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Sprague Dawley Rats Were Able To Ferment Purified Resistant Starch And Whole Grain Starch On Moderate And High Fat Diets

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SPRAGUE DAWLEY RATS WERE ABLE TO FERMENT PURIFIED
RESISTANT STARCH AND WHOLE GRAIN STARCH ON
MODERATE AND HIGH FAT DIETS

A Thesis

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
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in

The School of Nutrition and Food Sciences

by

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

~

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ABBREVIATIONS

ABF%: Abdominal fat percent

AMDR: Acceptable Macronutrient Distribution Range

CON: Control

CHD: Coronary Heart Disease

EBW: Emboweled body weight

ECW: Empty cecum weight

ELISA: Enzyme-linked immunosorbent assay

FAT: Fat factor

GI: Gastrointestinal

GLP1: Glucagon-like peptide 1

HAMRS: High-amylose maize resistant starch

HF: High Fat

HMWG: High amylose maize whole grain resistant starch

IACUC: Institutional Animal Care and Use Committee

MF: Moderate Fat

RS: Resistant Starch factor

SEM: Standard Error of the Mean

SCFA: Short chain fatty acid

SD: Sprague Dawley rat

WG: Whole Grain factor

WWG: Waxy Whole Grain

ZDF: Zucker Diabetic Fatty rat

ABSTRACT

Introduction: Whole grain (WG) and fat content of the diet have been previously shown to affect intestinal fermentation and phenotype conferred by high-amylose maize starch (HAM), a form of fermentable dietary fiber. The current study was 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 using a 2x2x2 factorial design.

Methods: MF and HF diets were prepared to contain the following diet sources: (1) control starch with no WG or RS [CON], (2) whole grain waxy corn flour with low RS [WWG], (3) purified HAM resistant starch (RS) [HAMRS], and (4) WG HAM flour rich in resistant starch (WG+RS) [HMWG]. The eight diet conditions were fed to Sprague Dawley rats for six weeks (n = 12 per group). After euthanasia, blood, cecal contents and cecal epithelial cells were collected and gastro-intestinal (GI) tract portions and fat pad (retroperitoneal, perirenal, and epididymal) weights recorded.

Results: The presence of purified RS2 resulted in greater fermentation as part of the RS*WG interaction whereas no WG with high RS had the lowest pH of cecal contents. There was a main effect of RS with the high RS groups having the lowest abdominal fat percent of body weight. The presence of WG resulted in consistency of fermentation as groups with WG had similar levels of short chain fatty acids with MF and HF diets as reflected by WG*FAT. No RS*FAT effect was observed because of the WG consistency. Also, a greater butyrate production with WG was demonstrated by RS*WG. Results were primarily driven by two major effects, reflected by the presence of and lack of some significant differences. Purified RS fermented better on MF than HF diets. Diets with RS+WG show similar fermentation on both types of

diets. These effects may be driven by RS1 vs. RS2 as high RS2 ferments better with MF diets, but presence of RS1 may ferment better with HF diets.

CHAPTER 1: INTRODUCTION

1.1. Significance of Research

Nutritional policies and recommendations regarding an adequate level of fiber (38g/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.

CHAPTER 2: LITERATURE REVIEW

2.1. Resistant Starch

Dietary fiber is defined as the “non-digestible carbohydrates and lignins that are intrinsic and intact in plants.” The non-digestible carbohydrates can include inulin, oligosaccharides, fructans, methylcellulose, polydextrose, resistant maltodextrose, resistant starch, and other compounds [1]. 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 [15]. 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” [16]. 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 [17].

Resistant starch resists enzymatic digestion in the small intestine and is fermented by bacteria in the large intestine [18]. 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. 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. 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 [19]. 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 [20].

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 [21]. 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 beyond the gut, cardiovascular diseases. [17, 22–24].

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 [25]. The two SCFAs are utilized by peripheral tissues (muscle, acetate) or by the liver (acetate, propionate) for metabolism [26–29]. Butyrate is especially important for gut health, and is a major source of energy for epithelial colonocytes [25]. 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 [30, 31]. 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 [32]. 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” [33, 34].

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 [34]. Current recommendations for whole grain consumption call for at least half the grains consumed to be whole grains [35]. Many studies have described the benefits of whole grains. Whole grains have been associated with

reduced risk for cardiovascular disease, type 2 diabetes [36, 37], cancers [38–40], and all-cause mortality [41, 42].

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 [38]. Similarly, phytochemicals have also been shown to provide protection against developing chronic diseases and cancers [43]. 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 [44].

Research regarding phytochemicals focuses primarily on prevention, while fiber research focuses on risk reduction [44, 45]. 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 [32]. 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 [46]. 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 [47], (2) transport of vitamins A, D, E, and K [48–50], and (3) formation of some steroid hormones [51]. 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 [52]. 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 [53]. 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 [54].

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 [55] 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 [56] and women [57]. 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, and 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 [58]. 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 understand how other nutritional components, such as whole grains, influence fermentation and health.

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 [59]. 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 [60]. 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 [61]. Resistant starch (RS) is a dietary fiber. Fermentation of RS stimulates the growth and maintenance of the gut microflora [21]. 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 microorganisms may populate the gut [62].

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 [63]. 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 humans tend to focus on consuming low (18–20% of energy) and very high (60–70% of energy) dietary fat intake [56, 57], 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) diets partially attenuated resistant starch fermentation in Sprague Dawley rats [9]. Zhou et al. (2009) demonstrated moderate fat (MF) diets were effective at reducing abdominal fat percentage (ABF %) as well as low fat (LF) 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 (WG) consist of three parts: the bran, the germ, and a starchy endosperm [64]. For a food to be considered whole grain, the bran, germ, and endosperm must be present in relative proportions found naturally in the kernel [34]. 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 [36, 37], cancers [38–40], and all-cause mortality [41, 42]. Despite the benefits derived from consumption and fermentation, whole grain intake has remained less than one-third of the recommendation [46]. 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 resistant starch, whole grains, and fat can improve gut health. 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.

3.2. Materials and Methods

Animals and diets

The protocol for this study was approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University. Male Sprague Dawley rats were purchased at six weeks of age from Harlan Laboratories Inc. (Indianapolis, IL). Rats were quarantined for one week and fed a standard chow diet. Following quarantine, animals were randomly stratified into eight groups by body weight (average 259 ± 8.4 grams). All animals were housed in individual stainless steel hanging cages with wire mesh bottom to prevent coprophagy and determine food spilled. Rats were housed in a locked facility in a room with a 12:12 h light–dark cycle, 21–22°C temperature, and 55% relative humidity. Rats were allowed *ad libitum* access to food and water, and body weight, food intake, and food spilled were measured twice per week for six weeks.

The experimental design for this study was a 2x2x2 factorial (Figure 3.1) with the following factors: (1) Resistant starch (High or Low/No), (2) Whole Grain (Present or Absent), and (3) FAT (Moderate, 30% of energy, or High, 42% of energy). For this study, 96 rats were stratified into 8 groups (n = 12 per group). 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 WG or RS [CON], (2) whole grain waxy corn flour with low RS [WWG], (3) purified high–amylose maize (HAM) resistant starch (RS) [HAMRS], and (4) WG HAM flour rich in resistant starch (WG+RS) [HMWG]. The eight diet conditions were fed for six weeks.

Diet treatments were based on the AIN–93M purified diet for rodents [65]. The compositions of the diets are listed in Table 3.1. All diets contained one major starch source as either a purified starch product or in whole grain flour, and starches and whole grain flours were analyzed by proximate analysis (Medallion Labs for Ingredion Incorporated). Starches consisted

of: (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 RS were calculated to contain 23% RS by weight. Diets with waxy whole grain starch were calculated to have 4.93% RS, because the whole grain kernel has the resistant starch component in the bran as long as it is not overly processed. RS content was determined by Ingredion Incorporated using the modified Englyst Assay [66].

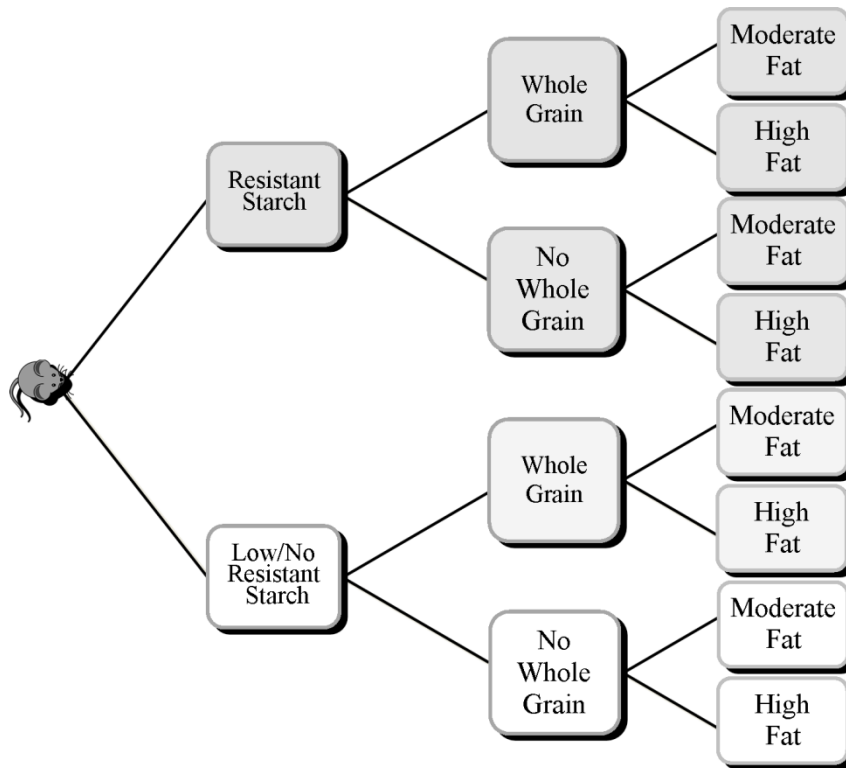


Figure 3.1 Experimental Design. Study was designed as a 2x2x2 factorial. Each of eight groups (n=12) contains a level of each factor: Resistant Starch, Whole Grain, FAT. Levels for factors are Resistant Starch (High or Low or No), Whole Grain (Present or Absent) and FAT (Moderate or High).

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. MF and HF diets were calculated to provide 3.75 ± 0.01 kcal/g and 4.2 ± 0.07 kcal/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

Ingredients	Moderate Fat			
	CON ¹ Grams	HAMRS Grams	WWG Grams	HMWG 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	1.40	1.40	1.40	1.40
L-Cystine	1.80	1.80	1.80	1.80
Total	1000.00	1000.00	1000.00	1000.00
Resistant Starch, % ⁷	0	23.37	0	23.45
Total Energy, kcal	3757	3750	3761	3754

Table 3.1. Diet Composition continued.

Ingredients	High Fat			
	CON ¹	HAMRS	WWG	HMWG
	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	1.40	1.40	1.40	1.40
L-Cystine	1.80	1.80	1.80	1.80
Total	1000.00	1000.00	1000.00	1000.00
Resistant Starch, % ⁷	0	23.32	4.93	23.41
Total Energy, kcal	4164	4136	4230	4209

¹Diets include: CON = Amylopectin control corn starch containing no resistant starch diet; HAMRS = Purified High-amylose resistant 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.

²AMIOCA® corn starch.

³HI-MAIZE® resistant corn starch.

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

⁵Casein was reduced in each diet based on the protein constituent in AMIOCA® 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.

⁶Corn oil was modified in each diet based on the fat content in AMIOCA® 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 ~ 30% of energy for moderate fat, and ~42% of energy for high fat.

⁷Diets 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 [66].

Euthanasia

Rats were euthanized and exsanguinated by cardiac puncture after inhalation of isoflurane anesthesia delivered by soaked cotton balls in a sealed bell jar. Several collections were made. For each rat, a blood sample was collected in a tube with dipeptidyl peptidase IV inhibitor for the measurement of serum active glucagon-like peptide 1 (GLP-1) levels. Active GLP-1 was measured with an enzyme-linked immunosorbent assay (ELISA) kit (ALPCO, NH). The gastrointestinal (GI) tract was removed from the base of the esophagus to the anus. The GI tract was then separated into individual parts: stomach, small intestine, cecum, and large intestine. GI tract divisions were 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 ($ABF = \frac{\text{Abdominal Fat Pads}}{\text{Body weight} - \text{Full GI} + \text{Empty GI}} * 100$). The denominator in the equation is referred to as emboweled body weight (EBW). Inguinal and epididymal fat pads were frozen in liquid nitrogen and stored at -80°C for later analysis. Cecal contents were collected and divided into 0.5 g aliquots. Cecal contents were frozen in liquid nitrogen for measurement of pH and short-chain fatty acids (SCFAs).

Cecal contents pH and short-chain fatty acids analysis

Cecal contents were thawed and 0.5 g of sample was vortexed with 5 ml of distilled water for pH measurements. Each sample was acidified with 1 ml 25% (wt/wt) solution metaphosphoric acid containing an internal standard for SCFAs, 2 g/L 2-ethyl-butyric acid. Samples were centrifuged at 8,000 X g for 10 minutes to separate solids and then the supernatant liquid was filtered through a Millipore filter (MILX HA 33 mm, 0.45 μm MCE STRL; Fisher

SLHA 033SS). The filtered supernatant liquid was carefully transferred to a gas chromatography (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 a previous publication from our lab [9].

Statistical Analysis

Statistical analysis was performed using Statistical Analysis Software SAS® version 9.4. A 2x2x2 factorial analysis was performed followed by an F–protected least significant difference (LSD) post–hoc means comparison test using the MIXED procedure. The three factors were resistant starch (RS, High or Low/No), whole grain (WG, Present or Absent), and fat (FAT, High or Moderate). Tests for normality, equal variance, and identification of outliers were conducted using the UNIVARIATE procedure. When normality assumption was not true, data were transformed to log₁₀. The following variables were transformed due to non–normal distribution ($w < 0.05$ for Shapiro–Wilk test): empty cecum weight (ECW); acetate, propionate, and butyrate in ceca.

An F–statistic of < 0.05 was considered statistically significant for interactions and main effects. Within interactions and main effects, a p–value < 0.05 was considered a statistically different observation, and expressed as means \pm pooled SE. If interactions were not significant, data were collapsed from three–way interactions, to two–way interactions, to finally main effects. Samples more than two standard deviations away from the pooled mean were considered as outliers and were removed only if their presence prevented normal distribution for statistical analysis.

3.3. Results

The study findings support two of the hypotheses, but do not support the third. Moderate fat diets were better than high fat diets for increasing markers of fermentation. High fat diets

attenuated fermentation, and negatively impacted food and energy intake and abdominal body fat. The whole grain resistant starch prebiotic did ameliorate the attenuation of fermentation by high fat diets, but was not more efficacious than a purified non-whole grain resistant starch. Whole grain combined with resistant starch was effective for maintaining, instead of reducing, fermentation, but not more effective than the purified resistant starch for increasing fermentation.

Data were examined and outliers were removed. One outlier was removed for ABF% (WWG HF: 4.459), Total Abdominal Fat (WWG HF: 25.359) mmol propionate produced (HAMRS MF: 0.0127), and mmol butyrate produced (HAMRS MF: 0.014). Four outliers were removed for cecal contents pH (CON HF: 6.57; HAMRS HF: 8.23; HMWG MF: 6.12, 7.78). Six outliers 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).

Main Effects

Descriptive data and analysis of main effect for the factors RS, WG, and Fat are shown in Table 3.2. Food and energy intake, fermentation-associated factors, and physiological variables were examined. Data for main effects are presented as means with pooled SEM in Table 3.2.

Table 3.2. Descriptive statistics about response to dietary factors¹.

Variables	Resistant Starch			p-value (F) ²
	High	Low/No	Pooled SEM	
Food Intake (g)	746.07	758.25	6.9200	0.2227
Energy intake (kcal)	2962.97	3013.30	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
Empty Cecum Wt. (g)	1.7672 ^a	0.4965 ^b	0.0227	<0.0001
Acetate (mmol)	0.4688 ^a	0.0916 ^b	0.0131	<0.0001
Propionate (mmol)	0.0732 ^a	0.0147 ^b	0.0024	<0.0001
Butyrate (mmol)	0.0870 ^a	0.0228 ^b	0.0036	<0.0001
Abdominal body fat % ³	2.0724 ^a	2.5548 ^b	0.0502	<0.0001
Total Abdominal Fat (g)	11.4133 ^a	14.2805 ^b	0.3198	<0.0001
Emboweled body weight (g)	383.88 ^a	400.20 ^b	3.4372	0.0012

Table 3.2. Descriptive statistics about response to dietary factors¹ continued.

Variables	Whole Grains			
	Present	Absent	Pooled SEM	p-value (F) ²
Food Intake (g)	758.85	745.48	7.0101	0.1814
Energy intake (kcal)	3013.66	2962.60	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)	1.0788	1.1849	0.0260	0.8288
Acetate (mmol)	0.2596 ^a	0.3008 ^b	0.0162	0.0066
Propionate (mmol)	0.0504	0.0374	0.0031	0.4870
Butyrate (mmol)	0.0648 ^a	0.0449 ^b	0.0044	<0.0001
Abdominal body fat % ³	2.3452	2.2821	0.0502	0.3796
Total Abdominal Fat (g)	13.0343	12.6596	0.3201	0.4101
Emboweled body weight (g)	394.44	389.64	3.4422	0.3262
Variables	Fat			
	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)	1.2100 ^a	1.0537 ^b	0.0261	0.0145
Acetate (mmol)	0.3040	0.2564	0.0162	0.1417
Propionate (mmol)	0.0440	0.0439	0.0031	0.9230
Butyrate (mmol)	0.0592	0.0505	0.0044	0.0145
Abdominal body fat % ³	2.2231 ^a	2.4042 ^b	0.0500	0.0126
Total Abdominal Fat (g)	12.3191 ^a	13.3748 ^b	0.3201	0.0220
Emboweled body weight (g)	391.00	393.08	3.4422	0.6711

¹Data are shown based on factors, resistant starch (RS, High; or Low or No), whole grains (WG, Present or Absent) and fat (FAT, High or Moderate).

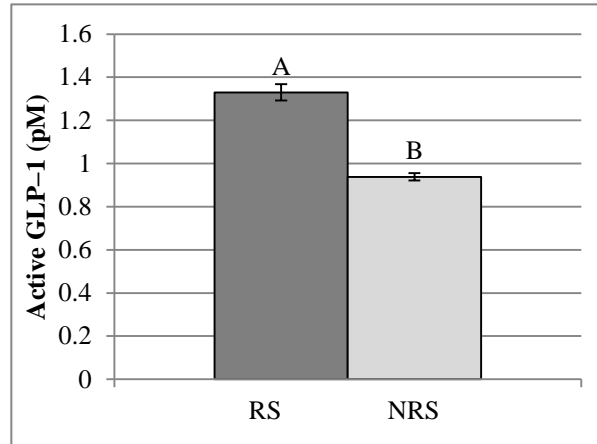
²An F-ANOVA statistic p<0.05 indicates a significant measurement. Means with different letters attached to numbers denote significant differences between groups (p<0.05).

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

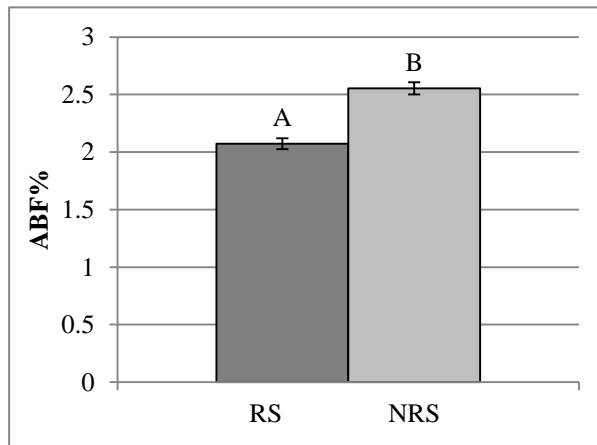
Resistant Starch

While food and energy intake were not significantly different between the two RS groups, other variables differed. As animals fed diets high in RS vs. no or low RS demonstrated increased serum active GLP-1, ECW, mmoles of SCFA produced, and decreased cecal contents pH, EBW, total abdominal fat and ABF%. Empty cecum weight and mmoles SCFAs produced

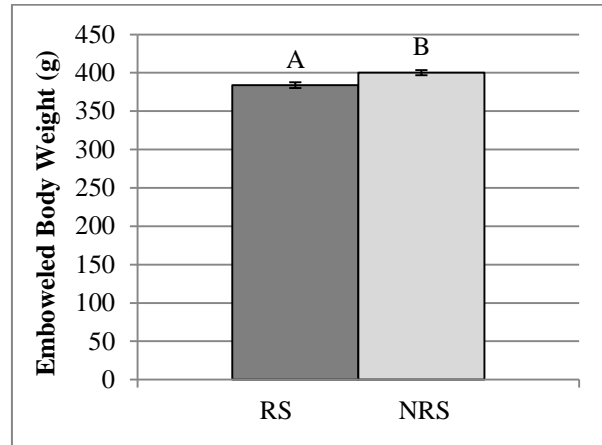
are two of several indicators of increased fermentation in the gut of rodents. These significant effects were evident when levels of FAT and WG were collapsed into high RS and no or low RS. However, active GLP-1, ABF%, and EBW were the only significant main effects not included in interaction effects (Figures 3.2A–C).



A.



B.



C.

Figure 3.2. Variables significantly different between RS and NRS that are present only as main effects. Grouping includes: RS = high resistant starch, NRS = low or no resistant starch. Data are shown collapsed to one factor, resistant starch (RS, High; or Low or No), 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 \pm standard error. Different letters above each bar denote significant differences at $p < 0.05$.

Whole Grain

Most variables did not differ between animals fed diets with and without whole grains.

Those fed whole grains, however had increased cecal contents pH, and decreased acetate, and increased butyrate production. These significant effects were evident when levels of RS and FAT were collapsed into WG presence and WG absence. These main effects are part of interaction effects presented later.

Fat

Differences were observed between rats fed diets with high and moderate levels of fat. Animals fed HF diets had reduced food (Figure 3.3A) and increased energy intake (Figure 3.3B).

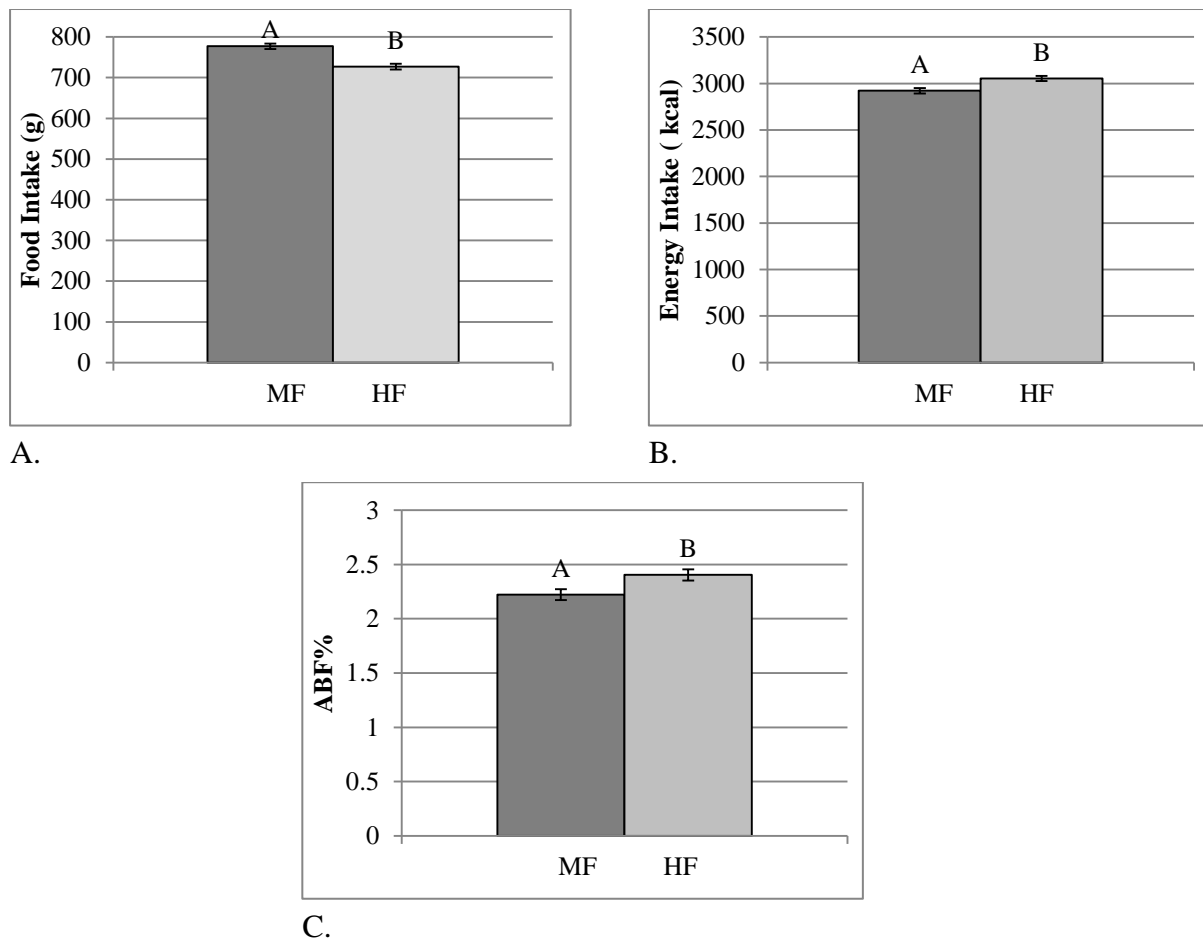


Figure 3.3. Variables significantly different between MF and HF present only as main effects. Grouping includes: MF = moderate fat and HF = high fat. Data are shown collapsed to one factor, fat (FAT, High or Moderate), with the other factors resistant starch (RS, High; or Low or No) and whole grain (WG, Present or Absent) present in both levels of fat. Data are expressed in their original form as means \pm standard error. Different letters above each bar denote significant differences at $p < 0.05$.

Those fed HF diets also had decreased ECW and increased ABF% (Figure 3.3C). These significant effects were evident when levels of RS and WG were collapsed into MF and HF. The significant effect for FAT for increased ECW with MF diets is presented below in interaction effects with RS and WG and approaching significance for RS with WG and with FAT.

Two-way Interactions

Resistant Starch * Whole Grain interaction

Several two-way interactions were noted for RS and WG fermentation variables. Interactions were observed for cecal contents pH, ECW, and mmoles SCFA per cecum. Food and energy intake and body composition variables did not interact. Data for two-way interactions are presented as means with pooled SEM in Table 3.3.

Table 3.3. Two-way interactions for fermentation variables¹.

Variables	RS * WG Interaction				Pooled SEM	p-value (F) ²
	RS WG	RS NWG	NRS WG	NRS NWG		
Food Intake (g)	750.58	741.56	767.11	749.40	9.7863	0.6619
Energy intake (kcal)	2980.73	2945.20	3046.59	2980.00	39.3940	0.6986
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)	1.62474 ^a	1.9312 ^b	0.5302 ^c	0.4628 ^d	0.0067	<0.0001
Acetate (mmol)	0.4060 ^a	0.5316 ^b	0.1132 ^c	0.0700 ^d	0.0115	<0.0001
Propionate (mmol)	0.0581 ^a	0.0882 ^b	0.0167 ^c	0.0127 ^d	0.0022	<0.0001
Butyrate (mmol)	0.1002 ^a	0.0738 ^a	0.0295 ^b	0.0160 ^c	0.0031	0.0200
Abdominal body fat % ³	2.1153	2.0295	2.5751	2.5346	0.0709	0.7504
Total Abdominal Fat (g)	11.7018	11.1248	14.3667	14.1943	0.4523	0.6561
Emboweled body weight (g)	386.32	381.43	402.57	397.84	4.8680	0.9872

Variables	RS * FAT Interaction				Pooled SEM	p-value (F) ²
	RS MF	RS HF	NRS MF	NRS HF		
Food Intake (g)	775.13	717.01	778.95	737.55	9.7863	0.4017
Energy intake (kcal)	2914.48	3011.45	2928.87	3097.73	39.3940	0.3713
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)	1.9161	1.6183	0.5039	0.4891	0.0321	0.1380

Table 3.3. Two-way interactions for fermentation variables¹ continued.

Variables	RS * FAT Interaction				Pooled SEM	p-value (F) ²
	RS MF	RS HF	NRS MF	NRS HF		
Acetate (mmol)	0.5148	0.4227	0.09315	0.09000	0.1850	0.1508
Propionate (mmol)	0.07256	0.07375	0.01536	0.01398	0.0034	0.3262
Butyrate (mmol)	0.09486	0.07908	0.02350	0.02200	0.0050	0.4660
Abdominal body fat % ³	1.9929	2.1520	2.4533	2.6564	0.0709	0.7577
Total Abdominal Fat (g)	10.9128	11.9139	13.7254	14.8356	0.4523	0.9043
Emboweled body weight (g)	382.35	385.40	399.65	400.75	4.8680	0.8426
Variables	WG * FAT Interaction				Pooled SEM	p-value (F) ²
	WG MF	WG HF	NWG MF	NWG HF		
Food Intake (g)	788.57	729.12	765.51	725.44	9.9137	0.3314
Energy intake (kcal)	2965.02	3062.31	2878.33	3046.87	39.9722	0.3756
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)	1.0936	1.0641	1.3264	1.0433	0.0185	0.2388
Acetate (mmol)	0.2559 ^a	0.2633 ^a	0.3521 ^a	0.2495 ^b	0.0114	0.0154
Propionate (mmol)	0.0335 ^a	0.0413 ^{ab}	0.0544 ^c	0.0464 ^{bc}	0.0022	0.0052
Butyrate (mmol)	0.0665	0.0631	0.0518	0.0380	0.0031	0.0775
Abdominal body fat % ³	2.2416	2.4488	2.2047	2.3595	0.0709	0.7132
Total Abdominal Fat (g)	12.2103	13.8582	12.4278	12.8913	0.4527	0.1943
Emboweled body weight (g)	388.11 ^{ab}	400.78 ^a	393.90 ^{ab}	385.38 ^b	4.8680	0.0322

¹Data are shown as full or collapsed interactions based on significant factors, resistant starch (RS, High; or Low or No), whole grain (WG, Present or Absent), and fat (FAT, High or Moderate).

²An F-ANOVA statistic p<0.05 indicates a significant measurement.

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

The two-way interaction between WG and RS for pH of cecal contents (Figure 3.4) indicated that the presence of WG had a different effect with low or no RS present than when there was high RS in the diet. The cecal content pH values were higher in the animals fed low or no levels of RS and in the RSWG group compared to RSNWG diets, and a lower pH indicates increased fermentation in the cecum. In the waxy whole grain control flour groups there was a low amount of RS (4.93% of diet) only as RS1. These two waxy whole grain control flour

groups (HF and MF included) have a lower pH ($p < 0.0001$ for LSD mean comparison) than the NWG groups that had essentially no RS. This means that the presence of RS resulted in some degree of fermentation. With high RS in the diet, the groups with no WG (HF and MF diets combined) had a significantly lower cecal contents pH than groups with WG present ($p < 0.0001$ for LSD mean comparison). This indicates that the high RS diet with only RS2 may ferment better than the combination of RS1 and RS2 in the WG high RS diets. The HAMRS groups had 23% resistant starch as RS2, but HMWG groups had an estimated 18.2% RS2, calculated by subtracting 4.93% (RS1) from 23% (total RS) in the diets. The WG resistant starch RS1 value of 4.93% was only an estimate because of the difference in corn variety (waxy vs. HI-MAIZE corn).

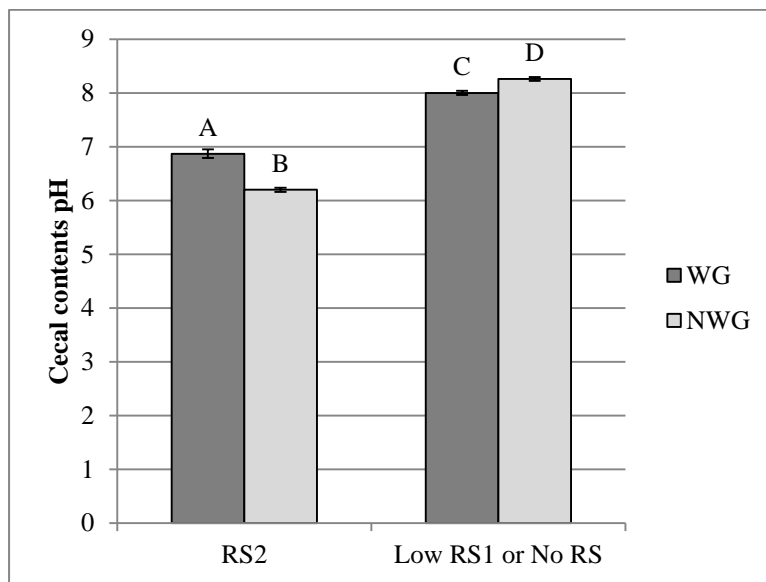
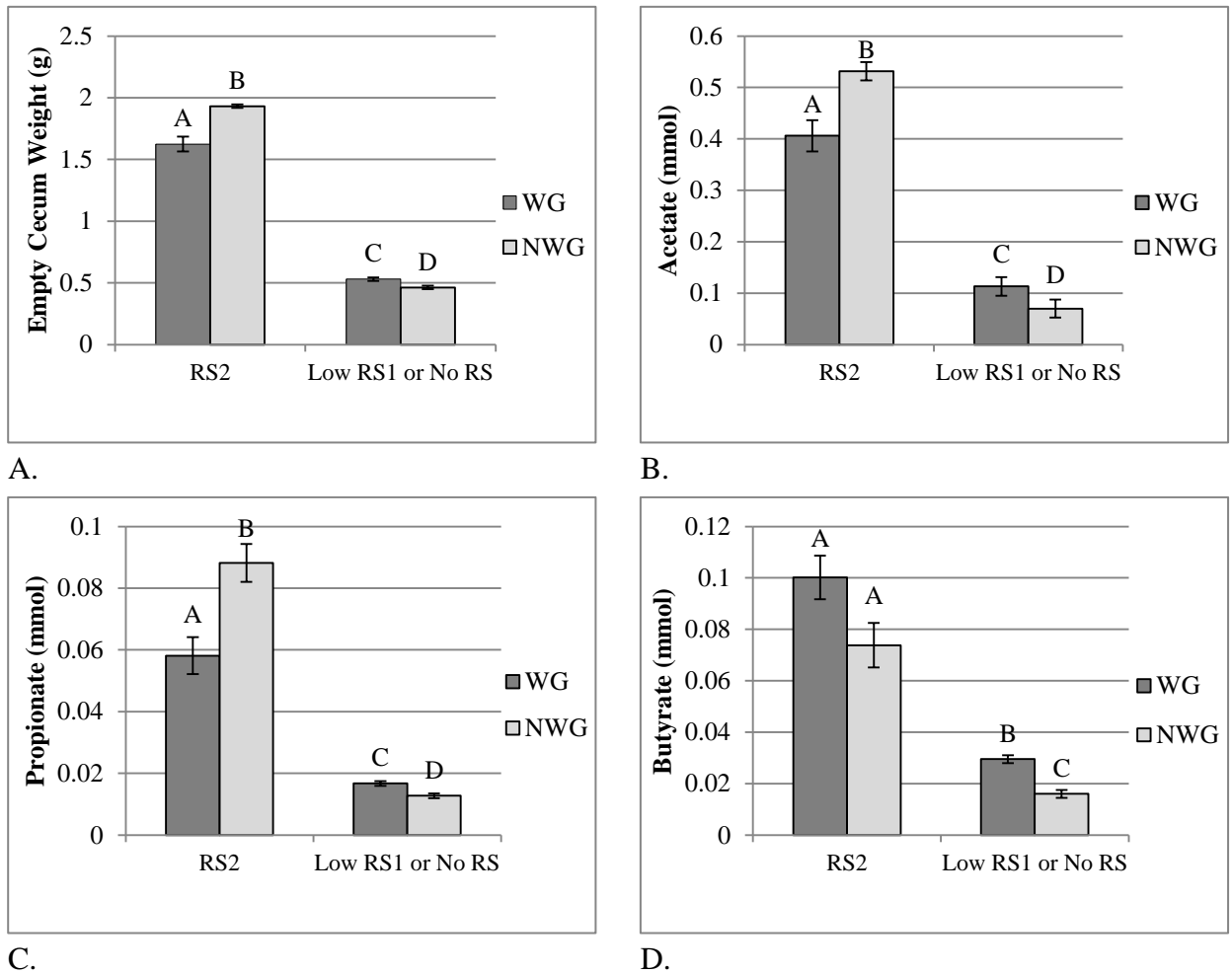


Figure 3.4. Two-way interaction of RS*WG on pH of cecal contents. Grouping includes: RS2 = high resistant starch, NRS = low resistant starch 1 or no resistant starch, WG = whole grain flour as waxy whole grain or high amylose maize whole grain, and NWG = no whole grain. Data are shown collapsed on two factors, resistant starch (RS, High; or Low or No) and whole grain (WG, Present or Absent) with no significant interaction with the third factor fat (FAT, High or Moderate). Data are expressed in their original form as means \pm standard error. Different letters above each bar denote significant differences at $p < 0.05$.

As previously stated, increased ECW is an indicator that greater fermentation has occurred in the

cecum (Figure 3.5A). Increased amounts of SCFA in cecal contents also indicate greater fermentation. The possible explanation for the significant two-way interactions between RS and WG for ECW (Figure 3.5A), acetate (Figure 3.5B) and propionate (Figure 3.5C) in cecal contents is the same as that for cecal contents pH (except in the opposite direction).



Figures 3.5. Two-way interactions of RS*WG on ECW and mmol SCFAs. Grouping includes: RS = high resistant starch, NRS = low resistant starch 1 or no resistant starch, WG = whole grain flour as waxy whole grain or high amylose maize whole grain, and NWG = no whole grain. Data are shown collapsed on two factors, resistant starch (RS, High; or Low or No) and whole grain (WG, Present or Absent) with no significant interaction with the third factor fat (FAT, High or Moderate). Data are expressed in their original form as means \pm standard error. Different letters above each bar denote significant differences at $p < 0.05$.

The presence of RS1 in the waxy whole grain control groups (HF and MF groups) had greater fermentation than the groups with no WG and no RS ($p < 0.0013$ for ECW, $p < 0.0001$ for acetate

and propionate for LSD mean comparison). The data from the high RS groups (HF and MF groups) with only RS2 indicated that fermentation was better with RS2 only than with the combination of RS1 and RS2 in the whole grain ($p < 0.0018$ for ECW, $p < 0.0001$ for acetate and propionate for LSD mean comparison).

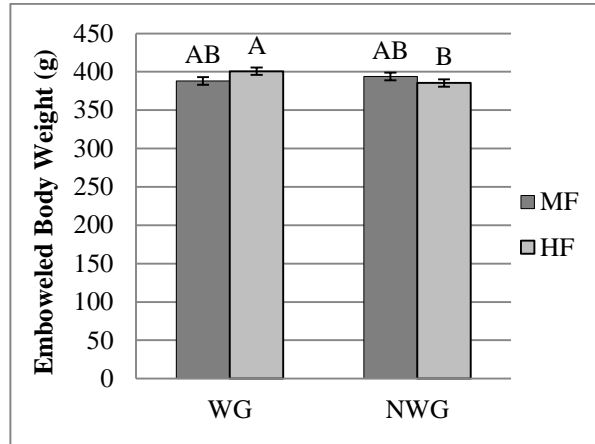
A significant interaction was noted between RS and WG for butyrate in cecal contents (Figure 3.5D). It was in the opposite direction from the relationships observed for pH of cecal contents, ECW, and acetate and propionate in cecal contents. The presence of WG in both high RS and low or no RS resulted in numerical or significantly increased amounts of butyrate in cecal contents, respectively ($p < 0.0001$ for LSD means comparison).

Resistant Starch * FAT interaction

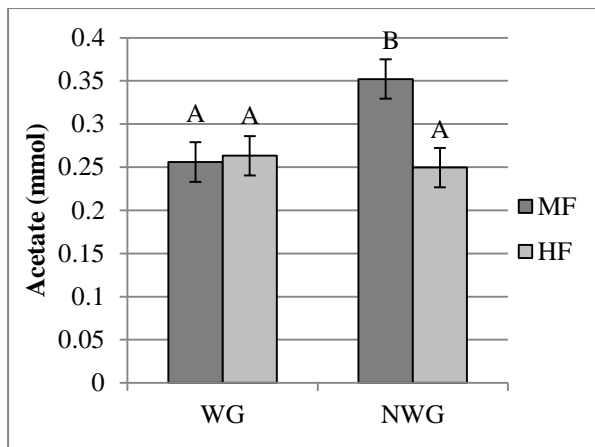
No statistical differences were observed for the interaction between RS and FAT for any of the parameters. No dependent variables demonstrated a p -value approaching significance for this interaction. Differences observed in RS and FAT on several dependent variables are independent of the other factor.

Whole Grain * FAT interaction

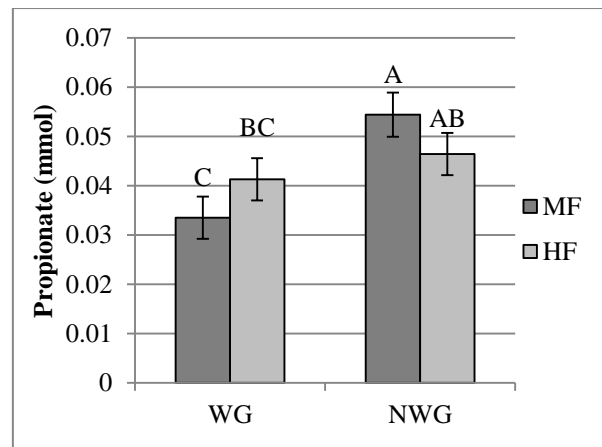
Two-way interactions were noted for factor WG with factor FAT for EBW and acetate and propionate cecal contents (Figure 3.7). Data for two-way interactions are presented as means with pooled SEM in Table 3.3. The explanation as to why the factor WG interacts with the factor FAT for EBW may be because the WG groups (high and low RS combined) have a greater EBW ($p < 0.0283$ for LSD mean comparison) with consumption of a high fat diet than NWG, but a lower EBW (numerical) than NWG groups (high and low RS combined) with consumption of a MF diet.



A.



B.



C.

Figure 3.7. Two-way interactions of WG*FAT on EBW and mmoles SCFAs acetate and propionate. Grouping includes: WG = whole grain flour as waxy whole grain or high amylose maize whole grain, NWG = no whole grain, MF = moderate fat and HF = high fat. Data are shown collapsed on two factors, whole grain (WG, Present or Absent) and fat (FAT, High or Moderate) with no significant interaction with the third factor resistant starch (RS, High; or Low or No). Data are expressed in their original form as means \pm standard error. Different letters above each bar denote significant differences at $p < 0.05$.

Acetate and propionate produced had similar responses for the WG * FAT interaction. Production of acetate was greater ($p < 0.0033$ LSD mean comparison) for NWG groups (high and low or no RS combined) with consumption of a MF diet than with consumption of a HF diet; but for WG there was no difference in production of acetate in cecal contents with either a MF or HF diet. This resulted in a significant interaction for acetate in cecal contents. Production of acetate was consistent for WG groups regardless of level of fat in the diet, but the high RS NWG group

fed a MF diet produced greater amounts of acetate than when fed high RS NWG with a HF diet ($p < 0.0063$, LSD mean comparison). Although not significant, a similar hypothesis explains the interaction between WG and Fat with greater production of propionate in cecal contents in rats fed high RS in a MF diet compared to the HF diet.

Three-way Interactions

Resistant Starch * Whole Grain * FAT interaction

Empty cecum weight was only one three-way interaction that approached significance ($p = 0.0526$) among the three factors (Figure 3.8). The explanation of these findings suggest a consistent fermentation of WG in both MF and HF diets with and without RS2, but high RS NWG fermented best on a MF diet compared to a HF diet demonstrated by ECW. Data for all three-way interactions are presented as means with pooled SEM in Table 3.4.

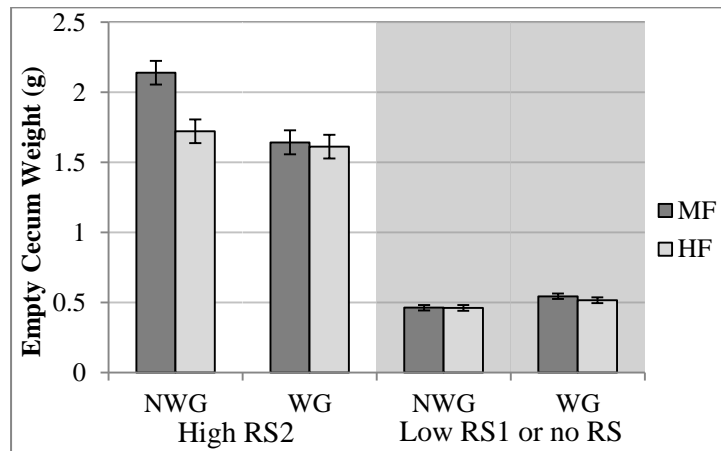


Figure 3.8. Three-way interaction of RS*WG*FAT on ECW. The interaction for RS*WG*FAT ($p = 0.0526$) is presented. Grouping includes: RS = high resistant starch type 2 or high RS2 plus low RS1, NRS = low resistant starch 1 or no resistant starch, NWG = no whole grain, WG = whole grain flour as high amylose maize (High RS2) whole grain or waxy whole grain (Low RS1), MF = moderate fat and HF = high fat. Data are shown as three factors, resistant starch (RS, High; or Low or No) and whole grain (WG, Present or Absent) with the third factor fat (FAT, High or Moderate). Data are expressed in their original form as means \pm standard error.

Table 3.4. Three-way interactions for fermentation variables¹.

Variables	RS * WG * Fat Interaction				Pooled SEM	p-value (F) ²
	RS WG MF (HF)	RS NWG MF (HF)	NRS WG MF (HF)	NRS NWG MF (HF)		
Food Intake (g)	780.41 (720.74)	769.84 (713.29)	769.72 (737.50)	761.19 (737.60)	13.8399	0.4147
Energy intake (kcal)	2934.36 (3027.11)	2894.60 (2995.80)	2995.67 (3097.52)	2862.07 (3097.94)	55.7115	0.4346
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)	0.0182	0.0526
Acetate (mmol)	0.3984 (0.4136)	0.6312 (0.4319)	0.1134 (0.1130)	0.07291 (0.0670)	0.0261	0.3004
Propionate (mmol)	0.0501 (0.0661)	0.0950 (0.0814)	0.0168 (0.0166)	0.0139 (0.0114)	0.0048	0.3953
Butyrate (mmol)	0.1032 (0.0972)	0.0866 (0.0610)	0.0299 (0.0291)	0.0171 (0.0149)	0.0071	0.5485
Abdominal body fat % ³	2.0298 (2.2008)	1.9560 (2.1031)	2.4533 (2.6968)	2.4533 (2.6159)	0.1002	0.8416
Total Abdominal Fat (g)	10.9734 (12.4303)	10.8521 (11.3975)	13.4472 (15.2862)	14.0035 (14.3851)	0.6396	0.7638
Emboweled body weight (g)	379.98 (392.66)	384.73 (378.14)	396.24 (408.90)	403.06 (392.61)	6.8844	0.8439

¹Data are shown as full or collapsed interactions based on significant factors, resistant starch (RS, High; or Low or No), whole grain (WG, Present or Absent), and fat (FAT, High or Moderate).

²An F-ANOVA statistic p<0.05 indicates a significant measurement.

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

3.4. Discussion

Results from the current study demonstrated that three factors (RS, WG, FAT) produce individual main effects and interaction effects with each other. Strong independent effects on fermentation and phenotype were observed as well as interactions between factors that shape the parameters of fermentation. There was an observed effect on ECW that approached significance

in a three-way interaction among the RS, WG and FAT factors. RS and FAT appear to be the primary factors driving differences observed for many of the dependent variables analyzed because of main effects for variables not included in interactive effects. Two-way interactions between WG and FAT and RS and WG were observed but not between RS and FAT.

Several of our previous studies demonstrated that consumption of RS was associated with a reduction in normalized abdominal body fat hypothesized to be the result of increased fermentation [3, 4, 9]. Similar results occurred in the present study with main effects of RS for increased ECW and active GLP-1, and decreased ABF% and EBW. In the current study, RS and WG interactions were demonstrated for cecal contents pH, ECW and cecal contents acetate, propionate and butyrate. These suggested that the WG control fermented better than the non-WG control because of the presence of a small but significant amount of RS1. However, the presence of WG resulted in greater amounts of butyrate, but lower amounts of acetate and propionate. Within these interactions there was consistent production of acetate and propionate with consumption of WG with a MF or a HF diet, but reduced amounts produced with the consumption of purified high RS product with a HF diet. This means that the purified high RS product was negatively affected by the HF diet. Although the interaction was statistically significant, the high RS WG groups and the high RS non-WG groups only had numerically higher values for butyrate. Increased amounts of butyrate with WG diets may be beneficial as butyrate is a major energy source for the colonocytes [25] and butyrate is considered beneficial to the health of the gut [67]. 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 [30, 31]. This may be a reason why acetate was significantly reduced in the WG groups.

The SCFAs measured have other distinct properties. Acetate and propionate produced in the colon can be found in the small and large intestines, portal, hepatic and peripheral blood [25]. These SCFAs are utilized by peripheral tissues (muscle, acetate) or by the liver (acetate, propionate) for metabolism [26–29]. Acetate and butyrate may have a role in modulating glucose metabolism [68]. Propionate has an important role as a precursor to metabolites in gluconeogenesis [69], and influences regulation of blood pressure through Olfactory receptor 78 and G protein coupled receptor 41 [70].

We hypothesized that a moderate fat diet would provide a similar phenotype (body weight, ABF%, food and energy intake, etc.) to low fat diets, and would lie between low and high fat diets in fermentation parameters (cecal contents pH, SCFAs, ECW, etc.). If these hypotheses were valid then we would be able to show that moderate dietary fat consumption provides greater health benefits than high dietary fat consumption. Our lab group has shown that moderate fat diets were comparable to low fat diets on ABF% in C57Bl/6J mice [10]. 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 MF diet was associated with a lower ABF% and a greater ECW.

FAT and WG had significant interactions on EBW and acetate and propionate in cecal contents. The interactions were the result of opposite effects of HF and MF diets depending on the presence or absence of WG.

We also hypothesized that a whole grain resistant starch prebiotic would ameliorate the negative effects caused by high fat diets. Whole grains have been shown to have many positive effects on gut and whole body health [13, 14, 36–42]. In this study, we observed that WG had a complicated role. For instance, WG improved fermentation variables (ECW and SCFA) for HF

diets when compared to diets without WG. However, the presence of WG in high RS groups reduced fermentation as indicated by increased cecal contents pH, and reduced ECW, and SCFA produced except for butyrate. These results suggest that RS2 is better fermented than RS1 because the high RS groups without WG had 100% RS2, but the high RS groups with WG had a combination of RS1 and RS2. The amount of RS2 in high RS groups with WG was ~5 g less than in high RS groups without WG.

There are two possible explanations as to why RS2 appears to be more fermentable than RS1. First, the physical arrangement of the starches differs between resistant starch forms. In RS2, the starch forms granules to resist digestion in the small intestine. The starch in RS1 is a component of the food matrix which acts as a barrier to amylolysis [16]. Bacteria feeding upon these starches can rapidly ferment the RS2, whereas the starch in RS1 requires more time to access [71]. Secondly, the site of measurement is important for determining the fermentability of RS. Starch without the bran (e.g. purified 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 [71]. The current study measured the effects of RS2 in the cecum and resulted in a substantial degree of fermentation using RS2 over RS1. This distinction is useful to examine 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 [38].

This RS2 vs. RS1 finding is in stark contrast to a previous study conducted by our lab [72]. In that study, obese Zucker Diabetic Fatty (ZDF) rats fermented the WG prebiotic better with and without RS compared to groups without WG in low fat diets. Although no changes in

body fat were observed, ZDF rats demonstrated substantial fermentation and microbiota changes. Before conducting the study, the ZDF rats were thought to be dysbiotic and poor fermenters of RS, whereas Sprague Dawley (SD) rats had previously been shown to ferment RS robustly. Sprague Dawley rats can be separated as obese-prone (OP) and obese-resistant (OR) based on consumption of high fat diets, i.e. by phenotype. 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, we did not separate by phenotype. Our previous studies appeared to not be affected by possible different phenotypes. Also, the dominant phenotype in the current study may not ferment RS in a WG product as well as the obese ZDF rats [72]. For greater consistency in results, especially regarding WG, future studies should take advantage of the Charles River colonies of cesarean-derived (CD) rats from the SD line of rat that have been separated into two separate colonies based on the OP and OR phenotypes.

The results from the current study support our previous studies and continue to demonstrate the benefits of consuming RS. In addition, benefits of MF diets and WG products were also demonstrated. Moderate fat diets appear to be as effective as LF diets in promoting fermentation of RS and other effects including normalization of abdominal body fat. Whole grain products with and without RS demonstrated consistent fermentation in both HF and MF diets and appear to promote increased butyrate production in the gut. However, the phenotype of rodent (OP, OR) may affect the response to RS and WG products. This suggests that the SD rat is likely a good model for investigating prebiotic substances, but the two phenotypes should be separated for more consistent results.

CHAPTER 4: CONCLUSION

This investigation demonstrated the importance of studying food components and their interactions, rather than examining specific outcomes from single factors. In this study, there were fermentation differences between rodents fed high RS in MF diets similar to previous studies, mirroring significant differences between rodents fed LF and HF diets. The differences occurred in diets without whole grain. With whole grain diets the fermentation was similar for MF and HF diets. Also, the high RS whole grain groups had similar fermentation effects as the group fed high RS with no whole grain as part of a HF diet. Since this level of fermentation is still significantly greater than groups with no or low RS, these results suggest that Americans may be able to consume the higher levels dietary fat (42% of energy) that some Americans consume and still benefit from consumption of RS. High fat diets may attenuate fermentation, but the addition of other bioactive components, provided by a high resistant starch whole grain product, were hypothesized to help maintain the fermentation process. Although gut health was improved with high RS whole grain diets as demonstrated by increased fermentation compared to low or no RS, high RS whole grain diets did not ferment as well as purified RS2 with the feeding of MF. This suggests that the combination of RS1 and RS2 present in the whole grain kernel compared to an equivalent amount of RS as non-whole grain purified RS2 is not as effective as the purified RS2. However, the WG products appear to increase butyrate production and may benefit the health of the colonocytes. Increased butyrate may indicate a greater utilization of acetate for butyrate production. The results from this study and our previous investigations also may indicate that there may be variation in response to dietary RS in a whole grain product in people as there have been in rodents. Future obesity studies will require multigenic models. One approach is to separate multigenic genotypes by phenotypes of obese-

proneness and obese–resistance into subgroups using caesarean–derived SD rats. This will address the variability found in people as opposed to studying monogenic models such as the obese ZDF rats that have a defective leptin receptor to induce obesity.

Future studies are also needed to determine lower dietary doses of resistant starch and whole grain high RS products that will most benefit the host. Dose response studies with RS can be performed with purified RS or whole grain high RS products. These studies should be performed separately with MF and HF diets to demonstrate benefits at recommended levels of dietary fat for Americans, and the upper range of consumption of dietary fat by Americans. This will enable increased health benefits from fiber and over a range of fat intakes, while not extensively modifying the dietary intake of Americans. As the path to combat obesity has many inroads, health behavior changes that do not require people to exhaustively alter or restrict their dietary intake will be necessary to ensure that compliance and moderation remain feasible in the face of ever evolving messaging and recommendations.

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