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RATS FED DIETARY BIOACTIVE COMPONENTS (RESISTANT STARCH, WHOLE GRAINS, AND FAT) UNDERGO ALTERED BIOMETRICS AND GENE EXPRESSION AS A RESULT OF GUT FERMENTATION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Nutrition and Food Sciences

by
Justin Lamont Guice
B.S., Louisiana State University, 2013
M.S., Louisiana State University, 2016
December 2018

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To those who believe I am capable of much more than I give myself credit for

~

ACKNOWLEDGMENTS

I would like to genuinely thank several persons who were instrumental in my development into a consummate professional. First, I would like to thank my mentor and advisor, Dr. Michael J. Keenan, who without his vast knowledge, patience, and selflessness, I would not be forging this path today. Secondly, I would like to express my thanks and gratitude to my advisory committee members, Drs. Jack Losso, Georgianna Tuuri, Brian Marx, and Donald Thompson, who all agreed to advise me to help make my research and this document a powerful example of what students are capable of when we are given the opportunity and tools to succeed. Next, I must thank Ms. Anne Raggio, who not only helped me gain competency in traditional and exotic laboratory techniques, but helped me solve the myriad of "problems" I tended to unearth throughout my graduate career.

I must thank my former and current lab mates, Dr. Felicia Goldsmith and Dr. Diana Carvajal de Grau, who showed me how to be an adept researcher, and Mr. Ryan Page and Dr. Diana Obanda who helped me clarify thoughts and ideas. Similarly, I owe thanks to Dr. Diana Coulon for her expansive knowledge and logistics about animal research that has aided me tremendously in the studies I've conducted. To the many people who have entered my life and made me a better person today by having at least a modicum of faith in me, I thank you all. While I cannot mention you all, please know that I appreciate your contributions to my development.

Finally, Dr. Courtney Passow, thank you for always having been there for me. Thank you for your support and encouragement. I don't feel as if I'll ever be able to show you the amount of gratitude, respect and love I have for you and that you deserve. Certainly though, I will try.

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ABBREVIATIONS

ABF%: Abdominal fat percent

ADM: Adrenomedullin

AIN–93M: American Institute of Nutrition (AIN) 1993 improved AIN–76A standard diet

AMDR: Acceptable Macronutrient Distribution Range

ANGPTL4: Angiopoietin–like 4

B–H FDR: Benjamini–Hochberg False Discovery Rate

cDNA: complementary DNA

CHD: Coronary Heart Disease

CON: Control

CRP: C–reactive protein

C_t: Cycles–to–threshold

CV: Critical value

DUSP1: Dual specificity protein phosphatase 1

EBW: Emboweled body weight

ECW: Empty cecum weight

ELISA: Enzyme–linked immunosorbent assay

FAT: Fat factor

GALE: UDP–Galactose–4–epimerase

GAPDH: glyceraldehyde–3–phosphate dehydrogenase

GC: Gas Chromatography

GI: Gastrointestinal

GLP1: Glucagon–like peptide 1

GMN: Global Mean Normalization

HAMRS: High–amylose maize resistant starch

HF: High Fat

HMWG: High amylose maize whole grain resistant starch

IACUC: Institutional Animal Care and Use Committee

IGN: Intestinal gluconeogenesis

IL–10: Interleukin 10

IL–6: Interleukin 6

LEP: Leptin

MCT1: Monocarboxylate transporter member

MF: Moderate Fat

mRNA: messenger RNA

MUC1: Mucin 1

OP: Obese–prone

OR: Obese–resistant

OTU: Operational taxonomic unit

PC: Pyruvate carboxylase

PCK1: Phosphoenolpyruvate carboxykinase 1

PCR: polymerase chain reaction

PPIF: Cyclophilin–F / Peptidyl–prolyl cis–trans isomerase F

QIIME: Quantitative Insights Into Microbial Ecology

qPCR: real–time PCR

RG: Reference Gene

RS: Resistant Starch factor

RT–PCR: reverse transcription PCR

RT–qPCR: reverse transcription real–time PCR / Quantitative reverse transcription PCR

SCFA: Short chain fatty acid

SD: Sprague Dawley rat

SEM: Standard Error of the Mean

SLC25A25: Solute carrier family 25 member 25

TBHQ: *tert*–butylhydroquinone

TNF–α: Tumor necrosis factor alpha

WG: Whole Grain factor

WWG: Waxy Whole Grain

ZDF: Zucker Diabetic Fatty rat

ABSTRACT

The whole grain and fat content of the diet have been previously shown to affect intestinal fermentation and phenotype conferred by high–amylose maize resistant starch (HAMRS), a form of fermentable dietary fiber. The current studies were designed to compare rodent gut health following consumption of whole grain and non–whole grain prebiotics on moderate fat (MF) and high fat (HF) diets, and to optimize health effect based on dosage of whole grain resistant starch prebiotics.

Study 1: Diets were prepared to contain the following factors RS (Present/Absent), WG (Present/Absent), and Fat (HF/MF). A three–way ANOVA was performed with statistical slice on interactions and main effects. *Study 2*: Isocaloric diets (3.7 kcal/g) were prepared as follows: non–RS non–WG control, non–RS WG control, or with increasing WGRS (5, 10, 15, 20% wt.)]. One–way ANOVA with *a priori* contrasts (WG vs. all individually) were performed at $p<0.05$ *Both:* Diets were fed to Sprague Dawley and lean Zucker Diabetic Fatty rats respectively, for six weeks. After euthanasia, blood, cecal contents and cecal epithelial cells were collected and gastro–intestinal (GI) tract portions and fat pad weights recorded. RT–qPCR was performed to analyze gluconeogenic enzymes, response to oxidative stress, and gut barrier resilience.

For study 1, a few interactions were significant, but the RS main effect provided the most substantial changes in biometric and gene expression parameters. WG presence resulted in consistency of fermentation. Results were primarily driven by two major effects: purified RS fermented better on MF than HF diets and diets with RS+WG show similar fermentation on both levels of dietary fat.

Although the 10–15% dosages were best for initiating benefits from fermentation in study 2, WG flour alone promoted fermentation with RS1 (a WG kernel component), and WGRS

(RS1+RS2) promoted greater fermentation. Replacing traditional starch with a whole grain with resistant starch, as low as 5%, had some beneficial effects. These results suggest that a lower level of intake of fermentable fiber as RS is beneficial, but show that substantial WG (only low RS1) also had beneficial effects.

CHAPTER 1. INTRODUCTION

1.1. Significance of Research

Nutritional policies and recommendations regarding an adequate level of fiber (38 g/day and 25 g/day for men and women respectively) [1] have not been successfully met as most Americans fail to meet even half the Adequate Intake [2]. New approaches must be employed to maximize nutritional benefits within this reduced fiber consumption profile. Fiber itself is not a singular substance and is understood to have complex chemical arrangements with a variety of functions. One function is the degree to which the fiber is fermentable. Fermentable fiber has a greater bioactive or biological effect than a non–fermentable fiber. It can act as a prebiotic to promote gut health by elevating the growth of beneficial bacteria, which increases the production of short–chain fatty acids [3–5].

The recommendations for dietary fat intake are within an acceptable macronutrient distribution range (AMDR) between 20% and 35% of energy [1], and the average dietary fat intake for Americans is approximately 33% of energy [6]. Studies examining the effects of fat intake on changes in the microbiota have focused on low fat (18–20% of energy) or very high fat (60–70% of energy) diets [7, 8]. The effects of high fat diets on the gut microbiota and the host have been characterized, with a reduction in fermentation as a primary result [9]. Simultaneously, there exists a void in the literature when examining the effects of a moderate fat diet on gut health. However, our lab group has begun to address this issue. In one study, we examined the effects of a moderate fat (26% of energy) diet, and found that low and moderate fat diets had similar effects on the fermentation of a non–whole grain resistant starch prebiotic fiber for reducing body fat [10] and improving bacterial population (unpublished data). However, a robust characterization of fermentation parameters produced from intake similar to what Americans consume (moderate fat versus high fat) does not currently exist.

Recommendations for whole grains initially appeared in the Dietary Guidelines for Americans 2005 [11], and current recommendations promote making at least half the grains consumed whole grains. As with many recommendations, Americans do not meet the federal dietary recommendations, and this is especially true for whole grains [12]. While no consensus on whole grain consumption has been reached, many reports describe a correlation with whole grain consumption and better health [13, 14].

It is important to determine how these bioactive components act individually, but as well as also how they interact with each other within a dynamic system dedicated to maintaining homeostasis. Characterizing how fat intake at levels similar to the typical American diet affect gut health remains incomplete. Determining how moderate fat diets compare to low and high fat diets needs to be examined. Similarly, simplifying whole grains to a singular substance begets the confusion regarding fiber. Yet, other questions remain unanswered. Can other bioactive components mitigate negative effects associated with high fat diets? Our lab is interested in investigating if lower levels of fiber intake (in the form of fermentable fiber) than the current recommendations promote a healthy gut phenotype when fed as part of a moderate fat diet comparable to the average dietary fat intake for Americans. In the future, more people may be able to benefit from these bioactive components without drastically altering their diet. Of course, those who partake in more of these components may see more benefits, but those who do not may still benefit even at reduced levels of intake.

1.2. Objectives

1. Use three bioactive components (resistant starch, whole grains, and fat) to improve gut health.

2. Determine if moderate dietary fat consumption provides greater health effects than high dietary fat consumption.
3. Determine if a whole grain version of resistant starch is more efficacious than a non–whole grain resistant starch.
4. Examine how gene expression changes in response to fermentation of dietary fermentable fibers.
5. Determine the optimal dose for a whole grain resistant starch product to elicit changes to biometric, fermentation, and gene expression parameters.

CHAPTER 2. LITERATURE REVIEW

2.1. Resistant Starch

Dietary fiber is defined as the "non–digestible carbohydrates and lignin that are intrinsic and intact in plants [15]." The non–digestible carbohydrates can include inulin, oligosaccharides, fructans, methylcellulose, polydextrose, resistant maltodextrose, resistant starch, and other compounds. The property of a starch depends on the arrangement of glycosidic bonds linking the glucose monomers that make up the amylose or amylopectin molecules in the granule. Using in vitro assays, Englyst et al. (1992) classified starches into three fractions: (1) rapidly digestible starch, digested to glucose within 20 minutes, (2) slowly digestible starch, digested between 20 and 120 minutes, and (3) resistant starch, any starch remaining after 120 minutes [16]. One function of dietary fiber is the degree to which it is fermentable. Resistant starch is one such fermentable fiber. In the early days of fiber research, observational studies noted a decreased risk for colorectal cancer and other bowel diseases after consuming a diet high in unrefined grains and cereals, attributed primarily to dietary fiber. Cassidy et al. (1994) reported one such benefit of consuming resistant starch finding a "strong inverse association between starch consumption and large bowel cancer incidence" [17]. Topping et al. (2001) agreed, but further attributed the benefits found in those studies primarily to resistant starch and to a lesser degree, non–starch polysaccharides [18].

Resistant starch resists enzymatic digestion in the small intestine and is fermented by bacteria in the large intestine [19]. Resistant starch can be classified into four major types. Resistant starch 1 (RS1) is a component of whole– and partially milled grains, seeds and legumes. The RS1 is found in the starch granule, and the intact cell wall enclosing the granule physically limits accessibility to enzymatic hydrolysis. Resistant starch 2 (RS2) is a highly compacted starch in granules with reduced accessibility to enzymes that digest the glycosidic

bonds. The RS2 found in raw starch can be gelatinized after heating, allowing amylases access to the starch and thus, the starch becomes digestible. High–amylose maize (HAM) is high in RS2 due to the high amylose content and having a higher gelatinization temperature that increases its resistance against enzymatic hydrolysis. Resistant starch 3 is formed by retrograded (gelatinized and crystallized) amylose and amylopectin. When heated, the starch's crystalline structures dissociate. Upon cooling, the crystalline structures are restored, returning stability to the molecule. Resistant starch 4 is a chemically modified starch. Modifications can emanate from direct addition of functional groups or cross–linking other chemical reagents to starch using novel bonds other than α–(1–4) and α–(1–6) glycosidic linkages [20]. Recently, another fraction of resistant starch, resistant starch 5, has been described. Resistant starch 5 is produced from the addition of lipid complexes (free fatty acids) to amylose. The pairing leads to a helical structure that is resistant to enzymatic hydrolysis [21].

The fraction of starch that escapes enzymatic digestion in the small intestine, resistant starch, is potentially capable of being fermented by the gut microbes in the large intestine. Fermentation of resistant starch stimulates the growth and maintenance of the gut microflora [22]. In this capacity, resistant starch is considered to be a prebiotic, because it is a non–digestible food component that provides benefits to the host via microbial fermentation. The end products of resistant starch fermentation are gases (CO_2, H_2 and CH_4), heat, and short–chain fatty acids (SCFAs), primarily acetic, propionic, and butyric acid, commonly called acetate, propionate, and butyrate. Through these SCFAs, resistant starch has been shown to provide many health benefits. Short–chain fatty acids contribute to gut health by improving energy homeostasis and metabolism, preventing pathology in the lumen, reducing risk for a variety of colon cancers, gastrointestinal (GI) disorders, and cardiovascular diseases [18, 23–25].

The short–chain fatty acids vary in mode and site of actions. Acetate and propionate produced in the colon can be found in the small and large intestines, and portal, hepatic and peripheral blood [26]. The two SCFAs are utilized by peripheral tissues (muscle, acetate) or by the liver (acetate, propionate) for metabolism [27–30]. Butyrate is especially important for gut health, and is a major source of energy for epithelial colonocytes [26]. Furthermore, acetate and lactate produced by bacteria in the gut can be utilized by bacteria in the Clostridium cluster IV, Clostridium cluster XIV and other genera to produce butyrate [31, 32]. Resistant starch fermentation provides benefits to the host mediated through the production of SCFAs.

2.2. Whole grains

Initially, a food or product containing more than 25% whole grain or bran content could be defined as whole grain. This definition included high fiber bran cereals, and did not precisely calculate the amount of whole grain present [33]. The newer definition, established with the Food and Drug Administration Modernization Act (1997), set the criteria for manufacturers to make health claims regarding whole grains. Under these criteria, a whole–grain food is one that contains more than "51% or more whole grain ingredient(s) by weight per reference amount customarily consumed [34, 35]."

A whole grain kernel consists of three parts: the bran, the germ, and a starchy endosperm. For a food to be considered whole grain, the bran, germ, and endosperm must be present in relative proportions as found naturally in the kernel [35]. Current recommendations for whole grain consumption call for at least half the grains consumed to be whole grains [36]. Whole grains have been associated with reduced risk for cardiovascular disease, type 2 diabetes [37, 38], cancers[39–41], and all–cause mortality [42, 43].

While the benefits of whole grains are numerous, it is not immediately clear if the benefits stem from the fiber or phytochemicals present. As previously mentioned, consumption

of dietary fiber in unrefined grains and cereals is associated with reduced risk for several types of cancer and bowel diseases [39]. Similarly, phytochemicals have also been shown to provide protection against developing chronic diseases and cancers [44]. Phytochemicals, chemicals derived from plants, are a large class of compounds that represent thousands of possibly bioactive molecules. Phytochemicals include carotenoids, organosulfur compounds, alkaloids, phenolics and other nitrogen–containing compounds [45].

Research regarding phytochemicals focuses primarily on prevention, while fiber research focuses on risk reduction [45, 46]. These concepts, while similar in thought, differ in execution. Risk reduction focuses on strategies that mitigate harm to people who are potentially susceptible. Furthermore, risk reduction focuses on reducing expected loss from a specific type of risk (e.g. aphasia from a stroke). Prevention strategies focus on reducing the likelihood of an event occurring. Although fiber and phytochemical research does overlap, the research for both fractions examines a different endpoint. Whole grain research can combine these strategies to examine benefits to health. Some suggest that without the fiber component of whole grains, the effect would be minimal [33]. This suggestion has not been explicitly tested, as the process of separating the components would result in a product that is not whole grain.

Whole grains are capable of fermentation as is resistant starch. Similarly, this fermentation occurs in the large intestine by gut microbes and promotes the production of SCFAs, gases and heat. Both the fiber component and the phytochemical component of whole grains have the capacity for fermentation, although some portions of the whole grain kernel may be non–fermentable (e.g. cellulose). Despite the benefits derived from consumption and fermentation, whole grain intake has remained less than one–third of the recommendation [47].

However, more research is needed to elucidate the role of whole grains as both a standalone component and mode of action affected by other nutrients and systems in the body.

2.3. Fat

Fat is a necessary macronutrient required for normal operation of the body. Fat is a convenient and economical way to store energy in the body, but has functions well beyond the notable energy storage. Fat is required for: (1) proper functioning of nerve cells [48], (2) transport of vitamins A, D, E, and K [49–51], and (3) formation of some steroid hormones [52]. Dietary fat consists primarily of triacylglycerol molecules with one glycerol molecule with three esterified fatty acid molecules attached. Dietary fats differ in many properties including degree of saturation, cis–trans isomerism, variability in attached moiety, and conjugation.

Dietary fat has many effects on whole body health. There is evidence that some low fat, high carbohydrate diets may modify lipoprotein and glucose/insulin metabolism in such a way that risk for chronic disease increases [53]. Krauss (2001) described a low fat, high carbohydrate diet lipoprotein profile, or atherogenic lipoprotein phenotype, that is minimally expressed in healthy individuals, but is promoted in sedentary, overweight/obese populations. This profile is associated with increased risk for coronary heart disease (CHD) when expressed in the general American population [54]. Although the diet was low in fat, it was also high in simple sugars as the carbohydrate source. Thus, the diet was low in fiber which, may contribute to the atherogenic lipoprotein phenotype. Diets high in fat, where fat is the major source of excess energy, tend to be energy dense. These diets consumed in excess exacerbate energy control in obese or overweight persons. Mechanisms influencing energy density's effect on total energy intake have been explored.

One tenet confounding the role of fat in promoting chronic disease is the designation of total energy intake in comparison to percentage of fat. Diets may be high or low in fat, but may

or may not alter total energy intake. The terms hypocaloric, isocaloric, and to a lesser extent, hypercaloric impart a distinction that is important in understanding the impact of fat on body weight. Roy et al. (2003) tested if adult female rats would adapt to lower and higher energy density at the same level of fat. Rats in the study adjusted food intake to defend a body weight previously adapted to a high or low energy density [55].

Regarding dietary fat content in fermentation studies in rodents, most focus on the extreme positions. Studies focus on low (18–20% of energy) and very high (60–70% of energy) dietary fat diets [7, 8], neglecting an intake representative of the average American (~33% of energy). Perhaps this neglect comes from the desire to design mechanistic studies that aim to tease out a specific outcome with a specific independent variable. Still, high fat diets (>40% of energy) have been shown to attenuate the beneficial effects of fermentation [9]. It is suspected that the impact of consuming a moderate fat diet (~30% of energy) on fermentation and body fat will lie between the low and high fat diets.

2.4. Factor Comparisons

Studies have focused on producing resistant starch from various components, examining whether whole grains are efficacious or not, and testing how fat affects the diet. Few studies attempt to compare resistant starch, whole grains, or fat as factors that may affect each other. For example, Lopez et al. (2000) showed that resistant starch improved mineral absorption from wheat bran [56] and Behall et al. (2006) tested plasma glucose and insulin responses after the addition of resistant starch and barley β–glucan to the diets of men [57] and women [58]. Still, considering the potential combinations of the five resistant starch types and hundreds of compounds that make up whole grains (vitamins, minerals, phytochemicals, lignans, fiber, phenolics, phytosterols, etc.), only a few studies have attempted to compare how these bioactive components interact. Furthermore, of the few studies that do attempt to compare the

components, many are not mechanistic in nature. It is important to understand how these factors work alone, yet nutrients have polyvalent effects [59]. To this degree, many studies have examined the effects of these factors at low or extremely high doses. This includes studies using resistant starch, whole grains, or fat. Studies that examine how bioactive components interact with each other at physiological doses similar to a typical human (American) diet are needed. A more complete characterization of how moderate and fat diets affect fermentation and gut health in a rodent model is required. Similarly, more exploration is needed to understand how other nutritional components, such as whole grains, influence fermentation and health.

2.5. Reference Gene Expression

The measurement of gene expression is important because it is one part of the path from gene to functional protein in biological systems [60–62]. Genes are the functional unit of heredity and are made of DNA. Gene expression spans the processes of transcription and translation; starting from DNA to messenger RNA (mRNA) to proteins. Although most genes do not code for proteins, they are integral for control of other gene activity. For gene expression experiments, researchers are interested in determining how expression of a gene changes under experimental conditions. Messenger RNA transcripts exported out of the cell nucleus are used as templates for the basis of gene expression experiments. These experiments are termed polymerase chain reaction (PCR), since they exponentially amplify minute DNA sequences to larger quantities of DNA. However, gene expression experiments typically use a combination of variants of PCR, termed real–time PCR (qPCR), reverse transcription PCR (RT–PCR), and the combined technique reverse transcription real–time PCR (RT–qPCR). Quantitative reverse transcription PCR, (RT–qPCR) uses RNA as the starting material to be reverse transcribed to complementary DNA (cDNA). This reverse transcription is required, because DNA is amplified in PCR. Quantifying the mRNA of a sample in this way is known as gene expression analysis.

Expression alone does not provide an accurate indication of gene activity. Researchers look to compare the gene targeted under various experimental conditions. In order to do this, a reference gene (RG) is used for generating equivalent comparisons, a process called normalization. This reference gene (RG), previously known as a housekeeping gene, should be stably expressed, as any variation to the normalizer will invariably produce artifacts [63]. Reference genes should have non–regulated constitutive (constant) stable expression and must exhibit equivalent expression under different treatment conditions [64]. Bustin et al. (2002) describe how glyceraldehyde–3–phosphate dehydrogenase (GAPDH) [65], although commonly used, should not be considered for most experimental conditions because mRNA levels of GAPDH are not always constant [66]. Moreover, RGs normally thought to provide stable expression have been shown not to retain that stability under broader testing conditions [67].

Due to the limitations of stability in RGs, guidelines have been established to aid researchers for publishing results of gene expression analyses where RGs are vetted through selection and validation techniques [68]. These guidelines also recommend avoiding normalization with a single RG, unless the RG has been confirmed to be invariant under the described testing conditions. New techniques for normalization have been developed to aid with testing and validation of RGs. Software packages for selecting between several candidate genes, such as geNORM or BestKeeper, aim to provide accurate normalization of RT–PCR data using the geometric mean of multiple reference genes [67, 69]. Use of such strategies has been recently employed to successfully incorporate PCR efficiency with amplification efficiency to reduce error when normalizing RT–qPCR data.

CHAPTER 3. A STUDY OF THREE INDEPENDENT DIETARY FACTORS IN SPRAGUE DAWLEY RATS: RESISTANT STARCH, WHOLE GRAIN AND FAT (MODERATE, 30%, OR HIGH, 42%)

3.1. Introduction

Nutritional recommendations for fiber and whole grain consumption suggest amounts that will deliver optimal nutrition to the consumers who stand to benefit from them [70]. These policies promote increased fiber and whole grain consumption, and decreased fat intake (Acceptable Macronutrient Distribution Range estimated for total fat is 20 – 35% of energy), specifically saturated and *trans* fatty acids [1] . The health benefits of fiber and whole grains have been increasingly studied in recent years. Epidemiological studies continue to demonstrate inverse associations between biomarkers of fiber and whole grain consumption and obesity and chronic disease risk [71] . These nutritional factors may act to promote health by several mechanisms, and fermentation in the gut is an important process where these components may be synergistic or antagonistic.

Dietary Fiber: Current policies and recommendations promote optimal levels of fiber for U.S. adults (38g/day and 25 g/day for men and women, respectively) [1]. Fiber is understood to have complex chemical arrangements and health benefits in addition to its original role as bulking agent. Fibers are mainly composed of plant constituents, such as polysaccharides and lignin, that resist hydrolysis by the digestive enzymes present in man, and some fibers are capable of being fermented by bacteria in the large intestine [72]. Resistant starch is a dietary fiber. Fermentation of resistant starch stimulates the growth and maintenance of the gut microflora [22]. The microflora produces many end products, including heat, gases, and short chain fatty acids (SCFAs), and may stimulate gut hormone production. In this capacity, resistant starch is unofficially considered to be a prebiotic, because it is a non–digestible food component

that provides benefits to the host via microbial fermentation. Prebiotics are important to the health of the gastrointestinal (GI) tract, providing the symbiotic link between host and the gut ecosystem. This ecosystem, the microbiota, can respond to dietary intake and provide health benefits as a "normobiosis." In contrast, a "dysbiosis" is a landscape where potentially harmful micro–organisms may populate the gut [73].

Dietary Fat: Dietary fat plays an important role in body health. It is a convenient and economical way to store energy in the body, but has additional physiologically active roles. It has been established that fat alone is not responsible for increasing adiposity, but consuming fat in conjunction with a relatively unrestricted energy intake contributes to increased weight gain [74]. Dietary fat has a complex role in the body and is useful for determining the roles of other bioactive components in food to determine how gut health is affected. Diets that contain fiber–rich carbohydrate and low levels of fat are both lower in calories and believed to be more satiating. Lower energy from fat appears to be important in the prevention and treatment of obesity. Still, many studies in models for humans tend to focus on consuming low (18–20% of energy) and very high (60–70% of energy) dietary fat intake [57, 58], but neglect an intake representative of the average American (~33% of energy).

In rodent models, studies have examined other levels of fat in the diet, improving the characterization of dietary fat as it affects other bioactive components. Charrier et al. (2013) demonstrated high fat (HF, 42% of energy) diets partially attenuated resistant starch fermentation in Sprague Dawley rats [9]. Zhou et al. (2009) demonstrated moderate fat (MF, 28% of energy) diets were effective at reducing abdominal fat percentage (ABF%) as well as low fat (LF, 18% of energy) diets when combined with resistant starch in C57bl/6J mice [10]. These studies showed how dietary fat had different effects on rodent health, but human studies using diets containing

fat at doses akin to average intake still need more exploration for their roles in fermentation and chronic disease.

Whole Grains: Whole grains consist of three parts: the bran, the germ, and a starchy endosperm [75]. For a food to be considered whole grain, the bran, germ, and endosperm must be present in relative proportions found naturally in the kernel [35] . Present in the bran, are dietary fiber and phytochemicals, chemicals derived from plants that include a large class of compounds that represent thousands of possibly bioactive molecules. One of the dietary fibers present in whole grains is resistant starch. The germ and endosperm contain other necessary macro– and micronutrients. Whole grains have been associated with reduced risk for cardiovascular disease, type 2 diabetes [37, 38], cancers [39–41], and all–cause mortality [42, 43]. Despite the benefits derived from consumption and fermentation, whole grain intake has remained less than one–third consumed while the recommendation is to make one–half of all grains consumed [47]. However, more research is needed to elucidate the role of whole grains as both a standalone component and how its mode of action is affected by other nutrients and systems in the body.

Identifying rodent models that respond to these dietary treatments may prove valuable to research on human health. It is important to understand how these bioactive components work in isolation, but only as a prelude to understanding how they work with or against each other. The purpose of this study was to determine if combinations of resistant starch, whole grains, and fat can improve gut health and biometric phenotypic measures. The objectives were to determine if moderate dietary fat consumption provided greater health effects than high dietary fat consumption, and if a whole grain diet with increased resistant starch was more efficacious than a non–whole grain resistant starch diet. In order to accomplish this, we designed a study to

determine how these bioactive components acted individually and to examine the compatibility of the components in regards to gut fermentation and biometric measures by determining possible interaction in a factorial study.

3.2. Methods

The experimental design for this study was a three–way ANOVA (Figure 3.1) with the following factors: (1) Resistant starch (RS) (Present or Absent), (2) Whole Grain (WG) (Present or Absent), and (3) FAT (Moderate or High).

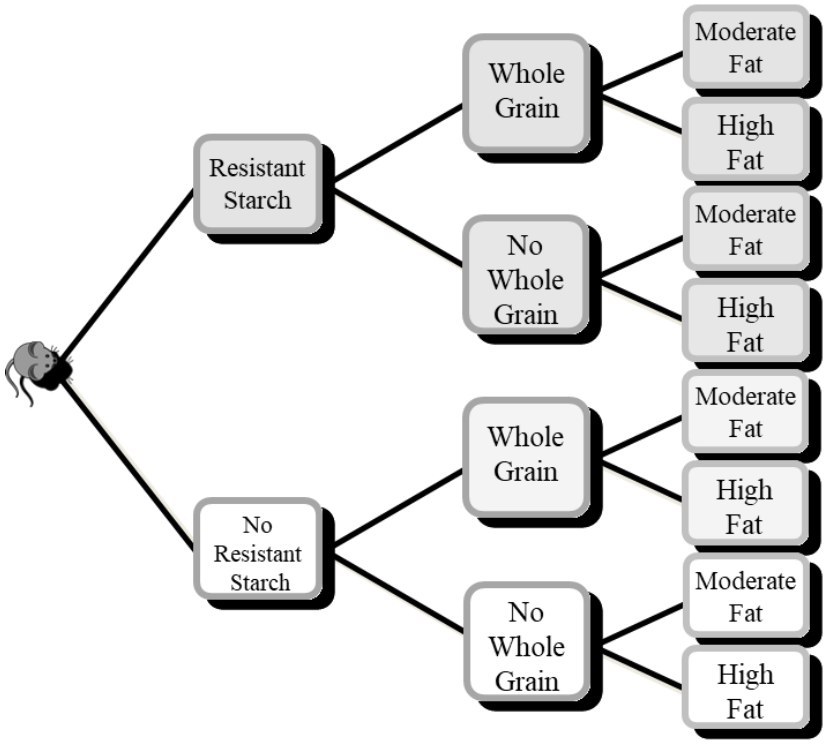


Figure 3.1. Experimental design. Study was designed as a three–way ANOVA. Each of eight groups (n=12) contains a level of each factor: Resistant Starch, Whole Grain, FAT. Levels for factors are Resistant Starch (Present or Absent), Whole Grain (Present or Absent) and FAT (Moderate or High).

Diets

Diet treatments were adapted from AIN–93M purified diets for rodents (Table 3.1) [76]. Diets contained one major starch source as either an isolated starch product or as whole grain flour. Starches and whole grain flours were analyzed by proximate analysis (Medallion Labs for Ingredion Incorporated). Starches included: (1) AMIOCA® waxy corn starch, (2) HI–MAIZE® resistant corn starch, (3) Waxy whole grain corn flour, or (4) HI–MAIZE® whole grain resistant corn flour. Diets with resistant starch were calculated to contain 23% resistant starch by weight. Diets with waxy whole grain starch were calculated to have 4.93% resistant starch, due to the whole grain kernel containing a resistant starch component because of the whole grain matrix when not overly processed. The whole grain resistant starch has both resistant starch type 1 (RS1) and resistant starch type 2 (RS2). The RS1 exists because the matrix of the whole grain kernel in the flour prevents access of the amylase enzymes to the starch; and the RS2 exists because of the granular structure of the high–amylose starch granules [77]. Thus, whole grain resistant starch would have a combination of RS1 and RS2, presumably mostly RS2. The whole grain control group was fed a diet that included a waxy whole grain flour product. This product also comes from a natural corn variety, but this product has 100% amylopectin for its starch component. Therefore, the waxy whole grain product has RS1, but no RS2. The amount of the waxy whole grain product used in the study resulted in ~5% of the diet as RS1. Resistant starch content was determined by Ingredion Incorporated using the modified Englyst Assay [78]. Assays for resistant starch do not distinguish between types of resistant starch.

Cellulose and AMIOCA® waxy corn starch were used to moderate the energy of each diet so that all diets within moderate fat or high fat, respectively, were isocaloric. Moderate and high fat diets were calculated to provide 3.75±0.01 kcal/g and 4.2± 0.07kcal/g respectively. Casein

was the major source of protein for the diets. Casein present in the diet differs from the typical 140 g/kg found in AIN–93M diets because the starches contain small amounts and whole grain corn flours do contain considerable amounts of protein. Corn oil and lard were used to provide the major source of fat in the diets. Fats were calculated to provide ~30% of energy for MF and ~42% of energy for HF diets. Fats were chosen to represent a ratio of saturated and unsaturated fats of $\frac{1}{3}:\frac{2}{3}$ for MF and $\frac{1}{2}:\frac{1}{2}$ for HF. Corn oil was used instead of soybean oil (AIN–93M) to better reflect fats present in the corn kernel used to derive the corn starches and corn flours used and was adjusted by the amount of fat present in starches and whole grain flours. A small amount of *tert*–Butylhydroquinone (TBHQ) was present in the corn oil as a preservative. Vitamins and minerals were in accordance with the AIN–93M diets, except for choline bitartrate, which was substituted with choline chloride.

Table 3.1. Diet composition.

	Moderate Fat			
	CON[1]	HAMRS	WWG	HMWG
Ingredients	Grams	Grams	Grams	Grams
Waxy corn starch[2]	473.30	72.31	67.83	143.74
High–amylose corn starch[3]	0.00	524.00	0.00	0.00
High–amylose whole grain starch	0.00	0.00	0.00	520.00
Waxy whole grain starch[4]	0.00	0.00	500.00	0.00
Sucrose	100.00	100.00	100.00	100.00
Casein[5]	136.00	133.12	99.42	80.56
Cellulose	115.00	0.00	78.00	24.00
Corn oil[6]	85.00	79.87	64.05	41.00
Lard[6]	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 chloride	2.80	2.80	2.80	2.80

(table cont'd.)

	Moderate Fat			
	CON[1]	HAMRS	WWG	HMWG
Ingredients	Grams	Grams	Grams	Grams
L–Cystine	1.80	1.80	1.80	1.80
Total	1000.00	1000.00	1000.00	1000.00
Resistant Starch, %[7]	0	23.37	4.93	23.45
Total Energy, kcal	3757	3750	3761	3754
	High Fat			
	CON[1]	HAMRS	WWG	HMWG
Ingredients	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
High–amylose whole grain starch	0.00	0.00	0.00	525.00
Waxy whole grain starch[4]	0.00	0.00	517.00	0.00
Sucrose	100.00	100.00	100.00	100.00
Casein[5]	136.75	133.70	98.74	80.58
Cellulose	110.00	0.00	56.91	10.00
Corn oil[6]	99.25	93.44	79.15	58.37
Lard[6]	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 chloride	2.80	2.80	2.80	2.80
L–Cystine	1.80	1.80	1.80	1.80
Total	1000.00	1000.00	1000.00	1000.00
Resistant Starch, %[7]	0	23.32	5.03	23.41
Total Energy, kcal	4164	4136	4230	4209

[1]Diets include: CON = Amylopectin control corn starch containing no resistant starch diet; HAMRS = Isolated high–amylose starch (HAMRS) corn starch diet; WWG = waxy whole grain amylopectin control corn flour containing low resistant starch diet; HMWG = whole grain HAMRS corn starch diet.

[2]AMIOCA® corn starch containing 100% amylopectin starch that is digestible.

[3]HI–MAIZE® resistant corn starch.

[4]Waxy & high–amylose corn starches and whole grain flours were gifts from Ingredion Incorporated (Bridgewater, NJ).

[5]Casein was reduced in each diet based on the protein constituent in AMICOA® and HI–MAIZE® corn starches and whole grain flours analyzed by proximate analysis performed by Medallion Labs for Ingredion Incorporated, and differs from the AIN–93M standard 140 g/kg.

(table cont'd.)

[6]Corn oil was modified in each diet based on the fat content in AMICOA® and HI–MAIZE® corn starches and whole grain flours analyzed by proximate analysis performed by Medallion Labs (Minneapolis, MN) for Ingredion Incorporated (Bridgewater, NJ). Corn oil and lard were calculated to adjust fat present in all diets to ~ 30% of energy for moderate fat, and ~42% of energy for high fat. The aim was to have corn oil be ~20% and lard ~10% of the energy for the moderate fat diets; and for high fat diets corn oil and lard each contributed ~21% of energy each. These values differ from the AIN–93M standard 40 g/kg.
[7]Diets with high amylose starch contain resistant starch type 2, but the whole grain flour with high amylose has both resistant starches types 1 and 2. Diets with waxy whole grain flour contain only resistant starch type 1. Resistant starch content of experimental starches was determined by Ingredion Incorporated using modified Englyst assay [78].

Animals & Euthanasia

Ninety–six male Sprague Dawley rats were purchased from Envigo (Somerset, NJ) at six weeks of age, and maintained on a chow diet during a one week quarantine. Rats were then stratified randomly by body weight into eight groups (n=12, average 259±8.4 grams). Treatment groups consisted of moderate fat (MF) and high fat (HF) diets prepared to contain each of the following starch sources: (1) control starch with no whole grains or resistant starch [79], (2) whole grain waxy corn flour [WWG], (3) isolated high–amylose maize (HAM) with high resistant starch [HAMRS], and (4) WG HAM flour rich in resistant starch (WGRS) [HMWG].

Animals were housed in a locked facility in individual stainless steel hanging cages with wire mesh bottoms to measure food spilled and prevent coprophagy. Housing environmental conditions included a 12:12h light–dark cycle, 21–22°C ambient temperature with a 55% relative humidity. Animals were allowed *ad libitum* access to food and water for six weeks. Food intake, food spilled, and body weight were measured twice per week.

Rats were euthanized and exsanguinated by cardiac puncture after inhalation of isoflurane anesthesia delivered by soaked cotton balls. For each rat, blood samples were collected with dipeptidyl peptidase IV inhibitor for additional analyses. The gastrointestinal (GI) tract was removed from the base of the esophagus to the anus, separated into individual parts (stomach, small intestine, cecum, and large intestine) and weighed full and empty. Subcutaneous

inguinal fat and abdominal fat pads (epididymal, perirenal, and retroperitoneal) were collected and weighed to determine percentage of abdominal fat (ABF%). Abdominal fat percent was calculated as the abdominal fat pads divided by the body weight of the rat with the GI tract contents weight removed ($ABF = \frac{\text{Abdominal Fat Pads}}{\text{Body weight} - \text{Full GI} + \text{Empty GI}} * 100$). Cecal contents were collected and divided into 0.5 g aliquots, and frozen in liquid N_2 for measurement of pH and short–chain fatty acids (SCFAs). Cells lining the ceca, inguinal and epididymal fat pads were flash frozen in liquid N_2 and stored at –80°C until later analysis. The protocol for this study was approved by the Louisiana State University Institutional Animal Care and Use Committee, protocol 13–088.

Blood measurements

Serum active glucagon–like peptide 1 (GLP–1, ALPCO, NH), and C–reactive protein (CRP), tumor necrosis factor alpha (TNF–α), and interleukin–10 (IL–10, ThermoFisher, Waltham, MA) levels were measured with enzyme–linked immunosorbent assay (ELISA) kits.

Cecal contents pH and short–chain fatty acids analysis

Cecal contents were thawed and 0.5 g of sample was homogenized with 5 ml of distilled water for pH measurements. Wet and dry weights were measured for each sample. Each wet sample was then acidified with 1 ml 25% (wt/wt) solution metaphosphoric acid containing a 2 g/L 2–ethyl–butyric acid internal standard. Solids were separated by centrifugation at 8,000 X *g* for 10 minutes and filtered through a Millipore filter (MILX HA 33 mm, 0.45 μm MCE STRL; Fisher SLHA 033SS). The filtered liquid was transferred to a gas chromatograph (GC) autosampler vial. SCFAs were analyzed by gas–liquid chromatography for quantitative determination. Detailed methods for quantification of SCFAs via GC have been described in previous publications from our lab [9].

Quantitative real–time PCR (qPCR)

RNA was extracted from cecal cells and inguinal adipose tissue using the RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit, respectively (Qiagen, Valencia, CA). Samples with a poor 260/230 absorption spectra ratio (lower than 1.8) were purified using GeneJet RNA Cleanup and Concentration Micro Kits (ThermoFisher Scientific, Waltham, MA). TaqMan® Gene Expression Assays (ThermoFisher, Waltham, MA) were used to measure intestinal gluconeogenesis (IGN) (*glucose–6–phosphatase (G6pc), pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase 1 (PCK1)*), colonic barrier and goblet cell function (*UDP–galactose–4–epimerase (GALE), monocarboxylate transporter member 1 (MCT1), mucin 1 (MUC1)*), and response to oxidative stress (*adrenomedullin (ADM)*) in cecal cells. TaqMan® Gene Expression Assays (ThermoFisher, Waltham, MA) were used to measure pro–inflammatory status (*IL–6, TNF–α*), anti–inflammatory status (*IL–10*), and bacterial manipulation (*angiopoietin–like 4 (ANGPTL4), leptin (LEP), solute carrier family 25 member 25 (SLC25A25)*) in inguinal adipose tissue. Cecal cell and inguinal adipose gene expression were normalized using cyclophilin–F (PPIF) and 18S rRNA, respectively.

DNA extraction and Next Generation DNA Sequencing and Bioinformatics

DNA was extracted by Louisiana State University Health Sciences Center. Purified DNA was sequenced using a MiSeq instrument after massive parallel PCR amplification was used to incorporate primers with barcodes to identify individual samples. Relative abundance of bacteria was determined using Quantitative Insights Into Microbial Ecology (QIIME) 1.9 and DaDa2 package pipeline assembly to assign operational taxonomic units (OTU) for use in determination of alpha– and beta–diversity.

Statistical Analysis

Statistical analysis was performed using Statistical Analysis Software SAS® version 9.4 (SAS Institute Inc., Cary, NC, USA). A 2x2x2 factorial analysis was performed using the MIXED procedure. The three factors were resistant starch (RS, Present or Absent), whole grain (WG, Present or Absent), and fat (FAT, High or Moderate). The model used the three factors as fixed effects, and did not use random effects. The linear model tested was:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 + \epsilon$$

where $\begin{cases} RS \\ NRS \end{cases} X_1 = \begin{cases} 1 \\ 0 \end{cases}$, $\begin{cases} WG \\ NWG \end{cases} X_2 = \begin{cases} 1 \\ 0 \end{cases}$, and $\begin{cases} HF \\ MF \end{cases} X_3 = \begin{cases} 1 \\ 0 \end{cases}$. Denominator degrees of freedom for fixed effects used the Kenward–Roger approximation. An F–test with $p<0.05$ was considered statistically significant for interactions and main effects. Benjamini–Hochberg false discovery rate (B–H FDR) was then used to screen dependent variable F–test p–values for interactions and main effects [80]. The B–H FDR procedure consists of ranking (indexing) the raw p–values from lowest to highest and then comparing each to the critical value (CV), which is determined by the formula "I/M*Q". "I" is the rank number, "M" is the total number of dependent variables, and "Q" is the chosen false discovery rate. The largest p–value less than the CV and all p–values smaller pass the FDR test. A Q = 0.05 was chosen as the false discovery rate.

If a dependent variable passed the FDR test, a statistical slice on two 2–way interactions (RS*WG, RS*Fat) was performed for 3–way interactions, and a statistical slice on main effects was performed for 2–way interactions. Main effects used only the F–statistic and subsequent p–value. The described slices return eight pairwise comparisons for 3–way interactions and four pairwise comparisons for 2–way interactions. To correct for multiple comparisons, a Bonferroni adjustment to the significance level was performed. A Bonferroni corrected p–value < 0.00625 (α=0.05/8) was considered statistically significant for pairwise comparisons within 3–way

interactions, and a corrected p–value < 0.0125 (α=0.05/4) was considered statistically significant for pairwise comparisons within 2–way interactions. Data are expressed as means ± pooled SE.

Homogeneity of variance and influence diagnostics were tested within the MIXED procedure, while tests for normality were conducted using the UNIVARIATE procedure. A null model likelihood ratio test (χ^2) was performed to determine if variance was homogeneous or heterogeneous. If variance was homogeneous, then normality testing of residual values used pooled group residuals. Heterogeneous variance proceeded with variance calculated for each group. Influential outliers, tested using studentized residuals and leverage, were removed only if their presence prevented normal distribution for statistical analysis. Influences on parameter estimates were examined using Cook's *D*. Data from dependent variables that violated the normality assumption were transformed to $\log_{10}$. Following $\log_{10}$ transformation, normality and homogeneity of variance testing were reapplied. Data violating the normality assumption after transformation was considered not normally distributed and reverted to raw data. The following variables were transformed due to non–normal distribution ($p<0.05$ for Shapiro–Wilk test): weight of empty ceca (ECW); each of the three major SCFA in the ceca. Transformed dependent variables were back–transformed by taking the antilog. When no significant interactions were observed, only the main effect was reported. If an independent variable was dominant (much lower p–value) as a main effect, this was noted even if an interaction(s) was (were) significant.

3.3. Results

3.3.1 Biometric Analysis

All rats fed whole grain resistant starch, one rat fed isolated resistant starch with high fat, and two rats fed isolated resistant starch with moderate fat diet had loose stools during week 1 of the six week study. Rats fed the control diets (NRS) had no loose stools throughout the study. As the study continued, several of the resistant starch fed rats intermittently had soft stools.

During week 6, one whole grain resistant starch rat fed moderate fat diet and one isolated resistant starch rat fed high fat diet had loose stools. Since the loose stool occurrences in week 1 occurred for all whole grain resistant starch fed rats and three isolated resistant starch fed rats, the greater reduction in ABF% for the isolated resistant starch moderate fat diet group was likely not the result of loose stools. If loose stools had a significant effect on ABF%, the whole grain resistant starch fed rats would have greater reductions in ABF% than the high isolated resistant starch fed rats. During intervals of loose stools, affected rats continued to gain weight and did not appear dehydrated. No RS groups (CON, WWG) experienced no loose stools. Intermittent stool softness did not persist noticeably as the study progressed.

Data were examined and influential outlier measurements were removed to achieve a normal distribution with or without $\log_{10}$ transformation of the data. One data point was removed for ABF% (WWG HF: 4.459), µmol propionate produced in total amount of cecal contents (HAMRS MF: 0.0127), and µmol butyrate produced (HAMRS MF: 0.014). Four influential data points were removed for cecal contents pH (CON HF: 6.57; HAMRS HF: 8.23; HMWG MF: 6.12, 7.78). Six data points were removed for active GLP–1 (CON HF: 2.053; HAMRS HF: 2.442; HAMRS MF: 1.971, 3.218; HMWG HF: 1.823; HMWG MF: 2.831).

Following the PROC MIXED factorial analyses, no 3–way interactions were found to be significant. However, the empty cecum weight (ECW, $F_{1,87} = 3.85$, $p > .05$) was approaching significance at p=0.0529 and warranted further examination looking into the components within. All dependent variables were also ranked by raw p–values below in Table 3.2 using the B–H FDR test. The lowest critical value CV for the B–H FDR test was 0.0045 and no p–values were less than the CV.

Table 3.2. Biometric analysis F–test p–values sorted by B–H FDR[1].

RS*WG*FAT		RS*WG	
Variables[2]	*p*	Variables	*p*
Empty Cecum Wt. (g)	0.0529	Acetate (µmol)	<0.0001[†]
Cecal contents pH	0.2046	Propionate (µmol)	<0.0001[†]
Acetate (µmol)	0.3025	Cecal contents pH	<0.0001[†]
Propionate (µmol)	0.3923	Empty Cecum Wt. (g)	<0.0001[†]
Food Intake (g)	0.4147	**Butyrate (µmol)**	**0.0075**[†]
Energy intake (kcal)	0.4346	Active GLP–1 (pM)	0.3630
Abdominal body fat %[3]	0.4948	Inguinal Fat (g)	0.6537
Inguinal Fat (g)	0.6225	Food Intake (g)	0.6619
Emboweled body weight (g)	0.8439	Energy intake (kcal)	0.6986
Butyrate (µmol)	0.9177	Abdominal body fat %[3]	0.8269
Active GLP–1 (pM)	0.9189	Emboweled body weight (g)	0.9872
Most relevant CV[4]	**0.0045**	**Most relevant CV[4]**	**0.0227**
RS*FAT		WG*FAT	
Variables	*p*	Variables	*p*
Empty Cecum Wt. (g)	0.1386	**Propionate (µmol)**	**0.0050**[†]
Acetate (µmol)	0.1523	Acetate (µmol)	0.0155[†]
Propionate (µmol)	0.3231	Emboweled body weight (g)	0.0322[†]
Energy intake (kcal)	0.3713	Inguinal Fat (g)	0.1727
Food Intake (g)	0.4017	Butyrate (µmol)	0.2169
Butyrate (µmol)	0.4324	Empty Cecum Wt. (g)	0.2395
Abdominal body fat %[3]	0.4355	Food Intake (g)	0.3314
Cecal contents pH	0.8086	Cecal contents pH	0.3331
Emboweled body weight (g)	0.8426	Energy intake (kcal)	0.3756
Inguinal Fat (g)	0.8662	Abdominal body fat %[3]	0.4051
Active GLP–1 (pM)	0.9506	Active GLP–1 (pM)	0.5888
Most relevant CV[4]	**0.0045**	**Most relevant CV[4]**	**0.0045**
RS		WG	
Variables	*p*	Variables	*p*
Active GLP–1 (pM)	<0.0001[†]	Butyrate (µmol)	<0.0001[†]
Empty Cecum Wt. (g)	<0.0001[†]	Cecal contents pH	0.0017[†]
Acetate (µmol)	<0.0001[†]	**Acetate (µmol)**	**0.0066**[†]
Propionate (µmol)	<0.0001[†]	Active GLP–1 (pM)	0.0775
Butyrate (µmol)	<0.0001[†]	Food Intake (g)	0.1814
Cecal contents pH	<0.0001[†]	Abdominal body fat %[3]	0.1961
Abdominal body fat %[3]	<0.0001[†]	Energy intake (kcal)	0.2052
Inguinal Fat (g)	<0.0001[†]	Emboweled body weight (g)	0.3261
Emboweled body weight (g)	**0.0012**[†]	Propionate (µmol)	0.4845

(table cont'd.)

RS		WG	
Variables	*p*	Variables	*p*
Energy intake (kcal)	0.2117	Empty Cecum Wt. (g)	0.8290
Food Intake (g)	0.2227	Inguinal Fat (g)	0.8402
Most relevant CV[4]	**0.0409**	**Most relevant CV[4]**	**0.0136**
Fat		B–H Critical Values (Q = 0.05)	
Variables	*p*	Rank	CV
Food Intake (g)	<0.0001†	1	0.0045
Energy intake (kcal)	0.0013†	2	0.0091
Abdominal body fat %[3]	0.0064†	3	0.0136
Empty Cecum Wt. (g)	**0.0147†**	4	0.0182
Inguinal Fat (g)	0.0613	5	0.0227
Acetate (µmol)	0.1431	6	0.0273
Butyrate (µmol)	0.2472	7	0.0318
Active GLP–1 (pM)	0.5896	8	0.0364
Cecal contents pH	0.6438	9	0.0409
Emboweled body weight (g)	0.6710	10	0.0455
Propionate (µmol)	0.9312	11	0.0500
Most relevant CV[4]	**0.0182**		

[1]Data are shown based on factors, resistant starch (RS, Present or Absent), whole grains (WG, Present or Absent) and fat (FAT, High or Moderate).

[2]An ANOVA F–test F<0.05 indicates a significant measurement with † superscript denoting significant difference.

[3]ABF%: $\frac{\text{Abdominal Fat Pads}}{\text{Body weight} - \text{Full GI} + \text{Empty GI}} * 100$

[4]Most relevant CV is determined from the greatest significant p–value of dependent variables (ranked lowest to highest) that is less than the CV at each rank, given I/M*Q. "I" is the rank number, "M" is the total number of dependent variables (11), and "Q" is the chosen false discovery rate (0.05).

3.3.1.1 Three–way Interactions

Resistant Starch * Whole Grain * FAT interaction

Empty cecum weight (ECW) approached significance (p=0.0529) for the interaction among the three factors (Figure 3.2). Increased empty cecum weight is a marker of increased fermentation as described in the introduction section. Thus, these findings suggest a consistent fermentation of whole grains in both moderate and high fat diets in the presence of high resistant starch as RS2, but resistant starch without whole grains fermented best on a moderate fat diet as

compared to a high fat diet and better than whole grain on either a moderate or high fat diet. Data for all three–way interactions are also presented as means with pooled SEM in Table 3.3.

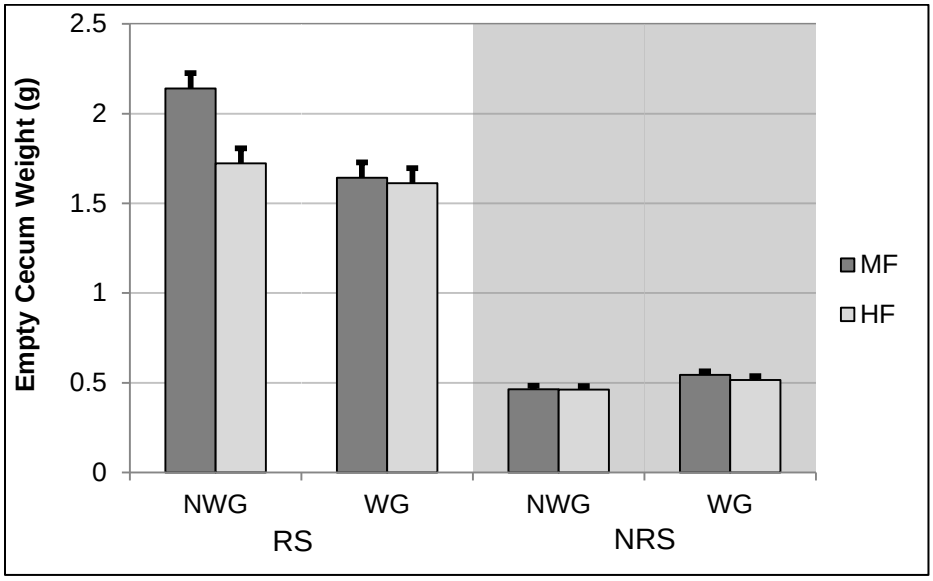


Figure 3.2. Three–way interaction of RS*WG*FAT on ECW. The interaction for RS*WG*FAT (p=0.0529) is presented. Groupings include: RS = resistant to digestion starch type 2 that has high amylose, NRS = no resistant to digestion starch type 2, WG = Whole grain present, NWG = whole grain absent, MF = moderate fat and HF = high fat. Data are shown as three factors, resistant starch (RS, Present or Absent) and whole grain (WG, Present or Absent) with the third factor fat (FAT, High or Moderate). Data are expressed in their original form as antilog means ± standard error.

Table 3.3. Three–way interactions for fermentation variables[1].

	RS * WG * Fat Interaction					
Variables	RS WG MF (HF)	RS NWG MF (HF)	NRS WG MF (HF)	NRS NWG MF (HF)	Pooled SEM	p–value (F)[2]
Food Intake (g)[3]	780.41 (720.74)	769.84 (713.29)	769.72 (737.50)	761.19 (737.60)	13.8399	0.4147
Energy intake (kcal)[3]	2934.36 (3027.11)	2894.60 (2995.80)	2995.67 (3097.52)	2862.07 (3097.94)	55.7115	0.4346
Active Glucagon–like peptide 1 (pM)	1.2696 (1.2765)	1.3646 (1.4083)	0.9234 (0.9165)	0.9332 (0.9803)	0.0552	0.9189
Cecal contents pH	6.9480 (6.7925)	6.1350 (6.2627)	8.0133 (7.9882)	8.2900 (8.2264)	0.0830	0.2046
Empty Cecum Wt. (g)	1.0936 (1.6118)	2.1981 (1.6248)	0.5441 (0.5164)	0.4637 (0.4168)	0.0490	0.0529

(table cont'd.)

	RS * WG * Fat Interaction					
Variables	RS WG MF (HF)	RS NWG MF (HF)	NRS WG MF (HF)	NRS NWG MF (HF)	Pooled SEM	p–value (F)[2]
Empty Cecum Wt. (g)	1.0936 (1.6118)	2.1981 (1.6248)	0.5441 (0.5164)	0.4637 (0.4168)	0.0490	0.0529
Acetate (µmol)	385.21 (398.38)	588.44 (406.82)	112.49 (121.90)	71.04 (65.30)	21.76	0.2998
Propionate (µmol)	45.21 (62.14)	89.85 (73.94)	16.67 (17.81)	13.71 (11.08)	4.48	0.3923
Butyrate (µmol)	96.69 (91.18)	78.92 (63.12)	29.15 (31.27)	16.26 (14.36)	5.40	0.9188
Abdominal body fat %[4]	2.0298 (2.2008)	1.9560 (2.1031)	2.4533 (2.6968)	2.4533 (2.6159)	0.1002	0.8416
Inguinal Fat (g)	2.9515 (3.3932)	2.9902 (3.1336)	3.6132 (4.2792)	3.9715 (4.0048)	0.2358	0.6225
EBW (g)	379.98 (392.66)	384.73 (378.14)	396.24 (408.90)	403.06 (392.61)	6.8844	0.8439

[1]Data are shown as full or collapsed interactions based on significant factors, resistant starch (RS, Present or Absent), whole grain (WG, Present or Absent), and fat (FAT, High or Moderate).
[2]An ANOVA F–test $p<0.05$ indicates a significant measurement.
[3]Total food and energy intakes over the total study of 6 weeks.
[4] Abdominal body fat %: $\frac{\text{Abdominal Fat Pads}}{\text{Body weight} - \text{Full GI} + \text{Empty GI}} * 100$

3.3.1.2 Two–way Interactions

Significant differences for several dependent variables were observed for two of the two–way interactions RS*WG and WG*FAT. The results are as follows: RS*WG – SCFAs acetate ($F_{1,63} = 44.96$, $p < .0001$), propionate ($F_{1,57} = 31.87$, $p < .0001$), and butyrate ($F_{1,55} = 7.33$, $p < .05$), ECW ($F_{1,87} = 22.62$, $p < .0001$), and cecal contents pH ($F_{1,57} = 54.89$, $p < .0001$), and WG*FAT – SCFAs acetate ($F_{1,63} = 6.20$, $p < .05$), propionate ($F_{1,57} = 8.53$, $p < .01$), and EBW ($F_{1,88} = 4.74$, $p < .05$). The RS*WG interaction was further analyzed using a statistical slice on RS and WG factors, while the WG*FAT interaction was sliced on the WG and FAT factors (Table 3.4). Test of slices were conducted, and adjusted using a Bonferroni corrected alpha level of 0.0125 per test (0.05/4), i.e. four *post hoc* comparisons were used for the dependent variables

Table 3.4. Two–way interactions test of effect slices for fermentation variables[1,2].

	RS * WG Interaction			
	RS		WG	
Variables	NRS WG vs. NWG	RS WG vs. NWG	WG RS vs. NRS	NWG RS vs. NRS
Acetate (µmol)	<0.0001†	0.0072†	<0.0001†	<0.0001†
Propionate (µmol)	<0.0001†	0.0012†	<0.0001†	<0.0001†
Butyrate (µmol)	<0.0001†	0.0070†	<0.0001†	<0.0001†
Cecal contents pH	<0.0001†	<0.0001†	<0.0001†	<0.0001†
Empty Cecum Wt. (g)	0.0020†	0.0007†	<0.0001†	<0.0001†

	WG * FAT Interaction			
	WG		FAT	
Variables	NWG HF vs. MF	WG HF vs. MF	HF WG vs. NWG	MF WG vs. NWG
Acetate (µmol)	0.0062†	0.4872	0.0004†	0.8255
Propionate (µmol)	0.0460	0.0505	0.1390	0.0136
Emboweled body weight (g)	0.2192	0.0691	0.0278	0.4028

[1]Data are shown as full or collapsed interactions based on significant factors, resistant starch (RS, Present or Absent), whole grain (WG, Present [WG] or Absent [NWG]), and fat (FAT, High [HF] or Moderate [MF]).
[2]After Bonferroni correction, an adjusted ANOVA F–test p<0.0125 indicates a significant measurement.

that had a significant p–value for ANOVA F and also passed the B–H FDR test for the two–way interactions. There were no significant two–way interactions for RS*FAT and no dependent variables demonstrated a p–value for ANOVA F approaching significance for this interaction. Differences observed in RS and FAT on several dependent variables are independent of the other factor. Data for two–way interactions are presented as means with pooled SEM in Table 3.5.

Table 3.5. Descriptive statistics for two–way interactions for fermentation variables[1].

	RS * WG Interaction					
Variables	RS WG	RS NWG	NRS WG	NRS NWG	Pooled SEM	p–value (F)[2]
Food Intake (g)[3]	750.58	741.56	767.11	749.40	9.7863	0.6619
Energy intake (kcal)[3]	2980.73	2945.20	3046.59	2980.00	39.3940	0.6986
Active Glucagon–like peptide 1 (pM)	1.2730	1.3864	0.9200	0.9568	0.0390	0.3630
Cecal contents pH	6.8702[a]	6.1989[b]	8.0008[c]	8.2582[d]	0.0311	<0.0001
Empty Cecum Wt. (g)	1.6181[a]	1.8867[b]	0.5265[c]	0.4276[d]	0.0286	<0.0001

(table cont'd.)

	RS * WG Interaction					
Variables	RS WG	RS NWG	NRS WG	NRS NWG	Pooled SEM	p–value (F)[2]
Acetate (µmol)	391.74[a]	489.22[b]	117.08[c]	68.11[d]	15.26	<0.0001
Propionate (µmol)	53.00[a]	81.51[b]	17.23[c]	12.33[d]	3.21	<0.0001
Butyrate (µmol)	93.89[a]	70.58[a]	30.19[b]	15.28[d]	3.81	0.0090
Abdominal body fat %[4]	2.1153	2.0295	2.5751	2.5346	0.0709	0.7504
Inguinal Fat (g)	3.1723	3.0619	3.9462	3.9882	0.1667	0.6537
Emboweled body weight (g)	386.32	381.43	402.57	397.84	4.8680	0.9872
	RS * FAT Interaction					
Variables	RS MF	RS HF	NRS MF	NRS HF	Pooled SEM	p–value (F)[2]
Food Intake (g)[3]	775.13	717.01	778.95	737.55	9.7863	0.4017
Energy intake (kcal)[3]	2914.48	3011.45	2928.87	3097.73	39.3940	0.3713
Active Glucagon–like peptide 1 (pM)	1.3171	1.3424	0.9283	0.9484	0.0390	0.9506
Cecal contents pH	6.5415	6.5276	8.1517	8.1073	0.0587	0.8086
Empty Cecum Wt. (g)	1.8578	1.6429	0.4985	0.4834	0.0286	0.1380
Acetate (µmol)	476.10	402.53	89.39	89.23	15.12	0.1505
Propionate (µmol)	63.74	67.80	15.12	14.05	3.15	0.3231
Butyrate (µmol)	87.36	75.86	21.77	21.19	3.75	0.4389
Abdominal body fat %[4]	1.9929	2.1520	2.4533	2.6564	0.0709	0.7577
Inguinal Fat (g)	2.9709	3.2634	3.7923	4.1420	0.1667	0.8662
Emboweled body weight (g)	382.35	385.40	399.65	400.75	4.8680	0.8426
	WG * FAT Interaction					
Variables	WG MF	WG HF	NWG MF	NWG HF	Pooled SEM	p–value (F)[2]
Food Intake (g)[3]	788.57	729.12	765.51	725.44	9.9137	0.3314
Energy intake (kcal)[3]	2965.02	3062.31	2878.33	3046.87	39.9722	0.3756
Active Glucagon–like peptide 1 (pM)	1.0965	1.0965	1.1489	1.1943	0.0417	0.5888
Cecal contents pH	7.4807	7.3903	7.2125	7.2445	0.0313	0.3331
Empty Cecum Wt. (g)	0.9418	0.9046	0.9835	0.8779	0.0286	0.2388
Acetate (µmol)	208.16[a]	220.34[a]	204.46[b]	162.97[a]	11.43	0.0155
Propionate (µmol)	27.45[a]	33.27[ab]	35.10[b]	28.26[a]	2.07	0.0050
Butyrate (µmol)	53.09	53.39	35.83	30.10	3.11	0.2240
Abdominal body fat %[4]	2.2416	2.4488	2.2047	2.3595	0.0709	0.7132
Inguinal Fat (g)	3.2823	3.8362	3.4809	3.5692	0.1692	0.1727
Emboweled body weight (g)	388.11[ab]	400.78[a]	393.90[ab]	385.38[b]	4.8680	0.0322

[1]Data are shown as full or collapsed interactions based on significant factors, resistant starch (RS, Present or Absent), whole grain (WG, Present or Absent), and fat (FAT, High or Moderate).
[2]An ANOVA F–test $p<0.05$ indicates a significant measurement.
[3]Total food and energy intakes over the total study of 6 weeks.
[4] Abdominal body fat %: $\frac{\text{Abdominal Fat Pads}}{\text{Body weight} - \text{Full GI} + \text{Empty GI}} * 100$

Cecal Contents pH (α<0.0125)

The presence of resistant starch with WG (6.87±0.08) resulted in a significantly greater cecal contents pH than when isolated resistant starch (6.20±0.08) was in the diet without whole grains (Figure 3.3). In the absence of resistant starch, the presence of whole grains (8.00±0.04) significantly reduced pH compared with NWG with NRS (8.26±0.04). The other two comparisons between RS and NRS with either WG (6.87±0.08 vs. 8.00±0.04) or NWG (6.20±0.08 vs. 8.26±0.04) were also significant. The lower cecal content pH values in rats fed resistant starch versus no resistant starch indicate increased fermentation in the cecum. In the waxy (no amylose starch) whole grain control flour groups (NRSWG) there was a low amount of RS (4.93 of diet) only as RS1 as part of the whole grain matrix, but the NRS NWG groups (HF and MF) had essentially no resistant starch. The presence of a small amount of RS1 resulted in some degree of fermentation.

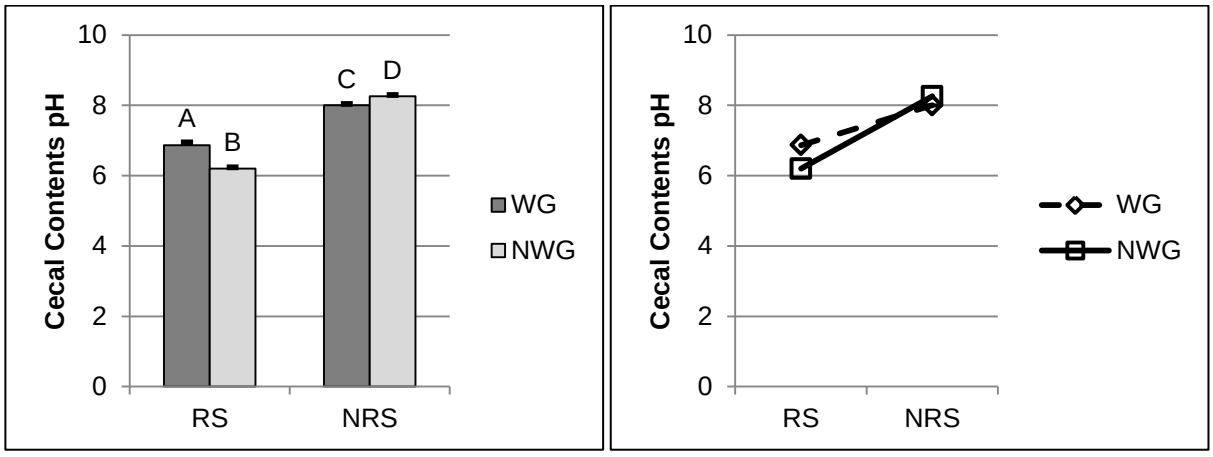


Figure 3.3. Two–way interaction of RS*WG for pH of cecal contents. Grouping includes: RS = resistant to digestion starch type 2 that has high amylose, NRS = no resistant to digestion starch type 2, WG = whole grain flour NWG = no whole grain flour. Data are shown collapsed on two factors, resistant starch (RS, Present or Absent) and whole grain (WG, Present or Absent). Data are expressed in their original form as means ± standard error. Different letters above each bar in the left figure denote significant differences at p<0.0125. The line graph figure on the right has the same data and is included for additional visualization.

Empty Cecum Weight (α<0.0125)

Increased ECW is an indicator that greater fermentation has occurred in the cecum (Figure 3.4). The presence of resistant starch without whole grains resulted in a significantly greater ECW (1.88 g±0.06) and fermentation than resistant starch with whole grains (1.62 g±0.05). In the absence of resistant starch, whole grains had significantly greater fermentation (0.53 g±0.02) than without whole grains (0.46 g±0.01). Comparing the resistant starch groups to appropriate whole grain or non–whole grain controls resulted in significant differences.

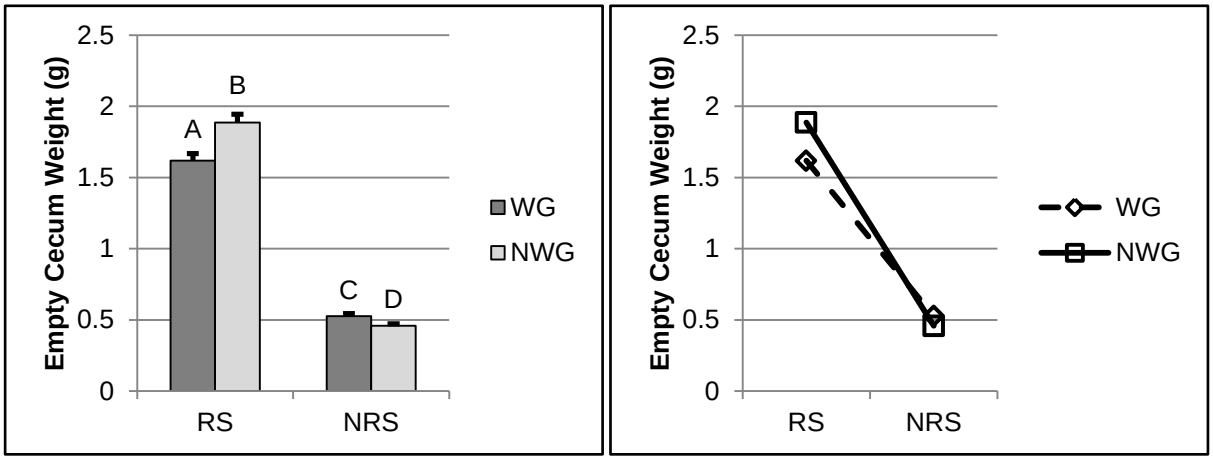
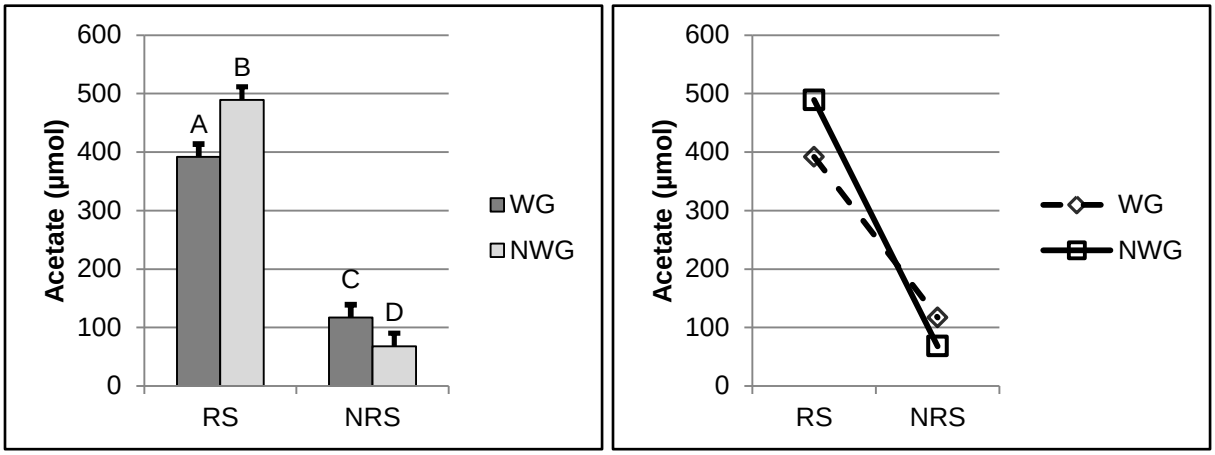


Figure 3.4. Two–way interaction of RS*WG for ECW. Grouping includes: RS = resistant to digestion starch type 2 that has high amylose, NRS = no resistant to digestion starch type 2, WG = whole grain flour NWG = no whole grain flour. Data are shown collapsed on two factors, resistant starch (RS, Present or Absent) and whole grain (WG, Present or Absent). Data are expressed in their original form as means ± standard error. Different letters above each bar in the left figure denote significant differences at p<0.0125. The line graph figure on the right has the same data and is included for additional visualization.

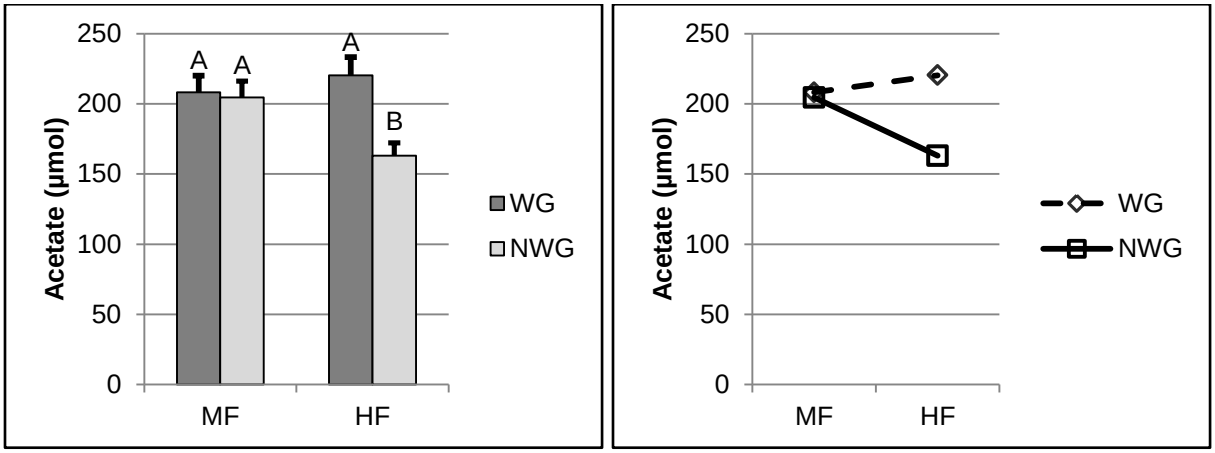
Short–chain fatty acids acetate, propionate, and butyrate (α<0.0125)

RS*WG: The presence of resistant starch without whole grains had greater production of acetate (489.22 µmol±27.94) than whole grain resistant starch (391.74 µmol±22.37), but was not statistically significantly at p>0.0125 (p<0.0295). In the absence of resistant starch, whole grain had significantly greater acetate production (117.08 µmol±6.84) than without whole grains (68.11 µmol±3.89). Acetate production was significantly greater when examining the

comparisons of whole grain resistant starch (391.74 µmol±22.37) to whole grain non–resistant starch (117.08 µmol±6.84), and the presence of non–whole grain resistant starch (489.22 µmol±27.94) to the non–whole grain no resistant starch (68.11 µmol±3.89) group (Figure 3.5A).



A.



B.

Figure 3.5. Two–way interactions of A. RS*WG and B. WG*FAT on µmol acetate produced. Grouping includes: RS = resistant to digestion starch type 2 that has high amylose, NRS = no resistant to digestion starch type 2, WG = whole grain flour, NWG = no whole grain flour, MF= moderate fat, and HF = high fat. Data are shown collapsed on two factors, resistant starch (RS, Present or Absent) and whole grain (WG, Present or Absent). Data are expressed in their original form as antilog means ± standard error. Different letters above each bar denote significant differences at $p<0.0125$. The line graph figures on the right have the same data and are included for additional visualization.

WG*FAT: In the presence of whole grains, there was no significant difference between MF (208.16 µmol±11.89) and HF (220.34 µmol±12.87) for µmol of acetate produced. However,

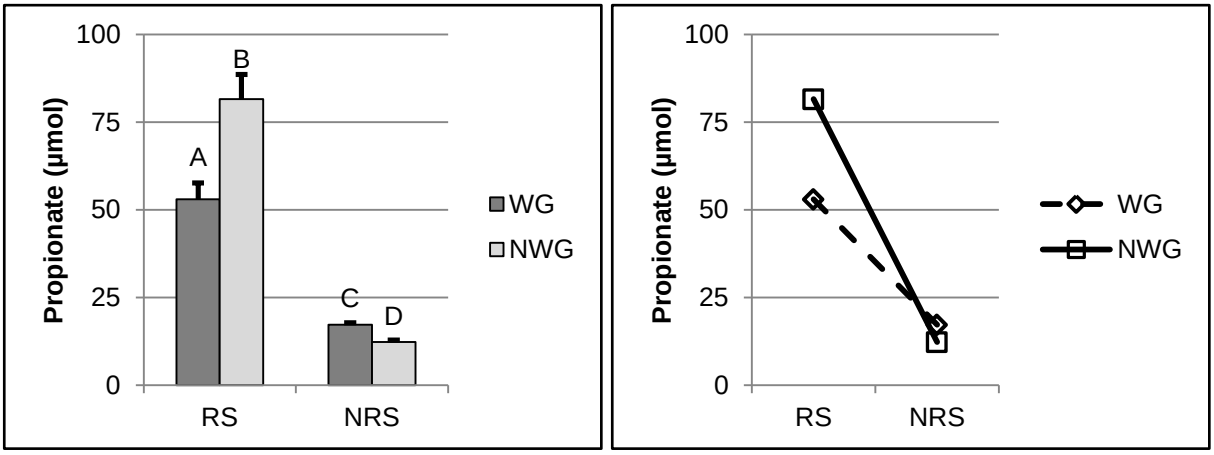
in the absence of whole grain, µmol acetate produced was significantly greater with MF diets (204.46 µmol±11.68) than HF diets (162.97 µmol±9.31). Upon examining MF diets, there was no difference between whole grain presence and absence. However, the in HF diets, presence of whole grains HF (220.34 µmol±12.87) was significantly greater than without whole grains (162.97 µmol±9.31) (Figure 3.5B).

RS*WG: The presence of resistant starch without whole grain had significantly greater production of propionate (81.51 µmol±7.08) than with whole grains (53.00 µmol±4.64) (Figure 3.6A). In the absence of resistant starch, whole grain had significantly greater propionate production (17.23 µmol±0.58) than without whole grains (12.33 µmol±0.56). Propionate production with whole grains present was significantly increased with resistant starch (53.00 µmol±4.64) than without resistant starch (17.23 µmol±0.58). The comparison of resistant starch (81.51 µmol±7.08) and non–resistant starch with no whole grains (12.33 µmol±0.56) resulted in significantly greater propionate production for the isolated resistant starch group.

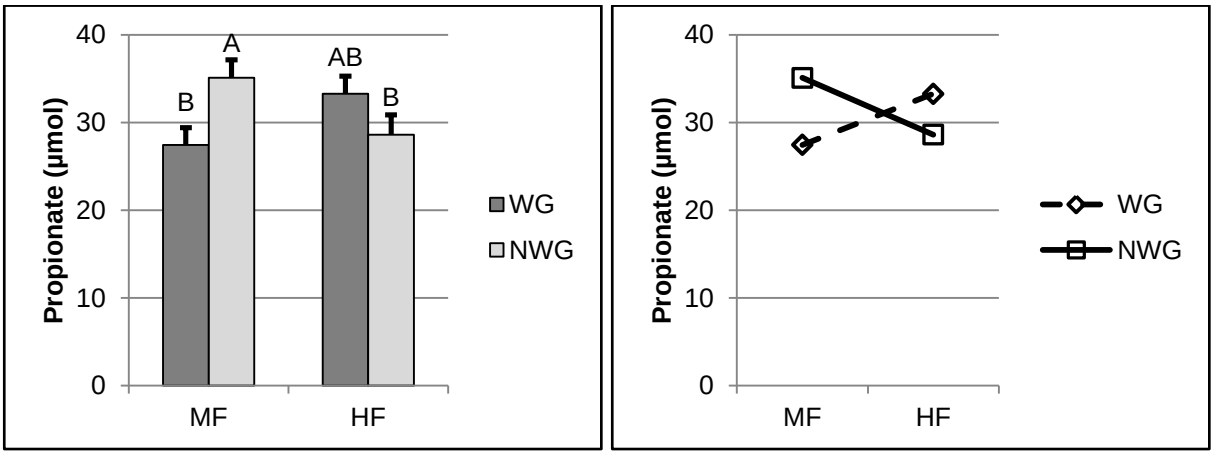
WG*FAT: There were no significant differences for propionate production at $p<0.0125$. However, for MF diets, the absence of whole grain led to numerically higher production of production of propionate, while for HF diets, whole grain presence led to higher production to result in the significant interaction (Figure 3.6B).

RS*WG: The presence of resistant starch without whole grains (70.58 µmol±5.21) was significantly different for production of butyrate compared to whole grain resistant starch (93.89 µmol±6.78) (Figure 3.7). In the absence of resistant starch, whole grains had significantly greater butyrate production (30.19 µmol±2.18) than without whole grains (15.28 µmol±1.08). This dependent variable was unique in that whole grains had greater values with both presence and absences of resistant starch. Butyrate production with whole grains present was significantly

increased with resistant starch (93.89 µmol±6.78) than without resistant starch (30.19 µmol±2.18); and in the absence of whole grains, resistant starch had significantly greater production of butyrate (70.58 µmol±5.21) than in the absence of resistant starch (15.28 µmol±1.08).



A.



B.

Figure 3.6. Two–way interactions of A. RS*WG and B. WG*FAT on µmol propionate produced. Grouping includes: RS = resistant to digestion starch type 2 that has high amylose, NRS = no resistant to digestion starch type 2, WG = whole grain flour, NWG = no whole grain flour, MF= moderate fat, and HF = high fat. Data are shown collapsed on two factors, resistant starch (RS, Present or Absent) and whole grain (WG, Present or Absent). Data are expressed in their original form as antilog means ± standard error. Different letters above each bar denote significant differences at $p<0.0125$. The line graph figures on the right have the same data and are included for additional visualization.

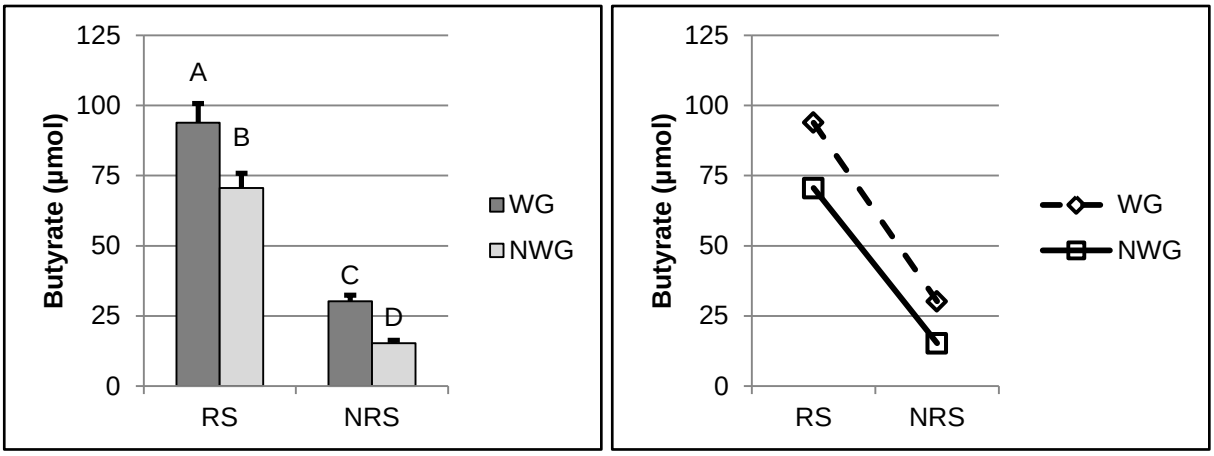


Figure 3.7. Two–way interaction of RS*WG on µmol butyrate produced. Grouping includes: RS = resistant to digestion starch type 2 that has high amylose, NRS = no resistant to digestion starch type 2, WG = whole grain flour NWG = no whole grain flour. Data are shown collapsed on two factors, resistant starch (RS, Present or Absent) and whole grain (WG, Present or Absent). Data are expressed in their original form as antilog means ± standard error. Different letters above each bar in the left figure denote significant differences at $p<0.0125$. The line graph figure on the right has the same data and is included for additional visualization.

Emboweled Body weight

The WG*FAT two–way interaction for EBW was statistically significant but within the interaction, no measurement met significance at $p<0.0125$.

3.3.1.3 Main Effects

Descriptive statistics for main effects for the factors RS, WG, and FAT are shown in Table 3.6. Food and energy intake, fermentation–associated factors, and physiological variables were examined. Data for all main effects are presented as means with pooled SEM.

Table 3.6. Descriptive statistics about response to dietary factors – Main Effects[1].

	Resistant Starch			
Variables	Present	Absent	Pooled SEM	p–value (F)[2]
Food Intake (g)	746.07	758.25	6.920	0.2227
Energy intake (kcal)	2962.97	3013.3	27.8558	0.2117
Active Glucagon–like peptide 1 (pM)	1.3297[a]	0.9384[b]	0.0276	<0.0001
Cecal contents pH	6.5346[a]	8.1295[b]	0.0415	<0.0001

(table cont'd.)

	Resistant Starch			
Variables	Present	Absent	Pooled SEM	p–value (F)[2]
Empty Cecum Wt. (g)	1.7470[a]	0.4909[b]	0.0224	<0.0001
Acetate (µmol)	437.72[a]	89.31[b]	12.05	<0.0001
Propionate (µmol)	65.74[a]	14.57[b]	2.23	<0.0001
Butyrate (µmol)	81.41[a]	21.48[b]	2.97	<0.0001
Abdominal body fat %[3]	2.0724[a]	2.5548[b]	0.0502	<0.0001
Inguinal Fat (g)	3.1171[a]	3.9672[b]	0.1180	<0.0001
Emboweled body weight (g)	383.88[a]	400.20[b]	3.4372	0.0012
	Whole Grains			
Variables	Present	Absent	Pooled SEM	p–value (F)[2]
Food Intake (g)	758.85	745.48	7.0101	0.1814
Energy intake (kcal)	3013.66	2962.6	28.2646	0.2052
Active Glucagon–like peptide 1 (pM)	1.0965	1.1716	0.0295	0.0775
Cecal contents pH	7.4355[a]	7.2285[b]	0.0443	0.0017
Empty Cecum Wt. (g)	0.9230	0.9292	0.0202	0.8288
Acetate (µmol)	214.19[a]	182.56[b]	7.74	0.0067
Propionate (µmol)	30.22	31.70	1.48	0.4845
Butyrate (µmol)	53.24[a]	32.84[b]	2.25	<0.0001
Abdominal body fat %[3]	2.3452	2.2821	0.0502	0.3796
Inguinal Fat (g)	3.5593	3.525	0.1196	0.8402
Emboweled body weight (g)	394.44	389.64	3.4422	0.3262
	FAT			
Variables	Moderate	High	Pooled SEM	p–value (F)
Food Intake (g)	777.04[a]	727.28[b]	7.0101	<0.0001
Energy intake (kcal)	2921.68[a]	3054.59[b]	28.2646	0.0013
Active Glucagon–like peptide 1 (pM)	1.1227	1.1454	0.0295	0.5896
Cecal contents pH	7.3466	7.3174	0.0443	0.6438
Empty Cecum Wt. (g)	0.9624[a]	0.8911[b]	0.0202	0.0145
Acetate (µmol)	206.30	189.50	7.97	0.1414
Propionate (µmol)	31.04	30.86	1.48	0.9312
Butyrate (µmol)	43.61	40.10	2.14	0.2543
Abdominal body fat %[3]	2.2231[a]	2.4042[b]	0.0500	0.0126
Inguinal Fat (g)	3.3816	3.7027	0.1196	0.0613
Emboweled body weight (g)	391.00	393.08	3.4422	0.6711

[1]Data are shown based on factors, resistant starch (RS, Present or Absent), whole grains (WG, Present or Absent) and fat (FAT, High or Moderate).

[2]An ANOVA F–test $p<0.05$ indicates a significant measurement. Means with different letters attached to numbers denote significant differences between groups ($p<0.05$).

[3]ABF%: $\frac{\text{Abdominal Fat Pads}}{\text{Body weight} - \text{Full GI} + \text{Empty GI}} * 100$

Resistant Starch

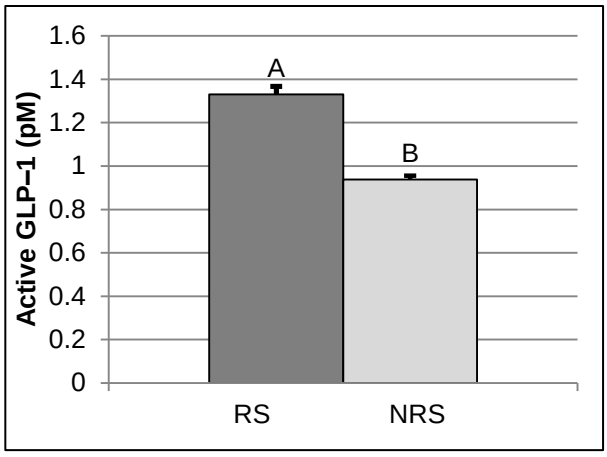
While food and energy intake were not significantly different between the two RS groups, other variables differed. Rats fed diets high in RS vs. NRS demonstrated increased serum active GLP–1, ECW, µmoles of SCFA produced, and decreased cecal contents pH, ABF%, inguinal fat, and EBW. Serum active GLP–1, ABF% and inguinal fat dependent variables were only significant as the main effect of RS (Figures 3.8A–C). However, all significant RS main effects are shown because RS demonstrated a dominant role in all dependent variables that also had interaction effects. Empty cecum weight and µmoles SCFAs produced are two of several indicators of increased fermentation in the gut of rodents. These significant effects were evident when levels of FAT and WG were collapsed into high RS and NRS.

Whole Grain

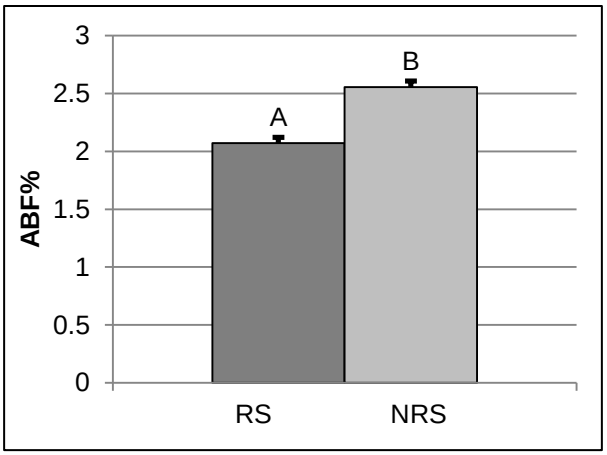
Rats fed WG had increased cecal contents pH, acetate, and butyrate production. Significant effects were evident when levels of RS and FAT were collapsed into WG presence and absence. These main effects are part of interaction effects presented prior.

Fat

Three dependent variables that had observed differences between rats fed diets with high and moderate levels of fat that were not part of interactions. Animals fed HF diets had reduced food intake, increased energy intake (Figure 3.9A, B), and increased ABF% (Figure 3.9C). Rats fed HF diets also had decreased ECW and this significant effect was evident when levels of RS and WG were collapsed into MF and HF. The significant effect for FAT for increased ECW with MF diets was presented prior.



A.



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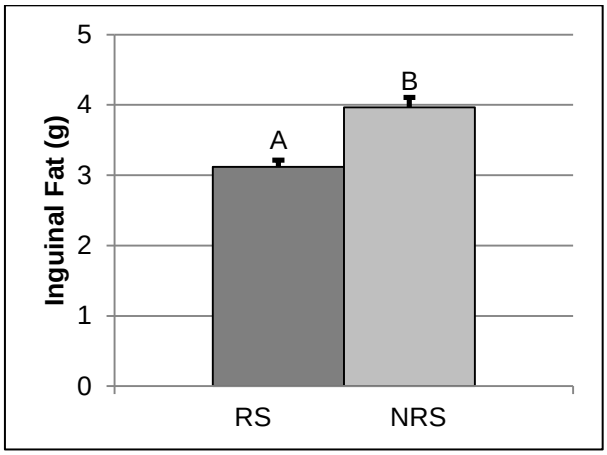
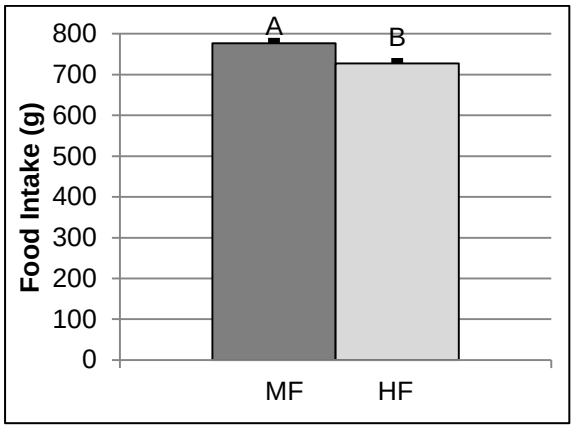


C.

Figure 3.8. Main effect of ABF%, active GLP–1, and inguinal fat. Variables significantly different between RS and NRS that are present only as main effects. A. active GLP–1, B. abdominal fat percent (ABF%), and C. inguinal fat. Grouping includes: RS = high resistant starch, NRS = no resistant starch. Data are shown collapsed to one factor, resistant starch (RS, Present or Absent), with the other factors whole grain (WG, Present or Absent) and fat (FAT, High or Moderate) present in both levels of RS. Data are expressed in their original form as means ± standard error. Different letters above each bar denote significant differences at $p<0.05$.



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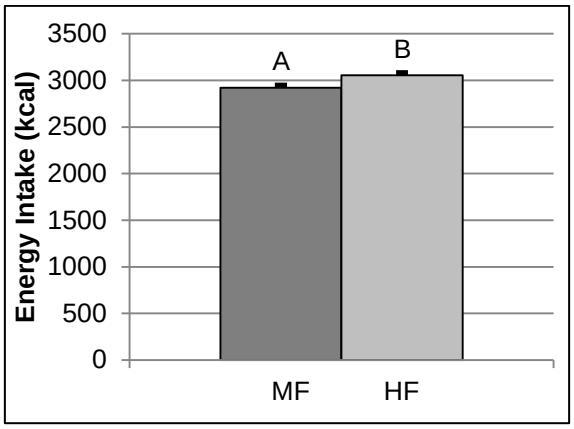
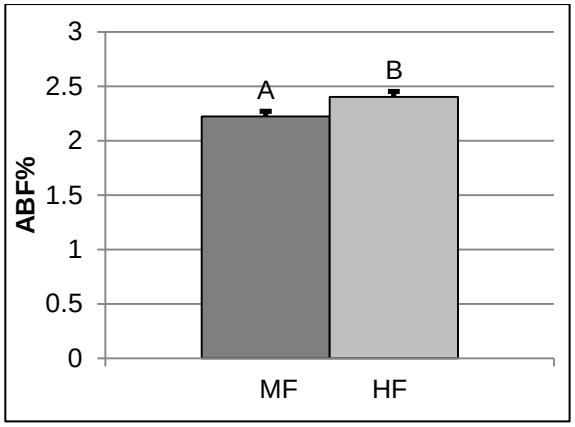


B.



C.

Figure 3.9. Main effect of food intake, energy intake, and abdominal fat percent (ABF%). Significant difference between MF and HF presented only as main effects. Grouping includes: MF = moderate fat and HF = high fat. Data are shown collapsed to one factor, fat (FAT, High or Moderate), with the other factors resistant starch (RS, Present or Absent) and whole grain (WG, Present or Absent) present in both levels of fat. Data are expressed in their original form as means ± standard error. Different letters above each bar denote significant differences at $p<0.05$.

Rats fed HF diets also had decreased ECW and this significant effect was evident when levels of RS and WG were collapsed into MF and HF. The significant effect for FAT for increased ECW with MF diets was presented prior.

3.3.2 Real–time quantitative polymerase chain reaction (qPCR)

3.3.2.1 Cecal cells

Amplification and efficiency for RT–qPCR is reported in Table 3.7. All standard curves for genes expression performed well. The slope and percent efficiency were within acceptable

ranges, –3.1 to –3.6 and 90 to 110% respectively. Slopes for PPIF and ADM were at the acceptable range, resulting in 90% percent efficiency. All other slopes were closer to 100%.

Table 3.7. RT–qPCR amplification efficiency in cecal cells.

Gene Targets	Standard Curve Equation ($y=C_t$)[1]	(R^2)	% Efficiency[2]
Adrenomedullin	–3.5914 * (X) + 36.76	0.9932	90
Cyclophilin–F[3]	–3.5852 * (X) + 26.35	0.9934	90
Galactose–4–epimerase	–3.3575 * (X) + 32.61	0.9916	99
Glucose–6–phosphatase	–3.3385 * (X) + 35.81	0.9848	99
Monocarboxylate transporter member 1	–3.2778 * (X) + 31.94	0.9906	102
Mucin 1	–3.3932 * (X) + 38.92	0.9804	97
Phosphoenolpyruvate carboxykinase 1	–3.2828 * (X) + 28.81	0.9982	102
Pyruvate carboxylase	–3.4675 * (X) + 34.99	0.9778	94

[1]Linear equation of standard curve where C_t = (m)*X + b; m = slope, C_t = cycles to threshold on Y axis, b = y–intercept; solve for copy number (X) which is in terms of arbitrary RNA.
[2]The variable % Efficiency is the amplification efficiency. % Efficiency is calculated as % E = (E–1)*100%, where $E = 10^{\frac{-1}{slope}}$.
[3]Cyclophilin–F was used as the reference gene (normalizer) for gene expression.

Factorial Interactions

Data were examined and underwent $\log_{10}$ transformation to achieve a normal distribution. No data points were considered influential or outliers. Dependent variables were ranked by raw p–values below in Table 3.8 using the B–H FDR test. The most relevant critical value (CV) for the B–H FDR test was calculated to where p–values were less than the CV.

Table 3.8. Cecal cells gene expression F–test p–values sorted by B–H sorted by B–H FDR[1].

RS*WG*FAT		RS*WG	
Variables[2]	*p*	Variables	*p*
Adrenomedullin	0.1484	Phosphoenolpyruvate carboxykinase 1	0.0746
Galactose–4–epimerase	0.1745	Galactose–4–epimerase	0.3094
Pyruvate carboxylase	0.1772	Adrenomedullin	0.4985
Phosphoenolpyruvate carboxykinase 1	0.2810	Mucin 1	0.5108
Glucose–6–phosphatase	0.2965	Pyruvate carboxylase	0.6620
Monocarboxylate transporter member 1	0.3631	Monocarboxylate transporter member 1	0.8180
Mucin 1	0.9320	Glucose–6–phosphatase	0.9831
Most relevant CV[3]	**0.0071**	**Most relevant CV[3]**	**0.0071**

(table cont'd.)

RS*FAT		WG*FAT	
Variables	*p*	Variables	*p*
Pyruvate carboxylase	0.0117†	Adrenomedullin	0.0902
Phosphoenolpyruvate carboxykinase 1	0.0369†	Galactose–4–epimerase	0.1082
Adrenomedullin	0.0839	Phosphoenolpyruvate carboxykinase 1	0.1215
Mucin 1	0.3903	Mucin 1	0.2396
Monocarboxylate transporter member 1	0.6018	Pyruvate carboxylase	0.3062
Galactose–4–epimerase	0.7354	Glucose–6–phosphatase	0.3255
Glucose–6–phosphatase	0.9385	Monocarboxylate transporter member 1	0.3764
Most relevant CV[3]	**0.0071**	**Most relevant CV[3]**	**0.0071**

RS		WG	
Variables	*p*	Variables	*p*
Adrenomedullin	<0.0001†	**Phosphoenolpyruvate carboxykinase 1**	**0.0002†**
Phosphoenolpyruvate carboxykinase 1	<0.0001†	Pyruvate carboxylase	0.0293†
Monocarboxylate transporter member 1	<0.0001†	Monocarboxylate transporter member 1	0.1080
Mucin 1	<0.0001†	Mucin 1	0.1812
Pyruvate carboxylase	<0.0001†	Glucose–6–phosphatase	0.5199
Glucose–6–phosphatase	<0.0001†	Adrenomedullin	0.8328
Galactose–4–epimerase	**0.0006†**	Galactose–4–epimerase	0.9540
Most relevant CV[3]	**0.0500**	**Most relevant CV[3]**	**0.0071**

Fat		B–H Critical Values (Q = 0.05)	
Variables	*p*	Rank	CV
Monocarboxylate transporter member 1	**0.0285†**	1	0.0071
Adrenomedullin	0.0684	2	0.0143
Phosphoenolpyruvate carboxykinase 1	0.1631	3	0.0214
Galactose–4–epimerase	0.3997	4	0.0286
Pyruvate carboxylase	0.4460	5	0.0357
Glucose–6–phosphatase	0.7999	6	0.0429
Mucin 1	0.9982	7	0.0500
Most relevant CV[3]	**0.0071**		

[1]Data are shown based on factors, resistant starch (RS, Present or Absent), whole grains (WG, Present or Absent) and fat (FAT, High or Moderate).

[2]An ANOVA F–test F<0.05 indicates a significant measurement with † superscript denoting significant difference.

[3]Most relevant CV is determined from the greatest significant p–value of dependent variables (ranked lowest to highest) that is less than the CV at each rank, given I/M*Q. "I" is the rank number, "M" is the total number of dependent variables (7), and "Q" is the chosen false discovery rate (0.05).

Following the PROC MIXED factorial analyses, no RS*WG*FAT, RS*WG, and WG*FAT interactions were found to be significant. Significant differences for two dependent variables were observed for the two–way interaction RS*FAT. The results are as follows: PC ($F_{1,76} = 6.66$, $p < .0118$) and PCK1 ($F_{1,77} = 4.51$, $p < .0369$) (Figures 3.10A, B). The RS*FAT interactions were further analyzed using a statistical slice on RS and FAT factors (Table 3.9), but did not pass the B–H FDR test, and are considered falsely detected significant differences.

Table 3.9. Cecal cell gene expression two–way interaction test of effect slices for significant RT–qPCR variables[1,2].

	RS * FAT Interaction			
	RS		FAT	
	NRS	RS	HF	MF
Variables	HF vs. MF	HF vs. MF	RS vs. NRS	RS vs. NRS
Phosphoenolpyruvate carboxykinase 1	0.5244	0.0389	<0.0001†	<0.0001†
Pyruvate carboxylase	0.1047	0.0517	<0.0001†	<0.0001†

[1]Data are shown as collapsed interactions based on significant factors, resistant starch (RS, Present [RS]; or Absent [NRS]) and fat (FAT, High [HF] or Moderate [MF]).
[2]After Bonferroni correction, an adjusted ANOVA F–test p<0.0125 indicates a significant measurement, denoted with the † symbol.

Factorial Main effects

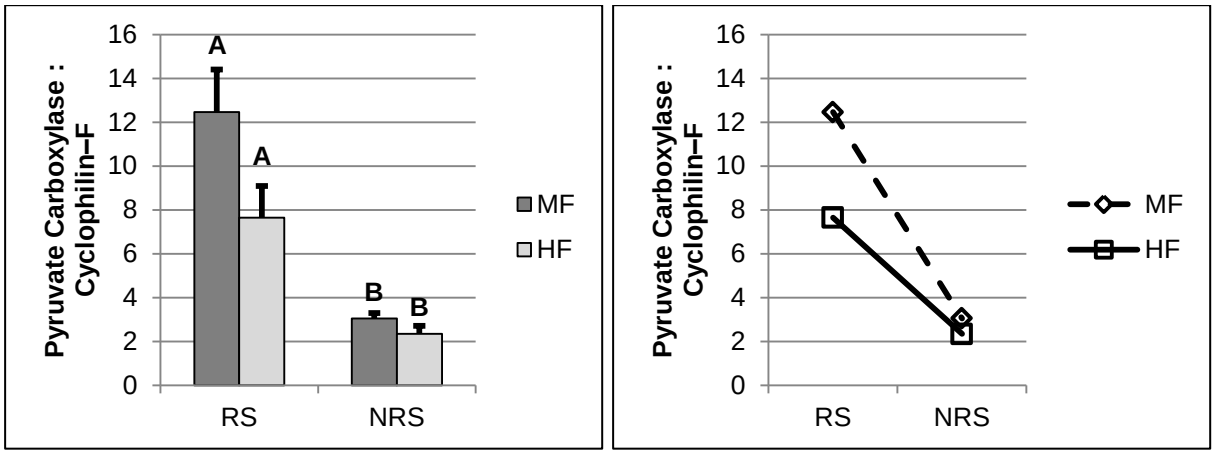
All genes examined were significant for resistant starch treatment. ADM ($F_{1,45} = 25.82$, $p < .0001$), G6pc ($F_{1,77} = 105.08$, $p < .0001$), GALE ($F_{1,88} = 12.54$, $p < .001$), MCT1 ($F_{1,88} = 40.79$, $p < .0001$), MUC1 ($F_{1,67} = 27.56$, $p < .0001$), PC ($F_{1,76} = 78.89$, $p < .0001$), PCK1 ($F_{1,77} = 126.00$, $p < .0001$). In all measurements, presence of resistant starch resulted in higher gene expression than without resistant starch. Whole grain presence increased PC ($F_{1,76} = 25.82$, $p < .05$) and PCK1 ($F_{1,77} = 25.82$, $p < .0005$) gene expression, while MF diets increased MCT1 ($F_{1,45} = 25.82$, $p < .05$) gene expression (Table 3.10).

Table 3.10. Descriptive statistics for targeted genes of interest in cecal cells using RT–qPCR[1].

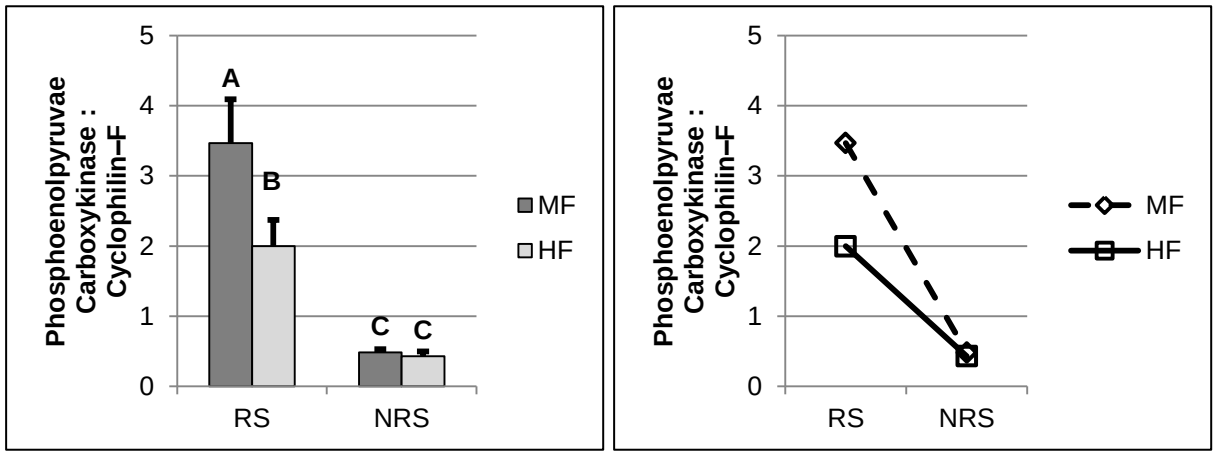
Variables (arbitrary RNA)	Resistant Starch			
	Present	Absent	Pooled SEM	p–value (F)[2]
Adrenomedullin	6.9626[a]	1.8424[b]	0.5042	<0.0001
Galactose–4–epimerase	3.3166[a]	1.5489[b]	0.2574	0.0006
Glucose–6–phosphatase	7.4548[a]	0.4483[b]	0.5631	<0.0001
Monocarboxylate transporter member 1	2.1805[a]	0.3784[b]	0.2234	<0.0001
Mucin 1	6.7737[a]	2.7526[b]	0.4898	<0.0001
Phosphoenolpyruvate carboxykinase 1	3.8133[a]	0.5958[b]	0.2617	<0.0001
Pyruvate carboxylase	13.8082[a]	1.8125[b]	1.6224	<0.0001
	Whole Grains			
Variables (arbitrary RNA)	Present	Absent	Pooled SEM	p–value (F)[2]
Adrenomedullin	4.0224	4.7827	0.5095	0.8328
Galactose–4–epimerase	2.4714	2.3941	0.2565	0.9540
Glucose–6–phosphatase	3.7304	4.1727	0.5241	0.5199
Monocarboxylate transporter member 1	1.6234	0.9355	0.2232	0.1080
Mucin 1	4.6561	4.8702	0.4279	0.1812
Phosphoenolpyruvate carboxykinase 1	2.4372[a]	1.9719[b]	0.2616	0.0002
Pyruvate carboxylase	9.3947[a]	7.6068[b]	0.929	0.0293
	Fat			
Variables (arbitrary RNA)	Moderate	High	Pooled SEM	p–value (F)[2]
Adrenomedullin	5.4221	3.3829	0.5095	0.0684
Galactose–4–epimerase	2.2203	2.6452	0.2565	0.3997
Glucose–6–phosphatase	3.8528	4.0503	0.5242	0.7999
Monocarboxylate transporter member 1	1.3206[a]	1.2383[b]	0.2232	0.0285
Mucin 1	4.8065	4.7198	0.4276	0.9982
Phosphoenolpyruvate carboxykinase 1	2.6281	1.781	0.2616	0.1631
Pyruvate carboxylase	9.6111	7.3904	0.929	0.4460

[1]Data are shown based on factors, resistant starch (RS, Present or Absent), whole grains (WG, Present or Absent) and fat (FAT, High or Moderate).

[2]An ANOVA F–test $p<0.05$ indicates a significant measurement. Means with different letters attached denote significant differences between groups ($p<0.05$).



A.



B.

Figure 3.10. Two–way interactions of RS*FAT on pyruvate carboxylase and phosphoenolpyruvate carboxykinase 1 gene expression. Data are shown collapsed on two factors in A. PC and B. PCK1 genes expressed. Grouping includes: RS = resistant starch, NRS = no resistant starch, MF = moderate fat and HF = high fat. Data are expressed as means ± standard error. Different letters above each bar denote significant differences at $p<0.0125$. The line graph figures on the right have the same data and are included for additional visualization.

3.3.2.2 Inguinal Fat

Data could not be appropriately analyzed for inguinal fat gene expression measurements. Samples either did not amplify sufficiently or standard curves had slopes and R^2 measurements with too poor quality to provide proper normalization and subsequent statistical analyses (Table 3.11). The samples for the 18S rRNA reference gene did amplify well, but the standard curve

had poor amplification not sufficient enough to provide meaningful normalization. Sufficient amplification was measured for ANGPTL4 but was unable to be normalized to the reference gene. Gene targets IL–6, IL–10, LEP, SLC25A25 and TNF–α efficiency was poor due to serial dilution pipetting error or non–specific product amplification. The gene targets had poor slopes and were not applicable to further analyzing. Samples adequately amplified for LEP, SLC25A25 and TNF–α. Gene targets IL–6 and IL–10 did not have adequate amplification of standards or samples, and slope and efficiency measurements were extremely elevated, indicative of non–specific amplification.

Table 3.11. RT–qPCR amplification efficiency in inguinal fat adipose tissue.

Variables	Standard Curve Equation ($y=C_t$)[1]	(R^2)	% Efficiency[2]
18S rRNA	–2.8559 * (X) + 11.61	0.9985	124
Angiopoietin–like 4	–3.1040 * (X) + 29.22	0.9913	110
Interleukin–6	–1.1871 * (X) + 35.79	0.5221	596
Interleukin–10	–0.7724 * (X) + 36.46	0.4977	1871
Leptin	–2.7310 * (X) + 29.84	0.9849	132
Solute carrier family 25 member 25	–2.9154 * (X) + 33.09	0.9621	120
Tumor necrosis factor–α	–1.7858 * (X) + 34.33	0.8045	236

[1]Linear equation of standard curve where C_t = (m)*X + b; m = slope, C_t = cycles to threshold on Y axis, b = y–intercept; solve for copy number (X) which is in terms of arbitrary RNA.
[2]The variable % Efficiency is the amplification efficiency. % Efficiency is calculated as % E = (E–1)*100%, where $E = 10^{\frac{-1}{slope}}$.

3.3.3 Serum

No significant differences for interaction or main effects were observed for serum levels of two pro–inflammatory markers, CRP and TNF–α, and one anti–inflammatory marker IL–10. All standards fit the standard curves well and all sample values were within the highest and lowest standards. Table 3.12 shows the results for these three serum inflammation markers.

Table 3.12. Serum inflammation markers.

Variables	Dietary treatments: RS WG MF (HF)	RS NWG MF (HF)	NRS WG MF (HF)	NRS NWG MF (HF)	Pooled SEM	p–value (F)[1]
C–Reactive protein (ng/ml)	282142 (256660)	304067 (294025)	296975 (313542)	315987 (233144)	12855	> 0.25
Interleukin–10 (pg/ml)	16.6032 (13.9558)	19.2855 (18.6176)	23.2878 (16.6105)	21.6948 (16.4150)	1.6297	> 0.25
Tumor necrosis factor–α (pg/ml)	8.8460 (8.0018)	9.9826 (7.2388)	7.0597 (7.3537)	9.1180 (8.2119)	0.3358	> 0.10

[1]The ANOVA F–test p–value shown is the lowest p–value between the interactions (RS*WG*FAT, RS*WG, RS*FAT, WG*FAT) and main effects (RS, WG, FAT).

3.4. Discussion

The study findings support two of the hypotheses, but do not support the third. The moderate fat diets were better than high fat diets for increasing markers of fermentation with the high resistant starch diet with no whole grains. High fat diets attenuated this fermentation, and negatively (increased) energy intake and abdominal body fat, but decreased food intake. The whole grain resistant starch (flour) prebiotic did not ameliorate the attenuation of fermentation by high fat diets, and was consistent for both levels of fat. This means that whole grain combined with resistant starch was effective for maintaining, instead of reducing, fermentation on moderate and high fat diets, but was not more effective than the isolated resistant starch for increasing fermentation compared to their respective control group.

Serum markers of inflammation were no different as a response to resistant starch, whole grain, or dietary fat. The major anti– and pro–inflammatory cytokines, IL–10 and TNF–α respectively, were similar in concentration, suggesting that gut fermentation of the dietary factors did not alter systemic levels of cytokines. Similarly, as CRP is elevated as a response to inflammation, no differences in concentration suggests that whole–body inflammation was not affected by dietary treatments. Studies with human subjects also exhibit inconsistent changes to

markers when consuming dietary resistant starch. Johnston et al. (2009) reported no changes in inflammatory markers in insulin resistant subjects [81], while Gargari et al. (2015) reported reductions in TNF–α in patients with Type 2 diabetes patients [82]. However in our studies, Sprague Dawley rats do not have a "disordered state" where resistant starch may be able to mediate changes in inflammatory status. Future studies using obese models or transgenic animals may further elucidate the role of fermentable fiber in altering inflammatory cytokines and proteins.

Biometric Analyses

Results from the current study demonstrated that the three factors (RS, WG, and FAT) produced various individual main effects and interactions with each other. Robust effects on fermentation and phenotype were observed between factors that shape the parameters of fermentation. There was an observed effect on ECW that approached significance in a three–way interaction that was primarily driven by the presence or absence of resistant starch. For many of the dependent variables analyzed, resistant starch, and to a lesser extent fat, appear to be the primary factors driving observed differences due to large changes in biometric responses identified in the main effects that were also similar in magnitude in the interactions.

Several of our previous studies demonstrated that consumption of resistant starch was associated with a reduction in body weight–normalized abdominal body fat. This was hypothesized to be the result of increased fermentation because diets with resistant starch and control diets were isocaloric [3, 4, 9]. Similar observations were noted in this present study through high resistant starch increasing ECW and active GLP–1, and decreasing ABF% and EBW. Fermentative ability is observed to be responsive to the interactions of the factors. The interactions that include resistant starch and whole grains for cecal contents pH, ECW and cecal

contents acetate, propionate and butyrate illustrate the efficacy of dietary resistant starch for increasing gut fermentation. These findings suggest that the whole grain control fermented better than the non–whole grain control because of the presence of a relatively small, but appreciable amount of RS1. However, the presence of whole grains resulted in greater amounts of butyrate, but lower amounts of acetate and propionate, suggesting additional utilization of the whole grain kernel by butyrate producing bacteria.

Within the two–way interactions, there was consistent production of acetate and propionate with the consumption of whole grains on moderate fat or high fat diets, but reduced production with the consumption of isolated high resistant starch product under high fat conditions. This consistent reduction illustrates that fermentation of isolated high resistant starch was negatively affected by the high fat diet. Increased amounts of butyrate with whole grain diets may be beneficial as butyrate is a major energy source for the colonocytes [26] and butyrate is considered beneficial to the health of the gut [83]. Along with lactate produced by the microbiota, acetate can be utilized by bacteria, in genera such as Clostridium cluster IV and Clostridium cluster XIV to produce butyrate [31, 32]. Acetate and propionate produced in the colon can be found in the portal, hepatic and peripheral blood in greater amounts than butyrate [26]. These SCFAs are utilized by peripheral tissues (muscle, acetate) or by the liver (acetate, propionate) for metabolism [27–30]. Propionate and butyrate may have a role in modulating glucose metabolism as propionic acid and butyric acid act as stimulators of intestinal gluconeogenesis (IGN) [84]. Butyric acid is a direct stimulator of IGN gene expression and propionic acid activates a gut–brain neural circuit for IGN gene expression and is a substrate for IGN. One of the main effects of fermentable fibers is to improve insulin sensitivity by reducing

hepatic glucose output because of the glucose signaling from IGN. Propionate also influences regulation of blood pressure through olfactory and G–protein coupled receptors [85].

We hypothesized that a moderate fat diet would result in a similar phenotype (body weight, ABF%, food and energy intake, etc.) as previously observed with low fat diets, and possibly lie between low and high fat diets in fermentation parameters (cecal contents pH, SCFAs, ECW, etc.). Validation of these hypotheses would show that moderate dietary fat consumption provides greater health benefits than high dietary fat consumption. Our lab group has shown that moderate fat diets are comparable to low fat diets on ABF% in C57Bl/6J mice [10] but the ceca and cecal contents were used for microbiota analyses and not analyzed for routine fermentation dependent variables. In our previous studies, diets low in fat contributed to a healthier gut (Zhou et al., 2009), while a high fat diet attenuated fermentation and phenotype effects (Charrier et al., 2013). In the current study, the moderate fat diet was associated with a lower ABF% and a greater ECW in rats consuming high resistant starch diets compared to those consuming a high fat diet. However, presence of whole grain with either the presence or absence of resistant starch showed no differences between high fat and moderate fat consumption. The latter result indicated our hypothesis that a whole grain resistant starch prebiotic would ameliorate the negative effects caused by high fat diets was partially validated. However, we expected that the whole grain resistant starch would ferment better than the isolated resistant starch with both moderate fat and high fat diets, but it did not.

Whole grains in diets have been shown to have many positive effects on gut and whole body health [13, 14, 37–43]. However, we observed that whole grains had a complicated role. For instance, whole grains increased the fermentation variable SCFA butyrate for high fat diets when compared to diets without whole grains. The disappointing results for whole grains were

that its presence with resistant starch reduced had fermentation in a moderate fat diet as indicated by greater cecal contents pH, lower ECW, and SCFA produced except for butyrate compared to the no whole grain with resistant starch. These results suggest that RS2 is better fermented than RS1, especially with moderate dietary fat. The isolated resistant starch groups had high–amylose starch composed of 100% RS2 starch granules. The whole grain resistant starch diet groups had whole grain flour that was the source of the high–amylose starch, and, therefore, had a combination of high–amylose starch RS2 granules and RS1 from the whole grain matrix. The assay for resistant starch does not distinguish between types of resistant starch, only time of digestion. Thus, our diets were prepared based on total resistant starch. Based on the content of RS1 in the waxy whole grain flour, that has no high–amylose RS2 starch granules (100% amylopectin) and only RS1, we estimate that our whole grain resistant starch diets had ~5% RS1 and ~18% RS2 for a total of ~23% resistant starch.

Possible explanations as to why RS2 appears to be more fermentable than RS1 must be considered. First, the physical arrangement of the starches differs between resistant starch forms. The high–amylose starch forms granules of RS2 that resist digestion in the small intestine. Unlike the granules of RS2, the resistant starch in whole grains (RS1) is a component of the food matrix which acts as a barrier to amylolysis [17]. So we speculate that it is unlikely that RS1 was digested to a greater extent, and it is more likely that bacteria feeding upon these starches can rapidly ferment the RS2, whereas the whole grain matrix that protects starch as with RS1 requires more time to access and more of it may not be fermented [86]. Secondly, the site of measurement is important for determining the fermentability of resistant starch. Starch without the bran (e.g. isolated RS2) is rapidly fermented in the cecum and proximal colon. Govers et al. (1999) determined that starch with the bran, such as a whole grain starch, is fermented slowly

and exhibits greater fermentation in the distal colon [86]. The current study measured the effects of RS2 in the cecum and likely resulted in a substantially greater degree of fermentation using isolated high–amylose RS2 granules over a combination of RS2 and RS1. This distinction is useful when examining the differences between resistant starch and whole grain. Regional differences in fermentation mean differing implications for risk of bowel diseases and SCFA distribution. Regional fermentation may substantially contribute to the finding that whole grains can reduce risk of colorectal cancers in the distal colon where most colon cancers occur [39]. This mix of two different types of RS, as well as a variety of fermentable fibers in a varied diet, has implications for better health of the entire GI tract. In addition, better health of the whole GI tract would likely lead to a better microbiota, and this in turn would lead to greater physical and mental health. Improved mental health is now correlated with differences in composition of the microbiota [87–89].

This RS2 versus RS1 finding is in stark contrast to a previous study conducted by our lab [90]. In that study, obese Zucker Diabetic Fatty (ZDF) rats fermented the whole grain prebiotic better with and without resistant starch compared to groups without whole grains in low fat diets. However, they also fermented the non–whole grain resistant starch well. Although no changes in body fat were observed, ZDF rats demonstrated substantial increases in fermentation and alterations to the microbiota. Prior to conducting the study, ZDF rats were thought to be dysbiotic and poor fermenters of resistant starch, whereas Sprague Dawley (SD) rats had previously been shown to ferment resistant starch robustly and have reduced ABF% [89, 91, 92]. The study with obese ZDF rats was our first study using the whole grain high resistant starch product and we switched to Sprague Dawley rats anticipating a similar response to whole grain

high resistant starch, but to also observe the reduced ABF%. Thus, our results with the whole grain resistant starch were unexpected.

Charles River company had developed separate Sprague Dawley colonies by phenotype with obese–prone (OP) and obese–resistant (OR) based on consumption of high fat diets but both had more body fat than typical Sprague Dawley colonies [93]. The genotype behind this phenotypic difference has not been delineated (personal communication with the local representative of Charles River animal supplier company) and is likely multigenic and more complex than many other rodent obesity models like the obese ZDF rat. Obesity in ZDF rats is a monogenic trait, where the leptin receptor is defective. In the current study, used Sprague Dawley rats from our usual supplier of these rats (Envigo, Somerset, NJ) as in our previous studies that are we did not separate by phenotype. Our previous studies with Sprague Dawley rats appeared not to be affected by possible different phenotypes as all except one used low fat diets. In the one study in which we did compare low fat and high fat diets fed to Sprague Dawley rats [9], we only used diets with isolated high–amylose RS2 starch granules and were not aware of an effect of the different phenotypes. Our current results with Sprague Dawley rats demonstrate that the rats with an unknown mix of phenotypes we received for the study appear not to ferment RS1 in a whole grain product as well as the obese ZDF rats [90], especially as part of a moderate fat diet.

The results from the current study support our previous studies and continue to demonstrate the benefits of consuming resistant starch. In addition, the benefits of moderate fat diets and whole grain products were also demonstrated. Moderate fat diets appear to be as effective as low fat diets in promoting fermentation of resistant starch and other effects including reduction of abdominal body fat normalized to body weight. Whole grain products demonstrated

consistent fermentation in both high and moderate fat diets and appear to promote nourishing the gut by increasing butyrate production. However, the phenotype mix of Sprague Dawley rats (OP, OR) may affect the response to resistant starch and whole grain products. This suggests that the Sprague Dawley rat is likely a good model for investigating prebiotic substances, but the two phenotypes possibly should be separated for more consistent results. . Additionally, our results indicate that different rat types appear to have different microbiota that affects their responses to the whole grain high resistant starch product

Gene Expression

The role of the gut as a gluconeogenic organ has been not been resolved. Currently, as evidence accumulates, the consensus is moving towards supporting the hypothesis. Here we provide additional evidence that several genes, likely through microbial fermentation, are upregulated in the gluconeogenesis pathway in the cecum. Pyruvate Carboxylase (PC, pyruvate to oxaloacetate), phosphoenolpyruvatecarboxykinase 1 (PCK1, oxaloacetate to phosphoenolpyruvate) and further along the pathway glucose–6 phosphatase (G6pc, glucose–6–phosphate to glucose) genes demonstrate increased gene expression when rats were fed resistant starch, and to lesser extent, whole grain diets. Although we did not measure insulin sensitivity in the current study, the increased gene expression of cecal gluconeogenic enzymes suggests an enhancement of insulin sensitivity through improved glucose homeostasis. Though gluconeogenesis genes are known to be expressed in the small intestine [94], we report upregulation of IGN gene expression at the site of fermentation likely through utilization of and RS2 as a fermentable substrate in the cecum.

Increased gene expression does not always mean increased translation to the protein and increased amounts of the protein. Therefore, we measured the major regulatory protein in the

gluconeogenesis pathway in the rat, PCK1 (cytoplasmic). It was observed that resistant starch, especially in whole grain diets, had increased amounts of the protein, which further indicated increased IGN in the cecum. This is somewhat surprising because we believe that the RS1 was fermented to a lesser extent in the cecum and would be fermented in the distal colon.

Increased oxidative stress is reported to contribute to insulin resistance [95, 96]. Adrenomedullin (ADM) suppresses reactive oxygen species which inflame adipose tissue due to overutilization of mitochondrial oxidative phosphorylation in adipose tissue [97, 98]. We observed a four–fold increase in ADM gene expression when rats were fed resistant starch. Though we did not measure insulin sensitivity, our observation indicates the potential for improved insulin sensitivity. An additional role of ADM as a potent vasodilator and a stimulator of angiogenesis imply a reduction to hypoxic injury and increased blood flow to the gut with increased fermentation and healthy growth of the cecum. The possibility of increased blood flow, reduced oxidative stress and increased gluconeogenic capacity, as a result of microbial fermentation of resistant starch, illustrates possible improved gut health and the potential to lead to improvements in physical and mental health.

Microbial fermentation also improves the colonic barrier and improves goblet cell function. The intestinal epithelium is important for innate host defense, primarily for its barrier between the host and microbial communities, pathogenic or not. *Akkermansia muciniphilia,* a mucin degrading bacterium, is considered beneficial for gut health, however in sufficiently large quantities, colonization of *A. muciniphilia* is detrimental to gut health with patients afflicted with IBD [99]. Aiding this barrier is the colonic mucus layer formed from goblet cells secretions. Galactose–4–epimerase (GALE) catalyzes the first step in the mucin biosynthesis pathway, while membrane–bound mucin 1 (MUC1) expression is partly responsible (with other mucins) for the

O–glycosylated proteins that form the protective mucus barrier lining the epithelial surface. Dietary resistant starch upregulated gene expression of GALE and MUC1, demonstrating a twofold increase over the non–resistant starch condition. Resistant starch induced mucin biosynthesis provides a better barrier for the intestinal epithelium, and may improve heath for patients afflicted with IBD. A relatively recent report [100] demonstrated that fermentable fiber is needed in the diet to support the maintenance of bacteria that limit by competition, the mucin–degrading bacteria to maintain a healthy mucus barrier to prevent bacterial access to the epithelial cells of the GI tract.

Coinciding with increased gut fermentation is the production of SCFAs. Uptake of produced SCFAs is partly facilitated by monocarboxylate transporters located on the epithelial plasma membrane. As butyrate is the main energy source for the colonocytes, increased gut fermentation provides an abundance of energy for the microbial community and one of its products provides energy for these cells. Increased expression of monocarboxylate transporter 1 (MCT1) illustrates increased capacity for uptake of SCFAs, and should improve gut health. We observed increased expression of the transporters when rats were fed high resistant starch, but lower amounts with the high fat diet that attenuates fermentation parameters with the high–amylose RS2. Fermentation of dietary resistant starch provided a fivefold increase in gene expression, suggesting that availability of energy is monitored by the colonocytes and uptake capacity is equivalent with microbial SCFA production. However, this also suggests that diets higher in fat produce less gut fermentation overall, with decreased production of SCFAs leading to decreased capacity for uptake being more energetically favorable for the cells.

Ravussin et al. (2012) demonstrated correlations between inguinal adipose adipokines (ANGPTL4, IL–10, TNF–α, and SLC25A25) that were negatively related to increases in

bacterial genus *Allobaculum* [8]. In this study, we were unable to replicate these findings. Our RT–qPCR experiments did not produce acceptable amplification for individual samples, even for ANGPTL4 which had a good standard curve. Unlike the measurements in cecal cells, RNA extracted from inguinal adipose required additional cleanup and concentration steps to obtain appropriate quality. It is not understood why Ravussin et al. had successful results with inguinal fat and our lab could not replicate their results despite what appeared to be clean and concentrated RNA.

Although isolated high–amylose RS2 granules in the diet had its greatest effect with a moderate fat diet and exhibited greater fermentation than whole grain high resistant starch, we observed a substantial benefit to gut health through feeding diets that included fermentable fibers. Dietary resistant starch, whole grains, and both moderate and high dietary fat improved gut health through microbial induced fermentation. Subsequently, improved gut health resulting from the enhanced ability to nourish and protect itself furthers the co–expression of genes that benefit from the availability of fermentable fiber. Potentially increased blood flow, reduced oxidative stress and gluconeogenic capacity, as a result of microbial fermentation of resistant starch, illustrated improved gut health that can indirectly lead to the improvement of whole body health.

CHAPTER 4. A DOSE RESPONSE ANALYSIS OF FERMENTATION IN LEAN ZUCKER DIABETIC FATTY RATS FED WHOLE GRAIN RESISTANT STARCH

4.1. Introduction

Epidemiological studies continue to demonstrate an association between decreased risk for obesity and chronic disease and increased consumption of fiber and whole grains [71]. However, current policies for fiber intake recommend twice the fiber amount that the average U.S. adult consumes [1, 12]. This issue is further exacerbated, because fiber intake is directly mediated by food intake. The prevalence of sugar– and fat–rich products available commercially, combined with diets that recommend low carbohydrate intake, produce an environment where dietary fiber intake has been an afterthought in consumer's purchasing and consumption habits. Regrettably, to abstain from dietary fiber is also to diminish the potential benefits induced from the availability of fermentable fibers.

Consumption of fermentable fiber stimulates the growth and maintenance of the gut microflora [22]. The microbial community, in turn, stimulates or produces many end products, including metabolically useful gut hormones and short chain fatty acids (SCFAs). Resistant starch (RS), a fermentable fiber, resists enzymatic digestion in the small intestine and is capable of fermentation in the lower gut, the colon [19]. Diets including RS have been shown to induce a suite of metabolically favorable outcomes, including improved plasma glucose and insulin responses [57, 58], mineral absorption [56], and reduced risks of cardiovascular disease and cancers [39–41].

Similarly, whole grain (WG) consumption has been associated with reduced risk for cardiovascular disease, type 2 diabetes [37, 38], cancers [39–41], and all–cause mortality [42, 43]. Like dietary fiber, whole grain intake has remained less than the recommended amounts

despite the benefits derived from consumption and fermentation [47]. At the time of our studies, a whole grain flour product from a natural corn variety with lower amylopectin than typical corn was available for comparison with the isolated starch from this same corn product. This comparison was addressed in chapter 3. In the current study, this whole–grain resistant starch (WGRS) product was more thoroughly studied in a dietary dose response design using lean Zucker Diabetic Fatty (ZDF) rats. The change from Sprague Dawley rats to lean ZDF rats was because Sprague Dawley rats fermented the isolated RS product better than the WGRS. In our previous study, obese ZDF rats fermented the WGRS better than the isolated RS product [90]. However, the obese ZDF rats had no reduction in ABF% likely due to their defective leptin receptor. We hypothesized that the lean ZDF rats would also ferment the whole grain product to a greater extent than the isolated RS product.

The WGRS has both RS1 and RS2. The RS1 exists because the matrix of the whole grain kernel in the flour prevents access of the amylase enzymes to the starch; and the RS2 exists because of the granular structure of the high–amylose starch granules [77]. Thus, WGRS has a combination of RS1 and RS2. The whole grain control group was fed a diet that included a waxy whole grain flour product. This product also comes from a natural corn variety, but this product has 100% amylopectin for its starch component. Therefore, the waxy whole grain product has RS1, but no RS2. The amount of the waxy whole grain product used in the study resulted in ~2% of the diet as RS1.

Identifying a minimal level of fiber intake to improve gut health remains imperative. It is estimated that ~10% of the weight of the diet in rats is equivalent to the recommendation for fiber consumption for humans [101]. Mechanistic, proof–of–concept doses of fiber designed to elicit strong responses to gut fermentation in rats have already shown favorable results [9, 23,

93]. In the current study, the aim was to determine if an effective lower dose was beneficial for gut health. The objectives were to (1) determine if whole grain alone (RS1 without RS2) was effective as a fermentable fiber compared to non–whole grain or RS fiber, and (2) determine which dose of WGRS was different from the waxy whole grain product without RS2. The hypothesis was that WGRS would be effective in providing health benefits at all doses included in the study that included 5, 10, 15, and 20% compared to the waxy whole grain control.

4.2. Methods

Diets

Diet treatments were adapted from AIN–93M purified diets for rodents (Table 4.1) [76]. Diets contained one major starch source as either an isolated starch product or as whole grain flour. Starches and whole grain flours were analyzed by proximate analysis (Medallion Labs for Ingredion Incorporated). Starches included: (1) AMIOCA® waxy corn starch, (2) Waxy whole grain corn flour, or (3) HI–MAIZE whole grain resistant corn flour. Diets with RS were calculated to contain increasing delineations by weight. The waxy whole grain starch was calculated to have 2.0% RS, due to the whole grain kernel containing a resistant starch component in the bran when not overly processed. RS content was determined by Ingredion Incorporated using the modified Englyst Assay [78]. Isocaloric diets were formulated to provide 3.70±0.001 kcal/g energy.

Table 4.1. Diet composition.

	CON[1]	WG	5% HMWG	10% HMWG	15% HMWG	20% HMWG
Ingredients	Grams	Grams	Grams	Grams	Grams	Grams
Waxy corn starch[2]	454.80	166.20	396.10	337.20	278.50	219.60
Waxy whole grain flour[3]	0.00	350.9	0.00	0.00	0.00	0.00

(table cont'd.)

Ingredients	CON[1]	WG	5% HMWG	10% HMWG	15% HMWG	20% HMWG
	Grams	Grams	Grams	Grams	Grams	Grams
High–amylose whole grain starch	0.00	0.00	93.10	186.20	279.30	372.40
Sucrose	100.00	100.00	100.00	100.00	100.00	100.00
Casein[4]	140.00	112.63	131.71	123.43	115.15	106.90
Cellulose	129.50	109.17	109.11	88.72	68.32	47.89
Corn oil[5]	85.00	70.40	79.40	73.80	68.10	62.50
Lard[5]	42.50	42.50	42.50	42.50	42.50	42.50
Mineral mix	35.00	35.00	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00	10.00	10.00
Choline chloride	1.40	1.40	1.40	1.40	1.40	1.40
L–Cystine	1.80	1.80	1.80	1.80	1.80	1.80
Total	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00
Resistant Starch, %[6]	0	2.00	5.00	10.00	15.00	20.00
Total Energy, kcal	3700.10	3700.00	3700.20	3700.00	3700.10	3700.20

[1]Diets include: CON = Amylopectin control corn starch containing no resistant starch diet; WG = waxy whole grain amylopectin control corn flour containing low resistant starch diet; X% HMWG = whole grain high–amylose maize resistant corn starch diet.

[2,3]Waxy & high–amylose corn starches and whole grain flours were gifts from Ingredion Incorporated (Bridgewater, NJ).

[4]Casein was reduced in each diet based on the protein constituent in AMICOA® and HI–MAIZE® corn starches and whole grain flours analyzed by proximate analysis performed by Medallion Labs for Ingredion Incorporated, and differs from the AIN–93M standard 140 g/kg.

[5]Corn oil was modified in each diet based on the fat content in AMICOA® and HI–MAIZE® corn starches and whole grain flours analyzed by proximate analysis performed by Medallion Labs (Minneapolis, MN) for Ingredion Incorporated (Bridgewater, NJ), and differ from the AIN–93M standard 40 g/kg. Corn oil and lard were calculated to adjust fat present in all diets to ~ 18% of energy.

[6]Diets with high amylose starch contain resistant starch type 2, but the whole grain flour with high amylose has both resistant starches 1 and 2. Diets with waxy whole grain flour contain only resistant starch type 1. Resistant starch content of experimental starches was determined by Ingredion Incorporated using modified Englyst assay [78].

Animals & Euthanasia

Sixty–eight male Lean Zucker rats (Fa +/?) were purchased from Envigo (Somerset, NJ) at six weeks of age, and maintained on a chow diet during a one week quarantine. Rats were then stratified randomly by body weight into six groups (n=11; n=12 5%, 10% HMWG; average 159.4±12.6 grams). Treatment groups consisted (1) control starch with no WG or RS [CON], (2)

whole grain waxy corn flour with low RS [WG], (3–6) WG high–amylose maize flour rich in resistant starch (WGRS) [x% HMWG] (Figure 4.1). All animals were housed in a locked facility in individual stainless steel hanging cages with wire mesh bottoms to measure food spilled and prevent coprophagy. Housing environmental conditions included a 12:12h light–dark cycle, 21–22°C ambient temperature with a 55% relative humidity. Animals were allowed *ad libitum* access to food and water, and for six weeks. Food intake, food spilled, and body weight were measured twice per week. Rats were then euthanized and exsanguinated by cardiac puncture after inhalation of isoflurane anesthesia delivered by soaked cotton balls. For each rat, blood samples were collected with dipeptidyl peptidase IV inhibitor for additional analyses. The gastrointestinal (GI) tract was removed from the base of the esophagus to the anus, separated into individual parts (stomach, small intestine, cecum, and large intestine) and weighed full and empty. Subcutaneous inguinal adipose and abdominal fat pads (epididymal, perirenal, and retroperitoneal) were collected and weighed to determine percentage of abdominal fat (ABF%). Abdominal fat percent was calculated as the abdominal fat pads divided by the body weight of the rat with the GI tract contents weight removed ($ABF = \frac{\text{Abdominal Fat Pads}}{\text{Body weight – Full GI + Empty GI}} * 100$). Cecal contents were collected and divided into 0.5 g aliquots, and frozen in liquid N_2 for measurement of pH and short–chain fatty acids (SCFAs). Cells lining the ceca, inguinal and epididymal fat pads were flash frozen in liquid N_2 and stored at –80°C until later analysis. The protocol for this study was approved by the Louisiana State University Institutional Animal Care and Use Committee, protocol 13–088.

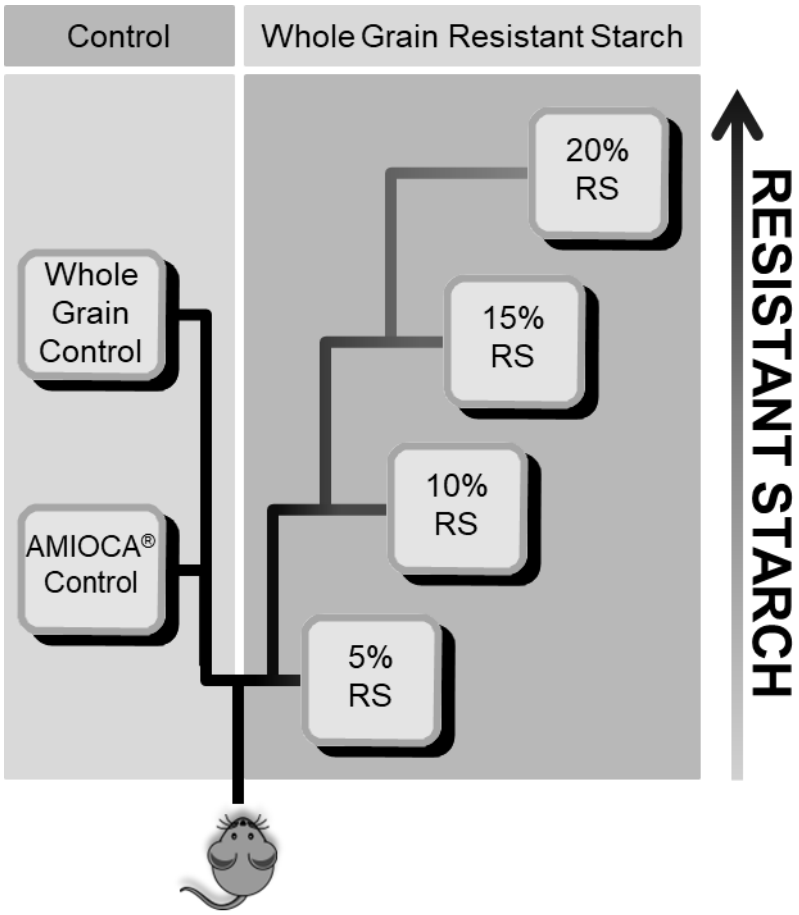


Figure 4.1. Experimental design. Study was designed as a dose–response to increasing percentage of whole grain resistant starch. Each whole grain resistant starch group (n=12; n=11 for 10% and 15% RS) contains an increased level of fermentable fiber. AMIOCA® and Whole grain controls (n=12) contain digestible fiber.

Blood measurements

Serum active glucagon–like peptide 1 (GLP–1, ALPCO, NH), and C–reactive protein (CRP), tumor necrosis factor alpha (TNF–α), and interleukin–10 (IL–10, ThermoFisher, Waltham, MA) levels were measured with enzyme–linked immunosorbent assay (ELISA) kits.

Cecal contents pH and short–chain fatty acids analysis

Cecal contents were thawed and 0.5 g of sample was homogenized with 5 ml of distilled water for pH measurements. Wet and dry weights were measured for each sample. Each sample was then acidified with 1 ml 25% (wt/wt) solution metaphosphoric acid containing a 2 g/L 2–

ethyl–butyric acid internal standard. Solids were separated by centrifugation at 8,000 X *g* for 10 minutes and filtered through a Millipore filter (MILX HA 33 mm, 0.45 μm MCE STRL; Fisher SLHA 033SS). The filtered supernatant was transferred to a gas chromatograph (GC) autosampler vial. SCFAs were analyzed by gas–liquid chromatography for quantitative determination. Detailed methods for quantification of SCFAs via GC have been described in previous publications from our lab [9].

Quantitative real–time PCR (qPCR)

RNA was extracted from cecal cells and inguinal adipose using the RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit respectively (Qiagen, Valencia, CA). Samples with a poor 260/230 absorption spectra ratio (lower than 1.8) were purified using GeneJet RNA Cleanup and Concentration Micro Kits (ThermoFisher Scientific, Waltham, MA). TaqMan® Gene Expression Assays (ThermoFisher, Waltham, MA) were used to measure intestinal gluconeogenesis (IGN) (*glucose–6–phosphatase (G6pc), pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase 1 (PCK1)*), colonic barrier and goblet cell function (*UDP–galactose–4–epimerase (GALE), monocarboxylate transporter member 1 (MCT1), mucin 1 (MUC1)*), and response to oxidative stress (*dual specificity protein phosphatase 1 (DUSP1)*) in cecal cells. TaqMan® Gene Expression Assays (ThermoFisher, Waltham, MA) were used to measure pro–inflammatory status (*IL–6, TNF–α*), anti–inflammatory status (*IL–10*), and bacterial manipulation (*angiopoietin–like 4 (ANGPTL4), leptin (LEP), solute carrier family 25 member 25 (SLC25A25)*) in inguinal adipose tissue. Cecal cell and inguinal adipose gene expression were normalized using Cyclophilin–F (PPIF) and 18S rRNA, respectively.

DNA extraction and Next Generation DNA Sequencing and Bioinformatics

DNA was extracted by Louisiana State University Health Sciences Center. Purified DNA was sequenced using a MiSeq instrument after massive parallel PCR amplification was used to incorporate primers with barcodes to identify individual samples. Relative abundance of bacteria was determined using Quantitative Insights Into Microbial Ecology (QIIME) 1.9 and DaDa2 package pipeline assembly to assign operational taxonomic units (OTU) for use in determination of alpha– and beta–diversity.

Statistical Analysis

Statistical analysis was performed using Statistical Analysis Software SAS® version 9.4 (SAS Institute Inc., Cary, NC, USA). A one–way ANOVA with *a priori* contrasts comparing each treatment to WG was performed using the MIXED procedure. The model used the dietary treatment as a fixed effect, and did not use random effects. Denominator degrees of freedom for fixed effects used the Kenward–Roger approximation. Benjamini–Hochberg false discovery rate (B–H FDR) was then used to screen dependent variable F–test p–values [80]. The B–H FDR procedure consists of ranking (indexing) the raw p–values from lowest to highest and then comparing each to the critical value (CV), which is determined by the formula "I/M*Q". "I" is the rank number, "M" is the total number of dependent variables, and "Q" is the chosen false discovery rate. The largest p–value less than the CV and all smaller p–values pass the FDR test. A Q = 0.05 was chosen as the false discovery rate.

If a dependent variable passed the FDR test, the F–statistic and subsequent p–value were considered valid. Homogeneity of variance and influence diagnostics were tested within the MIXED procedure, while tests for normality were conducted using the UNIVARIATE procedure. A null model likelihood ratio test (χ^2) was performed determine if variance was

homogeneous or heterogeneous. If variance was homogeneous, then normality testing of residual values used pooled group residuals. Heterogeneous variance proceeded with variance calculated for each group. Influential outliers, tested using studentized residuals and leverage, were removed only if their presence prevented normal distribution for statistical analysis. Influences on parameter estimates were examined using Cook's *D*. Data from dependent variables that violated the normality assumption were transformed to $\log_{10}$. Following $\log_{10}$ transformation, normality and homogeneity of variance testing were reapplied. Data violating the normality assumption after transformation was considered not normally distributed and reverted to raw data. The following variables were transformed due to non–normal distribution ($p<0.05$ for Shapiro–Wilk test): SCFAs acetate, propionate, and butyrate in ceca. Transformed dependent variables were back–transformed by taking the antilog. An F–statistic of $F<0.05$ was considered statistically significant for fixed effects and results are expressed as means ± pooled SE.

4.3. Results

A dose response relationship in observed when feeding whole grains and whole grain resistant starch. All doses related to fermentation parameters (SCFA, cecal contents pH, empty cecum weight) were effective, even if nominally, when RS was present. Whole grain alone is beneficial for increased production of short–chain fatty acids. Rats fed high RS (HMWG) had no soft or loose stools during the study. This was the same as our previous study with obese ZDF rats [90].

4.3.1. Biometric Analysis

Data were examined and influential outliers removed. Two data points were removed for ECW (AC 0.6515, 20% RS: 1.0228). One AC treatment rat was missing measurements for SCFA and pH measurements and was not included in the analysis. The F–test p–values were subjected to the B–H FDR procedure (Table 4.2). Seven of the eleven dependent variables

appeared to not be false discoveries. For these variables, we proceeded to perform multiple comparisons using *a priori* contrasts.

Empty Cecum weight and cecal contents pH

Planned contrasts of each of five groups compared to the waxy whole grain control revealed an increase in fermentation beginning at the 5% dietary RS level as indicated by a decrease in cecal contents pH (Figure 4.2A), but an increase in ECW was only evident at 10% and above (Figure 4.2B). At the 10% RS level and beyond, ECW increased linearly. No statistical differences were noted between the waxy whole grain control, the non–whole grain control, and 5% RS treatment.

Table 4.2. Biometric analysis ANOVA F–test p–value sorted by B–H FDR procedure.

		B–H Critical Values (Q = 0.05)	
Variables	p[1]	Rank	CV
Acetate (µmol)	<0.0001†	1	0.0045
Propionate (µmol)	<0.0001†	2	0.0091
Butyrate (µmol)	<0.0001†	3	0.0136
Emboweled body weight (g)	<0.0001†	4	0.0182
Empty Cecum Wt. (g)	0.0010†	5	0.0227
Cecal contents pH	**0.0010**†	6	0.0273
Abdominal body fat %[2]	0.3383	7	0.0318
Inguinal fat (g)	0.5225	8	0.0364
Active GLP–1 (pM)	0.8476	9	0.0409
Food Intake (g)[3]	0.9382	10	0.0455
Energy intake (kcal)	0.9382	11	0.0500
Most relevant CV[4]	**0.0273**		

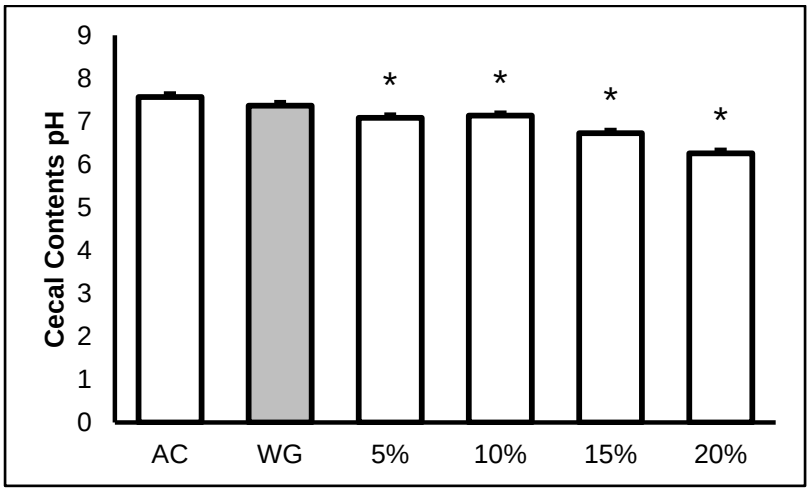
[1]An ANOVA F–test $p<0.05$ indicates a significant measurement with † superscript denoting significant differences among the six groups.

[2]ABF%: $\frac{\text{Abdominal Fat Pads}}{\text{Body weight – Full GI + Empty GI}} * 100$

[3]Food intake over the whole study of 6 weeks.

[4]Most relevant CV is determined from the greatest significant p–value of dependent variables (ranked lowest to highest) that is less than the CV at each rank, given I/M*Q. "I" is the rank number, "M" is the total number of dependent variables (11), and "Q" is the chosen false discovery rate (0.05).

A.



B.

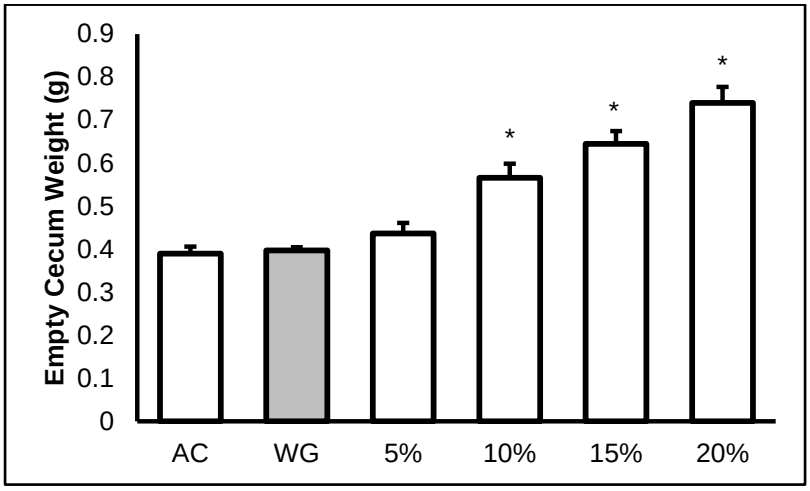


Figure 4.2. Response to increasing dosage of RS on weight of empty cecum and cecal contents pH. Grouping includes: AC = non–whole grain, non–RS control, WG = waxy whole grain, [5, 10, 15, 20%] RS = whole grain resistant starch. Significant differences as compared to WG are denoted with an asterisk. Data are expressed in their original form as means ± standard error.

Short chain fatty acids acetate, propionate, and butyrate

Planned contrasts of each of five groups compared to the waxy whole grain control demonstrated an increased production of acetate at the whole grain level vs. the non–whole grain control and increased sequentially as dosage increased (Figure 4.3A). Contrasts for propionate production continued the trend, although 5% RS was visually higher than 10% RS (Figure 4.3B).

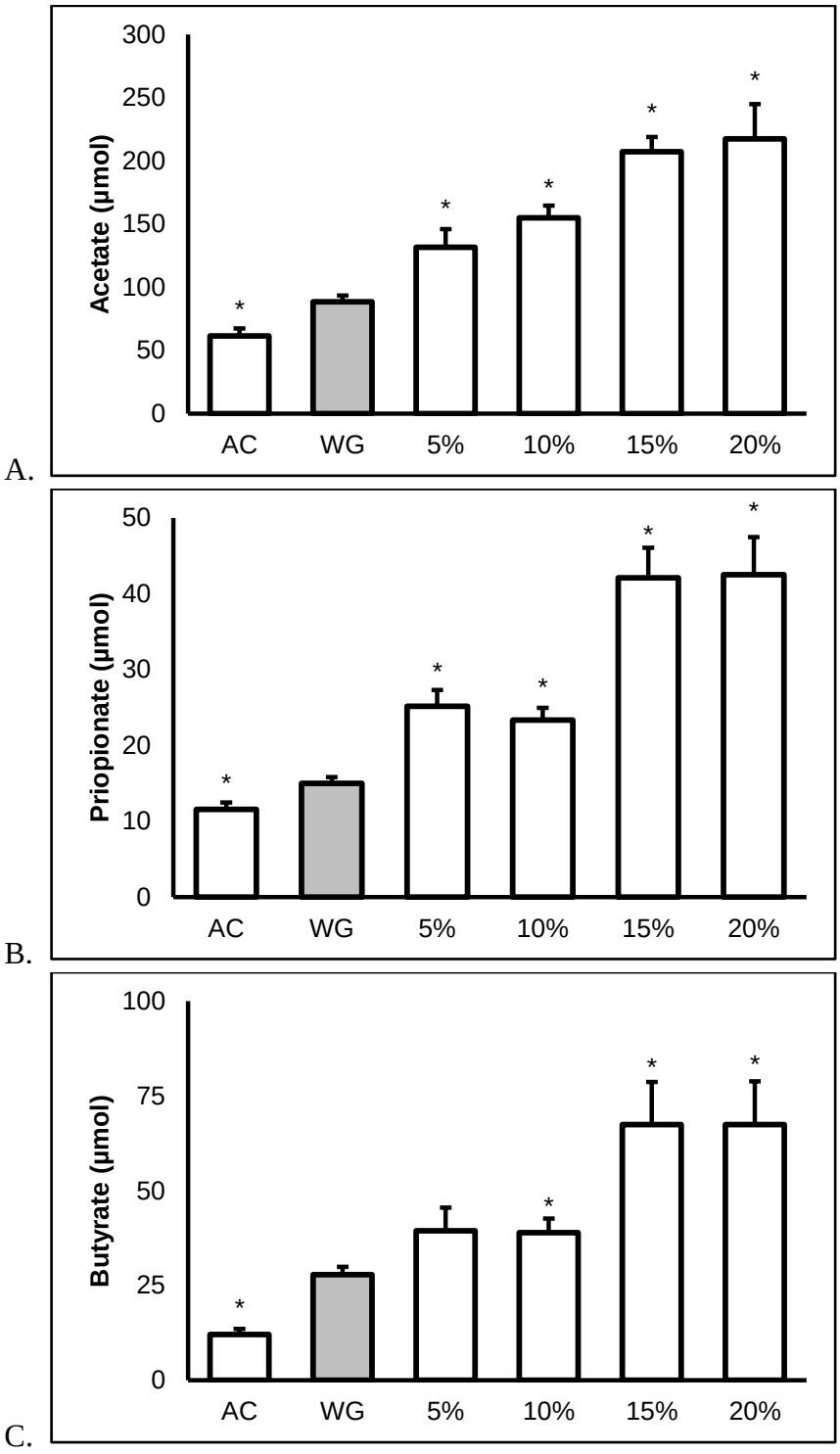


Figure 4.3. Response to increasing dosage of RS on short chain fatty acid production. Grouping includes: AC = non–whole grain, non–RS control, WG = waxy whole grain, [5, 10, 15, 20%] RS = whole grain resistant starch. Significant differences as compared to WG are denoted with an asterisk. Data are expressed in their original form as antilog means ± standard error.

Butyrate production saw no statistical differences at the 5% RS levels, but was increased similarly for the 15% and 20% RS levels (Figure 4.3C) as visually determined as these two groups were not statistically compared. However, the waxy whole grain control had greater amounts of butyrate than the non–whole grain control.

Emboweled Body weight

Planned contrasts of each of five groups compared to the waxy whole grain control showed a non–linear reduction in EBW starting as low as 5% RS (Figure 4.4). There was no difference, or even a whole grain effect, to reduce EBW when there was no presence of the high amylose, whole grain flour.

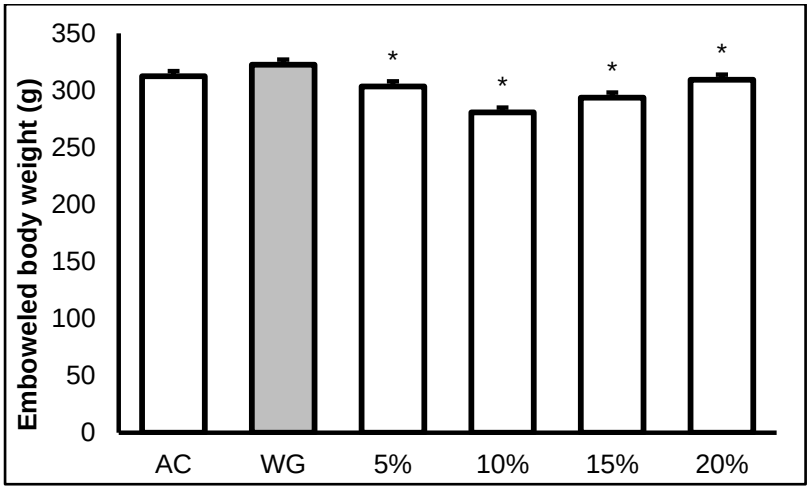


Figure 4.4. Response to increasing dosage of RS on emboweled body weight. Grouping includes: AC = non–whole grain, non–RS control, WG = waxy whole grain, [5, 10, 15, 20%] RS = whole grain resistant starch. Significant differences as compared to WG are denoted with an asterisk. Data are expressed in their original form as means ± standard error.

Food Intake, Energy Intake, Abdominal body fat percentage and inguinal adipose

Planned contrasts of each of five groups compared to the waxy whole grain control showed no differences in food intake and energy intake (Table 4.3). Additionally, the percentage of abdominal fat and inguinal adipose were not different from the WG control.

Table 4.3. Intake parameters and body fat descriptive statistics.

Variables	WG	AC	5%	10%	15%	20%	Pooled SEM	p–value (F)
Food Intake (g)[1]	789.35	798.02	803.58	805.66	796.06	810.96	120.63	0.9382
Energy Intake (kcal)	2920.60	2952.66	2973.23	2980.95	2945.41	3000.57	446.31	0.9382
Abdominal body fat %[2]	4.86	4.22	4.57	4.64	4.31	4.26	1.86	0.3380
Inguinal fat (g)	8.00	6.60	7.49	7.40	6.76	6.87	1.77	0.5225

[1]Food intake over the whole study of 6 weeks.

[2]ABF%: $\frac{\text{Abdominal Fat Pads}}{\text{Body weight – Full GI + Empty GI}} * 100$

4.3.2. Real–time quantitative polymerase chain reaction

4.3.2.1. Cecal cells

Amplification and efficiency for RT–qPCR is reported in Table 4.4. All standard curves for genes expression performed well. Slope and percent efficiency were within acceptable ranges, –3.1 to –3.6 and 90 to 110% respectively. The slope for PC was slightly above the acceptable range, resulting in percent efficiency slight below 90%.

Table 4.4. RT–qPCR amplification efficiency in cecal cells.

Variables	Standard Curve Equation ($y=C_t$)[1]	(R^2)	% Efficiency[2]
Cyclophilin–F[3]	–3.4334 * (X) + 24.67	0.9897	96
Dual specificity protein phosphatase 1	–3.4630 * (X) + 34.76	0.9916	94
Galactose–4–epimerase	–3.5507 * (X) + 34.76	0.9986	91
Glucose–6–phosphatase	–3.3160 * (X) + 40.02	0.9836	100
Monocarboxylate transporter member 1	–3.3000 * (X) + 35.02	0.9987	101
Mucin 1	–3.1736 * (X) + 41.70	0.9153	107
Phosphoenolpyruvate carboxykinase 1	–3.1692 * (X) + 32.63	0.9983	107
Pyruvate carboxylase	–3.6042 * (X) + 34.06	0.999	89

[1]Linear equation of standard curve where $C_t = (m)*X + b$; m = slope, C_t = cycles to threshold on Y axis, b = y–intercept; solve for copy number (X) which is in terms of arbitrary RNA.

[2]The variable % Efficiency is the amplification efficiency. % Efficiency is calculated as % E = (E–1)*100%, where $E = 10^{\frac{-1}{\text{slope}}}$.

[3]Cyclophilin–F was used as the reference gene (normalizer) for gene expression.

Data were examined and underwent log10 transformation. Under transformation, data were normally distributed. No influential measures were present for PC ($F_{1,30} = 1.41$, $p > 0.250$) and MCT1 ($F_{1,30} = 2.60$, $p < 0.0500$). Influential data were removed from DUSP1 ($F_{1,9} = 1.75$, p

> 0.200) and GALE ($F_{1,9}$ = 1.20, p > 0.300), but results were not significant. Two influential measurements were removed from PCK1 ($F_{1,28}$ = 4.28, p < 0.0100). Samples, unknowns, did not sufficiently amplify for statistical analysis of G6pc and MUC1 genes. Five dependent variables were ranked by p–values below in Table 4.5 using the B–H FDR procedure.

Table 4.5. Cecal cell gene expression ANOVA F–test p–value sorted by B–H FDR.

		B–H Critical Values (Q = 0.05)	
Variables	p[1]	Rank	CV
Phosphoenolpyruvate carboxykinase 1	**0.0051**†	1	0.0100
Monocarboxylate transporter member 1	0.0455†	2	0.0200
Pyruvate carboxylase	0.1824	3	0.0300
Dual specificity protein phosphatase 1	0.2171	4	0.0400
Galactose–4–epimerase	0.4056	5	0.0500
Glucose–6–phosphatase[2]	N/A		
Mucin 1[2]	N/A		
Most relevant CV[3]	**0.0100**		

[1]An ANOVA F–test p<0.05 indicates a significant measurement with † superscript denoting significant differences among the six groups.
[2]Target gene in samples did not amplify sufficiently enough for meaningful statistical analysis.
[3]Most relevant CV is determined from the greatest significant p–value of dependent variables (ranked lowest to highest) that is less than the CV at each rank, given I/M*Q. "I" is the rank number, "M" is the total number of dependent variables (5), and "Q" is the chosen false discovery rate (0.05).

The PCK1 gene was upregulated only under 10% RS treatment. The MCT1 gene was upregulated in the AC, 5% RS, and 20% RS treatments. Although the MCT1 gene was significant, it did not pass the B–H FDR procedure and was considered a falsely detected significant difference. Untransformed data are shown below in Table 4.6.

Table 4.6. Cecal cell gene expression descriptive statistics reported as arbitrary RNA[1].

Variables	WG	AC	5%	10%	15%	20%	Pooled SEM	p–value (F)[2]
Dual specificity protein phosphatase 1	1.2145	1.9411	0.501	0.8337	0.7578	1.0913	0.4985	0.2171
Galactose–4–epimerase	0.2873	0.2716	1.592	0.3944	0.3566	0.4151	0.1372	0.4056
Glucose–6–phosphatase	–	–	–	–	–	–	–	–
Monocarboxylate transporter member 1	0.1212	1.6279†	1.0664†	0.2385	0.5926	0.9836†	0.2003	0.0455†
Mucin 1	–	–	–	–	–	–	–	–

(table cont'd.)

Variables	WG	AC	5%	10%	15%	20%	Pooled SEM	p–value (F)[2]
Phosphoenolpyruvate carboxykinase 1	0.2462	0.0937	0.1196	0.6686†	0.3242	0.4206	0.0632	0.0051†
Pyruvate carboxylase	0.6365	0.175	0.2424	0.7567	0.6037	0.7751	0.1199	0.1824

[1]Data presented untransformed. Statistical significance was determined using $\log_{10}$ transformation.
[2]An ANOVA F–test $p<0.05$ indicates a significant measurement with † superscript denoting significant differences when compared individually to WG.

4.3.2.2. Inguinal fat

Data could not be completely analyzed for inguinal adipose gene expression measurements. Samples either did not amplify sufficiently or standard curves had slopes and R^2 measurements of too poor of quality to provide proper normalization and subsequent statistical analyses (Table 4.7). Standard curves for the 18S rRNA reference gene, ANGPTL4, LEP, and SLC25A25 had good amplification, but samples have not been analyzed to determine gene expression due to new normalization techniques being employed. ANGPTL4 efficiency was outside of acceptable error with a value of 113% either due to serial dilution pipetting error or non–specific product amplification. The TNF–α gene target had a very poor slope and was not applicable for further analyzing. Gene targets IL–6 and IL–10 did not see amplification of standards or samples, and slope and efficiency measurements could not be calculated.

Table 4.7. RT–qPCR amplification efficiency in inguinal adipose tissue.

Variables	Standard Curve Equation ($y=C_t$)[1]	(R^2)	% Efficiency[2]
18S rRNA	–3.2680 * (X) + 11.70	0.9925	102
Angiopoietin–like 4	–3.0543 * (X) + 32.23	0.9973	113
Interleukin–6	–	–	–
Interleukin–10	–	–	–
Leptin	–3.6008 * (X) + 30.32	0.9866	90
Solute carrier family 25 member 25	–3.1969 * (X) + 31.41	0.9621	105
Tumor necrosis factor–α	–8.7944 * (X) + 38.11	0.5877	30

[1]Linear equation of standard curve where $C_t = (m)*X + b$; m = slope, C_t = cycles to threshold on Y axis, b = y–intercept; solve for copy number (X) which is in terms of arbitrary RNA.
[2]The variable % Efficiency is the amplification efficiency. % Efficiency is calculated as % E = (E–1)*100%, where $E = 10^{\frac{-1}{\text{slope}}}$.

4.3.3. Serum Analysis

No significant differences for interaction or main effects were observed for serum levels of two pro–inflammatory markers, CRP and TNF–α, and one anti–inflammatory marker IL–10. All standards fit the standard curves well and all sample values were within the highest and lowest standards. Table 4.8 shows the results for these three serum inflammation markers.

Table 4.8. Serum inflammation markers.

Variables	WG	AC	5%	10%	15%	20%	Pooled SEM	p–value (F)[1]
C–reactive protein (ng/ml)	260830	274760	307690	284570	244440	249740	16428	0.8826
Interleukin–10 (pg/ml)	10.9373	14.7208	9.09	9.4265	16.1375	6.943	0.4397	0.5762
Tumor necrosis factor–α (pg/ml)	3.5918	3.9132	4.5262	3.9654	4.9518	4.5658	1.7338	0.9502

[1]An ANOVA F–test $p<0.05$ indicates a significant measurement with † superscript denoting significant differences when compared individually to WG.

4.4. Discussion

We hypothesized that significant increases in fermentation and beneficial health effects would be observable in as low as the 5% whole grain resistant starch treatment level. We used lean Zucker Diabetic Fatty (ZDF) rats in this study because we wanted to further test the whole grain resistant starch product and obese ZDF rats previously fermented whole grain resistant starch better than the isolated resistant starch. In our previously unpublished study, Sprague Dawley rats fermented the isolated resistant starch product better than the whole grain version. Another reason for using the lean ZDF rats rather than the obese was to be able to observe reduced abdominal fat with the better fermentation. Obese ZDF have monogenic obesity based on a defective gene for their leptin receptor. However, we observed that lean ZDF rats were heavier than Sprague Dawley rats. In practice, benefits were found to be typically conferred at 5% RS, but depending on the parameter, a minimum of 10% RS was required to elucidate other benefits in lean ZDF rats. Unpublished estimates cited in a review article [101] using the

average weight of food consumed by humans and the average of the average (31.6 g) fiber requirement for men (38 g) and women (25 g) estimate ~10% of the weight of the diet as fiber for rats as corresponding to the human fiber requirement. This was the reason for the undertaking of the dietary dose response study to move beyond mechanistic, proof–of–concept studies previously done. However, high doses of 15% and 20% RS were included for comparison with previous studies.

Serum markers of inflammation were no different as a response to resistant starch, whole grain, or dietary fat. The major pro– and anti–inflammatory cytokines, TNF–α and IL–10 respectively, were similar in concentration, suggesting that gut fermentation of the dietary factors did not alter systemic levels of cytokines. Similarly, as CRP is elevated as a response to inflammation, no differences in concentration suggests that whole–body inflammation was not affected by dietary treatments. Studies with human subjects also exhibit inconsistent changes to markers when consuming dietary RS. Johnston et al. (2009) reported no changes in inflammatory markers in insulin resistant subjects, while Gargari et al. (2015) reported reductions in TNF– α in patients with Type 2 diabetes patients. However in our previous studies, SD rats do not have a "disordered state" where RS may be able to mediate changes in inflammatory status. Here, using lean ZDF rats, we observed no differences using transgenic animals. Future studies using are required to further elucidate the role of fermentable fiber in altering inflammatory cytokines and proteins.

Biometric

No differences were observed in food or energy intake, inguinal or percent abdominal body fat when any treatment was compared to the low RS whole grain control. Likened to the previous study where there were no differences *within* moderate and high fat diets, all diets being

isocaloric and equivalent in macronutrient composition had similar response to consumption of moderate fat diets. Although we used lean ZDF rats, differences observed in biometric parameters were driven by resistant starch fermentation, while whole grains alone was typically numerically better than the non–WG non–RS control (AC, AMIOCA® control).

The observed effects on increased ECW and decreased cecal contents pH illustrate this trend. Whole grain alone was capable of a response in magnitude in the mean, but did not differ statistically from the other control. However, at 10% RS and greater, marked differences in fermentation parameters became apparent. The SCFAs to abilities nourish the gut and gut microbes (e.g. acetate to butyrate) were significantly different as compared to the whole grain control. This suggests that although pH and ECW did not change substantially from AMIOCA® control, the presence of RS1 in the whole grains provided fermentable substrate for the gut microbes. As a result, the whole grain control was always numerically better (in mean magnitude) than the AMIOCA® control for production of SCFAs. However, in the case of whole grain resistant starch, the presence of RS2 complemented fermentation more readily. The whole grain high RS diet had whole grain flour that is high–amylose starch, and, therefore has a combination of high–amylose starch RS2 granules and RS1 from the whole grain matrix. This suggests that gut microbes were not fully capable of accessing the fermentable fiber in the whole grain matrix but could better utilize the starch granules in the WGRS treatments, though this finding is speculation and not absolute. Gut microbes utilized the fermentable fiber in increasing dosage to increase SCFA production. Butyrate production increased with all RS treatments, but significant differences were observed only at 15% RS and above. As a whole, the benefits from microbial fermentation seem to be derived from consumption of dietary RS (RS1 and RS2), where higher doses produce more pronounced effects.

Gene Expression

Only one gene of the three measured in the gluconeogenesis pathway was increased by feeding of high RS whole grain. Pyruvate Carboxylase (PC, pyruvate to oxaloacetate), and glucose–6–phosphatase (G6pc, glucose–6–phosphate to glucose) genes did not differ in expression as compared to WG. Phosphoenolpyruvate Carboxykinase 1 (PCK1, oxaloacetate to phosphoenolpyruvate) demonstrated an increased expression when rats were fed the 10% WGRS diet. In our previous unpublished study (Chapter 3), we observed upregulation of all three genes measured from the gluconeogenesis pathway in cecal cells when rats were fed dietary RS. Here, it appears that WGRS only promotes increased gene expression for the major regulatory protein of the gluconeogenesis pathway [102]. This result may be due to the different strain of rat used. Sprague Dawley rats displayed increased expression of the three gluconeogenic genes measured. Increased gluconeogenic activity which correlates to improved insulin sensitivity as hepatic gluconeogenesis is reduced [103]. It is possible, that increased gene expression of the major regulatory enzyme may be enough to increase gluconeogenesis. Further studies would be required to elucidate this. However, lean ZDF rats may not produce as much glucose via the gluconeogenic pathway.

Uptake of produced SCFAs is partly facilitated by monocarboxylate transporters located on the epithelial plasma membrane. Butyrate, the main energy source for the colonocytes, is increased by gut fermentation and provides an abundance of energy for the microbial community and is utilized by epithelial cells. Increased expression of monocarboxylate transporter 1 (MCT1) illustrates increased capacity for uptake of SCFAs, and may improve gut health and possibly health of the whole body [26]. We observed inconsistent expression of the transporters

when rats were fed WGRS and the greatest expression occurred for rats fed isolated non–RS starch. Fermentation of 5% and 20% RS provided an eight to nine–fold increase, respectively, in expression over WG, while AC increased over ten–fold. As other genes examined were not different from WG, these findings suggest that WG is less capable of inducing gene expression than an isolated high RS product in our previously unpublished study. The alternative is that protein expression may have increased without a concomitant increase in gene expression. This result may be due to the fact that the current study used lean ZDF rats rather than Sprague Dawley rats which were used in our previously unpublished study. The benefits of whole grain consumption in our current study appear to be limited to biometric parameters rather than to alterations in gene expression. Ravussin et al. (2012) demonstrated correlations with inguinal adipose adipokines (ANGPTL4, IL–10, TNF–α, and SLC25A25) that were negatively correlated with increases in bacterial genus *Allobaculum* [8]. Unlike the measurements in cecal cells, RNA extracted from inguinal adipose required additional cleanup and concentration steps to obtain appropriate quality. We expect to find similar results once selection techniques for reference genes are refined.

Measureable benefits to gut health through feeding diets that included fermentable fibers were observed. Dietary resistant starch and whole grains improve gut health through microbial–induced fermentation. All WGRS dosages tested improved fermentation parameters, but recommendations for optimal health benefits appear to manifest primarily at ~10–15% weight of diet as fermentable fiber. Consumption of fiber at the dietary reference intake recommended levels has the potential to improve gut health and can indirectly lead to the improvement of whole body health.

CHAPTER 5. VARIABILITY IN REFERENCE GENE EXPRESSION DUE TO DIETARY RESISTANT STARCH TREATMENTS

5.1. Introduction

Though most foodstuffs are typically digested in the stomach and small intestine, some components in foods may escape digestion and reach the lower gut. Food components, such as fermentable fibers, that reach the lower gut may provide substrates for gut microbes. Gut microbes ferment these fibers and release many bioactive molecules that, if absorbed into the host's blood, may invoke metabolic responses. These molecules can affect tissues throughout the body by providing therapeutic or protective responses by affecting metabolic pathways [87–89].

Gene expression has become a popular way to enumerate internal responses in the host to biological and environmental stimuli. Gene expression has shown that nutrients and other food compounds can stimulate or inhibit transcription of genes [104]. Patterns of expression, as a response to food consumption, are also important for insights into many biological pathways. Real–time quantitative reverse transcription polymerase chain reaction (RT–qPCR) analyses have become the tool of choice to elucidate these outcomes, but are dependent upon accurate and validated normalization techniques.

Normalization occurs by adjusting the expression of the target gene to a common scale through use of endogenous controls. Afterwards, comparisons between treatments are allowed to determine if any independent variables have benefits or harm. The expression of endogenous controls should remain stable for individual samples, treatment groups or time points. Theoretically, the only difference in the endogenous control would be differences in pipetting. This occurred with Northern gel blots with two radioactive RNA probes used for the same lane of the gel. However, unless there is multiplexing, the endogenous control from the same sample

is assessed in a separate well on the plate for real time RT–qPCR. By using triplicate replicates, pipetting errors are likely minimized. Our molecular lab had been using Cyclophilin–F (PPIF) for several years, before we began research with resistant starch (RS). We have reported gene expression using Cyclophilin–F as the endogenous control for many years comparing RS versus control groups [91, 105, 106]. For validation of a gene array study, we used a panel of 94 genes from the array (each in separate wells and 4 samples per 384 well plate). The analysis by a scientist from Applied Biosystems (now Life Technologies within ThermoFisher, Waltham, MA) made use of the 18S rRNA as the reference cDNA after a separate reverse transcription for each sample [91]. Proper validation of endogenous controls, which are now referred to as reference genes (RGs), is an absolute requirement for any meaningful outcome. Many report variability in commonly used RGs for gene expression analyses [107–110].

Many options are available for RGs selection. Although only one RG is required to provide meaningful results, the impetus for use of single RG normalization has diminished. Selecting multiple RGs provides use of newer strategies that require validation of reference genes, or defining geometric mean to centralize expression to an unbiased average [67].

With the expansion of new foods, fibers, and supplements available commercially, detecting changes to the current and previous suitability of common RGs will be necessary. In this study, we examine if consumption of dietary bioactive components may alter gene expression of two commonly used RGs and analyze their expression in cecal epithelial cells. This allows the further consideration of single RG normalizations, the prospect of multiple RG validation techniques, and utilizing central tendency of multiple RG for expression analyses.

5.2. Methods

Study Design

The study was performed as a three–way ANOVA with the following factors: Resistant starch (Present or Absent), Whole Grain (WG: Present or Absent), and FAT (FAT: Moderate or High). Combinations of the three factors were cross classified to make eight diets, all using one level of each factor (RS: RS/NRS, WG: WG/NWG, FAT: MF/HF). Four isocaloric moderate fat treatments: NRSNWGMF (CONM), NRSWGMF (WGCM), RSNWGMF (HAMRSM), RSWGMF (HMWGM); and four isocaloric high fat treatments: NRSNWGHF (CONH), NRSWGHF (WGCH), RSNWGHF (HAMRSM), RSWGHF (HMWGH) resulted.

Animals and diets

Ninety–six male Sprague Dawley rats were purchased from Envigo (Somerset, NJ) at six weeks of age, and maintained on a chow diet during a one week quarantine. Rats were then stratified randomly by body weight into eight groups (n=12, average 259±8.4 grams). Animals were allowed *ad libitum* access to food and water for six weeks. Food intake, food spilled, and body weight were measured twice per week. Rats were euthanized and exsanguinated by cardiac puncture after inhalation of isoflurane anesthesia delivered by soaked cotton balls. The gastrointestinal (GI) tract was removed from the base of the esophagus to the anus, separated into individual parts (stomach, small intestine, cecum, and large intestine) and weighed full and empty. Cells lining the ceca were flash frozen in liquid N_2 and stored at –80°C until later analysis. RNA was extracted from cecal cells using the RNeasy Mini Kit (Qiagen, Valencia, CA).

Gene Expression Analysis

TaqMan® Gene Expression Assays (ThermoFisher, Waltham, MA) were used to measure intestinal gluconeogenesis (IGN) (*glucose–6–phosphatase (G6pc), pyruvate carboxylase (PC),*

phosphoenolpyruvate carboxykinase 1 (PCK1)), colonic barrier and goblet cell function (*UDP–galactose–4–epimerase (GALE), monocarboxylate transporter member 1 (MCT1), mucin 1 (MUC1)*), and response to oxidative stress (*adrenomedullin (ADM)*) in cecal cells and normalized using Cyclophilin–F (PPIF) or 18S rRNA (TaqMan® Gene Expression Assays; ThermoFisher, Waltham, MA).

Concentration of extracted RNA was determined using a NanoDrop™ ND–1000 microvolume spectrophotometer (ThermoFisher, Waltham, MA). Samples were diluted to 40 ng/µl, and 3 µl combined with 6 µl TaqMan™ Universal Master Mix II, no uracil–N–glycosylase (UNG) (Applied Biosystems™, Waltham, MA), 0.05 µl MuLV RT Transcriptase, 0.05 µl RNase inhibitor, 0.6 µl of the TaqMan® Gene Expression Assay primers and probe, and 0.3 µl of double distilled water. The complete reaction mixture (10 µl) was incubated in an ABI PRISM 7000HT Sequence Detection System (Applied Biosystems, Foster City, USA) with the following thermal profile: Stage 1: 1 cycle, 30 min at 48ºC, Stage 2: 1 cycle 10 min at 95ºC, Stage 3: 40 cycles, 15 sec at 95ºC, 1 min at 60ºC.

Quantification of RGs was determined by pooling aliquots of extracted RNA from all samples, and using ten–fold dilutions for development of standard curves, The standard curves were set up as cycles to threshold (C_t) on the Y axis and ng arbitrary RNA on the X axis. The real time RT–qPCR was done as a one–step procedure with a reverse transcriptase step for conversion of RNA to cDNA prior to qPCR. With each PCR cycle the cDNA is doubled. Standards or samples with greater amounts of starting mRNA for a gene reach an arbitrarily set threshold in fewer cycles than standards or samples with lower amounts of starting mRNA.

For individual samples, target genes and RGs were measured in separate plate wells with each measured in triplicate wells. Generally, it is recommended to have samples reach the

threshold between 5 to 35 PCR cycles. RNA samples for PPIF were diluted 1:2.5 and 3 µl used in the qPCR mix; for 18S rRNA, samples were diluted 1:4.

Statistical Analysis

Statistical analysis was performed using Statistical Analysis Software SAS® version 9.4 (SAS Institute Inc., Cary, NC, USA). Absolute quantity of arbitrary RNA and C_t were examined for each RG prior to normalization with gene targets in cecal epithelial cells. C_t values were analyzed for variation in RG expression. A 2x2x2 factorial analysis was performed using the MIXED procedure. The three factors for were described above in "Animals and Diets." The model used the three factors as fixed effects, and did not use random effects. The linear model tested was:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 + \epsilon$$

where $\begin{cases} RS \\ NRS \end{cases} X_1 = \begin{cases} 1 \\ 0 \end{cases}$, $\begin{cases} WG \\ NWG \end{cases} X_2 = \begin{cases} 1 \\ 0 \end{cases}$, and $\begin{cases} HF \\ MF \end{cases} X_3 = \begin{cases} 1 \\ 0 \end{cases}$. Denominator degrees of freedom for fixed effects used the Kenward–Roger approximation. An F–test with $p<0.05$ was considered statistically significant for interactions and main effects. A p–value less than 0.05 will be indicative of non–stable RG gene expression for treatment groups.

Homogeneity of variance and influence diagnostics were tested within the MIXED procedure, while tests for normality were conducted using the UNIVARIATE procedure. A null model likelihood ratio test (χ^2) was performed determine if variance was homogeneous or heterogeneous. If variance was homogeneous, then normality testing of residual values used pooled group residuals. Heterogeneous variance proceeded with variance calculated for each group. Influential outliers, tested using studentized residuals and leverage, were removed only if their presence prevented normal distribution for statistical analysis. Influences on parameter estimates were examined using Cook's *D*. Data from dependent variables that violated the

normality assumption were transformed to $\log_{10}$. Following $\log_{10}$ transformation, normality and homogeneity of variance testing were reapplied. Data violating the normality assumption after transformation was considered not normally distributed and reverted to raw data. When no significant interactions were observed, only the main effect was reported.

5.3. Results

Both RGs examined had a normal distribution ($p>0.05$ for Shapiro–Wilk test) and did not require transformation. There were no influential outliers or leverage measurements. Amplification and efficiency for RT–qPCR is reported in Table 5.1. Standard curves for reference genes had good amplification and slopes and percent efficiency were within acceptable ranges, –3.1 to –3.6 and 90 to 110% respectively.

Table 5.1. RT–qPCR amplification efficiency of reference genes.

Variables	Standard Curve Equation ($y=C_t$)[1]	(R^2)	% Efficiency[2]
18s rRNA	–3.4034 * (X) + 9.87	0.9892	97
Cyclophilin–F	–3.5852 * (X) + 26.35	0.9934	90

[1]Linear equation of standard curve where C_t = (m)*X + b; m = slope, C_t = cycles to threshold on Y axis, b = y–intercept; solve for copy number (X) which is in terms of arbitrary RNA.
[2]The variable % Efficiency is the amplification efficiency. % Efficiency is calculated as % E = (E–1)*100%, where $E = 10^{\frac{-1}{\text{slope}}}$.

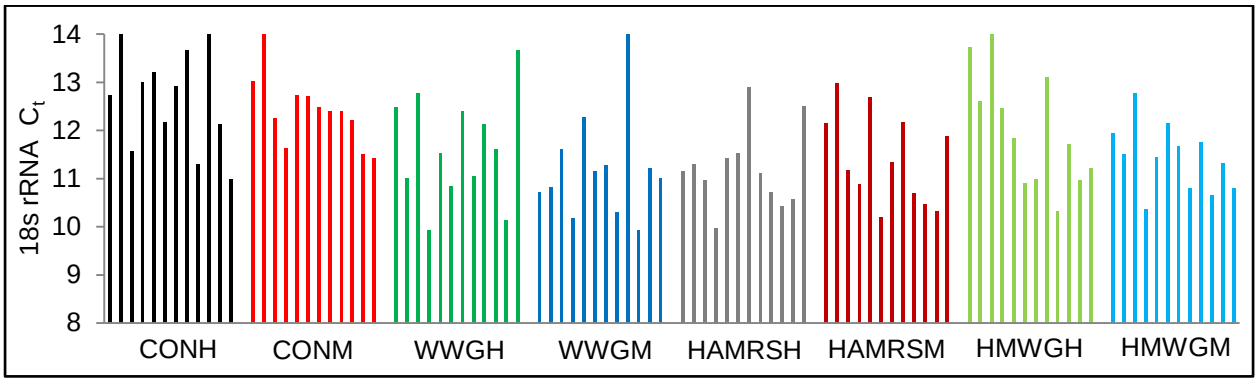
No interactions for 18S rRNA C_t were observed for the study: RS*WG ($F_{1,85} = 3.45$, $p > 0.05$), RS*Fat ($F_{1,85} = 0.26$, $p > 0.50$), WG*Fat ($F_{1,85} = 0.61$, $p > 0.10$), and RS*WG*Fat ($F_{1,85} = 2.35$, $p > 0.10$). No interactions for PPIF C_t were observed for the study: RS*WG ($F_{1,81} = 0.04$, $p > 0.50$), RS*Fat ($F_{1,81} = 0.01$, $p > 0.50$), WG*Fat ($F_{1,81} = 0.10$, $p > 0.50$), and RS*WG*Fat ($F_{1,81} = 0.41$, $p > 0.50$). The 18s rRNA and PPIF C_t were significant for the RS main effect ($F_{1,85} = 16.03$, $p < 0.0001$) and ($F_{1,81} = 30.35$, $p < 0.0001$), respectively. No other main effect was significant: *18s rRNA* (WG, ($F_{1,85} = 3.05$, $p > 0.05$), FAT: ($F_{1,85} = 3.14$, $p > 0.05$)), *PPIF* (WG, ($F_{1,81} = 0.82$, $p > 0.05$), FAT ($F_{1,81} = 1.66$, $p > 0.20$)). Main and two–way interaction C_t values

are shown in Table 5.2. Raw C_t values differed for each rat and did not correspond directly from 18s rRNA to PPIF genes. Figure 5.1 illustrates the distribution values for C_t per rat before statistical analyses were performed. A difference of one Ct corresponds to a doubling of starting material (PPIF: mRNA, 18S: rRNA) when comparing the higher Ct to the lower Ct (e.g. 12 vs. 11 for 18S rRNA), as Ct is measured in log2 fold change (2^{12} vs. 2^{11}).

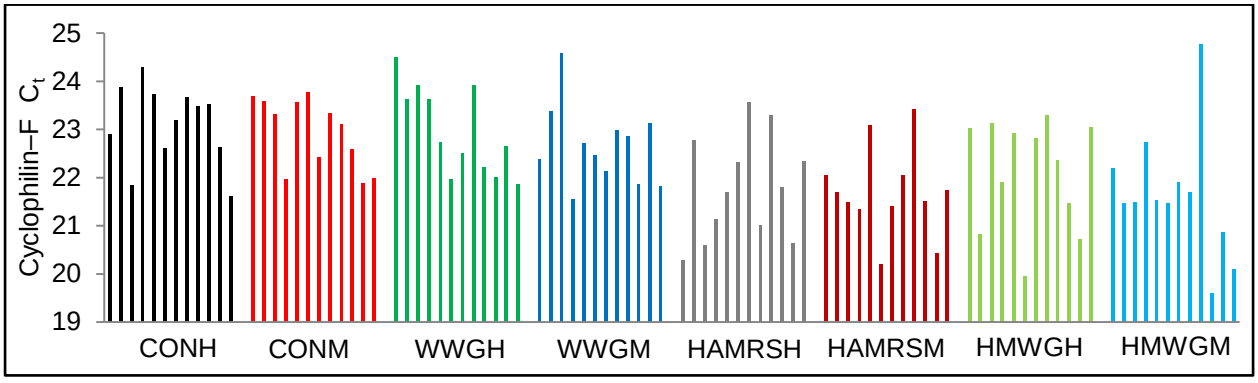
Table 5.2. RT–qPCR C_t two–way interaction and main effects for reference genes[1,2].

	RS * WG Interaction					
Reference Gene (C_t)	RS WG	RS NWG	NRS WG	NRS NWG	Pooled SEM	p–value (F)[2]
18s rRNA	11.3078	11.2870	11.7125	12.3926	0.0943	0.0666
Cyclophilin–F	21.7381	21.8815	22.8038	23.0229	0.1002	0.8506
	RS * FAT Interaction					
	RS MF	RS HF	NRS MF	NRS HF	Pooled SEM	p–value (F)[2]
18s rRNA	11.1788	11.4161	11.8370	12.2681	0.0943	0.6087
Cyclophilin–F	21.6710	21.9486	22.7938	23.0330	0.1002	0.9239
	WG * FAT Interaction					
	WG MF	WG HF	NWG MF	NWG HF	Pooled SEM	p–value (F)[2]
18s rRNA	11.4168	11.6035	11.5989	12.0807	0.0943	0.4361
Cyclophilin–F	22.1741	22.3678	22.2907	22.6137	0.1002	0.7478
	Resistant Starch					
	Present	Absent	Pooled SEM	p–value (F)[2]		
18s rRNA	11.2974	12.0525	0.0954	<0.0001†		
Cyclophilin–F	21.8098	22.9134	0.1013	<0.0001†		
	Whole Grain					
	Present	Absent	Pooled SEM	p–value (F)[2]		
18s rRNA	11.5102	11.8398	0.0949	0.0841		
Cyclophilin–F	22.2710	22.4522	0.1008	0.3683		
	FAT					
	Moderate	High	Pooled SEM	p–value (F)[2]		
18s rRNA	11.5079	11.8421	0.0949	0.0799		
Cyclophilin–F	22.4908	22.2324	0.1008	0.2009		

[1]Data are shown as collapsed interactions based on factors resistant starch (RS, Present [RS] or Absent [NRS]), whole grains (WG, Present [WG] or Absent [NWG]) and fat (FAT, High [HF] or Moderate [MF]).
[2]A ANOVA F–test $p<0.05$ indicates a significant measurement, denoted with the † symbol.



A.



B.

Figure 5.1. Illustration of C_t distribution of values per rat for 18s rRNA and Cyclophilin–F genes. Measurements are depicted by the same groups and order in A. and B. Measurements are exaggerated (i.e. do not start at 0) to be able to illustrate differences between individual rats, treatment groups, and reference genes.

5.4. Discussion

Upon analyzing RG C_t prior to use for normalization of target genes, we discovered a significant effect of RS when using PPIF. To our knowledge, this has not been reported in studies using high–amylose maize resistant starch when measuring gene expression. This finding represents a two–fold difference in expression when using the PPIF to normalize target genes with resistant starch groups. This difference becomes an inherent bias against resistant starch fed groups when comparing expression of target genes. Lower C_t values denote higher amounts of initial transcripts in the sample. Samples with resistant starch have more transcripts initially, such that when quantitation is performed the assumption that starting transcript

concentration being equivalent is violated. The reason for this bias against the resistant starch treatments is because we use a standard curve in every study and determine quantities in ng of arbitrary RNA, which are non–log numbers. Normalization is then accomplished by division by a greater number for resistant starch group samples versus non– resistant starch group samples. The bias is further present in the normalization for gene targets, such that normalizing with PPIF gives a biased result for total quantity of complementary DNA. The normalization expression utilizes the absolute quantity of the target gene and divides it by the absolute quantity of the RG. As proper reference gene expression is not affected by treatment, the bias discovered here will propagate through all subsequent genes normalized with PPIF. These errors can be further compounded when examining multiple tissues.

To counteract this bias, we examined another typical RG, 18s rRNA gene expression, for normalization. Unfortunately, the use of 18s rRNA also resulted in bias against resistant starch fed groups, indicating the need to find a truly constitutively expressed RG when using dietary resistant starch, or another approach to normalization. However, our findings indicate that even with a bias against resistant starch fed groups, gene expression is still robust with resistant starch treatments. Target genes have exhibited fold–changes of 2x to 10x even with RG bias against resistant starch. Interactions and main effects that were nearly significant in our prior studies may prove to be significant if better normalization techniques were utilized. Additionally, other commonly examined genes may be tested with resistant starch treatments to determine stability and suitability as reference genes. A low density array plate with replicate wells with probe and primers for many candidate RGs can be used to find RGs for treatments versus controls. The generated C_t values will be compared to other candidates, selecting the most stably expressed genes to produce accurate results. Future studies could employ both of these plates to provide an

accurate representation of gene expression when feeding dietary resistant starch or testing treatments that affect commonly used reference genes. Custom plates can be used with several RGs for studies to determine global mean expression (described below), however, use of custom plates may be too costly for many labs. The best approach may be to use many RGs (~32) on routinely produced plates for endogenous assays to find single RGs or groups of RGs for each study. This would also be cost–prohibitive for most labs.

Recently, new techniques have been introduced to specifically address issues with normalization. Global mean expression normalization (GMN) or common base normalization may suffice for unstable expression of RGs. Gene stability, GMN, is a normalization factor based on averaging to develop the geometric mean of multiple RG [67]. Multiple RGs should be used, instead of a single gene, to calculate a normalization factor for normalization of target genes. Vandesompele et al. (2002) describe the potential for erroneous normalization (3–fold to 6–fold) when single RG normalization strategies are performed [67]. These approaches all develop a single mean value for a group of RGs. Thus, a possible approach in our study may be to use the average of PPIF or 18S rRNA for all samples as our normalization factor; or the average of the combination of both RGs.

CHAPTER 6. CONCLUSIONS

These investigations demonstrate the importance of studying food components, such as resistant starch, whole grains, and levels of fat, and their interactions, rather than examining specific outcomes from single factors. In the first study, there were fermentation differences between rodents fed high resistant starch in moderate fat diets compared to high fat diets. This result was similar to previous studies, mirroring significant differences between rodents fed low fat and high fat diets. The differences occurred in diets without whole grain. With whole grain diets the fermentation of high resistant starch was similar for moderate fat and high fat diets. However, the whole grain high resistant starch groups had similar fermentation effects for both moderate and high fat diets, which were also similar to the group fed high resistant starch with no whole grain as part of a high fat diet. Since this level of fermentation is still significantly greater than groups with no resistant starch (non–whole grain control) or low resistant starch as resistant starch type 1 (whole grain control), these results suggest that Americans may be able to consume the higher levels of dietary fat (42% of energy). About 25% of the American population [1] consuming a high fat diet apparently would still benefit from consumption of resistant starch. High fat diets may attenuate fermentation, but the addition of other bioactive components, provided by a whole grain high resistant starch product, were hypothesized to help maintain the fermentation process. Although gut health was improved with whole grain high resistant starch diets as demonstrated by increased fermentation compared to low resistant starch (as resistant starch type 1), whole grain resistant starch diets did not ferment as well as resistant starch with the feeding of moderate fat. This suggests that, although the two different products with high resistant starch had the same amount of resistant starch, the combination of resistant starch type 1 and resistant starch type 2 present in the whole grain kernel compared to an

equivalent amount of resistant starch as non–whole grain high resistant starch is not as effective as the isolated starch with high resistant starch all as RS2. Since the scientific literature indicates that resistant starch type 1 takes longer to be fermented by gut bacteria, we may have missed its fermentative effects because we focused only on the cecum. The current results indicate that future studies using whole grain high resistant starch products, should measure fermentation effects in the rest of the large intestine in addition to the cecum. Continued fermentation along a greater length of the large intestine would be advantageous to gut health and, thus, would favor the use of the high resistant starch whole grain product. However, in the cecum, the whole grain product appears to increase butyrate levels compared to the non–whole grain high resistant starch product and may benefit the health of the colonocytes that use butyrate as a source of energy. Increased butyrate may indicate a greater utilization of acetate for butyrate production resulting in lower cecal acetate that was observed. The results from this study and our previous investigations also may indicate that there may be variation in response to dietary resistant starch in a whole grain product for humans as observed in rodent studies.

Although there were some effects of the whole grain and fat factors that included two–way interactions (WG*RS and WG*Fat), the dominant factor in the first study was the RS factor. In particular, the presence of high dietary resistant starch (no whole grain or whole grain) versus no resistant starch in no whole grain control or low resistant starch type 1 only in the whole grain control. The whole grain control groups had no resistant starch type 2 because the starch component of waxy whole grain flour has 100% amylopectin and no amylose with the latter in granules making up resistant starch type 2. Thus, high resistant starch stood out despite the interactions and has been addressed thoroughly in the results and discussion.

In the first study the single factor, resistant starch, was also responsible for most differences observed in cecal cell gene expression. Resistant starch fermentation induced significantly increased beneficial gene expression changes. Increased gluconeogenic capacity apparently promoted by intestinal fermentation of dietary resistant starch may improve insulin sensitivity and glucose homeostasis. The improved ability to provide nourishment to the gut, mediate oxidative stress, potentially further induce angiogenesis, and fortification of the epithelial lining were benefits indicated by increased cecal cell gene expression from dietary resistant starch fermentation.

The other factors, whole grain and fat, were associated with some changes in gene expression, but no significant interactions were noted. Whole grains may be capable of eliciting an improved insulin sensitivity response as cecal cell glucose production may lead to inhibition of liver gluconeogenesis, potentially through the major gluconeogenic regulatory enzyme phosphoenolpyruvate carboxykinase (PCK1). However, gene expression of other enzymes in the gluconeogenesis pathway was not significantly increased by the presence of whole grain. However, PCK1 increase may be the only increase necessary for increased gluconeogenesis. To better determine these results future studies should measure not only gene expression, but also protein levels of the enzymes and potentially production of radioactive glucose from radioactive oxaloacetate (starting metabolite for gluconeogenesis) in primary cultures of cecal cells. Additionally, whole grain high resistant starch diets appeared to be beneficial for uptake of SCFAs with increased expression of SCFA transporters, and along with increased production of butyrate may promote the health of the upper large intestine, the cecum. Diets high in fat attenuated the ability of the gut to maintain increased amounts of gene transcripts for SCFA transporters. This suggests the contributions to gut fermentation and whole body health were

driven primarily by fermentation of fibers that reached the cecum in our study. While whole grain promoted increased levels of a few products of gene expression including gene expression for gluconeogenesis and uptake of butyrate, resistant starch type 2 with its granular form appeared to be more readily accessible for fermentation in the cecum than the resistant starch type 1 and resistant starch type 2 combination or resistant starch type 1 alone in the whole grain control groups. There may be other pathways where the consumption of whole grains and fat are beneficial as factors, however resistant starch was the major modulator of biometric and gene expression parameters measured in the current first study. In future studies, scientists may wish to include measurement of gene expression of pathways that may be potentially affected by fat and also gene expression in the distal large intestine.

Future studies should continue to investigate fermentative effects and especially effects of fermentation on body fat in a variety of rodent models. Our previous study with obese Zucker Diabetic Fatty (ZDF) rats demonstrated that this rodent model fermented the whole grain high resistant starch product in the cecum better than the non–whole grain starch with high resistant starch. However, no reduction in abdominal body fat caused us to switch to Sprague Dawley (SD) rats that we had used in several previous studies using the non–whole grain high resistant starch product, and had observed reduction in abdominal body fat. We hypothesized that we would see better fermentation of the whole grain high resistant starch product and reduced abdominal body fat. Thus, our result of greater fermentation of the non–whole grain high resistant starch product by the SD rats was unexpected. The SD rat better models the vast majority of the American population than the obese ZDF rats, which are a model of monogenic obesity having a defect in their leptin receptor. Monogenic obesity in humans exists, but this population is very small. Since the SD rat model has a contrasting effect with feeding of a high

fat diet with some becoming obese and others remaining relatively lean, researchers and companies have developed colonies of obesity–prone and obesity–resistant rats. The obesity–prone SD rats are obese when consuming a high fat diet because of a poorly understood interaction of several genes and thus are a multigenic model, which models the majority of the obese human population.

For the second study, we did not want to give up on the possibility of the whole grain high resistant starch product so lean ZDF rats were chosen for the dose response. It was anticipated they would ferment the high resistant starch whole grain product similarly to their related obese ZDF rats. Since the lean ZDF rats had a functioning leptin receptor, it was also hypothesized that the lean ZDF rats would respond to fermentation with reduced abdominal body fat.

In the second study, it appeared that the lean ZDF rats were better fermenters of the high resistant starch whole grain product. However, we did not do a comparison with the non–whole grain high resistant starch product or a comparison with SD rats. The key result for the dose response study was that we demonstrated effective dietary doses for fermentation and these were at physiologically relevant doses as related to human consumption. Several dependent variables had significant outcomes versus the whole grain control group for all doses of whole grain resistant starch even at the lowest dose of 5%. With other variables, the 5% RS dose was not significantly different from the whole grain control, but its value was in line with the increases of the other three doses. Thus, it is not clear exactly, which is the lowest effective dose of whole grain resistant starch. Our aim was to assign that designation to the lowest dose that was significantly different from the whole grain control. Depending on the dependent variable, 5% RS fits that designation, but for one (ECW) it is 10% RS. One interesting result occurred with

butyrate levels in cecal contents where only 10, 15 and 20% whole grain resistant starch had significantly greater amounts than the whole grain control. The dose of 10% whole grain resistant starch is equivalent to the human fiber requirement when using the dry weight of daily food consumption and metabolic body size of the rat and the human [101]. Therefore, in regards to fermentation parameters, our study successfully demonstrated that doses of whole grain resistant starch at or below the equivalent of the human fiber requirement were effective.

One unexpected result for our second study with lean ZDF rats was that there was no reduction in body fat with any of the doses of whole grain resistant starch. In the first study, a whole grain resistant starch level of 23% resulted in reduced abdominal body fat in SD rats. Our observation (no statistical comparison) was that it appeared that the lean ZDF rats had more body fat than SD rats. An interesting future study would be to compare the lean ZDF rats with the SD rats using the dose response approach; and to use both the whole grain resistant starch product as well as the isolated starch with high resistant starch type 2.

In both of our studies, use of whole grain resistant starch, whether in a dose response of 5, 10, 15 or 20% RS, or a single dose of 23% RS resulted in limited benefits as far as increased gene expression for apparent health promoting proteins. The high single dose increased levels of messenger RNA for the PCK1 and MCT1 genes. Results were inconsistent with doses in our second study and may be due changing from SD to lean ZDF rats or possibly experimental error.

Future studies are also needed to determine how isolated starch with resistant starch and whole grain resistant starch products will most benefit the host by measuring other dependent variables than in our current two studies. Our studies have successfully demonstrated the whole grain resistant starch is an effective product for fermentation in the cecum in both moderate and high fat diets. Other studies may want to focus on following other compounds in the whole grain

product with moderate and high fat diets. It was encouraging to observe that there was a healthy amount of fermentation on the high fat diet, which was in the upper range of consumption of dietary fat typically consumed by Americans. Thus, the consumption of adequate amounts of fermentable fiber in recommended amounts in the form of isolated starch with resistant starch or whole grain resistant starch appears to be a very good approach to promote health benefits. More studies are needed to determine if this is enough to declare that under these dietary circumstances an individual would be relatively healthy.

The availability of the whole grain corn flour for these studies, provided by Ingredion Incorporated, has been discontinued, primarily as a result of instability and shorter shelf life. The whole grain corn flour requires a cool, dry, relatively airtight storage location to minimize enzymatic activity on oils found in the germ resulting in spoilage. Shelf life degradation is further enhanced by heat, light, and moisture. From a purely fermentation perspective, the whole grain components were unnecessary as the resistant starch fermentable fiber component appears to promote fermentation in the cecum. This parallels findings by Cho et al. (2013), which suggest that the fiber component of whole grains is the most important and accounts for most of the whole grain health benefit. Our studies show that isolated starch with resistant starch (as resistant starch type 2) ferments well and is believed to store relatively indefinitely. However, future studies should not forgo the use of whole grains. Govers et al. (1999) determined that whole grain starch is fermented slowly and exhibits greater fermentation in the distal colon. This would indicate that utilization of resistant starch type1 in the whole grain matrix may produce regional fermentation that reduces risk of colorectal cancers in the distal colon, where most colon cancers occur [39].

Whole grains also contain other metabolically active components and discarding their use may serve to limit knowledge on the activity of whole grains for improving health. Future research stabilizing the volatile and labile components of whole grains may improve shelf life and allow for long–term storage for greater use in studies and may result in demonstration of future beneficial health effects. From our study and other studies the resistant starch components of the whole grain product appeared to be stable. Teaming with food scientists to develop stabilization methods for whole grain corn flour may allow for further utilization of both resistant starch types 1 and 2 to promote fermentation in both the proximal and distal colon. Studies successfully demonstrating stabilization of whole grain flour would warrant restoring the availability of whole grain corn flour to consumers. Likewise, investigation into the stability of non–corn whole grain sources (e.g. barley, oats, rice, wheat, etc.), may also be fruitful and reduce limitations to procuring and examining their effects on metabolism if the availability of whole grain corn flour remains discontinued.

In summary: Our two studies have demonstrated beneficial health effects from consuming dietary resistant starch and have added important results to the body of knowledge regarding its fermentative and metabolic effects, and our results demonstrate that the effects measured in our studies were likely due to the amount of resistant starch fermentable fiber and not the result of other bioactive compounds in the whole grain resistant starch product. Our first study with Sprague Dawley rats, demonstrated that isolated starch with resistant starch type 2 fermented better than whole grain resistant starch with resistant starch types 1 and 2 with the feeding of a moderate fat diet. To better investigate the whole grain flour, lean ZDF rats were used in the second study with increasing doses of the high resistant starch whole grain flour. Our

results demonstrated that lower doses that are physiologically relevant for humans were effective in producing beneficial health effects in regard to fermentation in rats.

Future studies should also examine fecal matter repeatedly during the study to discern fermentation effects of resistant starch type 1 from whole grain flour from available sources in the distal colon. These studies would examine the entire large intestine as a fermentative organ. These should also include the characterization of microbes in the entire colon at euthanasia of the animals, to delineate the differences in resistance starch types 1 and 2. These studies should be performed in different types of rats that respond to the whole grain with variable degrees of fermentation. Dose response studies with different non–whole grain and whole grain sources, a variety of rats and mice, and different levels of fermentable fiber will be necessary to validate the recommendations proposed to consumers.

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