bridgewater, NJ).
[6]Casein was reduced from AIN-93M amount (140 g/kg) based on the protein in the AMIOCA® corn starch, HI-MAIZE® resistant corn starch, HI-MAIZE® whole grain corn flour, and waxy whole grain as analyzed by proximate analysis by Medallion Labs for Ingredion Incorporated.

[7] In moderate fat (30% of energy) diets, one-third of the fat was lard and two-thirds was corn oil. For high fat (42.48% of energy) diets one-half of the fat was lard and the other half was corn oil. Based on the proximate analysis of AMIOCA® corn starch, HI-MAIZE® resistant corn starch, HI-MAIZE® whole grain corn flour, and waxy whole grain flour as analyzed by proximate analysis by Medallion Labs for Ingredion Incorporated, the corn oil was reduced.

[8]Resistant starch content of the four experimental starch ingredients determined by Ingredion Incorporated using the modified Englyst assay [94].

Procedures

Study 2.1. During week 8, fed state blood samples were collected by retro-orbital bleeding to measure GLP-1 active with ELISA kit (ALPCO, NH). At week 10 another fasted blood collection was performed in order to repeat calculations for HOMA-IR. After 12-weeks GK rats were fasted again for euthanasia. Fifteen minutes prior to euthanasia the next day, 6 rats per treatment were given an intraperitoneal (IP) injection of saline solution (fasted state) and the other 6 rats were injected with 1.00 unit/kg of insulin to model the fed state.

In studies 2.1 and 2.2, rats were euthanized by cardiac puncture using inhalation of isoflurane soaked cotton balls as anesthesia in a bell jar. Fat pads (peritoneal, retroperitoneal, and epididymal) were removed and weighed for total abdominal fat percentage (ABF %) calculation. The gastro-intestinal (GI) tract was removed from esophagus to anus and weighed after removal of mesenteric fat. Emboweled weight for ABF% was obtained after subtraction of GI contents from body weight. Cecal contents and cecal epithelial cells, collected by scraping, were frozen in liquid nitrogen to analyze later. For studies 2.1 and 2.2, gene expression for GCG, G6Pase and PCase enzymes for IGN were measured, but that is the only data reported here for study 2.2.

Cecal contents pH and short-chain fatty acid analysis

For study 2.1, cecal contents were thawed and homogenized in distilled water (0.5 g wet sample to 5 ml of water), for pH measurements. Next, each sample was acidified with 1 ml of a 25% (w/w) solution of metaphosphoric acid containing 2 g/L 2-ethyl-butyric acid as an internal standard for SCFA contents. Solids were separated by centrifugation and filtration. The supernatant was transferred to a GC auto-sampler vial. Concentrations of SCFA were quantitatively determined by gas chromatography by a method described in a previous publication from our laboratory [66].

Measurement of mRNA expression

Approximately, 20 to 30 mg of cecal epithelial cells were cut using a super-cold (dry ice) blade for each rat's sample and placed into an ice cold 2 ml Lysing Matrix D tube (MP Biomedicals, OH) with 1.4 mm ceramic spheres and 600 µl RLT lysis buffer from RNeasy Mini Kit (Qiagen, Germany). Immediately, the tubes with the epithelial cells, lysis buffer and ceramic spheres were homogenized using a FastPrep®-24 Instrument (MP Biomedicals, OH) for 30

seconds at speed setting 6.0 m/s. The lysate was centrifuged for 3 minutes at 14,000 rpm, and total RNA was extracted from the supernatant by following the RNeasy Mini Kit protocol. RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (Wilmington, DE) and diluted to 40 ng/ µl. The gene transcription for GCG as positive control, PCase, and G6Pase for both studies, were determined using real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) using ABI PRISM 7900 Sequence Detection System, and SDS 2.4 software (Life Technologies, NY). Standard curves were produced for each mRNA by pooling aliquots of samples for cycles to threshold versus RNA amount. The results were expressed as a ratio to the expression of cyclophilin (CYC). The sequences of TaqMan probes and primers for cyclophilin (GenBank accession no. M15933) were: (5'-3') forward primer, CCCACCGTGTTCTTCGACAT; reverse primer, TGCAAACAGCTCGAAGCAGA; and probe, CAAGGGCTCGCCATCAGCCG. The probe and primers for proglucagon, G6Pase, and PCase were from Life Technologies (Foster City, CA). Each sample was tested in triplicate. The real-time RT-PCR conditions for all genes were 48°C for 30 min, 95°C for 15 s and 60°C for 1 min for 40 cycles.

Statistical Analysis

Data for both studies were analyzed using Statistical Analysis Software SAS® version 9.3. Equal variance, normal distribution, and possible outliers were tested by an UNIVARIATE procedure used in the MIXED procedure. Food intake, HOMA-IR at week 10, and GLP-1 data sets from study 2.1 were analyzed and statistical differences among groups were determined by a 2 x 2 factorial followed by F-protected LSD post-hoc mean comparison tests using the MIXED procedure. A $p < 0.05$ was considered statistically significant. The two factors were resistant starch (R, low or high), and whole grain (W, + or -). These data were collected before insulin injection. Only HOMA-IR was log 10 transformed from original data for statistical analyses due to non-

normal distribution (w<0.05) according to Shapiro-Wilk test. Data variables from study 2.1 collected at euthanasia were analyzed as 2 x 2 x 2 factorials. The three factors were resistant starch (R, low or high), whole grain (W, + or -), and injection (I, insulin or saline). Outliers were detected and removed for GLP-1 active (CON: 1.51, 1.97; RS: 2.52; WG: 1.49; WG+RS: 0.31), pH (WG+RS: 7.31), acetate (RS: 0.89; WG: 0.21), propionate (RS: 0.15; WG: 0.03), GCG:CYC (CON: 3.99), G6Pase:CYC (CON: 19.21, 6.93; RS: 3.50; WG: 0.06; WG+RS: 7.97). Acetate, propionate, butyrate, GCG:CYC, PCase:CYC, and G6Pase:CYC dependent variables were log 10 transformed from original data for statistical analyses due to non-normal distribution (w<0.05) according to Shapiro-Wilk test. GLP-1 active, ABF%, cecal contents pH, and empty cecum weight (ECW) data sets were not log 10 transformed. Data for analysis of IGN for study 2.2 reported here were a 2 x 2 x 2 factorial and the variables were R (low or high), W (+ or -), and Fat (F, moderate or high). GCG:CYC, PCase:CYC, and G6Pase:CYC dependent variables were log 10 transformed for statistical analyses because they were not normally distributed (w<0.05) according to Shapiro-Wilk test from original data. All factorial data were followed by F-protected LSD post-hoc mean comparison tests. Data are presented in their original form and expressed as means ± standard error. Correlations were performed using the CORR procedure.

4.3. Results
Study 2.1.

Food intake, HOMA-IR, and GLP-1 Active

Food intake (Fig 4.1.) over the study was increased by presence of dietary whole grain (p<0.0348) in the diet. WG and WG+RS groups registered the highest consumptions and they were not significantly different (p=0.7576) from one another. No significant effect for dietary resistant starch (p=0.2886) or interaction effect of dietary whole grain*dietary resistant starch (p=0.1374)

were documented. CON group consumed less food compared to WG (p<0.0123) and WG+RS (p<0.0263) groups. Additionally, the RS group approached a significant increase in food intake from CON group (p=0.0741). The RS group was not significantly different from WG (p=0.4391), and WG+RS (p=0.6405) groups.
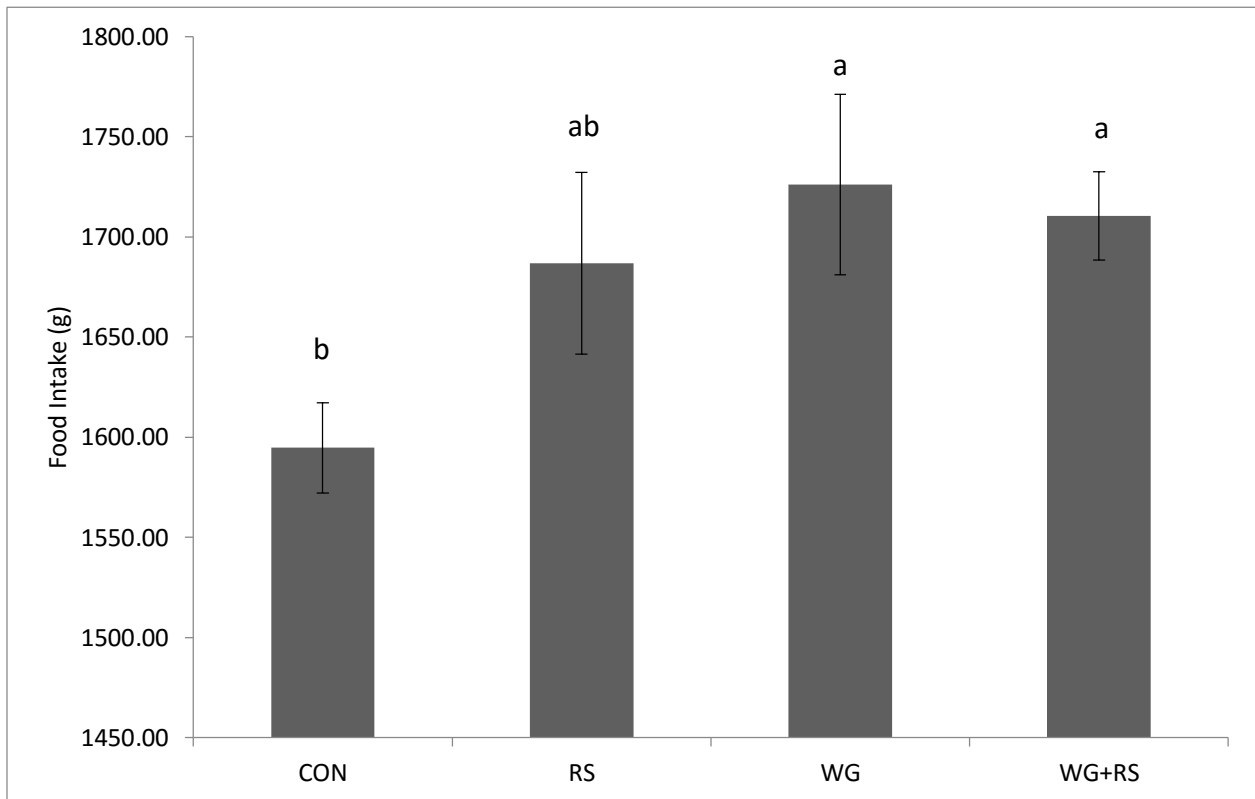

Figure 4.1. Food Intake for Study 2.1. Groups include: CON = Amylopectin control corn starch with no whole grain or resistant starch, RS = isolated HAMRS corn starch, WG = whole waxy corn flour with low RS, and WG+RS = whole grain HAMRS corn flour. Data are presented in their original form and expressed as means ± standard error. Different letters above columns indicate a statistically significant difference p<0.05 for a 2 x 2 factorial. A main effect of W (p<0.0348) was observed.

There was a main effect of feeding high resistant starch (p<0.0001) as dietary high resistant starch resulted in lower HOMA-IR (greater insulin sensitivity in fasted state) for RS and WG+RS groups compared to the control groups (CON, WG). However, WG+RS had a greater value than RS (p<0.0014) as a result there was a whole grain effect (p<0.0057) dominated by high resistant starch in whole grain diet and resistant starch effect (p<0.0001). This resulted in an interaction for

50

wholegrain*resistant starch (p=0.0628) approaching significance. CON and WG groups had the highest HOMA-IR and were not significantly different (p=0.4839) from each other (Fig. 4.2).
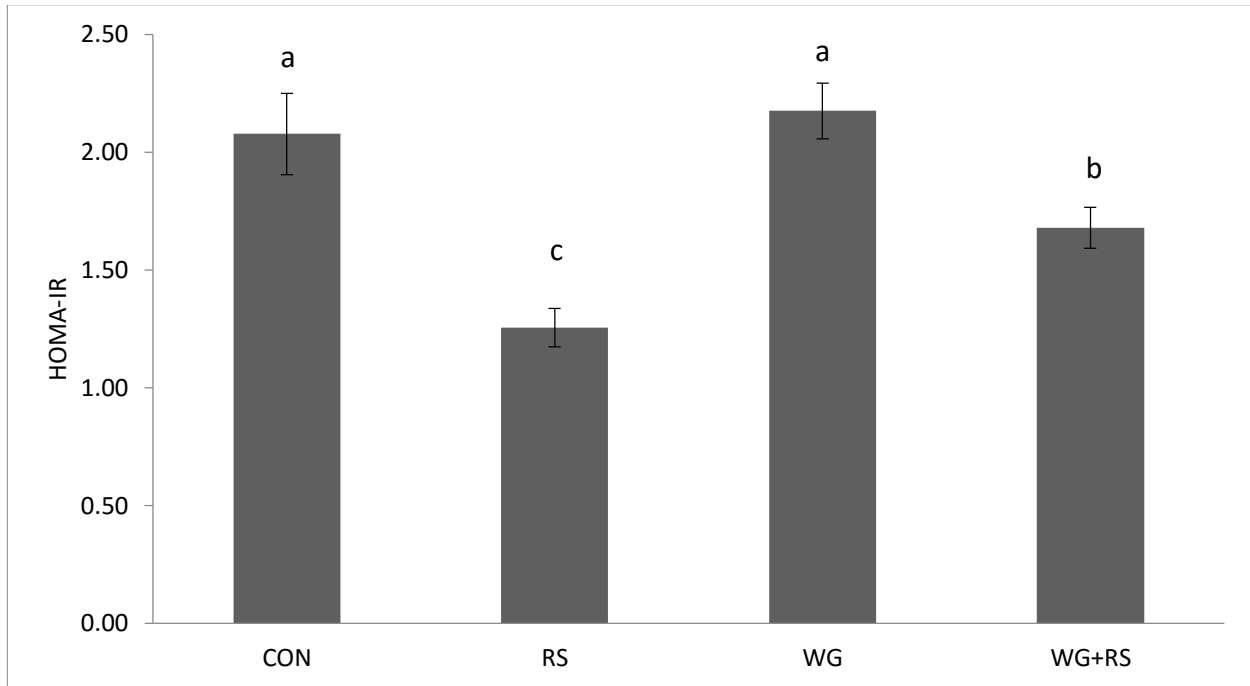

Figure 4.2. HOMA-IR for Study 2.1. Groups include: CON = Amylopectin control corn starch with no whole grain or resistant starch, RS = isolated HAMRS corn starch, WG = whole waxy corn flour with low RS, and WG+RS = whole grain HAMRS corn flour. Data are presented in their original form and expressed as means ± standard error. Different letters above columns indicate a statistically significant difference p<0.05. Data are shown on two factors, resistant starch (R, low or high) and whole grain (W, + or -) from 2 x 2 factorial. Main effects for W (p<0.0057) and R (p<0.0001), but no interaction for W*R (p=0.0628) were documented.

GLP-1 active (Fig 4.3.) measured in the fed state demonstrated a resistant starch effect (p<0.0001) as the RS and WG+RS groups fed high resistant starch were greater compared to CON and WG groups. There was no effect of whole grain (p=0.5929) as WG+RS was no different than RS (p=0.4876) and the control groups CON and WG were the lowest and not significantly different (p=0.5873) from one another. This also resulted in no interaction of whole grain*resistant starch (p=0.4179). GLP-1 active was negatively correlated with HOMA-IR (p=0.0361).
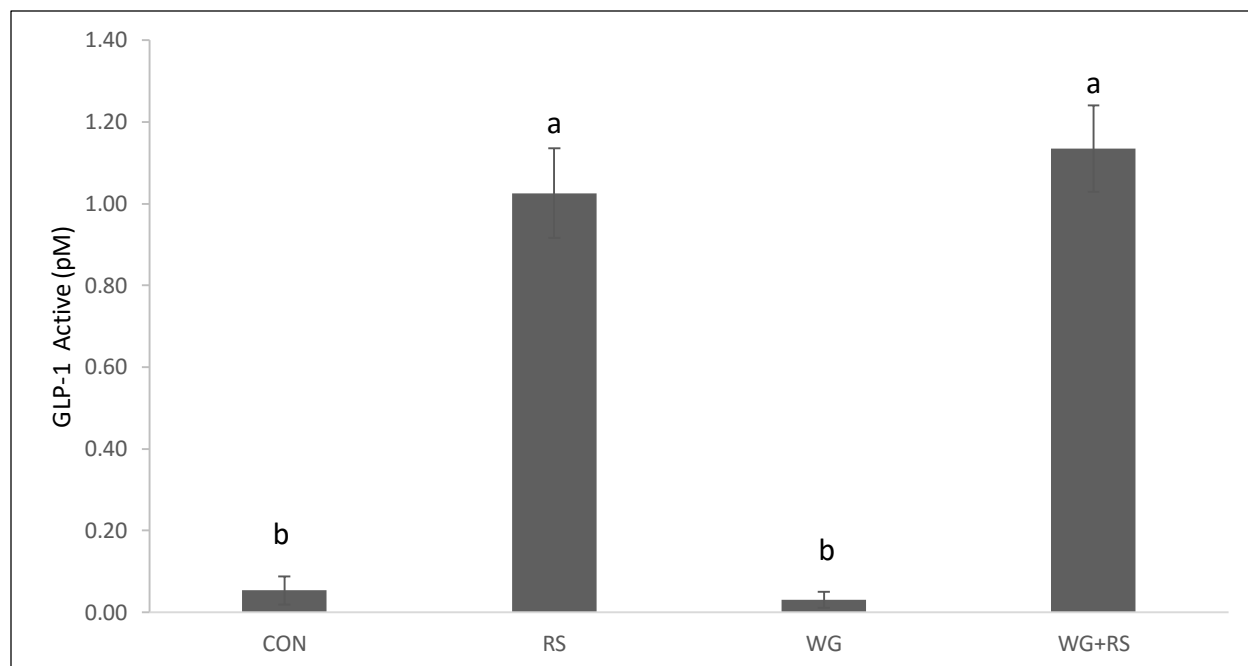
Figure 4.3. GLP-1 active for Study 2.1. Groups include: CON = Amylopectin control corn starch with no whole grain or resistant starch, RS = isolated HAMRS corn starch, WG = whole waxy corn flour with low RS, and WG+RS = whole grain HAMRS corn flour. Data are presented in their original form and expressed as means ± standard error. Different letters above columns indicate a statistically significant difference 0.05. Data are shown on two factors, resistant starch (R, low or high) and whole grain (W, + or -) from 2 x 2 factorial. Main effect for R ($p<0.0001$) was observed.

Abdominal fat percent (ABF %), cecal contents pH, SCFAs

For ABF % there was a main effect of dietary resistant starch ($p<0.0001$), and interaction effects for dietary whole grain*dietary resistant starch ($p<0.0119$) and dietary whole grain*injection ($p<0.0327$). The significant effect for resistant starch is the result of the RS group having the lowest value of all groups with saline injection, and RS group having a numerically lower value than WG and WG+RS and significantly lower than CON with insulin injection. The whole grain*resistant starch interaction was the result of the WG+RS group having a higher value than RS group with saline injection. The significant whole grain*injection interaction was the result of the WG+RS and WG groups having numerically lower values with the insulin injection compared with saline injection (Fig. 4.4.).
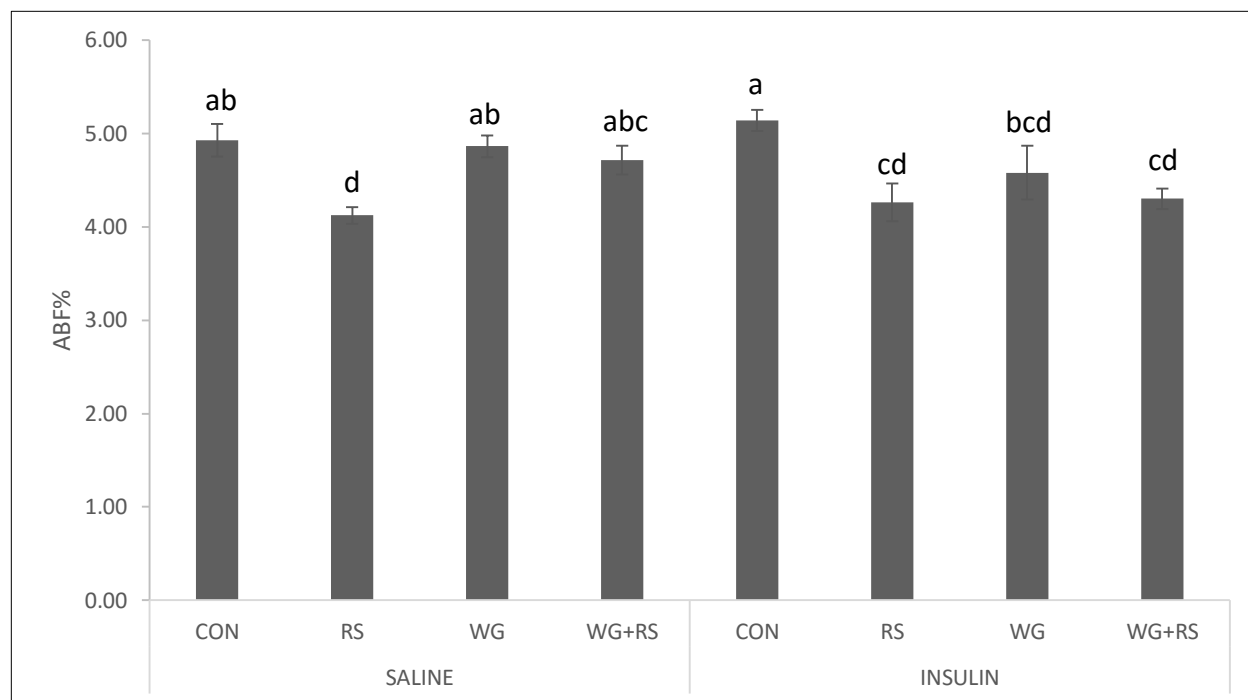
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Figure 4.4. Abdominal fat percent (ABF %) for Study 2.1. Groups include: CON = Amylopectin control corn starch with no whole grain or resistant starch, RS = isolated HAMRS corn starch, WG = whole grain waxy corn flour with low resistant starch, and WG+RS = whole grain HAMRS corn flour. Data are presented in their original form and expressed as means ± standard error. Different letters above columns indicate a statistically significant difference at p<0.05. Data are shown on three factors, resistant starch (R, low or high), whole grain (W, + or -), and Injection (I, insulin or saline) for a 2 x 2 x 2 factorial. A main effect for R (p<0.0001), and interactions for W*R (p<0.0119) and W*I (p<0.0327) were documented.

For cecal contents pH (Fig. 4.5) there were significant main effects for dietary whole grain (p<0.0001), dietary resistant starch (p<0.0001), and injection (p<0.0089), as well as interaction effects of dietary whole grain*dietary resistant starch (p<0.0001), dietary whole grain*injection (p<0.0027) and dietary whole grain*dietary resistant starch*injection (p<0.0048). The resistant starch effect occurred because the lowest cecal contents pH was for the RS group with both saline and insulin injections. The whole grain main effect and interaction effects are the result of the WG+RS group having lower cecal contents pH than the two control groups (CON, WG), but not the RS group with the insulin injection. Additionally, the WG control group had lower pH than the isolated starch group CON with both types of injections (insulin p<0.0363, saline p<0.0095).
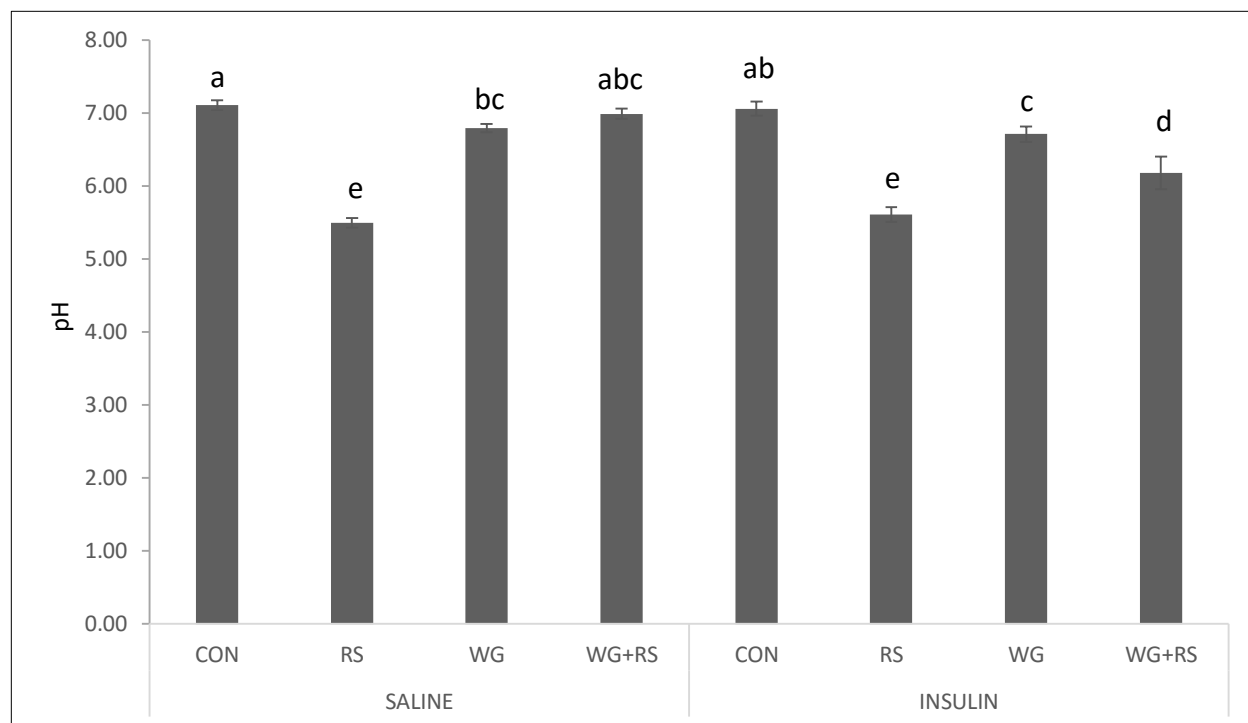
Figure 4.5. Cecal contents pH, and SCFA propionate for Study 2.1. Groups include: CON = Amylopectin control corn starch with no whole grain or resistant starch, RS = isolated HAMRS corn starch, WG = whole grain waxy corn flour with low resistant starch, and WG+RS = whole grain HAMRS corn flour. Data are presented in their original form and expressed as means ± standard error. Different letters above columns indicate a statistically significant difference at $p<0.05$. Data are shown on three factors, resistant starch (R, low or high), whole grain (W, + or -), and Injection (I, insulin or saline) for a 2 x 2 x 2 factorial. Main effects for W ($p<0.0001$), R ($p<0.0001$) and I ($p<0.0089$); and interactions for W*R ($p<0.0001$), W*I ($p<0.0027$) and W*R*I ($p<0.0048$) were observed.

There were significant main effects for dietary whole grain ($p<0.0248$), dietary resistant starch ($p<0.0001$) and injection ($p<0.0477$) and significant interactions for dietary whole grain*dietary resistant starch ($p<0.0001$) for production of the SCFA propionate in cecal contents (Fig 4.6.). The reasons for the significant main effects and interaction effect are: propionate was produced to a much greater extent in cecal contents of the RS group regardless of the type of injection compared to the other groups, but the amount was reduced with insulin injection compared to saline injection; and the WG+RS (saline or insulin $p<0.0001$) and WG (saline $p<0.0312$, insulin $p<0.0075$) groups had greater amounts than CON regardless of type of injection.
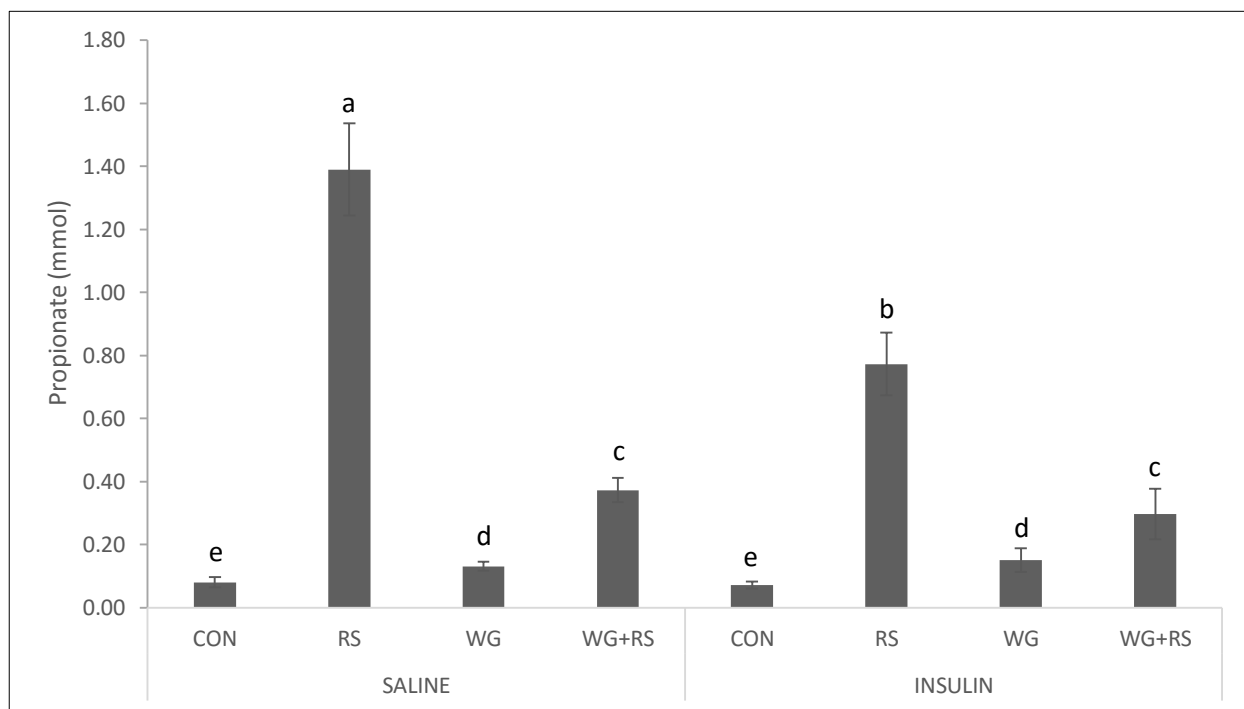
54

Figure 4.6. Millimoles of SCFA propionate in cecal contents Study 2.1. Groups include: CON = Amylopectin control corn starch with no whole grain or resistant starch, RS = isolated HAMRS corn starch, WG = whole grain waxy corn flour with low resistant starch, and WG+RS = whole grain HAMRS corn flour. Data are presented in their original form and expressed as means ± standard error. Different letters above columns indicate a statistically significant different at $p<0.05$ Data are shown on three factors, resistant starch (R, low or high), whole grain (W, + or -), and Injection (I, insulin or saline) for a 2 x 2 x 2 factorial. A main effect for I ($p<0.0477$) was reported, for that reason data were not collapsed. There were main effects of W ($p<0.0248$) and R ($p<0.0001$), and an interaction effect for W*R ($p<0.0001$) observed.

Statistical analysis for ECW and the SCFAs acetate and butyrate (Table 4.3.) were collapsed from eight to four groups, since no main effect or significant interaction was documented for injection. The ECW was increased in the presence of high resistant starch in the diet ($p<0.0001$) as RS and WG+RS groups had higher empty cecum weights compared to CON and WG groups. The RS group had a greater value than WG+RS group ($p<0.0001$) and there was also a dietary resistant starch*dietary whole grain effect ($p<0.0001$). This interaction was dominated by high resistant starch because its addition to a whole grain diet increased the ECW. The presence of high resistant starch (dietary resistant starch effect, acetate $p<0.0001$ and butyrate $p<0.0001$) in diets increased the production of acetate and butyrate for groups fed high resistant starch diets (RS,

WG+RS). There was also a resistant starch*whole grain effect (p<0.0001) because acetate and

butyrate in cecal contents were greater when there was whole grain resistant starch in the diet.

Additionally, the WG group had greater production than CON group (acetate p<0.0004, and

butyrate p<0.0009).

Table 4.3. ECW, Acetate, and Butyrate for Study 2.1[1].

Variables	Groups[2]				P value[1, 3,4]		
	CON	RS	WG	WG+RS	W	R	W*R
ECW[5] (g)	0.52c	1.64a	0.54c	1.15b	0.0001	<.0001	<.0001
Acetate (mmol)	0.32d	4.22a	0.6c	1.92b	0.5924	<.0001	<.0001
Butyrate (mmol)	0.04d	0.53a	0.11c	0.23b	0.8348	<.0001	<.0001

[1]Data are shown collapsed on two factors, resistant starch (R, low or high) and whole grain (W, + or -) for a 2 x 2 factorial because there were no significant effects of the third factor, Injection (I, insulin or saline). All markers of fermentation had a significant effect of R and some had significant effects of W. Acetate and butyrate data were log10 transformed for statistical analysis.
[2]Groups include: CON = Amylopectin control corn starch with no whole grain or resistant starch, RS = isolated high amylose maize resistant starch (HAMRS) corn starch, WG = WG waxy corn flour with low RS, and WG+RS = WG HAMRS corn flour. Data are presented in their original form and expressed as means.
[3]P value< 0.05 was considered statistically significant and it is represented with different letters horizontally.
[4] There were no interaction effects for: W*I, R*I and W*R*I (p>0.05).
[5]ECW = Empty cecum weight.

Transcript levels in cecal cells

The statistical analyses for mRNA expression measurements were collapsed and listed for

the independent variables dietary resistant starch and dietary whole grain because there were no

main or interactive effects for injection. There was higher GCG:CYC gene expression for RS and

WG+RS groups (Fig. 4.7A) compared to CON and WG groups for a resistant starch effect

(p<0.0001). The RS group had greater (p<0.0075) GCG:CYC gene expression than WG+RS

resulting in whole grain effect (p<0.0299), which was dominated by resistant starch effect and

there was no interactive effect for whole grain*resistant starch (p=0.0999). Additionally, the

injection effect approached significance (p=0.0518) because it was dominated by resistant starch effect and whole grain effect. The PCase:CYC (Fig. 4.7B) and G6Pase:CYC (Fig. 4.7C) gene expression had no significant main or interactive effects; therefore no significant differences were found among the groups. However, for PCase:CYC the resistant starch effect approached significance (p=0.0984).

A.

B.

C.

Figure 4.7. Transcript levels in cecal cells for Study 2.1. Groups include: CON = Amylopectin control corn starch with no whole grain or resistant starch, RS = isolated HAMRS corn starch, WG = whole grain waxy corn flour with low RS, and WG+RS = whole grain HAMRS corn flour. Data are presented in their original form and expressed as means ± standard error. Different letters above columns indicate a statistically significant difference at p<0.05. Data are shown collapsed on two factors, resistant starch (R, low or high) and whole grain (W, + or -) for a 2 x 2 factorial because there was no significant effect of the third factor, Injection (I, insulin or saline). **A**. Glucagon (GCG):Cyclophilin (CYC) gene expression in cecal cells. Main effects for W (p<0.0299) and R (p<0.0001) were documented. **B**. Pyruvate Carboxylase (PCase):CYC gene expression in cecal cells. There were no main or interaction effects observed. Also, there were no significant differences among treatment groups. **C**. Glucose-6 Phosphatase (G6Pase):CYC gene expression in cecal cells. No main or interactions were observed. There were no significant differences among groups.

Study 2.2

Transcript levels in cecal cells

mRNA expression measurements for GCG and G6Pase were collapsed to resistant starch and whole grain independent variables, because the level of fat in the diets did not affect the GCG and G6Pase ratios to CYC for gene expression. Results from GCG:CYC ratio (Fig.4.8A.) demonstrated that the RS group had the highest proglucagon gene expression resulting in a main effect of resistant starch (p<0.0002). A main effect for whole grain (p<0.0367) and interactive effect for whole grain*resistant starch (p<0.0012) were also observed, however, CON, WG and WG+RS groups were not significantly different from one another. For G6Pase:CYC ratio there was a significant resistant starch (Fig. 4.8B) effect (p<0.0001) as RS and WG+RS groups were not significantly different (p=0.6952) from one another, but had greater ratios than control groups (CON and WG). CON and WG groups were not significantly different (p=0.5853) from one another, and these were reflected in no main effect for whole grain (p=0.5192) or interactive effect for whole grain*resistant starch (p=0.9836). A higher PCase:CYC ratio was obtained for RS and WG+RS groups (resistant starch effect, p<0.0001) compared to CON or WG with a moderate fat diet (Fig. 4.8C). The PCase:CYC ratio was reduced for the RS group (resistant starch*fat, p<0.0118), but not reduced significantly with WG+RS with the feeding of the high fat diet (resistant starch*whole grain, p=0.6620). Also, the WG+RS and RS groups were not significantly different (p=0.0847) from one another with high fat diet. There was a significant whole grain effect (p<0.0294) because the WG group was numerically greater than CON for both moderate and high fat diets and WG+RS group was numerically greater than RS with the high fat diet.
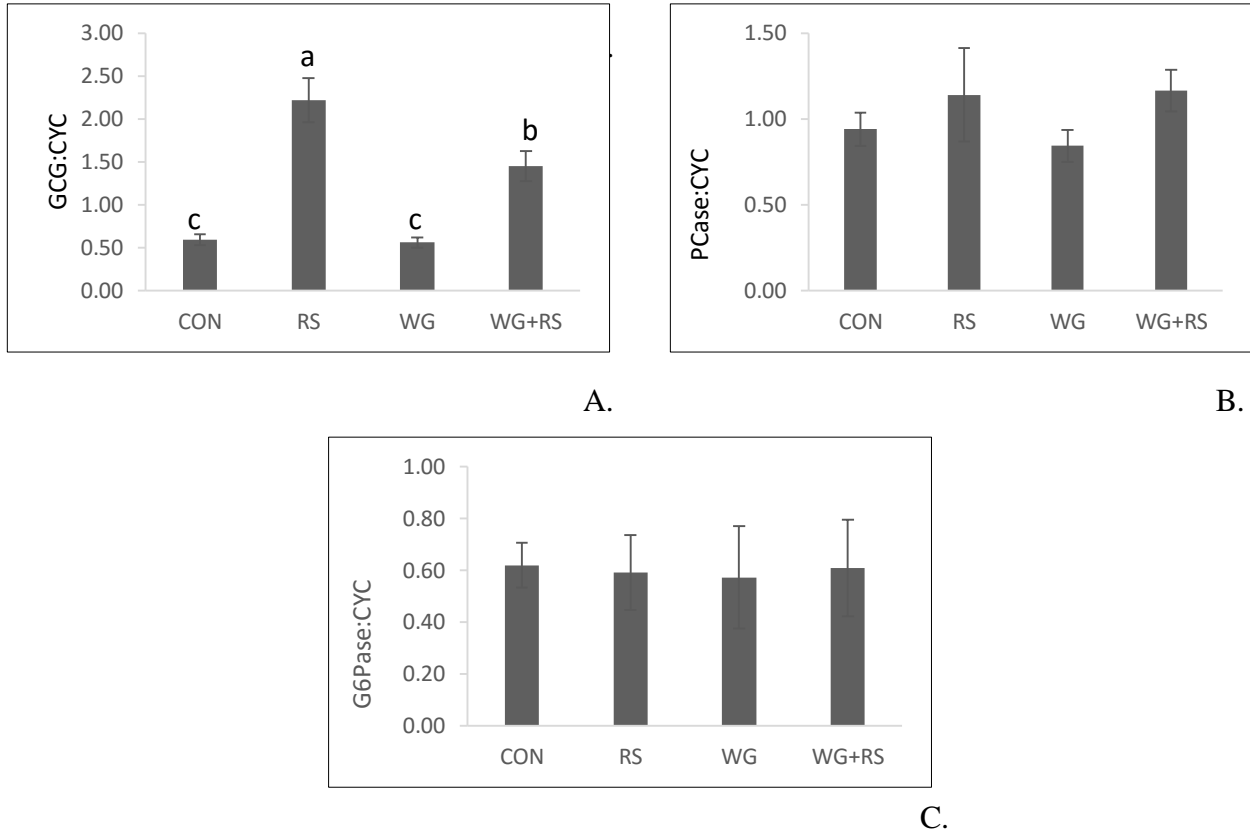
A.

B.

C.
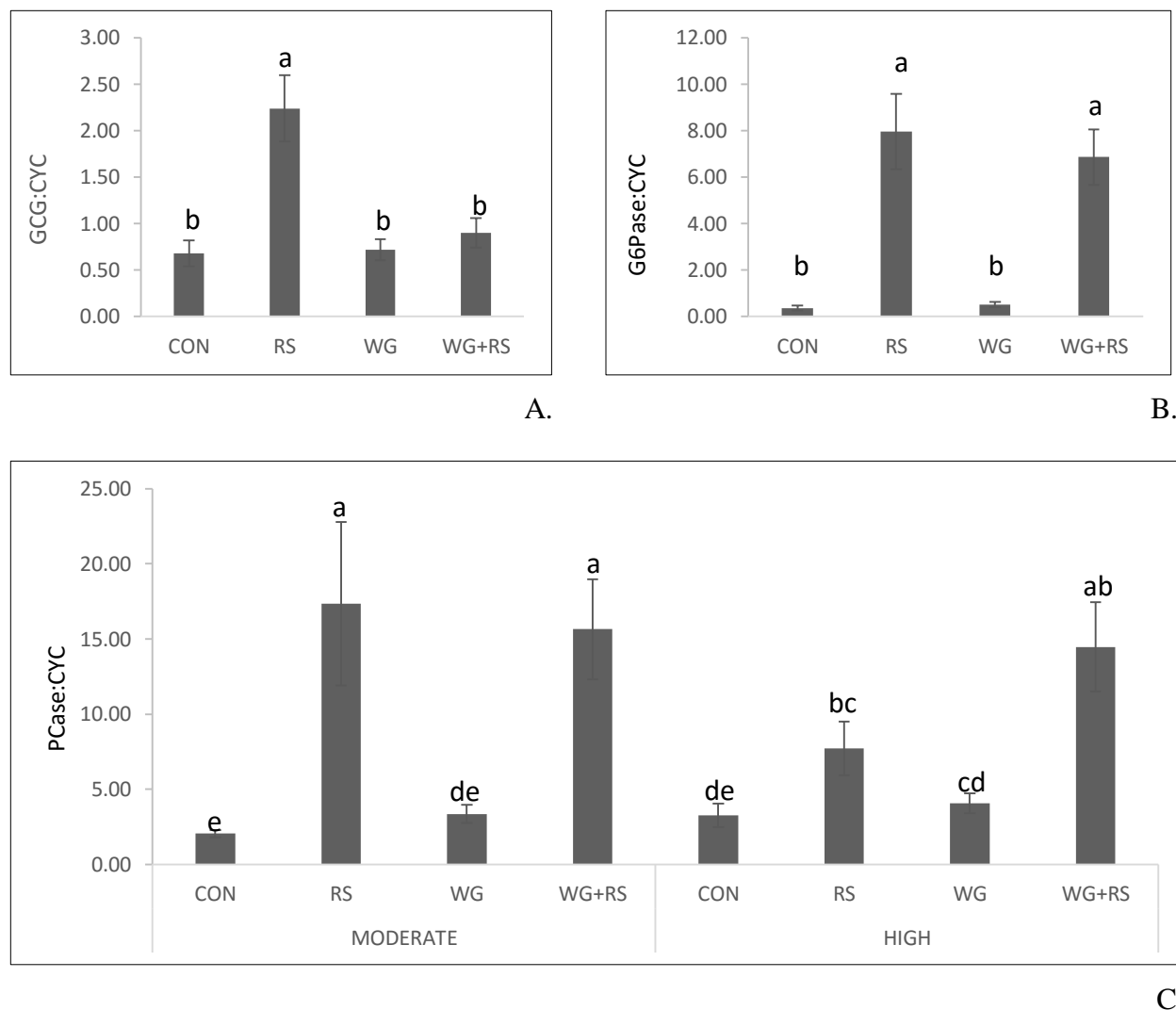
Figure 4.8. Transcript levels in cecal cells for Study 2.2. Groups include: CON = Amylopectin control corn starch with no whole grain or resistant starch, RS = isolated HAMRS corn starch, WG = whole waxy corn flour with low RS, and WG+RS = whole grain HAMRS corn flour. Data are presented in their original form and expressed as means ± standard error. Different letters above columns indicate a statistically significant difference at 0.05. Glucagon (GCG):Cyclophilin (CYC) and Glucose-6 Phosphatase (G6Pase):CYC data are shown collapsed on two factors, resistant starch (R, low or high) and whole grain (W, + or -) for a 2 x 2 factorial because there was no significant effect of the third factor, Fat (F, moderate or high). **A**. GCG:CYC gene expression in cecal cells. Main effects for W ($p<0.0367$) and R ($p<0.0002$) and interaction for W*R ($p<0.0012$) were documented. **B**. G6Pase:CYC gene expression in cecal cells. There was a main effect of R ($p<0.0001$) observed. **C**. Pyruvate Carboxylase (PCase):CYC gene expression in cecal cells. Main effects of W ($p<0.0294$) and R ($p<0.0001$) and an interaction of R*F ($p<0.0118$) were observed.

4.4. Discussion

As was stated in the introduction, humans fed resistant starch diets have exhibited the

beneficial health effect of improvement of insulin sensitivity, whether or not there was increased

59

production of GLP-1 active [18, 81]. Therefore, other mechanisms such as IGN might be involved. Rodent studies have reported insulin sensitivity improvements and decreased hepatic gluconeogenesis with increased IGN [54]. In contrast, human studies have reported insulin sensitivity improvements, increased glucose uptake in skeletal muscle and adipose, but no reduction of hepatic gluconeogenesis [18, 33]. Decreased hepatic gluconeogenesis in rodents could be a response of increased AMPK phosphorylation and activity due to SCFAs [14], and this mechanism might not occur in humans. Additionally, there is no scientific research done in humans for increased IGN as a result of a resistant starch feeding. It is known that not all findings in rodent studies can be translated to humans, but there is strong evidence that production of SCFAs during fermentation of resistant starch and other fermentable fibers provide beneficial health effects for rodents and humans.[29] Our results demonstrated that increased IGN gene expression occurred in the fed state of Sprague Dawley rats, therefore we propose IGN as a possible mechanism that improves insulin sensitivity in humans, but this needs to be confirmed in humans.

In Study 2.1, we investigated the fermentation effects of high resistant starch (isolated or whole grain HAMRS forms), compared to highly digestible isolated starch and waxy whole grain low resistant starch, on improving insulin sensitivity and gluconeogenic gene expression in cecum for GK rats, a non-obese type 2 diabetic model [95]. This model has a reduced pancreatic mass [32]. Our results demonstrated that high resistant starch as either isolated or in whole grain form had similar effects with increased GLP-1 active, greater insulin sensitivity (lower HOMA-IR) and greater fermentation (greater empty cecum weights, lower cecal contents pH, and greater SCFAs) in GK rats. GLP-1 active secretion in plasma was stimulated by RS and WG+RS diets in comparison with WG and CON diets. The increase of GLP-1 active was significantly negatively correlated to HOMA-IR (p=0.0361). GLP-1 secretion is associated with improvements in glucose

metabolism by stimulating glucose-dependent insulin secretion[80] and inhibition of glucagon secretion [96]. We also previously demonstrated that isolated resistant starch feeding to GK rats significantly improved insulin sensitivity [32], but hypothesized that whole grain may have a greater effect. These results indicate that the beneficial effect on insulin sensitivity results from presence of high resistant starch and was not due to other components in the whole grain. Also, increased GLP-1 secretion is proposed to be the result of increased SCFAs (product from fermentation of resistant starch or other fermentable fibers in the large intestine) binding to G-protein-coupled receptors on GLP-1-secreting L cells in the colon [97]. Human studies have also demonstrated that dietary resistant starch intake improves insulin sensitivity [98].

GK rats fed RS or WG+RS diet showed a decrease for ABF%. The reduced body fat percent for RS or WG+RS groups suggested increased fat oxidation as food intake was increased significantly for WG+RS and numerically for RS. Previous data from our lab demonstrated dietary resistant starch boosted fat oxidation in mice [29]. No scientific literature was found to support that insulin injection prior to euthanasia may affect ABF%, especially for whole grain diets and for that reason further research is suggested. However, the WG+RS rats that had the insulin injection had a lower cecal contents pH. Thus, by chance, the rats given the insulin injection appear to have greater fermentation and this may be the reason for the lower ABF% with insulin injection. Also, the presence of whole grain increased food intake in GK rats and presence of resistant starch approached significance so there may have possibly been increased fat oxidation and reduced abdominal fat pads [32] and reduced fat accretion in stores [98].

Body weight (data not shown) was not significantly different between the rats designated for injection of saline or insulin, as there was no main effect for dietary whole grain (p=0.5655), dietary resistant starch (p=0.7885) or interactive effect for dietary whole grain*dietary resistant

starch (p=0.2485). In another rodent study there was also no decrease in total body weight in response to resistant starch feeding [27]. These results might be a consequence of increased total bowel contents, thickness of the lumen, and mass of microbiome [27]. However, rodent studies have demonstrated reduction in total body fat percentage for resistant starch diets compared to a highly digestible starch diet [29, 83, 99].

The WG group exhibited less fermentation compared to the RS and WG+RS groups which demonstrated a greater or dominant effect of resistant starch compared to a whole grain effect on empty cecum weight, and SCFAs. The WG+RS group had a lower cecal contents pH compared to WG only with insulin injection. We hypothesized greater fermentation for whole grain because whole grain has more complex dietary fiber composed of oligosaccharides, resistant starch, and non-starch polysaccharides, and it has been shown to augment the production of SCFAs in the large intestine, especially butyrate [100]. However, this did not occur in the current study. This result did not occur in the present GK rat study likely because of the fasted state of the rats. It may be suggested that the WG+RS diet may have a shorter transit time than the RS diet so that more isolated resistant starch was present in the cecum at euthanasia.

IGN is a regulator of glucose and energy homeostasis [88]. De Vadder et al. reported that the production of propionate and butyrate from fermentation of FOS activates IGN genes such as G6Pase in small intestine (jejunum) and large intestine (colon) [54]. Sun et al. demonstrated that duodenal-jejunal bypass upregulated G6Pase in small intestine in GK rats [101]. In studies 2.1 and 2.2 we examined the gene expression of G6Pase and PCase in cecum as indicators of IGN in large intestine. The results of the studies demonstrated that resistant starch feeding results in increased IGN gene expression only in the fed state with dietary resistant starch. In addition GCG gene expression was measured as a positive control for real-time RT-PCR. In study 2.1, the increase of

GLP-1 active was significantly positively correlated to GCG:CYC. RS and WG+RS had higher gene expression of GCG compared to CON and WG as well as increased serum GLP-1 active. Increased GCG [31] and GLP-1 [29] active have also been reported in previous studies. Furthermore, in study 2.2 greater expressions of GCG gene and GLP-1 active in serum in Sprague Dawley rats fed the RS diet were observed independent of fat level. However, the WG+RS group did not have increased GCG gene expression, which suggests that this group was more efficient in translation as the rats in the RS group because both groups similarly increased serum GLP-1 active (data not shown).

Study 2.1 was planned to determine if resistant starch feeding would increase IGN gene expression in the fasted and fed states, with injection of insulin as a model for the fed state. We hypothesized that since FOS increased IGN, another fermentable fiber, resistant starch, should also stimulate IGN. Then in study 2.2, we measured IGN gene expression in Sprague Dawley rats only in the fed state. There was increased, gene expression of PCase and G6Pase, independent of fat level, in cecal cells in rats fed the RS and WG+RS diets. Previous results demonstrated that high fat diet partly diminished the fermentation in Sprague Dawley rats fed resistant starch [66], but in study 2.2 there were similar increases in IGN gene expression regardless of level of dietary fat. These results indicate that high resistant starch increased IGN gene expression in the large intestine only in the fed state and that injection of insulin associated with the fasted state is not a good model for the fed state in regard to IGN gene expression.

In summary, the present research study demonstrated that gene expression for two IGN enzymes are increased in the fed state in the large intestine of Sprague Dawley rats fed RS and WG+RS diets with either moderate or high fat. No increase in IGN enzyme gene expression was detected in the large intestine of fasted GK rats fed high resistant starch or whole grain in a low

fat diet, even though the insulin sensitivity was improved. IGN in the large intestine appears to occur in the fed state and not if the rats are fasted. It also appears that insulin injection does not model the fed state, in regards to IGN gene expression. However, the current data indicate that Americans consuming a moderate or high fat diet can include resistant starch as a fermentable fiber in their typical diet in order to increase IGN in the large intestine and benefit from better glycemic control and insulin sensitivity. Further research needs to be done for IGN in large intestine for Sprague Dawley rats to determine if increased IGN gene expression translates to increased enzyme levels; and to measure IGN gene expression in the fed state in GK rats in response to resistant starch consumption. Also, research is warranted to determine if IGN is increased in human subjects fed resistant starch and may account for increased insulin sensitivity when serum GLP-1 is not increased.

CHAPTER 5: CONCLUSIONS

The results obtained in study 1.2 demonstrated that the usage of very potent antibiotics can be avoided to reduce fermentation of resistant starch in the rat cecum for future mechanistic studies with dietary resistant starch. In phase 1 of study 1.1 we determined that fermentation of resistant starch was not knocked down if low potency antibiotics, neomycin and ampicillin, are given prior to feeding resistant starch. In study 1.2 it was demonstrated that low potency antibiotics were able to reduce total fermentation when they were given simultaneously with resistant starch feeding. In future studies low potency antibiotics can be used to study beneficial effects of resistant starch besides fermentation. Additionally, it was observed that neomycin and ampicillin stimulated the secretion of GLP-1. This side effect might improve insulin resistance, but this and other effects of antibiotics may not allow mechanistic studies with dietary resistant starch. However, it was encouraging to us that effects of antibiotics were reversed by subsequent feeding of resistant starch in phase 2 of study 1.1.

In study 2.2, it was demonstrated that high amylose corn starch and whole grain high amylose corn flour in moderate and high fat diets promoted increased gene expression of enzymes PCase and G6Pase in the fed state in the cecum of Sprague Dawley rats. Further study is necessary to determine enzyme levels, but the current results are similar to results with feeding of fructo-oligosaccharide. Also, insulin injection prior to euthanasia of GK rats was not able to model the fed state. Future studies are necessary to study if resistant starch in the diet promotes IGN also in humans. Due to these findings, it was demonstrated that resistant starch in isolated form or whole grain flour might promote a mechanism for better glycemic control and improvement of insulin sensitivity for human subjects that do not produce increased GLP-1 active secretion in response to the feeding of resistant starch.

Nowadays, people's life style is characterized by consumption of diets high in saturated fat and almost no physical activity. This has consequences such as obesity and inflammatory diseases. Therefore, it is important to add resistant starch as an ingredient in food products of daily consumption not only to decrease energy density, but also to promote a healthier microbiota and improve glucose control and insulin sensitivity. Also, the result of our research indicates another mechanism for beneficial effects of resistant starch besides increasing GLP-1 and offers a possible explanation for human subjects that have improved insulin sensitivity with the feeding of resistant starch without increased GLP-1 compared to control subjects.

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